Preserved Immune Functions and Controlled Leukocyte Oxidative Stress in Naturally Long-lived Mice: Possible Role of Nuclear Factor Kappa B

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In order to verify the survival biomarker role of several immune functions, and to determine the oxidation and inflammatory mechanisms underlying variability in the aging process, we have investigated a variety of immune functions and oxidative stress parameters as well as activation of the nuclear factor kappa B (NFκB) peritoneal leukocytes from four different age groups of mice, including natural extreme longevity. Immune cells from naturally long-lived animals showed preservation of immune function in response to stimuli and controlled oxidative stress as well as nuclear factor kappa B activation in resting conditions. Moreover, leukocytes from extreme long-lived animals showed increased catalase activity when compared with the adults. In contrast, the old and very old animal groups showed impaired immune function and increased oxidation as well as NFκB activation. Our results support preserved immune function as a biomarker of extended survival and point to controlled regulation of NFκB activity as a key mechanism restraining oxidative stress in immune cells and contributing to reach longevity.

Key Words: Aging—Immune function—Longevity—NFκB—Oxidative stress.

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THE capacity to preserve an adequate immune function throughout aging has been suggested as a positive health life-span marker, whereas the age-related decline in several immune functions has been related to decreased longevity (1,2). The dysregulation and overall impairment in host immunity that occur with aging are evidenced by the higher risk and severity of infections and the increased susceptibility to cancer among aged individuals (3,4), which exert a great influence on age-related morbidity and mortality (1). Indeed, specific age-related changes in immune function have been shown to correlate with increased mortality in humans, such as low T-cell proliferative response to mitogens among others, defining the immune risk phenotype in humans (5). In addition, a low level of natural killer (NK) cytotoxicity in the elderly has been shown to be a significant predictor of mortality (6). Similar age-related changes reported in human peripheral blood leukocytes have been found in mouse peritoneal immune cells and related to higher morbidity and mortality in aged rodents (7,8). Thus, old mice showing impaired immune competence levels have been found to die prematurely (2,7,8), and in humans, centenarians exhibit a high degree of preservation of several immune functions, which may be related to their ability to reach a very advanced age in healthy condition (9–12). However, there are few field studies in experimental animals that have achieved natural extreme longevity, and crucially, the primary biological processes that influence healthy aging of the immune system remain poorly understood. A previous study performed by us showed, in naturally long-lived BALB/c mice, values of lymphocyte functions similar to those in young adults (13).

Aging is characterized by a chronic low-grade inflammatory status, so-called “inflammaging” (14), and oxidative stress (15,16). Recently, both theories of aging have been integrated in the oxidative inflammatory theory of aging (12), according to which overactivation of immune cells, through persistent activation of transcription factors leading to sustained expression of a wide range of proinflammatory genes and long-term oxidative stress in leukocytes, would play a key role in immunosenescence and in the aging rate. Chronic oxidative stress (increase in oxidants and decrease in antioxidant compounds) leads to damage of cell components, including proteins, lipids, and DNA, contributing to the age-related decline of physiological functions, including immune function (17). Indeed, older humans and mice show higher levels of inflammation and oxidative stress in their leukocytes, coincident with the impaired immune responses (12). In contrast, recent studies have shown a lower expression of genes resulting in inflammation and oxidation in human centenarians, who show preserved immune functions (11,18). However, the mechanisms underlying preservation of the immune response and controlled oxidative stress in successful aging remain unclear.
Nuclear factor kappa B (NFκB) is a strong candidate for a potential mediator of the effects of inflammation and oxidative stress upon immune function. The activity of this pathway is increased in many chronic inflammatory disease states such as multiple sclerosis and acute lethal endotoxic shock (19,20). Furthermore, NFκB was found to be down-regulated by glutathione precursors such as N-acetylcysteine, which then prevented excessive oxidation and inflammation in animal models of these conditions (20,21). However, the activation status of NFκB in leukocytes of aging individuals has not been resolved with the few field studies performed reporting contradictory results (22,23).

The aim of the present work was therefore to study several immune functions and oxidative stress parameters, which show age-related changes, as well as NFκB activation in peritoneal leukocytes of ICR/CD1 female mice of different ages, that is, adult, old, very old and natural extreme long lived.

