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Arginine metabolism during macrophage autocrine activation and infection with mouse hepatitis virus 3

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Abstract

In contrast to BALB/c mouse macrophages (MΦ), MΦ from the A/J mouse strain, upon activation by exogenous interferon gamma (IFNγ), develop an anti-mouse hepatitis virus 3 (MHV3) state which correlates with resistance to virus infection. To investigate the autocrine activation of BALB/c and A/J MΦ, we activated them with interleukin-12 (IL-12) and/or IL-18, and quantified IFNγ production, the anti-MHV3 state and arginine metabolism. Synergistic activation by IL-12/IL-18 induced the expression of the IFNγ gene in MΦ from both mouse strains. In bone marrow (BM) or peritoneal (P) MΦ of specific pathogen-free (*spf*) mice of both strains, IFNγ synthesis occurred only with a synergistic IL-12/IL-18 activation and showed increasing levels from 24 to 72 h of activation. In contrast, when non-*spf* mice were used in the assay, their PMΦ synthesized higher IFNγ levels upon activation with only IL-12 or only IL-18 or both. The BALB/c MΦ were always capable of synthesizing higher amounts of IFNγ than the A/J MΦ. An anti-MHV3 state was observed only in A/J MΦ upon activation with IL-12/IL-18 or IFNγ regardless of their origin from the peritoneum or bone marrow. Arginine metabolism in activated and/or virus infected BMMΦ was investigated through nitric oxide (NO) and arginase induction as well as the consumption of arginine and synthesis of citrulline, ornithine and spermine. The results showed that both BALB/c and A/J BMMΦ populations released NO only after activation with IL-12/IL-18 or IFNγ. Arginase was not induced in BMMΦ from both strains by IL-12/IL-18 or IFNγ but only by IL-4/IL-10. Higher arginine consumption was observed in BMMΦ from both strains upon activation with IL-4 or IFNγ which further increased, in this case, when the cells were infected with MHV3. As a consequence of nitric oxide synthase synthesis and arginine consumption in IFNγ activated BMMΦ, we observed a higher synthesis of citrulline. High levels of ornithine were induced only upon IL-4 activation. Polyamine synthesis was higher in A/J BMMΦ than in BALB/c ones, which correlated with the slightly lower levels of ornithine observed. Upon infection with MHV3, we observed a higher synthesis of spermine. IL-12/IL-18 or IFNγ activation, mainly in MHV3 infected cells, led to a decreased synthesis of polyamines, notably spermine, only in A/J BMMΦ. Difluoromethylornithine treatment, which leads to inhibition of polyamine synthesis, induced a decreased MHV3 multiplication in

Abbreviations: BMMΦ, Bone marrow macrophages; DFMO, Difluoromethylornithine; MΦ, Macrophages; MHV3, Mouse hepatitis virus 3; NOS, Nitric oxide synthase; ODC, Ornithine decarboxylase; PMΦ, Peritoneal macrophages; SPF, Specific pathogen-free; uvMHV3, Ultraviolet inactivated MHV3

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both BALB/c and A/J BMMΦ. Altogether these data show the relevance of IFNγ, from the autocrine or paracrine pathway, and arginine metabolism for the control of MHV3 replication in MΦ of a resistant mouse strain.
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Keywords: Arginine; Macrophages; Interferon-γ; MHV3; Polyamines

Introduction

Mouse hepatitis virus 3 (MHV3) is a natural murine viral infection that, in experimental conditions, has a resistance pattern dependent on the genetic background of the animal. BALB/c mice are susceptible and adult A/J mice are resistant to MHV3. Our previous studies have shown that resistance to MHV3 infection in mouse populations can be a consequence of a T-cell dependent mechanism, in which the production of interferon-γ (IFNγ) and the sensitivity of macrophages (MΦ) to IFNγ play an essential role (Vassão et al., 2000). We have shown that the resistance of A/J mice to an MHV3 isolate that we have cloned, selected and amplified, is acquired after immunization with ultraviolet inactivated MHV3 (uvMHV3) and that IFNγ activation induces an antiviral state only in MΦ from resistant mice, leading to a partial restriction of multiplication by significantly delaying virus replication. Recently, we have demonstrated that murine MΦ are capable of secreting IFNγ upon a synergistic stimulation with interleukin (IL)-12 and IL-18, or an autocrine and T-cell independent MΦ activation mechanism (Munder et al., 1998a, 2001).

In MΦ, nitric oxide synthase (NOS) and arginase are inducible enzymes that act on a common substrate, arginine. As a consequence, arginine is oxidized by NOS producing citrulline and nitric oxide (NO) or is hydrolyzed by arginases producing ornithine and urea. Ornithine is in turn catabolyzed by ornithine decarboxylase (ODC) for polyamine (putrescine, spermidine and spermine) biosynthesis or ornithine amino transferase (OAT) for glutamate or proline synthesis (Fig. 1). Difluoromethylornithine (DFMO), an analog of ornithine that inhibits ODC, has been used in the study of polyamine depletion (Wang and Wong, 2003). Polyamines, such as spermine, play several roles in cell differentiation and proliferation (Thomas and Thomas, 2001). Despite contradictory observations, it is generally accepted that polyamines contribute to virus maturation (Cohen, 1998). We have recently shown that a competition between NOS and arginase for their substrate takes place in mouse bone marrow-derived MΦ (BMMΦ) and that they appear to define two alternate functional states of MΦ, induced by, respectively, Th1 (IFNγ) and Th2 cytokines (IL-4 and IL-10) (Munder et al., 1998b, 1999). These cytokines would also differentially regulate NOS2 and arginase 1 in vivo (Hesse et al., 2001).

NO has been considered an important molecule with protective or harmful effects on the organism and is directly involved in antiviral activities (Benencia and Courreges, 1999; Guillemard et al., 1996; Kreil and Eibl, 1995; Pereira et al., 1997; Pope et al., 1998; Saura et al., 1999; Tucker et al., 1996). Protective effects of NO have been related to infections involving ectromelia, vaccinia and herpes simplex 1 viruses (Harris et al., 1995; Melkova and Esteban, 1995), Coxsackie B3 virus (Lowenstein et al., 1996; Saura et al., 1999), Epstein-Barr virus (Kawanishi, 1995) and Japanese encephalitis virus (Saxena et al., 2000, 2001). On the other hand, there are indications that NO may not be essential for virus clearance in vaccinia virus-infected mice (Rolph et al., 1996) or tick-borne encephalitis virus infection (Kreil and Eibl, 1996). Although arginase induction in MΦ and its consequences has been poorly studied (Corraliza et al., 1995, 1997; Modolell et al., 1995), some reports implicate arginase in antiviral activities in MΦ, tumor cell cytotoxicity and immune response (Bansal and Ochoa, 2003; Mistry et al., 2001; Ochoa et al., 2001). More recent studies indicate that arginase induction is involved in host response to microbial infections although its protective or harmful role is uncertain (Morris, 2000; Ryo et al., 2000, Wu and Morris, 1998).

Concerning MHV3 infection, we have suggested in a previous publication that the anti-MHV3 state induced by IFNγ in MΦ, which may contribute to resistance, was not related to arginine metabolism (Pereira et al., 1997). Later published data indicate that resistance to MHV3 is dependent on the production of NO (Pope et al., 1998). Besides the fact that different mechanisms may contribute to resistance, a controversial finding seems to be a defect of NO production in susceptible BALB/c mice. Nevertheless, recent studies indicate that BALB/c mice are capable of producing NO in vivo (Fabre et al., 2004; Kwon et al., 2004). Unpublished data from our group show also that, in certain conditions, BALB/c mice are capable of producing NO in vivo, as evaluated by direct Electron Paramagnetic Resonance (EPR) spectroscopy in blood and liver. Genetic differences of the virus isolate or mouse strain used, as well as the basic mechanism considered for resistance may account for the divergent findings.

