LIPOXIN A4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION

Journal:	*International Journal of Cancer*
Manuscript ID	IJC-16-0527
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	21-Mar-2016
Complete List of Authors:	Simões, Rafael Loureiro; Universidade do Estado do Rio de Janeiro, Cell Biology de Brito, Natália Mesquita; Universidade do Estado do Rio de Janeiro, Cell Biology Cunha da Costa, Hayandra ; Universidade do Estado do Rio de Janeiro, Cell Biology Morandi, Verônica; Universidade do estado do Rio de Janeiro, Cell Biology Fierro, Iolanda Margherita; Universidade do Estado do Rio de Janeiro, Cell Biology Roitt, Ivan Maurice; Middlesex University, School of Health & Social Sciences Barja-Fidalgo, Christina; Universidade do Estado do Rio de Janeiro, Cell Biology
Key Words:	Inflammation, tumor, Macrophage, lipoxin

LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION

[1]RAFAEL LOUREIRO SIMÕES; [1]NATÁLIA MESQUITA DE BRITO; [1]HAYANDRA CUNHA DA COSTA, [2]VERÔNICA MORANDI; [1]IOLANDA MARGHERITA FIERRO; [3]IVAN MAURICE ROITT; *[1]CHRISTINA BARJA-FIDALGO.

[1]Laboratório de Farmacologia Celular e Molecular, [2]Departamento de Biologia Celular, IBRAG, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[3]School of Health & Social Sciences, Middlesex University, London, UK.

Running title: Lipoxin analog modulates tumor-associated macrophages.

Key words: tumor, inflammation, lipoxin, macrophage

Research article - Section: Tumor Immunology and Microenvironment

Our study demonstrates, for the first time, that lipoxin, an important anti-inflammatory mediator, selectively down-modulates the tumor progression stimulated by tumor-associated macrophages, inducing the shift of macrophage profile and leading to tumor cell apoptosis. These unexpected results reveal important differences in M2 macrophage subsets particularly those associated with tumors. This work suggests that this lipid mediator may become a new tool for reducing the development and progression of cancer.

*To whom correspondence should be addressed:

Christina Barja-Fidalgoemail: barja-fidalgo@uerj.br

Laboratório de Farmacologia Celular e Molecular
Instituto de Biologia - UERJ
Av. 28 de Setembro 87 fundos
Vila Isabel – Rio de Janeiro – RJ – Brasil – 20551-030.
Tel.: 55 21 2868 8298; Fax: 55 21 2868 8629;

Abbreviations used in this article:

ALX/FPR2; formyl-peptide receptor type 2;

ATL, aspirin-triggered lipoxin – 15-epi-LXA_4;

ATL-1, stable analog of ATL

BOC-1,N-t-butoxycarbonyl-methionine-leucine-phenylalanine;

DPI, Diphenyleneiodonium;

EC, endothelial cells;

FITC, fluoresceinisothiocyanate;

GPCR, G-protein-coupled receptor;

HMEC-1, human dermal microvascular endothelial cell line;

iNOS, inducible nitric oxide synthase;

LX, lipoxin;

LXA_4, one of the lipoxins derived from arachidonic acid

Mϕ, macrophages;

MRC1, mannose receptor, M2 macrophage marker

MV3, melanoma cell lineage;

NO, nitric oxide;

PE, phycoerythrin;

Per-CP, peridinin chlorophyll

PMA, phorbolmyristate acetate;

ROS, reactive oxygen species;

SPMs, specialized pro-resolving lipid mediators;

TAM, tumor-associated macrophages;

Abstract

In tumor microenvironments, pro-inflammatory macrophages (M1) acquire anti-inflammatory and pro-tumor characteristics. These tumor-associated macrophages (TAMs), often referred to as myeloid suppressors, exhibit an M2-like profile, with low cytotoxic properties and a deficient modulation of NO and ROS production. Lipoxins (LX) and 15-epi-lipoxins are lipid mediators inducing anti-inflammatory and pro-resolution activities in mononuclear cells, but their effects on TAMs remain to be elucidated. This study tested the hypothesis that ATL-1, a synthetic analogue of 15-epi-lipoxin A_4, could modulate TAM activity profile. We show that human macrophages (MΦ) differentiated into TAMs after incubation with conditioned medium from MV3, a human melanoma lineage cell. In contrast with the effects observedin the other M2 subset and M1 profile macrophages, ATL-1 selectively decreased M2 surface markers in these TAM, suggesting unique behaviour of the M2d subset. The effect was dependent on VEGF signaling and importantly, reproduced by the natural lipoxins, LXA and 15-epi-LXA_4. In parallel, ATL-1 stimulated TAM to produce NO by increasing the iNOS/arginase ratio and activated NADPH oxidase, triggering ROS production. These alterations in TAM profile induced by ATL-1 led to loss of the anti-apoptotic effects of TAMs on melanoma cells and increased their cytotoxic properties. Finally, ATL-1 was found to inhibit tumor progression *in vivo*, which was accompanied by alterations in TAM profile and diminished angiogenesis. Together, the results suggest unexpectedly that ATL-1down-modulates tumor progression stimulated by TAM probably by inducing a change in the TAMs from an M2- to an M1-like profile thereby triggering tumor cell apoptosis.

1. Introduction

The relationship between the inflammatory response and cancer has been extensively investigated.In some types of cancer, the microenvironment is similar to that observed in inflammatory processesand tissue repair, with the presence of inflammatory cells and mediators, including chemokines, cytokines, and growth factors, as well as changes in the processes of tissue remodeling and angiogenesis[1]. An early event in tumor progression is the recruitment of monocytes to the tumor site, where they differentiate into macrophages (MΦ)[2,3]. These cells, as important effectors of the inflammatory response and its resolution, can provide an immediate defense against neoplastic elementsbut maypromote tumor development through actionat different stages[4-5]. This paradoxical role of MΦ in cancer could be explained by their functional plasticity, which may result in the polarized expression of either pro- or anti-tumoral functions. During the acute inflammatory response, MΦ with an M1 profile acquire a potent cytotoxic profile, with increasing production of nitric oxide (NO) and reactive oxygen species (ROS), which mediate the elimination of pathogens or neoplastic cells[4]. However, evidence has shown that tumor cells can suppress these immune functions, primarily through down-regulation ofMΦ activity, allowing growth and spread of the cancer throughout the body, resulting in death of the host[6-7]. Tumor cells create a microenvironment that signals the pro-inflammatory and anti-tumoral M1 MΦ to acquire an M2-like profile. These MΦ, designated tumor-associated macrophages (TAMs), possess anti-inflammatory and pro-tumor characteristics, and, in contrast to M1 MΦ, exhibit low cytotoxic properties with defectiveproduction of NO and ROS, which allow tumor progression[8-9]. A number of studies have reported a positive correlation between high TAM density and poor prognosis in several human tumors[10-12].

Thedual role of MΦ in both endogenous tumor defense and tumor progression suggests that a better understanding of the molecular and cellular mechanisms involved in macrophage–tumor interactions is critical to developing new tools to control cancer progression.

The balance between pro- and anti-inflammatory mediators may modulate the outcome of the inflammatory response and consequent tumor development. Lipoxins (LX) are specialized pro-resolving lipid mediators (SPMs), produced by lipoxygenase interactions under a variety of conditions, including infection and inflammation. They can be produced by different biosynthetic routes in particular biological settings[13-14] and can exert selective effects on monocytes and Mϕs, polarizing these cells towards an anti-inflammatory profile, *in vitro* and *in vivo*[15-19] with potential for regulation of tumor growth. Although some studies reveal down-regulation of lipoxins in certain tumors[20-21], the anti-inflammatory actions of lipoxins indicate that they play unique roles as mediators in there solution of inflammation and could be important targets for the therapeutic control of cancer, although at present little is known about their effects on TAM behavior in the tumor microenvironment.

