UNIVERSIDADE D
COIMBRA

Inês Alexandra Ferreira Rodrigues

Evaluation of the Ability of CIAD7, a Natural Compound, to Induce the Pro-Resolution Phenotype in Murine Macrophages.

Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica, orientada pela Professora Doutora Armanda Emanuela Castro Santos e pela Professora Doutora Alexandrina Maria Ferreira Santos Pinto Mendes e apresentada à Faculdade de Farmácia Universidade de Coimbra.

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A ti, minha irmã…

Por seres o exemplo, a força e a inspiração de
todos os dias!

"The mind is a beautiful servant but a terrible master."

Robin Sharma

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ABSTRACT

Interactions between humans and microorganisms, through time, led to a developed and evolved immune system. In an early immune response, neutrophils and macrophages are important cells to phagocyte the an extracelullar pathogen. Macrophages are vital to maintain homeostasis, sensing environment alterations and inducing an immunologic response in order to support organs' needs or the attack to a pathogen invasion. They present phagocytosis capacity and phenotype plasticity that can give origin to two different polarization states, M1 macrophages and M2 macrophages. Macrophages are crucial agents in whole inflammation process. The switching balance between the M1 and M2 polarization states is essential to a well-managed and spatially- and temporally-controlled process. An imbalance between these two states may be associated with chronic inflammation and is involved in several diseases. Therapeutic strategies targeting macrophage polarization might be an efficient approach to stop inflammation and induce its resolution. CIAD7 a natural compound, was identified in a previous study as having anti-inflammatory properties. In this study, we evaluated the ability of CIAD7, to induce the anti-inflammatory phenotype in immature macrophages and in M1 macrophages, and to potentiate M2 macrophages. For that, NO production, iNOS protein levels and the surface markers CD80 and CD86 were studied as M1 markers. As M2 markers, total arginase activity and the cell surface markers, CD163 and CD206, were studied. The results show that CIAD7 reduced the effect of an inflammatory stimulus, lipopolyssacharide from Gram negative bacteria, in all M1 markers studied, confirming previous results. On the other hand, in imature macrophages, treatment with CIAD7significantly reduced arginase activity induced by treatment with IL-4 for 18 h, but, increased the expression of CD163 and CD206 in cells treated with IL-4 for 72 h. Nonetheless, these results need to be confirmed, since only one experiment could be performed. In summary, thes results obtained confirm the anti-inflammatory activity of CIAD7 and suggest that it can also induce macrophage differentiation into the M2 phenotype and, thus, may be able to promote the resolution of inflammation.

Keywords: Macrophages; Polarization; Inflammation; Resolution.

RESUMO

Ao longo do tempo, interações entre humanos e microorganismos permitiram o desenvolvimento e evolução do sistema imunológico. Numa primeira resposta imune inicial, os neutrófilos e macrófagos são células importantes para fagocitar patógeneos. Os macrófagos são vitais para manter a homeostase, detetando alterações do ambiente e induzindo uma resposta imunológica de forma a apoiar as necessidades dos órgãos ou combater o ataque a uma invasão de patógeneos. Apresentam capacidade fagocítica e plasticidade fenotípica responsável por dois estados de polarização, macrófagos M1 e macrófagos M2. Os macrófagos são agentes cruciais em todo o processo de inflamação. O equilíbrio na troca entre os estados de polarização é essencial para um processo bem controlado, espacial e temporalmente. Um desequilíbrio entre estes dois estados está associado à inflamação crónica que está relacionada com várias doenças. Estratégias terapêuticas direcionadas à polarização de macrófagos podem constituir uma abordagem eficaz para interromper a inflamação e induzir a sua resolução. Num estudo anterior do nosso laboratório foi identificado um composto de origem natural com actividade anti-inflamatoria, designado CIAD7. O objectivo do presente estudo, foi avaliar a capacidade do CIAD7 para induzir o fenótipo anti-inflamatório em macrófagos imaturos e em macrófagos M1, e/ou para potenciar a aquisição do fenótipo M2. Para tal, estudámos como marcadores M1, a produção de NO, os níveis da proteína iNOS, e os marcadores de superfície CD80 e CD86. Como marcadores M2, estudámos a atividade total da arginase e a expressão dos marcadores de superfície CD163 e CD206. Os resultados obtidos mostram que o CIAD7 diminuiu o efeito de um estímulo inflamatório, o lipolissacarídeo de bactérias Gram negativas, em todos os marcadores M1 estudados, confirmando os resultados obtidos anteriormente. Além disso, os resultados obtidos mostram também que em macrófagos imaturos, o tratamento com CIAD7 reduziu significativamente a actividade da arginase induzida por adição de IL-4 durante 18 h, mas aumentou significativamente a expressão de CD163 e CD206 induzida por tratamento das células com IL-4 durante 72 h. No entanto, estes resultados necessitam de ser confirmados, uma vez que só foi possível realizar uma experiência. Em suma, os resultados obtidos confirmam a actividade anti-inflamatória do CIAD7 e sugerem que é também capaz de induzir a diferenciação de macrófagos no fenótipo M2 e, portanto, que poderá apresentar actividade pró-resolutiva da inflamação.

Palavras-chave: Macrófagos; Polarização; Inflamação; Resolução.

ABBREVIATIONS LIST

APC - Antigen presenting cells

Arg1 - Arginase 1

Arg2 - Arginase 2

CCL17 - C-C Motif Chemokine Ligand 17

CCL22 - C-C Motif Chemokine Ligand 22

COX-2 - Cyclo-oxygenase 2

DAMPs - Damage-associated molecular patterns

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

FAO - Fatty acid oxidation

FBS - Fetal bovine serum

HIF-1α - Hypoxia-Inducible Factor 1-alpha

IC - Immune complexes

IFN-γ - Interferon gamma

IkB-α - Nuclear Factor -kappa-light-chain- enhancer of B-cells inhibitor

IL-10 - Interleukin 10

IL-13 - Interleukin 13

IL1-α - Interleukin 1 alpha

IL1-β - Interleukin 1 beta

IL-4 - Interleukin 4

IL-6 - Interleukin 6

iNOS/NOS2 - Inducible Nitric Oxide synthase or Nitric Oxide Synthase 2

JAK-STAT - Janus Kinase - Signal Transducer and Activator of Transcription

LPS - Lipopolysaccharides

M1 - Classically activated macrophages

M2 - Alternatively activated macrophages

MAPK - Mitogen-Activated Protein Kinase

MFI - Median fluorescence intensity

NAD^+ - Nicotinamide Adenine Dinucleotide oxidized form

NADPH - Nicotinamide Adenine Dinucleotide Phosphate reduced form

NED - N-(1-Naphthyl)ethylenediamine

NF-κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells

NO - Nitric oxide

OXPHOS - Oxidative phosphorylation

P - Value indicative of confidence interval

PAMPs - Pathogen-Associated Molecular Patterns

PRRs - Pattern-Recognition Receptors

PVDF - Polyvinylidene difluoride

RIPA - Radioimmunoprecipitation assay

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RVT - Resveratrol

SDS - PAGE - Sodium sodecyl sulfate - polyacrylamide gel electrophoresis

SDS - Sodium dodecyl sulfate

SEM - Standard error of the mean

SIRT-1 - Sirtuin 1

T2D - Type 2 Diabetes

TBS – T - Tris-buffered saline with Tween

Tc - T cytotoxic -cells

TGF-α - Transforming Growth Factor-alpha

Th1 - T helper -cells type 1

Th2 - T helper -cells type 2

TLR - Toll-Like Receptors

TNF-α - Tumor ecrosis Factor-alpha

Tris-HCL - 2-Amino-2-hydroxymethyl-propane-1,3-diol

V - Vehicle

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1. INTRODUTION

The human organism is a complex ecosystem, composed of roughly 10^{13} human cells and also about 10^{14} microorganisms that are part of the normal flora. In addition, humans are in a constant interaction with other microorganisms that can be pathogenic and result in a disease infection (Health *et al.*, 2018). Therefore, humans developed a immune system that evolved through time. The immune system responds to the presence of antigens, such as cell surface proteins of pathogenic microorganisms, toxins and other foreign particles. This system also senses cell mutations that comprise the normal cell functions (Chaplin, 2010; Nicholson, 2016).

Nowadays, we know that an effective immune system discriminates the self from non-self and distinguish inoffensive non-self from threatening non-self (Nicholson, 2016).

The branch of science dedicated to the study of the immune system is known as immunology. Immunology gained attention when Elie Metchnikoff and Paul Ehrlich were recognized with a Nobel Prize award, in the last century. Metchnikoff was the first to study phagocytosis by macrophages as a critical host-defense mechanism, elucidating the a major mechanism of innate immunity. Ehrlich first described antibody formation and their mechanisms to neutralize toxins, thus revealing the fundamentals of adaptative immunity (H. E. Kaufmann, 2008).

