Granulocyte Macrophage–Colony-Stimulating Factor-Dependent Proliferation Is Impaired in Macrophages From Senescence-Accelerated Mice

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A senescence-accelerated (SAMP8) mouse model was used to determine the effect of aging on the immune system. We produced in vitro bone marrow-derived macrophages from SAMP8 mice and compared them against senescence-resistant, long-lived mice (SAMR1). Although macrophages from both strains of mice proliferated in a similar manner in response to monocyte–colony-stimulating factor (M-CSF), SAMP8 macrophages showed an impaired response to granulocyte macrophage–colony-stimulating factor (GM–CSF). Similar levels of external regulated kinases (ERK)1/2 and signaling transducer and activator of transcription 5 (STAT5) phosphorylation were observed in macrophages from both strains of mice. The lack of proliferation was not caused by the induction of apoptosis. Differentiation of bone marrow cells into dendritic cells was similar in both strains of mice, as was the induction of major histocompatibility complex (MHC) class II molecules by interferon-gamma (IFN–γ). Finally, we determined the density of Langerhans cells in vivo in the skin of the two mouse strains, but no differences were found.

Key Words: Macrophages—Proliferation—Differentiation.

Immuno senescence is an age-dependent dysregulation of immune function and is associated with increased susceptibility to viral and bacterial infections, reactivation of latent viruses, and decreased response to vaccines (1). Traditionally, immunosenescence has been considered to be associated with defects in adaptive immunity in relation to a dysfunctional activity of T-cell (2,3) or B-cell functions (4,5). More recently, a number of defects have been found in innate immunity (6,7).

Macrophages are a heterogeneous group of phagocytes that originate from hematopoietic stem cells in the bone marrow. They are transported in blood to the diverse tissues where they differentiate. Depending on their functional activity and their morphology, these cells receive distinct names (Kupffer cells in the liver, microglia in brain, osteoclasts in bone, etc.). Macrophages are part of the natural immunity and also act as a link between innate and adaptive immune responses through their capacity to process and present antigens (8).

Few studies have addressed macrophages and their functional capacities during aging (9). Aged humans show a decreased number of macrophages (CD68+) as well as a reduced number of precursors in the bone marrow (10). In mice, macrophage activation is the most commonly reported effect of aging. The phagocytic activity of aged mice is impaired (11). We previously reported that interferon-gamma (IFN–γ)-induced expression of major histocompatibility complex (MHC) class II molecules at the cell surface is lower in older mice than in young ones (12). This observation is attributed to a decrease in the transcription factors that bind to the promoter of these genes. This observation has been confirmed in humans, where a decrease in human leukocyte antigen (HLA)-DR/DP expression in monocytes has been reported in elderly individuals (13), suggesting that this decrease contributes to poor T-cell response.

To study the effects or the mechanisms involved in aging, a number of animal models have been used. Several strains of mutant mice present premature senescence phenotypes. This is the case of the senescence-accelerated mouse (SAM) produced during maintenance of AKR/J mice (14). The SAM shows relatively strain-specific, age-associated phenotypic pathologies such as a shortened life span and early manifestation of senescence (including loss of activity, alopecia, lack of hair glossiness, skin coarseness, periphthalmic lesions, increased lordokyphosis, and systemic senile amyloidosis), similar to several geriatric disorders in humans (14,15). Compared with the senescence-resistant, long-lived mice (SAMR1), SAMP8 mice display a shorter life span (14,15). The defects that give rise to this accelerated senescence phenotype are not clear, but several studies suggest that it originates from an increase in oxidative stress (16). To characterize the immune response in this model, we analyzed the functional activity of macrophages and found a defect in granulocyte monocyte–colony-stimulating factor (GM–CSF)-induced proliferation.
MATERIALS AND METHODS

Reagents
Recombinant murine macrophage–colony-stimulating factor (M–CSF), GM–CSF, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The antibodies used were anti-β-actin (Sigma, St. Louis, MO), anti-MHC class II (I-A) FITC conjugate (Chemicon, Temecula, CA), anti-CD11c phycoerythrin conjugate, and anti-CD16/CD32 (BD Pharmingen, San Diego, CA). Peroxidase-conjugated antirabbit (Jackson ImmunoResearch Labs, Cambridgeshire, UK) or anti-mouse (Sigma) were used as secondary antibodies. All other chemicals were of the highest purity grade available and were purchased from Sigma. Deionized water further purified with a Millipore Milli-Q system A10 (Billerica, MA) was used.