METHODS

Animals

We used 44 ex-reproductive females of outbred ICR/CD-1 mice (Mus musculus) strain, purchased from Harlan Iberica (Barcelona, Spain) at the young adult age (28 ± 4 weeks). The mice were specifically pathogen free as tested by Harlan and according to the Federation of European Laboratory Science Associations recommendations. In our Animal Facility, placed at the Faculty of Biology (UCM), they were housed at 6 ± 1 per cage and maintained at a constant temperature (22 ± 2°C) in sterile conditions inside an aseptic air negative pressure environmental cabinet (Flufrance, Cachan, France), on a 12/12 hour reversed light/dark cycle (lights on at 8 a.m.). Mice had access to tap water and standard Sander Mus pellets (A04 diet; Panlab, Barcelona, Spain) ad libitum. Diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. This cross-sectional study was performed simultaneously on mice of different ages, namely adult (44 ± 4 weeks, n = 14), old (69 ± 4 weeks, n = 10), very old (92 ± 4 weeks, n = 10) and extreme long lived (125 ± 4 weeks, n = 10), which had aged in our Animal Unit under the above specified conditions from the adult age. Each age group category was formed by animals that had been purchased in the same set. The extreme long-lived mice had naturally achieved healthy and successful aging because the average life span for females of ICR/CD1 mice strain in our Animal House is 91.9 ± 5.6 weeks (7). The percentage of these females that reach exceptional longevity is approximately 7%–10%. Not all animals provided the full set of data. Mice were treated according to the guidelines of the European Community Council Directives (86/609 EEC). The animals were marked for their individual follow-up.

Collection of Peritoneal Leukocytes

Peritoneal suspensions were collected between 8 a.m. and 10 a.m., to minimize circadian variations of the parameters studied, without killing the animals. Mice were held by the cervical skin, the abdomen was cleansed with 70% ethanol and 3 mL of sterile Hank’s solution, previously tempered at 37°C, was injected intraperitoneally. After massaging the abdomen, 80% of the injected volume was recovered. Peritoneal leukocytes, mainly macrophages, and NK cells (13) were identified by their morphology and quantified in Neubauer chambers using optical microscopy (×40). Additionally, leukocyte counts were confirmed by immunostaining and flow cytometry, with cells stained for expression of CD11b (CALTAG Laboratories, Burlingame, CA), CD3, CD4, CD8, CD19, and CD56 (BD Pharmingen, San Diego, CA). Fluorescence was measured using a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) immediately after staining. Results were analyzed with Cell Quest Pro software (BD Biosciences, San Jose, CA).

Cellular viability, routinely checked before and after each experiment by the Trypan Blue (Sigma, St Louis, MO) exclusion test, was higher than 99 ± 1% in all cases.

Lymphocyte Proliferation

A previously described method (24) was used with minor modifications. The peritoneal suspension was adjusted to 5 × 10^5 lymphocytes/mL in complete medium containing RPMI-1640 (PAA, Pasching, Austria), 10% fetal bovine serum (Gibco, Paisley, UK) and 1% gentamicin (PAA), and 200 μL were cultured in 96-well plates with concanavalin A (ConA; 1 μg/mL per well; Sigma) or lipopolysaccharide (LPS; Escherichia coli, 055:B5, 1 μg/mL per well; Sigma) as stimulated samples or in complete medium alone as nonstimulated samples. Incubations were performed at 37°C in a sterile and humidified atmosphere of 5% CO₂. After 48 hours, 5 μL ³H-thymidine (MP Biomedicals) were added to each well (2.5 μCi per well), 100 μl of culture supernatants were collected for cytokine measurements and medium was renewed. Cells were harvested 24 hours thereafter. The results were expressed as counts per minute.

Interleukin 2

ConA-stimulated interleukin 2 (IL-2) levels were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) in supernatants of peritoneal leukocytes cultured as described previously. The results were expressed as picogram per milliliter. Sensitivity and range of detection were <0.3 pg/mL and 15.6–1000 pg/mL, respectively.

