Arginine transport is impaired in C57Bl/6 mouse macrophages as a result of a deletion in the promoter of *slc7a2* (CAT2) and Leishmania infection is reduced

Gloria Sans-Fons[1], Andrée Yeramian[1], Selma Pereira-Lopes[1], Luis Santamaría-Babi[1], Manuel Modolell[2], Jorge Lloberas[1] and Antonio Celada[1]

[1]Macrophage Biology Group, Department of Physiology and Immunology, Universitat de Barcelona, 08028 Barcelona, Spain

[2]Max-Planck Institute for Immunobiology and Epigenetics, 79108 Freiburg, Germany

Running title: Macrophage activation and arginine transport

Correspondence: Antonio Celada, Parc Cientific Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain; Phone 34 93 4037165; Fax 34 93 4034747; E-mail: acelada@ub.edu

Words count: 3484

ABSTRACT

Host genetic factors play a crucial role in immune response. To determine whether the differences between C57Bl-6 and BALB/c mice are due only to the production of Th1 or Th2 cytokines, we obtained bone marrow-derived macrophages from both strains and incubated them with these cytokines. Although the induction of *nos2* and *arginase 1* was similar in the two strains, infectivity to *L. major* differed, as did macrophage uptake of arginine, this being higher in BALB-c macrophages. The levels of the IFN-γ- and IL-4-dependent induction of the cationic amino acid transporter SLC7A2 (cationic amino acid transporter 2 or CAT2) were decreased in macrophages from C57Bl/6 mice. This reduction was a result of a deletion in the promoter of one of the four AGGG repeats. These results demonstrate that the availability of arginine controls critical aspects of macrophage activation and reveal a factor for susceptibility to Leishmania infection.

Key words: Macrophage; Leishmania; arginine; amino acid transporter; immune response; susceptibility.

INTRODUCTION

Through epidemiological and population studies, it has been established that with many pathogens host genetic factors play an important role in the onset, progression of infection, type of disease developed and the ultimate outcome of infection [1]. In some murine models of human pathogens, the pathogenesis of the infection and the immune response are extremely well reproduced [2, 3].

Host genetics modulate the clinical manifestations of patients with diverse infections, such as leishmaniasis [4]. Human leishmaniasis has been mimicked in the laboratory by infection of mice with *Leishmania major*. Most mouse strains control *L. major* infection, but some, such as BALB-c, develop progressive lesions and systemic disease [5]. The genetic predisposition to this infection in mice correlates with the production of Th2 cytokines (mostly IL-4) while resistance corresponds to Th1 cytokines (predominantly IFN-γ) [3]. However, recent data have challenged the simplicity of this model and have revealed a much greater complexity in the mechanisms of acquired resistance [5, 6].

Treatment of macrophages with Th2 cytokines induces alternative activation or M2, as opposed to Th1, which is termed classical activation or M1 [7]. The way in which arginine is catabolized is crucial. Th2 cytokines induce Arginase 1 that degrade arginine in polyamines and proline that are used for cellular proliferation and collagen production. In contrast, one of the hallmarks of classical macrophage activation is the generation of nitric oxide (NO) by an inducible nitric oxide synthase (NOS2) [8].

The cationic amino acid transporter (CAT) family is composed by CAT-1, -2A, -2B and -3. The substrate transported is almost identical for cationic amino acids. The major difference between these transporters is at the level of tissue specificity and the regulation of their expression. On macrophages, *slc7a1* is constitutively expressed and is not modified by activating agents, while *slc7a3* is not detected and *slc7a2a* is induced during classical and alternative activation [9].

To determine whether the differences between C57Bl-6 and BALB/c mice are due only to the production of Th1 or Th2 cytokines, we obtained bone marrow-derived macrophages from both strains and incubated them with these cytokines. Infectivity to *L. major* differed as did macrophage uptake of arginine. The decreased arginine transport in C57Bl/6 mice was due to a reduction in the expression of the transport system *slc7a*2 (CAT2) [9] as a result of a deletion in the promoter.

MATERIALS AND METHODS

Reagents

Recombinant IL-4, IL-10 and IFN-γ were purchased from R & D (Minneapolis, MN). All the other products were of the highest grade available and were purchased from Sigma.

Mice and cell culture

BALB-c and C57Bl/6 mice were purchased from (Charles River Laboratories, Inc., Wilmington, MA) and 6-8-week-old females were used that was approved by the Animal Research Committee of the University of Barcelona (number 2523). Bone marrow-derived macrophages were isolated as described [9].

Determination of *L. major* growth *in vitro*

L. major LV39 (MRHO/SU/59/P-strain) was kindly provided by Dr. I. Muller (Imperial College London, United Kingdom). *In vitro* studies with *L. major* were carried out as described [10]. Macrophages were activated in the presence or in the absence of 100 μM of nor-NOHA (Bachem, Switzerland). After 4 h the cultures were infected with *L. major* parasites. After 96 h, the macrophages were washed and lysed and a limiting dilution assay was performed to determine the number of viable parasites.

Determination of arginase activity and NO production

Arginase activity was measured in macrophage lysates as described [11]. Cells were lysed and arginine hydrolysis was conducted by incubating the lysate with L-arginine at 37°C. NO was measured as nitrite using Gries reagent [11].

Quantitative RT-PCR analysis

Real-time PCR was performed as described [12]. Data were expressed relative to β-actin. The primer sequences are described in Supplementary Material.

Catabolism of L-arginine

Catabolism of arginine was determined as described [13] by incubating macrophages with L-(U-^{14}C) arginine. The catabolic products were evaluated by thin-layer chromatography.

Arginine transport

Transport of L-[3^{H}]arginine (Amersham) was measured as described using radioactive arginine [9, 14].

Transfection of small-interfering RNA (siRNA)

siRNA was obtained from Dharmacon and transfected by electroporation as described [15]

Northern blot

Total RNA was extracted and separated by electrophoreses in an agarose/formaldehyde gel as described [16]. Samples were transferred by capillarity to a nylon membrane. RNA was then fixed in the membrane by UV irradiation. The probe was prepared as indicated in Supplementary materials.

Transient transfection and dual reporter luciferase-renilla assays

The construction of reported plasmids is described in Supplementary material. For luciferase-renilla assays RAW264.7 cells were used as described [15].

Electrophoretic Mobility Shift Assay (EMSA)

Cells were lysed and nuclear extracts obtained and EMSA assay was used as described [15]. The probes were synthesized by Sigma and correspond to a STAT6 binding element 2.86 Kb upstream of the *arginase-1* transcription start site [17].

Promoter Analysis

To determine the promoter(s) used to transcribe *CTN-RNA*, total RNA was reverse-transcribed using a RT-primer specific designed from the unique 3'UTR. These cDNAs were further PCR-amplified using forward PCR primers from each of the exon 1 variants representing promoters A to E [18] and a reverse primer from exon 3.

