Arginine transport via CAT2 plays a critical regulatory role in classical or alternative activation of macrophages[1]

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Abstract

Arginine is processed by macrophages in response to the cytokines to which these cells are exposed. Th1-type cytokines induce nitric oxide synthase (NOS)2, which metabolises arginine into nitrites while the Th2-type produces arginase, which converts arginine into polyamines and proline. Activation of bone marrow-derived macrophages by these two types of cytokines increases L-arginine transport only through the y^+ system. Analysis of the expression of the genes involved in this system showed that *Slc7A1,* encoding cationic amino acid transporters (CAT)1, is constitutively expressed and is not modified by activating agents, while *Slc7A2,* encoding CAT2, is induced during both classical and alternative activation. Macrophages from *Slc7A2* knock-out mice showed a decrease in L-arginine transport in response to the two kinds of cytokines. However, while NOS2 and arginase expression were unmodified in these cells, the catabolism of arginine was impaired by both pathways, producing smaller amounts of nitrites and also of polyamines and proline. In addition, the induction of *Slc7A2* expression was independent of the arginine available and of the enzymes that metabolize it. In conclusion, the increased arginine transport mediated by activators is strongly regulated by CAT2 expression, which could limit the function of macrophages.

Introduction

To perform their function macrophages must be activated either by Th1-type cytokines, such as IFN-γ, which is called "classical activation or M1", or by Th2-type cytokines, such as IL-4, IL-10, IL-13, etc, referred to as "alternative activation or M2" (1). Activation induces biochemical and morphological modifications in the macrophages that allow them to perform their functional activity (2). Activation blocks the proliferation of these cells (3). The two types of activation are characterized by the way in which arginine is processed. Th1-type cytokines induce nitric oxide synthase (NOS)2, causing the production of NO, while Th2-type produce arginase, which metabolizes arginine in polyamines and triggers the urea pathway (4, 5).

Cell activation requires of arginine for the synthesis of proteins, for the production of NO via classical activation and for the production of polyamines and proline through alternative activation (1, 5). The extracellular milieu is the main source of arginine. The functional significance of arginine transporters was demonstrated in macrophages, in which NOS2-mediated NO synthesis largely depends on the extracellular supply of L-arginine (6-8). Arginine crosses the plasma membrane through several transport systems. Depending on the cell type, a number of transport activities may be induced (9). The SLC7 family of transporters is divided into two subgroups, the cationic amino acid (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 family includes 4 members, CAT-1 to CAT-4, whose corresponding genes 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 comprises 7 proteins, whose genes are *SLC7A5* to *SLC7A11*; however, only y^+LAT2 (*SLC7A6*), y^+LAT1 (*SLC7A7*) and b^{o+}AT (*SLC7A9*) transport

cationic amino acids (9). In addition, the $B^{0,+}AT$ transporter (*SLC6A14*), belonging to the SLC6 family, also transports arginine but in a sodium- and chloride-dependent fashion (10).

Macrophages require arginine to elaborate gene products when they become activated, for example, IFN-γ induces the expression of more than 300 genes (11). Consequently, to meet their metabolic demands, macrophages require the uptake of exogenous arginine; this process may therefore be a key regulatory step for physiological responses in these cells. Here we studied how classical and alternative activation affects arginine transport activity. To this end, we used bone marrow-derived macrophages, which are non-transformed cells that respond to both Th1- and Th2-type activating stimuli. We showed that macrophage activation leads to an increase in arginine transport. For both types of activation, the only gene to show increased expression was *Slc7A2*, the product of which is a limiting factor that regulates the catabolism of arginine and the production of NO and polyamines.

Material and methods

Reagents

LPS and recombinant cytokines were purchased from Sigma Chemical Co. (St. Louis, MO). N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) was purchased from Bachem (Bubendorf, Switzerland). 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) (12), 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 as well as peritoneal macrophages were isolated from six-week-old BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) as previously described (13). Bone marrow-derived 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 KO for *Slc7A2* has been reported (14), while the *NOS2 KO* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Peritoneal exudate cells were harvested by lavage from mice that had been injected intraperitoneally with 3 ml of 10% protease peptone (Difco Laboratories, Detroit, MI) 3 d earlier. Macrophage monolayers were prepared by seeding the exudate cell suspension into flat-bottomed tissue culture plates. The cells were adhered for 2 h at 37ºC and the plates washed vigorously to remove non-adherent cells. Animal experiments were performed in accordance with institutional and government guidelines (University of Barcelona).

