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Implication of amino acid metabolism in monocyte differentiation

O impacto do metabolismo dos aminoácidos na diferenciação de monócitos

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ABSTRACT

Monocytes are circulating blood leukocytes that arise from the bone marrow and are responsible for a wide array of homeostatic functions upon recruitment to different tissues. These phagocytes are able to mount an anti-microbial response, they are responsible for the production of a large array of cytokines and, importantly, they represent a pool of myeloid precursors that ultimately may originate tissue macrophages and dendritic cells. Recently, several reports have shed light on the relation between metabolism and immune function, which has been vastly explored in groundbreaking research on the field of immunometabolism. The development of a specific immune response relies on the accurate utilization of certain nutrients, thus determining distinct metabolic phenotypes and consequently distinct effector functions. Although several reports state the importance of the metabolic environment for the correct function of macrophages, little is known about the impact of amino acids during monocyte differentiation. We are interested in studying the metabolic requirements of human $CD14^+$ monocytes derived from peripheral blood mononuclear cells during differentiation in macrophage populations. The analysis of amino acid catabolism/anabolism by these populations, using high performance liquid chromatography, revealed distinct metabolic needs, depending on the differentiation profile. In order to assess the contribution of non-essential amino acids during differentiation, we cultivated the purified monocytes in conditioned media, where we selectively depleted L-aspartate. Moreover, the role of L-aspartate as a modulator of monocyte differentiation was assessed for several functional characteristics of macrophages such as phenotypical markers and other effector functions by flow cytometry. We observed that the differential availability of this amino acid during monocyte-to-macrophage differentiation impacts their final fate regarding function, thus showing a role for amino acid metabolism in the modulation of macrophage response.

RESUMO

Os monócitos são leucócitos circulantes originários da medula óssea responsáveis por várias funções homeostáticas após recrutamento para diferentes tecidos. Estes fagócitos são capazes de desenvolver uma resposta anti-microbiana, produzir diversas citoquinas pró-inflamatórias e representam uma reserva de precursores mieloides, que podem eventualmente originar macrófagos tecidulares e células dendríticas.

Recentemente, diversos trabalhos expuseram a relação entre o metabolismo e as funções imunes, um conceito que tem vindo a ser aprofundado na área do imunometabolismo. O desenvolvimento de uma resposta imune específica depende da correta utilização de certos nutrientes, o que por sua vez dita a existência de diferentes fenótipos metabólicos e, consequentemente, funções efetoras distintas. Apesar de diversos estudos demonstrarem a importância do microambiente metabólico para o desenvolvimento de uma função adequada dos macrófagos, pouco se sabe acerca do papel dos aminoácidos durante a diferenciação de monócitos. Este trabalho tem como objetivo estudar os requisitos metabólicos de monócitos humanos $CD14^{+}$, derivados de células mononucleares do sangue periférico, durante a sua diferenciação em populações macrofágicas. A análise do catabolismo/anabolismo de aminoácidos, através de cromatografia liquída de alta performance, revelou diferentes necessidades metabólicas, consoante o perfil de diferenciação induzido nos macrófagos. De forma a entender a contribuição dos aminoácidos não essenciais durante este processo, os monócitos foram purificados e cultivados em meios condicionados em que o L-aspartato foi seletivamente depletado. O papel deste aminoácido como modulador da diferenciação de monócitos foi avaliado através do estudo de várias características funcionais dos macrófagos, como os marcadores fenotípicos e outras funções efetoras, que foram avaliados por citometria de fluxo. Neste estudo foi possível observar que a biodisponibilidade deste aminoácido durante a diferenciação dos monócitos em macrófagos tem um impacto no seu perfil final, nomeadamente em termos de função, o que demonstra um novo papel do metabolismo de aminoácidos na modulação da resposta dos macrófagos.

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LIST OF ABBREVIATIONS

7-AAD, 7-aminoactinomycin D

Acetyl-CoA, Acetyl-coenzyme A

AMPK, Adenosine monophosphate-activated protein kinase

APC, Allophycocyanin

Arg, Arginase

ASAT, Aspartate aminotransferase

ATP, Adenosine triphosphate

BMDM, Bone marrow-derived macrophages

CCL, Chemokine (C-C motif) ligand

CCR2, C-C chemokine receptor type 2

CD, Cluster of differentiation

CDP, Common dendritic cell precursor

CLR, C-type lectin receptor

CMP, Common myeloid progenitor

CO_2, Carbon dioxide

CSF, Colony-stimulating factor

CSF-1R, Colony-stimulating factor -1 receptor

CX3CR1, CX3C chemokine receptor 1

DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein

DAMPs, Damage-associated molecular patterns

DC, Dendritic cell

DHE, Dihydroethidium

DHR, Dihydrorhodamine

ECAR, Extracellular acidification rate

ELISA, Enzyme-linked immunosorbent assay

EMP, Erythrocyte-myeloid progenitor

FACS, Fluorescence-activated cell sorting

FAO, Fatty acid oxidation

FAS, Fatty acid synthesis

FBS, Fetal bovine serum

FcγRIII, Fc fragment of IgG receptor III

FITC, Fluorescein isothiocyanate

GLUT, Glucose transporter

GM-CSF, Granulocyte-macrophage colony-stimulating factor

GMP, Granulocyte macrophage progenitor

G6PD, Glucose-6-phosphate dehydrogenase

GSH, Glutathione

H_2O_2, Hydrogen peroxide

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α, Hypoxia-inducible factor-1 alpha

HLA-DR, Human leucocyte antigen – antigen D related

HPLC, High performance liquid chromatography

HSC, Hematopoietic stem cell

IDO1, Indoleamine 2, 3-dioxygenase 1

IFN, Interferon

IL, Interleukin

iNOS, inducible Nitric oxide synthase

LPS, Lipopolysaccharide

Mφ, Macrophage

M-CSF, Macrophage colony-stimulating factor

MDM, Monocyte-derived macrophages

MDP, Macrophage/DC progenitors

MDSC, Myeloid-derived suppressor cells

MEM, Minimum Essential Medium

mTOR, mammalian Target of rapamycin

mTORC1, mTOR complex 1

NAD^+, Nicotinamide adenine dinucleotide

NADH, Nicotinamide adenine dinucleotide hydrogen

$NADP^+$, Nicotinamide adenine dinucleotide phosphate

NADPH, Nicotinamide adenine dinucleotide phosphate hydrogen

NAO, Nonyl acridine orange

NF-κB, Nuclear factor- κB

NK, Natural killer

NLR, NOD-like receptor

NO, Nitric oxide

O_2^-, Superoxide anion

OPA, ortho-phthalaldehyde

OXPHOS, Oxidative phosphorylation

PAMPs, Pathogen-associated molecular patterns

PBMCs, Peripheral blood mononuclear cells

PBS, Phosphate-buffered saline

PDC, Plasmacytoid dendritic cells

PE-Cy7, Phycoerythrin-cychrome 7

PHD, Prolyl hydroxylase

PI, Propidium iodide

PI3K/Akt, Phosphatidylinositide 3-kinase/Protein kinase B

PGC-1β, PPARγ-coactivator-1 beta

PPAR, Peroxisome proliferator-activated receptor

PPP, Pentose-phosphate pathway

Pre-cDC, pre-classical dendritic cells

PRRs, Pattern recognition receptors

RLR, RIG-I-like receptor

ROS, Reactive oxygen species

RPMIc, Roswell Park Memorial Institute complete

RPMI w/o ASP, RPMI without L-aspartate

RT, Room temperature

SLE, Systemic lupus erythematous

SOD, Superoxide dismutase

STAT, Signal Transducer and Activator of Transcription

TAM, Tumor-associated macrophages

TCA, Tricarboxylic acid

TF, Transcription factor

Th, T helper

TLR, *Toll like*-receptor

TMRE, Tetramethylrhodamine ethyl ester perchlorate

TNF, Tumor necrosis factor

INTRODUCTION

1. The impact of cellular metabolism on immune function

The immune system is a crucial player on the homeostatic maintenance of any organism. It comprises adaptive and innate immune responses responsible for the protection of organisms against exogenous and endogenous pathogens or danger signals[1]. The adaptive system requires several days to mount a specific effective humoral and cellular response (characterized by T and B cells), which will recognize a particular antigen leading to the development of immunological memory[2]. While humoral responses are characterized by the production of specific antibodies by B cells, cellular responses are mediated by T cells after recognition of specific antigens delivered by antigen presenting cells. Adaptive immunity can provide long-time protection due to an acquired response that prepares the immune system for future challenges. On the other hand, the innate immune system, composed by proteins and cells involving phagocytes (dendritic cells (DCs), monocytes, macrophages (Mφ), neutrophils) or natural killer (NK) cells, is characterized by very rapid responses upon recognition of features common to pathogens or cellular damage[3,4]. The typical inducers of innate immunity are pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which instruct the initiation of effector responses through the activation of pattern recognition receptors (PRRs) such as "Toll-like" receptors (TLRs), "nucleotide-binding oligomerization domain receptors (NOD)-like" receptors (NLRs), C-type lectin Receptors (CLRs) or "retinoic acid inducible gene 1 (RIG-I)-like" receptors (RLRs). The recognition of intracellular or extracellular danger signals by these receptors allows the induction of distinct intracellular signaling to develop an efficient immune response. The combination of innate and adaptive responses allows the formation of a complex and robust immune response capable of eliminating the pathogen or recovering from the endogenous damage that originated a particular danger signal.

Although innate immunity lacks the specificity of adaptive immunity, being this interpreted as antigen-specific, recent evidences have been changing the paradigm that only adaptive immunity can build immunological memory[5]. Indeed, both "innate immune memory" and "trained immunity" have been coined to characterize the innate immunological memory of past insults. The concept of trained immunity implies that monocytes and/or Mφ exhibit memory characteristics orchestrated by

epigenetic reprogramming, which mediate protective effects after a second signal or encounter with a pathogen. As an example, β-glucan was describe to train monocytes to produce increased levels of pro-inflammatory cytokines after contact with a secondary and distinct stimulus[6]. This enhanced response confers protection against some infections such as by *Candida albicans*[7]. Importantly, the modulation of these functional programs may be used as a cellular and molecular basis for vaccine development[8]. In opposition, monocytes can undergo a distinct functional program during inflammatory conditions known as tolerance. This term has been described as the unresponsiveness state of cells to repeated or prolonged stimulus such as lipopolysaccharide (LPS). The LPS tolerance leads to inhibition of TLR signaling and consequent to a decreased production of inflammatory cytokines[9]. Interestingly, exclusive epigenetic signatures have been considered responsible for the distinct phenotype between trained and tolerant monocytes[10]. The trained and tolerant cells display distinct metabolic requirements, which will fulfill different energetic needs. If in one hand, naïve and tolerant cells use preferentially fatty acid β-oxidation and oxidative phosphorylation (OXPHOS) as energy sources, trained monocytes rely mostly on enhanced glycolysis and fermentation[11]. Associated with distinct metabolic choices and epigenetic signatures, different effector functions are also associated with these subsets. Although the epigenetic and metabolic reprograming of these cells may render groundbreaking and innovative mechanisms for the development of protection against infections and mucosal tolerance, trained immunity programs may also originate maladaptive states, as in what happens during *immune paralysis* in sepsis[5]. During acute inflammation, as seen in septic conditions, leukocytes shift towards fermentation, with upregulation of glycolytic genes involved in the mammalian target of rapamycin-Hypoxia-inducible factor-1 alpha (mTOR-HIF-1α) pathway and increased secretion of lactate and nicotinamide adenine dinucleotide (NAD^+) production. However, tolerant leukocytes (either induced *in vitro* through LPS exposure or extracted from septic patients), enter a state of *immunometabolic paralysis*[11]*.* This later stage of the disease is characterized by a shift to an anti-inflammatory stage, ultimately triggered to induce tissue repair but consequently leading to a premature inhibition of host defense pathways. These cells showed a defect in glycolytic and oxidative metabolism which is, on the contrary, characterized by an impairment of all major metabolic pathways, as seen by the decreased utilization of lipids and a diminished activation of mTOR[11].

2. Monocyte and Macrophage development

2.1. Monocytes: origin, development and fates

Circulating monocytes are a heterogeneous population of cells that initiate the maturation process in the blood and, when recruited to the sites of inflammation, undergo further maturation and activation[12]. The heterogeneity of this population is associated with different origins and fates[13]. In the bone marrow, blood monocytes and DCs are originated from hematopoietic stem cells (HSCs) and this differentiation includes the transition to a common myeloid progenitor (CMP), which will subsequently generate granulocyte macrophage progenitor (GMP) and then the macrophage/dendritic cell progenitors (MDP)[14]. Afterwards, MDP gives rise to monocytes or to the common DC precursors (CDPs) (Figure 1)[15]. Thus, MDPs give rise to distinct types of blood monocytes characterized by the surface expression of Ly6C (glycosylphosphatidylinositol-anchored molecule) in mice or CD14 in humans. These cells are able to leave bone marrow to circulate in the blood vessels and then give rise to Mφ or to inflammatory DCs upon entrance in tissues. Upon recruitment, monocytes populate different tissues as Mφ displaying significant heterogeneity in terms of phenotype, homeostatic turnover and function[15]. If monocytes have not been recruited to tissues after two days in circulation, they die and are removed from the blood. Indeed, this short half-life has suggested these myeloid precursors for optimal replenishment of tissue Mφ and DCs[16]. However, recent studies have shown that tissue-resident Mφ can self-maintain independently of HSCs and thus do not depend on monocytes originated from bone marrow myeloid precursors[17,18,19]. These resident Mφ originate from yolk-sac-derived erythrocyte-myeloid progenitors (EMPs) distinct from HSCs. The vast majority of adult tissue-resident Mφ in liver (Kupffer cells), brain (microglia), epidermis (Langerhans cells) and lung (alveolar Mφ) originate from the EMP in the yolk sac that migrate to the fetal liver during development[20]. Tissue-resident Mφ promote tissue homeostasis and present different characteristics and transcriptional profiles depending on the tissue[21]. For example, red-pulp Mφ in spleen regulate iron recycling, promoting red blood renewal; alveolar Mφ regulate pulmonary surfactant replenishment; osteoclasts promote bone regeneration[22]. Although these recent findings changed the initial paradigm that bone marrow progenitors were entirely responsible for replenishment of tissue Mφ through monocyte differentiation, it also reinforces the idea that Mφ are

a heterogeneous population, which display distinct functional programs depending on several factors, as the differentiation factors present in the microenvironment and epigenetic signatures originated though development.

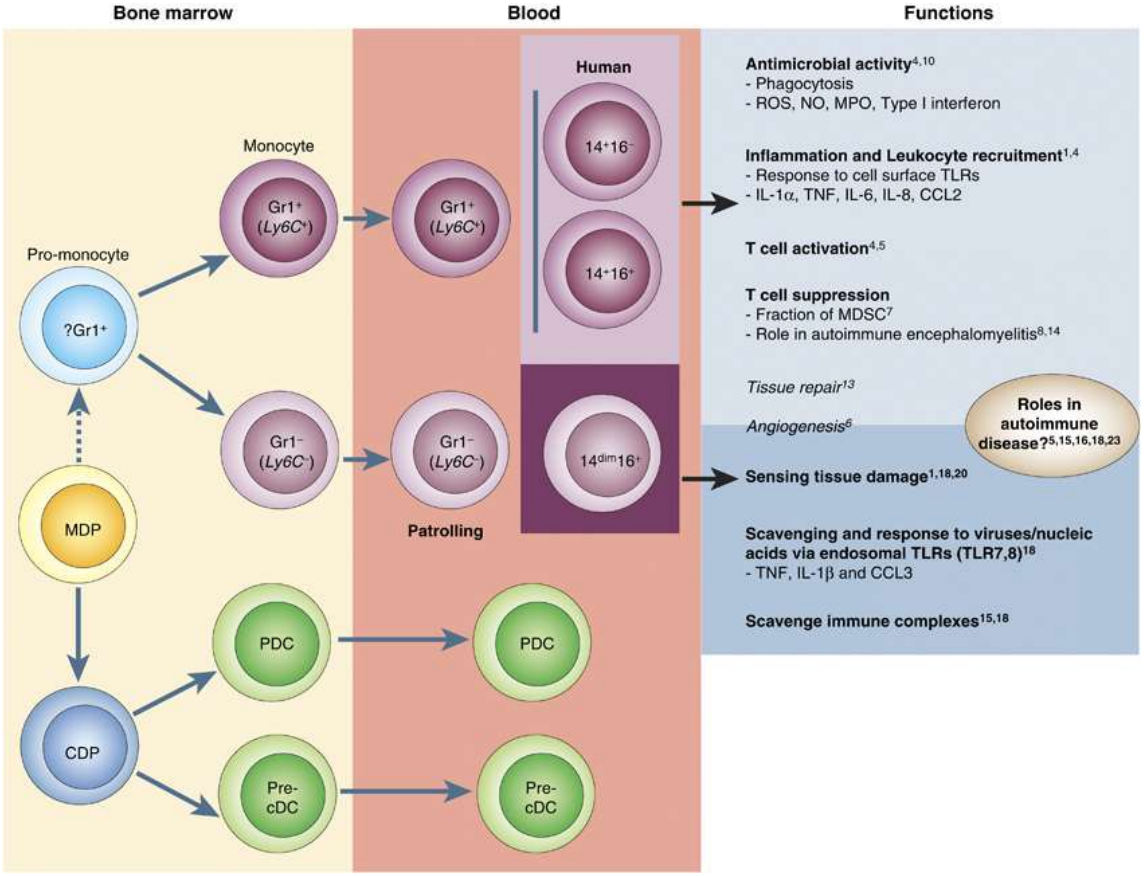


Figure 1. Development of blood monocytes from hematopoietic precursor cells. Hematopoietic stem cells produce monocytes and dendritic cells precursors (MDP) in the bone marrow via a myeloid committed precursor. MDPs give rise to monocytes, possibly through a pro-monocyte (dashed line), and pre-classical dendritic cells (Pre-cDC) and plasmacytoid dendritic cells (PDC) via a common dendritic cell precursor (CDP). In the mouse, two monocyte subsets Ly6C$^+$ and Ly6C$^-$ leave the bone marrow to enter the circulation. The corresponding human monocyte subsets are shown in the light and dark purple boxes: inflammatory murine Ly6C$^+$ and two human CD14$^+$ subsets; and murine Ly6C$^-$ and human CD14^{dim} monocyte subsets. Known subset specific functions are shown in bold in the light and dark grey boxes, while suspected functions are in italics. CD14$^+$ monocytes respond to cells surface TLRs and are involved in inflammation and leukocyte recruitment, while CD14^{dim} monocytes sense tissue damage and respond to viruses and nucleic acids via endosomal TLRs (TLR7 and TLR8). Monocytes may play an important role in autoimmune disease: patrolling mouse Ly6C$^-$ and human CD14^{dim} via the production of tumor necrosis factor (TNF), interleukin (IL)-1β and chemokine (C-C motif) ligand 3 (CCL3) after recognition of nucleic acids, while murine Ly6C$^+$ and human CD14$^+$ monocytes can either activate or suppress T-cell responses. (Adapted from[23])

Circulating monocytes are heterogeneous blood leukocytes responsible for a wide array of homeostatic functions[24,25]. They constitute 10% of all leukocytes present in a healthy human blood and approximately 4% of blood leukocytes in mice. During the process of development there is a clear definition of different subsets of these monocytes. In humans, peripheral blood monocytes are characterized based on their morphology and expression of different antigenic markers. The expression of CD14 (a surface marker involved in innate immune response to LPS) and CD16 (the Fc fragment of IgG receptor III - FcγRIII) surface markers on blood monocytes allow the discrimination and identification of three functional subsets of human monocytes[26]. The classic monocytes, $CD14^{++}CD16^{-}$ represent approximately 90% of the human monocyte blood population under physiological conditions and are characterized by a higher phagocytic activity as suggested by the high levels of CD36 and CD163 scavenger receptors. This subset also present an optimal antimicrobial activity as demonstrated by the increased production of reactive oxygen species (ROS) and nitric oxide (NO) in response to bacteria. These monocytes also have an important role in leukocyte recruitment expressing high levels of C-C chemokine receptor 2 (CCR2) and low levels of CXC3 chemokine receptor 1 (CX3CR1). In opposition, the remaining 10% of human monocytes are characterized by the expression of CD16 marker, an important molecule for cytokine production. This population is sub-divided in the intermediate subset ($CD14^{+}CD16^{+}$) that expresses normal levels of CD14 marker and the non-classical subset ($CD14^{low}CD16^{++}$), which presents lower levels of CD14[26]. Depending on the phenotypic expression levels of CD14, these two monocyte subsets play different functions in circulation. Both subsets expressed more TLRs 2, 4, 5, co-stimulatory molecules CD80, CD86 and Human Leukocyte Antigen – antigen D Related (HLA-DR) than the classical subset, suggesting their role in antigen presentation. Yet, while $CD14^{low}CD16^{+}$ monocytes present 'inflammatory' features on activation and have high antigen presenting capability, $CD14^{+}CD16^{+}$ intermediate monocytes appear to be transitional monocytes between classical and non-classical subsets displaying both phagocytic and inflammatory functions[27]. Moreover, the expression of high levels of CX3CR1 and low levels of CCR2, in opposite to classical monocytes, are characteristic of non-classic monocytes, being associated to the patrolling of the endothelium of blood vessels functions in steady state[28]. Some studies have also suggested that the expansion of the $CD16^{+}$ pool of monocytes as an indicator of inflammatory diseases, being the main producers of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β upon activation[29]. However, while in an acute

inflammation such as sepsis both classical and non-classical subsets increases, in a chronic inflammatory context as systemic lupus erythematous (SLE), only the non-classical subset is expanded[27].

