Targeted adenovirus-induced expression of IL-10 decreases thymic apoptosis and improves survival in murine sepsis

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Sepsis remains a significant clinical conundrum, and recent clinical trials with anticytokine therapies have produced disappointing results. Animal studies have suggested that increased lymphocyte apoptosis may contribute to sepsis-induced mortality. We report here that inhibition of thymocyte apoptosis by targeted adenovirus-induced thymic expression of human IL-10 reduced blood bacteremia and prevented mortality in sepsis. In contrast, systemic administration of an adenovirus expressing IL-10 was without any protective effect. Improvements in survival were associated with increases in Bcl-2 expression and reductions in caspase-3 activity and thymocyte apoptosis. These studies demonstrate that thymic apoptosis plays a critical role in the pathogenesis of sepsis and identifies a gene therapy approach for its therapeutic intervention.

Despite continuing progress in the development of antibiotics and other supportive care therapies, sepsis remains a leading cause of the high morbidity and mortality in the intensive-care unit. Septic shock and sequential multiorgan failure or dysfunction syndrome correlate with poor outcome. Overproduction of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and IL-1, are critical in the development of septic shock, but clinical trials with inhibitors of proinflammatory cytokines, such as IL-1 receptor antagonist and TNF receptor immunoadhesins and antibodies, have in general failed to improve outcome in septic patients (1, 2).

There is growing recognition, however, that a large number of patients with sepsis syndrome do not sustain an exaggerated proinflammatory cytokine response, but rather, manifest anergy and immune suppression (3). In animal models of sepsis, as well as in septic patients, increased apoptosis in lymphoid organs and decreased numbers of peripheral lymphoid cells have been reported to correlate with an adverse outcome (4, 5). Lymphocyte apoptosis can be induced by several different mediators produced during sepsis, including glucocorticoids and FasL (6, 7). The resulting apoptosis may lead to an impaired acquired immune response and increased susceptibility to invading microorganisms.

Recent studies in animal models of sepsis have demonstrated that inhibition of lymphocyte apoptosis increases survival. For example, transgenic mice overexpressing Bcl-2 are protected from lethality secondary to generalized peritonitis (8). Similarly, treatment of mice with a broad-acting caspase inhibitor also can protect mice from sepsis-induced mortality (9).

The current study was undertaken to directly test whether inhibition of thymocyte apoptosis could improve outcome in a model of generalized peritonitis. We hypothesized that targeted overexpression of IL-10 in the thymus might increase Bcl-2 expression, reduce caspase-3-dependent thymocyte apoptosis, and improve survival. IL-10, an anti-inflammatory cytokine, has been previously shown *in vitro* to reduce T cell apoptosis in part through up-regulation of Bcl-2 (10). Bcl-2 inhibits the release of cytochrome *c* from the mitochondria, and thus, inhibits caspase-9 and caspase-3 activation (11). To test this hypothesis, mice were injected intrathymically 24 h before cecal ligation and puncture (CLP) with a recombinant adenovirus (Adv) expressing human IL-10 (Adv/hIL-10). Because Adv does not readily transfect lymphocytes, but shows tropism for epithelial and mesenchymal cells, secreted protein by thymic accessory cells would act on adjacent T cells through a local bystander effect.

We report here that direct intrathymic instillation of a recombinant Adv expressing hIL-10 reduced blood bacteremia, improved outcome, abolished the increase in caspase-3 activity, and decreased thymic apoptosis in mice subjected to an otherwise lethal generalized peritonitis. The survival benefit was associated with up-regulation of the antiapoptotic protein Bcl-2. Surprisingly, i.v. administration of the same recombinant Adv/hIL-10 had no effect on outcome, despite a similar abrogation of the systemic proinflammatory response.

Materials and Methods

Animals. Female C57BL/6 mice between 5 and 7 weeks of age were obtained from The Jackson Laboratory. Additionally, transgenic mice overexpressing Bcl-2 primarily in T lymphocytes (C57BL/6-TgN(Bcl2)25 Wehi; Jackson Laboratory) were used. In this latter case, heterozygotic mice were bred to wild-type C57BL/6 mice, and offspring carrying *bcl-2* on the simian virus 40 promoter were identified by PCR analysis. The studies were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine.

CLP Model of Polymicrobial Sepsis. CLP was performed as described (9). Sham-treated animals received anesthesia and a laparotomy, but the cecum was neither ligated nor punctured.

