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Journal of Cellular Biochemistry 116:721–729 (2015)

Journal of Cellular Biochemistry

Involvement of Purinergic System in the Release of Cytokines by Macrophages Exposed to Glioma-Conditioned Medium

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ABSTRACT

Macrophages are involved in cancer progression. M1 macrophages have an antitumor effect, whereas M2 phenotype are associated with tumor growth. The progression of gliomas involves the participation of an inflammatory microenvironment. Adenosine triphosphate (ATP) can act as pro-inflammatory signal, whereas adenosine has opposite properties. The biological effects of extracellular nucleotides/nucleosides mediated by purinergic receptors are controlled by ectonucleotidases. In the present work, we evaluated whether glioma-conditioned medium (GL-CM) modulates macrophage differentiation and the participation of ATP and adenosine in the release of pro-and anti-inflammatory cytokines by these cells. The results show that macrophages exposed to GL-CM were modulated to an M2-like phenotype. HPLC analysis of GL-CM demonstrated the presence of significant amounts of ATP and its metabolites. Macrophages exposed to GL-CM presented decreased ATP and AMP hydrolysis and increased IL-10 and MCP-1 secretion, effects that were diminished by P1 or P2 antagonists. GL-CM did not alter the release of IL-6 by macrophages, although treatment with ATP promoted an increase in the release of IL-6, which was prevented by a P2X7 antagonist. In summary, we found that A_{2A} and P2X7 activation is necessary for IL-10, MCP-1, and IL-6 release by macrophages exposed to GL-CM, which, in turn, modulates the macrophages to M2-phenotype. The present study establishes a relationship between M2-like polarization, cytokine release and purinergic receptor activation in macrophages exposed to GL-CM. Therefore, the data presented herein contributes to advancing in the field of cancer-related inflammation and point specific purinergic receptors as targets for modulation of the phenotype of glioma-associated macrophages. J. Cell. Biochem. 116: 721–729, 2015.

KEY WORDS: GLIOMA; CONDITIONED MEDIUM; MACROPHAGES; PURINERGIC SYSTEM

Distinct macrophage populations have been related to cancer inhibition or progression [Sica and Bronte, 2007]. Macrophages exhibit a spectrum of activation ranging from M1/pro-inflammatory to M2/anti-inflammatory phenotypes [Gordon, 2003; Edwards et al., 2006; Mosser and Edwards, 2008]. In general, an antitumor effect has been ascribed to M1 macrophages, which are characterized by nitric oxide (NO) production and tumor-killing activity. In contrast, M2 macrophages exhibit an increased release of

Grant sponsor: CNPq-Brasil, FAPERGS; Grant number: Processo 10/0286-2; Grant sponsor: CAPES, FIPE-HCPA; Grant number: Projeto 11-0101.

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Manuscript Received: 10 April 2014; Manuscript Accepted: 13 November 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 24 December 2014
DOI 10.1002/jcb.25018 • © 2014 Wiley Periodicals, Inc.

anti-inflammatory cytokines and angiogenic effects, which are associated with promotion of tumor growth [Coussens and Werb, 2002; Edwards et al., 2006; Mosser and Edwards, 2008]. It is of note that previous studies have shown that tumor associated macrophages (TAMs) show an M2-like phenotype [Mantovani et al., 2013], and, by modulating multiple signaling pathways closely related to tumor malignancy, TAMs are considered key elements in the tumorigenesis process [Coussens and Werb, 2002; Sica and Mantovani, 2012; Mantovani et al., 2013].

Glioblastoma multiforme is the most common and devastating primary brain tumor [Dai and Holland, 2001]. The tumor microenvironment is heterogeneous, composed mainly of proliferating neoplastic cells and immune cells such as lymphocytes, neutrophils and macrophages [Solinas et al., 2009]. Several lines of evidence suggest that the presence of immune cells in the glioma microenvironment is an essential component of the processes of proliferation, migration and cell survival [Watters et al., 2005; Komohara et al., 2008; Gabrusiewicz et al., 2011]. Furthermore, it has been shown that inflammatory infiltration is directly correlated with the degree of malignancy of gliomas [Watters et al., 2005; Komohara et al., 2008; Gabrusiewicz et al., 2011].

There is growing interest in the effects of extracellular nucleotides on the development of different tumors including gliomas [Spychala, 2000; Melani et al., 2003; Morrone et al., 2005, 2006; Pellegatti et al., 2008; Braganhol et al., 2009; Bergamin et al., 2012; Di Virgilio, 2012]. High extracellular nucleotide levels are released into the extracellular medium due to cell damage, hypoxia or mechanical stress [Verkhrasky et al., 2009]. Adenosine triphosphate (ATP) and other nucleotides act in the immune response by controlling leukocyte traffic from the blood to tissues and by alerting the immune system to sites of cell damage/injury [Burnstock, 2004; Bours et al., 2006]. It has also been shown that extracellular ATP increases the release of the chemokines MCP-1 and IL-8 by C6 glioma cells, which may be important for the recruitment of macrophages and neutrophils, respectively, to tumor sites [Jantaratnotai et al., 2009]. In contrast, adenosine (ADO) increases the liberation of IL-10, an anti-inflammatory cytokine, by microglial cells and macrophages [Németh et al., 2005; Csóka et al., 2007; Koscsó et al., 2012]. Furthermore, this nucleoside has been reported to be a mediator of cell proliferation and angiogenesis, and it also acts in tumor progression [Spychala, 2000]. The biological effects of extracellular nucleotides/nucleosides are mediated by purinergic receptor activation and are controlled by ectonucleotidases, which efficiently hydrolyze ATP to adenosine in the extracellular space [Zimmermann et al., 2012].