The monocyte heterogeneity is conserved among mammals, as these different monocyte subsets have already been reported for other species[30,31]. For instance, in mice, circulating monocytes can be separated in two distinct subsets, according to their expression of Ly6C[31]. $Ly6C^{hi}$ $CX3CR1^{low}$ $CCR2^{+}$ $CD62L^{+}$ (L-selectin) monocytes present a gene expression profile that resembles the one observed in human classical monocytes. They comprise approximately 40% of the blood monocyte population, presenting higher migratory properties and being the main source of Mφ and DCs. On contrary, $Ly6C^{lo}$ $CX3CR1^{hi}$ $CCR2^{-}$ $CD62L^{-}$, that resemble non-classical monocytes, represent 60% of monocytes and consist in a patrolling and long-lived cell population that maintain the homeostasis of endothelium[32,31]. As the human subset, $Ly6C^{hi}$ monocytes, when recruited to inflamed tissues, undergo activation and produce TNF-α, NO and ROS, and are able to stimulate effector T-cell proliferation[33]. However, it has been described that these cells may also have an important role in the suppression of T-cell activation[34]. Furthermore, $Ly6C^{lo}$ monocytes have also been associated with autoimmune diseases by the release of IL-1β and TNF-α in response to nucleic acids and viruses[35]. Similar to what happens with human $CD16^{+}$ monocyte subsets, these cells also patrol the endothelium and protect the vasculature from tissue damage and infection and may be involved in the sensing of dying or infected cells through TLR7[36].

Overall, the identification of these subsets and the understanding of their developmental program allow us to study their function in a physiological context and associate them with a specific fate mapping[18].

2.2. Monocyte Differentiation and Macrophage Polarization

Blood monocytes and DCs, derived from $CD34^{+}$ myeloid progenitor cells in the bone marrow, are always being replenished by the bone marrow precursors, even during adulthood[37]. These cells are regulated by colony-stimulating factors (CSFs) that mediate the survival, proliferation, differentiation and functional modulation of blood precursors and mature blood cells[38]. Macrophage

colony-stimulating factor (M-CSF, also known as CSF-1) and granulocyte–macrophage colony-stimulating factor (GM-CSF) are the predominant promoters of monocyte differentiation[39]. The CSFs are distributed differently on myeloid cell populations[40] and are capable of activate monocytic and macrophagic lineages[41,42].

M-CSF is ubiquitously produced by several tissues and controls Mφ frequency and number in many tissues[43]. M-CSF and IL-34 growth factors instruct cells to leave the bone marrow and to circulate in the bloodstream[44]. During this developmental stage, monocytes and their precursors express CSF-1 receptors (CSF-1R) that bind to M-CSF. The level of CSF-1R expression increases from hematopoietic precursors to monocytes and Mφ and the maturation process is controlled by the presence of M-CSF being its removal of circulation deleterious for monocyte proliferation, differentiation and survival[45]. GM-CSF plays also an important role in the Mφ and DCs network, since it presents low basal circulating levels that are often elevated during immune/inflammatory conditions[46]. GM-CSF receptors are also expressed on $CD34^+$ progenitor cells and maturation of these progenitors into monocytic and granulocytic lineages leads to an increase in the expression of these receptors[38,47]. GM-CSF induces growth, differentiation, and survival of Mφ, granulocyte, erythrocyte and megakaryocyte cells from bone marrow progenitors and modulates the functions of mature effector cells such as neutrophils, Mφ and DCs[38].

Although monocyte subsets present different functions and have distinct fates, the monocyte and later Mφ plasticity allows them to respond to a variety of environment signals and undergo distinct differential profiles to maintain homeostasis[48]. Consequently, Mφ mature and become polarized to a particular specialized functional phenotype, usually clustered under the umbrella of the canonical pro-inflammatory M1 and anti-inflammatory M2 subsets[49]. These *in vitro* phenotypes are a clear reflection of Mφ function specialization. M1-Mφ (classically activated Mφ) result from the exposure to classical activating signals such as interferon-gamma (IFN-γ) and TLR ligands (e.g. LPS). These Mφ respond to further stimulation by secreting TNF-α, IL-12 and IL-6 and through the upregulation of surface expression of co-stimulatory molecules as CD86. This phenotype is associated with killing and degradation of intracellular pathogens due to the production of large amounts of NO and ROS. In the opposite extreme, M2-Mφ (alternatively activated Mφ) are induced

by certain anti-inflammatory cytokines as IL-4 and IL-13 and are associated to tissue remodeling, helminth infection clearance, tumor progression, adaptive immunity regulation and modulation of inflammation[50,51,52]. These Mφ also produce IL-10 and have an increased expression of scavenger and mannose receptors. In a similar manner, monocyte differentiation in response to M-CSF or GM-CSF growth factors leads to Mφ with distinct phenotypes[53,54]. Human monocytes are usually differentiated in monocyte-derived Mφ (MDM) using M-CSF (M-MDM) or GM-CSF (GM-MDM) as models for tissue Mφ[39,55]. GM-CSF in combination with IL-4 is also often used to induce the differentiation of human and mouse monocytes into DCs[56]. However it has been shown that the transcriptome analysis of these GM-MDM is more similar to Mφ than to DCs[57]. GM-MDM have been referred as M1-like Mφ and characterized by the production of pro-inflammatory cytokines. They exhibit potent microbicidal properties, thus promoting a strong IL-12-mediated T helper 1 (Th1) responses. On the other hand, M-MDM have been shown to have a repertoire of M2-like transcriptome with production of anti-inflammatory cytokines with acquisition of certain functions that support T helper 2 (Th2)-associated effector functions and resolution of inflammation[55]. A similar profile has been associated to the polarization phenotypes of murine bone marrow-derived Mφ (BMDM)[58,59]. BMDM, after differentiation in the presence of M-CSF (M-BMDM), are an excellent model for studying M2-Mφ function and signaling[60]. Nevertheless, upon differentiation with GM-CSF (GM-BMDM) it originates a cluster of cells that display similarities with DCs but, like in humans, they have a transcriptome similar to M1-Mφ[58]. Hence, a comparative analysis of M-CSF- and GM-CSF-treated human and murine Mφ populations is essential to fully understand the function of these CSFs in MØ polarization and acquisition of biological functions, particularly in inflammatory diseases[61].

3. Immunometabolism

3.1. Overview of macrophage metabolism

Growing evidences suggest that the polarization of Mφ is associated with a metabolic and bioenergetic reprogramming. Furthermore, the adopted metabolic pathways are a clear reflection of Mφ function and phenotype[62]. The accurate control of the involved metabolic pathways, which allow the fulfillment of the required biological activities, is essential to maintain homeostasis and a correct

functioning of Mφ. During the different stages that include development, proliferation, differentiation and the achievement of a particular effector function, immune cells demand an appropriate bioenergetic supply. For that to happen harmoniously, a crosstalk between the extracellular signals sensed by the immune system and the uptake and catabolism/anabolism of nutrients (glucose, amino acids, and fatty acids) is essential[63]. Upon a stimulus, the modulation of certain metabolic pathways is intrinsically connected to the regulation of cellular bioenergetics, thus supporting the development of an immune response. While before activation Mφ maintain a basal metabolic state, after a response to extracellular signals they undergo an extensive metabolic reprogramming [63]. Yet, the anabolic and catabolic choices strictly depend on the type of stimulus received and are associated with the establishment of an effector response. During this process, the acquired substrates and their intracellular fate will be crucial for the synthesis of adenosine triphosphate (ATP), to feed the synthesis of macromolecules such as RNA, DNA, proteins or even lipids and glucose and to maintain the control of redox state of the cell (Figure 2)[63]. During the glycolytic process, the uptake of glucose via glucose transporters (GLUT) nourish distinct metabolic pathways: anabolic pathways, such as the pentose-phosphate pathway (PPP) that generates riboses (for RNA or DNA synthesis) and nicotinamide adenine dinucleotide phosphate (NADPH) (for fatty acid synthesis (FAS) and the respiratory burst of phagocytes) or the glycogen synthesis for increasing the energetic storages; and catabolic pathways, committed to the production of pyruvate, ATP and nicotinamide adenine dinucleotide (NADH). Under normoxic conditions, pyruvate may be used in the Tricarboxilic Acid (TCA) cycle for the formation of Acetyl-Coenzyme A (acetyl-CoA), which via the electron transport chain of OXPHOS leads to the production of ATP and co-factors important for other metabolic pathways. Instead, in hypoxic conditions, the pyruvate is reduced to lactate, leading to the acidification of the extracellular environment. The Warburg effect, present mainly in cancer cells, is characterized by the production of ATP through high rates of glycolysis and subsequent production of lactate by fermentation. Even in the presence of oxygen, the pyruvate oxidation rate in mitochondria is low in comparison to the fermentative rate[64]. Similarly to what happens with carbohydrates, amino acids and fatty acids also can enter the TCA cycle and be catabolized to acetyl-CoA that replenishes the OXPHOS and allows ATP production. Besides its role in the synthesis of several macromolecules, acetyl-CoA is also involved in posttranslational modifications (namely acetylation) of histones and proteins, thus showing a great importance in other cellular processes apart from metabolism[65]. The

use of non-essential amino acids as glutamine provides a reinforcement of the TCA cycle and the PPP. Two anaplerotic reactions are used to fulfill the TCA cycle: the first converts pyruvate to oxaloacetate via pyruvate carboxylase, whereas the second converts glutamate to α-ketoglutarate via glutamate dehydrogenase.

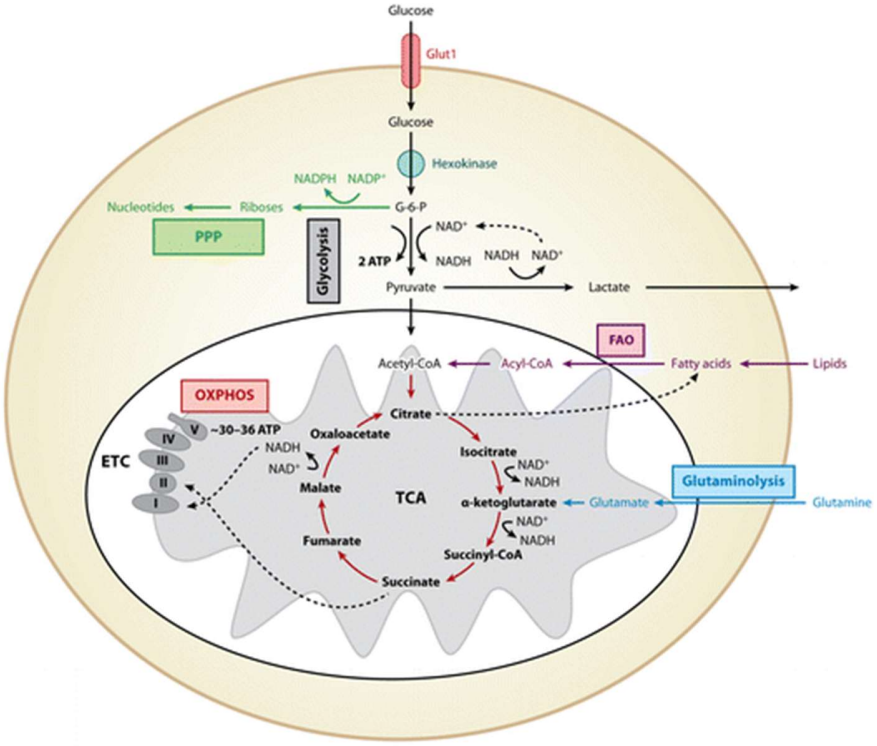


Figure 2. Major metabolic pathways of immune cells. Glucose enters the cells preferably through the GLUT1 and is phosphorylated to G-6-P by hexokinases. During glycolysis, G-6-P is metabolized to pyruvate, reducing NAD^+ to NADH and generating two ATP. In hypoxia, pyruvate is reduced to lactate, restoring NAD^+ levels in the cell. In normoxia, pyruvate is metabolized to acetyl-CoA, which is oxidized in the TCA cycle to generate NADH. In the redox reactions of OXPHOS, electrons are sequentially transferred to generate a H^+ gradient across the inner mitochondrial membrane, which drives the synthesis of ATP. In contrast to glycolysis, mitochondrial OXPHOS is a highly efficient form of generating ATP, yielding ∼30–36 ATP per molecule of glucose. Three additional pathways are shown; the PPP, glutaminolysis, and FAO. G-6-P is the entry point for PPP, which generates riboses for nucleotide synthesis. During this process, $NADP^+$ is reduced to NADPH, forming the critical cofactor required for ROS production via the NADPH oxidase system in neutrophils and Mφ. During glutaminolysis, glutamine is metabolized to glutamate and subsequently to α-ketoglutarate, which then enters the TCA cycle. The fate of glutamine depends on the activation state of the immune cell; it can either be oxidized completely to generate ATP or used to replenish the metabolic intermediates of TCA cycles, which are diverted for macromolecule biosynthesis. The FAO yields acetyl-CoA, which enter the TCA cycle and OXPHOS pathways to generate ATP. (Additional abbreviations: GLUT1, Glucose transporter 1; G-6-P, glucose 6-phosphate; ATP, adenosine triphosphate; TCA, Tricarboxylic acid; ETC, electron transport chain; FAO, fatty acid oxidation; ROS, reactive oxygen species; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogen; OXPHOS, oxidative phosphorylation; PPP, pentose-phosphate pathway; FAO, fatty acid oxidation; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen.) (Adapted from [63]).

Moreover, the glycolysis and TCA cycle pathways also provide intermediates for the biosynthesis of non-essential amino acids. For instance, the glycolysis intermediates 3-phosphoglycerate and pyruvate are used as precursors for the synthesis of serine, cysteine, glycine, and alanine, whereas the TCA cycle intermediates oxaloacetate and α-ketoglutarate are used to synthesize aspartate, asparagine, proline, and arginine[63]. The biosynthetic reactions obtain energy by the continuum replenishment of the TCA cycle and glycolysis metabolic pathways[66]. Their existence in different immune metabolic pathways display a role in regulatory immune networks to control proliferation and immune cells function[67,68].

Importantly, another homeostatic control of cell is the redox state. The reduction of pyruvate to lactate allows the generation of NAD^+ that is essential to sustain the redox state of the cell. The balance NAD^+/NADH is maintained by a control of regulated pathways as TCA cycle and glycolysis. In mitochondria, the malate-aspartate shuttle and $NADP^+$-dependent malate dehydrogenase also allows the balance of $NADP^+$/NADPH, which is required for generation of ROS. The regulation of these distinct metabolic pathways on MØ permits the efficient polarization into distinct phenotypes that are associated to different metabolic requirements.

3.2. Metabolic reprogramming and macrophage polarization

The control of nutrient metabolism and the maintenance of cellular redox state are essential for a homeostatic performance of immune cells. The sensing of extracellular signals through cell-surface receptors (as TLRs) leads to the activation of several metabolic regulators, such as the phosphatidylinositide 3-kinase/Protein kinase B (PI3K/Akt)/mTOR pathways, to initiate the required metabolic adaptations. The mTOR complex 1 (mTORC1) integrates the received signals and sense amino acids availability in order to control its import[69,70]. Nevertheless, the antagonism of 5'-adenosine monophosphate-activated protein kinase (AMPK) and mTOR activity appears to play a significant role in the regulation of the intermediary metabolism as bioenergetic sensors of cells. AMPK is modulated by hormonal and nutrient signals that in turn regulate food intake and energy availability, being a bioenergetic sensor essential in the regulation of cellular energy homeostasis. The role of this kinase in sensing energetic breaks lead to activation of catabolic pathways to obtain energy. On the other

side, AMPK inhibits mTOR, leading to inhibition of anabolic processes and to regulation of this intracellular nutrient sensor that controls protein synthesis, cell growth and metabolism[71]. Consequently, an equilibrium between these nutrient sensors allow nutrients to trigger metabolic switches that prime immune cells for their cell fate functions[63]. Genetic perturbations that prime or inhibit immune cell metabolism have a profound effect on their ability to undergo activation. For instance, deletion of c-Myc in mature T cells lead to inhibition of glycolysis and glutaminolysis pathways, which are crucial for the establishment of an effective proliferation process by these cells[63]. Besides, reprogramming of cellular metabolism is required for the proper polarization and functions of activated Mφ, as previously explained[72]. Recent evidences have begun to illustrate the mechanisms by which metabolism influences the functional phenotype acquired by Mφ under physiological and pathological conditions[62]. The metabolic variations impact the different polarization profiles adopted by Mφ that involve a continuum of functional phenotypes, canonically characterized by the *in vitro* M1 pro-inflammatory and M2 anti-inflammatory Mφ. The conception of metabolic requirements and effector functions of these Mφ are essential to understand the distinct phenotypes adopted by these cells (Figures 3 and 4).

3.2.1. M1-macrophage polarization

The induction of M1-Mφ upon activation by TLRs and pro-inflammatory cytokines, as IFN-γ, is associated with a pro-inflammatory and microbicidal phenotype[73]. In mice, one of the hallmarks of M1 polarization is the IFN-γ dependent expression of high levels of inducible nitric oxide synthase (iNOS) that leads to the production of high quantities of NO[74]. M1-Mφ present a metabolic reprogramming associated with a high glycolytic rate involving an increase in glucose consumption with consequent high levels of lactate secretion (Figure 3)[75]. The fermentation of glucose is the preferred metabolic choice, since it allows these Mφ to rapidly meet their high energy requirements. Accordingly, the glucose transporter GLUT1 is the rate-limiting transporter on pro-inflammatory polarized Mφ[76].

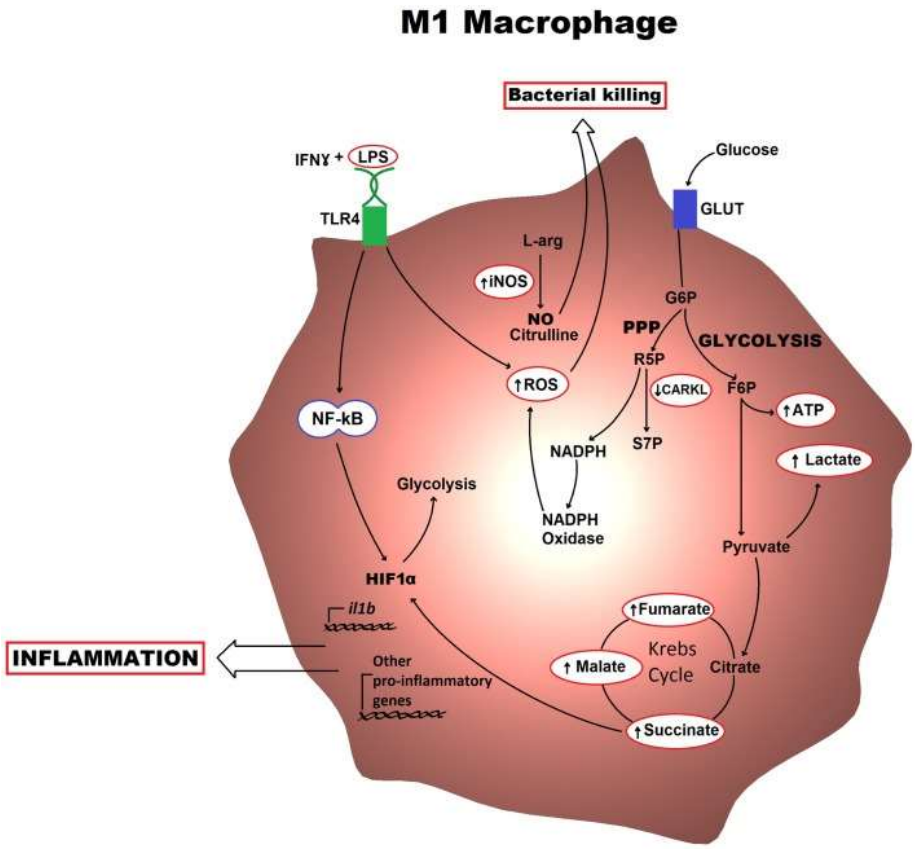


Figure 3. Metabolic profile of an M1 macrophage. Classically activated Mφ present a fermentative program that results in lactate production and increased levels of intermediates of the TCA cycle. The HIF-1α transcription factor becomes activated and drive production of pro-inflammatory cytokines. The key functional consequences are intracellular pathogen killing, mostly through the production of ROS and NO, and inflammation, which occurs via cytokine production. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribulose-5-phosphate; S7P, sedoheptulose phosphate; NO, nitric oxide; ROS, reactive-oxygen species; HIF-1α, hypoxia-inducible factor alpha. (Adapted from[77]).

During phagocytosis, there is also an increase in glycolysis that may be explained by an induction of PI3K/Akt/mTOR pathway. The high glycolytic rate that M1-Mφ adopt allows their survival under hypoxic conditions given their lower necessity on oxygen-dependent mitochondrial ATP production functions[78]. Although glycolysis is less efficient in ATP production, the rapid production of ATP by cells allows the quick replenishment of energetic pools, which is important for cellular homeostasis[64]. Indeed, these high glycolytic rates practiced by Mφ permit a production of intermediaries that are used for the biosynthesis of other macromolecules. For instance, it has been

shown that classically activated Mφ present an increased activity of hexokinase and glucose-6-phosphate dehydrogenase (G6PD), which suggests a metabolic shift between glycolysis and PPP in these Mφ[79], essential for the synthesis of nucleic acids. In addition, the biosynthesis of other macromolecules such as lipids is also important for the membranes restructuration and, consequently, for the establishment of an effective immune response. In parallel, there is a decrease in turnover rates of TCA cycle, which are revealed by an accumulation of citrate and succinate intermediates[80,81]. For instance, in LPS-stimulated Mφ, the mitochondrial membrane potential is reduced to allow the cells to survive and maintain their function during an immune response[82]. It has also been shown that LPS upregulates the cationic amino acid transporter of L-arginine in these Mφ and the consequent NO production leads to a suppression of cellular oxidative metabolism by inhibition of electron transport chain enzymes[83]. The suppression of mitochondrial functions and this accumulation of intermediaries such as succinate in the cytosol leads to a stabilization of hypoxia-inducible factor alpha (HIF-1α) that binds to IL-1β promoter and activates the sustained production of high levels of IL-1β, TNF-α and IL-6 by Mφ[81]. On the other hand, the stabilization of HIF-1α by inhibition of prolyl hydroxylase (PHD) protein, in the presence of succinate in cytosol, also leads to a higher glycolytic rate that is responsible for inducing ROS production[81,76]. The elevated GLUT1-driven glucose metabolism increases PPP that leads to the production of NADPH involved in ROS production. Lipidomics has also contributed to the understanding of the plasticity of lipid metabolism during Mφ activation. In that sense, citrate is another TCA cycle intermediate that accumulates upon LPS stimulation, resulting in its transport to the cytosol, where it serves as a precursor for fatty acid and phospholipid synthesis. In cytosol occurs the expansion of the Endoplasmic Reticulum and Golgi compartments to accommodate the increased secretory demand of M1 Mφ[80]. During Mφ response to TLRs, fatty acids may increase intracellular nuclear factor- κB (NF-κB) signaling. It has been shown that, in response to TLR4 stimulation, Mφ suffer lipid remodeling of glycerolipids, glycerophospholipids and prenols, which may impact its functions[84]. Therefore, the metabolic pathways chosen by immune cells contributes with rapid energy and reducing equivalents, which are crucial for their effector functions.