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 (15). 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 *NOS2,* GCCACCAACAATGGCAACA and CGTACCGGATGAGCTGTGAATT; for *arginase 1,* AACACGGCAGTGGCTTTAACC and GGTTTTCATGTGGCGCATTC; for *Slc7A1,* 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 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.

Nitrite production and arginase activity

NO was measured as nitrite using the Griess reagent (16). 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 (16) 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 MnCl2 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.

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.

Results

M1 and M2 activation catabolizes arginine differently.

In our experiments, we used bone marrow-derived macrophages, a homogeneous population of primary and quiescent cells. Treatment of these cells with several cytokines causes a series of modifications that allow them to develop their functional activities (3). To study M1 activation, we used IFN-γ or LPS, while IL-4 and IL-10 were used to examine M2 activation. Although these two types of activation regulate distinct sets of genes (17), both require arginine, either for NO production in classical activation or as a substrate for arginase induced during alternative activation. Only molecules from M1 activation induced nitrite production while only cytokines involved in M2 activation stimulated arginase activity (Fig. 1A and B). IFN-γ and LPS, but not IL-4 or IL-10, led to *NOS2* expression. In contrast, Th2-type but not Th1-type cytokines induced *arginase* (Fig. 1A and B). To determine the metabolism of arginine, we incubated activated macrophages with radiolabelled substrate and then separated the distinct products by thin layer chromatography. When macrophages were incubated with Th2-type cytokines, an increased amount of ornithine and spermine was produced as a result of the catabolic activity of arginase (Fig. 1C). However, when these cells were treated with IFN-γ or LPS, the amount of citrulline increased, indicating the processing of arginine by NOS2. Therefore, the reagents used in this study determined macrophage activation via the M1 or M2 pathway. No production of NOS2 was observed when cells were incubated with IL-4 or IL-4+IL-10 (Fig. 1A). However, an increased amount of citrulline was produced (Fig. 1C). This was probably mediated through the urea cycle, which is active in macrophages (18). Through the action of carbamylphosphate and ornithinecarbamyltransferase, ornithine may be converted into citrulline.

M1 and M2 activation increases arginine transport through the y^+ system, thereby inducing Slc7A2 expression.

Because arginine is required for macrophage metabolism, here we examined whether M1 and M2 activation modulates the transport of this amino acid. In a dose-dependent manner, both LPS and IL-4 produced an increase in arginine transport (Fig. 1D). Next, among the transport systems that mediate arginine uptake (y^+, B^{o+}, b^{o+} and y^+L) (9), we identified the system induced in macrophages activated by Th1- or Th2-type cytokines. Arginine transport was measured in the absence and in the presence of 5 mM L-leucine, in medium containing sodium, to distinguish between total and y^+ system-mediated transport. L-leucine inhibited most of the basal L-arginine transport, indicating that very little y^+ activity is present in these macrophages under basal conditions. In contrast, the increase in transport that occurred in the presence of Th1- or Th2-type cytokines was mediated by the y^+ system (Fig. 1E).

The y^+ system is encoded by the CAT genes *Slc7A1* (19, 20), *Slc7A2* (21, 22) and *Slc7A3* (23). Using a quantitative PCR reaction, we determined the levels of the three members of the y^+ system in macrophages activated by Th1- and Th2-type cytokines. Low levels of *Slc7A1* were found in untreated cells. Expression of this gene was not modified by the treatments (Fig. 2). *Slc7A2* was not detected in quiescent cells but was induced by LPS and LPS+IFN-γ and also by IL4 and IL-4+IL-10. *Slc7A3* was not detected. In addition, the expression of the genes of the y^+ system was measured in arginine-free media; however, no differences in induction by the distinct cytokines were observed in relation to the controls (Fig. 2).

Our results so far indicate that in basal conditions CAT1 mediates the transport of arginine through the y^+ system while CAT2 is the main L-arginine transporter for

activated macrophages. To confirm these data, we used macrophages from mice with disrupted *Slc7A2* (14). In basal conditions, without stimulation, the amount of arginine transported was similar in macrophages from *Slc7A2* KO mice and controls (Fig. 3A). However, while classical or alternative activators produced an increase in arginine transport in controls, no increase in cells from *Slc7A2* KO mice were observed (Fig. 3A). 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 they were treated with activators, and *Slc7A3* was not expressed in this model (data not shown). These results confirm that macrophage activation induces *Slc7A2* expression, which is responsible for increased arginine transport.