The purinergic receptors are divided into two classes, P1 and P2, which are activated by adenosine and ATP, respectively [Di Virgilio et al., 2001]. P2 receptors are subclassified as P2X ionotrophic (P2X1–7) and P2Y metabotrophic ($P2Y_{1,2,4,6,11-14}$) receptors [Abbracchio and Burnstock, 1994; Di Virgilio et al., 2001; Burnstock, 2004], while the P1 receptors are subclassified as A_1, A_{2A}, A_{2B}, and A_3 [Palmer and Stiles, 1995].

In the present work, we aimed to evaluate whether glioma-conditioned medium could modulate the differentiation of macrophages and the participation of ATP and adenosine on this modulation via P2X7 and A_{2A} activation.

MATERIALS AND METHODS

ANIMALS

Male CF1 mice (6–8 weeks) were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room-controlled temperature ($22 \pm 2\,^{\circ}C$). Mice had free access to chow and water. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH) and were approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (CEUA/UFRGS) under the registration number 18128.

GL261 CELL CULTURES AND GLIOMA-CONDITIONED MEDIUM PREPARATION

Mouse glioma cell line GL261 was a generous gift from Doctor Steve Lacroix (Department of Anatomy and Physiology, Université Laval, QC, Canada). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco). Cell cultures were maintained in 5% CO_2/95% air at 37 °C. To prepare the glioma-conditioned medium (GL-CM), GL261 cells were seeded at a density of 1×10^5 cells/well in 6 multi-well plates. Once the sub-confluence stage, the cell medium was replaced with fresh DMEM/10% FBS, and the cells were cultured for 24 h. Then, the glioma-conditioned medium was collected, centrifuged (1,000 x*g* for 10 min), and the contents were transferred to new tubes and stored at −80 °C until use.

MOUSE PERITONEAL MACROPHAGE CULTURES AND EXPOSURE TO GLIOMA-CONDITIONED MEDIUM

Mouse peritoneal macrophages were collected by lavage of the peritoneal cavity with 5 mL of sterile FBS-free DMEM culture medium. The cells were centrifuged and suspended in FBS-free DMEM, and the obtained cells were seeded in 6 or 48 multi-well plates at densities of 1×10^5 or 3×10^4 cells/well in a final volume of 1000 μL or 250 μL of culture medium, respectively. Macrophages were allowed to attach for 30 min, and then, any unattached cells were washed out with FBS-free DMEM. The attached cells were used for the subsequent experiments [Zanin et al., 2012]. Cell cultures were evaluated by flow cytometry (FACS Calibur, BD Biosciences, Mountain View, CA) using a CD11b Ab PE (BD Phamingen), indicating macrophage purity greater than 80% (Fig. 1). The obtained macrophages were exposed to glioma-conditioned medium for 24 h (Mφ-CM). Macrophages exposed to DMEM/10% FBS (Mφ) for 24 h and glioma-conditioned medium (GL-CM) were used as controls.

In experiments to evaluate the contribution of purinergic receptors on macrophage cytokine release, macrophage cultures were exposed to GL-CM for 24 h in the presence or absence of 1 mM ATP (Sigma–Aldrich, St. Louis, MO), 100 μM adenosine (ADO; Sigma–Aldrich), 10 μM A740003 (a selective P2X7 receptor antagonist; Tocris Bioscience, Bristol, UK), 30 μM and 100 μM caffeine (a non-selective P1 antagonist; Sigma–Aldrich) and 50 nM SCH58261 (a selective A_{2A} receptor antagonist; Tocris Bioscience). The antagonists were added 30 min before the addition of ATP or ADO.

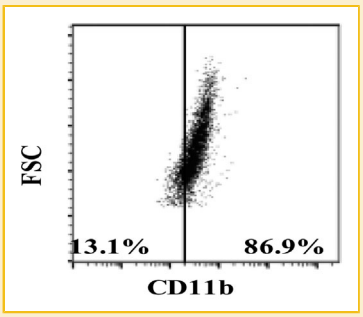

Fig. 1. Purity of macrophages in the peritoneal exudates. Peritoneal macrophages obtained from 6–8 mice were collected by lavage of the peritoneal cavity and cell culture purity was evaluated by flow cytometry using a CD11b antibody as described in Materials and Methods.

ARGINASE AND NITRITE DETERMINATION

Arginase activity in the cell lysates was measured based on the conversion of L-arginine to L-ornithine and urea [Corraliza et al., 1994] with minor modifications. Briefly, cells were lysed for 15 min with 100 μL of 0.1% Triton-X-100. Then, 100 μL of Tris-HCl (pH 7.4) and 10 μL of 10 mM $MnCl_2$ were added. One hundred microliters of the mixture was removed and placed in a new tube, and the enzyme was heat-activated (56 °C–7 min). The enzyme reaction was started by adding 100 μL of 0.5 M L-arginine (pH 9.7) as a substrate, and the mixture was incubated for 1 h at 37 °C. The reaction was stopped by adding 900 μL of H_2SO_4 (96%), H_3PO_4 (85%), and H_2O (1/3/7, v/v/v). Urea production was measured at 540 nm after the addition of 40 μL of 6% α-isonitropropiophenone, followed by heating at 95 °C for 30 min. The values were compared with a standard curve for urea.