3.2.2. M2-macrophage polarization

In contrast, the metabolic shift observed during M2 polarization is associated with low glycolytic rates while relying on fatty acid oxidation and oxidative metabolism for energy production (Figure 4)[85]. Cell-intrinsic lysosomal lipolysis has been considered essential in degradation of external triacylglycerol substrates via the scavenger receptor CD36, having a critical role in M2-activation[86]. However, endogenous lipid metabolism also contributes to Mφ phagocytosis by regulating membrane fluidity for the process. Nevertheless, M2-Mφ still present oxidative glucose metabolism through pyruvate oxidation in mitochondria to provide sustained energy for tissue remodeling and repair. This shift to oxidative metabolism allows the Mφ to meet the bioenergetics demands for long-term activation. In mice, M2-Mφ are characterized by the hallmark expression of arginase (Arg) 1dependent of signal transducer and activator of transcription (STAT) 6 and peroxisome proliferator-activated receptor (PPAR)[49,87], in particular due to a significant upregulation of PPARγ, a genetic sensor of fatty acids[88]. The latter plays an important role in Mφ lipid homeostasis since their deletion in Mφ impairs their ability to induce oxidative metabolism[89]. In response to IL-4 signal, PPARγ-coactivator-1β (PGC-1β) has been shown to induce fatty acid oxidation and mitochondrial biogenesis in Mφ[90]. Indeed, expression of PGC-1β primes Mφ for alternative activation and strongly inhibits pro-inflammatory immune response. Moreover, human Mφ present high levels of PPARα responsible for the regulation of cholesterol efflux[91,87]. PPARα also induces apoptosis and downregulates the expression of metalloproteinase in alternatively activated Mφ[92]. Besides, PPARδ controls an inflammatory switch through its association and disassociation with transcriptional repressors[93].

Moreover, IL-4-activated M2-Mφ are characterized by the high expression of mannose, scavenging and galactose receptors. These Mφ also present an efficient phagocytic activity and an increased production of polyamines by arginase pathway[94]. In contrast to what happens in M1-stimulated Mφ, HIF-2α activation is detected in M2-Mφ, which leads to an induction of the expression of Arg1 and consequent suppression of NO production[95]. A significant induction of mTOR pathway inhibits M2 polarization, suggesting this molecule is also closely involved in the regulation of anti-inflammatory polarization[96].

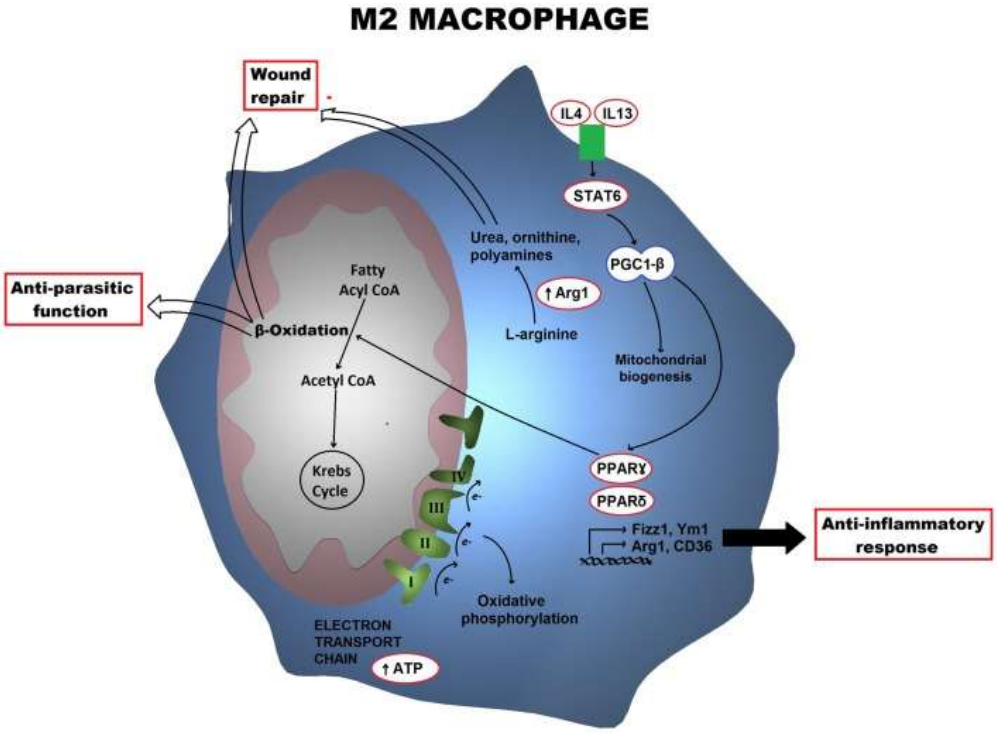


Figure 4. Metabolic profile of an M2 macrophage. Alternatively activated Mφ trigger a metabolic program including the electron transport chain as well as fatty acid β-oxidation, which is orchestrated by STAT6 and PGC-1β. Arg1 also drives the production of polyamines and ornithine. The key functional consequences are tissue repair and anti-parasitic responses. PGC-1β, Peroxisome proliferator-activated receptor gamma coactivator 1-beta; Arg1, arginase 1. (Adapted from[77]).

Overall, the plasticity related to metabolic modulation is exemplified by the blockage of oxidative metabolism, which prevents M2 polarization and reverts it to a M1 phenotype. Similarly, forcing oxidative metabolism in M1-Mφ potentiates the M2 phenotype[75]. Thus, the metabolic changes occurring during Mφ polarization appear to play an essential role in the determination of the activation and effector status of cells[97]. Although the key metabolic differences between differentially activated Mφ are currently accepted, the mechanisms and players responsible for orchestrating these different profiles at the molecular level remain largely unknown.

3.3. Amino Acid metabolism and Macrophage function

Amino acid metabolism is largely associated with the establishment of an effective immune response. The molecular pathways involved in amino acid catabolism have shown to be important in the regulation of innate and adaptive immune responses during physiological and pathological conditions[68]. Nevertheless, the consequence of alterations in amino acid catabolism or anabolism remains unclear. Contrarily to essential amino acids, non-essential amino acids can be synthetized by the cells of the organism. Yet, immune cells are auxotrophs for several of these nonessential amino acids[98]. Glutamine and asparagine represent nonessential amino acids required by immune system to regulate immune responses. For instance, T cells present an obligatory requirement for high levels of glutamine for achieve an effective proliferation[99].Besides, asparagine auxotrophy has also been described to be involved in cancer cell metabolism and the modulation of this amino acid may prove itself as a groundbreaking therapy against cancer cells. In fact, asparaginase, the enzyme responsible for catalyzing asparagine in aspartate, is already in clinical trials for several neoplastic diseases and it has been shown to inhibit tumor growth[100]. Due to the importance of these nutrients in several cell types, some studies intend to use catabolic enzymes for *in vivo* depletion of these indispensable amino acids[101,102].

The presence of amino acids may also be important for the development of effector functions. Regarding essential amino acids, leucine has been shown to activate mTORC1, thus leading to metabolic alterations in cell growth functions[103]. The mechanism of mTOR activation may be different in the presence of leucine, leading to an effective nutrient sensing mechanism essential for catabolism of cells[103]. In addition, another essential amino acid involved in immunometabolic regulation is tryptophan. It is a L-arginine-derived metabolite involved in regulation of immunosuppressive activity of myeloid-derived suppressor cells (MDSCs). Through activation of indoleamine 2, 3-dioxygenase 1 (IDO1), induced by TNF-α and IFN-γ stimulation, Mφ suppress T cell proliferation, thus decreasing inflammation[104]. Moreover, tryptophan is important for the *de novo* synthesis of NAD^+. The rate-limiting enzyme IDO1 converts L-tryptophan in N-formylkynurenine, a biosynthetic precursor of NAD^+, which in homeostatic levels assures an adequate environment for immune cells[105]. In spite of its known importance in the maintenance of redox cell homeostasis, NAD^+ is also important in regulation of the production of inflammatory cytokines as TNF-α and IL-6 by

phagocytes[106]. It has also been shown that Mφ are able to block tumor growth through the consumption of arginine, thus increasing competition for this semi-essential amino acid[107]. However, a dual effect of L-arginine has been described in the context of Mφ polarization. Arginine is imported by activated Mφ through cation transporters as SLC7A2[108] and then can be metabolized by two enzymes involved in its metabolism: iNOS and Arg1. iNOS metabolizes L-arginine leading to the production of NO and L-citruline and Arg1 uses L-arginine to the production of ornithine and urea[109]. Arg1 and iNOS compete with each other for arginine, however, the expression of these two enzymes is associated with different Mφ-polarized functions. Arg1 has an important role in the urea cycle and the deletion of this gene originates a lethal phenotype in M2-Mφ. In opposition, deletion of Arg2, the mitochondrial isoform of the enzyme, only leads to defects in biosynthesis of other amino acids such as glutamate, proline and citruline[110]. Overall, metabolic enzymes such as IDO1 and Arg1 that regulate amino acids availability are linked to the control of immunological mechanisms.

Regarding non-essential amino acids, glutamine, the most abundant amino acid in circulation, is important for the establishment of an effective immune response by Mφ[111]. It is crucial for the development of an M2-like phenotype but it has no major effects in M1-Mφ profile[112]. Depletion of glutamine has been described to decrease the expression of genes associated to M2 polarization, as chemokine (C-C motif) ligand (CCL) 22 gene. Additionally, glutamine shows an important role in inducing anaplerotic reactions that fulfill the TCA cycle[113]. Glutamine-driven glutamate is converted to α-ketoglutarate via glutamate dehydrogenase, which is an essential intermediate that completes TCA cycle contributing to the mitochondrial production of ATP. However, aspartate also contributes to anaplerosis by its conversion in oxaloacetate and consequent entry in the TCA cycle for activation of malate-aspartate shuttle, which transfers electrons to the inner mitochondrial membrane and produce ATP in the electron transport chain. Despite its importance in the production of ATP in mitochondria and biosynthesis of other amino acids, less is known about the effects of aspartate in immunometabolism. The induction of TCA cycle or, in contrast, the blockage of this amino acid catabolism may shift the metabolism in order to command cells to adopt different functions. Although the activation pathways involved in amino acids metabolism remain unclear in immune cells, new insights about amino acid metabolism may lead us to develop new

strategies that will allow a further understanding of their biological importance in immunoregulatory pathways.

AIMS

Monocytes are cells of the innate immune system essential in the regulation of homeostatic functions upon recruitment to the tissues. The monocyte differentiation is an immune process with associated bioenergetic costs, being the utilization of certain nutrients indispensable for the accurate development of effector cells. However, how the acquisition of distinct metabolic phenotypes is handled during monocyte differentiation remains unclear. Moreover, less is known about the nutrients required during the differentiation process and in the acquisition of effective functional phenotypes. Accordingly, our main goals are to characterize the amino acid and carbohydrate metabolism requirements of human monocytes during the differentiation process and to understand how an altered amino acid metabolism may impact the acquisition of a metabolic and functional phenotype. Overall, our final objective is to deplete L-aspartate from the differentiation medium and assess how the differential availability of this amino acid during monocyte-to-macrophage differentiation impacts their metabolic and functional phenotype, thus showing a role for amino acid metabolism in the modulation of macrophage response.

MATERIAL AND METHODS

1. Biological samples

All experiments were conducted using buffy coats from healthy blood donors (n=23) supplied by *Hospital de Braga*. Ethical approval reference SECVF014/2015.

2. Culture medium

RPMI 1640 without amino acids (US Biological Life Sciences) was supplemented with 10% of heat-inactivated dialyzed FBS, Penicillin (100 U/mL), streptomycin (100 mg/mL) and 10 mM HEPES (all from Gibco, Life Technologies). Complete RPMI (RPMIc) was finalized by the addition of essential (MEM Amino Acids Solution, Gibco, Life Technologies) and non-essential amino acids suitable for cell culture: 2 mM L-Glutamine, 128 µM L-Alanine, 378 µM L-Asparagine, 136 µM L-Glutamic Acid, 133 µM Glycine, 174 µM L-Proline, 285 µM L-Serine and 150 µM L-Aspartic Acid (all from Sigma Aldrich).

3. Generation of monocyte-derived macrophages and dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats using a density gradient centrifugation step (400g, 30 min at RT, Multifuge 3 S-R, Heraeus) with Histopaque-1077 density solution (ratio 1:1) (Sigma Aldrich). Monocyte isolation was performed by immunomagnetic separation using anti-CD14-labelled microbeads (Miltenyi Biotec) according to the manufacturer's instructions. *In vitro* differentiation into Mφ and DCs was performed during 7 days at 37°C, 5% CO_2. Isolated $CD14^+$ monocytes were washed in PBS, counted using a Neubauer chamber and cultured at a concentration of $5x10^5$/mL in RPMIc with or without aspartate. Mφ were differentiated by cultivating monocytes in the presence of 20 ng/mL of GM-CSF or M-CSF, while DCs were obtained by cultivating monocytes with 20 ng/mL of IL-4 plus 20 ng/mL of GM-CSF (Peprotech). At day 4, the media was renewed using similar concentrations of each differentiating factor. At the 7^{th} day of differentiation, Mφ were stimulated with 10 ng/mL lipopolysaccharide (LPS) (*Escherichia coli* O26:B6, Sigma Aldrich) for 24 hours. Unstimulated cells were used as control. The

supernatants were stored at -20°C for posterior analysis and the cells were recovered for phenotypic analysis and effector function evaluation.

4. Flow Cytometry Analysis

The phenotypical and functional characterization of monocyte-derived Mφ and LPS-stimulated Mφ was performed by flow cytometry (Supplementary figure 1). For the assessment of the phagocytic activity, fluorescent yellow-green latex beads (1.0 μm mean particle size) (Sigma-Aldrich) were added to Mφ cultures at a ratio 1:10 (Mφ /beads) and incubated for 4 hours at 37°C, 5% CO_2. Mφ were washed three times with FACS buffer (PBS with 2% FBS) to remove non-phagocytized or adherent latex beads and acquired at the flow cytometer. Mφ with no incubation with the fluorescent beads were used as control. Mitochondrial mass and membrane potential were assessed by staining Mφ with 2.5 μM of 10 nonyl acridine orange (NAO) during 30 minutes at 37°C and 400 nM of tetramethylrhodamine ethyl ester perchlorate (TMRE) during 15 minutes at 37°C, respectively. Mφ intracellular nitric oxide was quantified by staining with 5 μM of 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (Daf-FM diacetate) during 60 minutes at 37°C. Total intracellular Mφ superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production was evaluated with 0.1 μM Dihydroethidium (DHE) and 10 μg/mL Dihydrorhodamine 123 probe (DHR 123), respectively, during 10 minutes at 37°C (all from Molecular Probes, Life Technologies). For the phenotypical characterization, $2.5x10^5$ Mφ were stained for surface antigens during 30 minutes at 4°C in the dark with the following monoclonal antibodies: PE-cy7 mouse anti-human CD163 1:25 (clone GHI/61), APC mouse anti-human CD206 (MMR) 1:25 (clone 15-2), Brilliant Violet 711 mouse anti-human CD197 (CCR7) 1:12.5 (clone G043H7) and Pacific Blue mouse anti-human HLA-DR 1:25 (clone 307633) (all from Biolegend). Subsequently, cells were washed FACS buffer to remove unbound antibodies and resuspended in FACS buffer prior to flow cytometric analysis. Single stainings for each fluorochrome was performed in unstimulated cells for fluorescence compensation and unstained cells were used as auto fluorescence control. Cell viability was assessed by 7-aminoactinomycin D (7-AAD) staining labeling Mφ prior to analysis. In acquisition, cells were selected on the basis of forward scatter/side scatter values disregarding the dead cells. Data was

acquired on a LSRII flow cytometer with Diva Software and analyzed using FlowJo version 10 software (TreeStar Inc.).

5. Cytokine production evaluation by Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of human IL-6, TNF-α and IL-10 were quantified on the supernatant of monocyte-derived Mφ in the presence of M-CSF or GM-CSF or upon a 24-hour LPS stimulation using colorimetric ELISA MAX kits (Biolegend), according to the manufacturer's instructions.

6. Amino Acid Quantification by High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was used to quantify the amino acid content in supernatants. For this, precolumn derivatization method using ortho-phthalaldehyde (OPA with methanol ≥99.9%, potassium borate 1M pH=9.5, and 2-mercaptoethanol ≥99.0%) 1:5 (Sigma Aldrich) was used to detect amino acids by a Gilson UV/vis_155 detector (338nm). Culture supernatants were filtered by Acrodisc 13mm syringe filters with 0.2µm supor membrane (Pall Corporations) previously to analysis and all mobile phases for elution were degasified for 30 minutes: inorganic mobile phase A, pH=7.8, was composed by $Na_2HPO_4.2H_2O$ 350mM:propionic acid 250mM (1:1) mixture (Merck) with acetonitrile HPLC grade in water (10:2:13) and organic mobile phase B was composed by acetonitrile, methanol and water (3:3:4) (HPLC grade, HiPerSolv Chromanorm, VWR Chemicals). Standard solutions of each amino acid were prepared in MilliQ water (Millipore): L-aspartate, L-glutamate, L-asparagine, L-serine, L-glutamine, L-arginine, Glycine, L-threonine L-alanine, L-tyrosine, L-lysine, L-isoleucine, L-phenylalanine, L-methionine, L-tryptophan, L-cistine and L-leucine (all from Sigma Aldrich). A Gilson bomb system (Gilson) was used with a 40°C Hi-Chrom C18 (model HI-5C18-250A) 5µm particles column (HiCrom). All data was analyzed in Gilson Uniprot Software, version 5.11.

7. Glucose and Lactate Quantification by High Performance Liquid Chromatography (HPLC)

The quantification of glucose and lactate by HPLC was performed in supernatants previously filtered. A Gilson bomb system (Gilson, USA) was used with a 54°C HyperREZ XP Carbohydrate H+ 8µM (TermoFisher) column and a Refractive index detector (IOTA 2, Reagentes). The 0.0025 M of H_2SO_4 mobile phase was degasified for 30 minutes before being used. Standard solutions were prepared in MilliQ water (Millipore, Germany) and the calibration curves were previously made. All data was analyzed in Gilson Uniprot Software, version 5.11.

8. Statistical Analysis

Statistical analyses were performed in GraphPad Prism 6 using Oneway ANOVA test with a Bonferroni multiple-comparison posttest for multiple group comparisons and differences between groups were analyzed using Student's t-Test. Statistically significant values are as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

RESULTS

1. Metabolic and phenotypical characterization of human macrophages and dendritic cells derived from CD14$^+$ blood monocytes

1.1. Amino acid metabolism evaluation in monocyte differentiation

Amino acids play a key role in regulation of Mφ metabolism, cell survival, defense and effector mechanisms[98]. However, due to the lack of knowledge regarding the importance of amino acid metabolism during monocyte differentiation and in the acquisition of a phenotypical profile, we decided to evaluate amino acids metabolism during differentiation of human blood monocytes into Mφ or DCs. For that, we quantified the concentration of amino acids in the cell culture supernatants after 7 days of *in vitro* differentiation of human CD14$^+$ monocytes with GM-CSF and M-CSF (both for Mφ differentiation) or IL-4 + GM-CSF (for DC differentiation). At the end of the differentiation process the levels of essential and non-essential amino acids were quantified by HPLC and compared to the initial concentration of each amino acid in the differentiation medium (Figure 5A-C and Supplementary figure 2). Positive values correspond to consumption while negative values indicate production of individual amino acids. Mφ differentiated from monocytes in the presence of GM-CSF or M-CSF presented similar amino acid catabolic and anabolic profiles. L-glutamine and L-aspartate were consumed in the presence of GM-CSF or M-CSF, while no differences were observed on L-glutamate levels when compared to the initial culture medium. Our results showed a similar consumption of L-aspartate by Mφ on average of approximately 29 μM and 24 μM upon differentiation with GM-CSF or M-CSF, respectively, while no variation in concentration levels of L-glutamate was observed. Although the consumption of L-glutamine was found to be significantly higher in the presence of GM-CSF when compared to M-CSF (350 μM and 130 μM, respectively; $p \leq 0.01$), the overall consumption profiles of these three amino acids appear to be independent of the factor used to differentiate Mφ from monocytes. During the DC differentiation process, we detected a significant increase on the consumption of L-glutamine (average of 480 μM; $p \leq 0.0001$) comparing to M-CSF differentiated Mφ. Consequently, and in opposition to monocyte derived-Mφ, a production of 110 μM on average of L-glutamate ($p \leq 0.0001$) was observed during dendritic cell differentiation. Also, we did not observe any modification on the L-aspartate levels during

differentiation, in striking contrast to Mφ differentiated in the presence of GM-CSF or M-CSF (statistical significance of $p \leq 0.001$ and $p \leq 0.01$, respectively). Moreover, no major alterations were detected regarding the metabolism of other non-essential and essential amino acids between monocyte differentiation in the presence of the different growth factors (Supplementary figure 2). Overall, our data suggested a distinct metabolic necessity for L-glutamate, L-glutamine and L-aspartate between the differentiation of monocytes to Mφ, both with GM-CSF or M-CSF, and to DCs. Particularly, our data indicates L-aspartate as an amino acid whose consumption is significantly different between Mφ and DC differentiation.

