Macrophages require distinct arginine catabolism and transport systems for proliferation and for activation

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Abbreviations used in this paper: CAT, cationic amino acid transporters; gpaAT glycoprotein-associated amino acid transporters; M-CSF, macrophage colony-stimulating factor; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; NOS, nitric oxide synthase, *SLC*, Solute carrier.

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Abstract

In murine macrophages, as a result of arginine catabolism during activation, citruline is produced under the effect of IFN-γ and LPS and ornithine and polyamines by IL-4 and IL-10. For proliferation, arginine is required from the extracellular medium and is used for protein synthesis. During activation most arginine (>95% in 6 hours) was metabolized while under proliferation only half was incorporated into proteins. Under basal conditions, this amino acid was preferentially transported by y+L activity. During activation, arginine transport increased drastically (4- to 5-fold) through y^+ (CAT) activity. By contrast, M-CSF induced only a modest increase in uptake (0.5-fold). The increase in arginine transport during activation but not proliferation was mediated by the *SLC7A2*/Cat2 gene. *SLC7A1*/Cat1 is constitutively expressed and is not modified by proliferating or activating agents. M-CSF dependent proliferation was not affected in the macrophages of *SLC7A2* KO mice; however, these cells showed a drastic reduction in the production of citruline or ornithine and polyamines during activation. The data show that a large increase in a specific transport system (CAT2) is necessary to fulfil activation-induced arginine metabolism, while arginine is in excess for the requirements of proliferation and a modest increase in transport occurs.

INTRODUCTION

Macrophages originate from undifferentiated stem cells in the bone marrow and are transported through the blood to all body tissues where, in most cases, they undergo apoptosis [1]. Depending on the stimuli received, in tissues these phagocytes differentiate in numerous cell types including liver Kupffer cells, dermal Langerhans cells, bone osteoclasts, brain microglia, and several interdigitating and follicular dendritic cells from lymphoid organs [2]. In response to M-CSF, macrophages proliferate in tissues [1]. To carry out their functional activities, these cells must become activated either by type Th1 cytokines, such as IFN-γ, which we call "classical activation", or by type Th2 cytokines, such as IL-4, IL-10 and IL-13, which we refer to as "alternative activation" [3]. Activation induces biochemical and morphological modifications that allow macrophages cells to perform their function [4]. Activation blocks the proliferation of these cells [5].

Cell proliferation and activation require high concentrations of arginine either for protein synthesis or to elaborate either nitric oxide (NO) in classical activation or to produce polyamines and praline in alternative activation [3]. The extracellular milieu is main source of arginine and several transport systems are involved in carrying this amino acid across the plasma membrane. Depending on the cell type, a number of transport activities may be expressed [6]. The *SLC7* (Solute carrier) family is divided into two subgroups, the cationic amino acid transporters (the CAT family, *SLC7A1-4*) and the glycoprotein-associated amino acid transporters (the gpaAT family, *SLC7A5-11*), also called light chains or catalytic chains of the hetero(di)meric amino acid transporters (HAT). The CAT group includes 4 members, CAT-1 to CAT-4, whose gene products are *SLC7A1* to *SLC7A4*. The first 3 members transport cationic L-amino

acids while the function of CAT-4 is not known. The HAT family is composed by 7 proteins, whose genes are *SLC7A5* to *SLC7A11*, but only y^+LAT2, y^+LAT1 and b^{o+}AT transport cationic amino acids [6].

Macrophages require arginine to proliferate and to elaborate gene products when they become activated, e.g. IFN-γ activates more than 300 genes [7]. Consequently, to meet their metabolic demands, macrophages require the uptake of exogenous arginine. Therefore, the uptake of this amino acid may be a key regulatory step for physiological responses in macrophages. Here we studied how M-CSF-induced proliferation as well as classical and alternative activation affects arginine metabolism and transport activity. To this end, we used bone marrow-derived macrophages, which are non-transformed cells that respond to both proliferative and activating stimuli separately. Here we show that most arginine is metabolized in activation while in proliferation it is used for protein synthesis. Under basal conditions, arginine is incorporated through the y+L system, an arginine efflux transport system [8-10]. Activation induces a considerable increase in arginine transport mediated by the y+ system, while proliferation produces only a modest increase. For activation, but not for proliferation, the expression of *SLC7A2*/Cat2 is induced.

RESULTS

Proliferation requires arginine import by macrophages.

We used bone marrow-derived macrophages, which are a homogeneous population of primary and quiescent cells. Treatment with M-CSF induces the proliferation of these cells while incubation with several cytokines blocks this process and causes a series of modifications that allow them to develop their functional activity [1]. Because

proliferation and activation are opposite effects in macrophage biology, here we examined the metabolism of arginine under these two conditions. In the presence of increasing amounts of M-CSF, thymidine uptake increased in a dose-dependent manner, which correlated with macrophage growth (Fig. 1A) [11]. In arginine-free media, macrophages did not incorporate thymidine (Fig. 1A). An explanation for this observation is that arginine, like many other amino acids, is required for protein synthesis and this would explain why, in arginine-free media, M-CSF-dependent macrophage proliferation was almost abrogated. To study classical activation, we used IFN-γ and LPS (Th-1-type) while for the alternative activation IL-4 and IL-10 (Th-2-type) were used. Although these two types of activation regulate distinct set of genes [12], both require arginine, either to produce nitrites in the former or as substrate for arginase in the latter. Only molecules of classical activation produced nitrites through the induction of NOS2 while only cytokines of alternative activation stimulated arginase activity (Fig. 1B and 1C). M-CSF, which induced proliferation, did not produce either nitrites or arginase activity. Moreover, neither of the activators used led to macrophage proliferation. Therefore, the reagents used in this study determine whether macrophages proliferate or become activated in the classical or alternative way.