Intrathymic and i.v. Gene Therapy. After anesthesia, a 1- to 2-cm midline incision was made from the angle of the mandible to the level of the fourth rib. An upper median sternotomy was created from the incision to the second rib. Using a 50-μl Hamilton syringe and a 30-gauge needle, 20-μl intrathymic injections were made by direct visualization. Mice received injections of either 10^5 or 10^{10} particles of a recombinant Adv construct expressing hIL-10 (Adv/hIL-10), green fluorescent protein (GFP) as a reporter gene (Adv/GFP), or an identical recombinant Adv vector (E1a, E1b, E3 deleted) containing an empty cassette (Adv/empty) in buffer (1× PBS, 2 mM $MgCl_2$, and 3% sucrose). Intravenous injections (100 μl) were made with the same doses of Adv in the tail vein by using a tuberculin syringe.

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Abbreviations: Adv, adenovirus; CLP, cecal ligation and puncture; GFP, green fluorescent protein; hIL-10, human IL-10; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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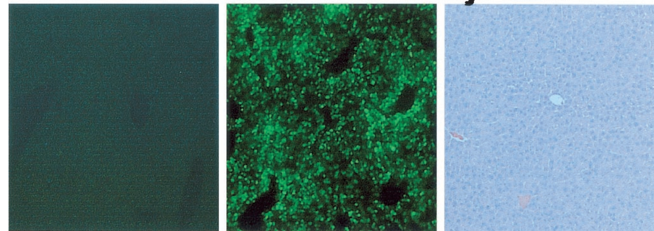

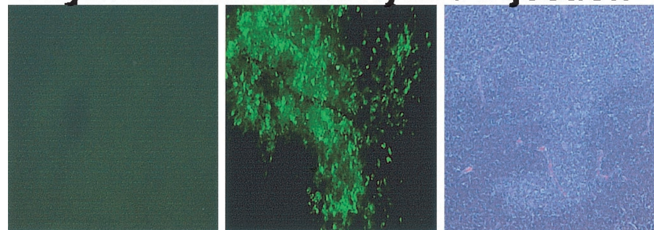

Fig. 1. Histological GFP-positive cells. Mice were injected i.v. (*Upper*) or intrathymically (*Lower*) with 10^{10} particles of an adenoviral vector containing an empty cassette or expressing GFP. Tissues were examined 24 h later for GFP fluorescence or by hematoxylin and eosin (H&E) staining. (Magnification: ×100.)

Construction of a Recombinant Adv Expressing hIL-10 or GFP. A derivative of human Adv serotype 5 was used as the source of viral DNA backbone. The construct was deleted in early region 1, polypeptide IX, and early region 3, as described (12). Recombinant Advs were constructed by using standard homologous recombination methods. hIL-10 and GFP were expressed with a cytomegalovirus early promoter enhancer (12).

Detection of GFP Expression. Mice were anesthetized, and a midline incision was made to expose the thorax. The right atrium was cannulated, and mice were perfused through the left ventricle first with PBS and then with 4% paraformaldehyde in PBS (pH 7.0) until the animals became rigid. Organs were harvested and suspended in 30% sucrose for 2 h, before frozen sections were made. The tissues were sectioned at 20 microns and then photographed by using a fluorescent microscope (Zeiss Axioskop wide-field fluorescent microscope).

Histological Examination of Apoptosis by 3′ End Labeling. At death, the organs from additional mice were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were affixed to slides and deparaffinized. *In situ* terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed by using an *in situ* apoptosis detection kit (Apoptag, Oncor) as described (5). Hematoxylin and eosin-stained tissue sections also were examined by light microscopy.

Caspase-3 Activity Assay. Protein extracts were prepared by homogenization of tissues, and caspase-3 activity was determined by a fluorogenic assay (Enzyme Systems Products, Livermore, CA) as described (5).

Cytokine Measurements. Bioactive TNF was measured in plasma by using the TNF-sensitive WEHI 164 clone 13 murine fibrosarcoma cell line. Human and murine IL-10 and murine IL-6 in the plasma and homogenized thymus were measured by specific ELISA, using commercially available reagents (human IL-10 and IL-6 by Endogen, Woburn, MA; mouse IL-10 by R & D Systems).

Flow Cytometry. Thymi were gently dispersed through a 40-mm mesh filter into RPMI 1640 and centrifuged to pellet the cells. Residual erythrocytes were lysed in ice-cold hypotonic ammonium chloride. After washing twice with 4% BSA in PBS, T cells were identified by using anti-$CD3^+$ or anti-$CD11C^+$ antibody conjugated with either phycoerythrin or allophycocyanin (PharMingen). Samples were analyzed by FACSCalibur and LYSIS II software (Becton Dickinson).