Animal Model
Female SAMP8 and their control SAMR1 breeding pairs were obtained from the Council for SAM Research, Kyoto, Japan, through Harlan (Barcelona, Spain). The animals were provided by Dr. J. Mallol (Medical School, Universitat Rovira i Virgili, Reus, Spain) and maintained under a 12-hour light/dark cycle (lights on at 7:00 AM) at 23 ± 1°C. Feeding consisted of regular chow and tap water, under the supervision of veterinarians. The use of animals was approved by the Animal Research Committee of the University of Barcelona (protocol number 2523). Mice were killed at 10 months of age, under anesthesia with an i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline, by exsanguination through cardiac puncture.

Cell Culture
Bone marrow-derived macrophages were isolated as previously described (17). The cells were cultured in plastic tissue culture dishes (150 mm) in 40 mL of Dulbecco’s modified Eagle medium (DMEM) containing 20% fetal bovine serum (FBS) and 30% L-cell-conditioned medium as a source of M-CSF. They were then incubated at 37°C in a humidified 5% CO2 atmosphere. After 7 days of culture (37°C and 5% CO2), a homogeneous population of adherent macrophages was obtained (99% Mac-1+). To render cells quiescent, at 80% confluence macrophages were deprived of M–CSF and maintained in DMEM and 10% FCS for 16–18 hours before the distinct treatments. To be differentiated into dendritic cells (DCs), bone marrow cells were cultured in DMEM, 10% FCS, and 5 ng/mL of GM–CSF (18). At days 2 and 4, the plates were shaken and the culture supernatant collected and replaced by fresh medium with GM–CSF. At days 6 and 8, plates were fed aspirating supernatants (without shaking) and medium, and GM–CSF was then added. At day 8, cells were stimulated with lipopolysaccharide (LPS) from Escherichia coli (Sigma) at 1 μg/mL for 48 hours. At day 10, plates were shaken, the supernatant was collected, and DCs were separated from adherent macrophages.

Proliferation Assay
Macrophages were cultured for 30 hours in 24-well plates (0.5 × 10^6 cells/well) with distinct amounts of recombinant (r)M–CSF or rGM–CSF or without stimulus. Cell proliferation was measured by 3H-thymidine incorporation, as previously described (19). Each step was performed in triplicate, and the results are expressed as mean ± standard deviation. Proliferation was also determined by cell counting using a hemocytometer. In this case, macrophages were cultured with the growth factors for 48 hours and then counted.

Apoptosis Assay
To determine the protection against apoptosis derived from the absence of stimuli, we performed an apoptosis assay using M–CSF and GM–CSF as protectors, and cells deprived of growth factors (DMEM and 10% FCS) or with actinomycin D (Sigma) were used as inducers of apoptosis. Macrophages were cultured for 48 hours in 24-well plates (0.5 × 10^6 cells/well). Cells were collected with trypsin–EDTA (Biological Industries, Beit Haemek, Israel) and incubated with 4 μL of annexinFITC and 2 μL of propidium iodide from an rhAnnexin V/FITC Kit (Bender MedSystems, Vienna, Austria) for 10 minutes prior to fluorescence-activated cell sorting (FACS) analysis.

Protein Cell Surface Expression
Surface expression of IA-β was analyzed with monoclonal anti-mouse IAa,b antibodies (06281D; BD Pharmingen) as described (12). The cells were activated with saturating amounts of IFN-γ (300 U/mL) (17) at a range of times, and then harvested and washed in ice-cold phosphate-buffered saline (PBS). After fixing with 2% paraformaldehyde for 30 minutes at 4°C, the cells were resuspended in 50 μL of PBS containing 5% FBS and then incubated at 4°C for 15 minutes with 1 μg/10^6 cells of anti-CD16/CD32 monoclonal antibody (BD Pharmingen) to block Fc receptors. This antibody was made in rats and is not recognized by the secondary antibody directed against mice (20). Cells were then incubated for 1 hour at room temperature with murine IAa,b specific antibody. Cells were washed by centrifugation through an FBS cushion and finally incubated with FITC-conjugated antimouse immunoglobulin G antibody for 1 hour at 4°C. Stained cell suspensions were analyzed using an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL). The parameters used to select cell populations for analysis were forward and side light scatter. A nonrelated antibody of the same isotype was used as control for specificity.