Statistical analyses

Experimental results were analyzed using a two-tailed Mann-Whitney test and the Wilcoxon paired test [19].

RESULTS

Distinct responses in macrophages of BALB-c and C57Bl/6 mice

To determine whether the distinct susceptibility of BALB-c and C57Bl/6 mice to leishmaniasis is attributable exclusively to the cytokines produced, we incubated bone marrow-derived macrophages produced *in vitro* with Th1 or Th2 cytokines. These primary

cultures of macrophages represent a homogeneous population of cells that respond *in vitro* to activating stimuli. Using this approach, we circumvented the distinct cytokine production profiles of the two strains by comparing only the response of macrophages. Macrophages were stimulated with IFN-γ and 4 h later the cultures were infected with *L. major*. After 96 h, these cells were washed and lysed, and a limiting dilution assay was performed to determine the number of viable parasites. Interestingly, C57Bl/6 macrophages showed *L. major* proliferation similar to that of BALB-c ones (Figure 1*A*). However under IL-4 incubation, parasites showed a higher proliferation in BALB/c than in C57Bl/6 macrophages (Figure 1*A*). We have shown [10] that treatment of macrophages with N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) inhibits the activity of arginase and therefore the conversion of L-arginine into ornithine and spermine, both of which are required for *L. major* growth. In our conditions, nor-NOHA abolished the effect of IL-4 on parasite growth in the macrophages of both strains, thereby suggesting that arginase is critical for this growth.

The differences in parasite growth observed in the macrophages could be attributed to the distinct expression of arginase in BALB/c and C57Bl/6 mice. However, no differences in the induction of mRNA between these two phenotypes were found when macrophages were incubated with IL-4 or IL-4 with IL-10 (Figure 1*B*). To explore the extent of arginase activity, we determined the production of urea [20]. After activating macrophages, we lysed them and then added arginine. Again, no differences were found between the macrophages of the two mouse strains (Figure 1*B*).

Next we determine the catabolism of arginine when macrophages were induced by IL-4. After activation, these cells were incubated with radiolabelled arginine for 2 and 6 h. The products of degradation were then resolved using thin-layer chromatography. Although arginase activity was similar in macrophages from both strains, the consumption of arginine was lower in the C57Bl/6 strain while the production of ornithine, citruline, spermine or

proline was higher in BALB-c mice (Figure 1*C*). As macrophages do not accumulate putrescine, spermidine or glutamate, no differences were detected between the two strains.

Given that NO plays a major role in killing *L. major* [8], we examined the expression of *nos2* in C57Bl/6 and BALB-c macrophages after activation with IFN-γ or after addition of LPS. No differences were found in the expression of *nos2* (Figure 1*D*) or its protein (data not shown). Interestingly, a significant difference was observed in NO production. BALB-c macrophages produced more NO than C57Bl/6 ones (Figure 1*D*).

Distinct arginine transport in macrophages from BALB-c and C57Bl/6 mice

So far, the data show that although the amounts of NOS2 and arginase were similar in macrophages of the C57Bl/6 and BALB-c mouse strains, there was a significant difference in the catabolism of arginine as NO or as proline and spermine. This finding implies that if these strains show similar amounts of these enzymes and fewer products of the catabolism of the substrate, the amount of arginine inside the macrophages of these strains differs. This notion led us to further explore the transport of this amino acid in macrophages. For this purpose, we determined the uptake of radiolabelled arginine by these cells in the two mouse phenotypes [9]. Several transporters handle arginine in macrophages. We previously showed that in basal conditions >75% of the total transport rate corresponds to system y^+L. There is a second component of arginine transport into macrophages that is insensitive to L-leucine, even in the presence of Na^+, which is inhibited by treatment with the sulfhydryl-specific reagent N-ethyl maleimide (NEM). This NEM-sensitive component corresponds to system y^+. The participation of the B^{o+} and b^{o+} systems was excluded by measuring transport in media with or without sodium [9, 14].

BALB-c macrophages treated with Th-1 or Th-2 cytokines showed a drastic increase in arginine transport. This effect was not inhibited by treatment with NEM, thereby indicating

that the increase was due to system y^+L (Supplementary Figure 1). Given that the treatment with cytokines did not modify the amount of arginine transported by system y^+L, we calculated the difference that corresponds to the inducible y^+ system (Figure 2*A*). In C57Bl/6 macrophages the cytokines also induced an increase, but significantly lower, in arginine transport through system y^+ (Figure 2*A*). These results explain why parasite growth decreased in BALB-c macrophages stimulated with Th1 cytokines and showing an increased production of NO. In contrast, in the presence of Th2 cytokines, and thus an increased production of polyamines, which are required for the growth of the parasite [21], the number of parasites increased.

Distinct induction of *slc7A2* in activated macrophages from BALB-c and C57Bl/6 mice

We showed that the increase in arginine transport induced in the macrophages by the two types of cytokines was mediated by the y^+ system and the gene induced was *slc7A2* [9, 22]. Using quantitative PCR, we determined the induction of this gene in macrophages from the two mouse phenotypes. Th-1 and Th-2 cytokines induced the expression of *slc7A2* in both strains; however, in BALB-c mice this induction was greater (Figure 2*B*).

To determine the contribution of *slc7A2* to the functional activities of macrophages, we inhibited its expression using siRNA (Figure 2*C*). The macrophages of both animal models showed a significant decrease in *slc7A2* expression as well as in the amount of arginine taken up (Figure 2*C*). As a functional consequence of *slc7A2* inhibition, the amount of NO produced in response to treatment with IFN-γ, with or without LPS, was drastically reduced (Figure 2*C*), without modifications in the amount of *nos2* induced *or arginase-1* (Figure 3). This observation confirmed our previous results using the *slc7A2* KO model [9]. These findings demonstrate that the differences in the functional activity of macrophages of these two strains of mice are due to the differential expression of the *slc7a2* arginine transporter.