In this study, we investigated the role of lipoxins in the activity of TAMs, using ATL-1(15-epi-16-(para-fluoro)phenoxy-LXA_4), a more stable analog of 15-epi- lipoxinA_4, an aspirin-inducible lipoxin. We show that this LXA_4 analog down-modulates the pro-tumor activity of TAM, inducing the shift from the M2 to the M1 profile, promoting tumor cell apoptosis and controlling tumor growth *in vivo*.

2. Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and penicillin/streptomycin/amphotericin B solution were purchased from Life Technologies-Invitrogen (Carlsbad, CA). SDS and type IV horseradish peroxidase (HRP) was purchased from Sigma (St Louis, MO). Ficoll-Hypaque and Percoll were purchased from Amersham Biosciences (Piscataway, NJ). Antibodies were purchased from ABCAM (Cambridge, MA) and Biolegend (San Diego, CA). All other reagents and chemicals were purchased from Sigma (St Louis, MO). ATL-1, the stable 15-epi-LXA_4 analog, was a generous gift from Dr. John F. Parkinson, Bayer Healthcare Pharmaceuticals.

2.2. Melanoma cell culture

MV3 human melanoma cells were obtained from the Temple University Center for Neurovirology and Cancer Biology (PA, USA). B16F10 murine melanoma cells were obtained from American Type Culture Collection (ATCC). MV3 cells were maintained in DMEM and B16F10 were maintained in RPMI, both enriched with 10 % fetal bovine serum), 3.7 g/L sodium bicarbonate, 5.2 g/L HEPES, 0.5 U/mL penicillin, and 0.5 mg/mL streptomycin at 37°C in a humidified atmosphere of 5 % CO_2. Cells were grown to 80-90 % confluence in 75-cm^2 culture flasks and were then cultured for 3 days to generate conditioned medium.

2.3. Murine melanoma model

C57BL/6 mice at 6–7 wk of age were provided by the Department of Pharmacology and Psychobiology and all experimental procedures were performed according to guidelines

of the Committee on Ethical Use of Laboratory Animals of the State University of Rio de Janeiro, Rio de Janeiro, Brazil (CEUA/077/2012/, UERJ, Rio de Janeiro, Brazil). Mice were caged with free access to food and fresh water in a temperature-controlled room (22–24°C) on a 12-hr light/dark cycle. For the induction of tumors, mice (n = 6-8) were administered B16 melanoma cells (2 x10^5 cells/mouse suspended in 30 µl of saline) by the subcutaneous (s.c.) route. ATL-1 (1 µg/mouse) or ethanol (Vehicle) were injected intravenously 14 days after melanoma cell injection. For experimental analysis, mice were sacrificed on day 14 or 21 after melanoma induction and tumor mass was removed for histological analysis, morphometry, immunohistochemistry, and cellular profile. Lung and liver were analyzed to detect metastasis.

2.4. Macrophage isolation and TAM differentiation

Isolated PBMCs were obtained from EDTA (0.5 %)-treated venous blood of healthy volunteers by Ficoll-Hypaque density gradient, as described previously[18]. Cells were plated for 1 hr to isolate lymphocytes and monocytes were cultured for 7 days for MΦ maturation. To obtain TAMs, the MΦ were cultured for an additional 3 days with RPMI 1640 medium plus conditioned medium from MV3 cultures (MV3-CM; 1:1), as described above.

2.5. Measurement of TAM survival

A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify the effect of lipoxins on TAM survival. This method measures mitochondrial function as described previously[18]. Briefly, MΦ and TAM (2x10^5 cells/well) were incubated with or without ATL-1 (10-100 nM),LXA_4 (10nm) and 15-

epi LXA_4 (10nM) for 48 hr. Survival was assessed and expressed as % of live cells above the negative control.

2.6. FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was conducted with an ACCURI C6 flow cytometer with CFLOW software (Becton Dickinson, Heidelberg, Germany). MΦ and TAMs ($1x10^6$ cells/well) cultured in 24-well plates were treated with ATL-1 (1-100 nM), LXA_4 (10nM), and 15-epi-LXA_4 (10nM) for 48 hr. Following incubation, the cells were stained with conjugated monoclonal antibodies MRC1-phycoerythrin (PE), CD68-fluorescein (FITC) or CD86-PerCP-Cy5.5(Biolegend) with which they were incubated for 60 min at RT in the presence of blocking solution (2%bovine serum albumin,, 5%fetal bovine serum, and 0.1%sodium azide). An isotype control antibody was used as the negative control.

2.7. RNA isolation and RT-PCR

MΦ and TAMs ($3x10^6$ cells/well) were incubated with or without ATL-1 (10 nM) for 6 hr. Total RNA was isolated from TAMs using RNeasy Mini kits (Quiagen®, Hilden, Germany) . After DNase treatment (RQ1 RNase-Free DNase), the mRNA was reverse transcribed using high-capacity cDNA reverse transcription kits as described previously[22]. Primers were designed based on the sequence of human IL-10 (GeneBank accession nº NM_000265 - IL-10: sense (5'-AATAAGGTTTCTCAAGGGGCT-3'); anti-sense (5'- AGAACCAAGACCCAGACATCAA-3'), GAPDH sense (5´-CAGTCAGCCGCATCTTCTTT-3´); anti-sense (5´-AATTCCGTTGACTCCGACCTT-3´)). PCR was performed with the following program: 1 cycle of 95°C for 5 min and 32

cycles of denaturation at 95°C for 45 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 30 sec. Primers were used to validate the cDNA in each reaction). PCR products were separated by 2% agarose gel electrophoresis and visualized by UV exposure on a transilluminator. Relative band densities were determined by densitometric analysis using National Institutes of Health Image Software, and the ratios calculated.

2.8. *NO production*

Nitrite release by TAM was determined by the accumulation of a stable nitrite end-product in cell-free culture supernatants using a modified Griess reaction method, as performed by Silva *et al.*(22). Briefly, TAMs (2×10^6 cell/mL) were incubated with ATL-1 (1-100 nM) for 8 hr, and the samples incubated with the Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphtylethylenodiamine). The nitrite concentration was then determined as previously described[22].

2.9. ROS production assays

MΦ and TAM were suspended in Hank´s balanced salt solution (HBSS) and placed in a white, 96-well plate ($2x10^5$ cells/well, final volume 200 µL). Following pretreatment with or without DPI (10 µM) or BOC-1 (10 µM) for 15 min, cells were loaded with Luminol (50 µM) and hydroxyphenyl-fluorescein (HPF, 10µM), and stimulated with ATL-1 (10 nM) or PMA (10 ng/mL), a known NADPHox inducer, as a positive control. Cells that remained unstimulated were considered the control group. ROS production was assessed as described before[23]. The area under the curve is expressed as the mean ± standard deviation (S.D.).

2.10. MV3 apoptosis

MV3 apoptosis was assessed via cell cycle analysis by flow cytometry. MV3 cells were distributed into 24-well plates at a density of $5x10^5$ cells/well in 10 % FBS medium. MΦ and TAM ($1x10^6$ cells/mL) were cultured in inserts (0.4μm) of transwell platesand the TAMs incubatedfor 15 min with or without DPI (10 μM), aminoguanidine (AG, 50 μM), or PEG-catalase (30 U/mL), followed by treatment with ATL-1 (10 nM) for 15 min. The analog and inhibitors were then removed, and TAM were co-cultured with MV3 cells for 72 hr. MV3cells treated with cycloheximide (5 μM) was used as the positive control. Thereafter, the MV3 cells were detached with 5 mM EDTA in HBSS. DNA content was accessed by propidiumiodide (PI) staining (50 μg/ml PI; 0.1 mg/ml RNase A; 0.05% Triton X-100) and analyzed by flow cytometry with Accuri C6® (Becton Dickinson), as previously described[23].

2.11. Preparation of cell extracts

MΦ and TAM ($1x10^6$/mL) were incubated with different concentrations of ATL-1 (1-100 nM) and then frozen in an ice bath to stop the reaction. To obtain whole cell extracts, cells were resuspended in lysis buffer as previously described[18]. The total protein contents of the cell extracts were determined by the Bradford method[26].

