J. Amer. Aging Assoc., Vol. 25, 3-10, 2002

# SURVIVAL AND CELL MEDIATED IMMUNITY AFTER BURN INJURY IN AGED MICE[1]

**Elizabeth J. Kovacs,[a,b,c,d] Kristy A. Grabowski,[b] Lisa A. Duffner[b], Timothy P. Plackett[b], and Meredith S. Gregory[be]**

[a]Immunology and Aging Program, [b]Department of Cell Biology Neurobiology and Anatomy, and [c]Burn and Shock Trauma Institute, [d]Department of Surgery, Loyola University Chicago, Maywood, IL.

## ABSTRACT

**The elderly are less able to survive burn injury than young healthy individuals. Regardless of age, burn victims often succumb to secondary infections rather than the primary injury. Since immune responses diminish with age, it is likely that aged individuals are predisposed to a poor outcome by virtue of their weak immune system. Elevated production of macrophage-derived mediators, including interleukin-6 (IL-6), may lead to post-injury immunosuppression in young adults. Healthy aged individuals produce high circulating levels of these mediators; therefore, the combination of the age and burn trauma could further suppress immune responses and contribute to the rapid demise of aged burn patients. Herein, the effects of age and burn trauma using a murine scald injury model were examined. After injury, aged mice are less likely to survive, are unable to mount immune responses, and produce more IL-6 when compared to young adult mice given the same size injuries. Enhancing our understanding of the mechanisms responsible for regulating cell-mediated immune responses after injury could lead to the development of therapies designed to treat aged burn patients.**

## INTRODUCTION

Aged individuals who suffer a burn trauma exhibit greater mortality than young adult burn victims (Pruitt et al, 1964; Rittenbury et al, 1965; Linn, 1980; Griffiths and Laing,1981; Deitch and Clothier, 1983; Tran et al, 1990). A moderate size burn covering 20% of the total body surface area (TBSA) has been associated with a mortality of only 20% in healthy young adult patients, while elderly patients with the same burn size have a mortality of 75% (Griffiths and Laing, 1981). While many factors may contribute to the increased lethality of moderate size burns in the elderly population, the well documented reduction in immune function associated with the natural aging process is likely to be involved in this phenomenon (Pawelec et al, 1998; Chakravarti and Abraham, 1999; Ginaldi et al, 1999).

Regardless of age, burn patients usually succumb to secondary infectious complications rather than their primary injury (Pruitt, 1990; Ramzy et al, 1999). Thus, the functional integrity of immune responses after injury is of paramount importance. Many studies have documented that, like other injuries, burn trauma leads to suppression of cell mediated immunity which is critical for resistance to infection (O'Sullivan and O'Connor, 1997; Lederer et al, 1999).

While it is clear that lymphocyte functions are impaired after burn injury in patients and in animal models (Miller and Baker, 1979; Wood et al 1984; O'Sullivan et al, 1995; Zedler et al, 1997), evidence from a number of laboratories reveals that macrophages control many of these lymphocyte functions through the production of proinflammatory cytokines and arachidonic acid metabolites (Wood et al 1987; Mandrup-Poulsen et al, 1995; Faist et al, 1996; Faunce et al, 1998a; Gregory et al, 2000a; Gregory et al, 2000b). Macrophage-derived mediators, including interleukin-1, IL-6, tumor necrosis factor- (TNF- ), and prostaglandin E 2 (PGE 2), have been found in the sera and tissues of burned animals and patients (Ogle et al, 1994; Mester et al 1994; Drost et al, 1993; Zhou et al, 1992; Schluter et al, 1991; Rodriguez et al 1993; Kowal-Vern et al, 1994). In addition, it is thought that aberrant production of these mediators is responsible, in part, for the decreased ability of neutrophils and macrophages to perform their bactericidal function in burn patients leading to increased susceptibility to infection (Alexander, 1990; Winkelstein, 1984; O'Riordain et al, 1992).