slc7a2 has various isoforms, the expression of which depends on the usage of 5' and 3' untranslated regions. However, the translated region is the same for all the transcripts. In macrophages, the 5' unstranslated region used is 1A. Independently of the 5' region transcribed, the 3' region differs in length as a result of the presence of two distinct polyadenylation sites. These sites are separated by almost 4 kb and they determine the length of the two isoforms expressed in macrophages, which have been identified as *CTN-RNA* and *mCAT-2* (8 and 4.4 Kb respectively) [23]. The isoform *CTN-RNA* is diffusely distributed in nuclei and is also localized in paraspeckles [23]. Under stress, *CTN-RNA* is post-transcriptionally cleaved to produce protein-coding *mCAT2* mRNA. In our experiments, we used an exon probe hybridizing both *CTN-RNA* and *mCAT2* [23]. As described in several cell lines, including macrophages [23], no detectable mRNA was found in the non-transformed macrophages before activation, as shown by Northern blot. As a control, we used mRNA from the liver, which expressed both species of mRNA (Figure 4*A*). Incubation of macrophages from both strains with Th1 or Th2 cytokines induced mRNA, *CTN-RNA* and *mCAT-2* (Figure 4*A*). However, BALB-c cells showed greater amounts of these products than C57Bl/c ones. To exclude different kinetics of *CTN-RNA* and *mCAT-2* induction in the two strains, we performed time-course experiments. After 3 h of incubation with IL-4, macrophages simultaneously expressed both species of mRNA, reaching maximum expression at 6 h (Figure 4*B*). On the basis of these results, we conclude that there is a quantitative difference in the expression of *CTN-RNA* and *mCAT-2* between C57Bl/6 and BALB-c mice, thereby confirming the results on arginine transport and quantitative PCR. In the absence of available antibody, the distinct amounts of mRNA shown by the two mouse strains could explain the differences in the functional capacities of macrophages in these phenotypes.

To confirm the role of the SLC7A2 arginine transporter in the infectivity of macrophages to Leishmania, we inhibited the *slc7a2* expression using siRNA. While in

macrophages from C57Bl/6 treated with IL-4 the infectivity was not reduced, it was drastically diminished in macrophages from BALB-c mice (Figure 4*C*).

A deletion in the promoter of C57Bl/6 impairs the cytokine-induction of *slc7A2*.

To examine whether the increase in *slc7A2* expression induced by cytokine treatment occurred at the transcriptional level or was due to mRNA stabilization, we determined the half-life of *slc7A2* transcripts in cells treated with IFN-γ and LPS. Macrophages were treated with IFN-γ and LPS for 9 h, thereby inducing *slc7A2*. Actinomycin D was then added at a concentration sufficient to block all further mRNA synthesis, as determined by [3H]UTP incorporation [15]. We then isolated mRNA from aliquots of cells at a range of intervals. Northern blot measurement of *slc7A2* expression allowed us to estimate that the half-life of *CTN-RNA* and *mCAT-2* in resting cells was very stable (Figure 5*A*). Treatment with IFN-γ and LPS did not modify the stability of *CTN-RNA* or *mCAT-2*, thus indicating that the induction of *slc7A2* in response to cytokines was at the transcriptional level. Similar results were found when macrophages were activated with IL-4 and IL-10 (Figure 5*A*).

For the transcription of *slc7A2,* multiple promoters (A to E, each having unique exon variants: exons 1A to 1E, comprising the 5'UTRs) are used in a tissue-specific manner [18]. RT-PCR analysis showed that *CTN-RNA* was exclusively transcribed by the distal promoter A in macrophages from both strains of mice, (Figure 5*B*), as described in the macrophagic cell line RAW264.7 [23].

To determine the presence of mutations in the regulatory region next to exon 1A, we sequenced 1300 nucleotides of the two mouse strains. Alignment of these sequences allowed us to observe that between 352 and 348 bp upstream of exon A, the sequence AGGG was absent in C57Bl/6 mice in relation to the BALB-c (Figure 6*A*). Interestingly, these four bases in BALB-c mice were repeated four times while the C57Bl/6 strain had only three repeats.

These sequences are binding sites for a number of transcription factors, such as SP1, LYF1 and MZF1, and the AGGG deletion abrogates the binding of these factors [24].

Next, we analyzed the functional activity of the A promoters of BALB-c and C57B1/6 mice. For this purpose, a fragment from their A promoters was linked to the luciferase reporter gene. Due to the difficulty to transfect non-transformed macrophages, we used the macrophagic cell line RAW264.7. The vectors were transfected and luciferase activity was measured. Each construct was cotransfected with the renilla expression vector. All luciferase activity values were normalized to the level of renilla expression to correct for any differences in transfection efficiency. In unstimulated macrophages, the construct comprising 1193 bp of the A promoter of BALB-c and C57Bl/6 mice showed little activity (Figure 6*B*). However, macrophage stimulation with Th1 or Th2 cytokines induced high expression of the promoter of BALB-c mice. In contrast, induction was low when the same treatments were made with the promoter of the C57Bl/6 strain. To confirm that the deletion of AGGG was responsible for the decreased activity of *slc7A2* in the macrophages of C57Bl/6 mice, we deleted the AGGG motif in the promoter of BALB-c mice. The promoter with this mutation was not induced when macrophages were treated with either IFN-γ or IL-4 (Supplementary Figure 2). To determine the areas of the promoter that are important for induction by Th1 or Th2 cytokines, we performed several deletions. An area between -773 and -473bp was observed to be critical to elicit induction (Figure 6*B*). However, the four AGGG repeats are also required since mutation of one of these repeats abolished the induction. Therefore, we conclude that the deletion of the AGGG motif is responsible for the distinct expression of *CTN-RNA* and *mCAT-2* shown by BALB-c and C57Bl/6 macrophages.

To establish whether distinct protein complexes were associated with the promoters of BALB-c and C57Bl/6 mice, we performed gel electrophoresis DNA binding assays. Nuclear extracts were prepared from IFN-γ-treated macrophages from the two mouse strains.

When the extracts of these cells were incubated with a probe corresponding to the BALB-c mice, two types of DNA-protein complexes were obtained, one weak and the other strong. These complexes were obtained when we used either the nuclear extracts from macrophages from BALB-c or C57Bl/6 mice (Figure 6*C*). When we used a probe comprising an oligonucleotide corresponding to the region where the AGGG motif was deleted, only the weak band was detected while the stronger one was absent. Given that no differences were found using proteins of either of the two mouse strains, we conclude that the DNA binding proteins are present in the nuclear extracts of macrophages from BALB-c and C57B1/6 mice and the defect of the binding proteins that bound to the C57B1/6 promoter is due to the deletion in the AGGG motif.

DISCUSSION

Using cultures *in vitro* of macrophages, we reveal that arginine transport is a critical factor for genetic predisposition to *L. major* infection in animal models and that this transport system could partly explain the different susceptibility of BALB-c and C57Bl/6 strains of mice to this infection. Our results demonstrate that arginine transport in activated BALB-c and C57Bl/6 macrophages differs. These differences were due to differentially transcribed *slc7a2*, which encodes a cationic amino acid transporter called CAT2. The decreased expression of *slc7a2* in C57Bl/6 cells was caused by a deletion in the promoter in an area with four AGGG repeats. This deletion abrogates the formation of a palindromic sequence where several transcription factors, such as SP1, LYF1 or MZF1, can bind [25].

