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Role of IL-23/IL-17 axis in the development of atherosclerotic plaque

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1) ABSTRACT

Atherosclerosis is the main cause of death in industrialized countries and, more recently, it has also started to affect developing countries. The immune system plays a key role in the disease progression. Both innate and adaptive immunity cells are involved in the inflammatory response. Macrophages, belonging to the innate immunity, are plastic cells and their phenotype can be influenced by the surrounding microenvironment. Indeed, these cells can polarize into two types of macrophages: one characterized by a proinflammatory phenotype, called M1, which promotes inflammation by releasing proinflammatory cytokines, and one characterized by an anti-inflammatory phenotype, M2, which releases anti-inflammatory cytokines. In human atherosclerotic plaques the localization of M1 and M2 is different. Indeed, M1 macrophages are aggregated on the shoulders of the vulnerable plaques while M2 macrophages are present in the stable region away from the lipid core. Cytokines can drive macrophages' phenotype and functions. IL-23 is a proinflammatory cytokine involved in various inflammatory and autoimmune diseases, which may also play a role in the development of atherosclerosis. IL-23 binds to its receptor IL-23R and IL-12Rβ1 and activates the JAK/STAT signaling pathway via STAT3, releasing cytokines such as IL-17A, IL-22 and IL-6. Our study focused on investigating the role of IL-23 in atherosclerosis. In particular, we analysed the role of IL-23 in macrophage polarization by Real-Time PCR, immunoenzymatic assay, flow cytometry and immunofluorescence. The analysis was carried out both on macrophages derived from THP-1 cell line models and on macrophages derived from monocytes obtained from healthy donors. Results regarding macrophages derived from human leukemia THP-1 cell line showed that IL-23 promotes the expression of the pro-inflammatory cytokines IL-1ß, IL-6 and TNF-α in M0 macrophages, suggesting the induction of macrophage activation. It also led to an increase in the expression of IL-10 in all populations studied. In inflammatory conditions, after the stimulation with lipopolysaccharide (LPS), IL-23 increased the expression of IL-1β, IL-6 and TNF-α and IL-10 levels in all macrophage phenotypes, suggesting a synergic action with LPS. Experiments on macrophages derived from healthy donors' monocytes did not give the same results. Indeed, IL-23 induced the expression and release of IL-6 in all subpopulations examined, while the expression of IL-1β, IL-6, TNF-α and IL-10 only increased in M1 macrophages in pro-inflammatory conditions. Furthermore, IL-23 induced M1 macrophages migration suggesting a chemotactic function. Another surprising phenomenon observed was that all macrophages produced IL-17A, with resting macrophages representing the main source of production. IL-23 also induced an increase in the expression of IL-22 and IL-10 in resting macrophages and, in association with LPS and IFN-γ, it potentiated the expression and production of IL-17A and IL-10, suggesting a synergic action between LPS and IL-23. Indeed, the stimulation with IL-23 increased both CD86 and CD206 surface markers, although, CD86 appeared to be prevalent. The immunofluorescence analysis showed that all macrophages produce IL-17A and that IL-23 increases its production, while the immunohistochemistry analysis confirms the presence of macrophages and IL-17A in atherosclerotic plaques and their co-localization.

2) INTRODUCTION

2.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory immune-mediated disease of the arterial wall and is a major cause of cardiovascular diseases (CVD) such as strokes and myocardial infarctions. Atherosclerotic lesions are initiated by the accumulation of plasma lipoproteins [apolipoprotein B-containing lipoproteins (apoB-LPs)], including low-density lipoproteins (LDLs) and remnants of triglyceride-rich lipoproteins, in the intima layer of the vessel. Their aggregation, oxidation and modification result in the activation of the endothelial cells to express adhesion and chemotactic molecules to recruit monocytes. Upon the local differentiation of the latter into macrophages, they take up modified lipoproteins and transform into foam cells. Over time, the foam cells die and generate necrotic cores consisting of cell debris and cholesterol (Businaro R. et al., 2012). Typically, lesion growth can reduce blood flow in the lumen or cause clots that may completely obstruct the blood flow locally or in the brain upon migration (Björkegren J.L.M. et al., 2022).

Hyperlipidemia, hypertension, cigarette smoking, hyperglycemia, obesity and inactive lifestyle represent the most well-known risk factors for developing this pathology (Rafieian-Kopaei M. et al., 2014). Moreover, substantial progress continues to be made in the understanding of the interplay of genetic and environmental risk factors of atherosclerosis. Genome-wide association studies of CVD have identified about 200 loci, harboring candidate genes that contribute to common, complex forms of the disease. Although a large number of these genes do not fit into any CVD-related category, many of them are involved into inflammation, plasma lipid levels (Graham E.L. et al., 2021), hypertension (Cabrera et al., 2019), and diabetes (Mahajan A. et al., 2018). However, the environment also appears to play a major role in atherosclerosis as shown by the fact that modifications to lifestyle and the influence of local cultures can greatly alter CAD risk despite the same genetic background. Nutrition and obesity, exercise and physical activity, sleep and stress, smoking, pollution, intestinal microbiota, alcohol consumption, infections are listed as the main environmental risk factors of atherosclerosis (Björkegren J.L.M. et al., 2022)

Although atherosclerotic cardiovascular disease was previously considered a disease affecting mainly the industrialized world, it now spans the globe becoming the leading cause of mortality

worldwide. At the same time, the clinical profile of patients with atherosclerosis has evolved. Although atherosclerosis is largely a disease of the elderly, it now affects also younger people, and more women (Libby P. et al, 2021). Chronic inflammation is a key component of atherogenesis. In sufficient quantities it is essentially protective, but if the inflammatory response is excessive it can lead to tissue damage. Oxidized LDL, various infectious agents, interaction between leukocytes and endothelium, chronic inflammatory cellular infiltration and related cytokines and chemokines are the fundamental components of this inflammatory process. At the same time, the presence of immune components such as B lymphocytes, mostly present in the adventitial tunic surrounding the lesions, and T lymphocytes, a relevant component both in the early and in the late phase of atherosclerotic lesions, suggests the hypothesis of an autoimmune response (Surma S. et al., 2022; Sima P. et al., 2018¸Matsuura, E.et al., 2014). Atherosclerosis is a dynamic inflammatory process and the surrounding microenvironment can influence the composition of the plaque itself. Indeed, atherosclerotic plaques can be subject to instability and the factors that most influence their stability are both intrinsic and extrinsic as well as mechanical and hemodynamic factors. Therefore, components of innate and adaptive immunity can influence the local inflammatory response and can therefore play an important role in the progression or regression of atherosclerosis leading to the transition from the chronic phase of the disease to the acute one, in which there is plaque rupture and formation of the thrombus also inducing a state of irreversibility (Razeghian-Jahromi I. et al., 2022; Moriya J. 2018; Raggi P.et al., 2018; Hansson GK et al., 2006).