In young adults, the level of production of these macrophage-derived proinflammatory mediators in the absence of injury is minimal and increases markedly after burn or trauma. In contrast, the normal aging process is associated with increased levels of these cytokines (Doria and Frasca, 1994), most notably IL-6 (Ershler et al, 1994; Daynes et al, 1993; Fagiolo et al, 1993; Ershler and Keller, 2000). Thus, it is likely that

[1] Corresponding Author:
Elizabeth J. Kovacs, Ph.D.,
Professor, Department of Cell Biology,
Neurobiology, and Anatomy, Professor,
Department of Surgery,
Loyola University Chicago,
Stritch School of Medicine,
Building 110, Room 4221,
2160 South First Avenue,
Maywood, IL 60153,
Office: 708-327-2477,
Fax: 708-327-2813,
Email: ekovacs@lumc.edu

[e] Present address:
Meredith S. Gregory, Ph.D.,
The Schepens Eye Research Institute,
20 Staniford St.,
Boston, MA 02114

aged individuals are predisposed to a poor outcome after injury by virtue of an overproduction of mediators like IL-6 and their weakened immune system. Herein, we utilize a dorsal scald injury model to compare the effects of injury on survival and cell mediated immune responses in young and aged mice.

## MATERIALS AND METHODS

Induction of burn injury. Young (3-4 month) and aged (18 - 24 month) female BALB/c mice from the National Institute on Aging's breeding colonies at Charles River (Portage, MI) and Harlan Sprague Dawley, Inc (Indianapolis, IN) were maintained on a 12 hour light/dark cycle with food and water available ad libitum. Mice were subjected to a 15% TBSA dorsal scald or sham injury as previously described (Faunce et al, 1997). In brief, after induction of anesthesia (Nembutal 40 mg/kg, intraperitoneally (i.p.)), clippers were used to remove the hair from the dorsum of each animal. Anesthetized mice receiving scald injuries were placed on their dorsum in a plastic template that exposed 15% TBSA, as calculated according to the method of Spector (1956), and immersed in a 100 C water bath for 8 seconds. Following burn injury, mice were immediately dried off to prevent any further scalding. Sham-injured animals were anesthetized, shaved, held in a plastic template and placed into a room temperature water bath for the same time period. Both burned and sham injured animals were resuscitated with 1.0 ml of 0.9% normal saline (i.p.) and allowed to recover under a heat lamp. After recovery from anesthesia, mice were returned to their cages and maintained under barrier conditions in the animal facility. The animal studies described herein were performed in strict accordance with the guidelines set forth by the Loyola University Chicago Institutional Animal Care and Use Committee. At the time of sacrifice, all mice were dissected and the organs screened for visible tumors and/or gross abnormalities. Animals with visible tumors or abnormalities were excluded from these studies.

Analyses of cell-mediated immune responses. Delayed-type hypersensitivity (DTH) responses were measured as previously described (Faunce et al, 1997). In brief, five days prior to injury all groups of experimental mice were sensitized to the hapten 2,4-dinitrofluorobenzene (DNFB) (Sigma Chemicals, St. Louis, MO) by applying 20 ul of a 0.5% solution in acetone:olive oil (4:1) directly to the shaved skin of the abdomen. Immediately after burn or sham injuries, ear thickness measurements were made with engineering calipers and then an eliciting dose (20 l of 0.2% DNFB) was applied to the pinna of the right ear. Ear thickness measurements were made 24 hours after elicitation, at the time of sacrifice. The magnitude of ear swelling was expressed as percent change in ear thickness using the following formula: (in thickness/pre-elicitation thickness) x 100%, where in thickness = post-elicitation - pre-elicitation ear thickness. A group of naïve animals received only the elicitation dose of DNFB for the determination of nonspecific ear swelling due to application of the hapten in acetone:olive oil. The naive animals exhibited only a 4% change in ear thickness. Left ear (completely unmanipulated) measurements were also obtained and served as internal controls for each animal.