Arginine is critical for the innate immune response. This essential amino acid is required for macrophage growth [26] and for classical and alternative activation [9]. When macrophages are alternatively activated, arginase 1 is induced and then arginine is degraded to proline and polyamines, both types of molecules being required for *L. major* growth [10]. Our

experiments point to a link between decreased arginine uptake and reduced *L. major* growth. Nevertheless, to be certain of this relationship, we should compare the growth of *L. major* in identical macrophages with four or three AGGG repeats in the promoter of *slc7a2*. However, this experiment is technically impossible. The data presented here provide a new explanation for the susceptibility of BALB-c mice to intracellular parasite replication in non-healing *L. major* infections. In this mouse model, the amount of arginine that entered the macrophages was much higher that in C57Bl/6 mice. This increased uptake may favour the growth of *Leishmania*, being thus another factor that influences susceptibility to the disease.

To evade immune responses some pathogens generate their own arginases [10, 27, 28] or induce arginase expression in the host [29, 30]. Arginine then becomes limited for the production of NO by NOS2, an essential mechanism for host defense against many pathogens [8]. High expression of Arginase 1 blocks the immune response locally at the site of pathology, causing local depletion of arginine, which impairs the capacity of T cells in the lesion to proliferate and to produce IFN-γ [6].

In fact, the catabolism of arginine by macrophages has emerged as a critical mechanism for the regulation of the immune response, not only in the case of *L. major* but also in several other parasitic diseases [31, 32]. If the amount of arginine available is important, then the system through which to introduce this amino acid into macrophages is also critical. After interaction with cytokines, these cells show a considerable increase in arginine cellular uptake as a result of the induction of the SLC7A2 transport system, which is the limiting factor for NO production and for arginine catabolized by Arginase 1 [9, 22]. Our results demonstrate that the availability of arginine reveal a factor for susceptibility to Leishmania infection.

Notes

Acknowledgments. *L. major* LV39 was kindly provided by Dr. I. Muller, Imperial College London. We thank Manuel Palacin and Annabel F. Valledor for comments and Tanya Yates for editing the manuscript.

G.S.; A.Y.; S.P.L; L.S.B. did experiments; M.M. did experiments, and supervised the research, J.L. and A.C. designed and supervised the research and wrote the manuscript.

Financial support. This study was supported by grants BFU2007-63712/BMC and BFU2011-23662 and a MEICA award (Genoma España).

Potential conflict of interest. The authors declare no conflict of interests.

References

1. Hill AV. Aspects of genetic susceptibility to human infectious diseases. Annu Rev Genet **2006**; 40:469-86.

2. Lipoldova M, Demant P. Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. Nat Rev Genet **2006**; 7:294-305.

3. Solbach W, Laskay T. The host response to Leishmania infection. Adv Immunol **2000**; 74:275-317.

4. Blackwell JM, Fakiola M, Ibrahim ME, et al. Genetics and visceral leishmaniasis: of mice and man. Parasite Immunol **2009**; 31:254-66.

5. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol **2002**; 2:845-58.

6. Modolell M, Choi BS, Ryan RO, et al. Local suppression of T cell responses by arginase-induced L-arginine depletion in nonhealing leishmaniasis. PLoS Negl Trop Dis **2009**; 3:e480.

7. Gordon S. Alternative activation of macrophages. Nat Rev Immunol **2003**; 3:23-35.

8. Bogdan C. Oxidative burst without phagocytes: the role of respiratory proteins. Nat Immunol **2007**; 8:1029-31.

9. Yeramian A, Martin L, Serrat N, et al. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. J Immunol **2006**; 176:5918-24.

10. Kropf P, Fuentes JM, Fahnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. Faseb J **2005**; 19:1000-2.

11. Classen A, Lloberas J, Celada A. Macrophage activation: classical versus alternative. Methods Mol Biol **2009**; 531:29-43.

12. Marques L, Brucet M, Lloberas J, Celada A. STAT1 regulates lipopolysaccharide- and TNF-alpha-dependent expression of transporter associated with antigen processing 1 and low molecular mass polypeptide 2 genes in macrophages by distinct mechanisms. J Immunol **2004**; 173:1103-10.

13. Comalada M, Yeramian A, Modolell M, Lloberas J, Celada A. Arginine and macrophage activation. Methods Mol Biol **2012**; 844:223-35.

14. Martin L, Comalada M, Marti L, et al. Granulocyte-macrophage colony-stimulating factor increases L-arginine transport through the induction of CAT2 in bone marrow-derived macrophages. Am J Physiol Cell Physiol **2006**; 290:C1364-72.

15. Serra M, Forcales SV, Pereira-Lopes S, Lloberas J, Celada A. Characterization of Trex1 induction by IFN-gamma in murine macrophages. J Immunol **2011**; 186:2299-308.

16. Valledor AF, Xaus J, Marques L, Celada A. Macrophage colony-stimulating factor induces the expression of mitogen-activated protein kinase phosphatase-1 through a protein kinase C-dependent pathway. J Immunol **1999**; 163:2452-62.

17. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM, Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene **2005**; 353:98-106.

18. Finley KD, Kakuda DK, Barrieux A, Kleeman J, Huynh PD, MacLeod CL. A mammalian arginine/lysine transporter uses multiple promoters. Proc Natl Acad Sci U S A **1995**; 92:9378-82.

19. Snedecor G, Cochran W. Statistical methods. Iowa State University Press, Ames, Iowa, **1967** (edition t, ed.

20. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive

regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J Immunol **1998**; 160:5347-54.

21. Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in Leishmania. Characterization of gene deletion mutants. J Biol Chem **2004**; 279:23668-78.

22. Nicholson B, Manner CK, Kleeman J, MacLeod CL. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. J Biol Chem **2001**; 276:15881-5.

23. Prasanth KV, Prasanth SG, Xuan Z, et al. Regulating gene expression through RNA nuclear retention. Cell **2005**; 123:249-63.

24. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res **1995**; 23:4878-84.

25. Ihn H, LeRoy EC, Trojanowska M. Oncostatin M stimulates transcription of the human alpha2(I) collagen gene via the Sp1/Sp3-binding site. J Biol Chem **1997**; 272:24666-72.

26. Yeramian A, Martin L, Arpa L, et al. Macrophages require distinct arginine catabolism and transport systems for proliferation and for activation. Eur J Immunol **2006**; 36:1516-26.

27. Das P, Lahiri A, Chakravortty D. Modulation of the arginase pathway in the context of microbial pathogenesis: a metabolic enzyme moonlighting as an immune modulator. PLoS Pathog **2010**; 6:e1000899.

28. Fitzpatrick JM, Fuentes JM, Chalmers IW, et al. Schistosoma mansoni arginase shares functional similarities with human orthologs but depends upon disulphide bridges for enzymatic activity. Int J Parasitol **2009**; 39:267-79.