Due to the overall irreversibility of the atherosclerotic lesions, the clinically most important goals are prevention and early diagnosis. However, the advances in understanding of the biology of atherosclerosis have opened avenues to the development of novel medical therapeutic applications.

2.2 Healthy artery

Atherosclerosis is a pathological process that affects large and medium-size arteries with a diameter of more than 2 mm, such as the abdominal aorta, coronary, carotid and cerebral arteries (Circle of Willis). The lesions consist of a thickening of the innermost portion of the wall of these arteries.

The wall of an artery, whether it is a large and elastic artery or a medium and muscular artery or a small artery or arteriole, is made up of a tunica intima, tunica media and adventitia, which are separated from one another by an internal elastic lamina and an external elastic lamina.

The tunica intima consists of a single layer of endothelial cells resting on a basal lamina. The sub-endothelial layer lies just below these cells and is composed of loose connective tissue and few smooth muscle cells. To separate the intima from the media there is an elastic lamina composed of elastic fibers organized in a fenestrated lamina which allows the diffusion of nutrients from the lumen of the vessel towards the deeper layers of the arterial wall.

The tunica media composed of circularly arranged smooth muscle cells, elastin, collagen fibrils, and proteoglycans is the muscular portion of the arterial wall. Larger muscular arteries also have a prominent external elastic lamina that separates the media from the underlying adventitia.

The tunica adventitia mainly contains fibroblasts, collagen, and longitudinally arranged elastic fibrils. This tunic is the outermost layer of the vessel and its composition guarantees rigidity, with the function of protecting it and determining its anchorage to the adjacent anatomical structures.

2.3 Plaque formation

The initial stage of atherosclerotic plaque formation is endothelial dysfunction, especially in high-risk areas, caused by mechanical and molecular stimuli. This endothelial dysfunction allows low-density lipoproteins (LDLs) to migrate into the sub-endothelial region of the vessel. Once they reach the intima of the vessel, they are attacked by enzymes and, consequently, they undergo modifications such as oxidation and glycosylation. The Oxidation of LDLs generates Oxidized-LDLs (oxLDLs) which are responsible for activating inflammatory responses in macrophages and vascular wall cells. Indeed, OxLDL have an important role in atherosclerosis, they activate endothelial cells following interaction with the lectin-like receptor LOX-1 and are recognized through the scavenger receptors by monocytes and macrophages (Vekic J. et al., 2022). The activation of endothelial cells induces an increased expression of adhesion molecules such as E-selectin and vascular adhesion molecule 1 (VCAM-1) that acts in synergy with chemokines such as CCL2, CCL5, CXCL10 and CX3CL1 to attract monocytes, dendritic cells (DCs) and T cells into the intima (Gencer S., et al, 2021; Zernecke A., et al., 2008). Monocytes, recalled by the circulatory stream, carry out a rolling process through P-selectin, adhere to the endothelium

through ICAM-1 and VCAM-1 and migrate into the subendothelial space thanks to chemotactic factors such as MCP-1 and IL-8 (Linton M.R.F., et al., 2000). Once the intima of the vessel is reached, monocytes differentiate into macrophages, recognize oxLDL through CD36 receptors and scavenger A receptors, engulf the oxLDL transforming into foam cells and produce cytokines and adhesion molecules, thus amplifying the inflammatory loop. In addition to macrophages, other cells of the innate and adaptive immune system are also involved in the development of a plaque (Razeghian-Jahromi I. et al., 2022). Among those, dendritic cells and mast cells are worth being mentioned. Dendritic cells act in a pro-atherogenic manner: activating reactive T cell clones, secreting pro-inflammatory cytokines and following lipid absorption, promoting the activation of the inflammasome with the consequent production of pro-inflammatory cytokines (Razeghian-Jahromi I. et al., 2022). Mast cells also have a pro-atherogenic behaviour, releasing pro-inflammatory cytokines such as IFN-γ and IL-6 and promoting plaque development (Razeghian-Jahromi I. et al., 2022). There are also other immune cells such as T and B cells which have role in the development of the atherosclerotic lesion. These acquired immunity cells can both have pro-atherogenic roles: promoting plaque formation by releasing pro-inflammatory cytokines (IFN-γ) and antibodies IgA, IgG and IgE, both anti-atherogenic roles; promoting the release of anti-inflammatory cytokines (IL-10 and TGF-β) and natural antibodies (NAbs) (Razeghian-Jahromi I et al., 2022; Linton M.R.F., et al., 2000). The reduction of non-HDL cholesterol and the selective elevation of HDL cholesterol lead to the decrease in plaque size, reduction of M1 macrophage content and enrichment of M2 anti-inflammatory macrophages, hence promoting the regression of atherosclerosis. However, the stage of the plaque can affect the evolution of the plaque itself. Indeed, in early lesions, dying cells are eliminated by nearby macrophages that perform efferocytosis, transforming them into smaller lesions with fewer macrophages. The removal of advanced injuries is inefficient (Brophy M. L. et al., 2017). The regression or progression of an atherosclerotic plaque is affected by the migration and proliferation of smooth muscle cells. Growth factors and chemoattractants released by macrophages allow these cells to proliferate and migrate into the vessel's intima and thus form a complex extra-cellular matrix consisting of collagen, proteoglycans and elastin, named "fibrous cap". As the inflammatory condition progresses, the plaque becomes vulnerable, resulting in the formation of a necrotic core and the thinning of the fibrous cap (Alonso-Herranz L. et al., 2023; Linton M.R.F., et al., 2000).