Adaptative immunity is mediated by immune cells known as lymphocytes, that rapidly adapt to the situation generating subpopulations of specialized cells, cytokines, and antibodies (Yatim e Lakkis, 2015). This type of immunity is based on an antigen-specific recognition and results in a long-lasting immunologic memory. Antigen-recognition receptors at the surface of T lymphocytes bind processed antigens that are presented to them by antigen-presenting cells (APC), such as macrophages. The helper T lymphocytes (Th) may differentiate into different subpopulations whereas cytotoxic T cells (Tc) may become active Tc lymphocytes, supporting the immune response and eliminating infected cells, respectively. In B lymphocytes receptors that specifically bind an antigen. The receptor with bound antigen internalized by endocytosis and, after enzymatic processing it is then exposed on the cell surface of the lymphocyte B in order to be recognized by cognate T lymphocyte. These activated B cells differentiate in plasma cells that produce and secrete antibodies which neutralize pathogens and mark them for destruction by phagocytic cells (Molnar e Gair, 2015; Nishimura, 1952; Yatim e Lakkis, 2015). In contrast, innate immunity is characterized by a set of disease-resistance mechanisms mediated, among others, by pattern recognition receptors (PRRs) present in immune cells,

being the first defense line of the immunologic system (Richard A. Goldsby , Thomas J. Kindt , Barbara A. Osborne, 2000). In an early immune response, neutrophils and macrophages are important cells to phagocyte the pathogen (Nicholson, 2016). These last ones can be divided into two types according to their origin. They can be embryonic macrophages or adult-derived macrophages. The embryonic type can be found in tissue compartments in adulthood. By contrast, adult-derived macrophages result from tissue-infiltrating monocytes that differentiate into macrophages (Varol, Mildner e Jung, 2015).

Macrophages are vital to maintain homeostasis. They sense environment alterations and induce a response in order to support organs' needs or fight a pathogen invasion. Their phagocytosis capacity is very important in host defense, as already mentioned, but are also fundamental to eliminate and recycle dead cells and tissue debris, preserving organ functions(Mosser, Hamidzadeh e Goncalves, 2021). The macrophages' capacity to respond to different stimuli causes phenotypic changes due to their phenotype plasticity, that can give origin to two different polarization states, M1 macrophages (classically activated) and M2 macrophages (alternatively activated). This process is known as macrophage polarization and it is well-accepted that microRNA (miRNA) plays an important role in this (Mosser, Hamidzadeh e Goncalves, 2021; Shapouri-Moghaddam *et al.*, 2018; Wu *et al.*, 2015).

Inflammatory stimuli with lipopolysaccharide (LPS), interferon gamma (IFN-γ) or Tumor necrosis factor (TNF-α), activate inflammatory genes and stimulate polarization into M1 macrophages which present an inflammatory phenotype. At the metabolic level, these macrophages present major demands, depending mainly on glycolysis (Kang, Kim e Shong, 2019). They produce high levels of pro-inflammatory molecules, for example TNF-α, interleukin-1 (IL-1α and IL-1β), IL-6, cyclooxygenase-2 (COX-2), and generate reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) (Mantovani, 2012; Martinez e Gordon, 2014; Murray, 2017; Wang *et al.*, 2019; Wu *et al.*, 2015). M1 macrophages overexpress CD80, CD86, and CD40 on their cell surface (Aron-Wisnewsky *et al.*, 2009; Shapouri-Moghaddam *et al.*, 2018; Wang *et al.*, 2019; Yunna *et al.*, 2020). Conversely, macrophages that differentiate into M2 are subjected to by anti-inflammatory stimuli such as IL-4, IL-10, IL-13, immune complexes (IC) and glucocorticoids (Martinez e Gordon, 2014; Mosser, Hamidzadeh e Goncalves, 2021; Wang *et al.*, 2019, 2019). In contrast to M1 macrophages, their energy requirements are sustained by mitochondrial oxidative phosphorylation (OXPHOS) and by fatty acid oxidation (FAO) (Kang, Kim e Shong, 2019). M2 subtype produces high levels of anti-inflammatory molecules like IL-10, TGF-α, C-C Motif Chemokine Ligand (CCL)-17 and CCL22

(Wang *et al.*, 2019). On the cell surface, M2 overexpress CD206 and CD163 (Aron-Wisnewsky *et al.*, 2009; Dyken, Van e Locksley, 2013).

Another difference to take into account on M1/M2 macrophage classification is the metabolism of the amino acid arginine. In M1 macrophages, arginine is metabolized by inducible Nitric oxide Synthase (iNOS), and nitric oxide (NO) and citrulline are produced. While in M2 macrophages, arginine is converted via arginase to ornithine and urea (Mills, 2012). The arginase isoform that seems upregulated in M2 is Arginase-1 (Dyken, Van e Locksley, 2013). NO production by iNOS activity inhibits cell proliferation and eliminates pathogens, whereas ornithine generation promotes cell proliferation and repairs tissue damage through generation of polyamines and collagen. The intermediates in one arginine enzymatic pathway inhibit the opposing pathway thus determining whether NO or ornithine will be produced (Mills, 2012). Apart from the M1 and M2 macrophages functions described above these cells interact with lymphocytes to induce type 1 (Th1) or type 2 (Th2) helper T Cells responses, respectively, that in turn further amplify M1 or M2 type responses, defining the predominant immune phenotype in a situation of infection, tumor, or inflammation (Rath *et al.*, 2014). Indeed, macrophages are crucial agents in inflammation.

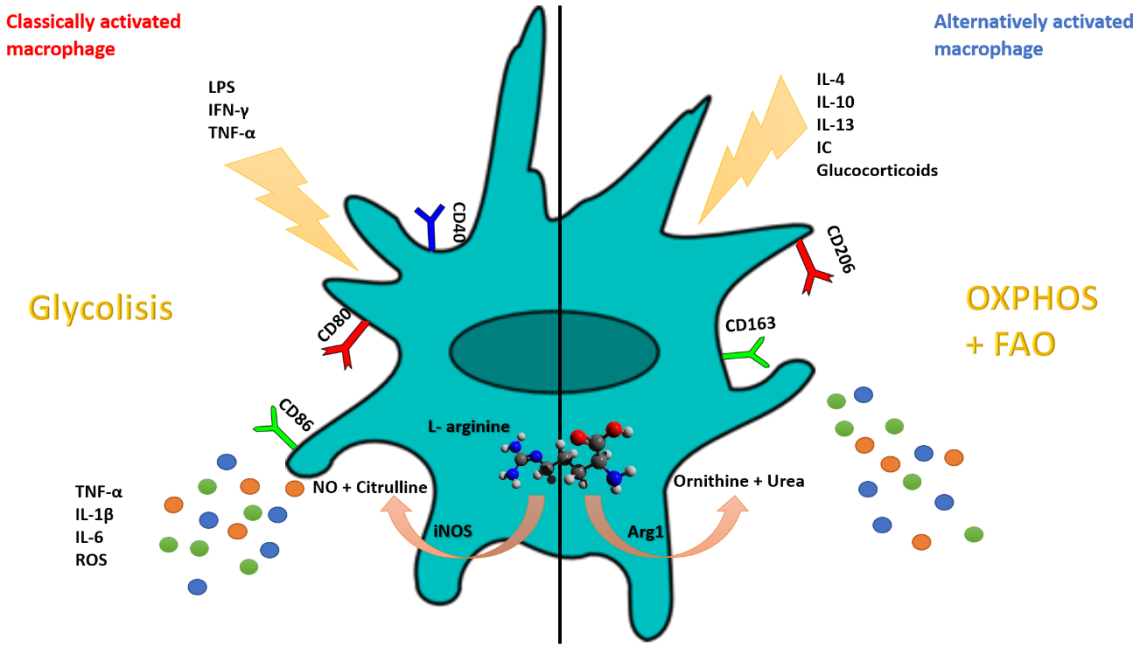

Figure 1: Representative image of the two macrophage polarization states. M1 can be induce by LPS, IFN-γ and TNF-α. They metabolize glucose through glycolysis and produce high levels of pro-inflammatory molecules, for example TNF-α, IL-1β, IL-6, COX-2 and ROS. On their cell surface, overexpress CD80, CD86, and CD40. Regarding, L-arginine metabolism, arginine is metabolized by iNOS, as a consequence, NO and citrulline are released. M2 stimulated by, for instance, IL-4, IL-10, IL-13, IC and glucocorticoids. M2 metabolism is sustained by OXPHOS and by FAO. This subtype produces high levels of IL-10, TGF-α, CCL17 and CCL22. On the cell surface, M2 overexpress CD206 and CD163. M2 express Arg1 and this is the enzyme responsible to convert arginine into ornithine and urea.