Western Blot Analysis for Bcl-2. After harvesting organs, thymi were placed in lysis buffer (10 mM Tris·HCl, pH 7.6/150 mM NaCl/1% Triton X-100/170 mg/ml PMSF/2 mg/ml leupeptin/2 mg/ml aprotinin) for 10 min and then homogenized and sonicated. Cellular protein (40 mg) was fractionated by SDS/PAGE and transferred electrophoretically to poly(vinylidene difluoride) membranes (Millipore). Blots were blocked (0.05% Tween, 5% nonfat dry milk in PBS) and then incubated in the same buffer for 2 h with anti-mouse Bcl-2 antibody (PharMingen). After washing (0.3% Tween in PBS), the membrane was incubated with horseradish peroxidase-conjugated anti-hamster IgG (PharMingen) for 1 h. Western blot analysis was conducted according to standard procedures by using enhanced chemiluminescence detection.

Blood Bacteremia. Twenty-four hours after induction of generalized peritonitis, mice were euthanized and bled aseptically by cardiac puncture. Heparinized whole blood was log-serially diluted in sterile PBS, and 100-μl aliquots were cultured overnight at 37°C on Trypticase soy agar, 5% sheep blood plates (Becton Dickinson). Colonies were counted, and data are presented as colonies/ml of whole blood.

Presentation of Data and Statistics. Results are presented as the mean ± SEM. Differences between two experimental groups were considered significant at $P < 0.05$ as determined by the Rank sum test. For multiple comparisons, a one-way ANOVA was used. Posthoc analyses were performed with Fischer's least significant difference test. Differences in the survival study were determined with Kaplan-Meier log-transformed survival analysis.

Results

Adv Readily Transfects Thymic Accessory Cells and Hepatocytes. To confirm the identity of Adv-transfected cells, thymi and livers were harvested 24 h after intrathymic and i.v. injection of a recombinant Adv (12) expressing GFP in healthy mice. In the thymus, the distribution of cells expressing GFP was consistent with transduction limited primarily to accessory cells, and not to thymocyte populations, as determined by phenotype and localization (Fig. 1). In contrast, after the i.v. injection of the same adenoviral recombinant, GFP expression could not be detected in the thymus, but was widespread in hepatocytes, although concentrated around perivascular spaces (Fig. 1).

To identify the phenotype of thymic cells transduced with the Adv, flow cytometric analysis was performed. Only 0.8% of the total $CD3^+$-positive thymocytes expressed GFP (data not shown), suggesting that the transfected cells were accessory cells in the thymus, such as dendritic cells, natural killer cells, or macrophages. In contrast, 25.3% of the $CD11c^+$ cells expressed GFP, indicating that dendritic-like cells were one target of the Adv. Thus, it was obvious that although Adv gene therapy could be used to target ectopic expression in the thymus, forced overexpression in a significant number of thymocytes could not be achieved with this adenoviral recombinant vector. This failure to transduce lymphocytes directly eliminated the possibility of

Table 1. Human IL-10 levels in plasma and thymus of healthy and septic mice depending on dose and route of administration (n = 5)

		24 h Posttransduction		48 h, 24 h post-CLP	
Route of injection	Dose	Plasma, ng/ml	Thymus, ng/g wet weight	Plasma, ng/ml	Thymus, ng/g wet weight
Intrathymic	10^5	0.55 ± 0.27	7.81 ± 2.19*	≤0.03	0.85 ± 0.23*
	10^{10}	65.6 ± 13.3†	825.4 ± 327.4*†	3.2 ± 0.2†	38.1 ± 7.8*†
Intravenous	10^5	≤0.03	≤0.45	ND	ND
	10^{10}	232.8 ± 17.2†	2.26 ± 0.84*†	ND	ND

The time intervals chosen represent 24 and 48 h postadenoviral transduction. Data are presented as mean ± standard error of the mean. ND, not determined. *, $P < 0.05$ plasma vs. thymus. †, $P < 0.05$ 10^5 vs. 10^{10}.

direct Adv gene transfer of antiapoptotic proteins to thymocytes such as Bcl-2 and would require a "bystander" effect to achieve increased expression of antiapoptotic proteins.

Because IL-10 is a secreted protein with antiapoptotic properties on T and B lymphocytes (10, 13), it represented an ideal therapeutic approach to use with Adv. Furthermore, biological responses to IL-10 often are seen with low concentrations, often in the range of 0.1 to 10 ng/ml or ng/g wet weight. In healthy mice, intrathymic injections of 10^5 and 10^{10} particles/mouse of the recombinant Adv/hIL-10 resulted in detectable tissue levels of hIL-10 (Table 1). After 24 h, tissue concentrations ranged from 7 to 800 ng/g wet weight, whereas plasma concentrations were more than a log less. As anticipated, mice treated with 10^{10} particles had higher IL-10 concentrations than levels seen in mice treated with 10^5 particles.