Arginine catabolism in macrophage proliferation and activation differs.

To determine arginine catabolism, macrophages were incubated with [^{14}C]radiolabelled arginine. The distinct products of degradation were then measured in the cell and in the supernatant together using thin-layer chromatography (Fig. 2A). After 6 hours, cells treated with IFN-γ and LPS had converted most of the arginine in citruline while those treated with IL-4 and IL-10 metabolized argine into ornithine and spermine. In macrophages incubated with M-CSF or control media, most of the arginine was not

catabolized. Even after 6 hours of incubation, less than 10% was converted to spermine. Next, we incubated macrophages for 30 minutes with radiolabelled arginine. Cells were washed, incubated for 6 additional hours and the amount of radioactivity in the supernatant and in the cells was then measured as bound or non-bound protein. In control cells and those activated with IFN-γ and LPS, most of the radioactivity (77 and 89%, respectively) was extracellular and was not bound to proteins (Fig. 2B). In contrast, in M-CSF-treated cells, approximately half of the radiolabelled material was extracellular (52%) and half intracellular (47%). However, while the radioactivity outside was not protein-bound (49% of the total), that inside was associated with proteins (43%). These data suggest that during activation arginine is depleted while during proliferation there is more arginine available than that required for protein synthesis and arginine not bound to protein is exchanged with that present in the media. In starvation conditions, arginine is required for protein synthesis, and the non-bound protein is also exchanged with that in the extracellular media.

Distinct transport systems in macrophage proliferation and activation.

The differences in arginine metabolism in activation and proliferation prompted us to study the L-arginine transporters that are active in macrophages. The transport systems that mediate arginine uptake (y^+, B^{o+}, b^{o+} and y^+L) are well characterized [6]. Under basal conditions, in the presence of sodium, L-leucine inhibited most of the L-arginine transport, indicating that very little y^+ activity is present in these cells and most of the transport is due to the y+L system. The participation of the B^{o+} and b^{o+} systems was excluded by measuring the transport in the presence and absence of medium containing sodium and leucine. The y^+ system was strongly induced in response to Th1- or Th2-

type cytokines. In contrast, M-CSF treatment resulted in only a modest increase in the activity of this system (Fig. 3A).

The y^+ system is encoded by the CAT genes *Slc7A1* [13, 14], *Slc7A2* [15, 16] and *Slc7A3* [17]. Using a quantitative PCR reaction, we determined the levels of the three members of the y^+ system in macrophages treated with M-CSF or with Th1- and Th2-type cytokines. Low levels of *Slc7A1* were found in untreated cells. The expression of this gene was not modified by any treatment (Fig. 3B). *Slc7A2* was not detected in quiescent or M-CSF-treated cells but was induced by LPS+IFN-γ and also by IL-4+IL-10. *Slc7A3* was not detected. In addition, the expression of the genes of the y^+ system was measured in arginine-free medium; however, no differences in induction by the distinct cytokines were observed in relation to the controls (Fig. 3B).

Our results so far indicate that in basal conditions and in proliferation most argine transport is mediated by the y+L system and the small increase caused by M-CSF is produced by the y^+ system, which mediates arginine transport through CAT1. In contrast, CAT2 is the main L-arginine transporter for activated macrophages. To confirm these data, we used macrophages from mice with disrupted *Slc7A2* [18]. In *Slc7A2* KO mice and controls, untreated macrophages and cells stimulated with M-CSF showed similar amounts of arginine transport (Fig. 4A). However, while classical and alternative activators produced an increase in the transport of this amino acid in controls, no increase in cells from *Slc7A2* KO mice was observed (Fig. 4A). When analyzed in more detail, the increased transport activity corresponded to the y^+ system (data not shown). *Slc7A1* expression was not modified in *Slc7A2* KO macrophages or in controls when treated with activators, and *Slc7A3* was not expressed in this model (data not shown). The lack of the CAT2 transport system in M-CSF-treated macrophages was demonstrated because in cells from Cat2 -/-, although arginine was required for

proliferation, this activity was not affected (Fig. 4B). After incubation with activating agents, *Slc7A2* KO macrophages showed a reduced catabolism of arginine and the amounts of citruline and ornithine produced were decreased in relation to controls (Fig. 4C). However, in the M-CSF-treated cells the same amounts of citruline or ornithine were produced by cells from wild type or *Slc7A2* KO. These results confirm that macrophage proliferation and activation use distinct arginine transport systems.

The production of polyamines by degradation of arginine is not a limiting factor for macrophage proliferation.

Polyamines are critical mediators of cell growth and division because of their capacity to bind directly to DNA and to modulate DNA-protein interactions [19]. Moreover, it has been proposed that polyamines are involved in tumor progression [20]. Because we detected spermine, spermidine and putrescine as products of arginine metabolism, we next examined their roles in proliferation. Macrophages cultured with M-CSF incorporated thymidine in similar amounts when polyamines were present in the media (Fig. 5). At doses of 25 μM of spermidine or spermine, the polyamines inhibited thymidine uptake, which is caused by the induction of apoptosis [21], as shown by Annex V determination, which is an indicator of phosphatidyl serine exposure in an early apoptotic event. Combinations of the polyamines did not increase macrophage proliferation (data not shown).

