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AUTOPHAGY AND INFLAMMSOME: HOW ARE THEY RELATED?

UNIVERSIDADE DE COIMBRA

Mestrado em Investigação Biomédica

2013

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Dissertação de Mestrado em Investigação Biomédica, na especialidade de Infecção e Imunidade apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

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Coimbra, 2013

UNIVERSIDADE DE COIMBRA

O trabalho apresentado nesta dissertação foi realizado no Centro de Oftalmologia e Ciências da Visão, Instituto de Imagem Biomédica e Ciências da Vida (IBILI) da Faculdade de Medicina da Universidade de Coimbra, sob a orientação da Doutora Teresa e do Doutor Henrique Girão, no âmbito do Mestrado de Investigação Biomédica (MIB) da Faculdade de Medicina da Universidade de Coimbra.

Dedico esta tese à minha Aidinha.

Da tua primeira, que te adora!

Agradecimentos:

Este trabalho é meu, mas eu não sou mais que o resultado das pessoas que me rodeiam, portanto agradeço-lhes pelo papel que tiveram durante esta fase do meu percurso.

Primeiro aos orientadores, claro, por me darem esta oportunidade e acreditarem que eu era capaz. Teresa e Henrique, além do vosso encorajamento e estímulo científico, agradeço essencialmente a vossa imensa compreensão e paciência para com: a demora nos resultados, as muitas teimosias, a terrível desorganização e a necessidade de "espaço" para manter o entusiasmo...por me aceitarem tal como sou e verem potencial onde eu não sabia que tinha. Cresci imenso com este processo ao vosso lado. Ajudaram-me a reconhecer as minhas falhas e virtudes. Levo-vos no pensamento para o resto do meu percurso na ciência.

Aos colegas de trabalho com quem contactei diariamente durante esta experiência. Foram essenciais para que eu me sentisse confortável neste laboratório, nesta família científica que temas sido ao longo de muitos meses. Deste alguns merecem um agradecimento extra. Ahah! O João, meu companheiro de bancada, que me distraías e animavas com as nossas conversas sobre coisas "esquisitas" sobre outras ciências e sobre o mundo, enquanto eu fazia a milionésima variação da mesma experiência a ver se era "desta, que ia ter resultados". A Teresinha, minha office-mate, minha inspiração de responsabilidade e sensatez, por partilhares as lutas e as frustrações deste percurso a meu lado, pela parceria nas choradeiras, pela nossa amizade. A minha Ana Margarida, soul-mate do meu coração, por compreenderes melhor que ninguém a loucura da minha essência e ainda assim a encorajares, não tenho palavras para descrever o teu contributo…aos nossos cigarrinhos.

Depois a quem está longe…

Aos amigos por me manterem a sanidade mental. Às meninas que me aliviavam as amarguras da vida laboratorial com risos e palhaçadas quando me levavam a dançar. E

ao Portinho, pelas conversas filosóficas que aligeiravam o peso da ciência na minha mente e me abriram os horizontes a outros mundos. Estar convosco era o meu prazer de fim-de-semana, a minha fonte especial de felicidade e tranquilidade, aquilo que me recarregava as baterias para voltar com energia ao trabalho difícil que me esperava todas as segundas-feiras.

E finalmente, à mãe e ao Xico, porque deixar-vos orgulhosos foi essencial para a minha motivação naqueles dias em que nada resultava. Agradeço-te mãe, por sempre teres sido uma lutadora e me inspirares a tornar-me numa mulher tão independente e corajosa nas minhas batalhas como tu, e por acreditares que eu tenho o potencial para alcançar tudo o que sonho. Xico, obrigada por seres uma pessoa tão maravilhosa e me surpreenderes tanto com a tua maturidade, ser uma fonte de inspiração como irmã mais velha assim, é não só um privilégio como um desafio. Se tu és capaz de ser adulto sem perderes a tua doçura, eu só tinha de o ser também.

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Abbreviations:

Acb1 – acyl coenzyme A binding protein 1 (yeast)

ATP – adenosine 5'-triphosphate

CD 63 – cluster of differentiation 63

CD 8^{+} – cluster of differentiation 8 positive

CUPS – compartment for unconventional protein secretion

DAMP – damage-associated molecular pattern

DC – dendritc cell

ELISA – enzyme-linked immunosorbent assay

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

HMGB1 – high mobility group box 1

IL-1 – interleukin 1

IL-1b – interleukin 1 beta

IL-18 – interleukin 18

LC3 B – microtubule-associated protein 1 light chain 3 beta

LPS – lipopolysaccharide

MHC – major histocompatibility complex

mRNA – messenger ribonucleic acid

microRNA – micro-ribonucleic acid

MVB – multivesicular body

NLR – nod-like receptor

NLRP3 – nod-like receptor family, pyrin domain containing 3

PAMP – pathogen-associated molecular pattern

RNA – ribonucleic acid

RT-PCR – real-time polymerase chain reaction

TLR – toll-like receptor

WGA-PE – phycoerythrin- conjugated wheat germ agglutinin

Resumo

Um número crescente de doenças tem vindo a ser associado à inflamação prolongada, muitas vezes ligada a um aumento dos níveis de IL-1b extracelular. Assim, a redução desta via pró-inflamatória tem sido sugerida como um importante alvo para terapias de protecção contra estas patologias. Contudo, encontrar um mecanismo eficaz para a inibição da secreção de IL-1b continua a ser um desafio neste campo de investigação. Tem sido amplamente demonstrado que esta citocina é exportada a partir de células, após a activação do inflamossoma, através de várias vias não-convencionais de secreção, incluindo por exossomas. Estas vesículas, que representam uma sub-classe específica de vesículas membranares provenientes da fusão de corpos multivesiculares com a membrana plasmática, foram isoladas de diversos fluidos corporais e a sua acumulação foi descrita em associação a uma variedade de doenças humanas. Os exossomas são veículos importantes para a comunicação intercelular, uma vez que permitem a entrega a longa distância de cargas efetoras. Portanto, no contexto da inflamação conduzida pela IL-1b, é razoável especular que estas vesículas sejam cruciais para a estimulação de células distantes e para a amplificação de respostas imunitárias. Para além da sua fusão com a membrana plasmática que resulta na libertação exossomas, os corpos multivesiculares também pode interagir com autofagossomas gerando um organelo híbrido denominado anfissoma, o qual pode subsequentemente fundir com o lisossoma (formando um autolisossoma) para degradar o material incorporado. Assim, é concebível sugerir que a estimulação da fusão com lisossomas constitua uma estratégia para a inibição da secreção de exossomas. Um dos mecanismos que é conhecido por promover essa fusão é autofagia.

Tomados em conjunto, estes dados levaram-nos a investigar o papel da indução de autofagia como um mecanismo para modulador da libertação de exosomes contendo IL-1b pela linha celular monocítica THP-1, depois da activação do inflamossoma. Começámos por demonstrar que a activação do inflamossoma, depois da incubação com LPS ou LPS e ATP, em células THP-1 induz a secreção de exosomes contendo não

só a citocina IL-1b, mas também o seu ARNm. Além disso, a incubação de células não estimuladas com essas vesículas levou a um aumento da resposta pró-inflamatória sem qualquer outro estímulo imunológico. Finalmente, verificou-se que a estimulação da actividade autofágica pela rapamicina em células THP-1, previamente activadas com LPS e ATP, foi capaz de inibir tanto a libertação de exossomas como os níveis totais de IL-1b (proteína e ARNm) produzidos e secretados para exterior.

De acordo com o nosso conhecimento, os resultados obtidos neste estudo mostram, pela primeira vez, que a indução da autofagia pode ter um efeito anti-inflamatório através da inibição da libertação e posterior propagação de exossomas contendo IL-1b. No seu conjunto, os resultados obtidos no presente estudo, não só constituem uma contribuição importante para o estudo dos mecanismos envolvidos nas respostas inflamatórias, mas também para o campo da biologia celular em geral, amplificando o conhecimento sobre os mecanismos envolvidos na secreção através exosomes.

Abstract:

A growing number of diseases have been established to be linked to prolonged or exacerbated inflammation, often associated with increased levels of extracellular IL-1b. Hence, reducing this pro-inflammatory pathway has been suggested as a major target for protective therapies in these pathologies. However finding an effective mechanism for the inhibition of IL-1b secretion remains a challenge in the field. It has been extensively demonstrated that this cytokine is exported from cells, upon activation of the inflammasome, through several unconventional secretory pathways, including via exosomes. These vesicles, which represent a specific subclass of membrane vesicles originated from the fusion of multivesicular bodies (MVBs) with the plasma membrane, have been isolated from many body fluids and were described to accumulate in association with a variety of human diseases. Exosomes are important vehicles for intercellular communication, since they allow long-distance delivery of effector cargo. Therefore, in the context IL-1b driven inflammation, it is reasonable to speculate that those vesicles would be crucial for the stimulation of distant cells and the amplification of immune responses. Besides their fusion with the plasma membrane resulting exosome release, MVBs can also interact with autophagosomes generating a hybrid organelle termed amphisome, which may subsequently fuse with lysosomes (forming an autolysosome) to degrade the incorporated material. Therefore, it is conceivable to suggest that stimulating the fusion with lysosomes constitutes a strategy to prevent exosome secretion. One of the mechanisms that is known to promote such fusion is autophagy.