Following aseptic removal of the spleens, splenocyte proliferation was assessed as previously described (Faunce et al, 1997) with minor modifications (Gregory et al, 2000a). Briefly, splenocytes were plated in triplicate in 96-well microtiter plates at a density of 200,000 cells per well in RPMI supplemented with L-glutamine (2 mM), penicillin-G (100 U/ml), streptomycin (100 ug/ml), and 10% fetal bovine serum. The viability of the cells was confirmed to be >98% by trypan blue exclusion. Splenocyte cultures were incubated at 37 C in the presence or absence of concanavalin A (Con A; 2 ug/ml) for 72 hours. In the absence of Con A, proliferation of splenocytes was minimal in all treatment groups (data not shown). For the final 4 hours of culture, the medium was removed and replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) which, in the presence of the mitochondrial enzyme succinate dehydrogenase, is cleaved into a blue colored product. The O.D. of each well was measured using an automatic plate reader at a 540 nm wavelength. Data are presented as mean absorbance for each group.

Determination of circulating levels of IL-6. For measurement of circulating IL-6, blood was obtained by cardiac puncture at the time of sacrifice and serum was stored at -80 C prior to assay, as described previously (Faunce et al, 1998). The IL-6 content in serum was determined by ELISA (Endogen Inc., Cambridge, MA) according to the manufacturers' specifications. The lower level of detection of the kit was 25 pg/ml.

Statistical analyses. Data are expressed as mean ± SEM unless otherwise noted. In all data shown, N represents the number of individual animals. Differences between groups were determined by ANOVA followed by Fisher's LSD post hoc test. For mortality studies, a Fischer's Exact Test was used. A difference of $p<0.05$ was considered significant.

## RESULTS

Percent survival following burn trauma. There was a marked difference in the survival rate of young adult and aged mice following a 15% TBSA dorsal scald injury (Figure 1). While none of the young adult mice succumbed following burn trauma, 67% of the aged mice died within the 10 day period of time examined ($p<0.01$). This significant increase in mortality in the aged burned mice was not observed until 6 days after injury. In contrast, no mortality was observed among aged or young mice receiving sham injury at any time point examined (data not shown). Because the mortality of the aged burn injured mice was observed at >48 hours after injury, further studies were conducted at 24 hours after injury in order to allow us to examine early changes in immune responses and cytokine production, which may contribute to the decreased

survival of

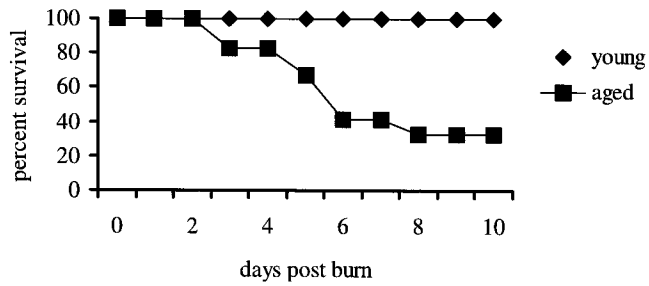


**Figure 1.** Ability of young and aged mice to withstand burn trauma. Young and aged mice were subjected to an 8 second 15% TBSA scald and allowed to recover. Data are expressed as % survival over 10 days. N=12 mice per group.

burn injured aged mice.

Cell-mediated immune responses after burn trauma. In order to determine whether there are age-related differences in cell-mediated immune responses after burn trauma, DTH and mitogen-induced splenocyte proliferative responses were examined in young and aged mice subjected to burn or sham injuries. Young sham-injured mice were able to mount a normal DTH response, as shown by a 52% increase in ear thickness (Figure 2). However, young female mice did not exhibit depressed DTH responses 24 hours after burn injury. These finding are consistent with those of Gregory et al (2000a), which showed that burn induced suppression of the DTH response in young BALB/c female mice was not observed until 7 days post injury. In contrast to young animals, aged sham injured animals had only a 30% increase in ear thickness, which was significantly less than that of sham injured young mice ($p<0.05$). Moreover, the DTH response observed of aged burn injured mice was severely impaired averaging only one-fifth that of sham injured aged mice ($p<0.01$), and only one-tenth that of young burn injured animals. The response of the burned aged mice was comparable to that obtained in naïve animals (4% increase in ear thickness). Thus, these results indicate decreased immune responsiveness in sham injured aged mice in comparison to sham injured young mice, and a more profound decrease in immunity after burn injury in aged mice.

