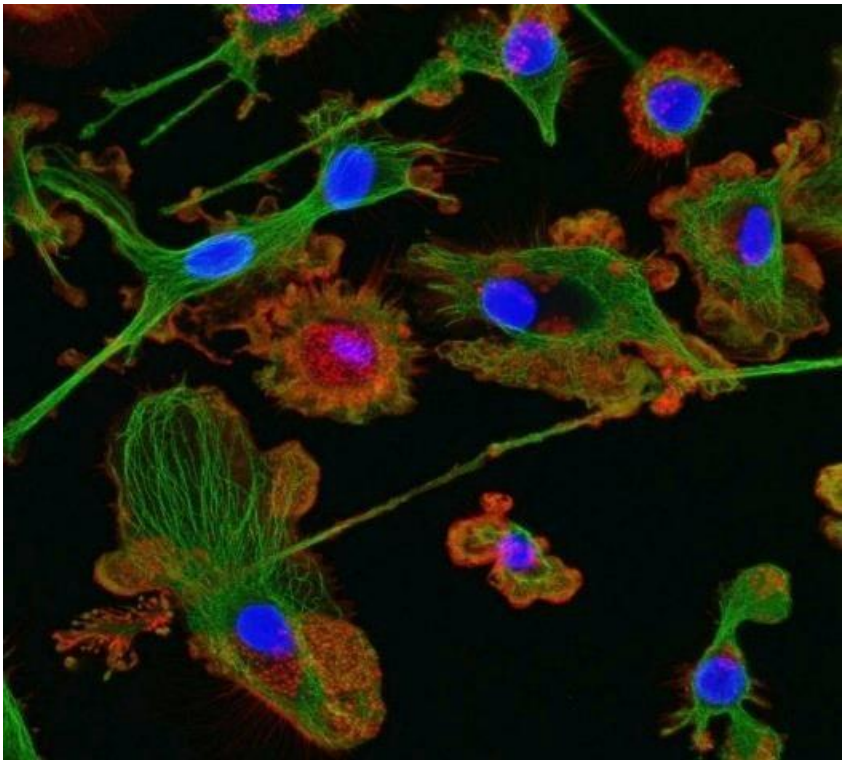

Adrenergic control of human macrophages polarization and its putative impact in cancer progression

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Mestrado em Bioquímica

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2017/2018

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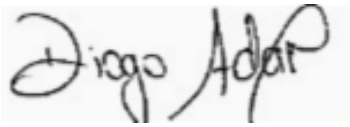

Acknowledgements

Em primeiro lugar quero fazer um agradecimento especial ao professor Jorge Gonçalves, por ter abraçado este projeto, por todos os ensinamentos que me deu durante este ano, mas principalmente por todas as ferramentas que me transmitiu para fazer sempre mais e melhor. Obrigado por toda a orientação e otimismo.

Agradeço também à Maria José Oliveira. Um obrigado por me ter recebido de braços abertos no grupo. Obrigado pelo conhecimento, pela paciência, por todos os ensinamentos e horas despendidas a falar comigo. Obrigado pelo apoio durante o ano, pelas grandes conversas e discussões sobre o trabalho, mas sobretudo obrigado pelos conselhos e os grandes serões passados no instituto.

Quero agradecer a toda o grupo de investigação. Ângela, por tudo o que me ensinaste, pela paciência e pelos brilhantes conselhos e opiniões. Patrícia, pela grande ajuda na iniciação das experiências, pelas opiniões e pela paciência que tiveste comigo. Um obrigado muito grande às duas. Obrigado à Flávia Castro pelas conversas, dicas e palhaçadas que mesmo estando longe, conseguiu ajudar tanto! Agradeço também à Rosa, à Flávia Pereira, à Andreia Matos e a Flávia Martins pela espirito de equipa e companheirismo! Quero ainda agradecer a todos os colegas do laboratório, à Xana, Cristina, Hugo e ao resto da equipa, pelos almoços, cafés e conversas.

Um enorme obrigado a todos os meus mais próximos! Aos da faculdade que percorreram esta jornada comigo, obrigado por tudo! Aos que estão comigo desde sempre obrigado pelas conversas, momentos e pela enorme amizade, agradeço a prontidão em atenderem o telefone, principalmente naqueles momentos mais desesperantes.

Por fim, um agradecimento especial à minha família, pelo apoio incondicional, a força e porque se não fossem vocês não chegava até aqui.

Resumo

O cancro é composto por células malignas, pela matriz extracelular, fibroblastos, células endoteliais, células imunes, vasos e nervos. A relação e a interação entre todos os componentes contribui para criar o microambiente tumoral. As catecolaminas (adrenalina e noradrenalina), neurotransmissores/hormonas libertadas em condições de stress, podem estar presentes no microambiente e promover a tumorigénese e prejudicar a vigilância imunológica. A noradrenalina e adrenalina exercem os seus efeitos através de uma família de recetores acopladas à proteína G, designados de recetores adrenérgicos. Três famílias de recetores adrenérgicos são reconhecidos, mas o subtipo β2-adrenérgico tem sido particularmente apontado como um imunomodulador e um recetor tumorigénico, devido à sua expressão em vários tipos de células imunes, como os macrófagos, um dos elementos mais abundantes no microambiente tumoral. Os macrófagos podem assumir diferentes fenótipos e podem agir como supressores tumorais (tipo M1) ou anti-inflamatórios e promotores tumorais (tipo M2), de acordo com os estímulos a que estão expostos.

A fim de compreender como a ativação β2-adrenérgica influencia a polarização de macrófagos humanos e se pode causar um impacto na invasão de células cancerígenas. Foram utilizados macrófagos humanos derivados de monócitos obtidos a partir de dadores saudáveis. Os macrófagos foram caracterizados pelos métodos apropriados (morfologia, expressão de genes, expressão de marcadores de superfície e libertação de citocinas) e estudados os efeitos do isoproterenol (um agonista dos recetores beta2 adrenérgicos) em macrófagos polarizados com LPS para um fenótipo tipo M1 ou por IL-10 para um fenótipo semelhante a M2. Além disso, analisou-se a capacidade desses macrófagos em promover a invasão de células cancerígenas.

O perfil de caracterização dos macrófagos revelou que diferentes estímulos (LPS ou IL-10) contribuem para a aquisição de diferentes fenótipos de macrófagos. A polarização dos LPS promovem um perfil pro-inflamatório, com altos níveis de citocinas pró-inflamatórias e células mais alongadas. Em contraste, os macrófagos polarizados com IL-10 apresentam uma forma arredondada e um perfil anti-inflamatório com elevados níveis de citocinas anti-inflamatórias.

Em relação ao efeito do isoproterenol, observou-se que, além das alterações na área celular dos macrófagos, este agonista induz alterações na expressão de marcadores de

polarização, impedindo um aumento de níveis de secreção de TNF, IL-6 e IL-12/23(p40) e também prevenindo o aumento da expressão de CD80/86, enquanto aumenta a secreção de IL-10. O mecanismo de transdução pode envolver, pelo menos em parte, a via do NF-kB. O isoproterenol reduz a fosforilação da p65 e do IkBa e, consequentemente a transcrição dos genes pró-inflamatórios.

Observou-se ainda que os efeitos do isoproterenol no fenótipo dos macrófagos tem impactos funcionais: (i) a atividade fagocítica é menor em macrófagos estimulados com LPS, mas tende a aumentar com o isoproterenol e (ii) a capacidade dos macrófagos em prevenir invasão de células cancerígenas é maior em macrófagos polarizados com LPS, mas parece estar diminuída pelo isoproterenol, fazendo com que os macrófagos assumam um perfil semelhante aos macrófagos com IL-10.

Os nossos resultados sugerem que estímulos que promovem o aumento de catecolaminas podem favorecer tumorigénese, por induzir a polarização de macrófagos, presentes no microambiente tumoral, em direção a um fenótipo imunossupressor. Alem disso, acreditamos que este conhecimento pode contribuir para o desenvolvimento de terapias anti tumorais mais eficientes, utilizando β-bloqueadores para reeducar macrófagos associados a tumores.

Palavras-chave: Microambiente tumoral, macrófagos, catecolaminas, recetores β2-adrenérgico, polarização, invasão de células cancerígenas.

Abstract

The cancer is composed by malignant cells, by extracellular matrix (ECM), fibroblasts, endothelial cells, immune cells, vessels and nerves. The relation and crosstalk between all components contribute to create the tumour microenvironment. Catecholamines (noradrenaline and adrenaline) neurotransmitters/hormones released under stress conditions, may be present in the microenvironment and may promote tumorigenesis and impair immune surveillance. Noradrenaline and adrenaline exert their effects through activation a family of G-protein coupled receptors, named adrenoceptors. Three families of adrenoceptors are recognized, but the β2-adrenoceptor subtype has been particularly pointed as an immunomodulator and tumorigenic receptor, because of its expression in several immune cells, such as macrophages, one of the most abundant element at the tumour microenvironment. Macrophages can assume different phenotypes and can act as tumour suppressors (M1-like) or anti-inflammatory and tumour promotors (M2-like), according to the stimuli they are exposed.

In order to understand how β2-adrenoceptor activation influence human macrophage polarization and if can cause an impact on cancer cell invasion, human macrophages derived from monocytes obtained from healthy donors were used. The macrophages were characterized by the appropriate methods (morphology, gene and surface markers expression and cytokine release) and studied the effect of isoproterenol (a β-adrenoceptor agonist) on macrophages polarized by LPS to a M1-like phenotype or by IL-10 to a M2-like phenotype. Additionally, the ability of these macrophages to promote the cancer cell invasion was analysed.

Macrophage characterization profile revealed that different stimuli (LPS or IL-10) contribute to acquiring different macrophage phenotypes. LPS polarization promotes a pro-inflammatory profile, with high levels of pro-inflammatory cytokines and more elongated cells. In contrast, the IL-10 polarized macrophages present a round shape and an anti-inflammatory profile with high levels of anti-inflammatory cytokines.

Concerning the effect of isoproterenol, it was observed that, in addition to changes in the cellular area of macrophages, this agonist induces alterations in the expression of polarization markers, preventing an LPS-induced increase in TNF-α, IL-6, and IL-12/23(p40) secretion levels, and also preventing the increase of CD86/80 expression, while increasing IL-10 secretion. The transduction mechanism may involve, at least in

part, the NF-kB pathway. Isoproterenol reduces p65 and IkBα phosphorylation and consequently, the transcription of the pro-inflammatory genes.

It was further observed that isoproterenol effects on macrophage phenotype has functional impacts: (i) the phagocytic activity is lower in LPS stimulated macrophages, but tends to enhanced by isoproterenol and (ii) the ability of macrophages to prevent cancer cell invasion is higher in LPS polarized macrophages but seems to be decreased by isoproterenol, suggesting that this β2-adrenoceptor agonist modulates macrophages to assume a profile similar to IL-10 polarized macrophages.

Our results suggest that stimuli that promote the increase of catecholamines may favour tumorigenesis, by inducing the polarization of macrophages, present at the tumour microenvironment, towards an immunosuppressive phenotype. Furthermore, we believe that this knowledge may contribute to the development of more efficient anti-tumour therapies using b β-blockers to re-educate tumour-associated macrophages.

Keywords: Tumour microenvironment, macrophages, catecholamines, β2-adrenoceptors, polarization, cancer cell invasion.

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List of Abbreviations

APC – Allophycocyanin

APCs – Antigen Presenting Cell

BSA – Bovine Serum Albumin

CAF – Cancer Associated Fibroblasts

cAMP - cyclic adenosine monophosphate

CBP – CREB-binding protein

CCL18 – C-C Chemokine Ligand

CD - Cluster of Differentiation

CREB – cAMP response element binding

CXCL – C-X-C Chemokine Ligand

DAPI – 4´, 6-Diamidino-2-Phenylindole

DCs – Dendritic Cells

ECM – Extracellular Matrix

EGF – Epidermal Growth factor

ELISA – Enzyme-linked Immunosorbent Assay

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FGFs – Fibroblast Growth Factors

FITC- Fluorescien Isothiocyanate

GRK – G-protein coupled receptor Kinase

HLA-DR – Human Leukocyte Antigen – D Related

IFN-ϒ – Interferon-gamma

IL- Interleukin

LPA – Lysophosphatidic Acid

LPS - Lipopolysaccharide

MAPK – Mitogen-activated protein kinase

M-C SF – Macrophage Colony-Stimulating Factor

MDSCs – Myeloid-derived Suppressor Cells

MFI – Mean Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMPs – Matrix Metalloproteinases

NF-kB – Nuclear Factor kappa B

NK - Natural Killers

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate-Buffered Saline

PDGFs- Platelet-derived Growth Factors

PDL1 - Programmed death-ligand 1

PE - Phycoerythrin

PFA - Paraformaldehyde

PI – Propidium Iodide

PKA – Protein Kinase A

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

rpm – Rotations per minute

RT – Room Temperature

SNS – Sympathetic Nervous System

STAT- Signalling Transducer an Activator of Transcription

TAMs – Tumour-associated Macrophages

TGF-β – Transforming Growth Factor – beta

TIMPs - Tissue Inhibitors of Metalloproteinases

TLR4 – Toll-Like Receptor 4

TNF-α – Tumour Necrosis Factor - alpha

VEGF – vascular endotelial Growth Factor

β2-ADR – β2-adrenoceptor

Introduction

1. The tumour microenvironment

Tumours are very complex structures. They are composed by malignant cells but also by a variety of stromal cells and non-tumour-cells, such as fibroblasts, endothelial and immune cells but also nerves, all embedded within an enriched extracellular matrix (Fig.1) (1,2). All these elements are in a microenvironment which contains soluble growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), which are key regulators of cell migration, adhesion, and angiogenesis but also modulators of immune surveillance (3,4). Therefore, to understand the complex interactions established between these elements, a holistic approach is required, to highlight the contribution of each of them for homeostasis, and to retrieve information on their role on tumorigenesis. This integrative knowledge may provide novel avenues for the development of more efficient therapeutic strategies. Unfortunately, the role of these components in cancer development is, still, largely unknown. In these chapter, an attempt will be made to summarize some of the known contributions of each element to the so-called, tumour microenvironment.

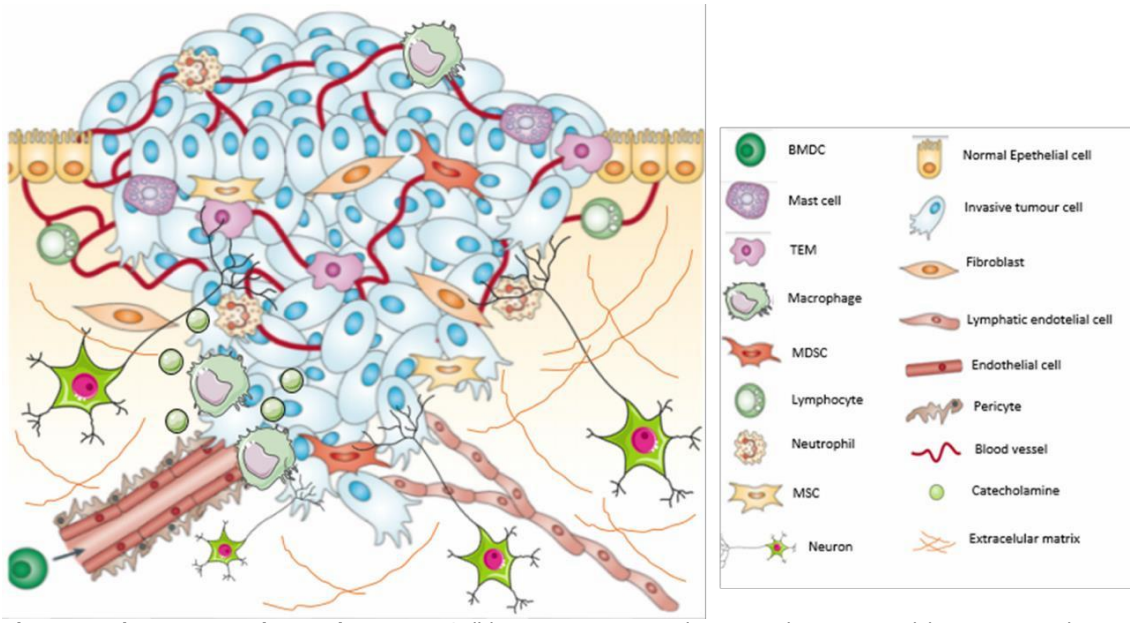


Figure 1. The tumour microenvironment. Solid tumours are complex networks composed by cancer and non-malignant cells, supported by extracellular matrix components. The microenvironment is composed by fibroblasts, endothelial cells, and a variety of immune cells, namely macrophages. Another important component are the nerves that produce catecholamine within the tumour microenvironment. Adapted from Joyce and Pollard et al., 2009; and Hayakawa and Wang et al., 2009.

1.1. Cellular components

1.1.1. Fibroblasts

In normal tissues, fibroblasts are distributed along the stroma. They have a crucial role on extracellular matrix (ECM) production and remodelling, wound healing repair and, when activated, on cell proliferation, differentiation, migration and survival (1). In cancer, fibroblasts are called cancer-associated fibroblasts (CAFs) and their activities are described to support cancer cell invasion and metastasis (5). In parallel, the activation of stromal fibroblasts is associated with factors secreted by tumour cells, such as transforming growth factor-beta (TGF-β), interleukin-6 (IL-6), IL-1β and lysophosphatidic acid (LPA), with the ability to transform precancerous fibroblasts into CAFs (6).
The presence of CAFs was proposed to precede the onset of invasion and to contribute to tumour growth and progression (7). CAFs secret pro-migratory ECM components, upregulate the expression of metalloproteinases (MMP)s, which degrade and remodel the ECM, drives angiogenesis and recruits inflammatory cells (8). Additionally, CAFs express a range of growth factors and cytokines, such as CXCL12, a chemokine that can promote growth and survival of cancer cells, and chemoattractant properties that stimulate the migration of other stromal cell types. CAFs also express TGF-β, which induces epithelial-mesenchymal transition in malignant cells, and contributes to the immunosuppressive microenvironment (9).

1.1.2. Endothelial cells

Vascular endothelial cells are also crucial for tumour growth, supporting oxygen and nutrients access, and permitting the diffusion of the metabolites produced within the highly proliferative tumour(1,8). Importantly, the new vasculature formed (angiogenesis) is abnormal in almost all aspects of their structure and function, lacking pericytes and presenting fenestrations, which facilitate tumour cell intravasation and metastasis (10). Angiogenesis is induced by VEGFs, FGFs, PDGFs (platelet-derived growth factors), and chemokines, produced and sustained by tumour cells but also by the other elements within the tumour microenvironment (9,11). Such regulation may be altered along cancer development since, in advanced stages, tumour angiogenesis can become VEGF-independent and supported by the production of other pro-angiogenic molecules (12).

1.1.3. Adipocytes

Adicpocytes are a cell type able to store excess of energy in the form of lipids. These cells can produce hormones, growth factors and adipokines (13). The release of the growth factors and adipokines has been associated with promotion of cancer cell proliferation, invasive potential, survival and angiogenesis. Adipokines secretion aid in recruitment of tumour cells, and fatty acids provided by adipocytes work as source of energy for cancer cells, promoting tumour progression (14,15).

1.1.4. Extracellular matrix

The major component of the tumour microenvironment is the extracellular matrix (ECM), a complex network of macromolecules with different physical, biochemical and biomechanical properties (16). The ECM is composed by numerous proteins that regulate tissue homeostasis, organ development, inflammation and disease. It is produced in excess by CAFs, other stromal cells, and by cancer cells (17). Structurally supports the tumour microenvironment and interacts with cancer cells through molecular and mechanical mechanisms (18).

ECM is composed by fibrous proteins (as collagen and elastin), adhesive proteins (as fibronectin and laminin), proteoglycans (as hyaluronic acid and aggrecan) and several growth factors that are secreted locally (19). Collagens are the most abundant ECM components, and they offer the structural integrity the tensile strength of tissues and organs. In general, these ECM components regulate the physical and the biochemical properties of the tumour microenvironment, modulating cancer survival, polarity, migration, invasion, metastasis and signalling (16).

Structurally, the ECM can be organized in the basement membrane and interstitial matrix. The basement membrane is produced by epithelial, endothelial and stromal cells and is mainly composed by collagen type IV, laminins, fibronectin and proteoglycans. The interstitial matrix is produced by stromal cells and constituted by fibrillar collagens, proteoglycans and glycoproteins. It is less compact and more porous than the basement membrane (19).

The ECM remodelling is regulated by the expression of enzymes, as MMPs and tissue inhibitors of MMPs (TIMPs) (20) which, when uncontrolled, can have devastating destructive consequences on tissues (16). In tumours, an altered ECM remodelling, with

enhanced proteolysis and increased stiffness, is frequently associated with poor prognosis (21).

1.1.5. Immune cells

The existence of a functional interaction between inflammation and cancer is not new, but recent studies have demonstrated that inflammation is a critical component for tumour progression (22).

In the tumour microenvironment, a multitude of immune cells might be found, as macrophages, polymorphonuclear leukocytes natural killer cells (NK cells), T lymphocytes, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) and, occasionally, B cells (23).