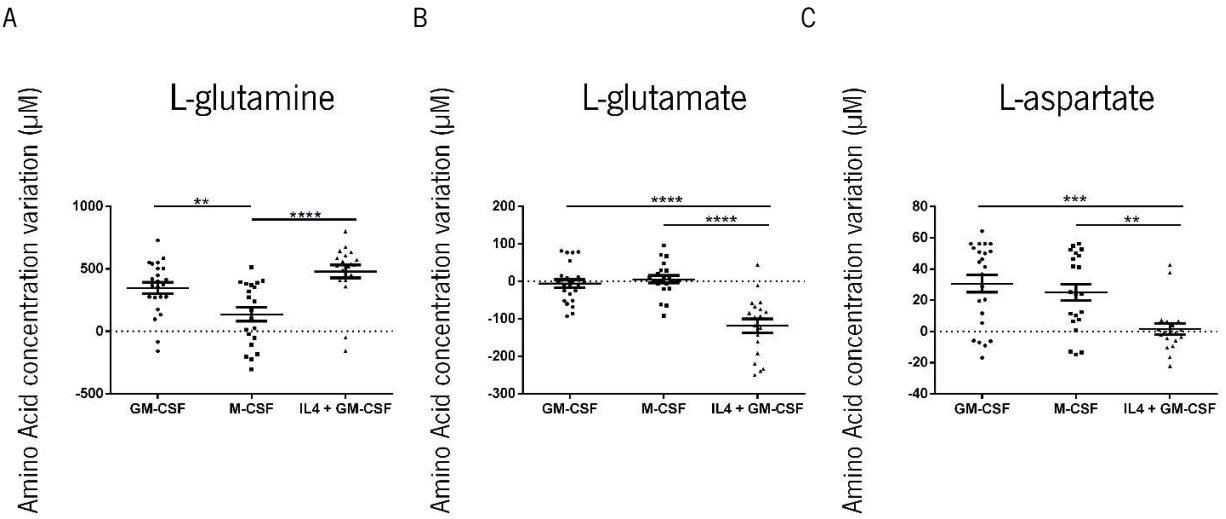


Figure 5. Characterization of amino acids metabolism by human Mφ and DCs derived from CD14+ peripheral blood monocytes. The consumption or production of amino acids was quantified by HPLC in the supernatants of 7 days *in vitro* differentiated monocyte-derived Mφ (GM-CSF or M-CSF) or DCs (IL-4 + GM-CSF). The results are expressed as the concentration variation (µM) subtracting the concentration at the end of the differentiation process with the initial value of the differentiation medium. Positive values correspond to consumption levels, while negative values indicate production of L-glutamine (A), L-glutamate (B) or L-aspartate (C) (µM). Data was obtained from 23 different donors. *p* values were determined and differences were considered significant for ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

The similarities observed between the amino acid metabolic profiles of Mφ allowed to positively and negatively correlate different amino acids profiles (Figure 6 and Supplementary figures 3 and 4).

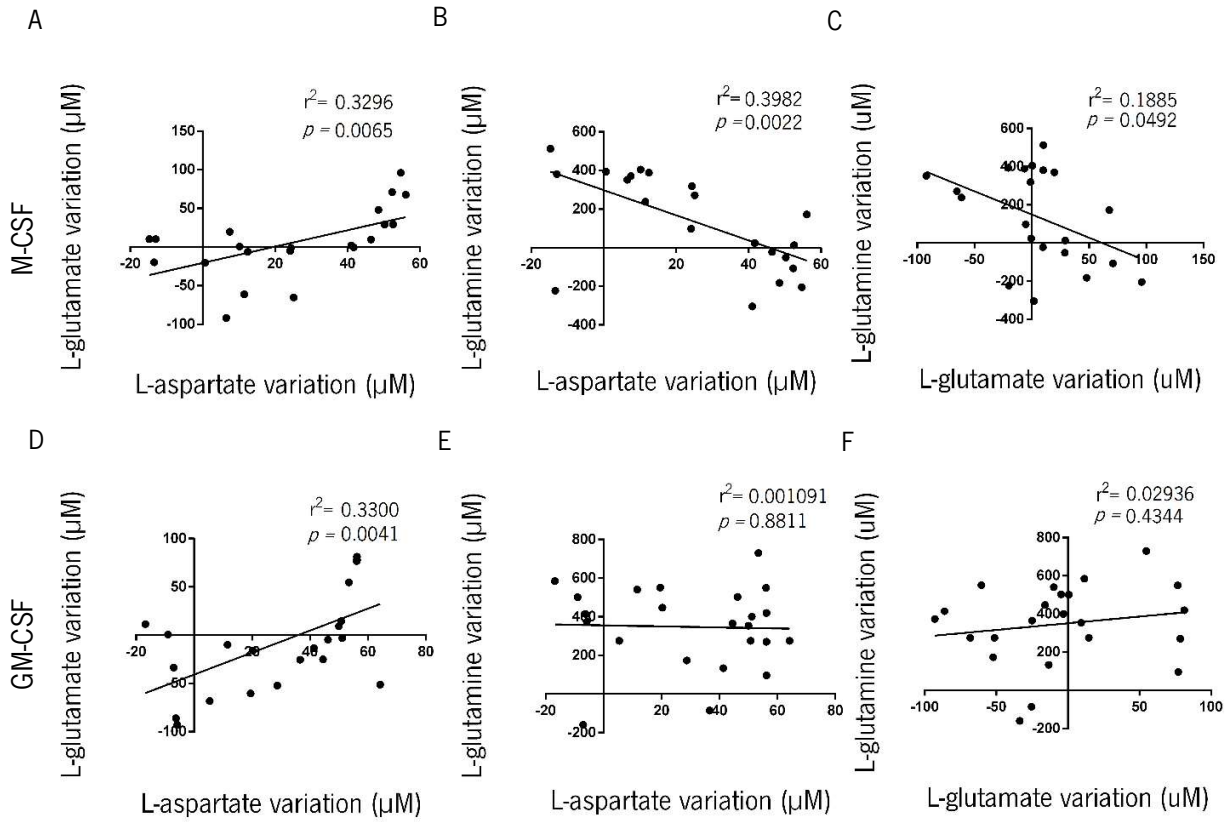


Figure 6. Correlation of amino acids metabolic profiles of human Mφ derived from CD14⁺ peripheral blood monocytes. The consumption or production of amino acids was quantified by HPLC in the supernatants of monocyte-derived Mφ differentiated *in vitro* during 7 days with M-CSF (A, B, C) or GM-CSF (D, E, F). The concentration variation (µM) is represented as the subtraction of the initial concentration in the differentiation medium and the concentration of culture supernatant at the end of the differentiation process. Linear regression between the metabolic profiles of each amino acid in the culture supernatant is shown. Data was obtained from 23 different donors. r^2 and *p* values were determined and differences were considered significant for $p \leq 0.05$.

We observed a positive correlation between L-aspartate and L-glutamate consumption/production profiles in the presence of GM-CSF and M-CSF (r^2=0.3300, p=0.0041 and r^2=0.3296, p=0.0065, respectively) (Figure 6 A and D). In contrast, the results displayed negative correlations between L-glutamine and L-aspartate/L-glutamate during differentiation with M-CSF (r^2=0.3982, p=0.0022 and r^2=0.1885, p=0.0492, respectively) (Figure 6 B-C), which was not observed in the presence of GM-CSF (r^2=0.0011, p=0.8811 and r^2=0.0294, p=0.4344, respectively) (Figure 6 E-F). Moreover, neither positive nor negative significant correlations were found between these three amino acids after DC differentiation (Supplementary figure 3A-C). Although this suggests a role for L-aspartate, L-glutamate and L-glutamine during Mφ differentiation process, we also detect some other additional correlations between catabolic and anabolic profiles of other amino acids after differentiation in the presence of GM-CSF or M-CSF (Supplementary figure 4A-F). Correlations between L-aspartate and other amino acids such as L-serine, L-threonine and L-leucine were considered statistical significant in GM-CSF and M-CSF-differentiated Mφ. However, we decided to focus this work on L-aspartate, L-glutamate and L-aspartate catabolic and anabolic profiles. Based on these observations, we hypothesized that there is a metabolic link between the consumption and production levels of L-aspartate, L-glutamate and L-glutamine in the differentiation of human Mφ that could have an impact on their immune functions.

1.2. Carbohydrate metabolism during monocyte differentiation

The carbohydrate metabolism has been well studied during Mφ polarization, particularly upon stimulation with pro-inflammatory molecules. However, the role of carbohydrates and their metabolism during human monocyte differentiation into Mφ and DCs still remains unclear. Thus, we decided to perform a metabolic characterization of carbohydrate utilization during differentiation of human $CD14^+$ monocytes in the presence of GM-CSF, M-CSF or IL-4 + GM-CSF. After differentiation with GM-CSF, Mφ present a significant increase in glucose consumption and lactate production when comparing to DCs (p=0.0060 and p=0.0290, respectively), which display lower levels of lactate production (approximately 2.5 mM on average) and irrelevant glucose consumption levels (Figure 7A-B). These Mφ consume in average approximately 3.4 mM of glucose and produce 7.9 mM of lactate.

Although not significantly different, this glucose consumption and lactate production levels are higher when compared to the 1.3 mM of glucose consumption and 3.5 mM of lactate production levels obtained in Mφ differentiated in the presence of M-CSF. Despite these differences on individual glucose consumption and lactate secretion among the distinct stimuli, both types of Mφ and dendritic cells present similar lactate/glucose ratios (Figure 7C).

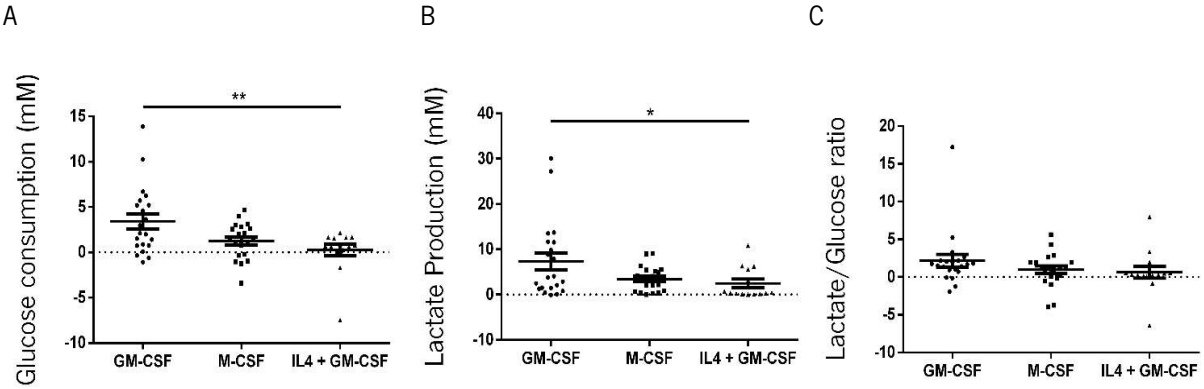


Figure 7. Characterization of glucose consumption and lactate production by Mφ and DCs derived from CD14⁺ peripheral blood monocytes. Glucose consumption and lactate production levels (mM) were evaluated in culture supernatants after 7 days of *in vitro* differentiation with GM-CSF, M-CSF or IL-4 + GM-CSF growth factors. Positive values correspond to glucose consumption levels (A) and lactate production levels (B), while negative values indicate production of glucose (A). The ratio between lactate production and glucose consumption is also represented (C). Scatter charts reflect data obtained with cells derived from 23 different donors. *p* values were determined and differences were considered significant for * $p \leq 0.05$ and ** $p \leq 0.01$.

1.3. Phenotypical evaluation of macrophages and dendritic cells after monocyte differentiation

Different subsets of Mφ can be evaluated through the quantification of distinct surface markers associated to Mφ differentiation and polarization. After monocyte differentiation, we analyzed the phenotypical profile of these cells through a flow cytometry panel of M1 and M2 associated markers (CD197/HLA-DR and CD163/CD206, respectively) typically used to characterize populations of human Mφ and DCs. Indeed, CD206 and CD163 markers have been associated with a more anti-inflammatory profile of Mφ, while CD197 and HLA-DR have been linked to a more inflammatory profile of Mφ or to an activation state of DCs. When comparing GM-CSF and M-CSF differentiated Mφ, the proportion of cells positive to M2 markers CD206/CD163 was greater in the differentiation with M-CSF although not significant (Figure 8A). Yet, the percentage of Mφ positive to M2 markers in the presence of M-CSF was significantly higher when compared to differentiated DCs. Although a tendency was observed with the quantification of M1 markers, we did not observe any significant difference (Figure 8B). These findings show that M-CSF differentiated Mφ are to some degree more polarized towards an M2 profile than GM-CSF differentiated ones, although this division appears to be ambiguous. A further analysis on the mean fluorescent intensity of each marker consequently demonstrated that among the M2 markers, Mφ differentiated in the presence of GM-CSF present a significant increase in CD206 expression, when compared to differentiation with M-CSF (p=0.0386; Figure 8C). On the other hand, M-CSF-differentiated Mφ present a significant increase in CD163, comparing with GM-CSF differentiated Mφ and to DCs, which express only residual levels of this marker ($p \leq 0.01$ and $p \leq 0.001$, respectively; Figure 8D). Moreover, no significant differences were detected in CD197 and HLA-DR expression levels (Figure 8E-F).

All together, these results contribute to a characterization of human monocyte differentiation in Mφ and DCs, which will allow a further understanding on how different growth factors may induce changes in human monocytes during the acquisition of different metabolic and phenotypical profiles.

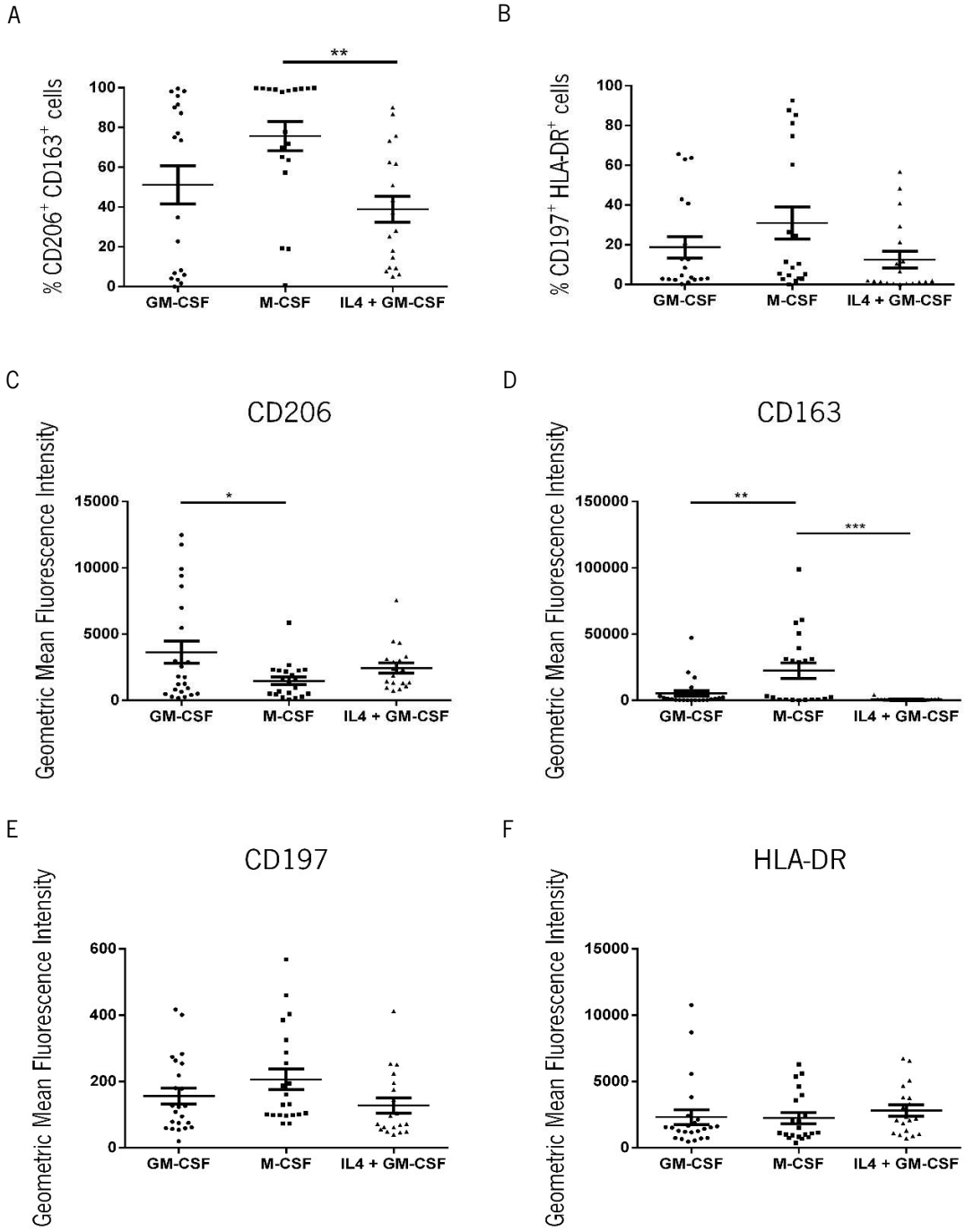


Figure 8. Phenotypical characterization of surface markers of human Mφ and DCs derived from CD14⁺ peripheral blood monocytes. Flow cytometry phenotypical analysis of Mφ and DCs after 7 days of *in vitro* differentiation with GM-CSF, M-CSF or IL-4 + GM-CSF. Scatter charts represent percentage of $CD206^{+}$ $CD163^{+}$ (A) and $CD197^{+}$ $HLA\text{-}DR^{+}$ cells (B) and geometric mean fluorescence intensity values for phenotypical markers CD206 (C), CD163 (D), CD197 (E) and HLA-DR (F), considering data obtained with cells derived from 23 different donors. *p* values were determined and differences were considered significant for * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

2. Metabolic and phenotypical characterization of human macrophages derived from CD14+ blood monocytes in conditioned media

After an initial characterization of CD14+ circulating blood monocyte-derived Mφ and DCs in a metabolic and phenotypical perspective, we recognized a correlation between aspartate catabolism and monocyte differentiation into Mφ. Interestingly, since this phenotype was specific for Mφ and not DCs, we were impelled to address the exact contribution of L-aspartate for the phenotypic and metabolic profile acquired by Mφ. For that, we differentiate *in vitro* monocytes into Mφ using GM-CSF or M-CSF in a L-aspartate depleted media to address how this amino acid influence the phenotype, metabolism and effector function of Mφ after *in vitro* differentiation. Therefore, we started by characterizing amino acid and carbohydrate metabolism during *in vitro* monocyte differentiation in complete medium comparing to L-aspartate-depleted medium. We analyzed the phenotype acquired by the monocytes upon differentiation in these different media and evaluated the role of L-aspartate in metabolic requirements of Mφ differentiation.

2.1. Amino acid metabolism evaluation during monocyte differentiation in conditioned media

We started by evaluating the catabolic and anabolic profiles of amino acids in the conditioned media. Our goal was to understand if amino acid utilization was altered upon the conditioned deprival of L-aspartate during the differentiation process. Non-essential and essential amino acids catabolic and anabolic profiles were analyzed after 7 days of *in vitro* monocyte differentiation with GM-CSF or M-CSF in the presence of complete RPMI medium or in RPMI medium without L-aspartate. As a control, we quantified the L-aspartate levels in both media. As expected, no L-aspartate was found in the differentiated cells on conditioned media while on complete RPMI, Mφ consume approximately 20 and 34 μM of L-aspartate on average, with M-CSF and GM-CSF, respectively (Figure 9A and D). In the absence of L-aspartate, M-CSF-differentiated Mφ increase to approximately 40 and 200 μM of L-glutamate and L-glutamine consumption (p=0.0368 and

p=0.0134, respectively; Figure 9B-C) while GM-CSF-differentiated Mφ presented an increased consumption of L-glutamate (60 μM) but not of L-glutamine (p=0.0118 and p=0.1566, respectively; Figure 9E-F).

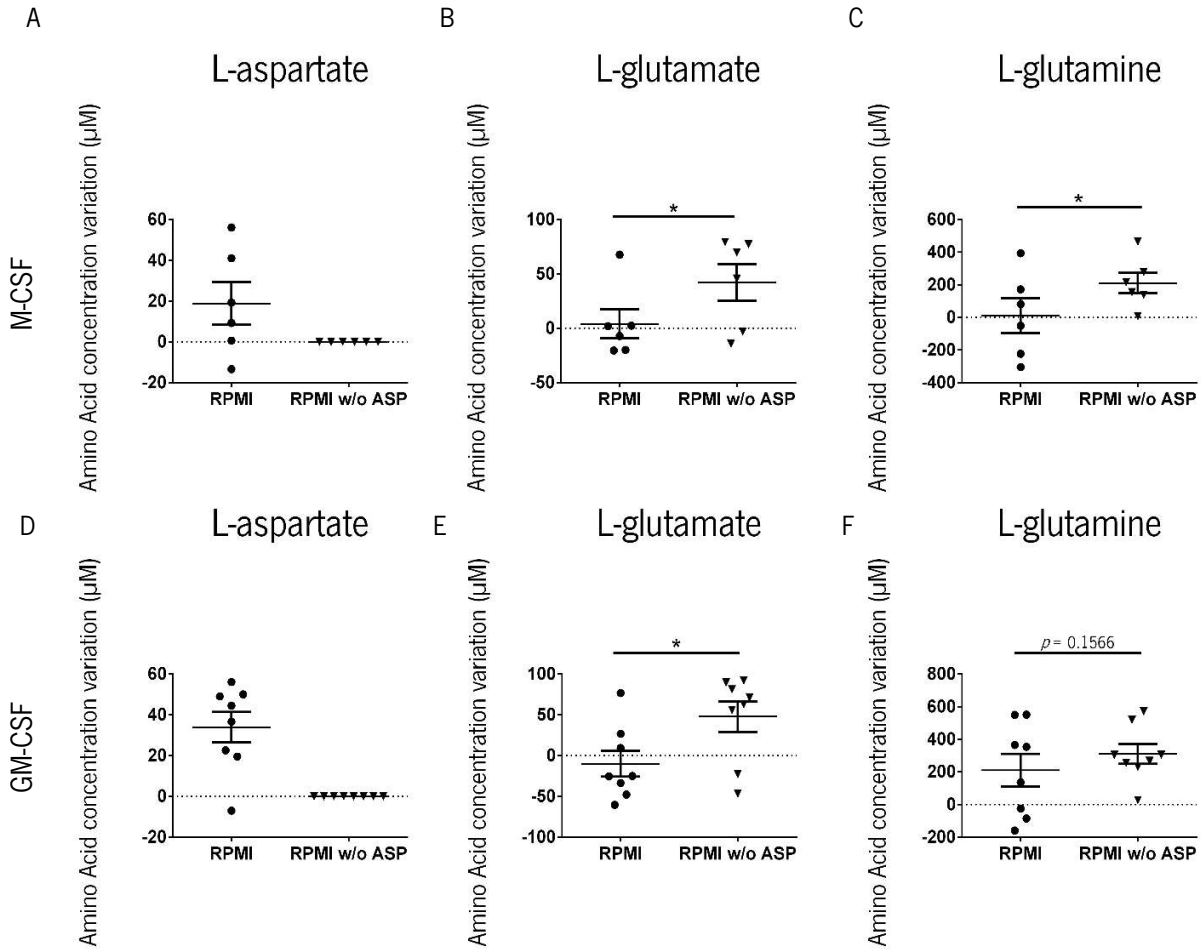


Figure 9. Characterization of amino acids metabolism by human Mφ derived from CD14⁺ peripheral blood monocytes in conditioned media. Amino acid concentration variation (μM) in culture supernatants after 7 days of *in vitro* differentiation with M-CSF (A-C) or GM-CSF (D-F) in conditioned media. Positive values correspond to consumption, while negative values indicate production of L-aspartate (A and D), L-glutamate (B and E) or L-glutamine (C and F). Data show mean values of Mφ differentiated in complete RPMI (RPMI) and mean values of Mφ differentiated in RPMI without L-aspartate (RPMI w/o ASP). Scatter charts represent data obtained with cells derived from 6 and 8 different donors. p values were determined and differences were considered significant for * $p \leq 0.05$.

