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Abstract

The limited amount of information on the primary age-related deficiencies in the innate immune system led us to study the production of inducible nitric oxide synthase (iNOS), arginase, and cytokines in macrophages of young (8 weeks old) and old (72 weeks old) female BALB/c mice. We first evaluated iNOS and arginase inducers on peritoneal (PMΦ) and bone marrow-derived (BMMΦ) macrophages of young BALB/c and C57BL/6 mice, and then investigated their effects on macrophages of old mice. Upon stimulation with lipopolysaccharide (LPS), resident and thioglycolate-elicited PMΦ from young mice presented higher iNOS activity than those from old mice (54.4%). However, LPS-stimulated BMMΦ from old mice showed the highest NO levels (50.1%). Identical NO levels were produced by PMΦ and BMMΦ of both young and old mice stimulated with interferon-γ. Arginase activity was higher in resident and elicited PMΦ of young mice stimulated with LPS (48.8 and 32.7%, respectively) and in resident PMΦ stimulated with interleukin (IL)-4 (64%). BMMΦ of old mice, however, showed higher arginase activity after treatment with IL-4 (46.5%). In response to LPS, PMΦ from old mice showed the highest levels of IL-1α (772.3 ± 51.9 pg/mL), whereas those from young mice produced the highest amounts of tumor necrosis factor (TNF)-α (937.2 ± 132.1 pg/mL). Only TNF-α was expressed in LPS-treated BMMΦ, and cells from old mice showed the highest levels of this cytokine (994.1 ± 49.42 pg/mL). Overall, these results suggest that macrophages from young and old mice respond differently to inflammatory stimuli, depending on the source and maturity of the cell donors.

Key words: Aging; iNOS; Arginase; Cytokines; Murine macrophages; Inflammatory stimuli

Introduction

Aging is accompanied by a reduced efficiency of the immune system, thus compromising the general health of older individuals (1-3). Although the age-dependent changes in the adaptive immune system are well documented, changes in the innate immune system have been poorly described. However, the interdependency of the adaptive and innate immune systems is becoming increasingly clear, since they appear to cooperate at various levels to ensure an efficient immune response against microbes and tumor cells (4). Thus, the malfunctions in one aspect of immunity may severely hinder the functioning of the whole immune system, especially in the elderly.

Cells of the immune system deal efficiently with microbes by producing antimicrobial molecules, as well as free radicals (for a review, see Ref. 5). Nitric oxide (NO) is a toxic gas produced by the action of the enzyme family nitric oxide synthase (NOS), which converts L-arginine to citrulline and NO (6,7). Cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), as well as bacterial lipopolysaccharide (LPS), are responsible for the expression of an inducible form of NOS (iNOS) and the generation of large amounts of NO in neutrophils and macrophages (8-12). Under certain circumstances, NO reacts with superoxide anion to form peroxynitrite, a potent oxidizing molecule that contributes to tissue injury during inflammatory processes (13).

Arginase also metabolizes L-arginine to ornithine and urea; it exists in two forms, arginase 1 and 2 (Arg 1 and Arg 2) (14-16). These arginase isoforms differ in terms of tissue distribution, subcellular localization, immunologic cross-reactivity, and physiologic functions. During early developmental stages, Arg 1 is expressed predominantly in the peripheral nervous system, the digestive system and in tissue leukocytes, whereas Arg 2 is found only in the intestines (16). Cytosolic Arg 1 is involved in ammonia degradation in the urea cycle, and its absence in deficient

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patients leads to hyperammonemia. Arg 2, on the other hand, is a mitochondrial isoform and is mainly involved in ornithine biosynthesis, with its absence not resulting in any phenotypic deficiency (17). The arginases are also involved in the generation of polyamines, e.g., putrescine, spermidine, and spermine, which are important for the regulation of cell proliferation, cell differentiation, and cell death (15,18).

Macrophages may be able to express both arginase isoforms in response to LPS, although the literature is controversial in this regard (19,20). Another way of inducing arginase production is the stimulation of macrophages with Th2 cytokines such as interleukin-4 (IL-4), IL-10, and IL-13. These murine macrophages up-regulate the expression of Arg 1, but not that of Arg 2 (21). On the other hand, inflammatory cytokines such as IFN-γ and TNF-α, alone or in combination with LPS, are able to induce iNOS. It has been suggested that the production of iNOS is usually accompanied by arginase expression, and that this arginase may contribute to buffering the overproduction of toxic NO (17). However, it is unclear whether this balance is maintained with aging. In the present study, we analyzed iNOS and arginase activities, as well as cytokine production, in macrophages from young and old mice submitted to different stimuli. Our results suggest that significant changes take place in macrophages of old mice, depending on the source and maturity of the cell donors.

Material and Methods

Animals

Four-week-old female BALB/c and C57BL/6 mice were supplied by the Multi-Institutional Center for Biological Investigation (CEMIB) of the University of Campinas (UNICAMP). Mice were housed under specific pathogen-free conditions in the animal facilities of the Department of Genetica, Evolution and Bioagents, Institute of Biology, UNICAMP, and were provided with autoclaved food and water *ad libitum*. Both mouse strains were used at 8 weeks of age. Seventy-two-week-old BALB/c mice were also used. The study was approved by the institutional Committee for Ethics in Animal Experimentation (Protocol No. 450-1).

Reagents

LPS from *Escherichia coli* and thioglycolate medium were purchased from Difco Laboratories (USA); RPMI 1640, HEPES, Hank's balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} (H6648), 2-mercaptoethanol, L-arginine, urea, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride, Triton-X, aprotinin, antipain, and α-isonitrosopropiophenone (ISPF) were purchased from Sigma-Aldrich Chemical (USA); IL-4 and IFN-γ were purchased from R&D (USA) and from Genzyme (USA), respectively.

Cells

Macrophages were isolated from the peritoneal cavity of naive (N = 15) or thioglycolate-treated mice (N = 5), as described elsewhere (22). Peritoneal cells were cultured at a density of 2 x 10^5 cells in 96-well plates, 1 x 10^6 cells in 24-well plates or 5 x 10^6 cells in 6-well plates in RPMI-1640 medium containing 10% heat inactivated fetal calf serum (FCS; Brazil) for 2 h at 37°C in 5% CO_2. Non-adherent cells were washed out, and adherent cells were cultured in the presence of appropriate stimuli as indicated below.

