Lymphokine Activated Killer Cells Enhance IL-2 Prevention of Sepsis-Related Death in a Murine Model of Thermal Injury

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It has previously been shown by this laboratory that immunomodulation of thermally injured animals with low-dose interleukin-2 (IL-2) and indomethacin (Indo) improves survival following septic challenge. Lymphokine-activated killer (LAK) cells have been shown to be effective in certain viral infections and to act in synergy with IL-2 in the treatment of certain types of cancer. We have studied the effect of LAK cells in combination with IL-2 and Indo in a murine model of thermal injury and sepsis. Male A/J mice received a 25% scald burn injury or sham burn and were randomized into five groups: (a) sham/vehicle, (b) burn/vehicle, (c) burn/IL-2 (250 U) + Indo (5 μg), (d) burn/LAK cells (2 × 10⁶ cells), or (e) burn/LAK cells + IL-2 + Indo and were treated accordingly for 6 days following injury. LAK cells were generated by *in vitro* IL-2 treatment of syngeneic spleen cells for 72 hr and cytotoxic activity was confirmed by standard ^{51}Cr release assay using natural killer (NK)-sensitive and NK-resistant targets. In the groups receiving LAK cells they were administered on Day 1 and Day 6 postinjury. On Day 10, septic challenge by cecal ligation and puncture (CLP) or splenectomy, for *in vitro* studies, was performed. Five-day survival after CLP was 80% in the sham/vehicle group compared to 0% in the burn/vehicle group ($P < 0.01$). IL-2/Indo and LAK/IL-2/Indo improved survival to 25% ($P < 0.05$) and 57.1% ($P < 0.01$), respectively. *In vitro* lymphocyte IL-2 production in response to mitogens was increased in the LAK/IL-2/Indo group compared with the burn/vehicle group (3 U/ml vs 2 U/ml, respectively, $P < 0.05$). IL-2/Indo alone had no significant effect on these *in vitro* parameters. As shown previously, monokine (IL-1, IL-6, and TNF) secretion by adherent splenocytes in response to LPS was markedly increased following thermal injury; no change was observed with any of the therapeutic protocols. We conclude that combined LAK/IL-2/Indo therapy is an effective regime for reduction of sepsis-related mortality and that LAK cells act synergistically with IL-2 to improve *in vitro* parameters of immune function in this model. © 1993 Academic Press, Inc.

INTRODUCTION

Despite modern techniques in intensive care and newer and more powerful antibiotics, development of sepsis and subsequent mortality occurs very frequently in burn patients [1].

Many abnormalities in both specific and nonspecific mechanisms of defense against microorganisms have been described [2–8]. Of relevance, natural killer (NK) cell activity and production of interleukin-2 (IL-2), in severely burned patients, have been found to be significantly depressed [2, 3, 8, 9] while a marked elevation of the cytokines IL-1, IL-6, and TNF has been found [10].

In a murine model of burn injury and sepsis, immunomodulation has proven beneficial in reducing mortality by using different schemes of therapy [10, 11]. Mice treated with low doses of IL-2 in combination with the prostaglandin inhibitor indomethacin (Indo) showed increased survival after septic challenge compared to the vehicle-treated mice [12]. Immunotherapy using high doses of IL-2 has also proven to be beneficial in the treatment of certain metastatic tumors in mouse [13] and humans [14, 15]. Addition of lymphokine-activated killer (LAK) cells to this modality of treatment has shown increased beneficial effects [16, 17]. This combination has also been used in the prevention and treatment of certain viral infections [18, 19]. Increased production and sensitivity of immune cells to products of the cycloxygenase pathway have been found in major burn injury [20], prompting the use of inhibitors of this pathway such as indomethacin or ibuprofen in therapeutic protocols. Finally, ibuprofen has been shown to reduce toxic effects of IL-2 and LAK cells therapy in patients with metastatic cancer [21].

This study was designed to evaluate (i) whether the addition of LAK cells to the IL-2 + indomethacin scheme of immunotherapy might further increase survival after septic challenge in the burn mouse model, (ii) whether addition of LAK cells to the IL-2 + Indo treatment modified *in vitro* parameters of immune function (lymphocyte proliferation and cytokine production) of

resting and activated lymphocytes, and (iii) whether there is a correlation between (i) and (ii).