Although we did not observe any alteration regarding the other analyzed amino acids consumption or production profiles in GM-CSF-differentiated Mφ, we detected increased consumption of L-serine, L-alanine and L-leucine after differentiation in the presence of M-CSF (Supplementary figure 5 and 6). This demonstrates that the amino acids L-glutamate and L-glutamine are avidly consumed when Mφ are differentiated in medium L-aspartate depleted medium, which may represent a compensatory mechanism for Mφ differentiation. Nevertheless, we observed that while GM-CSF-differentiated Mφ showed a higher increase in the consumption of L-glutamate, M-CSF-differentiated Mφ showed an increase in both the consumption of L-glutamine and L-glutamate.

2.2. Evaluation of carbohydrate metabolism during monocyte differentiation in conditioned media

We demonstrated that during monocyte differentiation in a medium deprived of L-aspartate Mφ compensate by upregulating the uptake and potentially the catabolism of other amino acids. We have previously observed that glucose is being consumed during monocyte-to-Mφ differentiation independently of the growth factor, which indicate that it might serve as a precursor for the production of ATP, NADPH and the synthesis of macromolecules such as nucleic acids. Thus, our next goal was to realize if macrophages also use carbohydrates as a compensatory mechanism in the absence of L-aspartate. To accomplish this, we decided to evaluate the levels of glucose consumption and lactate production in complete RPMI medium and compare it with glucose and lactate levels in supernatants of cells differentiated in the absence of L-aspartate. The results showed that the abolishment of L-aspartate during monocyte differentiation does not alter glucose consumption and lactate production profiles of Mφ during differentiation (Figure 10). The lactate/glucose ratios also showed no significant differences ($p \geq 0.05$), which suggest that L-aspartate abolishment do not alter the carbohydrate metabolism of Mφ differentiated in the presence of GM-CSF or M-CSF.

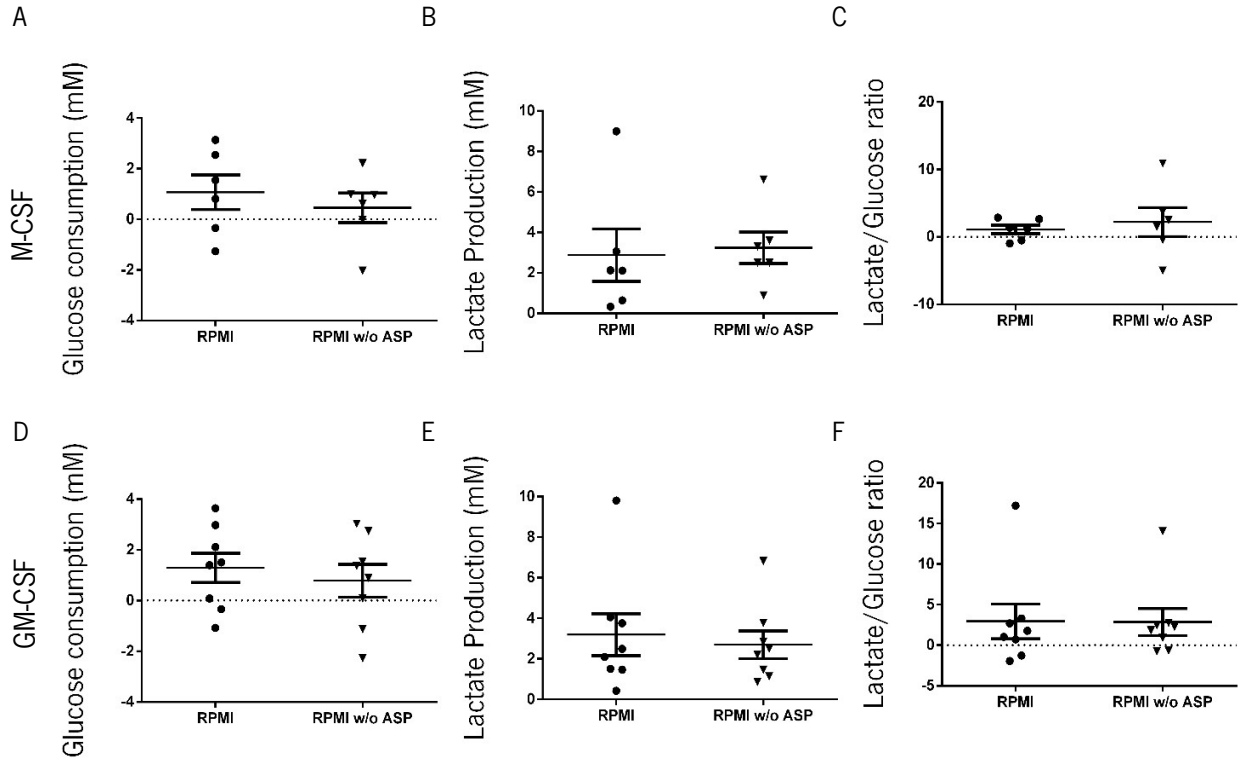


Figure 10. Characterization of glucose consumption and lactate production by human Mφ derived from CD14$^+$ peripheral blood monocytes in conditioned media. Glucose consumption and lactate production levels (mM) were evaluated in culture supernatants after 7 days of *in vitro* differentiation with M-CSF (A-C) or GM-CSF (D-F) growth factors in conditioned media. Positive values correspond to consumption levels of glucose (mM) (A, D), production levels of lactate (mM) (B, E) or the ratio between lactate production and glucose consumption (C, F). Data show mean values of Mφ differentiated in complete RPMI (RPMI) and mean values of Mφ differentiated in RPMI without L-aspartate (RPMI w/o ASP). The charts reflect data obtained with cells derived from 6 and 8 different donors. *p* values were determined and no significant statistical differences were obtained.

2.3. Phenotypical evaluation of macrophages after monocyte differentiation in conditioned media

In order to assess the role of L-aspartate in the phenotypical characterization of monocytes differentiated with M-CSF or GM-CSF, we explored by flow cytometry the markers previously analyzed and compared their expression in Mφ after differentiation in complete RPMI medium or in RPMI medium without L-aspartate (Figure 11). Our results showed no statistical significant differences

between expression levels of CD206 (Figure 11A and E), CD163 (Figure 11B and F), CD197 (Figure 11C and G) and HLA-DR (Figure 11D and H) markers upon monocyte differentiation in conditioned media, in the presence of M-CSF or GM-CSF.

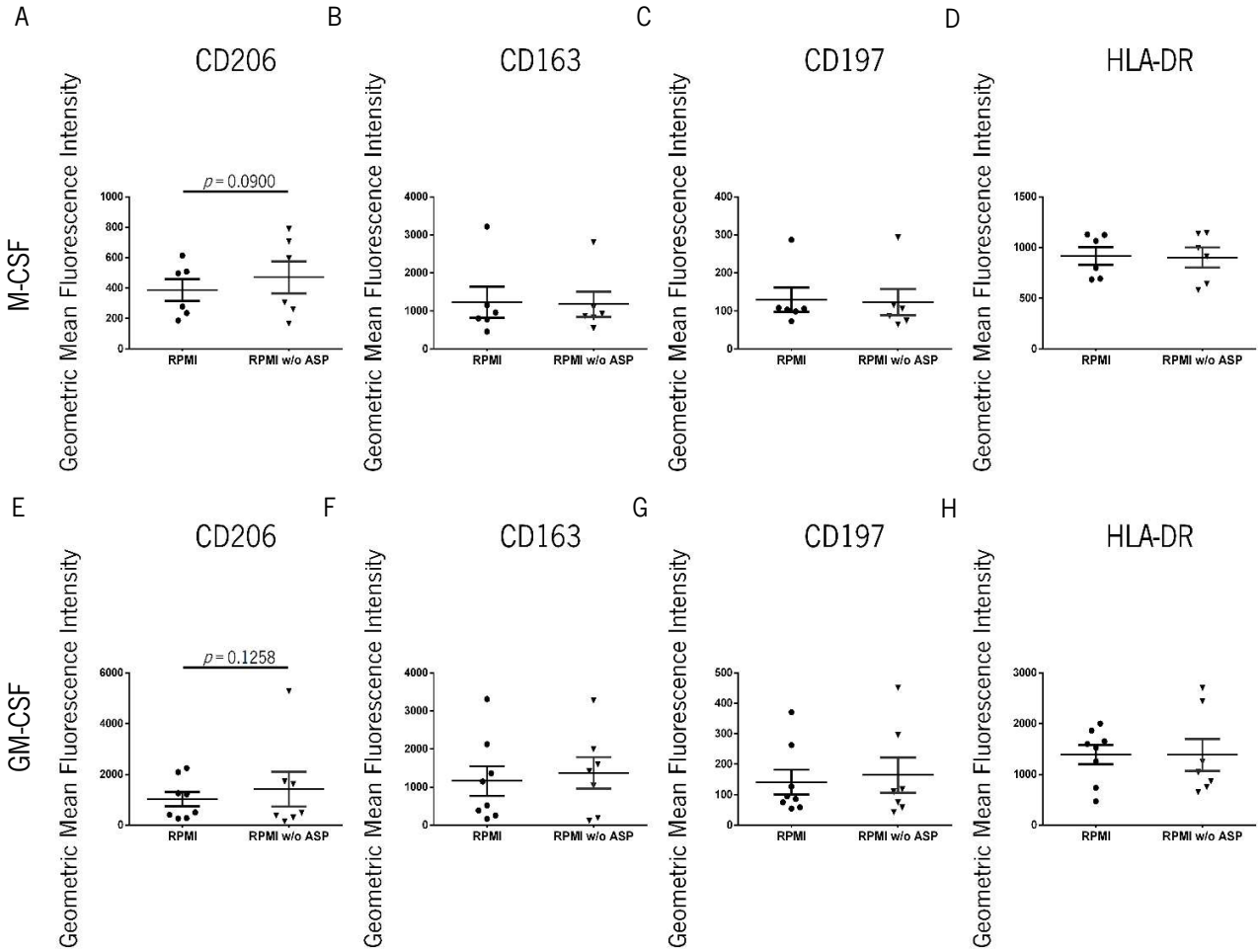


Figure 11. Phenotypical characterization of human Mφ derived from CD14⁺ peripheral blood monocytes in conditioned media. Flow cytometry phenotypical analysis of Mφs after 7 days of *in vitro* differentiation with M-CSF (A-D) or GM-CSF (E-H) growth factors in conditioned media. The data represent mean values of geometric mean fluorescence intensity for phenotypical markers CD206 (A, E), CD163 (B, F), CD197 (C, G) and HLA-DR (D, H) of Mφ differentiated in complete RPMI (RPMI) or in RPMI without L-aspartate (RPMI w/o ASP). The charts consider data obtained with cells derived from 6 and 8 different donors. *p* values were determined and no significant statistical differences were obtained.

Although not statistical significant, we observe a tendency to increase the surface expression of CD206 marker when monocytes are differentiated in the absence of L-aspartate either in the presence of M-CSF (p = 0.0900) or GM-CSF (p = 0.1258). Overall, L-aspartate, a non-essential amino acid that can be synthesized by humans, influences the choice of amino acid catabolism without any impact on carbohydrate metabolism. Despite the absence of statistical significance, the observed tendency of increased surface levels of the M2 marker CD206 in the absence of L-aspartate led us to perform further studies to characterize functionally the impact of L-aspartate on the differentiation of monocytes.

3. Functional characterization of human monocyte-derived macrophages differentiated in conditioned media

The metabolic and phenotypical characterization of $CD14^+$ monocyte differentiated Mφ in medium lacking L-aspartate suggested an amino acid metabolic reprograming of monocytes. Thus, we decided to address how this metabolic adaptation may influence a functional phenotype of human monocyte-derived Mφ, once the functional characterization of these cells is essential for the understanding of their role upon recruitment to the tissues.

3.1. Effector function evaluation of macrophages differentiated in conditioned media

In order to evaluate the effector functions of Mφ differentiated in conditioned media, we started to measure the levels of cytokines production by Mφ after a 7 days *in vitro* differentiation with GM-CSF or M-CSF growth factors. Although not significant, M-CSF differentiated Mφ display a tendency to reduce the levels of IL-6 and TNF-α upon differentiation (p = 0.1407 and p = 0.0807, respectively; Figure 12A-B). No significant differences were found with the production and secretion of IL-10 (Figure 12C). The absence of L-aspartate during differentiation with GM-CSF do not alter the secretion levels of the pleiotropic IL-6 cytokine (p = 0.2987; Figure 12A). In opposition, we observed

a significant reduction on the release levels of TNF-α (p = 0.0334; Figure 12B) and a tendency to upregulate the IL-10 levels (p = 0.0723; Figure 12C). Overall, our data suggests that the absence of L-aspartate modifies the profile of secreted cytokines with a tendency for decreasing the inflammatory TNF-α cytokine secretion.

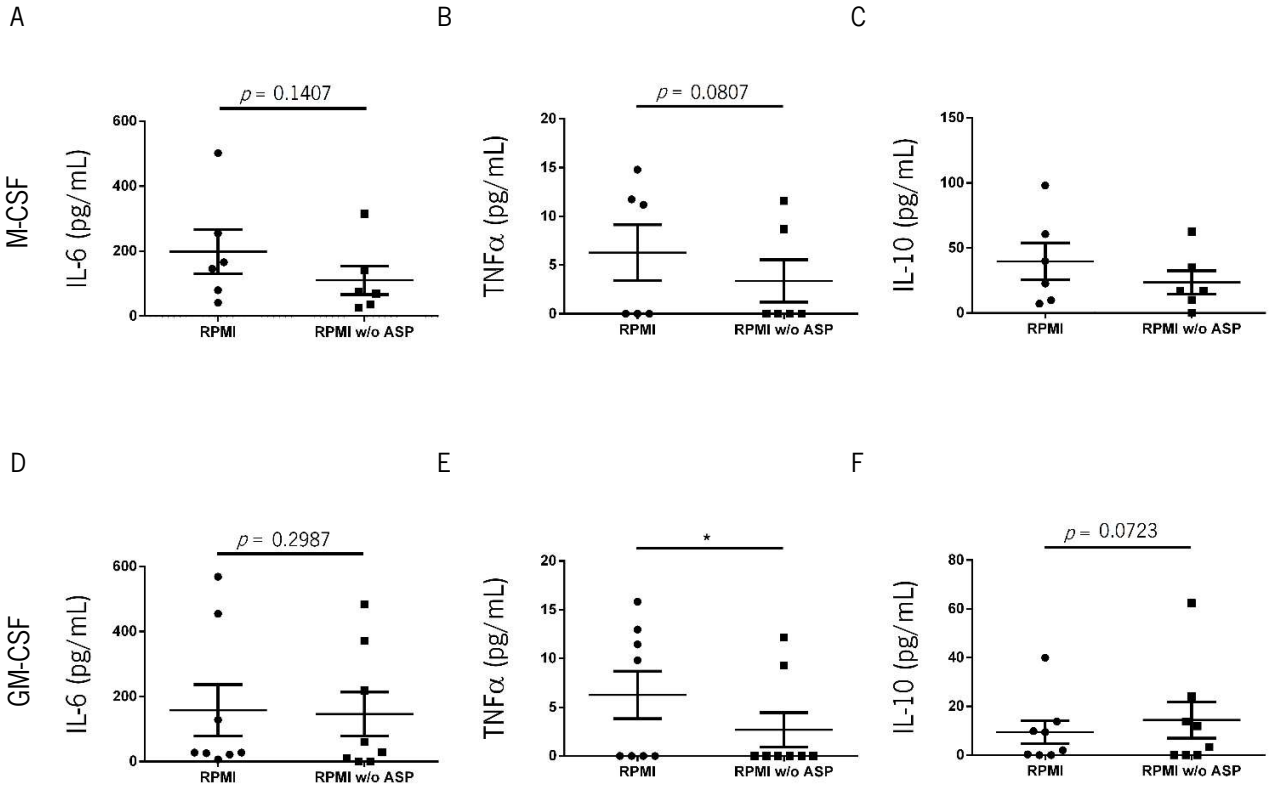


Figure 12. Effector function evaluation measuring cytokine production levels after human monocyte differentiation into Mφ in conditioned media. Effector function was evaluated by the quantification of cytokine production in Mφ differentiated with M-CSF (A-C) or GM-CSF (D-F) factors in conditioned media. IL-6, TNF-α and IL-10 concentrations (pg/mL) were measured by ELISA after differentiation in complete RPMI (RPMI) or in RPMI lacking L-aspartate (RPMI w/o ASP). Scatter charts reflect data obtained with cells derived from at least 6 and 8 different donors. p values were determined and differences were considered statistical significant for * $p \leq 0.05$.

To further assess differences in the functional profile of Mφ after differentiation we pursuit our study by evaluating by flow cytometry specific functions of these cells, such as the production of ROS, NO and the percentage of phagocytosis (Figure 13). Due to the reduced number of samples tested so far and the similarities of the results obtained with M-CSF and GM-CSF differentiated Mφ, we choose to combine the data obtained with both stimuli in these following sections.

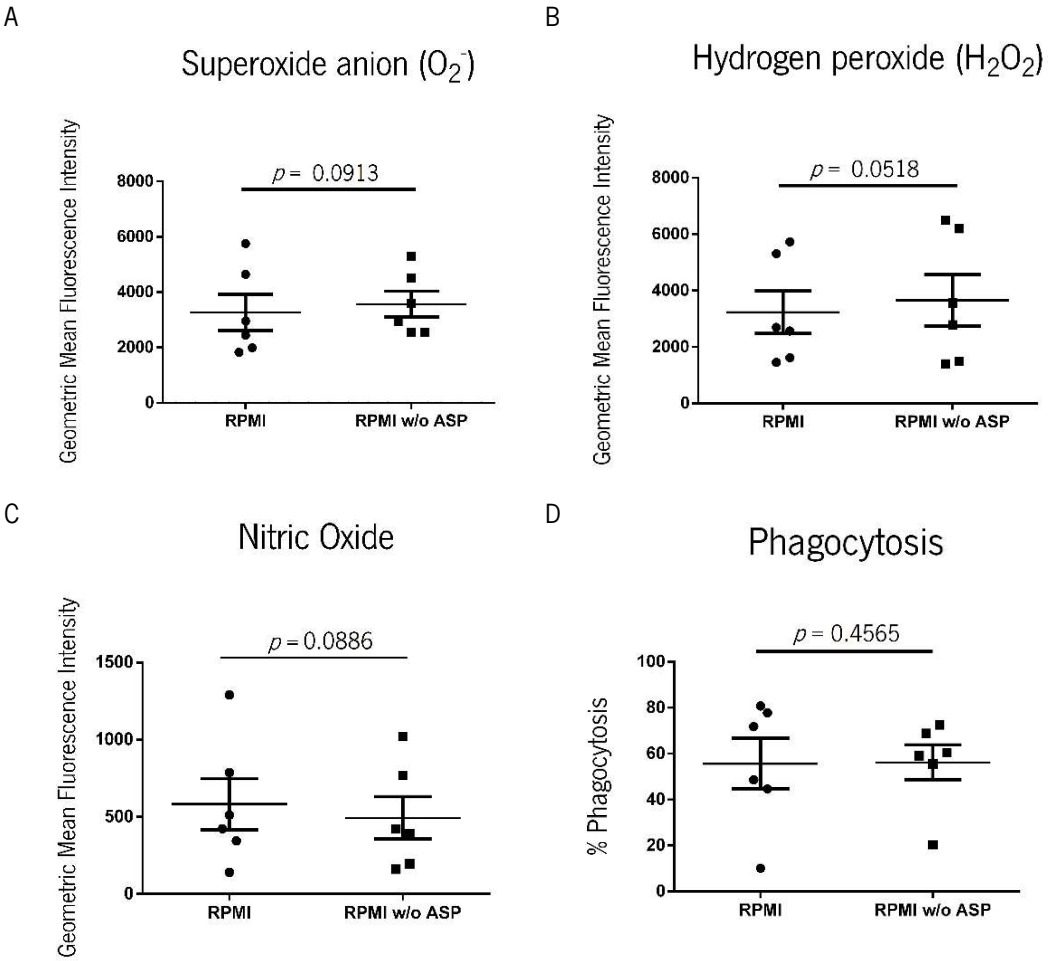


Figure 13. Effector function evaluation of human Mφ differentiated in conditioned media. Effector functions of Mφ were evaluated by flow cytometry analysis after *in vitro* differentiation with GM-CSF or M-CSF in RPMIc (RPMI) or RPMI lacking L-aspartate (RPMI w/o ASP). The production of ROS was evaluated measuring the production of superoxide anion (O_2^-) (A) and hydrogen peroxide (H_2O_2) (B). The production of nitric oxide (C) and the percentage of phagocytosis (D) were also assessed. The data was obtained derived from 6 different donors. *p* values were determined and no significant statistical differences were obtained.

