Granulocyte-macrophage colony-stimulating factor increases L-arginine transport through the induction of CAT2 in bone marrow-derived macrophages


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IN ADDITION to its role as a precursor in protein synthesis, L-arginine is a substrate for two key enzymes in macrophage activation, the inducible nitric oxide (NO) synthase 2 (NOS2) and arginase I. To perform their function, macrophages must be activated by either type I cytokines, such as IFN-γ, which results in classic activation, or type II cytokines, such as IL-4, which results in alternative activation (reviewed in Ref. 11). These activations correspond to different physiological modifications. NOS2 participates in classic (type I) activation, whereas arginase I is induced in alternative (type II) activation (11, 12, 22).

The challenge of macrophages with either type I or type II factors results in increased L-arginine transport (21, 25). Four distinct transport systems account for L-arginine flux through the plasma membrane in mammalian cells (5, 8). These are the following: 1) system B0,+; a Na+- and Cl−-dependent transporter for neutral and cationic amino acids; 2) system b0,+; which handles both neutral and cationic amino acids in a Na+-independent fashion; 3) system y+, which interacts with cationic amino acids and only very weakly (Km > 10 mM) with neutral amino acids in either the absence or presence of Na+; and 4) system y+L, which handles cationic amino acids in a Na+-independent fashion and neutral amino acids in the presence of Na+. Known proteins have been assigned to these distinct transport activities. System B0,+ is due to the activity of the ATB0,+ transporter (33), whereas system b0,+ is caused by the activity of the heteromeric amino acid transporter b0,+AT/rBAT (10). System y+L is the result of the activity of the heteromeric amino acid transporters y+LAT1/4F2hc and y+LAT2/4F2hc (34), where LAT1 and LAT2 refer to L-arginine transporters-1 and -2. Finally, system y+ arises from the activity of cationic amino acid transporters (CAT)1–3 (36).

Studies using macrophage-derived cell lines or primary differentiated macrophages have shown that macrophages express CAT1 transporters under resting conditions and CAT2 transporters upon type I or type II activation (21, 25). L-Arginine transport is crucial for NOS2 activity, and, in mouse peritoneal macrophages, NO production requires the activity of the CAT2 transporter (25). CAT1, which has a very similar transport activity, cannot compensate for CAT2 in supplying these cells with L-arginine for NO production (25). In contrast,
fibroblasts derived from CAT2-knockout mice show only a 19\% reduction in the production of NO compared with wild-type controls (26). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important factor in the regulation of macrophage function. In fact, in knockout mouse models for this factor or its receptor present pulmonary alveolar proteinosis because of macrophage malfunction in surfactant clearance (9, 28, 32). In addition, GM-CSF plays a crucial role in macrophage differentiation (3) and proliferation (3, 24) and induces arginase I activity as in alternative activation (18).

As a model system to study macrophage biology, bone marrow cells can be grown and differentiated ex vivo by being cultured in petri dishes in the presence of fetal calf serum and GM-CSF. After 7 days in culture, the cells attached to the plastic actively proliferate, express macrophage markers, and respond to the expected regulatory factors (2). These cells are referred to as bone marrow-derived macrophages (BMM).

The participation of L-arginine transporters other than the CATs in basal L-arginine transport into macrophages has not been addressed, and the effect of GM-CSF on L-arginine transport is unknown. In this study, we examined the L-arginine transporters that are active in BMM both under resting conditions and after treatment with GM-CSF. We show that before activation, system y⁺ L is the main L-arginine transport activity present at the plasma membrane. GM-CSF treatment leads to an increase in L-arginine metabolism through arginase. This increase depends on the expression of the CAT2 transporter, resulting in higher system y⁺ transport activity.