Under the perspective of the innate immunity, macrophages play a crucial role in the defence against foreign pathogens and in the modulation of the adaptive immune response (24) and their role will be discussed in more detail in the next section.

The link between innate and the adaptive immunity involves antigen presenting cells (APCs). Tumour cells contribute to the recruitment of innate immune cells through the expression of antigens that can activate APCs, as macrophages and dendritic cells, which in turn, mediate the proliferation and activation of host CD8+ T cells. (25). Additionally, pro-inflammatory cytokines, released by dying tumour cells, potentiate DCs and macrophages recruitment and activation. These APCs will present the antigens captured on major histocompatibility complex (MHC-I and MHC-II),originating a T cell response against the presented tumour-specific antigens (Fig.2) (26).

T cell response involves migration of DCs-activated T cells from the lymph nodes to the tumour microenvironment where they may recognize, bind and kill cancer cells, a process that may induce the release of additional tumour-associated antigens and further recruitment of more T cells (27).

B cells can also be found in tumours, particularly at the invasive margin. However, it is more common to found B cells in draining lymph nodes and lymphoid structures adjacent to the tumour microenvironment (9).

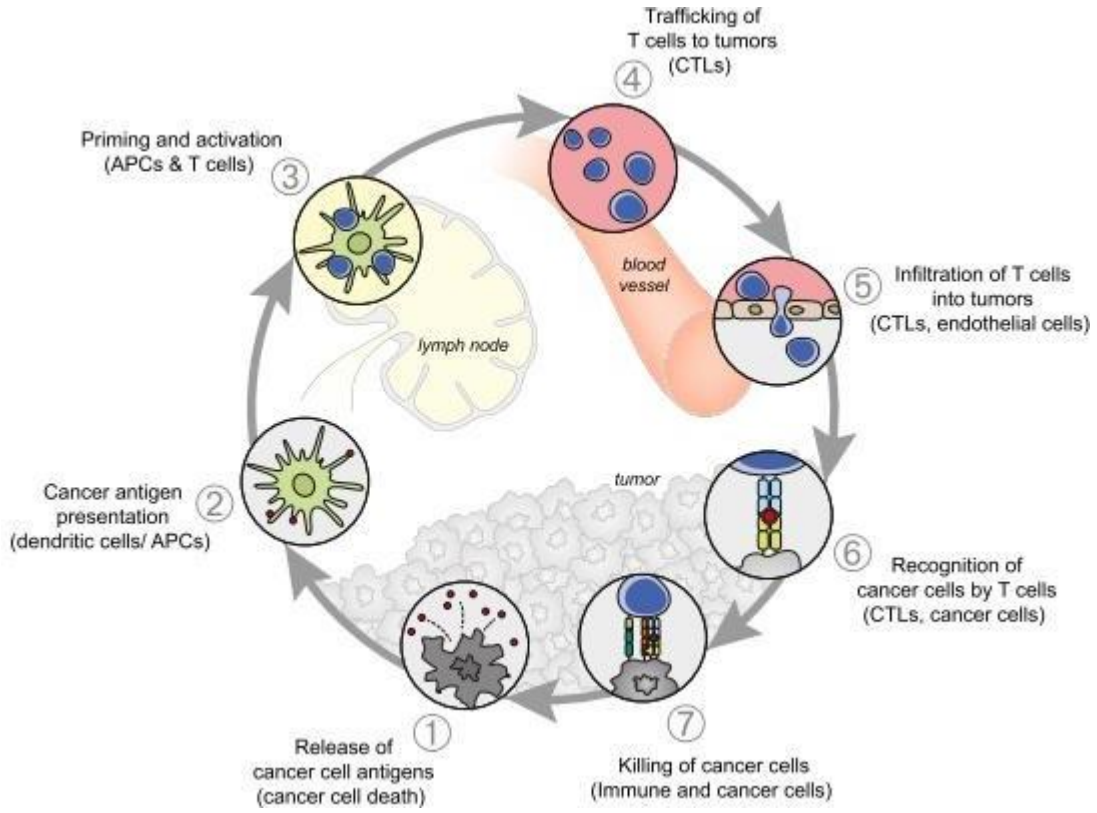


Figure 2. The immune cells contribution for tumour progression. In the tumour microenvironment, several studies, evidenced of a T-cell-infiltrated phenotype. It has been proposed that a series of steps must occur in order to mount an anti-tumour immune response able to effectively kill the cancer cells (26).

2. Macrophages

One of the main characteristics of macrophages are their diversity and plasticity. These characteristics are crucial to the acquisition of distinct phenotypes and biological functions, depending on the surrounding microenvironment (24,28,29).

In a simplistic manner, macrophages exist in manly two different subtypes: the classically activated (M1-like) or the alternatively activated (M2-like), a nomenclature inspired by the Th1 and Th2 classification for T helper cells. M1 and M2 correspond to extreme populations because polarization causes a wide spectrum of cells containing a mix of characteristics from pro- to anti-inflammatory phenotypes (30,31).

For a proper characterization of macrophage subpopulations, it is necessary to evaluate gene expression profile, protein surface markers, the pattern of cytokines and chemokines produced and even their cytoskeleton organization. According to Cardoso and colleagues, M1-like macrophages display a more elongated morphology than M2

macrophages (32). In general, M1-like macrophages are polarized in response to interferon-gamma (IFN-ϒ), lipopolysaccharides (LPS) and cytokines, such as tumour necrosis factor -α (TNF-α). M1-like are able to secrete high levels of pro-inflammatory mediators, such as interleukins (IL)-1, IL-6, IL-12, IL-23, TNF- α, nitric oxide and reactive oxygen intermediates, and release low levels of immunosuppressive cytokines such as IL-10, CCL18 and TGF-β (28,33,34).

Functionally, M1-like macrophages are recruited in response to viral and bacterial infections, but also to the abnormal tumour microenvironment, at initial stages of tumour development. They are seen as pro-inflammatory, but are also designated as anti-tumour since they may act, as APCs to cytotoxic T cells, fighting against tumour growth (24,30,35). Activated M1 macrophages express high levels of co-stimulatory and antigen presenting molecules such as CD80, CD86 and MHC-I and II molecules on their surface (31,36), and their presence in the tumour microenvironment may favour activation of adaptive immune responses.

On their turn, M2-like macrophages are considered anti-inflammatory and lack the ability to inhibit tumour growth. Polarization to M2 phenotype may be caused by IL-4, IL-13, IL-10 and also by IL-33, which amplifies IL-13-induced polarization. M2-polarization may also be favoured by other factors, as glucocorticoids and vitamin D3. M2 macrophages release anti-inflammatory cytokines, with a profile characterized by high levels of IL-10, TGF-β and low levels of IL-12; and expressing high levels of mannose (CD206) and scavenger (CD163) receptors (28,31,37,38).M2-like macrophage phenotype can be subcategorized in different subtypes: M2a, M2b, M2c and M2d (39). Generally, the M2a subtype is induced by IL-4 and IL-13, and produces high levels of CD206. The M2b are activated by IL-1 receptor ligand and LPS; M2c are polarized by IL-10, TGF-β and glucocorticoids, and the M2d subtype is induced by IL-6 and adenosine. Each M2 subtype present different functions: M2a are associated with parasite killing; M2b with immunoregulatory functions; M2c with immune response and tissue remodelling; while M2d have some features of the so called tumour-associated macrophages (TAMs) (Fig.3) (30,37).

2.1. Tumour-associated macrophages

TAMs are abundantly present in the tumour microenvironment of distinct types of cancers, and have been suggested to contribute to tumour progression (40). TAMs are recruited by several messengers produced by tumour and stromal cells, such as tumour-derived C-C Motif Chemokine Ligand 2 (CCL2), CCL3, CCL4, CCL5, CCL7, CCL8, C-X-C Motif Chemokine Ligand 12 (CXCL12), macrophage colony stimulating factor (M-CSF), VEGF, and IL-1 (41).TAMs infiltration is strongly associated with poor prognosis and reduce survival rates in cancer patients and any therapy to prevent TAMs polarization or its re-polarization towards the pro-inflammatory M1-like phenotype is expected to represent a favourable anti-cancer therapy (31,42).

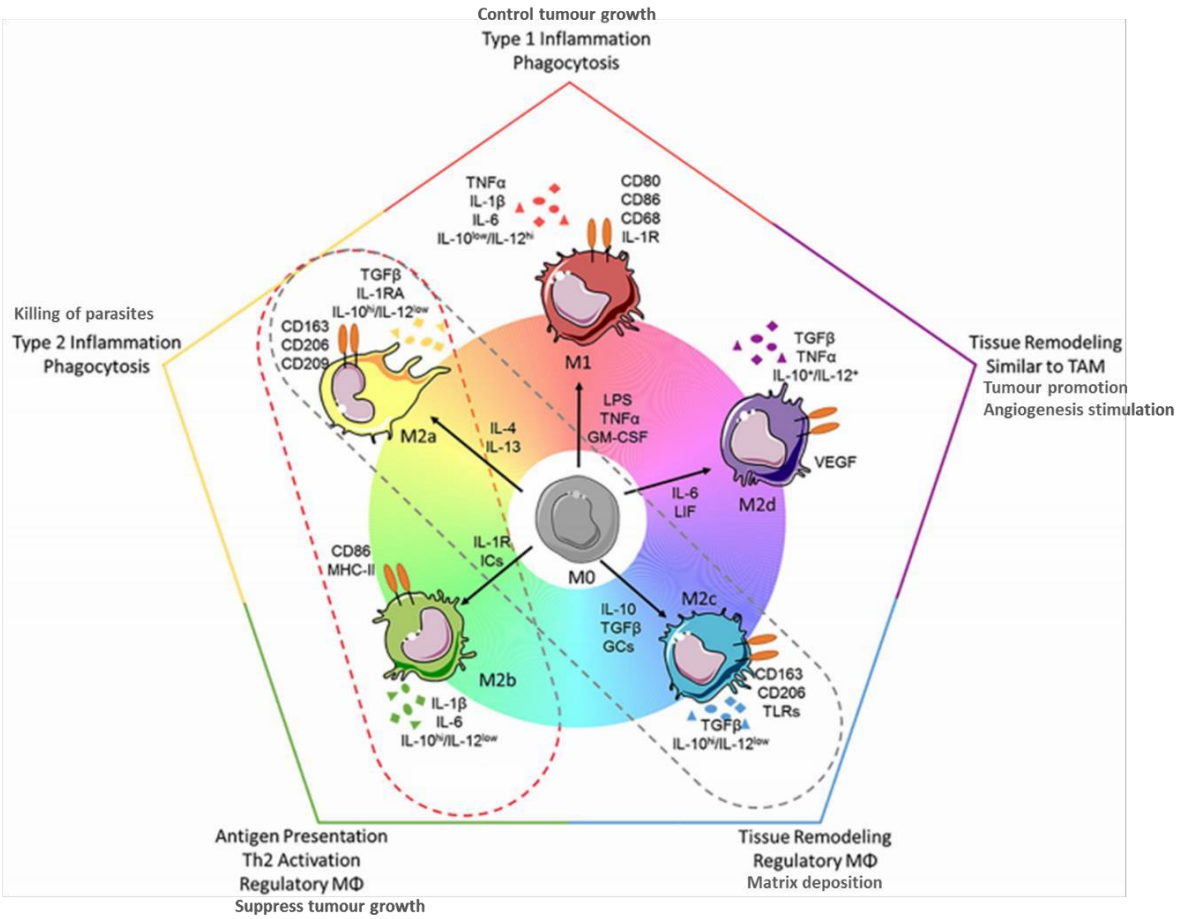


Figure 3. Spectrum of macrophage polarization. Macrophage polarization is nowadays considered as a continuum spectrum. Accordingly, macrophages are divided into four main categories: the pro-inflammatory M1 and the anti-inflammatory M2, subdivided into M2a, M2b, M2c and M2d macrophages. M1 or different M2 macrophage subtypes may be identified by the surface receptors expressed, the messengers secreted and their function. Adapted from Schliefsteiner et al., 2017

3. Adrenergic system, cancer and macrophages

3.1. Adrenergic system and receptors

The sympathetic nervous system (SNS) is a division of the autonomic nervous system known to mediate the “flight-or-fight” response. It acts by releasing noradrenaline and adrenaline among other co-transmitters, from the postganglionic sympathetic nerve terminals and from the adrenal gland (43).

Effects of noradrenaline and adrenaline are mediated by a family of receptors, called adrenoceptors (44). Adrenoceptors are a class of membrane-bound receptors that are widely dispersed throughout the human body, including in immune cells (45,46) and thus involved in the modulation of the immune response (47,48).

Adrenoceptors are a type of G protein-coupled receptors (49). Three families of adrenoceptors are accepted: alpha1 (α1), alpha2 (α2) and beta (β) adrenoceptors, based on their genetic and molecular characterization and on their pharmacological profile (50). Each of these families can be subdivided into three subtypes: α_{1A}, α_{1B} and α_{1D}; α_{2A}, α_{2B} and α_{2C}; and β1, β2 and β3 (51), which differ on the potency of adrenaline and noradrenaline response. For the purposed of this work, the higher potency of adrenaline (released predominantly from the adrenal medulla) (52) for the β2 subtypes should be kept in mind.

Since all adrenoceptors subtypes are G-protein coupled receptors, they share similarities on their structure. The extracellular and transmembrane domains are responsible for forming and stabilizing the ligand-binding site. On the other hand, the intracellular regions are associated with G-proteins, which are coupled to different signalling cascades (53). The activation of different second messenger systems can promote to a variety of outcomes, such as intracellular Ca^{2+} release, ion channel activation, kinase activation, protein phosphorylation and gene transcription. In spite of the similar structure, it is important to note that each subtype is specific for certain ligands, activating specific coupled G-proteins and consequently particular second messenger signalling systems (48,54).

Adrenoceptors are involved in a wide range of physiological activities including the pro- and anti-inflammatory immune response. Importantly, β2-adrenoceptors have been identified on several immunocompetent cells and are known to influence their inflammatory immune response (55). Because of the relevance of β2-adrenoceptors for the modulation of the immune cells, particularly of macrophage responses, its transduction mechanism is described in more detailed, namely its desensitization and internalization mechanisms.

The β2-adrenoceptor activation leads to the increase of the cyclic adenosine monophosphate (cAMP) levels due to the activation of adenylate cyclase by the α-subunit of the receptor associated Gs-protein. The increase of the cAMP leads, on its turn, to the stimulation of protein kinase A (PKA) (56). Another mechanism implicates the Gi-dependent pathway that results in the activation of the mitogen-activated protein kinase (MAPK) cascade(57,58). This pathway requires the phosphorylation of the β2-adrenoceptor by PKA and is mediated by the βϒ-subunits of the associated G-protein, The cytoplasmic regions are also involved in functional regulation of β2-adrenoceptors signalling, either on receptor desensitization or on activation of distinct transduction pathways (59).

The process of receptor desensitization is very important to prevent overstimulation of the β2-adrenoceptors in presence of excessive receptor ligand (Fig.4). The desensitization can occur via receptor phosphorylation, receptor internalization and receptor uncoupling (60), involving G-protein coupled receptor kinases (GRK) and β-arrestins. The receptor internalization drives β2-adrenoceptors into endocytic vesicles, which are recycled back to the cell surface or sorted into lysosomal vesicles where they are degraded. Sometimes, this degradation results in debris that trigger the activation of intracellular signalling pathways (61). Therefore, this “desensitization” may be, under some circumstances, a mechanism to displace the signalling to cytoplasmic compartments different from that reached by the membrane bound receptors. For both processes, β-arrestin seems to play a very important role, not only to promote internalization but also as a scaffold for other signalling molecules, such as SOS, cSrc, RAS and Raf (62).

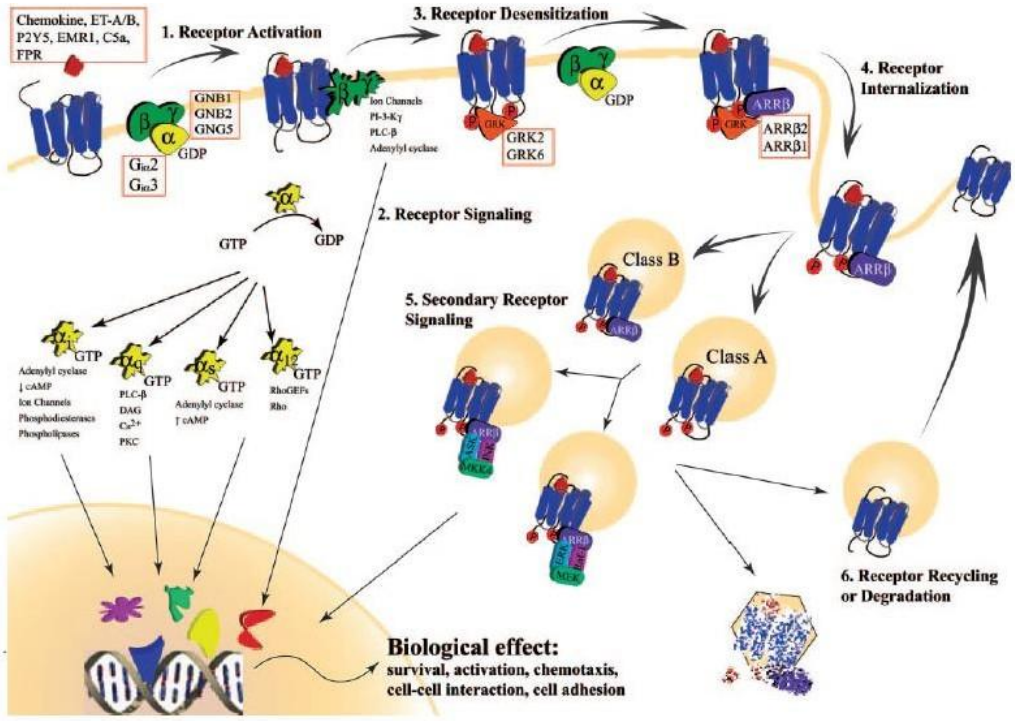


Figure 4. G-protein coupled receptor signalling pathway. The agonist binds the receptor which is coupled to the G-protein and promotes the exchange of GDP for GTP on α-subunit. The GTP-bound α-subunit dissociates from βϒ-subunit; the free subunits then regulate effector enzymes positively or negatively, ultimately leading to a biological response. G-protein signalling is terminated by receptor desensitization and internalization, mediated by GPCR kinases (GRK) and β-arrestins. Finally, the receptor is then recycled to the membrane to undergo further rounds of signalling or is targeted for degradation (61)

3.2. Catecholamines influence on macrophages

Macrophage polarization may also be influenced by neurotransmitters and by hormones, namely by those released during stress responses (63), as glucocorticoids, adrenaline and noradrenaline, the “classical” messengers of the SNS.

It has been also suggested that SNS-mediated effects might be associated with an increased vulnerability to tumour growth and metastasis (64,65). I*n vivo* studies have showed that stress conditions can accelerate the progression of prostate, breast, ovarian carcinomas, neuroblastomas, malignant melanomas, pancreatic carcinoma and some haematopoietic cancers (66).

The SNS stimulation can regulate tumour microenvironment through different pathways. It may act distally, by influencing the immune response through regulation of biological processes such as myelopoiesis, in bone marrow and lymph nodes, and lymphocyte differentiation in secondary lymphoid organs (spleen and lymph nodes) (67). SNS can also influence directly the tumour microenvironment by releasing catecholamines,

produced by the adrenal gland or by the sympathetic nerve terminals, which activate adrenergic receptors on the surface of adipocytes, immune, stromal, and cancer cells. However at the tumour microenvironment, other cells than those of neuroendocrine origin, as cancer and immune cells, may sustain paracrine or autocrine catecholamines production. Curiously, recent studies reported that macrophages when stimulated, can generate and release their own catecholamines (55,68,69).

The adrenoceptor-mediated effects on cancer cells seem to involve activation of β-adrenoceptors (mainly β_2), promoting tumour proliferation, angiogenesis, invasion, migration and metastasis (70). In breast and ovarian cancers, these tumorigenic effects are mediated by β2-adrenoceptors (71,72). The tumorigenic effect involves an increase of VEGF secretion (73), protection of tumour cells from programmed cell death by inhibiting anoikis (66,74,75), and activation of the transcription factor-3 (STAT-3), leading to its downstream effects on cell proliferation, survival, angiogenesis, and inhibition of apoptosis (60). In addition a number of different other cell types present within the tumour microenvironment express β-adrenoceptors, and thus are able to response to stress signalling. These include adipocytes, immune and endothelial cells (2,76,77). In immune cells, β2-adrenergic receptor is recognized to be the main subtype of adrenoceptors expressed (70,78).

Macrophages are targets of the adrenergic stimulation (79). In murine peritoneal macrophages, adrenaline and noradrenaline promote a M2-like macrophage phenotype, inhibiting LPS-induced TNF-α and increasing IL-10 secretion (80), through a β2-adrenoceptor mediated mechanism. That may also be observed in man, creating favourable conditions to polarize macrophages to a M2-like phenotype, anti-inflammatory and favourable to tumorigenesis. Blockade of this β2-adrenoceptor-mediated immunosuppression could constitute a potential pharmacological alternative to other immunomodulators already in use in cancer.