Inflammation is defined as a non-specific immune system's response to infection by pathogens, a physical trauma, such as a wound, or endogenous signals such as damaged cells. The inflammatory response is regulated by activation of various signaling pathways responsible for coordinating the expression of pro- and anti-inflammatory mediators in resident tissue cells and recruited leukocytes. Acute inflammation results in the elimination of the injurious stimuli and the induction of tissue repair, being an essential process to maintain tissue homeostasis (Atri, Guerfali e Laouini, 2018; Chen *et al.*, 2018; Medzhitov, 2010; Molnar e Gair, 2015). During this event, PRRs present in immune and nonimmune cells recognize alarming stimuli. PRRs recognize pro-inflammatory cytokines, pathogen-associated molecular patterns (PAMPs), which are microbial structures, and danger-associated molecular patterns (DAMPs), endogenous biomolecules that are able to induce a non-infectious inflammatory response. As a consequence, inflammatory pathways are activated, including, mitogen-activated protein kinase (MAPK), nuclear factor of kappa light chain polypeptide gene enhancer in B-cells (NF-κB) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways (Chen *et al.*, 2018; Kyriakis e Avruch, 2001; Lawrence, 2009; Medzhitov, 2008). The activation of these pathways, induces the release of inflammatory mediators by leukocytes, such as tissue-resident macrophages, and by local tissue cells. These inflammatory mediators cause blood vessels dilatation and changes in its permeability, allowing the recruitment of leukocytes to the site of infection or injury (Chen *et al.*, 2018; Medzhitov, 2008; Molnar e Gair, 2015). Macrophages express many scavenger receptors and Toll-like receptors (TLR), among other PRRs. Interaction with scavenger receptors can lead to phagocytosis, while engagement of TLRs results in the activation of NF-κB and MAPK pathways, in the production of ROS and in the release of cytokines which exaggerate the local inflammatory response (Lawrence, 2009; Libby, 2008; Rao, 2015). At this point, M1 macrophages are the dominant phenotype at the injury site (Atri, Guerfali e Laouini, 2018).The acute inflammatory response ends once the stimuli is eliminated, the infection is cleared, and damaged tissue is repaired. The resolution of inflammation consists in the transition to the homeostatic state and includes the reduction of pro-inflammatory mediators, macrophage polarization from classically to alternatively activated cells, and initiation of healing. At this stage, the major population of macrophages is of the M2 phenotype, that will induce production of anti-inflammatory mediators (Atri, Guerfali e Laouini, 2018; Chen *et al.*, 2018; Medzhitov, 2010).

The switching balance between the M1 and M2 polarization states is essential to a well-managed and spatially- and temporally-controlled process. It protects against overwhelming uncontrolled inflammation. An imbalance between these two states of polarization is

associated with chronic inflammation. Chronic inflammation is known to be involved in several diseases (Atri, Guerfali e Laouini, 2018; Chen *et al.*, 2018; Mantovani, 2012; Medzhitov, 2010). Rheumatoid arthritis (RA) is an autoimmune disease, in macrophages which increase their population in the synovium and are predominantly of the M1 phenotype. M1 phenotype is induced by ROS and by the Hypoxia-inducible factor (HIF-1α), which inhibits macrophage polarization to M2 (Shapouri-Moghaddam *et al.*, 2018; Yunna *et al.*, 2020). Chronic inflammation can also be found in metabolic diseases such as type 2 Diabetes Mellitus (T2D). In this scenario, the high levels of glucose in the environment induce M1 polarization, and even though molecular mechanisms responsible for this process remain unclear, M1 macrophages generate inflammation. The result is insulin resistance in liver, adipose and musculoskeletal tissues and dysfunction of pancreatic beta cells. It was also observed that, in T2D the three inflammatory pathways, NF-κB, MAPK, and JAK-STAT are activated (Chen *et al.*, 2018; Shapouri-Moghaddam *et al.*, 2018).

Therapeutic strategies targeting macrophage polarization can be an efficient approach to stop chronic inflammation and induce its resolution (Mantovani, 2012). Natural compounds, namely polyphenolic, are well known for their antioxidant properties and recent evidences indicate that certain polyphenols also present anti-inflammatory properties (Joseph, Edirisinghe e Burton-Freeman, 2016). One polyphenol with potent anti-inflammatory properties is Curcumin. Curcumin is able to suppress macrophage inflammatory responses, it significantly reduces the expression of co-stimulatory molecules and, modulates MAPK and NF-κB pathways. This compound can repolarize macrophages toward the M2 phenotype (Mohammadi *et al.*, 2019). Another example of a polyphenol with potent anti-inflammatory properties is Resveratrol (RVT) (Dai *et al.*, 2018; Yoshizaki *et al.*, 2010). Among other effects, it inhibits the degradation of the NF-κB inhibitor- alpha (IκBα), an endogenous natural inhibitor of NF-κB, and, therefore, NF-κB can not translocate to the nucleus to active pro-inflammatory gene expression (Holmes-McNary e Baldwin, 2000).

Accordingly, RVT decreases the expression of pro-inflammatory M1 macrophage markers and increases the anti-inflammatory M2 macrophages in a rheumatoid arthritis model by inducing AMPKα phosphorylation and subsequent NF-κB downregulation (Park *et al.*, 2017).

Objectives

Recently, our group identified a natural compound that we named CIAD7, with anti-inflammatory activity, namely by inhibiting the expression of inflammatory markers in a murine macrophage cell line (Yeung *et al.*, 2004). The present study aims to further characterize the pharmacological properties of CIAD7 and understand if it can modulate macrophage phenotypes, namely: i) if it can inhibit the inflammatory M1 macrophage phenotype; ii) if it can induce the M2 phenotype in naïve macrophages and, finally; iii) if it can potentiate the pro-resolution effect of IL-4, that is, its ability to induce the M2 phenotype. For this, we began by evaluating potential cytotoxic or anti-proliferative effects of various concentrations of CIAD7 using the metabolic activity as an indicator of cell death or proliferation. Then, we measured the effect of CIAD7 on NO production and iNOS protein levels to confirm that the experimental conditions used corroborate previous results from our group. Finally, we used flow cytometry to evaluate the expression of surface markers characteristic of the M1 (CD80 and CD86) and M2 (CD163 and CD206) phenotypes and measured arginase activity as another indicator of M2 polarization. RVT was used as a positive pharmacological control.

2. MATERIALS AND METHODS

2.1 Macrophage cell culture and treatments

The mouse macrophage cell line, Raw 264.7 (ATCC No. TIB71), was cultured in 75 cm^2 flasks, in Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK) supplemented with 10% non-heat inactivated fetal bovine serum (FBS; Gibco, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin (Grisp, Portugal), 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., USA) and 3.5 g/L D-glucose (Sigma-Aldrich Co., USA). Cells were maintained in a humidified 5% CO2–95% air atmosphere at 37 °C. For experiments, macrophages were plated at a density of 3×10^5 cells/mL and left to stabilize for about 20 h. Raw 264.7 cells were used from passages 32 to 40.

For cell treatments, CIAD7 and RVT (Extrasynthese, France) were dissolved in dimethyl sulfoxide (DMSO; Honeywell, Germany). Lipopolysaccharides from Escherichia coli 026:B6 (LPS; Sigma-Aldrich Co., USA) were dissolved in phosphate buffered saline (PBS) and recombinant murine interleukin 4 (IL-4; Peprotech, USA) was dissolved in PBS containing 0.1% Bovine Serum Albumin (Sigma-Aldrich Co., USA). The concentrations of each compound and the experimental treatment periods are indicated in figure legends. DMSO was used as vehicle and added to control and LPS or IL-4-treated cell cultures to match the same concentration (0.1% *v/v*) as in cells treated with the compounds indicated above. The test compounds or the vehicle were added to macrophage cell cultures 1h before the pro-inflammatory stimulus, 1 µg/mL LPS or the anti-inflammatory stimulus, 10 ng/mL IL-4 and maintained for the rest of the experimental period.

2.2 Resazurin Reduction Assay

The metabolic activity of Raw 264.7 cells was assessed by the resazurin reduction assay. Resazurin is a synthetic non-toxic, non-fluorescent redox-sensitive compound and it can be used as an indicator of cellular metabolic activity to evaluate cell viability, proliferation and toxicity. In metabolically active cells, resazurin (blue) is irreversibly reduced to resorufin, a pink colored and fluorescent compound. This reaction is catalyzed by NADPH dehydrogenases which are enzymes that couple the conversion of resazurin to resorufin with the conversion of NADH to NAD+ (Devi e Dutta, 2017; Silanikove e Shapiro, 2013). In order to select non-cytotoxic concentrations of the test compounds, the resazurin solution was added to each well to a final concentration of 50 µM, 90 minutes before the end of the treatment. Then, absorbance at 570 nm and 620 nm (reference wavelength) were read in a

Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA). The reduction of resazurin was expressed as percentage of the absorbance mean value obtained in control cells, which was considered to be 100%.

