IL-15 Prevents Apoptosis, Reverses Innate and Adaptive Immune Dysfunction, and Improves Survival in Sepsis

Shigeaki Inoue, Jacqueline Unsinger, Christopher G. Davis, Jared T. Muenzer, Thomas A. Ferguson, Katherine Chang, Dale F. Osborne, Andrew T. Clark, Craig M. Coopersmith, Jonathan E. McDunn and Richard S. Hotchkiss

*J Immunol* 2010; 184:1401-1409; Prepublished online 21 December 2009; doi: 10.4049/jimmunol.0902307
http://www.jimmunol.org/content/184/3/1401

Supplementary Material  http://www.jimmunol.org/content/suppl/2009/12/18/jimmunol.0902307.DC1

References  This article cites 41 articles, 17 of which you can access for free at:  http://www.jimmunol.org/content/184/3/1401.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:  http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:  http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:  http://jimmunol.org/alerts
IL-15 Prevents Apoptosis, Reverses Innate and Adaptive Immune Dysfunction, and Improves Survival in Sepsis

Shigeaki Inoue,* Jacqueline Unsinger,* Christopher G. Davis,* Jared T. Muenzer,† Thomas A. Ferguson,‡ Katherine Chang,* Dale F. Osborne,* Andrew T. Clark,§ Craig M. Coopersmith,*§ Jonathan E. McDunn,* and Richard S. Hotchkiss*§

IL-15 is a pluripotent antiapoptotic cytokine that signals to cells of both the innate and adaptive immune system and is regarded as a highly promising immunomodulatory agent in cancer therapy. Sepsis is a lethal condition in which apoptosis-induced depletion of immune cells and subsequent immunosuppression are thought to contribute to morbidity and mortality. This study tested the ability of IL-15 to block apoptosis, prevent immunosuppression, and improve survival in sepsis. Mice were made septic using cecal ligation and puncture or *Pseudomonas aeruginosa* pneumonia. The experiments comprised a 2 × 2 full factorial design with surgical sepsis versus sham and IL-15 versus vehicle. In addition to survival studies, splenic cellularity, canonical markers of activation and proliferation, intracellular pro- and antiapoptotic Bcl-2 family protein expression, and markers of immune cell apoptosis were evaluated by flow cytometry. Cytokine production was examined both in plasma of treated mice and splenocytes that were stimulated ex vivo. IL-15 blocked sepsis-induced apoptosis of NK cells, dendritic cells, and CD8 T cells. IL-15 also decreased sepsis-induced gut epithelial apoptosis. IL-15 therapy increased the abundance of antiapoptotic Bcl-2 while decreasing proapoptotic Bim and PUMA. IL-15 also decreased sepsis-induced gut epithelial apoptosis. IL-15 therapy increased the abundance of antiapoptotic Bcl-2 while decreasing proapoptotic Bim and PUMA. IL-15 increased both circulating IFN-γ, as well as the percentage of NK cells that produced IFN-γ. Finally, IL-15 increased survival in both cecal ligation and puncture and *P. aeruginosa* pneumonia. In conclusion, IL-15 prevents two immunopathologic hallmarks of sepsis, namely, apoptosis and immunosuppression, and improves survival in two different models of sepsis. IL-15 represents a potentially novel therapy of this highly lethal disorder.

Interleukin-15 is a member of the common receptor γ-chain family of cytokines, which includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (1–5). IL-15 is unique among IL-2 family members because of its pattern of receptor expression (i.e., DC [dendritic cell], NK, and CD8 T cells). As a result, IL-15 coordinates the response of these innate and adaptive immune cells for host protection (6–10). Mice without IL-15 or IL-15Ra have no NK cells and markedly decreased numbers of CD8 T cells, NK T cells, γδ T cells, and DCs (4, 6). IL-15 is essential in promoting the differentiation of activated CD8 T cells into effector CD8 T cells, and is required for DC priming of NK cells (5); these activities are necessary for an effective host response to many microorganisms. In addition to these key immunomodulatory effects, IL-15 is a potent inducer of antiapoptotic Bcl-2 family member proteins Bcl-2 and Bcl-XL in lymphocytes and DCs, thereby reducing their susceptibility to apoptotic stimuli (11, 12).

Sepsis, a disorder responsible for over 200,000 deaths annually in the United States, is characterized by an initial net hyperinflammatory response, followed by a period of immunosuppression that has been termed “immunoparalysis” (13–16). During the immunosuppressed phase of sepsis, patients may have difficulty eradicating the invading pathogens and are susceptible to life-threatening secondary hospital-acquired infections. This immunosuppression of sepsis is characterized by numerous defects in both the innate and adaptive immune system (13–16). Given the broad ranging effects of IL-15 to enhance function of multiple diverse immune effector cells, as well as its efficacy in blocking cell apoptosis, a hallmark and critical pathogenic factor in sepsis, IL-15 represents a logical therapeutic candidate in sepsis (17, 18). For example, the ability of IL-15 to augment IFN-γ production may be beneficial in sepsis by preventing the monocyte/macrophage dysfunction that contributes to the immunosuppression (15, 16). The effect of IL-15 to enhance the lytic function of CD8 T cells is expected to be beneficial in control of many microorganisms.