Regarding the production of ROS, we measured by flow cytometry the levels of superoxide anion (O^{2-}) and peroxide hydrogen levels (H_2O_2). Although not statistically significant, we detected a tendency for an increased production of these molecules in absence of L-aspartate (p =0.0913 and p =0.0518, respectively; Figure 13A-B). In opposition, the production levels of NO by these cells appear to decrease when differentiated in the absence of L-aspartate (p =0.0886; Figure 13C). Finally, no differences were found about the percentage of phagocytosed fluorescent beads between Mφ differentiated in the presence or absence of L-aspartate (p =0.4565; Figure 13D). Therefore, these results suggest that the absence of L-aspartate during differentiation may have an impact, although minor, on the production of oxygen and nitrogen reactive species, yet with opposite outcomes. To gain insight on a potential effect on mitochondria biogenesis and function, we also analyzed by flow cytometry the mitochondrial potential and mitochondrial mass after differentiation in conditioned media. Yet, no significant alterations on mitochondrial mass and membrane potential were found suggestive that the absence of L-aspartate do not interfere with mitochondrial biogenesis and structural integrity (p =0.1863 and p =0.1534, respectively; Figure 14A-B).

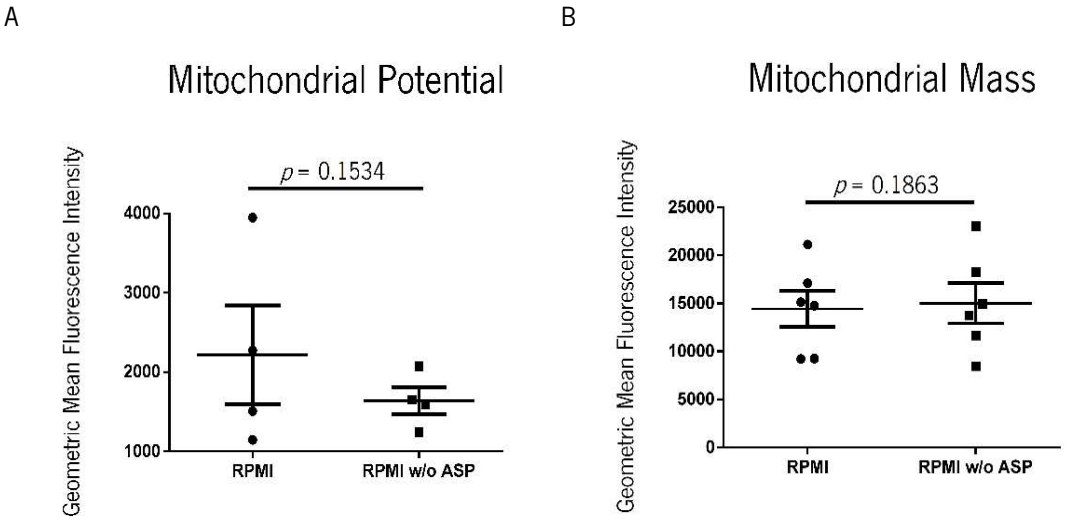


Figure 14. Mitochondrial evaluation of human Mφ differentiated in conditioned media. Mitochondrial potential (A) and mass (B) were evaluated by flow cytometry analysis in monocyte-derived Mφ after *in vitro* differentiation with GM-CSF or M-CSF in RPMIc (RPMI) or RPMI lacking L-aspartate (RPMI w/o ASP). The data was obtained with cells derived from 4 and 6 different donors. p values were determined and no significant statistical differences were obtained.

Although we did not observed significant modifications on Mφ metabolic profile upon differentiation in the absence of L-aspartate, our data revealed a minor impact on several tested effector functions. Yet, we detected several tendencies that will be further explored in the future by increasing the number of individuals. Nevertheless, we further address how the absence of L-aspartate impact Mφ functions upon an inflammatory stimulus.

3.2. Assessment of effector functions of macrophages differentiated in conditioned media after stimulation with LPS

We started to address the production of cytokines of Mφ differentiated in conditioned media upon stimulation with LPS during 24 hours. For that, we measured the production of IL-6, TNF-α and IL-10 cytokines by Mφ in order to unveil if there is any functional difference of these cells in response to a stimulus after differentiation in complete media or in media without L-aspartate. As referred previously, M-CSF differentiated Mφ display a more M2-like profile in opposition to GM-CSF differentiated ones. Indeed, the quantification of IL-6, TNF-α and IL-10 on the culture supernatants of 24 hours LPS-stimulated cells perfectly show the higher levels of IL-6 and TNF-α achieved by the GM-CSF differentiated Mφ while the opposite is observed with IL-10 levels (Figure 15). Similarly to what was obtained upon the analysis of the profile of cytokines produced at the end of the differentiation process, and probably due to the reduced number of individuals tested so far, we failed to obtain any statistical significance. Nevertheless, a similar tendency of decreased IL-6 and TNF-α secretion and increased levels of IL-10 was observed upon LPS stimulation on M-CSF differentiated Mφ in the absence of L-aspartate (p = 0.2143, p = 0.2672 and p = 0.1285, respectively; Figure 15A-C). Yet, this phenotype was not observed with GM-CSF differentiated Mφ (Figure 15D-F).

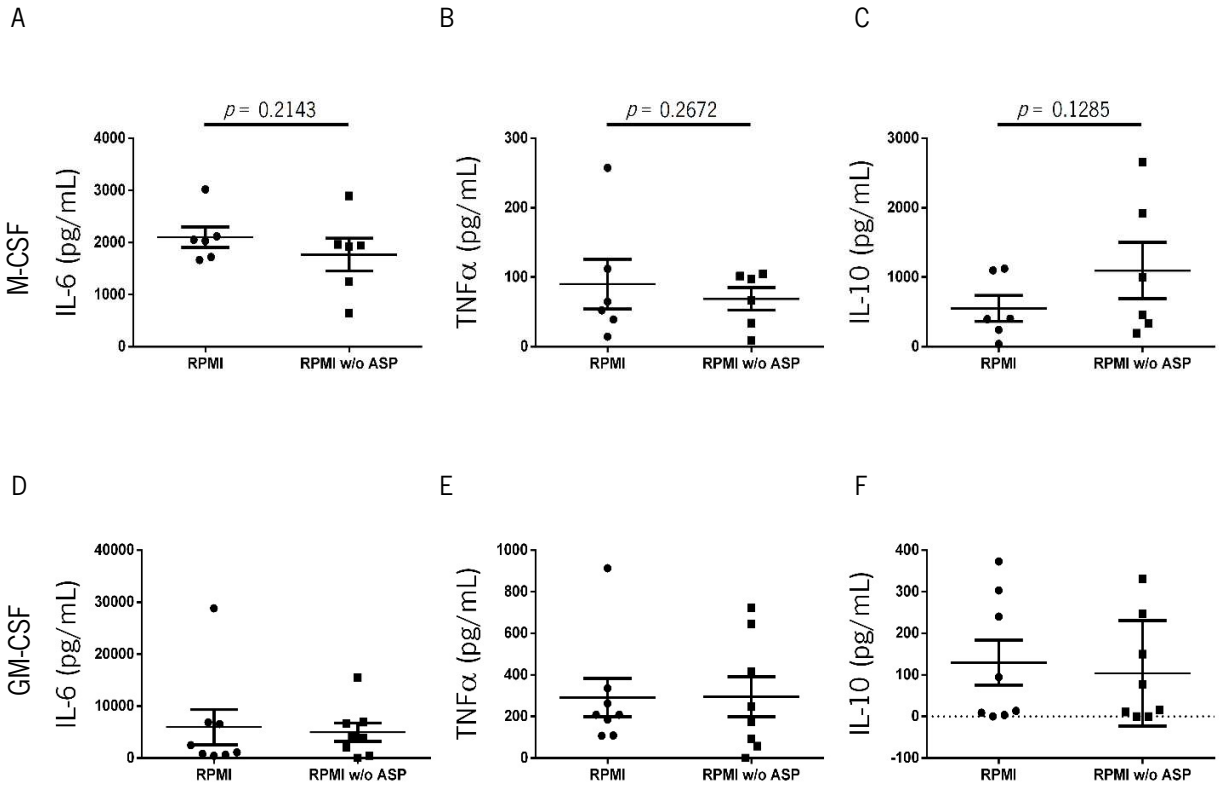


Figure 15. Effector function evaluation measuring cytokine production levels after human monocyte differentiation into Mφ in conditioned media upon stimulation with LPS. Effector function was evaluated by the quantification of cytokine production in 24 hours LPS-stimulated Mφ after 7 days of *in vitro* differentiation with M-CSF (A-C) or GM-CSF (D-F) in conditioned media. IL-6, TNF-α and IL-10 concentrations (pg/mL) were measured by ELISA after differentiation of Mφ in RPMIc (RPMI) or in RPMI lacking L-aspartate (RPMI w/o ASP). Scatter charts reflect data obtained with cells derived from at least 6 and 8 different donors. *p* values were determined and no significant statistical differences were obtained.

We also assessed specific effector functions of Mφ after stimulation with LPS, such as ROS and NO production, and phagocytosis (Figure 16). As before, Mφ stimulated with LPS in a medium deprived of L-aspartate presented a significant increase of the superoxide anion levels (p =0.0271; Figure 16A) although the production of hydrogen peroxide was similar in both media (p =0.2115; Figure 16B). On the other hand, we did not detect any difference regarding the production of nitric oxide and phagocytosis after LPS-stimulation of these Mφ (Figure 16C-D).

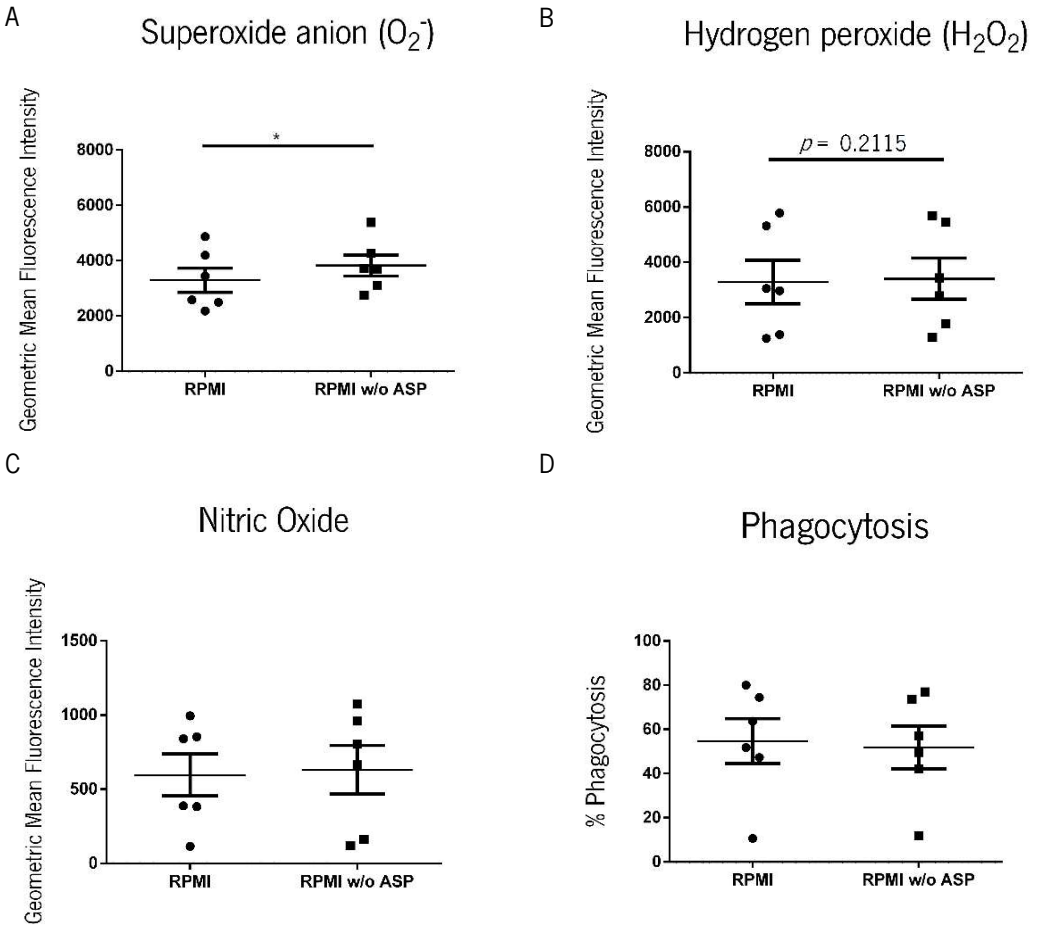


Figure 16. Effector function evaluation of human Mφ differentiated in conditioned media after stimulation with LPS. Effector functions were evaluated by flow cytometry analysis in 24 hours LPS-stimulated Mφ after *in vitro* differentiation with GM-CSF or M-CSF in RPMIc (RPMI) or RPMI lacking L-aspartate (RPMI w/o ASP). The production of ROS was evaluated measuring the production of superoxide anion (O_2^-) (A) and hydrogen peroxide (H_2O_2) (B). The production of nitric oxide (C) and the percentage of phagocytosis (D) were also assessed. The data was obtained with cells derived from 6 different donors. *p* values were determined and differences were considered significant for * $p \leq 0.05$.

Regarding mitochondrial analysis, we did not observed any significant differences in mitochondrial mass and potential indicating that Mφ differentiated in absence of L-aspartate do not present changes in mitochondrial biogenesis, even after LPS-stimulation (Figure 17A-B).

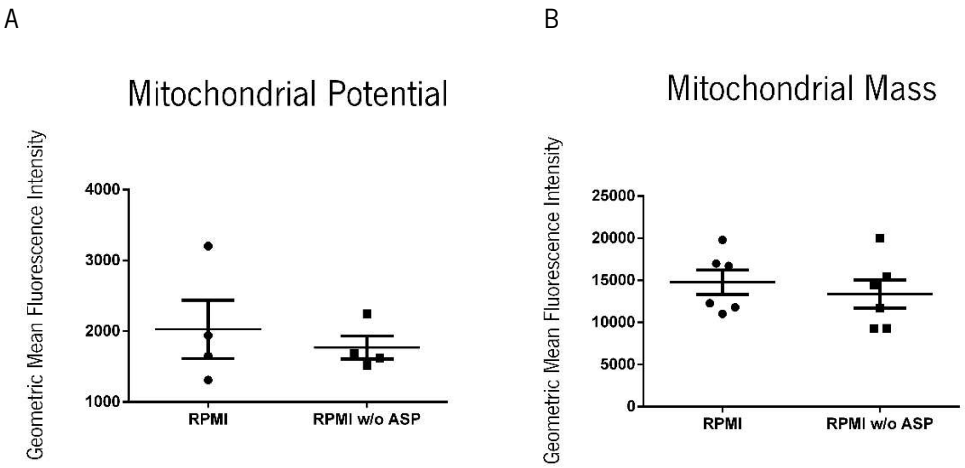


Figure 17. Mitochondrial evaluation of human Mφ differentiated in conditioned media after stimulation with LPS. Mitochondrial potential (A) and mass (B) were evaluated by flow cytometry analysis in 24 hours LPS-stimulated Mφ after *in vitro* differentiation with GM-CSF or M-CSF in RPMIc (RPMI) or RPMI lacking L-aspartate (RPMI w/o ASP) media. The data was obtained with cells derived from 4 and 6 different donors. *p* values were determined and no significant statistical differences were obtained.

Although these Mφ differentiated in conditioned media present distinct metabolic profiles, with the reduced numbers of independent samples tested so far we only detected minor fluctuations on their effector functions. However, the interaction between these different subsets of Mφ in a biological context may present a different functional phenotype.

Discussion and Future Perspectives

1. Metabolic and phenotypical characterization of human macrophages derived from CD14+ blood monocytes

CD14+ blood monocytes consist in a heterogeneous population of circulating leukocytes that may ultimately originate Mφ or DCs upon recruitment to tissues. While some studies have started to elucidate the impact of different phenotypical profiles of these cells in physiologic and pathological conditions, the understanding of the role of metabolism and its impact on the acquisition of a certain phenotype is still scarce. Metabolic alterations are associated with the polarization profiles adopted by Mφ, as it has been shown recently with the expansion of the field of immunometabolism[77]. Mφ polarization is characterized by a continuum of functional phenotypes, canonically categorized by the *in vitro* M1 pro-inflammatory and M2 anti-inflammatory Mφ. These extremes of polarization are also associated with specific and divergent metabolic requirements. In order to understand how the metabolic alterations may impact the acquisition of a functional phenotype, we started by characterizing amino acid and carbohydrate metabolism during *in vitro* monocyte differentiation into Mφ and DCs. Our main objective was to associate specific metabolic requirements with the phenotype acquired by the CD14+ monocytes upon differentiation with different growth factors. Therefore, GM-CSF and M-CSF were used to differentiate blood monocytes into M1 and M2 Mφ, respectively[59], while IL-4 + GM-CSF cocktail was used to differentiate monocytes into DCs[114]. The combination of IL4 and GM-CSF triggers the differentiation of DC through the inhibition of Mφ differentiation, since these differentiation pathways appear to be mutually exclusive.

After comparison of the catabolic and anabolic profiles of each amino acid during monocyte differentiation, we evaluated the specific modifications occurring with Mφ comparing between them and with DCs. We observed that L-glutamine and L-aspartate are consumed during Mφ differentiation in the presence of GM-CSF or M-CSF, although L-glutamate levels do not suffer major alterations. On the other hand, monocyte-derived DCs show a higher consumption of L-glutamine and production of L-glutamate during differentiation, possibly derived from L-glutamine deamination. During the maturation process, DCs use glutamine to fuel the TCA cycle through glutaminolysis process, in which intramitochondrial L-glutamine is converted to α-ketoglutarate. This metabolic

process is induced by mTORC1 after GM-CSF signaling through PI3K/AKT, being this pathway essential in regulation of MYC complex expression, which is an essential step for the maturation of DCs[115]. The increase in mitochondrial mass and mitochondrial DNA is associated with a higher oxygen consumption and increased mitochondrial function that may explain the requirement of higher levels of L-glutamine to fulfill the TCA cycle. GM-CSF signaling and consequent regulation of these metabolic processes also explain the higher consumption levels of L-glutamine found in GM-CSF differentiated Mφ when compared to Mφ differentiated with M-CSF. However, the observed consumption of L-glutamine after differentiation with GM-CSF is still lower when compared to monocytes differentiated with IL-4 + GM-CSF. Thus, it appears that the combination of these growth factors is important for the modulation of DCs and consequent metabolic requirements. Nevertheless, M-CSF has also been described to be responsible for slightly increased levels of glucose and L-glutamine catabolism in Mφ, through a Myc-dependent induction of cell cycle entry, that has been associated to a M2-profile of human Mφ or tumour associated Mφ (TAM) and this may explain the amino acid consumption levels by these Mφin our results[116]. Since a pro-inflammatory stimulus, as LPS, is able to induce a complete opposite engagement of HIF-1α-dependent glycolysis in Mφ, we hypothesize that GM-CSF, being associated to M1-Mφ profile, may induce a similar LPS-driven modulation of the metabolic activities of Mφ through a higher increase in glycolysis[117]. Therefore, the assessment of carbohydrate metabolism of these Mφ is important to understand their metabolic reprogramming after differentiation with M-CSF or GM-CSF.

Our results suggest that both L-glutamine and L-aspartate may play a role during Mφ differentiation from monocytes. Interestingly and in opposition to L-glutamine, L-aspartate is specifically consumed by Mφ but not by DCs. Therefore, our data led us to hypothesize that L-aspartate might play an important role during Mφ differentiation. On contrary, we only observed glutamate production during DC differentiation. L-aspartate and L-glutamate are important amino acids for fuelling anaplerotic reactions in the TCA cycle and for metabolic processes such as the malate-aspartate shuttle, which is involved in the mitochondrial control of cellular bioenergetics status. Nonetheless, our results suggest that human Mφ and DCs present different amino acid requirements during their differentiation process from monocyte, putting forward a tempting and valuable field to explore. A positive correlation between the consumption of L-aspartate and L-

glutamate was found when cells are differentiated in the presence of GM-CSF or M-CSF, which may be explained by the expression of glutamate aspartate transporter at plasma membrane of human Mφ[118]. This transporter is encoded by *SLC1A3* gene and co-transport glutamate and aspartate to the cytoplasm by a symport mechanism. This transporter is also localized in the inner mitochondrial membrane as constituent of the malate-aspartate shuttle and it is crucial for the exchange of these amino acids with protons, which are essential for the production of ATP in the mitochondrial electron transport chain[119]. Our data suggest that in Mφ this symport mechanism is performed at a higher extension than in DCs, which is in accordance with the lack of correlations between L-aspartate and L-glutamate consumption or production profiles. However, regarding L-glutamine profiles, Mφ and DCs display opposite behaviors. A consumption of L-aspartate and L-glutamate is negatively correlated with L-glutamine consumption by M-CSF-differentiated Mφ. One possible explanation for these correlations is the deamination process of L-glutamine that upon arrival to the cell is converted to L-glutamate with production of ammonia (NH_4^+). The consequent production of L-glutamate is important for a variety of cellular functions, including the production of glutathione (GSH), which is essential for the maintenance of the antioxidant status of the cell. Accordingly, a higher consumption of L-glutamate and L-aspartate may limit the consumption of L-glutamine. In case of GM-CSF-differentiated Mφ, there are no correlations with L-glutamine, probably because this amino acid may be used for other functions of cells as regulation of mitochondrial metabolic pathways. In contrast to Mφ, we observe no correlations between these amino acids in DCs, which indicate a distinct amino acidic metabolic profile for these cells. Future studies will address the metabolic fate of these amino acids in M-CSF or GM-CSF-differentiated Mφ. Overall we hypothesize that a metabolic link between the consumption and production of these non-essential amino acids is present during human Mφ differentiation, being the consumption and production profiles of DCs distinct during monocyte differentiation.