DISCUSSION

Macrophages either proliferate or, under the effect of distinct cytokines, stop proliferation and become activated [1]. In both cases, these cells require exogenous

arginine to meet their metabolic demands. Here we used murine bone marrow-derived macrophages because they are a unique primary culture model in which proliferation and activation can be studied separately [5, 22]. Moreover, these cells, unlike peritoneal or alveolar macrophages, are not previously activated. For proliferation, arginine is predominantly required for protein synthesis. While for classical activation (Th1 cytokines) it is needed to produce NO and citruline, in alternative activation (Th2 cytokines) it is required to produce ornithine. and polyamines. Interestingly, while most of the arginine was catabolized and the products were found outside the cell during activation, in proliferation this amino acid was not catabolized and only half returned to the media, the other half being incorporated into the proteins of the macrophages (Fig. 6). In quiescence, most of the arginine was not catabolized and returned to the media while a small amount was incorporated into proteins. These data suggest that arginine is a limiting factor for the production of NO or polyamines during activation but this is not the case for proliferation. The availability of arginine appears to be essential in the regulation of cellular immune response and the inflammatory process during critical illness [23]. Mixed leukocyte cultures are suppressed by the addition of excess macrophages because they deplete arginine from the media [24]. Arginine consumption by arginase-expressing macrophages modulates the expression of the CD3ζ chain in T lymphocytes [25] and this may have a suppressive role in tumor immunology [26, 27].

The catabolism of arginine by macrophages through NOS2 or arginase generates several crucial products for immune regulation. For example, NOS2 produces NO while arginase gives proline and polyamines, thereby inducing the synthesis of collagen, which enhances inflammation [28]. However, in some cases the degradation products of arginine have a beneficial effect on intracellular microbes. For example, polyamines are required for the intracellular growth of Leishmania [29]. Also, mycobacterium enhances

arginine transport in infected macrophages and acquires the metabolites required for bacterial growth and inhibition of killing [30].

Given the interest in arginine catabolism during proliferation and activation of the macrophages, we examined the transport of this amino acid across the plasma membrane as an essential regulatory first step. The distinct arginine requirements during activation and proliferation correlate with the different amounts of arginine incorporated into cells under these conditions. Consequently, the increase in arginine transport in M-CSF-treated macrophages is very modest and, as in quiescent conditions, is mostly due to the y+L system with little activity of y+ through the CAT1 transporter. In activated macrophages, the drastic increase corresponds to an induction of the CAT2 transporter.

To date, the data on arginine transport in macrophages are very limited and are related to activation but not to proliferation. In peritoneal macrophages, this transport is induced by LPS+IFN-γ through the y^+ system and particularly by CAT2 [18], while in human monocytes, IFN-γ stimulates this transport through the y^+L system [31]. In rat alveolar macrophages, an increase in CAT2 has been described after LPS treatment [32].

Our data support the notion of distinct metabolic pathways in macrophage proliferation and activation. Recently, we reported that the transport systems for nucleosides required for DNA and RNA synthesis in macrophages differ when cells proliferate or when they become activated [33, 34]. Similarly, we found differential voltage-dependent K+ channel responses during proliferation and activation in macrophages [35], indicating that early responses differ. This observation shows that the arginine transport systems used in macrophages differ when these cells are activated or when they proliferate. The regulation of *Slc7A2* is a limiting factor for the activation of macrophages as the product of this gene restricts the supply of the substrate to the

machinery that triggers classical or alternative activation. In contrast, in quiescence or under proliferative situations arginine is in excess.

Arginine is required for cell proliferation, as shown here in non-transformed cells, and in tumor models [36, 37]. Accordingly, arginase activity has been reported as a limiting factor for cell proliferation [38, 39]. In our cellular model, most of the arginine was used in protein synthesis, but after 6 hours of incubation with M-CSF 10% was converted in polyamines. Given the potential role of polyamines in cell-cycle progression [19] and in cancer development [20, 40], a hypothetical role as limiting factor for proliferation was considered. However, the addition of polyamines to the media did not increase macrophage proliferation. The discrepancies between our results and those reported in previous publications may be related to the distinct cell types tested. Also, although polyamines play a crucial role in binding to nucleic acids and proteins, which affects their conformation and biological activity, [41] perhaps only small intracellular amounts may be required. This would explain why the addition of polyamines did not increase proliferation. *In vivo*, the role of polyamines may be related to the production of collagen or other extracellular matrix proteins that are involved in cellular growth, development and tissue repair. Furthermore, polyamines modulate the functional activities of macrophages [42]. For example, spermine inhibits pro-inflammatory cytokine synthesis [43-46] and down-regulates arginine transport and NOS2 expression in rat alveolar macrophages [47].

In conclusion, there is a clear distinction between arginine transport systems used in macrophage proliferation and activation. This finding is of particular interest because the transporters required for activation may offer suitable new drug targets for the clinical management of aberrant macrophage activation in several diseases.