Taken together, this information led us to explore the role of autophagy induction as a modulatory mechanism for the release of IL-1b-containing exosomes by the monocytic cell line THP-1, after inflammasome activation. We started by demonstrating that activation of the inflammasome, after incubation either with LPS or LPS and ATP, in THP-1 cells up-regulates the secretion of exosomes containing the not only IL-1b cytokine but also its mRNA. Moreover, incubation of unstimulated cells with these IL-1b-containg vesicles led to the amplification of the pro-inflammatory response without

any other immune challenge. Finally, we found that in THP-1 cells, previously activated with LPS and ATP, the stimulation of autophagic activity by rapamycin was able to inhibit both release of exosomes and total levels of IL-1b (protein and mRNA) produced and secreted.

According to our knowledge, the results obtained in this study show, for the first time, that induction of autophagy may have an anti-inflammatory effect by inhibiting the release and further spreading of IL-1b-containing exosomes. Altogether, the results obtained in this study constitute not only an important contribution for the study of the mechanisms involved in inflammatory responses, but also for the field of cell biology in general, broadening our understanding of the mechanisms involved in secretion through exosomes.

Introduction

IL-1b

More than any other cytokine family, the IL-1 family of ligands and receptors is primarily associated with acute and chronic inflammation[1]. In that way, microbial invasion and tissue injury activate innate immune cells and promote overexpression and secretion of pro-inflammatory cytokines, such as Il-1b which is particularly known to evoke a constellation of host responses within hours or days after the recognition of "danger" signals. Hence, this cytokine regulates systemic and local responses to immunological challenges by generating fever, activating lymphocytes and promoting leukocyte transmigration into sites of injury or infection[2].

However, although these processes are meant to protect the body from insults, prolonged or exacerbated inflammatory responses associated with increased levels of extracellular IL-1b, have been linked to a growing number of diseases. Augmented IL-1b production has been found in patients with viral, bacterial, fungal and parasitic infections. Increased IL-1b levels have also been related to atherosclerosis, type2 diabetes and various autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis and Crohn's disease. Neurodegenerative diseases that are accompanied by inflammatory processes, such as Alzheimer's disease or Parkinson's disease, have also been characterized by augmented IL-1b production[3].

IL-1b production

The cytokine IL-1b is known to be primarily produced by cells of the monocytic lineage, including monocytes, macrophages and dendritic cells. Its production and release are induced by a wide variety of stimuli, which can be divided into pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs). For example, invading microorganisms are recognized through

PAMPs, such as lipopolysaccharide (LPS), while DAMPs, such as ATP, are endogenous ligands released by damaged or dying cells. Triggering signals mediated by recognition of PAMPs and DAMPs involve the activation of membrane-associated toll-like receptors (TLRs) or cytosolic nucleotide binding and oligomerization domain (nod)-like receptors (NLRs), respectively[4]. In turn, once activated, NLRs induce the assembly of multiprotein complexes called inflammasomes that trigger innate immune defenses through the maturation and secretion of IL-1b (fig. 1).

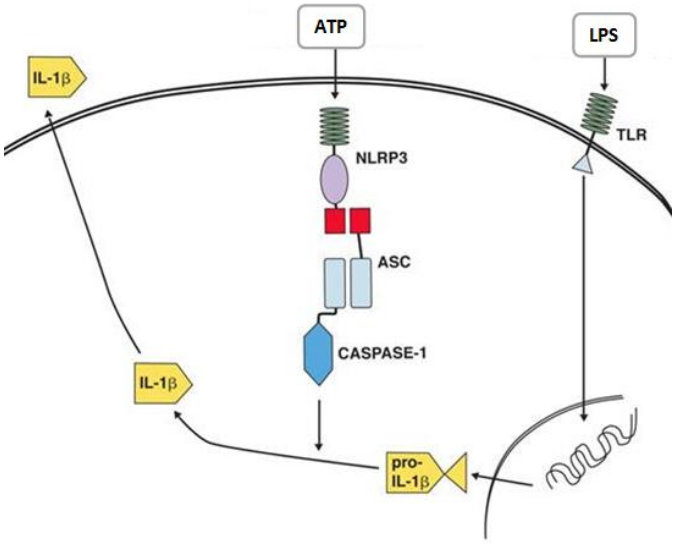

Figure I – Example of IL-1b production activation after ligation of a TLR receptor by LPS and activation of the NLRP3 inflammasome by ATP. (Adapted from Wolff, 2011)

Indeed, there is in fact a large body of experimental evidence demonstrating that serial treatment of cells with LPS and ATP provides a very powerful stimulus to induce rapid and efficient release of IL-1b from monocytes, macrophages and dendritic cells (DCs) [5,6,7].

IL-1b secretion

In general, secretion of proteins can occur through classical or non-classical secretory pathways. Since, IL-1b lacks a conventional hydrophobic signal sequence for classical externalization and this so called leaderless protein is released through a non-classical

secretory pathway (also termed unconventional secretion) independent of endoplasmic reticulum and Golgi apparatus. Five different models of the mechanisms of IL-1b release have been proposed: (i) exocytosis of secretory lysosomes that accumulate cytosolic IL-1b via undefined protein transporters; (ii) release of membrane-delimited microvesicles derived from plasma membrane blebs formed by evaginations; (of the surface membrane that entrap cytosolic IL-b); (iii) release of membrane-delimited exosomes secondary to the exocytosis of multivesicular bodies formed by invaginations of recycling endosomes that entrap cytosolic IL-b; (iv) exocytosis of autophagosomes or autophagolysosomes that accumulate cytosolic IL-1b via entrapment during formation of the initial autophagic isolation membrane or omegasome and (v) direct release of cytosolic IL-1b secondary to regulated cell death by pyroptosis or necroptosis[8] (fig. 2).

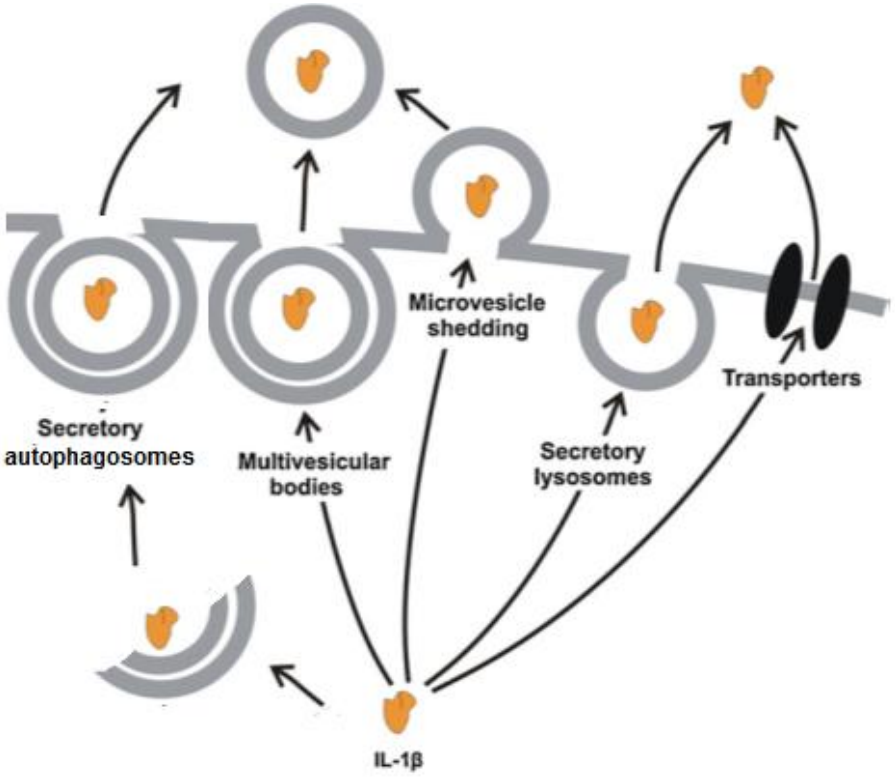

Figure II – Five major pathways of non-classical secretion by which the cytosolic IL-1b can be exported to the extracellular compartment: secretory autophagosomes, MVBs, microvesicle shedding, secretory lysosomes and membrane transporters. (Adapted from: Duitman et al, 2011)

Lastly, it is important to note that even though the stimuli and the mechanisms that lead to IL-1b secretion are well characterized, finding an effective way of reducing this

pro-inflammatory pathway is still a major challenge. On this regard, one can speculate that interfering with the specific release of IL-1b-containing vesicles might be a more powerful approach to modulate systemic inflammatory pathologies, since it would reduce the stimulation of distant cells and therefore the amplification of immune responses.