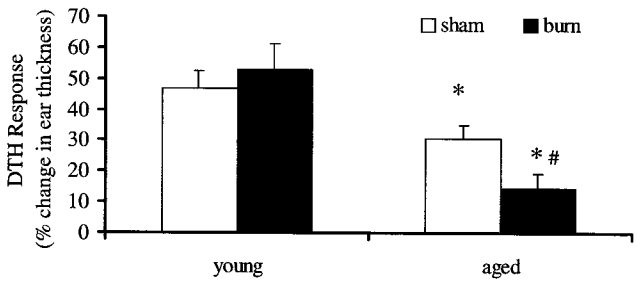


**Figure 2.** Effect of age on the delayed type hypersensitivity response after burn. Mice were sensitized to DNFB 5 days prior to injury. Elicitation of DTH was performed immediately following injury on the pinna of the right ear. Naïve mice mounted only a 4% increase in ear thickness after elicitation (data not shown). Data are compiled from five independent experiments and are expressed as mean % increase in ear thickness ± SEM. N=13 mice for young sham and 16-18 mice per group for other groups. *p<0.05 from young groups, #p<0.01 from all other groups, determined by ANOVA with Fisher's LSD post hoc analysis.

Like the DTH responses, splenocytes obtained from young adult mice 24 hours after burn or sham injury did not differ in their mitogen-induced proliferative responses (Figure 3). In contrast, splenocytes obtained from sham injured aged mice had a significant suppression of Con A-induced proliferation, which was approximately one-half that of the level observed in sham injured young mice ($p<0.01$). While proliferation of splenocytes from both aged sham and burn injured mice was suppressed, there was no significant difference in the magnitude of suppression.

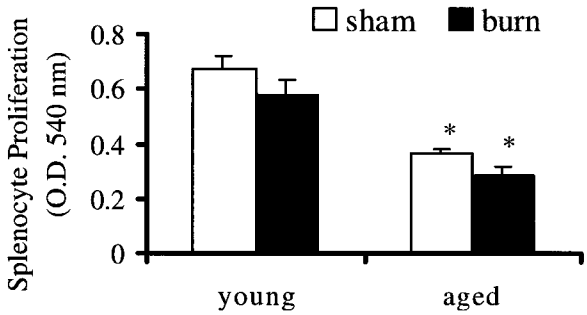


**Figure 3.** Effect of age and injury on the splenocyte proliferative response. Total splenocytes were harvested 24 hours post injury and cultured 72 hours in the presence of Con A (2 ug/ml) as described in the Methods. Splenocyte proliferation was measured using the MTT assay. Data are compiled from five independent experiments and are shown as mean values ± SEM. N=17 mice for sham groups and 19 mice for the burned groups. *p<0.01 from young groups, determined by ANOVA with Fisher's LSD post hoc analysis.

IL-6 levels in aged burn injured mice. IL-6 was measured in the serum of young and aged mice 24 hours after burn or sham injury. Circulating IL-6 was undetectable in sham injured young mice (Figure 4). However, following burn injury, young mice exhibited a significant increase in circulating IL-6 levels (152 ± 25 pg/ml) in comparison to sham injured young mice ($p<0.05$). The IL-6 levels measured in young mice were consistent with our previous studies in which burn injury resulted in a significant elevation in IL-6 levels in young adult male BDF mice (Faunce et al, 1998) and female

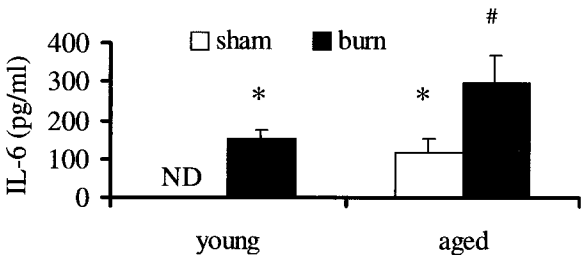


**Figure 4.** Elevation in circulating levels of IL-6 following burn trauma. Twenty-four hours post injury, blood was collected by cardiac puncture and serum assayed for IL-6 by ELISA. Data, compiled from four independent experiments, are shown as mean serum IL-6 levels ± SEM. ND = not detectable. N=12 young and 15-18 aged mice per group for all other groups. *p<0.01 from young groups, #p<0.05 from all groups, as determined by ANOVA with Fisher's LSD post hoc analysis.

