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Interleukin-2 Receptor Expression and Function Following Thermal Injury

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Background/Objective: Serious traumatic or thermal injury is associated with depression of cellular immunity, including the failure of T-lymphocyte proliferation in response to stimulation that depends both on production of interleukin-2 (IL-2) and on expression of functional IL-2 receptors (IL-2R). While decreased IL-2 production following thermal injury is undisputed, the status of IL-2R expression and function in this setting is controversial; therefore, we sought to investigate this issue.

Design: A total of 220 male A/J mice (n=22 per group) were subjected to a 20% scald burn injury or sham burn, killed 4, 7, 10, 14, or 21 days later, and splenocytes harvested. In vitro parameters of both IL-2R expression and function were measured.

Results: On day 7, splenic lymphocyte proliferation and IL-2 production in response to mitogenic stimulation were

both suppressed following burn injury to 50% and 60% of controls, respectively. Northern blot analysis revealed normal IL-2R p55 messenger RNA expression in response to mitogenic stimulation on days 7, 10, and 14 in thermally injured animals. Phenotypic IL-2R p55 expression in concanavalin A-stimulated CD3⁺ cells was unchanged following burn injury. Binding of fluorescein-labeled IL-2 to cell membranes was increased in burned animals at days 10 and 14. The addition of IL-2 to cultures of spleen cells from burned mice consistently restored the mitogenic response to that of the controls.

Conclusions: Thermal injury in this model does not result in either quantitative or functional suppression of IL-2R. Suppression of T-cell activation and proliferation, seen following thermal injury, appears primarily related to abnormal IL-2 production.

(Arch Surg. 1995;130:165-170)

SEVERE TRAUMATIC or thermal injury results in the depression of cellular immunity, including failure of T-lymphocyte (T-cell) proliferation in response to mitogenic or antigenic stimulation.^{1,2} These immunologic changes predispose to the high incidence of sepsis, with resultant morbidity and mortality seen after this type of injury. Interleukin-2 (IL-2), a product of T cells, is the major growth factor for T lymphocytes, and its action on T cells is mediated through binding with IL-2 receptor (IL-2R) proteins on the cell surface, with resultant cellular proliferation. This autocrine mechanism is of critical importance to the cell-mediated immune response and is obviously dependent both on expression of functional IL-2R on the cell surface and on normal production and secretion of the IL-2 protein.

Structurally, the IL-2 binding complex consists of a number of polypeptide

chains, the principal two of which are a 55-kd, Tac, or IL-2R α chain and the 70- to 75-kd, IL-2R β chain. The β -chain is of intermediate affinity and is expressed on resting cells.³ The α -chain, on the other hand, is not expressed in the resting state, is of low affinity when expressed alone, but, together with IL-2R β , forms a high-affinity IL-2R.⁴ Expression of high-affinity IL-2R, and thus cell proliferation, is therefore significantly regulated by IL-2R α gene expression.

One of the primary immunologic defects in severely burned patients is decreased production of IL-2.⁵ While decreased IL-2 production following thermal injury is undisputed, the status of IL-2R expression and activity in this setting is controversial. Previous studies have vari-

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METHODS

ANIMAL MODEL

All animal studies were performed with the approval and under the guidance of Harvard Medical School's Standing Committee on Animal Research and the National Institutes of Health. As previously described,¹ male A/J mice (Jackson Laboratories, Bar Harbor, Me), 7 to 8 weeks old, were caged in groups of five in a controlled environment with water and mouse food allowed ad libitum. Animals were randomized into groups, anesthetized with pentobarbital sodium (60 mg/kg), and their dorsa shaved. The animals then were placed in a plastic template, and a 20% body surface area burn injury or sham burn was produced by immersion for 9 seconds in water at 90°C or at room temperature, respectively. The former produced a localized, full-thickness burn.¹ Animals were resuscitated with 1 ml. of 0.9% saline solution, recaged, killed 4, 7, 10, 14 or 21 days later, and their spleens harvested. Experiments were performed on two occasions, with 10 groups each time, each consisting of 10 or more mice.