MATERIALS AND METHODS

Burn Model

Male A/J mice, 7 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME). They were caged in groups of five and given standard mouse chow and water *ad libitum*. After acclimatization for 1 week, mice were randomized into burn and sham burn groups and entered into the following protocol.

All animal procedures were reviewed and approved by the Harvard Standing Committee for the Protection of Research Animals under guidelines of the U.S. Public Health Service, National Institutes of Health.

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital in a dose of 1.25 mg/g administered in a total of 0.25 ml of sterile 0.9% saline solution. This procedure was followed by shaving of the dorsum of the animal. Thermal injury was a scald burn using a mold partially submerged in water at 90°C for 9 sec. This results in a 25% body surface area, full-thickness burn, confirmed histologically. Sham-burn groups were anesthetized, shaved, and submerged in water at room temperature. All mice were carefully dried and resuscitated immediately with 1 ml of 0.9% saline solution given intraperitoneally.

The mortality rate was less than 20% after the burn injury.

Septic Challenge

Ten days after burn, thermally injured or sham-injured mice were again anesthetized following the same protocol as above. Celiotomy was performed after shaving the abdomen and the cecum was localized and mobilized. After mobilizing the cecum, its base was ligated with 3-O silk sutures and then perforated twice with a 27-gauge needle (cecal ligation and puncture or CLP). A small amount of feces was expressed and the cecum returned to the peritoneal cavity. The wounds were closed with 6-O nylon suture in a continuous fashion. Animals were resuscitated with 1 ml of 0.9% saline solution, subcutaneously.

Induction of LAK Cells

Leukocytes were harvested from spleens removed from normal 8-week-old male A/J mice. Spleens were teased apart with sterile forceps and washed in RPMI 1640 three times. Cells were suspended in complete medium (CM), which consisted of RPMI 1640 medium with 2 mM L-glutamine, 5 × 10^{-5} M 2-mercaptoethanol, 1% antibiotic antimycotic (penicillin, 5000 units; streptomycin, 5000 μg, amphotericin, 2.5 μg/ml), 10 mM Hepes, and 5% heat-inactivated (56°C, 30 min) fetal calf serum

(FCS). All reagents for cell washing and culture were obtained from Grand Island Biological Co. (Grand Island, NY). Cells were adjusted to a concentration of 2 × 10^6 viable cells/ml. Cell viability was assessed using trypan blue dye exclusion. Cells were incubated in a volume of no more than 15 ml and exposed to recombinant human IL-2 (a gift from Amgen) at 1000 units/ml for 72 hr in 50-mm^2 plastic tissue culture flasks at 37°C in a 5% CO_2-enriched atmosphere. Cells were harvested from flasks and washed three times in Hanks' balanced salt solution (HBSS), and viable cells counted and adjusted to 4 × 10^6 cells/ml in HBSS, before intraperitoneal infusion.

Assessment of LAK Cell Activity

Assessment of LAK cell activity was performed in each of the samples infused. The rIL-2-activated splenocytes were tested for *in vitro* cytotoxicity against the cell lines YAC-1 (NK sensitive) and EL-4 (NK resistant) (American Type Culture Collection, Rockville, MD). Target cells were labeled with chromium 51 (200 μCi; Dupont/NEN Research, Boston, MA) in a 37°C water bath for 60 min. The cells were washed with CM and distributed into round-bottom plates (5 × 10^3 cells/well in a volume of 50 μl). Total radioactivity was determined by adding 100 μl of 0.1 M HCl to target cells. Background radioactivity was established by adding 100 μl of CM. Effectors were added in a volume of 100 μl in effector to target ratios of 40:1, 20:1, 10:1, 5:1, 2.5:1, or 1.25:1 and incubated at 37°C. After 4 hr, the supernatants were collected (Skatron Inc., Sterling, VA) and counted in a gamma counter (Gamma Track 1191; TM Analytic, Elk Grove Village, IL). Results were converted to percentage specific release = (^{51}Cr test − ^{51}Cr background/^{51}Cr total − ^{51}Cr background), ^{51}Cr being measured in cpm.