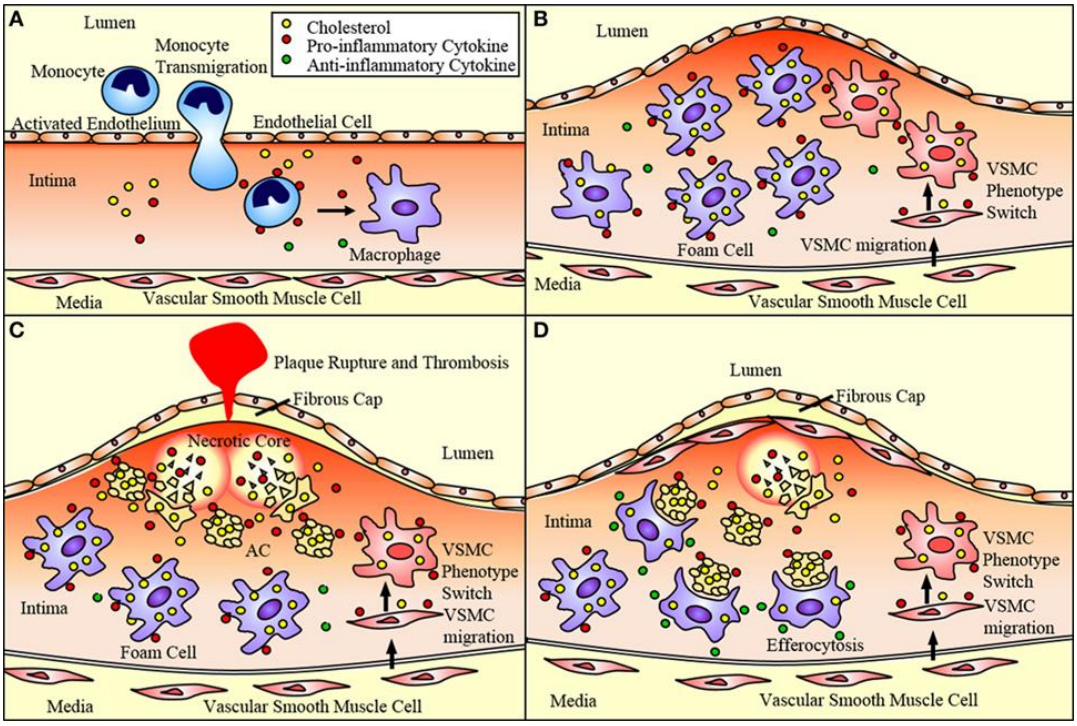


Figure 1

(Brophy M. L. et al., 2017) Schematization of the process of formation of an atherosclerotic plaque. From the dysfunction of the vascular endothelium, the retention of lipoproteins, the recall of cells of the immune system and the self-maintenance of the pro-inflammatory process, primary events in the pathophysiology of atherosclerosis, and its progression or regression.

2.4 Classification of an atherosclerotic plaque

The scientific committee of the American Heart Association (AHA) has identified a numerical classification system for atherosclerotic lesions, taking into consideration the evolution of the type of lesion and the development of clinical manifestations. This numerical system, currently used, classifies lesions into 6 evolutionary types.

Type I lesions, typical lesions of infants and children, are characterised by a small and isolated presence of macrophages containing lipid deposits (foam cells). This type of lesion is also present in adults in arteries at low risk of lesion.

Type II lesions are characterised by layers of foam cells and lipid-rich vascular smooth muscle cells (SMCs). These types of injuries can be further classified into type IIa and type IIb injuries. The former consists of a large number of SMCs, extracellular matrix, macrophages and lipids in

regions distant from the endothelium. The latter is made up with a smaller number of SMCs. Indeed, the latter does not progress or the progression occurs very slowly.

Following the destruction of a part of the connective tissue, near or at the base of the plaque, a central nucleus or core is formed, composed of a large amount of extracellular lipids. This type III lesion, called pre-atheroma, represents the transition between the fatty streak in which the core is completely lipidic and acellular.

These three types of lesions are small, clinically silent and can be evident already in the first decade of life while they can occupy a third of the arterial surface in the third decade of life.

Type IV lesions are characterised by the increase and confluence of small isolated pools of extracellular lipids that develop a lipid core, called atheroma, in which small calcium particles are visible. Between the lipid core and the endothelial surface, the intima contains macrophages and smooth muscle cells with or without lipid droplet inclusions as well as lymphocytes and mast cells. At the periphery of the lesion (e.g. in the plaque shoulder) macrophages, foam cells and lymphocytes are often found; this localization can predispose the lesion to complications such as fissures and ruptures.

When the layer of tissue between the lipid core and the endothelial surface undergoes an increase of fibrous tissue (mainly collagen), the lesion is then called type V. If the new tissue is part of a lesion with a fatty core (type IV) it may be referred to as a fibroatheroma, or type Va lesion; if the fatty core and other parts of the lesion are calcified it is a type Vb lesion while an absence of a lipid core and lipids in general identify a type Vc lesion. The mechanical forces of flow can shape the architecture of multilayered fibroatheromas inducing repeated surface ruptures, hematomas and thrombotic deposits.

The complicated lesions, called type VI lesions, are characterised by fibrous and calcified areas with evident ulceration and with possible adherent thrombi and sub-intimal hemorrhages with intraparietal hematoma or partial "detachments" of the wall.

Type VI can be divided into three subtypes: VIa based on surface disruption; VIb hematoma or haemorrhage, VIc thrombosis. If they have all three characteristics, it is called a type VIabc lesion. An updated version of the classification of atherosclerotic lesions also includes type VII

and VIII lesions. These additional lesion types better describe the morphological change that Vb and Vc undergo following a lipid regression.