29. El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol **2008**; 9:1399-406.

30. Qualls JE, Neale G, Smith AM, et al. Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. Sci Signal **2010**; 3:ra62.

31. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol **2001**; 167:6533-44.

32. Vincendeau P, Gobert AP, Daulouede S, Moynet D, Mossalayi MD. Arginases in parasitic diseases. Trends Parasitol **2003**; 19:9-12.

Legend to the figures

Figure 1. Distinct infectivity to *L. major* in activated macrophages from BALB-c and C57Bl/6 mice. (A) Macrophages from BALB-c or C57Bl/6 mice were stimulated with IL-4 (10 ng/ml) plus/minus nor-NOHA or IFN-γ (10 ng/ml) and infected with *L. major* parasites. After 96 h, the number of viable parasites was determined by limiting dilution analysis. (B) Expression of *arginase 1* and arginase activity was measured in macrophages cultured for 24 h in the absence or presence of IL-4 or IL-4 plus IL-10 (10 ng/ml), or in the presence of IFN-γ or IFN-γ and LPS (10 ng/ml). (C) Degradation of arginine was determined in macrophages stimulated with IL-4. (D) Expression of *nos2* and NO production was measured in macrophages under the same conditions as in B. In all the figures data are representative of at least four experiments. Each determination was made in triplicate, and the values shown correspond to the mean + SD. * $p<0.01$ when the results of the four experiments were compared.

Figure 2. BALB-c macrophages show higher arginine transport than those of the C57Bl/6 strain. (A) Arginine transport mediated by the y^+ system was measured in macrophages cultured for 24 h in the absence or in the presence of IFN-γ with or without LPS or in the presence of IL-4 with or without IL-10. (B) The expression of *slc7a2* was measured by RT-PCR using macrophages treated in the same conditions as in A. (C) Macrophages were electroporated with siRNA to *slc7a2,* scrambled control siRNA or with media (Mock), then they were activated as in A, and the levels of *slc7a2*, arginine uptake and NO production were measured.

Figure 3. Inhibition of *slc7a2* does not inhibit the expression of *nos2* or *arginase 1*. Macrophages were electroporated with siRNA to *slc7a2,* scrambled control siRNA or with media (Mock). They were then cultured for 24 h in the absence or in the presence of IFN-γ with LPS, or in the presence of IL-4 with IL-10. *nos2* and *arginase 1* expression was then measured using quantitative PCR. Data are representative of at least four experiments. Each determination was made in triplicate, and the values represented correspond to the mean±SD.

Figure 4. Increased expression of *slc7a2* by macrophages from BALB-c mice in relation to C57Bl/6 mouse macrophages. (A) Northern blot analysis of total RNA using an exon 11 probe that detects both the 8 kb *CTN-RNA* and the 4.2 kb *mCAT2* isoforms in liver as well as in activated macrophages. Macrophages were cultured for 12 h in the absence or in the presence of IFN-γ with or without LPS or in the presence of IL-4 with or without IL-10. (B) Time-course determination of *slc7a2* in macrophages treated with IL-4. The quantification of *CTN-mRNA* and *mCAT2*At is shown at the bottom. (C) Infectivity of macrophages with *L. major* parasites depends of SLC7A2 expression. Macrophages were treated with IL-4 and proceed as in Figure 1*A* and 2*C*.

Figure 5. *slc7a2* is induced transcriptionally in macrophages through the promoter A. (A) Macrophages from BALB-c and C57Bl/6 mouse strains were treated with the indicated activators for 6 h and then DRB (20 µg/ml) and actinomycin D (5 µg/ml) were added. CTN-mRNA and mCAT2 were measured by Northern blot after the indicated times. Cell viability was >95% for all culture conditions. The figure shows one representative result of three independent experiments. (B) Promoter analysis of CTN-RNA was performed using RT primers from a CTN-RNA specific region, followed by PCR with all the exon 1 variant specific primer pairs. On top a map of slc7a2 is shown. Macrophages from both strains of

mice were treated with IFN-γ or IL-4 for 9 h. Starved are macrophages cultured in the absence of cytokines. Control represents the PCR without cDNA. The schematic representation of mCAT2 and CTN-RNA is shown at the top. The arrows represent the primer pairs used to amplify the RT products with specific forward primers for each exon 1 variant (representing each promoter A to E having unique exon 1A to E1 respectively) and common reverse primer (from exon 3).

Figure 6. The decreased expression of *slc7a2* by macrophages from C57Bl/6 mice is due to a deletion in the promoter. (A) Sequencing analysis of the A promoter of *slc7a2* in macrophages from BALB-c and C57Bl/6 strains. (B) The expression of *slc7a2* A promoter was determined in the macrophage cell line RAW264.7 transiently transfected with the reporter plasmids containing the A promoter from BALB-c and C57Bl/6 strains as well as from BALB-c with the deletion found in the C57Bl/6. (C) For the DNA binding assays, as probes we used fragments of the A promoter of the BALB-c and C57Bl/6 strains. The sequences of the oligonucleotides are indicated in the figure. Nuclear extracts were obtained from macrophages of both strains of mice treated for 9 h with IFN-γ or IL-4.