Despite the potential beneficial effects of IL-15 in infectious disease, few studies have been conducted. IL-15 administration improved survival in a murine model of *Mycobacterium tuberculosis* and appears to play a beneficial role in clearance of malarial parasites, *Cryptococcus* neoformans, and *Salmonella* (8–10, 19, 20). In addition, mice transgenic for IL-15 (under the control of a MHC class I promoter) have improved survival in *Escherichia coli*-induced shock (10). Although the preceding studies suggest that IL-15 might be helpful in selected infectious disease models, one study reported that IL-15 knockout mice had improved survival in sepsis (21).

The purpose of this investigation was to examine whether therapeutic administration of IL-15 prevented sepsis-induced immunosuppression, decreased sepsis-induced apoptosis, and improved survival in two widely used models of lethal sepsis, that is, the cecal ligation and puncture (CLP) and *Pseudomonas aeruginosa* pneumonia, the most common Gram-negative pathogen in nosocomial pneumonia.

Abbreviations used in this paper: CLP, cecal ligation and puncture; DC, dendritic cell; MFI, mean fluorescence intensity.

*Department of Anesthesiology, †Department of Pediatrics, ‡Department of Ophthalmology, and §Department of Surgery, Washington University School of Medicine, St. Louis, MO 63110. E-mail address: hotch@wustl.edu

Received for publication July 16, 2009. Accepted for publication November 12, 2009.

This work was supported by National Institutes of Health Grants GM44118 (to R.S. Hotchkiss), EY015570 (to T.A.F.), EY06765 (to T.A.F.), and by the Alan A. and Edith L. Wolff Foundation.

Address correspondence and reprint requests to Dr. Richard Hotchkiss or Dr. Jonathan E. McDunn, Campus Box 8054, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: hotch@wustl.edu or mcdunn@wustl.edu

The online version of this article contains supplemental material.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10 $16.00

Materials and Methods

**Mice**

Male CD-1 mice (Charles River Laboratories, Wilmington, MA) ~20–25 g body weight and 6–8 wk of age were used for all studies. Mice were housed for at least 1 wk prior to use.

**Abs**

Abs were purchased from BD Pharmingen (San Diego, CA), Cell Signaling Technologies (Danvers, MA), eBioscience (San Jose, CA), or Jackson Immunoresearch Laboratories (West Grove, PA).

**BiD Pharmingen.** CD4-FITC (Cat. no. 553729), CD8-PECy5 (Cat. no. 553405), CD45-PerCP (Cat. no. 131239), CD122-Fc (Cat. no. 553901); CD11c-PE (Cat. no. 553801); and HMC2-PE (Cat. no. 557000)—these two Abs were used to identify DCs; T cell activation markers CD44-PE (Cat. no. 553134), CD69-PE (Cat. no. 01505B), and CD25-PE (Cat. no. 553075); the cell proliferation marker Ki-67-PE (Cat. no. 556027); antiapoptotic Bcl-2-PE and its isotype control (Cat. no. 556557).

**Cell Signaling Technologies.** Bcl-2 family members Bcl-xL (Cat. no. 2764), Bim (Cat. no. 2819), and PUMA (Cat. no. 4976); and the apoptosis marker, cleaved caspase-3 (Cat. no. 9661).

**eBioscience.** DX5-FITC (a marker to identify NK cells) (Cat. no. 11-5971-85).

**Jackson Immunoresearch Laboratories.** A secondary PE-labeled donkey anti-rabbit IgG (F(ab’))2-fragment (Cat. no. 711-116-152).

**IL-15 and IL-15Rα subunit**

Recombinant mouse IL-15 was purchased from eBiosciences (San Jose, CA, Cat. no. 34-8151-85). Mouse IL-15Rα subunit Fc chimera (IL-15 Rα) was purchased from R&D Systems (Minneapolis, MN, Cat. no. MN-551-MR-100). Recombinant IL-15Rα subunit could be markedly enhanced by combining IL-15 with its IL-15Rα subunit (22–24). Combining IL-15 with the IL-15Rα subunit assists in presenting IL-15 to the target cells termed "transpresentation" and thereby converts it to a superagonist. The IL-15 and IL-15Rα stabilization protocol detailed by Rubenstein et al. was used for this study (23). Briefly, 75 μg IL-15 and 350 μg IL-15 Rα-Fc were incubated together in 1.5 ml PBS to preform the IL-15/IL-15Rα complex for 20 min at 37°C. Samples were diluted 10-fold in PBS to a total volume of 15 ml, then aliquoted, and frozen. In the current study, each mouse was injected s.c. with 300 μl stabilized IL-15 solution (1.5 μg IL-15/7.0 μg IL-15Rα). In the current study, injection of IL-15 refers to the combination of IL-15 and its IL-15 Rα-Fc unless stated otherwise.