MATERIALS AND METHODS

Macrophages. Bone marrow-derived macrophages were isolated from 6-wk-old Swiss or FVB mice, as described elsewhere (2). FVB mice were used in the experiments involving CAT2-knockout mice. The animals were euthanized, and femurs and tibias were dissected after adherent tissue was removed. The ends of the bones were cut off, and the marrow tissue was flushed by irrigation with medium. The marrow plugs were passed through a 25-gauge needle for dispersion. Cells were cultured in nontreated petri dishes in DMEM containing 20\% fetal bovine serum and 30\% cell-conditioned medium as a source of M-CSF (2). Macrophages were obtained as a homogeneous population of adherent cells after 7 days of culture and were maintained at 37°C in a humidified 5\% CO₂ atmosphere. The purity of the culture was checked regularly by flow cytometry using an anti-F4/80 antibody (Serotec). The animal experiments were performed in accordance with University of Barcelona and governmental animal care guidelines, and the animal protocols used were approved by the Animal Experimentation Ethics Committee of the University of Barcelona, with final approval granted by the Animal Experimentation Commission of the Generalitat de Catalunya.

Transport measurements. BMM (5×10⁵) were plated in 24-well plates with culture medium deprived of l-cell-conditioned medium and were either treated or not with GM-CSF. Transport of L-arginine (Amersham) was measured under linear conditions (1 min incubation). Briefly, the cells were washed twice in warm (37°C) uptake solution (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, pH 7.4). Uptake solution containing the desired amino acid concentration and radioactive amino acid (5 µCi/ml) was then added to the cells. The transport process was stopped by a wash (repeated 3 times) with 1 ml of uptake solution containing 10 mM L-amino acids at 4°C. To measure Na⁺ independent transport, NaCl in the uptake solution was replaced by N-methyl d-glucamine brought to pH 7.4 with HCl. After being washed, the cells were lysed by the addition of 200 µl of 0.1\% SDS and 100 mM NaOH. From this amount, 100 µl were used to count the radioactivity in a beta-scintillation counter, and 20 µl were used to determine the protein content in the well using the BCA Protein Assay kit (Pierce Biotechnology). To distinguish transport activities, we measured the transport of L-arginine in the absence or presence of Na⁺ and either a 100-fold excess of l-leucine or a 100-fold excess of l-arginine. No Na⁺ dependent transport of L-arginine was detected, thereby ruling out the participation of system B₄⁺. To obtain the y⁺ component, the uptake of 50 µM l-arginine in the presence of cold l-arginine 5 mM was subtracted from the uptake in the presence of 5 mM l-leucine. The y⁺ L component was inhibited by l-leucine in a Na⁺ dependent fashion and was obtained by subtracting the transport remaining in the presence of l-leucine from the total transport rate evaluated in the absence of inhibitors. To evaluate putative b₄⁺ system activity, inhibition of l-arginine transport by l-leucine in the absence of Na⁺ was measured.

Real-time PCR analysis. BMM (2×10⁵) were plated in 60-mm diameter plates, deprived of l-cell-conditioned medium, and treated with GM-CSF for 16 h or left untreated. Total RNA was then isolated using the RNEasy kit (Qiagen), retro-transcribed at 42°C 1 h, and subjected to real-time PCR sequence detection with the use of ABI Prism 7900 (AME Bioscience). PCR was performed using the following cycling parameters: activation at 95°C for 10 min; PCR cycling, 40 cycles at 95°C 15 s (denaturation), and 60°C 1 min (amplification-extension). The primers used were the following: for CAT1, GTTTCATGCCCGAGGTATCTAT and ATTACCGGTTCGTTTGTGCTATTGTG; for CAT2 GTTTCCCATGCCCCGAGTTATCTAT and ATTACGGGT- LAT1, CCTTGGCCATTGGGATTTCT; for y⁺LAT2, CTGCCCTTACTTCTTTCATCACTCA and CTCTCATTCCATCAAGTCATTTCT; for y⁺LAT2, CTTGCGCATTTGGGATTCTAT and ACAGGCCAAGCTGCTACTTCTTATG; and for cyclophilin, CAAAATGGTCGACCAACACACACAC and TGCCCATCCAGCATCAGTCT, which hybridize to the corresponding murine cDNAs. Experiments were performed in triplicate.