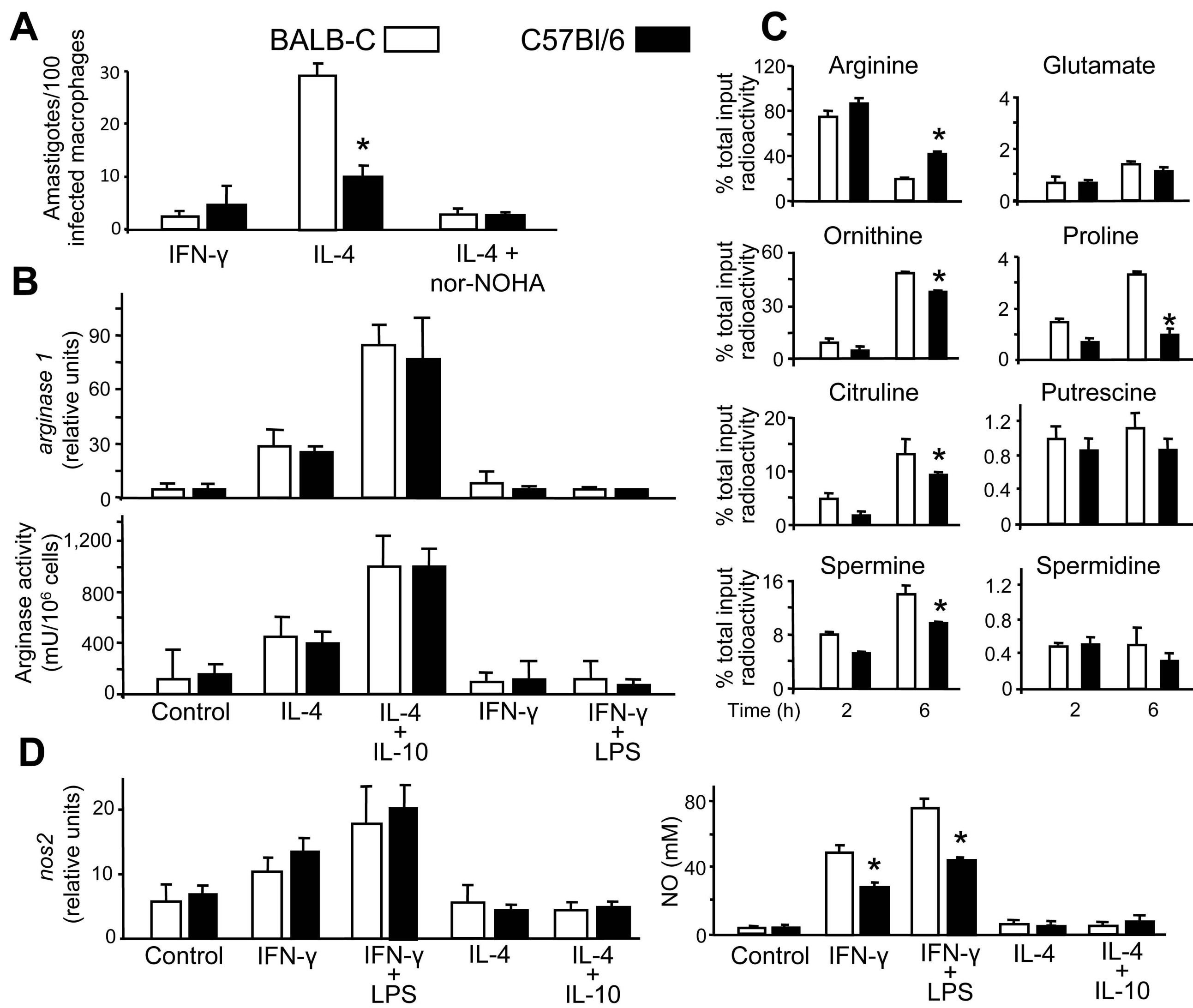


Figure 1

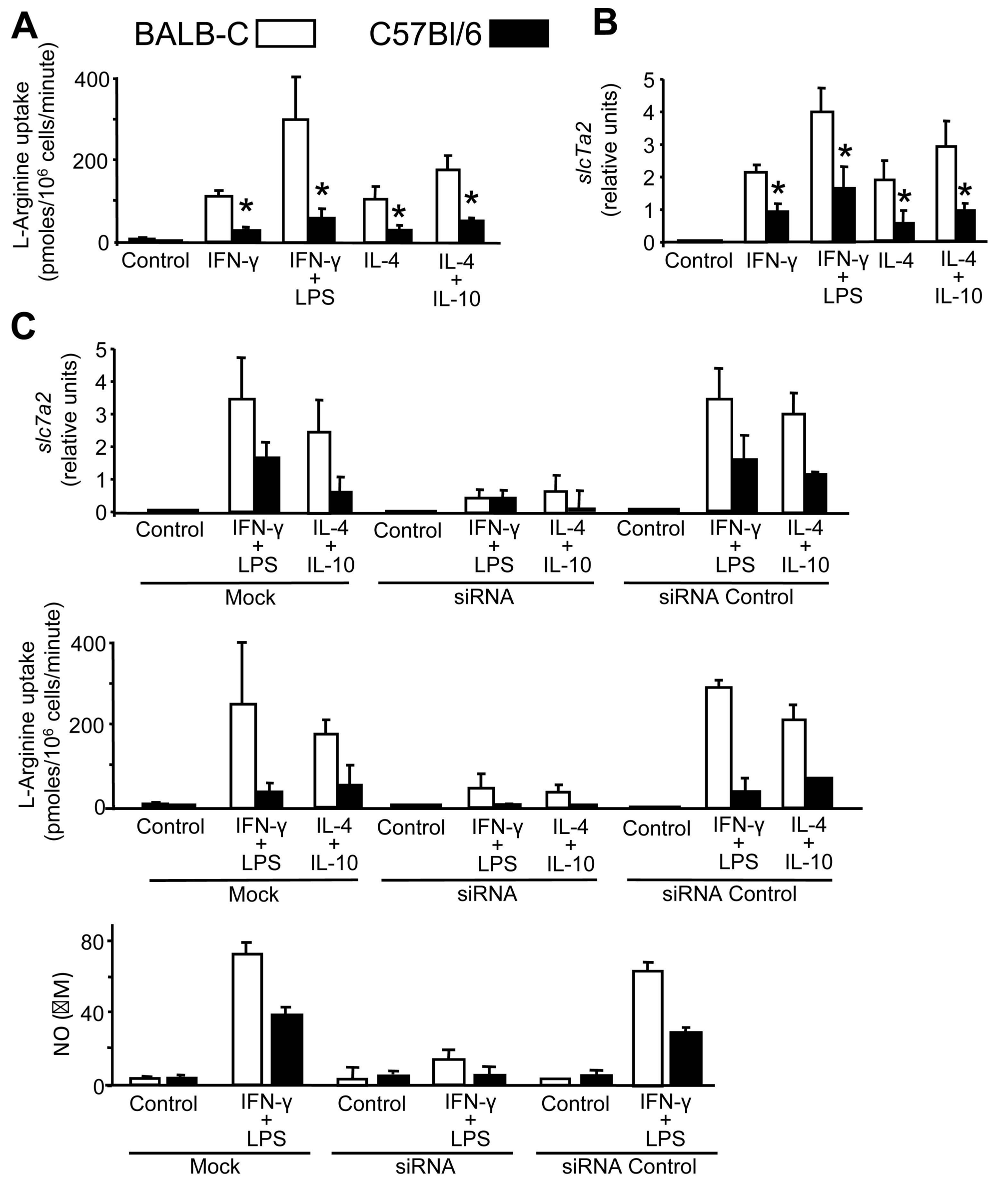


Figure 2

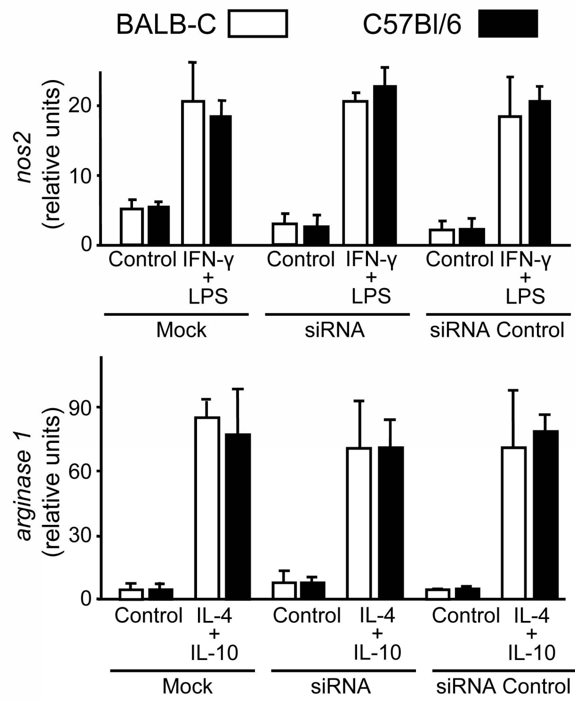