In the absence of Slc7A2, macrophage activation is limited.

We next addressed the functional consequences of *Slc7A2* disruption on macrophages. M1 activation produced *NOS2* and M2 activation induced *arginase 1* in *Slc7A2* KO and control cells at similar levels (Fig. 3B). Comparable results were found when we measured the expression of protein by Western blot and when we used peritoneal macrophages (data non shown). When macrophages were incubated with activating agents, *Slc7A2* KO macrophages showed a reduced catabolism of arginine (Fig. 4A). Also, the amounts of ornithine, spermine and citrulline produced by these cells were decreased in relation to controls (Fig. 4B).

On the basis of the decreased catabolism of arginine in macrophages from *Slc7A2* KO mice, we next studied the functional activity of these cells. Although the amounts of NOS2 were similar to those of controls, a significant decrease in NO production was detected in both bone marrow-derived and peritoneal macrophages (Fig.

4B). As the measurement of NO production is made with the entire cell, we conclude that the low amount of NO produced is due to the lower amount of intracellular arginine available. The enzymatic assay for arginase activity requires the rupture of the cell and the addition of external substrate. Under these conditions, arginase activity in *Slc7A2* KO macrophages was similar to the controls. However, because the metabolic conversion of arginine into ornithine and polyamines is impaired in the entire cell, these data show that the amount of arginine available is the limiting factor for arginase activity. Therefore, CAT2 limits the activation of macrophages by Th1- and Th2-type cytokines.

Arginine transport is not regulated by the levels of NOS2 or arginase

We next studied whether the induction of the enzymes that catabolize arginine produces an increase in the transport of this amino acid. To explore the role of arginase 1, we used the specific inhibitor nor-NOHA, which blocks arginase activity by inhibiting the degradation of arginine into ornithine but does not block the production of NO after IFN-γ treatment. Treatment with nor-NOHA did not affect the transport of arginine induced by the IL-4+IL-10 or LPS+IFN-γ treatments (Fig. 5A). As expected, the induction of *Slc7A2* expression in macrophages via classical and alternative activation was not modified by incubation with nor-NOHA (Fig 5B).

To determine the role of NOS2, we used macrophages from *NOS2* KO mice. As in the case of arginase, the elimination of *NOS2* did not modify the increase in arginine transport induced by M1 and M2 activators (Fig. 5C). No significant difference in *Slc7A2* expression was observed when we compared *NOS2* KO macrophages with the controls (Fig. 5D).

Discussion

Arginine is one of the metabolic and signalling functions required for protein synthesis by macrophages. In addition, it is the key amino acid involved in both M1 and M2 activation (1, 5). Macrophages require exogenous arginine to meet their metabolic demands. Therefore, the transport of this amino acid across the plasma membrane is an essential regulatory first step during activation of the macrophage, which synthesizes a large number of proteins (11). In addition, these cells catabolize arginine to NO by NOS2 induced by Th-1-type cytokines or to ornithine, proline and polyamines by arginase induced by the Th-2-type (1, 5, 24).

Stimulation of L-arginine transport by only Th1-type inductors has been reported in several macrophage populations, including murine peritoneal (14, 25-27), rat alveolar (28), human monocyte-derived (27), and the murine cell lines J774 (27, 29-31) and RAW264 (32). However, data on arginine transport in macrophages is limited. In peritoneal macrophages, this transport is induced by LPS+IFN-γ through the y^+ system and particularly by CAT2 (14), while in human monocytes, IFN-γ stimulates this transport through the y^+L system (33). In rat alveolar macrophages, the y^+ and y^+L transport systems participate in arginine transport (28). Previous observations in rat alveolar macrophages described an increase in CAT2 after LPS treatment (28).

Here we used murine bone marrow-derived macrophages because they are primary cells and precursors of the different types of macrophages (peritoneal, alveolar, dendritic cells, etc). Moreover, these cells constitute a unique culture model, which, unlike peritoneal or alveolar macrophages, are not previously activated and which respond to Th1- and Th2-type cytokines (2). Since the two types of macrophage activation require extracellular arginine, we studied the transport systems and their regulation in this model. Under basal conditions, y^+-mediated L-arginine transport is

due only to CAT1, and after activation, the increase in transport is due to CAT2. Interestingly, although CAT1 is functional, the absence of CAT2 caused a decrease in NO production and also in the production of polyamines and proline. This observation indicates that arginine transported by CAT1 does not fulfil the requirements of the machinery responsible for macrophage activation.