**CLP sepsis model**

All animal studies were approved by the Washington University Animal Studies Committee. The CLP model as developed by Chaudry et al. (25) was used to induce intra-abdominal peritonitis, as described previously (26, 27). Mice were anesthetized with isoflurane and a midline abdominal incision was made. The incision was ligated, ligated below the ileocecal valve, and punctured twice with a 25-gauge needle. The abdomen was closed in two layers, and the mice were injected s.c. with 1 ml 0.9% saline. In one group, IL-15 was injected s.c. 30 min after the operation. Sham-operated mice were handled in the same manner, except the incision was not ligated or punctured. Cohorts of mice used for acute studies were treated with IL-15 or the saline diluent 30 min after sham or CLP surgery. Mice used to determine absolute cell counts, apoptosis, and cytokine production were sacrificed 22–24 h after surgery. For survival studies, mice underwent CLP as described previously and IL-15 or the saline diluent was injected s.c. 30 min, 24 h, and 48 h after the operation. A second time course of administration of IL-15 was performed in which IL-15 was given at 6 h after sepsis and survival recorded. The broad spectrum antibiotic imipenem (25 mg/kg body weight) was administered s.c. 6 h postoperatively and every 24 h times two additional doses. For cell proliferation and activation marker studies, mice underwent CLP with a single puncture using a 27-gauge needle or sham surgery. IL-15 was injected 30 min, 24 h, and 48 h after surgery. Mice were killed and cells harvested 96 h after surgery for staining for cell surface markers and Ki-67.

**Pneumonia model of sepsis**

*P. aeruginosa* pneumonia was induced as previously described (28). *P. aeruginosa* was selected because it is a Gram-negative bacteria that is one of the most common causes of nosocomial pneumonia. *P. aeruginosa* (ATCC 27853) were grown overnight in tryptase soy broth. A 10-ml volume of the culture medium was placed in a 50-ml conical tube and bacteria were harvested by centrifugation. The pellet was resuspended, centrifuged, and the density of inoculum adjusted to 0.5 A600 mm corresponding to between 5 × 10^8 and 1 × 10^9 CFU/ml, as determined by serial dilution and colony counts.

Mice were anesthetized with isoflurane and placed in the supine position with neck extended. A midline neck incision was made and exposed trachea. Using an insulin syringe (Terumo, Tokyo, Japan), 30 μl *P. aeruginosa* suspension was slowly injected intratracheally and observed to be aspirated on inhalation. IL-15 or the saline diluent was injected s.c. 30 min, 24 h, and 48 h after the operation. Survival was recorded for 8 d after pneumonia.

**Spleen harvest**

Immediately prior to sacrifice, spleens were surgically removed. Isolated splenocytes were prepared by gently pressing the organ through a 70-μm filter; cells were then washed and red cells lysed as previously described (26, 27).

**Quantification of absolute cell counts and apoptosis**

Total cell counts per spleen were determined via the Vi-Cell counter (Beckman Coulter, Fullerton, CA). The percentage of individual cell phenotypes, that is, CD4, CD8, B, etc., were determined via flow cytometric analysis (FACScan, BD Pharmingen) as described previously (26). The absolute cell counts for each splenic subset population were calculated by the following formula: cell counts of cell subpopulations = total cell counts (determined by the Vi-Cell counter) multiplied by the subset population percentage (determined by flow cytometer). Apoptosis was quantified by flow cytometry using both FACS for cleaved caspase-3 and the TUNEL assay-APC-BRDU kit as described previously (26–29).

**Determination of intracellular Bcl-2, Bcl-xL, Bim, and PUMA protein abundance**

Splenocytes were prepared as described previously and labeled with fluorescently tagged Abs to surface markers (26). Cells were then fixed, permeabilized, washed, and stained for intracellular proteins as described previously. Briefly, the primary Ab for Bcl-2 immunostaining was PE-labeled and therefore no fluorescent-labeled secondary Ab was necessary. Abs for Bcl-xL, Bim, and PUMA were not fluorophore labeled, therefore after incubation with the primary Ab, cells were washed and stained (30 min) with the PE-labeled secondary donkey anti-rabbit F(ab’)_2. The mean fluorescence intensity (MFI) of the protein of interest, that is, Bcl-2, Bcl-xL, Bim, or PUMA, was determined by FACS analysis and presented on a relative scale.

**Determination of cell proliferation—Ki67 staining**

Sham- or CLP-operated mice were treated with 1.5 μg IL-15 or saline diluent at 30 min after surgery and again at 24 and 48 h later. At 96 h after surgery, spleen cells were harvested from sham and CLP mice and Ki67 staining determined, as described previously (26).

**Bright-field microscopy of H&E-stained tissue sections**

In addition to the flow cytometric method TUNEL assay to detect apoptosis, bright-field microscopy of H&E-stained tissue sections was also performed. Splenic tissue section were obtained at 20–22 h after sham or CLP surgery and fixed overnight in 10% buffered formalin. Tissue sections were then processed and stained with H&E. The degree of spleen apoptosis was evaluated by bright-field microscopy using a Nikon Eclipse E600 (Tokyo, Japan). Microscopic evaluation of apoptosis was used as a confirmatory method to evaluate whether the ability of IL-15 to inhibit sepsis-induced apoptosis was observed throughout the architecturally distinct regions of the spleen. Apoptotic splenocytes exhibit characteristic findings of nuclear compaction (pyknosis) and nuclear fragmentation (karyorrhexis). These morphological features are readily apparent on bright-field light microscopy by staining determined, as described previously (30). A minimum of five to seven random fields was evaluated for each organ section (×200 magnification). Higher magnification (×400–600) was used to visualize the finer details of cellular apoptotic changes.