MATERIAL AND METHODS

Reagents

LPS and recombinant cytokines were purchased from Sigma Chemical Co. (St. Louis, MO). IL-4 was obtained from R+D systems (Minneapolis, MN). In several experiments, the results obtained with commercial LPS were compared with highly purified LPS from Salmonella *abortus equi*, kindly donated by Dr. C. Galanos (Max Planck Institute, Freiburg, Germany) [48], and no differences were found. All other chemicals were of the highest purity grade available and were purchased from Sigma. Deionized water that had been further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Cell Culture

Bone marrow-derived macrophages were isolated from six-week-old BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) as previously described [49]. Macrophages were cultured in plastic tissue-culture dishes (150 mm) in 40 ml DMEM containing 20% FBS (Sigma) and 30% L-cell conditioned media as a source of macrophage colony-stimulating factor (M-CSF). Penicillin and streptomycin were added. Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. After 7 days of culture, macrophages were obtained as a homogenous population of adherent cells (>99% Mac-1^+). To render cells quiescent, at 80% confluence, cells were deprived of L-cell-conditioned medium for 16-18 hours before treatment. Macrophages from knock-out (KO) mice and the corresponding wild-type (WT) controls were isolated under the same conditions. The *Slc7A2* KO has been reported [18]. Animal experiments were performed in accordance with institutional and government guidelines (University of Barcelona).

Proliferation assay

Cell proliferation was measured as previously described [11] with minor modifications. Quiescent cells (10^5) were incubated for 24 hours in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA) in 1 ml of medium with the concentrations of M-CSF indicated. The media was aspirated and replaced with 0.5 ml of media containing ^{3}H-thymidine (1 μCi/ml) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After 4-6 hours of incubation at 37ºC, the media was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% trichloroacetic acid (TCA), the cells were solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was measured by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter. Each point was performed in triplicate and the results were expressed as the mean ± SD.

Quantitative RT-PCR analysis

Cells were washed twice with cold PBS, and total RNA was extracted with the acidic guanidinium thiocyanate-phenol-chloroform method, as described [50]. RNA was treated with DNAse (Ambion, Austin, TX) to eliminate DNA contamination. For cDNA synthesis, 1 μg RNA and *Taq*Man reverse transcription reagents (including Multiscribe reverse transcriptase and random hexamers) were used, following the manufacturer's instructions (Applied Biosystems, Foster City, CA). The primers used to amplify mouse *Slc7A1* were CTTGGACCAGTGCAAATGACG and TGATCCTGAGGCATGAGTGCA; for *Slc7A2,* GTGAAGAGGTTCGGAATCCACA and CGTTAAAGCTGCAGA; and for *Slc7A3,* GGCTCCCTCTGTGCACTTTCTA and TAGCAAGGACACGGAACAGGA. Real-time monitoring of PCR amplification of cDNAs was done using the *Taq*Man Universal master mix (Applied Biosystems) in an

ABI Prism 7700 sequence Detection System (Applied Biosystems). Relative quantification of gene expression was performed using β actin, as described in the TaqMan users manual (GCACCACACCTTCTACAATGAGCTGT and CTGCTGGAAGTCTAGAGCAACATA). The threshold cycle (C_T) was defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR arbitrary units of each gene were defined as the mRNA levels normalized to the β actin expression level in each sample. The RT-PCR analysis was controlled by sequencing the amplification products. In addition, we included a sample without RNA in each reaction.

Transport Measurements

Cells were plated in 6-well plates 1-2 days before the transport assay (10^6 cells/well) and treated as indicated in the Figures. To measure L-arginine uptake, cells were washed three times in preheated (37°C) uptake solution (10 mM HEPES, 5.4 mM KCl, 1.2 mM $MgSO_4$ 7 H_2O, 2.8 mM $CaCl_2$ 2 H_2O, 1 mM KH_2PO_4, and 137 mM NaCl, pH 7.4). They were then incubated at 37ºC with 0.5 ml of uptake solution containing 50 μM L-[^{3}H] arginine (5 μCi/ml) in the presence or absence of L-leucine (5 mM) or L-arginine (5 mM) for 1 minute. Uptake was stopped by removing the uptake solution and washing cells with 2 ml of ice-cold stop solution (10 mM HEPES, 10 mM Tris and 137 mM NaCl, pH7.4 with 10 mM non-radioactive L-arginine) three times. After the third wash, cells were lysed in 200 μl of 0.1% SDS and 100 mM of NaOH and 100 μl was used to measure the radioactivity associated with the cells. Values obtained in the presence of 5 mM L-arginine as competitor were always below 10% of the total transport and were subtracted to estimate y^+ activity.

Catabolism of L-arginine in macrophages

Macrophages were incubated with a number of cytokines in a microplate (10^5 cells per well). After 24 hours, cells were washed and incubated for 2 or 6 hours at 37ºC in 0.1 ml arginine-free DMEM containing 2% FCS, 0.1μCi of L-(U-^{14}C) arginine (Amersham, UK). Cells were subsequently lysed by two freeze-thaw cycles. The remaining arginine and synthesis of metabolic products were evaluated by thin-layer chromatography. To identify the spots, 10 μl of a solution containing 2.5 mg/ml of arginine, ornithine and spermine was added to the cell lysates. 20μl of the samples were spotted onto thin-layer chromatographic (TLC) plates (Cromatoplates TLC 20x20 cm, Silica Gel 60 F254, Merck, Germany) and dried for 1 hour at 42°C. Plates were developed in the solvent system chloroform/methanol/ammonium hydroxide/water 0.5/4.5/2.0/1.0 (vol/vol) and dried. Spots were developed with ninhydrin (Spray Solution, Merck) by heating at 120°C for 5 min and scraped into scintillation tubes containing 6 ml EcoscintA™ (National Diagnostics, GA). Radioactivity was determined by scintillation counting (Beckman Instruments) and the values for each compound were expressed as percentage of the total radioactivity measured in triplicate cultures ± standard deviation.