Exosomes

Intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell–cell contact, secretion of molecules which then bind to receptors on surrounding cells or release of membrane vesicles that allow long-distance delivery of effector cargo[9]. Exosomes represent a specific subclass of such membrane vesicles and their production is initiated when cell membrane proteins transfer to early endosomes by inward budding (Fig. 3). In early endosomes, molecules can be either recycled to the plasma membrane or incorporated into internal vesicles, commonly called multivesicular bodies (MVBs). These vesicles are formed when the limiting membranes of endosomes invaginate and bud into the lumen of the vesicle. During this process, several proteins can be sorted into the forming vesicles. In the degradation pathway, the MVBs fuse with lysosomes, but for exosome production, the MVBs fuse with the plasma membrane and consequently release the produced vesicles into the extracellular milieu as exosomes[10] (Fig. 3).

Since their discovery almost 30 years ago, exosomes have been isolated from several body fluids and were described to accumulate in association with a variety of diseases. Additionally, it was also found that cells can tightly regulate the secretion and molecular composition of these vesicles in response to different stimuli. More recently, these vesicles were shown to be able to deliver functional RNA (both messenger and microRNA) to target cells, suggesting the existence of a transfer of genetic information between cells, and thus elevating their role in the induction, amplification and/or modulation of pathological processes[11].

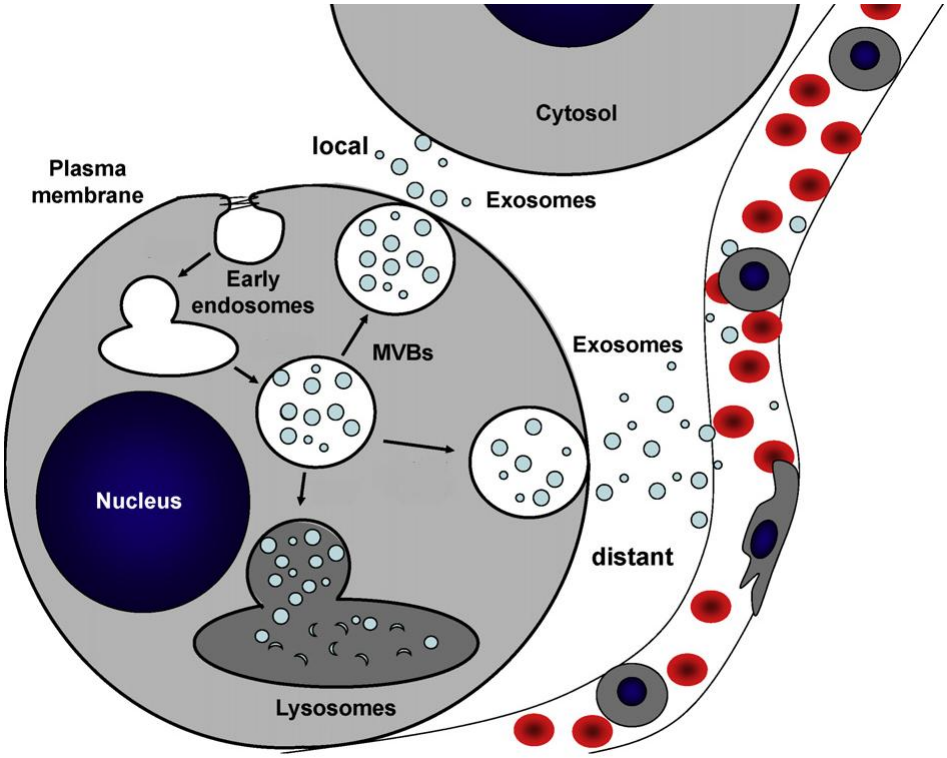

Figure III - Endocytosis, exosomes and intercellular communication. MVBs can either fuse with lysosomes to degrade their cargo, or with the plasma membrane to release their intra-luminal vesicles as exosomes. Exosomes can locally mediate intercellular signaling or at distant sites when secreted into the blood stream. (Adapted from: Ludwig et al, 2012)

Immunologist's interest in exosomes came from the discovery in 1996 that cells of the immune system, B lymphocytes, were able to secrete exosomes by fusion of MVBs with the plasma membrane. Exosomes secreted by these cells harbor MHC class II dimers bound to antigenic peptides, molecules essential for the adaptive immune response. These exosomes were also shown to present the MHC–peptide complexes to specific T cells, suggesting that they could play a role in adaptive immune responses[12]. Two years later, the groups of Raposo, Amigorena and Zitvogel[13] showed that DCs also secrete exosomes bearing functional MHC class I–peptide complexes, which could promote induction of $CD8^+$ T-lymphocyte-dependent antitumor immune responses in mice *in vivo*. These results set the basis for the exploration of exosome trafficking and cargo characterization in the context of immune responses to cancer, as they can constitute powerful vehicles for anti-tumoural therapies[14]. However the role of exosomes in the modulation of inflammatory responses and innate immunity

processes has been left on the sidelines of scientific interest and is still very much underexplored, despite their previously mentioned role in the secretion of pro-inflammatory cytokine IL-1b after inflammasome activation.

Autophagy:

Autophagy and exocytosis can have opposing or synergistic roles in cell function. For example, during times of reduced nutrient availability, autophagy is stimulated, allowing cells to recycle cytoplasmic components, and exocytosis is reduced to conserve cellular constituents and energy. In response to several stimuli, specialized secretory cells divert energy utilization towards the exocytic pathway. Conversely, when a cell is presented with an immune challenge, both exocytosis and autophagy can be up-regulated; exocytosis for the release of immune response factors and autophagy to clear invading pathogens from cells[15].

Degradative/secretory Autophagy

Autophagy is a degradation mechanism in which a cupped-shape structure, also referred to as omegasome, isolation membrane or pre-autophagosome, encloses a portion of cytoplasm or even organelles, forming a double-membraned vacuole called autophagosome. To carry out degradative functions, autophagosomes fuse with lysosomes to form autolysosomes where the captured cargo is eventually degraded[16]. However, the notion of autophagy as a purely degradative pathway was recently challenged by the emergence of three independent reports on the role of autophagy in unconventional secretion of Acb1 in yeast and IL-1b and other cargo (e.g. HMGB1 and IL-18) in mammalian cells. These new insights assign a non-degradative function to autophagy manifested as unconventional protein secretion[17] (fig. 4).

Here, a membrane structure, termed compartment for unconventional protein secretion (CUPS) has been identified as the source of organelles or trafficking intermediates for autophagy-based unconventional secretion. It is still unknown if this CUPS, the term used in yeast, is related to the mammalian cell omegasome, which acts as a cradle for generating nascent autophagosomes normally associated with degradative autophagy[20].

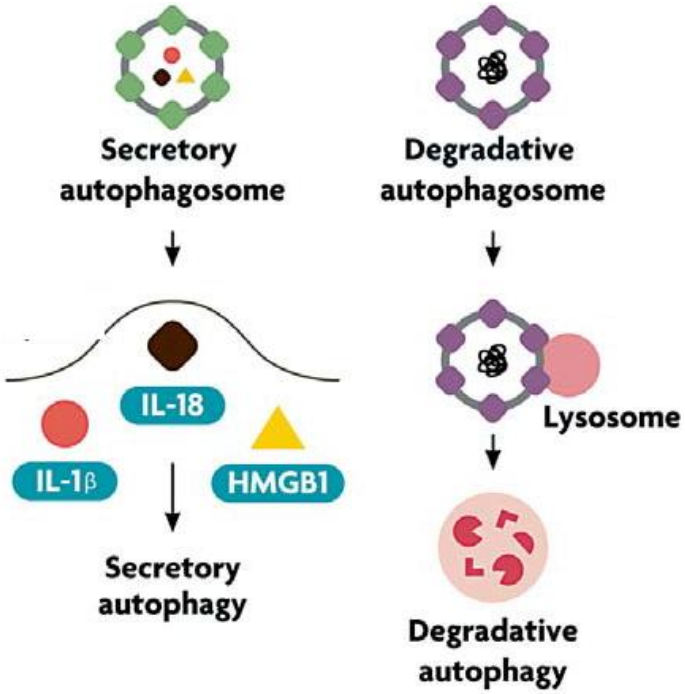

Figure IV – Hypothetical model divergence between degradative and secretory autophagy as a result of the formation of different autophagosomes. (Adapted from Jiang et al, 2013)

Nonetheless, it was already shown that although serum starvation induces both CUPS and omegasomes, CUPS is reportedly not induced by rapamycin, which suggests that it is probably distinct from omegasomes[18].

