Arginine metabolic pathways involved in the modulation of tumor-induced angiogenesis by macrophages

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Abstract Neovascularization, an essential step for tumor progression and metastasis development, can be modulated by the presence of macrophages (Mps) in the tumor microenvironment. The ability of Mps to regulate the angiogenicity of the LMM3 tumor cell line was studied. Peritoneal Mps from LMM3 tumor-bearing mice (TMps) potentiate in vivo LMM3 angiogenicity. These results were confirmed by CD31 immunoblotting assays. The activity of TMps depended on nitric oxide synthase (NOS) and arginase (A) activity. By immunoblotting we evidenced that AI and AII isoforms were up-regulated in TMps while the inducible and neuronal NOS isoforms were highly expressed in normal Mps. TMps might positively modulate tumor growth by stimulating angiogenic cascade mainly through polyamine synthesis.

Key words: Tumor angiogenesis; Macrophage; Arginase; Nitric oxide synthase; CD31

1. Introduction

The role of nitric oxide (NO) in tumor biology has been extensively studied. Several authors have evidenced that NO plays a major role in angiogenesis and tumor progression [1,2]. It is well established that angiogenesis is essential for tumor and metastasis growth. LMM3 cells, derived from a murine mammary adenocarcinoma, induce a strong angiogenic response in vivo, that partially depends on arginine metabolic products via nitric oxide synthase (NOS), as it diminishes by the addition of NOS inhibitors (N^{ω}-nitro-L-arginine methyl esther, L-NAME; and aminoguanidine, AG) [3].

Malignant tumors contain macrophages (Mps) as a major component of the host leukocytic infiltrate and the role of Mps in tumor progression have given off contradictory evidence [4]. It has been recognized that Mps can act either as negative regulators by achieving tumor cytotoxicity, or as positive regulators by promoting tumor growth. Both actions can be exerted via L-arginine metabolism [5].

Here we investigate the modulatory role of peritoneal Mps from LMM3 tumor-bearing mice (TMps) on the angiogenic response induced by LMM3 cells in relation to arginine metabolism through NOS/arginase (A) pathway.

2. Materials and methods

2.1. Animals and tumor cell line

BALB/c mice (females, 12 weeks old) from our Animal Division were used. Animal care was provided in accordance with that outlined in the Guide for Care and Use of Laboratory Animals (NIH Publication, 1986). The tumor-cell line LMM3 was previously selected from a spontaneous syngeneic mammary adenocarcinoma MM3 [6]. Cell suspensions with more than 90% viability (assessed by trypan blue) were used. Tumor-bearing mice (TBM) were obtained by s.c. inoculation of 4×10^5 LMM3 cells into the flank.

2.2. Purification of peritoneal macrophages

Peritoneal cells from normal and TBM (7 days post inoculation, with similar palpable tumors) were obtained by washing the peritoneal cavity with 5 ml MEM supplemented with 10% FCS. The adherent Mps from normal (NMps) and TMps were purified by adhesion to plastic for 2 h. After washing twice with PBS, adherent cells were scraped and resuspended in culture medium. Cell viability ($>95\%$) was assessed by trypan blue exclusion test.

2.3. Tumor-induced angiogenesis

2.3.1. Bioassay. Tumor-induced angiogenesis was quantified using an in vivo bioassay previously described [7]. Briefly, 2×10^5 LMM3 cells were inoculated i.d. alone or with NMps or TMps (2×10^3) in both flanks of female normal mice ($n=4$ for each group). In another set of experiments, previous to the inoculation, Mps were treated during 2 h with: 2×10^{-3} M L-NAME, 10^{-3} M aminoguanidine (AG), 5×10^{-2} M valine (Val) or 10^{-4} M N^{ω}-hydroxy-L-arginine (NOHA). On day 5, animals were killed with ether. The inoculated sites were photographed and the slides were projected onto a reticular screen to count the number of vessels/mm^2 skin.

