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Impact of chronic inflammatory responses on the biology of monocytes/macrophages

Impacto de respostas inflamatórias crónicas na biologia de monócitos/macrófagos

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e do

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ABSTRACT

Behçet´s disease is a relapsing, multisystemic and inflammatory condition characterized by systemic vasculitis of small and large vessels. The recurrence of oral and genital ulcers as well as uveitis are hallmarks of this disease, being the involvement of gastrointestinal and nervous systems associated with worst cases of this condition. This disease has been associated with the silk-road, where it shows higher prevalence, especially in Turkey. Although the prevalence in Portugal is not high, the morbidity and mortality rates make this disease an important issue. The etiology and pathophysiology are still unclear but the current hypothesis states that individuals with genetic predisposition (mainly linked to HLA-B51 and SNPs in other genes, including *IL-10*) develop a systemic inflammatory type of response upon contact with some bacteria or viruses. This systemic inflammation, although with unknown origin, has been associated with an hyperactivation of monocytes/macrophages with release of proinflammatory cytokines, elevated Th1 and Th17 local levels and neutrophilia. Neutrophils show higher chemotaxis, adhesion molecules and ROS levels, which is thought to lead to the vessel damage. Although many studies have addressed the contribution of different cells to Behçet's disease, monocytes remain poorly studied. Monocytes are a heterogeneous population, being recently subdivided into classical, intermediate and non-classical subsets based on the differential expression of CD14 and CD16. These three subsets have been associated with different functions in health, and shown to be disturbed in inflammatory disorders, such as systemic lupus erythematosus. In this study, we performed a phenotypic and functional monocyte characterization of 22 individuals from a cohort of 50 Behçet's disease patients from Hospital de Braga, and age- and gender-matched controls. While the relative percentage of the different monocyte subsets was similar in the two groups, in patients we observed an increase in the expression of CD86 and CD206 in intermediate and non-classical monocytes, respectively. We have also observed an increase in the levels of superoxide anion and a decrease in the mitochondrial mass, being the last observed in the three subsets of monocytes. Interestingly, all these differences were lost upon *ex-vivo* differentiation to macrophages. Our results show that the biology of monocytes from Behçet's patients is conditioned by the chronic inflammatory environment, suggesting a role of these cells in the pathophysiology of Behçet's disease.

RESUMO

A doença de Behçet é uma condição inflamatória, multi-sistémica com episódios de surto-remissão, caracterizada por uma vasculite sistémica. Os sintomas que caracterizam a doença passam por ulceração recorrente a nível oral e genital, bem como uveíte, sendo que o sistema gastrointestinal e nervoso central também podem ser afetados em casos mais severos da doença. Esta doença tem sido associada à rota da seda devido a uma maior prevalência nessa área, especialmente na Turquia. Apesar desta condição não ser muito prevalente em Portugal, a morbidade e mortalidade associadas tornam esta doença um assunto de interesse elevado. A sua etiopatologia não é clara, no entanto é aceite que indivíduos com predisposição genética (ligada principalmente a HLA-B51 e a polimorfismos em outros genes como *IL-10*) desenvolvem uma resposta inflamatória sistémica exacerbada após contacto com algumas bactérias ou vírus. Esta resposta inflamatória, apesar de ter origem desconhecida, parece estar associada a uma híper-ativação de monócitos/macrófagos com libertação de citocinas pro-inflamatórias, elevados níveis de células Th1 e Th17 locais, assim como neutrofilia. Os neutrófilos apresentam elevada quimiotaxia, adesão ao endotélio e produção de espécies reativas de oxigénio, levando a dano no endotélio. Muitos estudos têm sido realizados com o objetivo de avaliar o estado de populações celulares na doença de Behçet, no entanto os monócitos continuam pouco estudados. Os monócitos são uma população bastante heterogénea, tendo sido recentemente subdividida em clássicos, intermediários e não clássicos, de acordo com a sua expressão de CD14 e CD16. Estas três subpopulações dos monócitos estão também associadas a diferentes funções em indivíduos saudáveis, tendo sido demonstradas alterações nestas células a nível de doenças inflamatórias, como é o caso de Lúpus. No estudo realizado, foram recrutados 22 doentes de Behçet a partir de um cohort de 50 indivíduos, sendo também incluídos controlos saudáveis com idade e sexo coincidente com o grupo de doentes. Foi realizada uma análise fenotípica e funcional de monócitos e não foram encontradas diferenças relativamente à percentagem relativa de casa subtipo de monócitos, tendo sido encontrada uma sobreexpressão de CD86 e CD206 nos monócitos intermediários e não clássicos, respetivamente. Adicionalmente, também observamos um aumento nos níveis de superóxido e uma diminuição da massa mitocondrial dos monócitos dos doentes, sendo o ultimo parâmetro observado em todos os subtipos de monócitos. Os mesmos parâmetros foram avaliados em macrófagos derivados de monócitos, no entanto não foram encontradas diferenças significativas. Os resultados deste trabalho dão indicações que a biologia dos monócitos se

encontra condicionada pelo ambiente inflamatório sistémico, sugerindo que estas células possam ter um papel importante na patofisiologia da doença.

CONTENTS

ABBREVIATIONS LIST

BD	Behçet's disease
CD	Cluster of differentiation
CO_2	Carbon Dioxide
DAMPs	Damage associated molecular patterns
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt dehydrate
FACS	Flow activated cell sorting
FBS	Fetal bovine serum
FMO	Fluorescence minus one
FSC	Forward scatter
HLA	Human leukocyte antigen
HSP	Heat shock protein
HSV-1	Herpes simplex virus 1
IFN-γ	Interferon gamma
IL	Interleukin
ISGB	International study group of Behçet
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NAO	Acridine orange 10-nonyl bromide

NETS	Neutrophil extracellular traps
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
SNP	Single nucleotide polymorphism
SSC	Side scatter
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

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1. INTRODUCTION

1.1 CLINICAL CHARACTERISTICS OF BEHÇET'S DISEASE

Behçet's disease (BD) was first described by Benediktos Adamantiades in 1930. He presented a clinical case during a lecture entitled "A case of relapsing iritis with hypopyon" (Zouboulis, 2002). In 1937, Hulusi Behçet while working with similar cases presented at the meeting of the dermatological association of Istanbul, a 34-year-old female patient with recurrent oral aphthous ulcers, genital ulcers, and ocular lesions, the triple symptom complex that characterizes Behçet (Behcet, 1937). Although Adamantiades was the first to describe this disease, the name Behçet became linked with the disease after several authors had used it when referring to this condition.

BD is a relapsing, multisystemic and inflammatory condition characterized by systemic vasculitis of small and large vessels. The recurrence of oral and genital ulcers as well as uveitis are hallmarks of this disease (Behcet, 1937; Saadoun & Wechsler, 2012). BD affects people worldwide even though its prevalence is higher in the Mediterranean, Middle East, and Far East, suggesting an association with the "silk route" (Yoshikawa, Kotake, Sasamoto, Ohno, & Matsuda, 1991). Although recurrent ulcers and uveitis are the key symptoms of this disease, other common problems include skin rashes and arthritis (Park, 1999; Saadoun & Wechsler, 2012). As the disease evolves to more severe states, other complications involving the gastrointestinal and nervous systems may develop, such as giant ulcers in the ileocecal and the involvement of the esophagus and small intestines, ophthalmoparesis, cranial neuropathy, cerebellar or pyramidal dysfunction, myelopathy, encephalopathy, hemiparesis, hemisensory loss, seizures and dysphasia, intracranial hypertension syndrome, cerebral venous thrombosis and acute meningeal syndrome (Kokturk, 2012).

Besides high morbidity mainly associated to complications in eye involvement, BD can also cause mortality where the main cause is usually due to major vessel involvement, particularly in patients with pulmonary artery aneurysms (Saadoun et al., 2010). According to Hamuryudan *et al*. the overall survival rate of Behçet's patients presenting pulmonary artery aneurysms is 50% for patients diagnosed before 1992 and 62% after that date. This improved survival rate is thought to be associated with the improvement of the therapeutic approaches (Hamuryudan et al., 1994).

1.2 EPIDEMIOLOGY OF BEHÇET'S DISEASE

BD has higher prevalence along the silk route, an ancient network of trade routes that connects the east and west of china to the Mediterranean Sea. Even though this disease affects people worldwide, the prevalence of BD varies around the world, with a stronger incidence in Turkey, with 420 Behçet patients per 100000 people. Still within the silk road, Israel and northern China present prevalence higher than 100 patients per 100000 people (Figure 1) (Cho, Cho, & Bang, 2012).

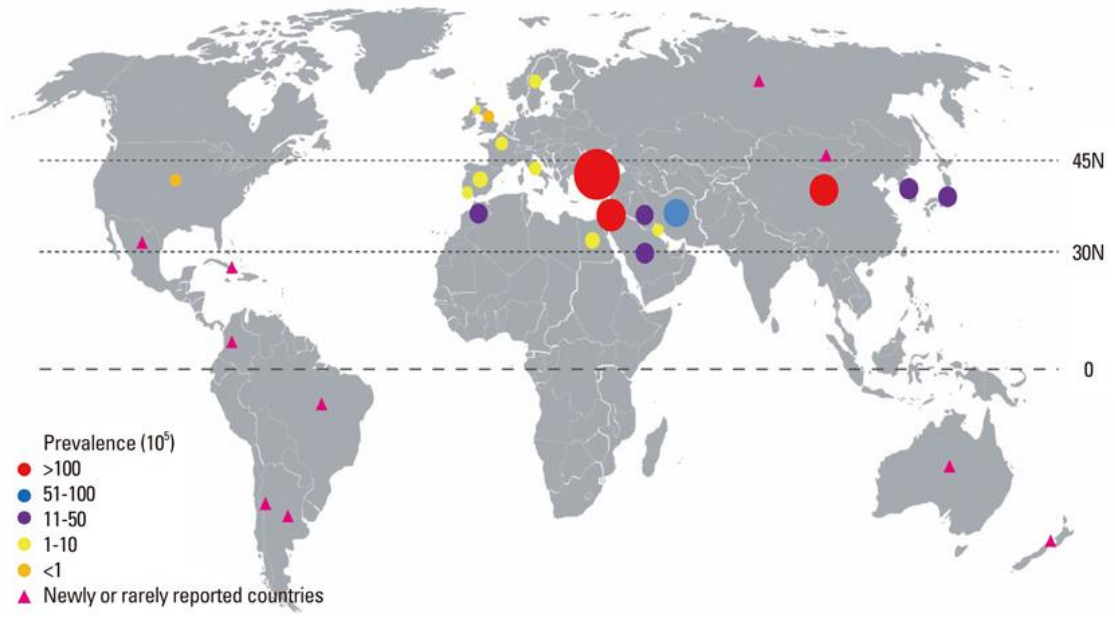

Figure 1: **Behçet´s disease prevalence distribution around the world**. The prevalence of Behçet is higher between 30N and 45N, being associated with the ancient trade route, the Silk road. Dot size reflects prevalence. The image was adapted from Bang, D *et al*, 2012

Besides the different prevalence around the world, it is well established that the age of onset occurs around the third or fourth decade of life, however it can also affect both children and patients above the age of 50 (Bang et al., 2012). Despite affecting both children and older people, the complications associated with this disease are more severe in children. According to Melikoğlu and Melikoğlu, both the oral ulceration rates and the BD current activity form scores in children are higher in comparison to patients with more than 40 years old (Melikoğlu & Melikoğlu, 2008). Accordingly, a clinical study performed in Portugal (in 2010) reported an average age on diagnosis of 31.3 years (Costa, Castanheira, Coelho, & Dias, 2010). The

association between gender and prevalence is still controversial. Although several studies have demonstrated a higher prevalence among females, others show no difference (Sula, Batmaz, Ucmak, Yolbas, & Akdeniz, 2014; Zouboulis, 1999). As to disease outcome there is also some controversy with some studies showing that males often have more severe forms of the disease (Davatchi et al., 2011).