Cell extraction and Western blot analysis. After the appropriate treatment, cells were harvested and a membrane-rich fraction was obtained in 250 mM sucrose, 2 mM EDTA, and 20 mM HEPES, pH 7.5, in the presence of protease inhibitors. Briefly, cells were disrupted by passing the suspension 20 times through a 25-gauge needle. After elimination of cell debris at 300 g (4°C; 10 min), the supernatant was centrifuged at 100,000 g (4°C; 90 min) to obtain the membrane-rich fraction. Lysates were then treated with peptide:N-glycosidase F (Roche Molecular Biochemicals) ratio of 4 U/80 µg protein, for 1 h at 37°C. Proteins were separated on 8\% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were blocked for 2 h with Blotto, composed of 5% nonfat dry milk, 0.1% SDS, and Tween 20, pH 7.5, and were either treated or not with 30 µl of 10 mM MnCl₂ were added, and 100 µl were used to count the radioactivity in a beta-scintillation counter, and 20 µl were used to determine the protein content in the well using the BCA Protein Assay kit (Pierce Biotechnology). To distinguish transport activities, we measured the transport of L-arginine in the absence or presence of Na⁺ and either a 100-fold excess of l-leucine or a 100-fold excess of l-arginine. No Na⁺ dependent transport of L-arginine was detected, thereby ruling out the participation of system B₄⁺. To obtain the y⁺ component, the uptake of 50 µM l-arginine in the presence of cold l-arginine 5 mM was subtracted from the uptake in the presence of 5 mM l-leucine. The y⁺ L component was inhibited by l-leucine in a Na⁺ dependent fashion and was obtained by subtracting the transport remaining in the presence of l-leucine from the total transport rate evaluated in the absence of inhibitors. To evaluate putative b₄⁺ system activity, inhibition of L-arginine transport by l-leucine in the absence of Na⁺ was measured.

Arginase activity. Arginase activity was measured in cell lysates. Cells (10⁵) were lysed in 100 µl of 0.1\% Triton X-100 by being shaken for 30 min. Next, 100 µl of 25 mM Tris·HCl, pH 7.5, were added. To 30 µl of this lysate 3 µl of 10 mM MnCl₂ were added, and the enzyme was activated by heating at 55°C for 10 min. This activated lysate was used to measure arginase activity by the addition of 30 µl of 0.5 M L-arginine, pH 9.7, and incubating at 37°C for 120
min. The reaction was stopped with 240 μl of a solution containing 1 vol H2SO4 (96%), 3 vol H3PO4 (85%), and 7 vol H2O. The urea concentration in the reaction tube was measured spectrophotometrically at 550 nm after the addition of 10 μl of 6% α-isotrodnisopropiophenone (dissolved in 100% ethanol), followed by heating at 95°C for 30 min.

Catabolism of l-arginine in macrophages. Macrophages (10⁵ cells per well) were incubated in a 96-well plate with GM-CSF (10 ng/ml), IL-4 + IL-10 (10 U/ml each) or LPS (10 ng/ml) + IFN-γ (500 U/ml) (Peprotech). After 24 h, cells were washed and incubated at 37°C for 6 h in l-arginine-free DMEM containing 2% fetal bovine serum and 0.1 μCi of l-[U-¹⁴C]arginine (Amersham). Under these conditions, the concentration of l-arginine in the medium at the beginning of the 6-h incubation was ~3 μM. Cells were subsequently lysed in the incubation medium by two freeze-thaw cycles. Metabolic products were evaluated by thin-layer chromatography (TLC). To identify the spots, 10 μl of a solution containing 2.5 mg/ml arginine, ornithine, spermine, spermidine, putrescine, proline, and glutamate were added to the cell lysates. The samples (20 μl) were spotted onto TLC plates (Chromatoplates TLC, 20 × 20 cm, Silica Gel 60 F254; Merck). The products were separated in the solvent system chloroform/methanol/ammonium hydroxide/water (0.5/4.5/2.0/1.0; vol/vol), and the plates were dried. Spots were developed with ninyhydrin (Spray Solution, Merck) by being heated at 120°C for 5 min and scraped into scintillation tubes containing 6 ml of EcoscintA (National Diagnostics). Radioactivity was determined by scintillation counting, and the values for each compound were expressed as a percentage of the total radioactivity. Experiments were done in triplicate.

