Age-Related Alterations in the Inflammatory Response to Dermal Injury

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Previous studies have documented that the ability to heal wounds declines with age. Although many factors contribute to this age-associated deficit, one variable that has not been carefully examined is leukocyte recruitment and function in wounds. This investigation compares the inflammatory response in excisional wounds of young (age 8 wk) and aged (age 22 mo) mice. In the early inflammatory response, neutrophil content of wounds was similar for both aged and young mice. In contrast, macrophage levels were 56% higher in aged *versus* young mice (81 ± 20 *vs* 52 ± 13 cells per mm^2). In the later inflammatory response, wounds of aged mice exhibited a delay in T cell infiltration, with maximum T cell levels at day 10 in aged mice *versus* day 7 in young mice. Despite this delay, the eventual peak concentration of T cells was 23% higher in the wounds of aged mice (152 ± 11 cells per mm^2 *vs* 124 ± 21cells per mm^2). The observed alterations in inflammatory cell content suggested that chemokine production might be altered with age. An elevation of monocyte chemoattractant protein (MCP-1) levels was observed in wounds of aged mice. RNase protection studies, however, revealed that the production of most chemokines, including MIP-2, MIP-1α, MIP-1β, and eotaxin, tended to decline with age. Because optimal wound healing requires both appropriate macrophage infiltration and phagocytic activity, phagocytosis was examined. Compared to young mice, wound macrophages from aged mice exhibited a 37%–43% reduction in phagocytic capacity. Taken together, the data demonstrate age-related shifts in both macrophage and T cell infiltration into wounds, alterations in chemokine content, and a concurrent decline in wound macrophage phagocytic function. These alterations may contribute to the delayed repair response of aging. *Key words: inflammation/macrophages/neutrophils/T cells/wound healing. J Invest Dermatol 117:1027–1035, 2001*

The wound repair process is a highly ordered series of events that encompasses hemostasis, inflammatory cell infiltration, tissue regrowth, and remodeling. An important component of normal wound healing is the generation of an inflammatory reaction, which is characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes (Ross and Benditt, 1962). Although neutrophils are the first leukocyte in the wound, these cells are not necessary for normal wound healing (Simpson and Ross, 1972). In contrast, the macrophage has been shown to be a critical mediator of tissue repair (Leibovich and Ross, 1975). Within a few days after injury, macrophages are recruited to sites of injury by various chemoattractants, including chemokines (DiPietro *et al*, 1995; 1998). Previous studies have demonstrated that macrophages perform two distinct functions vital to the healing process. Initially, macrophages engulf and phagocytose wound debris, clearing the way for the growth of new dermal matrix. Subsequently, macrophages produce angiogenic and fibrogenic growth factors that promote the growth phase of repair (Polverini *et al*, 1977; Hunt *et al*, 1984; DiPietro, 1995). As neutrophil and macrophage levels decrease, the later inflammatory phase of wound healing is typified by the ingress of T lymphocytes (Ross and Odland, 1968).

Laboratory and patient studies dating back almost a century describe an age-related decline in the rate of repair (DeNouy, 1916). The majority of more recent studies define age-related alterations in the proliferative aspects of repair, including keratinocyte proliferation (Gilchrest, 1983), extracellular matrix synthesis (Ashcroft *et al*, 1997), and angiogenesis (Reed *et al*, 1998). Previous studies in our laboratory have documented the effects of aging on multiple proliferative aspects of wound healing in a defined murine excisional wound model (Swift *et al*, 1999). These many studies have provided detailed information regarding changes in the wound proliferative response in aging. In contrast, age-related changes in the function of inflammatory cells within wounds are less well studied.

Our experiments compare inflammatory cell infiltration at sites of injury in young and aged mice in a defined and well-described model of excisional wound healing. Previous studies by our laboratory have shown that excisional wounds of aged mice exhibit significantly delayed wound repair (Swift *et al*, 1999). In aged mice, complete wound reepithelialization was delayed by 3 d. Rates of collagen synthesis and angiogenesis were similarly delayed in the wounds of aged mice. The present study examines changes in the functional status of wound macrophages by evaluating the ability of

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Abbreviations: MCP-1, monocyte chemoattractant protein 1; MPO, myeloperoxidase.

wound macrophages from young and aged mice to perform phagocytosis. The studies provide new information about how the sequence of wound inflammatory cell infiltration changes with age, and provide possible mechanisms for the altered repair response that is observed in aging.

MATERIALS AND METHODS

Animals and excisional injury model All procedures were reviewed and approved by the Loyola University Institutional Animal Care and Use Committee. Female BALB/c mice aged 8 wk and 22 mo (NIA/Charles River, Stone Ridge, NY) were used in all studies except flow cytometry. Male BALB/c mice aged 8 wk (Jackson Laboratory, Bar Harbor, ME) and 27 mo (NIA/Charles River) were used for flow cytometry. Female BALB/c mice reach sexual maturity at 7–8 wk of age, and have a mean lifespan of 28 mo (Goodrick, 1975). Animals were anesthetized by methoxyflurane inhalation, and the dorsal hair was shaved. Six full-thickness dermal wounds (3 mm) were produced on the dorsum of each mouse with a standard biopsy punch (Acuderm, Fort Lauderdale, FL). Wounds were placed in three pairs on opposite sides of the midline, and were approximately 5 mm apart. The most caudal wounds were located approximately 2 cm from the cervical vertebrae. At various intervals between 1 d and 21 d after injury, the mice were killed by halothane inhalation. Wounds and surrounding tissue were removed and embedded in TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC), or snap frozen in liquid nitrogen, and stored at −80°C for analysis. For RNA, myeloperoxidase (MPO), and chemokine analysis, wounds were harvested with a 5 mm diameter dermal biopsy punch.

Immunohistochemistry and tissue analysis Frozen embedded wounds were sectioned at a thickness of 10 μm for immunohistochemical analysis. To identify the central portion of the wound, section width was monitored as serial sections were produced. Only intact sections from the widest portion of the wound were used for analysis. Sections that were torn or incomplete were discarded. All incubations and washes were carried out at room temperature.