1.3 ETIOLOGY AND PATHOPHYSIOLOGY OF BEHÇET'S DISEASE

1.3.1 THE PROCESS OF INFLAMMATION AND ITS RESOLUTION IN HEALTH AND DISEASE

Inflammation is a natural process to maintain tissue homeostasis. When challenged/damaged by pathogens, foreign bodies or injuries, an inflammatory type of response is initiated to clear the infection and/or repair the damaged tissue (Medzhitov, 2008). According to the definition of inflammation, there are several events that characterize the inflammatory process, such as vascular dilation, enhanced permeability of capillaries, increased blood flow and leukocyte recruitment (Ryan & Majno, 1977). Neutrophils are among the first cell type to migrate to the inflamed tissue, where they act as a line of defense of the innate immune system due to their phagocytic and microbicidal functions (Jones, Robb, Perretti, & Rossi, 2016).

The initiation of the inflammatory response relies on the binding of pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) to pattern recognition receptors (PRR), namely Toll-like receptors (TLR´s), on resident macrophages which elicits the production of inflammatory cytokines, chemokines and proinflammatory lipid mediators (Tang, Kang, Coyne, Zeh, & Lotze, 2012). These mediators are essential in establishing an effective inflammatory response and the proper clearance of pathogens. In fact, the production of inflammatory mediators such as chemokines and complement components are the basis of the chemotaxis observed during inflammation. Once blood leukocytes exit a blood vessel, they are attracted by gradients of chemotactic factors to the site of infection (Harris, 1954). These cells are followed by mononuclear cells, such as monocytes and macrophages that enter the inflammatory site to help in the clearance of pathogens, cellular debris, apoptotic cells, as well as to resolve inflammation. Efficient initiation and resolution of the inflammation are crucial to

prevent damage and therefore maintenance of tissue homeostasis. It is interesting to notice that, the type and degree of the inflammatory response depends on many factors, namely the type of the trigger, the dose as well as its endurance in the host system (Nathan, 2002).

1.3.2 AUTOIMMUNE VERSUS AUTOINFLAMMATORY DISEASES – WHERE DOES BEHÇET'S DISEASE FIT IN?

Autoinflammatory disease is a concept used when referring to rare genetic disorders, with recurrent and unprovoked inflammatory states in the absence of any infection, the so called sterile inflammation. These conditions usually have self-directed inflammation, whereby local factors in predisposed individuals lead to the activation of the immune system, such as macrophages and neutrophils, resulting in tissue damage. Nevertheless, there is still a group of other diseases that can be classified as autoinflammatory. These disorders present episodes of acute, apparently inexplicable, inflammation, including some conditions such as pyogenic disorders, immune-mediated granulomatous diseases (such as Crohn's disease), and idiopathic febrile syndromes (such as pharyngitis) (Doria et al., 2012). On the other hand, autoimmune disorders are characterized by self-directed inflammation caused by immune reactivity towards self-antigens, usually due to aberrant B and T cell responses (Doria et al., 2012). Regarding BD, the classification is not clear, however, McGonagle and McDermott have proposed a different classification of several conditions, including BD, as a mixed pattern disease, explained by the involvement of both the innate and acquired immune system (McGonagle & McDermott, 2006).

1.3.3 GENETIC PREDISPOSITION TO BEHÇET'S DISEASE

Although the pathophysiology of BD is still unclear, there are some characteristics that have already been associated with this condition. Regarding the host, some criteria support evidence for a genetic predisposition to BD: the geographical distribution, correlation with class I human leukocyte antigen (HLA) and polymorphisms in genes involved in the immune response (Remmers et al., 2010). The HLA corresponds to the human version of the major histocompatibility complex (MHC) and it is known to be the most polymorphic genetic system. This complex comprises HLA class I, II and III genes (Choo, 2007). These first two classes are

functionally related and are associated with the presentation of processed peptide antigens to T cells, contributing to the development of an adaptive immune response. Although functionally related, class I genes differ from class II in several aspects. While class I genes encode proteins that are expressed by most somatic cells, class II genes are usually expressed by a small group of immune cells, including B cells, activated T cells, macrophages, dendritic cells and thymic epithelial cells. Furthermore, these two classes also present antigens at the cellular surface through different mechanisms. HLA class I molecules are initially manufactured in the endoplasmic reticulum where they are loaded with antigens that undergo proteasome degradation (Pamer, 1998). HLA class II molecules are also manufactured in the endoplasmic reticulum however they are associated with other proteins, the invariant chains, and only then can they be enclosed in membranous vesicles that fuse with endosomes filled with exogenous proteins and be loaded with the peptide fragments for presentation (Cresswell, 2003). Additionally, there is another difference between HLA class I and II, in terms of presentation. While HLA class I presents antigens to cluster of differentiation (CD) 8^+ T cells, HLA class II molecules present antigens to $CD4^+$ T cells (Both $CD4^+$ and $CD8^+$ T cells will be approached in more detail further on) (Cresswell, 2003; Pamer, 1998). Concerning BD, HLA-B5 allele, more specifically HLA-B51, has been shown to be a predisposition factor (Wallace, 2014). Even though there are still differences between studies, this association was first described by Ohno et al. in 1982 and has been proposed since then as the strongest immunogenetic predisposition factor for BD (Ohno et al., 1982). In fact, according to a systematic review including 78 independent studies, the population-attributable risks percentage suggests that the presence of the HLA–B51/B5 allele accounts for 32–52% of BD cases within different geographic subgroups (De Menthon, Lavalley, Maldini & Guillevin, 2009).

Besides HLA related genes, genes encoding cytokines and cytokine receptors have been widely studied. A genome-wide association study found the interleukin (IL) 10 region to have five single nucleotide polymorphisms (SNPs) strongly associated with Behçet's disease, identifying as well common variants in interleukin 23 receptor/interleukin 12 receptor subunit beta 2 locus associated with predisposition to BD. Furthermore, several SNP haplotypes of the *IL-10* gene promoter have been reported to be associated with regulation of the gene's expression. In the same study, Remmers et al. have found that lipopolysaccharide (LPS)-activated mononuclear cells from healthy Turkish donors homozygous for the disease associated *IL-10* variant (the rs151811 A allele) show significantly lower amounts of IL-10 protein (Remmers et al., 2010). In

fact, this finding is in accordance with the disease phenotype. IL-10 is an anti-inflammatory cytokine that mainly acts by inhibiting the activity of type 1 T helper (Th) cells, natural killer (NK) cells, macrophages and dendritic cells (Couper, Blount, & Riley, 2008). Being BD characterized by an excessive inflammatory environment (Zhou, Chen, Shen, & Lu, 2012), lower production of IL-10 is translated into a defective regulation of this environment, which is thought to be associated with an overresponse of immune system's cells.

1.3.4 TRIGGERING CUES - ENVIRONMENTAL FACTORS

Although there are strong evidences for a genetic predisposition to BD, the inflammatory reactions that characterize the disease seem to be initiated by environmental cues. The main environmental factors described to lead to the development of BD is the exposure to infectious agents, such as viruses (Herpes simplex virus 1 (HSV-1), Hepatitis virus, Parvovirus B19 and bacteria (*Streptococcus* spp., Mycoplasma, *Helicobacter pylori*) (Galeone, Colucci, D'Erme, Moretti, & Lotti, 2012). Indeed, the infectious model is supported by observations that oral ulcers often precede the establishment of disease. The viral association to the development of BD was first postulated in 1937 (Behcet, 1937) and it is still a matter of disagreement. However, HSV-1 is currently the most common virus associated with BD. Supporting this information, studies found HSV deoxyribonucleic acid (DNA) and serum antibodies against the virus to be in a higher proportion in patients with BD in comparison to controls (Direskeneli, 2001). Regarding the bacterial infections, *Streptococcus* spp. has also been widely reported. Clinical observations found unhygienic oral conditions including periodontitis, decayed teeth, and chronic tonsillitis frequently noted in the oral cavity of BD patients. Additionally, uncommon oral *Streptococcus sanguinis* serotype (called KTH-1) and antibodies against bacteria are significantly increased in the oral flora and serum as well as hypersensitivity reactions against streptococcal antigens in skin tests. Some studies have also found an activation of T cells from BD patients by *S. sanguinis* and heat shock proteins (HSP) 60/65 that is not observed in healthy controls (Yoshikawa et al., 1991).

HSPs are very conserved proteins among nearly all the organisms and were first associated with the cellular response that is triggered when the environment temperature increases above the homeostatic temperature of the organisms (Lindquist & Craig, 1988). Several other studies have been conducted and now it is known that HSPs are produced not only

when the temperature increases but also upon different cellular stress situations, such as infection, inflammation, elevated levels of reactive oxygen species (ROS) and similar events (Zugel & Kaufmann, 1999). They protect cells from severe damage and premature death by its involvement in transcription, translation and posttranslational modifications, protein folding, and aggregation and disaggregation of proteins (Tyedmers, Mogk, & Bukau, 2010). Regarding BD, these proteins have been described to be associated with BD etiopathogenesis. Although the mechanisms are not clear yet, increased T and B cell activity against HSP60/65 is observed in this disease (Direskeneli, 2003). Furthermore, it has been reported that HSP60 serves as a "danger signal" activating the innate immune system through the TLR system. In fact, when macrophages are stimulated with HSP60, these cells elicit a pro-inflammatory phenotype, releasing cytokines and overexpress several adhesion molecules (Chen, Syldath, Bellmann, Burkart, & Kolb, 1999).