2.3.2. Detection of CD31 (PECAM-1) by Western blot. Skin strips from the angiogenic site devoided of hair, were obtained. Tissues were homogenized at 4°C in four volumes of 20 mM Tris–HCl, 10 mM EGTA, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, pH 7.4. Homogenates were centrifuged at 4°C, 10 000 rpm for 10 min. Supernatants were stored at −80°C until use and protein concentration was determined by the method of Lowry [8]. Samples were subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes and washed. The nitrocellulose strips were blocked in 20 mM Tris–HCl buffer, 500 mM NaCl, 0.05% Tween 20 (TBST) with 5% skimmed milk for 1 h at room temperature and subsequently incubated overnight with a goat polyclonal antibody anti-PECAM-1 (Santa Cruz Biotechnology). After several rinses, membranes were incubated 1 h with alkaline phosphatase-conjugated secondary antibody anti-goat IgG. The proteins were visualized using a mixture of nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt (BCIP). Quantification was performed us-

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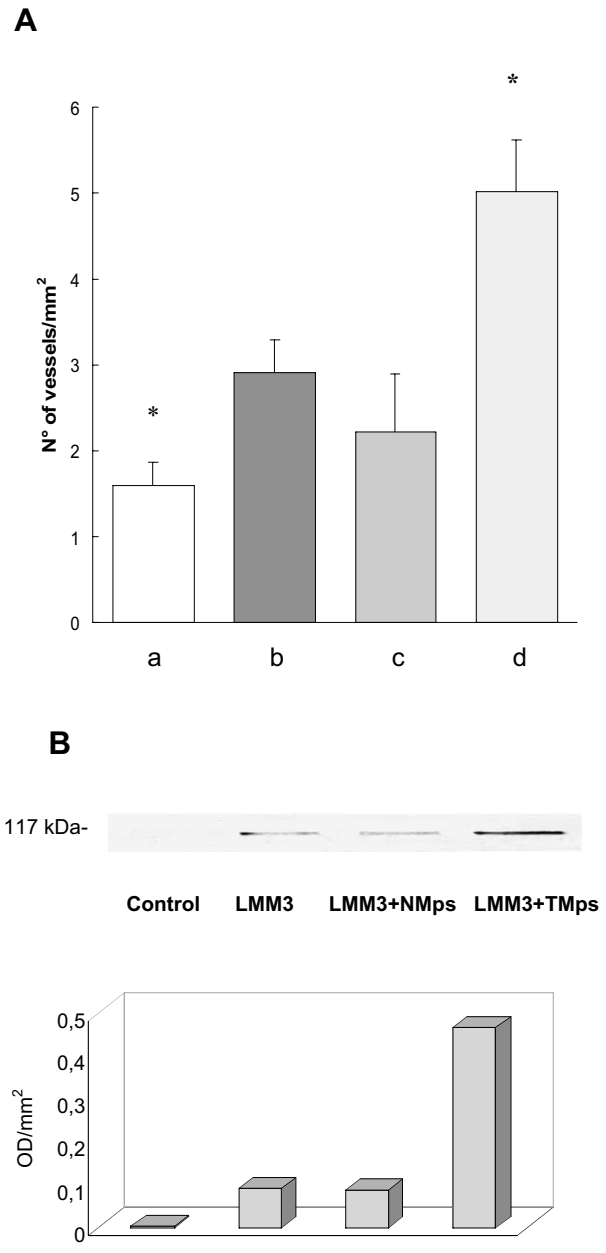

Fig. 1. A: LMM3 tumor cells (2×10^5) were injected alone (b) or with NMps (2×10^3, c) or TMps (2×10^3, d) as stated in Section 2. Angiogenic response was measured as number of vessels per square millimeter (δ). $^*P<0.0001$ vs. δ of normal skin (a) 1.65 ± 0.20 according to Kruskal–Wallis test. B: Western blot for CD31 expression in angiogenic skin. Protein molecular weight is indicated on the left. Control corresponds to normal skin. Lower panel shows densitometric analysis of the bands expressed as optical density units (OD)/mm^2.

ing a computerized densitometer connected to an image analyzer (Bio-Rad GS 700).

2.4. Determination of nitric oxide

Levels of NO released into the cell culture media by NMps or TMps were determined by Griess reaction [9]. Briefly, 10^5 cells were incubated in triplicate in 96-well plates in 200 μl MEM alone or with the inhibitors mentioned above. After 2 h incubation, medium was replaced by fresh MEM. After 24 h, 100 μl of the supernatants were added to an equal volume of Griess reagent (1% sulphanyl amine in 30% acetic acid with 0.1% N-1-naphtyl ethylenediamine dihydrochloride in 60% acetic acid). Absorbance was measured at 540 nm with an ELISA Reader (Labsystem). Results are expressed in micromolar per million cells (μM/10^6 cells).