When treated i.v. with the same doses of Adv, expression in the thymus was markedly reduced. Mice treated i.v. with 10^5 particles/mouse had no detectable hIL-10 in either plasma or thymus, whereas at the higher dose (10^{10} particles) concentrations were significantly higher in the plasma than in the thymus (Table 1).

Twenty four hours later, corresponding to 48 h after Adv transduction and 24 h after induction of CLP, plasma hIL-10 expression had significantly ($P < 0.05$) declined in mice transduced intrathymically with the Adv/hIL-10 (Table 1). Although the values represent the means for the entire group, nondetectable levels (≤0.45 ng/g wet wt) were observed in approximately one-third of the mice 24 h after CLP.

Intrathymic Expression of hIL-10 Improves Survival and Reduces Bacteremia in Septic Mice. We chose 10^5 particles of Adv as a therapeutic dose because the goal of these studies was to achieve therapeutic levels in the thymus (1–10 ng/ml) without significant appearance in the systemic circulation. However, as a control, similar doses of Adv were administered i.v.. Mice were pretreated with the i.v. or intrathymic instillation of adenoviral vectors, and 24 h later, the animals underwent CLP and were observed for 6 days. Animals receiving no pretreatment had a survival rate to CLP of 30% (9/30) (Fig. 2). In contrast, mice pretreated intrathymically with 10^5 particles of the Adv/hIL-10 had a significantly increased survival rate of 75% (15/20, $P <$ 0.05) compared with the untreated group. Intrathymic administration of a recombinant Adv/empty also improved outcome (14/30, 47%), but the degree of improvement was significantly less ($P < 0.05$) than compared with mice treated intrathymically with the Adv/hIL-10 and was not statistically different from the untreated CLP group. Transgenic mice overexpressing Bcl-2 primarily in lymphocytes demonstrated no mortality to CLP (10/10 survived).

To confirm that the beneficial effect seen with intrathymic administration of Adv was not caused by systemic appearance of hIL-10, but by targeted expression in the thymus, additional mice were injected i.v. with 10^5 particles of both recombinant Adv vectors and compared with septic mice receiving no treatment. There was no significant difference in survival among the three study groups: 3/11 mice (27%) survived CLP without treatment, 5/11 (45%) and 5/12 (42%) survived in groups receiving Adv/empty or Adv/hIL-10, respectively.

The improved outcome in mice receiving intrathymic injections of Adv/hIL-10 was associated with a reduced blood bacteremia 24 h after induction of CLP. Blood bacteria counts were 2.7×10^4 and 5.6×10^4 colonies/ml in the untreated septic (n = 10) and Adv/empty-treated septic mice (n = 9), respectively. In contrast, blood bacteremia levels were reduced more than 10-fold in septic mice pretreated with Adv/hIL-10 (1.3×10^3 colonies/ml; $n = 9$, $P < 0.05$ by ANOVA and Fischer's least significant difference posthoc versus both groups). Levels were essentially undetectable in healthy controls.

Increased Survival Is Associated with Reduced Thymic Apoptosis. Reductions in thymic apoptosis were associated with improved outcome in mice receiving intrathymic instillation of Adv/hIL-10. Septic mice were killed 24 h after CLP and 48 h after Adv instillation. As expected, sepsis induced by CLP increased caspase-3 activity in the thymus of these mice more than 50-fold (Fig. 3). Pretreatment of mice with intrathymic injections of 10^5 particles of Adv significantly reduced caspase-3 activity in a pattern similar to that seen for survival. The recombinant Adv/empty significantly decreased caspase-3 activity (56% reduction), but the increased caspase-3 activity was completely abolished by intrathymic treatment of 10^5 particles of Adv/

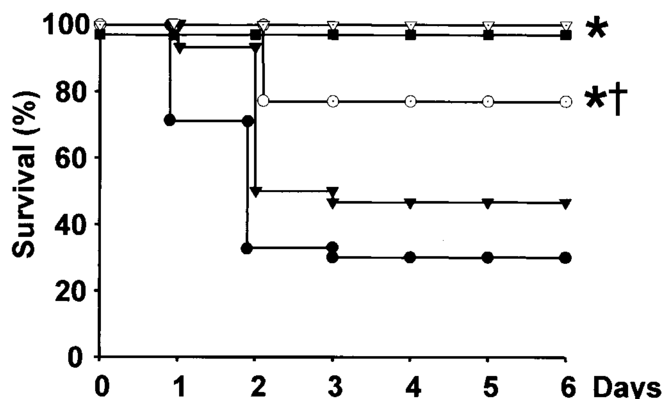

Fig. 2. Survival rate of different treatment and nontreatment groups. Survival rate of mice undergoing CLP without treatment (●) was compared with that of animals pretreated intrathymically with 10^5 particles of an adenoviral vector expressing hIL-10 (Adv/hIL-10) (○) 24 h before CLP and mice pretreated with an equivalent number of adenoviral particles containing an empty cassette (▼). Transgenic mice overexpressing Bcl-2 in T cells (■) as well as sham mice (▽) had a survival of 100%. *, $P < 0.05$ CLP vs. treatment or sham; †, $P < 0.05$ Adv/hIL-10 vs. Adv/empty, by Kaplan-Meier, log rank.