In addition to evaluating the effect of IL-15 on splenocyte apoptosis, the effect of IL-15 on intestinal epithelial cells was also examined as reported previously (31). Briefly, apoptotic crypt cells were identified in H&E-stained intestinal sections using morphological criteria of cell shrinkage with nuclear condensation and fragmentation. Apoptosis was quantitated in 100 contiguous well-oriented crypt-villus units by an examiner blinded to section identity.

**IFN-γ intracellular and secretion assay**

Mice underwent sham or CLP surgery and were treated with 1.5 μg IL-15 30 min after surgery. Spleens were harvested 24 h later, splenocyte suspensions prepared, and cells stimulated as described previously (27). Dissociated splenocytes (10^5/ml) were stimulated overnight with anti-CD3 and anti-CD28, and Golgi blocker then was added for 4 h, followed by staining for CD8 T and NK cells and quantitation of intracellular IFN-γ.
by flow cytometry. Supernatant fluid was obtained from the incubation media prior to harvesting the cells at 24 h after incubation. The concentration of IFN-γ in the media was quantitated by ELISA (30).

Quantification of peritoneal polymorphonuclear neutrophil
Mice received 1.5 μg IL-15 30 min after sham or CLP surgery and 24 h later the peritoneal cavity was lavaged with 10 ml warmed 0.09% saline. The peritoneal fluid was harvested and neutrophils and monocyte/macrophages identified by FACS analysis using cell surface markers as previously described (26, 27).

Cytokine analysis
At ~20–22 h postsurgery, mice were anesthetized and blood obtained by cardiac puncture in heparinized syringes. Plasma cytokines were quantitated using BD FACSAarray and the Inflammation Kit per the manufacturer’s recommendations as previously described (26, 27). The lower limits of detection were IL-6 (5 pg/ml), TNF-α (7.3 pg/ml), MCP-1 (22.7 pg/ml), IL-10 (17.5 pg/ml), and IFN-γ (2.5 pg/ml). In addition to plasma cytokines, IFN-γ from the supernatant of stimulated splenocytes was also analyzed.

Statistical analysis
Data were analyzed with the statistical software Prism (GraphPad, San Diego, CA). Data are reported as the mean ± SEM. Two-way ANOVA examining the main effects of surgery (CLP versus sham) and treatment (IL-15 versus vehicle) as well as their interaction was performed. A Bonferroni posttest was used to determine whether there was a treatment effect that was dependent on which surgery (sham or CLP) the animals received. For survival studies, a log-rank test was used. Significance was reported at p < 0.05. For data comparing apoptosis in spleens from septic mice examined by conventional bright-field microscopy, a Student t test was used.

Results
IL-15 reduced the sepsis-induced loss of total splenocytes and CD8 T cells
Sepsis caused the loss of >50% of total splenocytes compared with sham-operated animals and this loss was significant in all cell subsets (Fig. 1). Using a Bonferroni post hoc test, we determined that for total splenocytes, B cells, and DCs, the differences between septic- and sham-operated mice were significant (p < 0.05) only in the vehicle-treated mice, whereas the depletion of NK cells was significant in both vehicle- and IL-15–treated mice. IL-15 treatment significantly increased the number of CD8 T cells. The only significant interaction between surgery and treatment, that is, a difference in the effect of IL-15 in sham-operated mice versus CLP-operated mice, was the number of splenic DCs sug-

FIGURE 1. IL-15 attenuated the loss of total splenocytes, CD8 T cells, B cells, and DCs in sepsis. Mice underwent sham or CLP surgery and at 30 min were injected s.c. with IL-15 or vehicle. At ~20–22 h postsurgery, splenocytes were harvested for determination of absolute cell counts (see Materials and Methods). The comparison of CLP versus sham was significant (two-way ANOVA, p < 0.05) for all groups presented. The comparison of vehicle versus IL-15 treatment had statistically significant effects (two-way ANOVA, p < 0.05) on both total splenocytes and CD8 T cells. There was a statistically significant interaction between surgery and treatment on the total number of dendritic cells. Data expressed as mean ± SEM; n = 16–23 mice in each group; *p < 0.05 after two-way ANOVA with a Bonferroni post hoc test.

IL-15 protected CD4, CD8, NK, and DCs from sepsis-induced apoptosis
Examination of H&E-stained splenic tissue sections (×200 magnification) from sham mice showed <3 apoptotic splenocytes per high-powered field (data not shown). In contrast, spleen sections from septic mice demonstrated extensive apoptosis with focal regions in the white pulp and, to a lesser degree, red pulp in which >25% of cells demonstrated classical features of apoptosis, including pyknosis and karyorrhexis (Fig. 2A). Compared with wild-type mice with sepsis that did not receive IL-15, septic mice that were treated with IL-15 had decreased splenocyte apoptosis in both white and red pulp (>10% of splenocytes showed evidence of apoptosis in these animals); p < 0.01, n = 10 CLP mice and n = 8 CLP plus IL-15. Quantitative evaluation of apoptosis in these samples found that compared with sham-treated animals, sepsis caused a significant increase in the percentage of apoptotic splenic CD4, CD8, NK, and DC cells (Fig. 2B). For all cell types, these differences were only significant between vehicle-treated animals and not between IL-15–treated animals. There was a significant interaction term between surgery and treatment, namely, a difference in the effect of IL-15 in sham-operated mice versus CLP-operated mice. These findings indicate that sepsis
causes an increase in gut epithelial apoptosis that is partially ameliorated by IL-15 therapy. IL-15 treatment had no effect on the incidence of apoptotic gut epithelial cells in the sham-operated animal, indicating that IL-15 does not prevent physiologic gut epithelial apoptosis (Fig. 3).