Cytotoxicity

Murine lymphoma YAC-1 cells were used as targets in the NK activity assay (24). An enzymatic colorimetric
test was used for cytolysis measurements of target cells (Cytotox 96; Promega, Madison, WI) based on lactate dehydrogenase determination using tetrazolium salts. Aliquots of 100 μL of peritoneal leukocytes, previously adjusted to 10^6 leukocytes per mL in RPMI 1640 without phenol red (PAA) were cultured at an effector/target rate of 10/1 and incubated for 4 hours. Thereafter, lactate dehydrogenase enzymatic activity was measured in 50 μL of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. The results were expressed as percentage of lysis of target cells.

**Macrophage Chemotaxis**

The chemotactic index was evaluated by the capacity of macrophages to migrate through a filter, with a pore diameter of 3 μm (Millipore, Madrid, Spain), included in a Boyden chamber (25), and toward a chemoattractant agent (10^-8 M f-Met-Leu-Phe: fMLP; E.coli; Sigma), following a method previously described (26). After a 3-hour incubation of aliquots of 300 μL of the peritoneal suspension adjusted to 5 × 10^5 macrophages per mL in Hank’s medium, filters were fixed and stained, and the macrophage chemotactic index was calculated by counting, in an optical microscope (×100), the total number of macrophages in one third of the lower face of the filters.

**Macrophage Phagocytosis**

Phagocytosis of inert particles (latex beads; Sigma; diluted to 1% in phosphate-buffered saline) was carried out following a method previously described (26). Aliquots of 200 μL of the peritoneal suspension adjusted to 5 × 10^5 macrophages per mL Hank’s medium were incubated in migratory inhibitory factor plates (Kartell, Noviglio, Italy) for 30 minutes. The adhered monolayer was washed and resuspended in 200 μL of Hank’s medium plus 20 μL of latex solution. After 30 minutes of incubation, plates were washed, fixed, and stained, and the phagocytosis was expressed as the number of latex beads ingested per 100 macrophages (phagocytic index), which was assessed by optical microscopy (×100). The number of ingesting macrophages per 100 macrophages (phagocytic efficiency) was also determined.

**Reactive oxygen species**

Reactive oxygen species (ROS) levels were measured by fluorescence following a method previously described (26) with some modifications, using the probe deacetylated 2',7'-dichlorodihydrofluorescein (Molecular Probes, Paisley, UK), which is oxidized in the cytoplasm by ROS into 2',7'-dichlorofluorescein, a highly fluorescent compound. Aliquots of 200 μL of the peritoneal suspension adjusted to 10^6 leukocytes per mL in Hank’s medium were incubated for 30 minutes and 20 μL of deacetylated 2',7'-dichlorodihydrofluorescein (1 mM per well) were added to each well. After 15 minutes of incubation, 20 μL of phorbol myristate acetate (5 ng/mL in well; Sigma) and 20 μL of Hank’s medium were added to stimulated and nonstimulated samples, respectively. Samples were incubated for 15 minutes and fluorescence was recorded with excitation and emission wavelengths of 485 and 535 nm. The results were expressed as percentage of stimulation with respect to the basal value without phorbol myristate acetate.

**Total and Oxidized Glutathione**

Total glutathione (mainly formed by reduced glutathione or GSH) as well as its oxidized form (GSSG) were assayed by the enzymatic recycling method of Tietze (27), with some modifications (28), by monitoring the change in absorbance at 412 nm. Briefly, aliquots of 1 mL of the peritoneal suspension (10^6 leukocytes per mL Hank’s medium) were centrifuged at 1200 g for 10 minutes at 4°C. Pelleted cells were resuspended in previously degassed media, containing 5% of trichloroacetic acid in HCl 0.01 N for GSH measurements, and phosphate buffer 50 mM pH 7.0 plus ethylene diamine tetraacetic acid (1 mM; Sigma) and N-ethylmaleimide (12.5 mM; Sigma) to block reduced GSH for samples aimed to measure GSSG. Then, samples were sonicated and centrifuged at 3200 g for 5 minutes at 4°C. Aliquots of supernatants of immune cells were measured using the reaction mixture: 5,5'-dithiobis (2-nitrobenzoic acid); 6 mM; Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH; 0.3 mM) (Sigma) and glutathione reductase (10 U/mL; Sigma). The reaction was monitored for 240 or 140 seconds for GSH or GSSG determinations, respectively, and the results were expressed as nmol per 10^6 leukocytes.