Bone marrow-derived macrophages were differentiated from precursor cells in a modification of the procedure described by Munder et al. (21). Briefly, bone marrow cells were obtained by flushing the femurs of mice (N = 5). They were cultured in 6-well plastic plates at a density of 5 x 10^6 cells/well, in 3 mL RPMI-1640 medium containing 10% heat inactivated FCS, 5% horse serum, and a 30% (v/v) concentration of L929 fibroblast culture supernatants, as a source of colony stimulating factors (CSFs) to drive cell differentiation. On day 4, the plates were swirled vigorously, and 2.5 mL of the medium was discarded and replaced with fresh CSF-containing medium. The adherent cells were harvested on day 8 with cold saline, sedimented by centrifugation and then cultured at a density of 2 x 10^5 in 200 µL medium in 96-well plates, of 1 x 10^6 in 1 mL medium in 24-well plates or of 5 x 10^6 in 3 mL medium in 6-well plates.

The strain of *Leishmania major* was supplied by Dr. Fernando de Queiróz Cunha (Faculdade de Medicina de Ribeirão Preto, USP, Brazil) and grown in our laboratory by passages at 5-7-day intervals in an Iscove medium containing 10 mM HEPES, 2 g/L sodium bicarbonate, 25 mg/L gentamicin and 20% FCS. For the phagocytosis assays, the promastigotes (stationary phase) were collected from culture flasks, transferred to centrifuge tubes and centrifuged at 200 *g* for 20 min. The pellet was resuspended in incomplete RPMI medium and the parasites were pelleted at 200 *g* for 20 min. The final sediment was suspended in complete RPMI and counted in a Neubauer chamber, and the suspension was adjusted to 3 x 10^6 forms/mL.

Thymocytes of BALB/c or C57BL/6 mice were treated with dexamethasone to induce apoptosis as described by Fadok et al. (23). Briefly, the thymuses were collected aseptically and placed on sterile Petri dishes containing incomplete RPMI. The organs were macerated and the suspension obtained was filtered through a sieve to separate cell debris. After centrifugation of 200 *g* for 10 min, cells were resuspended in a culture medium. An aliquot of the suspension was labeled with anti-CD3 (clone 2C11)-fluorescein for analysis by flow cytometry (FACScalibur, USA), and 81-99.7% of $CD3^+$ cells were detected in three separate preparations. Cells were plated onto 6-well culture plates (Corning) at a density

of 2 x 10^7 cells in 3 mL/well RPMI containing 10% FCS, 50 mL/L gentamicin and 10 µM dexamethasone, and incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere for 15 to 18 h. Apoptotic cells were then collected from culture plates by gentle pipetting, centrifuged at 200 *g* for 10 min, resuspended in culture medium, and adjusted to a concentration of 5 x 10^6 cells/mL.

Zymosan was prepared from the cell wall of *Saccharomyces cerevisiae*. Briefly, 5 mg baker's yeast (Fleischmann's Yeast; Fermix, Brazil) was transferred to a glass flask, which was filled with 150 mL sterile 0.15 M saline, and heated in a water bath at 100°C (Dubnoff bath; Marconi, Brazil) for 1 h. Aliquots of 50 mL were separated and filtered through a funnel with sterile gauze, and centrifuged several times at 200 *g* for 10 min until the supernatants became clear. The particles were resuspended in 0.15 M saline, adjusted to a concentration of 2 x 10^8/mL and kept at -20°C until use. A 2-mL aliquot of the standard zymosan suspension was added to 200 µL normal rabbit serum and incubated for 15 min at 37°C with occasional shaking. The reaction was stopped by reducing the temperature on an ice bath and by diluting the suspension in cold 0.15 M saline. After centrifugation at 200 *g* for 5 min at 4°C, the supernatant was discarded. Then, the zymosan was washed again in cold 0.15 M saline and the sediment was resuspended in 2 mL HBSS (Sigma). The suspension was adjusted to a concentration of 10^8 cells/mL.

Cell cultures for the evaluation of NO production, arginase activity and cytokines

Peritoneal (PMΦ) and bone-marrow-derived (BMMΦ) macrophages were cultured for 48 h in the presence of LPS (1 to 1000 ng/mL), IFN-γ (0.5 to 50 IU/mL) and IL-4 (0.1 to 10 ng/mL), alone or in combination. The supernatants were then harvested for the determination of NO and cytokine production. Cell extracts were used to measure arginase activity as described below. In some experiments, adherent cell monolayers were cultured simultaneously in the presence of IL-4 and LPS, as indicated in the figures.

NO production and arginase activity were also analyzed in PMΦ harvested from the peritoneal cavities of BALB/c and C57BL/6 mice, and cultured in the presence or absence of LPS, IL-4 and IFN-γ after the phagocytosis of *L. major*, apoptotic lymphocytes and zymosan. Briefly, adherent cells from the peritoneal cavity of mice were seeded at a density of 1 x 10^6 cells/mL in 24-well plates and cultured for 18 h with suboptimal doses of LPS (100 ng/mL), IL-4 (4 ng/mL) or IFN-γ (5 IU/mL). After washing the cultures to remove the stimulus, opsonized zymosan (5 x 10^6), apoptotic lymphocytes (5 x 10^6) or promastigotes of *L. major* (3 x 10^6) were added to culture wells and the plates were incubated for 3 h at 37°C in a CO_2 incubator. After this period, free particles were removed by washing with incomplete medium, and cultures were re-incubated in complete RPMI medium for 48 h in a CO_2 incubator. After this period, the supernatants were collected for the determination of nitrite, and macrophage monolayers were prepared to obtain cell extracts for the determination of arginase activity by conversion of L-arginine to urea.

Measurement of NO

NO was measured as nitrite in the supernatants of macrophage cultures using the Griess reagent, as described elsewhere (10). Briefly, each 50-µL aliquot was mixed with 50 µL 1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, and 2.5% H_3PO_4. Absorbance was measured at 540 nm in a microplate reader (Multiskan II, MS, Labsystem, Finland), and the concentration of nitrite in the samples was determined by comparison with a standard curve of sodium nitrite (5 to 320 µM).