In this paper we investigate arginine metabolism associated with the autocrine pathway of IFNγ activation in MΦ of mice resistant and susceptible to MHV3.

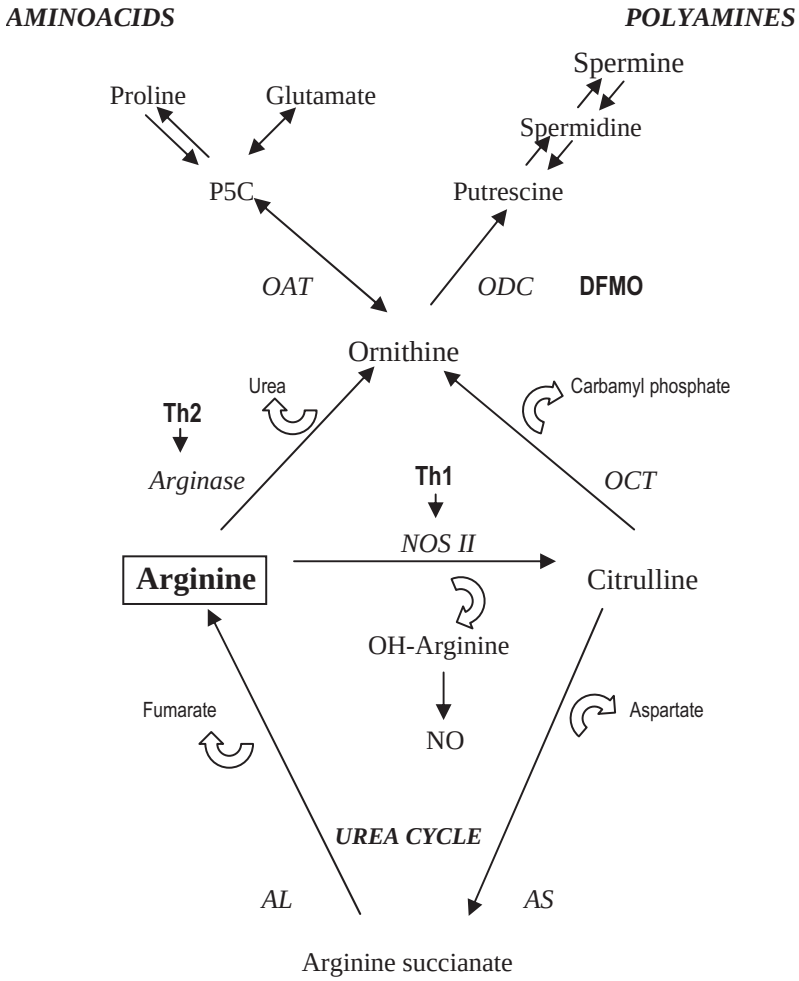


Fig. 1. Scheme of arginine metabolism. *AL*, arginine succianate lyase; *AS*, arginine succianate synthase; NO, nitric oxide; *NOS II*, nitric oxide synthase II; *OAT*, ornithine amino transferase; *ODC*, ornithine decarboxilase; *OCT*, ornithine carbamyl transferase; OH-, Hydroxi-; DFMO, DFMO; Th1/Th2, T helper lymphocytes types 1 and 2 cytokines.

Materials and methods

Mice, macrophages and virus

BALB/c and A/J mice were obtained from the mouse colonies of the Instituto Butantan, Instituto de Ciências Biomédicas of the University of São Paulo or Max-Planck Institute for Immunobiology, Freiburg, Germany. They were kept under conventional conditions and used when 4–8 weeks old. Care of animals followed the appropriate guidelines.

BMMΦ of mice were obtained from bone marrow cells collected from femurs by flushing of cavities with Dulbecco's modified MEM (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 100 U/ml of penicillin (Sigma) and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, MO). As clearly described in another publication (Munder et al., 2001) these BMMΦ population are homogeneously F4/80+, MAC-1+, I-A+, B7.1+, CD16/32+ with no detectable cells expressing T, B or NK cell markers (CD3, CD4, CD8, CD19, CD5, B220, DX5, NK1.1). Cells were cultured in non-toxic Teflon bags (fluorinated ethylene propylene, Biofolie 25; Heraeus GmbH, Hanau, Germany) as described (Munder et al., 1998a). The Teflon foils were folded and sealed with a diathermal sealing apparatus (Polystar 100 B and Polystar 401 M-RPA; Rische + Herfurth GmBH, Hamburg, Germany) to give rectangular bags, and subjected to gas sterilization. Cells (5×10^4/ml) in DMEM with 10% fetal bovine serum (FBS) (Biochrom AG), 5% horse serum (Invitrogen-Gibco Co., Carlsbad, CA), 15% L929 supernatant as a source of colony-stimulating factor, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate (Invitrogen-Gibco) and 0.5% 2-mercaptothanol (Sigma) were incubated for 12 days at 37 °C in the presence of 10% CO_2. Cells were detached by repeated careful stretching of the Teflon bags, washed once with

medium, transferred to 96-well microplates at a concentration of 2×10^5 cells/100 μl and used in the experiments. Peritoneal macrophages (PMΦ) were prepared from peritoneal exudate cells collected by peritoneal lavage with DMEM containing 10% FBS and cultured in 96-well microplates at a concentration of 2×10^5 cells/100μl. The cells were incubated for 2 h at 37 °C in 5% CO_2, washed three times with DMEM after vigorous shaking to remove non adherent cells and used in the experiments. Although carefully treated, the PMΦ population may contain a few other cells.

MHV3, originally obtained by the Institute of Virology, University Louis Pasteur, Strasbourg from Dr. J.L. Virelizier, Institut Pasteur, Paris, was cloned by limiting dilution; one plaque was selected and amplified on L929 cells to serve as the inoculum for future stocks to limit spontaneous mutations. Samples were then transferred to the Max Planck Institute of Immunobiology, Freiburg and to the Laboratory of Virus Immunology, Institute Butantan, São Paulo. Aliquots containing 2×10^5 plaque-forming units per milliliter (PFU/ml) were stored at −80 °C and used in all experiments. The stocks as well as experimental samples were always titrated by plaque assay on L929 cells as previously described (Vassão et al., 2000).

IFNγ assays

In order to study the synthesis of IFNγ, BALB/c or A/J mouse PMΦ or BMMΦ cultures were stimulated with 10 ng/ml of IL-12 (R&D Systems Europe Ltd., Abingdon, UK) and/or 50 ng/ml of IL-18 (PeproTech EC Ltd., London, UK) for the indicated periods. The total cellular RNA was then extracted to evaluate the expression of the IFNγ gene and the supernatants collected to quantify the level of IFNγ secreted. RNA was extracted using the TriFast reagent (PEQLAB Biotechnologie GmBH, Erlangen, Germany), which briefly includes the following steps: homogenization, phase separation, RNA precipitation, RNA wash and RNA solubilization. The final RNA concentrations and degree of purity were determined by optical spectroscopy. Only highly purified preparations ($OD_{260}/OD_{280} = 1.8–2.0$) were used. Reverse transcription was performed by standard procedures using Moloney murine leukemia virus reverse transcriptase (Amersham Biosciences Europe GmbH, Freiburg, Germany) and 1 μl of a dilution of the reverse transcription mixture was used for amplification by polymerase chain reaction (PCR). The primers, the fragment obtained after 35 cycles and the annealing temperature were as follows: up, 5′-GCT CTG AGA CAA TGA ACG CT-3′; down, 5′-AAA GAG ATA ATC TGG CTC TGC-3′, 227 bp, 58 °C. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide.

IFNγ secreted in the supernatants was determined by the OptEIA, mouse IFNγ Set, a commercially available (BD Biosciences-Pharmingen, San Diego, USA) sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol. The data are expressed in pg/ml of IFNγ measured in triplicate cultures ± standard deviation.