In several experiments, macrophages were plated in 6 well plates (10^6 cells/well) and incubated for 24 hours in media containing M-CSF, IL4+IL10 or IFN-γ +LPS. After removal of incubation media, cells were washed twice with PBS and ^{14}C-L-arginine in PBS (0.5 μCi/well; 300 mCi/mmol of specific activity, 200 μl per well final volume) was added to the wells. Loading of ^{14}C-L-arginine was allowed to proceed for 30 minutes. Cells were then washed three times with PBS and the corresponding media were freshly replaced for the next 6 hours. After this incubation, media were harvested into tubes and cells were lysed with 0.06% tritonX-100, 0.06% SDS, 0.03% sodium deoxycholate and 0.03% BSA in PBS containing protease inhibitors (PMSF, leupeptin

and pepstatin). Media and lysates were cleared of debris by a brief centrifugation and the resulting starting materials were counted to assess the total radioactivity loaded in each condition. Aliquots were used for TCA precipitation by adding FCS to 2% final concentration and TCA 20% final concentration. Next, mixing tubes were centrifuged at 12000 *g* for 10 minutes at 4°C and the supernatants were collected for radioactivity counting. Radioactivity in the pellets was estimated by subtracting the supernatant count from the total count in the sample prior to precipitation.

Nitrite production and arginase activity

NO was measured as nitrite using the Griess reagent [51]. Culture supernatant was mixed with 100 µl of 1% sulphanilamide, 0.1% *N*-(1-naphthyl)ethyl-enediamine dihydrochloride, and 2.5 % H_3PO_4. Absorbance was measured at 540 nm in a microplate reader (Molecular Devices, Ismaning, Germany). Arginase activity was measured in cell lysates, as previously described [51] but with slight modifications. Briefly, cells were lysed with 100 µl of 0.1% Triton X-100. After 30 minutes on a shaker, 100 µl of 25 mM Tris-HCl was added. We then added 10 µl of 10 mM $MnCl_2$ to 100 µl of this lysate and the enzyme was activated by heating for 10 minutes at 56ºC. Arginine hydrolysis was conducted by incubating the lysate with 100 µl of 0.5 M L-arginine (pH 9.7) at 37ºC for 15-120 minutes. The reaction was stopped with 900 µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O_2 (1/3/7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 ml α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95ºC for 30 minutes. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of urea per minute.

Apoptosis assay

Cell viability in culture conditions was assessed by particle counting using FACS (Coulter Multisizer II, Midland, Canada) and confirmed by trypan blue exclusion. Cell death was also assessed by FACS analysis using the rAnnex V-FITC kit (Bender MedSystems, Burlingame, CA), following the manufacturer's instructions. Each point was performed in triplicate and the results were expressed as the mean ± S.D.

Statistical analysis

To calculate the statistical differences between the control and treated samples, we used the Student's paired t-test. Values of $p < 0.05$ or lower were considered significant.

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Figure legends

Figure 1. Arginine is required for M-CSF-dependent macrophage proliferation and M-CSF does not induce activation. A) Quiescent macrophages were incubated with increasing amounts of M-CSF for 24 h and thymidine incorporation was then measured. This figure represents one of three experiments with the mean ± SD of triplicates for each point. $p<0.01$ between a concentration of 300 U/ml and above and controls. B and C) Macrophages were cultured for 48 hours in the presence M-CSF (300 U/ml), IFN-γ (500 U/ml) and LPS (10 ng/ml) or IL-4 (10 U/ml) and IL-10 (10 U/ml) and nitrites (B) or arginase activity (C) were then determined. The values shown correspond to the mean ± SD of three independent experiments. In B) $p< 0.01$ between IFN-γ+LPS-treated samples and controls, M-CSF and IL-4+IL-10-treated samples. In C) $p< 0.01$ between IL-4+IL-10-treated samples and controls, M-CSF and IFN-γ+LPS-treated samples.

Figure 2. Arginine metabolism differs between macrophage proliferation and activation. A) Macrophages were incubated for 2 (open bars) or 6 hours (black bars) with radiolabelled arginine. The total products from outside and inside the cells were then separated by thin-layer chromatography. The results are indicated as percentage of arginine added at the beginning of the assay. The values shown correspond to the mean ± SD of three independent experiments. After 6 hours, a significant difference for arginine degradation between controls or M-CSF with IFN-γ+LPS or IL-4+IL-10 ($p<0.01$) is detected. There is a significant difference for ornithine and spermine production between controls or cells treated with M-CSF and those treated with IFN-γ+LPS or those treated with IL-4+IL-10 ($p<0.01$). Also, a significant difference for citruline was found between the controls or M-CSF-treated cells and those treated with IFN-γ+LPS or IL-4+IL-10 ($p<0.01$). B) Macrophages were incubated for 24 hours with the reagents indicated and for 30 minutes radiolabelled arginine was added. Then cells

were washed and incubated for 6 hours. Media and cells were obtained separately and radioactivity was determined in the pellets and supernatants after TCA treatment of the samples. Protein bound was considered the TCA precipitable material. The results are indicated as percentage of arginine remaining inside the cells before 6 hours of incubation. The values shown correspond to the mean ± SD of a representative experiment run in triplicate. Two additional experiments gave similar results. A significant difference was found for extracellular free and metabolized arginine and for protein-bound intracellular arginine between the controls or the cells treated with IFN-γ+LPS and those stimulated with M-CSF ($p<0.01$).