4. Therapeutically approach

The so-called check-point inhibitors, such as anti-PDL1 and anti-PD1 drugs, are examples of such strategy (81,82). However, the costs of these therapies are very high and their efficacy and safety are still questioned. If proved a relevant role of β2-adrenoceptors on macrophage polarization in human macrophages, such pathway could be explored by using the wide offer of β-adrenoceptor antagonists, used for several cardiovascular diseases, as repurposed drugs in cancer. Such possibility already has a scientific ground (83). Several pharmacoepidemiologic and retrospective studies suggest a decrease in cancer progression for several solid tumours in patients that were exposed to β-blockers, which do appear as a novel adjuvant to existing therapeutic strategies in clinical oncology (84).

5. Aim of this thesis

The aim of the present study was to investigate the effect of isoproterenol, a β2-adrenoceptor agonist, on human macrophages polarization, and to evaluate its impact on macrophage-mediated cancer cell invasion and phagocytosis. We believe that this knowledge will contribute to exploit the use of β2-adrenoreceptors antagonists as modulators of the immune response in cancer.

Although the ultimate goal would be the therapeutic use of β-adrenoceptor antagonist for cancer patients' treatment, the approach used was based on the effects of an agonist on macrophage polarization. This can be seen as an indirect approach. However, it was considered the only feasible. Theoretically, a more direct approach using antagonists on patients would be more appropriate. However, it would require a clinical study relating the plasma catecholamine levels and the macrophage phenotype that predominate in each donor, with and without taking β-adrenoceptor antagonists. It would require time and resources incompatible with a work like this. In spite of such limitations, the results obtained with an agonist have, in our view, the merit to provide evidence that allow us to anticipate what could happen *in vivo* when endogenous released catecholamines activate macrophage β2-adrenocetors in patients. They have also the merit to provide an explanation of the above referred antitumor effects of β-adrenoceptor antagonists and, hopefully, an additional stimulus for the urgency of develop more clinical studies to define when and in which patients with cancer may profit from the use of these drugs.

Materials and Methods

Ethics Statement

Human monocytes were obtained from buffy coats, a leukocyte-enriched waste-product that results from a whole blood donation, from healthy blood donors. A protocol was established between our Institute and Centro Hospitalar São João (CHSJ), where blood donations were performed, allows the use of these products for investigation purposes. Studies using this human material were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), respecting the Helsinki declaration. Informed consent was obtained from all subjects before each blood donation.

Human peripheral blood monocyte isolation and macrophage differentiation

Human monocytes were isolated from buffy coats from healthy blood donors, provided by Centro Hospitalar São João-Service of Hematology. Briefly, buffy coats were centrifuged at 1200 g for 20 minutes at room temperature, without brake. The peripheral blood mononuclear cells (PBMCs) were then collected, from a whitish layer, and incubated, during 20 minutes under rotation, with the RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies). This mixture was then diluted (1:1) in PBS and 2% FBS (Biowest), added carefully over Ficol-Histopaque 1077 (Sigma), and centrifuged at 1200 g during 20 minutes at room temperature, without brake. After this procedure, an intermediate layer, enriched in human monocytes, was collected, washed in PBS and centrifuged three times at 700 rpm for 17 minutes. Then, cells were resuspended in complete RPMI medium and $1,2x10^6$ macrophages were plated in 6-well culture plates with glass coverslips inserted and incubated at 37°C and 5% CO_2 humidified atmosphere. Macrophages were allowed to differentiate in the presence of 50 $ngmL^{-1}$ of M-CSF (ImmunoTools) during 7 days. After this period, cell culture media were replaced for media without M-CSF.

Macrophage polarization and reagents

After 10 days of seeding, macrophages were stimulated for 3 additional days with 10 ng/mL LPS (sigma-Aldrich) or 10 ng/mL IL-10 (ImmunoTools) towards a pro- or anti-inflammatory phenotype, M1- and M2-like, respectively. In parallel, these cells were treated or not with 100 nM isoproterenol (Sigma), a β2-adrenoceptor agonist (Fig.5).

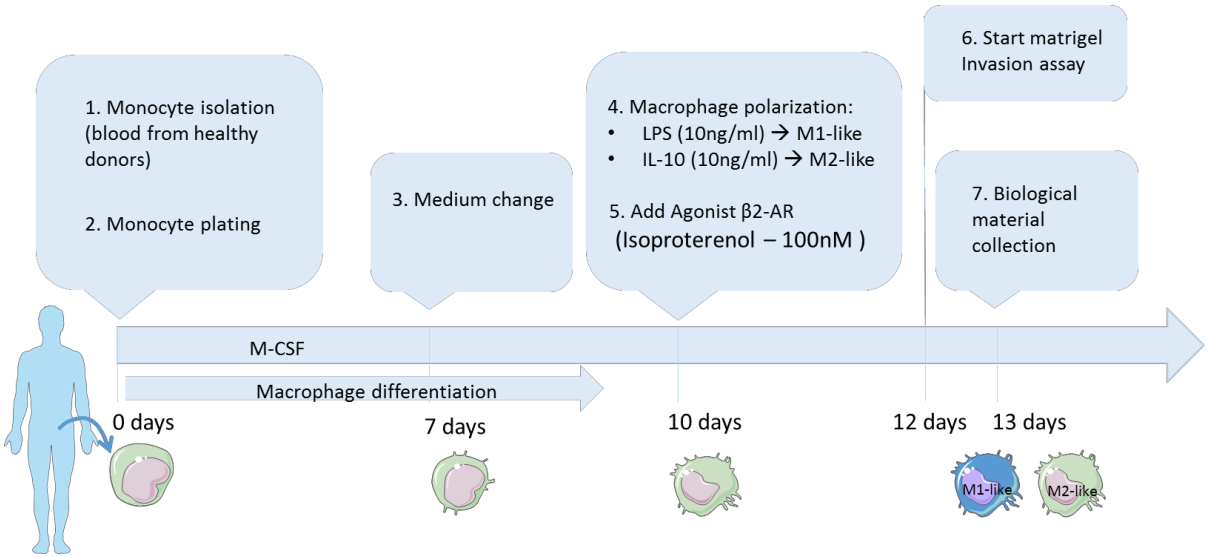


Figure 5. Experimental design used along this work. Monocytes were isolated from healthy blood donors, provided by Hospital de São João. Briefly, monocytes were plated (1,2x10^6) on culture plates with RPMI medium with GlutaMax supplemented with 10% FBS, PenStrep and M-CSF for 7 days. After this period of differentiation, cell culture media were renewed, and monocytes were left differentiating for additional 3 days with medium RPMI without M-CSF. Ten days after monocyte isolation, macrophages were stimulated with LPS or IL-10 towards a pro- (M1-like) or anti-inflammatory (M2-like) phenotype, respectively. At this time, these cells were treated or not with 100nM Isoproterenol, a β2-adrenoceptor agonist, for 3 additional days. Then, biological materials were collected. Matrigel invasion assays were performed at the 12th day for 24h.

Calcein-AM/Propidium Iodide assay

The impact of the different treatments on macrophage viability was determined using a fluorescence-based live and/or dead cell assay. Therefore, a viable staining for calcein-acetoxymethyl (calcein-AM) and positive death staining for Propidium iodide (PI) were performed. Calcein-acetoxymethyl (Calcein-AM) is permeable and highly lipophilic. It easily diffuses into the cells, and is hydrolysed by nonspecific esterases into green fluorescent products that are retained by cells with intact plasma membranes. Instead, propidium Iodide (PI) cannot pass through viable cell membranes. It reaches the nucleus

of non-viable cells by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix to emit red fluorescence. At the 13th day of culture, macrophages were washed with PBS and incubated for 15 minutes at 37°C and 5% CO_2 humidified atmosphere with 1 µM calcein-AM (λ_{ex}488 and λ_{em}530) (Molecular Probes, Invitrogen). After this incubation, the calcein-AM solution was removed, and macrophages were incubated with 4µg/mL PI (λ_{ex}535 and λ_{em}617) (Sigma) for 5 minutes at 37°C and 5% CO_2. Cells were then washed with PBS and, in order to prevent cell drying, complete RPMI medium was added. Cells were finally visualised using a fluorescence microscopy (Axiovert 200M, CarlZeiss) and the number of live (green) and dead (red) cells were counted with Fiji software.

Resazurin assay

The metabolic activity of unstimulated, LPS- and IL-10- stimulated macrophages, treated or not with isoproterenol was measured by the resazurin reduction assay. Resazurin is a cell permeable redox indicator that can be used to monitor the metabolic activity of viable cells. Viable cells with active metabolism can reduce resazurin into the resorufin product, which is pink and fluorescent and can be quantified using a microplate fluorometer. At the end of the experiments, resazurin redox dye (0.01 mgmL^{-1}, Sigma-Aldrich) was added (1/10 of the total volume of culture medium) to cell culture wells, which were then incubated for 4h at 37°C and 5% CO_2 humidified atmosphere. Fluorescence intensity was measured (λ_{ex} 530 nm, λ_{em} 590 nm) using the multi-mode microplate reader Synergy Mix (BioTek).

Conditioned media processing

Conditioned media collected from unstimulated and LPS or IL-10-stimulated macrophages, treated or not with isoproterenol, were centrifuged at 1500 rpm for 5 minutes at room temperature. Supernatant were then transferred to a new eppendorf and frozen at -20°C.

Enzyme-linked Immunosorbent assay (ELISA)

TNF-α, IL-6, IL-10 and IL-12/23(p40) cytokines, present in conditioned media from unstimulated, LPS- and IL-10- stimulated macrophages, in the presence or absence of isoproterenol, were measured by ELISA kits (Biolegend, LEGEND MAX), according to

the manufacturer´s instructions. At the end, absorbance levels were measured using the multi-mode microplate reader Synergy Mix (BioTek).

Flow Cytometry

For cell surface receptor expression analysis, unstimulated, LPS- and IL-10- stimulated macrophages, in the presence or absence of isoproterenol, were harvested by incubation with accutase (BD) for 30 minutes at 37°C. Cells that remained adhered were then washed with PBS, and gently detached by scraping, and centrifuged at 1500 rpm for 5 minutes at 4°C. Pellets were then resuspended in flow cytometry buffer (PBS, 2%FBS, 0,01% sodium azide) and stained with anti-human CD14-APC, CD86-FITC, CD163-PE (Invitrogen), for 40 minutes at 4°C in the dark. After additional washing steps were performed with flow cytometry buffer, macrophages were fixed with 1% PFA for 20 minutes. Subsequently, samples were filtered and acquired with FACS Canto Flow Cytometry (BD Biosciences) using FACS Diva software. Data analysis was performed using the FlowJo software.

Immunocytochemistry

Macrophage cytoskeleton organization was visualized by immunocytochemistry staining for actin/tubulin. At the thirteenth day of culture, unstimulated, LPS- and IL-10-stimulated macrophages, polarized in the presence or absence of isoproterenol, were fixed with 4% PFA for 20 minutes at room temperature. Cells were then washed in PBS, and quenched with 50 mM NH_4Cl for 10 minutes, to block free aldehyde groups. After washing with PBS, macrophages permeabilization was performed with 0.2% Triton X-100 for 5 minutes, followed by another wash with PBS, and 5% BSA (Sigma-Aldrich) blocking for 30 minutes, to minimize unspecific antibody binding. After this procedure, macrophages were incubated, for 1 hour, with primary and secondary antibodies, mouse anti-α-tubulin (1:4000, Sigma Aldrich), Alexa Fluor 488 goat anti-mouse (1:1000, Invitrogen, Molecular Probes), respectively. At the end, F-actin was stained with Alexa Fluor 568 Phalloidin (1:40, Invitrogen, Molecular Probes) for additional 20 minutes. In order to visualize the presence and distribution of β2-adrenoceptors (β2-ADR) in the distinct phenotypes, macrophages were incubated, for 1hour, with the primary rabbit anti-β2ADR antibody (1:100, Proteintech) and during 45 minutes with the secondary Alexa 488 goat anti-rabbit antibody (1:1000, Invitrogen, Molecular Probes). After staining, coverslips were mounted on Vectashield with DAPI (Vector Laboratories) and visualized with a Zeiss Axio Imager

Z1 fluorescence microscope (CarlZeiss) or with the high resolution Leica TCS-SP5 AOBS confocal microscope. In this case, images were obtained with a 63X 1.4 numerica aperture objective, at zoom 1.3 and Zstacks were performed with an interval of 1µm. At the end, a maximum projection was performed to facilitate overall visualization.

Calculation of macrophage aspect ratio and area measurement

FiJi software was used to quantify the cell aspect ratio and the area of actin/tubulin stained macrophages upon LPS- and IL-10-stimulation and treatment or not with isoproterenol. Cell aspect ratio was calculated as the quotient between the length of each cell major and minor axes. A minimum 100 cells/condition were analysed for each condition.

Phagocytosis assay

To evaluate the phagocytic activity of macrophages, pHrodo green *Staphylococcus aureus* BioParticles conjugate (Molecular Probes) were used. Briefly, pHrodo green *Staphylococcus aureus* Bioparticles conjugate were resuspended in PBS up to 1 mg/mL, gently vortexed and sonicated for 5 minutes to acquire a homogeneous dispersion. After engulfment by macrophages, red bioparticles were encapsulated in endocytic vesicles with an acidic environment and due to the reduced pH they emitted green fluorescence, measured under a fluorometer. Unstimulated, LPS- and IL-10-stimulated macrophages, polarized in the presence or absence of isoproterenol ($2.4x10^5$), were incubated with $9,6x10^6$ *S.aureus* particles at 37°C and a 5% CO_2 humidified atmosphere. After 1hour, macrophages were washed in PBS and fixed with 4% PFA for 20 minutes at room temperature. To facilitate cell identification, macrophages were incubated with mouse anti-α-tubulin antibody (1:4000, Invitrogen, Molecular Probes) following by incubation with the secondary goat anti-mouse 594 antibody (1:1000, Invitrogen, Molecular Probes) for 45 minutes, after previous quenching with 50 mM NH_4Cl for 10 minutes, permeabilization with 0.2% Triton X-100 for 5 minutes and blocking with 5% BSA for 30 minutes. Subsequently, coverslips were mounted on Vectashield with DAPI (for nuclei visualization). Images were acquired IN Cell Analyzer 2000 (GE Healthcare, United States), a high throughput wide field fluorescence microscope. Images analysis and quantification of the number of the cells able to phagocyte *S.aureus* particles were performed using Ilastik, a machine learning segmentation software and CellProfiler, an

image processing software. A minimum of 2500 cells/condition were analysed for each condition.

Invasion assays

Invasion assays were performed using Matrigel-coated invasion inserts of 8µm pore-size filters (Corning) to mimic the basement membrane, having AGS cells seeded in the upper compartment, and unstimulated, LPS- and IL-10- stimulated, in the presence or absence of isoproterenol in the lower compartment, as invasive stimuli. Before the experiment, filters were re-hydrated in RPMI medium for 1 hour at 37°C and a 5% CO_2 humidified atmosphere. Confluent AGS cells were then detached by trypsinization and resuspended in complete RPMI medium, and $5.0x10^4$ cells were seeded the upper compartment of Matrigel-coated filters. To discard any influence of soluble factors released along macrophage differentiation, media was renewed before invasion assays performance. Cells were then incubated for 24 hours at 37°C and a 5% CO_2 humidified atmosphere. The inserts were then washed in PBS and fixed with PFA 4%. Non-invasive cells, presents on the top of the insert, were removed by swabbing Invasive cells were mounted in Vectashield Mounting Medium with DAPI (Vectashield, Vector Laboratories) for nucleic staining, and visualized through a Leica DM2000 fluorescence microscope (Leica Microsystems).

Western Blot

Cells lysates were prepared to perform Western Blot. Briefly, macrophages were washed twice with cold PBS, and incubated in cold Rippa buffer [20mM Tris-HCl; 1% NonidetP (NP)-40; 150mM NaCl and 1% Triton-X-100] with proteases/phosphatases inhibitors [10mM NaF; 200mM phenylmethylsulfonyl fluoride (PMSF); 1 $mgmL^{-1}$ aprotinin and leupeptin; 50mM Na4VO3 and 50 $mgmL^{-1}$ Na4P2O7], on ice, during 15 minutes. After this incubation, cells were gently detached by scraping, supernatants collected and centrifuged at 14000 rpm for 10 minutes at 4°C. Protein concentration was determined using the DCProtein assay kit (BioRad) and 50µg of protein were mixed with Laemmli sample buffer [0.5 M Tris-HCl pH 6.8, 9.2g SDS, 40mL Glycerol, 5% β-mercaptoethanol, 5% bromophenol blue] and PBS, and for denaturation purposes, boiled for 5 minutes at 95°C. Thirty five micrograms of whole cell lysates were loaded on a 7.5% SDS-polycrylamide gel and run at 120V. Following electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare) for 2h at 100V. Membranes were then

incubated with Ponceau (Sigma) solution for 3 minutes. Subsequently, membranes were washed n PBS 1X and blocked with 5% non-fat powder milk in PBS+0.5% Tween-20 (PBS-T 0.5%) for 30 minutes while shaking and incubated overnight at 4°C with primary antibodies according to table 1. The next day, membranes were washed with PBS-T 0.5% and incubated with secondary antibodies during 1hour at room temperature while shaking, in agreement with table 2 selected antibodies. After the last wash, membranes were incubated with Clarity Western ECL Susbtrate (BioRad) for signal detection. Densitometry analysis of protein bands was performed with the QuantityOne software.

Table 1. *Primary antibodies for Western Blot*

TARGET MOLECULE	Species of origin	Molecular Weight (kDa)	Dilutions	Supplier	Incubation conditions
IkB-α	Rabbit	40	1.10000	Cell Signalling	4°C overnight
Phospho-IkB-α	Mouse	40	1.1000	Cell Signalling	4°C overnight
Src	Rabbit	60	1.1000	Cell Signalling	4°C overnight
Phospho-Src	Rabbit	60	1.1000	Cell Signalling	4°C overnight
p38	Mouse	38	1:500	Cell Signalling	4°C overnight
Phospho-p38	Mouse	38	1.1000	Cell Signalling	4°C overnight
NF-kB p65	Rabbit	65	1:500	Santa Cruz	4°C overnight
Phospho-NF-kB p65	Rabbit	65	1.1000	Cell Signalling	4°C overnight
β2-adrenoceptor	Rabbit	50-55	1.1000	Proteintech	4°C overnight
α-tubulin	Mouse	55	1.10000	Sigma	4°C overnight

Table 2. Secondary antibodies for Western Blot

Target species	Dilutions	Supplier	Incubation conditions
Mouse	1:2500	Santa Cruz	45 minutes at RT
Rabbit	1:2500	Santa Cruz	45 minutes at RT

RNA extraction

To extract RNA from the different macrophages conditions, 500 µL of TriPure Isolation Reagent (Roche) was used according to manufacturers' instructions. Macrophages were then detached, incubated for 5 minutes at room temperature to dissociate nucleoprotein complexes. Hundred µL of chloroform were then added and tubes were inverted several times, following incubation for 15 minutes at room temperature. After this incubation, samples were centrifuged for 12000 g, during 15 minutes at 4°C to obtain separation into three phases: the upper one, which is transparent and contains the RNA; the middle one that contains proteins and DNA, and the lower one that contains DNA. The upper phase, that contains RNA, was rescued without disturbing the others. RNA was then precipitated with 250 µL of isopropanol, tubes were inverted several times and incubated during 10 minutes at RT. Samples were then centrifuged at 12000g for 10 minutes at 4°C. Subsequently, the residual pellet was resuspended in 500 µL of 75% ethanol, vortexed and centrifuged at 7500g for 5 minutes at 4°C. The supernatant obtained was discarded and the excess of ethanol was completely removed by pellet air drying for 1 hour at RT. Subsequently, the pellet was resuspended in 20 µL of RNase-free water and incubated for 1 hour at 4°C. To finish this procedure, RNA concentration and purity were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). All purified RNA samples were stored at -80°C until further use.

Complementary DNA (cDNA) synthesis

The cDNA synthesis was performed using 1µg of RNA, using SuperScript II Reverse Transcriptase kit (Invitrogen). Briefly, two independent mixes were prepared. The annealing mix was composed by 1µg of RNA random primers and RNase/DNase free water, and incubated for 10 minutes at 70°C in a thermocycler (MyCyder, BioRad). The RT-MIX, composed by 4µL of 5x first-strand buffer, 2 µL of DTT (0.1M), 1µL of dNTPs

(10mM, Bloron), 0.2µL of RNasin (8U, Promega), 0.5 µL SuperScript II RT (200 U) and 1µL RNase/DNase free water, was added to the annealing mix and incubated for 37°C during 1h in a thermocycler.