Intracellular amino acid content. Macrophages plated in 12-well plates were washed three times with 1 ml of ice-cold phosphate-buffered saline. For deproteinization, 200 μl of 10% (wt/vol) sulfosalicylic acid was added to each well. Samples were collected in tubes and centrifuged at 12,000 g for 5 min. The supernatant was removed and stored at −20°C until amino acid measurement. Pellets were dissolved in 0.1 M NaOH for protein determination. Total intracellular amino acid content was determined by HPLC. Amino acid content in cells is expressed as nanomoles per milligram of protein.

Nitrite/nitrate quantification assay. NO production was quantified by assaying for nitrite and nitrate accumulation in the culture media. Briefly, after the cells were stimulated with the agents indicated, culture media were treated with nitrite reductase to convert all nitrates to nitrite before 100 μl of the Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% phosphoric acid) were added. Absorbance was measured at 540 nm, and nitrite concentration was determined using a standard curve of Na⁺ nitrite.

RESULTS

BMM express distinct carriers for l-arginine transport. Several transporters handle l-arginine in animal cells (see Ref. 8 for a review). We evaluated the l-arginine transport systems that are active in BMM. In the presence of Na⁺, a component of the entry of l-arginine was blocked by L-leucine (Fig. 1A). This component corresponded to system y⁺L and accounted for >75% of the total transport rate. The concentration of L-leucine (5 mM) used in the cis-inhibition studies was 100-fold greater than that of l-arginine (50 μM) and was sufficient for maximal inhibition (Fig. 1B, inset). Figure 1B, inset, shows that inhibition of 50 μM l-arginine uptake by L-leucine is concentration dependent and maximum at 2 mM L-leucine. L-Leucine did not inhibit the entry of l-arginine into BMM in the absence of Na⁺, thereby ruling out a contribution of systemic b⁰⁺⁺ (Fig. 1A). Neither was system B⁰⁻⁺ activity present in BMM because the transport rate in the absence of Na⁺ was the same as in its presence (Fig. 1A). There was a second component of l-arginine transport into BMM insensitive to L-leucine, even in the presence of Na⁺, which was inhibited by pretreatment with the sulfhydryl-specific reagent N-ethyl maleimide (NEM) (Fig. 1B). The NEM-sensitive component, corresponding to system γ⁺ (7), accounted for only 8% of the total 50 μM l-arginine uptake in our BMM preparations. Inhibition by NEM and leucine led to total blockage of the saturable uptake of l-arginine in BMM, thereby demonstrating the presence of system γ⁺ and γ⁺⁺ L activities (Fig. 1B). Finally, a third component of uptake was detected that was not inhibited by even a 200-fold (10 mM) excess of l-arginine. This component, which can be attributed to either a carrier with a very low affinity for l-arginine or to passive diffusion, accounted for 10–20% of the total entry at 50 μM l-arginine in the different experiments (Fig. 1B).

GM-CSF increases arginase activity and l-arginine transport into BMM through system γ⁺. GM-CSF is an important factor that has diverse effects on macrophage biology. GM-CSF increased arginase I activity in BMM without affecting...
NOS2 activity (Table 1). Nitrite concentration values of 2 μM in untreated cells were just at the detection limit of the assay. Cells treated with lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) as a control for type I activation (11, 12, 22), gave values close to 50 μM nitrite in the same assay (Table 1). In contrast, activation with interleukin-4 (IL-4), a typical type II activator (11, 12, 23), led to an increase in arginase activity without affecting nitrite production (Table 1). These data indicate that GM-CSF induces arginase activity similar to type II activation in BMM.

Because GM-CSF induces L-arginine consumption through arginase I, we studied the effect of GM-CSF on L-arginine transport. Treatment of BMM with GM-CSF increased L-arginine transport, but no significant change in L-alanine transport was detected, indicating that the effect is specific (Fig. 2A). The total entry of L-arginine into BMM increased 1.8-fold in response to GM-CSF. This effect was concentration dependent, having been saturated at 10 ng/ml (Fig. 2B).