MHV3 replication in macrophages

Experiments were carried out in order to evaluate, in the MΦ, the induction of anti-MHV3 state, mediated by exogenous IFNγ or the endogenous IFNγ synthesized upon IL-12/IL-18 activation. BALB/c or A/J mouse PMΦ or BMMΦ cultures were activated for 24 h with 100 U/ml of IFNγ (Genentech Inc., South San Francisco, CA) or 10 ng/ml of IL-12 and/or 50 ng/ml of IL-18 and then infected with MHV3 at multiplicity of infection (moi) of 0.1. Cell supernatants were collected 18 h later and the virus titers determined by plaque assay as indicated above. The data are expressed as plaque-forming units per milliliter (PFU/ml) measured in triplicate cultures ± standard deviation. In order to investigate the influence of polyamine depletion by DFMO, BALB/c or A/J mouse BMMΦ cultures were treated for 24 h with 10 mM DFMO (EMD Biosciences Inc., Calbiochen, San Diego, CA) and then infected with 0.1 moi of MHV3 for 18 h. Supernatants were titrated and the results expressed as described above.

Measurement of NO synthesis and arginase activity in macrophages

To evaluate the induction of NO synthesis and arginase activity, BALB/c or A/J mouse BMMΦ cultures were activated for 24 h with 100 U/ml of IFNγ or 10 ng/ml of IL-12 and/or 50 ng/ml of IL-18 or with 20 U/ml of IL-4 (R&D Systems Europe) and 40 U/ml of IL-10 (PeproTech EC) and then the supernatants and the cells were collected to measure, respectively, the NO synthesis and arginase activity.

NO was measured as nitrite using the Griess reagent as previously described (Modolell et al., 1995). Briefly, culture supernatants (100 μl) were mixed with 100 μl of 1% sulfanilamide, 0.1% naphtylethylenediamine dihydrochloride (NED) and 2.5% H_3PO_4 and absorbance was measured at 540 nm in an ELISA reader (Biochrom AG). The data are expressed in μM measured in triplicate cultures ± standard deviation.

Arginase activity was measured in cell lysates as previously described (Corraliza et al., 1994). Briefly, 10^6 cells were lysed with 0.5 ml 0.1% Triton X100 and after 30 min 0.5 ml 25 mM Tris–HCL 5 mM $MnCl_2$, pH 7.4, was added. The enzyme was then activated

by heating for 10 min at 56 °C. Arginine hydrolysis was carried out by incubating 25 µl of the activated lysate with 25 µl of 0.5 M arginine, pH 9.7 at 37 °C for 60 min. The reaction was stopped with 400 µl of an acidic mixture (H_2SO_4, H_3PO_4 and H_2O, 1:3:7 v/v). The urea was then measured at 540 nm after addition of 25 µl of 9% alpha-isonitrosopropiophenone (dissolved in 100% ethanol) and then heating at 100 °C for 45 min. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 µmol of urea/min. The data are expressed as mU/10^6 cells measured in triplicate cultures ± standard deviation.

Arginine consumption and polyamine synthesis in macrophages

With the aim of investigating arginine metabolism, BALB/c or A/J mouse BMMΦ cultures were activated for 24 h with 20 U/ml of IL-4 or 100 U/ml of IFNγ (Genentech Inc.) or 10 ng/ml of IL-12 and/or 50 ng/ml of IL-18 and then infected with 0.1 moi of MHV3 for 2 h. The cultures were then washed and incubated with arginine free DMEM containing 2% FBS and 0.1 µCi of L-(U-^{14}C) arginine (Amersham) per well. Microplates were then incubated for 4 h at 37 °C and freeze-thawed twice at −80 °C for cell lysis.

Arginine consumption and the synthesis of its products were evaluated by Thin-Layer Chromatography (TLC) (Sessa et al., 1990). To identify the spots, 10 ml of a solution containing 2.5 mg/ml of citrulline, glutamate, proline, ornithine, putrescine, spermidine and spermine was added to the cell lysates. 20 ml of the samples were spotted onto TLC plates (25-Cromatoplates TLC 20 × 20 cm, Silica Gel 60 F254) (Merck KGaA, Darmstadt, Germany) and dried for 1 h at 42 °C. They were then developed in the solvent system chloroform/methanol/ammonium hydroxide/water 1:9:4:2 (vol/vol) and dried. Spots were developed with Ninhydin (Spray Solution Merck) by heating at 120 °C for 5 min and scraped into scintillation tubes containing 6 ml Ecoscin-tATM (National Diagnostics, Atlanta, GA). Radioactivity was determined by scintillation counting (Beckman Instruments Inc., Fullerton, CA) and the values for each compound are expressed as percentage (%) of the total radioactivity measured in triplicate cultures ± standard deviation.

Statistical analysis

Mean and standard deviations (bars) were calculated. The significance levels were assessed by Student's *t*-test at a confidence level of $p < 0.01$

Results

IFNγ induction and MHV3 replication in BALB/c and A/J mouse macrophages upon activation with IL-12 and IL-18

Studies performed to investigate IFNγ induction in activated MΦ are shown in Fig. 2. Only upon a synergistic IL-12/IL-18 activation, BMMΦ from *spf* BALB/c and A/J mice were capable of synthesizing the IFNγ gene as shown by RT-PCR or secrete IFNγ into the supernatant culture as shown by specific ELISA assay. The treatment of BMMΦ from these *spf* mice with only IL-12 or IL-18 failed to either induce expression of the IFNγ gene or secretion of IFNγ during the first 48 h. BALB/c mouse MΦ were shown to regularly synthesize higher amounts of IFNγ (3200 ± 60 pg/ml for BALB/c BMMΦ and 1600 ± 20 pg/ml for A/J BMMΦ after 72 h of activation) and the studies showed that, in BMMΦ of both mouse strains, the IFNγ mRNA and secreted IFNγ could be detected after 24 h of IL-12/IL-18 activation. The amount of secreted IFNγ present in supernatants increased consistently from 24 to 72 h (from 1500 ± 20 to 3200 ± 60 pg/ml in BALB/c BMMΦ and from 500 ± 10 to 1600 ± 20 pg/ml in A/J BMMΦ). At that time (72 h) some IFNγ could be detected in cultures treated only with IL-18 (1200 ± 20 pg/ml in BALB/c BMMΦ and 750 ± 30 pg/ml in A/J BMMΦ). When PMΦ of non-*spf* BALB/c or A/J mice were used in the experiments, secreted IFNγ could be detected in high amounts upon activation with IL-12 or IL-18 alone (3100 ± 200 or 3800 ± 180 pg/ml for BALB/c PMΦ and 1500 ± 100 or 3200 ± 60 pg/ml for A/J PMΦ) in addition to the synergistic IL-12/IL-18 activation (4000 ± 50 pg/ml for BALB/c PMΦ and 3600 ± 40 pg/ml for A/J PMΦ).

Cultured macrophage populations activated with IL-12, IL-18 or exogenous IFNγ were assayed for their ability to restrict MHV3 after infection. The data shown in Fig. 3 demonstrate that both BMMΦ and PMΦ from resistant A/J mice were capable of restricting MHV3 multiplication only when synergistically activated with IL-12/IL-18 (inhibition of 90.9% and 94% for PMΦ and BMMΦ, respectively) ($5.5 \pm 0.5 \times 10^5$ PFU/ml and $3 \pm 1.1 \times 10^5$ PFU/ml for control PMΦ and BMMΦ, respectively, and $5 \pm 3.1 \times 10^4$ PFU/ml and $3 \pm 0.8 \times 10^4$ PFU/ml for IL-12/IL-18 activated PMΦ and BMMΦ, respectively) or with exogenous IFNγ (inhibition of 92.8% and 96% for PMΦ and BMMΦ, respectively) ($4 \pm 0.7 \times 10^4$ PFU/ml and $2 \pm 0.4 \times 10^4$ PFU/ml for IFNγ activated PMΦ and BMMΦ, respectively). In contrast, no MHV3 multiplication restriction was observed in macrophages from the susceptible BALB/c mice (values from 5 to 7.5×10^5 PFU/ml).