**Xanthine Oxidase**

Xanthine oxidase (XO) activity was determined by fluorescence using a commercial kit (Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes) in 50 μL of the peritoneal suspension adjusted to 10^6 leukocytes per mL Hank’s medium. In the assay, XO catalyses the oxidation of purine bases (xanthine) to uric acid and superoxide. The superoxide spontaneously degrades in the reaction mixture to H2O2, which in the presence of horseradish peroxidase, reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. After 30 minutes of incubation, measurement of fluorescence was performed in a microplate reader using excitation at 530 nm and emission detection at 595 nm. Levels as low as 0.1 mM/mL of XO can be detected. Protein content of the same samples was evaluated following bicinchoninic acid Protein Assay Kit protocol (Sigma). The results were expressed as international milliunits (mU) of enzymatic activity per milligram of protein.
NFκB Activation

Nuclear and cytoplasmic extracts were isolated as previously described (29) and frozen at −80°C until used in electrophoretic mobility shift assays (EMSAs). The electrophoretic mobility shift assays were performed as previously described by Tato and coworkers (30). Briefly, double-stranded oligodeoxynucleotides corresponding to the palindromic κB target site (5'-GGGAATTCCC-3'; MWG-Biotech AG, Ebersberg, Germany) were labeled by filling the overlapping ends with the Klenow fragment of DNA polymerase I (Invitrogen, Paisley, UK) and [32P]dCTP (Amersham Biosciences, Buckinghamshire, UK). Unincorporated nucleotides were removed and labeled oligonucleotide (100,000 counts per minute) was incubated with 15 μg nuclear protein extract and 2 μg poly(dI-dC) (Amersham Biosciences) in buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl2, 17% glycerol and, just before use, 1 mM dithiothreitol (Sigma) and 0.7 mM phenylmethylsulfonyl fluoride (Sigma) added, in a final volume of 23 μl for 15 minutes at 20°C. Complexes were separated on 5.5% polyacrylamide gels run on 0.25% Tris–borate–ethylene diamine tetraacetic acid. Gels were dried and exposed using a Phoimager. Bands were analyzed by ImageQuant Program. The results were expressed as densitometry arbitrary units.

Antioxidant Enzymes

The peritoneal suspension was adjusted to 10⁶ leukocytes per mL in Hank’s medium and aliquots of 1 mL were used to perform the enzymatic assays. The cells were centrifuged at 1200g for 10 minutes at 4°C, and the pellets were resuspended in 50 mM phosphate buffer pH 7.0 previously degassed. Thereafter, the samples were sonicated and centrifuged at 3200g for 20 minutes at 4°C. Glutathione peroxidase (GPx) enzyme activity was measured using the original technique described by Lawrence and Burk (31) with some modifications (28). This method is based on the oxidation of glutathione in the presence of cumene hydroperoxide (0.71 mM in phosphate buffer; Sigma) by glutathione peroxidase. A reaction solution was used, containing GSH (4 mM; Sigma), glutathione reductase (1 U/mL; Sigma), β-NADPH (0.2 mM; Sigma), ethylene diamine tetraacetic acid (1 mM; Sigma) and sodium azide (4 mM; Sigma). The reaction was followed spectrophotometrically during 300 seconds by the decrease of the absorbance at 340 nm due to NADPH oxidation. The results were expressed as mU of enzymatic activity per 10⁶ cells.

Catalase activity (CAT) was determined following the method described by Beers and Sizer (32) with slight modifications previously introduced by us (28). The enzymatic assay was followed spectrophotometrically for 80 seconds at 240 nm through the decomposition of H₂O₂ (14 mM in phosphate buffer) into H₂O + O₂. The results were expressed as International Units (U) of enzymatic activity per 10⁶ cells.