The carbohydrate metabolism is also a gold example of how different metabolic requirements are associated with distinct polarizations phenotypes of Mφ. Thus, in order to understand the role of carbohydrates and how they are metabolized during human monocyte differentiation into Mφ and DCs, we decided to perform a characterization of carbohydrate utilization during differentiation of human $CD14^+$ monocytes. Our results showed similar levels of glucose

consumption and lactate production during differentiation of Mφ. Nonetheless, when differentiated with GM-CSF, Mφ present a higher consumption of glucose and a higher production of lactate, when compared to DCs subset. However, comparing the different subsets of differentiated cells, the lactate production/glucose consumption ratios are similar. Higher glucose consumption and production of lactate levels are associated to higher glycolytic and fermentative rates that lead to a higher extracellular acidification rate (ECAR) of the medium by GM-CSF differentiated-Mφ[120]. The enhanced glycolysis and glutamine consumption observed in our results may indicate that during Mφ differentiation and specially with GM-CSF a metabolic stabilization of HIF-1α may occur as shown previously[121]. Succinate derived from glutamine-fueled aneplerotic reactions was shown to play a role in the enhancement of glycolytic process and in the production of interleukin-1β during inflammation[81]. It was previously described that M-CSF differentiated Mφ exhibit a significant higher oxygen consumption rate/ECAR ratio[120]. The increase in this ratio implies a higher mitochondrial activity, in detriment to fermentation. Although our results showed a similar ratio of lactate/glucose between differentiated Mφ and DCs, M-CSF differentiated-Mφ may present a lower glycolytic rate, especially if the mitochondrial OXPHOS process happens to be the main source of ATP production, as it happens to DCs. Our data also demonstrate that IL-4 + GM-CSF differentiated-DCs presented a lower glycolytic rate with consequent lower glucose consumption and lactate production. It was previously shown that after stimulation with TLRs agonists, DCs also present an increased consumption of glucose and production of lactate, which probably leads to an equally rapid production of ATP, essential for the activation state of these cells[122]. Nevertheless, before activation, the differentiation process of DCs may require mitochondrial OXPHOS to obtain energy. In line with our data, it has been described that after differentiation, DCs present increased content in mitochondrial DNA, as well as more efficient antioxidant systems and a higher expression of genes associated to mitochondrial biogenesis, such as PGC-1α[123]. Therefore, distinct bioenergetic profiles during monocyte differentiation may be important depending on the activation status of these cells.

Mφ phenotypic markers have also been associated with different Mφ subsets upon stimulation and consequent polarization. In order to characterize DCs or Mφ after differentiation, we assessed by flow cytometry some phenotypical markers associated to human monocytes and Mφ: CD206, the mannose receptor C type 1 (MRC1), CD163, a scavenger receptor with high affinity for

the hemoglobin-haptoglobin complex, CD197, a C-C chemokine receptor type 7 (CCR7), and HLA-DR, human leukocyte antigen - antigen D related. These markers are generally used to characterize populations of the monocyte/Mφ lineage and they are known to be associated with specific functions of these cells. CD206 and CD163 markers have been associated with anti-inflammatory profiles, while CD197 and HLA-DR have been linked to a more inflammatory profile of Mφ or to an activation state of DCs. Our characterization showed an increase of CD206 expression by GM-CSF differentiated Mφ and an increase in CD163 in M-CSF differentiated Mφ. DCs does not display CD163 expression that may be explained by the fact that GM-CSF and IL-4 factors strongly reduce CD163 expression during monocyte differentiation[124]. In contrast, CD206 is strongly induced by these two factors, which may justify the increase of CD206 expression in GM-CSF differentiated Mφ and DCs comparative to M-CSF-differentiated Mφ. A higher expression of CD206 marker and the higher percentage of $CD206^{+}CD163^{+}$ Mφ upon differentiation in the presence of M-CSF resemble an alternative activation of monocytes, which supports the reprograming of monocyte function to be more prone to act in an inflammatory context.. Indeed, the CD206 expression has been shown to be increased in $CD14^{+}$ monocytes of patients with sepsis and was also correlated with the production of IL-17 pro-inflammatory cytokine characteristic of these patients[125]. Nevertheless, in this condition a modulation of monocyte function occurs with a consequent reduction of pro-inflammatory cytokines upon stimulation with LPS, which is present in alternatively activated Mφ. The analysis of CD197 and HLA-DR showed no alterations between the distinct growth factors-induced profiles, being these markers associated to DCs or with LPS-stimulated Mφ. However, it has been described that tissue Mφ, such as alveolar Mφ differentiated in the presence of M-CSF, increase the expression of CD197, which could have a role in promoting the adaptive immune response through induction of migration to lymph nodes by a CCR7-dependent mechanism, what could be important during the early stages of an infection[126]. On the other hand, HLA-DR is a molecule involved in promoting adaptive immune responses and it is largely associated with DCs and antigen presentation[127]. Although our results show no statistical significance, there is a tendency for an increase of HLA-DR expression in DCs. The basal levels of this marker expression in DCs is associated to its maturation, since complete mature and functional DCs present higher expression of HLA-DR and are ready to complete their function in inducing adaptive immune responses. This phenotypical evaluation is essential for the characterization of human derived Mφ and DCs, crucial to understand how different growth factors

may induce changes in human monocytes, especially regarding the acquisition of different metabolic and phenotypical profiles. Moreover, the understanding of these functional and metabolic changes in tissues is essential for modulation of these differentiation profiles of cells during physiologic and pathologic conditions.

2. Metabolic and phenotypical characterization of human macrophages derived from CD14+ blood monocytes in conditioned media

The metabolic and phenotypical analysis of monocyte-derived Mφ and DCs led us to hypothesize about the exact contribution of amino acids for the phenotype and metabolic profile acquired by these cells. Our previous results showed a positive correlation of L-aspartate catabolism with other amino acids metabolism during Mφ differentiation. Also, the increase on L-aspartate consumption was specific to Mφ, irrespective of the growth factor, while it was not observed during DCs differentiation. Therefore, we decided to explore the metabolic and phenotypic profiles acquired by Mφ during monocyte differentiation in the absence of L-aspartate. This approach allowed us to explore how this amino acid may influence or not the phenotype and metabolic requirements of monocytes during *in vitro* differentiation. Accordingly, we started by characterizing amino acid and carbohydrate metabolism during *in vitro* monocyte differentiation in complete medium or medium lacking L-aspartate and compare the phenotype acquired by the CD14+ monocytes upon differentiation in these different media.

Our results showed an increase in L-glutamate and L-glutamine consumption levels during differentiation in absence of L-aspartate, which may suggest a compensatory mechanism to obtain the required amino acid source for survival and differentiation. However, relevant differences were observed among Mφ differentiation with both factors. M-CSF differentiated Mφ present increased levels of L-glutamate and L-glutamine, while GM-CSF only present an increase in the consumption of L-glutamate. L-aspartate is a non-essential amino acid involved in several biosynthetic processes such as biosynthesis of proteins and synthesis of other amino acids such as methionine, threonine, isoleucine and lysine. Besides, this amino acid may also be involved in other homeostatic processes

of cells such as redox equilibrium. Our data lead us to speculate that different subsets of Mφ may use amino acids in a different way, ultimately resulting in the activation of different metabolic pathways. It is known that L-glutamate may originate L-aspartate by conversion of oxaloacetate into α–ketoglutarate, in the presence of aspartate aminotransferase (ASAT), which may enter in TCA cycle leading to the production of energy or other amino acids. In addition, L-glutamine may also originate L-glutamate by deamination reaction, important for the aneplerotic reactions. So, the increase in consumption of these two amino acids may be important to fulfill the TCA cycle leading to the production of energy by Mφ in order to maintain the basic functions of the cells. We also hypothesize that a deregulation of L-aspartate levels may lead to a dysruption of the malate-aspartate shuttle and a consequent break in ATP production in mitochondria. The L-aspartate supply may be replenished by L-glutamate and oxaloacetate. However, oxaloacetate pools have to be capable of transaminate and produce L-aspartate to maintain the conversion of malate with production of NAD^+, essential for the malate-aspartate shuttle transport of electrons process and for the equilibrium of redox state of cell. This NAD^+ produced is essential to the replenishment of the cytosolic pool of NAD^+ essential for diverse metabolic pathways such as glycolysis. On the other hand, in the absence of L-aspartate the consumption levels of L-glutamine are particularly higher comparing to the consumption levels of L-aspartate in RPMIc. This L-glutamine is being used as a compensatory mechanism for L-aspartate in the oxidative pathways to produce energy, but probably is also being used for reductive pathways such as FAS, essential for membrane cell remodeling and proliferation processes.

Accordingly to our results, in the absence of L-aspartate Mφ differentiate using diverse amino acids as substrate to maintain cell survival. However, we do not know if this compensatory mechanism is effective for the nutrient supplying of the acquiring of a functional phenotype by Mφ. Glucose is used as a carbon substrate in the production of ATP, NADPH and precursors for the synthesis of macromolecules such as nucleic acids. Then, our next goal was to realize how monocytes use carbohydrates as a compensatory mechanism upon Mφ differentiation in medium without L-aspartate. The results showed that the abolishment of L-aspartate during monocyte differentiation do not alter glucose consumption and lactate production profiles of cells during differentiation. These results may explain the higher consumption of L-glutamine and L-glutamate

during differentiation in the absence of L-aspartate, once it is expected that these amino acids are used to originate TCA cycle intermediaries and produce energy. The glutamate originated from glutaminase reaction originates α–ketoglutarate in mitochondria that is oxidized to malate in TCA cycle. Malate can be exported to cytosol for conversion to pyruvate, which is used to the production of lactate through lactate dehydrogenase A, previously induced by MYC oncogene[128]. Another study also show that B cells may have a glucose-independent glutamine metabolism, being important for acquisition of different functions[129]. Although it seems that L-aspartate has a short effect on metabolism of M-CSF differentiated Mφ, we do not know how this amino acid is really involved in the metabolic changes and in the consequent phenotyping acquisition process by Mφ. Nevertheless, the metabolic characterization of these different subsets of differentiated Mφ allow us to realize that during the differentiation process the monocytes have different metabolic requirements depending on the differentiation factor and the nutrients that they receive.

In order to assess the role of L-aspartate in the phenotypical characterization of monocytes differentiated with GM-CSF or M-CSF, we compared the expression of the markers previously analyzed in Mφ after differentiation in complete RPMI medium or in RPMI medium lacking L-aspartate. Our results showed no differences between expression levels of different markers upon monocyte differentiation, leading us to suggest that no major phenotypical alterations occur in the absence of L-aspartate during monocyte differentiation into Mφ. L-aspartate, a non-essential amino acid that can be synthesized by humans, showed to have a key role in modulation of amino acid metabolism, perceptible by higher increases in other amino acids consumptions profiles. However, the absence of this amino acid did not bring any alterations to carbohydrate metabolism or to phenotypical characterization of Mφ. Likewise, less is known about the reprogramming of the functional phenotype of these Mφ and its link to a physiologic or pathophysiologic context.

3. Functional characterization of human monocyte-derived macrophages differentiated in conditioned media

The metabolic and phenotypical characterization of $CD14^+$ monocyte differentiated Mφ in complete media and media lacking L-aspartate suggested a reprograming amino acid metabolism of monocytes. Thus, we decided to address how this metabolic reprogramming may influence a functional phenotype of human monocyte-derived Mφ, once the functional characterization of these cells is essential for the understanding of their role upon recruitment to the tissues. To evaluate the effector functions of Mφ differentiated in media with or without L-aspartate, we started to measure the levels of cytokines produced by Mφ after *in vitro* differentiation. We observed a decrease in the production of TNF-α in media lacking L-aspartate that may suggest these Mφ as reducing the production of inflammatory cytokines after differentiation without L-aspartate. These differences only were observed for GM-CSF-differentiated Mφ, having the Mφ differentiated with M-CSF a trend to produce lower levels of TNF-α also. Although these results are no statistical significant, GM-CSF differentiated Mφ also show a tendency to produce higher levels of IL-10 in the absence of L-aspartate during monocyte differentiation. This decreased production of pro-inflammatory cytokines like TNF-α and increased production of anti-inflammatory cytokines production such as IL-10 may suggest an anti-inflammatory functional profile for these Mφ when differentiated in medium lacking L-aspartate. Although further studies as needed to increase the number of samples to ascertain this phenotype, this anti-inflammatory role of Mφ may be important in a biologic context, for instance in infection or tumoral settings. How these cells respond to a myriad of signals or even pathogens in different environments is paramount to determine the success of their effector functions, since it has been shown that anti-inflammatory Mφ are less efficient in pathogen clearance during infections. On the other hand, the role of these Mφ in a tumoral context may determine the progression and the severity of the tumor, once M2-Mφ may be associated to TAM[51]. We decided to evaluate other effector functions of Mφ and for that we assessed the mitochondrial status of these cells after differentiation in complete RPMI and in RPMI lacking L-aspartate. We analyzed by flow cytometry specific functions of Mφ such as phagocytosis, NO and ROS production in order to understand how

the effector functions of these Mφ may be altered or not after differentiation. We detected a tendency for a higher production of superoxide anion (O^{2-}) and peroxide hydrogen (H_2O_2) molecules in the absence of L-aspartate. Although these differences are not statistical significant, the increase of ROS levels may be associated to a deregulation of mitochondrial function and, in a less extension, to a lower production of GSH which have an important antioxidant activity for the cell. We hypothesize that in absence of L-aspartate, L-glutamate probably is used in order to compensate the absence of L-aspartate, leading to a decrease in the production of GSH and consecutively to an increase in ROS. We also assessed the mitochondrial potential and mitochondrial mass to assess mitochondrial biogenesis and functionality of these organelles after differentiation of Mφ without L-aspartate. The results showed no alterations in mitochondrial status, being similar levels of mitochondrial mass indicative of no differences in mitochondrial biogenesis. However, it appears that mitochondrial potential is lower in absence of L-aspartate during differentiation of these cells. Although this difference in mitochondrial potential is not statistically significant probably due to the reduced number of donors, this result may indicate that the mitochondria are not functionally active and this may shift the metabolism in other direction for the successful production of energy. Indeed, the deregulation of mitochondria may also explain the higher levels of ROS production observed. ROS levels may also have a cytosolic origin and not exclusively mitochondrial. Deregulated levels of GSH and other antioxidant enzymes such as superoxide dismutase and catalase (for degradation of O_2^- and H_2O_2, respectively) may lead to an inefficient response in dealing to ROS reduction or an upregulation of systems producers of cytosolic ROS such as NADPH oxidase system may also be involved in increased levels of ROS. Therefore, it is important to address how these ROS are being produced and their role in the effector functions of these macrophages. In opposition, our results showed a trend to a decreased production of NO by these Mφ when differentiated in the absence of L-aspartate. As a note, the phagocytic capacity do not appear to be affected by the absence of L-aspartate.

Alltogether, these results suggest that Mφ differentiated in absence of L-aspartate present some altered effector functions that may lead to a lower performance in an inflammatory context, such as infections or tumors. Indeed, the decreased NO production for these cells and the lower levels of pro-inflammatory cytokines production in contrast to higher levels of anti-inflammatory

cytokines production lead us to speculate that these Mφ present some anti-inflammatory characteristics that may be prejudicial in an infection or tumoral context, but may be important in an non-reversible inflammatory environment as happens in autoimmune diseases.

Accordingly, in order to assess the effector functions of Mφ in a context of stimulation, we decided to stimulate differentiated Mφ during 24 hours with LPS to see if there are any differences in effector functions upon stimulation. The results showed no significant differences in the production levels of IL-6, TNFα and IL-10 cytokines between Mφ differentiated in complete medium or medium lacking L-aspartate. Although it appears to exist a tendency to a lower production of IL-6 and a higher production of IL-10 in M-CSF-differentiated Mφ in the absence of L-aspartate, this difference is not statistical significant probably due to the heterogeneity of these human cells. However, with an increase of the number of donors, possibly the differences between the differentiation of Mφ in conditioned media will become more reliable. In this way, we speculate that these Mφ show a profile of cytokines that is consistent to a modulation of Mφ functions proper of a metabolic and functional reprograming. So, we decided to assess ROS and NO production and phagocytosis after stimulation with LPS. Differences in the production of O_2^- were observed, which may indicate that a higher production of O_2^- may be associated to deregulation of mitochondria activity or to an increase of cytosolic ROS. On the other hand, no differences were obtained in H_2O_2,, which may indicate different expression of catalase in comparison to superoxide dismutase (SOD) antioxidant enzyme. After analysis of mitochondrial function, we observed no differences in mitochondrial potential and in mitochondrial mass. These results suggest that Mφ differentiated in absence of L-aspartate do not have changes in mitochondrial biogenesis and the observed ROS may be originated by deregulation of superoxide dismutase antioxidant enzyme or upregulation of systems producers of ROS such as NADPH oxidase system leading to an increase of cytosolic ROS. Moreover, we did not detect any difference regarding the production of NO, which may lead us to speculate that in the presence of LPS these Mφ lose their capacity to produce NO. As before, the percentage of phagocytosis in the LPS-stimulation do not exhibit alterations, which may indicate that upon differentiation and consequent stimulation with LPS Mφ are prepared to respond in the same way.

After addressing the bioenergetic state of macrophages differentiated in conditioned media we observed that Mφ differentiated in absence of L-aspartate present some altered effector functions. In the future, we intent to increase the number of samples for monocyte differentiation into Mφ in conditioned media, in order to validate our results. Therefore, it is also paramount to address how ROS are being produced and their role in the effector functions of Mφ and how dysfunctional mitochondria may influence the energetic state of cell and consecutively may influence the functional phenotype of these cells in a biological context. In a metabolic perspective, it will be crucial to study the lipid metabolism in order to understand how the L-aspartate is being used or what metabolic compensatory mechanisms are being used in the absence of this amino acid. However, since the interaction between these different subsets of Mφ and a pathogen in an infection context, or since Mφ may display a distinct performance in a tumoral or inflammatory context, our main goal is to address the role of these Mφ in distinct inflammatory, infectious and tumoral contexts, in order to evaluate the effectiveness of the functional profiles of these Mφ.

CONCLUSION

Nutrient availability is essential for any immunologic process, being crucial for cell differentiation processes such as monocyte differentiation. A characterization of metabolic requirements of human $CD14^+$ monocytes derived from peripheral blood mononuclear cells during differentiation in Mφ populations was performed in order to understand the different phenotypes acquired by these cells. The phenotypical and metabolic differences may originate functional changes in Mφ and DCs that are essential or deleterious depending on the tissues for where they are recruited. Moreover, the understanding of these functional and metabolic changes in tissues is essential for modulation of these differentiation profiles of cells during physiologic and pathologic conditions. During the characterization of amino acid and carbohydrate metabolism, we assessed and correlated different non-essential amino acids catabolic and anabolic profiles upon differentiation with different growth factors, comparing it with the different phenotypic profiles acquired by these cells. We revealed that amino acid catabolism of L-aspartate is important in monocyte-to-Mφ differentiation, but not for monocyte differentiation into DCs. Using a conditioned media, the absence of L-aspartate during monocyte differentiation cells led to compensatory mechanisms as demonstrated by the use of different amino acids, mainly L-glutamine and L-glutamate, as substrate to maintain cell survival for an effective supplying of nutrients in the acquiring of a functional phenotype by Mφ. Although it appears to induce a reprograming of monocytes into a different phenotype of Mφ, the assessment of Mφ effector functions was essential to understand the acquired phenotypic profiles of these cells during differentiation in a fully supplied environment or in an environment lacking a specific nutrient as L-aspartate. In the future, we want to assess the lipid metabolism in order to see how these different phenotypes of Mφ modulate lipid metabolism after monocyte differentiation in the absence of L-aspartate. Furthermore, we also want to address how these Mφ with distinct phenotypes may deal and present an effective response in distinct inflammatory, infectious and tumoral contexts in order to understand their functional phenotypes in a biological context.