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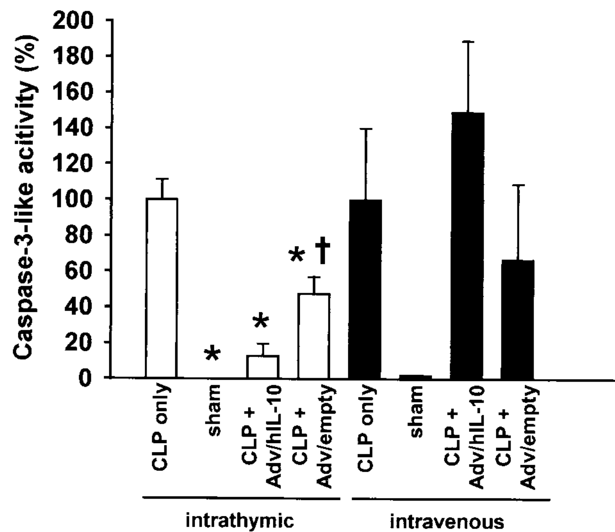

Fig. 3. Caspase-3-like activity in the thymus of septic mice. Caspase-3-like activity was determined in the thymus from septic mice ($n = 10$/group) 24 h after CLP and 48 h after intrathymic or i.v. pretreatment. Data from several experiments were combined, and the rate of caspase-3 activity in the thymus of septic mice was normalized to 100%. Statistical comparisons could not be performed between groups of mice treated intrathymically and i.v. because of the effect of a prior surgery (thoracotomy) on subsequent thymic caspase activity. Data were normalized (100%) for values from sham, septic mice [2,329 relative fluorescence intensity (RFI) for intrathymic and 301 RFI for i.v. injections]. *, $P < 0.05$ CLP vs. treatment or sham; †, $P < 0.05$ Adv/hIL-10 vs. Adv/empty, by one-way ANOVA and Fischer's least significant difference posthoc test.

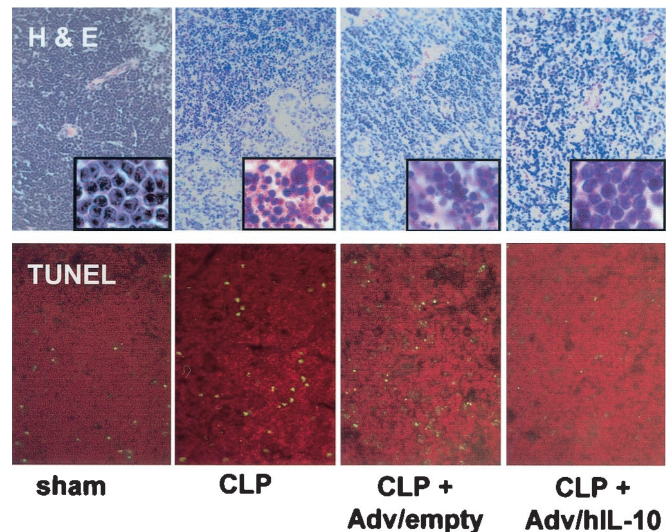

Fig. 4. *In situ* TUNEL staining and histological examination of thymi from mice after CLP. Thymi were harvested from mice 24 h after CLP, and tissues were stained with hematoxylin and eosin (H&E), or by 3′ end labeling of apoptotic nuclei (TUNEL) staining as described in *Materials and Methods*. Increased numbers of cells undergoing apoptosis were seen in mice after CLP. Mice pretreated intrathymically with 10^5 particles of Adv/empty had similar numbers of apoptotic cells, as determined by TUNEL staining. In mice pretreated with 10^5 particles of Adv/hIL-10, there was a marked reduction in the numbers of apoptotic cells. In the hematoxylin and eosin-stained sections (see *Insets* for greater detail), apoptotic cells (fragmented and pyknotic) were seen primarily in the thymus of untreated mice and mice pretreated with Adv/empty. [Magnification: ×100 and ×1,000 for *Insets*].)

hIL-10 (92% reduction). The reduction in caspase-3 activity in mice treated with Adv/hIL-10 was significantly greater than the reductions seen in the mice treated with the Adv/empty vector (Fig. 3). Transgenic mice overexpressing Bcl-2 also demonstrated no increase in caspase-3 activity in the thymus (data not shown).