T Cell Mitogen Responses

Splenocytes were harvested by gentle teasing with two forceps and suspended in RPMI with additives as above but without FCS. Cell suspensions were washed three times in this medium, centrifuging at 1500 rpm for 10 min and finally suspending in CM. Mononuclear cells were counted using Turk's solution, adjusted to 2 × 10^6 cells/ml and cultured in microtiter plates at 2 × 10^5 cells per well in 200 μl of CM, with or without phytohemagglutinin (PHA; Sigma Chemical Co. St. Louis, MO) at 5 μg/ml final well concentration. Assessment of cell viability was made using trypan blue and was always >95%. The plates were incubated at 37°C in 5% CO_2 for 48 hr. At 48 hr the plates were pulsed with 1 μCi/well of tritiated thymidine ([^{3}H]Tdr) (New England Nuclear, Boston, MA), and following a further 18 hr of incubation, the plates were frozen. Cells were harvested in a multiautomated sample harvester (Cambridge Technology, Cambridge, MA). Incorporation of [^{3}H]Tdr was

measured in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD).

Production and Assay for Cytokines

Measurements of different cytokines were performed on supernatants harvested from resting lymphocyte cultures and activated lymphocyte cultures after 48 hr incubation under optimal conditions.

IL-2 was generated by culturing splenocytes from mice at 1×10^5 cells per well in a 200-μl volume for 48 hr in CM with or without 2.5 μg purified concavalin A (Con A; Sigma Chemical Co., St. Louis, MO)/well. IL-1, IL-6, and TNF-α were generated by adherent splenocytes obtained from 200 μL of splenocytes at 5×10^6 per ml of medium when allowed to adhere to each well of flat-bottom plastic microtiter plates for 1 hr at 37°C, 5% CO_2, and 95% air. Nonadherent cells were removed by washing the plate three times in RPMI 1640 medium containing 1% antibiotics, L-glutamine, and 10 mM Hepes buffer without FCS and the adherent cells were cultured for 48 hr in 200 μl of the same medium in the presence of 0.2 μg per well lipopolysaccharide (LPS; from *Escherichia coli* 055:B5) or with no additions. The supernatants were removed at 48 hr and similar wells pooled and frozen at −70°C until tested.

Bioassay of IL-2. For measurement of biological activity of the IL-2, the supernatants were diluted from 1:2 to 1:128 in complete medium in a 100-μl volume and incubated for 1 hr at 37°C to ensure proper pH. The CTLL-2 cells were washed free of T-cell growth factor three times, diluted to 5×10^4/ml, and 100 μl of CTLL-2 cells added to each well. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye reduction method was used to determine proliferation as explained below. Units of IL-2 production were determined by comparison to a standard normal recombinant IL-2 (gift from Amgen) using probit analysis according to the method of Gillis *et al.* [22] with a computer program kindly provided by Brian Davis (Immunex Corporation, Seattle, WA).

IL-1, IL-6, and IL-2 were measured by bioassay and TNF-α by ELISA method.

IL-1 bioassay. This assay uses the cell line NOB-1 obtained from Dr. A. Gearing (London, UK) and the IL-2-dependent cell line CTLL-2. The NOB-1 cell lacks thymidine kinase; therefore, only proliferation of the CTLL-2 cell is measured in response to IL-2 produced by the NOB-1 cells upon stimulation by the IL-1 present in the supernatants tested, when [³H]Tdr is added after 20 hr of culture. Measurement of IL-2 was made as outlined above. This assay has been reconfirmed in our laboratory not to respond to TNF-α or -β, IL-2, -3, -4, -5 or -6, GM–CSF, or interferon-α and -γ.

IL-6 bioassay. This assay uses the IL-6-dependent cell line B9, obtained from Genetics Institute (Cambridge, MA) with the kind permission of Dr. Lucien Aar-

den. Proliferation of this cell line in the presence of IL-6 was determined by the MTT dye reduction method [23]. For this 25 μl of MTT solution (5 mg/ml) was added to each well, and the plates were incubated for 4 hr. Formazan crystals were dissolved in acidified 10% SDS and color was read on a multiscanning ELISA reader (Molecular devices, Mountain View, CA). IL-6 in samples was determined from the curve obtained by a standard of recombinant IL-6 and compared with a standard monocyte supernatant obtained from normal human monocytes stimulated with LPS and known to contain IL-1, TNF, and IL-6.