1.3.5 OXIDATIVE STRESS IN BEHÇET'S DISEASE

Nowadays, there have been described several roles for ROS in living organisms with evidence showing that these organisms have adapted to the coexistence with free radicals, and most important, have developed mechanisms to use these radicals efficiently. In fact, important physiological functions involving free radicals as well as their derivatives have been described. These include the regulation of vascular tone, the enhancement of signal transduction, sensing oxygen tension as well as the maintenance of redox homeostasis. The most relevant radicals include the superoxide anion and the nitric oxide (NO) (Droge, 2002). Superoxide can be produced either by enzymes, such as nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases and xanthine oxidase or redox-reactive compounds, such as semi-ubiquinone present in the mitochondrial electron transport chain. In fact, semi-ubiquinone is a major site of superoxide production through the reduction of molecular oxygen, being present in practically all cells and tissues. NO on the other hand, can be produced by nitric oxide synthase (NOS), through an oxidation reaction of terminal guanidine nitrogen atoms of L-arginine (Droge, 2002). These radicals usually exist in biological tissues in low concentrations, whose concentrations must be tightly balanced in physiological conditions. This balance is done by the production of radicals and its clearance by antioxidant compounds and enzymes. The clearance of free radicals by enzymes are carried by superoxide dismutase (SOD), glutathione peroxidase and catalase. In

addition to the enzymatic clearance, nonenzymic compounds include α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C) and glutathione (Droge, 2002). Regarding BD, several studies have identified an increased oxidative state. Besides this increased oxidative state, it has also been reported a decrease in the levels of antioxidants. With this, authors have suggested that the damage associated to this disorder is caused, at least in part, by the oxidative stress observed (Isik, Koca, Ustundag, & Selek, 2007; Köse, Doğan, Aşçioğlu, Erkiliç, & Aşçioğlu, 1995). Furthermore, this oxidative state observed has been suggested as another parameter to be assessed in the follow up of these patients.

1.3.6 ENDOGENOUS CONTRIBUTIONS – NEUTROPHILS, T CELLS AND MONOCYTES

Along with the acknowledgement of infectious agents as triggers of BD, there are a series of endogenous processes that lead to the development of the common symptoms observed in BD. As previously described, people suffering from this condition show often tissue damage due to an unregulated activation of the immune system. Upon normal conditions, exposure to microorganisms leads to physiological processes such as chemotaxis and release of cytokines and chemokines, contributing to the development of inflammation that is beneficial to the clearance of the pathogens (Medzhitov, 2008; Nathan, 2002; Stein & Nombela-Arrieta, 2005). However, in BD patients, these processes are "exacerbated" leading to tissue damage. The biology of neutrophils and lymphocytes has already been described to be altered in this condition, helping to explain how the inflammatory reaction associates with the problems seen in BD patients (Takeno et al., 1995).

Neutrophils are a type of polymorphonuclear leukocytes associated with the acute phase of inflammation. These cells are recruited to an inflammatory site where they can eliminate microorganisms in several ways: i) through phagocytosis leading to the production of ROS through the NAD(P)H oxidase and/or antibacterial peptides, such as cathepsins, defensins, lactoferrin and lysozyme that are released inside the phagosome; ii) by the release of antibacterial peptides in the extracellular milieu, acting on both intra and extracellular pathogens, and iii) by the formation of NETS - neutrophil extracellular traps, that consist of a core DNA element attached with histones, proteins and enzymes - that either act by immobilizing pathogens, which facilitates their phagocytosis, or killing them directly by antimicrobial histones and proteases (Kaplan & Radic, 2012; Kolaczkowska & Kubes, 2013). Due to its implications in

inflammation, these cells have been extensively studied in the context of BD. Several studies have been conducted regarding functions associated with neutrophils' activation. Among these, chemotaxis and adhesion have been the most reported alterations. Studies report an increase in the chemotaxis and adhesion capacity of neutrophils, some studies claim that these enhanced functions are only observed when these cells are stimulated with BD plasma or serum, suggesting a hyperactivation of neutrophils by soluble factors present in plasma. Furthermore, these cells also present an increased accumulation of superoxide in active BD without any stimulation which also points to an hyperactivation of these cells in peripheral blood (Takeno et al., 1995), that may result from an altered composition of the plasma of these individuals.

Considering that this is a chronic disease and that it has been associated with HLA complex molecules and infection, it is not surprising that the biology of T cells has been previously addressed. Unlike neutrophils, the principal function of T lymphocytes is to produce cytokines and be involved in cell-cell interactions (Ross, 1994). Regarding their role in immunity, T cell-mediated immunity is based on the development of antigen-specific T lymphocytes to eliminate viral, bacterial, or parasitic infections or malignant cells. These cells present a T cell receptor (TCR) that is responsible for the recognition of MHC-associated specific antigens. Upon recognition, T cells become activated, acquiring either a memory or effector phenotype. During development, T cells can produce two general types of TCR. The majority express antigen-binding $\alpha\beta$ chains in the TCR while a small fraction express $\gamma\delta$ chains. Apart from the TCR differences, T cells can still be subdivided according to the expression of the surface markers CD4 and CD8 as cytotoxic ($CD8^+$) or helper ($CD4^+$) T lymphocytes. Cytotoxic $CD8^+$ T cells are very effective in direct apoptosis of infected or malignant cells, while $CD4^+$ T helper cells produce different cytokine profiles that can stimulate other T cell effector functions and B cell antibody production, as well as contribute to inflammatory mechanisms. Regarding the helper $\alpha\beta$-T cells, there are also subpopulations according to the cytokine patterns that each cell produces. For example, Th1 cells, one of the helper T cell subsets, produces mainly interferon gamma (IFN-γ) while Th2 produces IL-4 and IL-5 (Luckheeram, Zhou, Verma, & Xia, 2012; Müller et al., 2012; Zhang & Bevan, 2011).

In the context of BD, T cell activation and its phenotypes have been extensively studied, including the cytokine production profiles. Cytokines are small peptides that are secreted by cells and influence cellular interactions and communications. According to their functions during

inflammation, cytokines can be classified as pro-inflammatory, when they act by augmenting the inflammation, or anti-inflammatory, when their actions aim to resolute the inflammation (J.-M. Zhang & An, 2007). Th1 associated cytokines, such as the proinflammatory cytokines IL-12, IFN-γ and tumor necrosis factor alpha (TNF-α) as well as Th1 associated chemokine receptors CC chemokine receptor 5 and CXC chemokine receptor 3 were found to be increased in Behçet patients, indicating an increase on the activation of Th1 type of cells, which is in accordance with the increased inflammatory state of BD (Dalghous, Freysdottir, & Fortune, 2006.; Hamzaoui et al., 2002). As mentioned before, the Th1 type of cells is a subset of $CD4^+$ T lymphocytes associated with the immune response against intracellular bacteria and some viruses. Regarding their effects, Th1 cytokines act mainly on the induction of cell-mediated immunity, predominantly producing a phagocyte-dependent inflammation by activating macrophages, stimulating the production of opsonizing and complement-fixing antibodies by B cells, and cell cytotoxicity (Romagnani, 2000). The involvement of Th1 cells in BD may help to explain the hyperactivation of the innate immune system observed in this condition. Besides Th1 cells, Th17 have also been associated with the pathophysiology of BD. This cellular subset increased in the course of this disease associated to an increased amount of IL-17 (Al-Zifzaf, Mokbel, & Abdelaziz, 2015). Furthermore, there is an increased percentage of γδ T cells in peripheral blood of patients with BD and a great proportion of these cells are in an activated stage. In fact, it has been described that these cells express activation markers (namely CD69 and CD29) and produce higher levels of the proinflammatory cytokines IFN-γ and TNF-α. All of these observations suggest a dysfunction of T cell regulatory mechanisms which contributes to the pathophysiology of this disease (Dalghous et al., 2006.; Shimizu, Yoshikawa, Takada, Hirotsu, & Suzuki, 2011).

Monocytes macrophages derive from a common myeloid progenitor cell in the bone marrow and account for 2-12% of the leukocytes. In the absence of stimulation, monocytes circulate in the bloodstream. Even though, these cells can be recruited, from the blood stream to the tissue, where they mediate antimicrobial defenses or tissue healing. Size, trafficking ability, expression of immune receptors, phagocytic ability and the ability to be activated and differentiate in response to either cytokines or antigens, are among the most common characteristics that make monocytes a heterogenous population (Hume, 2006; Shi & Pamer, 2011). This heterogeneity, along with the plasticity with which cells acquire different phenotypes, led to studies in which three major subsets were identified: classical, intermediate and non-classical monocytes that differ mainly in the expression of the surface markers CD14 and CD16 and their

functions (Wong et al., 2012). Norata et al. stated that monocytes have their functions influenced/altered during both homeostasis or inflammation. Additionally, they associate this alteration to the intracellular metabolic changes, as well as the microenvironmental cues monocytes are exposed (Norata et al., 2015). The contribution of monocytes to BD has not been studied in detail, however, it has been reported that monocytes from patients spontaneously secrete higher amounts of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8, which suggests an activated phenotype (Mege et al., 1993).

In fact, BD seems to present a multifactorial pathogenesis, with the involvement of both genetic predisposition as well as several exacerbated responses by immune system's cells to infection. Although several studies have been conducted to understand the role of both neutrophils and T cells, the role of monocytes as well as its functions and biology during this disease are still unclear and require further research. Indeed, further studies are required to address whether the soluble inflammatory mediators influence the activation of circulating monocytes behind the aforementioned production of cytokines.

2. RESEARCH OBJECTIVES

The hypothesis behind this work is that the inflammatory environment in the blood stream will impact the phenotype of monocytes. In this line it is expectable that, both circulating monocytes and monocyte-derived macrophages are functionally different and with a more activated functional phenotype. This work is relevant since it brings new knowledge about a cellular population in BD that has not been well described yet, which will help better understand the pathology of this disease.

To answer this question, in a cohort of BD from Hospital de Braga, we aimed to:

1. Phenotypically characterize the leukocytes of BD, regarding the different monocyte subsets and their activation status, by flow cytometry.
2. Analyze the monocyte and monocyte-derived macrophages, regarding some of their functions, by flow cytometry.

3. MATERIAL AND METHODS

3.1 STUDY POPULATION

Patient recruitment was obtained from the Autoimmune disease unit, internal medicine department – Hospital de Braga, with no restriction of age or gender. For this prospective study, a total of 22 patients with either definitive or probable diagnostic of BD (according to the international study group of Behçet (ISGB) criteria) were recruited. Individuals with other systemic inflammatory conditions were excluded (Inclusion and exclusion criteria described in table 1). Patients already diagnosed will also be stratified in those who are currently under treatment, those without any pharmacological approach and patients presenting a BD flare during the period of study (Not discussed in this thesis). Samples from age- and sex-matched healthy individuals were used as healthy controls. The controls showed no inflammatory or auto-immune conditions neither any pharmacological therapy on going during the study period. The patients sample was selected in a non-probabilistic manner. All participants were informed about the aims of the experimental study and signed the informed consent. Both patients and health controls were punctioned in the median cubital vein, after 8 hours of fasting, and the blood was collected to vacuette tubes K3E K3EDTA (Greiner Bio-One, Kremsmünster, Austria) (9mL). The experiment protocol has been approved by the "Subcomissão de Ética para as Ciências da Vida e da Saúde (SECVS) - 059/2014" and the "Comissão de Ética para Saúde do Hospital de Braga (CESHB) in July 22nd 2014", with an upgrade for the metabolomic studies in July 4th 2016 by the SECVS.