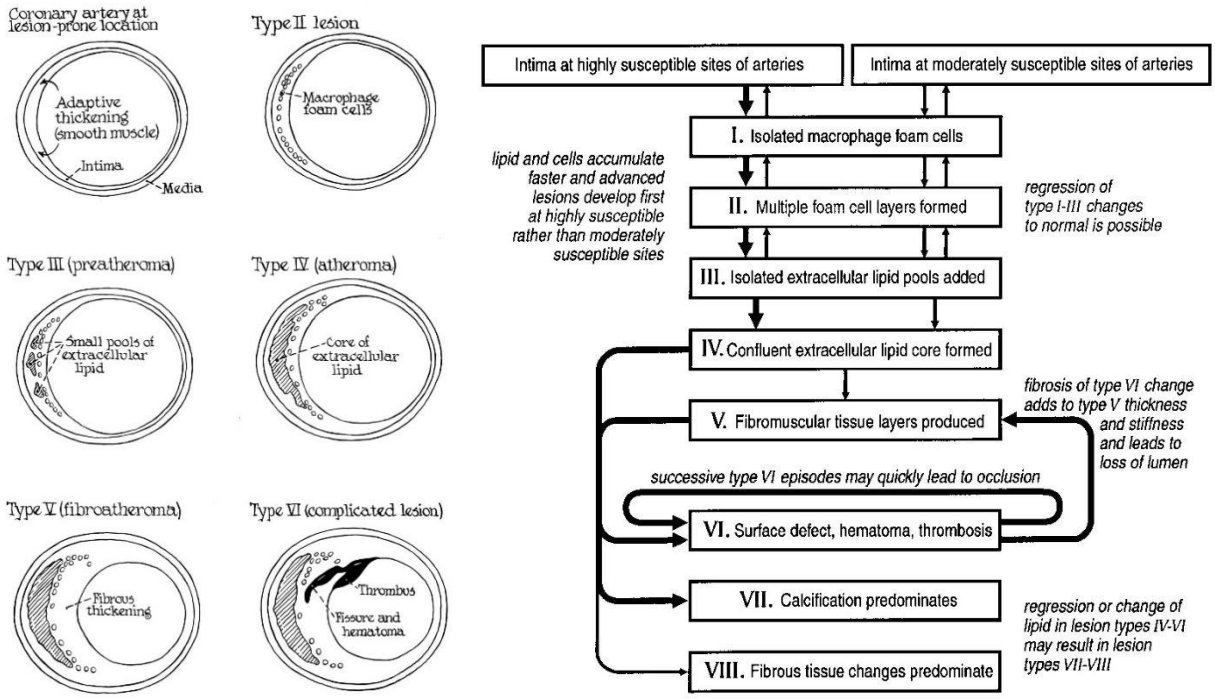


Figure 2

(Stary H.C et al., 2000) Evolution of atherosclerotic lesions from type I to type VI and the various possible subsequent pathways of progression to lesion types beyond type VI. The main histological characteristics of each sequential step (type of lesion) are represented.

2.5 Macrophages

The immune system plays an important role in the progression or regression of atherosclerosis. The inflammatory response is mediated by the innate immune system, such as macrophages and dendritic cells, and by the adaptive immune system, such as lymphocytes. Macrophages, first discovered in 1882 by Metchnikoff, are mononuclear cells belonging to the phagocyte system. Recent studies have shown that these cells of the innate immune system can have two different origins: they can be derived from the differentiation of circulating monocytes as well as from embryonic origins. (Sreejit G. et al., 2020; Stremmel C. et al., 2017; Perdiguero E.G. et al., 2015). In the case of embryonic origin, macrophages can originate from hematopoietic stem cells, which are produced in the bone marrow, enter the circulatory stream and differentiate into monocytes in the presence of growth factors such as GM-CSF (stimulating factor the Granulocyte-

Macrophage Colonies), M-CSF (Macrophage colonies stimulating factor) and IL-3. Embryonic origin macrophages can also originate as a result of waves of hemopoiesis during the development of the fetus: a first wave of primitive hematopoiesis of the yolk sac allows the formation of "primitive" macrophages; the second wave, that always originates in the yolk sac, allows the formation of fetal monocytes that, once they reach the tissue, transform into adult resident macrophages; finally the third wave will allow the formation of the first stem cell Embryonic hematopoietic, which will reach the fetal liver (Sreejit G. et al., 2020; Stremmel C. et al., 2017; Perdiguero E.G. et al., 2015). There are numerous types of macrophages based on the tissue in which they reside and assume morphologically different forms after activation by external stimuli. Depending on the migration site they take different names:

• Epithelioid cells: macrophages present in the skin with abundant cytoplasm;

• Microglia: in the central nervous system;

• Kupffer cells: macrophages that line the vascular sinusoids of the liver;

• Alveolar macrophages: macrophages in the pulmonary airways;

• Osteoclasts: the multinucleated phagocytes in the bone.

Macrophages play a central role in the development of homeostasis, the immune response and the reparative process. They represent the line of defense against external pathogens. These cells are equipped with receptors that allow them to discriminate between the antigens of our organism, "self-antigens", and foreign antigens, "non-self-antigens". The primary role of macrophages is to engulf antigens, produce pro-inflammatory and anti-inflammatory cytokines and reactive oxygen species (ROS) then attracting and activating other inflammatory cells. Macrophages recognize only a part of the antigens, the pathogen-associated molecular patterns “PAMP”, through the Toll-like receptors. Once the antigen is recognised, macrophages present it to the T lymphocytes, thus activating the cell-mediated response. Moreover, the bond between the macrophage and the antigen activates, through the translation of the signal carried out by FCyRIs, phagocytosis and, successively, the production of microbial molecules which clear the microorganism. In atherosclerotic plaque, macrophages play a central role in the development and maintenance of lesion with pro-inflammatory roles; they phagocytize the subendothelial lipid accumulation becoming "foam cells" (Blagov A.V. et al., 2023; Chinetti Gbaguidi G. et al., 2011; Moore K.J.

and Tabas I., 2011); they contribute to the maintenance of local inflammatory response by secreting pro-inflammatory cytokines and chemokines and producing reactive oxygen species (ROS) (Blagov A.V. et al., 2023; Bae Y.S. et al., 2009); they are also responsible for the formation of the necrotic nucleus of a plaque in progression (Williams J-W. et al., 2018; Seimon T.and Tabas I. 2009); finally, they also have anti-inflammatory roles by secreting anti-inflammatory factors such as IL-10 and TGF-β which reduce the plaque inflammation , promoting tissue remodeling, repairing and clearing dying cells and debris via efferocytosis, thereby reducing apoptotic and necrotic cells within the plaque and promoting plaque stability . (Blagov A.V. et al., 2023; Tabas I., 2010a).