We next studied the component of L-arginine transport that is affected by GM-CSF. The y+ component increased >10-fold in response to GM-CSF (Table 2), whereas the y+L component was not significantly affected by this treatment (Table 2). Consequently, the repertoire of transport activities at the plasma membrane was modified such that y+ activity accounted for ~40% of the total transport rate in treated cells, when measured at 50 μM L-arginine. Changes in the transport of L-arginine did not lead to significant alterations in its intracellular content between control and cells treated with GM-CSF for 16 h (1.04 ± 0.19 vs. 1.08 ± 0.12 nmol/mg protein, respectively).

The increase in y+ activity correlates with an increase in CAT2 mRNA and protein. The increase in L-arginine transport paralleled an increase in CAT2 mRNA and the corresponding protein (Fig. 3). RNA analysis revealed that y+LAT1 and y+LAT2 were expressed in BMM and therefore may be responsible for the y+L activity observed (Fig. 3A). System y+ can be explained by the activity of the transporters CAT1, CAT2, and CAT3 (5, 36). BMM expressed CAT1 and CAT2 mRNAs (Fig. 3A). CAT3 expression is restricted to the brain in mice (14, 16, 27) and was not detected in BMM. The increment in mRNA was observed only for CAT2. In fact, y+LAT1 mRNA was significantly reduced after 16 h of exposure to GM-CSF (Fig. 3B). In agreement with this finding, Western blot analysis revealed a 6.1 ± 1.7 (n = 9)-fold increase in the amount of CAT2 protein in response to GM-CSF (Fig. 3B).

LPS treatment, known to cause a large increase in CAT2 protein expression (19), was used as a control in the Western blot analysis and resulted in a 21.7 ± 4.9 (n = 3)-fold increase. Treatment with GM-CSF in the presence of cycloheximide did not increase the transport of L-arginine, indicating that the effect is dependent on protein synthesis (data not shown).

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<tr>
<th>Table 1. GM-CSF increases arginase activity in BMM</th>
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<td><strong>Arginase Activity</strong>, nmol·h⁻¹ urea/mg protein⁻¹</td>
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<tr>
<td>Control</td>
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<tr>
<td>GM-CSF</td>
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<td>IFNγ + LPS</td>
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<td>IL-4</td>
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Values are means ± SE of 3 independent experiments. GM-CSF, granulocyte macrophage colony-stimulating factor. Bone marrow-derived macrophages (BMM) were plated in 24-well plates (5 × 10⁵ cells/well) and incubated overnight (16 h) in the presence or absence of GM-CSF (10 ng/ml). Cells were then lysed and arginase activity was measured, as described in MATERIALS AND METHODS. Nitrites (NO₃) in the culture media were determined, as described in MATERIALS AND METHODS, to assess differences in nitric oxide production. IFNγ + LPS and IL-4 treatments were used as controls. *P < 0.001, differences are statistically significant.

<table>
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<th>Table 2. GM-CSF increases L-arginine transport through system y⁺</th>
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<td><strong>L-Arginine Transport</strong>, pmol·mg protein⁻¹·min⁻¹</td>
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<tr>
<td><strong>System</strong></td>
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<tr>
<td>y⁺</td>
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<td>y⁺L</td>
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<td>Total</td>
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Values are means ± SE of 10 independent experiments. BMM were used to assess the transport of L-arginine after treatment with GM-CSF. Controls were left untreated. Uptake conditions were set to distinguish y⁺ from y⁺L components as described in MATERIALS AND METHODS. Total uptake and transport corresponding to systems y⁺ and y⁺L are presented. *P < 0.01, differences are statistically significant.
CAT1 compensates only partially for CAT2 activity in BMM in the response to GM-CSF. Because CAT2 appeared to be the only transporter in BMM responsible for the increased \( y^+ \) /H11001 activity in response to GM-CSF, we evaluated the effect of this factor on cells from mice lacking the CAT2 protein (25). In basal conditions, BMM derived from CAT2 knockout animals transported L-arginine mainly through \( y^+ \) /H11001 transporters, as revealed by L-leucine inhibition studies. Thus 50 /H9262 ML-arginine transport was reduced from 479 /H11006 44 to 83 /H11006 8 pmol /H18528 mg protein /H11002 1 by L-leucine in the presence of Na\(^+\). Therefore, \( y^+ \) /H11001 activity was 396 /H11006 45 pmol /H18528 mg protein /H11002 1 by L-leucine (n = 3 independent experiments). GM-CSF treatment did not lead to significant changes in \( y^+ \) /H11001 activity (320 /H11006 46 pmol /H18528 mg protein /H11002 1; n = 3 paired independent experiments). In contrast, there was an increase in \( y^+ \) activity in response to