Determination of arginase activity

Arginase activity was measured in cell lysates in the presence of urea, which is a conversion product of L-arginine, according to the procedure described by Corraliza et al. (24), with some modifications. Briefly, cells in 96-well flat-bottomed plastic plates were incubated with 50 µL/well lysis solution (0.1% Triton X-100 solution containing 5 µg aprotinin and 5 µg antipain) in a shaker, for 30 min, at room temperature. Then, 50 µL 50 mM Tris-HCl, pH 7.5, containing 2 mM $MnCl_2$ was added to each lysate, and the enzyme was activated by heating the plates for 10 min at 56°C. The lysates were transferred to centrifuge tubes (25 µL/tube) containing 25 µL 0.5 M L-arginine, pH 9.7, and substrate hydrolysis was performed by incubating the tubes for 60 min at 37°C. The reaction was stopped with 400 µL H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). Then, 25 µL 9% α-isonitrosopropiophenone (dissolved in 100% ethanol) was added to the tubes, which were heated for 30 min at 95°C. After cooling, 50 µL of each reaction mixture was transferred to microplates in triplicate, and absorbance was measured at 540 nm with an ELISA reader (Multiskan II, MS). The concentration of urea was determined by comparison with a standard curve of 1.5 to 300 µg/mL urea.

Cytokine determination

The levels of IL-1α (Quantikine M murine; R&D Systems; test sensitivity, 5 pg/mL), IL-10 (Pharmingen, USA; test sensitivity, 30 pg/mL) and TNF-α (DuoSet ELISA Kit; R&D Systems, Inc.; test sensitivity, 62.5 pg/mL) were measured in macrophage supernatants according to manufacturer instructions. Absorbance was read at 450 nm, with the wavelength of 540 nm used for correction.

Statistical analysis

Data were analyzed with the GraphPad Prism software using the Student *t*-test to compare two groups. One-way ANOVA followed by the Bonferroni test was used for the comparison of more than two groups. Statistical significance was set at $P < 0.05$.

Results

iNOS and arginase activities in PMΦ and BMMΦ of young BALB/c mice

The peritoneal and bone marrow-derived macrophages were cultured for 48 h with LPS, IFN-γ and IL-4, alone or in combination. iNOS activity was measured on the basis of nitrite accumulation in the culture supernatants, and arginase activity was determined by measuring arginine-derived urea in the cell extracts.

LPS and IFN-γ induced nitrite accumulation in the culture supernatants of naive and elicited PMΦ in a dose-dependent manner (Figure 1, Panels A and C, respectively). Arginase activity was already present in the non-stimulated macrophages, since extracts obtained from these cells were able to convert L-arginine to urea (Figure 1, Panels B and D). The addition of IL-4 to peritoneal macrophage cultures