BALB/c mice (Gregory et al, 2000a). These levels are also comparable to the elevated IL-6 levels which were reported to increase with burn size and correlate positively with rates of mortality and septic or infectious complications (Schluter et al, 1991; Rodriguez et al 1993). In contrast to young animals, sham injured aged mice had elevated circulating concentrations of IL-6 (143 ± 37 pg/ml) which increased significantly (328 ± 73 pg/ml) following burn injury ($p<0.05$).

## DISCUSSION

Aged burn patients exhibit a higher rate of mortality after burn trauma than their younger counterparts (Pruitt et al, 1964; Rittenbury, et al, 1965). In the current studies, we utilized a murine model of thermal injury and found that aged mice succumb more readily after being subjected to a moderate size burn injury than young adult mice. While the exact mechanisms responsible for this age-dependent difference in survival have yet to be elucidated, the results of the present study suggest, for the first time, a possible mechanism responsible for the enhanced morbidity among aged mice subjected to a moderate size scald injury.

Data shown herein confirm that the cell-mediated immune responses of uninjured aged mice were diminished when compared to younger animals, as reported by others (Miller, 1991; Weigle, 1989; Saltzman and Peterson, 1987). The observations are also consistent with the observed temporal difference in the post burn immunosuppression in male and female mice subjected to identical size 15% total body surface area scald injuries (Gregory et al, 2000a). Gregory and coworkers showed that the DTH response was intact in young adult female BALB/c mice at 24 hours after injury, but was suppressed during the second week post injury. In contrast to the timing of post-burn immunosuppression in female mice, the DTH response in male BALB/c mice occurred early (24 hours to 4 days post injury), after which it recovered (Gregory et al, 2000a; Messingham et al, 2001a). Thus, the early suppression of the DTH response seen after burn injury in aged female mice is reminiscent of the temporal pattern observed injured males. However, a larger percentage of the aged mice do not survive their injuries. Gregory et al (2000b) went on to show that the gender difference in the timing of post burn immunosuppression was dependent on the gonadal steroid hormone, estrogen. Whether this age-dependent difference in responses to burn injury involve differences in the levels of hormones, such as estrogen, remains to be seen.

While the DTH response in aged mice subjected to burn trauma was markedly suppressed relative to other treatment groups, there was neither a synergistic nor an additive effect of age and injury on mitogen-mediated splenocyte proliferation, as recently shown in aged mice subjected to hemorrhage (Kahlke et al, 2000a). The diminished proliferative response of splenocytes from aged mice relative to younger animals may be due, in part, to the age-associated decrease in the production of lymphocyte derived cytokines, such as interleukin-2, described in other systems in uninjured mice (Haynes et al, 1999; T.P. Plackett and E.J. Kovacs, unpublished observation) and after hemorrhage (Kahlke et al, 2000a). The observation that the magnitude of mitogen-induced proliferation is not different in splenocytes from sham and burn injured aged mice, whereas the DTH response does differ between these two groups, may reflect the fundamental differences between the two assays. The DTH response is performed in vivo and is antigen specific, while Con A-induced splenocyte proliferation is ex vivo and non-specific.