In addition, thymic caspase-3 was determined in mice pretreated with i.v. injections of the Adv vectors. Caspase-3 activities among CLP mice receiving no pretreatment or pretreatment with Adv/empty or Adv/hIL-10 were not significantly different (Fig. 3).

These findings were confirmed with *in situ* 3′ end labeling and hematoxylin and eosin staining. *In situ* 3′ end labeling, although not quantitative, indicated a similar number of apoptotic cells in the untreated septic mice, as well as in the septic mice treated with intrathymic instillation of Adv/empty (Fig. 4). Conversely, in mice treated with 10^5 particles of an Adv/hIL-10, there was a dramatic reduction in the number of apoptotic cells. The physical characteristics of apoptosis, such as shrinking and fragmentation of cells, were evident in the hematoxylin and eosin staining. These apoptotic cells were found predominantly in the cortex. In contrast, mice receiving i.v. administration of Adv/empty or Adv/hIL-10 showed no significant reduction of either caspase-3 activity or the numbers of apoptotic nuclei (data not shown).

Bcl-2 Is Up-Regulated in the Thymus of hIL-10-Treated Mice. Transgenic mice overexpressing Bcl-2 were protected from the lethality associated with CLP (Fig. 2). We therefore examined whether the decreases in apoptosis seen in the thymi of mice treated intrathymically with adenoviral constructs expressing hIL-10 also were associated with an up-regulation of the antiapoptotic protein Bcl-2. Six mice per group underwent the procedures as described earlier. Samples were obtained from three representative mice in the groups receiving intrathymic administration of Adv/empty or Adv/hIL-10, as well as in a CLP group not receiving a pretreatment. In both the untreated septic mice and septic mice pretreated with the Adv/empty vector, Bcl-2 expression was significantly reduced compared with both the healthy group and mice pretreated with the Adv/hIL-10 (Fig. 5 *C* and *D*). Interestingly, there was no apparent difference in thymic Bcl-2 levels between this latter group and the healthy animals. To rule out any reduction in Bcl-2 expression secondary to the surgical procedure, Bcl-2 expression between healthy animals and ani-

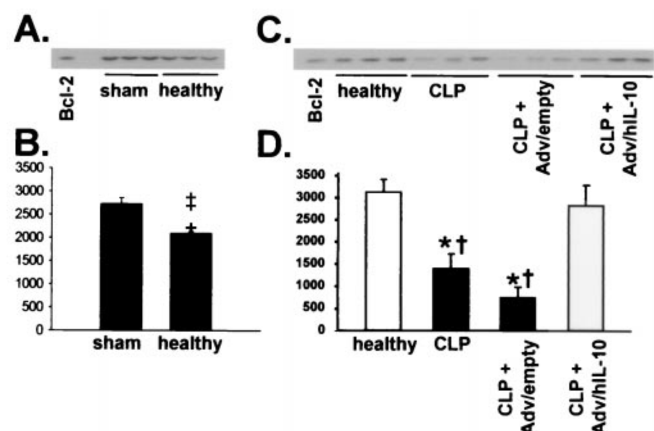

Fig. 5. Bcl-2 expression in thymus as determined by Western blot analysis. Bcl-2 levels were examined in the thymus of healthy mice and mice after a CLP. Mice were pretreated intrathymically with either Adv/hIL-10 or Adv/empty at a dose of 10^5 particles. Bcl-2 levels were determined by Western blot analysis (*A* and *C*), and the relative quantities of Bcl-2 were determined by densitometric analysis (*B* and *D*). *, $P < 0.05$ healthy vs. CLP; †, $P < 0.05$ Adv/hIL-10 vs. Adv/empty or CLP; ‡, $P < 0.05$ healthy vs. sham. As a positive control for detecting murine Bcl-2, M1 cell lysate (ATCC TIB 192) was used.

Table 2. Plasma IL-6 and murine IL-10 levels in plasma and thymus of septic mice depending on dose and route of administration (n = 7)

	Plasma IL-6, ng/ml		Murine IL-10 Intrathymic administration	
Treatment group	Intravenous administration	Intrathymic administration	Plasma, pg/ml	Thymus, pg/g wet wt
Sham	≤0.07		<32	1,126 ± 42
CLP	512 ± 264		4,157 ± 2,609*	1,063 ± 125
CLP + Adv/empty	139 ± 111*	99 ± 63*	2,240 ± 889*	2,283 ± 494*
CLP + Adv/hIL-10	18 ± 12	34 ± 32	339 ± 149*	2,845 ± 315*†

The time intervals chosen represent 24 hours post cecal ligation and puncture. Data are presented as mean ± standard error of the mean. *, $P < 0.05$ vs. sham. †, $P < 0.05$ CLP vs. CLP + Adv.

mals undergoing sham procedure was compared. Surprisingly, Bcl-2 expression appeared to increase in the thymus of mice after a modest sham treatment, when compared with that of healthy mice (Fig. 5 *A* and *B*).