Analysis of DNA Content with DAPI
Cells (10^6) previously subjected to a specific treatment were resuspended and fixed in ice-cold 70% ethanol (21). They were then washed in PBS; resuspended in 0.2 mL of a solution containing 150 mM NaCl, 80 mM HCl, and 0.1% Triton X-100; and incubated at 4°C for 10 minutes. Afterwards, 1 mL of a solution containing 180 mM Na2HPO4, 90 mM citric acid, and 4’,6-diamidino-2-phenylindole (DAPI) at 2 μg/mL, pH 7.4, was added to each sample. After incubating the cells at 4°C for 24 hours, fluorescence was measured with an Epics Elite flow cytometer (Coulter Corporation). For this analysis, we used an ultraviolet laser with a 25 mW excitation beam at 333–364 nm, and fluorescence was collected with a 525-nm bandpass filter. Cell doublets were gated out by comparing
pulse area versus pulse width. Twelve thousand cells were counted for each histogram, and cell-cycle distributions were analyzed using the Multicycle program (Phoenix Flow Systems, Inc., San Diego, CA).

Western Blot Analysis

Total cytoplasmic extracts were obtained, and western blotting was performed as previously described (22). The antibodies used were antisignaling transducer and activator of transcription 5a (anti-STAT5a), anti-STAT5b (R&D Systems), anti-phospho-STAT5a/b Y694/Y699 (Upstate Biotechnology, Lake Placid, NY), and anti \( \beta \)-actin (Sigma). Detection was conducted using an EZ-ECL kit (Biological Industries). \( \beta \)-Actin expression was measured as a control for differences in loading and transfer.

Determination of External Regulated Kinase Activity by In-Gel Kinase Assay

External regulated kinase (ERK) activity was analyzed as described (23) using 50–100 \( \mu \)g of total protein obtained and separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing myelin basic protein at 0.1 mg/mL (Sigma) as substrate copolymerized in the gel. After several washes, denaturing, and renaturing, a phosphorylation assay was performed with 50 \( \mu \)M adenosine-5’-triphosphate (ATP) and 100 \( \mu Ci \) of \( { }^{32} \)P ATP (GE Healthcare Biosciences, Piscataway, NJ).

Langerhans Cells

After sacrificing the mice, we depilated the dorsal skin with a commercial cream (Veet, Reckitt Benckiser, UK) and rinsed it with tap water. The method to determine Langerhans cells is based on the ATPase reaction, as described (24).

We processed four to six epidermal samples from each animal, and with the aid of an ocular grid we observed 10–20 fields in each sample, so that the variability coefficient was \(< 10\%\). The number of Langerhans cells was expressed per square millimeter.

Statistical Analysis

The nonparametric Mann–Whitney test was used for unpaired differences in all calculations (25).

RESULTS

To determine whether SAM macrophages responded correctly to proliferative, activating, or differentiating stimuli, we used bone marrow-derived macrophages from the SAMP8 strain whereas those from SAMR1 were used as a control. These macrophages were produced in vitro in medium containing M–CSF as a growth factor and constitute a homogeneous population of nontransformed quiescent cells. In addition, the production of macrophages in vitro prevents the presence of exogenous factors that could modulate the functional activity of macrophages in these two strains. In these conditions, we analyzed the effect of premature aging on the genomic expression of macrophages without the interactions of other cell types that may modulate such expression. The number, size, and DNA content of macrophages obtained from SAMP8 and SAMR1 were similar (data not shown). Also, cell surface markers expressed during macrophage maturation, such as Mac 1 (26), were similar in macrophages from both strains. Therefore, the procedure used to obtain macrophages in vitro allowed us to compare the effects of distinct genetic backgrounds only.

To determine whether macrophages of aging animals respond differently to proliferative stimuli, we carried out \(^{3}\)H-thymidine incorporation as a means to measure proliferation. Cells were stimulated for 24 hours with non-saturating amounts of rM–CSF (100 U/mL) (17) or rGM–CSF (3 ng/mL), and then proliferation was measured. In the presence of M–CSF or GM–CSF, macrophages proliferated in a dose-dependent manner (Figure 1A). When we compared each independent experiment for M–CSF-dependent proliferation of macrophages, we found that in three cases the number of counts increased for SAMP8 macrophages in relation to the controls, whereas in four cases they decreased. Therefore, no significant differences were detected when we compared M–CSF-dependent