Nitrite concentrations were measured using a Greiss reaction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

CYTOKINE RELEASE DETERMINATION

The cytokine release was analyzed in glioma-conditioned medium (GL-CM), the supernatant of macrophages exposed to DMEM/10% FBS (Mφ) or the supernatant of macrophages exposed to glioma-conditioned medium (Mφ-CM) with a cytometric bead array (CBA) mouse inflammation kit (IL-6, IL-10, MCP-1, TNF-α, and IL-12p70; R&D Systems) by flow cytometry using the FACSCalibur system, according to the manufacturer's instructions.

HPLC ANALYSIS OF GL261 GLIOMA CELL BASAL RELEASE OF NUCLEOTIDES AND NUCLEOSIDES

For HPLC analysis, glioma-conditioned medium (GL-CM) was prepared as described above, except that DMEM/10%FBS was replaced with DMEM/10%FBS without phenol red (Gibco). Aliquots of 20 μL were applied to a reversed-phase C_{18} column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek, USA) on a Shimadzu LC-10AD HPLC (Shimadzu, Japan). The elution was carried out with a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30 min period (flow rate of 1 mL/min) [Voelter et al., 1980]. The amounts of purines were measured by absorption at 260 nm. The retention time of the standards was used as a parameter for identification and quantification. Purine concentrations were expressed as μM.

ECTONUCLEOTIDASE ACTIVITY DETERMINATION

ATPase, ADPase, and AMPase activities were evaluated in 48 multi-well plates containing macrophages exposed to GL-CM that had been washed three times with incubation medium in the absence of nucleotides. The enzymatic reaction was started with the addition of 200 μL of incubation medium containing 2 mM $CaCl_2$ (for the ATPase and ADPase assays) or 2 mM $MgCl_2$ (for the AMPase assay), 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES (pH 7.4), and 1 mM ATP, ADP or AMP as substrates, at 37 °C. After incubation for 10 min, an aliquot of the incubation medium was transferred to a pre-chilled tube containing trichloroacetic acid (final concentration of 5% v/v). The release of inorganic phosphate (P_i) was measured by the malachite green method [Chan et al., 1986] using KH_2PO_4 as a P_i standard. Controls to determine non-enzymatic P_i release were performed by incubating the substrate in the absence of cells. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as a standard [Bradford, 1976]. Specific activity was expressed as nmol Pi released/min/mg of protein.

STATISTICAL ANALYSIS

The results were presented as the mean ± SD (standard deviation). Data were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's test. The differences between the means were considered significant for P values <0.05.

RESULTS

MACROPHAGES EXPOSED TO GLIOMA-CONDITIONED MEDIUM ARE MODULATED TO AN M2-LIKE PHENOTYPE

In general, M1 macrophages are characterized biochemically by increased production of nitric oxide (NO) and pro-inflammatory cytokines, while M2 macrophages exhibit increased arginase activity and production of anti-inflammatory cytokines [Gordon, 2003; Edwards et al., 2006; Mosser and Edwards, 2008; Mantovani et al., 2013]. Therefore, in the first set of experiments, we evaluated the effects of glioma-conditioned medium (GL-CM) on macrophage activation. We observed that the exposure of macrophages to glioma-conditioned medium (Mφ-CM) for 24 h promoted a significant increase in arginase activity (Fig. 2A) and in the release of IL-10, an anti-inflammatory cytokine (Fig. 2B). Interestingly, no difference in nitrite production was observed (Fig. 2C), and macrophages exposed to glioma-conditioned medium did not secrete detectable levels of TNF-α or IL-12, two pro-inflammatory cytokines (data not shown). Taken together, these results indicate that macrophages were modulated to an M2-like phenotype when exposed to conditioned medium obtained from glioma cells.

GLIOMA CELLS SECRETE ADENINE NUCLEOTIDE/NUCLEOSIDE INTO THE CONDITIONED MEDIUM

Extracellular nucleotides and nucleosides, such as ATP and ADO, act as endogenous signaling molecules, and these molecules are secreted at sites of inflammation associated with tumors [Melani et al., 2003;