Our results disagree partially with those described by Nicholson *et al.* (14), who reported the absence of NO production in *Slc7A2*-/- macrophages after activation. In these macrophages, the arginine content was unchanged in relation to controls. This finding indicates that two defined pools of L-arginine may be present in these cells, only one of which is accessible for NOS2 while the other covers the CAT1-dependent metabolic requirements of the cell. Nicholson *et al.* (14) proposed that CAT2 and NOS2 co-associate. However, no physical interaction was demonstrated. The disagreement between these and our results may be attributable to technical differences in experimental design. In fact, in our case, NO production was determined after incubating macrophages for 19 hours in the presence of cytokines, while in the study performed by Nicholson et al. (14), macrophages were incubated with cytokines and then washed and incubated in fresh cytokine-free media for an additional 24 hours, after which NO production was measured. Therefore, under these conditions, in the absence of cytokines, the levels of NOS2 decline (34) and NO production also decreases. However, we cannot exclude other explanations for the discrepancy such as differences in culture conditions or the measurement method used. However, the background of KO animals (C57Bl/6 or Balb/c) does not explain these discrepancies because similar results were found when we used the two strains (data not show). It should be noted that for these authors, the production of NO in fibroblasts and astrocytes is not completely dependent on CAT2 (35, 36).

Recently, we reported that the transport system of nucleosides in macrophages differs when cells proliferate or when they become activated by either IFN-γ or LPS (37, 38). This finding indicates distinct pools for different activities. When we studied the effect of M-CSF on arginine transport, no increase was observed in relation to the controls and no expression of *Slc7A2* was detected (Manuscript in preparation). This observation shows that the arginine transport systems used in macrophages differs when these cells are activated or when they proliferate. Therefore, the regulation of *Slc7A2* is a limiting factor for the activation of macrophages by restricting the supply of the substrate to the appropriate machinery that triggers M1 or M2 activation.

Regarding the control of arginine transport by NOS2, there are contradictory data in the literature. While some studies report that the LPS-induced *NOS2* expression regulates arginine transport (27, 39), in other cases, the regulation of this transport is independent of NOS2 activity (30). These differences may be related to the distinct populations of macrophages used in the studies, as well as to the methods applied to inhibit NOS2 activity. Using macrophages from *NOS2* KO animals, we found that M1 and M2 activators induce *Slc7A2* expression, which is responsible for the increase in L-arginine transport. Furthermore, no modifications of *Slc7A2* expression or transport induced by activators were detected when we blocked arginase activity, thus demonstrating that the activators control over *Slc7A2* is independent of the induction of either *NOS2* or arginase activity. However, several signal transduction pathways may be common for the regulation of *NOS2* and *Slc7A2* genes, such as NF-κB (40).

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 fibrinogen, which plays a role in inflammation (41). 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 (24). Also, mycobacterium enhances arginine transport in infected macrophages and acquires the metabolites necessary for bacterial growth (42). Furthermore, polyamines modulate the functional activities of macrophages (43). For example, spermine inhibits pro-inflammatory cytokine synthesis (44-47) and has been reported to down-regulate arginine transport and NOS2 expression in rat alveolar macrophages (48).

Several of the genes involved in the control of growth or amino acid metabolism are regulated by amino acid availability (49). In our studies, in the absence of arginine, no differences were found for the induction of genes regulated by M1 (MHC class II *IA-β*, *NOS2* or *TNF-α*) or M2 activators (*arginase 1* or *mannose receptor*). In addition, the absence of arginine did not affect the regulation of the transporter genes *Slc7A1* or *Slc7A2*.

In conclusion, there is a clear association between CAT2 and macrophage activation. This finding is of particular interest because arginine transporters may offer a suitable new drug target for the clinical management of aberrant macrophage activation in diseases.