Figure 3

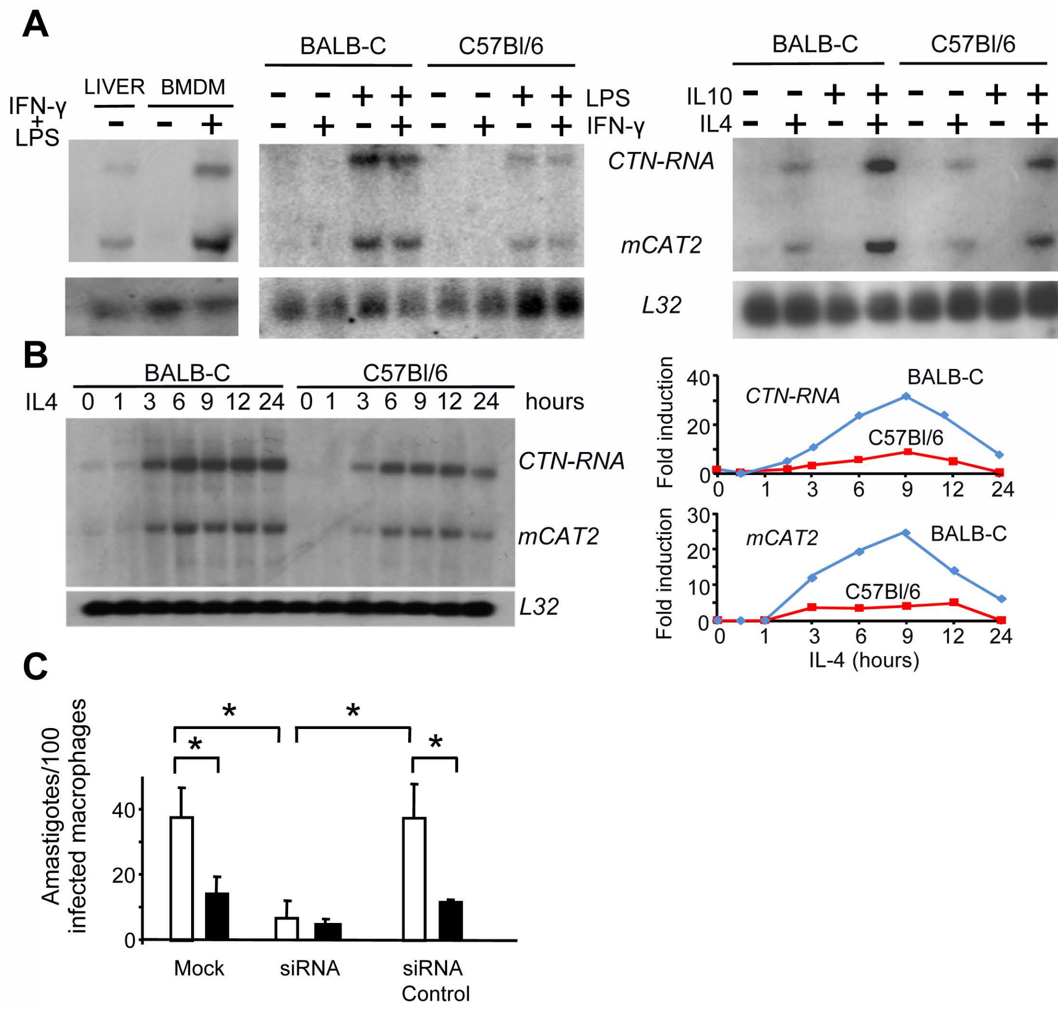
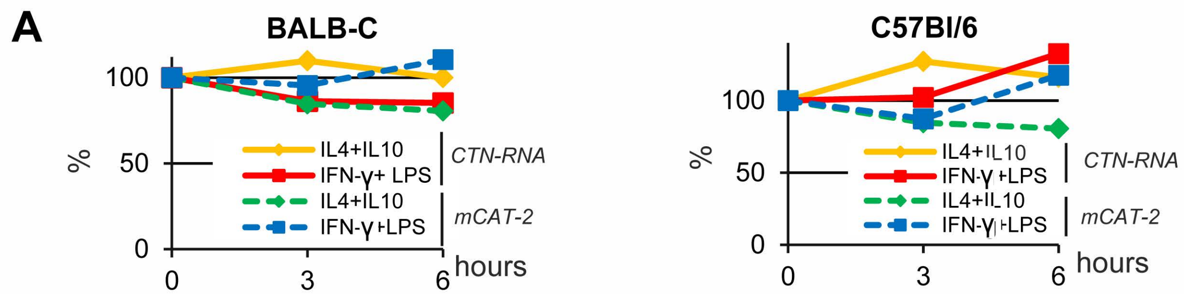
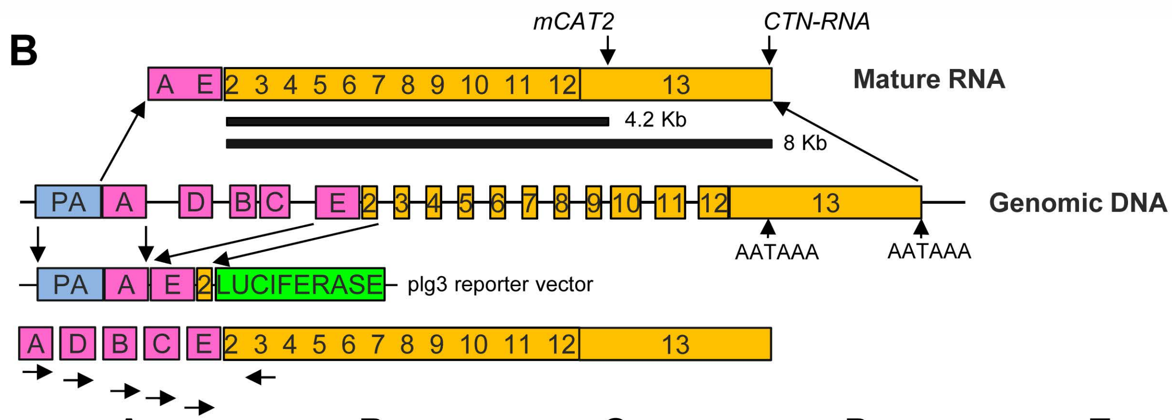