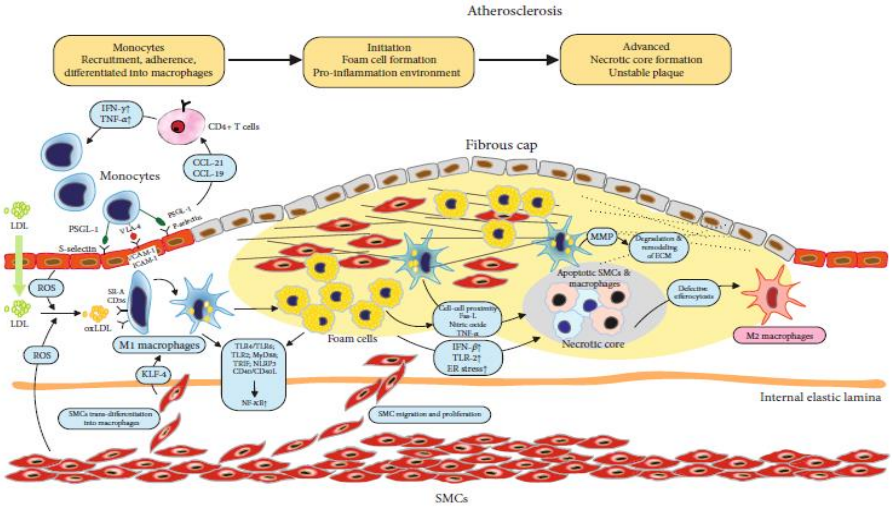


Figure 3

(Hailin Xu et al., 2019) In this image the role of M1 and M2 macrophages in the progression and regression of atherosclerotic plaque is shown.

2.6 Macrophage phenotypes and functions

As previously mentioned, one of the key characteristics of macrophages is their high degree of plasticity, which allows them to produce a specific response based on the stimuli coming from the microenvironment. On the basis of in vitro experiments, "classically activated" or pro-inflammatory M1 macrophages are those which are stimulated with Th1-type cytokines (IL-1β, TNF-α and IFN-γ) and lipopolysaccharide (LPS) (Blagov A.V. et al., 2023; Wu J. et al., 2023; Shapouri-Moghaddam A., et al., 2018). In response to these stimuli, macrophages are activated

and differentiate into macrophages with a pro-inflammatory activity. The canonical signaling pathway is the one mediated by IRF/STAT through the recruitment of STAT1 which ends with the activation of the transcription factor NF-kB which, in turn, regulates the expression of cytokines and chemokines such as IL-1β, TNF-α, IL-6, IL-23 which contribute to further recruitment of inflammatory immune cells and the spread of inflammation (Blagov A.V. et al., 2023; Wu J. et al., 2023; Shapouri-Moghaddam A. et al., 2018). Th2-type cytokines (IL-4, IL-13) induce M2 or "alternately activated" macrophages via the canonical IRF/STAT signaling pathway with the recruitment of STAT6. The activated macrophages release anti-inflammatory cytokines such as IL-10 and the IL-1β receptor antagonist. The cytokines IL-4 and IL-13 induce a subtype of M2 characterised by the expression of high levels of CD206 (subtype M2a), while the interaction of macrophages with immune complexes and LPS produces the subtype M2b which, in turn, produces both pro- and anti- inflammatory cytokines (IL-10, IL-1β, IL-6 and TNF-α). The stimulation of macrophages with glucocorticoids and IL-10 induces the M2c subtype, which predominantly produces the anti-inflammatory cytokines IL-10 and TGF-ß. TLR agonists via the adenosine receptor promote polarization of macrophages towards the M2d subtype that releases anti-inflammatory cytokines and growth factor VEGF. (Blagov A.V. et al., 2023; Wu J. et al., 2023; Lin P. et al., 2021; Liu N. et al., 2021; Mohmmad-Rezaei M., et al., 2021). There are additional subsets of macrophages beyond those already mentioned: MOX macrophages induced by membrane phospholipids, M4 macrophages induced by platelet factor 4 and, finally, $TREM^{hi}$ macrophages characterised by an increase in the expression of the Trem2, Cd9, Ctsd and spp1 genes and a decrease in the expression of inflammatory cytokines. This subset also plays an important role in the development and progression of atherosclerotic plaque, having pro-atherogenic actions as in the case of MOX and M4 and $TREM^{hi}$ which effectively represent the lipid-laden foam cells and have biological functions attributed to lipid metabolism and cholesterol efflux (Barret J.T. 2020; Kim K. et al., 2018).