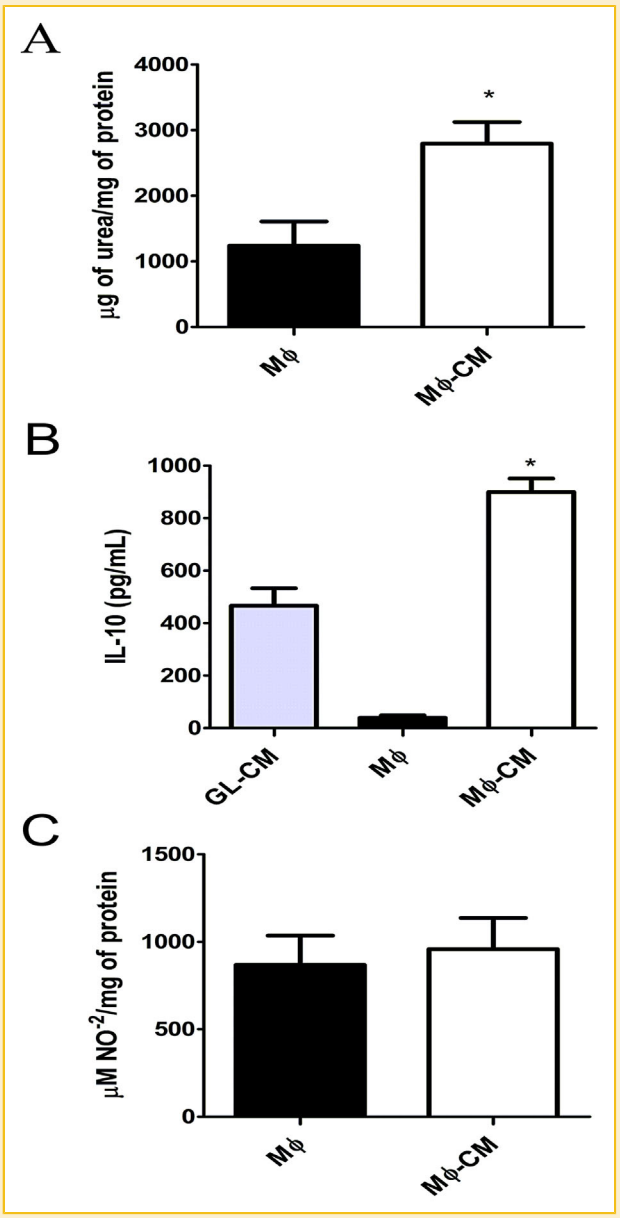

Fig. 2. Characterization of macrophage activation following exposure to glioma-conditioned medium. Glioma-conditioned medium (GL-CM) was prepared as described in the Material and Methods section. Then, mouse peritoneal macrophages were exposed to DMEM/10%FBS (Mφ) or glioma-conditioned medium (Mφ-CM) for 24 h, and the macrophage phenotype was evaluated as follows: (A) Arginase activity was estimated by measuring the formation of urea from arginine in macrophage lysates . The values represent the mean ± SD from five independent experiments. Macrophages were pooled from 6 to 8 mice per experiment. Data were analyzed by Student's *t* test. *, significantly different from the Mφ group at $P < 0.05$ (B) IL-10 release was measured by CBA. The values are expressed as pg/mL and represent the mean ± SD. Macrophages obtained from 6 to 8 mice were pooled for each experiment. Data were analyzed by ANOVA followed by Tukey's test. *, significantly different from Mφ and GL-CM groups at $P < 0.05$. (C) iNOS activity was estimated by the accumulation of nitrite (NO^{2-}) in the supernatant of cell cultures. The values represent the mean ± SD from three independent experiments. Macrophages obtained from 6 to 8 mice were pooled for each experiment. Data were analyzed by Student's *t*-test.

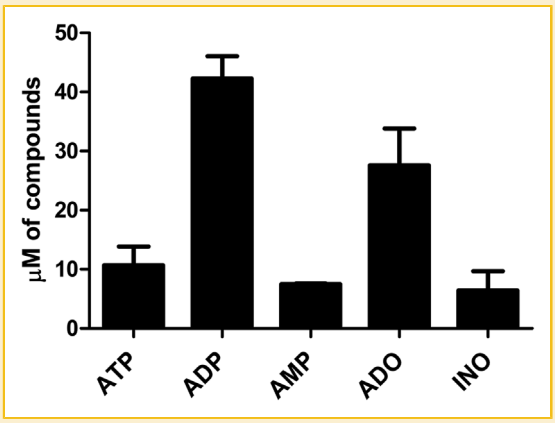

Fig. 3. Analysis of nucleotide and nucleoside levels in glioma-conditioned medium. Glioma-conditioned medium (GL-CM) was prepared by incubating GL261 glioma cells for 24 h in DMEM without phenol red and supplemented with 10% FBS. The amounts of purines were measured by HPLC. Purine concentrations are expressed as μM. The values represent the mean ± SD from three independent experiments.

Pellegatti et al., 2008; Bours et al., 2011]. Thus, to evaluate the levels of adenine nucleotide/nucleoside secreted by glioma cells, GL261 cells were cultured for 24 h, the cell medium was collected, and the extracellular nucleotide/nucleoside levels were determined by HPLC. The analysis of the GL-CM revealed that glioma cells secreted significant amounts of ATP (10.7 ± 3.2 μM), ADP (42.3 ± 3.8 μM), AMP (7.5 ± 0.13 μM), ADO (27.6 ± 6.2 μM), and INO (6.43 ± 3.3 μM) (Fig. 3). These results mean that glioma-conditioned medium contains significant amounts of secreted ATP and its metabolites, which, among other secreted substances, could be involved in the modulation of macrophages to the M2-like phenotype.

EXTRACELLULAR NUCLEOTIDE METABOLISM IN MACROPHAGES IS MODULATED BY GLIOMA-CONDITIONED MEDIUM

Considering that the ectonucleotidase activities are an important mechanism in the control of purinergic receptor activation [Zimmermann et al., 2012], the effect of GL-CM on nucleotide metabolism of macrophages was then evaluated (Fig. 4). Colorimetric analysis of nucleotide hydrolysis showed that the ATP and AMP hydrolysis were decreased by 27% and 44%, respectively, while the ADP hydrolysis was unchanged in macrophages exposed to glioma-conditioned medium (Mφ-CM) when compared to control macrophages (Mφ). It is possible to correlate these results to the data presented in Fig. 3 with two explanations: first, it is possible to assume that AMP hydrolysis was inhibited by the significant levels of ATP and ADP present in the glioma-conditioned medium. Second, the ATPase inhibition could be negative feedback caused by ADP, which is the product of ATP hydrolysis.