**IL-15 alters Bcl-2 family protein expression to favor cytoprotection in select immune effector cells**

Intracellular Bcl-2, Bim, and PUMA abundance were measured by intracellular protein staining and flow cytometry in splenic CD4, CD8, and NK cells 20 h after surgery and treatment. Sepsis caused a significant decrease in intracellular Bcl-2 protein staining in CD4 splenocytes in vehicle-treated but not IL-15–treated animals (Fig. 4). IL-15 treatment increased Bcl-2 abundance in all cell types assayed (splenic CD4, CD8, NK, and DC cells).

Fig. 5 shows representative flow cytometry histograms demonstrating the effect of IL-15 treatment on Bim and PUMA expression after CLP. For Bim expression in CD4 and CD8 splenocytes and PUMA expression in splenic CD8 and NK cells, there was a significant interaction term between surgery and treatment. In these cell types, IL-15 treatment had no effect on the intracellular abundance of these proapoptotic proteins if the animals were sham-operated, but prevented the increase in protein expression because of sepsis. Both surgery and treatment independently affected PUMA staining in CD4 splenocytes, whereas neither surgery nor treatment had a significant effect on intracellular Bim staining in NK cells. Because of technical limitations, intracellular Bim and PUMA staining was not performed on DCs.

**IL-15 increased cell proliferation in sham and, to a lesser degree, in septic mice**

Sham or CLP mice were treated with IL-15 on the day of surgery and for 2 consecutive days. On day 4 after surgery, splenocytes were harvested and stained for Ki67, a measure of cell proliferation. IL-15 treatment increased cell proliferation of CD8 and NK cells for both sham and CLP mice (Fig. 6). There was a significant interaction between surgery and treatment for all cell types studied that can be interpreted as CLP significantly attenuated the effects of IL-15 treatment with regard to the total number of Ki67+ CD4, CD8, and NK cells.

**IL-15 induces expression of cell activation markers in sham but not septic mice**

The effect of IL-15 to increase cell activation was evaluated at 96 h after surgery. In sham-operated mice, IL-15 caused an increased cell activation as detected by the increase in the absolute numbers of CD25+, CD69+, and CD25+/CD69+ (double positive) in both CD4 and CD8 T cells (Supplemental Fig. 2). Non–IL-15–treated septic mice had a similar increase in these activation markers in CD4 but not CD8 T cells. IL-15 treatment of septic mice did not further increase the number of CD25+, CD69+, or CD25+/CD69+ (double positive) CD4 or CD8 splenocytes (Supplemental Fig. 2).

**IL-15 modulates pro- and anti-inflammatory cytokines**

Mice were subjected to sham or CLP surgery and plasma harvested ∼24 h later. Surgery had a significant effect on all cytokines measured (Table I). The Bonferroni post hoc test established that these differences were significant comparing vehicle- with IL-15–treated groups for TNF-α, IL-6, and IL-10 in the sham-operated animals and IL-6 and INF-γ in the septic animals. There was a significant treatment effect on TNF-α, IL-6, and INF-γ. Although both TNF-α and IL-6 production were decreased with IL-15 treatment in septic mice, there was an increase in circulating INF-γ in septic mice treated with IL-15 compared with vehicle.

In addition to evaluating circulating cytokines, the percentage of immune effector cells that produced INF-γ was determined by intracellular staining of IFN-γ. Representative flow cytometry data
IL-15 improves survival in two models of sepsis

The efficacy of IL-15 was tested in two widely used models of sepsis, that is, the CLP and the P. aeruginosa pneumonia model. IL-15 was tested in these two models because the immune pathogenesis differs in the two insults and therefore the effects of IL-15 might be different in the two disparate models (28). CLP was performed and IL-15 (IL-15 plus the IL-15Rα; see Materials and Methods), saline, or IL-15Rα alone were injected s.c. 30 min, 24 h, and 48 h after surgery. Survival was recorded for 7 d. CLP-operated mice treated with IL-15 had a >3-fold improvement in survival compared with CLP mice that did not receive IL-15, p = 0.016 (Fig. 8). A second trial of IL-15 was undertaken in which IL-15 was administered 6 h after CLP and mice treated with IL-15 had a >3-fold improvement in survival compared with CLP mice treated with vehicle (29, 30).