2.12. Immunoblotting

The membranes were blocked with 5 % BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20, and then probed with anti-iNOS (1:500), anti-ALX/FPR2, anti-arginase-1, anti-tubulin or anti-actin (1:1000) antibodies for 2 hr and developed as described previously[18]. Bands were visualized via an enhanced chemiluminescence

system (ECL, Amersham, Arlington Heights, IL). In all instances, band density values were corrected by subtracting the background values.

2.13. Immunohistochemistry

Twenty-one days after administration of B16F10 cells, mice were killed, the tumor mass removed and immersed in the fixative solution for 24 hr. It was then embedded in optimum cutting temperature (O.C.T., Tissue-tek®) and sliced (10 μm) perpendicular to the tumor base (apical-basal axis) and stained with H&E. Sections were hydrated and the slides washed in TBS with 0.05% Tween 20 (T-PBS, Sigma-Aldrich), blocked with block solution (BSA 5% in T-TBS), and immunostained with antibodies to endothelial cells (CD105), MΦ (F480) and TAM (MRC1), used at a 1/100 dilution in PBS/Tween buffer overnight at 4°C. Immunofluorescent imagesweredeveloped with antibodies conjugated with fluorescein or phycoerythrin and counterstained with DAPI for nuclear staining. An irrelevant isotype IgG was used as negative control.

2.14.Statistical analysis

The data are representative of different experiments and expressed as the mean ± S.D. The means from different treatments were compared by Student´s *t*-test and ANOVA, followed by the *Bonferroni t*-test for unpaired values. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characterization of a TAM phenotype induced by a simulated tumor microenvironment *in vitro*

Characteristic TAM markers are the macrophage mannose receptor (MRC1), and CD163, which are usually expressed on M2 cell membranes[27]. For induction of a TAM phenotype by a simulated tumor microenvironment *in vitro*, MΦ derived by maturation of peripheral blood monocytes were incubated with conditioned medium obtained from cultures of melanoma cells. MΦ were converted to a TAM profile expressing higher levels of MRC1 and CD163 than do naïve MΦ (Fig. 1A and B).

One key feature of TAM is the high level of expression of IL-10, an immunosuppressive cytokine produced by tumor cells and TAMs that allows these cells to assume M2-related functions[2]. Indeed, under our experimental conditions, the TAM also presented high levels of IL-10 mRNA (Fig. 1C).

LX and their analogs exert their anti-inflammatory activities via activation of ALX, a GPCR expressed in a variety of cellular types[16]. We demonstrated that TAM express ALX and the treatment of the cells with ATL-1 (10 nM) did not change this expression (Fig. 1D). Furthermore, in the presence of ATL-1, the cells maintained viability even after 48 hr of incubation, as demonstrated by MTT assays (Fig. 1E).

3.2. ATL-1 selectively decreasesM2 marker expression by TAM

To investigate the effects of ATL-1 on the TAM phenotype, we treated the cells with the analog for 72 hr. As demonstrated in Figure 2A, ATL-1 treatment decreased MRC1 expression to levels similar to those observed in MΦ. Furthermore, ATL-1 inhibited IL-10 release by TAMs (Fig. 2B). The natural lipoxin, LXA_4 and its aspirin-induced analogue, 15-epi-LXA_4induced similar effects to ATL-1 (Fig 2C). Instriking

contrast, ATL-1 had no effect on the M2 MRC1 phenotype induced by other M2 stimuli, such as IL-4, TGF-β and VEGF, and even induced an M2 profile in M1 cells (Fig 2D). This suggests that TAM represent a subset of M2 macrophages which are selectively down-modulated by ATL-1 to an M1-like profile *in vitro*. A further response of TAM was the significant reversal of MRC1 expression by an M1 stimulus.

3.3. ATL-1 effect on TAM is vascular endothelial growth factor (VEGF)-dependent.

In the tumor microenvironment, VEGF is the most important angiogenic factor and is involved in several steps of tumor progression[28]. Recently, our group demonstrated that ATL-1impairs VEGF actions both *in vitro* and *in vivo*[29-30]. On this evidence, we investigated the involvement of VEGF in the ATL-1-induced TAM effects. First, we found that conditioned medium from MV3 (CM-MV3) induced an M2 profile independent of VEGF, since treatment with a neutralizing anti-VEGF antibody did not affect MRC1 expression on TAM (Fig. 2E). On the other hand, when M2 polarization triggered by CM-MV3 was performed in the absence of VEGF, the ability of ATL-1 to alter the TAM profile was impaired (Fig.2E). These results suggest that the effects of ATL-1 on the TAM profile are dependent of VEGF actions.

3.4. ATL-1 induces NO production by increasing theiNOS/Arginase-1 ratio in TAM

In the tumor microenvironment, TAMs,in contrast to M1 cells, exhibit deficient production of NO due toinhibition of iNOSactivity byelevated levels of arginase-1[8,31]. Figure 3B shows that TAMinduced in the present study express high levels of arginase-1, in accord with these observations. Treatment of TAM with ATL-1 decreased

arginase-1 and enhanced iNOS expression, thus increasing the iNOS/arginase-1 ratio (Fig.3A,B and C). Corroborating these results, ATL-1 (10 nM) also induced significant NO production by TAM, an effect that was inhibited by BOC-1, a selective inhibitor of ALX/FPR2 (Fig. 3D).

3.4. ATL-1 induces ROS production by TAMs

In addition to their limited ability to produce NO[8], TAM also produce low amounts of ROS (Fig.4A and B). After treatment with ATL-1 (10nM), TAM generated significantly larger amounts of ROS, an effect impaired by both BOC-1 and DPI, an NADPH oxidase inhibitor (Fig. 4A and B). Furthermore, ATL-1 had no effect on ROS production by MΦ. Additionally, we show that ATL-1,as a possible consequence of an increased production of ROS and NO[32], induces peroxynitrite formation, an effect inhibited by BOC-1 (Fig 4C).

3.6. ATL-1 induces MV3 apoptosis in a ROS- and NO-dependent manner

Since the activation of NO and ROS could lead to the recovery of the cytotoxic properties of TAM[2], we analyzed the melanoma cell cycle after 72 h of co-incubation with ATL-1-treated TAM. Compared to MΦ-co-cultured tumor cells, MV3 melanoma cells co-cultured with TAM were protected from apoptosis (Fig.5A). By contrast, the treatment of TAM with ATL-1 (10nM) reversed this anti-apoptotic activity of TAM on MV3 to levels comparable to those seen withMΦ and after an M1 stimulus (Fig. 5A). The incubation of tumor cells with supernatant from the ATL-1-treated TAM culture did not interfere with MV3 apoptosis (Fig. 5A), suggesting that the pro-apoptotic effect depends on the presence of the TAM.

To investigate the contribution of the NADPH oxidase-derived ROS to the pro-apoptotic effects of ATL-1-treated TAM, cells were pretreated with DPI (10 μM) before incubation with the analog (10nM). DPI impaired ATL-1-induced MV3 apoptosis, indicating that the analog induces tumor cell death via activation of the NADPH oxidase system in TAM (Fig. 5B).

A simultaneous increase in the production of superoxide/H_2O_2 and NO leads to potent cytotoxic properties displayed by ATL-1-stimulated TAM.To test this hypothesis, TAM were treated with aminoguanidine (AG,50 μM) and PEG-catalase (30U/mL), inhibitors of iNOS and H_2O_2, respectively, followed by the treatment with ATL-1 (10 nM). Treatment with the inhibitors completely blocked the pro-apoptotic effects of ATL-1, but did not potentiate the inhibitory effect of either one alone, suggesting that NO and H_2O_2 act through a common pathway (Fig. 5C).