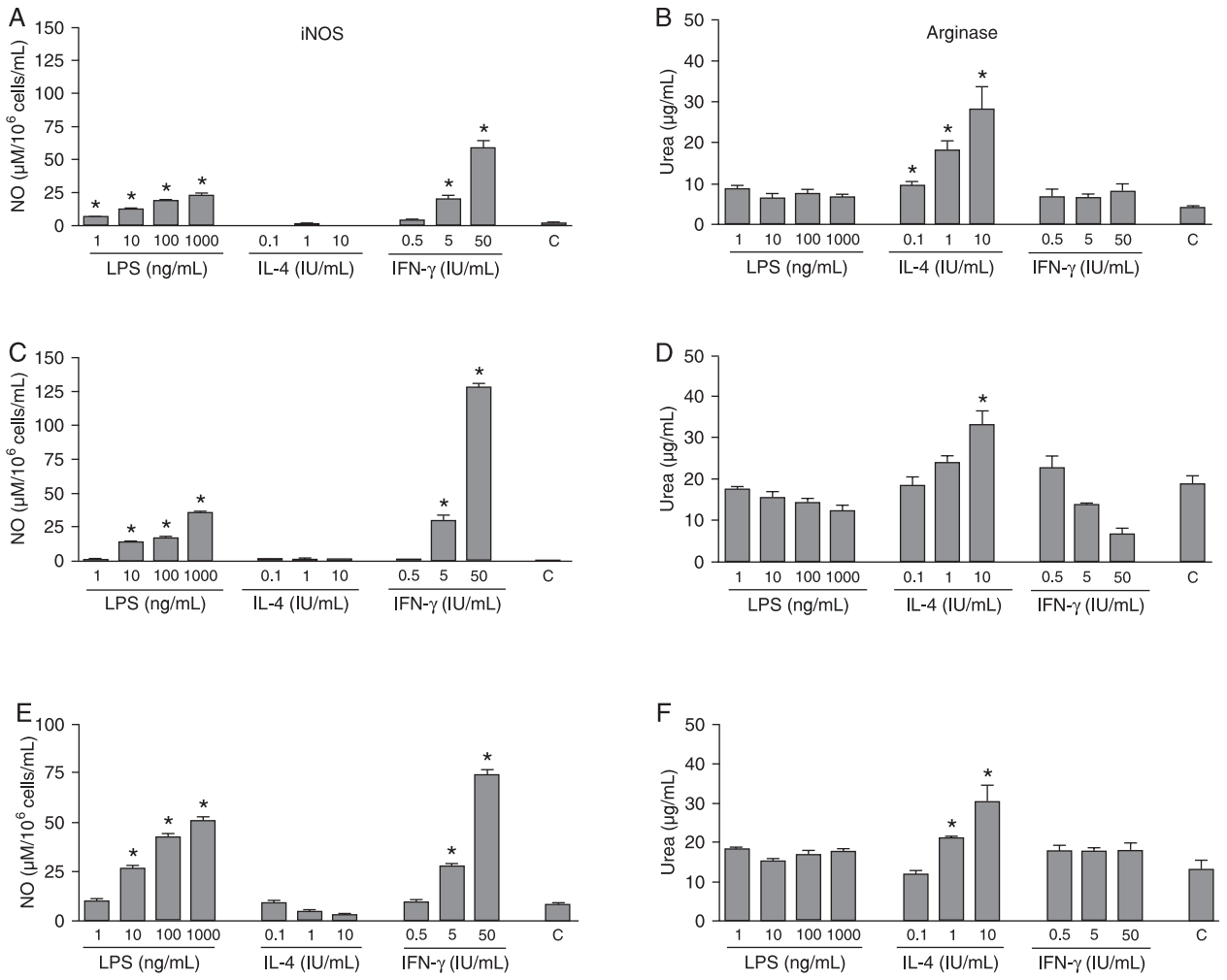

Figure 1. Inducible nitric oxide synthase (iNOS) and arginase activities in macrophages of young BALB/c mice: effects of lipopolysaccharide (LPS), interleukin-4 (IL-4) and interferon-γ (IFN-γ). *A* and *B*, Peritoneal macrophages from naive mice; *C* and *D*, peritoneal macrophages from thioglycolate-elicited mice; *E* and *F*, bone marrow-derived macrophages. Adherent cells from 6 mice per group were incubated in the presence of LPS (1, 10, 100, and 1000 ng/mL), IL-4 (0.1, 1, and 10 IU/mL) or IFN-γ (0.5, 5, and 50 IU/mL) for 48 h; all studies were conducted in triplicate. Cells of control groups (C) were incubated in the absence of stimuli. Nitrite was measured in the culture supernatants using Griess reagent, and arginase activity was determined in cell lysates on the basis of the amounts of urea produced from L-arginine. Bars indicate the means ± SEM of nitrite (Panels A, C, and E) and urea (Panels B, D, and F) levels in three independent experiments. *P ≤ 0.05 compared to control (Student *t*-test).

increased arginase expression in a dose-dependent manner, as observed by the increase in urea conversion rates in these extracts (Figure 1, Panel C), whereas no such results were obtained with the addition of LPS or IFN-γ (Figure 1, Panel D).

BMMΦ cultured in the presence of LPS or IFN-γ also produced elevated levels of NO (Figure 1, Panel E). The exposure of these macrophages to IL-4 also resulted in the elevation of their arginase activity (Figure 1, Panel F).

The treatment of cell monolayers with IL-4 simultaneously with LPS significantly reduced the production of NO in PMΦ of naive mice but not of elicited mice (Figure 2, Panel A). Conversely, treatment with IL-4 parcialy reversed the inhibitory effect of LPS on arginase activity observed in elicited PMΦ (Figure 2, Panel B). Treatment with IL-4 significantly reduced the LPS-induced NO production in BMMΦ (Figure 2, Panel C), but had no effect on arginase activity when added to the cultures concomitantly with LPS (Figure 2, Panel D).

Effects of phagocytosis on iNOS and arginase activities in peritoneal macrophages of young mice

To investigate the role of phagocytosis in arginase activity and NO production, adherent cells from BALB/c and C57BL/6 mice were treated with three different kinds of particles: *L. major*, C3b opsonized zymosan and apoptotic lymphocytes. Figure 3 illustrates the results obtained in these experiments. Phagocytosis of zymosan particles resulted in an elevation of arginase activity in peritoneal macrophage lysates of both naive and elicited BALB/c mice (Figure 3, Panels B and H, respectively), as well as those from elicited C57BL/6 mice (Figure 3, Panel K). The phagocytosis of apoptotic lymphocytes also increased arginase activity in elicited PMΦ of both BALB/c and C57BL/6 mice, but not in those of naive mice (Figure 3, Panels I and L, respectively). Treatment with *L. major* did not alter the arginase levels in