TNF-α ELISA. In this assay a hamster monoclonal antimurine TNF-α antibody (Genzyme), at a previously determined appropriate dilution in 0.1 M carbonate buffer, pH 9.6, was added to wells of ELISA plates (Costar, Cambridge, MA) incubated overnight at 4°C and washed in washing solution, phosphate-buffered saline (PBS) plus 0.05% Tween 20. Active sites on the plates were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 hr and washed as above. The samples and standards to be tested were added in 50-μl volumes. The plates were incubated 60 min at room temperature and washed three times and 50 μl rabbit antimouse TNF-α antibody appropriately diluted in PBS, 0.1% BSA, was added per well. The plates were incubated 1 hr and washed and appropriately diluted alkaline phosphatase conjugated goat antirabbit immunoglobulin antibody in washing solution was added to appropriate wells. The plates were again incubated for 60 min at room temperature and washed as above. The substrate, paranitrophenylphosphate disodium, 1 mg/ml in substrate diluent (pH 9.8, 0.05 M sodium carbonate buffer with 1 mM $MgCl_2$), was added, 100 μl/well, and incubated at room temperature for 1 hr. The results were read at 405 nm in an ELISA reader (UVmax; Molecular Devices, Mountain View, CA). Standards of TNF-α and negative controls for all antibodies and antigens were used in all assays. All assays have been tested for sensitivity and specificity using recombinant TNF-α and mixtures of cytokines to control for synergy. Data were obtained from standard curves generated by the ELISA reader software "Softmax" (Molecular Devices), using four-parameter curves.

Treatment Protocol

Survival study. This group consisted of 60 burned mice and 15 sham-burned mice. The burned mice were further allocated to one of four treatment groups of 15 mice each. Treatment groups received daily ip injection (beginning 1 day after burn injury) of: (i) vehicle (HBSS), (ii) 2×10^6 LAK cells on Day 1 and Day 6, (iii) 250 units rhIL-2 + 5 μg indomethacin (Merck and Co., West Point, PA)/mouse, or (iv) 250 units rhIL-2 + 5 μg Indo + 2×10^6 LAK cells on Day 1 and Day 6. After 6 days of therapy the mice were allowed to rest for 3 days
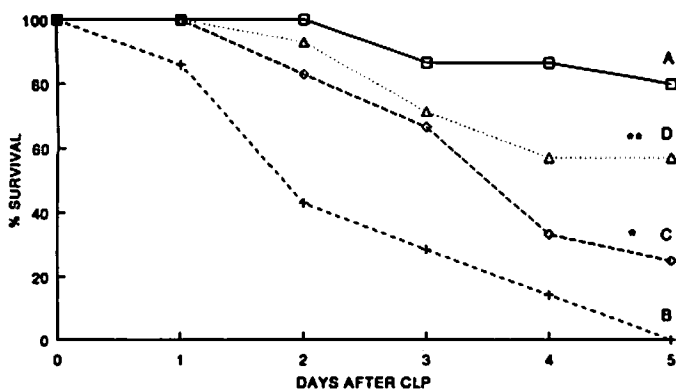
FIG. 1. Five-day survival curve after CLP. Group A, sham/vehicle (80%); Group B, burn/vehicle (0%); Group C, burn/IL-2/Indo (25%); and Group D, burn/LAK/IL-2/Indo (57.1%). *$P < 0.05$, **$P < 0.01$. No statistical difference was found between survival curves of A when compared to D.

and then subjected to septic challenge by CLP as above. Sham-burned mice received treatment with vehicle for 6 days and were subjected to CLP.

In vitro study. A second experimental group was used to determine if the effects on mortality noted in the first part of the experiment were reflected by changes in the splenocyte proliferative response to the mitogen PHA, splenocyte IL-2 production when challenged with Con A, and adherent cell production of IL-1, IL-6, and TNF-α when challenged with *E. coli* LPS (055:B5). This group consisted of 21 burned mice allocated to one of three equal-size treatment groups. One group received ip injections of HBSS. The second group received 250 units rhIL-2 + 5 μg indomethacin/mouse/day and the third group received 250 units rhIL-2 + 5 μg indomethacin + 2 × 10⁶ LAK cells on Day 1 and Day 6 after injury. Seven sham-burned mice received ip injections of HBSS. All mice were treated for 6 days and their spleens harvested on Day 10 postburn. All injections were given ip and were 1 ml in volume.

Statistical Analysis

The Wilcoxon–Gehan test was used to study the survival data. Mann–Whitney test was used for results on cytokine production and proliferation. All results were considered to be significant if $P < 0.05$.