Table 1: **The inclusion and exclusion criteria for the selection of Behçet's disease patients**.

Inclusion criteria:	***Exclusion criteria:***
– Patients with definitive or very probable diagnostic of Behçet's disease according to ISGB;	– Patients diagnosed with other chronic inflammatory conditions;
– Patients that were being followed at Hospital de Braga.	– Patients with infection complications during the research period;

3.2 PHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD LEUKOCYTES

3.2.1 EXTRACELLULAR STAINING FOR FLOW CYTOMETRY

Two hundred microliters of human peripheral blood were incubated with 4 mL of Ammonium-Chloride-Potassium red blood cell lysis buffer (0.155M Ammonium Chloride, 0.01M potassium bicarbonate, 0.0001M ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)) at room temperature (RT) for 10 min. Upon centrifugation for 5 minutes at 300g RT, the supernatant was removed and the pellet resuspended in flow activated cell sorting (FACS) buffer (2% fetal bovine serum (FBS) in phosphate buffered saline (PBS), both from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The cells were put in a 96-round well plate and centrifuged 5 min at 300g RT. The supernatant was removed and the antibody mix (table 2) was added. The cells were incubated for 20 min at 4°C in the dark. After incubation, cells were centrifuged at RT and resuspended in FACS buffer for flow cytometry analysis. For the controls, unstained, single staining and fluorescence minus one (FMOs) were used. The unstained control was performed by incubating cells with only FACS buffer and no antibodies. This control was used to determine the autofluorescence of the cells. For the single staining, cells were incubated with each antibody alone to adjust the voltages of each channel and correct the spillover effect of the fluorochromes. FMO's were performed by incubating cells with every fluorochrome except for one, allowing the determination of the negative and positive populations for each marker.

Table 2: **Antibody panel for the cell surface staining for phenotypic characterization of peripheral blood leukocytes**.

	Cell surface staining				
Panel	**Antigen**	**Fluorochrome**	**Supplier**	**Concentration**	**Clone**
Exclusion	CD3	BV711	Biolegend	1.6 µg/mL	OKT3
	CD19	BV786	Biolegend	2 µg/mL	HIB19
	CD56	BV605	Biolegend	4 µg/mL	HCD56
	CD66b	FITC	Biolegend	8 µg/mL	G10F5
Subsets	CD16	APC-Cy7	Biolegend	4 µg/mL	3G8
	CD14	Brilliant violet 650	Biolegend	2 µg/mL	M5E2
Activation	CD86	Brilliant Violet 510	Biolegend	4.8 µg/mL	IT2.2
	CD163	PE-Cy7	Biolegend	4 µg/mL	GH1/61
	CD206	APC	Biolegend	8 µg/mL	15-2
	HLA-DR	Pacific Blue	Biolegend	3 µg/mL	L243

3.2.2 FLOW CYTOMETRY GATING STRATEGY

The gating strategy used in the phenotypic characterization of peripheral blood leukocytes was based on the gating strategy used from Mukherjee, R. et al. with minor modifications (Mukherjee et al., 2015). Based on the forward (FSC) and side scatter (SSC), we selected the lymphocytes followed by the selection of single cells based on the FSC-area and FSC-height. Then we excluded neutrophils and NK cells using SSC versus CD66b and CD56, respectively. Then, excluded B and T lymphocytes, using CD3 and CD19. Using HLA-DR against SSC, we selected the HLA-DR positive cells. Finally, using CD14 against CD16 we selected the monocytes. The three monocyte subsets were gated based on their differential expression of CD14 and CD16: $CD14^{++}$/CD16- for classical monocytes, $CD14^{++}/CD16^{+}$ for intermediate monocytes and $CD14^{+}$/CD16- for non-classical monocytes (Appendix 1).

3.3 FUNCTIONAL ANALYSIS OF MONOCYTES AND MONOCYTE-DERIVED MACROPHAGES

3.3.1 PBMC ISOLATION USING HISTOPAQUE

Peripheral blood mononuclear cells (PBMC) consist on the leukocytes that present a round nucleus, including lymphocytes and monocytes. To isolate PBMC, human peripheral blood was thoroughly placed on top of an equal volume of histopaque 1077 (MilliporeSigma, St Louis, Missouri, USA) so that both solution would not mix. The solutions were then centrifuged at 400g for 30 minutes, RT with minimum acceleration and without brake. After centrifugation, three layers are easily visible, with erythrocytes on bottom, followed by histopaque 1077, plasma on top and between the last 2, a ring of PBMC. The plasma was stored at -80°C for further analysis and the ring of PBMC was carefully removed to clean falcons. These cells were washed with sterile PBS and centrifuged at 300g for 10 min at RT. Total number of cells and viability were determined by counting upon trypan blue 0.04% staining.

3.3.2 MONOCYTE ISOLATION USING CD14$^+$ MAGNETIC BEADS

Monocytes were isolated from the PBMC's suspensions by immunomagnetic separation with the human anti-CD14 purification kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, after PBMC counting, the cells were left to incubate for 30 minutes at 4°C with the CD14+ beads in a ratio of 2.5µL of CD14$^+$ beads and 80 µL miltenyi buffer (0.5% Bovine serum albumin, 2 mM EDTA in PBS) per 1.0x10^7 PBMCS, mixing the tube twice during this period. After a washing step with miltenyi buffer, the cells were centrifuged and resuspended in 500 µL of miltenyi buffer. Magnetic columns were placed on the manual magnetic activated cell sorter (Octo MACS) and equilibrated with miltenyi buffer. The PBMC suspensions were then placed on the columns followed by three washes with 500µL of miltenyi buffer. The CD14$^+$ cells trapped in the magnetic columns where then recovered using 1 mL of miltenyi buffer and counted with trypan blue

0.04%. The purity of the separation was randomly confirmed by flow cytometry and was superior to 95%.

3.3.3 MONOCYTE DIFFERENTIATION

Purified monocytes were differentiated *in vitro* into monocyte-derived macrophages by plating in 96-flat well plates at a concentration of $5x10^6$ cells/mL in Roswell Park Memorial Institute (RPMI) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) medium supplemented with 10% FBS, PenStrep, (100U/mL of penicillin and 100 µg/mL of streptomycin) and 0.01M hydroxyethyl piperazineethanesulfonic acid with macrophage- colony stimulating factor (M-CSF) (20 ng/mL) (Peprotech, Fulham, London, UK) at 37°C and 5% CO_2. The medium was replaced for new medium with M-CSF at day four.

3.3.4 CELLULAR SUPEROXIDE AND MITOCHONDRIAL MASS

The levels of superoxide and mitochondrial mass were measured both in monocyte-derived macrophages and peripheral blood leukocytes. FACS buffer was added to the cells and these were scraped. After the cells were detached, we proceeded to the staining using dihydroethidium (DHE) (45µM) for 10 minutes at 37°C and acridine orange 10-nonyl bromide (NAO) (1.25 µM) for 30 minutes at 37°C (Sigma, St. Louis, Missouri, EUA). For peripheral blood, the cells were first stained for extracellular markers as described above and then with DHE in the same conditions as in monocyte-derived macrophages.

3.3.5 PHAGOCYTOSIS

Monocyte-derived macrophages were incubated with fluorescent latex beads (Sigma, St. Louis, Missouri, EUA) in a ratio of 10 beads:1 macrophage for 6 hours. After the incubation time, the medium was discarded and cells were recovered in FACS buffer and analyzed by flow cytometry.

3.4 FLOW CYTOMETRY DATA ANALYSIS

All samples were acquired on a BD LSR II flow cytometer using FACS DIVA software and the data was analyzed using FlowJo Software (Tree Star, OR, USA).

3.5 STATISTICAL ANALYSIS

All samples were tested for normality using the Shapiro-Wilk normality test. According to the results of the test, if the samples were normal, an unpaired t-test with Welch corrections was made. When samples did not have a normal distribution, a Mann-Whitney U test was used. Significance was considered in results were the *p*-value had values below 0.05.

4. RESULTS

4.1. SOCIODEMOGRAPHIC AND CLINICAL CHARACTERIZATION OF THE COHORT

The cohort contains 50 diagnosed BD patients from Minho region of Portugal. Regarding the sociodemographic characterization, the population present a mean age of 39.3 years old (SD=10.2), with 62.0% being females (n=31) and 38.0% males (n=19). The male/female ratio within the whole cohort was 0.61 (Table 3).

Table 3: **Sociodemographic characterization of the cohort**.

Males	38% (19)
Females	62% (31)
Male/Female ratio	0.61
Age, mean ± standard deviation (years)	39.3 (± 10.2)
Age range (years)	19-67

This cohort was also characterized on basis of median age of first symptoms and diagnosis. This characterization shows a median age of 24 years old for the appearance of the first symptoms (24 ± 18.5 years) with a mean age of diagnosis of 34.4 years old (34.4 ± 10.9). The diagnosis of these patients was performed based on the ISGB criteria, with 66% (n=33) showing a definitive diagnosis. Furthermore, 24% (12) of the cohort show family history of recurrent oral ulceration and 18% (9) family history of other autoimmune disorders (table 4).

Table 4: **Clinical characterization of the cohort**

Age at first symptoms, median ± IQR[1] (years)	24 ± 18.5
Age at diagnosis, mean ± SD (years)	34.4 ± 10.9
Definitive diagnosis, % (n)	66% (33)
Probable diagnosis, % (n)	44% (17)
Family history of recurrent oral ulceration, % (n)	24% (12)
Family history of other autoimmune disorders, % (n)	18% (9)

At the beginning of the disease course, 92.0% of the patients presented oral ulcers (n= 46), 10.0% presented genital ulcers (n=5), 10.0% cutaneous lesions (n=5) and 6.0% joints complications (n=3) (Table 5). As the disease evolves, the symptoms' profile is different from patient to patient, however, all patients have documented oral ulcers. The cutaneous lesions, genital ulcers and joints complications were also reported very often. Some of the patients had also more severe complications such as uveitis and vascular, gastrointestinal and nervous system complications (Table 6).