Thymic Expression of hIL-10 Is Associated with a Generalized Reduction in the Magnitude of the Systemic Inflammatory Response. Plasma proinflammatory cytokine concentrations were determined 24 h after induction of sepsis in an effort to determine whether the magnitude of the systemic inflammatory response was abrogated by intrathymic therapies that suppressed apoptosis. Plasma TNF-α concentrations in mice treated with 10^5 particles of an Adv/hIL-10 could not be detected (<110 pg/ml) and were no different from sham mice or mice pretreated with Adv/empty. Only in the untreated septic mice was TNF-α detectable in the plasma (1,344 ± 776 pg/ml). Interestingly, mice pretreated with both adenoviral constructs i.v. also did not show any detectable plasma TNF-α level.

There was also evidence that intrathymic administration of the recombinant Adv/hIL-10 reduced the magnitude of more distal markers of the systemic inflammatory response. For example, both plasma IL-6 and murine IL-10 concentrations were significantly reduced by intrathymic instillation of Adv/hIL-10 (Table 2). Moreover, plasma IL-6 concentrations decreased by more than 90% in septic mice treated with Adv/hIL-10 (Table 2). Pretreatment with Adv/empty also produced reductions in plasma IL-6, although not to the degree seen in animals treated with Adv/hIL-10. Similarly, plasma IL-6 concentrations also significantly decreased in mice treated i.v. with Adv/empty, as well as in mice receiving the viral construct expressing hIL-10.

Plasma murine IL-10 concentrations were markedly reduced in animals receiving the intrathymic instillation of Adv/hIL-10 compared with CLP group (Table 2). Although not significant, murine IL-10 concentrations also were decreased in the plasma of mice pretreated intrathymically with Adv/empty. Murine IL-10 concentrations in the thymus actually followed a different pattern. The animals treated intrathymically with the adenoviral construct expressing hIL-10 had the highest murine IL-10 concentrations and the untreated CLP group the lowest. The mice receiving Adv/empty had levels intermediate to these two groups.

Discussion

Sepsis syndrome often is characterized by both a systemic proinflammatory response and defects in antigen presentation, macrophage "paralysis," and reduced T cell proliferation to mitogenic stimulation (14). Current therapeutic approaches aimed at blocking the proinflammatory response generally have failed to show any improvements in outcome in either mice with CLP (15) or patients with sepsis syndrome (1, 2). Rather, recent studies have focused on the observation that increased apoptosis of lymphoid cells also is seen in animal models of sepsis, burn injury, and trauma (16). Hotchkiss *et al*. (4) recently reported the presence of increased numbers of apoptotic lymphocytes in the spleen, as well as in intestinal epithelial cells of patients dying from sepsis.

Although increased apoptosis in lymphoid and epithelial tissues has been demonstrated to accompany sepsis syndromes, increased apoptosis may also directly contribute to the adverse outcome and be a potential therapeutic target for intervention. For example, the role of caspase-dependent apoptosis in the survival response to sepsis recently has been clarified. Hotchkiss *et al*. (9) observed that systemic injection of Z-VAD, a broad acting caspase inhibitor, into mice receiving a similar CLP improved survival and reduced apoptosis in the thymus. Although such studies confirm a direct involvement of caspase activation in outcome to sepsis syndrome, the systemic administration of a broad acting caspase inhibitor, like Z-VAD, cannot identify either the specific target (such as the individual caspase) or anatomical site of action (such as the thymus).

Caspase-dependent apoptosis is regulated in part by a family of mitochondrial proteins of the Bcl-2 family. Bcl-2 and its other family members, such as Bcl-xL and the proapoptotic proteins, Bid, Bad, and Bax, are important regulators of cellular apoptosis (8, 17–20). Members of this family can inhibit or promote apoptosis via homodimerization and heterodimerization (21). Thymocytes overexpressing Bcl-2 are resistant to apoptotic stimuli *in vitro*, such as dexamethasone and γ-irradiation, and they survive apoptosis induced by the withdrawal of growth factors (18). In animal models performed with Bcl-2 overexpressing mice, such as the ischemia-reperfusion-injury model as well as in the CLP model, decreased numbers of apoptotic intestinal cells or lymphocytes of spleen and thymus have been observed, and a survival benefit was demonstrated in the latter group (8, 20). Furthermore, mice deficient in the proapoptotic protein Bid are resistant to Fas-induced liver injury and have an increased survival (19).