Arginine metabolism in BALB/c and A/J mouse macrophages upon activation with IL-12, IL-18, IFNγ and infection with MHV3

As shown in Fig. 4 the background levels of nitrites and arginase activity found in cultured BMMΦ of BALB/c and A/J mice were low (<4 μM). Nevertheless, high amounts of nitrites could be induced upon activation with IFNγ (70±5 and 50±5 μM for BALB/c and A/J BMMΦ, respectively) or by the synergistic treatment with IL-12/IL-18 (35±6 and 20±2 μM for BALB/c and A/J BMMΦ, respectively). In both situations higher amounts of nitrites were observed in BALB/c BMMΦ and no nitrite synthesis was observed upon treatment with IL-12 or IL-18 alone or with IL-4/IL-10. The MHV3 infection was capable of further enhancing the IFNγ or IL-12/IL-18 triggered-nitrite release from both BMMΦ populations (data not shown).

On the other hand, as also shown in Fig. 4, arginase activity was not induced in either BALB/c or A/J BMMΦ upon IFNγ activation or synergistic treatment with IL-12/IL-18 (< 40 mU/10^6 cells). Nevertheless high and comparable amounts of arginase activity could be induced in BMMΦ populations from both strains upon treatment with IL-4/IL-10 (2100±400 mU/10^6 cells and 1800±200 mU/10^6 cells for BALB/c and A/J BMMΦ, respectively). The background levels of arginase activity were higher in BALB/c BMMΦ than in A/J ones (15±4 mU/10^6 cells and 5±2 mU/10^6 cells for BALB/c and A/J BMMΦ, respectively) and the MHV3 infection did not influence significantly the arginase activity (data not shown).

The studies of arginine consumption and synthesis of citrulline, ornithine and spermine are shown in Fig. 5. The data show that highly enhanced arginine consumption was observed in BMMΦ of both BALB/c and A/J mice upon activation with exogenous IFNγ (17±1% or 18±2% for controls and 54±2% or 55±3% for IFNγ-activated in BALB/c and A/J BMMΦ, respectively) but not when the cells were treated with IL-12 and/or IL-18 (18±1% for BALB/c and 18±3% for A/J BMMΦ). In these cases, MHV3 always further enhanced the arginine consumption (30±2% or 35±2% for controls or 66±0.1% or 72±4% for IFNγ-activated BALB/c and A/J BMMΦ, respectively). Comparable values of arginine consumption were observed in BMMΦ of both BALB/c and A/J mice independent of the treatment.

Comparable basal levels of citrulline were observed in BALB/c and A/J BMMΦ, but upon IFNγ activation, it

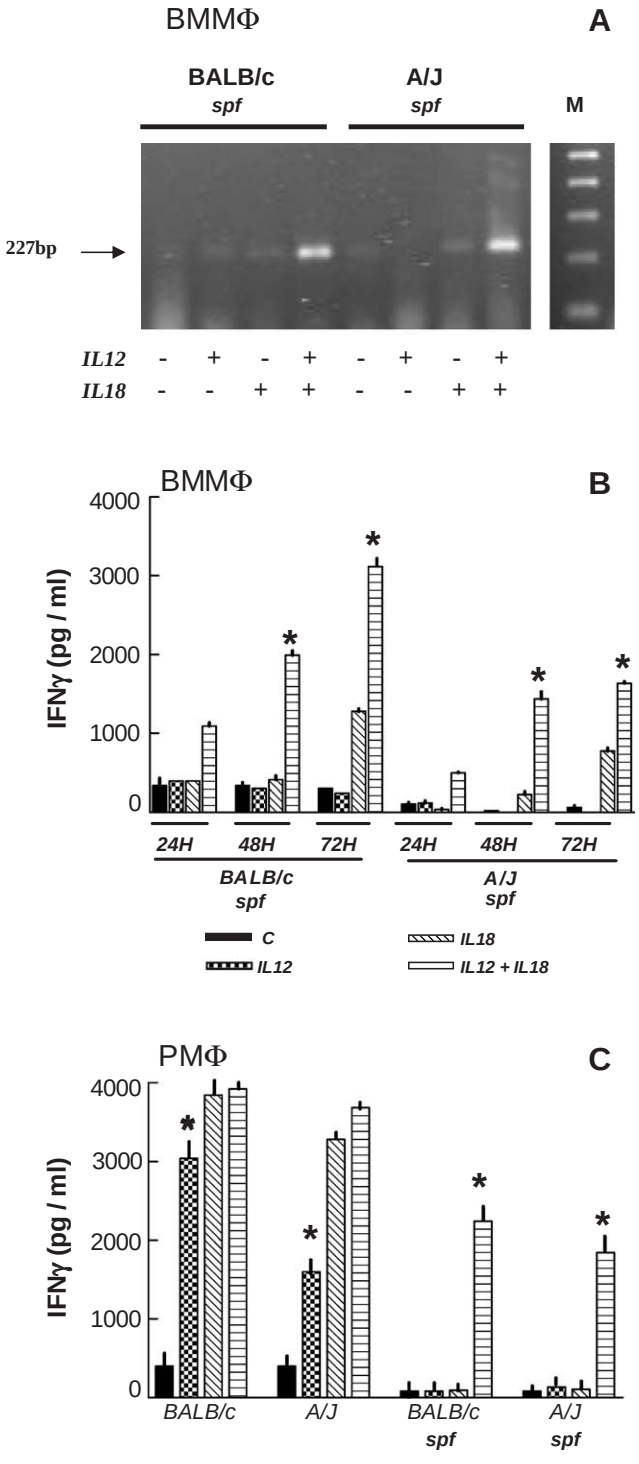


Fig. 2. Induction of IFNγ in BALB/c and A/J mouse macrophages upon activation with IL-12 and/or IL-18. (A) Agarose gel electrophoresis of RT-PCR product specific for IFNγ mRNA from bone marrow macrophages (BMMΦ) of BALB/c or A/J *spf* mice after 24 h treatment with IL-12 and/or IL-18. (B) Kinetics of IFNγ synthesis in BMMΦ of BALB/c or A/J *spf* mice after 24–72 h treatment with IL-12 and/or IL-18. IL-12 (10 ng/ml) and IL-18 (50 ng/ml). (C) IFNγ synthesis in peritoneal exudate macrophages (PMΦ) of BALB/c or A/J *spf* or non-*spf* mice after 24 h treatment with IL-12 and/or IL-18. M, size markers; arrow, 227 kb mRNA for IFNγ specific DNA product. Each bar represents the mean±SD of the results from independent experiments. *Significant ($p<0.01$), as compared to controls.