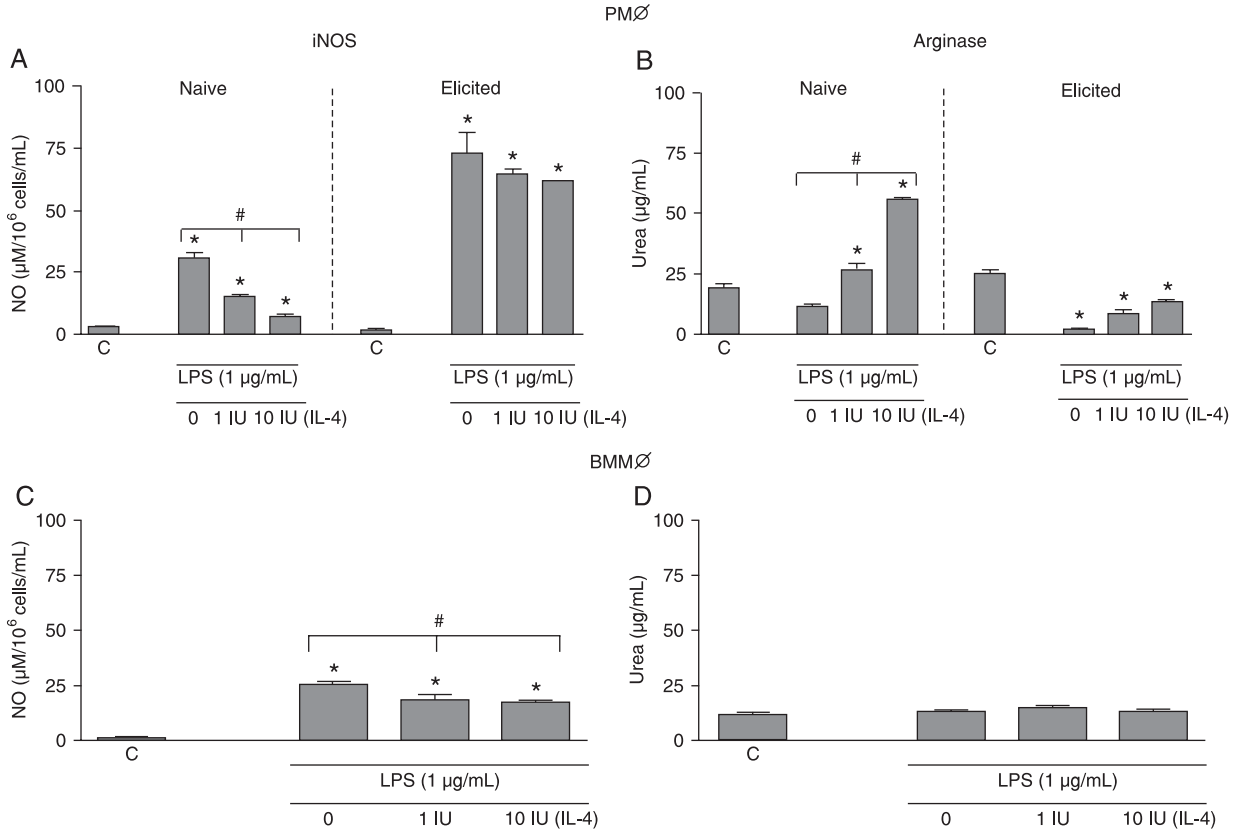

Figure 2. Effects of IL-4 on LPS-induced inducible nitric oxide synthase (iNOS) and arginase activities in macrophages of young BALB/c mice (N = 6 per group). Naive and elicited peritoneal macrophages (PMΦ) and bone marrow-derived macrophages (BMMΦ) from young BALB/c mice were incubated with lipopolysaccharide (LPS; 1 μg/mL) in the presence or absence of interleukin-4 (IL-4; 1 or 10 IU/mL) for 48 h, at 37°C, in 5% CO_2. Cells of the control groups (C) were incubated in the absence of stimuli. Nitrite was measured in the culture supernatants using Griess reagent, and arginase activity was determined in cell lysates based on the amounts of urea produced from L-arginine. All studies were conducted in triplicate. Bars indicate the means ± SEM of nitrite (Panels A, B) and urea (Panels C, D) levels in three independent experiments. *P ≤ 0.05 (comparison was carried out by the Student *t*-test) and #P ≤ 0.05 (comparison was carried out by ANOVA followed by the Bonferroni post-test).

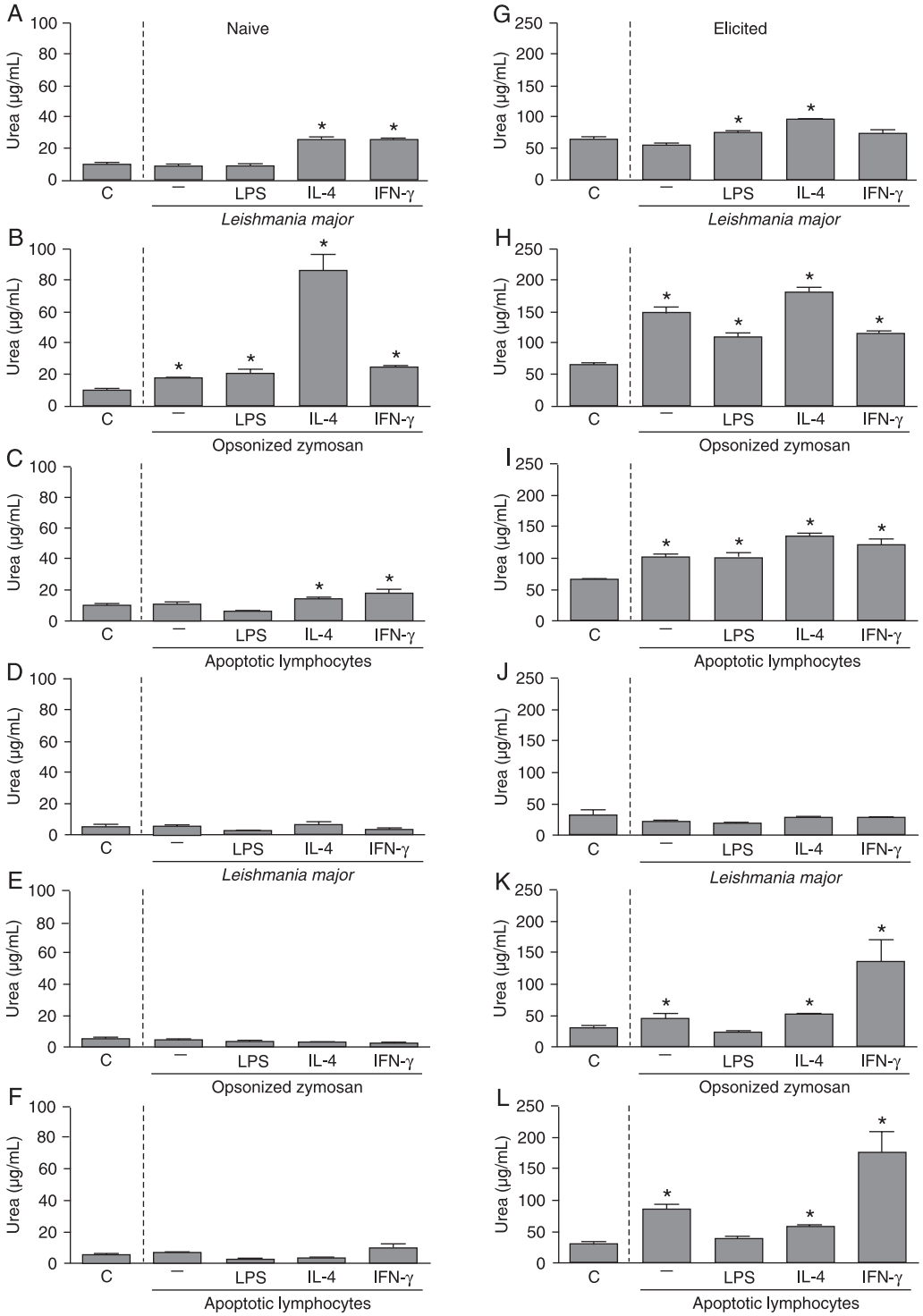

Figure 3. Effects of phagocytosis on arginase activity in peritoneal macrophages of mice (N = 6 per group). *A*, *B*, *C*, Naive BALB/c mice; *D*, *E*, *F*, naive C57BL/6 mice; *G*, *H*, *I*, elicited BALB/c mice; *J*, *K*, *L*, elicited C57BL/6 mice. Adherent peritoneal cells were incubated with lipopolysaccharide (LPS; 100 ng/mL), interleukin-4 (IL-4; 4 IU/mL) or interferon-γ (IFN-γ, 5 IU/mL) for 18 h, at 37°C, in 5% CO_2. Cells of the control groups (C) were incubated in the absence of stimuli. After removal of the stimuli, cells were incubated with *Leishmania major* (3 x 10^6 cells/mL), C3b opsonized zymosan (5 x 10^6 yeasts/mL) or apoptotic lymphocytes (5 x 10^6 cells/mL) for 3 h. Particles were then removed by washing, and the plates were re-incubated for 48 h. Arginase activity was determined in cell lysates on the basis of the amount of urea produced from L-arginine. All studies were conducted in triplicate. Bars indicate the means ± SEM of urea levels obtained in three independent experiments. *P ≤ 0.05 compared to control (Student *t*-test).

adherent cells of either BALB/c (Figure 3, Panels A and G) or C57BL6 mice (Figure 3, Panels D and J).