ORGAN HARVESTING AND CELL CULTURE

Spleens were teased apart, and single-cell suspensions were prepared for culture as previously described.¹ Cells were cultured on 96-well microtiter plates with 200 μ L per well of a solution containing 1×10^6 cells/mL at 37°C in 5% carbon dioxide and stimulated with either concanavalin A (ConA) (Sigma Chemical Co, St Louis, Mo) alone at a concentration of 2.5 μ g/ml. or in combination with human recombinant IL-2 at a concentration of 100 U/mL.

MEASUREMENT OF PROLIFERATIVE RESPONSE

Following 30 hours of stimulation, 0.037 mBq of tritiated thymidine (New England Nuclear, Boston, Mass) was added to each well and the cell cultures were rapidly frozen 18 hours later and subsequently harvested as previously described.¹ Mitogen responses were calculated by subtracting tritiated thymidine incorporation in unstimulated cell cultures from that in mitogen-stimulated cultures.

IL-2 BIOASSAY

Splenocytes from individual mice were cultured and stimulated with ConA as described above. At 48 hours, supernatants were harvested and frozen at -20°C for later assay. Supernatants were assayed for IL-2 using the IL-2-dependent cell line CTLL-2 as previously described.^{1,8} The IL-2 production was calculated from standard curves using probit analysis and expressed as units per milliliter.

IL-2R p55 PROTEIN EXPRESSION IN CD3⁺ CELLS

Splenocytes were harvested and were cultured in the presence of ConA as described above for 48 hours, after which viability was assessed with trypan blue stain. One million viable cells were incubated for 45 minutes at 4°C in the presence of fluorescein-conjugated monoclonal murine anti-CD3 antibody (Boehringer Mannheim, Indianapolis, Ind) and phycoerythrin-conjugated anti-IL-2R p55 chain antibody (Boehringer Mannheim) and then fixed in 1% paraformaldehyde in phosphate-buffered saline. Samples were processed using an Epics C flow cytometer (Coulter Electronics Inc, Hialeah, Fla) with a single 488-nm argon laser, counting 5000 cells per sample. Forward and 90° two-color analysis was used and the data were processed by com-

ously reported reduced⁶ or increased⁷ IL-2R p55 expression in severely burned patients. These studies looked only at cell surface expression of the p55 protein, without examining other parameters of IL-2R expression and function. While methodologic differences may contribute in part to the apparent discrepancies between these studies,⁷ it is apparent that, as yet, there is no clear answer as to the status of IL-2R in the setting of thermal injury.

The following study was designed to resolve this controversy through detailed investigation of both IL-2R expression and function in an established animal model of immunosuppression following thermal injury.

RESULTS

T-LYMPHOCYTE PROLIFERATION AND IL-2 PRODUCTION

The response of lymphocytes to mitogenic stimulation was clearly reduced in the burned animals compared with the sham burned animals on all days following injury. Proliferation in response to ConA stimulation in burn animals was consistently suppressed in com-

parison with control animals, maximally by 50% and 37% ($P < .05$, both values), respectively, on days 4 and 7 (**Table**). The production of IL-2 in the lymphocytes of ConA-stimulated burned mice was similarly suppressed, maximally by 40% and 36% ($P < .05$, both values) on days 7 and 10, respectively (**Table**).

IL-2R EXPRESSION

Cell surface IL-2R p55 chain expression in mitogen-stimulated CD3⁺ cells was not changed in the burn animals compared with the control animals at any day measured (**Figure 1**). The percentage of CD3⁺ cells expressing IL-2R p55 varied between 50% and 80%, and at any given time the results in burn and control groups were very similar, varying by no more than 9% on day 7.

IL-2R mRNA EXPRESSION

The expression of IL-2R p55 chain mRNA in response to *in vitro* stimulation, as measured by Northern blot analysis, showed no consistent change in thermally injured animals compared with controls when measured on days 7, 10, or 14 (**Figure 2**). Quantification by laser

puter using Howard Shapiro 4cyte software (Howard Shapiro, Cambridge, Mass). Nonspecific fluorescence and background staining were accounted for using appropriate controls including normal murine IgG and staining of unstimulated cells.