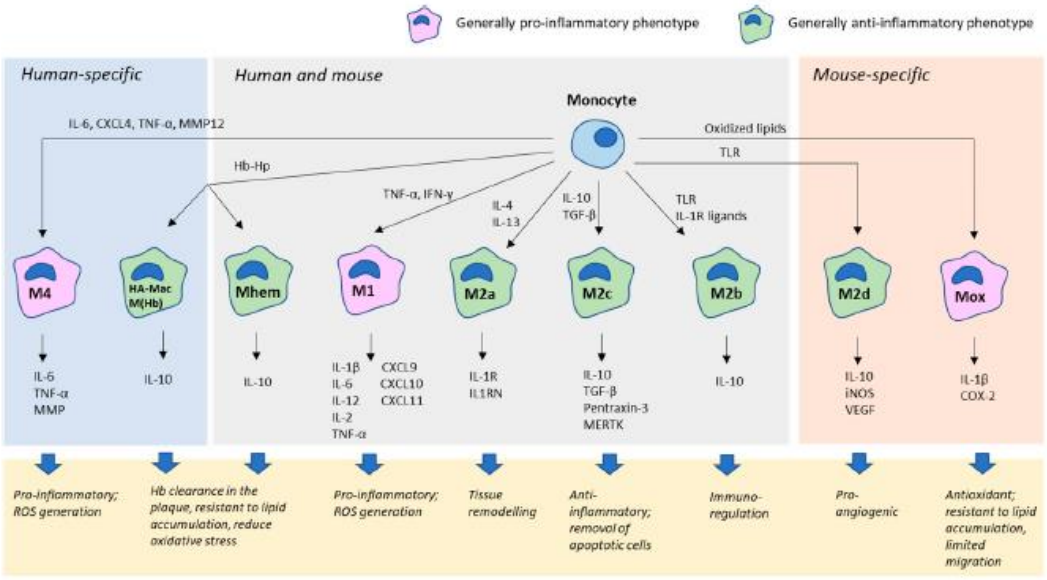


Figure 4

(Chistiakov D.A. et al., 2019) Representation of all macrophage populations. We can see the macrophages that have a pro-inflammatory action in pink and the macrophages with anti-inflammatory activity in green. The "classically activated" or pro-inflammatory "M1" macrophages are stimulated with Th1-type cytokines and lipopolysaccharide (LPS), and release cytokines and chemokines such as IL-1beta, TNF-alpha, IL-6, IL-23. Th2-type cytokines (IL-4, IL-13) induce M2 or "alternately activated" macrophages and once activated release anti-inflammatory cytokines such as IL-10 and IL-1beta receptor antagonist. The cytokines IL-4 and IL-13 induce a subtype of M2a characterised by the expression of high levels of CD206. Interaction of macrophages with immune complexes and LPS produces the M2b subtype which produces both pro- and anti-inflammatory cytokines (IL-10, IL-1βa, IL-6 and TNF-α). The stimulation of macrophages with glucocorticoids and IL-10 induces the M2c subtype which predominantly produces the anti-inflammatory cytokines IL-10 and TGF-beta. TLR agonists via the adenosine receptor promote polarization of macrophages towards the M2d subtype that releases anti-inflammatory cytokines and growth factor VEGF.

2.7 IL-23 and the IL-23/17 axis

Cytokines are a group of low molecular weight proteins which aid cell-to-cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection, and trauma. They are divided into several groups, such as interleukins, chemokines, CSFs (colony stimulating factors) and IFNs. IL-23, mainly produced by dendritic and macrophage cells, is a heterodimeric cytokine belonging to the IL-12 family such as IL-12, IL-35, IL-39 and its structure is formed by two subunits, the p40 subunit, in common with IL-12, and a specific p19 subunit. While IL-12 promotes the Th1 differentiation, IL-23, in collaboration with TGF-Beta and IL-6, promotes the Th17 differentiation. Furthermore, its action is mediated by the natural killer T subset (NKT) and innate lymphoid cell type 3 (ILC3). IL-23 binds its membrane receptor IL-23R

in complex with IL-12Rβ1 and activates the JAK/STAT signaling pathway via STAT3 and to a minor extent STAT1, STAT4 and STAT5. The phosphorylation of STAT3 induces the migration of the same transcription factor to the nucleus and an upregulation of the RORγt expression which, in turn, plays a key role in the production of IL-17. RORγt, in addition to driving the Th17 differentiation, also plays important roles in all IL-23 target cells (Ergberg A. et al., 2020). IL-17, the first cytokine identified downstream of the IL-23 pathway, is composed by 6 subtypes (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F) and plays an important role in various chronic inflammatory diseases. One of the major cytokines released in the IL-23/IL-17 axis is IL-17A. Both IL-17A and IL-17F bind to the IL17RA and IL-17RC receptor complex, present in both immune system cells (e.g. T lymphocytes) and non-immune system cells (e.g. endothelial, epithelial, and fibroblast cells). IL-17, once it has interacted with the respective receptor, releases antimicrobial peptides, cytokines and chemokines promoting the recruitment and activation of neutrophils and macrophages at the site of inflammation by activating NF-KB MAPK and C/EBP. Studies carried out on human keratocytes have shown that IL-17 and IL-22 bring to the increase of the expression and production of IL-1ß through the activation of NLRP3 through the NLRP3-caspase-1 pathway (Cho K.A. et al 2012). The role of IL-17 is still completely uncertain. Indeed, this cytokine can perform various pro-inflammatory and anti-inflammatory functions in numerous chronic inflammatory diseases. Another cytokine released by IL-23 when interacting with its receptor is IL-22. IL-22, a cytokine belonging to the IL-10 family, plays pleiotropic roles by promoting angiogenesis, inflammatory response, VSMC proliferation and migration, and cholesterol metabolism. This cytokine interacts with the IL-22R receptor, present in stromal and epithelial cells, consisting of two subunits: IL-22R1 and IL-10R2. Upon interaction with its receptor IL-22 activates the Janus tyrosine kinase (JAK) JAK1/Tyr2, leading to signal transduction and activation of the STAT3 phosphorylation. Although the pro-inflammatory role of IL-23 in various pathologies such as inflammatory bowel disease, rheumatoid arthritis and psoriasis has been widely reported (Sewell G.W. et al., 2022; Liu T. et al., 2020; Bianchi E. et al., 2019; Hou Y. et al, 2018; Hawkes JE et al. al, 2018; Abbas A. et al., 2015; Erbel C. et al., 2014) its role in the development and regression of atherosclerosis is yet unclear. It is now known that this cytokine, mainly produced by macrophages and activated dendritic cells, induces the differentiation and proliferation of Th17 lymphocytes, synergizing with IL-6 and TGF-β (Kleiner J.C. et al., 2022; Van Der Heijiden T. et al., 2019). Th17 lymphocytes, a subpopulation of CD4+T, promote the recruitment and activation of neutrophils and macrophages at the site of

inflammation as well as have an important role in the activation of immune responses against extracellular bacteria and fungi. Characterised by the expression of the transcription factor RORγ and the chemokine receptor CCR6, these cells produce IL-17A, IL-17F, IL-22 and GM-CSF when activated. Th17 lymphocytes have heterogeneous phenotypes and functions and may have a pro-inflammatory and anti-inflammatory activity (Binger K.J., et al., 2017). The molecular microenvironment in which they mature is capable of influencing their differentiation program, thus determining the development of a protective or pathogenic immune response.

The pathogenic or pro-inflammatory Th17 lymphocyte phenotype, driven by the cytokine IL-23 and inhibited by the cytokine IL-21, expresses high levels of IL23R and avβ3 integrins on the surface, produces the pro-inflammatory factor GM-CSF and IFNγ, and expresses the transcription factor Th1 "T-bet", in addition to the transcription factor RORγ. (Binger K.J., et al., 2017, Du F., et al., 2016).