2.3 Griess assay

The presence of nitrite (NO_2^-) was measured as the amount of nitrite accumulated in the culture supernatants by the Griess assay. Griess assay is a spectrophotometric assay based on the formation of a red-violet azo dye by reaction of NO_2^- with the Griess reagent. The NO_2^- under acidic conditions, reacts with sulfanilamide and N-(1-Naphthyl)ethylenediamine (NED) to form a pink colored compound (Green *et al.*, 1982). Briefly, 150 µL of cell culture medium were mixed with an equal volume of Griess reagent [equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) NED] in a 96-well plate and incubated at room temperature for 10 min in the dark. Then, the absorbance was read at 550 nm in a Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA). A series of sodium nitrite ($NaNO_2$) standards was analysed in parallel and used to set up the corresponding standard curve from which the concentration of nitrite in the media was calculated by interpolation of the absorbance of each sample.

2.4 Western Blot

After the treatment period, macrophages in monolayer culture were lysed with ice-cold RIPA buffer [150 mM sodium chloride, 50 mM Tris (pH 7.5), 5mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 1% Triton X-100, supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Germany) and a phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics, Germany), and incubated on ice for 30 minutes. The cell lysates were centrifuged at 14,000 rpm for 10 minutes at 4° C and the supernatants were stored at -20° C until use. Protein concentration was determined with the bicinchoninic acid kit (Sigma-Aldrich Co., USA) and total cell protein (25 µg) was denatured at 95° C in sample buffer (5% SDS, 0.125 M Tris–HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol and bromophenol blue) for 5 min. Proteins were separated by SDS-PAGE and were electrotransferred onto PVDF membranes using a wet transfer system at 350mA for 210 min. After membrane blocking with 5% nonfat dry milk in Tris-buffered saline (TBS)-Tween 20 (0.1%) for 2 h, membranes were probed overnight at 4° C with mouse monoclonal antibody against NOS2 (dilution 1:500; MAB9502, R&D Systems, USA). Then membranes were washed with TBS-Tween 20 and incubated anti-mouse alkaline phosphatase-conjugated secondary

antibodies (dilution 1:5000; sc-516102, Santa Cruz Biotechnology, Europe) for 1 h at room temperature. After washing, immune complexes were detected with Enhanced chemiluminescence (ECL) reagent (BioRad, USA) in the imaging system ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare, Sweden). The membranes were reprobed with a mouse monoclonal anti-β-Tubulin I antibody (dilution 1:20000; T7816, Sigma-Aldrich Co., USA), as a loading control, for 1 h at room temperature. Image analysis was performed with TotalLab TL120 software (Nonlinear Dynamics Ltd).

2.5 Arginase Activity Assay Kit

Arginase is an enzyme that catalyzes the conversion of arginine to urea and ornithine. In this assay, arginase activity was evaluated by quantifying the urea produced, which specifically reacts with the color development reagent to generate a colored product. The optical density of the resulting solution is proportional to the arginase activity. After treatment, Raw 264.7 cells in monolayer culture were washed twice with PBS, pH 7.4, harvested with the aid of cell scrapper and centrifuged at 1,000 g for 10 minutes at 4° C. Next, cells were lysed with ice-cold lysate buffer [10 mM Tris-HCl, pH 7.4, 0.4% (w/v) Triton X-100 and supplemented with protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Germany), then incubated on ice for 10 minutes. The cell lysates were centrifuged at 13,000 g for 10 minutes at 4° C and the supernatants were stored at -80° C until use. The Arginase activity assay kit (MAK 112, Sigma-Aldrich Co.,USA) was used to measure arginase activity in samples and standards, according to the manufacturer's protocol. The absorbance was measured at 450 nm using an absorbance microplate reader (Biotek, Winooski, VT, USA). The arginase activity of each sample was calculated using the formula Activity = [((A430)sample – (A430)blank) / ((A430)standard – (A430)water)] x [((1 mM x 50 x103) / (V x T))], where T corresponds to the reaction time in minutes, V to the sample volume (µL) added to well, 1 mM to the concentration of Urea Standard, 50 to the reaction volume (µL) and 10^3 to mM to µM conversion factor, and normalized to the protein concentration of each sample.

2.6 Flow Cytometry analysis

Flow cytometry is a technology that rapidly analyzes single cells or particles as they flow through single or multiple lasers while suspended in a buffered salt-based solution. To study the cell surface markers, after the treatment period, macrophages were washed twice with PBS, pH 7.4 and collected to eppendorfs, then centrifuged at 300 g for 3 minutes at 4° C. The resultant pellet was re-suspended in 400 µL of PBS, pH 7.4 supplemented with 1% FBS. Then, the cell suspension was distributed by two microcentrifuge tubes (100 µL each). One tube of

each sample received no further treatments and was used to assess the cells' autofluorescence. The other was incubated at 4° C, for 30 min, with FITC anti-mouse CD80, Phycoerythrin anti-mouse CD86, APC anti-mouse CD163 and PerCP/Cyanine 5.5 anti-mouseCD206 antibodies (BioLegend, USA). Then, 500 µL PBS, pH 7.4 were added to each sample and centrifuged at 300 g for 3 min at 4°C. The supernatants were discarded and 100 µL of PBS, pH 7.4 supplemented with 1% FBS were added to each microtube. Sample aliquots were analyzed in an Accuri C6 flow cytometer (BD Bioscience, San Jose, CA, USA). Results were analyzed with BD Accuri C6 software (BD Bioscience, USA) and presented as median fluorescence intensity (MFI) after subtraction of autofluorescence values.

2.7 Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Normality tests such as D'Agostino & Pearson omnibus, the Shapiro-Wilk and the Kolmogorov-Smirnov tests were performed and showed that the data follows a normal distribution. Therefore, statistical analysis was performed with one-way ANOVA and the Dunnett's post-test for multiple comparisons. A t-test was also performed whenever only two conditions needed to be compared.

Results were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Evaluation of the ability of CIAD7 to inhibit the LPS-induced M1 macrophage phenotype

3.1.1 Evaluation of the effect of CIAD7 on the metabolic activity of Raw 264.7 murine macrophages, in the presence and absence of LPS

To select non-cytotoxic concentrations of CIAD7 and RVT (used as a pharmacological control), in the absence or presence of LPS, were tested using the resazurin reduction assay (O'Brien *et al.*, 2000). Cytotoxicity was set at a decrease of metabolic activity greater than 30% relative to control cells, according to the standard for cytotoxicity assessment, ISO 10993-527 (Devi e Dutta, 2017). Furthermore, to determine whether any decrease of resazurin reduction is due to cell death or to decreased cell proliferation, untreated cell cultures were analyzed after the stabilization period (~20 h), that is, immediately before addition of vehicle or test compounds to other cultures (time 0 of the experiment). The results in figure 2 show that cells treated with the vehicle (0.1% DMSO) for 18 h reduced significantly more resazurin than cells left untreated at time 0, reflecting a significant increase in the number of metabolically active cells. Neither 10 μM RVT nor CIAD7 in concentrations ranging from 100 to 333 μM had any effect on the amount of resazurin reduced relative to cells treated with the vehicle alone (Fig. 2a). At the two highest concentrations, however, CIAD7 significantly decreased the number of metabolically active cells, relative to those treated with the vehicle (T=18 h). This suggests that CIAD7 reduced cell proliferation. Untreated cells at time 0 reduced considerably less resazurin than cells treated with the vehicle, demonstrating that at the begin of the treatment the number of metabolically active cells was significantly lower. Regarding concentrations tested in the presence of LPS, only the highest concentration of CIAD7 decreased cell viability significantly by more than 30%, compared to LPS alone (Fig. 2b).