3.7. ATL-1 inhibits tumor progression *in vivo*.

In view of these *in vitro* results, we investigated the effects of ATL-1 on tumor progression in a murine model *in vivo*. We observed that treatment with a unique dose of ATL-1 at the 14th day after implantation inhibits tumor growth, as observed in Figures 6A and B. This potent inhibition of ATL-1 was accompanied by the impaired angiogenic process and a decrease in the TAM marker, as shown in Figures 6C and D.

4. Discussion

The relationship between the inflammatory response and cancer has been widely investigated. In spite of the strong evidence for their anti-inflammatory and pro-resolution actions, reports on the effects of lipoxins on tumor progression are limited. In this study, we present novel and promising data on the effects of ATL-1 on the behavior of TAM in the tumor microenvironment. We show that the treatment with ATL-1 selectively alters TAM to an M1-like profile, increasing their tumoricidal activity and leading to control of tumor progression *in vivo*. These effects emphasize the important role of lipoxins in modulation of the macrophage profile in the tumor microenvironment with consequent induction of pro-resolutive properties. As described before it has been demonstrated by a number of groups, including ours, that lipoxins and other lipid mediators can induce anti-inflammatory properties in mononuclear cells[17-19]. Indeed, Li et al, screening the effects of lipoxins on naïve and inflammatory monocytes and macrophages derived from the U937 cell line, demonstrated that LXA_4 induces the M2 anti- inflammatory phenotypes[33]. Vasconcelos *et al* showed that lipoxins allowed *in vivo* the control of macrophage phenotypic profile, displaying a characteristic M2 phenotype[34].

Depending on the inducing stimulus, M2 macrophages can be classified into four subsets[35-36]. M2a and M2b, activated by IL4/IL13 or immune complexes/LPS, respectively, exert immune regulatory functions, whereas M2c macrophages activated by IL-10 and TGF-β have a predominant role in suppressing immune responses and in promoting tissue remodeling. TAM have been reported as a novel M2 subset, called M2d, that are apt to promote tumor growth, tissue remodeling, angiogenesis, and suppression of adaptive immunity[1,3,4,36]. Although there is a paucity of evidence for the lipoxin effects on macrophages present in the conditions of the tumor

microenvironment, we have demonstrated for the first time that the stable derivative of aspirin-induced LXA_4, ATL-1, alters the TAM profile to an M1-like phenotype. In contrast, we observed that ATL-1 induces an M2 profile in Mϕs activated by LPS and IFNɣ that exhibit an M1 profile, and maintains M2 polarization in Mϕ treated with IL4, a classical M2 stimulus, indicating a specificity of the LX effect on TAM polarization.

We demonstrated that the effect of lipoxins on TAM polarization appears to be dependent on VEGF released in the tumor microenvironment. Our data strongly suggest that prior signaling triggered by VEGF during the differentiation of Mϕs in TAMs would be an essential step for the specific effect of lipoxins on TAM. We propose that VEGF, in synergy with other factors released into the tumor microenvironment, such as TGF-β, VEGF, IL4 and IL10, may trigger distinct intracellular signaling, which induces the unique M2-like profile, which differentiates TAM from other M2-phenotypes. Corroborating this hypothesis, Linde *et al* showed that, beyond its chemotactic effect, VEGF induces the production of IL- 4 by a macrophage subset and of IL10 by tumor cells, so potentiating M2 polarization[30].

The recruitment, maturation, and differentiation of infiltrating leukocytes in tumors are dependent upon the local conditions in the tumor microenvironment, which play a central role in the activation of specific pathways expressed by TAM[37]. In this study, we observed that ATL-1, besides decreasing the expression of the mannose receptor MRC1, a characteristic surface antigen marker of TAM[27], also drastically inhibited IL-10 gene expression. IL-10, a known anti-inflammatory and immunosuppressive cytokine, reported to be present in the microenvironment of different tumors, inhibits the expression of pro-inflammatory mediators, including

IL-12, inducible cyclooxygenase, and nitric oxide synthase, by phagocytic cells[7,38-40]. This inhibitory effects of the ATL analog contrast clearly with other reports showing that lipoxins can induce IL-10 synthesis by macrophages in a non-tumor environment[41]. Moreover, this inhibition of IL-10 expression by ATL-1 suggests that the alteration in macrophage profile could facilitate the restoration of their potent cytotoxic activities, thereby increasing NO and ROS production.

Indeed, we demonstrated that ATL-1, in changing the TAM phenotype, increases the production of NO and induces ROS generation by activating the NADPHox system. These pro-oxidative effects triggered in TAM contrast, again, with those obtained in other Mϕ phenotypes, where inhibitory effects of lipoxins and other ALX/FPR2 agonists on reactive species production were demonstrated[42-44]. Corroborating the hypothesis of a selective effect of ATL-1 on TAM, we demonstrated that it had no effect on ROS production by peripheral blood-derived Mϕs. The NO production is consequence of a balance between iNOS and arginase-1 activity[9]. The arginase pathway limits arginine availability for iNOS action and NO synthesis. Interestingly, NO production by TAM triggered by ATL-1 seems to be more closely related to a decline in arginase-1 expression than to an augmentation in iNOS expression, resulting in an increase in the iNOS/arginase-1 ratio.

Several studies have demonstrated that malignant cells are able to grow indefinitely because of unregulated proliferative capacity and acquired resistance to apoptosis, allowing these cells to become invasive and metastatic[45-46]. Alterations in the TAM profile and the recovery of the cytotoxic properties of Mϕs have been proposed as part of an efficient strategy to control tumor progression[9]. Our data indicate that ATL-1, by triggering ROS and NO production, enables TAM to induce

apoptosis of MV3 melanoma cells. Inhibition of either NADPH oxidase or iNOS systems alone impaired the pro-apoptotic effect of ATL-1-stimulated TAMs on the tumor cells, suggesting that both reactive species, ROS and NO, share a common pathway. An increased production of superoxide/H_2O_2 and NO leads to the production of the highly cytotoxic mediator peroxynitrite[32] consistent with our observation that ATL-1 induces peroxynitrite production by TAM. Conceivably this metabolite could be the main factor responsible for the potent cytotoxic properties displayed by ATL-1- stimulated TAM. The data would appear to indicate the involvement of different M2 subsets under the same umbrella of the M2 markers used to define the M2 phenotype in this study.

Tumor cells need to avoid apoptosis, which is also induced by limited supplies of oxygen, nutrients, and growth factors[47]. ATL-1, in addition to its ability to alter TAMs to a tumor pro-apoptotic phenotype, also inhibited human endothelial cell tubulogenesis stimulated by TAM *in vitro* (Supplemental Figure S1). Furthermore, the treatment with ATL-1 decreased, in parallel with M2 cell population, angiogenic markers in the tumor microenvironment, *in vivo*. The data corroborate early studies that suggested ATL-1 as a potent inhibitor of angiogenesis by down-regulating VEGFR activation, and inhibiting migration and proliferation of endothelial cells[31-32]. Taken together, our data support the idea that the shift in macrophage profile can also contribute to the anti-angiogenic effects of ATL-1 *in vivo* .

The production of ATL by aspirin *in vivo* results from the inhibition of COX-2 through its acetylation[14]. Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, were reported to impair tumor progression through the suppression of proliferation, the induction of apoptosis, and the inhibition of the invasiveness of neoplastic cells[48-49]. Interestingly, Claria *et al* showed that ATL was produced by

aspirin-activated neutrophils co-cultured with tumor cells[50], and proposed that lipoxins may have a function in the control of cancer by affecting leukocyte activity. Corroborating this suggestion, we demonstrate for the first time that lipoxins, by inducing a shift from the M2-like (TAM) to the M1-like profile, promote inhibitory effects on tumor cell proliferation and survival, leading to tumor cell apoptosis and control of tumor progression *in vivo*. This work highlights an important role for lipoxins in tumor progression and suggests that these lipid mediators may become new targets for reducing the development and progression of cancer.

Acknowledgements

We acknowledge Genilson Rodrigues da Silva, Gabriele E. M. dos Santos and Renata R.P.Tureta for technical support.