Studies have shown that IL-17A plays an important role in autoimmune diseases. Since atherosclerosis is considered an immune disease, it could be influenced by this cytokine (Erbel C. et al., 2014). Therefore, although the role of IL-23 in atherosclerosis is still not fully understood and clarified, it is important to consider that a study by Abbas and his collaborators has shown that IL-23 significantly increases the release of IL-17 in patients with atherosclerosis (Abbas A. et al., 2015). Another study by Subramanian et al. shows that IL-23 induces apoptosis in macrophage cells $CD11c^{Hi}MHCII^{Hi}$. Indeed, this cytokine, in combination with a defective efferocytosis in advanced lesions, leads to an increase of the central necrotic core, which contributes to plaque instability (Subramanian M et al., 2015). A study has highlighted how the expression of IL-23R+ influences the atherogenic role of γδ T cells and how these localize in the early formation of lesions in the aortic root (Gil-Pulido J. et al 2022). Since we do not have much data regarding the role of IL-23 in relation with atherosclerosis, it can be useful to analyse studies on how it influences other inflammatory diseases. Indeed, several studies have shown how this cytokine can affect already existing inflammatory pathologies, such as Kawasaki's disease or psoriasis, leading to the development of cardiovascular diseases (Su Y. et al., 2022; Liao Y. et al., 2017; Khojasteh-Fard M. et al., 2012). For instance, a study has shown that patients with Kawasaki's disease have an increased serum presence of IL-23 and that the same is greater especially in patients who have suffered a coronary artery lesion (Su Y. et al., 2022). Moreover, Liao et al carried out a study on mice which suggested a pathogenic role of IL-23 in myocardial

I/R damage. Indeed, IL-23 releases IL-17A through the JAK/STAT3 signaling pathway and promotes inflammatory responses and myocardial apoptosis. However, through the use of a JAK2/STAT3 specific inhibitor, namely AG490, the pro-inflammatory and pro-apoptotic effect of IL-23 is attenuated (Liao Y. et al., 2017). Another interesting research carried out on murine models by Hou and colleagues has shown that IL-23 induces the production of IL-17A, IL-17F, IL-22 and IFN-γ in peritoneal macrophages whose phenotype is different compared to the classical ones described above (Hou Y. et al, 2018). The study of cytokines and their role in the pathogenesis of atherosclerosis has suggested that designing new drugs, specifically acting on the cytokines, could modify the course of the disease.

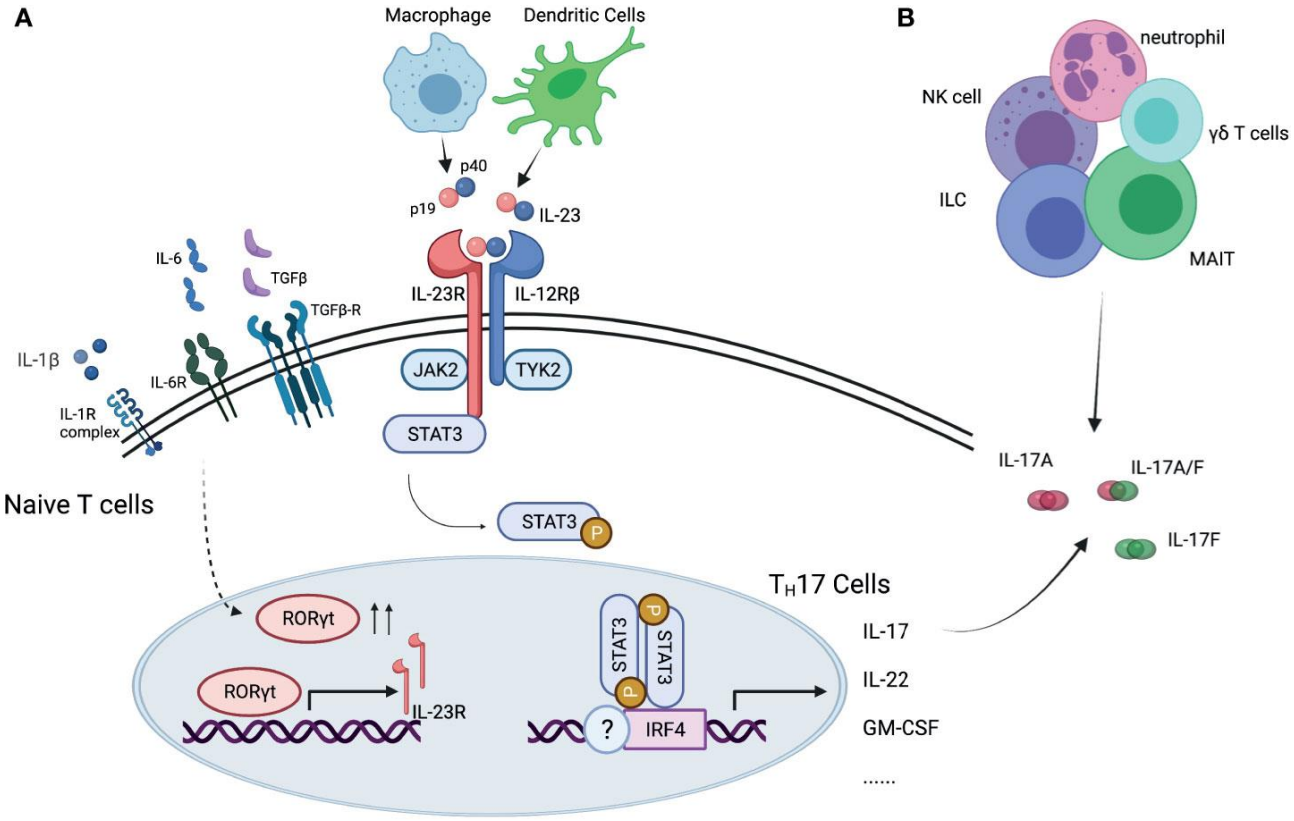


Figure 5

(Wang R. and Maksymowych W. P., 2021) IL-23/IL-17 pathway representation. IL-23, released by macrophage and dendritic cells, binds to its receptor IL-23R and IL-12Rβ1 in naïve T cells and activates the JAK/STAT signaling pathway via STAT3, releasing cytokines such as IL-17A, IL-22 and IL-6. IL-17 promotes the recruitment and activation of neutrophils and macrophages at the site of inflammation.