CELLULAR IL-2 BINDING

Splenocytes were stimulated with ConA (2.5 µg/mL) as above, after which 10⁶ viable cells were incubated for 1 hour at 4°C with biotinylated recombinant human IL-2 (Boehringer Mannheim), then washed and incubated for 45 minutes in the presence of an avidin-fluorescein complex. Samples again were analyzed by flow cytometry as above. Nonspecific fluorescence and background staining were accounted for using an irrelevant first-step antibody followed by the same second-step reagent.

RNA ISOLATION AND NORTHERN BLOT ANALYSIS

Northern blot analysis for cytokine messenger RNA (mRNA) expression was performed on days 7, 10, and 14 following injury. After 8 hours of in vitro stimulation with ConA, cells from animals in each group were pooled, lysed in guanidinium isothiocyanate and stored at -70°C. Total cellular RNA from 1×10⁶ cells per sample was isolated using phenol-chloroform extraction. RNA samples were subjected to electrophoresis on 1% agarose-formaldehyde gels and transferred to nylon membranes by capillary action.⁹ RNA was fixed to membranes by UV cross-linking.

COMPLEMENTARY (cDNA) PROBES

The cDNA probes were produced from plasmids for murine IL-2R p55¹⁰ and chicken β-actin and radiolabeled with

α-³²P-deoxycytidine 5'-triphosphate (New England Nuclear, Boston, Mass) using random sequence hexanucleotide priming.¹¹ Specific activity ranged from 5×10⁸ to 5×10⁹ cpm/µg. Probes were denatured using sodium hydroxide, and fish sperm DNA was added to decrease nonspecific binding.

HYBRIDIZATION

Hybridization was performed as previously described.⁸ Autoradiography was performed using Kodak XAR-5 film (Sigma) and intensifying screens at -70°C. Probes were stripped from membranes in a 1% glycerol solution at 80°C for 10 minutes and subsequently hybridized with β-actin cDNA as a control for RNA loading in each lane.

QUANTIFICATION AND STANDARDIZATION OF mRNA EXPRESSION

Autoradiographs were quantified using a laser densitometer (300 series computing densitometer, Molecular Dynamics, Sunnyvale, Calif). Signal intensity for cytokine mRNA expression was normalized using the β-actin signal and was expressed as a ratio of cytokine to β-actin mRNA expression.

STATISTICAL ANALYSIS

The results were expressed as percentage change in the burn group compared with the sham burn group. Proliferation, cytokine production, IL-2R expression, and IL-2 binding were compared between the different groups using the Mann-Whitney U test. Results were considered significant at P≤.05.

densitometry revealed changes in IL-2R p55 mRNA expression of -23%, +6%, and -12% in the burn animals on days 7, 10, and 14, respectively.

IL-2R FUNCTIONAL STUDIES

Binding of fluorescein-labeled IL-2 was unchanged in the burned animals compared with sham burned mice on days 4, 7, and 21 but was increased by 117% (P<.01) and 22% (P>.05) on days 10 and 14, respectively (**Figure 3**).

The addition of IL-2 to cultures of cells from the burned mice caused a significant increase in the proliferative response on all days following injury, indicating the presence of functional cellular IL-2R. On each day, IL-2 restored the response of the cells of burned animals to that of the control cells (**Figure 4**).

COMMENT

Abnormalities of T-lymphocyte function are central to the immune dysfunction that increases susceptibility to sepsis following thermal or traumatic injury.¹²⁻¹⁴ Our laboratory has previously focused on abnormal

Results of In Vitro Studies Expressed as Percentage Change in Burned Mice Compared With Sham Burned Mice

	Days Postburn				
	4	7	10	14	21
IL-2 production	-22	-40†	-36‡	-30†	+16
Lymphocyte proliferation	-50	-37†	-23†	-5	-12
IL-2R expression CD3 ⁺ cells	-6	+9	+1	+7	-5
Splenocyte IL-2 binding	-3	+2	+117‡	+22	-7

*IL-2 indicates interleukin-2; IL-2R, IL-2 receptor.