Figure 3. Arginine transport differs in macrophage proliferation and activation. A) Macrophages were incubated for 24 hours with the activators indicated and arginine uptake was then measured in the absence (open bars) or in the presence of 5 mM L-leucine (black bars) under linear conditions (one minute incubation). There is a significant difference between the control values or those after M-CSF treatment and the values after IFN-γ+LPS or IL-4+IL-10 treatment ($p<0.01$). Results are representative of three independent experiments. B) Macrophages were incubated with the activators for 24 hours and gene expression was then determined using real-time PCR. A significant difference for the *Slc7A2* expression was detected between control or M-CSF-treated cells and after treatment with IFN-γ+LPS or IL-4+IL-10 ($p<0.01$). No significant differences were found in gene expression in the presence or absence of arginine in the medium. Results are representative of at least three independent experiments.

Figure 4. The induction of arginine transport in macrophage activation but not in proliferation is due to CAT2. A) Arginine uptake was measured in macrophages from *Slc7A2* knock-out mice (black bars) and the corresponding wild type controls (open bars). A significant difference for the amount of transport between the controls and

knock-out macrophages after treatment with IFN-γ+LPS or IL-4+IL-10 ($p<0.01$) was detected. No significant differences were observed when we compared the corresponding values of the controls or M-CSF-treated cells. Results are representative of two independent experiments. B) CAT2 is not required for M-CSF-dependent macrophage proliferation. Quiescent macrophages from *Slc7A2* knock-out mice (black round) and wild type (black squares) were incubated with increasing amounts of M-CSF for 24 h and thymidine uptake was then measured. This figure represents one of three experiments with the mean ± SD of triplicates for each point. C) Macrophages from controls (white bars) and *Slc7A2* knock-out mice (black bars) were incubated for 6 hours with radiolabelled arginine. The products were then separated by thin-layer chromatography. Results are indicated as percentage of arginine added at the beginning of the assay. The values shown correspond to the mean ± SD of three independent experiments. The values between controls and *Slc7A2* knock-out macrophages were significantly different ($p<0.01$) for citruline when cells were treated with IFN-γ+LPS and for ornithine when treated with IL-4+IL-10.

Figure 5. Polyamines do not increase M-CSF-dependent macrophage proliferation. Quiescent macrophages were pre-treated for 1 h with the concentrations of polyamines indicated, before incubation with 1200 U/ml of M-CSF for 24 h. Thymidine incorporation was then measured. This figure represents one of three experiments with the mean ± SD of triplicates for each point. $p<0.01$ between concentrations of 25 μM and the other vales for spermidine and spermine.

Figure 6. Arginine uptake and catabolism by activated and proliferative macrophages. During activation CAT2 increases arginine uptake and most is metabolized by NOS2 (classic activation) to NO and citruline and by arginase (alternative activation) to ornthine and polyamines. In quiescence, most of the arginine returns to the media while

a small amount is used for protein synthesis. During proliferation, arginine is transported by a CAT2-independent system and about half is incorporated to protein synthesis while the other half, which is not catabolized, is exchanged with the media.

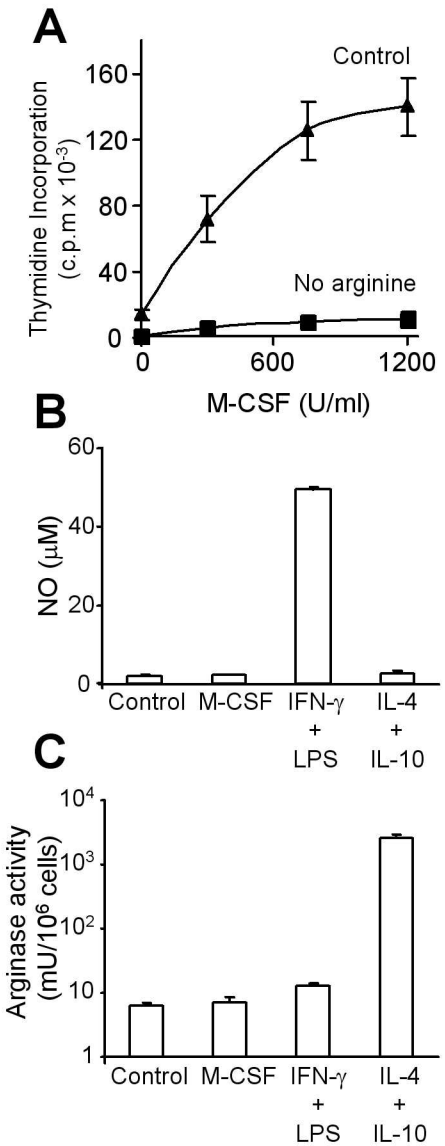
A
Control
No arginine
Thymidine Incorporation (c.p.m x 10-3)
160
120
80
40
0
0
600
1200
M-CSF (U/ml)
B
NO (μM)
60
40
20
0
Control
M-CSF
IFN-γ + LPS
IL-4 + IL-10
C
Arginase activity (mU/10^6 cells)
10^4
10^3
10^2
10
1
Control
M-CSF
IFN-γ + LPS
IL-4 + IL-10