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[2]*Abbreviations used in this paper:* CAT, cationic amino acid transporters; HAT, hetero(di)meric amino acid transporters; M1, Classical activation; M2, Alternative activation; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; NOS, nitric oxide synthase; ppaAT, glycoprotein-associated amino acid transporters;

Figure legends

Figure 1. IFN-γ and LPS induce M1 activation of macrophages while IL-4 and IL-10 lead to M2 activation Macrophage activation increases arginine uptake through the y^+ system. Macrophages were cultured for 24 hours in the presence of IL-4 (10 U/ml), IL-10 (10 U/ml), IFN-γ (500 U/ml) or LPS (10 ng/ml). Nitrites (A) or arginase activity (B) were then determined. The values shown correspond to the mean ± SD of three independent experiments. In A) $p< 0.01$ between IL-4 or IL-4+IL-10-treated samples and IFN-γ-, LPS- or IFN-γ+LPS-treated samples. In B) $p< 0.01$ between IFN-γ-, LPS- or IFN-γ+LPS-treated samples and IL-4 and IL-4+IL-10-treated samples. C) Macrophages were incubated for 2 (open bars) or 6 hours (black bars) with radiolabelled arginine. The products were then separated by thin layer chromatography. The results are indicated as percentage of the arginine added at the beginning of the assay. The values shown correspond to the mean ± SD of three independent experiments. There is a significant difference for ornithine and spermine production between macophage controls and those treated with IL-4 and IL-4+IL-10 ($p<0.01$). Also, a significant difference was found for citrulline between the controls and cells treated with IFN-γ or IFN-γ+LPS ($p<0.01$). D) Macrophages were incubated for 24 hours with the activators indicated and arginine uptake was then measured. There is a significant difference between the control values and the values after LPS treatment with 50 and 100 ng/ml or after 5 and 10 U/ml of IL-4 ($p<0.01$). Results are representative of three independent experiments. E) Macrophages were incubated for 24 hours in the presence of IFN-γ (500 U/ml) and LPS (100 ng/ml) or IL-4 (10 U/ml) and IL-10 (10 U/ml). Then, 50 μM L-arginine was added and uptake was 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

in arginine transport before and after treatment with LPS, IFN-γ+LPS or IL-4+IL-10 ($p<0.01$). Results are representative of three independent experiments.

Figure 2. M1 and M2 activators induce the expression of *Slc7A2*. Macrophages were cultured for 24 hours in the presence of the activators indicated and the expression of *Slc7A1*, *Slc7A2* and *Slc7A3* was then measured using real-time PCR. For the expression of *Slc7A2,* there is a significant difference between the values of the controls and those after treatment with LPS, LPS+IFN-γ, IL-4 and IL-4+IL-10 ($p<0.01$). No significant differences were observed when we compared the corresponding values in the presence or absence of arginine. Results are representative of two independent experiments.

Figure 3. The induction of arginine transport by M1 and M2 activators 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). There is a significant difference for the amount of transport between the controls and knock-out macrophages after treatment with LPS, LPS+IFN-γ, IL-4 or IL-4+IL-10 ($p<0.01$). No significant differences were observed when we compared the corresponding values of the controls. Results are representative of two independent experiments. B) Macrophages from *Slc7A2* knock-out mice (black bars) and the corresponding wild type controls (open bars) were incubated with the activators indicated for 24 hours and the expression of *NOS2* or *arginase* 1 was measured using real-time PCR. No significant differences were found between the corresponding values. Results are representative of two independent experiments.

Figure 4. CAT2 is a limiting factor for M1 and M2 activation. A) 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 the arginine added at the

beginning of the assay. The values shown correspond to the mean ± SD of three independent experiments. For arginine, ornithine and spermine, in macrophages treated with IL-4 and IL-4+IL-10, the values between controls and *Slc7A2* knock-out macrophages were significantly different ($p<0.01$). Also, for arginine and citrulline values were significantly different after treatment with IFN-γ, LPS or IFN-γ+LPS ($p<0.01$). B) Macrophages from *Slc7A2* knock-out mice and the corresponding wild type controls were incubated for 24 hours with the activators indicated and the activity was measured. Nitrites were determined and a significant difference ($p<0.01$) was found between the corresponding values for IFN-γ, LPS and LPS+IFN-γ. Results are representative of three independent experiments.