Autophagy and MVBs

Autophagosomes can also interact with MVBs, generating a hybrid organelle termed amphisome. Subsequently, the amphisome fuses with lysosomes (forming an

autolysosome) to degrade the incorporated material[19]. However, as previously referred, in some conditions, MVBs are diverged for fusion with the plasma membrane where they release their intraluminal vesicles into the extracellular compartment as exosomes (fig. 5).

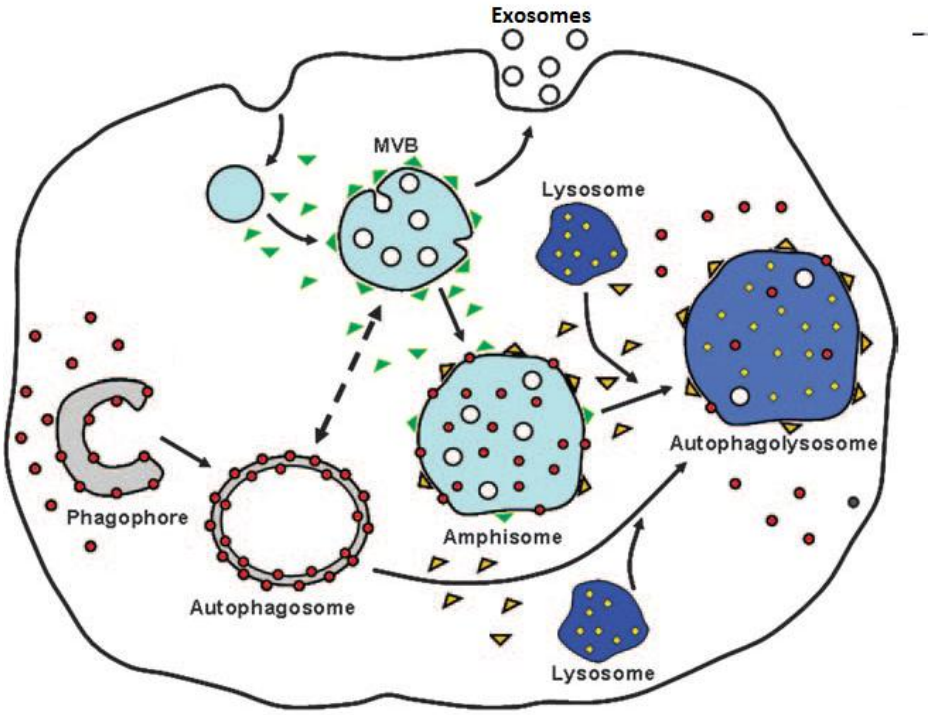

Figure V –Autophagy induction favors fusion between autophagic vacuoles and MVBs. The model proposes that autophagy induction promotes the fusion between autophagic vacuoles and MVBs the amphisome, which finally fuses with the lysosomal enzyme-containing compartment. (Adapted from: Fader et al, 2008)

Even though the convergence between the endocytic and the autophagic pathways has been widely characterized, and the morphological evidences suggest that MVBs are the main endocytic fusion partners that meet with the autophagosome, this process remains much underexplored as a means of modulating exosome secretion. There was, however, a report in 2008 by Colombo *et al.* where they presented evidence that in K562, a cell type that secretes exosomes through fusion of MVBs with the plasma membrane, autophagy induction caused fusion of these endocytic compartments with autophagic vacuoles. Interestingly, their results indicated that the convergence between MVBs and autophagosomes stimulated by autophagy induction also inhibited exosome secretion in all the conditions analyzed[20].

Aim:

Inflammation is widely recognized to be at the root of a host of serious human diseases from heart disease to diabetes and cancer. Acute inflammation occurs in response to pathogen attack (infection) or tissue injury and almost always involves inflammasome activation, a process that is supposed to clear up when the injury or infection is resolved. However, persistent inflammatory stimuli or dysfunction of the resolution phase results in chronic inflammation, which is known to underlie the development of various diseases.

Consequently, finding anti-inflammatory approaches capable of silencing these exacerbated immune responses is certainly an important step towards finding efficient therapies for such diseases. On that account, it is reasonable to speculate that the release of pro-inflammatory mediators via membrane vesicles, such as exosomes, might be able to induce systemic inflammation more potently, thus elevating this pathway of secretion as an important target for the referred anti-inflammatory therapies.

One of those pro-inflammatory mediators that is both secreted inside exosomes and also related to many diseases associated with increased inflammation is IL-1b. This cytokine is known to evoke a constellation of host responses after the recognition of "danger" signals by the inflammasome, being one of the major cytokines responsible for the regulation of systemic and local responses to immune challenges. Therefore, here we proposed to investigate a mechanism capable of dampening the secretion of IL-1b-containing exosomes after inflammasome activation.

For that, we started by examining if the monocytic cell line THP-1 released exosomes upon stimulation with classic inflammasome activators and evaluated the vesicles' cargo on their levels of IL-1b (for both protein and mRNA). Then, we confirmed that those exosomes isolated from the culture medium of activated cells were indeed functional in the amplification of the pro-inflammatory response when transferred to the culture of unstimulated cells inducing their production of IL-1b without any other

immune activation signal. Finally, the activation of autophagy was investigated as a means of lowering IL-1b levels and reducing exosome release by THP-1 cells after inflammasome activation.

Materials and Methodology:

Cell Culture

FBS (fetal bovine serum) (Invitrogen) used for cell culture was ultracentrifuged at 100,000 g for 16 h to remove exosomes present in FBS. THP-1, a human monocytic cell line derived from an acute monocytic leukemia (ATCC, TIB-202TM) was cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 0.05 mM β-mercaptoethanol and standard antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), in 37ºC, 5% CO_2 in air incubator.

For autophagic flux assay 2 x 10^6 cells were cultured per condition. To induce autophagy, rapamycin was added to the cultures at a concentration of 200 nM for either 1h or 3h. To evaluate autophagic flux cells were treated in the absence or presence of chloroquine (100 μM) to inhibit autophagosome-lysosome fusion, for the last 30 min of culture.

For inflammasome activation experiments 7,5 x 10^6 cells were cultured per condition. Cells were incubated with 200 ng/mL of LPS for 6h and 1 mM of ATP was added for the last 30 min before harvesting. After the immune stimuli, cells were washed with PBS and replated in fresh medium with or without 200 nM of rapamycin, for 1h or 3h additional hours.

For the exosome-conditioning assay, 2 x 10^6 cells were incubated with or without the addition to the culture medium of exosomes isolated from the supernatant of 1,5 x 10^7 cells incubated with LPS and ATP as described above. Exosomes were added at three different time points (0, 3, 6 h) and cultured for 24h.

Exosomes isolation

Exosomes secreted by THP-1 monocytes were isolated from the culture media by two-step centrifugation, always at 4ºC: The first step, at 15,000 g for 20min to remove

microvesicles and cell-debris, and then the collection step at 100,000 g for 1h. In the end the exosome rich pellet was either resuspended in 1mL of Trizol (Invitrogen Life Technologies) for RNA extraction or 200µL of cold phosphate saline buffer (PBS) and then stored at 4ºC for further analysis by western blot, flow cytometry and ELISA.

Western blot

After harvest, cells were washed with cold PBS, then lysed with 150µL of RIPA buffer, vortex vigorously 2-3 times and incubated on ice for 45 min. After centrifugation at 16,000 g *for* 10 min at 4°C, the lysate supernatants were mixed with Laemmli Buffer, boiled at 95°C for 10 min and then sonicated (3 pulses of 15 sec). For LC3 B flux analysis, 45µL of each sample were resolved on 15% SDS-acrylamide gel and transferred to 0.2 µm nitrocellulose membranes. Exosome samples (obtained from the supernatant of 1,5 x 10^7 cells incubated with LPS and ATP as described above) were mixed with Laemmli Buffer and boiled at 95 °C. 50µL of exosome samples and 45µL of cell samples were resolved on 10% SDS-acrylamide gel and transferred to 0.2 µm nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBS-T for 45 min, then incubated with anti-LC3 B (Cell Signaling Techonolgy) or anti-CD63 (Santa Cruz) primary antibodies at a dilution of 1:500 in TBST containing 5% skim milk for 12 h at 4°C. Then, the membranes were washed three times with TBST, incubated with HRP-conjugated secondary antibodies (Bio-Rad Laboratories, Inc.) at a dilution of 1:5000 in TBST containing 5% skim milk for 2 h, and then washed three times with TBST. Membranes were stripped and re-probed with anti-GAPDH antibody (SICGEN), to demonstrate equivalent protein loading, or anti-Lamin B antibody (*Oncogene* Research Products), to confirm that exosome samples were not contaminated with cell debris. The immuno-positive bands were visualized by enhanced chemi-luminescence. Densitometry was performed *using VersaDoc* (Bio-Rad).