Quantitative real-time PCR

The quantitative real-time PCR reaction was carried out using a mix composed by 0.5µL of each cDNA sample, 4µL of water and 5µL TaqMan Universal PCR Master Mix (Applied Biosystems). The qRT-PCR program used was composed by 2 holding stages of 50°C for 20 seconds and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The PCR was performed in a 7500 Real Time PCR System (Applied Biosystems) and each reaction was executed in triplicate. The following probes were used for qRT-PCR: 18s (used as housekeeping gene), CD80 and CD163 (Applied Biosystems). Relative mRNA expression of the target genes was normalized to the levels of the housekeeping gene using the comparative ΔΔCt method.

Statistical analysis

All graphs and statistical analysis were performed using the GraphPad Prism Software v7. Differences were tested with one sample t-test and statistical significance was achieved when $p < 0.05$.

Results

In order to study the influence of adrenergic system on human macrophages polarization and functions, a β2-adrenoceptor agonist (isoproterenol) was added to cultured macrophages under different stimuli. Therefore, unstimulated, LPS- and IL-10-stimulated macrophages were characterized regarding the expression of different pro- and anti-inflammatory cell surface receptors and intracellular mediators, in the presence or absence of isoproterenol. The impact of such treatments on macrophages cellular functions were also evaluated. Importantly, in the first part of this section, the characterization of human macrophage polarization profile was performed in the absence of isoproterenol; in the second part, the influence of this β2-adrenoceptor agonist on macrophage polarization and ability to modulate macrophage-mediated cancer cell invasion was evaluated.

1. Human macrophage polarization profile characterization

The phenotypic characterization of macrophages derived from $CD14^+$ peripheral blood monocytes was performed through different assays. The cytoskeleton organization, mRNA expression of pro- and anti-inflammatory genes, the expression of surface makers and production of cytokines were evaluated on unstimulated (MAC), LPS- (MAC-LPS) and IL-10-stimulated (MAC-IL-10) macrophages.

1.1. Macrophage morphology under different stimuli

After 10 days of differentiation, human macrophages were allowed to polarize, for 3 additional days in the absence of stimuli (unstimulated), or in the presence of 10ng/mL of LPS or IL-10. The morphology of polarized macrophages was then carefully observed under an inverted light microscope (Fig.6). Overall our results suggest that none of the treatments affected macrophage cell culture density, in agreement with our previous results evidencing that these stimuli do not affect macrophage viability nor metabolic activity (data not shown). Moreover, we observed that while unstimulated macrophages are heterogeneous, in the presence of LPS, the majority of them acquired an elongated morphology, with less rounded macrophages. In addition, in the presence of IL-10 some elongated macrophages are still visible, but the majority of them presented rounded-shape with a central nucleus.

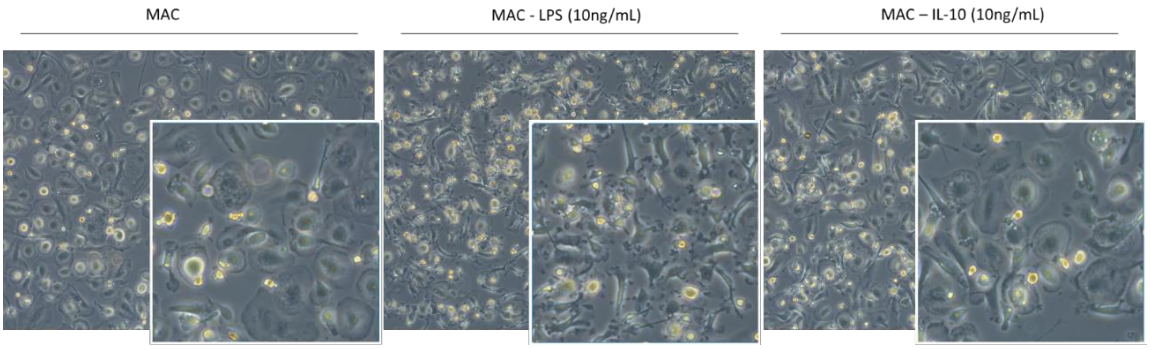


Figure 6. Phase contrast images of human macrophages and influence of LPS and IL-10 on macrophage morphology. Monocytes were plated and differentiated for 10 days. After this period, differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10). Data are representative from at least ten independent experiments.

1.2. Macrophage cytoskeleton organization under different stimuli

Since the microenvironment may modulate macrophages cytoskeleton organization with impact on their polarization and function, we decided to evaluate how the selected external stimuli (LPS and IL-10) could affect actin and tubulin network organization (Fig.7). Regarding the actin staining (red), unstimulated macrophages (MAC) with rounded morphology exhibited points of intense actin accumulation, corresponding to podosomes, basal-side adhesion structures. Their distribution is accompanying the basal side of the macrophage in a discoid-shape structure. These podosomes were almost absent in more elongated macrophages. In the presence of LPS, the majority of macrophages were elongated, presenting a rough basal side surface without visible podosomes. In this condition, only the rounded cells, although smaller, exhibited well-defined podosomes. Finally, in the presence of IL-10, macrophages preferentially showed a rounded morphology, with evident discoid podosomes displayed in concentric layers. For tubulin staining (green), in all conditions macrophages present a well-defined interlaced network, which extends towards the cell periphery and its projections. Nevertheless, IL-10 stimulated macrophages exhibited a more dense and nested meshwork.

Additionally, to better characterize and quantify macrophage morphology, the cell aspect ratio and cell area were evaluated (Fig.8). Cell aspect ratio was calculated as the quotient between cell major and minor axes length (Fig.8A). As evidenced, cell aspect ratio enhanced in MAC-LPS, indicating that this population is in fact more elongated. In contrast, unstimulated (MAC) and MAC-IL10 presented a cell aspect ratio closer to 1,

demonstrating the acquisition of a rounded phenotype. In addition, cell area of LPS-stimulated macrophages (MAC-LPS) was reduced in comparison with the other two macrophages populations (Fig.8B).

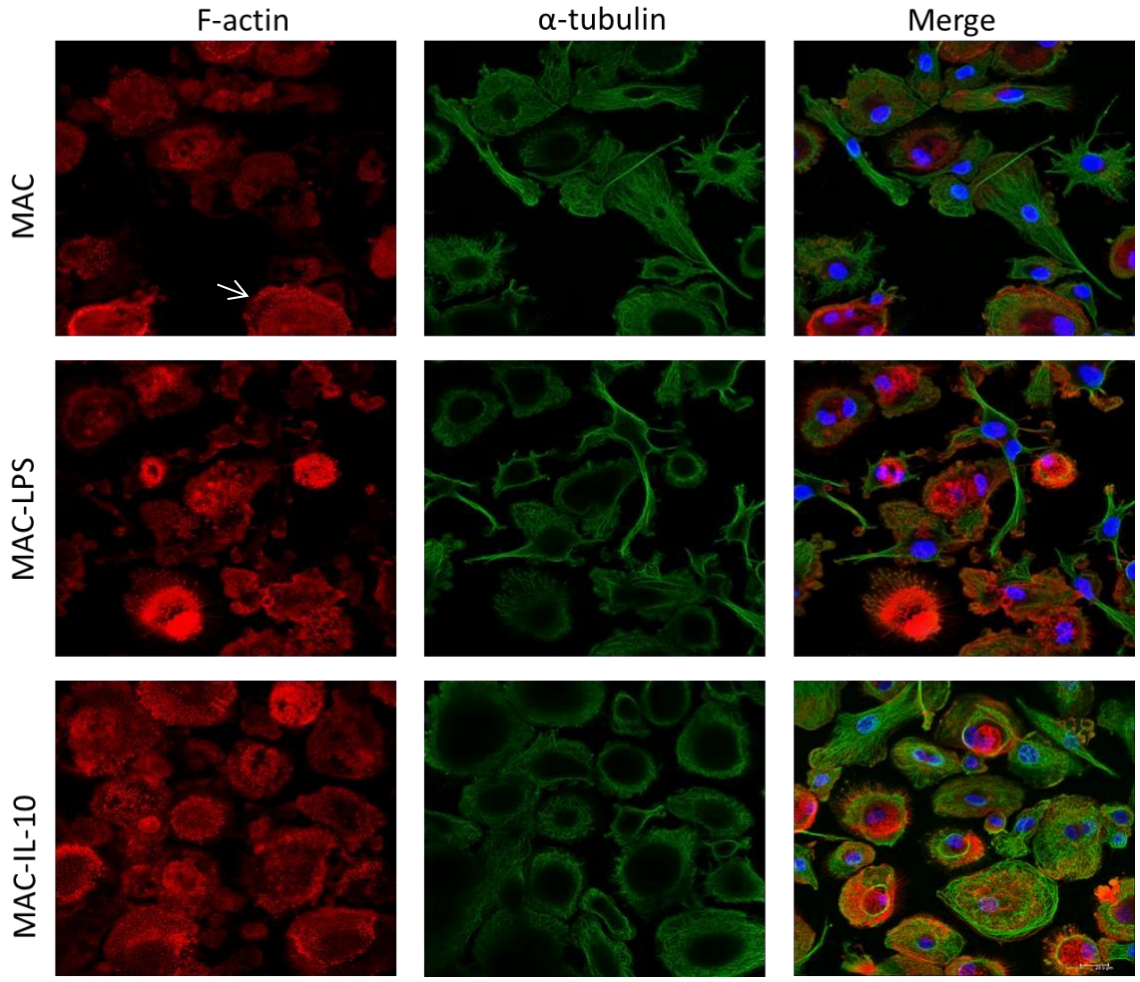


Figure 7. Influence of LPS and IL-10 on human macrophage cytoskeleton organization. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10). After this period, actin (red) and tubulin (green) stainings were performed as previously described and nuclei were counterstaining with DAPI (blue). Data are representative images, acquired by confocal microscopy, from at least two independent experiments. Arrows indicate podosomes like structures.

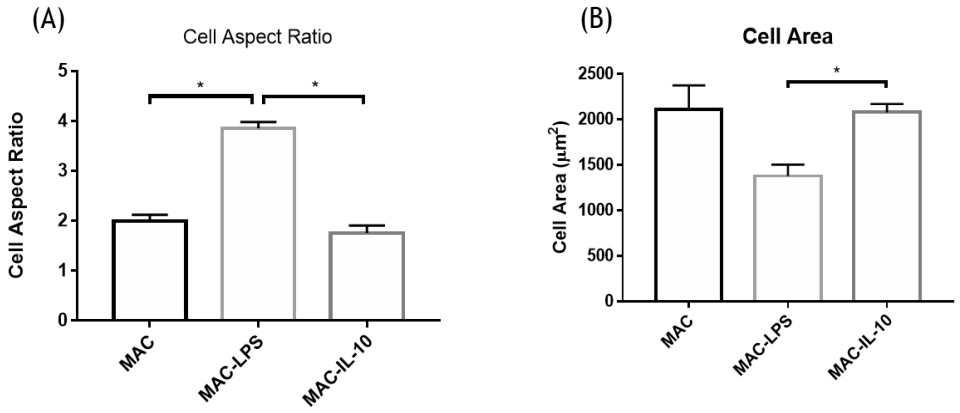


Figure 8. Influence of LPS and IL-10 on human macrophage cell aspect ratio and cell area. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10). (A) Represent the cell aspect ratio (major and minor cell axes ratio) of actin/tubulin stained cells and (B) total cell area from actin/tubulin stained macrophages. The bars represent mean values of at least four independent experiments and flags indicate standard error of the mean (100 cells/condition). Measurements were performed after the 13-days differentiation period. *: significantly different at $p<0.05$.

1.3. Characterization of LPS- and IL-10-stimulated macrophages phenotypes

Aiming to characterize the distinct macrophage phenotype achieved under LPS and IL-10 stimuli, qRT-PCR and flow cytometry analysis was performed. Analysis by qRT-PCR was oriented to evaluate the expression of pro- (CD80, a co-stimulatory molecule) and anti-inflammatory (CD163, a scavenger receptor) genes, markers of macrophage polarization status. As shown (Fig.9), unstimulated macrophages (MAC) are distinct from their LPS- and IL-10-stimulated counterparts. In spite of the present data represents a single donor experiment, it is possible to observe that LPS-stimulation induced an increase of CD80, almost absent in the other populations, while IL-10-stimulation enhanced the expression of CD163.

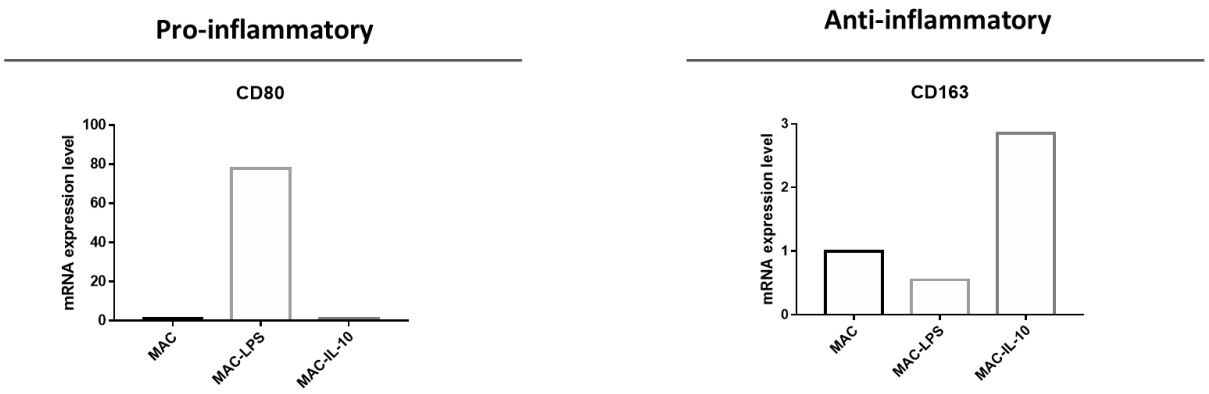
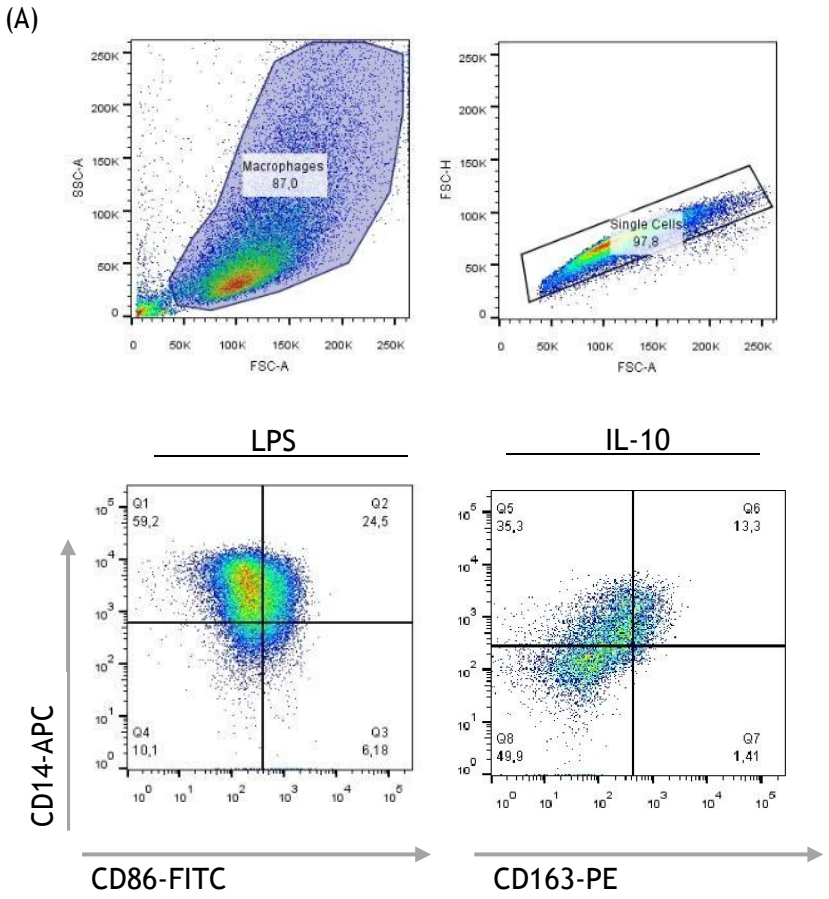
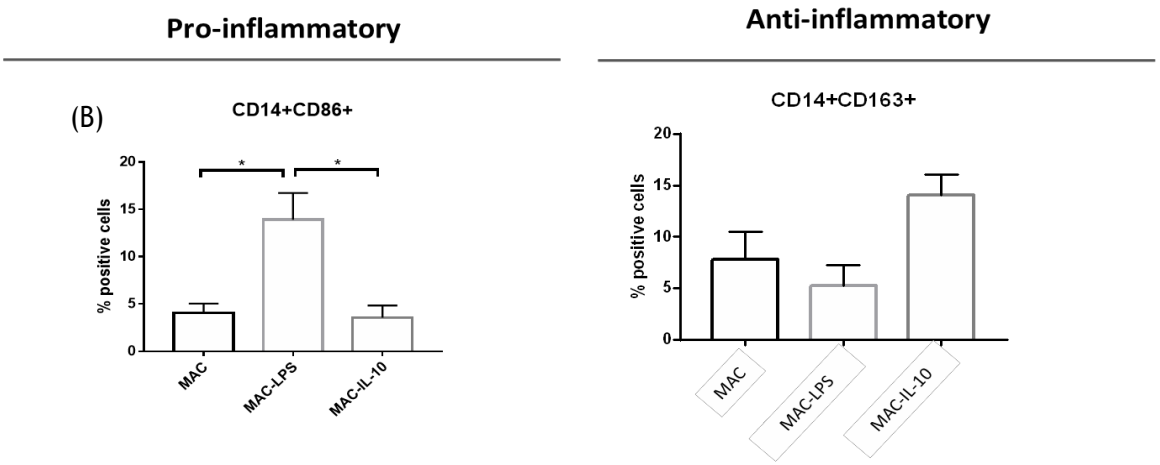


Figure 9. Influence of LPS and IL-10 on mRNA expression levels of the pro-and anti-inflammatory markers CD80 and CD163 of human macrophages cultures. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10). Shown are mRNA expression levels of CD80 (a pro-inflammatory marker) and CD163 (an anti-inflammatory marker) after normalization to 18s expression levels, of macrophage cultures, after the 13-day differentiation period. The bars represent data from a single donor experiment.

In parallel, flow cytometry was performed to analyse the expression of specific macrophage surface receptors. The receptor analysed were CD14, a macrophage lineage marker, CD86, a co-receptor M1-like marker and, CD163, a scavenger receptor M2-like marker. Our results evidence that on LPS-stimulated macrophages, the percentage of $CD86^+$ cells and CD86 MFI significantly increased, in comparison with MAC and MAC-IL-10. On the other hand, considering IL-10-stimulation, a tendency to increase the percentage of $CD163^+$ cells was observed, without differences at the MFI level (Fig.10). However, such tendency did not reach a statistically significance for the number of observations available so far.


(A)
Macrophages
87,0
SSC-A
FSC-A
Single Cells
97,8
FSC-H
FSC-A
LPS
IL-10
Q1
59,2
Q2
24,5
Q4
10,1
Q3
6,18
Q5
35,3
Q6
13,3
Q8
49,9
Q7
1,41
CD14-APC
CD86-FITC
CD163-PE


Pro-inflammatory
Anti-inflammatory
(B)
CD14+CD86+
% positive cells
MAC
MAC-LPS
MAC-IL-10
CD14+CD163+
% positive cells
MAC
MAC-LPS
MAC-IL-10

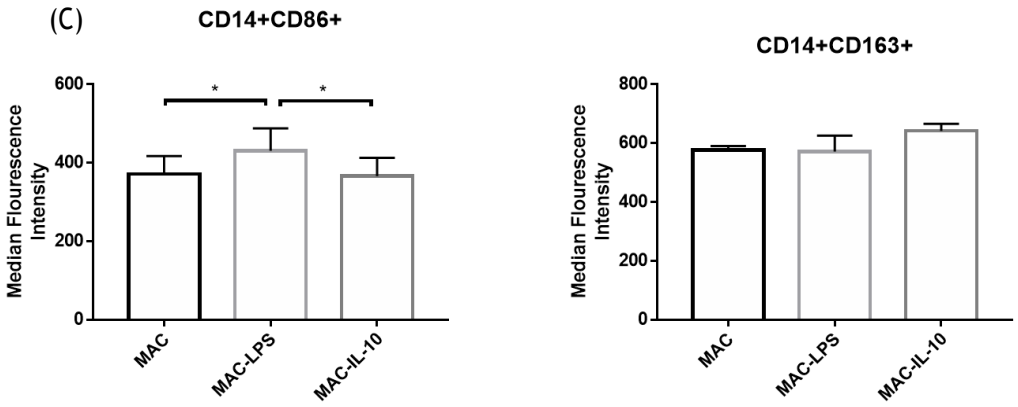


Figure 10. Influence of LPS and IL-10 on cell surface markers of macrophage polarization using flow cytometry analysis of human macrophage cultures. Expression of monocyte/macrophage lineage (CD14) and pro- (CD86) and anti-inflammatory (CD163) markers were performed in macrophages cultures, after a 13-day differentiation period. (A) pseudo-color plots of the cell distribution in the light scatter, based on cell size and granularity (FSA-A/SSC-A). FCS-A/FSC-H represents gatted single cells. CD14-APC/CD86-FITC and CD14-APC/CD163-PE represents positive cells. The percentage of $CD86^+$ and $CD163^+$ cells within $CD14^+$ cells is also shown (B). (C) Median fluorescence intensity of the $CD14^+CD86^+$ and $CD14^+CD163^+$ under different conditions. The bars represent mean values of at least seven independent experiments and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

In addition, the levels of soluble pro-inflammatory cytokines, as IL-6, IL-12/23(p40) and TNF-α, and of anti-inflammatory counterparts, such as IL-10, were evaluated by ELISA to further characterize the macrophage polarization profile. As expected, our results evidence that LPS-stimulated macrophages secret higher levels of the pro-inflammatory cytokines, in comparison with the other populations. In addition, IL-10-stimulated macrophages produced very low levels of these cytokines and secret significantly higher levels of IL-10 (Fig.11).