Figure 5. Arginine uptake and *Slc7A2* induction are independent of arginase or NOS2 activity. A) Macrophages were incubated for 2 hours with or without nor-NOHA (50 μM) and the activators indicated were then added and macrophages were incubated for 24 hours. Arginine uptake was then measured. B) *Slc7A1*, *Slc7A2* and *Slc7A3* expression was determined by real-time PCR under the same conditions as A. For arginine transport and gene expression, no significant differences were found between cells treated or not with nor-NOHA. C) Macrophages from *NOS2* knock-out mice and wild type controls were incubated for 24 hours with the activators indicated. Arginine uptake was then measured. D) *Slc7A1*, *Slc7A2* and *Slc7A3* expression was determined by real-time PCR under the same conditions as C. For arginine transport and gene expression, no significant differences were found between cells from *NOS2* knock-out mice and the controls. Results are representative of three independent experiments.

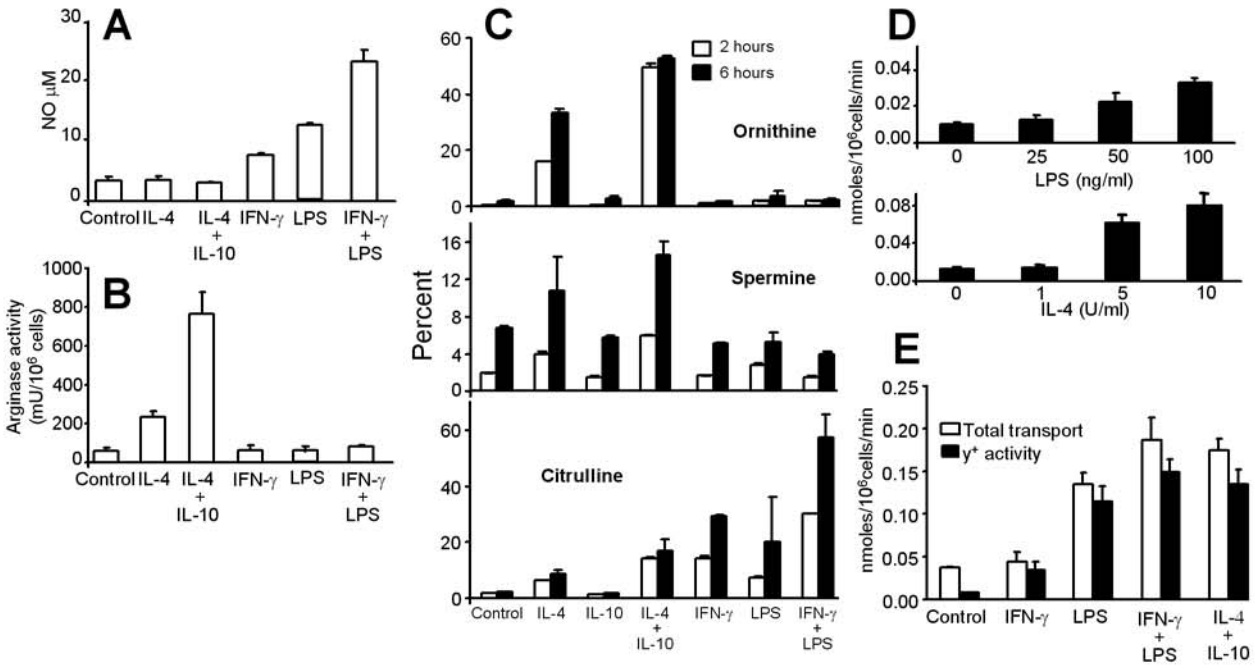
A
NO μM
Control
IL-4
IL-4 + IL-10
IFN-γ
LPS
IFN-γ + LPS
B
Arginase activity (mU/10^6 cells)
C
2 hours
6 hours
Ornithine
Spermine
Citrulline
Percent
D
nmoles/10^6cells/min
LPS (ng/ml)
IL-4 (U/ml)
E
Total transport
y+ activity