RESULTS

LAK Cell Activity

Cytotoxicity displayed by LAK cells generated following the previously described protocol was 78.38 (±4.52)% killing at an E:T ratio of 40:1 for NK-sensitive target YAC-1 and 22.5% killing at an E:T ratio of 40:1 for NK-resistant target EL-4. This cytotoxicity profile was similar to that reported elsewhere [24].

Survival after Burn and Sepsis (Fig. 1)

Five-day survival in the sham-burned group after CLP was 80%. In the same period of time, animals that were subjected to burn injury and treatment with vehicle had a survival of 0%. Therapy with low-dose rhIL-2 (250 U) plus indomethacin (5 μg/mouse/day) increased the survival to 25% ($P < 0.05$). Therapy with LAK cells alone did not increase survival significantly. However, the combination of LAK cells plus low-dose rhIL-2 plus indomethacin increased survival to 57.1% ($P < 0.01$).

Lymphocyte Proliferation (Fig. 2)

Proliferation of lymphocytes in complete medium without the presence of mitogens showed no significant difference between sham, burn/vehicle, and burn treated with IL-2/Indo groups (2444.7 ± 201.1, 4137 ± 1563, 2908 ± 310, respectively). Lymphocytes from the IL-2/Indo/LAK-treated group showed a significantly increased proliferation compared to all other groups (13,110.3 ± 157, $P < 0.01$). Proliferation in the presence of PHA showed a significant decrease in proliferation in the burn/vehicle and burn treated with IL-2/Indo alone groups when compared with the sham group (32,815 ± 4020 and 31,483 ± 3619.9 vs 44,647 ± 3056.4, respectively, $P < 0.05$). No significant difference was observed between the sham group and the burn treated with IL-2/Indo/LAK cells group (44,647.6 ± 3056.4 vs 39,823.3 ± 4250, respectively, $P > 0.05$).

Cytokine Production (Table 1)

IL-2. IL-2 production in response to Con A was increased in the LAK/IL-2/Indo group compared with the burn/vehicle group (3.0 ± 0.03 U/ml vs 2.0 ± 0.01 U/ml, respectively, $P < 0.05$). IL-2/Indo alone had no significant effect. As observed in proliferation experiments, IL-2 production of nonactivated lymphocytes was also significantly increased in the LAK/IL-2/Indo group compared to any of the other groups (2.9 ± 0.07 vs 0, $P < 0.01$).

IL-1, TNF-α, IL-6. As shown in Table 1, monokine production was significantly elevated in all burn groups compared to the sham group but no significant difference was observed between the burn/vehicle group and burn/IL-2/Indo- or burn/IL-2/Indo/LAK-treated groups ($P > 0.05$).

DISCUSSION

IL-2 is an NK cell activator, and mononuclear cells cultured in IL-2 will divide and generate cells that are capable of lysing autologous or syngeneic tumor cells (LAK cells). LAK cells are also capable of producing a wide spectrum of cytokines (e.g., TNF-α, INF-γ, IL-1, IL-2, and GM-CSF). Although there is still some controversy surrounding the phenotype of the cells that me-
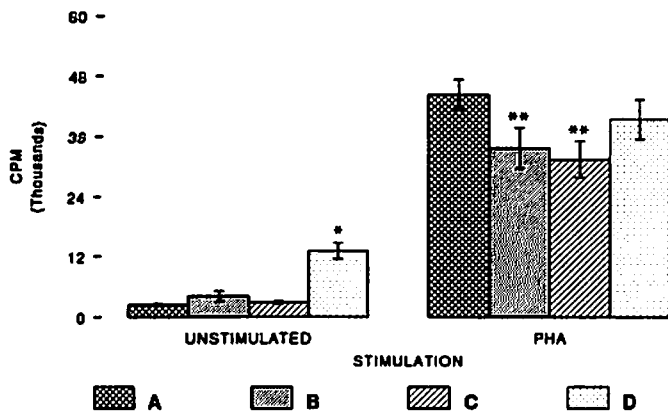
FIG. 2. Proliferation of 2 × 10⁶ splenocytes/ml, unstimulated and stimulated with PHA, after incubation for 48 hr under optimal conditions. A, sham/vehicle; B, burn/vehicle; C, burn/IL-2/Indo; D, burn/LAK/IL-2/Indo. Proliferation of unstimulated cells from D was significantly increased compared to all the other groups. Proliferation of stimulated B and C was significantly depressed compared to A. No significant difference was found between A and D in the stimulated group.

a chemoattractant is released from NK cells (NK-LCF) that can stimulate the migration of neutrophils, monocytes and LGL [30]. Some evidence suggests that NK cells can kill *Shigella* and *Salmonella* both directly and by an ADCC mechanism. A subpopulation of NK cells can both phagocytose gram-positive bacteria and release IL-1 following contact with certain bacterial species. Finally, evidence exists for the release of an NK macrophage-activating factor that can stimulate monocyte oxidative metabolism and intracellular killing of *Staphylococcus aureus* by macrophages [31].