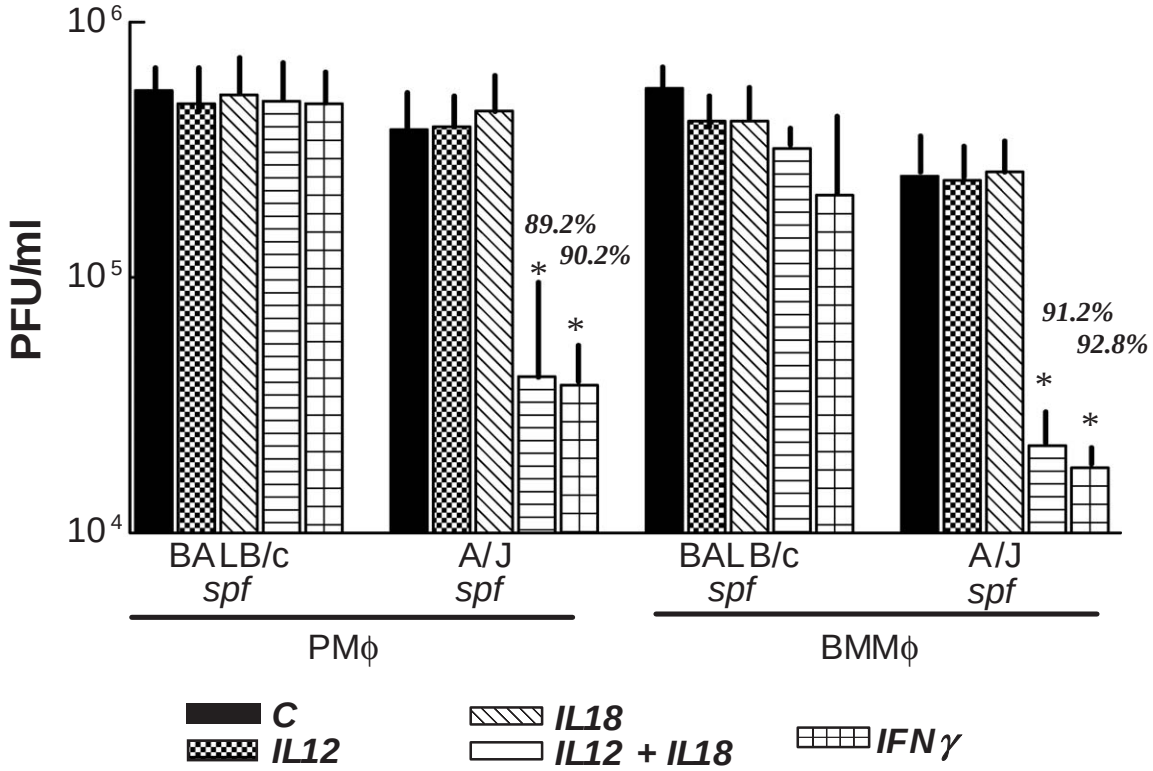


Fig. 3. Anti-MHV3 state induced in peritoneal (PMΦ) or bone marrow (BMMΦ) macrophages of BALB/c and A/J *spf* mice upon activation with IL-12, IL-18, IL-12 + IL-18 or IFNγ. Macrophage cultures were activated with IL-12 (10 ng/ml), IL-18 (50 ng/ml), IL-12 + IL-18 (respectively, 10 ng/ml + 50 ng/ml) or IFNγ (100 U/ml) for 24 h and then infected with 0.1 moi of MHV3 for 18 h. Supernatants were collected and virus titers evaluated. Data are reported as PFU/ml and each bar represents the mean $\pm$ SD of the results from independent experiments. C, control experiment of non-activated macrophages. *Significant ($p < 0.01$), as compared to controls. The percentage (%) of virus inhibition is indicated in italics.

was synthesized in high amounts in BMMΦ of both mouse strains (31 $\pm$ 4% and 40 $\pm$ 2% in BALB/c and A/J BMMΦ, respectively) and could be further increased by MHV3 infection (48 $\pm$ 3% and 55 $\pm$ 4% in BALB/c and A/J BMMΦ, respectively). It correlated with our previous data (Fig. 4) showing that IFNγ was capable of inducing NO synthesis, which was also further enhanced by MHV3 infection.

Overall polyamine synthesis was found to be lower in BALB/c BMMΦ than in A/J ones (6.9 $\pm$ 3% and 14 $\pm$ 2% of spermine in control BALB/c and A/J BMMΦ, respectively), which correlated with the levels of ornithine observed (4 $\pm$ 1.5% and 2 $\pm$ 1.5% in BALB/c and A/J BMMΦ, respectively). MHV3 infection was capable of enhancing spermine synthesis (20 $\pm$ 2% and 32 $\pm$ 3% of spermine in MHV3 infected BALB/c and A/J BMMΦ, respectively).

IFNγ activation or synergistic treatment with IL-12/IL-18, mainly in MHV3 infected cells, led to a decreased synthesis of spermine only in A/J BMMΦ (14 $\pm$ 2%, 8 $\pm$ 1%, 32 $\pm$ 3% and 12 $\pm$ 2% in control, IFNγ-activated, MHV3 infected and MHV3 infected/IFNγ-activated A/J BMMΦ, respectively).

Overall putrescine and spermidine synthesis (data not shown) showed the same pattern as that of spermine (Fig. 5) but at lower levels (0.69 $\pm$ 0.2% and 1.3 $\pm$ 0.6% of putrescine, and 0.49 $\pm$ 0.2% and 0.76 $\pm$ 0.3% of spermidine in control BALB/c and A/J BMMΦ, respectively; 1.12 $\pm$ 0.6% and 1.75 $\pm$ 0.5% of putrescine, and 1.1 $\pm$ 0.2% and 1.25 $\pm$ 0.4% of spermidine in MHV3 infected BALB/c and A/J BMMΦ, respectively).

Upon stimulation with IL-4 we could observe a high consumption of arginine in both BALB/c and A/J BMMΦ (95 $\pm$ 2% in both BALB/c and A/J BMMΦ), followed by lower synthesis of citrulline (17 $\pm$ 1% and 13 $\pm$ 1% in BALB/c and A/J BMMΦ, respectively) and higher synthesis of ornithine (38 $\pm$ 1% and 36 $\pm$ 2% in BALB/c and A/J BMMΦ, respectively) and spermine (34 $\pm$ 1% and 44 $\pm$ 1% in BALB/c and A/J BMMΦ, respectively) when compared to IFNγ activation (Fig. 5).

As shown in Table 1, the inhibition of ODC activity by DFMO, which leads to a depletion of polyamines, induced a decrease in the MHV3 replication in both BALB/c ($7 \pm 0.4 \times 10^6$ to $0.8 \pm 0.02 \times 10^6$ PFU/ml in control and DFMO-treated MΦ, respectively) and A/J