Figure 1. Granulocyte macrophage–colony-stimulating factor (GM–CSF)-dependent proliferation is impaired in macrophages from senescence-accelerated (SAMP8) mice. A, Macrophages from SAMR1 and SAMP8 proliferate in a dose-dependent fashion in response to macrophage–colony-stimulating factor (M–CSF) and GM–CSF. Quiescent macrophages were treated with increased amounts of growth factors for 24 hours, and then thymidine incorporation was determined. M–CSF– (B) and GM–CSF-induced (C) proliferation was determined after treatment with M–CSF at 1200 U/mL or GM–CSF at 5 ng/mL for 24 hours. Each point represents the mean of a triplicate of an individual mouse.
proliferation of macrophages with that of SAMP8 (Figure 1B). However, in the presence of GM–CSF, macrophages from the control mouse showed greater proliferation than those from SAMP8. Consequently, a significant decrease in proliferation occurred in SAMP8 (Figure 1C). Macrophages exposed to M–CSF for 48 hours increased in number in the controls, from $3 \times 10^6$ to 7.2 $\pm$ 0.3 $\times 10^6$, and in SAMP8 to 7.4 $\pm$ 0.6 $\times 10^6$ ($n = 6$). In the presence of GM–CSF, macrophages increased from $3 \times 10^6$ to 5.5 $\pm$ 0.6 $\times 10^6$ in control mice and to 3.9 $\pm$ 0.6 $\times 10^6$ in SAMP8 ($n = 6$). The difference between the increases is significant ($p < .01$).

In an attempt to establish the defect underlined in SAMP8 mice responsible for the impaired proliferation, we tested the signaling pathways activated by the GM–CSF receptor. This growth factor requires the activation of ERK to induce macrophage proliferation (23). GM–CSF-induced ERK activation showed similar levels and kinetics in macrophages from SAMP8 and from control mice (Figure 2A). Thus, this pathway is not responsible for the GM–CSF-dependent impaired proliferation.

In addition to ERK, binding of GM–CSF with its receptor also induces the activation of Janus kinase 2 (Jak2)/STAT5 pathway (27–29) that is involved in the proliferation of many cell types. To study this pathway, we first analyzed the expression of STAT5 proteins. Both STAT5a and STAT5b showed similar levels of protein in macrophages from SAMP8 and from control mice (Figure 2B). Like all the STATs, STAT5 needs to be phosphorylated to become functional. We then examined the phosphorylation status of STAT5; no defect in STAT5 phosphorylation was found in cells from SAMP8 mice (Figure 2C).

The decreased proliferative activity could be attributed to a putative toxic effect of GM–CSF on the macrophages from the aging model. To test this, we determined cell death using Annexin V staining. In the absence of growth factors, control macrophages underwent cell death by apoptosis, whereas macrophages treated with M–CSF or GM–CSF rescued these cells from their fate (22) (Figure 3). In SAMP8 macrophages, both anti-apoptotic stimuli caused an increase in unlabelled cells similar to that of controls.

We assayed a further GM–CSF-dependent activity, which is the differentiation of bone marrow cells into DCs. After a 12-day culture, we obtained an almost identical number of DCs as shown by the two markers, constitutive of MHC class II expression and CD11c, in both cases using macrophages from the two strains of mice (Figure 4). When we counted the number of DCs, the absolute numbers were similar in the SAMP8 strain ($5.6 \pm 0.4 \times 10^5$; $n = 5$) or in...
the control strain \( (5.9 \pm 0.7 \times 10^5; n = 5) \). Neither were differences found when at day 8 we did not stimulate the cells with LPS. For the SAMP8 strain, we obtained DC counts of \( 2.1 \pm 0.3 \times 10^5 \) \((n = 5)\) and for the controls \( 1.9 \pm 0.3 \times 10^5 \) \((n = 5)\).

We have previously reported a defect in the expression of MHC class II molecules in aged mice (30). Because SAMP8 is described as an aging model, we tested the induction of MHC class II molecules by IFN-\( \gamma \) in this strain. After 24 hours of incubation with the cytokine, the number of macrophages in all the mice increased significantly \((p < 0.01)\) (Figure 5). However, comparison of the levels of induction showed that in four SAMP8 animals, the levels increased in relation to the controls whereas in three they decreased, showing no significant differences between the two strains (Figure 5).

Finally, we determined the density of Langerhans cells in the skin of both strains. No significant differences were found between SAMP8 and the corresponding controls (Figure 6).

**DISCUSSION**

Progressive decline in immune function and chronic inflammation have been reported as the major causes of mortality and morbidity associated with old age. We do not yet fully understand the cellular and molecular mechanisms involved in aging-associated chronic inflammation and immune dysfunction. An attempt to clarify the mechanisms that underlie aging has been made using SAMP8, a strain that displays a shorter life span (14,15). As regards the immune status of these animals, high levels of pro-inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), and IL-6 have been reported in the SAM strain (31,32). One mechanism that promotes accelerated aging and death in the SAM model is related to the higher oxidative status of these animals (33).