RNA extraction and RT-PCR

Total RNA was extracted from cells and exosomes samples using Trizol according to manufacturer's protocols (Invitrogen Life Technologies). The RNA was eluted in RNA storage solution (Ambion) and stored at −80° until RT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry.

RT-PCR assay was performed using the two-step method. For the first-step of reverse transcription, we used iScript™ cDNA Synthesis Kit (Bio-Rad) with 1 µg of total RNA as template RNA, following the manufacturer's procedure. For the second step of real time PCR reactions, we employed SYBR® Green Supermix (Bio-Rad) and Primer Assays for human IL-1b and GAPDH according to manufacturer's protocol described for the iQ5 Real-Time PCR Detection System (Bio-Rad). PCR data obtained by the IQ5 Optical System were automatically analyzed by the IQ5 Optical System Software (Bio-Rad) and expressed as target/reference ratio. Our approach was based on the calibrator-normalized relative quantification including correction for PCR efficiency of each gene.

ELISA

Total IL-1b concentration in cell culture supernatants and exosomes was determined by sandwich ELISA (Quantikine assay; R&D). Intracellular IL-1b was obtained from cells that were first washed with PBS, and then lysed with 2 M NaCl, 1% Triton X100, pH 5.5. The lysate was centrifuged at 16,000 g for 15min at 4ºC and then added directly to the microtiter wells. The concentrations of intracellular IL-1b were analyzed as described above.

Flow-cytometry

Exosome preparations were incubated with 25 µl of 4.5-µm-diameter *Dynabeads® Human CD63* (Invitrogene) for 22h at 4ºC, then washed using a magnetic separator

(Magna GrIP™ Rack, Milipore) and resuspended into 300 µl PBS. For the staining procedure the mixtures of beads and exosome-coated beads were incubated at room temperature for 1h with wheat germ agglutinin (WGA)-FICT (*Sigma*-Aldrich) and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

The results are presented as mean ± S.D., and the statistic difference between two groups was determined by the two-sided unpaired Student's t test. The tests were performed using GraphPad Prism, version 5.02 (GraphPad Software). Statistically significant values are as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

It is known that some specialized cells can tightly regulate the secretion of a class of membrane vesicles of endosomal origin called exosomes in response to different stimuli. Although, it was already established that cells of the immune system release exosomes upon inflammatory challenge, however the regulation of this secretory mechanism is still very unclear. A main objective of this study is to investigate the release of exosomes in the context inflammasome activation using the monocytic cell line THP-1.

In order access that we were able to perform successfully the exosome isolation protocol by ultra-centrifugation method and that the samples we then examined were not contaminated with cell debris, we started by incubating the cells with LPS followed by ATP, which is a classic stimulus for inflammasome activation, and access the isolated exosome samples by western blot for the presence of the protein commonly enriched in these vesicles tetraspanin CD63, and the absence of the nuclear protein Lamin B (Fig. 1 – A).

Flow cytometric analysis of exosomes attached to anti-CD63 magnetic beads showed that activation of the inflammasome was indeed a powerful stimulus to boost the secretion of exosome. In fact, LPS alone was sufficient to induce an increase in the number of vesicles released to the extracellular medium, however the addition of ATP (1mM) for just the last 30 min of culture proved to be a much more powerful stimulus, promoting more than 2 fold-change in exosome counts (Fig. 1 – B).

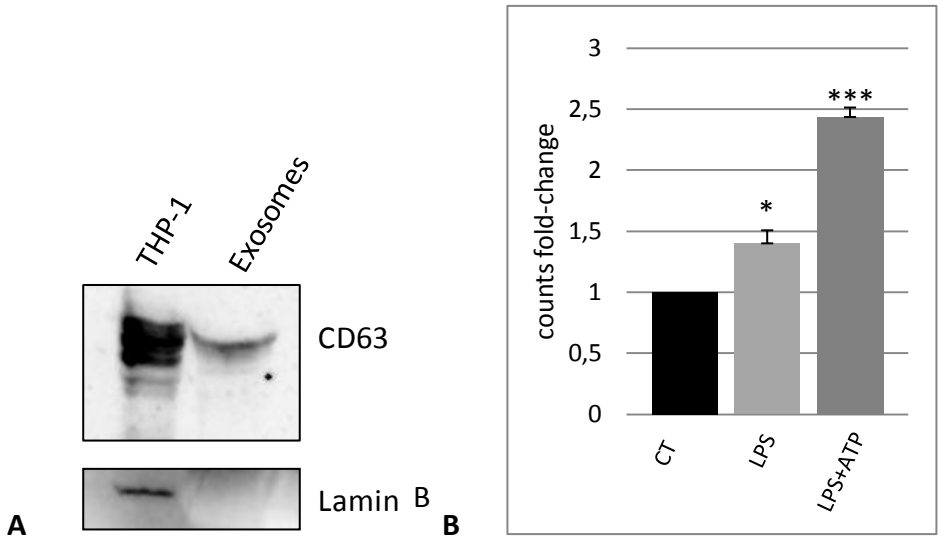

Figure 1 – Induction of exosome release after THP-1 treatment with LPS and ATP. THP-1 cells were incubated for 6h with 200 ng/mL LPS in the presence or in the absence of 1mM of ATP for the last 30 min of culture. Exosomes were isolated from culture medium by ultra-centrifugation method, as described above. (A) Western blot analysis of representative samples of cells and of exosomes isolated from the culture medium by the ultra-centrifugation method; (B) Fold-change in exosome counts relative to the control analyzed by flow cytometry. Exosomes were captured by anti-CD63 magnetic beads and stained with plasma membrane marker WGA-PE. Data represents the means of fold induction ± S.D. relatively to control, for three independent experiments. Statistically significant values compared to control are as follows: *p <0.05, ***p <0.001.

It is known that IL-1b is primarily produced by cells of the monocytic lineage upon activation of the inflammasome, and that this pro-inflammatory cytokine is secreted by a variety of non-classical secretory pathways[8]. Here we investigated and quantified the production (intracellular) and release (total and via exosomes) of IL-1b by THP-1 cells after incubation with either LPS or LPS and ATP using an ELISA assay (Fig. 2).

As expected, when treated with LPS alone, THP-1 monocytes show an up-regulation of IL-1b synthesis and consequent secretion, and the combined treatment with ATP further induces that production. However, although we could clearly identify an increase in exosomes release after addition of ATP to the cell culture (Fig. 1 – B), that increase, surprisingly, was not associated with cytokine levels in the vesicles, remaining

practically the same in both conditions (Fig. 2 – EXO). This result suggests that each exosome can carry less IL-1b.

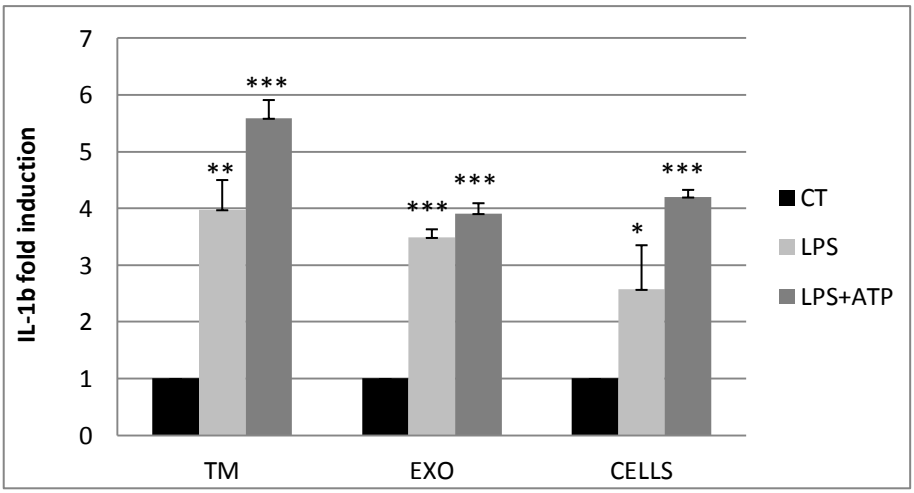

Figure 2 – Induction of IL-1b production after THP-1 treatment with LPS and ATP. THP-1 cells were incubated for 6h with 200 ng/mL LPS in the presence or in the absence of 1mM of ATP for the last 30 min of culture. Intracellular (cells), total release to the medium (TM) and secretion via exosomes (EXO) of the pro-inflammatory cytokine IL-1b were determined by ELISA. Data represents the means of fold induction ± S.D. relatively to control, for three independent experiments. Statistically significant values compared to control are as follows: *p <0,05, **p <0,01, ***p <0,001.