2.5. Detection of NOS isoforms by Western blot

Lysates were prepared by resuspending the cells (2×10^7 Mps) in 1 ml of lysis buffer containing 50 mM Tris–HCl (pH:8), 100 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 10 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor. Cell lysates were centrifuged at $10\,000\times g$ for 10 min. Protein concentration in supernatants was measured by the method of Lowry [8]. Samples were subjected to 7.5% SDS–PAGE electrophoresis, seeding 20–50 μg protein in each lane. Proteins were transferred to nitrocellulose membranes and incubated overnight with a rabbit polyclonal antibody anti-nNOS or anti-eNOS and a goat polyclonal antibody anti-iNOS (Santa Cruz Biotechnology). After several rinses strips were incubated at 37°C during 1 h with alkaline phosphatase-conjugated secondary antibody (rabbit anti-IgG or goat anti-IgG, respectively). Bands were visualized with NBT/BCIP and quantified by densitometry [10].

2.6. Arginase activity assay

Arginase (A) activity was determined in cell lysates according to previously described methods [11]. Briefly, 10^5 cells treated or not with 10^{-4} M L-NMMA, 10^{-3} M AG, 5×10^{-2} M Val or 10^{-4} M NOHA were lysed with 0.5 ml 0.1% Triton X-100, 25 mM Tris–HCl containing 5 mM $MnCl_2$, pH 7.4. The enzyme was activated at 56°C and 25 μl of the activated lysate were incubated with 25 μl of 0.5 M arginine, pH 9.7, at 37°C for 60 min. The reaction was stopped in acid medium. Urea concentration was measured at 540 nm with a microplate reader. Results are expressed as μmol of urea per hour per 10^6 cells (μmol/h/10^6 cells).

2.7. Detection of arginase isoforms by Western blot

Adherent cells were rinsed twice with ice-cold PBS and then scraped into 300 μl lysis buffer: 50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.1 mM PMSF. Lysis was completed by sonication. Samples (25 μg) were subjected to 10% SDS–PAGE as it was stated in previous methods [12]. Nitrocellulose membranes were incubated overnight with a monoclonal anti-mouse arginase I antibody (BD Transduction Labo-

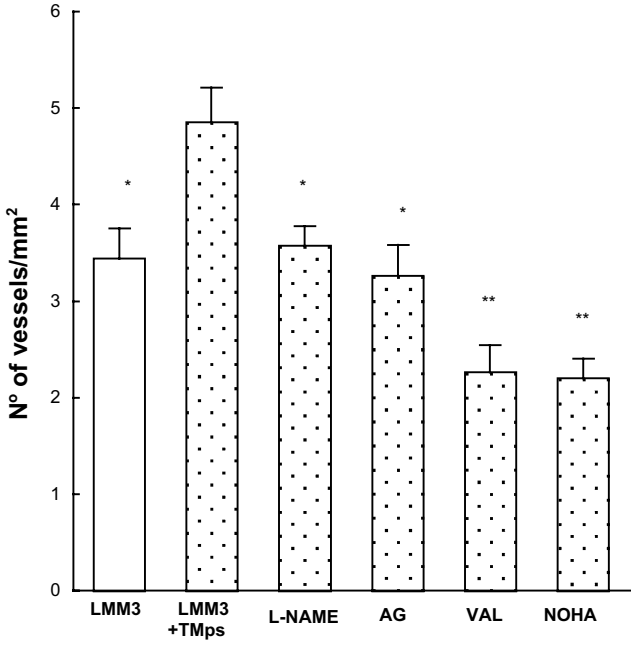

Fig. 2. LMM3 tumor cells alone or co-injected with TMps in the optimal relation tested, treated or not with NOS inhibitors (2×10^{-3} M L-NAME, 10^{-3} M AG) or arginase inhibitors (5×10^{-2} M Val or 10^{-4} M NOHA) during 2 h and inoculated to syngeneic recipients as was stated in Section 2. Each value represents the mean ± S.D. of four experiments. $^*P<0.001$ and $^{**}P<0.0001$.