†P<.05, Mann-Whitney U test.

‡P<.01, Mann-Whitney U test.

lymphocyte proliferation and reduced IL-2 production as being the primary deficiencies in T-cell function in this setting.^{1,5} Expression of functional high-affinity IL-2R is also critical to the normal lymphocyte response to antigenic or mitogenic stimulation and is closely linked to expression of the IL-2Rα (p55) gene product.

Other groups have previously addressed the question of IL-2Rα expression following trauma or thermal injury. Teodorczyk-Injeyan and colleagues^{2,6} have shown

a decrease in the percentage of T lymphocytes expressing IL-2R α in patients following thermal injury. On the other hand, there is evidence to dispute depressed cellular IL-2R expression following thermal injury. Schlieter and colleagues⁷ recently reported increased IL-2R α expression in severely burned patients. A number of studies have suggested the presence of functional cellular IL-2R by demonstrating a significant proliferative response of lymphocytes from burn victims, with the addition of recombinant IL-2 to *in vitro* cultures.^{2,15} It is clear that the status of IL-2R following thermal injury has not been satisfactorily determined. The present study addressed these questions in an established and well-controlled animal model of burn-induced immunosuppression. Our results clearly demonstrate normal expression of cell surface IL-2R α in mitogen-stimulated CD3⁺ cells following thermal injury. These data are strengthened further by the demonstration of normal IL-2R mRNA levels in this setting. That burn injury

was sufficiently severe to induce immunosuppression was demonstrated by reduced lymphocyte proliferation and by reduced IL-2 production; this is consistent with our previous reports in the same animal model.¹⁸ Additionally, we demonstrated in this study that IL-2R function is normal following this degree of thermal injury. Cellular binding of IL-2 was normal early and late following injury but actually increased on days 10 and 14, possibly reflecting an increased quantity of cell surface IL-2R α as demonstrated by Schlieter and colleagues.⁷ The addition of human recombinant IL-2 to *in vitro* cell cultures restored the proliferative response of suppressed lymphocytes to normal, demonstrating the presence of functional cellular IL-2R. Together, these morphologic and functional studies provide strong evidence that suppression of IL-2R does not play a significant role in immunosuppression following moderate thermal trauma. That we can induce abnormal lymphocyte proliferation and abnormal IL-2 production without suppressing IL-2R α

Figure 2. Northern blot showing interleukin-2 receptor p55 (α -chain) (IL-2R α) messenger RNA expression in concanavalin A-stimulated splenocytes of sham burn (S) and burn (B) animals on days 7, 10, and 14 following injury. IL-2R α message (top) and β -actin control (bottom) are shown.

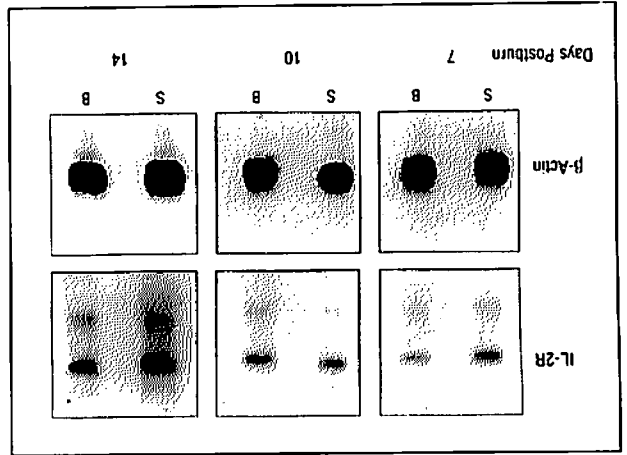


Figure 1. *In vitro* expression of interleukin-2 receptor p55 (α -chain) on the cell surface of CD3⁺ cells in response to concanavalin A stimulation of sham burn and burn animals on days 4 to 21 following injury. Percentages above bars represent the percentage change in burned mice compared with controls. Percentages below bars represent the percentage change in burned mice compared with controls. Astisks indicates $P < .01$.