Statistical Analysis

The data are expressed as mean ± standard error (SE). The normality of the samples and the homogeneity of variances were checked by the Kolmogorov–Smirnov and Levene analyses, respectively. Differences due to age were studied through the one-way analysis of variance. The Tukey test with a level of significance set at p < .05 was used for post hoc comparisons when variances were homogeneous, whereas its counterpart analysis Games–Howell set at the same significance level was used with unequal variances. Differences as compared with the adult age group are shown.

RESULTS

Preserved Immune Functions in Leukocytes of Naturally Long-lived Mice

These studies were performed using unfractionated peritoneal leukocyte isolates, containing mainly lymphocytes, macrophages, and NK cells as previously described (13), in order to better reproduce the in vivo immune response.

Lymphoproliferation, IL-2 levels, and NK activity

One of the most important functions of lymphocytes crucial for their role in immune responses is their proliferative capacity. Proliferation stimulated by the T-cell mitogen ConA (Figure 1A) was preserved in the extreme long-lived as compared with adults, whereas old animals showed a significantly decreased response (p < .001). In contrast, proliferation in response to LPS (Figure 1B), mimicking bacterial infection, declined in lymphocytes from extreme long-lived animals when compared with adults (p < .01), although the loss of proliferative capacity was much less than that seen in the old and very old age groups (p < .001). For Con-A-stimulated IL-2 production (Figure 1C), supernatants of 48-h cultures of peritoneal leukocytes from old and very old individuals showed significantly reduced levels when compared with younger animals (p < .001), whereas levels of IL-2 in the presence of ConA were found to be increased in extreme long-lived animals (p < .01). NK cytolytic activity (Figure 1D) was lower in very old animals as compared with adults (p < .05), but extreme long-lived animals showed levels of this function similar to the adults.

Macrophage Functions and ROS Levels

The chemotactic capacity of macrophages toward the formylated peptide fMLP (Figure 2A) was significantly impaired in very old animals (p < .001) when compared with adults. In contrast, peritoneal macrophages from extreme long-lived mice showed increased migration compared with macrophages from adult individuals (p < .05). Moreover, phagocytosis (Figure 2B and C), a key function of macrophages, was reduced in both old and very old animals (p < .001) as compared
IMMUNE FUNCTION, OXIDATION, AND NFKB IN LONGEVITY

with adults. Importantly, macrophages from old and very old individuals not only phagocytosed less beads (Figure 2B), but also these participants had reduced numbers of macrophages with phagocytic ability (Figure 2C). In contrast, the peritoneal macrophages from extreme long-lived animals showed intact phagocytic function. Stimulation of ROS levels by phorbol myristate acetate (Figure 2D), which is a powerful activator of NADPH oxidase responsible for oxidative burst, was decreased in extreme long-lived individuals when compared with the adult group ($p < .05$).

Taken together, these data confirm previous reports of reduced immune response with age but reveal that those animals surviving to extreme old age in fact display an immune response more similar to that seen in younger adults.

Preserved Redox State and NFκB Activation in Leukocytes of Naturally Long-lived Mice