Table 5: **The first symptoms presented by Behçet's disease patients**

Genital ulcers, % (n)	10% (5)
Cutaneous manifestations, % (n)	10% (5)
Joint manifestations, % (n)	6% (3)

Table 6: **Symptoms described along the disease course**

Gastrointestinal involvement, % (n)	4% (2)
Central nervous system involvement, % (n)	8% (4)
Vascular manifestations, % (n)	14% (7)
Erythema nodosum, % (n)	24% (12)
Acneiform/papule pustule lesions, % (n)	26% (13)
Eye involvement, % (n)	36% (18)
Joint manifestations, % (n)	56% (28)
Genital ulcers, % (n)	62% (31)
Pseudo folliculitis, % (n)	70% (35)
Oral ulcers, % (n)	100% (50)

4.2. PHENOTYPIC CHARACTERIZATION OF PERIPHERAL BLOOD LEUKOCYTES

4.2.1. SIMILAR LEUKOCYTES' RELATIVE PERCENTAGES BETWEEN HEALTHY CONTROLS AND BEHÇET'S DISEASE PATIENTS

Leukocytes are continuously renewed and are dependent on the regulation of hematopoiesis, the process by which hematopoietic stem cells are differentiated into several types of cells. The regulation of the normal numbers of each leukocyte however, needs to be balanced since it must respond to infections or other type of damage-inducers (Thomas, Vadas, & Lopez, 2004). Being one of the most studied cellular population, neutrophils have been reported to be increased in the peripheral blood of active BD. Furthermore, the changes in neutrophil percentages (Neutrophil to lymphocyte ratio) has also been used as an indicator of inflammation in other conditions, including diabetes mellitus, and other autoinflammatory disorders (Rifaioglu, Bülbül Şen, Ekiz, & Cigdem Dogramaci, 2014). In our study, we also evaluated each cell type to find out if there are any alterations in the relative percentages of leukocytes, including monocytes. The relative percentage of leukocytes in the peripheral blood was assessed by flow cytometry, using the gating strategy described before (Material and Methods). Within the total leukocytes gated, none of the cellular populations showed a statistical difference between patients and controls (Table 7). Despite the fact that none of the studied populations showed statistical difference, there was an increased percentage of neutrophils in BD patients which goes in accordance with the increase in neutrophils in active BD already described (Rifaioglu et al., 2014).

Table 7: **Leukocyte frequency on peripheral blood.**

Cellular population	***% in Controls (16)***	***% in Behçet patients (17)***	***Statistic data***
Neutrophils	38.60 + 9.30	45.00+10.80	$p = 0.078$
T cells	33.40 + 8.45	30.90+5.95	$p = 0.34$
NK cells*	6.92+4.09*	6.48+2.96*	$p = 0.36$*
Monocytes	4.66+1.82	3.79+1.70	$p = 0.17$

* The sample do not follow a normal distribution (according to Shapiro-Wilk test), and an unpaired Mann-Whitney test was used in this case. For the other samples, an unpaired t-test with Welch corrections was used.

4.2.2. THE MICROENVIRONMENT IN BEHÇET'S DISEASE PERIPHERAL BLOOD DOES NOT IMPACT THE NON-CLASSICAL AND INTERMEDIATE MONOCYTES' PERCENTAGES WITHIN MONOCYTES

As already mentioned, monocytes are a very heterogeneous population that responds to the surrounded microenvironment. Furthermore, monocyte subset alterations have been pointed out in many conditions, including inflammatory diseases such as lupus and sepsis (Mukherjee et al., 2015). Being BD a systemic inflammatory condition, we decided to assess the percentage of the different monocyte subtypes. For that, blood from healthy controls and BD was analyzed by flow cytometry. To increase the purity of the monocyte population, a gating strategy described by Mukherjee et al. was applied (Appendix 1). As observed for the relative percentages of the different leukocytes, regarding the three subsets, we did not find statistical differences between the percentage of each population in BD patients in comparison to the controls (p=0.5861 for classical monocytes, p=0.4389 for non-classical monocytes and p=0.3777 for intermediate monocytes) (Figure 2). Monocyte subsets have been suggested to be implicated in different conditions, with changes on different subsets. In fact, non-classical monocytes have been implicated in conditions such as cardiac surgery or lupus (Gawdat et al., 2017; Mukherjee et al., 2015) Even though, non-classical monocytes from BD patients, another inflammatory condition, show no difference in its percentage in comparison to healthy individuals.

4.2.3 MONOCYTES FROM BEHÇET'S DISEASE PATIENTS HAVE HIGHER EXPRESSION OF CD86 AND CD206

Besides the classification of the monocyte population based on the expression of surface markers, we also used commonly used M1 (CD86 and HLA-DR) and M2 (CD206 and CD163) activation markers. Monocytes and macrophages have been described to be very heterogeneous and acquire different phenotypes according to the stimuli they are exposed to. Although it is known that these cells actually present a broader spectrum of phenotypes, M1 and M2 have been pointed as the two opposite phenotypes easily inducible *in vitro* (Martinez, Gordon, Locati, & Mantovani, 2006). Regarding the expression of this markers on monocytes, CD86 was found to be overexpressed on intermediate monocytes from BD patients in comparison to healthy controls (p=0.0084) (Figure 3.A). CD206 is also overexpressed, however only on non-classical monocytes from BD patients (p=0.0135) (Figure 3.B). All the other subsets of monocytes show no difference on the expression of the surface markers CD86, HLA-DR, CD163 and CD206 (Table 8).

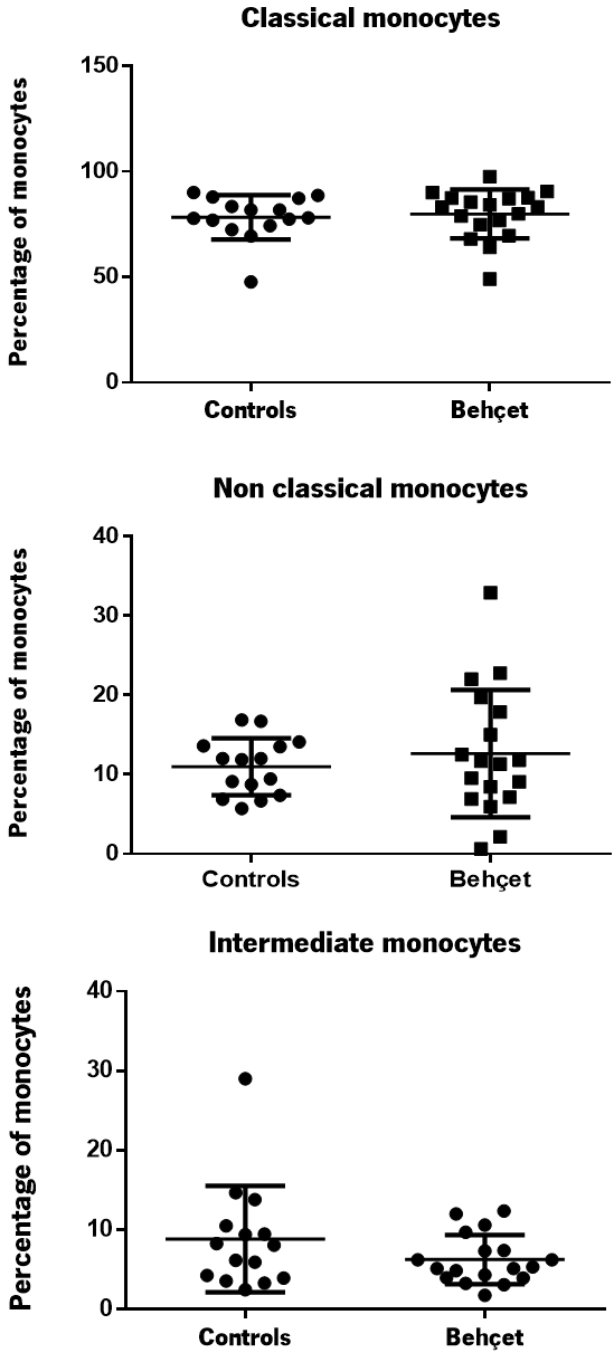

Figure 2: **The percentage of each monocyte subset remains unchanged in Behçet's disease**. The gating of monocytes was performed by an exclusion method of other cells containing CD16 expression. The Neutrophils were gated out through the expression of CD66b, followed by NK cells due to their expression of CD56. Both T and B lymphocytes were also excluded based on their expression of CD3 and CD19, respectively. The monocytes were then selected by gating the HLA-DR positive cells, followed by the removal of the $CD14^-$ and $CD16^-$negative population. Also, based on the expression of CD14 and CD16, after monocyte selection, monocytes were subdivided on the three subsets, $CD14^{++}/CD16^-$ (Classical monocytes), $CD14^{++}/Cd16^+$ (intermediate monocytes) and $CD14^+/CD16^{++}$ (non-classical monocytes). The different monocyte subsets show no statistical differences between the healthy controls and the Behçet's disease patients ($p>0.05$, using an unpaired t-test with Welch corrections or unpaired Mann-Whitney test for non-parametric distribution)

Table 8: **M1 and M2 markers' expression on monocyte subsets**. The expression of CD86 and HLA-DR (M1 markers) and CD163 and CD206 (M2 markers) was assessed by flow cytometry. The results are shown in mean ± standard deviation for samples with normal distribution or median ± interquartile range for samples without normal distribution. t: t-student test, U: Mann-Whitney test, p: *p* value.

	Controls	***Behçet patients***	***Statistic data***	
CD86				
Classical monocytes	849.9 ± 51.66	907.3 ± 41.88	$t = 0.8633$	$p = 0.3952$
Intermediate monocytes	1627 ± 676	1920 ± 333	$U = 63$	$p = 0.0084$
Non-classical monocytes	1094 ± 44.16	1194 ± 45.20	$t = 1.576$	$p = 0.1253$
HLA-DR				
Classical monocytes	2458 ± 201.1	2174 ± 172.9	$t = 1.072$	$p = 0.2923$
Intermediate monocytes	3188 ± 217	3220 ± 249.3	$t = 0.09548$	$p = 0.9245$
Non-classical monocytes	5530 ± 485	6179 ± 444	$t = 0.9873$	$p = 0.3314$
CD163				
Classical monocytes	6396 ± 4278	5313 ± 5096	$U = 133.5$	$p = 0.9648$
Intermediate monocytes	4088 ± 3471	4087 ± 3608	$U = 133$	$p = 0.9455$
Non-classical monocytes	1514 ± 69.65	1761 ± 96.50	$t = 1.858$	$p = 0.0739$
CD206				
Classical monocytes	1837 ± 100.7	1646 ± 96.50	$t = 1.372$	$p = 0.0084$
Intermediate monocytes	2437 ± 125.7	2664 ± 187.7	$t = 1.002$	$p = 0.3247$
Non-classical monocytes	1348 ± 271	1575 ± 242	$U = 67.5$	$p = 0.0135$