For analysis of macrophage content, sections from the central portion of each wound were stained with anti-F4/80, a rat monoclonal antibody specific for murine macrophages (Serotec, Raleigh, NC). Histologic sections were fixed in acetone for 30 min and pretreated with 0.3% H_2O_2 in methanol to block endogenous peroxidase. After three 5 min washes in phosphate-buffered saline (PBS), sections were incubated with normal mouse serum (1:10, Harlan, Indianapolis, IN) for 30 min. Sections were incubated in primary antibody anti-F4/80 (Supernatant, 1:50) for 30 min, washed three times in PBS, and incubated in secondary antibody (biotinylated mouse antirat Ig, 13.0 μg per ml, Jackson Laboratories, West Grove, PA) for another 30 min. Subsequently, sections were incubated with avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min, and again washed in PBS. Color development was performed with 3,3′-diaminobenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 10 min and sections were counterstained with Gills hematoxylin (Sigma, St. Louis, MO). To quantitate macrophages, F4/80 positive cells were counted in 10 random high power fields within the wound bed, and the number of macrophages per square millimeter was determined. At each time point, four wounds from each of six young and four aged mice were analyzed and compared.

For analysis of T cell content, sections were stained with anti-CD3ε (Dako, Carpinteria, CA). Sections were briefly thawed and fixed in acetone for 15 min, and then rehydrated in PBS and blocked with 10% normal goat serum. Samples were stained for CD3ε with a rabbit antihuman polyclonal primary antibody (Dako) at 2 μg per ml. After washing, peroxidase activity was blocked with 0.3% H_2O_2 in methanol. A biotinylated goat antirabbit secondary antibody (Sigma) was applied at 3.8 μg per ml followed by avidin–biotin–horseradish peroxidase complex from a Vectastain ABC Peroxidase kit (Vector). The slides were incubated in 3,3′-diaminobenzidine (Kirkegaard & Perry Laboratories) for 10 min and counterstained with Harris hematoxylin (Sigma). To quantify T cells, video images of wound sections were captured using Optronics Acquisition software (Optronics, Goleta, CA). Wound bed areas were outlined and measured using ScionImage Beta 3b Acquisition and Analysis software (Scion Corporation, Frederick, MD). The number of CD3ε-positive cells was counted in each wound bed, and the number of CD3ε-positive cells per square millimeter was calculated for each section.

Neutrophils were stained using anti-GR-1 (Ly-6 g; Pharmingen, San Diego, CA) following the identical protocol used to stain F4/80-positive cells. The primary antibody was applied at a concentration of 5 μg per ml. Gr-1-positive cells in the wound bed were counted and wound bed areas were determined as described above using ScionImage with counts expressed as neutrophil number per square millimeter.

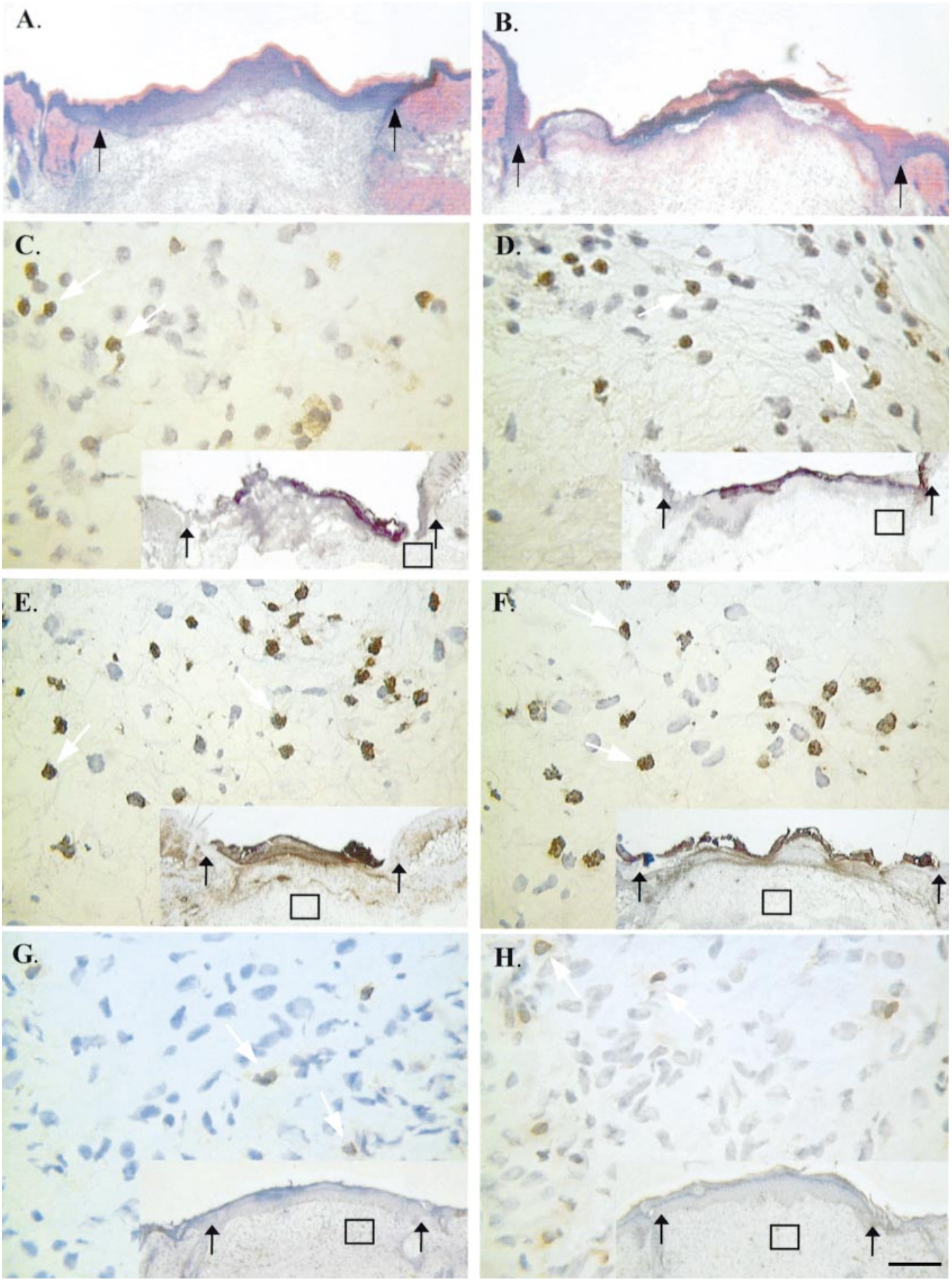
Analysis of neutrophil MPO MPO levels were determined as described by Suzuki *et al* (1983). To prepare samples, individual wounds were homogenized in 2.0 ml of 20 mM phosphate buffer, pH 7.4. Homogenates were centrifuged at 12,000*g* for 45 min, and the supernatant was decanted. The pellets were resuspended in 1.0 ml of 50 mM phosphate buffer containing 10 mM ethylenediamine tetraacetic acid and 0.5% hexadecyltrimethyl-ammonium bromide (HTAB). After a freeze–thaw cycle, the samples were sonicated briefly and incubated at 60°C for 2 h to release maximal MPO activity. The samples were centrifuged at 500*g* for 10 min and the supernatant was transferred to 1.5 ml tubes for storage at −20°C.