Oxidants and NFκB Activation.—GSH is the principal intracellular antioxidant and plays a major role in maintenance of the intracellular redox state. GSSG represents an important oxidant compound. The levels of GSSG in peritoneal leukocytes (Figure 3A) from old animals was increased compared with adults ($p < .05$), but not in the extreme long-lived animals. XO is an enzyme that catalyses the oxidation of purine bases to uric acid in a reaction that generates ROS and can also act as an NADH oxidase. XO activity (Figure 3B) was found to be increased in leukocytes from old individuals in comparison with adults ($p < .01$), but extreme long-lived mice showed similar levels to the adults. Sustained activation of NFκB results in expression of a large repertoire of proinflammatory genes leading to increased oxidative stress. Thus, nuclear extracts of peritoneal leukocytes were generated and the presence of binding activity of NFκB–DNA was assessed by electrophoretic mobility shift assays (Figure 3C). The results show a low level of basal NFκB activation in cells from adult and extreme long-lived animals. However, leukocytes from both old and very old animals showed greater activation of NFκB compared with the adult animals ($p < .001$), suggesting chronic activation of this transcription factor in resting leukocytes with aging, but not in those animals that achieved extreme old age. It is important to note that only 1 of 10 individuals analyzed from the old and very old age groups ultimately achieved extreme longevity (138 and 132 weeks, respectively), and interestingly these two mice were the ones showing controlled NFκB activation in their leukocytes.
Antioxidants.—GSH content (Figure 4A) was reduced in resting peritoneal leukocytes from old animals \( (p < .001) \) as compared with adults. However, leukocytes from extreme long-lived mice showed similar levels to those from adult animals. In addition, GPx activity (Figure 4B), which allows the antioxidant function of GSH and can also act as direct antioxidant, was lower in leukocytes from both old \( (p < .001) \) and very old \( (p < .01) \) animals when compared with adults. Again, leukocytes from extreme long-lived animals showed preserved enzymatic activity. Moreover, CAT (Figure 4C), which detoxifies from \( \text{H}_2\text{O}_2 \) and protects against severe oxidative stress, was also reduced in peritoneal leukocytes from very old mice \( (p < .05) \) in comparison with adults, whereas extreme long-lived animals showed increased values \( (p < .01) \).

**DISCUSSION**

By their ability to survive well beyond average life expectancy, extremely long-lived individuals of any species would be expected to have withstood the detrimental effects of the aging process better than those individuals who do not live to extreme old age. The study of such individuals may thus provide important insights into the aging process and the processes that result in frailty in a wide range of physiological systems. The dysregulation and overall decline in the immune response with age is well documented and is evidenced by the higher risk and severity of infections and the increased susceptibility to cancer in aged individuals (3,4). The present study examined immune cell functions in mice of four different age groups, including those reaching extreme longevity. The results demonstrate that peritoneal immune cells, including mainly lymphocytes, NK cells, and macrophages, from exceptionally long-lived animals preserved their function in response to stimuli and showed controlled oxidative status, which seems to be, at least in part, mediated by strengthened CAT, and minimal NFκB activation in resting conditions. In contrast, the old and very old groups of mice showed impaired leukocyte functions and increased oxidation/inflammation status, confirming previous findings of our group and others (12,17). Although this was a cross-sectional study, we did monitor the life spans of mice in the old and very old groups and only one animal in each group reached extreme old age. Interestingly, these were the only animals within these groups that showed controlled basal NFκB activation in their peritoneal leukocytes. These data suggest the importance of maintaining both redox status and low NFκB activity for preserving immune function and achieving extreme old age.
Age-related alterations in immunity have been extensively studied and T cells are considered to be extremely sensitive to immunosenescence (3,4,17). Longitudinal studies in elderly individuals have defined an immune risk phenotype in humans, which is predictive of significantly decreased survival, and includes a low lymphoproliferative response (5,6). Similar results have been reported in rodents (12). T-lymphocyte proliferative responsiveness to antigens or mitogens decreases with age, as does the expression of the costimulatory molecule CD28 and production of the T-cell growth factor, IL-2 (17). The lower production of IL-2 with aging has been related to a skewing of CD4 T-cell responses during infection from a T helper 1 toward a T helper 2 profile (33,34). The present work revealed that extremely long-lived mice showed preserved ConA-stimulated proliferation and increased IL-2 production. This suggests that extended longevity is associated with maintenance of immune homeostasis including a preserved T helper 1/T helper 2 profile dominance. The relevance of these data to longevity is supported by epidemiological studies in humans showing that maintenance of the lymphoproliferative response to ConA is predictive of low morbidity and mortality (6).

LPS is known to directly activate innate immune cells, such as macrophages, and can also activate B cells independently of antigen. B cells also reduce their proliferative response with aging (35,36). The present study confirms our previous data, in BALB/c mice, showing an effect of age on lymphocyte proliferation in response to LPS, revealing that the extremely long-lived mice showed a higher proliferative capacity than the old and very old groups (13).