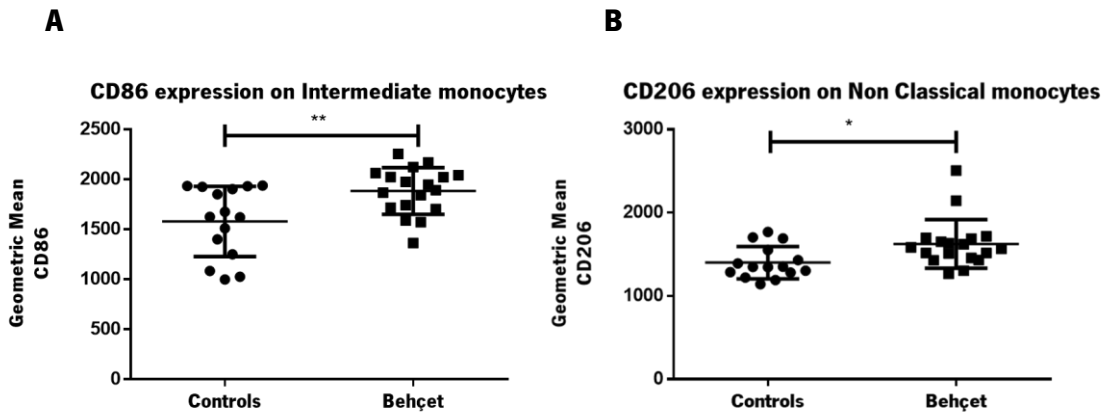

Figure 3: **The intermediate and non-classical monocytes show different expression levels of the surface markers CD86 and CD206**. The monocyte subsets gated as described above were also assessed to see the levels of CD86 and HLA-DR, as M1 markers, and CD163 and CD206, as M2 markers. The geometric mean of fluorescence intensity was determined in these subsets and found to be increased in the intermediate monocytes from Behçet's disease in the case of CD8 (A) and in the non-classical monocytes from Behçet's disease regarding CD206 (B). * $p<0.05$, ** $p<0.01$ (Unpaired Mann-Whitney test)

4.3. FUNCTIONAL CHARACTERIZATION OF PERIPHERAL BLOOD LEUKOCYTES

4.3.4 DIFFERENT LEVELS OF SUPEROXIDE ANION AND MITOCHONDRIAL MASS ARE FOUND ON MONOCYTES

Superoxide plays several modulating effects on inflammation as well as in the regulation of immune responses, being produced as a killing mechanism upon phagocytosis (Guzik, Korbut, & Adamek-Guzik, 2003). Due to its role in cell physiology, as well as the immune response, the study of superoxide in disease can be used to better understand the physiology of cells. Apart from this, superoxide production was already described to be increased on neutrophils but has not been described in monocytes. Taking this into account, we also checked for the levels of superoxide anion on *ex vivo* peripheral blood cells. To evaluate the levels of superoxide, we used DHE, which freely enters the cells where it can react with free superoxide radicals, making it widely used to measure total superoxide levels. We observed that the geometric mean intensity fluorescence of DHE was higher in BD monocytes in comparison to those from healthy controls (p=0.0206) (Figure 4.A). We also found differences in neutrophils (p=0.0111) (Figure 4.B). Regarding lymphocytes, no statistical differences were observed. These results are in line with the existent literature on oxidative state in Behçet's disease. As mentioned before, it has been described that the BD patients present lower levels of antioxidants as well as an increased oxidative state. These reports support the fact that we find increased levels of superoxide anion.

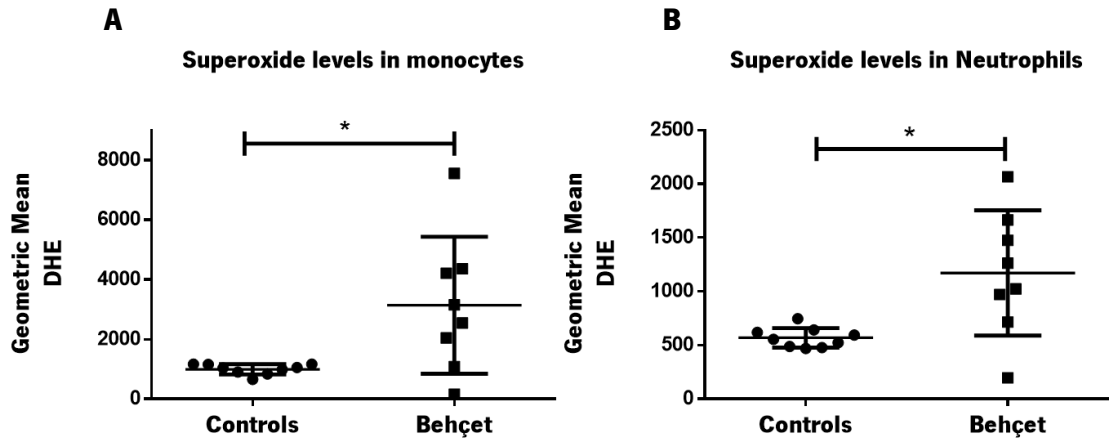

Figure 4: **Superoxide anion levels are increased in Behçet's disease**. The superoxide anion levels were measured in peripheral blood cells, using DHE by flow cytometry. The cells were gated based on their size and complexity, and the geometric mean fluorescence intensity was analyzed for lymphocytes, monocytes and neutrophils. The levels of superoxide anion were found to be increased in both monocytes (A) and neutrophils (B). *p< 0.05 (unpaired t-test with Welch corrections)

Mitochondria have several roles in cellular physiology and recently, some roles in immunity have also been suggested. In fact, mitochondrial outer membrane is a platform for signaling molecules, including the activation of the NLRP3 inflammasome, an assembly of proteins that activates caspase-1 leading to the release of IL-1β, playing a central role in the inflammatory response and in diverse human diseases. Furthermore, mitochondrial biogenesis, fusion and fission have been associated with aspects of immune-cell activation. Most important, Krebs cycle intermediates such as succinate, fumarate and citrate participate in several processes in immunity and inflammation, in both innate and adaptive immune cells (Mills, Kelly, & O'Neill, 2017). Additionally, it has been reported that there are metabolic changes in patients with sepsis, another inflammatory condition (Cheng et al., 2016). Bearing in mind all the roles that mitochondria play, we decided to measure the levels of the mitochondrial mass. Using flow cytometry, we assessed the mitochondrial mass, using NAO. NAO is a probe that binds to cardiolipin, a phospholipid that is mainly present in the inner mitochondrial membrane. We observed that the monocytes from BD patients present lower geometric mean intensity fluorescence for mitochondrial mass in comparison to healthy controls (p=0.0032) (Figure 5.A). Furthermore, B cells also present the same phenotype (p=0.0120) (Figure 5.B). Regarding the lymphocytes, there is no statistical difference between patients and healthy controls (p=0.0830). The reduction of mitochondrial mass, although not directly related, is also observed in other situations, including ageing. As individuals grow older, a decrease in function, distribution as well as in its dynamics is observed (Chistiakov, Sobenin, Revin, Orekhov, & Bobryshev, 2014).

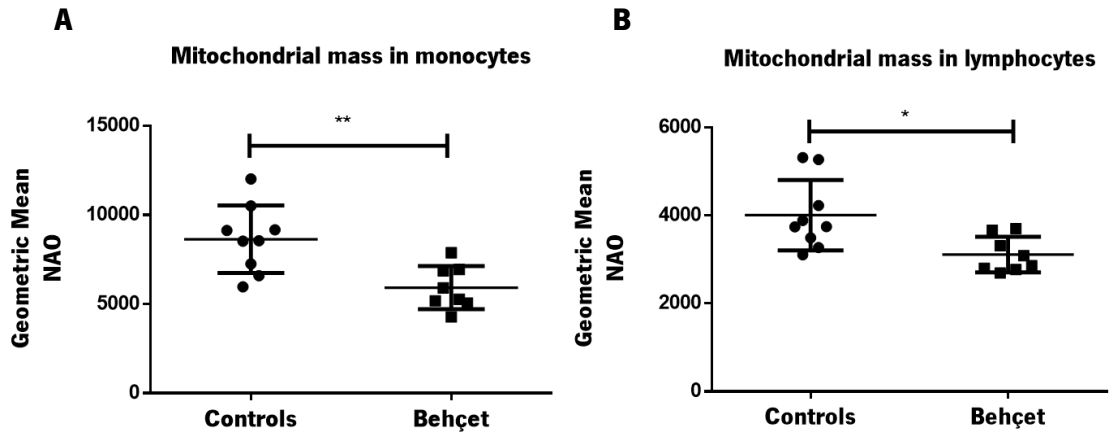

Figure 5: **Monocytes and lymphocytes show decreased mitochondrial mass**. The mitochondrial mass of leukocytes was assed using NAO by flow cytometry. The results show a decrease in the mitochondrial mass of monocytes (A) and lymphocytes (B) from BD patients. * p<0.05, ** p<0.01 (unpaired t-test with Welch corrections)

Besides to the analysis of mitochondrial mass in monocytes, which as shown a decrease in comparison to the healthy controls, we also analyzed the same parameter within the three subsets of monocytes, the classical, intermediate and non-classical monocytes, to see if the decrease of the mitochondrial mass is due to subsets in specific. Interestingly, we found that the decrease is observed in all subsets of monocytes (p=0.0019 for classical monocytes, p=0.0007 for intermediate monocytes and p=0.0025 for non-classical monocytes). Since no alterations were observed in the percentages of each monocyte subset, the reduction of the mitochondrial mass among all the subsets is not surprising.

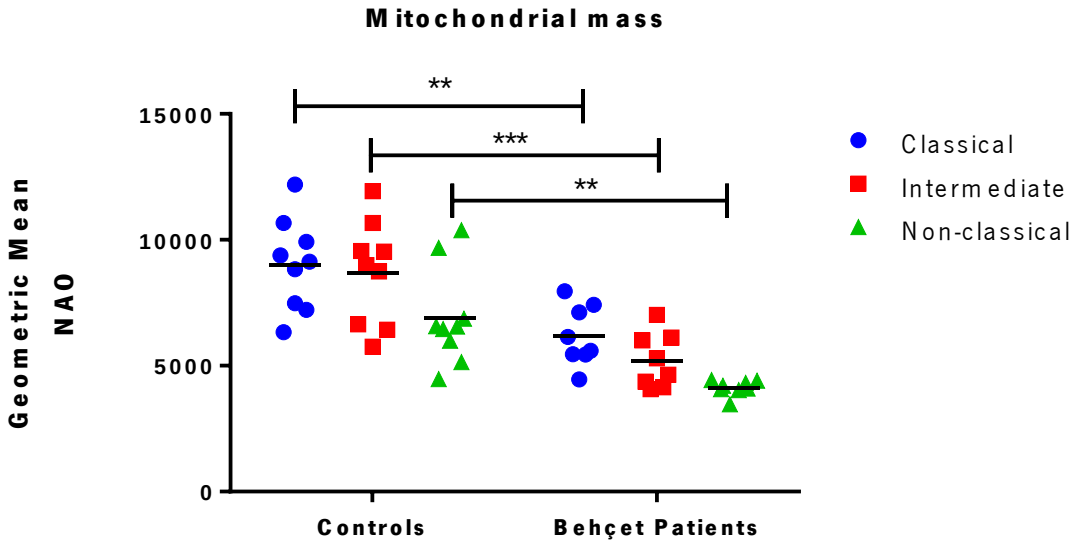

Figure 6: **Mitochondrial mass decreases in all monocyte subsets.** Monocyte subsets were gated based on the CD14 and CD16 expression. The mitochondrial mass was assessed by flow cytometry using NAO. The decrease in the mitochondrial mass of this individuals is not observed in a specific subset of monocytes, but instead is the result of the decrease of the mitochondrial mass in all subsets. **p<0.01, ***p<0.001 (Unpaired t-test with Welch corrections)

4.3.5 BD MONOCYTES' PHENOTYPE IS REVERTED AFTER DIFFERENTIATION INTO MACROPHAGES

Upon recruitment to tissues, usually due to injury or infection, monocytes cross the blood vessels where they further differentiate into either dendritic cells or monocyte-derived macrophages (Geissmann et al., 2010). Macrophages are found in all body tissues, both the so called resident macrophages, that are tissue-specific and have self-renewal properties, and the monocyte-derived macrophages. Besides several healing properties already described, macrophages also serve as sentinels in wait for pathogens (Hume, 2006). Once macrophages can derive from monocytes, and since monocytes from BD present changes in their normal

physiology, in comparison to the controls, we decided to evaluate if the peripheral blood's environment effects observed on monocytes are maintained after differentiation. For that, we differentiated these cells into monocyte-derived macrophages and after differentiation, we assessed the mitochondrial mass, total superoxide anion levels, as well as their phagocytic activity. In relation to the levels of superoxide anion, which has its accumulation increased in monocytes (Figure 4.A), we found no difference between healthy controls and patients when analyzing monocyte-derived macrophages. (p=0.3682).