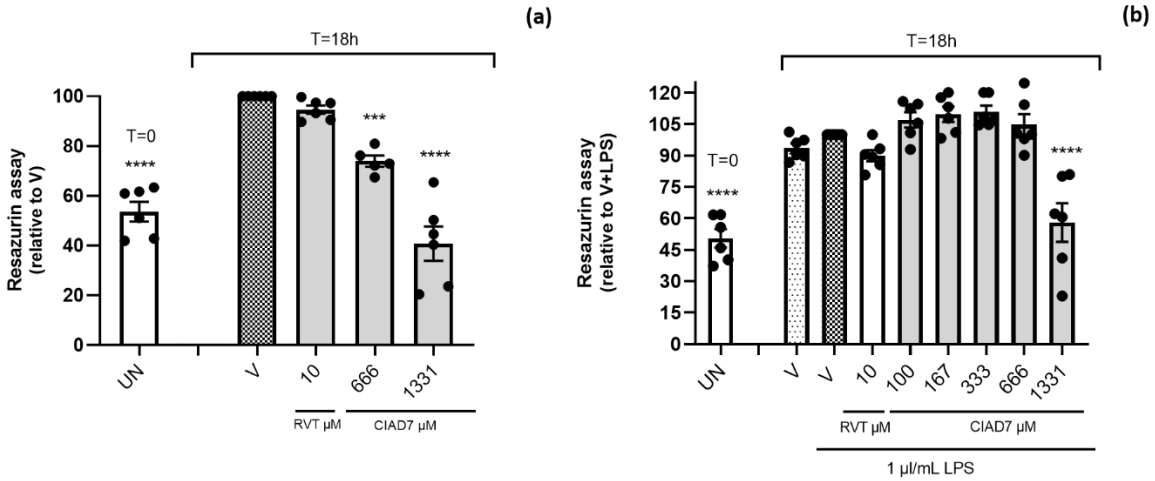

Figure 2: Effect of CIAD7 on resazurin reduction as an indicator of metabolically active cells, in the presence and absence of LPS, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then left untreated (a) or treated with 1 µg/mL LPS (b), respectively, for 18 h. Control cells for these groups were treated with vehicle alone (V: 0.1% DMSO) or with LPS in vehicle. Each column represents the mean ± SEM of, at least, three independent experiments. ****p<0.001 relative to cells treated with the vehicle alone (a) or to cells treated with vehicle and LPS (b).

3.1.2. Effect of CIAD7 on LPS-induced NO production, in murine macrophages

In the absence of LPS, neither RVT nor CIAD7 had any effect on basal NO production (Fig. 3a). On the other side, basal NO production was significantly enhanced in cells treated with LPS (V+LPS) compared to cells treated only with the vehicle (V). The three highest concentrations of CIAD7 reduced LPS-induced NO production in a concentration dependent manner. RVT had no significant effect on the NO levels (Fig. 3b).

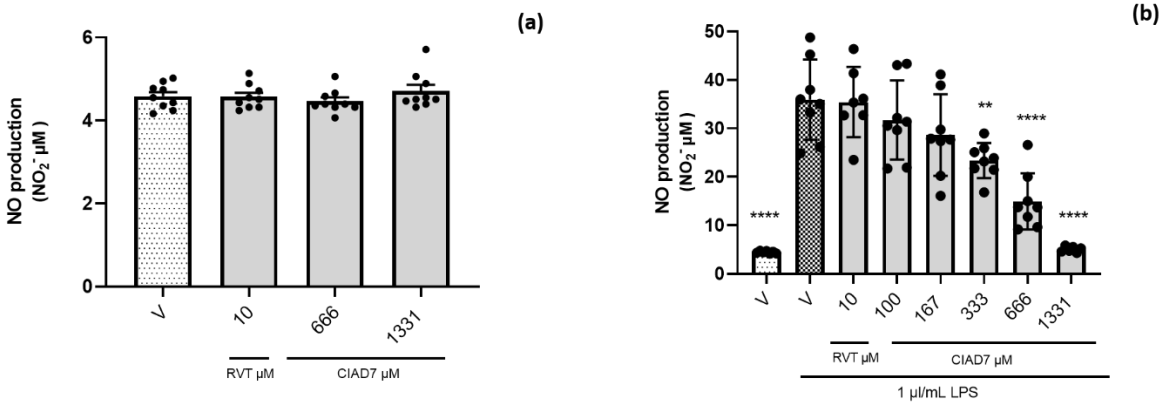

Figure 3: Effect of CIAD7 on nitric oxide (NO) production, in the absence and presence of LPS, in the murine macrophage cell line, Raw 264.7. NO production was evaluated as the amount of nitrite accumulated in the culture supernatants, collected 18 h after cell treatment. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then left untreated (a) or treated with 1 µg/mL LPS (b), respectively, for 18 h. Control cells for these groups were treated with vehicle (V: 0.1% DMSO) (a) or with the LPS in vehicle (V+LPS) (b). Each column represents the mean ± SEM of, at least, three independent experiments. **p<0.01, ***p<0.001 and ****p<0.0001 relative to V+LPS treated cells.

3.1.3 CIAD7 reduces LPS-induced iNOS protein levels in murine macrophages

To confirm whether the inhibition of NO production observed with CIAD7 results from a decrease in the iNOS levels inhibition of iNOS expression, its protein amount was assessed by western blot. Macrophage cells treated only with vehicle (V) have no detectable iNOS protein, whereas treatment with LPS (V+LPS) increased its levels which were reduced by all concentrations of CIAD7 tested. Importantly, the highest concentration of CIAD7 completely abolished the increase in iNOS protein levels induced by LPS (Fig. 4). Neither RVT nor CIAD7 had any effect on basal iNOS levels (data not shown).

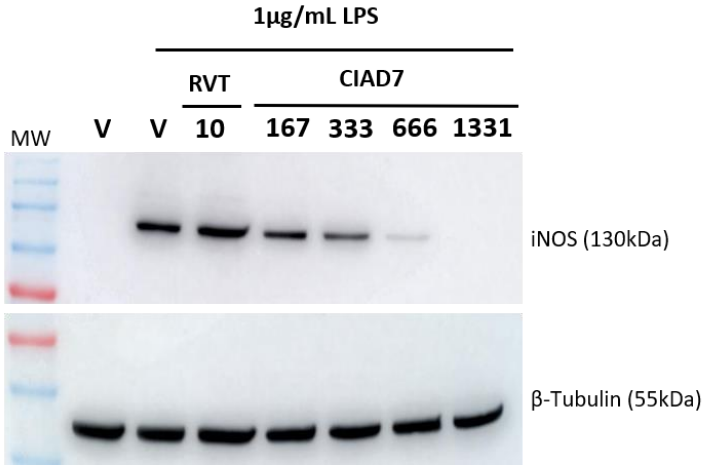

Figure 4: Effect of CIAD7 on iNOS protein levels in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then left untreated (V) or treated with 1 µg/mL LPS for 18 h.

3.1.4. Effect of CIAD7 on total Arginase activity, in the presence and absence of LPS, in murine macrophages

We investigated whether CIAD7 could influence arginase activity. In the absence of LPS, the two highest non-cytotoxic concentrations of CIAD7 tested, induced a significant increase in enzyme activity, although only the results obtained with 666 µM reached statistical significance relative to the control (V) (Fig. 5a). Treatment with RVT had no effect on basal arginase activity. Treatment with LPS increased basal arginase activity significantly, which was not modified by pretreatment with any of the concentrations of CIAD7 tested nor with RVT (Fig. 5b).

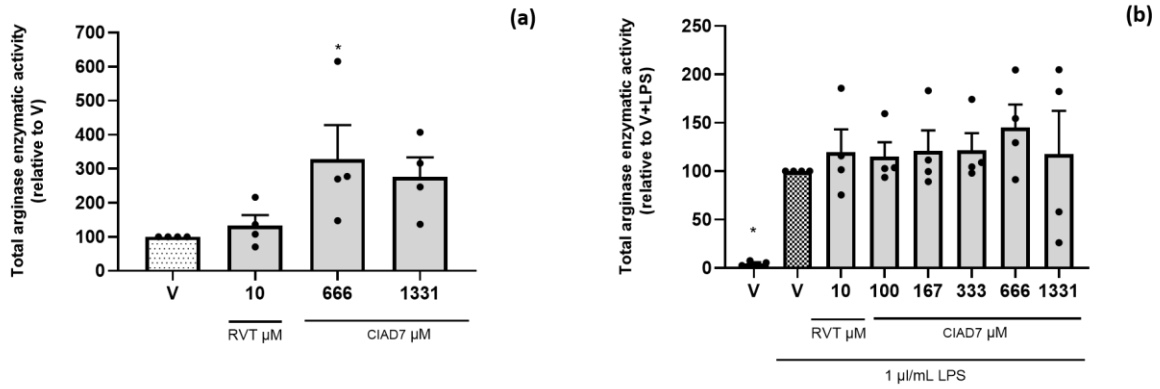

Figure 5: Effect of CIAD7 on Arginase activity, in the presence and absence of LPS in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then left untreated (a) or treated with 1 µg/mL LPS (b), respectively, for 18 h. Control cells for these groups were treated with vehicle (V: 0.1% DMSO) or with LPS in vehicle (V+LPS) and these groups correspond to 100% total arginase activity (a,b). Each column represents the mean ± SEM of, at least, three independent experiments. *$p<0.05$ relative to the control group (a).