	NOS activity (NO_2^-μM /10^6 cells)	Arginase activity (urea μmoles/ h/10^6 cells)
NMps	86.3 ± 2.8	10.2 ± 0.35
TMps	59.9 ± 2.2	22.2 ± 1.6

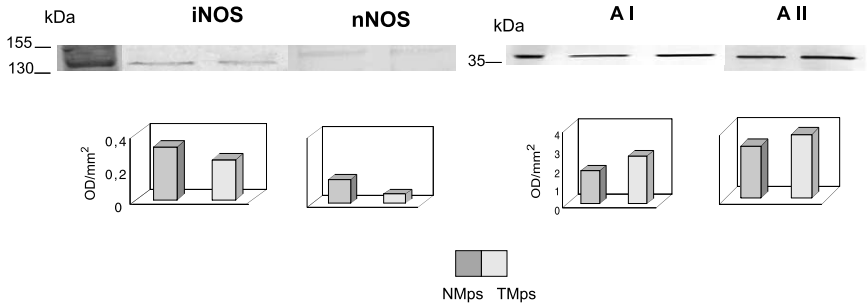

Fig. 3. Upper panel: NOS activity measured as NO_2^- (μM/10^6 cells) and arginase activity measured as urea (μmol/h/10^6 cells) were determined, in basal conditions, in the culture supernatants of NMps or TMps. Values are mean ± S.E.M. of four experiments performed. Lower panel: immunoblotting assay for NOS and arginase (A). Left: iNOS (130 kDa) and nNOS (155 kDa) isoforms in NMps and TMps. Right: Western blot assay to detect AI and AII isoforms. First lane: AI positive control. Bands were quantified by densitometric analysis. One representative experiment of four performed.

ratories) or with a rabbit anti-arginase II antibody (kindly gifted by Dr. Masataka Mori) [13,14]. The secondary antibody conjugated with alkaline phosphatase-anti-mouse or anti-rabbit IgG was added for 1 h at 37°C. Proteins were visualized using a mixture of NBT/BCIP and quantified by a densitometric analysis.

2.8. Drugs

All drugs were purchased from Sigma-Aldrich unless otherwise stated. Solutions were prepared fresh daily.

3. Results

3.1. Angiogenic activity of peritoneal Mps

Since LMM3 cells are strongly angiogenic, here we explored the ability of Mps to modulate the neovascular response. Peritoneal TMps (2×10^5) alone, elicited a positive angiogenic response (2.7 ± 0.14 vessels/mm^2) comparable to LMM3 cells (2.91 ± 0.38). When TMps were co-injected with LMM3 cells in a subthreshold dose (TMps:LMM3, 2×10^3:2×10^5), that was ineffective per se, they markedly potentiated tumor cell angiogenesis (Fig. 1A). NMps were devoided of angiogenic activity either alone (1.79 ± 0.14) or in the presence of LMM3 cells (Fig. 1A). These results were confirmed by studying CD31 expression in angiogenic skin. As is shown in Fig. 1B, CD31 expression is negative in normal skin and becomes evident in the LMM3 angiogenic site, increasing in the skin of animals inoculated with LMM3 plus TMps. By immunohistology we observed that anti-CD31 antibody only binds to neovessels (data not shown).

3.2. Participation of Mps NOS and A in tumor angiogenesis

Since L-arginine metabolism via the NOS pathway was involved in LMM3 cell angiogenesis [3], we investigated whether Mps could be stimulating angiogenic activity by arginine catabolites. As shown in Fig. 2, the preincubation of TMps with a non-selective inhibitor of NOS, L-NAME, or with AG (iNOS inhibitor), significantly blocked their stimulatory action on LMM3-induced angiogenic response. Furthermore, when TMps were treated with Val or NOHA, that inhibit A pathway, a greater reduction in the angiogenic response was detected (nearly 50% inhibition), pointing to a main role of NOS and A in TMp pro-angiogenic activity.