The pretreatment of adherent peritoneal cells with a suboptimal dose of IL-4 before the addition of phagocytic stimuli resulted in a more marked effect on arginase activity in PMΦ lysates obtained from BALB/c mice (Figure 3, Panels A, B, C and G, H, I), whereas such pretreatment had no effect on arginase activity in those from C57BL/6 mice. Conversely, pretreatment with a suboptimal dose of IFN-γ resulted in more elevated arginase activity in PMΦ lysates obtained from C57BL/6 after phagocytosis of zymosan and apoptotic lymphocytes (Figure 3, Panels K and L). NO production was not observed in macrophage supernatants after phagocytosis of the particles used here.

iNOS and arginase activity in peritoneal and bone marrow-derived macrophages of old BALB/c mice

iNOS and arginase activities were investigated comparatively in PMΦ and BMMΦ from both old (72 weeks of age) and young (8 weeks of age) BALB/c mice. Figure 4 illustrates the results obtained in these experiments. PMΦ from naive old mice exhibited a diminished production of NO in response to both LPS and IFN-γ (Figure 4, Panel A), and lower arginase activity after treatment with LPS and IL-4 (Figure 4, Panel B) than did those from young mice. Thyoglycolate-elicited PMΦ isolated from old mice also revealed slightly lower NO production following stimulation with LPS than did those from young mice. IFN-γ induced similar levels of NO in elicited PMΦ of both young and old mice (Figure 4, Panel A). The arginase activity in response to IL-4 increased equally in both naive and thyoglycolate-elicited PMΦ of young mice (Figure 4, Panel B), and basal levels of arginase activity were observed in naive PMΦ of old mice stimulated with IL-4. BMMΦ obtained from both young and old mice were equally capable of producing NO in response to IFN-γ, but old mice were more responsive to LPS (Figure 4, Panel C). Arginase activity was elevated only in BMMΦ from old mice stimulated with IL-4 (Figure 4, Panel D).

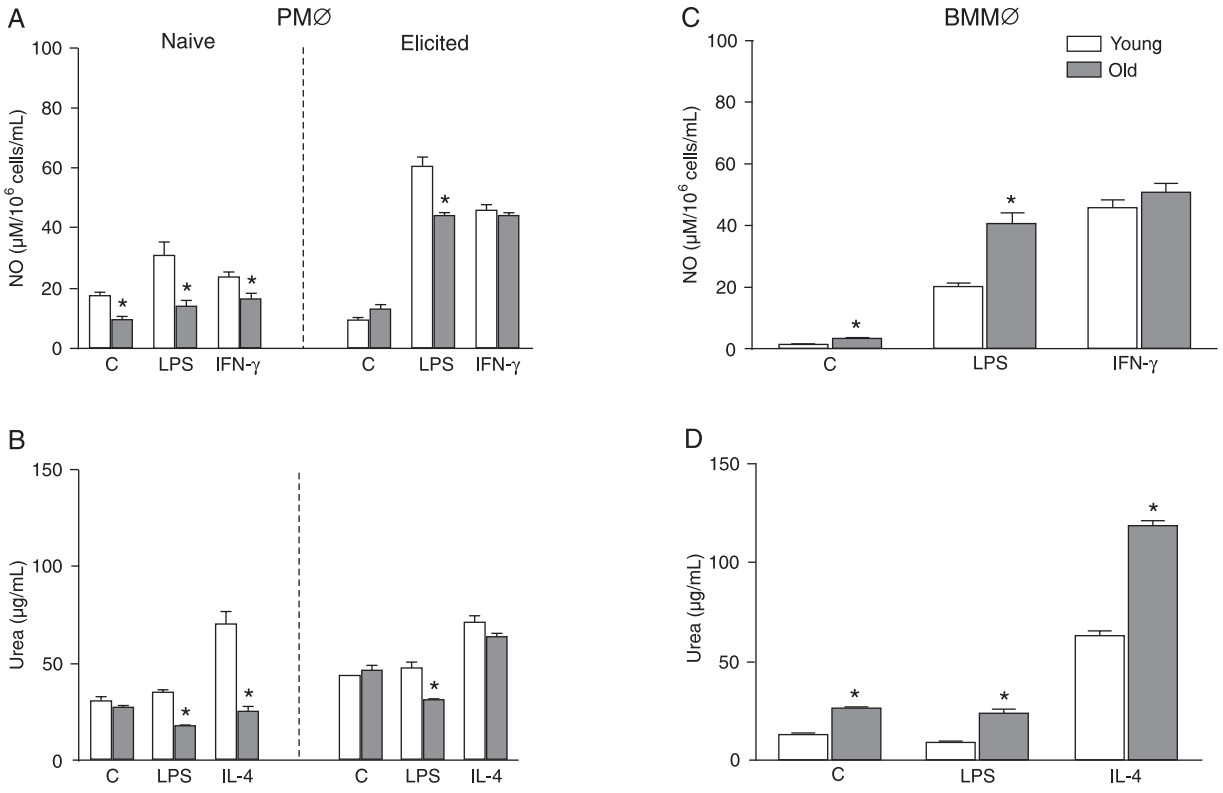

Figure 4. Aging affects inducible nitric oxide synthase (iNOS) and arginase activities in macrophages of BALB/c mice. Naive mice (N = 14) were the source of naive peritoneal macrophages and bone marrow-derived macrophages. Elicited macrophages were obtained from mice (N = 6) injected 4 days previously with thioglycolate medium. Young and old mice were used at the ages of 8 and 72 weeks, respectively. Adherent cells were cultured in the presence of lipopolysaccharide (LPS; 1 µg/mL), interleukin-4 (IL-4; 40 IU/mL) or interferon-γ (IFN-γ; 50 IU/mL) for 48 h at 37°C, in 5% CO_2. Cells of the control groups (C) were incubated in the absence of stimuli. Nitrite was measured in the culture supernatants using a Griess reagent, and arginase activity was determined in cell lysates based on the amounts of urea converted from L-arginine. All studies were conducted in triplicate. Bars indicate the means ± SEM of nitrite (Panels A, C) or urea (Panels B, D) levels in three independent experiments. *P ≤ 0.05 compared to control (Student *t*-test).