3.1.5. Effect of CIAD7 on the expression of CD80 and CD86 proteins on the cell surface in murine macrophages.

The M1 cell surface markers, CD80 and CD86, were studied in macrophages by flow cytometry. Neither CIAD7 at a concentration of 666 µM, nor 10 µM RVT affected the basal expression of CD80 and CD86 (Fig. 6). Treatment with LPS significantly increased the expression of both markers, which was reduced by treatment with CIAD7, but not with RVT (Fig. 6). Nonetheless, while both concentrations of CIAD7 tested decreased CD86 and CD80 expression, the lowest concentration (167 µM) only decreased significantly CD86 expression, while the highest concentration (666 µM) inhibited both markers expression in a statistically significant manner (Fig. 6).

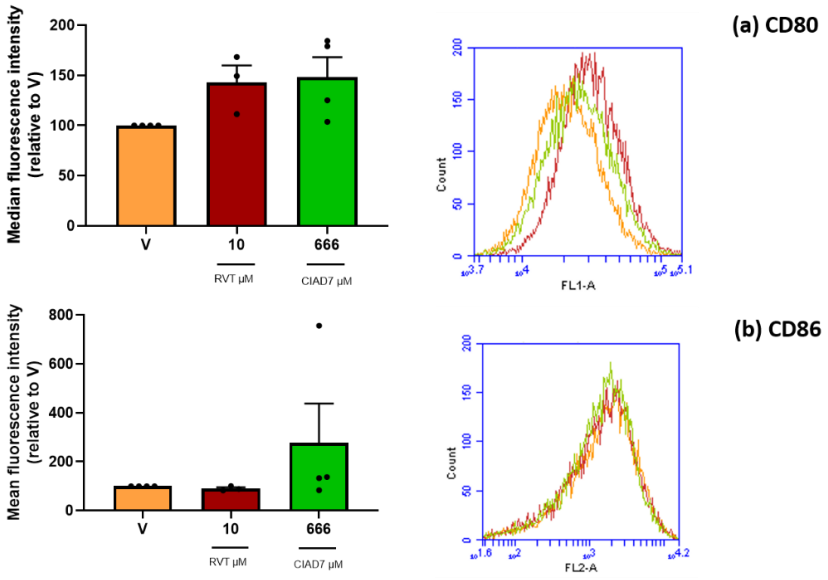

Figure 6: CIAD7 alone has no significant effect on the expression of M1 surface markers, CD80 and CD86, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with the indicated concentrations of CIAD7 or vehicle (0.1% DMSO) for 18 h. The expression of CD80 and CD86 was evaluated by flow cytometry. The histograms shown are representative of, at least, 3 independent experiments. Each column represents the mean ± SEM of, at least, three independent experiments.

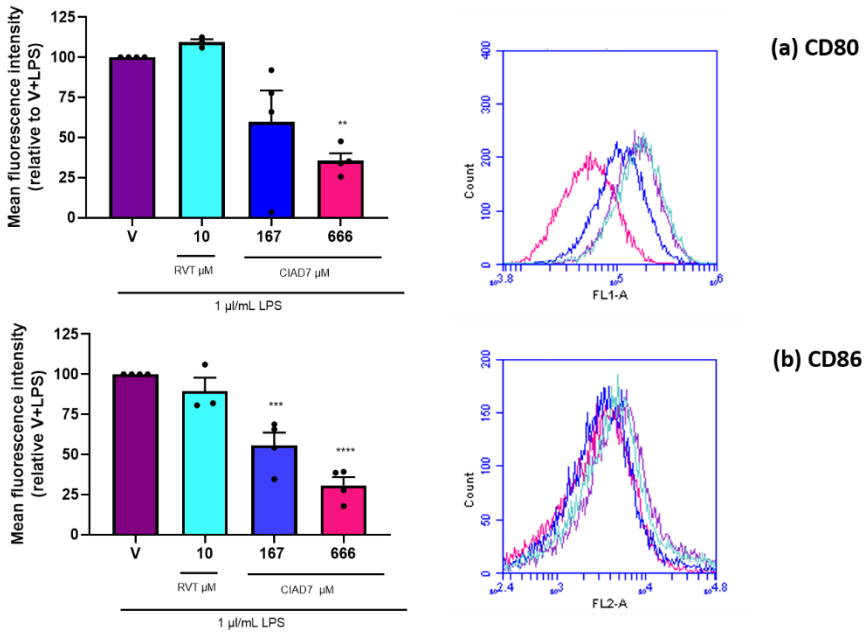

Figure 7: CIAD7 reduces the expression of M1 surface markers, CD80 and CD86, induced by LPS in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with the indicated concentrations of CIAD7 or vehicle (0.1% DMSO) for 1h followed by addition of 1 µg/mL LPS in vehicle for 18 h. The expression of CD80 and CD86 was evaluated by flow cytometry. The histograms shown are representative of, at least, 3 independent experiments. Each column represents the mean ± SEM of, at least, three independent experiments. **p<0.01, ***p<0.001 and ****p<0.0001 relative to V+LPS-treated cells.

3.2. Evaluation of the ability of CIAD7 to induce the M2 phenotype and/or to potentiate the pro-resolution effect of IL-4.

3.2.1 Evaluation of the effect of CIAD7 on the metabolic activity of Raw 264.7 murine macrophages, in the presence of IL-4

Five different concentrations of CIAD7, in the presence of IL-4, were evaluated for cytotoxicity using the resazurin reduction assay, as in the previous experiments in section 3.1.1. Relative to untreated cells (UN, time = 0), incubation for an additional 18 h with IL-4 and/or vehicle significantly increased the amount of resazurin reduced, reflecting an increase in the number of metabolically active cells. Treatment with CIAD7 in concentrations up to 333 µM or with 10 µM RVT, in the presence of IL-4, did not affect the number of metabolically active cells relative to cells treated with IL-4 and vehicle. The two highest concentrations of CIAD7 tested, 666 and 1331 µM, elicited a significant reduction of the number of metabolically active cells relative to cells treated with IL-4 and vehicle, but still above the result obtained in untreated cells at time 0. Therefore, these results suggest that the decrease in metabolic activity observed with 666 and 1331 µM CIAD7 are due to reduced proliferation, not cell death (Fig. 8).

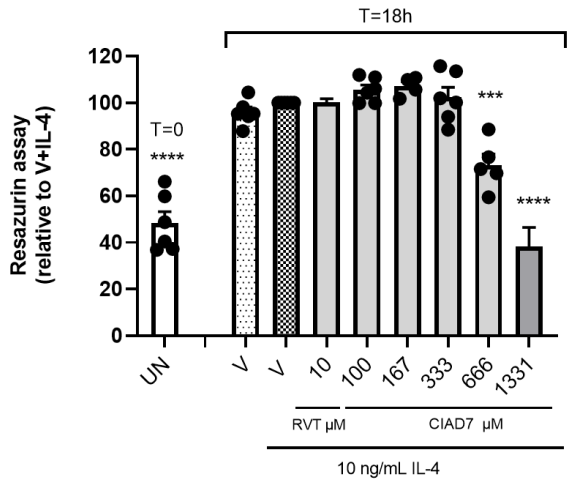

Figure 8: Effect of CIAD7 on the metabolic activity of cells treated with IL-4. The resazurin reduction assay was evaluated as an indicator of cell number. Raw 264.7 macrophage cultures were treated with vehicle (0.1% DMSO) or the indicated concentrations of CIAD7 or Resveratrol (RVT) for 1 h and then with 10 ng/mL IL-4 (V+IL-4) for 18 h. Control cells for these groups were treated with vehicle (V) or vehicle and IL-4 (V+IL-4). Each column represents the mean ± SEM of, at least, three independent experiments. ***p<0.001 and ****p<0.0001 relative to V+IL-4-treated cells.

3.2.2. Effect of CIAD7 on NO production, in the presence of IL-4, in murine macrophages

Macrophage treatment with IL-4 in the presence of the concentrations of CIAD7 or RVT did not affect basal NO production for any of the concentrations tested (Fig. 9).

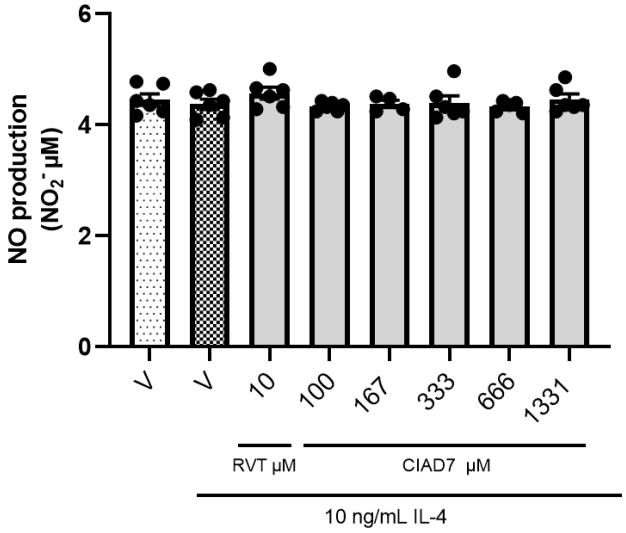

Figure 9: Effect of CIAD7 on nitric oxide (NO) production, in the presence of IL-4, in the murine macrophage cell line, Raw 264.7. NO production was evaluated as the amount of nitrite accumulated in the culture supernatants. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then with 10 ng/mL IL-4 (V+IL-4) for 18 h. Control cells for these groups were treated with vehicle (V) with or without IL-4 (V+IL-4). Each column represents the mean ± SEM of, at least, three independent experiments.