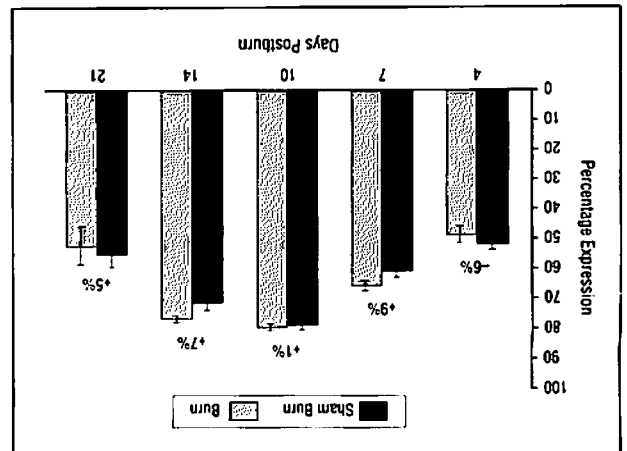


Figure 4. Effect of the addition of exogenous human recombinant interleukin-2 (IL-2) *in vitro* on the proliferation of concanavalin A-stimulated splenocytes. On each day, the addition of IL-2 restored the proliferative response in the burn mouse cells to that of the control cells.

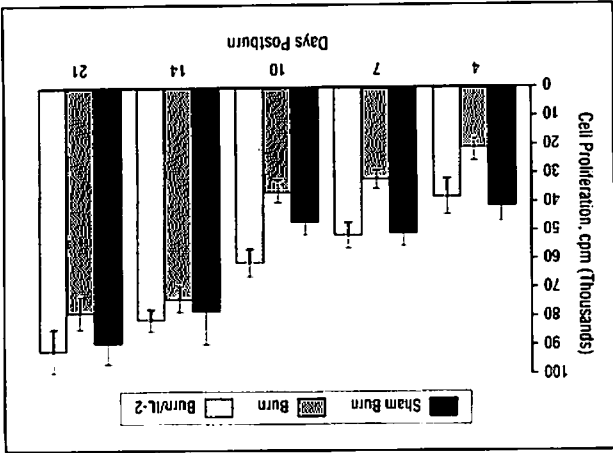
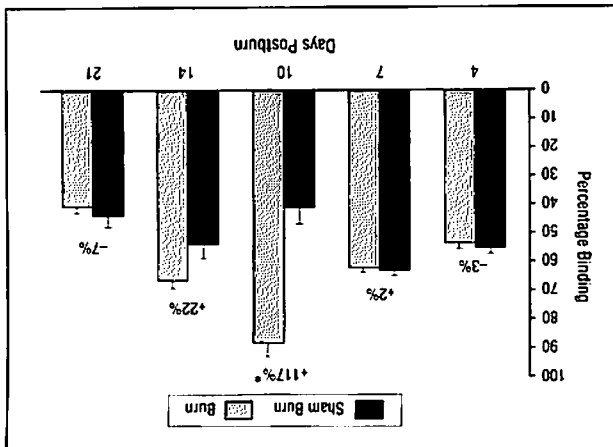


Figure 3. Binding of fluorescein-labeled interleukin-2 to concanavalin A-stimulated splenocytes of sham burn and burn animals on days 4 to 21 following injury. Percentages above bars represent the percentage change in burned mice compared with controls. Astisks indicates $P < .01$.



expression or function suggests that reduced IL-2 production, rather than an abnormality of the IL-2R, is the dominant T-cell defect in this setting.

In light of the present results, the findings of Teodorczyk-Injeyan and colleagues⁶ of reduced IL-2R expression following thermal injury are difficult to explain. The methods of measurement of the percentage of cells expressing IL-2R α used by this group are almost identical to ours. While our studies are in an animal model, and this could, theoretically, explain some differences, our previous extensive immunologic studies with this model have demonstrated a close parallel with the human situation. It is possible that the major reason for the difference in our findings lies in the extent of injury. It appears that the 20% total body surface area burn injury induced in our model is on average somewhat less severe than that in the patients reported by Teodorczyk-Injeyan et al (>35% total body surface area). This difference may well explain our different findings, especially when we consider that group's earlier report that indicated that IL-2R suppression was predominantly in the more severely burned nonsurvivors, with mild or no suppression occurring in the less severely burned survivors.² The present investigation of IL-2R is considerably more detailed than those of previous groups who have examined this receptor in the setting of thermal or traumatic injury. Earlier articles have concentrated on cellular expression of IL-2R α alone and have not examined other aspects of IL-2R expression or function. The finding of no significant reduction in IL-2R mRNA expression and of IL-2R function lends considerable additional weight to the observation of normal IL-2R α expression in this study.