In addition to an intact lymphoproliferative response, extreme longevity in the mice was also associated with preserved lymphocytotoxic activity. The latter plays a central role in combating both viral infections and cancers, and epidemiological data have shown that the majority of cancers occur in patients over the age of 65 years, but cancer incidence and mortality show a leveling off around the age of 85–90 years, and thereafter reach a plateau and decline (37). A growing literature shows the decrease of antitumour NK cell activity with aging (10,12), in agreement with the present work, resulting in higher morbidity and mortality (6). Importantly, extreme long-lived mice maintained their NK cytotoxic activity, a finding also reported for human centenarians (12,38,39).

Figure 3. Oxidants and nuclear factor kappa B (NFkB) activation. (A) Oxidized glutathione (GSSG, nmol per 10⁶ leukocytes x 10⁻³) and (B) xanthine oxidase activity (XO, mU/mg protein) in peritoneal leukocytes from adult (A), old (O), very old (VO) and extreme long-lived (EL) female mice. (C) Upper panel–NFkB activation in nuclear extracts of peritoneal leukocytes (Densitometry Arbitrary Units, DAU); and lower panel–representative electrophoretic mobility shift assays; the old and very old animals corresponding to the bands marked with arrows were the only individuals from their age groups achieving extreme longevity. Each column represents the mean ± standard error of 7–14 values corresponding to that number of animals and each value being the mean of duplicate assays. Notes: ***p<0.001, **p<0.01, *p<0.05 with respect to the value in adult animals.
affect membrane fluidity, for example, through increased 
rafts has been shown to be altered in neutrophils from 
older humans (44). Increased oxidative stress is known to 
found in membrane lipid raft function with age, which 
would be expected to modify immune receptor function. 
Interestingly, the signaling pathways affected were diverse 
chemotactic activity (41) and proliferative responses (43). 
Moreover, phagocytes from human centenarians have 
shown increased CAT as compared with 25- to 35-year-old 
individuals (11), and increased CAT in transgenic mice can 
delay aging and has been found to extend both median and 
maximum life span (46,47). The decrease in antioxidant 
defenses in leukocytes from old and very old animals 
compared with adults was coincident with the increase in 
oxidation markers, namely enhanced GSSG content and 
basal XO activity. GSSG levels in leukocytes have previ 
ously been described to be higher in old mice (24), in agree 
ment with the present results. In contrast, successfully aged 
animals showed GSSG levels and basal XO activity similar 
to adult individuals. Thus, taken together, extreme long-
ived animals show a well-preserved redox state, in terms of 
antioxidant defenses and oxidant compounds, in peritoneal 
leukocytes, which could be related to the well-conserved 
function in response to stimuli of these cells.

Although phagocytes were thought to play a less critical 
role in the immune dysfunction occurring in the elderly, 
more recent investigations have shown that the general 
decline in the function of these cells is a major reason for 
the increased susceptibility to bacterial and viral infections 
among aged individuals (12,34,40,41). However, little is 
known of phagocyte function in successful aging, though 
decreased phagocyte function has been found to be related 
to shorter life span in old individuals in both mice and hu-
mans (2,11,42). In the present study, extremely long-lived 
mice showed preserved macrophage phagocytic function, 
suggesting that preservation of this function could also con-
tribute to extend survival. Phagocyte migration was addi-
tionally found to be impaired in old and very old mice, 
confirming previous work in humans and mice (7,26,41), 
whereas extremely long-lived animals showed a well-
 preserved migratory function. Recent studies have reported 
a decrease with age in cell signaling elicited by ligation of a 
wide variety of immune cell receptors, leading to alteration 
of the receptor-driven functions of leukocytes, including 
chemotactic activity (41) and proliferative responses (43). 
Interestingly, the signaling pathways affected were diverse 
(41,43,44), suggesting that the effects of aging are not re-
ceptor specific and are targeted at events proximal to the 
membrane receptor. In this context, alterations have been 
found in membrane lipid raft function with age, which 
would be expected to modify immune receptor function. 
For example, recruitment of the receptor TREM-1 into lipid 
rafts has been shown to be altered in neutrophils from 
older humans (44). Increased oxidative stress is known to 
 affect membrane fluidity, for example, through increased 
peroxidation of lipids (45). The data reported here show that 
extreme longevity was associated with the preservation of 
antioxidant mechanisms and we propose that this would 
have a beneficial effect on cell signaling in leukocytes and 
contribute to the maintenance of a variety of functions in-
cluding proliferation, migration, and cytokine secretion. 