3.2.3. Effect of CIAD7 on total Arginase activity, in the presence of IL-4, in murine macrophages

IL-4-treated cells (V+IL-4), compared to cells treated with the vehicle alone (V), showed a significant increment of arginase activity. On the opposite, pretreatment with CIAD7 demonstrated a robust concentration-dependent reduction of IL-4-induced arginase activity relative to control cells (V). RVT had no impact in Arginase activity, in the presence of IL-4 (Fig. 10).

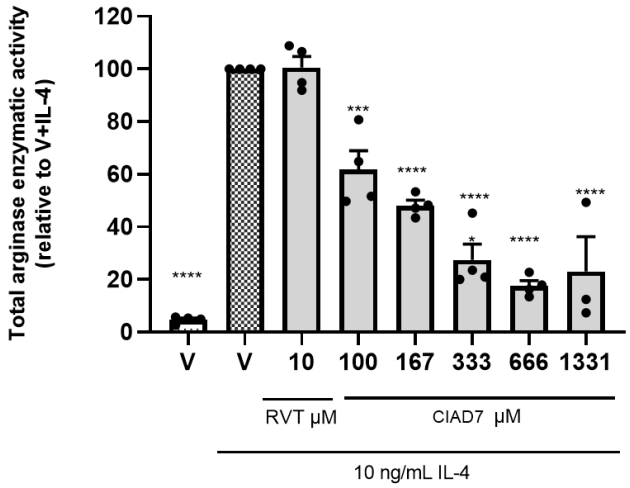

Figure 10: Effect of CIAD7 on Arginase activity in the presence of IL-4, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or the indicated concentrations of CIAD7 for 1 h and then with 10 ng/mL IL-4 (V+IL-4) for 18 h. Control cells for these groups were treated with vehicle with (V+IL-4) or without IL-4 (V). Each column represents the mean ± SEM of, at least, three independent experiments. ***p< 0.001 and ****p<0.0001.

3.2.4. Effect of CIAD7 on the expression of the M2 surface markers, CD163 and CD206, 18 h after treatment in murine macrophages.

On macrophages pre-treated with CIAD7 and stimulated or not with IL-4, the M2 cell surface markers, CD163 and CD206, were evaluated by flow cytometry. The results presented in fig. 11 and in fig. 12, show that none of the test compounds alone (Fig. 11), nor IL-4 (Fig. 12) had any effect on the expression of CD163 and CD206. Likewise, the expression of both markers remained unchanged, relative to cells treated with the vehicle alone, in cells treated with the test compounds and IL-4 (Fig. 11 and Fig. 12).

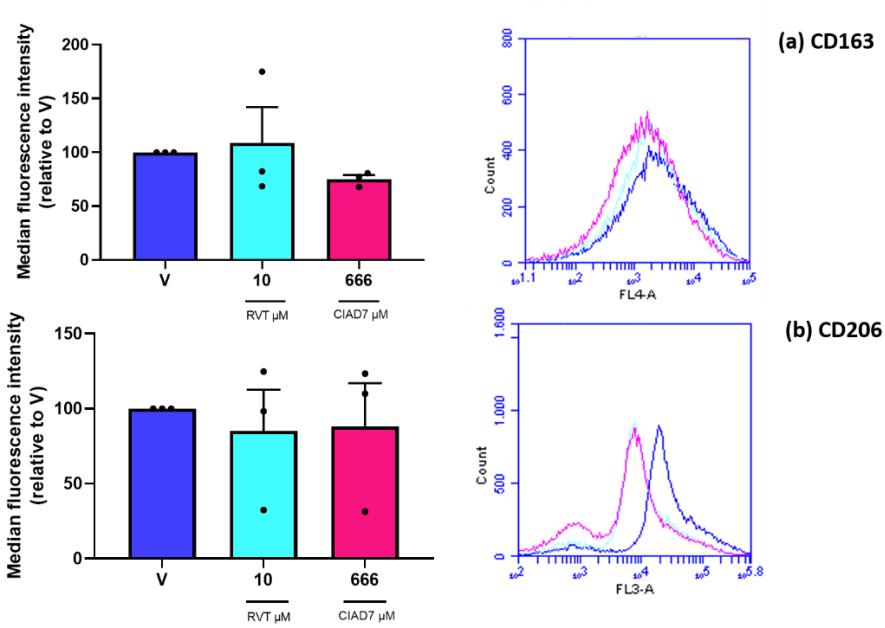

Figure 11: Effect of CIAD7 on the expression of M2 surface markers, CD163 and CD206, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with the indicated concentrations of CIAD7, vehicle (0.1% DMSO) or 10 µM resveratrol (RVT) for 18 h. The histograms shown are representative of, at least, 3 independent experiments. Each column represents the mean ± SEM of, at least, three independent experiments. No significant differences were detected relative to the control (V).

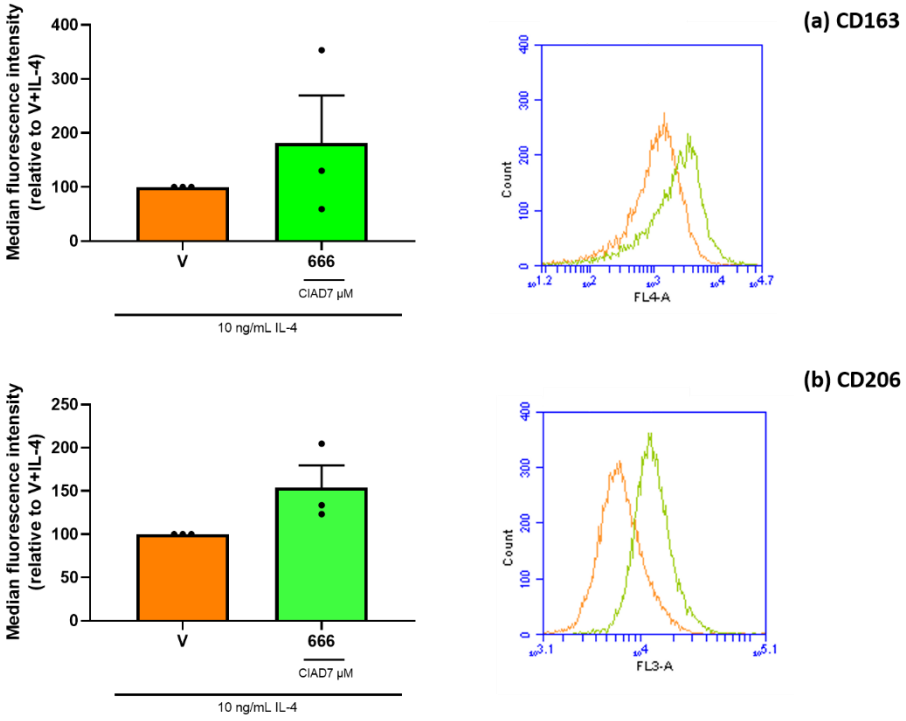

Figure 12: Effect of CIAD7 and IL-4 on the expression of M2 surface markers, CD163 and CD206, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with the indicated concentrations of CIAD7, vehicle (0.1% DMSO) or 10 µM resveratrol (RVT) for 1h followed or not by addition of 10 ng/mL IL-4, for 18 h. The histograms shown are representative of, at least, 3 independent experiments. Each column represents the mean ± SEM of, at least, three independent experiments. No significant differences were detected relative to the control V+IL-4.

3.2.5. Effect of CIAD7 on resazurin reduction 72h after treatment, in the presence of IL-4, in murine macrophages

Since under our experimental conditions, IL-4 failed to induce CD163 and CD206 expression and a recent study reported increased expression of these surface markers after treatment with IL-13 for 72h (Tariq *et al.*, 2017), we hypothesized that the response to IL-4 may also require a similar treatment period. Due to the previous inexistent data relative to CIAD7 cytotoxicity at 72h, this parameter was evaluated at this point by the resazurin reduction assay. Since we observed previously a significant reduction of proliferation in cells treated with 1331 µM CIAD7 for 18 h, only the concentration immediately below, 666 µM, was tested. RVT was tested at the same concentration used in previous experiments. Due to time constraints, each experiment in this section was performed only once, so there is no statistical data. Nonetheless, the metabolic activity of cells pretreated with CIAD7 and then with IL-4 for 72 h is identical to that observed in control cells with and without IL-4 treatment (Fig. 13). However, treatment with 10 µM RVT decreased resazurin reduction by approximately 50%, suggesting possible cytotoxic or anti-proliferative effects under these experimental conditions.