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**Fig. 3.** GM-CSF increases cationic amino acid transporter 2 (CAT2) mRNA and protein in macrophages. A: BMM were incubated for 16 h in 10 ng/ml GM-CSF or left untreated as controls. RNA was then isolated and used for real-time PCR analysis after retro-transcription. Data correspond to means ± SE of 12 BMM preparations normalized to cyclophilin amplification and are expressed in relative amounts with respect to amplification in samples from non-GM-CSF-treated cells. ***P < 0.001, vs. statistically significant differences. B: representative Western blot analysis using a polyclonal anti-mouse CAT2 antiserum (6). Samples correspond to membranes isolated from BMM that were either treated or not treated with GM-CSF and deglycosylated with endoglycosidase F. For quantification, Western blot data were normalized to the amount of tubulin signal, which was almost identical in the different lanes (data not shown).

**Fig. 4.** In BMM from CAT2-knockout (KO) mice, GM-CSF causes a modest increase in \( y^+ \) activity, which correlates with an increase in the level of CAT1 mRNA. A: BMM from CAT2-knockout mice (CAT2 KO) or paired wild-type controls were treated with GM-CSF (16 h, 10 ng/ml) or left untreated. Cells were then used to measure L-arginine uptake through system \( y^+ \), as described in MATERIALS AND METHODS. Data correspond to means ± SE of 3 preparations. **P < 0.01; ***P < 0.001, statistically significant differences. B and C: RNA was isolated and used in reverse transcription, followed by real-time PCR to analyze the amount of mRNA corresponding to CAT1 (B) and CAT2 (C) relative to cyclophilin. Results are expressed as the (relative) fold induction with respect to amplification in samples from non-GM-CSF-treated cells from wild-type mice. No amplification of CAT2 was detected in samples corresponding to KO cells. Data correspond to means ± SE of 3 preparations. *P < 0.05, statistically significant differences.
GM-CSF, which, in absolute terms, was about four times lower than that observed in paired controls corresponding to cells obtained from wild-type animals (Fig. 4A). Because the increase observed could not be attributed to CAT2 in deficient cells, we analyzed CAT1 mRNA. We detected an increase that was not observed in control cells from wild-type mice (Fig. 4B). As expected, cells from wild-type mice responded to GM-CSF by increasing CAT2 mRNA (Fig. 4C). Therefore, macrophages with inactivated CAT2 responded to GM-CSF treatment by rising CAT1 mRNA and moderately increasing system y⁺ activity.

Because CAT1 does not supply l-arginine for NOS2 in macrophages derived from CAT2-deficient mice (25), we studied whether the conversion of l-arginine to ornithine, and to other metabolites into which ornithine can be metabolized, is impaired in CAT2-knockout BMM after GM-CSF treatment. Metabolization of l-arginine was evaluated by analyzing the conversion of l-[14C]arginine to ornithine and to metabolites that can be synthesized from this primary product of arginase activity. During incubation for 6 h, GM-CSF-treated BMM metabolized ~90% of the l-arginine in the culture medium to mainly ornithine, citrulline, spermine, glutamate and proline, whereas untreated cells consumed only ~30% of the l-arginine (Fig. 5). Interestingly, CAT2-knockout cells showed a lower (20%) basal metabolization of l-arginine to these products and GM-CSF treatment, despite inducing equal arginase I activity (1.68 ± 0.1 and 1.78 ± 0.1-fold induction from an identical basal activity in wild-type and CAT2-knockout cell lysates, respectively; n = 3 paired independent experiments), caused only a very modest increase in l-arginine metabolization to ~30% (Fig. 5). In fact, the increase in conversion to citrulline and ornithine in CAT2-knockout BMM, although significant, was very low in absolute terms compared with wild-type cells. The amount of 14C in spermine showed a large increase in response to the GM-CSF in CAT2-knockout BMM, but was still much lower than that in treated wild-type BMM (Fig. 5).