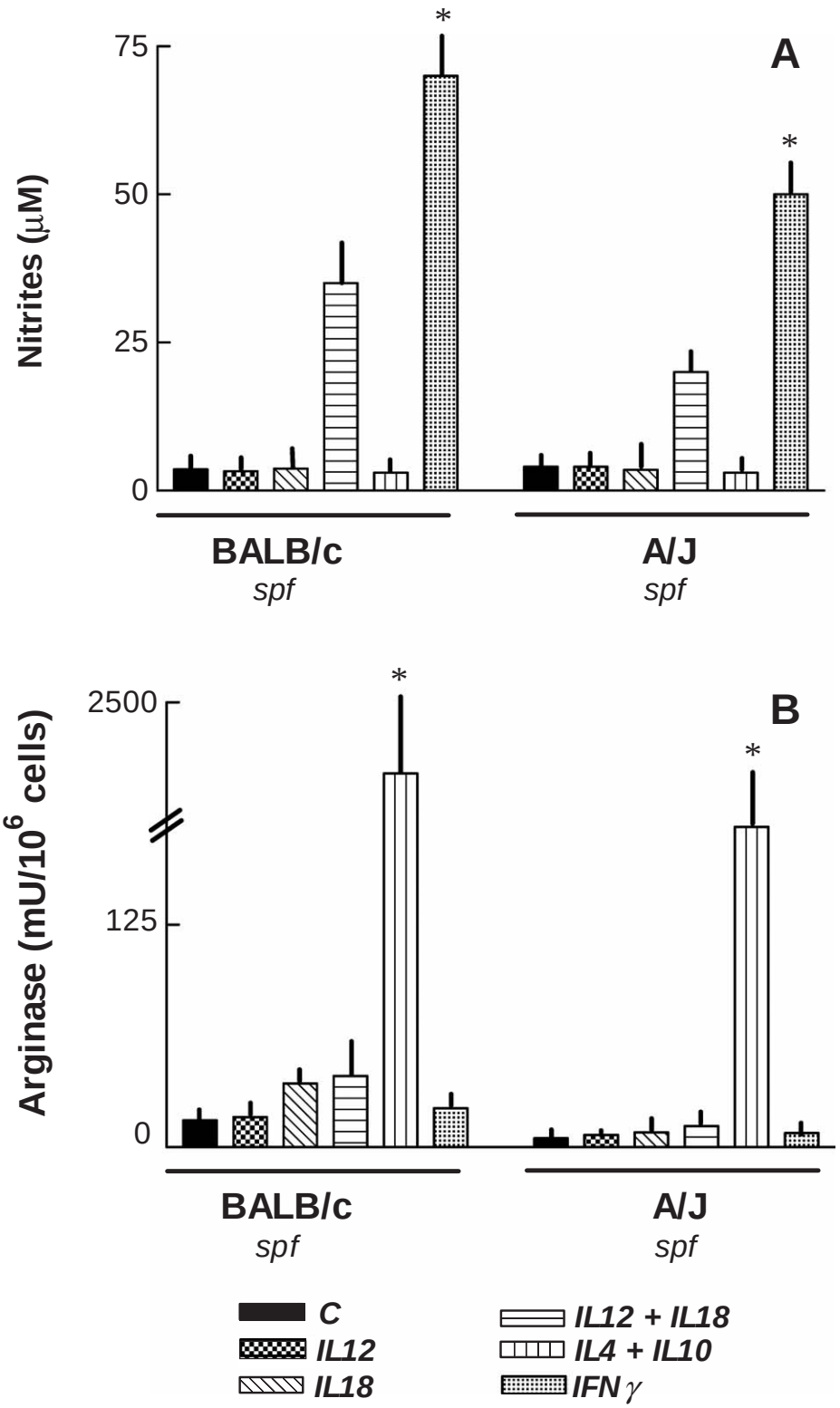


Fig. 4. Induction of NO synthesis and arginase activity in bone marrow derived macrophage cultures of BALB/c and A/J *spf* mice upon activation with IL-12, IL-18, IL-4, IL-10 or IFNγ. Macrophage cultures were activated for 24 h with IL-12 (10 ng/ml), IL-18 (50 ng/ml), IL-12 + IL-18 (respectively, 10 ng/ml + 50 ng/ml), IL-4 (20 U/ml), IL-10 (40 U/ml) or IFNγ (100 U/ml) and nitrite (A) or arginase (B) concentrations measured in the supernatants. Nitrites are expressed in μM and arginase in mU/10^6 cells. Each bar represents the mean ± SD of the results from independent experiments. C, control experiment of non-activated macrophages. *Significant ($p < 0.01$), as compared to controls.

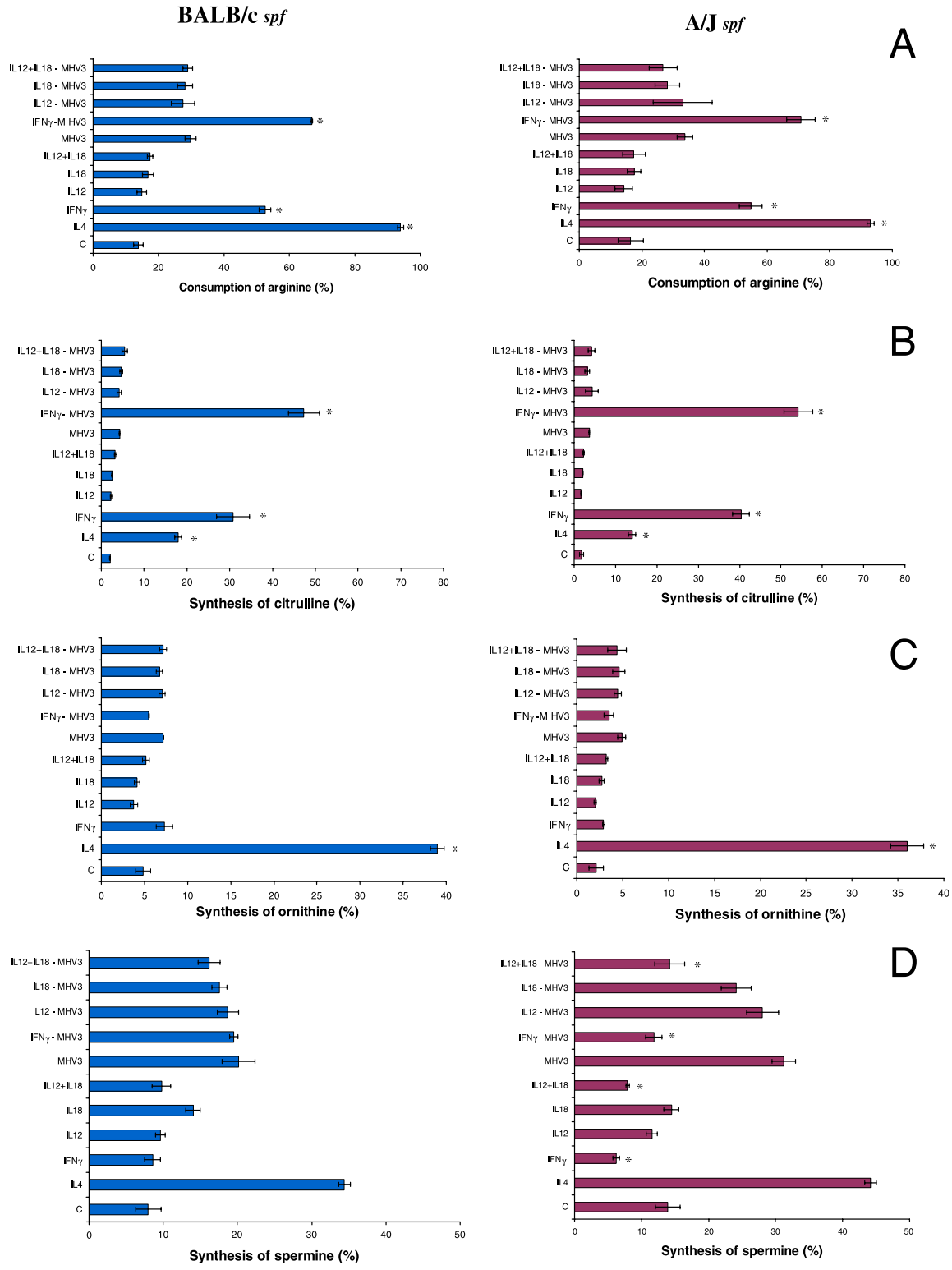


Fig. 5. Consumption of arginine (A) and synthesis of citrulline (B), ornithine (C) and spermine (D) in bone marrow derived macrophages of BALB/c and A/J *spf* mice upon activation with IL-12, IL-18, IL-12 + IL-18, IFNγ or IL-4 and infection with MHV3. Macrophage cultures were activated with IL-12 (10 ng/ml), IL-18 (50 ng/ml), IL-12 + IL-18 (respectively, 10 ng/ml + 50 ng/ml), IFNγ (100 U/ml) or IL-4 (20 U/ml) for 24 h and infected with 0.1 moi of MHV3 for 2 h. After washing and treatment for 4 h with L-(U-^{14}C) arginine the cultures were lysed and submitted to TLC. The results of arginine consumption or citrulline, ornithine or spermine synthesis are expressed in percent (%) of total radioactivity. Each bar represents the mean $\pm$ SD of the results from independent experiments. C, control experiment of non-activated nor infected macrophages. *Significant ($p < 0.01$), as compared to controls.

Table 1. MHV3 replication in bone marrow derived macrophages of A/J and BALB/c mice. Macrophage cultures were treated with 10 mM DFMO for 24 h and then infected with 0.1 moi of MHV3 for 18 h

Treatment	PFU/ml ± SD	
	BALB/c	A/J
—	$7.0 \pm 0.4 \times 10^6$	$4.0 \pm 0.3 \times 10^6$
DFMO	$0.8 \pm 0.02 \times 10^6$*	$0.3 \pm 0.01 \times 10^6$*

Supernatants were collected and virus titers evaluated. Data from independent experiments are reported as PFU/ml ± SD. *Significant ($p < 0.01$), as compared to controls.