Figure 1

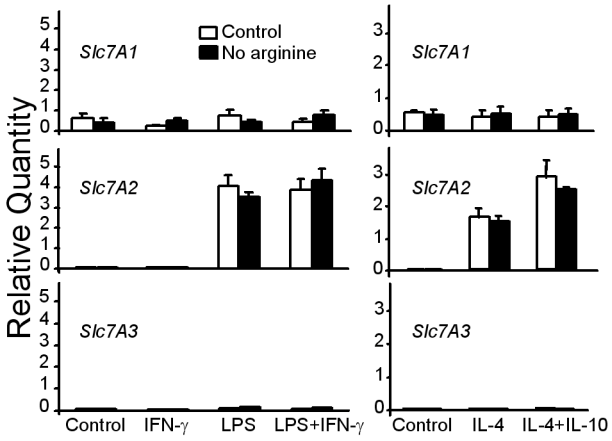
Relative Quantity
Control
No arginine
Slc7A1
Slc7A2
Slc7A3
Slc7A1
Slc7A2
Slc7A3
Control
IFN-γ
LPS
LPS+IFN-γ
Control
IL-4
IL-4+IL-10

Figure 2

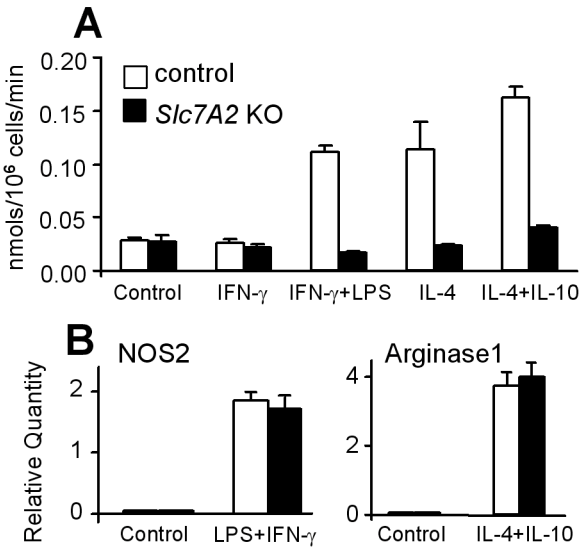
A
control
Slc7A2 KO
nmols/10^6 cells/min
0.20
0.15
0.10
0.05
0.00
Control
IFN-γ
IFN-γ+LPS
IL-4
IL-4+IL-10
B
NOS2
Arginase1
Relative Quantity
2
1
0
4
2
0
Control
LPS+IFN-γ
Control
IL-4+IL-10

Figure 3

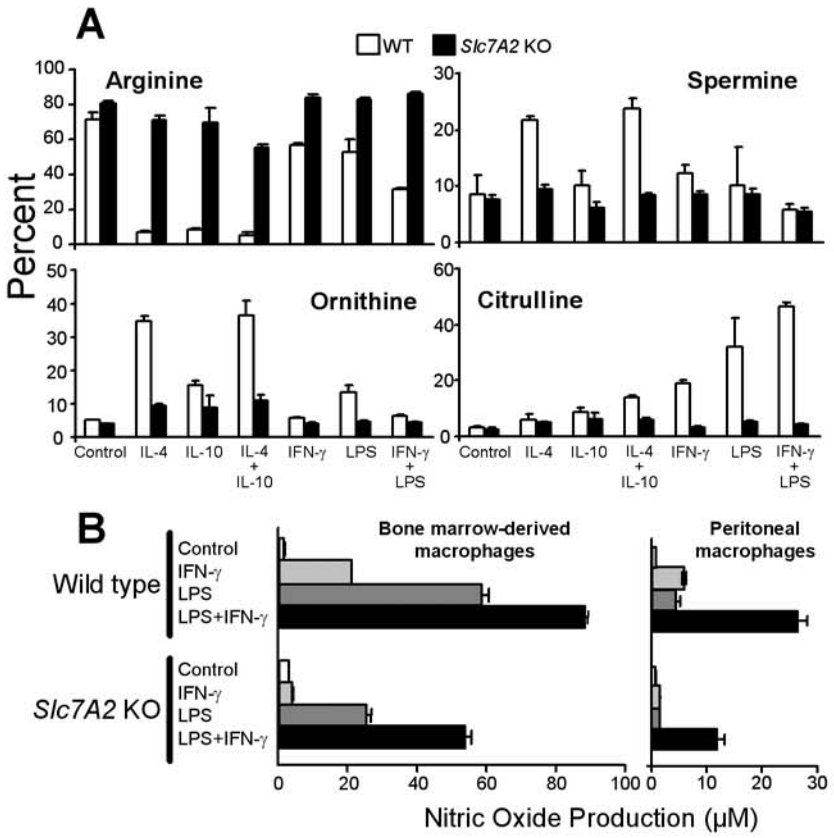
A
WT
Slc7A2 KO
Arginine
Spermine
Ornithine
Citrulline
Percent
Control
IL-4
IL-10
IL-4 + IL-10
IFN-γ
LPS
IFN-γ + LPS
B
Bone marrow-derived macrophages
Peritoneal macrophages
Wild type
Slc7A2 KO
Control
IFN-γ
LPS
LPS+IFN-γ
Nitric Oxide Production (μM)