This work received financial support from the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ - E-26/103.174/2011), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 302238/2009-3), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - 23038.38.006279/2011).

References

1- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454:436-444.

2-Lewis CE, PollardJW. Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006; 66:605-612.

3- Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 2007; 117:1155-1166.

4- Mantovani A, BottazziB, Colotta F, Sozzani S, Ruco L. The origin and function of tumor associated macrophages. Immunol Today 1992; 13:265-270.

5- Condeelis J, Pollard JW. Macrophages, obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006; 124:263–266.

6- Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature Immunology 2010; 11:889–896.

7- Mantovani A, Sica A, SozzaniS,Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol2004; 25:677–686.

8- Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, BiswasSK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. Seminars in Cancer Biology 2008; 18:349-355.

9- Dinapoli MR, Calderon CL, Lopez DM. The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduced expression of the inducible nitric oxide synthase gene. J Exp Med 1996; 183:1323–1329.

10- Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. Int J Oncol 2000; 17:445–451.

11- Nishie A, Ono M, Shono T, Fukushi J, Otsubo M, Onoue H, Ito Y, Inamura I, Ikezaki K, Fukui M, Iwaki T, Kuwano M. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. Clin Cancer Res 1999; 5:1107–1113.

12- Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC, FisherRI, Braziel RM, Rimsza LM, Grogan TM, Miller TP, Le Blanc M, Greiner TC, Weisenburger DD, Lynch JC, Vose J, Armitage JO, SmelandEB, Kvaloy S, Holte H, Delabie J, Connors JM, Lansdorp PM, Ouyang Q,ListerTA, Davies AJ, Norton AJ, Muller-Hermelink HK, OttG, Campo E, Montserrat E, Wilson WH, Jaffe ES, Simon R, YangL, Powell J, ZhaoH, GoldschmidtN, Chiorazzi M, Staudt LM. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl JMed 2004; 351:2159–2169.

13- Serhan CN, Hamberg M, Samuelsson B. Lipoxins, a novel series of biologically active compounds formed from arachidonic acid in human leukocytes. PNAS USA.1984; 81:5335-5339.

14- Fierro IM, Serhan CN. Mechanisms in anti-inflammation and resolution, the role of lipoxins and aspirin-triggered lipoxins.Braz J Med Biol Res 2001; 34:555-566.

15- Claria J, Serhan CN. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. PNAS USA 1995; 92:9475-9479.

16- Fiore S, Maddox JF, Perez HD, Serhan CN. Identification of a human cDNA encoding a functional high affinity lipoxin A_4 receptor. J Exp Med 1994; 180:253-260.

17- Simões RL, Fierro IM. Involvement of the Rho-Kinase/Myosin Light Chain Kinase Pathway on human monocyte chemotaxis induced by ATL-1, an aspirin-triggered lipoxin A_4 synthetic analog.JImmunol 2005; 175:1843-1850.

18- Simões RL, Niconi-de-Almeida Y, da-Fé AR, Barja-Fidalgo TC, Fierro IM. A synthetic analog of 15-epi-lipoxin A_4 inhibits human monocyte apoptosis, involvement of ERK-2 and PI3-kinase. Prostaglandins Other Lipid Mediat 2010; 91:10-17.

19- Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. Cutting edge, lipoxins rapidly stimulate non-phlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. J Immunol 2000; 15:1663-1667.

20- Stenke L, Nasman-GlaserB, Edenius C, Samuelsson J, Palmblad J, Lindgren JA. Lipoxygenase products in myeloproliferative disorders,increased leukotriene C4 and decreased lipoxin formation in chronic myeloid leukemia. Adv. Prostaglandin Thromboxane Leukot Res 1991; 21:883–886.

21- Stenke L, Edenius C, Samuelsson J, Lindgren JA. Deficient lipoxin synthesis, a novel platelet dysfunction in myeloproliferative disorders with special reference to blastic crisis of chronic myelogenous leukemia. Blood1991; 78:2989-2995.

22- Citelli MM, Bittencourt LL, da Silva SV, Pierucci AP, Pedrosa C. Vitamin A modulates the expression of genes involved in iron bioavailability. Bio. Trace Elem Res 2012; 149:64-70.

23- Silva SV, Garcia-Souza EP, Moura AS, Barja-Fidalgo C. Maternal protein restriction during early lactation induces changes on neutrophil activation and TNF-alpha production of adult offspring. Inflammation 2010; 33:65-75.

24- Barcellos-de-Souza P, Canetti C, Barja-Fidalgo C, Arruda MA. Leukotriene B_4 inhibits neutrophil apoptosis via NADPH oxidase activity:Redox control of NF-κB pathway and mitochondrial stability. Biochim Biophys Acta 2012; 1823:1990-1997.

25- Mhaidat NM, Wang YF,Kiejda KA, Zhang XD, Hersey P. Docetaxel-induced apoptosis in melanoma cells is dependent on activation of caspase-2. Molecular Cancer Therapeutics 2007; 6:752–761.

26- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.

27- Laoui D, MovahediK, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, Nikolaou A, Morias Y, De Baetselier P, Van GinderachterJA. Tumor-associated macrophages in breast cancer, distinct subsets, distinct functions. Int J Dev Biol 2011; 55:861-867.

28- Linde N, Lederle W, Depner S, van Rooijen N, Gutschalk CM, Mueller MM. Vascular endothelial growth factor-induced skin carcinogenesis depends on recruitment and alternative activation of macrophages. J Pathol 2012;227:17-28.

29- Fierro IM, Kutok JL, SerhanCN. Novel lipid mediator regulators of endothelial cell proliferation and migration, aspirin-triggered-15R-lipoxin A_4 and lipoxin A_4.J PharmacolExpTher 2002; 300:385-392.

30- Cezar-de-Mello PF, Vieira AM, Nascimento-Silva V, Villela CG, Barja-Fidalgo C, Fierro IM. ATL-1, an analogue of aspirin-triggered lipoxin A_4, is a potent inhibitor of several steps in angiogenesis induced by vascular endothelial growth factor. Br J Pharmacol 2008;153:956-965.

31- Odegaard JI, Chawla A. Mechanisms of macrophage activation in obesity-induced insulin resistance. Nature Clin Practice Endocrin and Metab 2008; 11:619-626.

32- Laskin JD, Heck DE, LaskinDL. Nitric oxide pathways in toxic responses.In: Ballantyne R, Marrs T, Syversen T., *General and Applied Toxicology.* New Jersey: B. Hoboken2009: pp. 425–438.

33- Y Li, Cai L, Wang H, Wu P, Gu W, Chen Y, Hao H, Tang K, Yi P, Liu M, Miao S, Ye1 D. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. Oncogene 2011;30:3887-3899.

34- Vasconcelos DP, Costa M, Amaral IF, Barbosa MA, Aguas AP, Barbosa JN. Modulation of the inflammatory response to chitosan through M2 macrophage polarization using pro-resolution mediators. Biomaterials 2014; 37:116-123.

35- Mantovani A, SozzaniS, Locati M, Allavena P, Sica A. Macrophage polarization, tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23:549–555.

36-Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel) 2014;13:1670-90.

37-Coussens LM, Werb Z. Inflammation and cancer. Nature 2002; 420:860-867.

38-Mertz PM, DeWitt DL, Stetler-Stevenson WG, Wahl LM. Interleukin 10 suppression of monocyte prostaglandin H synthase- 2: Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production. J BiolChem 1994; 269:21322–21329.

39- Cunha FQ, Moncada S, Liew FY. Interleukin-10 inhibits the induction of nitric oxide synthase by interferongamma in murine macrophages. Biochem Biophys Res Commun 2003; 182:1155–1159.

40- D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin-10 inhibits human lymphocyte interferon gammaproduction by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J ExpMed 2003; 178:1041-1048.