Our data demonstrate for the first time that GM–CSF-dependent proliferation of SAMP8 macrophages is impaired compared to that of SAMR1 controls. The experiments were carried out using bone marrow-derived macrophages produced in vitro, a homogeneous population of primary quiescent nontransformed macrophages. In addition, this method enables comparison of the effects of distinct genetic backgrounds on macrophages. The modification of the proliferation was not caused by an increased induction of apoptosis. No modifications were found when we measured the IFN-\( \gamma \)-dependent induction of MHC class II molecules, as observed in macrophages from aged mice (12). This discrepancy could be attributed to the distinct immune response of the two strains, for aged mice C57BL/6 were used, whereas in the present study we used SAMP8. In humans, a decreased expression of MHC class II molecules has been described in monocytes (13), but not in DCs (34–36). This observation may be due to the fact that the molecular mechanisms of MHC class II gene regulation differ between macrophages and DCs (18). A decreased response of macrophages to GM–CSF with age has not been previously reported. However, in neutrophils, several observations suggest an impaired response to this growth factor. GM–CSF fails to delay rapidly induced (37) or spontaneous apoptosis (38) in neutrophils of elderly humans. This finding may be due to an alteration in the signaling of phosphoinositol 3-kinase (PI 3-kinase) and ERK1/2. Recently, a similar defect has been described in DCs from elderly individuals (34).
Finally, a defect in GM–CSF-induced chemotaxis has been observed in neutrophils of aged individuals (39).

Precise control of the amplitude and duration of nuclear ERK signaling is fundamental for cell homeostasis and is achieved by the coordinated action of numerous proteins that participate in import, export, and ERK cytoplasmic and nuclear anchoring (23). Aged tissues and in vitro senescent cells show impaired ERK signaling. For example, terminally arrested senescent fibroblasts show a significant loss of nuclear ERK activity (40), which results in impaired activation of certain transcription factors, such as Elk-1, which in turn drives c-fos expression and cell-cycle progression (41). Restoration of nuclear ERK activity can bypass a critical senescence checkpoint and, thus, extend replicative life span (42). In our model with nontransformed macrophages, we found no modifications in the early steps of GM–CSF-dependent ERK activation between macrophages from the two strains of mice.

Another pathway activated by GM–CSF involves STAT5, and it has been reported that constitutively active STAT5A induces a p53- and Rb-dependent cellular senescence response (43). Our results showed that, in macrophages of both strains, STAT5 is induced in a similar way.

Interestingly, when we differentiated bone marrow into DCs using GM–CSF, no differences were found between the two strains of mice examined. This observation is supported by studies in vivo, in which we determined the population of Langerhans cells that represent a group of DCs (44). The lack of differences in DCs of the two strains in response to GM–CSF can be explained in two ways. First, M–CSF differentiation into macrophages may impair the receptor or signaling transmitted after GM–CSF engagement. A second possibility is that distinct pathways are used for proliferation or for differentiation. The GM–CSF receptor shares a common β-chain with the receptors of IL-3 and IL-5 (45). Also, the three ligands have three ligand-specific α-subunits that provide specificity and alone do not appear to transduce any of the biological activities ascribed to GM–CSF, IL-3, and IL-5. The β-chain subunit, in contrast, converts the ligand-bound subunit to a high-affinity state and is relevant for most, if not all, of signaling. GM–CSF activates at least three pathways: the JAK/STAT, the ras/mitogen-activated protein (MAP) kinase, and the PI 3-kinase. These pathways should not be viewed as being mutually exclusive, and may have substantial overlap. Although the β-chain lacks intrinsic kinase activity, GM–CSF induces tyrosine phosphorylation of the β-chain as well as a number of cytoplasmic proteins including: kinases (PI 3-kinase); adaptors (Gerb1, Cbl, SHC, etc.); guanine nucleotide exchange factors (Vav); phosphatases (Src homology 2 [SH2]-domain protein tyrosine phosphatase-2 and SH2-containing inositol phosphatase); and transcription factors (STAT5). This phosphorylation is mediated by receptor-associated kinases such as JAK2 and src-family kinases. The membrane proximal region of the β-chain contains a preserved proline-rich motif termed “box 1” and serves as a binding site for JAK2, and kinase activity occurs most likely because of JAK2 transphosphorylation. This phosphorylation is important in transmitting signals from the cell surface to the nucleus. One such example is the signal transducer and activator of transcription molecule, STAT5. The phosphorylation by JAK2 results in STAT5 activation, dimerization, and translocation to the nucleus, where it is directly involved in regulating gene transcription (28). It is also possible that the lower amount of activity of GM–CSF is compensated by a stronger activity of LPS (46), thereby resulting in the same number of differentiated cells. However, this possibility was excluded because, in the absence of LPS, we obtained the same number of DCs.

Other possibilities that explain our results are related to deactivation cascades, mostly through dephosphorylation.

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