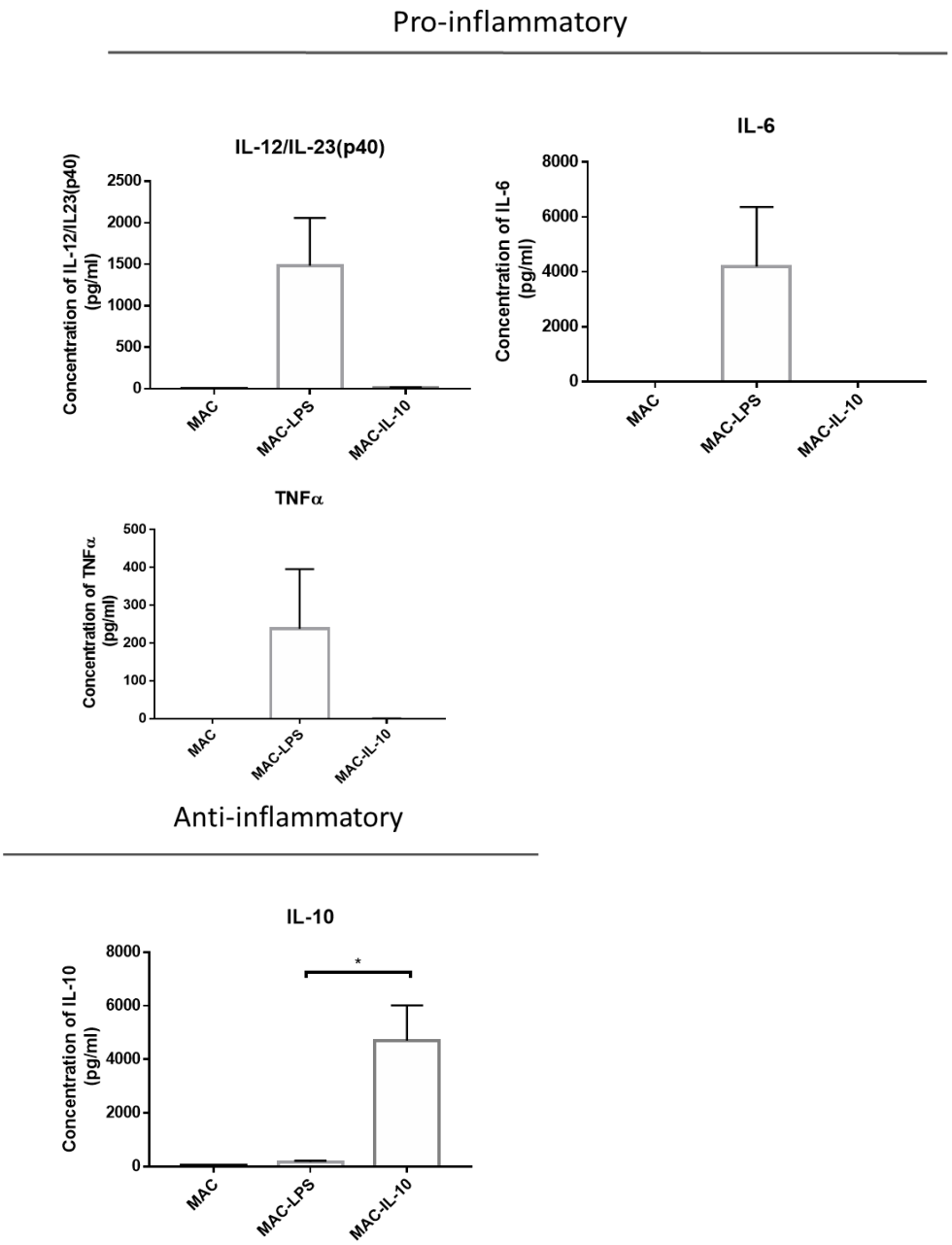


Figure 11. Production profile of pro- and anti-inflammatory cytokines by human macrophage cultures. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10). Shown are cytokine concentrations in the conditioned medium at the 13th day. The bars represent mean values of at least five independent experiments and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

Overall our data indicate that LPS- and IL-10 stimulated macrophages exhibit a pro-inflammatory and anti-inflammatory polarization phenotypes, respectively. Moreover, unstimulated macrophages are molecularly more similar to the M2-like anti-inflammatory phenotype.

2. Influence of isoproterenol on human macrophage polarization and on their ability to modulate macrophage-mediated cancer cell invasion

After the previous characterization of the macrophage phenotypes under different stimuli, the influence of isoproterenol, a non-selective β2-adrenoceptor agonist with also affinity for β1-adrenoceptor, on pro- and anti-inflammatory macrophages profile and functions was carefully investigated.

2.1. Isoproterenol does not affect the viability and metabolic activity of macrophages

To analyse whether isoproterenol (100nM) was affecting macrophage viability, the Calcein-AM/Propidium Iodide assay was performed (Fig.12). Calcein, a viable dye will only stain live macrophages, while propidium iodide, only permeable to disturbed plasma membranes, will only stained the death counterparts. As a cell death positive control, macrophages were fixed with 4% PFA and treated with 0.2% Triton X-100. In the presence of isoproterenol (100nM), no significant impact on macrophage viability was observed, indicating the absence of a direct cytotoxic effect induced by this adrenoceptor agonist.

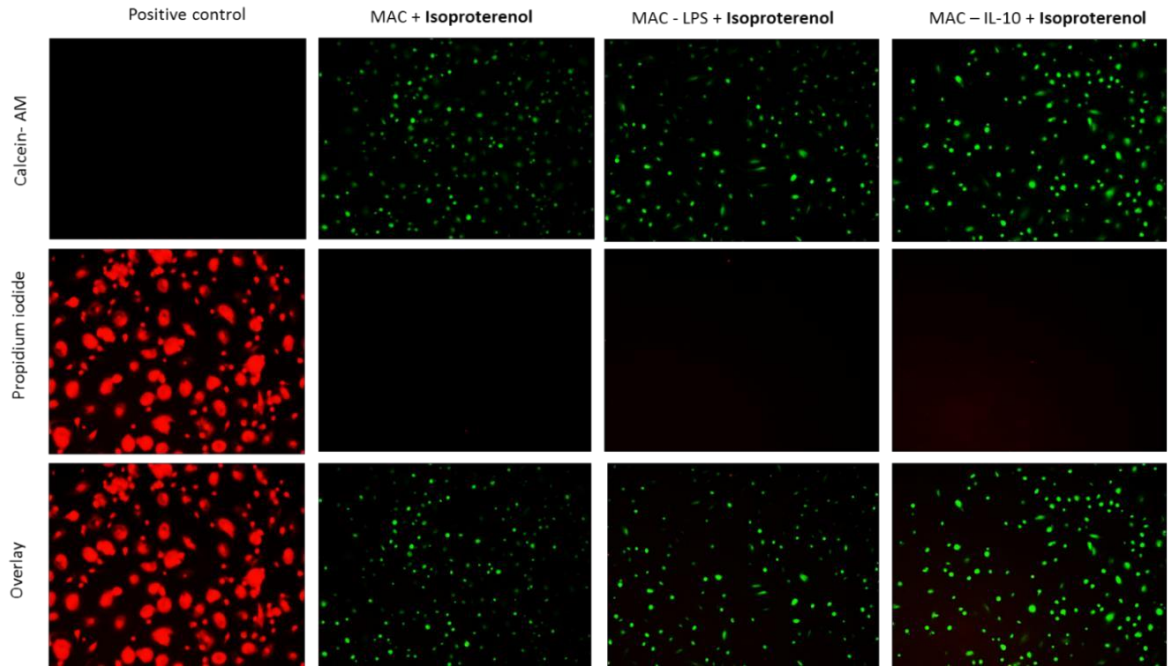


Figure 12. Infuence of isoproterenol on human macrophage viability. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10), in the presence or absence of isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Shown are representative images acquired by fluorescence microscopy of a positive staining with Propidium Iodide (red) and Calcein-AM (green) performed on dead and live macrophages, respectively, after a 13-day differentiation period. As a cell death positive control, macrophages were fixed with 4% PFA and treated with 0.2% Triton X-100. Data are representative images from at least two independent experiments.

In addition,the metabolic activity of the different macrophage phenotypes, incubated in the presence or absence of isoproterenol was evaluated by resazurin assay (Fig.13). Our results indicated that the presence of isoproterenol (100nM) did not affect the metabolic activity of unstimulated macrophages (MAC) or of anti- and pro-inflammatory macrophages, stimulated with IL-10 (MAC-IL-10) or LPS (MAC-LPS), respectively.

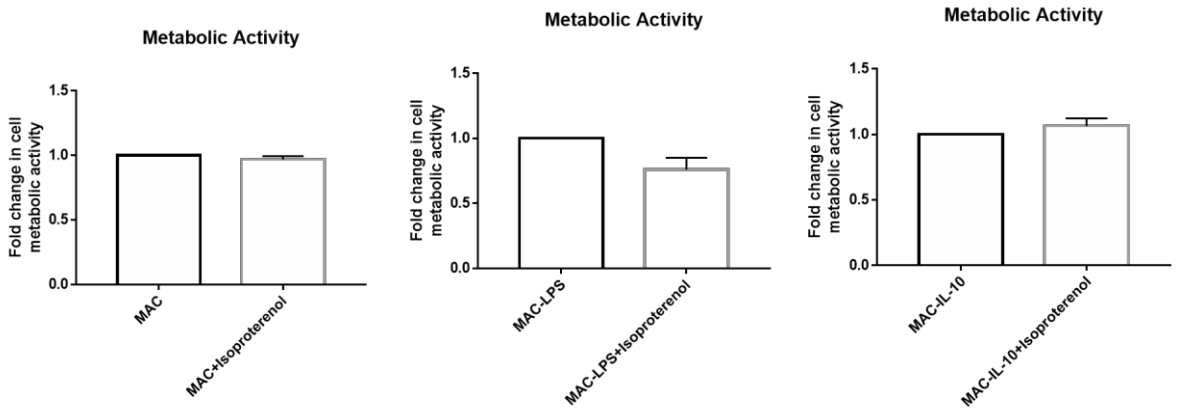


Figure 13. Effect of isoproterenol on human macrophage metabolic activity. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10), without or withisoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Graphs represent macrophage metabolic activity measured by resazurin reduction assay, after a 13-day differentiation period. Quantifications were normalized to unpolarised or polarized macrophages cultured in the absence of isoproterenol. Data are expressed in fold change, bars represent mean values of at least three independent experiments, and flags indicate standard error of the mean.

2.2. Isoproterenol modulates macrophage cytoskeleton organization

To understand if isoproterenol (100nM) influences pro- and anti-inflammatory macrophages cytoskeleton organization, F-actin and tubulin immunostainings were evaluated in macrophages polarized with LPS and IL-10 stimuli, respectively (Fig.14). As already mentioned, unstimulated macrophages (MAC) presented a heterogeneous morphology, in comparison with the more elongated LPS-stimulated macrophages (MAC-LPS) and their large rounded IL-10 stimulated counterparts (MAC-IL-10). In the presence of isoproterenol (100nM) seems to induce alterations on macrophage morphology. To better characterize and quantify these alterations, the cell aspect ratio and cell area were calculated (Fig.15 A e B), as previously. Interestingly, the cell aspect ratio of the distinct macrophage subpopulations was not affected by isoproterenol (100nM), maintaining the elongated and rounded morphology of the LPS- and of the IL-10 stimulated macrophages, respectively (Fig. 15A). Nevertheless, differences regarding cell area were observed (Fig.15B). Notably, macrophages when treated with isoproterenol (100nM) presented a significantly reduced cell area, irrespectively of the polarizing agent being LPS or IL-10. These morphological alterations may suggest differences on macrophage behaviour and on macrophage-matrix interactions.

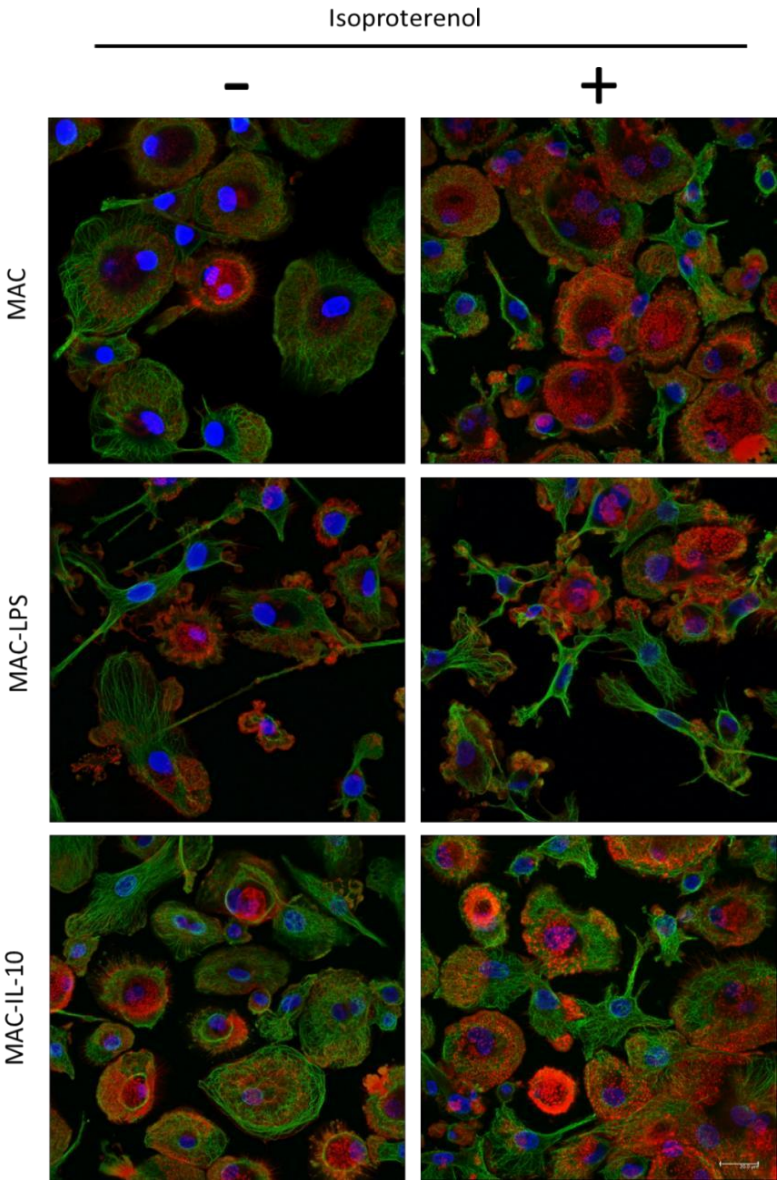


Figure 14. Influence of isoproterenol on human macrophage cytoskeleton organization. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10), in the presence or absence of isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. After this period, actin (red) and tubulin (green) stainings were performed, as previously described, and nuclei were counterstaining with DAPI (blue). Data are representative images, acquired by confocal microscopy, from at least two independent experiments.

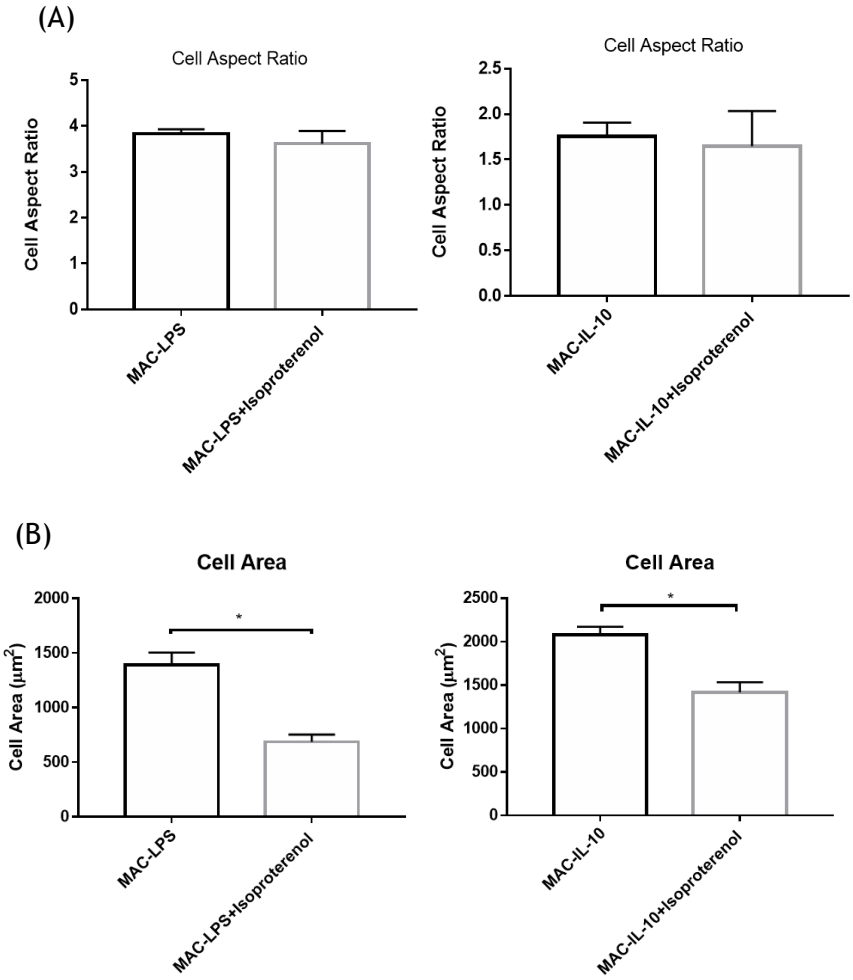


Figure 15. Influence of isoproterenol on human macrophage cell aspect ratio and cell area. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli, or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and, in the presence or absence of isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. (A) The Cell Aspect Ratio (major and minor cell axes ratio) of actin/tubulin stained cells and (B) the total cell area from actin/tubulin stained macrophage cultures after 13-day differentiation period. The bars represent mean values of at least four independent experiments (100 cells/condition) and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

2.3. Isoproterenol signals through the β2-Adrenoceptor

To confirm the expression and distribution of β2-adrenergic receptor in human macrophages, Western blot and immunocytochemistry analysis were performed. Western blot data suggests that LPS-stimulated macrophages exhibit higher endogenous receptor expression levels (Fig.16A). On their turn immunocytochemistry analysis performed by confocal microscopy suggest differences regarding beta2-adrenoceptor distribution, with IL-10-stimulated macrophage exhibiting a perinuclear

intense staining. Interestingly, the addition of isoproterenol intensified the staining in all phenotypes (Fig.16B).