41- Souza DG, Fagundes CT, Amaral FA, Cisalpino D, Sousa LP, Vieira AT, Pinho V, Nicoli JR, Vieira LQ, Fierro IM, Teixeira MM. The required role of endogenously produced lipoxin A_4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. J Immunol 2007; 179:8533-8543.

42- Prieto P, Cuenca J, Través PG, Fernández-Velasco M, Martín-Sanz P, Boscá L. Lipoxin A_4 impairment of apoptotic signaling in macrophages: implication of the PI3K/Akt and the ERK/Nrf-2 defense pathways. Cell Death Differ 2010,;17:1179-1188.

43- Lee HN, SurhYJ. Resolvin D1-mediated NOX2 inactivation rescues macrophages undertaking efferocytosis from oxidative stress-induced apoptosis. Biochem Pharmacol 2013; 86:759-769.

44- Jozsef L, Zouki C, Petasis NA, Serhan CN, Filep JG. Lipoxin A_4 and aspirin-triggered 15-epi-lipoxin A_4 inhibit peroxynitrite formation, NF-kB and AP-1 activation, and IL-8 gene expression in human leukocytes. PNAS USA 2002; 99:13266-13271.

45- Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. Nature Reviews Cancer 2004; 4:592-603.

46- Vousden KH, Lu X. Live or let die, the cell's response to p53. Nature Review Cancer 2002;2:594–604.

47- Weis SM, Cheresh DA. Tumour angiogenesis: molecular pathways and therapeutic targets. Nature Med 2011; 17: 1359–1370.

48- Abiru S, Nakao K, Ichikawa T, Migita K, Shigeno M, Sakamoto M, Ishikawa H, Hamasaki K, Nakata K, Eguchi K. Aspirin and NS-398 inhibit hepatocyte growth factor-induced invasiveness of human hepatoma cells. Hepatology 2002; 35:1117–1124.

49- Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. PNAS USA 1997; 94:3336-3340.

50- Clària J, Lee MH, SerhanCN. Aspirin-triggered lipoxins (15-epi-LX) are generated by the human lung adenocarcinoma cell line (A549)-neutrophil interactions and are potent inhibitors of cell proliferation. Mol Med 1996;2:583-596.

Figures Legends

Figure 1 - Characterization of a TAM phenotype induced by simulated tumor microenvironment *in vitro*. A-B) MΦwere cultured with or without conditioned medium from melanoma cells for 72hr to TAM differentiation. Expression of the M2 markers, MRC1**(A)** and CD163 **(B)** was assessed by flow cytometry using the anti-MRC1-PE and CD163-APC antibodies. CD68-FITC and CD80-PerCP-Cy5 were used as the MΦ marker. The figure is representative of three experiments performed separately. **C)**MΦ and TAM ($3x10^6$ cells) were treated with ATL-1 (10 nM) for 6 hours. Cells were lysed in TRIZOL® and IL-10 gene expression was investigated by RT-PCR analysis. GAPDH was used as load control. Figure is representative of three experiments performed separately. **D)** MΦ and TAM ($1x10^6$ cells) were treated with ATL-1 (10 nM) for 48 hr. ALX/FPR2 expression was investigated by Western blot. The figure is representative of three experiments performed separately. **E)** MΦ and TAM ($2x10^5$ cells) were treated with ATL-1 (10-100 nM) for 48 hr. Cell viability was assessed by the MTT assay. Results are expressed as the mean ± S.D of three independent experiments.

Figure 2 - ATL-1 selectively decreases M2 markers expression by TAMs.A)MΦ and TAMs ($1x10^6$ cells) were incubated with or without ATL-1 (1-100nM) for 72 hours. MRC1 expression was assessed by flow cytometry using the antibody anti-MRC1-PE.The results are expressed as the mean ± S.D of three independent experiments.*, $P < 0.05$ when compared to MΦ; **, $P < 0.05$ when compared to TAMs. **B)** MΦ and TAM ($1x10^6$ cells) were treated with ATL-1 (10 nM) for 24 hours. IL-10 release was assessed by ELISA using an anti-IL10 antibody. The results are expressed as the mean ± S.D of

three independent experiments.*, P < 0.05 when compared to TAM alone. **C)** MΦ and TAM (1x10^6 cells) were incubated with or without ATL-1(10nM), 15-epi-LXA$_4$(10nM), LXA$_4$ (10nM) and in the latter case, with M1 stimulus (LPS1μg/mL + IFNγ 40ng/mL) for 72 hr. MRC1 expression was assessed by flow cytometry using the antibody anti-MRC1-PE.The results are expressed as the mean ± S.D of three independent experiments.*, P < 0.05 when compared to control MΦ; **, P < 0.05 when compared to TAM. **D)** MΦ (1x10^6 cells) were incubated with medium alone, MV3 conditioned medium (TAM), M1 stimulus (LPS1μg /mL + IFNγ 40ng/mL), M2 stimuli: IL-4 (100ng/mL), TGF-β (10ng/mL) or VEGF (10ng/mL) for 3 days. After washing cells were then incubated in the presence or absence of ATL-1 (10 nM) for 72 hr. MRC1 expression was assessed by flow cytometry using the anti-MRC1-PE antibody. The results are expressed as the mean ± S.E. of three independent experiments.*, P < 0.05 when compared to control MΦ; **, P < 0.05 when compared to TAM. #, P < 0.05 when compared to M1. **E)** MΦ were incubated with MV3 conditioned-medium (CM-MV3) in the presence or absence of monoclonal anti-VEGF (0.1μg/mL) for 72 hours. The results of treating MΦ and TAMs (1x10^6 cells) with or without ATL-1 (10 nM) for 48 hr are shown for comparison. MRC1 expression was assessed by flow cytometry using the anti-MRC1-PE antibody. The results are expressed as the mean ± S.D of three independent experiments.*, P < 0.05 when compared to untreated MΦ; **,P< 0.05 when compared to untreated TAM.

Figure 3. ATL-1 induces NO production by increasing the iNOS/Arginase-1 ratio in TAMs. A-C) TAMs (1x10^6 cells) were incubated with ATL-1 (10-100 nM) for 2 hr. The cells were lysed in a specific buffer and analysed by SDS-PAGE. **A)** iNOS/actin ratio. **B)** Arginase-1/actin ratio. **C)** iNOS/Arginase-1 ratio. Protein expression was

evaluated by immunoblotting with specific antibodies. The results are expressed as the mean ± S.D. The data shown are representative of three independent experiments. *, $P<$ 0.05 compared with MΦ.**, $P<$ 0.05 compared with TAMs alone. **D)** TAM ($2x10^5$/mL) were cultured in 96-well plates, pretreated with BOC-1 (10 μM), and treated with ATL-1 (1-100 nM) for 8 hr. NO production was assessed by the Griess Reaction and analyzed on an Envision® plate reader, as described in the Methods. *, $P<$ 0.05 when compared with TAMs alone. **, $P<$ 0.05 when compared with TAMs treated with ATL-1.

Figure 4.ATL-1 induces ROS production by TAMs. A) MΦ and TAMs (2×10^5/mL) were pre-treated with BOC-1 (10 μM) or DPI (10 μM) and then incubated with or without ATL-1 (10 nM) for 1 hr in the presence of luminol as a chemiluminogenic probe (50 μM). BOC-1 alone had no effect. PMA was used as a positive control. The figure is representative of 4 experiments. **B)** ROS production. Mean ± S.D. of the area under the curve. **C)** MΦ and TAMs (2×10^5/mL) were pre-treated with BOC-1 (10 μM) and then incubated with or without ATL-1 (10 nM) for 30 min in the presence of HPF as a chemiluminogenic probe (10μM). The results are expressed as the mean ± S.D of four independent experiments. *, $P < 0.05$ compared with MΦ. **, $P < 0.05$ compared to cells treated with ATL-1.