Figure 1

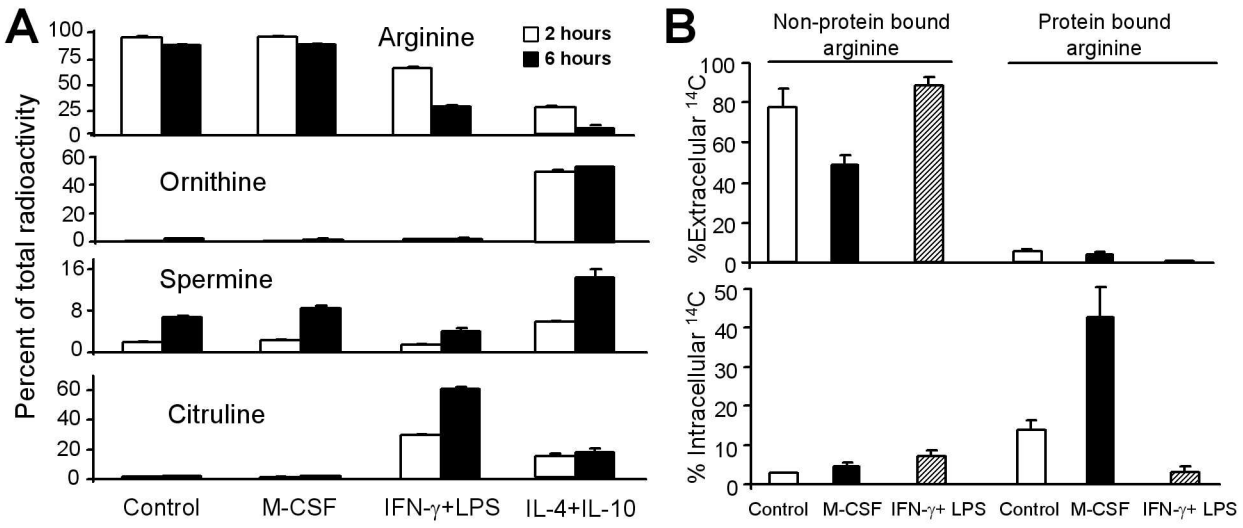
A
Arginine
2 hours
6 hours
Ornithine
Spermine
Citruline
Percent of total radioactivity
Control
M-CSF
IFN-γ+LPS
IL-4+IL-10
B
Non-protein bound arginine
Protein bound arginine
%Extracelular 14C
% Intracellular 14C
Control
M-CSF
IFN-γ+ LPS
Control
M-CSF
IFN-γ+ LPS

Figure 2

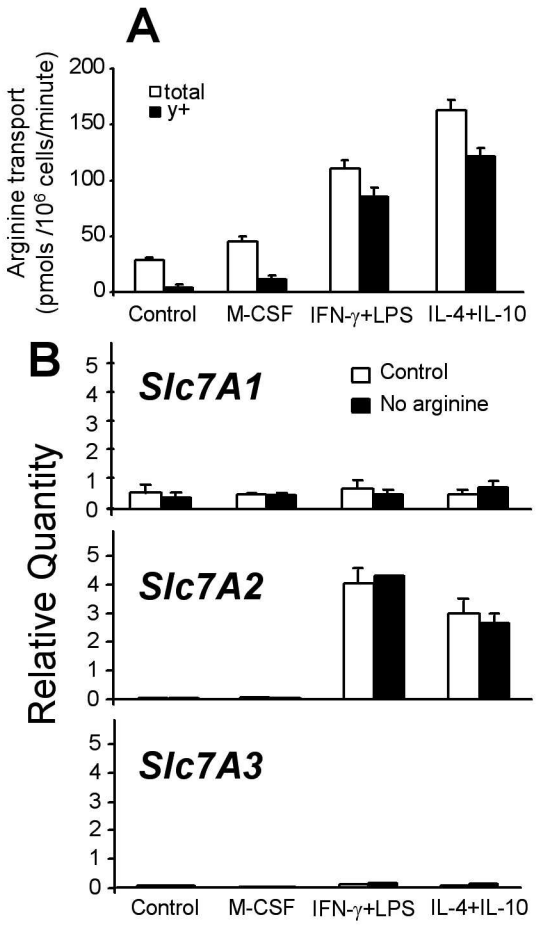
A
Arginine transport
(pmols /10^6 cells/minute)
200
150
100
50
0
total
y+
Control
M-CSF
IFN-γ+LPS
IL-4+IL-10
B
Relative Quantity
Slc7A1
Control
No arginine
Slc7A2
Slc7A3
5
4
3
2
1
0
Control
M-CSF
IFN-γ+LPS
IL-4+IL-10