For analysis, samples were thawed, and MPO standard (Sigma) was diluted to generate a standard curve ranging from 0 to 3.0 units per ml. Fifty microliter aliquots of sample or standard were placed in 12 × 75 mm glass tubes with 500 μl of assay buffer (0.1 M phosphate buffer, pH 5.4, 1% HTAB, 0.43 mg per ml 3,3′,5,5′-tetramethylbenzidine). The reactions were started by addition of 50 μl of 15 mM H_2O_2, incubated at 37°C for 15 min, and stopped with 1.0 ml of cold 0.2 M sodium acetate, pH 3.0. The absorbance of each sample was determined by reading absorbance at 655 nm within 10 min. The assay was performed on one wound from each of six young and four aged animals at each time point. All samples and standards were tested in duplicate.

Isolation of wound macrophages Wound macrophages were isolated using the subcutaneous sponge model. This model has been shown to closely replicate the environment of the wound, and has been used to study inflammation, angiogenesis, and granulation tissue formation (Holm-Pedersen and Zederfeldt, 1971; Fahey *et al*, 1991; Reed *et al*, 1998). Briefly, mice were anesthetized, and the dorsal hair was shaved. A 2.0 cm incision was made on the dorsum of the mouse, and a polyvinyl alcohol sponge (20 × 5 × 1 mm, Rippey, El Dorado Hill, CA) was placed under the skin. The skin edges were approximated and secured with surgical clips. At 3 d after placement, the sponge was removed and placed in 1 ml of Dulbecco's PBS (Gibco BRL, Grand Island, NY) in a 17 × 100 mm polypropylene tube. Sponges were compressed with forceps for approximately 15 s to free inflammatory cells from the sponge matrix. The barrel of a 3 ml syringe was inserted into the top of each tube and the sponge was placed into the syringe barrel. The syringe and the tube were subjected to centrifugation at 200*g* for 1–2 min to remove residual liquid from the sponge.

Latex bead phagocytosis The wound cell suspension was adjusted to a concentration of 2.5×10^5 cells per ml in RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum (Biologos, Montgomery, IL), 100 U per ml penicillin, 100 μg per ml streptomycin (Biologos), and 2.5 μg per ml fungizone (Biologos) and plated in eight-well chamber slides (10^5 cells per well) ($n = 10$ young; $n = 11$ old). After incubation at 37°C for 1 h, the chambers were washed three times with warm PBS to remove nonadherent cells. Fresh medium or medium containing 10 ng per ml lipopolysaccharide (*Escherichia coli* O26:B6, Sigma) was added to each chamber. Slides were incubated at 37°C for 60 min, followed by addition of 10^6 latex beads per well (6 μm diameter, Polysciences, Warrington, PA). The cells and beads were incubated at 37°C for 90 min, and the excess beads were washed away with PBS. In order to visualize the ingested beads, cells were fixed and stained with Wright Giemsa (Sigma). A total of 200 cells per chamber were examined and

Figure 1. Wound histology and immunhistochemical analysis. *Black arrows* demarcate wound margins. For (*C*)–(*H*), black boxes on low power (25×) insets indicate the region shown in the upper portion of the figure. *White arrows* indicate positively stained cells. (*A*, *C*, *E*, *G*) young mice; (*B*, *D*, *F*, *H*) aged mice. (*A*, *B*) Day 5 wound sections, hematoxylin and eosin staining. (*C*, *D*) Day 1 wound sections, immunohistochemical staining with the mature macrophage marker F4/80. (*E*, *F*) Day 2 wound sections, immunohistochemical staining with the neutrophil marker anti-GR-1. (*G*, *H*) Day 14 wound sections stained with the T cell marker anti-CD3ε. *Scale bar*: (*A*, *B*) 260 μm; (*C*–*H*) 25 μm.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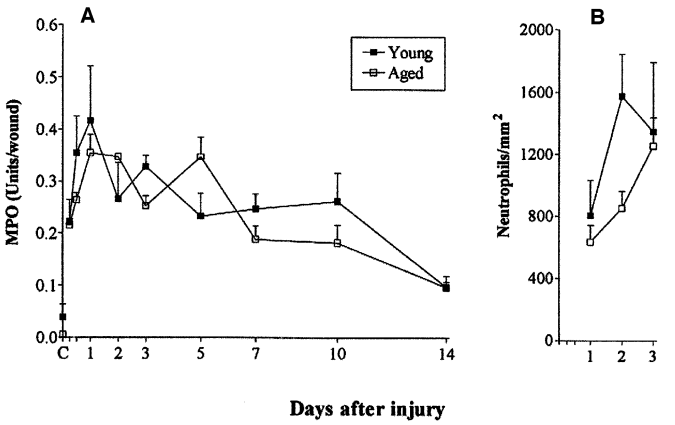

Figure 2. Neutrophil content of excisional wounds from young and aged mice as measured by total wound MPO level and the number of GR-1-positive cells. (*A*) The mean MPO ± SEM of wounds from young ($n = 6$), and aged ($n = 4$) mice is shown. C, control uninjured skin. (*B*) The mean number ± SEM of GR-1-positive cells per mm^2 is depicted for young and aged mice (day 1, 2, $n = 4$; day 3, $n = 3$). Differences between young and aged mice did not reach statistical significance.