Our data indicate that the production of the T-cell growth factor, IL-2, and the expression of the IL-2R are not necessarily coordinate events and can be dissociated in experimental situations. This finding is readily understandable, as the intracellular processes that regulate IL-2 production and IL-2R expression, although similar, show important differences with respect to both their inductive signals and negative feedback pathways. That IL-2R is unaffected but IL-2 production is reduced makes intuitive sense, as the signals required for IL-2R gene expression appear to be considerably less specific and less stringent than those required for IL-2 gene expression. A number of articles have explored differences in IL-2 and IL-2R induction and may provide clues as to the mechanism of dissociated IL-2 and IL-2R expression in our model. Stimulation of the T α /CD3 complex by CD3 antibodies gives rise to low-level stimulation of protein kinase C and can induce IL-2R expression, but the failure of this stimulus to induce IL-2 production suggests that higher signaling thresholds exist in the latter case.¹⁵ Short-term activation of protein kinase C is sufficient for IL-2R expression whereas either long-term activation or repetitive short-term activation is necessary for IL-2 synthesis.¹⁶ Furthermore, in contrast to IL-2 production, IL-2R expression appears to be independent of increased intracellular calcium levels.^{15,17} Therefore, alterations in the strength or duration of protein

kinase C activation or a subnormal intracellular calcium response to stimulation are possible explanations for decreased IL-2 production in the face of normal IL-2R expression following thermal injury.

Prostaglandin E₂ (PGE₂) release from macrophages is induced following thermal injury and results in suppression of T-cell function, most likely mediated through the intracellular messenger, cyclic adenosine monophosphate (cAMP). While it has been shown that increased PGE₂ product and cAMP concentration not only decrease lymphocyte proliferation and IL-2 production but also expression of high-affinity IL-2R,¹⁸⁻²¹ the effect on IL-2R α expression is controversial. Some investigators have shown down-regulation of IL-2R α by cAMP,¹⁸⁻²⁰ while others have shown normal or increased IL-2R α expression with increased cAMP.^{21,22} Exactly where these data fit in with our present experiment showing normal IL-2R α expression in the presence of elevations of PGE₂ and cAMP²³ is unclear. The jump from these in vitro experimental results to the in vivo situation is a large and uncertain one. We cannot make the assumption that because addition of cAMP analogues to in vitro cell cultures inhibits IL-2R α expression, the same applies to in vivo cellular exposure to more physiologic concentrations of PGE₂. It is quite possible that at physiologic concentrations, PGE₂ may have a strong enough inhibitory effect to reduce IL-2 production without having an appreciable effect on IL-2R expression.

The present study provides strong evidence for normal IL-2R expression and function in the presence of suppressed lymphocyte activation. Since IL-2 and IL-2R are the chief components of the T-cell proliferative response to antigenic stimulation, the suppression of T-cell proliferation that follows severe thermal injury or trauma appears primarily related to abnormal IL-2 production.

This work was supported by grant NIGMS 5R01GM35633-09 from the National Institutes of Health and grants from Ainsworth Scholarship, Cork, Ireland; Brigham Surgical Group Foundation; and the Cohen Fund, Boston, Mass.

The human recombinant IL-2 was the generous gift of Amgen, Thousand Oaks, Calif, and software for the protein analysis was generously provided by Brian Davis of Immunex Corp, Seattle, Wash.

The authors gratefully acknowledge the expert technical assistance of Kathryn Collins, Inna Saporoschetz, and Miriam Nestor. We also thank Christine Kelley for preparation of the manuscript.

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