Figure 5.ATL-1 induces MV3 apoptosis in a ROS and NO dependent manner. **A)** MΦ and TAM ($1x10^6$ cells) were cultured in *transwells*and the TAM treated with or without ATL-1 (10 nM)or an M1 stimulus (LPS1μg /mL + IFNγ 40ng/mL) for 15 min. The analog was then removed, and the cells were co-cultured with MV3 for 72 hours. MV3 apoptosis was assessed by cell cycle analysis via flow cytometry. Cycloheximide

(CHX)-treated MV3 cells were used as the positive control. SN TAM+ATL-1; supernatant from the ATL-1-treated TAM. Dashed line, MV3 cells alone. **B-C**) MΦ and TAM ($1x10^6$ cells) cultured in *transwells* were pretreated for 15 min as shown in the Figure with or without **B)**DPI (10 μM); or **C)** aminoguanidine (AG, 50 μM) and polyethyleneglycol-catalase (PEG-Cat) (30 U/mL), followed by treatment with ATL-1 (10 nM) for 15 min. The analog and inhibitors were then removed, and the cells co-cultured with MV3 cells 72 hr. MV3 apoptosis was assessed by cell cycle analysis via flow cytometry.The results are expressed as the mean ± S.D of three independent experiments.*, $P < 0.05$ when compared to MΦ; **, $P < 0.05$ when compared to TAMs; #, $P < 0.05$ when compared to TAM plus ATL-1.

Figure 6. ATL-1 inhibits tumor progression *in vivo*. Groups of 6-8 mice were inoculated with B16F10 tumor cells. The tumor mass was removed from the animals on the 14^{th} and 21^{st} day after B16F10 injection. ATL-1 (1 μg/mouse) or ethanol (vehicle alone) was injected on day 14, as described in Methods section 2.3. **A)** Tumor volume and **B)** weight were analyzed. The excised tumors were immunostained with antibodies for: **C)** blood vessels (CD105-PE) and **D)** whole macrophage (F4/80-FITC) or M2 macrophages (MRC1-PE). DAPI was used for nuclear staining shown in the upper right-hand square. An isotype IgG was used as negative control. Pictures are representative of each group. *, $P< 0.05$.**, $P< 0.01$.

Figure 1 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

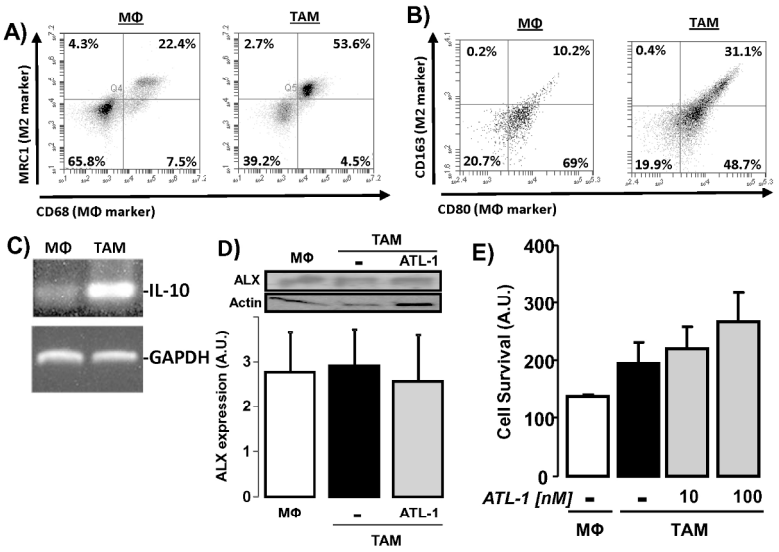

Figure 1
190x254mm (300 x 300 DPI)

Figure 2 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

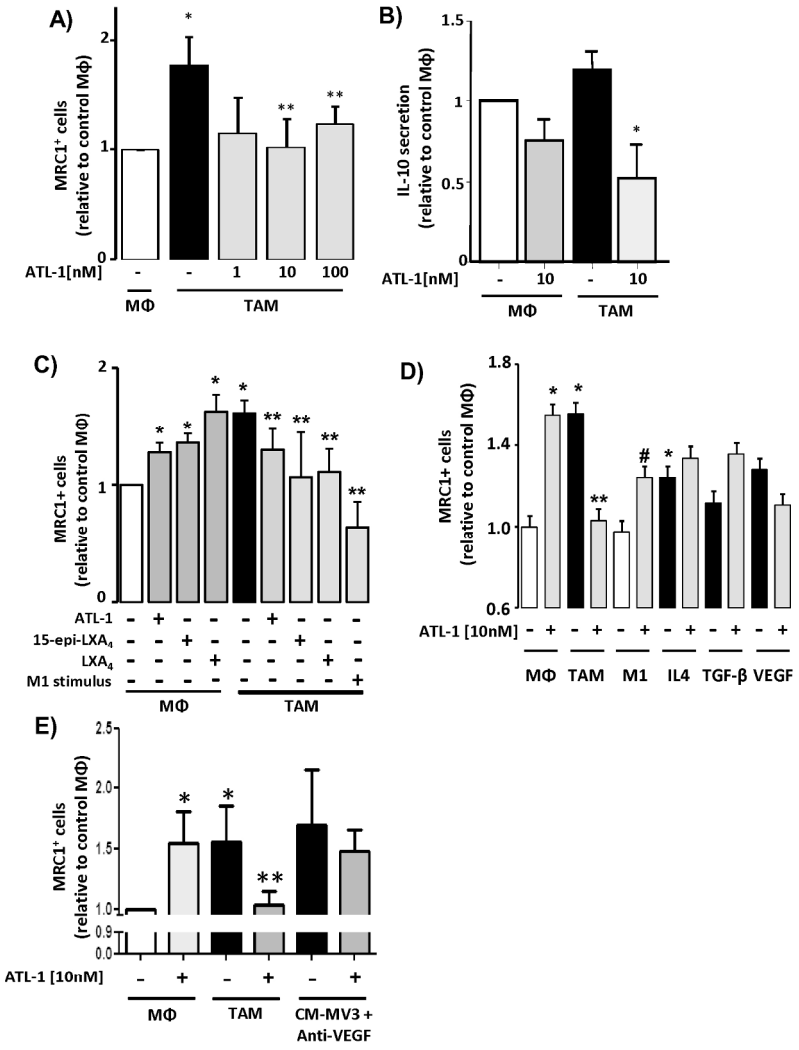

Figure 2

190x254mm (300 x 300 DPI)

Figure 3 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

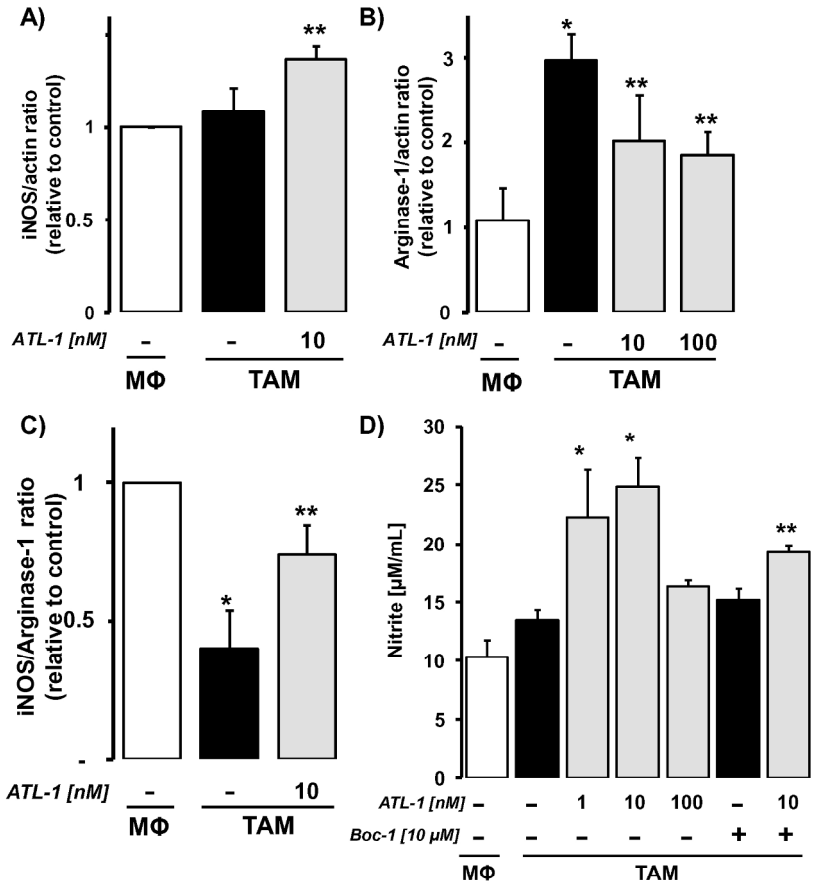

Figure 3
190x254mm (300 x 300 DPI)