THE P2X7 RECEPTOR IS INVOLVED IN CYTOKINE RELEASE BY MACROPHAGES EXPOSED TO GLIOMA-CONDITIONED MEDIUM

Studies from our laboratory have proposed the involvement of purinergic signaling in glioma cell proliferation and neurotoxicity [Morrone et al., 2006; Braganhol et al., 2009; Bergamin et al., 2012].

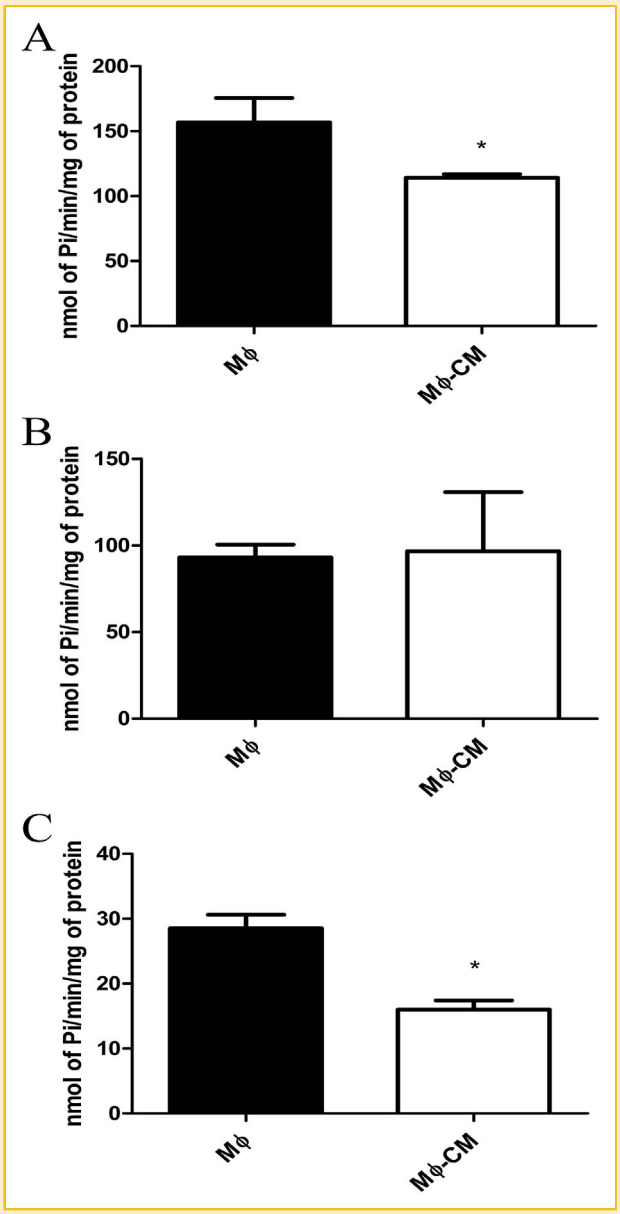

Fig. 4. Ectonucleotidase activity in macrophages exposed to glioma-conditioned medium. Macrophages were exposed to DMEM/10% FBS (Mφ) or to glioma-conditioned medium (Mφ-CM) for 24 h, and the hydrolysis of (A) ATP (B) ADP, and (C) AMP was evaluated as described in the material and methods section. The specific activity values were expressed as nmol Pi/min/mg protein. The values represent the mean ± SD from three independent experiments. Macrophages obtained from 6 to 8 mice were pooled for each experiment. Data were analyzed by Student's *t*-test. *, significantly different from the control group (Mφ) at $P < 0.05$.