In these experiments we were able to reproduce the previous findings reported by this laboratory, that immunotherapy with low-dose IL-2 plus indomethacin significantly reduces mortality after septic challenge in burned mice. We also were able to demonstrate that addition of LAK cells to IL-2 plus Indo immunotherapy improved survival to almost two times that of IL-2 plus Indo alone. This additional beneficial effect was correlated with improvement of *in vitro* parameters of immune function as shown by a significant increase in proliferation and IL-2 production by unstimulated and stimulated cells *in vitro*. Taken together, these observations argue that immunomodulation of the thermally injured mice using IL-2/LAK cells/Indo improves immune response to septic challenge and that the benefit of the addition of LAK cells may be related to their ability to home to sites of inflammation and act directly as antibacterial effectors or act indirectly as immunomodulators by producing many different cytokines that will eventually improve local or systemic immune responses. It also suggests that by giving LAK cells we are potentially replacing a population of cells known to be depressed as a consequence of thermal injuries, that is, the NK cell. Finally, another possible explanation could be that the mechanisms of generation of LAK cells modify their antigenic phenotypes and activate, upon transfer, a nonspecific immune reaction potentiated by release of different cytokines that leads eventually to a better capacity to respond to new antigenic challenge.

diate LAK cell activity, recent results suggest that the LAK cell is a natural effector cell likely to be predominantly of the NK lineage [24, 25].

NK cells possess the ability to kill certain tumor cells or normal cells infected by virus. Additionally recent evidence indicates that NK cells may also play an important role in host defense against bacterial infection and modulation of the inflammatory response [26]. NK cells appear to have many of the properties of an inflammatory cell. That is, they are highly motile and can respond by increasing their migratory activity in response to bacterial products, complement components, and lymphokines [27–29]. *In vivo*, it is a widely distributed cell that can home to a specific organ site in response to bacterial and viral infection and following stimulation by noninfectious bacterial extracts. The NK cell can synthesize cytokines (IL-1, TNF-α) that are chemoattractants, and

TABLE 1

Cytokine Production on Unstimulated and Stimulated Splenocytes, from the Different Groups, Harvested on Day 10 after Thermal Injury

	IL-2 (U/ml)		IL-1 (U/ml)		TNF-α (pg/ml)		IL-6 (U/ml)	
	Unstim.	Stim.	Unstim.	Stim.	Unstim.	Stim.	Unstim.	Stim.
Group A	0	2.3 ± 0.34	0.05 ± 0.03	0.32 ± 0.045	3.75 ± 3.47	292.1 ± 46.5	0	1.02 ± 0.23
Group B	0	2.0 ± 0.15	0.14 ± 0.09	1.32 ± 0.15†	40.49 ± 29.3†	611.6 ± 116.7†	0.26 ± 0.24†	4.80 ± 0.91†
Group C	0	2.6 ± 0.3	0.07 ± 0.02	1.06 ± 0.22†	10.4 ± 6.4	499.6 ± 150†	0.02 ± 0.01	2.72 ± 0.71†
Group D	3 ± 0*	3 ± 0.3¶	0.52 ± 0.17†	1.94 ± 0.81†	57.33 ± 31.3†	586.2 ± 128.1†	0.21 ± 0.09†	3.01 ± 0.88†

Note. Measurements were made on supernatants of 2 × 10⁶ cells/ml incubated for 48 hr under optimal conditions. Group A is sham/vehicle, Group B is burn/vehicle, Group C is burn/IL-2 + Indo, and Group D is burn/LAK + IL-2 + Indo. All typographic symbols are equivalent to $P < 0.05$. * D compared with B; ¶ D compared to all other groups; † Groups compared to A.

ACKNOWLEDGMENTS

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