The diagram in Fig. 6 indicates that citrulline, putrescine, spermidine, spermine glutamate, and proline can be synthesized from ornithine in macrophages (13, 17). As stated above, metabolization to ornithine, spermine, citrulline, proline, and glutamate from arginine after GM-CSF treatment was higher in wild-type than in CAT2-knockout cells (Fig. 5). Differences could not be attributed to total intracellular l-arginine content. In fact, the amount of l-arginine in BMM lysates, obtained after 7 days in the culture conditions described in MATERIALS AND METHODS, was 1.15 ± 0.16 vs. 1.15 ± 0.18 nmol/mg of protein for wild-type and CAT2-knockout cells, respectively. These amounts of l-arginine did not change significantly upon

Fig. 5. Metabolization of l-arginine in response to GM-CSF is reduced in BMM from CAT2-KO mice. BMM from wild-type or CAT2-KO mice were incubated for 24 h with GM-CSF (10 ng/ml) or left untreated. 14C-Labeled l-arginine was then added to the culture medium for another 6 h. Cells were then lysed, and the conversion of l-arginine into diverse metabolites was assessed after thin-layer chromatography, as described in MATERIALS AND METHODS. Conversion to ornithine (A), spermine (B), proline (C), glutamate (D), citrulline (E), and 14C (F) remaining as arginine. Data correspond to means ± SE of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, differences are statistically significant. 14C accumulation in putrescine was close to 3% and in spermidine ~1%, both in wild-type and CAT2-KO BMM. These values did not change significantly in response to GM-CSF.
treatment with GM-CSF in either cell type (1.07 ± 0.07 vs. 0.88 ± 0.11 nmol/mg of protein, respectively). These data indicate that transport limits the supply of substrate to arginase I, leading to lower amounts of metabolized L-arginine products. In conclusion, the increase in CAT1 activity did not completely compensate CAT2 activity regarding L-arginine transport or in providing arginase with enough substrate.

DISCUSSION

In this study, we have shown that L-arginine flux through the plasma membrane of resting BMM occurs mainly through the system y\textsuperscript{+}L. In addition, we have provided conclusive evidence of the regulation of L-arginine transport by GM-CSF in BMM. GM-CSF challenge led to a quantitatively different repertoire of L-arginine transport activities at the cell surface of BMM by upregulating the CAT2 transporter. This change is likely to have an important impact on the cell because of the distinct roles of L-arginine transport activities. The primary function of system y\textsuperscript{+}L is to remove cationic amino acids from the cell in exchange for neutral amino acids plus Na\textsuperscript{+} (4, 34). This has been clearly demonstrated for epithelial cells in the kidney and intestine, and, because of the obligatory exchange mechanism of transport for system y\textsuperscript{+}L, it might be extended to other cell types (4, 29, 34). In contrast, system y\textsuperscript{-}L moves cationic amino acids following an electrochemical gradient (20). Therefore, upon GM-CSF treatment, more L-arginine can be taken up by the cell to feed arginase, an enzyme upregulated by GM-CSF (18).