Figure 4

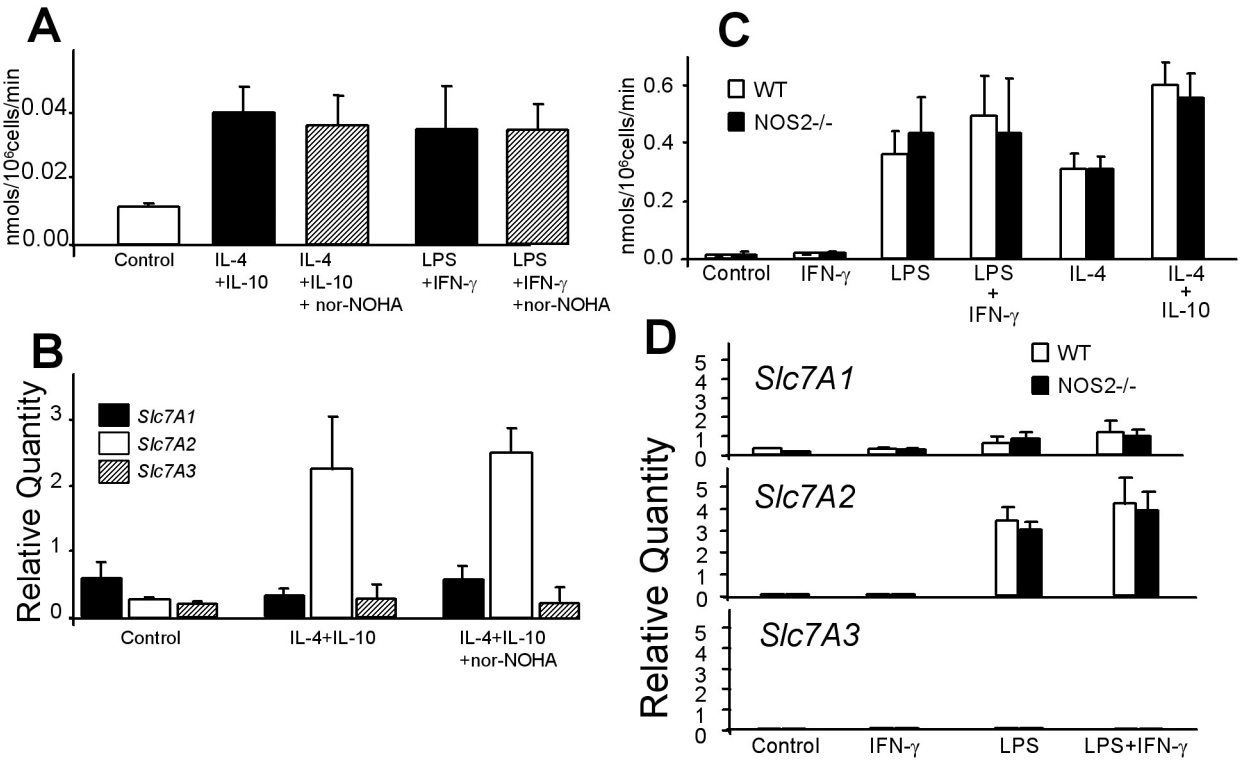
A
nmols/10⁶cells/min
0.04
0.02
0.00
Control
IL-4 +IL-10
IL-4 +IL-10 + nor-NOHA
LPS +IFN-γ
LPS +IFN-γ +nor-NOHA
B
Relative Quantity
Slc7A1
Slc7A2
Slc7A3
Control
IL-4+IL-10
IL-4+IL-10 +nor-NOHA
C
nmols/10⁶cells/min
WT
NOS2-/-
Control
IFN-γ
LPS
LPS + IFN-γ
IL-4
IL-4 + IL-10
D
Relative Quantity
Slc7A1
Slc7A2
Slc7A3
WT
NOS2-/-
Control
IFN-γ
LPS
LPS+IFN-γ

figure 5