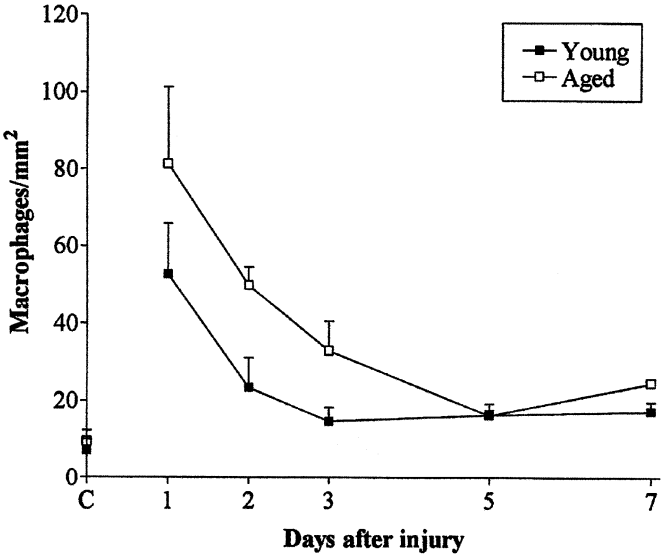

Figure 3. Macrophage infiltration in excisional wounds from young and aged mice. Macrophage infiltration was analyzed by immunohistochemical staining of wound sections and quantitation of F4/80 immunopositive cells. The mean number of macrophages per mm^2 ± SEM in wounds of young ($n = 6$) and aged ($n = 4$) mice is shown. C, control uninjured skin. $p < 0.05$ by two-way ANOVA comparing differences in groups over time.

categorized according to the number of beads ingested. All samples were tested in duplicate.

Sheep erythrocyte phagocytosis Wound cells were prepared and allowed to adhere, as described above. To prepare opsonized erythrocytes, sheep erythrocytes (Bio-Whittaker, Walkersville, MD) were adjusted to a concentration of 1×10^9 cells per ml in dextrose–glucose–veronal buffer (DGV; Bio-Whittaker) ($n = 9$ young; $n = 10$ old). An equal volume of antisheep red blood cell stroma (7S) antibody (Sigma, 1:100) was added, and the mixture was incubated at 37°C for 30 min followed by 30 min on ice. Opsonized erythrocytes were washed twice in fresh DGV and resuspended in RPMI 1640. A total of 2×10^6 opsonized red blood cells were added to each chamber containing wound macrophages and incubated at 37°C for 15 min. The excess erythrocytes were washed away with PBS. In order to visualize the ingested erythrocytes, cells were fixed and stained with Wright Giemsa (Sigma). A total of 200 cells per chamber were examined and categorized according to the number of erythrocytes ingested. All samples were tested in duplicate.

Immunostaining and flow cytometry Isolated wound cell suspensions from young and aged male mice were pelleted and erythrocytes were lyzed. The following steps were carried out on ice. 2–5×10^5 wound cells were incubated in 4% paraformaldehyde for 5 min. After extensive washing in PBS, cells were incubated for 10 min in PBS containing 1% bovine serum albumin (BSA, Sigma). Cells were incubated with anti-FcγRIII/II monoclonal antibody (2.4G2, Pharmingen) and fluorescein isothiocyanate (FITC) conjugated $F(ab')_2$ fragment mouse antirat IgG (Jackson Laboratories). At each step cells were washed with 1% BSA in PBS. Post staining cells were treated with PBS containing 1% saponin (Sigma) and 1% BSA for 10 min. Subsequently cells were incubated with rat antimouse macrophages/monocytes monoclonal antibody (MOMA-2, Serotec) followed by phycoerythrin (RPE) labeled goat antirat IgG (Caltag Laboratories, Burlingame, CA). After each step, cells were washed with 1% saponin/1% BSA/PBS. Flow cytometric analysis was carried out using a FACStar^{PLUS} (Becton Dickinson, San Jose, CA).

Determination of monocyte chemoattractant protein 1 (MCP-1) levels in wounds Murine MCP-1 enzyme-linked immunosorbent assay (ELISA) analysis was performed as previously described (Swift *et al*, 1999). Briefly, individual wounds were homogenized in 1.0 ml of PBS containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Homogenates were sonicated for 15 s, centrifuged to remove debris, and filtered through a 1.2 μm pore syringe filter. Analysis was performed with a commercially available murine MCP-1 specific ELISA kit (BioSource International, Camarillo, CA).

Ribonuclease protection assay Specific RNA species were detected using the RiboQuant Ribonuclease Protection Assay System (Pharmingen). A ^{32}P-UTP labeled probe was generated by *in vitro* transcription of the mCK5 template set. For hybridization, 2×10^5 CPM of probe and 5 μg of total wound RNA were incubated overnight at 56°C. RNA–probe hybrids were subjected to RNase digestion and proteinase K treatment, followed by phenol extraction. The resulting protected fragments were separated on a 5% polyacrylamide gel, and detected by autoradiography. The expression of the mouse chemokines RANTES, eotaxin, MIP-1α, MIP-1β, and MIP-2 was equalized to two housekeeping genes (L32 and glyceraldehyde-3-phosphate dehydrogenase) in a single RNase protection reaction. The assay was performed with RNA prepared from two wounds from each of four young and four aged animals at each time point.

Statistics For time course experiments, data were analyzed by a two-way ANOVA. This analysis does not determine the significance of differences between aged and young mice at individual time points, but instead detects the effect of age over the time course of wound healing. Inflammatory cell infiltration, RNase protection, and phagocytosis data were analyzed by a one-tailed *t* test. For all analyses, p-values less than 0.05 were considered significant.

RESULTS

Inflammatory cells in dermal wounds The infiltration of inflammatory cells into wounds from young and aged mice was determined using a standard dermal excisional injury model (Swift *et al*, 1999). Neutrophil infiltration was measured by counting the number of GR-1-positive cells in the wound bed, and by measuring neutrophil MPO levels in wound homogenates. Neutrophil infiltration was observed as early as 6 h post injury in wounds from both young and aged mice, peaking at day 1 to day 3 (**Figs 1**, **2**). Compared to young mice, the wounds of aged mice appeared to have decreased numbers of GR-1-positive infiltrating cells at days 1 and 2. MPO levels were similar in both groups, however, and declined slowly from day 3 after injury, reaching near baseline by day 14 after injury. The apparent discrepancy between cell counts and MPO activity may be due to differential MPO activity in neutrophils from aged and young mice. Another possible explanation is that whereas MPO analysis includes all neutrophils, even those trapped within the fibrin clot and scab, the GR-1 counts

are limited to the histologic wound bed. Despite the suggestion of an increased level of GR-1-positive neutrophils in the wounds of aged mice, neither the GR-1 counts nor wound MPO demonstrated statistically significant differences between young and old mice at any time.