Recent studies demonstrated that exposure of human microvascular endothelial cells to several stress conditions alter RNA content of exosomes released by these. This data suggests that stress related signaling via exosomes does not only occur through protein transfer but also through shuttling of RNA[21].

Additionally, it has been established that incubation with LPS, followed or not by ATP, results in up-regulation of IL-1b mRNA expression by monocytic cells[4]. Together these observations led us to propose that the exosomes secreted by THP-1 monocytes might also carry not only the protein but also IL-1b mRNA. To access this question, we evaluated the expression of IL-1b mRNA, by RT-PCR, both intracellularly and in exosomes isolated from cells cultured either with LPS or LPS and ATP.

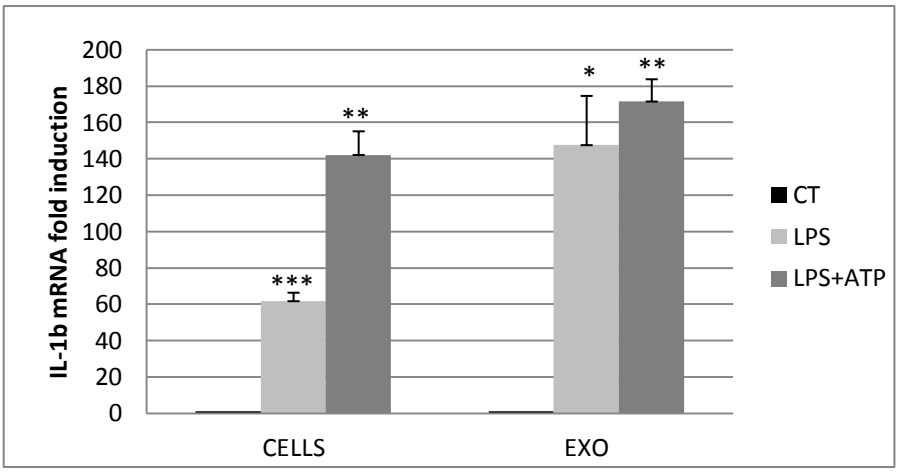

Figure 3 – Induction of IL-1b mRNA expression after THP-1 treatment with LPS and ATP. THP-1 cells were incubated for 6h with 200 ng/mL LPS in the presence or in the absence of 1mM of ATP for the last 30 min of culture. RT-PCR results of induction of IL-1b mRNA levels in cells and in exosomes (EXO) isolated from culture. Data represents the means of fold induction ± S.D. relatively to control, for three independent experiments. Statistically significant values compared to control are as follows: *p <0,05, **p <0,01, ***p <0,001. (CT=1)

Results obtained from gene expression analysis demonstrated that the induction on intracellular IL-1b mRNA expression, after the stimulation required for inflammasome activation with LPS or LPS and ATP, was, indeed, correlated with its secretion via exosomes (Fig. 3).

Moreover, in 2007 Valadi and colleagues reported that exosomes were also able to deliver functional mRNA to target cells that then translated it into protein, suggesting the existence of a transfer of genetic information between cells[22]. Based on that information we decided to investigate if unstimulated THP-1 cells were able to amplify this pro-inflammatory signal after incubation with the exosomes isolated from cells treated for inflammasome activation (LPS followed by ATP). ELISA assays were performed to evaluate the modulation on intracellular and total medium levels of IL-1b (Fig. 4).

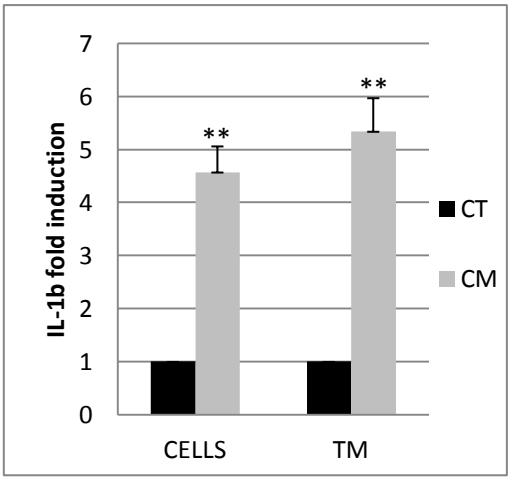

Figure 4 – Increase in IL-1b levels after exosome transfer. THP-1 cells were incubated with or without exosomes (24h) isolated from the medium of cells stimulated with LPS and ATP (CM). Intracellular (cells) and culture medium (TM) levels of IL-1b were determined by ELISA. Data represents as the means of fold induction ± S.D relatively to control, for three independent experiments. Statistically significant values compared to control are as follows: ***p* <0,01.

The results presented in Figure 4 show that incubation of THP-1 cells with IL-1b-mRNA containing exosomes leads to a dramatic increase in the amount of IL-1b produced and secreted by cells cultured in this conditioned medium. This data, proves that their up-take is enough to modulate pro-inflammatory responses without the need for any further immune challenge. Altogether, the results obtained up to this point demonstrate that inflammatory stimuli induces the release, by THP-1 monocytes, of exosomes containing both mRNA and the cytokine IL-1b, which can further "spread" this pro-inflammatory response throughout the organism.

Nonetheless, a main goal of this project was to find a mechanism capable of reducing the secretion of this "pro-inflammatory" exosomes. On that account, there was a report by Colombo *et al.* where they presented evidence that in K562 cells, stimulation of autophagy induced the convergence between MVBs and autophagosomes, which also inhibited exosome secretion[20]. So we considered using that approach as a means

of dampening the secretion of IL-1b-containing exosomes by THP-1 monocytes after inflammasome activation.

It has been described that one of the pathways of IL-1b secretion after inflammasome activation is based on secretory autophagy, which is a mechanism based on the release of autophagosomes[17]. Furthermore, serum starvation reportedly induces not only the commonly known degradative autophagy that leads to autophagosomal degradation in the lysosome, but also up-regulates this recently discovered secretory pathway. However, it was suggested that incubation with rapamycin only induces conventional macro-autophagy[18], and since our intent was to impair exosome release by divergence of MVBs to the degradative pathway, we selected the last as the treatment to be used after inflammasome activation in order to induce autophagy and potentially confirm our hypothesis.

First, in order to optimize the appropriate conditions that lead to autophagy induction, we incubated THP-1 cells with rapamycin, for 1 and 3 hours, either in the presence or absence of chloroquine. The accumulation of LC3B-II, observed by western blot analysis, in the presence of chloroquine (that inhibits both fusion of autophagosome with lysosome and lysosomal protein degradation) indicates increased autophagic flux (Fig. 5) under these conditions.

The results presented in Figure 5 demonstrate that THP-1 cells present high constitutive autophagy activity, as shown by the accumulation of LC3B-II in cells incubated with chloroquine. Furthermore, the accumulation of LC3B-II observed after incubation with rapamycin, for 1h or 3h, in the presence of chloroquine, demonstrates that autophagy is induced in our experimental conditions.

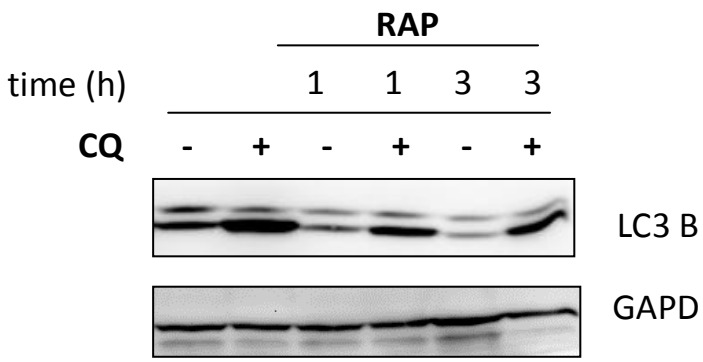

Figure 5 – Induction of the autophagic flux by rapamycin treatment in THP-1 cells. Cells were treated with 200nM of rapamycin for 1h or 3h, in the presence or absence of the lysosomal protease inhibitor chloroquine (100 µM) added for the last 30 min of treatment. Protein lysate was collected and analyzed for LC3B using Western blot analysis to detect autophagy flux. To ensure equal protein loading between samples, the membranes were stripped and reprobed with a GAPDH antibody. The immunoblot shown is representative of three independent experiments.