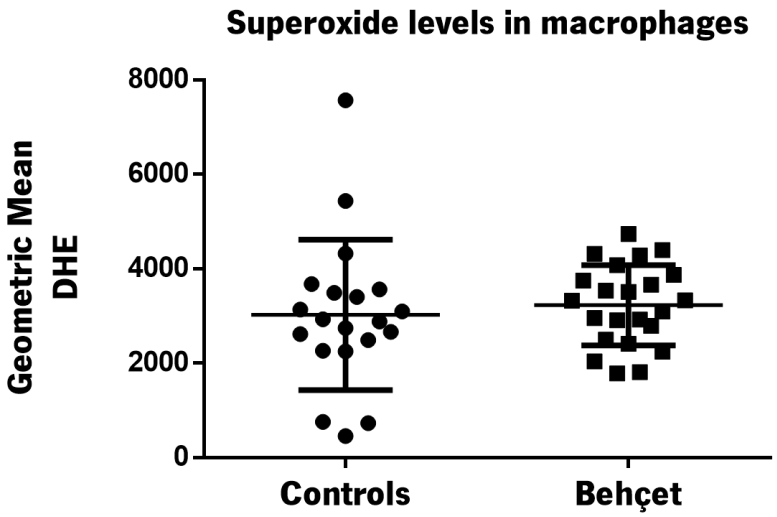

Figure 7: **Monocyte-derived macrophages do not have higher levels of superoxide.** The levels of superoxide were assessed by flow cytometry, using DHE. Monocytes were differentiated into monocytes using M-CSF for 7 days. Monocyte-derived macrophages from BD patients and healthy controls show similar levels of superoxide, instead of an increased accumulation of this ROS, as seen in monocytes. (Unpaired t-test with Welch corrections).

Besides the levels of superoxide anion, analyzing the mitochondrial mass, we did not find statistical differences between controls and BD patients (p=0.6900). Although monocytes present increased levels of superoxide anion and less mitochondrial mass, after differentiation of the monocytes into macrophages, there is no difference in the biology of these cells, looking at the same parameters as in monocytes.

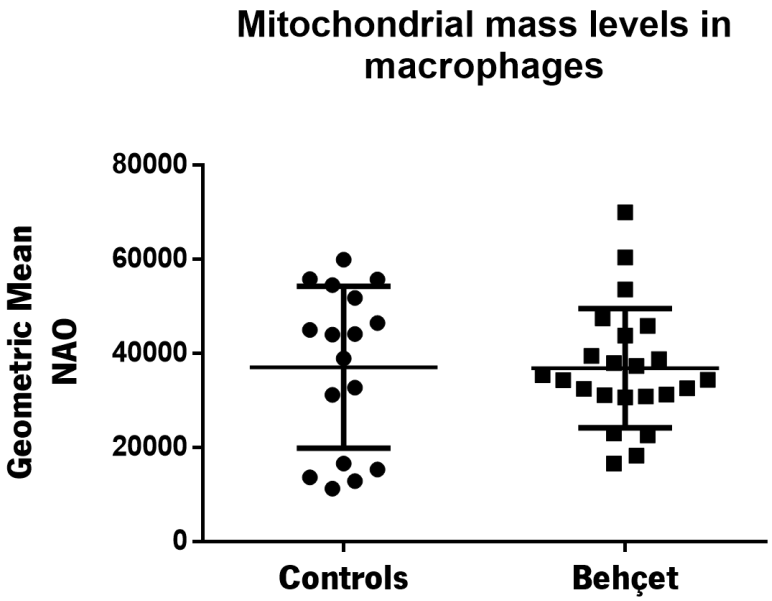

Figure 8: **Monocyte-derived macrophages show no alterations on mitochondrial mass.** Monocytes were differentiated into macrophages using M-CSF for 7 days. The mitochondrial mass was assessed by flow cytometry, using NAO. As in superoxide levels, the mitochondrial mass of BD patients is also no different from that of the healthy controls. (Unpaired t-test with Welch corrections).

Still regarding the effector functions of macrophages, the phagocytic capacity of macrophages was assessed using fluorescent latex beads and measuring the percentage of cells with phagocytized beads. Upon recognition of pathogens by macrophages, these cells bind the pathogens via phagocytic receptors that lead to the process of its internalization, called phagocytosis. Although phagocytosis is associated the removal of senescent cells and tissue remodeling, it is also the first step in the initiation of host responses and inflammation, with macrophages as the main cells of the immune system associated with phagocytosis (Hume, 2006). With this, the ability of macrophages to phagocytose is essential in the clearance of pathogens and the initiation of an efficient immune response. Furthermore, although we found similar levels of superoxide anion and mitochondrial mass in healthy controls and BD patients, the normal functioning of these cells could be impaired. Even though, and regarding the phagocytosis in macrophages, we found no statistical differences between the groups (p=0.3408).

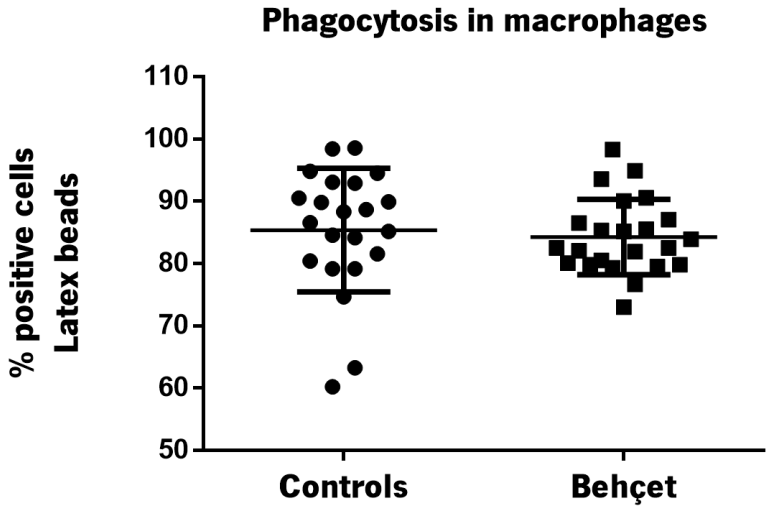

Figure 9: **Monocyte-derived macrophages do not have alterations on their phagocytic ability**. Monocytes were differentiated into macrophages using M-CSF for 7 days. Phagocytosis was assessed by flow cytometry using fluorescent latex beads. Regarding this parameter, macrophages show neither an increase or decrease phagocytosis. (Unpaired t-test with Welch corrections)

5. DISCUSSION

The study of monocyte biology on the context of BD has not been well explored. Our study attempts to elucidate the changes that occur on the monocyte population when this cell type is under the influence of a chronic inflammatory environment. Since several studies have described perturbations on this population regarding other inflammatory conditions, our study will open doors to better understand the pathophysiology of this disease that remains unclear. To do so, we have recruited patients from a cohort in Hospital de Braga. A sociodemographic and clinic characterization of this population was done and we found a ratio of male-to-female of 0.61 to 1. Although several studies have established a prevalence per gender in BD, accordance is not observed since some authors say that they found a prevalence of females, others say the opposite while some even say that there is no difference between gender (Davatchi et al., 2011; Zouboulis, 1999). Regarding BD in Portugal, a study performed in Porto, 2010, showed a higher prevalence in females (Costa et al., 2010), which is in accordance with our results. The fact that we cannot find agreement regarding this issue, suggests that the prevalence among gender may vary with the location of the population, among many other factors that may be influencing the results. Furthermore, we also see an average age of diagnosis of 34.4 years old, which is within the common range reported for BD (30-40 years old) (Costa et al., 2010). Regarding the symptoms observed, the pattern is the same as the one described for BD, with the appearance of oral ulceration as the principal first symptom, and the development of the disease for worst outcomes, such as the involvement of gastrointestinal and central nervous system, usually called gastro- and neuro-Behçet, respectively (Kokturk, 2012).

As we assess the analysis of peripheral blood we see a different pattern from that published by others (Rifaioglu et al., 2014) as we did not see any difference in cellular relative percentages within leukocytes. It has been described that neutrophils are increased in BD (Rifaioglu et al., 2014). With this, we expected an increase in neutrophils' percentage. Since neutrophil increase has been associated to inflammation and not BD specifically, it suggests that the increase in the neutrophils may be due to the inflammatory environment associated with BD. Since we do not know yet the levels of many inflammatory mediators in the blood at the moment of blood collection, it is expectable that some patients may have low-grade inflammation, and that neutrophil increase may associate only with active states of the disease. As mentioned before, the monocyte population can be further divided, according to the expression of CD14 and CD16, into non-classical, intermediate and classical monocytes (Wong et al., 2012). These three populations present different functions, with non-classical monocytes displaying 'inflammatory'

characteristics while the classical monocytes are phagocytic with no inflammatory attributes (Mukherjee et al., 2015). Following this, we expected, as observed for both sepsis and lupus, the population of non-classical monocytes to be increased due to the inflammatory state that characterizes BD (Mukherjee et al., 2015). However, looking at the inflammation parameters such as C-reactive protein levels and sedimentation rates measured in peripheral blood from BD and lupus patients, we saw that these parameters in inactive BD are as increased as in lupus. This fact may indicate that either the transition to or the recruitment of non-classical monocytes may depend on the severity of the inflammatory environment, which is observed in lupus (Gheita, El-Gazzar, Azkalany, El-Fishawy, & El-Faramawy, 2012; Müftüoğlu et al., 1986). Although no differences were found between the different monocyte subpopulations, we did find an overexpression of CD86 in the intermediate monocytes and CD206 in non-classical monocytes. The terms M1 and M2 have been used to describe the two main phenotypes observed in *in vitro* macrophages. M1 macrophages are associated with the pro-inflammatory state of these cells, while M2 with the anti-inflammatory. Although it is widely used, it is known that this classification is not accurate and there is a broader spectrum of phenotypes (Martinez & Gordon, 2014). Even though, M1 or M2 markers can be used to assess whether cells have acquired a more pro-inflammatory or anti-inflammatory phenotype. In our study, we used the M1 markers CD86 and HLA-DR and the M2 markers CD163 and CD206. CD206 is a mannose receptor expressed on the surface of many immune cells, mainly macrophages. Its function is associated with the recognition of terminal mannose, N-acetylglucosamine and fucose residues on glycans attached to proteins. These residues are found on many microorganisms, which makes CD206 an important player on innate immunity (Ezekowitz, Sastry, Bailly, & Warner, 1990). Since Behçet is an autoinflammatory associated with an exacerbated response to some microorganisms, the overexpression of this receptor may be associated with the increased response to some bacteria, as described already. CD86 is a surface protein expressed on antigen-presenting cells associated with the costimulatory signals necessary for T cell activation. Since intermediate monocytes have been associated with antigen presentation functions, an overexpression of CD86 may be due to its functions during T cell activation and antigen presentation.