Macrophage infiltration was assessed using immunohistochemical staining for the F4/80 antigen on mature macrophages. In wounds from both age groups, maximal macrophage counts were observed on the first day after wounding, with wounds from aged mice having 56% more macrophages than wounds from young (81 ± 20 *vs* 52 ± 13 macrophages per mm^2). Whereas macrophage infiltration followed the same time course in both young and aged animals, there were significantly greater numbers of macrophages in the wounds of aged mice than in young mice ($p < 0.05$, **Figs 1**, **3**). By day 5 after injury, macrophage numbers in wounds from both groups had declined, and were comparable to numbers seen in uninjured skin.

The infiltration of T cells into wounds was also significantly altered in aged mice ($p < 0.001$, **Figs 1**, **4**). In young mice, the number of CD3ε-positive cells reached a peak of 124 ± 21 cells per mm^2 at day 7 post injury and steadily declined through day 21. In contrast, the peak density of T cells in wounds of aged mice was delayed, occurring at day 10 (152 ± 23 cells per mm^2). T cell levels were also greatly sustained in aged mice, remaining increased by 74% at day 14 and 154% at day 21 over values seen in the wounds of young mice. Taken together, the results suggest that inflammatory cell recruitment is significantly perturbed with aging.

Chemokine levels in dermal wounds The considerable increase in macrophage infiltration in the wounds from the aged mice suggested that the level of macrophage chemoattractants in wounds might be altered with age. As previous studies have implicated MCP-1 as an important macrophage chemoattractant in wounds (DiPietro *et al*, 1995; 2001), the level of this CC chemokine was examined throughout the time course of wound repair (**Fig 5**). Because the amount of wound fluid that can be obtained from this small excisional wound is limited, chemokine levels were examined in wound homogenates. When measured in wound homogenates, wounds from aged mice contained greater amounts of MCP-1 than wounds from young mice ($p < 0.01$) at nearly all time points. Interestingly the peak levels of MCP-1 were reached at 12 h post injury, just prior to maximal macrophage infiltration. These data suggest that the increased levels of MCP-1 might be responsible for the increased macrophage infiltration that is observed in aged mice. Our previous studies of MCP-1 and MIP-1α production in wounds suggested that chemokine mRNA levels in wounds correlate well with chemokine protein levels (DiPietro *et al*, 1995, 1998, 2001; Low *et al*, in press). Thus we took advantage of RNase protection assays to examine the levels of a large number of additional chemokines in the wounds of the aged mice. As opposed to MCP-1, all other chemokines that were examined, including MIP-2, MIP-1α, MIP-1β, RANTES, and eotaxin, showed either no change with aging or decreased levels of mRNA with aging (**Fig 6**). This interesting result suggests that age-related alterations in chemokine production are specific to the individual chemokine.

Wound macrophage function To assess the functional activity of macrophages in wounds, wound macrophages were isolated from sponges implanted subcutaneously in incisional wounds. The ability of isolated wound macrophages to perform nonspecific phagocytosis and Fc-mediated phagocytosis was examined. First, macrophages were examined for their ability to phagocytose latex beads, a standard *in vitro* phagocytic target. Second, cells were assessed for their ability to carry out phagocytosis of opsonized sheep erythrocytes. When phagocytosis of latex beads was assessed, 27% fewer cells from aged mice were phagocytic ($p < 0.01$), and those phagocytic cells consumed 37% fewer particles than cells from young mice ($p < 0.001$) (**Fig 7**). A diminished phagocytic response was also observed when opsonized erythrocytes were used as the phagocytic target. Compared to

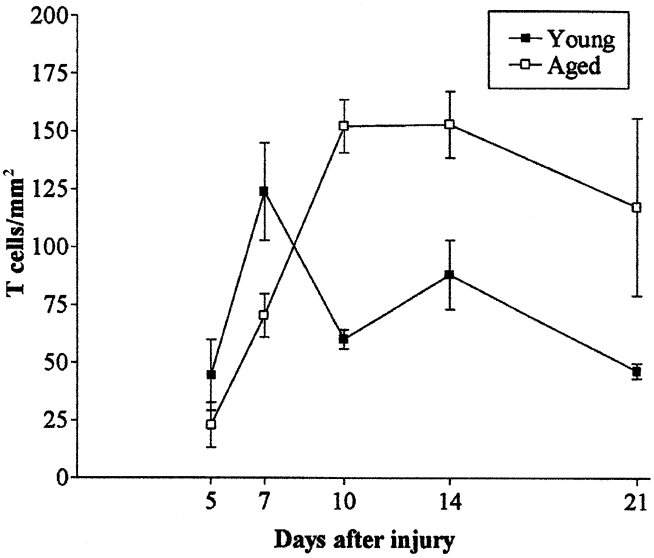

Figure 4. T cell infiltration in excisional wounds from young and aged mice. T cell infiltration was quantitated by immunohistochemical staining of wounds for CD3ε. The mean T cell number per $mm^2 \pm$ SEM in wounds of young and aged mice is shown. Young mice, $n = 8$ (days 5, 10, 14), 9 (day 7), or 5 (day 21); aged mice, $n = 5$ (days 5, 7) or 4 (days 10, 14, 21); $p < 0.001$ by two-way ANOVA comparing differences in groups over time.