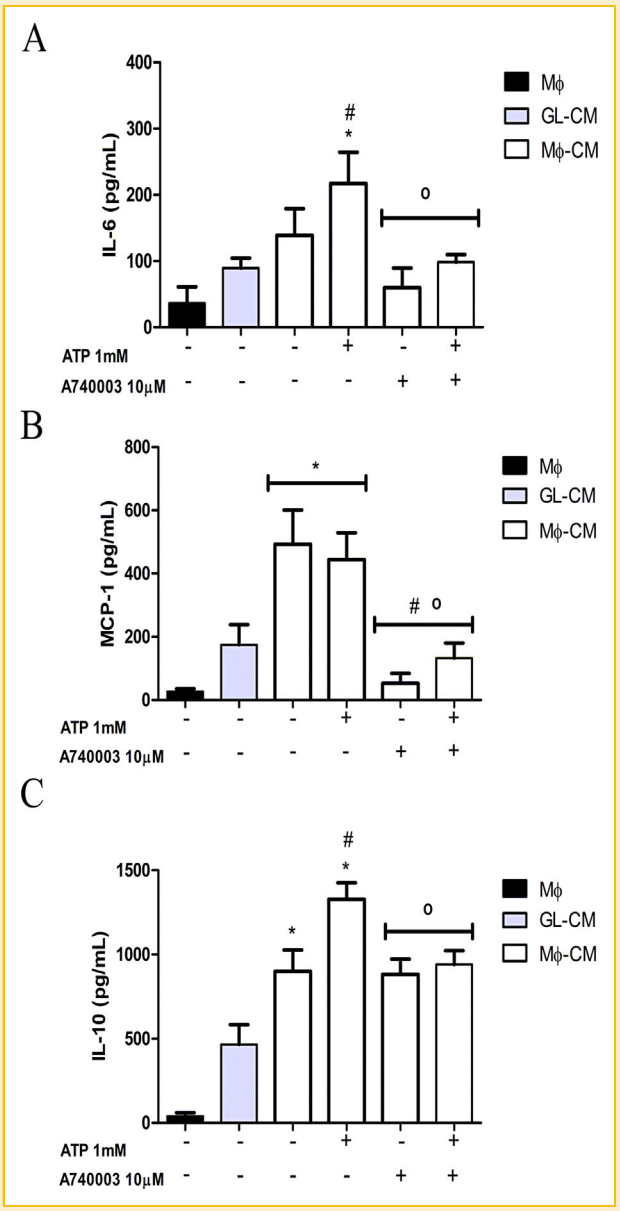

Fig. 5. Involvement of P2X7 receptor in cytokine release by macrophages exposed to glioma-conditioned medium. The cytokine release was measured in glioma-conditioned medium (GL-CM), the supernatant of macrophages exposed to DMEM/10% FBS (Mφ) and the supernatant of macrophages exposed to glioma-conditioned medium (Mφ-CM) in the presence or absence of 1 mM ATP or 10 μM A740003. The antagonist was added 30 min before the addition of ATP. (A) IL-6, (B) MCP-1 and (C) IL-10 release. The values represent the mean ± SD from three independent experiments. Experiments were performed using macrophages pooled from 6 to 8 mice. Data were analyzed by ANOVA followed by Tukey's test. *, # and °, significantly different from Mφ and GL-CM, Mφ-CM and Mφ-CM + ATP, respectively, at $P < 0.05$.

To further explore the functional relevance of decreased ATP metabolism exhibited by macrophages exposed to glioma conditioned medium, we investigated whether ATP or adenosine receptors could be involved in the secretion of IL-6, MCP-1 and IL-10. To this end, we investigated P2X7 and A_{2A} receptors. These cytokines and purinergic receptors were chosen because they are potentially involved in the inflammatory process associated with tumors [Allavena et al., 2008; Wei et al., 2008; Fang et al., 2011; Bergamin et al., 2012; Csóka et al., 2012; Koscsó et al., 2013]. The role of ATP in macrophage tumor-promoting actions was investigated through the exposure of macrophages to GL-CM in the presence or absence of exogenous ATP and a P2X7 antagonist (A740003); then, the release of IL-6, MCP-1 and IL-10 was evaluated (Fig. 5). We verified that there was no

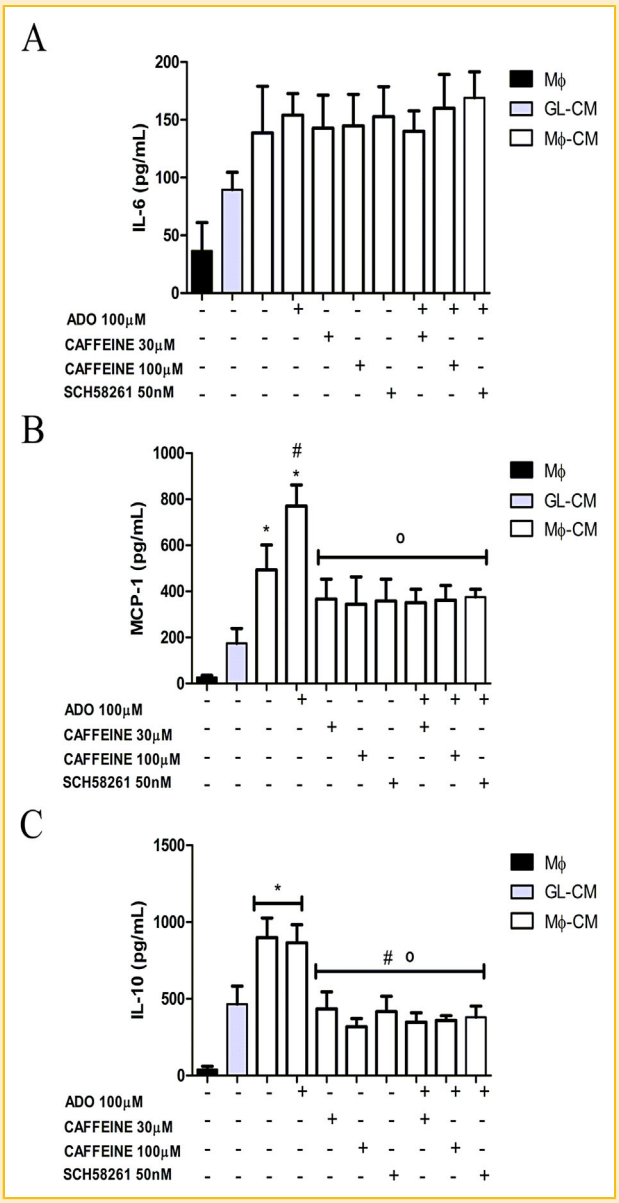

Fig. 6. Involvement of adenosine receptors in cytokine release by macrophages exposed to glioma-conditioned medium. Cytokine release was measured in glioma-conditioned medium (GL-CM), the supernatant of macrophages exposed to DMEM/10% FBS (Mφ) and the supernatant of macrophages exposed to glioma-conditioned medium (Mφ-CM) in the presence or absence of 100 μM ADO, 30 μM and 100 μM caffeine and 50 nM SCH58261. The antagonists were added 30 min before the addition of ADO. (A) IL-6, (B) MCP-1, and (C) IL-10. The values represent the mean ± SD from three independent experiments. Experiments were performed using macrophages pooled from 6 to 8 mice. Data were analyzed by ANOVA followed by Tukey's test. *, # and °, significantly different from Mφ and GL-CM, Mφ-CM and Mφ-CM + ADO, respectively, at $P < 0.05$.

significant difference in the IL-6 release by macrophages exposed to glioma-conditioned medium (Mφ-CM) when compared to glioma-conditioned medium (GL-CM). Importantly, the addition of 1 mM ATP to Mφ-CM promoted an increase in the release of IL-6 when compared to Mφ-CM alone. This effect was reversed by addition of a P2X7 antagonist (10 μM A740003) (Fig. 5A).