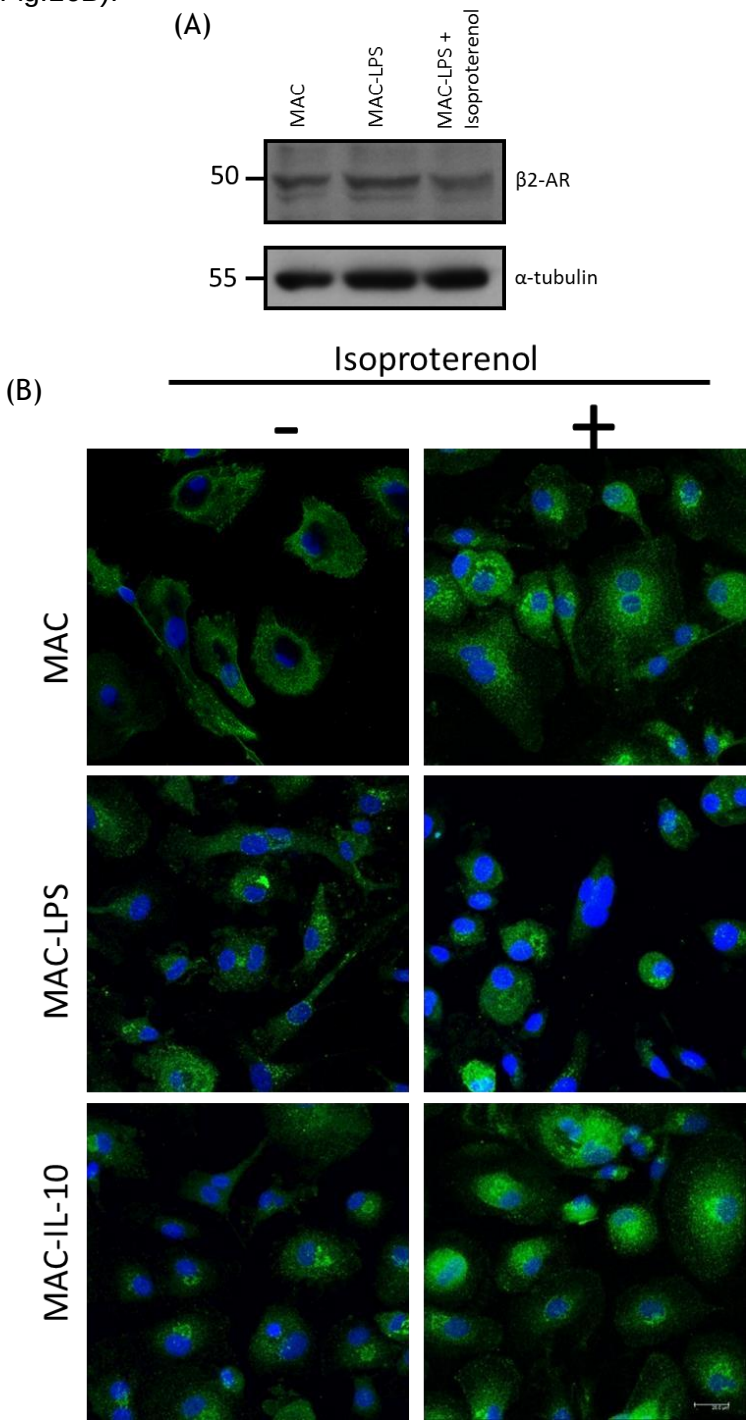


Figure 16. β2-Adrenergic receptor on human macrophage. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and, treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. (A) β2-adrenoceptor expression was evaluated in unstimulated macrophages and LPS stimulated in presence or absence of isoproterenol from a only single donor. Alpha-tubulin (α-tubulin) was used as loading control. (B) Are representative images acquired by confocal microscopy of β2-adrenergic receptor (green) on macrophage cultures after 13-day differentiation period. Nuclei were counterstaining with DAPI (blue). Data are representative images, acquired by confocal microscopy, from at least two independent experiments.

2.4. Isoproterenol-modulated macrophage inflammatory profile

As previously characterized by qRT-PCR, the co-stimulatory CD80 revealed to be an appropriate pro-inflammatory marker, while the scavenger receptor CD163 an appropriate anti-inflammatory marker. Considering this, we evaluated the influence of isoproterenol in expression of these cell surface receptors in LPS- and IL-10-stimulated macrophages, treated or not with isoproterenol. Because of methological limitations, only the material obtained from one donor was studied so far. As observed, isoproterenol did not seem to affect the polarization of unstimulated macrophages (MAC), but seem to induce an increase of CD80 mRNA expression levels on LPS-polarized macrophages (Fig.17A). The opposite was observed on the expression of CD163, where isoproterenol increased the expression of this anti-inflammatory marker, both in LPS- or IL-10-stimulated macrophages (Fig.17B).

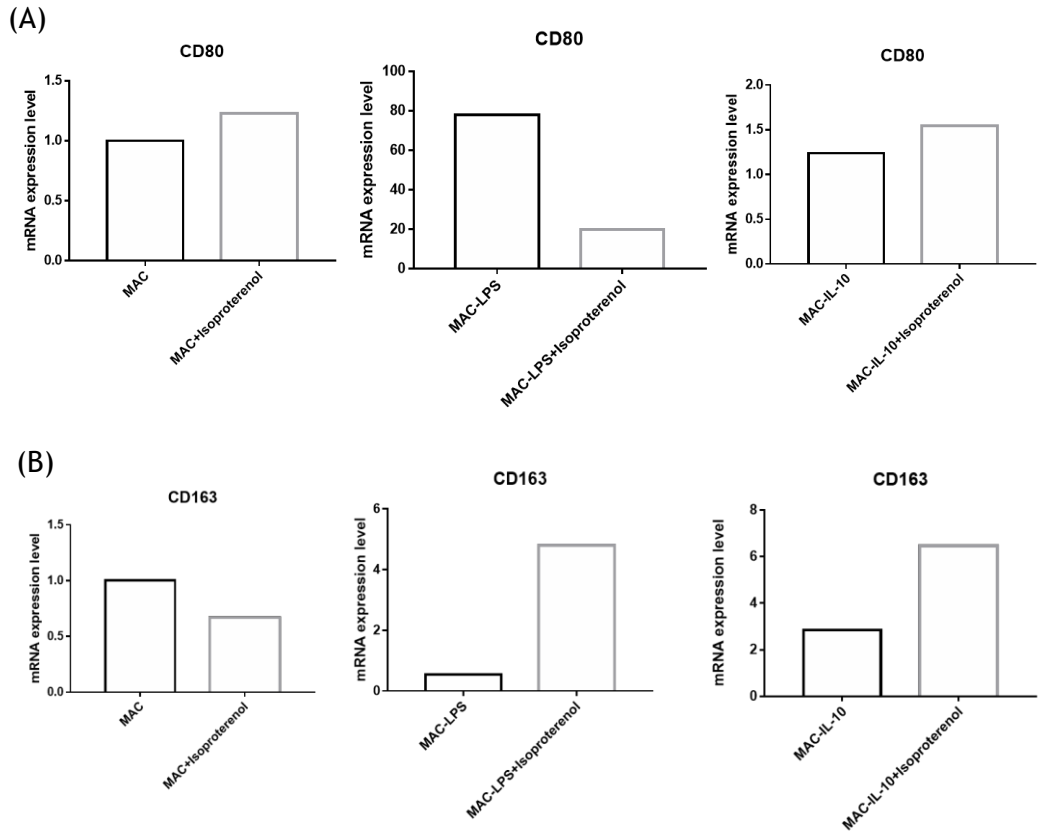


Figure 17. Influence of isoproterenol on mRNA expression levels of the pro- and anti-inflammatory markers CD80 and CD163 of human macrophage cultures. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. (A) are mRNA expression levels of CD80 (a pro-inflammatory marker) and (B) CD163 (an anti-inflammatory marker), after normalization to 18s levels, of macrophage cultures, after 13-day differentiation. Bars represent data from a single donor experiment

In order to analyse the influence of isoproterenol in cell surface expression of typical macrophage surface receptors, CD86 (pro-inflammatory marker) and CD163 (anti-inflammatory marker, flow cytometry was performed. The gating strategy is presented in Fig.18 and can be seen that in presence of isoproterenol the distribution of human macrophages is not equal for the different markers. The percentage of positive cells and the median fluorescence intensity for pro- (Fig.19) and anti-inflammatory marker (Fig.20) is shown. From the experiments performed so far with the donors available, the most clear effect is the reduction of CD86 (the pro-inflammatory marker) expression, caused by isoproterenol in LPS-polarized macrophages (Fig.19A). Isoproterenol also seems to enhance the expression of CD163 (the anti-inflammatory marker) on LPS-polarized macrophages (Fig.20A). However, because of the variability of the results, such difference is not significant. Importantly, the Median Fluorescence Intensity (MFI) of CD86 (Fig.19B) and CD163 (Fig.20B) were not altered in the presence of isoproterenol. The effect of isoproterenol to promote an anti-inflammatory profile can also be observed when the previous results considered the $CD86^+/CD163^+$ ratios. Interestingly, $CD86^+/CD163^+$ ratio was markedly lower in isoproterenol treated LPS-polarized macrophages, suggesting a decrease of the pro-inflammatory markers in favour of an acquisition of a more anti-inflammatory phenotype (Fig.21).

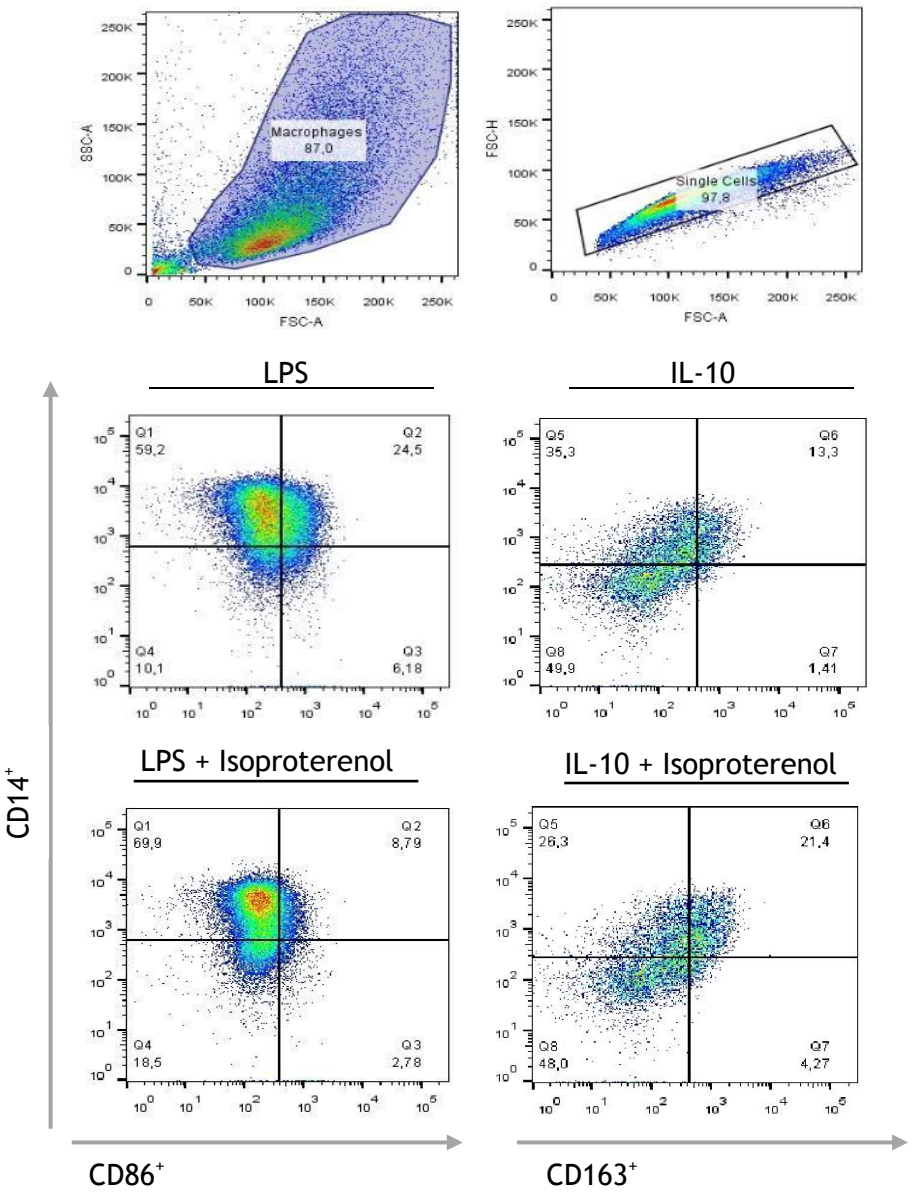


Figure 18. Influence of isoproterenol on the distribution of human macrophages in light scatter using flow cytometry analysis. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Expression of typical macrophage lineage polarization markers (CD86 and CD163) was determined in macrophages cultures, after a 13-day differentiation period. Shown are Pseudo-color plots exhibiting the distribution of cells in the light scatter based on cell size and granularity (FSC-A/SSC-A). FSC-A/FSC-H, represents single cells. CD14-APC/CD86-FITC and CD14-APC/CD163-PE represents double positive cells.

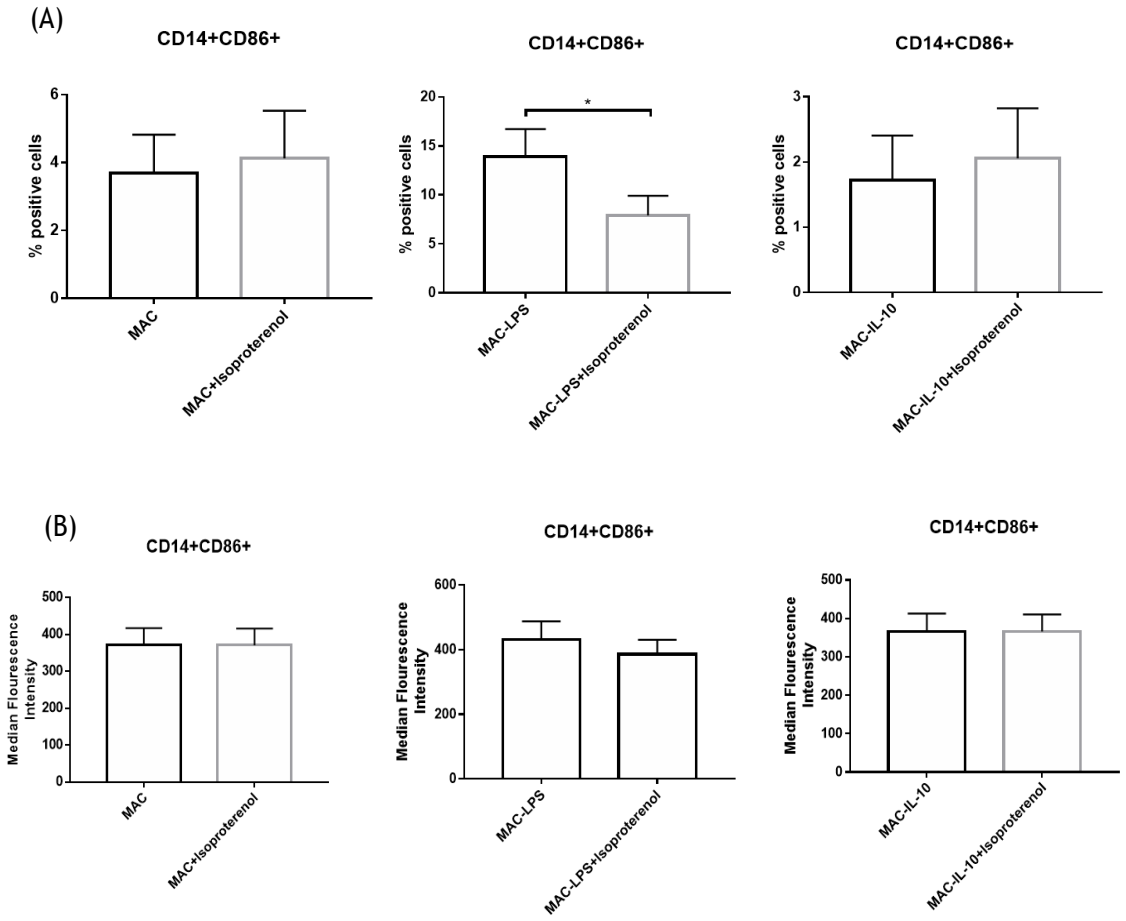


Figure 19. Influence of isoproterenol on pro-inflammatory markers of macrophage polarization using flow cytometry analysis of human macrophages cultures. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Expression of the pro-inflammatory marker (CD86) was determined in macrophages cultures, after a 13-days differentiation period. (A) Graphs represent the percentage of $CD86^+$ cells within the $CD14^+$ population. (B) Shown the median fluorescence intensity of the CD86 under different conditions. The bars represent mean values of at least seven independent experiments and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

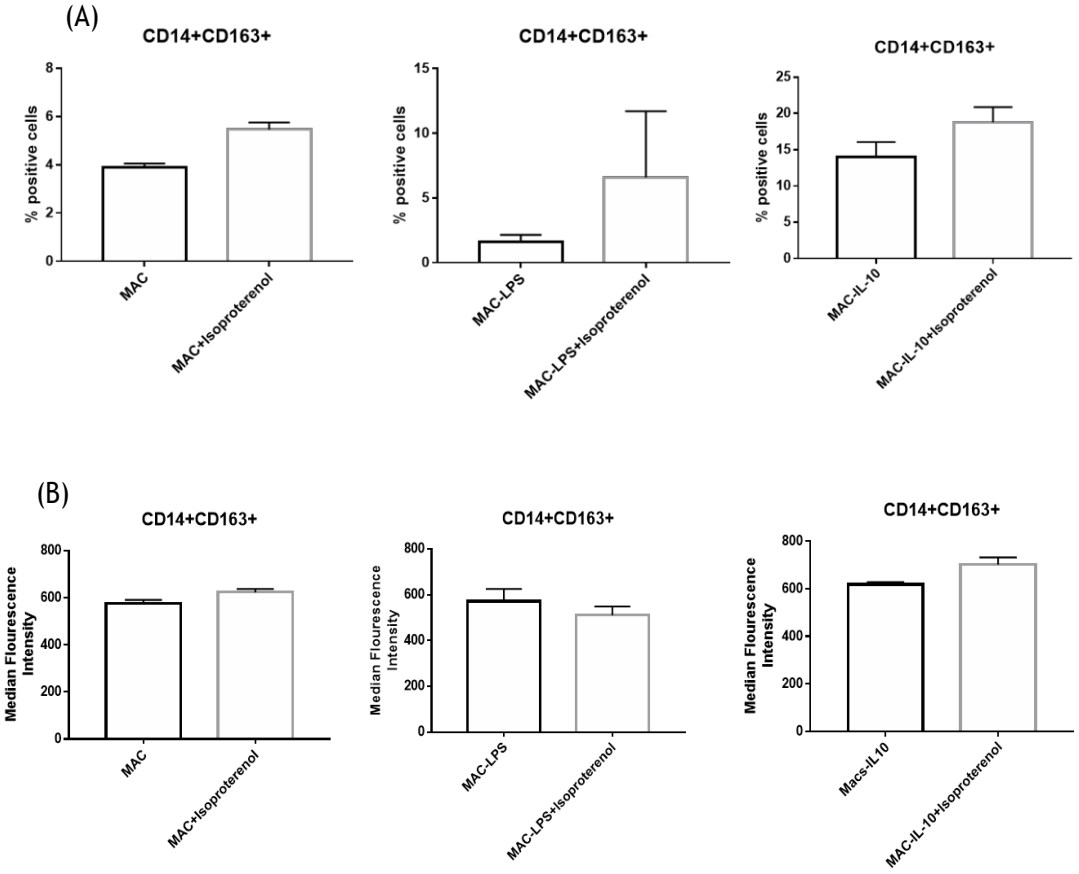


Figure 20. Influence of isoproterenol on anti-inflammatory markers of macrophage polarization using flow cytometry analysis of human macrophages cultures. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Expression of the anti-inflammatory marker (CD163) was determined in macrophages cultures, after a 13-days differentiation period. (A) Graphs represent the percentage of $CD163^+$ cells within the $CD14^+$ population. (B) Shown the median fluorescence intensity of the CD163 under different conditions. The bars represent mean values of at least seven independent experiments and flags indicate standard error of the mean.