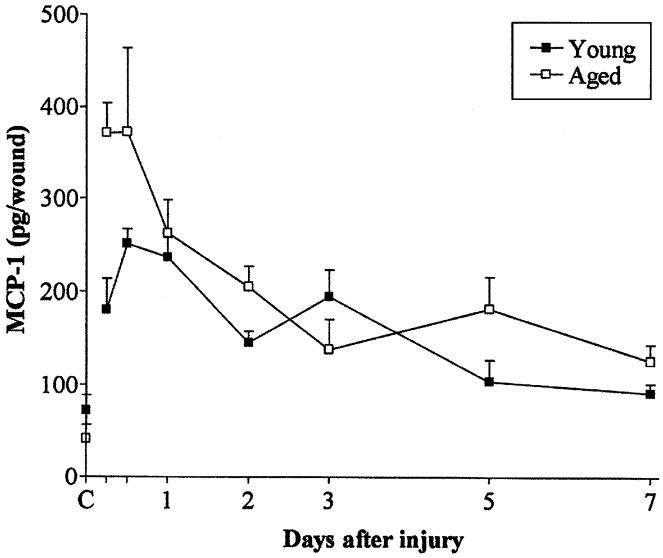

Figure 5. Time course of wound MCP-1 production in young and aged mice. MCP-1 content of wound homogenates was measured by ELISA. The mean pg of MCP-1 per wound ± SEM is shown for young ($n = 6$) and aged ($n = 4$) mice. One wound per mouse was analyzed. C, control uninjured skin. $p < 0.01$ by two-way ANOVA comparing differences in groups over time.

cells from young mice, 22% fewer wound macrophages from aged mice were phagocytic. A 43% decrease in the number of erythrocytes ingested per macrophage was also observed ($p < 0.001$) (**Fig 8**). Thus, in both the latex bead assay and the opsonized erythrocyte assay, macrophages from aged animals were found to have reduced phagocytic activity.

Macrophage Fcγ receptor levels One possible reason for the observed decrease in macrophage phagocytic activity might be a decrease in the levels of surface receptors critical to phagocytosis. To examine this possibility, wound macrophage Fcγ receptor status

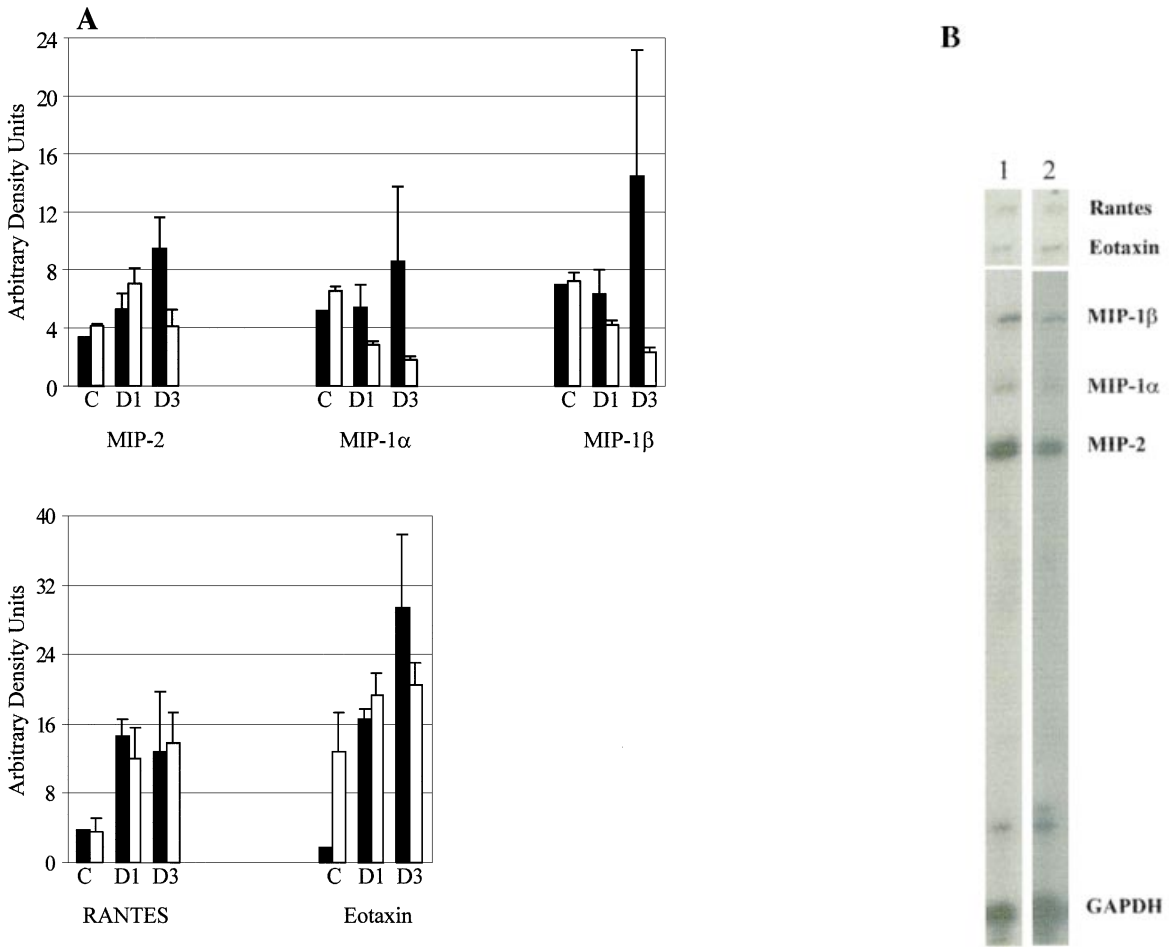

Figure 6. Chemokine RNA levels in wounds of aged and young mice. Chemokine mRNA levels in wounds were determined by RNase protection. (*A*) Densitometric analysis of chemokine expression. Values represent levels of mRNA relative to standard housekeeping genes (L32 and glyceraldehyde-3-phosphate dehydrogenase). Mean ± SEM of young (*closed bars*, $n = 4$) and aged (*open bars*, $n = 4$) are shown. C, control uninjured normal skin; D1, day 1, D3, day 3. (*B*) A representative RNase protection assay from day 3 wounds of young (*lane 1*) and aged (*lane 2*) mice.

was investigated using two-color immunofluorescence staining with monoclonal antibodies specific to mouse macrophage/monocytes (MOMA-2) and FcγRIII/II (**Fig 9**). These studies demonstrated no difference between the FcγRIII/II levels of young and aged mice. The percentage of macrophages (MOMA-2 positive cells) that were also highly FcγRIII/II positive was not significantly different between the wound cells of young and aged mice (9.9 ± 2.1 *vs* 10.3 ± 2.9%, NS). This result suggests that age-related decreases in Fc-mediated phagocytosis are not simply due to a decreased number of receptors.