The functions of multiple immune cell types, including lymphocytes and macrophages, are altered following traumatic injury (O'Sullivan and O'Connor, 1997; Lederer et al, 1999). After burn trauma, for example, many T lymphocyte functions, such as proliferation and cytokine production, are depressed (Miller and Baker, 1979; Wood et al 1984; O'Sullivan et al 1995). Further evidence reveals that the suppression of cell-mediated immune responses after burn injury is due, in part, to overproduction of macrophage-derived mediators (Wood et al 1987; Faist et al, 1996; Faunce et al, 1998; Schwacha and Somers, 1998; Gregory et al, 2000a; Ogle et al 1994; O'Riordain et al 1992). Interestingly, the same set of mediators that are aberrantly produced in patients and animals subjected to traumatic injury are synthesized and secreted spontaneously by monocytes and macrophages from aged individuals in the absence of injury, such as IL-1, IL-6 and PGE2. Of these proinflammatory mediators, IL-6 appears to play a key role in age-induced immune dysfunction (Ershler et al, 1994; Ershler and Keller, 2000; Daynes et al, 1993; Fagiolo et al, 1993). Low levels of these mediators are thought to be beneficial to immune function, however, higher concentrations are clearly immunosuppressive (Wood et al, 1987, Faunce et al, 1998; Gregory et al, 2000a; Zhou et al 1991) and correlate with a poor prognosis in trauma patients (Drost et al 1993; Zhou et al, 1992; Rodriguez et al 1993). The correlation is further supported by the findings that young adult burn patients who do not survive following burn trauma have circulating IL-6 levels that are almost 10-fold higher than in surviving patients (Schluter et al, 1991; Drost et al, 1993). Thus, it is possible that the dysregulation of baseline cytokine production in the elderly may contribute to the increased immunosuppression and subsequent mortality following relatively minor injuries.

Consistent with the work of others, the present studies show that circulating levels of IL-6 are significantly elevated in aged sham injured mice relative to young sham injured animals. In addition, these studies show for the first time that this pleuripotent cytokine is elevated to a higher extent in aged burn injured mice when compared to young burn injured animals. This observation, taken along with earlier work showing that marked elevation in circulating levels of this cytokine correlate with post-injury immunosuppression (Faunce et al 1998; Gregory et al, 2000a; Fontanilla et al, 2000; Messingham et al, 2001b), suggests that blocking IL-6

may be therapeutic in aged burn injured mice as it was in young adult mice subjected to traumatic injury (Gennari et al 1994; Gennari and Alexander, 1995; Gregory et el, 2000a; Fontanilla et al, 2000). Preliminary data from this laboratory suggest that the administration of anti-IL-6 antibody to burn injured aged mice partially restores the DTH response while it completely restores splenocyte proliferation (K.A. Grabowski, L.A. Duffner, and E.J. Kovacs, unpublished observation). This suggests that while IL-6 is an important mediator of post trauma immunity, other factors are likely to work in parallel or in series with this cytokine.

Additionally, levels of IL-6 receptors (both the membrane bound and soluble forms) may differ in number in young and aged subjects, thus, altering the target cell responsiveness to the mediator. In support of this, preliminary studies reveal there is a difference in the level of expression of IL-6 receptor on the surface of splenocytes from young and aged mice (K.A.N. Messingham, L.A. Duffner, and E.J. Kovacs, unpublished observation). In the absence of injury, approximately one third as many cells from aged mice express IL-6 receptor as from young adult mice. This is consistent with the observed higher levels of IL-6 in aged mice, which could result in ligand-induced down-regulation of receptors.

In summary, these studies reveal that mortality is higher in aged mice subjected to traumatic injury relative to young mice. Additionally, cell mediated immune responses are impaired in burn injured aged mice relative to other treatment groups and the have higher circulating levels of IL-6 when compared to uninjured aged mice and young adult mice regardless of injury. Further studies will need to be conducted to determine if treatments directed at blocking the production of IL-6, or the response to this cytokine, will be therapeutic in burned subjects of all ages.

## ABBREVIATIONS

ANOVA, analysis of variance, Con A, concanavalin A; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity (DTH); IL-6, interleukin-6; i.p., intraperitoneal; MTT, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; O.D., optical density; PGE 2, prostaglandin E 2, SEM, standard error of the mean; TBSA, Total body surface area; TNF- , tumor necrosis factor-

## ACKNOWLEDGMENTS

The authors wish to extend a special thank you to Alessandra Colantoni, M.D. and Kelly A. N. Messingham, Ph.D., for thoughtful discussion and suggestions. The authors wish to thank Dr. Pam Witte, Director of the Immunology and Aging Program at Loyola University Medical Center, and Heather Minges Wols who provided critical review of the manuscript. This work was supported by NIH GM55344, AG16067, AG18859, and AG00997.

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