Cytokine production

IL-1, IL-10 and TNF-α were measured by ELISA in supernatants of PMΦ and BMMΦ obtained from both young and old BALB/c mice, cultured in the presence of LPS. As depicted in Figure 5, peritoneal macrophages from both naive and elicited mice were capable of producing IL-1 and TNF-α when stimulated by LPS. However, elicited PMΦ from older mice showed a more elevated production of IL-1 (Figure 5, Panel A), whereas those from younger mice produced more TNF-α (Figure 5, Panel B). The production of IL-10, on the other hand, was not affected by LPS stimulation, independent of the age of the mice (Figure 5, Panel C).

Stimulation of BMMΦ with LPS led to production of only TNF-α, with macrophages from aged mice showing the highest levels of this cytokine (Figure 5, Panel E). As shown in Panel D, IL-1 was not produced by BMMΦ.

Discussion

Aging is accompanied by a low-grade elevation in the

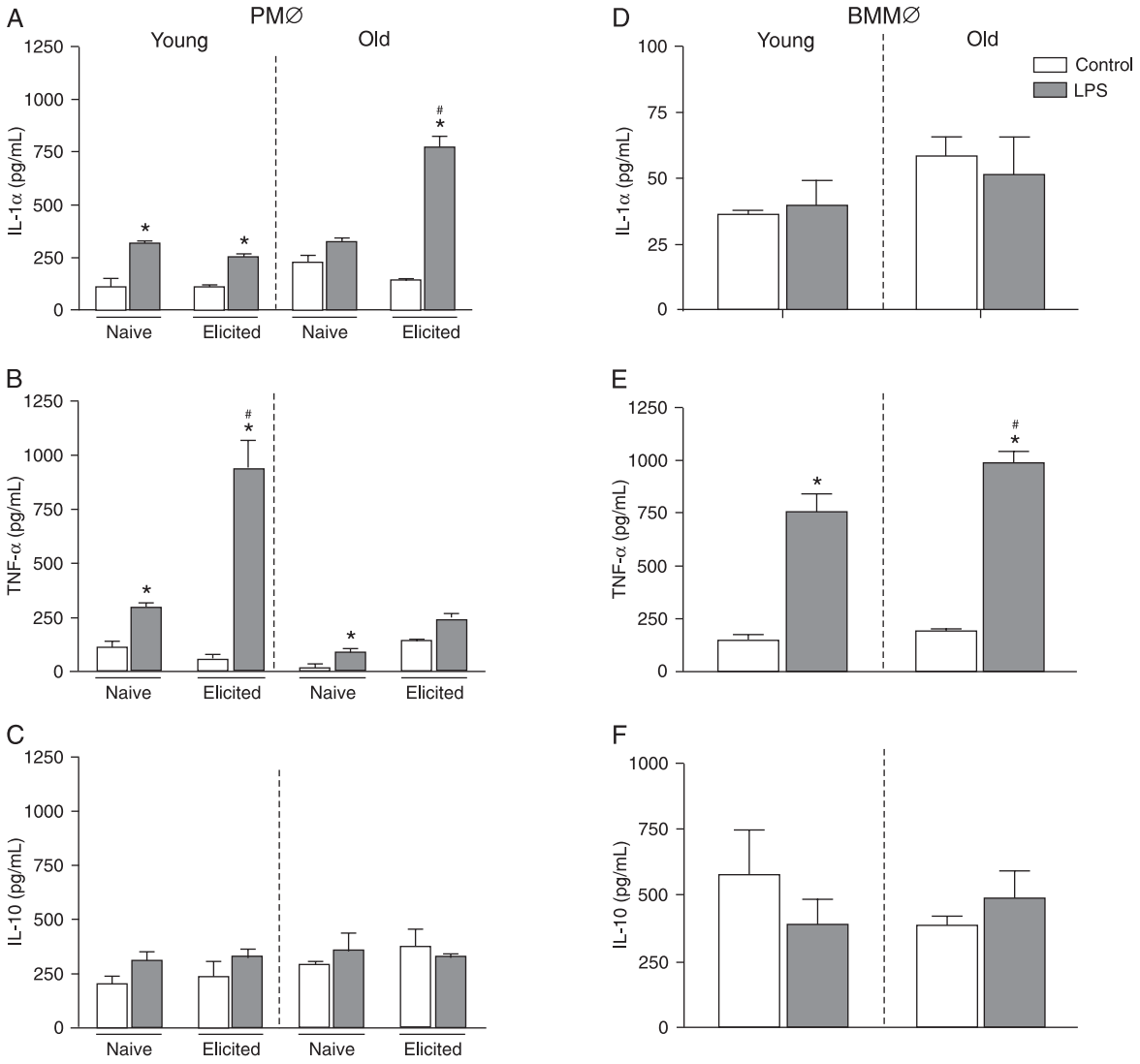

Figure 5. Aging affects cytokine production in peritoneal macrophages and bone marrow-derived macrophages of BALB/c mice (N = 6 per group). Naive and thioglycolate-elicited peritoneal macrophages (Panels A, B, and C) and bone marrow-derived macrophages (Panels D, E, and F) from young and old mice were prepared and cultured as described in the legend to Figure 4. The culture supernatants were used for the detection of interleukin-1α (IL-1α; Quantikine M murine; test sensitivity, 5 pg/mL), tumor necrosis factor-α (TNF-α; DuoSet ELISA Kit; test sensitivity, 62.5 pg/mL) and interleukin-10 (IL-10; Pharmingen; test sensitivity, 30 pg/mL). All studies were conducted in triplicate. Bars indicate the means ± SEM of the respective cytokines detected in three independent experiments. *P ≤ 0.05 compared to control (Student *t*-test).

concentrations of certain circulating inflammatory markers such as cytokines and their receptors, chemokines, and acute phase proteins (25). This state, also called "Inflamm-aging" (26), correlates with neurological conditions such as Alzheimer's disease and various cardiovascular diseases such as atherosclerosis (for a review, see Ref. 2).