Furthermore, the analysis of M1 and M2 markers did not give clear results regarding the phenotype of monocytes, which is in line with the fact that there is a broader spectrum of phenotypes, being the monocytes analyzed between the M1/M2 phenotypes. However, regarding the intermediate monocytes, that present an overexpression of CD86, it seems that these cells

present a phenotype closer to M1 than to M2. Being M1, a pro-inflammatory phenotype, our results suggest that intermediate monocytes in BD acquire a more pro-inflammatory phenotype in comparison to healthy controls. Additionally, we observe an overexpression of CD206 on non-classical monocytes. Interestingly, non-classical monocytes have been associated with a proinflammatory phenotype, with high production of proinflammatory cytokines, while intermediate monocytes are associated with antigen presentation (Wong et al., 2011). One would expect that, non-classical monocytes, being more inflammatory, would present M1 markers, and intermediate, that are more associated with antigen presentation, M2 markers. As it was mentioned above, the markers used have physiological roles. With this, the overexpression of these markers may be associated with their functions instead of their phenotype, as explained before.

Neutrophils, from BD patients, have already been described to have higher production of ROS, including superoxide anion (Takeno et al., 1995). Monocytes on the other hand have not yet been associated with higher levels of superoxide. Our results show an accumulation of superoxide in monocytes and neutrophils as well as a decrease on the mitochondrial mass on monocytes and lymphocytes. This increase in the levels of superoxide anion can either be due to its overproduction or the decrease of its neutralization by antioxidants and antioxidant enzymes. This accumulation of superoxide, along with the decrease of the mitochondrial mass suggests that these patients show mitochondrial dysfunctions, which helps explain the accumulation of the superoxide. In fact, Richter C. et al. have described that high levels of ROS leads to mitochondrial damage, mainly by DNA damage (Richter, Park, & Ames, 1988). Furthermore, Batandier, C. et al. as shown that upon mitochondrial damage, where is observed a permeability transition pore opening, there is an increase in the accumulation of reactive oxygen species (Batandier, Leverve, & Fontaine, 2004). The origin of the superoxide increase is still unknown; however, superoxide is known to be increased during the oxidative burst in leukocytes, leading to pathogen killing and causing endothelial damage that increases vascular permeability. Besides its implication in the pathogen killing, superoxide anion produced by NAD(P)H oxidases plays a key role modulating the release of other inflammatory mediators, including cytokines. In turn, inflammatory cytokines can increase the activity of NAD(P)H oxidases, contributing to more production of superoxide (Buetler, Krauskopf, & Ruegg, 2004). Besides the study of these functions in monocytes, monocyte-derived macrophages were also assessed. Our results have shown that, upon differentiation of monocytes in macrophages using M-CSF, these cells lose the phenotype

observed for *ex vivo* monocytes. Since monocytes present an increase of superoxide accumulation as well as a decrease in the mitochondrial mass in a basal state, we also expected the monocyte-derived macrophages to present the same characteristics. The peripheral blood of BD patients has been shown to present an inflammatory environment, mainly associated with an overproduction of proinflammatory cytokines. Furthermore, some studies regarding neutrophils have shown that *in vitro,* these cells show increased chemotaxis and adhesion only when stimulated with BD plasma, suggesting that their activation relies on soluble factors present on the bloodstream (Mege et al., 1993; Takeno et al., 1995). In the case of macrophages, since these cells undergo a process of differentiation of 7 days, without the contribution of BD-associated plasma, the effects observed on monocytes that were under the effect of the soluble factors, are lost. Also, these macrophages are stimulated with M-CSF and Martinez, FO. et al. have shown that differentiation of monocytes using this factor, drives monocyte-derived macrophages to a M2-like phenotype (Martinez & Gordon, 2014). Being macrophages a very heterogeneous population that responds very well to stimuli and acquire different phenotypes, the differentiation of these cells without inflammatory environment, as well as with an anti-inflammatory factor may explain the loss of the phenotype observed in BD monocytes.

Altogether, our results show that monocytes from BD patients present an altered phenotype, with evidences of mitochondrial dysfunction. Although no changes were found on the monocyte subsets, the whole population seems to be affected. As the pathophysiology of this disease is yet unclear, our results have contributed to a better understanding of a cellular population that is yet poorly characterized on the contexts of BD.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work developed during this thesis has resulted in a better understanding of how monocytes are being influenced by the inflammatory environment associated to Behçet's disease. Although we were unable to see differences in the percentages of the monocyte subsets, herein we describe significant alterations at a cellular level. We have shown that monocytes from BD patients show an accumulation of superoxide anion as well as a decrease in the mitochondrial mass. Although more studies shall be done regarding monocyte physiology to understand how the mitochondria are being affected, we suggest that these cells show mitochondrial dysfunction. Furthermore, considering the process of differentiation of monocytes into macrophages, we also show that there is a loss of the phenotype observed in monocytes. With this we also suggest that the monocytic changes in BD are dependent on the inflammatory environment from the peripheral blood of these patients.

Although we present associations with the inflammatory environment from Behçet's disease and the changes in monocyte biology, we still lack information regarding the inflammatory mediators that are present in the blood of Behçet's disease patients. For that, we will continue processing blood from these patients until we have studied the full cohort. Having all the individuals analyzed regarding the data presented in this document, a metabolome study of the plasma will be performed which will help characterized each patient based on the inflammatory mediators. Furthermore, since BD is a condition characterized by flare ups, the inflammatory environment presents high variations. It would help to the analysis to divide the patients based on the active stages of the disease, ongoing treatments, as well as the symptoms associated. With this information, it will be possible to correlate the inflammatory mediators with the functional status of the cells, helping to unravel the effects of the inflammatory environment on monocytes from Behçet's patients. Also, more studies, including fluorescence assays, regarding the mitochondria of these individuals will be performed to better understand the state of these organelles. With all the results, and with the data from the metabolomic studies, it will be possible to assess which metabolites and cytokines are associated with the induction of the phenotype observed, that might be used for clinical applications.

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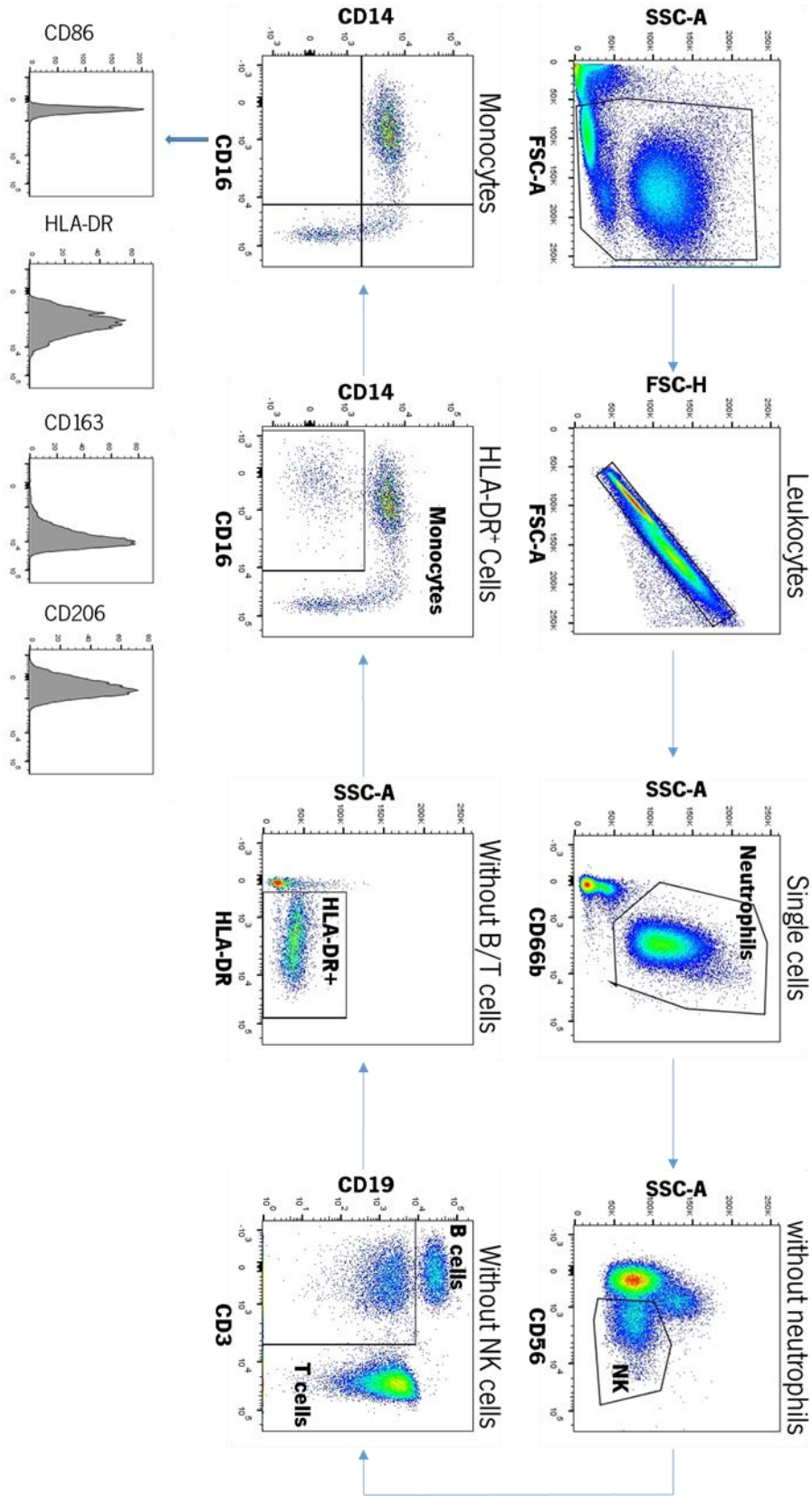
8. APPENDIX

Gating strategy



Supplementary figure 1: **Gating strategy used to select the monocyte subsets.** The gating strategy used was described by Mukherjee, R. et al. 2015. With only minor modifications.