In the present report, we have demonstrated that ectopic overexpression of IL-10 in the thymus of septic mice also improves survival. Improvements in outcome were associated with increases in thymic Bcl-2 expression and reductions in thymic apoptosis and caspase-3 activity. We have used an adenoviral construct to deliver hIL-10 to the thymus of mice before the induction of generalized peritonitis or sepsis syndrome. Adv readily transduces epithelial cells, fibroblasts, and dendritic cells, but has only a limited propensity for transducing thymocytes or other lymphocyte populations. This difficulty in transducing thymocytes with Adv necessitated an indirect approach to target thymocyte apoptosis. We chose to transduce thymic accessory cells with the cDNA for a secretable protein, hIL-10, and affect adjacent lymphocytes through a paracrine, bystander process.

Although IL-10 is a pleiotropic cytokine with both anti-inflammatory and immunosuppressive properties, IL-10 has been shown to suppress T cell apoptosis through a Bcl-2-dependent process. For example, Cohen *et al*. (10) reported that

IL-10 suppressed peripheral blood T lymphocyte apoptosis *in vitro* by increasing Bcl-2 expression. Similarly, Taga *et al*. (22) observed that IL-10 prevents T cell apoptosis after IL-2 withdrawal and Epstein–Barr virus infections through up-regulation of Bcl-2 expression.

In the present report, intrathymic expression of hIL-10 markedly increased Bcl-2 levels in the thymus, and this was associated with reductions in thymic caspase-3 activity and the numbers of apoptotic cells. The improved outcome in mice receiving intrathymic administration of adenoviral vectors expressing hIL-10 was caused by the local intrathymic expression, because mice injected i.v. with the same vector did not have improved outcome compared with untreated septic mice. This finding confirms that systemic hIL-10 expression cannot explain the improved outcome in this model or the reductions in thymocyte apoptosis. Rather, the beneficial effects appear to require localized hIL-10 expression in the thymus. In a recent report, Takakuwa *et al*. (23) systemically administered an Adv expressing IL-10 and saw improved outcome to a bacterial bolus. However, this peritoneal administration of live bacteria is predominantly a model of an exaggerated proinflammatory response and hypovolemic shock where exogenous administration of IL-10 protein is already known to be effective. In contrast, we have used a more clinically relevant model of sepsis and one in which systemic administratition of IL-10 or other cytokine inhibitors have not been effective (24).

To further demonstrate the influence of apoptosis on outcome in this model, we compared the results to those obtained from transgenic mice overexpressing Bcl-2 under a T cell-specific promoter (18). These mice were completely resistant to a CLP-induced mortality, suggesting that the improvements in outcome in mice receiving intrathymic instillation of Adv/hIL-10 were possibly through up-regulation of Bcl-2.

Unfortunately, IL-10 is a pleiotropic cytokine, and improvements in outcome may have been secondary to IL-10 activities not involving Bcl-2 expression and apoptosis. Although IL-10 has anti-inflammatory properties, the improvements in outcome cannot be easily attributed to any reductions in the magnitude of the systemic inflammatory response induced by IL-10. The attenuation of the plasma TNF-α and IL-6 responses after CLP also were seen when the mice were treated i.v. with the same dose of Adv vectors, but similar improvements in outcome were not observed. Thus, the proinflammatory cytokine response was attenuated by all of these treatments, but did not appear to be associated with any differences in survival. This latter finding is consistent with the inability of anti-TNF-α therapies (1, 15) or systemically administered IL-10 (25) as anti-inflammatory agents to improve outcome in this model of sepsis syndrome.

One surprising aspect of the current study was the observation that intrathymic, but not i.v., instillation of a recombinant Adv/empty vector modestly improved outcome and reduced thymic caspase-3 activity. This beneficial effect may be secondary to activation of the innate or acquired immune response to the adenoviral expression (19, 20). Consistent with this latter hypothesis is the observation that intrathymic levels of murine IL-10 significantly increased in septic mice pretreated with the Adv/empty when compared with sham-treated animals. Intrathymic Adv instillation may have induced a sufficient local IL-10 expression to partially attenuate the increases in caspase-3 activity.

In conclusion, intrathymic expression of hIL-10 with Adv leads to a reduction in thymocyte apoptosis and significant increases in survival after CLP. These results appear to be mediated at least in part through up-regulation of Bcl-2 and inhibition of caspase-3-dependent apoptosis. The study provides strong evidence that lymphocyte apoptosis plays a critical role in sepsis, indicating a potentially new therapeutical approach for treatment of sepsis syndrome. Further studies are required to demonstrate the feasibility of gene therapy in T cell-rich organs other than the thymus to prevent T cell apoptosis in humans.

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