In the present study, we analyzed the production of iNOS and arginase enzymes in PMΦ and BMMΦ from young and old BALB/c mice, as well as cytokine production by these cells. Initially, we evaluated the most favorable conditions for the induction of iNOS and arginase activity in PMΦ and BMMΦ from young mice stimulated with LPS, either alone or in combination with IFN-γ or IL-4. NO accumulated in a dose-dependent manner in both types of macrophages treated with LPS+IFN-γ, whereas arginase activity increased only in IL-4-stimulated cells. Moreover, IL-4 inhibited the NO production induced by LPS, thus confirming reports in the literature (24,27-28). LPS, however, did not increase arginase activity in either type of macrophages obtained from BALB/c mice, nor was IL-4 capable of reversing the inhibition of arginase activity induced by LPS. Our results partially contradict those reported by Mills et al. (29), who showed that LPS increased the metabolism of L-arginine to ornithine/urea in resident PMΦ from BALB/c mice at doses as low as 1 ng/mL, whereas macrophages from C57BL/6 mice metabolized L-arginine mainly via iNOS. In the present study, however, neither resident nor elicited PMΦ or BMMΦ from BALB/c mice significantly elevated the conversion rate of L-arginine to urea when LPS was used at doses from 1 to 1000 ng/mL. However, the concept of M-2 macrophages proposed by Mills et al. (29) is still applicable to BALB/c macrophages because of the clear contrasting effects of Th1 and Th2 cytokines on L-arginine metabolism in the macrophages of these mice.

Moreover, we tested the effects of phagocytosis on both arginase and iNOS activity in PMΦ from young BALB/c and C57BL/6 mice. Adherent cells were incubated with opsonized zymosan, *L. major*, and apoptotic lymphocytes, so that different surface receptors would be used for the internalization of the particles. Opsonized zymosan, a complement C3bi-coated insoluble polysaccharide, binds to its specific receptor of the β2 integrin family (CR3, CD11b/CD18) to trigger cytoskeletal reorganization (30) and the activation of many important cell functions, including phagocytosis, superoxide production, and chemotaxis (31). On the other hand, binding of apoptotic cells to the phosphatidylserine receptor on phagocytes stimulates cell internalization and simultaneous secretion of immunosuppressive cytokines (32). However, the initial process of *Leishmania* internalization remains unknown, and recent literature data have ruled out the mannose receptor as the way by which the parasite enters the macrophages (33). In the present study, we observed that NO was not produced in any of these situations; hence, we concluded that BALB/c and C57BL/6 PMΦ are unable to metabolize L-arginine via iNOS following phagocytosis of such different particles, even in the presence of suboptimal doses of LPS or IFN-γ. Arginase activity, however, was affected by phagocytosis and was dependent on the type of stimuli and strain of macrophage donors. In this regard, macrophages from BALB/c showed higher arginase activity after phagocytosis than those from C57BL/6 mice. Pretreatment with suboptimal doses of IL-4 increased the conversion of L-arginine to urea in both naive and elicited BALB/c phagocytic cells, whereas for C57BL/6 macrophages, such treatment was only effective for elicited cells and for the phagocytosis of opsonized zymosan and apoptotic lymphocytes. Similarly, Iniesta et al. (18) reported that BMMΦ from BALB/c mice treated with Th2 type cytokines (IL-4, IL-10, and TGF-β) before incubation with *L. major* presented higher levels of arginase activity than did C57BL/6 mice. Surprisingly, the suboptimal doses of IFN-γ induced an increase in arginase activity in the PMΦ of both BALB/c and C57BL/6 mice that phagocytized opsonized zymosan and apoptotic lymphocytes.

The cellularity in peritoneal washings from old BALB/c mice was higher than that observed in younger ones (data not shown). However, the adherent cells of young mice showed greater responses to LPS than those of older mice, as indicated by the amounts of NO and TNF-α measured in the cultures. On the other hand, BMMΦ from old mice exposed to LPS showed the highest levels of iNOS. In contrast, when the stimulus was IFN-γ, the differences as a function of age and of macrophage source were no longer observed. The responses to the anti-inflammatory cytokine IL-4 followed the same pattern as described for LPS, with the younger cells presenting the more elevated levels of arginase activity. Our results are similar to those reported by Kissin et al. (34) since stimulation of PMΦ with LPS led to a decrease in iNOS activity in older mice. Chen et al. (35), however, reported an increase in iNOS expression in older mice stimulated with LPS. Such discrepancies may be related to the specific experimental model, including factors such as mouse lineage, age range studied and inflammatory stimuli used. Moreover, the literature comparing iNOS and arginase activity in BMMΦ from young and old mice is very limited; such cells have been subjected to more studies involving their antigen-presenting activity, than their response to inflammatory agents (36).

Age also proved to be critical for the production of inflammatory cytokines by PMΦ stimulated with LPS. Cells elicited from older mice produced larger amounts of the pro-inflammatory IL-1α, whereas those from younger mice produced more TNF-α. IL-10 levels of older and younger mice were not changed by LPS treatment in PMΦ or BMMΦ. However, macrophages differentiated *in vitro* presented more elevated constitutive levels of IL-10 than those from the peritoneum, a characteristic that agrees with the tolerant profile of these immature cells (37). The only pro-inflammatory cytokine produced by BMMΦ in response to LPS was TNF-α, with the older mice producing more than

the younger ones. Although some studies have shown an elevation of inflammatory markers in older individuals that correlate with age-related diseases and increased risk of mortality, literature data are inconclusive about this matter in both animal models and human subjects, with some reports showing an age-associated decrease in IL-1 and TNF-α levels and others suggesting an elevation of pro- and anti-inflammatory cytokines after LPS stimulation of macrophages (for a review, see Ref. 38).

The results obtained in the present study indicate that the responses of macrophages from young and old animals may vary according to the microenvironment and to the stimuli to which they are subjected, without necessarily displaying a constitutive inflammatory profile. However, we also speculate that the changes in innate immunity observed here may contribute to the damages associated with the aging process.

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