In contrast, there was a clear increase in MCP-1 release in Mφ-CM when compared to Mφ or GL-CM, and the addition of 1 mM ATP did not promote a further increase in MCP-1 release in Mφ-CM. Interestingly, the P2X7 antagonist (10 μM A740003) decreased the liberation of MCP-1 to control levels in macrophages exposed to glioma-conditioned medium (Fig. 5B).

Similar to the pattern of MCP-1 release, GL-CM also positively modulated the release of IL-10 by macrophages when compared to Mφ or GL-CM alone (Fig. 5C). However, the addition of 1 mM ATP caused an additional increase in IL-10 release. Importantly, the addition of P2X7 antagonist (10 μM A740003) reverted only the ATP-stimulated increase of IL-10 (Fig. 5C). It is noteworthy that control macrophages exposed to DMEM/10% FBS in the presence or absence of 1 mM ATP or 10 μM A740003 exhibited very low IL-6 production, MCP-1 production and IL-10 production (data not shown). Taken together, these results indicate that the P2X7 receptor is involved, at least partially, in cytokine release by macrophages exposed to glioma-conditioned medium.

INVOLVEMENT OF A_{2A} RECEPTORS IN CYTOKINE RELEASE BY MACROPHAGES EXPOSED TO GLIOMA-CONDITIONED MEDIUM

Macrophages express all four P1 receptors, and adenosine increases the release of IL-10, an anti-inflammatory cytokine, by macrophages [Bours et al., 2006; Haskó and Cronstein, 2013]. Considering the observed effects of ATP on IL-6, MCP-1 and IL-10 release in macrophages exposed to glioma-conditioned medium and the presence of detectable adenosine levels in GL-CM, as well as the presence of the ectonucleotidase cascade in the cell surface of macrophages, we next investigated the participation of adenosine in cytokine release by macrophages after GL-CM exposure (Fig. 6). Adenosine (100 μM) treatment differentially modulated the cytokine release by Mφ-CM. In contrast to IL-6 release, which was neither affected by ADO nor by ADO antagonists (caffeine and SCH58261) (Fig. 6A), the production of MCP-1 by Mφ-CM was further increased by ADO treatment when compared to Mφ-CM alone (Fig. 6B). Notably, treatment with caffeine or an A_{2A}-selective antagonist reverted the effect of ADO (Fig. 6B).

Finally, ADO treatment did not cause any additional increase in the IL-10 release by macrophages exposed to GL-CM (Fig. 6C). Moreover, treatment with caffeine or A_{2A} antagonist reversed the secretion of this cytokine (Fig. 6C). It is noteworthy that the macrophages exposed to DMEM/10%FBS in the presence or absence of ADO, caffeine or SCH5826 presented very low IL-6, MCP-1 and IL-10 production (data not shown). Taken together, these results indicate that A_{2A} receptor is partially involved in the secretion of these cytokines.

DISCUSSION

The present study demonstrates that alterations in purinergic signaling in macrophages exposed to glioma-conditioned medium modulate cytokine release might contribute to an M2-like phenotype. These alterations may contribute to glioma immune escape, favoring chemoresistance, tumor growth and invasion. First, we showed that macrophages exposed to glioma-conditioned

medium release high levels of IL-10 and exhibit increased arginase activity. In agreement with the modulation of macrophages to an M2-like phenotype, there was no difference in nitrite production when macrophages were exposed to glioma-conditioned medium, nor did the macrophages secrete detectable levels of TNF-α and IL-12, two pro-inflammatory cytokines. These results are consistent with literature data showing that modulation of macrophages associated with tumors (TAM) to having a low release of IL-12, high IL-10 expression and up-regulated levels of M2-specific genes, such as arginase-1 [Coussens and Werb, 2002; Sica and Mantovani, 2012; Mantovani et al., 2013].

Immunosuppressive environments and macrophage phenotype modulation by tumor cells constitute key elements of cancer progression [Allavena et al., 2008; Solinas et al., 2009; Mantovani et al., 2013]. Extracellular nucleotides and nucleosides, such as ATP and adenosine, are found at sites of inflammation associated with tumors [Melani et al., 2003; Pellegatti et al., 2008; Bours et al., 2011]. Considering that nucleotides and nucleosides are currently considered true inflammatory mediators [Németh et al., 2005; Csóka et al., 2007; Jantaratnotai et al., 2009; Koscsó et al., 2012], the second objective of the present work was to investigate the possible involvement of ATP and adenosine in the modulation of macrophages to an M2-like phenotype. HPLC analysis demonstrated the presence of ATP, ADP, AMP, adenosine and inosine in the micromolar range in glioma-conditioned medium. A recent study [Csóka et al., 2012] showed that adenosine modulates macrophages to an M2-like phenotype through the activation of A_{2A} and A_{2B} receptors. Similarly, our results show that the concentration of adenosine in glioma-conditioned medium is sufficient to activate these receptors on macrophages. Furthermore, glioma-conditioned medium has high amounts of IL-10, IL-6 and adenosine, and these molecules are related to the modulation of macrophages to an M2-like phenotype [Allavena et al., 2008; Solinas et al., 2009; Koscsó et al., 2013; Mantovani et al., 2013]. Therefore, IL-10, IL-6 and adenosine, among other factors, such as PGE-2 and TGFβ, could be involved in the induction of macrophages to an M2-like phenotype [Allavena et al., 2008; Solinas et al., 2009; Mantovani et al., 2013].