BMMΦ ($4 \pm 0.3 \times 10^6$ to $0.3 \pm 0.01 \times 10^6$ PFU/ml in control and DFMO-treated MΦ, respectively).

Discussion

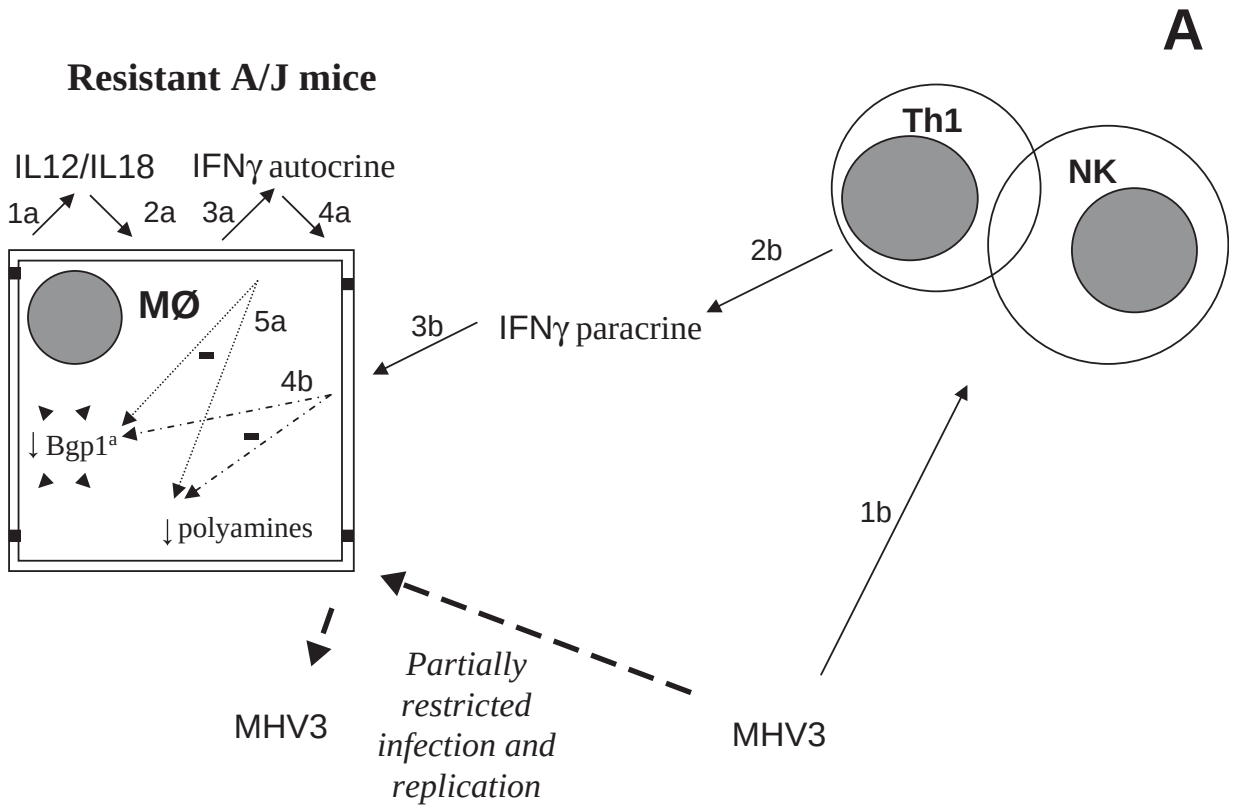
MHV3, a coronavirus found endemically in mouse colonies, has been used as a model of hepatotropic virus infection. Among the mechanisms proposed for resistance of some mouse strains to experimental MHV3 infection (Fingerote et al., 1996; Lamontagne et al., 1996), we have described a T-cell dependent one in which IFNγ synthesis and MΦ sensitivity to IFNγ play a central role. Susceptible mice would develop a fatal hepatitis by lacking either the ability to synthesize IFNγ or cells capable of responding to IFNγ. IFNγ would exert its activity by down-regulating the expression of the main viral receptor (Vassão et al., 2000). Recently, we have also shown a novel pathway of autocrine MΦ activation by demonstrating that MΦ, upon synergistic stimulation with IL-12 and IL-18, are a potent IFNγ-producing cell (Munder et al., 1998a). BMMΦ are a reliable non contaminated population which can support these findings (Munder et al., 2001). So, along with the classical or paracrine MΦ activation by IFNγ produced by lymphoid cells, an autocrine MΦ activation could take place, where IFNγ is produced by IL-12/IL-18-activated MΦ. Although their relative magnitudes are not yet known, both processes occurring in MΦ could play a crucial role in controlling infections.

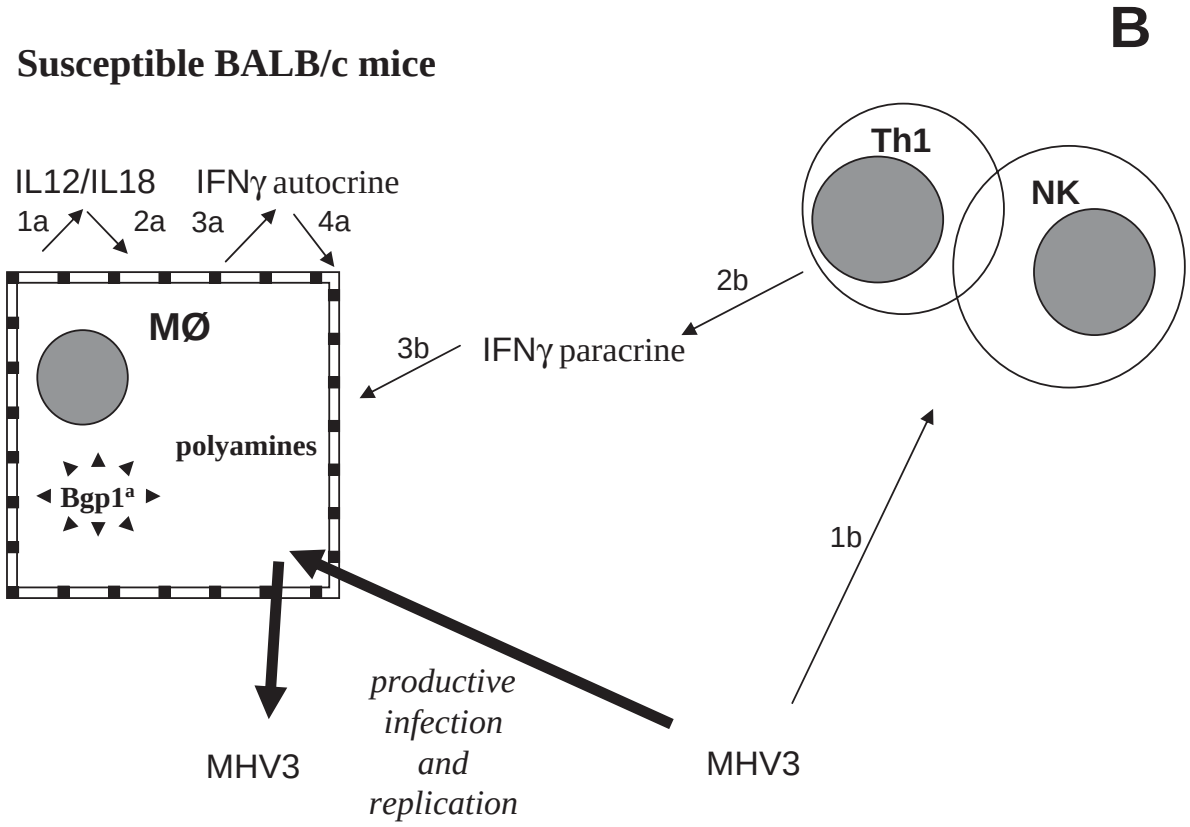
In the present work we extended our previous observations (Vassão et al., 2000) concerning MΦ activation by exogenous IFNγ, which leads to a partial restriction of MHV3 replication only in MΦ of resistant mice, to the participation of the autocrine MΦ activation in MHV3 restriction. The data presented show that synergistic activation by IL-12/IL-18 of MΦ from resistant (A/J) and susceptible (BALB/c) mouse strains induced the expression of the IFNγ gene (Fig. 2A). BMMΦ or PMΦ of both *spf* mouse strains secreted IFNγ upon a synergistic IL-12/IL-18 activation (Fig. 2C) and the levels of secreted IFNγ increased along the time of stimulation and were higher in BALB/c MΦ (Figs. 2B and C). Nevertheless, an anti-MHV3 state was observed only in A/J MΦ upon a synergistic activation with IL-12/IL-18 or with exogenous IFNγ (Fig. 3). As expected, in the PMΦ of non-*spf* animals, IFNγ synthesis could be observed also among MΦ stimulated only with IL-12 or IL-18. Taken together, these results demonstrate that autocrine MΦ activation takes place in both BALB/c and A/J MΦ and, as shown for paracrine IFNγ MΦ activation, only A/J MΦ respond with the expression of an anti-MHV3 state. Both BMMΦ and PMΦ behave similarly regarding IFNγ synthesis and anti-MHV3 state. Among non-*spf* mice, which better mirror the natural state of mice concerning this endemic virus infection, their PMΦ synthesize high levels of IFNγ upon treatment with only IL-12 or IL-18 possibly due to their already immunologically primed state or to the presence of other cells. The evidence that, after 72 h, the BMMΦ from *spf* mice synthesized some IFNγ upon stimulation of only IL-18, may rely on the autocrine pathway. The contribution of each, the paracrine, T-cell dependent, and the autocrine, T-cell independent, mechanism of MΦ activation during MHV3 infection remains to be determined. It is conceivable that autocrine-IFNγ synthesis participates in early stages of infection, prior to specific antigen recognition by lymphoid cells, not only by enhancing the MΦ ability to restrict the agent's infectivity but also by enhancing the MΦ ability to present antigens. By this way, autocrine MΦ activation would represent a link between the innate and acquired immune response (Frucht et al., 2001).