Figure 4





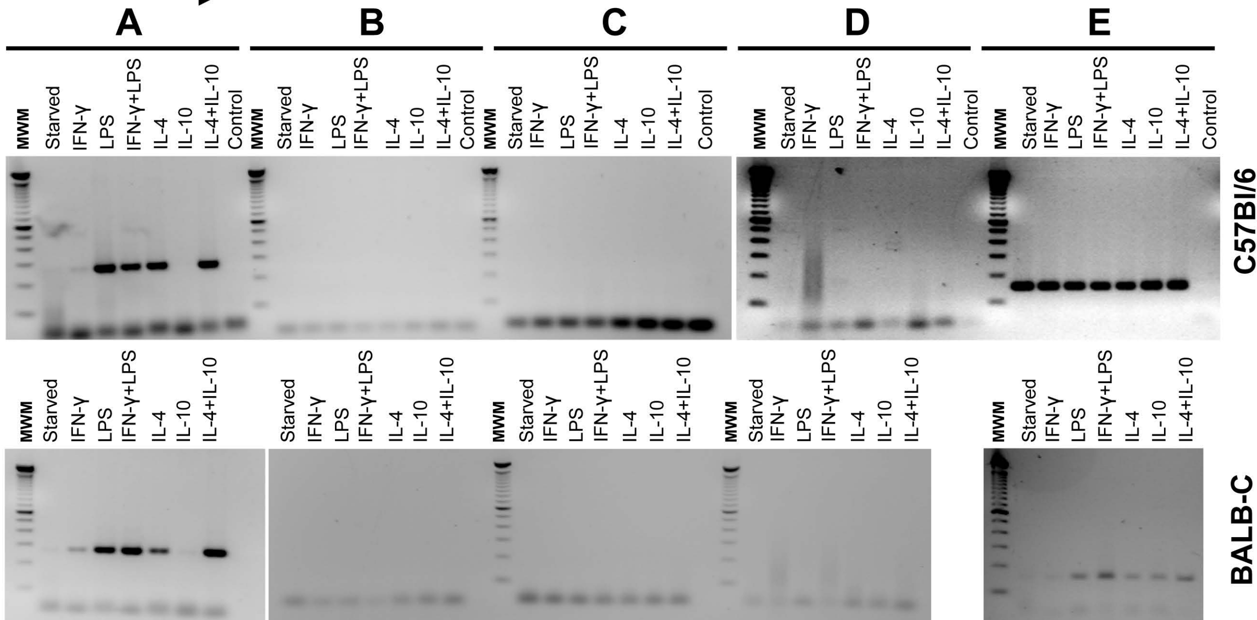


Figure 5

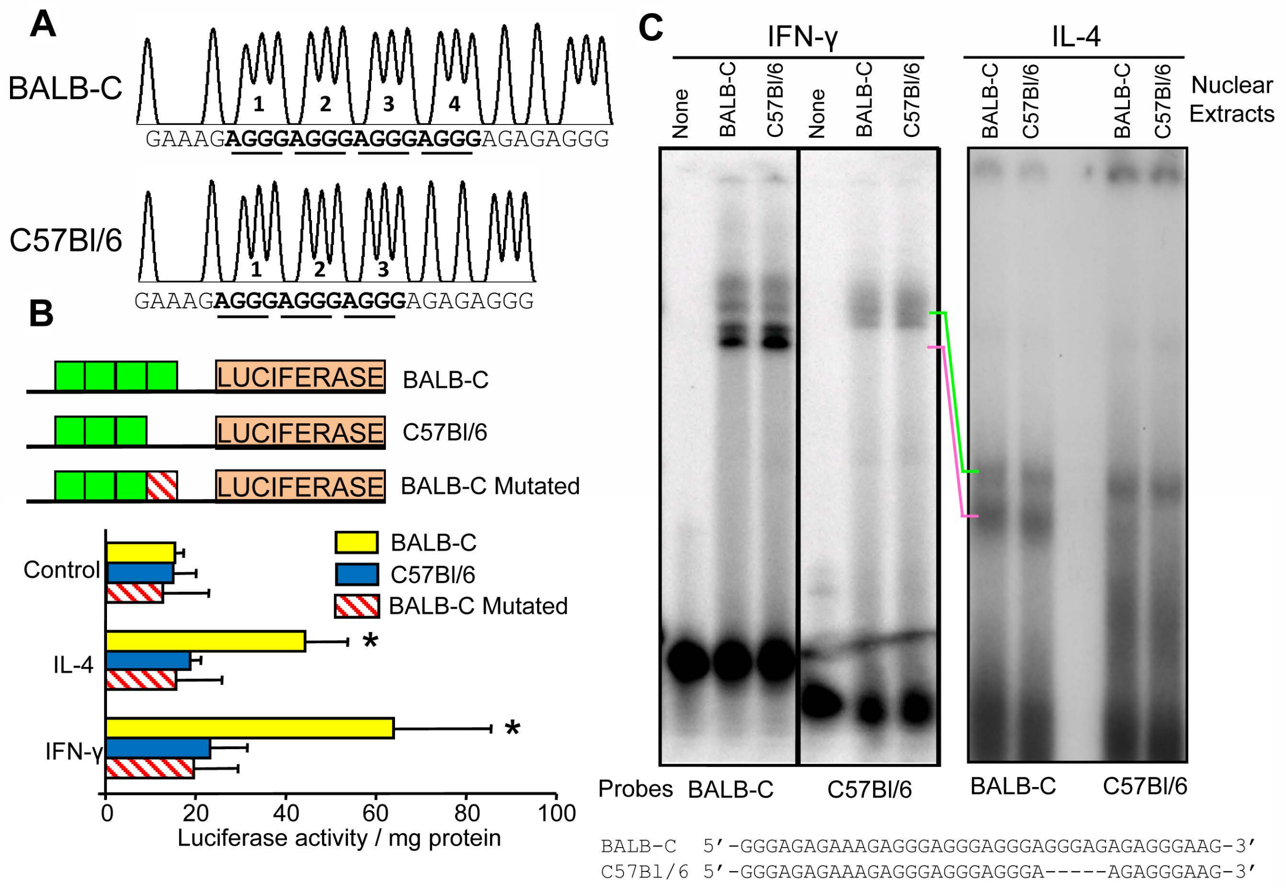


Figure 6