3.3. Expression of NOS and A isoenzymes in Mps

Studying NOS enzymes expression in NMps and TMps we detected iNOS and nNOS isoforms in both Mps populations, the former being more highly expressed than the latter. Endothelial NOS protein was undetectable in both populations. TMps exhibited lesser amounts of both isoenzymes in comparison to NMps (Fig. 3, lower panel). The decrement in protein expression was accompanied by a fall in NOS activity as it was confirmed by nitrite determination: TMps produced significantly lower amounts of NO (30%) than NMps (Fig. 3, upper panel).

Expression of A isoforms was also assayed by Western blot. Both AI and AII were expressed in NMps and in TMps but, contrary to NOS, both proteins were up-regulated in TMps (Fig. 3, lower panel). The increment in protein expression correlated with an increase by 100% in A activity, measured as urea production, in TMps compared to NMps (Fig. 3, upper panel).

3.4. Interactions between NOS and A in macrophages

According to our results, we investigated interactions between NOS and A pathways in NMps and TMps by measuring their respective products, nitrite and urea, in the presence of adequate inhibitors. As mentioned earlier, NMps were able to liberate 44% more nitrite to the culture medium than TMps (Fig. 3, upper panel). Both NOS inhibitors, L-NMMA and AG, were equipotent to inhibit NO production in both Mp populations (Fig. 4A). The ability of Val, which interferes

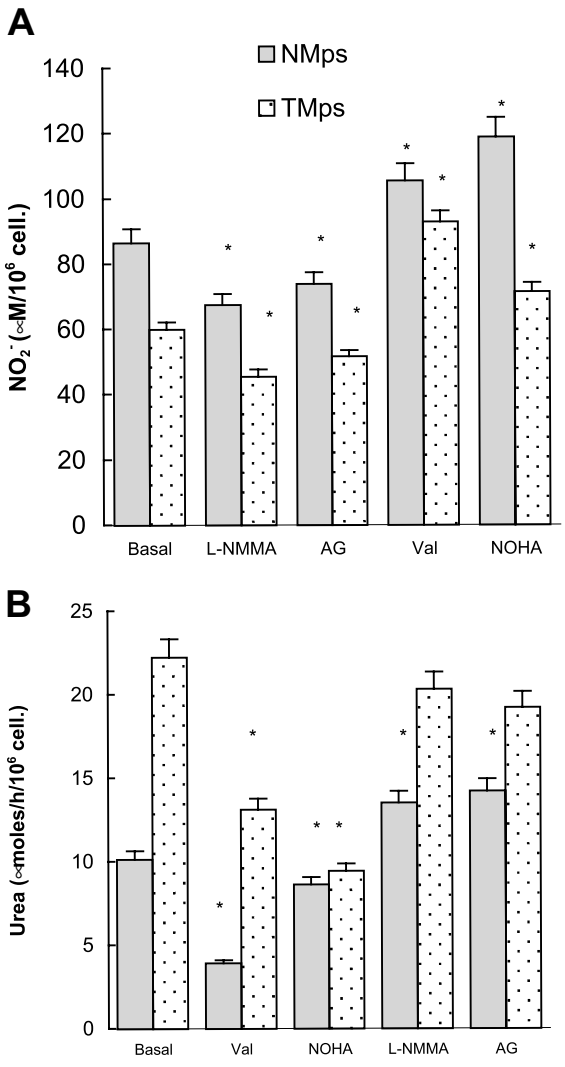

Fig. 4. Untreated NMps and TMps (basal) or treated with 2×10^{-3} M L-NAME, 10^{-3} M AG, or 5×10^{-2} M Val or 10^{-4} M NOHA were used to determine: NOS activity measured as NO_2^- (μM/10^6 cells) into the culture supernatants (A) or arginase activity measured as urea (μmol/h/10^6 cells) production (B). Values are mean ± S.E.M. of four experiments performed in duplicate. *Significantly different from basal at $P<0.05$.

with A pathway, to potentiate NO synthesis was significantly greater in TMps than in NMps, while NOHA potentiated NO production only in NMps. As mentioned above, TMps produced significantly higher amounts of urea than NMps in basal conditions. Val always inhibited urea production while NOHA was more effective in inhibiting A activity in TMps than in NMps (Fig. 4B). It is also shown in Fig. 4B that the addition of NOS inhibitors (L-NMMA or AG) induces urea formation over basal levels only in NMps.