Discussion

Sepsis is a highly lethal disorder that induces multiple defects in cells of both the innate and adaptive immune system (14–17). Sepsis targets T cells, B cells, DCs, NK cells, and monocytes for apoptotic destruction (17, 26, 27). Sepsis also induces an immunosuppressive phenotype consisting of decreased responsiveness of immune effector cells (13, 14). The net result of these sepsis-induced alterations is a severe impairment in immunity such that patients are often unable to eradicate their primary infection and are vulnerable to secondary nosocomial infections, often with organisms that are not pathogenic in patients with competent immune systems. Perhaps the strongest evidence for the profound immunosuppression in patients with sepsis is their loss of the delayed type hypersensitivity response to positive controls and their reactivation of latent viral pathogens (32–34). Two recent studies of critically ill immunocompetent patients (many of whom had sepsis) requiring prolonged...
intensive care unit stays showed a high incidence of reactivation of CMV (33%) and HSV (21%) (32, 33). Reactivation of these viral pathogens is presumably due to loss in T cell immunity, thereby allowing reactivation of latent virus.

Given the compelling evidence of immunosuppression as a major pathologic sequela of sepsis, immunostimulatory cytokines are rational agents to study in animal models of sepsis as potential therapeutics. In the current study, we evaluated the efficacy of therapeutic IL-15 in the disorder and identified cellular mechanisms for its salutary effects.

IL-15 can activate both innate and adaptive immunity (1–4) and is required for the differentiation of activated CD8 T cells into effector CD8 T cells, a requirement for an effective immune response to invading pathogens. IL-15 is also necessary for DC priming of NK cells (5) that may be beneficial in sepsis by production of IFN-γ, a key macrophage-activating cytokine. Eradicating pathogens in sepsis requires coordinated action of cells of both the innate and adaptive immune system and IL-15 is advantageous in this regard because of its broad effects on cells in both systems.

As an IL-2 family member, IL-15 also has potent antiapoptotic activity that we hypothesized would override the widespread apoptosis of immune cells in sepsis. Previous studies support this assertion; IL-15 was found to protect against lethal apoptosis in vivo and prevent death of lymphocytes and hepatocytes against multiple apoptotic stimuli (11). In the current study, IL-15 prevented the sepsis-induced apoptotic depletion of DCs, NK cells, and CD8 T cells. The potential significance of this antiapoptotic effect of IL-15 should be viewed in the context of numerous animal studies showing that prevention of sepsis-induced apoptosis by a variety of different means improves survival in the disorder (17, 18).

Attesting to its potent and diverse antiapoptotic effects, IL-15 also prevented sepsis-induced death of intestinal epithelial cells that have been shown to play a key role in sepsis (31, 35). Work from our group has shown that transgenic mice that overexpress Bcl-2 in the intestinal epithelium had markedly decreased gut apoptosis and conferred a 3-fold improvement in survival (35). Additional work from our group has shown that administration of epidermal growth factor ameliorated the increase in proapoptotic proteins, reduced gut apoptosis, and decreased mortality from 60% to 30% in the mouse CLP model (36). One theory of the role of the gut in sepsis relates to its barrier function. It is postulated that the loss of bowel integrity in sepsis results in translocation of bacteria or bacterial products, for example, endotoxin, into the circulation, leading to the theory that the gut represents the “motor” of the systemic inflammatory response.

Survival of hematopoietic cells is due to the antagonistic balance between pro- and antiapoptotic Bcl-2 family members (37). One of the antiapoptotic mechanisms of action of IL-15 in CD8 T cells in sepsis is due to its effects to increase Bcl-2 as demonstrated in the current study (Fig. 4). CLP mice treated with IL-15 had an increase in intracellular Bcl-2 staining in CD4 T, CD8 T, NK, and DC cells compared with non-IL-15–treated CLP mice (Fig. 4). In addition to
increasing antiapoptotic Bcl-2, IL-15 also prevented the sepsis-induced increase in proapoptotic Bim and PUMA protein (Fig. 5). Bim and PUMA protein abundance were unchanged in IL-15–treated septic mice compared with sham-operated mice. Previous work from our laboratory has shown that Bim null and PUMA null mice have a marked decrease in sepsis-induced apoptosis; Bim null mice also had an improved survival in sepsis (30). The current results in CD8 T cells are consistent with work by Huntington et al. who showed that IL-15–mediated survival of NK cells was due to the effect of IL-15 to decrease Bim (38). These investigators showed that IL-15 decreased Bim via its effects on phosphorylation of Erk1 and Erk2 kinases (38). To the best of our knowledge, the current work demonstrating that IL-15 decreases PUMA in CD8 T cells is the first report to note this particular mechanism of action of IL-15. These findings, showing the effects of IL-15 on Bim and PUMA protein in sepsis, are consistent with recent work from our laboratory in which IL-15 prevented the sepsis-induced increase in mRNA for both Bim and PUMA in CD8 T cells (data not shown). In short, antiapoptotic effects of IL-15 are due in part to increasing antiapoptotic Bcl-2 family member expression while preventing the injury-induced increases in proapoptotic Bcl-2 family members.