Our results also demonstrated a decrease in ATPase and AMPase activity, indicating less ATP breakdown and consequent ATP accumulation in the extracellular milieu, which could be related to the tumor-promoting effects of TAM. Similarly, previous results from our group showed that injection of apyrase (an ATP scavenger enzyme) in a rat glioma model resulted in decreased tumor progression [Morrone et al., 2006]. Additionally, despite the reduction in AMP hydrolysis, the results shown in Figure 2 that the glioma-conditioned medium contains adenosine at micromolar levels that are able to activate P1 receptors. Thus, it is possible to suggest that in an in vivo situation, the presence of adenosine in the extracellular milieu of gliomas could act as a mediator of cell proliferation and angiogenesis, processes that are involved in tumor progression [Spychala, 2000; Bergamin et al., 2012].

To better evaluate the effects of glioma-conditioned medium on macrophages, the secretion of different cytokines was determined. TAMs are reported to secrete factors that recruit and suppress immune cells, such as IL-10, IL-4, IL-6 and MCP-1 [Allavena et al., 2008; Jantaratnotai et al., 2009; Solinas et al., 2009; Mantovani et al., 2011]. We verified that MCP-1 and IL-10 were increased after exposure of macrophages to glioma-conditioned medium. In contrast, IL-6 secretion was not increased in macrophages exposed to glioma-conditioned medium, but the addition of ATP increased the release of this cytokine, and this effect was reversed by a selective antagonist of the P2X7 receptor. Treatment of macrophages exposed to glioma-conditioned medium with ATP did not cause a further increase in MCP-1 release; however, the addition of a selective antagonist of the P2X7 receptor decreased its release. The addition of ATP also caused an additional increase in the release of IL-10. P2X7 antagonists decreased only the ATP-stimulated increase of IL-10. A recent study [Jantaratnotai et al., 2009] showed that the treatment of glioma cells with low concentrations of ATP leads to an increase in MCP-1 release. Furthermore, other studies have suggested that activation of the P2X7 receptor is associated with enhanced cellular expression of pro-inflammatory factors including MCP-1, IL-6, IL-8, and VEGF [Bours et al., 2006; Wei et al., 2008; Fang et al., 2011].

Other P2-type receptors, especially $P2Y_{11}$ and $P2Y_{12}$, both expressed in macrophages, are also involved in the anti-inflammatory effects of ATP and ADP. The binding of ATP and ADP to the $P2Y_{12}$ receptor stimulated the production of IL-10 in blood cells [Swennen et al., 2006]. The results shown here demonstrated that the glioma-conditioned medium presents ADP levels that are able to activate the $P2Y_{12}$ receptor. Thus, the participation of other P2 receptors, in addition to P2X7, could be involved in in the secretion of pro-tumor cytokines.

Treatment with adenosine, caffeine or a selective A_{2A} receptor antagonist did not affect IL-6 secretion. Adenosine caused an additional increase in MCP-1 release, and treatment with caffeine or an A_{2A}-selective antagonist decreased the additional increase caused by adenosine. Finally, we verified that the treatment of macrophages exposed to glioma-conditioned medium with adenosine did not cause any additional increase in IL-10 secretion, but the addition of caffeine or A_{2A} antagonist partially reversed the release of this cytokine. Previous studies have demonstrated that adenosine augments IL-10 through A_{2A} or A_{2B} receptor activation [Németh et al., 2005; Csóka et al., 2007; Koscsó et al., 2012, 2013]. Furthermore, adenosine inhibits TNF-α, IL-6 and IL-12 release by LPS or bacteria-activated macrophages, and these effects are mediated by A_{2A} or A_{2B} receptors [Haskó et al., 2000; Pinhal-Enfield et al., 2003; Németh et al., 2005; Kreckler et al., 2006; Csóka et al., 2007; Csóka et al., 2012]. Moreover, inosine, a metabolite of adenosine, has been related to the suppression of macrophage, lymphocyte and neutrophil activation [Haskó et al., 2004]. Inosine inhibits the production of pro-inflammatory factors (IL-1, IL-6, IL-12, and TNF-α), most likely by A_{2A} and A_3 receptor activation in macrophages [Haskó et al., 2004; Bours et al., 2006].

In summary, we showed here that macrophages exposed to glioma-conditioned medium polarize to a TAM/M2-like phenotype and that ATP and adenosine increase protumoral cytokine release by these treated cells in vitro. These data provide further evidence for the involvement of the purinergic system in glioma progression.

ACKNOWLEDGMENTS

This work was supported by the CNPq-Brasil, FAPERGS (Processo 10/0286-2), CAPES, FIPE-HCPA (Projeto 11-0101). L. S. Bergamin and F. Figueiró were recipients of CNPq fellowships and J. Sévigny was the recipient of an FRQS senior scholarship. The authors thank Dr. L. Porciuncula for the donation of caffeine and SCH58261, and L.R. Blazina, F. Manica and M. S. Quevedo for their technical assistance.

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