According to our hypothesis, stimulation of autophagic flux, that was shown to impair exosome secretion in another model, can prevent the release of IL-1b-containing exosomes by cells treated with LPS and ATP, used as inflammasome activators. To further determine whether exosome secretion after inflammasome activation could be inhibited by treatment with rapamycin, THP-1 monocytes were incubated with LPS and ATP, as described above, then washed and replated in fresh medium (FM) with or without rapamycin (Rap), for 1h or 3h additional hours, after which exosome samples were isolated from each culture and quantified after flow cytometry analysis using anti-CD63 magnetic beads. The data presented in Figure 6, demonstrates that rapamycin treatment results in a significant decrease in the amount of exosomes secreted by THP-1 monocytes, previously activated to induce the expression and maturation of IL-1b, as well as the release of exosomes (Fig. 1 - B).

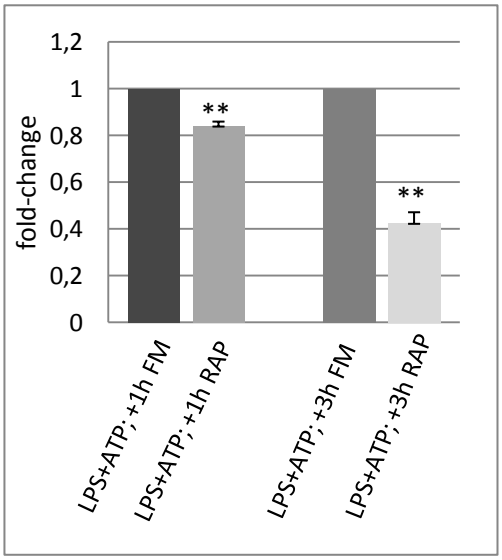

Figure 6 - Modulation of exosome release by autophagy induction, after inflammasome activation. After THP-1 treatment with LPS and ATP, cells were washed and replated in fresh medium (FM) with or without rapamycin (Rap), for 1h or 3h additional hours. Exosomes were captured by anti-CD63 magnetic beads and stained with plasma membrane marker WGA-PE, and total number of exosome-postive counts was obtained by flow-cytometry. Data are represented as the means of fold induction ± S.D. relatively to cells cultured in the presence of LPS and ATP and in the absence of rapamycin, for three independent experiments. Statistically significant values compared to same time of culture are as follows: $**p < 0,01$.

Although basal autophagy has been consistently reported to be essential for preventing inflammasome activation[4], the effect of its stimulation in cells whose inflammasome has been already fully activated is still not as clear. Thus, here we also intended to investigate the consequences of up-regulating the autophagic flux on the modulation of IL-1b production (which is the main consequence of inflammasome activation) by THP-1 cells that were previously incubated with LPS and ATP. ELISA measurements of IL-1b levels revealed that rapamycin treatment had a stastically significant, yet modest, effect on reducing intracellular cytokine leves (Fig. 7 - CELLS).

Nontheless, this down-modulatory mechanism showed to be more powerful in decreasing the total secretion of IL-1b and, speacialy, its secretion via exosomes (Fig. 7

– TM; EXO), agreeing with our previous results which showed that induction of autophagy reduces the number of exosomes released to the extracellular medium (Fig. 6). Taken together these results demonstrate that induction of autophagy, by rapamycin not only inhibits IL-1b production but also its release through exosomes.

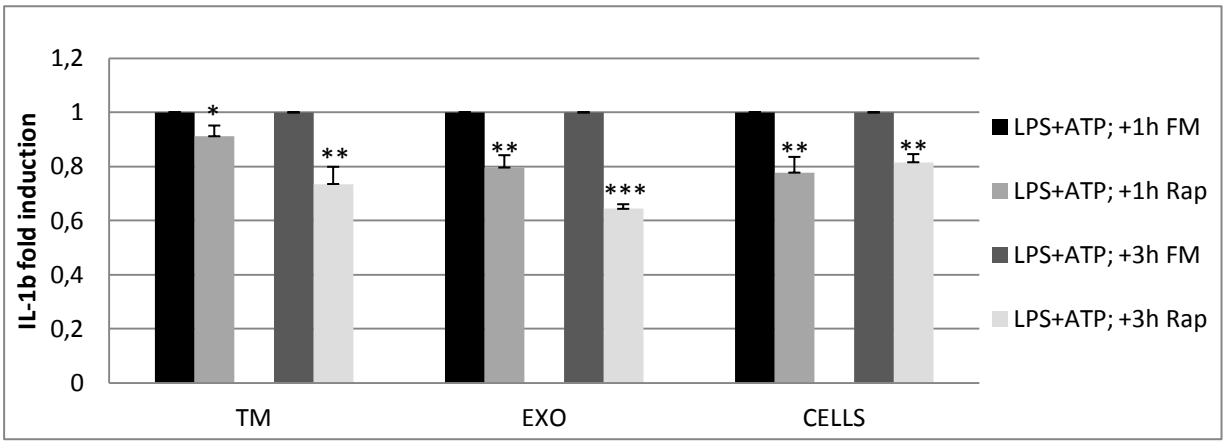

Figure 7 - Modulation of IL-1b production by autophagy induction, after inflammasome activation. After activation of IL-1b production by THP-1 treatment with LPS and ATP, cells were washed and replated in fresh medium (FM) with or without rapamycin (Rap), for 1h or 3h additional hours. Intracellular (cells), total release to the medium (TM) and secretion via exosomes (EXO) of the pro-inflammatory cytokine IL-1b were determined by ELISA. Data are represented as the means of fold induction ± S.D. relatively to cells cultured in the presence of LPS and ATP and in the absence of rapamycin, for three independent experiments. Statistically significant values compared to same time of culture are as follows: *p <0.05, **p <0.01, ***p <0.001.

We further pursued to explore if the induction of autophagic flux, after inflammasome activation, was able to decrease intracellular levels of IL-1b mRNA, and also if that was associated with a reduction of the cytokine mRNA concentration in the exosomes released by THP-1 monocytes.

Consistent with protein data (Fig. 7), RT-PCR analysis of gene expression, preformed as described previously, showed that rapamycin treatment down-regulated intracellular

expression of IL-1b mRNA (Fig. 8 - CELLS), although the decrease in exosomal concentration levels of the same mRNA was not as strong (Fig. 8 – EXO).

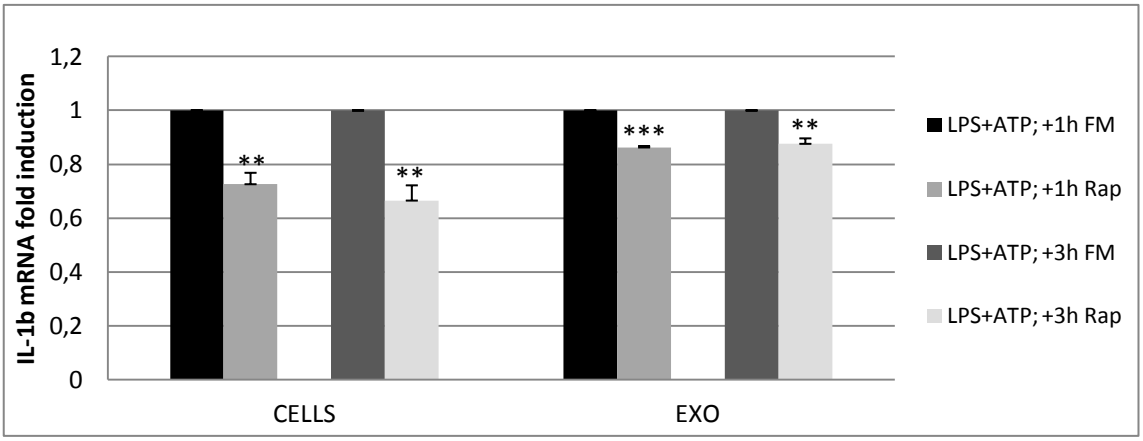

Figure 8 – Modulation of IL-1b mRNA levels by autophagy induction, after inflammasome activation. After activation of IL-1b mRNA expression by THP-1 treatment with LPS and ATP, cells were washed and replated in fresh medium (FM) with or without rapamycin (Rap), for 1h or 3h additional hours. RT-PCR results of the modulation of IL-1b mRNA levels in cells and in exosomes (EXO) isolated from culture, after treatment. Data are represented as the means of fold induction ± S.D. relatively to cells cultured in the presence of LPS and ATP and in the absence of rapamycin, for three independent experiments. Statistically significant values compared to same time of culture are as follows: $^{**}p$ <0,01, $^{***}p$ <0,001

Sulpemmentary Figure:

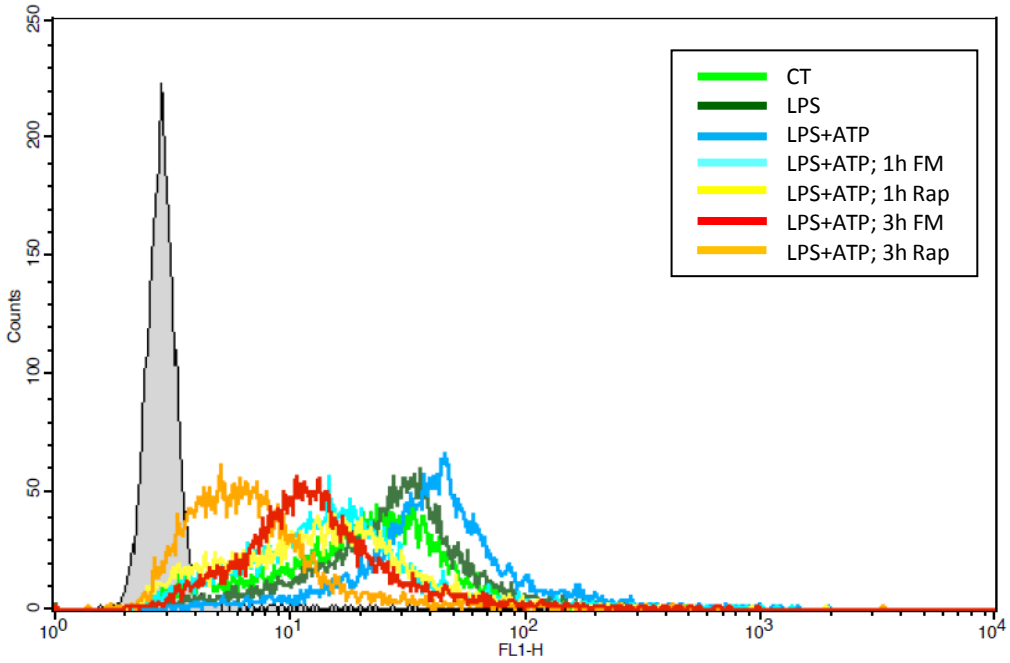

Figure S1 – Representative graph of flow cytometry analysis of exosome count change after THP-1 treatment with LPS and ATP, flowed by autophagy induction with rapamycin. Exosomes from each sample were capture by anti-CD63 magnetic beads. The bead-exosome complexes (positive counts) were stained with the plasma membrane marker WGA-PE to distinguish from exosome-free bead counts. Data collected from these experiments was use to quantify the modulation of exosome count modulation by inflammasome activation or autophagy induction after immune challenge.

Discussion:

The data presented in this work shows that activation of the inflammasome with LPS and ATP stimulated production and release of IL-b. Moreover, we demonstrate that treatment of THP-1 cells with LPS and ATP leads to exosome secretion. Interestingly, we observed that the increase in the amount of exosomes released after addition of ATP, was much higher than the increase in the concentration of IL-1b detected in exosomes isolated from the supernatant of cells cultured in the same conditions. This finding may indicate that for undifferentiated THP-1 cells, ATP treatment induces the release of exosomes containing IL-1b, but also stimulates the secretion of this cytokine through another pathway.

Surprisingly, we found that those exosomes secreted upon activation of the inflammasome contained not only the cytokine, but also IL-1b mRNA. This finding is of particular relevance, since it clearly highlights their importance in the physiological context of inflammasome-associated diseases, linked to increase levels of IL-1b. Indeed, these vesicles could thus prove to be part of a mechanism of facilitating the dissemination of inflammatory responses to other cells that did not directly contact with the danger signals, during immune challenge. To investigate the potential transfer of genetic information between cells via exosomes, which would provide an alternative pathway of efficient and rapid translation and maturation of IL-1b, we incubated unstimulated THP-1 monocytes with exosomes isolated from cells treated with LPS and ATP, after which we evaluated the production of the cytokine. The results obtained demonstrate a strong up-regulation of IL-1b after the addition of IL-1b mRNA-containing exosomes, suggesting that exosomes constitute a carrier for the transport of IL-1b mRNA that can be translated in cells localized far from the stimulated, mRNA producing, cells. Moreover, to prove that the increase in IL-1b is due to the translation of the cytokine's mRNA, and not a result of the up-take of IL-1b protein carried by these vesicles, we should have done a control where we would incubate the cells with exosomes and a translation inhibitor (e.g. Cycloheximide) and show that the up-regulation of IL-1b production was not as strong as without such inhibition. Even so,

inhibiting the secretion of vesicles capable of inducing a pro-inflammatory response at a distance and without any other immune challenge, might turn out to be a protective therapeutic mechanism for diseases associated with exacerbated inflammation.

In this study, our approach for reducing the secretion of exosomes after inflammasome activation was the stimulation of autophagy. The selection of this approach was based on a report by Fader et al. (2008) where they presented evidence that in K562 cells, induction of the autophagic process led to the convergence between MVBs and autophagosomes, which strongly inhibited exosome release[20]. Here, we also observed a marked inhibition of this secretory pathway by rapamycin treatment in activated THP-1 monocytes, apparently in a time dependent manner. Nonetheless, despite the successful reduction of the number of exosomes released after inflammasome activation, and because IL-1b is also exported via autophagy-based unconventional secretion, the relationship between stimulation of autophagy and total production of IL-1b in the context of inflammasome activation is still quite controversial.

Earlier studies have clearly linked basal autophagy with the inhibition of spurious activation of the inflammasome. Since dysfunctional organelles, including mitochondria, are removed by autophagosomes, inhibition of autophagy leads to an accumulation of ROS-producing mitochondria, which is accompanied by increased release of mitochondrial DNA, which are known “danger” signals that activate the inflammasome, consequently leading to the release of IL-1b. Moreover, autophagy is also involved in the removal of ubiquitinated inflammasomes and pro-IL-1b in activated cells. In contrast to the anti-inflammatory effects of basal autophagy, which suppresses unscheduled inflammasome activation, the consequences of induced autophagy in the secretion of the pro-inflammatory cytokine IL-1b are not as consistent[4].

In 2011, Harris et al demonstrated that in mouse macrophages primed with particulates and TLR agonists followed by 4h incubation with rapamycin, as well as in an LPS challenge *in vivo* with or without concomitant addition of rapamycin, the

induction of autophagy led to an inhibition the secretion of IL-1b[23]. Consistently, another group (Shi et al. 2011) also found a reduction in amount of IL-1β in the supernatants of differentiated THP-1 cells. Indeed, these authors demonstrated that induction of autophagy reduced the levels of IL-1b released by cells incubated with the NLRP3 inflammasome–inducers uric acid crystals or nigericin, coordinately treated either with amino-acid starvation or rapamycin for 6h, when compared to the levels found in cells treated only for inflammasome activation[24]. However, another study shows an opposite role for autophagy in modulation of IL-1b release during immune challenge. It was reported, by Dupont and colleagues (2011) that stimulation of autophagy by serum starvation, while activating the inflammasome with different danger signals, for 1h in LPS-primed mouse macrophages leads to an increase in the export of IL-b to the extracellular medium. The up-regulated export of IL-1b was found to happen as a result of an increase in its release via secretory autophagy, for which the pro-inflammatory cytokine is prototypical example of the newly found unconventional manifestation of autophagy[25].

The inconsistency of the previously described results may be due to the existence of temporal or treatment differences required to activate either degradative or secretory autophagy. It was indeed suggested that although serum starvation was capable of stimulating both pathways, rapamycin only induced the degradative autophagic process[20]. Still, our main goal in this study was to test the stimulation of autophagy as a means of dampening exosome secretion upon inflammasome activation, thus reducing the amplification of inflammation. Therefore, it was important to use a strategy that while attempting to inhibit IL-1b-containg exosome release, we were not increasing the export of this cytokine by another pathway. It was on that account that we chose rapamycin for the stimulation of degradative autophagy, which proved to be a treatment capable of reducing intracellular and secreted levels of IL-1b after inflammasome activation by THP-1 cells, not interfering with our model of protection against inflammation.

In summary, we have demonstrated that activation of the inflammasome by treatment with LPS and ATP in THP-1 monocytes, induces the secretion of exosomes containing the cytokine IL-1b and its mRNA. This secretion is inhibited by autophagy stimulation with rapamycin treatment, which also reduces total levels of IL-1b. It would now be interesting to access if we could mimic this mechanism of exosome inhibition after inflammasome activation *in vivo,* decreasing serum levels of IL-1b-containing exosomes and if that would be an effective mechanism to reduce the phenotypic and pathological consequences usually associated with elevated production of this pro-inflammatory cytokine. Moreover, we believe that our study opens a new therapeutic avenue for diseases associated with elevated secretion of exosomes, not only in the context of inflammation.

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