The proper host reaction to invading pathogens is a vigorous but controlled proinflammatory immune response (13, 15, 34). A failure of the host to mount an initial robust immune response results in further pathogen multiplication. Alternatively, an excessive unbridled proinflammatory response may result in “cytokine storm”–mediated organ injury and mortality. The effect of sepsis to increase CD4 T cell activation markers and to increase circulating proinflammatory cytokines is indicative of the establishment of a heightened host immune response to the severe infection. Because of the potent effects of IL-15 on CD8 T cells, DCs, and NK cells, the authors were concerned that IL-15 might exacerbate the proinflammatory response in sepsis and lead to worsened survival in the current study. The results reported in this study indicate that IL-15 does not cause excessive immune stimulation and “cytokine storm” using this dosing regimen. In this regard, IL-15 did not further increase CD4 or CD8 T cell activation markers in the septic animals (Supplemental Fig. 2). Furthermore, although IL-15 did increase circulating IFN-γ, it modestly decreased circulating IL-6 and TNF-α, two prototypical proinflammatory cytokines in sepsis (Table I). One possible explanation for the decreased IL-6 and TNF-α in IL-15–treated mice with sepsis could be that IL-15 resulted in a more rapid containment of the infectious process and thereby a less robust immune response.

As noted, IL-15 caused an increase in circulating IFN-γ, a key macrophage-activating cytokine. In addition, IL-15 more than doubled the percentage of IFN-γ–positive NK cells in both sham and septic mice as well as increased IFN-γ production in stimulated splenocytes (Fig. 7A–C). The effect of IL-15 on IFN-γ production may be particularly important. Sepsis impairs the production of IFN-γ by immune cells and although there is some controversy regarding exogenous administration of IFN-γ, studies have shown that restoration of production of endogenous IFN-γ can improve survival (39, 40).

To date there are few studies examining the role of IL-15 treatment in animal models of bacterial infection. Hiromatsu and associates demonstrated that mice treated with IL-15 immediately after injection of live E. coli had improved survival and reduced apoptosis in the peritoneal cavity, liver, spleen, and lung (10). Although the study by Hiromatsu et al. provides important data, the injection of live E. coli is not considered to be a clinically relevant animal model.
of sepsis (41). There is also an earlier report examining the role of IL-15 in the more widely accepted CLP model of sepsis using knockout mice. In that study Orinska and colleagues found that IL-15 null mice had improved survival in the CLP model of sepsis, and they attributed this improvement to an inhibitory role of intracellular IL-15 in mast cell cytokine maturation (21). The confounding nature of the findings between Orinska et al. and Hiromatsu et al. motivated the current study.

The current results are consistent with the report from Hiromatsu et al., suggesting that there is a predominant immunosupportive and immunostimulatory effect of therapeutically administered IL-15. In contrast, Orinska and associates showed a key role for mast cell-specific intracellular IL-15 (21). Their work established that mast cell-specific IL-15, that is, IL-15 that was confined intracellularly within the mast cells, inhibited mast cell chymase activity. Mast cell chymase activity is essential for mast cell antibacterial effects by regulating activation of several biological mediators (especially chemokines and cytokines) that assist in host defenses. Deletion of IL-15 resulted in enhanced mast cell chymase activity, increased chemokine/cytokine processing, and more efficient attraction of polymorphonuclear cells into the peritoneal cavity after CLP. In the current study, there were no differences in the recruitment of cells to the peritoneum in septic mice between IL-15 and vehicle treatment, suggesting that exogenous IL-15 does not alter mast cell-mediated recruitment of myeloid cells to the site of infection (Supplemental Fig. 3).

There is no clear explanation for the differences in the findings of Orinska et al. versus the findings in the current study. One possibility is that Orinska et al. used IL-15 null mice and it is known that the host immune system compensates for genetic deletion of various key components. Thus, the IL-15 null mice may have had other unknown compensatory mechanisms that were operative in sepsis. To further address this issue, we conducted studies testing an anti–IL-15 Ab in sepsis. Mice treated with anti–IL-15 Ab had a significantly worsened mortality compared with septic mice that did not receive the Ab (data not shown).

In conclusion, IL-15 had broad antiapoptotic effects and protected C8D8 T, NK, DC, and intestinal epithelial cells from sepsis-induced apoptosis and augmented IFN-γ production in septic animals. These effects were associated with improved survival in two widely used....

FIGURE 7. IL-15 reverses the sepsis-induced defect in IFN-γ production. Sham- or CLP-operated mice were treated with IL-15 or with the saline diluent immediately after surgery. Mice were sacrificed 24 h later and harvested splenocytes (2 × 10^5/well) were stimulated with soluble anti-CD3/anti-CD28 Abs for 24 h; 4 h before the cells were harvested, the Golgi inhibitor brefeldin A was added to the media. Cells were then evaluated by flow cytometry for IFN-γ expression. A, A typical FACs analysis of INF-γ in NK cells showing the effect of IL-15 to increase the number of NK cells in CLP mice. B, From a separate cohort of animals, splenocytes were processed in the manner described with the exception of the addition of brefeldin A. C, IFN-γ secreted into the supernatant after 24 h stimulation was measured by ELISA. Both surgery and treatment had significant, independent effects on the amount of IFN-γ present in the culture supernatant (two-way ANOVA, p < 0.05). Data expressed as mean ± SEM; n = 3–5 mice in each group; *p < 0.05 after two-way ANOVA with a Bonferroni post hoc test.