Figure 3

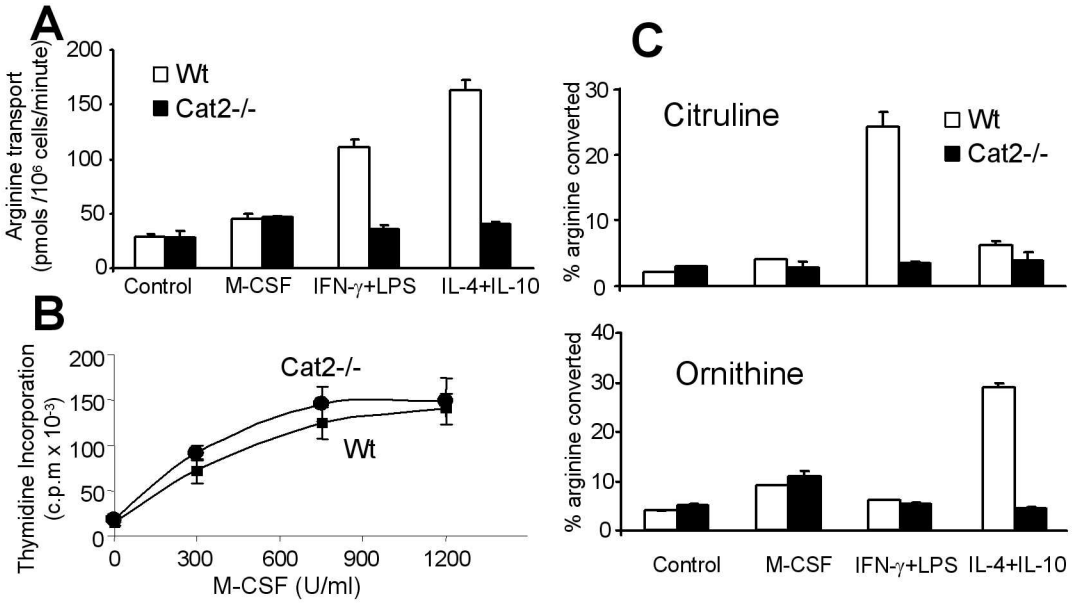
A
Arginine transport (pmols /10⁶ cells/minute)
200
150
100
50
0
Wt
Cat2-/-
Control
M-CSF
IFN-γ+LPS
IL-4+IL-10
B
Thymidine Incorporation (c.p.m x 10⁻³)
200
150
100
50
0
Cat2-/-
Wt
0
300
600
900
1200
M-CSF (U/ml)
C
Citruline
% arginine converted
30
20
10
0
Wt
Cat2-/-
Ornithine
% arginine converted
40
30
20
10
0
Control
M-CSF
IFN-γ+LPS
IL-4+IL-10

Figure 4

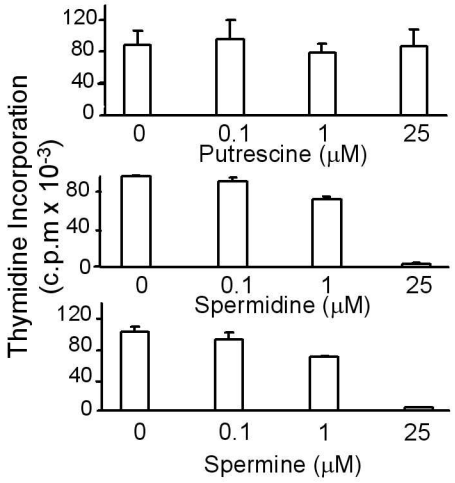
Thymidine Incorporation
(c.p.m x 10-3)
120
80
40
0
0
0.1
1
25
Putrescine (μM)
80
40
0
0
0.1
1
25
Spermidine (μM)
120
80
40
0
0
0.1
1
25
Spermine (μM)

Figure 5

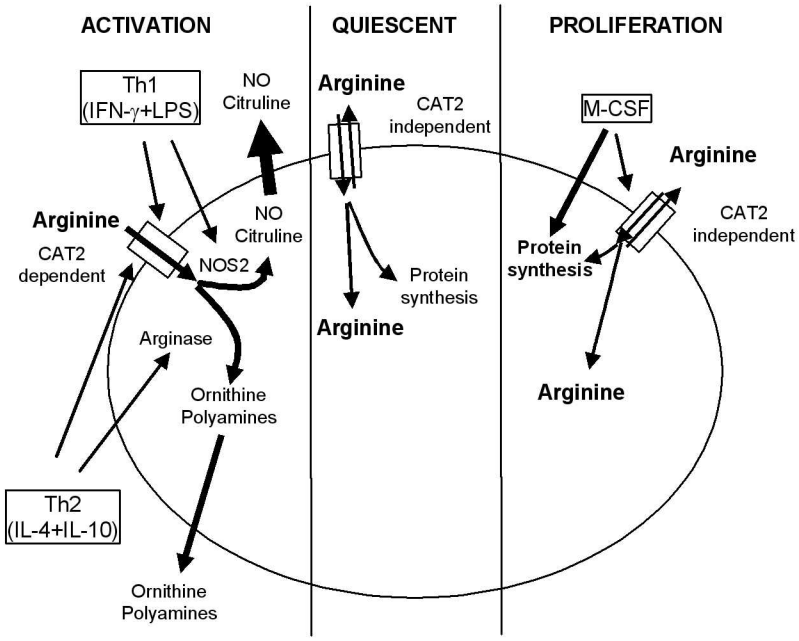
ACTIVATION
QUIESCENT
PROLIFERATION
Th1
(IFN-γ+LPS)
NO
Citruline
Arginine
CAT2
independent
M-CSF
Arginine
CAT2
independent
Arginine
CAT2
dependent
NO
Citruline
NOS2
Protein
synthesis
Protein
synthesis
Arginine
Arginase
Arginine
Ornithine
Polyamines
Th2
(IL-4+IL-10)
Ornithine
Polyamines

Figure 6