DISCUSSION

Within the spectrum of events that occur during healing, an adequate inflammatory response is key to appropriate tissue repair (Leibovich and Ross, 1975). Although many parameters of immune function have been shown to be altered with age, few studies have previously examined age-related changes in the immune response in the context of acute injury. This study presents a quantitative assessment of multiple inflammatory cells in a single defined murine model system. The ages studied represent roughly early adulthood and late maturity in mice, and probably correspond to the extremes of the adult wound healing response in this species. In humans, age-related changes in wound healing have been described as following a continuum, with decline beginning as early as the fourth decade of life (DeNouy, 1916). Whether the age-related deficits that occur in murine wound healing will directly correlate with age-related deficits in human wound repair remains to be investigated.

In excisional wounds, neutrophil infiltration was unchanged in aged mice. In contrast, macrophage infiltration was observed to be significantly greater in the wounds of aged mice than in young. This finding agrees with a recent study of human wounds in which an increase in the number of mature macrophages was noted (Ashcroft *et al*, 1998). Because the macrophage has been documented to enhance repair (Leibovich and Ross, 1975; Danon *et al*, 1989), the discovery of increased macrophage levels in the face of declining repair capacity is somewhat surprising. The most logical explanation is that, although the number of wound macrophages increases, the functional abilities of this cell decrease with age. An increased number of macrophages in wounds might therefore partially compensate for decreased function. Few previous studies have specifically examined wound macrophage function. Several previous studies utilizing other macrophage populations, however, have shown that specific macrophage functions are significantly altered with age (Sunderkotter *et al*, 1997). For example, the oxidative burst of macrophages is diminished with age (Davila *et al*, 1990; Alvarez and Santa Maria, 1996). The production of monocyte-derived cytokines, such as interleukin-1 and interleukin-6, is also altered with age (Bradley *et al*, 1989; Roubenoff *et al*, 1998). Pertinent to wound healing, macrophage production of the angiogenic factor VEGF also declines with age (Swift *et al*, 1999). With regard to phagocytic function, both age-related increases (Weeks and Kavas, 1979; Wustrow *et al*, 1984; Rosa *et al*, 1993) and decreases (Hazlett *et al*, 1990; Higashimoto *et al*, 1994; Ferrandez and De la Fuente, 1999) have been demonstrated. The apparent discrepancies in these results may arise from the fact that

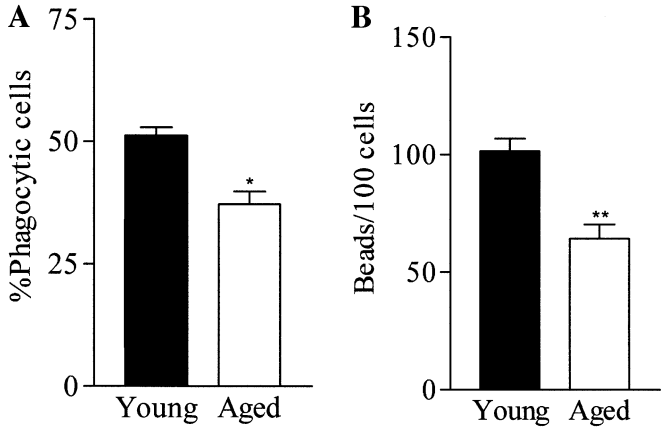

Figure 7. Phagocytosis of latex beads by wound macrophages of aged and young mice. Wound macrophages were assessed for their ability to phagocytose 6 μm diameter latex beads. (*A*) Percentage of total cells that were found to phagocytose one or more beads. (*B*) The total number of beads phagocytosed by 100 macrophages. $n = 10$ young, $n = 11$ aged. A total of 400 cells per sample were analyzed. * $p < 0.01$, ** $p < 0.001$ *vs* young by one-tailed *t* test.

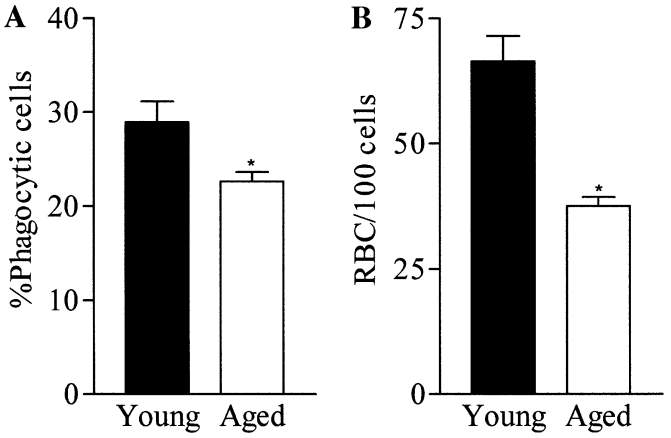

Figure 8. Phagocytosis of opsonized erythrocytes by wound macrophages of aged and young mice. Wound macrophages were assessed for their ability to phagocytose opsonized sheep erythrocytes. (*A*) Percentage of total cells that were found to phagocytose one or more erythrocytes. (*B*) The total number of erythrocytes phagocytosed by 100 macrophages. $n = 9$ young, $n = 10$ aged. A total of 400 cells per sample were analyzed. * $p < 0.01$ *vs* young by one-tailed *t* test.

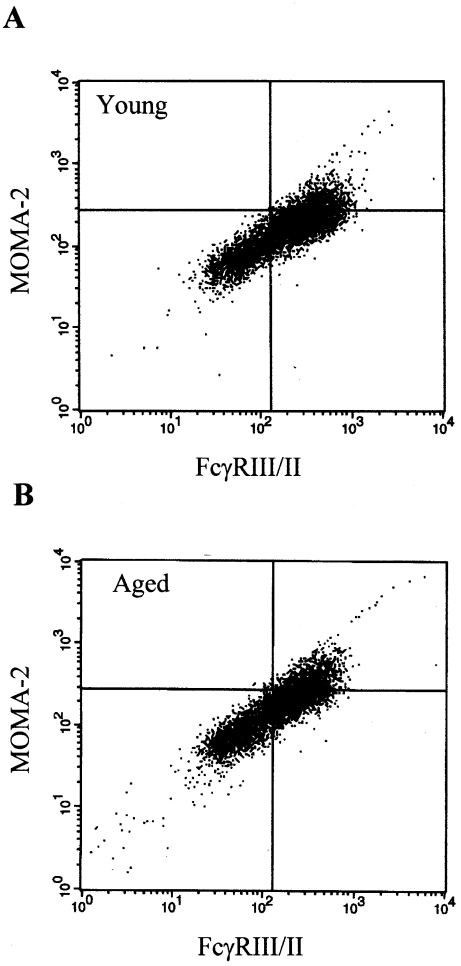

Figure 9. Two-color analysis of the expression of FcγRIII/II on wound macrophages from young and aged mice. The cells were stained with anti-FcγRIII/II monoclonal antibody followed by FITC antirat IgG, and then stained with antimacrophage/monocytes monoclonal antibody followed by RPE antirat IgG. Profiles representative of five separate experiments are shown. Flow cytometric analysis of wound inflammatory cells from (*A*) young and (*B*) aged mice. $n = 5$.