FIGURE 8. IL-15 improves survival in CLP and P. aeruginosa pneumonia. A, CLP was performed, and mice received formulated IL-15 (composed of IL-15 plus its receptor α subunit; see Materials and Methods), the IL-15α subunit alone, or saline diluted 30 min after surgery. Additional doses of IL-15, IL-15α subunit, or saline were administered at 24 and 48 h after the original surgery. Survival was recorded for 7 d. Mice receiving IL-15 had a significantly improved survival compared with mice treated with the IL-15α subunit or saline (n = 18 mice per group; p = 0.016). B, Delayed administration (6 h postsurgery and then once daily at 24 and 48 h postsurgery) of IL-15 or vehicle was also examined and survival was recorded for 7 d. Mice receiving IL-15 had significantly improved survival compared with vehicle-treated animals (n = 23 per group; p = 0.011). C, IL-15 was finally examined in a mouse model of P. aeruginosa pneumonia. After isoflurane anesthesia, the trachea was surgically exposed and 30 μl 0.5 McFarland of P. aeruginosa (ATCC 27853) suspension was slowly injected intratracheally and observed to be completely aspirated on inhalation. Thirty minutes, 24, and 48 h later, mice received IL-15 or saline diluted and survival was recorded for 7 d. Mice treated with IL-15 had a significant improvement in survival compared with saline-treated mice (n = 23 mice per group; p = 0.021).
models of sepsis. These attributes make IL-15 an attractive target for further development as a potential therapy for sepsis.

Disclosures

The authors have no financial conflicts of interests.

References


Supplemental Figure Legends

**Supplemental Figure 1.** *IL-15 protects CD8 T-cells from apoptosis.* Representative FACS data illustrating simultaneous surface marker and TUNEL staining in disaggregated splenocytes. Data from these studies were aggregated to populate Figure 2 in the manuscript.

**Supplemental Figure 2.** *IL-15 induced activation of cells from sham but not CLP operated mice.* Sham or septic mice were treated with IL-15 at 30 minutes, 24hrs and 48hrs after surgery. Spleens were harvested 96 hrs later and lymphocytes stained for CD4 or CD8 and CD69 and CD25, cell activation markers. IL-15 caused an increase in CD4 and CD8 T cell activation (both singly and doubly positive CD25 and CD69) in sham operated mice. Sepsis caused increased singly and doubly positive CD4 T cells but not in CD8 T cells. There was no effect of IL-15 to further activate CD4 T cells in CLP operated mice. * represents a statistically significant difference (*p*<0.05) compared to CLP. Brackets represent a statistical difference between the paired comparison. N = 3-5 mice per group for sham and CLP.

**Supplemental Figure 3.** *IL-15 does not alter total cell count, neutrophils, or macrophages in peritoneal fluid in sepsis.* Mice received IL-15 30 minutes after sham or CLP surgery and 24 hrs later the peritoneal cavity for lavaged with 10 ml of warmed saline. The peritoneal fluid was then aspirated and cells were harvested and stained for neutrophil markers (CD11b and Gr1) and the macrophage marker F4/80. Cells were
identified by examining staining of these 3 markers for each population. Sepsis caused dramatic increases in macrophages and neutrophils but there was no difference in CLP mice treated with IL-15 versus saline diluent in total cell count, neutrophils, or macrophages in the peritoneal cavity. $P^*$ represents a statistically significant difference ($p<0.05$) compared to CLP. N=3-5 mice in each group.

**Supplemental Figure 4. IL-15 requires lymphocytes but not neutrophils for its beneficial effect.** To determine the particular immune effector cells that were required for the beneficial effect of IL-15 on survival, we examined mice depleted of neutrophils using a neutrophil depleting antibody (250 micrograms/mouse - BioXcell, Cat. # BE0075) which was administered via i.p. As shown in supplemental Fig. 4A, the beneficial effect of IL-15 on survival in sepsis was also present in mice that were depleted in neutrophils. Thus, the salutary effect of IL-15 in sepsis does not require neutrophils. In contrast, as demonstrated in supplemental Fig. 4B, IL-15 did not improve survival in Rag 1 null mice which lack mature T and B cells. Rag 1 null mice were treated with IL-15 in a manner identical to that described previously. Thus, lymphocytes are required for the beneficial effects of IL-15 in sepsis.
Supplemental Figure 1

- Sham: (4.7%)
- Sham+IL-15: (4.8%)
- CLP: (10.8%)
- CLP+IL-15: (5.0%)

TUNEL vs. CD8
Supplemental Figure 3

**Total cells in peritoneal lavage**

![Bar chart showing total cells in peritoneal lavage](chart1)

**PMN in peritoneal lavage**

![Bar chart showing PMN in peritoneal lavage](chart2)

**Monocyte/macrophages in peritoneal lavage**

![Bar chart showing monocyte/macrophages in peritoneal lavage](chart3)
Supplemental Figure 4

A  Neutrophil Depleted mice

- CLP (n=15)
- CLP+IL-15 (n=15)

Percent survival

Day

Day

B  Rag mice

- CLP (n=7)
- CLP+IL-15 (n=7)

Percent survival

Day

Day

N.S.

p=0.044