Fig. 6. Scheme for the transport and metabolization of L-arginine in BMM on GM-CSF treatment. A diagram of the metabolic fate of arginine in activated macrophages from wild-type (A) or CAT2-knockout (B) BMM is presented. Because GM-CSF-treated macrophages activate arginase I, the pathways that convert ornithine into the diverse metabolites analyzed in the present study are succinctly indicated. The line between ornithine and citrulline corresponds to the urea cycle. The analysis of these compounds explained >90% of the total radioactivity added to the culture in the present study. Arrow width correlates with the increase in metabolic flow caused by GM-CSF treatment. The number of arrows next to metabolite or enzyme names correlates with metabolite accumulation or enzyme activation, respectively. Because NO synthesis is not induced by GM-CSF (Table 1), the accumulation of 14C in citrulline is presumably due to the urea cycle, which is active in macrophages (13). The role of y\textsuperscript{-}LAT1 as an obligatory exchanger in the flux of L-arginine in BMM is indicated. Transport of L-neutral amino acids (αααα) through system y\textsuperscript{-}L is Na\textsuperscript{+} dependent. Therefore, neutral amino acids use system y\textsuperscript{-}L to enter the cell in exchange for cationic amino acids that will be released from the cell. Because of the obligatory exchange mechanism of this transport system, uptake of cationic amino acids through system y\textsuperscript{-}L can be conceived only in the context of exchange with other cationic amino acids (4).
GM-CSF greatly increases the consumption rate of l-arginine through arginase I in BMM, and this increase is highly dependent on the induction of the CAT2 transporter. In our experiments, in cells treated with GM-CSF, >90% of the \[^{14}C\]arginine added was metabolized in 6 h. In nontreated cells or cells from CAT2-knockout animals, 70–80% of the radioactivity remained in the form of arginine at this incubation time. Interestingly, CAT2-knockout animals attempted to compensate for l-arginine demand by upregulating CAT1 in response to GM-CSF. CAT1 mRNA has been shown to be posttranscriptionally regulated by amino acid depletions, resulting in an increased CAT1 mRNA stability (1, 15). In the present study, we provide evidence for substrate limitation in CAT2-knockout BMM, when these cells are treated with GM-CSF. As a consequence, the induced arginase activity does not result in higher l-arginine metabolism. This cell situation might mimic amino acid starvation and lead to the observed higher amount of CAT1 mRNA. Notwithstanding, in our study, the transport activity at the plasma membrane was only partially compensated, thereby diminishing the impact of GM-CSF on the balance between y\[^+\]L and y\[^+\] activities and the capacity of the cell to direct extracellular l-arginine to arginase I. Therefore, the lack of total compensation of system y\[^+\] transport activity results in lower supply of substrate to arginase I and lower metabolism of l-arginine. The presence of a constitutively high systemic y\[^+\]L transport activity does not provide l-arginine to arginase I. In this sense, GM-CSF-treated CAT2-deficient BMM, which have noteworthy system y\[^+\]L and stimulated arginase I activities, present a low rate of consumption of extracellular l-arginine. This supports the hypothesis that system y\[^+\]L serves to remove arginine from macrophages, rather to mediate its uptake into these cells.

GM-CSF is also essential for the maintenance of pulmonary surfactant homeostasis through its action on alveolar macrophages, as revealed by knockout experiments (9, 28, 32). In fact, bone marrow transplantation in mice deficient in the GM-CSF receptor corrects the lung abnormalities observed in these mice, which include pulmonary alveolar proteinosis (28). The increase in CAT2 caused by GM-CSF in BMM results in changes in the l-arginine transporter repertoire, which may be critical for some macrophage functions. Therefore, it is plausible that the imbalance between CATs and y\[^+\]LATs in distinct conditions leads to alterations in macrophages that affect their activity. In this regard, mutations in y\[^+\]LAT1 cause lysinuric protein intolerance in humans (35). Several patients with this type of intolerance have developed pulmonary alveolar proteinosis, and lung transplantation resulted in relapse in one patient, indicating that the pathogenesis came from outside the lung (30, 31). The observations reported in the present study may contribute to our understanding of the pulmonary alveolar proteinosis associated with lysinuric protein intolerance.

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REFERENCES


2. Celada A, Gray PW, Rinderknecht E, and Schreiber RD. Evidence for a gamma-interferon receptor that regulates macrophage tumouricidal activi-