One of the main features occurring during MΦ activation involves arginine metabolism and the synthesis of key molecules such as NO on one side and arginase, which may lead to the formation of polyamines, on the other. NO is a highly reactive molecule

Fig. 6. Schema of proposed mechanisms for resistant A/J (A) and susceptible BALB/c (B) mouse macrophages, involving autocrine and paracrine activation by IFNγ and MHV3 restriction of replication. MΦ, macrophage; Th1, T helper lymphocyte type 1; NK, natural killer cell; a, autocrine pathway; b, paracrine pathway; 1a, IL-12 and IL-18 synthesis by MΦ; 2a, IL-12 and IL-18 activation of MΦ; 3a, IFNγ synthesis by activated MΦ; 4a, IFNγ activation of MΦ; 5a, intracellular response to autocrine IFNγ activation leading to biliary glycoprotein ($Bgp1^a$) virus receptor down-regulation and inhibition of polyamine synthesis; 1b, MHV3 activation of cellular immune response; 2b, synthesis of IFNγ by Th1 and NK cells; 3b, IFNγ activation of MΦ; 4b, intracellular response to paracrine IFNγ activation leading to $Bgp1^a$ down-regulation and inhibition of polyamine synthesis; ↓ $Bgp1^a$, down-regulation of $Bgp1^a$; ■, $Bgp1^a$ receptors on the cell membrane; ↓ polyamines, inhibition of polyamine synthesis.

produced from a guanidine-nitrogen of arginine in a reaction catalyzed by NOS. NO is synthesized at high levels by MΦ upon stimulation by Th1 cytokines (Hesse et al., 2001; Munder et al., 1999) and has been implicated in host defense (Guillemard et al., 1996; Harris et al., 1995; Kawanishi 1995; Lowenstein et al.,





1996; Melkova and Esteban, 1995; Tucker et al., 1996). Arginase, an enzyme induced in MΦ upon stimulation by Th2 cytokines (Hesse et al., 2001; Munder et al., 1999), drives the synthesis of ornithine, which is catalyzed by ODC to form polyamines (putrescine, spermidine and spermine). DFMO, an analog of ornithine, can irreversibly inhibit ODC causing polyamine depletion. Arginase has been implicated in virus inhibition and may potentially have an antimicrobial activity OR play an antimicrobial role (Corraliza et al., 1995; Modolell et al., 1995). Polyamines, such as spermine, play several roles in cell differentiation and proliferation and may contribute to virus maturation (Cohen, 1998; Thomas and Thomas, 2001; Wang and Wong, 2003). Although data have shown that polyamine depletion reduces cytomegalovirus or herpes simplex virus 2 infectivity, contradictory reports are found in the literature (Cohen, 1998).

Our data show that after activation with IL-12/IL-18 or IFNγ, BALB/c and A/J BMMΦ released NO (Fig. 4A). Arginase was induced only when IL-4/IL-10 was used for activation (Fig. 4B). Higher arginine consumption was observed in BMMΦ from both strains upon IFNγ or IL-4 activation and, as expected, IFNγ activation led to higher citrulline synthesis and IL-4 activation to higher ornithine synthesis (Figs. 1, 5B and 5C). Both arginine consumption and spermine synthesis increased during MHV3 infection. Although IL-12/IL-18 activation of both BALB/c and A/J BMMΦ led to NO synthesis (Fig. 4A), a higher consumption of arginine was not observed in this situation (Fig. 5A). IL-12/IL-18 or IFNγ activation, mainly in MHV3 infected cells, led to a decreased synthesis of spermine only in A/J BMMΦ (Fig. 5D). The putrescine and spermidine pattern levels followed that of spermine but at lower levels (data not shown). Intracellular polyamines were shown to be important during MHV3 replication since, in DFMO-treated BMMΦ, we detected a significant decrease in MHV3 titer (Table 1).

Our data of arginine metabolism in MΦ (Fig. 5) are consistent with the notion that the newly established autocrine MΦ activation leads to IFNγ synthesis which in turn induces NO synthesis (Fig. 2 and 4). The lack of arginase induction indicates the Th1 behavior of this type of autocrine activation. The fact that upon exposure to IL-12/IL-18, which induces endogenous IFNγ (Fig. 2) and NO (Fig. 4), we failed to observe the expected arginine consumption and citrulline synthesis, clearly observed when exogenous IFNγ was employed (Figs. 5A and B), may rely on inherent quantitative and kinetic characteristics of the process.

Regarding the mechanism involved in resistance/susceptibility of mice to MHV3 infection, our previous (Pereira et al., 1997; Vassão et al., 2000) and present data obtained with BALB/c and A/J MΦ, clearly link IFNγ synthesis, anti-MHV3 state induction and polyamine inhibition in A/J MΦ (Fig. 6A). In these MΦ, polyamines were induced during MHV3 infection and significantly inhibited upon activation by IFNγ or IL-12/IL-18. Supported by the data showing a decreased MHV3 replication in DFMO treated MΦ (Table 1), the polyamine modulation by IFNγ correlates with the anti-MHV3 state observed only among A/J MΦ. Nevertheless, the molecular basis of a mechanism underlying the influence of IFNγ on polyamine synthesis remains unclear.

Our findings show that both autocrine and paracrine processes of MΦ activation may participate in the inhibition of MHV3 replication and that the involvement of arginine metabolism in resistance to MHV3 may rely on the decreased synthesis of polyamines induced by IFNγ (Figs. 5D and 6)We can speculate that polyamines, besides their effects on cell differentiation, may participate directly in MHV3 maturation.

Altogether these data show the relevance of IFNγ, from autocrine or paracrine pathways, and suggest how arginine metabolism may be involved in the modulation of MHV3 replication in MΦ of a resistant mouse strain.

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