It must be mentioned that we could not detect cytotoxic effects of TMps against LMM3 cells at the effector:target ratio 1:100 used in the angiogenesis assays. However, cytotoxicity was exerted using target:effector ratios 1:25 and 1:50 (data not shown).

4. Discussion

Mps perform a multitude of functions essential for tissue remodeling, inflammation and immunity by secreting cytokines, growth factors and prostaglandins [15]. The majority of malignant tumors contain Mps as a major component of the host leukocytic infiltrate [4]. These tumor-associated Mps are recruited into the tumor mass from the circulation by the action of different chemotactic factors. Tumor-associated Mps not only exert cytotoxicity without stimuli but, to the contrary, they can also exert pro-tumor functions [16]. Concordantly, we observed that TMps were able to exert pro-tumor functions via a complex mechanism. We here demonstrated that a sufficient number of TMps not only induce per se a positive angiogenic response tested in vivo, but when co-injected in a TMp/LMM3 ratio less than 1/10 strongly up-regulate LMM3 tumor cell-induced angiogenesis. It is noteworthy that this same low concentration of TMps or NMps alone were ineffective to elicit angiogenesis; however, 100-fold higher number of TMps but not NMps were angiogenic per se. Akedo et al. [17] have reported that Mps had the potential to induce the invasiveness of tumor cells in vitro and in vivo, postulating that this effect occurred depending on the ratio of the number of Mps to that of tumor cells. Thus, Mps were stimulatory to tumor cells when the ratio remained between 0.1 and 1.0, attributing the positive influence on tumor progression and metastasis to the production of oxidative metabolites by active Mps.

Mps use L-arginine to synthesize NO through NOS isoforms and/or urea to produce ornithine via arginases activation. It has been largely discussed whether arginine metabolites act for or against tumor growth. It has been suggested that L-arginine-dependent reactive nitrogen products hold a major role in tumor cell killing by activated Mps [18]. iNOS expression and activity has been linked with pathological status as chronic inflammation and tumors [19]. In our model the stimulatory action of TMps on LMM3 angiogenesis was blunted by selective inhibiting iNOS with AG or using L-NAME as non-selective inhibitor. We cannot dismiss that nNOS might be also involved in this process. Thus, although TMps produce lower amounts of NO than NMps, with concomitantly lower expression of iNOS and nNOS proteins, it seems that this low level of NO is enough to enhance tumor angiogenesis.

Taking into account that L-arginine metabolism could be derived to the A pathway, we also tested the action of A inhibitors on TMps-stimulated tumor angiogenesis. We demonstrate that both Val and NOHA, which significantly inhibited urea formation, potently reduce stimulatory effect of TMps on LMM3-induced neovascularization. The synthesis of polyamines via AI and AII isoforms is essential for human tumor cell proliferation [20–22]. But other sources of polyamine precursors, such as those from Mps, can contribute to tumor growth and might not be discarded. The fact that NMps did not affect angiogenesis although producing high amounts of NO needs further studies.

It has been described that iNOS and arginases are co-expressed in murine Mps, resulting in complex interactions where the level of substrate and/or product of one enzyme may regulate the activity of the other enzyme [5]. By immunoblotting we evidenced the presence of iNOS, nNOS, AI and AII in peritoneal Mps. But while both NOS isoforms actively predominate in NMps, arginases are up-regulated in TMps. The preincubation of TMps with high doses of Val or NOHA strongly inhibited A activity, while only Val was effective in

enhancing NO synthesis. These results could probably be due to inhibitor selectivity for one or both A isoforms in these cells. Further experiments are needed to clarify this mechanism. On the contrary, the inhibition of A in NMps with Val or NOHA significantly increases nitrite production while NOS blockade stimulates urea production, pointing to a reciprocal regulation between both enzymes. This cross-regulatory mechanism seems to be more evident in NMps, since they express higher amounts of both active proteins.

Taken together, our results suggest that peritoneal Mps from TBM, far from exerting tumoricidal activity when present at low rates, contribute to enhance LMM3 angiogenesis. By switching L-arginine from NOS to A metabolic pathway, TMps diminish NO levels and probably provide polyamine precursors to favor neovascularization and tumorigenesis.

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