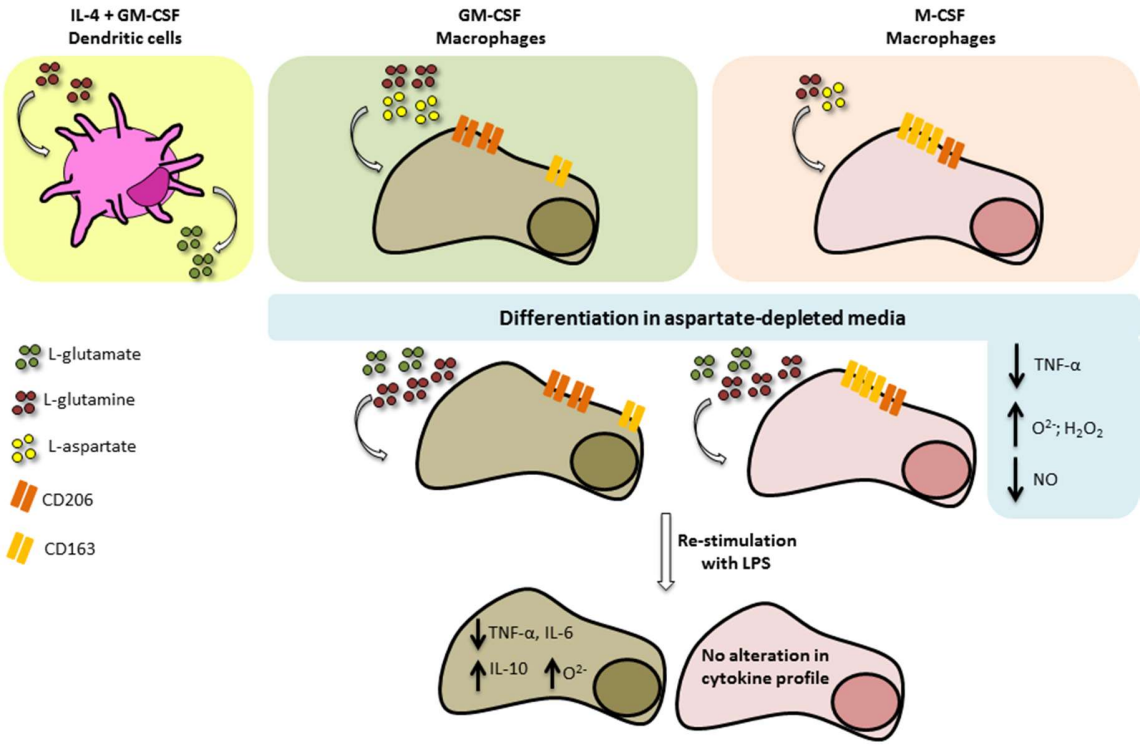
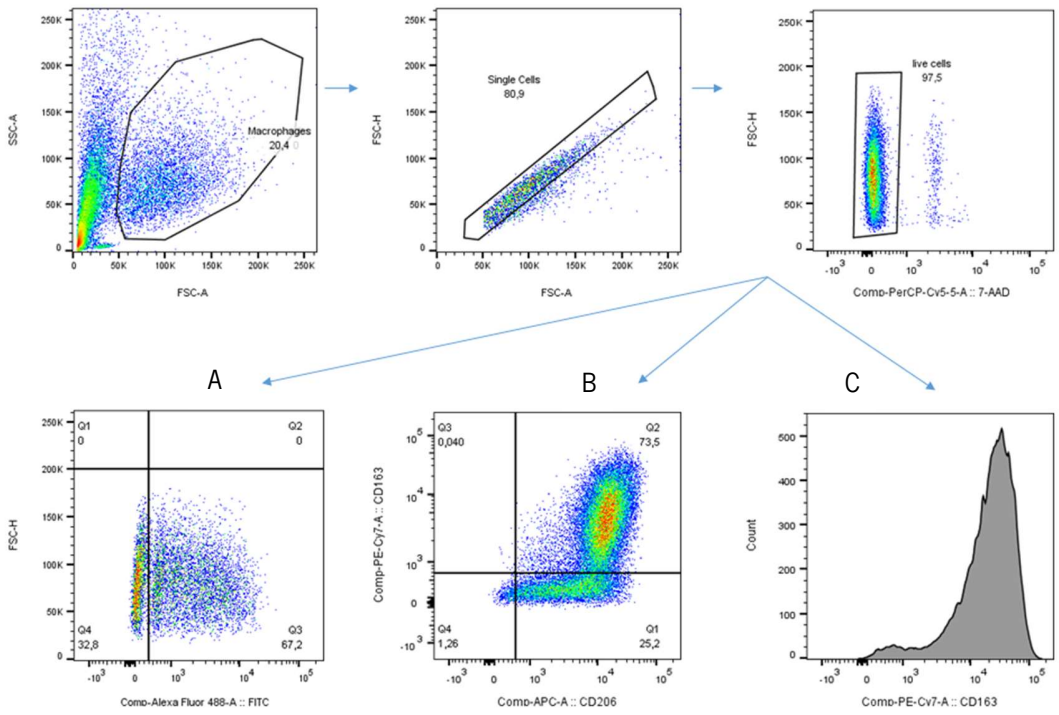
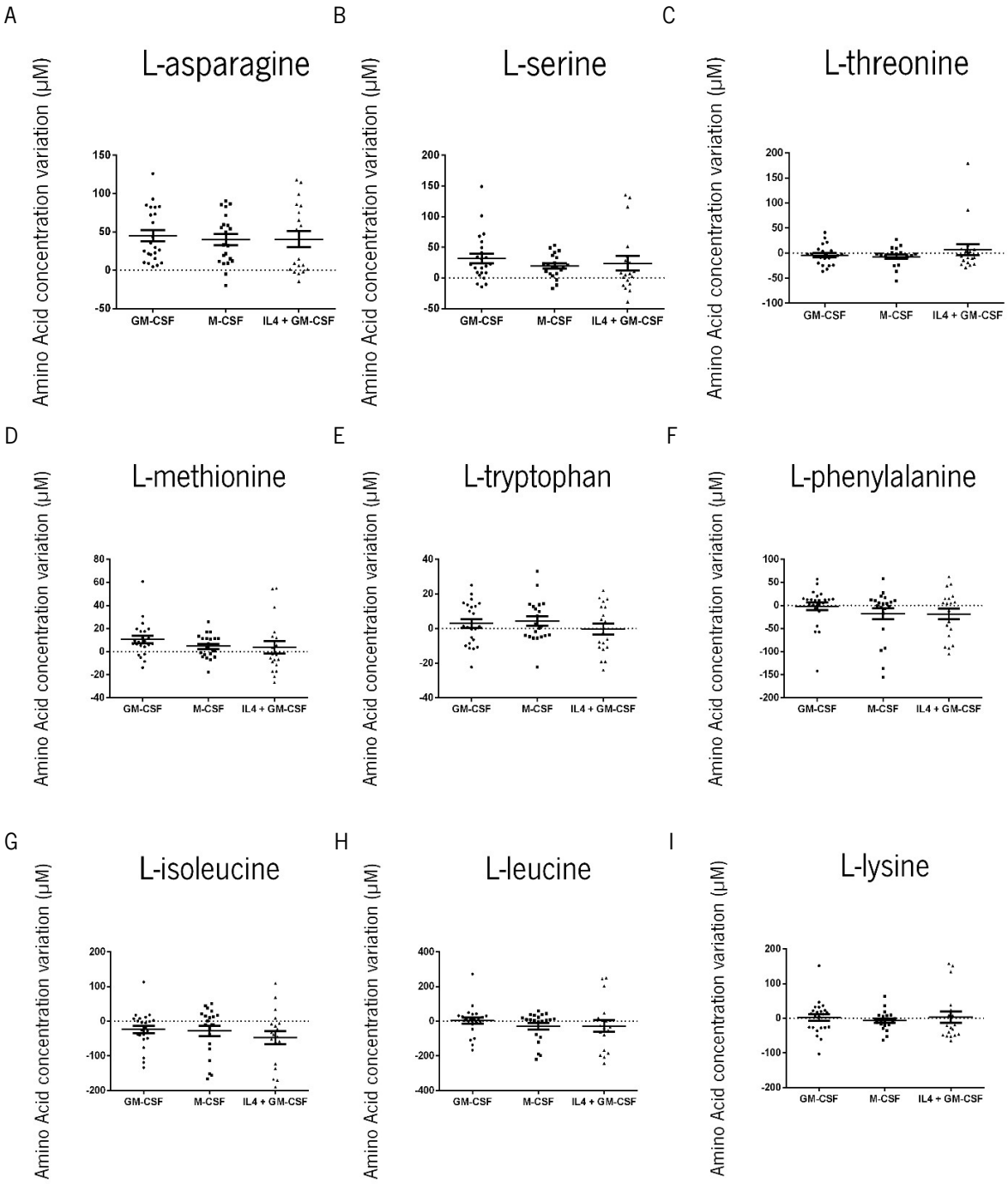


Figure 18. Graphical conclusion of the main results of this work. Briefly, a metabolic characterization of human Mφ and DCs derived from $CD14^+$ monocytes revealed distinct amino acid requirements upon differentiation with GM-CSF, M-CSF or IL-4 + GM-CSF. We revealed that amino acid catabolism of L-aspartate is important in monocyte-to-Mφ differentiation, but not for monocyte differentiation into DCs. Using a conditioned media, the absence of L-aspartate during monocyte differentiation into Mφ led to alterations in consumption levels of amino acids and differences in effector functions of Mφ differentiated with GM-CSF or M-CSF: a decrease in TNF-α and NO levels and an increase in O_2^- and H_2O_2 production. After stimulation with LPS the alterations of effector functions only were maintained for GM-CSF-differentiated Mφ revealed by a decrease in TNF-α and IL-6 levels and an increase in IL-10 and in O_2^- production levels.

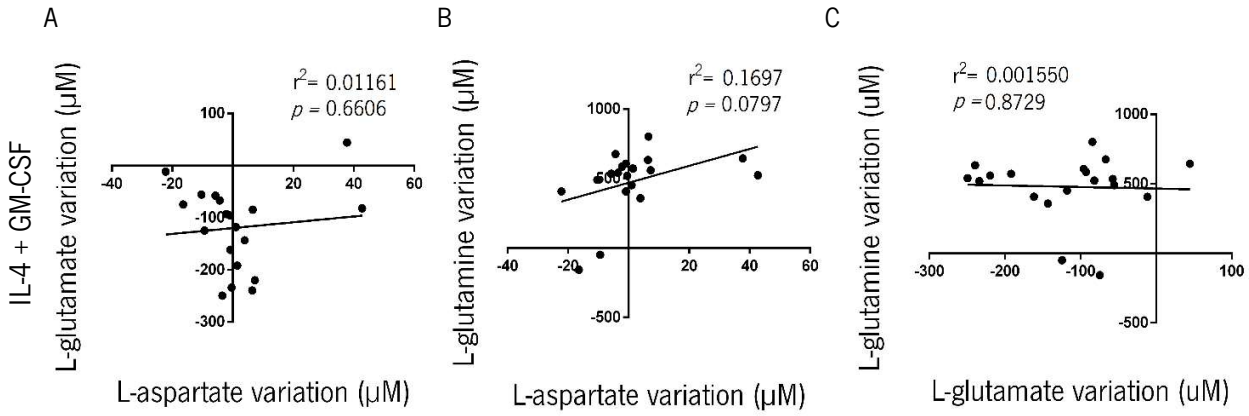
SUPPLEMENTARY DATA



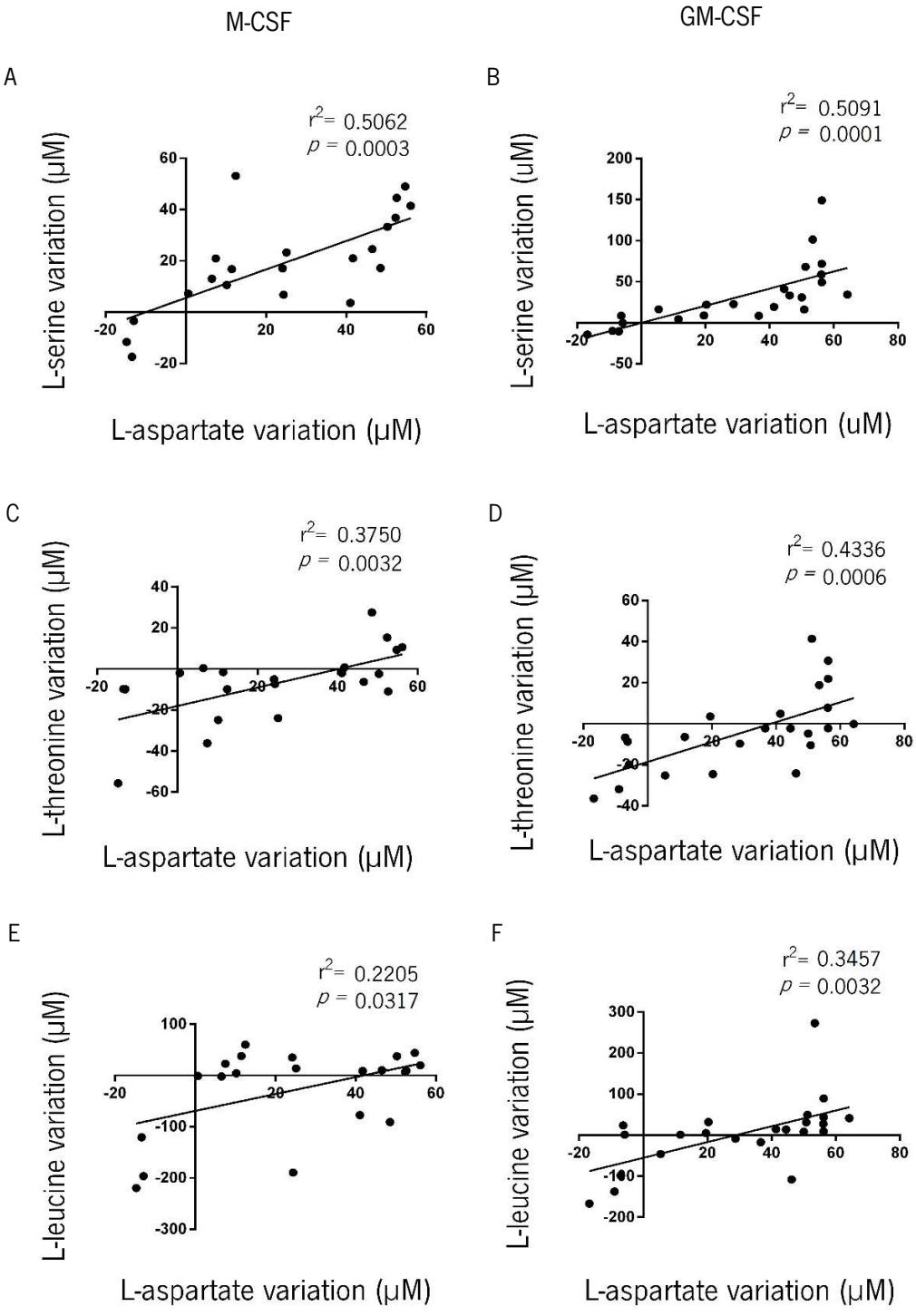
Supplementary figure 1. Gating strategy of Flow cytometry analysis. Gating strategy for the assessment of phagocytosis (A), for calculation of percentage of double positive cells (B) and for estimation of geometric mean fluorescence intensity of each phenotypic marker or probe used (C).



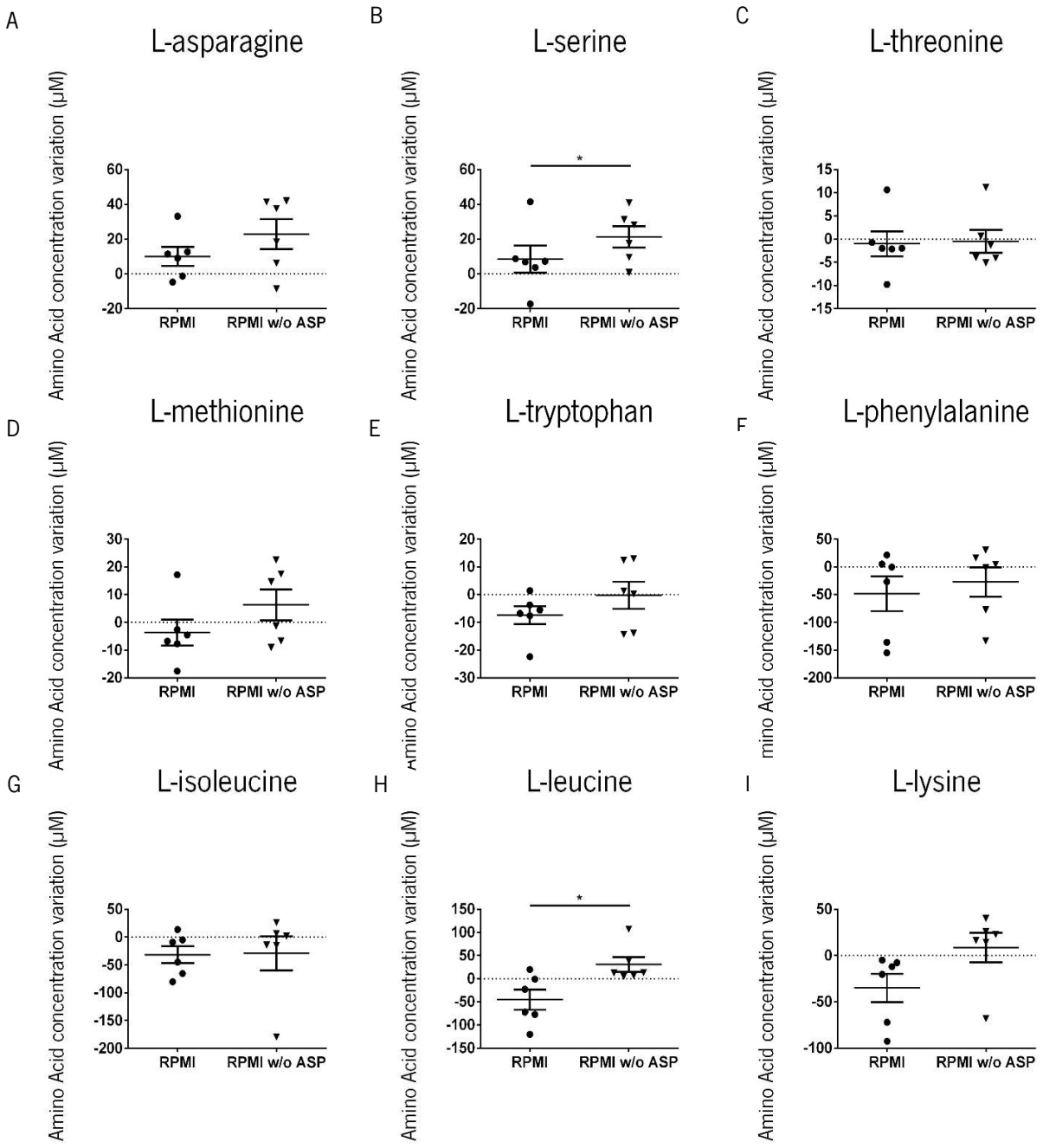
Supplementary figure 2. Amino acids consumption and production profiles upon human monocyte differentiation. The consumption (positive values) or production (negative values) of amino acids was quantified by HPLC (µM) in supernatants after 7 days of *in vitro* differentiation with GM-CSF, M-CSF or IL-4 + GM-CSF growth factors (A-I). Data was obtained from 23 different donors. *p* values were determined and no significant statistical differences were obtained.



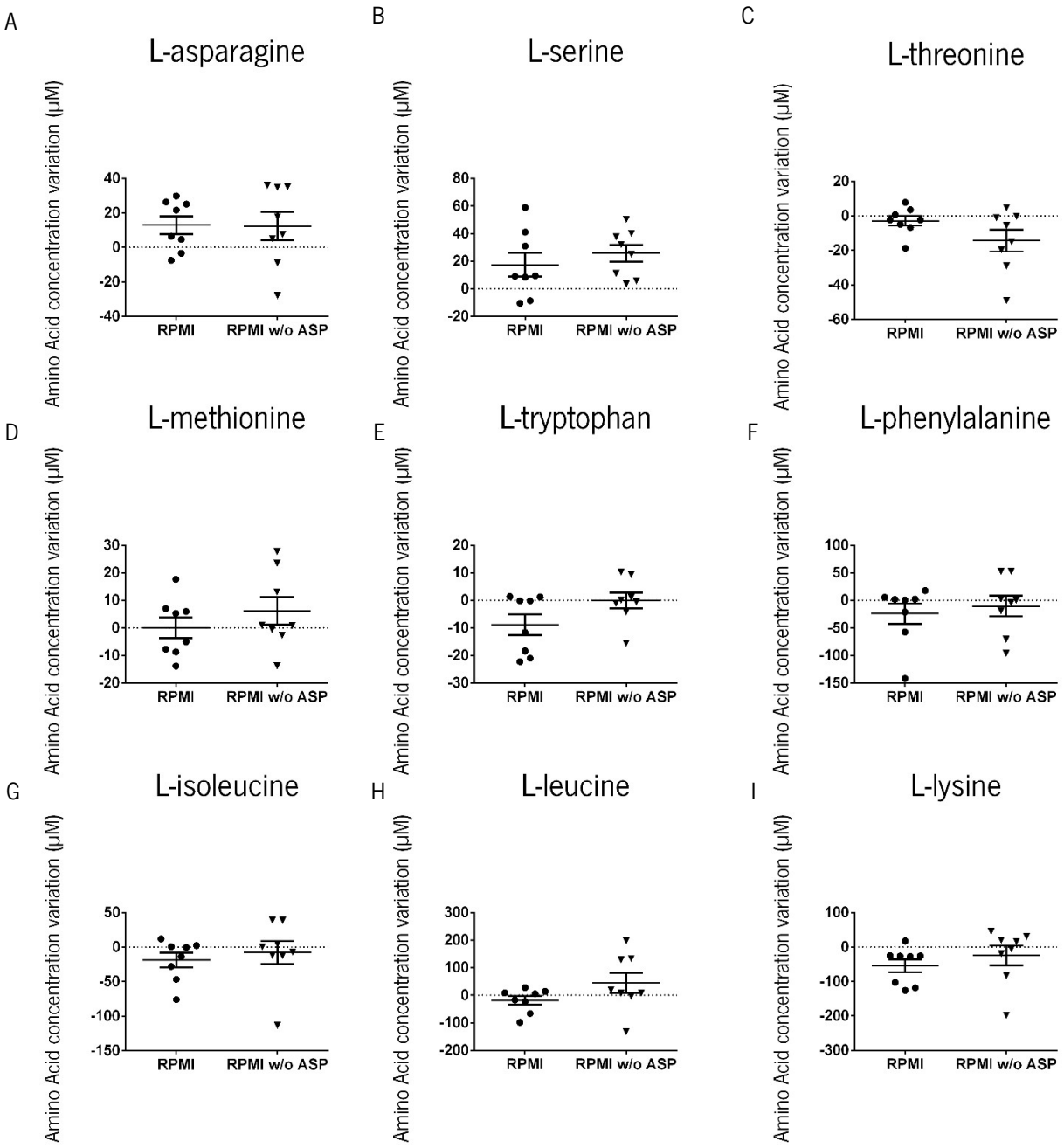
Supplementary figure 3. Correlations between amino acids consumption and production profiles in human-derived DCs. Linear regression between the consumption and production profiles of each amino acid in the culture supernatant (µM) after 7 days of *in vitro* differentiation with IL-4 + GM-CSF (A-C). Data obtained with cells derived from 23 different donors. r^2 and *p* values were determined and no significant statistical differences were obtained.



Supplementary figure 4. Correlations between amino acids consumption and production profiles in human monocyte-derived Mφ. Linear regression between the consumption and production profiles of each amino acid in the culture supernatant (µM) after *in vitro* differentiation with M-CSF (A, C ,E) or GM-CSF (B, D, F). Data obtained from 23 different donors. r^2 and *p* values were determined. Differences were considered significant for $p \leq 0.05$.



Supplementary figure 5. Characterization of other amino acids metabolism by human monocytes-derived Mφ in conditioned media in the presence of M-CSF. Amino acid concentration variation (µM) in culture supernatants after 7 days of *in vitro* differentiation with M-CSF in conditioned media (A-I). Data show mean values of Mφ differentiated in RPMIc (RPMI) and mean values of Mφ differentiated in RPMI without L-aspartate (RPMI w/o ASP). Scatter charts represent data obtained with cells derived from 6 and 8 different donors. *p* values were determined and differences were considered significant for * $p \leq 0.05$.



Supplementary figure 6. Characterization of other amino acids metabolism by human monocytes-derived Mφ in conditioned media in the presence of GM-CSF. Amino acid concentration variation (µM) in culture supernatants after 7 days of *in vitro* differentiation with GM-CSF in conditioned media (A-I). Data show mean values of Mφ differentiated in RPMIc (RPMI) and mean values of Mφ differentiated in RPMI without L-aspartate (RPMI w/o ASP). Scatter charts represent data obtained with cells derived from 6 and 8 different donors. *p* values were determined and no significant statistical differences were obtained.

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