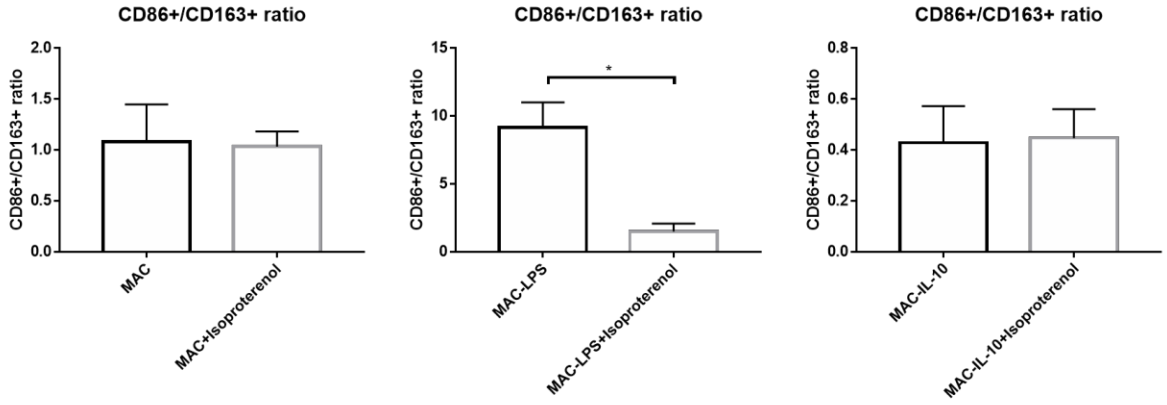


Figure 21. Influence of isoproterenol on cell surface markers of macrophage polarization using flow cytometry analysis of human macrophages cultures. Graphs represent the ratio between $CD14^+CD86^+/CD14^+CD163^+$ double positive macrophages. The bars represent mean values of at least seven independent experiments and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

In addition, pro-inflammatory cytokines levels (IL-6, IL-12/23(p40), TNF-α) and anti-inflammatory cytokine levels (IL-10) were evaluated in supernatants of LPS or IL-10 polarized macrophages, stimulated in the presence or absence of isoproterenol. As evidenced (Fig.22A), isoproterenol (100nM) caused a decrease on the release of all pro-inflammatory cytokines in LPS-polarized macrophages, while caused an increase on the release of the anti-inflammatory cytokine IL-10. In contrast, on IL-10 polarized macrophages, no effect of isoproterenol on cytokine release was observed (Fig.22B)

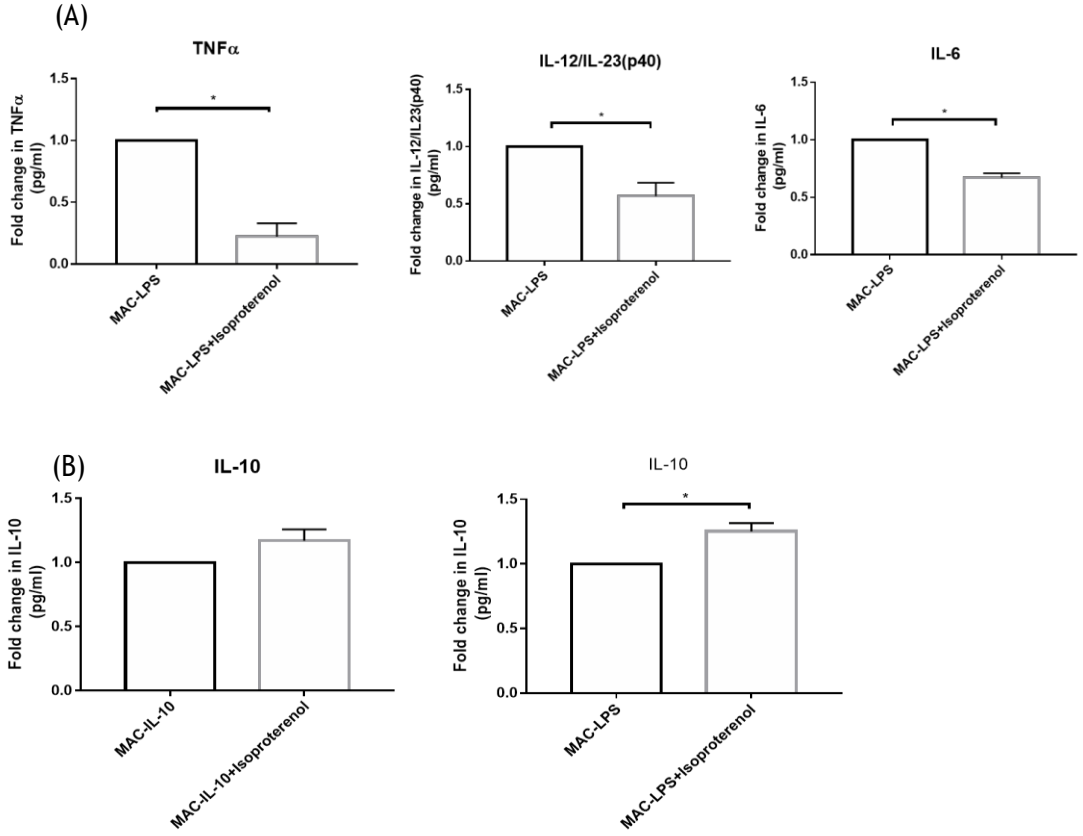


Figure 22. Effect of isoproterenol on release of inflammation modulatory cytokines [IL-12/23(p40), IL-6 and TNF-α or IL-10] from human macrophage cultures polarized. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10), in the presence or absence of isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. (A) Graphs represent the concentration of pro-inflammatory cytokines in conditioned media at 13th days and (B) graph represents the concentration of the anti-inflammatory cytokine IL-10 profile in conditioned media at 13th days. The bars represent mean values of at least five independent experiments and flags indicate standard error of the mean; *: significantly different at $p<0.05$.

2.5. Isoproterenol-modulated macrophage signalling pathway

The nuclear factor kappa B (NF-kB) pathway is a signalling pathway that has been associated with cancer-related inflammation, playing a role in the switch from anti- to pro-inflammatory macrophages. In order to explore this signalling pathway, the expression levels of phosphorylated and of total NF-kB p65 and IkBα were evaluated by western blot in unstimulated (MAC) and LPS stimulated macrophages (MAC-LPS), in presence or absence of isoproterenol. The α-tubulin was used as loading control. In spite of these results are representative of only one donor, data revealed an increase of phosphorylated p65 in LPS-stimulated macrophages, which decreased in the presence of isoproterenol treatment (Fig.23A). Similar differences seem to be applied to IkBα phosphorylation (Fig.23B).

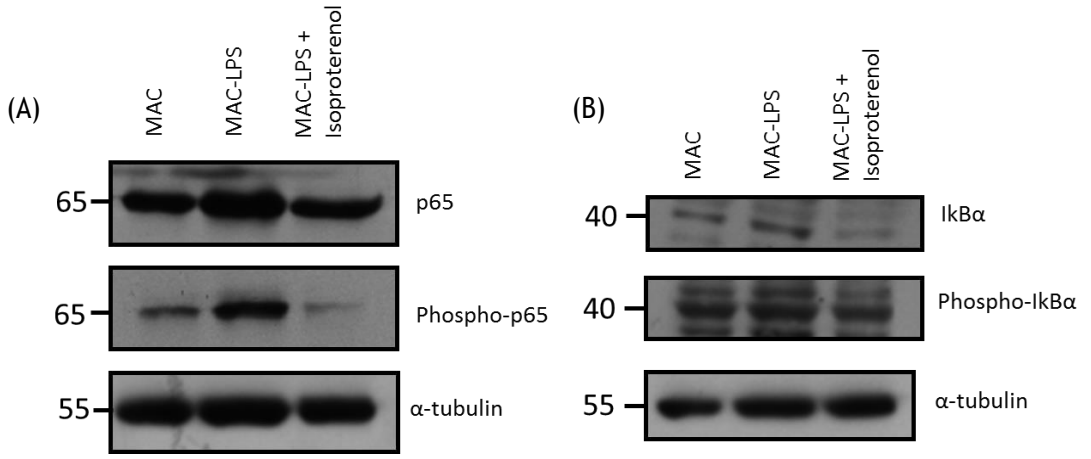


Figure 23. Analysis of macrophage signalling pathways. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), treated or not with isoproterenol (100nM) [(MAC, MAC-LPS) + isoproterenol]. **(A) and (B)** Total and phosphorylated NF-kB (p65), and IkBα protein levels, respectively, were evaluated by western blot in macrophages cultures, after a 13-days differentiation period. Alpha-tubulin (α-tubulin) was used as loading control. Data represent a single donor experiment.

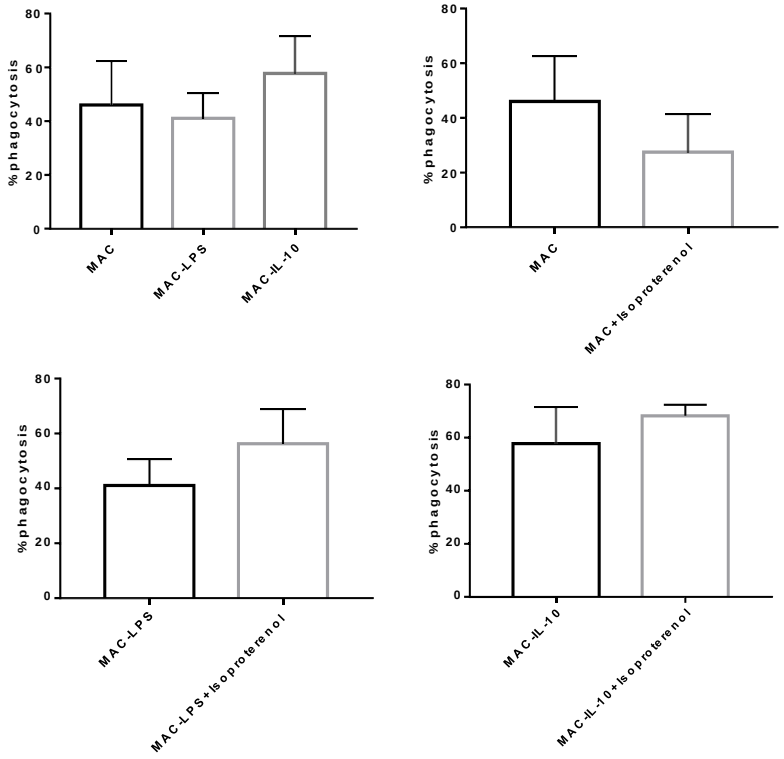
2.6. Isoproterenol-modulated macrophage functional role

In order to have an insight of other potential impacts of observed anti-inflammatory effect of isoproterenol, (i) the influence on phagocytic activity and (ii) modulation of cancer cell invasion began to be studied. Preliminary results are herein presented.

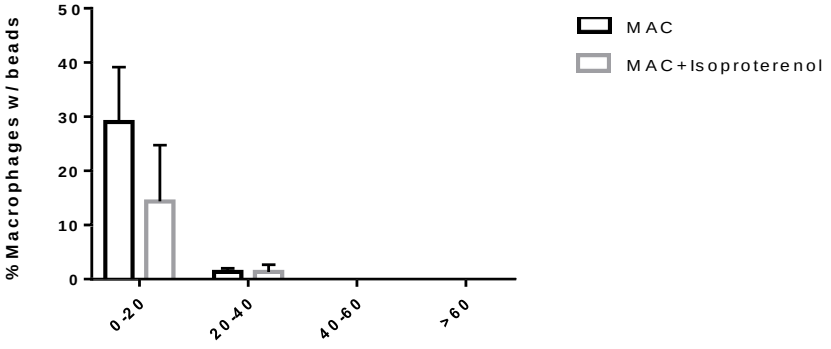
(i) Isoproterenol-modulated macrophage phagocytic activity

The phagocytic activity is being evaluated in LPS or IL-10 polarized macrophages in the absence or in the presence of isoproterenol (100nM). Figure 24 shows the results obtained so far. The phagocytic activity (Fig.24A) is estimated based on the number of cells with particles incorporated (Fig.24B) versus the total number of cells. As observed, a marked phagocytic activity is visible either in LPS or IL-10 polarized macrophages upon treatment with isoproterenol. It is also shown that the phagocytic activity was lower in LPS-polarized macrophages and that treatment with the β2-adrenoceptor agonist tends to increase their phagocytic capacity.

(A)


% phagocytosis
0
20
40
60
80
MAC
MAC-LPS
MAC-IL-10
MAC+Isoproterenol
MAC-LPS+Isoproterenol
MAC-IL-10+Isoproterenol

(B)


% Macrophages w/ beads
0
10
20
30
40
50
0-20
20-40
40-60
>60
MAC
MAC+Isoproterenol

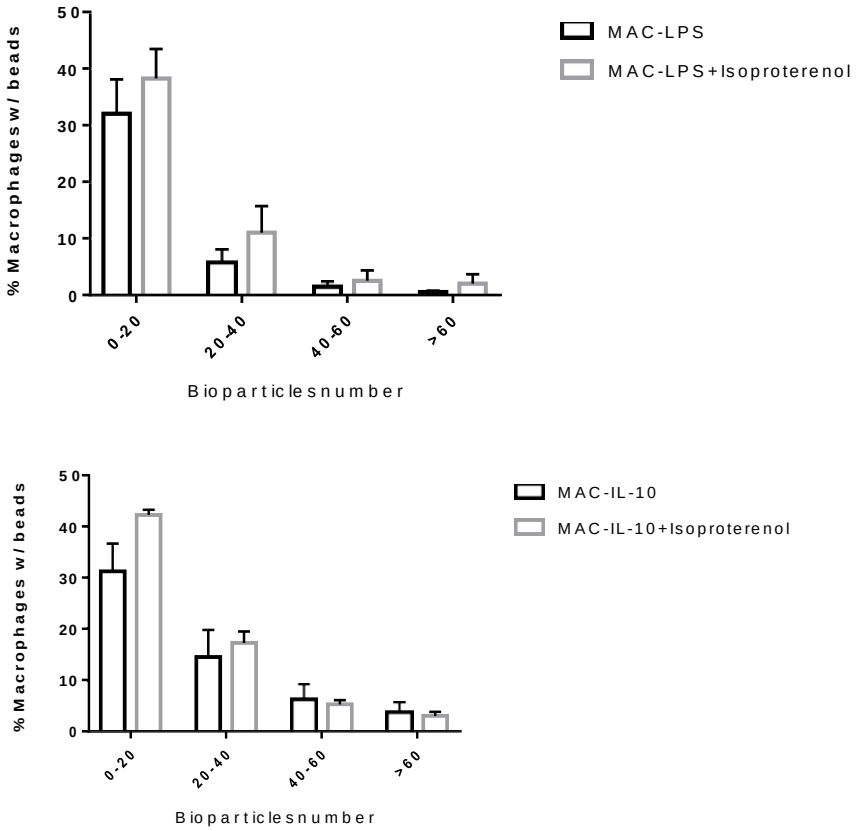


Figure 24. Effect of isoproterenol on human macrophage phagocytic activity. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and treated or not with isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. The phagocytic activity was performed in macrophages cultures, after 13-days differentiation period. After this period, macrophages were incubated with S.aureus bioparticles. The phagocytosis of the FITC-labelled beads by macrophages was measured using cell profile software. (A) Graphs represent percentage of phagocytosis, and (B) represent the percentage of macrophages that engulfed beads. Macrophages that have not been in contact with S. aureus bioparticles were used a positive control. The bars represent mean values of at least four independent experiments (2500 cells/condition) and flags indicate standard error of the mean.

(ii) Isoproterenol-modulated macrophage-mediated cancer cell invasion

It is know that invasion of cancer cells may be influenced by macrophages. This influence is different according to the macrophage phenotype. Therefore, in order to understand if the phenotype induced by isoproterenol influences cancer cell invasion, the effect of this b2-adrenoceptor agonist in cancer cell invasion was evaluated, using LPS or IL-10 polarized macrophages, in the absence or presence of isoproterenol (100nM) (Fig 26, Appendix 1). Matrigel invasion assays results, obtained so far, indicate that cancer cell invasion is lower only when LPS polarized macrophages are present whereas cell invasion is identical in the presence of IL-10 polarized and non-polarized macrophages (Fig.25A). However, isoproterenol (100nM) seems to enhance LPS polarized macrophages pro-invasive activity (Fig.25B). Ongoing experiments will provide additional data to confirm the relevance of the observations obtained so far.

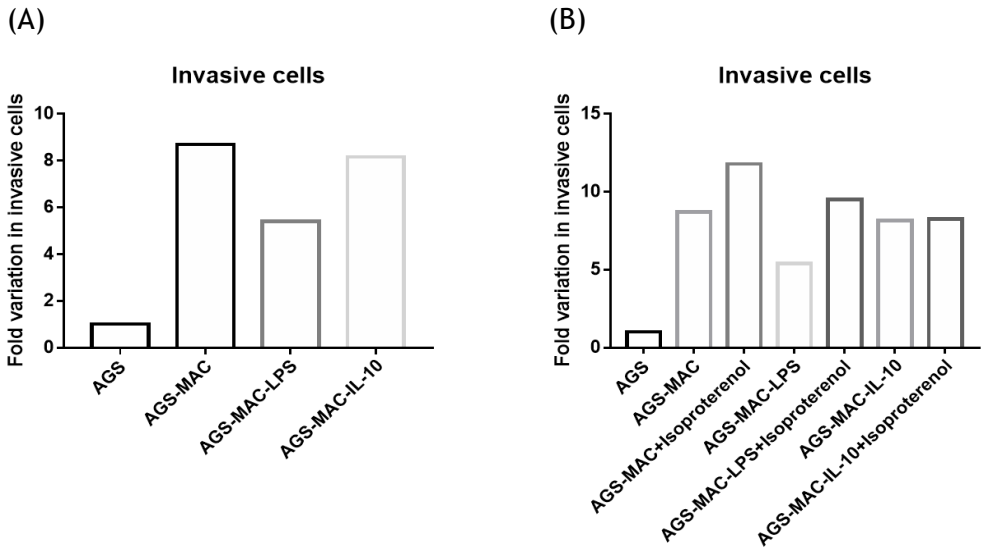


Figure 25. Impact of isoproterenol to modulate macrophage-mediated cancer cell invasion. Ten days differentiated macrophages were cultured, for 3 additional days, in absence of stimuli (MAC), or in the presence of 10ng/mL LPS (MAC-LPS), or of 10ng/mL IL-10 (MAC-IL-10) and isoproterenol (100nM) [(MAC, MAC-LPS, MAC-IL-10) + isoproterenol]. Invasion assay were performed using AGS cancer cell in Matrigel coated transwell filters and macrophages as stimuli to invasion. At the 12th day, invasion assays were performed during 24hours. Data represent the fold variation in the number of invasive cells. The bars represent data from a single donor experiment.

Discussion

The effect of the sympathetic nervous system on both tumour progression and macrophage inflammatory profile has been explored (85,86). The sympathetic nervous system exerts its control through the release of neurotransmitters from the postganglionic sympathetic nerves (mainly noradrenaline), and through the release of messengers from the adrenal medulla (mainly adrenaline), exerting their effects by acting according to a hormonal model of action. Like many other organs/tissues, the immune system is under this adrenergic influence.

Noradrenaline and adrenaline exert their actions through activation of a series of membrane G-protein coupled receptors which are grouped in three types, called α1, α2 and β-adrenoceptors(87). From all the adrenoceptor types and subtypes, the β2-adrenoceptors are those more associated with the control of the immune system, according to pharmacological data base consensus (88). β2-adrenoceptors are expressed on innate and adaptive immune cells of humans and rodents and are reported to have an immuno-modulating effect (64). Bacou *et al* evaluated the expression of adrenergic receptors in bone marrow-derived macrophages of pigs and showed that β2-adrenoceptors were more expressed than others subtypes (89). The expression of β2-adrenoceptor on murine peritoneal macrophages has been reported and associated with an anti-inflammatory profile and dampening of innate immune response (80).

Studies on human macrophages suggest no differences on β2-adrenoceptor expression, upon stimulation with a pro-inflammatory stimuli, as LPS (90). In the present study, the expression of β2-adrenoceptors in human macrophages was confirmed by immunocytochemistry and by Western blot. Although we have not explored the impact of an anti-inflammatory stimuli on β2-adrenoceptor expression, Western blot analysis suggests that LPS enhance the expression of such receptor. In spite of we have use the same LPS concentration than the previous study, differences might be justified by longer (24h vs 72h) exposure to the stimulus. Concomitantly, our immunocytochemistry analysis suggest differences regarding β2-adrenoceptors distribution, we cannot exclude that such effects are related to morphology differences.

Most of the studies carried out to investigate the influence of adrenoceptors activation on macrophage have used animal models and cell lines and showed that β2-adrenoceptor activation avoids polarization towards a pro-inflammatory phenotype (86,91). Direct translation of this knowledge to man is not trivial, namely because many

of the markers used for murine have not converted to human macrophages (33). Only indirect attempts have been made to promote this translation, namely though the use of pig macrophages, which are considered the most appropriate animal model to mimic the human system (80,89).

To our knowledge, the present study is the first attempt to investigate the effect of β2-adrenoceptor activation on human macrophage polarization. In previous studies, the macrophages used have a murine origin while we used macrophage isolated from blood monocytes. This detail is important because, on top of the advantage of using human cells, it allows a broader coverage of the macrophage populations present in humans, since many tissue resident populations are not of bone marrow origin. In our experiments, monocytes were differentiated in the presence of M-CSF, which is a growth factor known to be involved in the recruitment of monocytes/macrophages to tissues and also, in tumours, in the regulation of macrophage function (92,93).

It is known that adrenergic control of macrophages depends on the origin and state of macrophage activation and on the concentration of catecholamines (89,94). Some of these variables were inherent to donors and, therefore, out of our control, and a putative cause of result variability. In spite of this limitation, cells from all donors were exposed to the same concentration of isoproterenol, a full agonist for β-adrenoceptors, with pKi values of 6.6-7.0 for β1-adrenoceptors, 6.4 for β2-adrenoceptors and 4.2-6.2 for β3-adrenoceptors (95). The number of human donors imposed methodological limitations of the study and, therefore, only a single concentration of isoproterenol (100nM) was used.

On an initial approach to study the influence of the adrenergic activation on human macrophage polarization, the macrophage inflammatory profile was extensively characterized under different polarization stimuli, namely on expression of pro- and anti-inflammatory markers, release of immunological mediators, cytoskeleton organization and cell morphology . The question we aimed to address was if the phenotype “typical” features can be changed by β2-adrenoceptor activation.

Macrophages are cells with high plasticity and its cytoskeleton organization is susceptible to alteration by the composition of their microenvironment (96). LPS/IFN stimulated macrophages are, in general, more elongated, whereas polarization towards an anti-inflammatory phenotype (with IL-10 or IL-4/IL-13, for instance) induces a population of macrophage with a predominance of a more rounded form (32,97). An identical pattern of morphology was found in the present study where predominates macrophages with a more elongated form in LPS stimulated cells and macrophages with

a more rounded form in IL-10 stimulated cells. These changes in the macrophage cytoskeleton may be characterized and quantified by determining the cell aspect ratio and cell area. In our work, the LPS stimulation increased macrophages cell aspect ratio, while IL-10 stimulation increased cell area. However, the presence of isoproterenol seems to reduce cell area in both, LPS and IL-10 stimulated macrophages, although the aspect ratio was not altered, preserving the typical morphology. These results may indicate that actin and tubulin distribution is modulated by isoproterenol. However, in spite of the cell morphology alterations, we confirmed that the macrophages remained viable and metabolically active when cultured with isoproterenol.