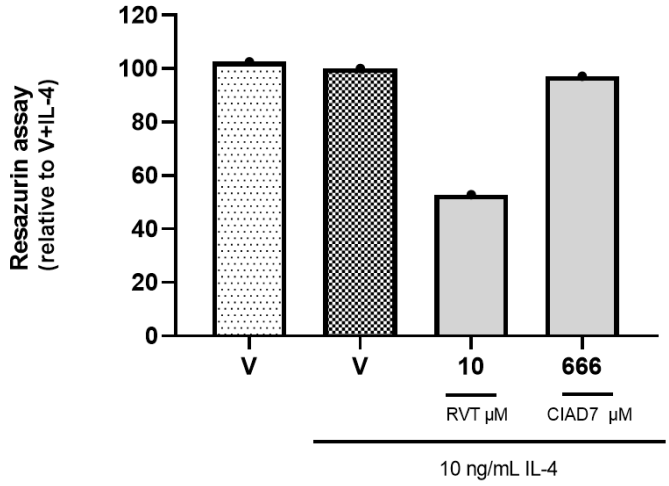

Figure 13: The effect of CIAD7 on resazurin reduction, 72h after treatment, in the presence of IL-4, in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 10 µM resveratrol (RVT) or 666 µM CIAD7 for 1 h and then with 10 ng/mL IL-4 (V+IL-4) for 72 h. Control cells for these groups were treated with vehicle and IL-4 (V+IL-4). Each column represents the result of one independent experiment.

3.2.6. CIAD7 increases the expression of CD163 and CD206 M2 surface markers, 72h after treatment, in murine macrophages.

Expression of CD163 and CD206 was evaluated 72h after treatment with the test compound or RVT w in cells stimulated with IL-4. Macrophages treated with CIAD7 and stimulated with IL-4 show higher expression of these M2 markers, relative to the control (V+IL-4). CIAD7 also seems to be more efficient than RVT. These results represent only one experience so no statistical data is available (Fig. 14).

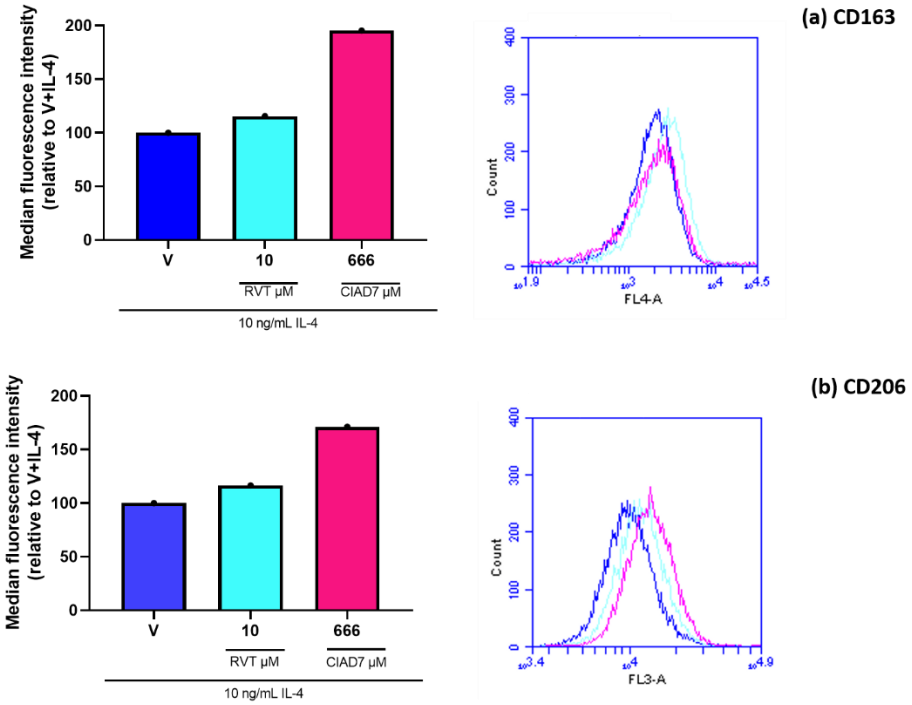

Figure 14: CIAD7 increases the expression of M2 surface markers, CD163 and CD206 induced by IL-4 in the murine macrophage cell line, Raw 264.7. Macrophage cultures were treated with vehicle (0.1% DMSO), 666 µM CIAD7 or 10 µM resveratrol (RVT) for 1h followed or not by addition of 10 ng/mL IL-4 in vehicle, for 72 h. The expression of CD163 and CD206 was evaluated by flow cytometry. Each column represents the result of a single independent experiment.

4. DISCUSSION AND CONCLUSIONS

Taken together, the results presented show that CIAD7 is effective in inhibiting the expression of inflammatory, M1 markers, namely NO production and the expression of iNOS, CD80 and CD86, induced by an inflammatory stimulus, LPS, in murine macrophages. These results confirm the anti-inflammatory properties of CIAD7 previously observed by our group. We also observed that the highest concentrations of CIAD7 tested have anti-proliferative effects in macrophages which may contribute to the anti-inflammatory activity of this natural compound. Treatment with CIAD7 alone was also effective in significantly increasing arginase activity, generally considered a M2 marker (Dyken, Van e Locksley, 2013; Mills, 2012). However, in macrophages stimulated for 18 h with IL-4, CIAD7 caused a significant reduction of the arginase activity induced by cytokine. Furthermore, in agreement with other studies (Louis *et al.*, 1999; Mori, 2007), we found that LPS also induced a significant increase of arginase activity which was not affected by pretreatment with CIAD7. Further experiments would be required to explain these intriguing observations. Since there are two isoforms of arginase, arginase 1 and 2, which seem to play different roles in inflammation and to be regulated by different stimuli (Louis *et al.*, 1999), it would be important to elucidate whether CIAD7 affects both or specifically one of the enzyme isoforms and if its effect occurs at the level of gene transcription and *de novo* synthesis or affects the enzyme activity directly.

On the other hand, pre-treatment with CIAD7 increased the expression of CD163 and CD206, two M2 markers, in cells treated with IL-4, suggesting that it can promote the M2 phenotype and the resolution of inflammation. However, under our experimental conditions, IL-4 alone did not increase the expression of CD163 and CD206. The results of these experiments need to be confirmed as we performed only one single experiment. Unfortunately, this was impossible due to time constraints.

Besides the present studies, it would also be important to consider that M2 macrophages can be induced by different stimuli and, as a result, four subgroups of M2 macrophages can be characterized. M2a macrophages are involved in wound healing and are induced by IL-4 and IL-13, expressing high levels of CD206 and secreting fibronectin that contributes to tissue repair. M2b macrophages, considered regulatory macrophages, can be induced upon combined exposure to IC and TLR agonists. These cells express and generate substantial amounts of the anti-inflammatory cytokine, IL-10 and are associated with cancer, promoting tumor development. The M2c subtype of macrophages are stimulated by IL-10 and exhibit strong anti-inflammatory activities by releasing large amounts of IL-10. This macrophage subset

efficiently phagocytes apoptotic cells. M2d macrophages are tumor-associated macrophages (TAMs) and are stimulated by IL-6, secreting high amounts of IL-10 and vascular endothelial growth factor (VEGF), which contributes to angiogenesis and cancer metastasis (Wang *et al.*, 2019).

Taking these features into account and to further elucidate the role of CIAD7 in modulating macrophage plasticity, it would be interesting to study its ability to modulate the phenotype induced by of other cytokines, namely IL-10. Moreover and considering the inability of IL-4 to induce CD163 and CD206 expression, it might be helpful to combine this cytokine with another one, like IL-13, to more efficiently induce the M2 phenotype and assess the effects of CIAD7. Other markers could also be assessed to better distinguish the M1 and M2 phenotypes, namely glycolysis and oxidative phosphorylation that are preferentially used for glucose metabolism by M1 and M2 macrophages, respectively. Finally, the Raw 264.7 cell line may not closely represent naïve macrophages and, thus, a more physiologic model should be used to further evaluate the anti-inflammatory and pro-resolution properties of CIAD7.

Despite these limitations, the present study confirms the anti-inflammatory properties of CIAD7 previously observed and provides evidence that it can also promote the pro-resolution phenotype in murine macrophages.

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