Figure 4 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

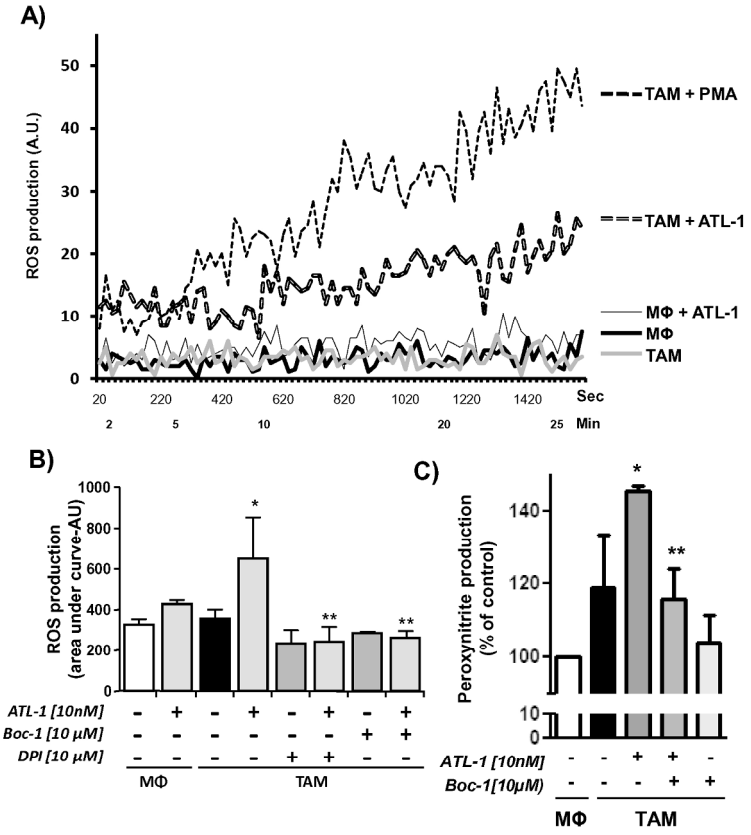

Figure 4
190x254mm (300 x 300 DPI)

Figure 5 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

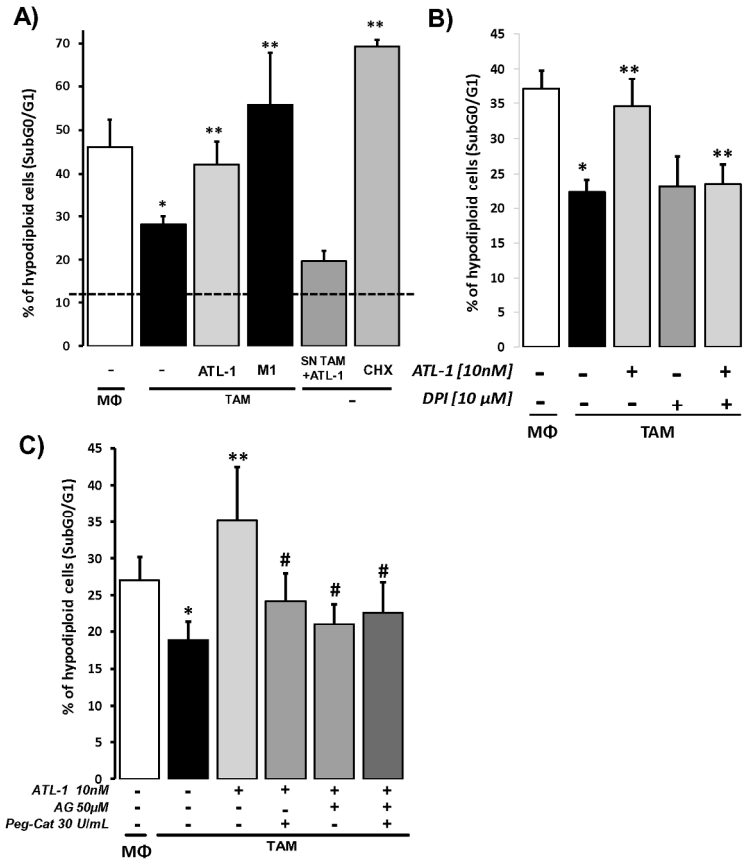

Figure 5
190x254mm (300 x 300 DPI)

Figure 6 – Simões R.L. et al - **LIPOXIN A_4 ANALOG SELECTIVELY ALTERS THE TUMOR-ASSOCIATED MACROPHAGE PROFILE LEADING TO CONTROL OF TUMOR PROGRESSION**

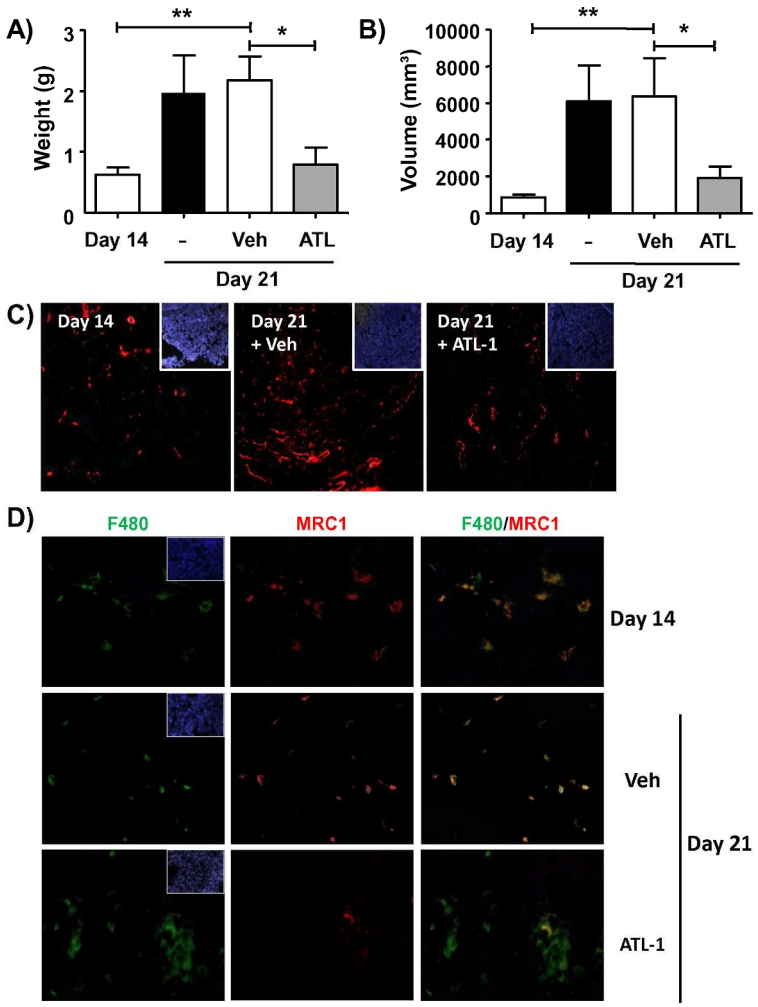

Figure 6
190x254mm (300 x 300 DPI)