The tumour microenvironment affects macrophage polarization. The process of polarization can be diverse because of the complex environment created that can interfere with the function (33). The inflammatory profile was measured by different parameters, such as mRNA levels and protein expression, to achieve a complete characterization. Different inflammatory signals can polarized macrophages to the M1 phenotype. LPS or IFN-Ɣ can interact with Toll-like receptor 4 (TLR4) on the surface of macrophages, inducing the activation of NF-kB pathway. Such induction can lead to the activation of essential genes necessary for a pro-inflammatory response (98), and associated with high levels of CD86 and HLA-DR cell surface expression (99,100). On the other hand, signals as IL-4 bind to IL-4R and activate signalling pathways, which regulate the expression of genes associated with an anti-inflammatory response, and express CD163 and CD206 receptors (100,101). In our study, we analysed the expression of M1-like and M2-like polarization markers in LPS- and IL-10 stimulated macrophages for 3 days, by flow cytometry analysis and qRT-PCR. Our results confirmed previous reports demonstrating that unstimulated macrophages are more similar to IL-10 than to LPS-stimulated (32). As mentioned, the different polarization status were associated with distinct markers. We confirmed that the LPS and IL-10 stimulated macrophages resulted in distinct profiles, with high expression of co-stimulatory molecule CD86 on M1 and high levels of CD163 receptor on M2 macrophages.

Our study evidence that the macrophage polarizations markers were altered in presence of isoproterenol. This result is not in agreement with what was described in mouse peritoneal macrophages where the CD206 receptor expression was not altered in LPS stimulated macrophages with isoproterenol (80), what may denote an interspecies difference on the role of β-adrenoceptors. Furthermore, our results show that the percentage of positive cells expressing the pro-inflammatory marker (CD86) reduces to about half of the population in the presence of isoproterenol. Interestingly, the LPS

stimulated macrophages present an increase of the anti-inflammatory marker in presence of these agonist, what supports that in human macrophages β2-adrenoceptor activation tends to counteract the M1-like phenotype. These results were confirmed by mRNA levels of pro-(CD80) and anti-(CD163) inflammatory markers obtained so far.

To complement the morphological characterization and surface marker expression of the macrophages and the influence of β2-adrenoceptor activation, the profile of cytokine release was determined to define polarization outcomes. The cytokines are small soluble proteins that are relevant to mediate communication between immune and non-immune cells. These mediators are important because when dysregulated are implicated in several diseases (102). The pro-inflammatory cytokines are produced when macrophages are exposed to inflammatory stimuli and release TNF-α, IL-6, IL-1, IL-8 and IL-12. On the other hand, IL-10 is associate to supress MHC-II expression in activated macrophages and inhibits the production of the TNF-α, IL-6 and IL-12, being associated an anti-inflammatory profile (103,104). Our results confirmed that M1-like macrophages release high amounts of TNF-α, IL-6 and IL-12/23(p40), while the IL-10 cytokine is upregulated in M2-like.

The role of β2-adrenoceptor activation in cytokine production has been addressed by different groups. Bacou et al (89) demonstrated that β2-adrenoceptor stimulation of porcine macrophages prevented the LPS induced increase in TNF-α and IL-8, while increasing IL-10 secretion. Thus, concluding that β2-adrenoceptor activation of porcine macrophages prevented polarization towards a pro-inflammatory phenotype. In addition, other authors confirmed that, in mouse peritoneal macrophages, the LPS stimulation in presence of noradrenaline or adrenaline resulted in a strong M2 phenotype with high levels of IL-10 (80). The results obtained in the present study in human macrophages are in line with those obtained in pigs and mouse. Notably, isoproterenol decreased LPS induced pro-inflammatory signal whereas increased IL-10 anti-inflammatory effect Therefore, this approach provided an additional evidence that, even on the messengers released by macrophages to the microenvironment, β2-adrenoceptor activation tends to counteract the cytokines profile typical of the M1-like phenotype, contributing to the creation of a more immunotolerant microenvironment.

These results gave us the idea to develop new strategies to repolarize M2-like macrophages towards an M1-like phenotype. Several approaches are implemented to repolarization, creating an environment less immunosuppressive. For example, the success of anti-angiogenics is partly due to repolarization of M2-like to M1-like, inhibiting the abnormal tumour vasculature (105). Therefore, the manipulation of

microenvironment stimuli to repolarize M2-like (TAMs) to a tumour-suppressive phenotype is a potential clinical strategy for cancer therapy (106).

To study the immunomodulatory mechanism of β2-adrenoceptor, it is important to remember the signalling pathway of β2-adrenoceptor activation (Fig.4). The increase of cAMP levels leads to PKA activation, and it is important to the phosphorylation of cAMP-responsive element binding protein (CREB). In unstimulated cells, NF-kB proteins are localized in the cytoplasm and associated with inhibitory proteins, such as IkBα. Several stimuli are able to activate the NF-kB pathway, therefore increasing phosphorylation of IkBα proteins by IKK complex, which results in its ubiquitination and proteosomal degradation, leading to NF-kB release, allowing their nuclear translocation and further activate gene expression (107). The literature reported that elevated cAMP levels can inhibit NF-kB mediated transcription via PKA pathway. Then, PKA-mediated phosphorylation of CREB leads to the recruitment of CREB-binding protein (CBP). This protein is a coactivator of NF-kB and interacts with p65 subunit of NF-kB to promote the transcription. However, the competition between p65 and CREB for CBP leads to reduce NF-kB activity (108). Another mechanism involves the regulation of IkB proteins. Literature demonstrated that, when β2-adrenoceptor was activated, IkBα levels were significantly increased. Then the IkBα levels accumulate, and NF-kB is inhibited. This mechanism can be explained because the β2-adrenoceptor agonist increases the cytoplasmic β-arrestin2 (109). Our results showed an increase in p65 phosphorylation in LPS stimulated macrophages, which in presence of isoproterenol decreased. Similar differences seem to be applied to IkBα phosphorylation. Given this, our results seem to indicate that in LPS stimulated macrophages, the NF-kB pathway is activated, probably recruited to the nucleus and leading to the expression of several pro-inflammatory genes. As evidenced, this pathway has been described as an important pathway to “re-educating” tumour-associated macrophages (110–112). In contrast, when the β2-adrenoceptor is simultaneously activated, IkBα and NF-kB phosphorylation decrease suggesting reduce nucleus translocation, impairing the transcription of pro-inflammatory genes and favouring the acquisition on an anti-inflammatory phenotype, which may contribute to stimulation of cancer cell invasion (Fig.29 Appendix 2). These possibilities will be further discussed in more detail.

One of the main functions of macrophages is their phagocytic activity. Phagocytosis seems to occur after a period in which functions are more oriented to killing the infectious agent. In spite of the debate about the relevance of each of these two functions (killing of the infectious agent and phagocytosis of debris) for the different macrophage

phenotypes, M1-like macrophages seem to be more related with microorganism killing, while M2-like macrophages are more linked with phagocytosis of cell debris (36,80) and less capable of bacterial phagocytosis (113). By using bacteria-conjugated particles, we observed that phagocytic activity was lower in LPS-polarized macrophages and treatment with the β-adrenoceptor agonist increased their phagocytic capacity. Identical results were obtained by others (80).

Immunosuppression have a crucial role on cancer cell invasion, promoting a tumour progression (114). So, we think that would be important to evaluate the alterations that macrophages can induce on cancer cells. As reported by our group, macrophages induce an increase in cancer cell (32). M2-like macrophages, which efficient in stimulating gastric cancer cell invasion than M1-like macrophages. Curiously, M1-like macrophages in presence of isoproterenol were more capable of inducing gastric cancer cell invasion, showing ability to promote cancer cell invasion similarly to M2-like macrophages. This data emphasize that isoproterenol drives pro-inflammatory macrophages into an anti-inflammatory phenotype. In addition, is very interestingly, contributing to exploit the use of β-antagonists in order to impair the cancer cell invasion. However, the results obtained so far strongly support such possibility.

Altogether, our data demonstrate that, in the presence of a β-adrenoceptor agonist, a danger signal able to cause a M1-like macrophage phenotype polarization will be more rapidly counteracted, favouring the creation of a more immunosuppressive environment (Fig.27 e 28, Appendix 2). Having in mind that endogenous adrenaline, a catecholamine release during stress which has higher affinity for β2-adrenocpetors, may be present in tumour microenvironment and the reports showing that β-adrenoceptor antagonists exert anti-tumour effects *in vivo*, the present study presents a contribution to explain the unexpected clinical effects of such class of drugs and should be seen as a stimulus for the development of clinical studies to define when and in which cancer patients may profit from the use of these drugs.

Conclusion and future perspectives

Our study was focused on the direct effect of isoproterenol on human macrophages polarization and on their ability to influence cancer cells invasion.

Macrophage characterization revealed that different stimuli promote different phenotypes. LPS polarization contributes to an M1-like pro-inflammatory phenotype. On the other hand, IL-10 polarization contributes to the acquisition M2-like anti-inflammatory phenotype. For this, important features of macrophages namely their morphology, cytoskeleton organization, pro- and anti-inflammatory markers, phagocytic activity, NF-kB signalling pathway and the ability to promote cancer cell invasion were evaluated. In spite of the cell area was affected, the use of β2-adrenoceptor agonist, the isoproterenol, did not cause a cytotoxic effect maintained macrophage metabolic activity.

In addition, we investigated if the β2-adrenoceptor activation can promote changes on human macrophage polarization profile and features. LPS stimulated macrophages were more responsive to isoproterenol, decreasing the pro-inflammatory markers, such as CD80, CD86, TNF, IL-6 and IL-12/23(p40), but also promoting an increase of anti-inflammatory markers, such as CD163 and IL-10. However, the IL-10 stimulated macrophages were not altered. To better characterize the effect of isoproterenol on macrophage polarization profile, numerous pro- and anti-inflammatory molecules and cytokines were analysed through flow cytometry, qRT-PCR and ELISA, as potential interesting tools to use in the future. This change on macrophage polarization can be justified, not only, through alterations on NF-kB pathway but also through the simultaneously activation of other molecular partners, such as MAPK. Future experiments will be require to deeply investigate these mechanisms, including their cross-talk with the β2-adrenoceptor pathway (Fig.29, Appendix 2).

Concerning the effect of isoproterenol on macrophage functionality, we also analysed important characteristics related to their ability to induce cancer cell invasion. We found that isoproterenol drives LPS stimulated macrophages towards a more anti-inflammatory and pro-invasive phenotype. The phagocytic activity is an important macrophage feature. Despite literature be controversy, isoproterenol promoted again the acquisition of a more phagocytic anti-inflammatory phenotype. In the future, it will be interesting to measure the ability of isoproterenol-modulated macrophages to phagocytes cancer cells.

Overall, these results provide evidence that the isoproterenol drives pro-inflammatory macrophages towards an anti-inflammatory phenotype and promoting macrophage-

mediated cancer cell invasion. These results opens a new opportunity to investigate, the use of β-blockers, as an anti-cancer therapeutic agents.

To get more detail on the impact of isoproterenol on the ability of macrophages to induce cancer cells invasion-associated activities, it would be also important to analyse the involved signalling pathways, finding novel targets to impair tumour progression.

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Appendix

Appendix 1. Schematic overview of the matrigel invasion assay

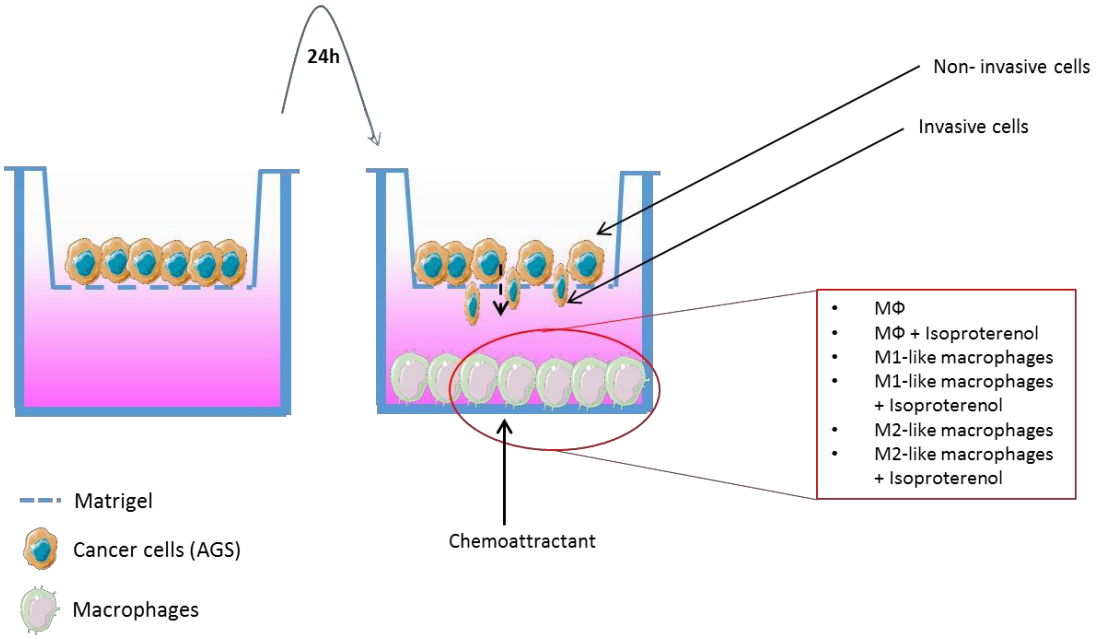

Figure 26. Experimental design of matrigel invasion assay. Overview of invasion assay with macrophages and gastric cancer cell. At 12th day of macrophages cultured, invasion assay was performed during 24h. The macrophages were used as a stimulus to invasion of the cancer cells. The cells that invaded are at bottom of matrigel membrane and those that did not invaded remain at the top of the membrane. The membrane of matrigel represents the basement membrane matrix.

Appendix 2. Schematic overview of the results obtained in this study

1. Human macrophage profile

M1-like macrophages	M2-like macrophages
CD86 ↑ *	CD163 ↑
TNF-α ↑	IL-10 ↑ *
IL-6 ↑	-
IL-12/23(p40) ↑	-
Elongated shape *	Rounded shape *
Cancer cell invasion ↓	Cancer cell invasion ↑
Phagocytic activity ↓	Phagocytic activity ↑
Phospho-p65↑	-
Phospho-IkBα↑	

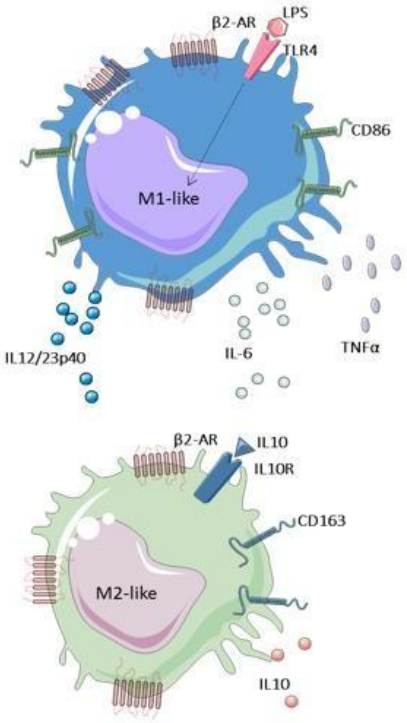

Figure 27. Characterization of macrophages under different stimuli toward a distinct polarization profile. Human macrophage polarization profile was characterized by evaluating morphology, cytoskeleton organization, phagocytic activity, the expression of pro- and anti-inflammatory markers, cytokine production, signalling pathways and ability to promote cancer cell invasion by several techniques; * : significantly different at $p<0.05$

2. Influence of isoproterenol on human macrophage polarization

ISOPROTERENOL

M1-like macrophages	M2-like macrophages
CD86 ↓ *	CD163 =
TNF-α ↓ *	IL-10 =
IL-6 ↓*	-
IL-12/23(p40) ↓ *	-
Cancer cell invasion ↑	Cancer cell invasion ↑
Phagocytic activity ↑	Phagocytic activity ↑
Phospho-p65 ↓	-
Phospho-IkBα↓	-
Cell area↓ *	Cell area↓*
Cell aspect ratio =	Cell aspect ratio =
CD163↑	-
IL-10↑ *	-

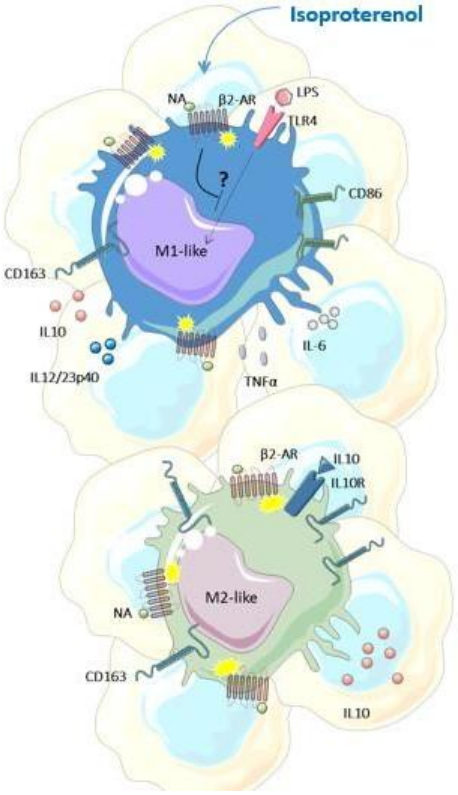

Figure 28. Isoproterenol-modulated human macrophage polarization. The influence of isoproterenol on human macrophage polarization profile was characterized by evaluating morphology, cytoskeleton organization, phagocytic activity, the expression of pro- and anti-inflammatory markers, cytokine production, signalling pathways and ability to promote cancer cell invasion by several techniques; * : significantly different at $p<0.05$

3. Influence of isoproterenol on NF-kB pathway

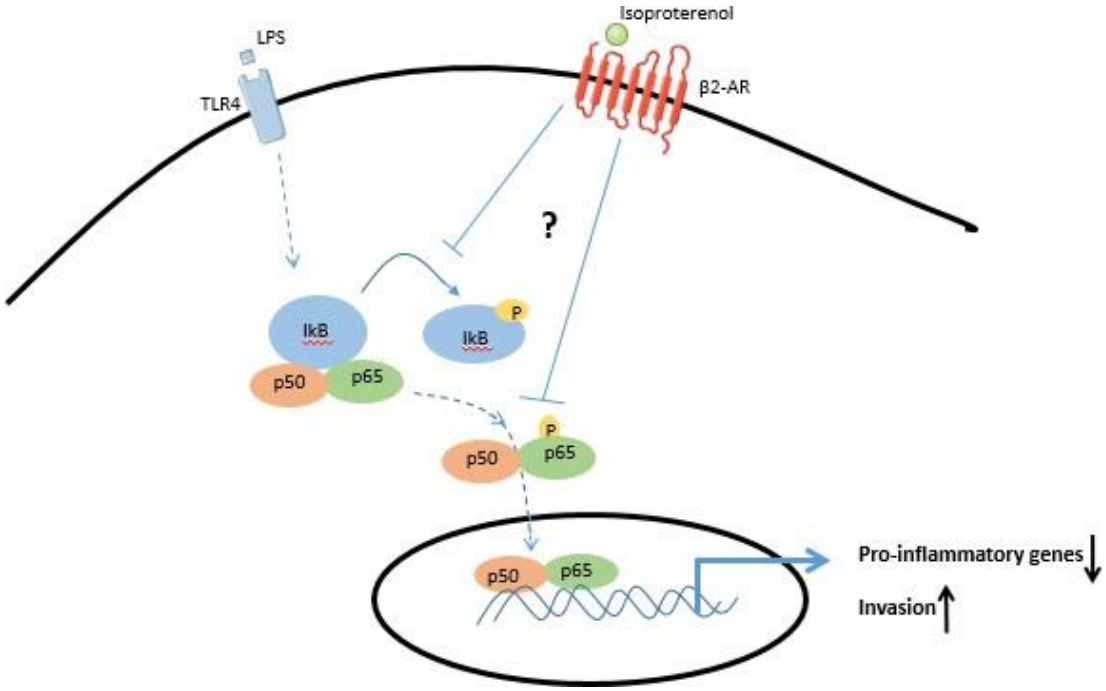

Figure 29. Hypothetical mechanism model established between NF-kB pathway and β2-adrenergic system. The LPS-binding to TLR4 induces the activation of the NF-kB pathway. The IkBα is phosphorylated, by IKK complex, which results in its degradation. So, the NF-kB (p50/p65) is released and translocated into nucleus, where it actives the transcription of pro-inflammatory genes. The NF-kB mediated transcription may be inhibited by β2-adrenoceptor decreasing the transcription of pro-inflammatory genes and enhancing cancer cell invasion.