Maintenance of low oxidative parameters was indeed 
observed in the leukocytes of extreme long-lived mice, which 
showed preserved leukocyte GSH levels and GPx activity, 
increased basal CAT, and decreased stimulated ROS levels. 
Increased activity of the potent antioxidant enzyme CAT 
could be a key mechanism in preventing endogenous 
damage caused by oxidative burst and chronic inflamma-
tion, leading to long-term preserved immune cell function 
and longevity. Previous studies from our group have found 
reduced CAT in leukocytes from prematurely aged mice, 
which further supports the impairment of this parameter 
as an early driver of aging of the immune system (28). 
Moreover, phagocytes from human centenarians have 
shown increased CAT as compared with 25- to 35-year-old 
individuals (11), and increased CAT in transgenic mice can 
delay aging and has been found to extend both median and 
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leukocytes, which could be related to the well-conserved 
function in response to stimuli of these cells.

Immune cells are constantly exposed to sublethal doses 
of different aggressors that could lead to the creation of a 
chronic low-grade inflammatory state and thereby contrib-
ute to tissue aging (48). This study showed that activation 
of NFκB in resting peritoneal leukocytes increases in old 
and very old individuals and is similar in extreme long-
ived animals to that of adult mice. Moreover, in the old and 
very old groups of mice, only animals showing controlled 
basal NFκB activation in leukocytes achieved extreme lon-
gevity, which further supports the key role played by this 
thranscription factor in aging of the immune system. NFκB 
is a ubiquitous transcription factor known to be activated 
by proinflammatory cytokines and oxidative stress, the lat-
ter present in the old and very old groups of animals. In 
turn, NFκB induces the expression of a variety of genes 
encoding molecules involved in the immune and inflamma-
tory responses such as proinflammatory cytokines and ad-
hesion molecules (49). Thus, activation of NFκB must be 
under tight control because adequate levels of NFκB acti-
vation both in basal conditions as well as in response to 

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**Figure 4. Antioxidants.** (A) Total glutathione (GSH, nmol per 10^6 leukocytes), 
(B) Glutathione peroxidase activity (GPx, nU per 10^6 leukocytes) and (C) cata-
lase activity (CAT, U per 10^6 leukocytes) in peritoneal leukocytes from adult, 
old, very old and extreme long-lived female mice. Each column represents the 
mean ± standard error of 7–14 values corresponding to that number of animals 
and each value being the mean of duplicate assays. Notes: ***p<.001, **p<.01, 
*p<.05 with respect to the value in adult animals.
stimuli are essential for the adequate preservation of cell homeostasis and consequent development of a proper immune response. In contrast, persistent high NFκB activation in basal conditions could be deleterious and has been related to a wide range of aging-related diseases, such as atherosclerosis (50).

It is interesting to note in the present results that very old mice are in several cases more similar to the long-lived and adult animals than to the old mice (see for instance ConA-stimulated lymphoproliferation, GSH and GSSG levels, XO activity), and this might have contributed to the attainment of a life span beyond the average. Conversely, those parameters that show a greater impairment in very old than in old mice as compared with the adult group, but are similar in these animals and the long-lived mice, could contribute to longevity achievement (ie, ConA-stimulated IL-2 levels, NK activity, macrophage chemotaxis and phagocytosis, CAT activity, and NFκB activation). In spite of the limitations derived from cross-sectional data, which do not allow the establishment of the direct relationship between immunity and longevity, our results suggest that the mice that reach much longer than average life spans, do so with an immune profile similar to that of younger adult mice and with few signs of immune decline. In conclusion, the present work supports the proposal that successfully aged individuals would be those who have the better capacity to adapt and deal with immunological stressors throughout life (51) and indicates that controlled NFκB activation in resting leukocytes could contribute importantly to a basal antioxidant environment, mediated at least in part by strengthened CAT, which would allow a well-preserved response to stimuli of immune cells that might help healthy aging and survival.

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**References**