diverse monocyte and macrophage populations, obtained from a variety of sources, were examined. Because site-specific differences in macrophage function seem to exist, the study of macrophages derived from the particular site of interest, such as the wound itself, might be critical to obtaining relevant functional information (Sunderkotter *et al*, 1997). Interestingly, age appears to be linked to decreases both in the percentage of wound macrophages that are phagocytic and in the number of particles consumed by each cell. Overall, this decreased phagocytic activity may be an important mechanistic factor in age-related changes in wound repair.

The reasons for diminished phagocytic capability with aging may be numerous. In the case of Fc-receptor-mediated phagocytosis, no significant change in surface FcγRIII/II receptor levels was seen in wound-derived macrophages. A previous report that examined macrophages from the peritoneal cavity noted an increase in the level of FcγRIII/II receptors in macrophages from aged mice (Kizaki *et al*, 1998). Both findings generally support the hypothesis that a decrease in macrophage phagocytic receptors is not a global feature of aging. One intriguing possibility is that the age-related functional deficit in phagocytosis involves a diminished signaling capacity of surface receptors. Multiple age-related changes in intracellular signaling, including protein phosphorylation and Ca^{2+} flux, have been described for the T cell receptor (Patel and Miller, 1992; Shi and Miller, 1993; Chakravarti and Abraham, 1999). Such age-related signaling alterations have yet to be described for the Fc receptor.

An additional novel observation of this study is the delayed, yet ultimately increased, T cell infiltration in the wounds of aged mice. Ashcroft *et al* also noted that wound T cell content is altered in aging, although their study examined much later time points in the healing process (Ashcroft *et al*, 1998). The delayed but increased T cell content in the wounds of aged mice raises new questions about how T cell migration is modulated with age. If wound inflammatory cell ingress is perturbed with age, inflammatory cell infiltration in other circumstances, such as infective processes, may also exhibit age-related alterations.

The significance of altered T cell content to age-related delays in wound healing is at present unknown. Speculation on this finding

is complicated by the fact that a definitive understanding of T cell function in normal wounds has proved elusive. In normal mice, T cell depletion impairs the healing response (Peterson *et al*, 1987). Athymic nude mice exhibit an increase in wound breaking strength over that of normal mice, however (Barbul *et al*, 1989). The delay in T cell infiltration seen in the wounds of aged mice is most consistent with the notion that T cells function to augment the healing process. The ramifications of the sustained T cell content in the resolving wound of the aged animal are less understandable. Previous studies of age-related changes in T cell function have shown that several cellular functions, including cytokine production and responsiveness, may be altered with age (Hefton *et al*, 1980; Gillis *et al*, 1981; Chakravarti and Abraham, 1999). These findings suggest that, in tandem with changes in the number of wound T cells, particular T cell functions that are critical to wound healing may also be altered with age.

In addition to examining the pattern of neutrophil, macrophage, and T cell infiltration into dermal wounds, the levels of leukocyte chemoattractants in wounds were also examined. Chemokine production has been shown to be critical to optimal wound repair in several experimental systems (Fahey *et al*, 1990; DiPietro *et al*, 1995; 1998; Fivenson *et al*, 1997; Devalaraja *et al*, 2000; Jackman *et al*, 2000). In this study, the age-related increase in wound MCP-1 production paralleled an increase in macrophage infiltration. This finding implies that age-related alterations in chemokine production might be mechanistically significant to age-related changes in the inflammatory response. Previous studies in our laboratory have shown that topical application of recombinant MCP-1 to incisional wounds in young mice enhances macrophage infiltration (DiPietro *et al*, 2001). Similarly, the neutralization of MCP-1 by antibody treatment caused a reduction in macrophage recruitment into wounds (DiPietro *et al*, 2001). These studies support the notion that MCP-1 levels can strongly influence the influx of macrophages into wounds. Our examination of a panel of chemokines, however, failed to demonstrate a consistent pattern of increasing wound chemokine levels with age. The increase in MCP-1 content in the wounds of aged mice was observed only early in the repair process, at day 1 after injury. By day 3, many of the chemokines that we examined, including MIP-2, MIP-1α, MIP-1β, and eotaxin, appear to be decreased in the wounds from aged mice, although these trends did not reach significance. Whereas the role of each of these specific chemokines in healing wounds has not yet been discovered, a growing body of evidence suggests that the appropriate production of both CC and CXC chemokines is critical to optimal wound repair. Mice deficient in CC chemokines, such as MCP-1, display defective wound repair (Low *et al*, in press). Similarly, mice deficient for either the CXC chemokine IP-10 or the CXC receptor CXCR2 also exhibit delayed wound healing (Luster *et al*, 1998; Devalaraja *et al*, 2000). Taken together, these findings suggest that the delay in repair that is seen with age may derive, at least in part, from selective changes in chemokines. This hypothesis is supported by studies in other models of inflammation where specific chemokines are known to critically regulate inflammatory cell function (Lukacs *et al*, 1993; Karpus *et al*, 1995). In addition, because chemokines can influence not only inflammation but also proliferative events, such as angiogenesis, the observed decrease in the levels of chemokines in the wounds of aged animals may have multiple effects. The examination of a very broad panel of chemoattractants by contemporary molecular methods, such as gene array analysis, may assist in establishing the critical chemokine coordinators of tissue repair. This information will ultimately provide a new understanding of the consequences of aging on both the acute inflammatory and reparative responses.

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