3) AIMS

The expression and production of cytokines in differently polarized macrophages

As previously stated, atherosclerosis is a chronic inflammatory disease and the inflammatory response drives its progression. Among the actors involved in atherosclerosis we find macrophages. These cells of the innate immune system are involved in all stages of the disease development, from the lesion formation to its expansion and rupture. However, they may also play a role in the regression of the disease. Macrophages exhibit numerous phenotypes and can change their phenotype based on intrinsic, extrinsic and tissue stimuli, transforming into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. Cytokines are molecules that play an important role in the polarization process, so the aim of the first part of our research was to evaluate the role of IL-23 in the polarization and migration of all macrophage populations.

Expression and production of cytokines and transcription factor of the IL-23/IL-17 axis in primary monocyte-derived macrophage cells

As previously mentioned, IL-23, mainly produced by dendritic and macrophage cells, binds to the heterodimeric receptor composed by IL-12Rβ1 and IL-23R and activates the JAK/STAT signaling pathway by STAT3, releasing pro-inflammatory cytokines such as IL-17A (Ergberg A. et al., 2020). Although, the pro-inflammatory role of IL-23 in various pathologies such as inflammatory bowel disease, rheumatoid arthritis and psoriasis has been widely reported (Erbel C. et al., 2014; Abbas A.et al., 2015; Hawkes J.E. et al., 2018; Hou Y. et al, 2018; Bianchi E and Rogge L., 2019; Liu T. et al., 2020) its role in the development or regression of atherosclerosis is yet unclear. Referring to a study carried out on murine models which observed that IL-23 induces the production of IL-17A, IL-17F, IL-22 and IFN-γ in peritoneal macrophages (Hou Y. et al, 2018) and given that the latter express IL-23R, although to a minor extent, the IL-23/IL-17 axis was analysed in macrophages derived from peripheral blood.

Characterization of macrophages and IL-17A in human atherosclerotic plaques

To confirm the data obtained in the first part of our research, which was conducted in vitro, we used an immunohistochemical and immunofluorescence approach in order to identify the existing macrophage populations and to verify if the same produce IL-17A within human carotid atherosclerotic plaques.

4) MATERIALS AND METHODS

Cell cultures

THP-1 cells were provided by Abcam (Milan, Italy) and were grown in RPMI 1640 (GIBCO, Paisley, UK) supplemented with 1% nonessential amino acids (Gibco, Karlsruhe, Germany), 1% sodium pyruvate (Sigma Aldrich, St. Louis, Missouri, USA), 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Karlsruhe, Germany), 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 10% Foetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT) "complete medium". M0 Macrophages were obtained using 10 ng/ml of PMA in "complete medium" (Sigma Aldrich, St. Louis, Missouri, USA) for 24 hours. Cells were then seeded in 6-well flat-bottom tissue culture plates (2×10^6 cells/well, 3 mL/well; Corning New York, NJ) and maintained in "complete medium" without PMA at 37°C in 5% CO2.

We also used monocytes derived from peripheral human blood which were purified by incubating PBMC, obtained from a buffy coat, with microspheres coated with CD14 (MiniMacs, Miltenyi Biotec). In this case, M0 macrophages were obtained by incubating CD14+ monocytes for 6 days in the "complete medium", previously described, in presence of 10 ng/ ml (rh) monocyte-colony stimulating factor (M-CSF) (R&D System, Minneapolis, MN, USA). Following such treatment, cells were seeded in 6-well flat-bottom tissue culture plates (2×10^6 cells/well, 3 mL/well) and maintained at 37°C in 5% CO2.

We used two different stimuli to create M0 macrophages because the stimulation through PMA could be too strong for monocytes derived from peripheral human blood. Moreover, a great number of studies follow the protocol explained above (Ruder A.V. et al., 2023; Tedesco S. et al., 2018; Star T. et al., 2018).

Polarization cells and treatment

M0, M IFN, M IL-4 and M IL-10 macrophages were used during this research.

THP-1-derived macrophages were polarized towards M1-like phenotypes using 25 ng/ml IFN-γ (Miltenyi Biotec, Gladbach, Germany) and 200 ng/ml LPS (M IFN) (Lipopolysaccharide; from Escherichia coli strain 0111:B4 Sigma Aldrich, St. Louis, Missouri, USA) for 18h. Other THP-1-derived macrophages were polarized towards M2-like phenotypes using 10 ng/ml of IL-4 (M

IL-4) (Miltenyi Biotec, Gladbach, Germany) or using 10ng/ml of IL-10 (M IL-10) (R&D System,Minneapolis, MN, USA) for 18 h. These cultured cells were then washed with warm phosphate-buffered saline (PBS) (Gibco, Karlsruhe, Germany) and harvested using 0.01% trypsin (MP Biomedicals, Santa Ana, CA, USA).

The macrophages obtained from monocytes derived from peripheral human blood were either left untreated or polarized. The polarization was either towards M1, obtained using 10 ng / ml of rh-IFN-γ and 10 ng / ml of LPS, or towards M2a, obtained using 10 ng / ml of rh-IL-4 for 18 hours. The cultured monocyte-derived macrophages were then washed with warm phosphate-buffered saline (PBS) and harvested using 0.01% trypsin.

Following this procedure M1 and M2a macrophages were treated once again, this time with IL-23 (100ng/ml) (R&D system, Minneapolis, Minnesota, USA), with and without the pro-inflammatory stimulus LPS (10 ng / ml) for 8h.

During the experiments performed on the undifferentiated population "M0", macrophages were treated with IL-23 (100ng/ml), with or without pro-inflammatory stimulus (LPS 200ng/ml and IFNγ 25ng/ml) for 8h.

Cytotoxicity

To determine whether IL-23 induces toxicity in the macrophage population obtained from monocytes derived from peripheral human blood, the viability of the cells was evaluated by the trypan blue exclusion test. In preparation for such test, M0, MIFN and MIL-10 were seeded in 24-well plates at a density of $1x10^6$/well. The cells were treated with different concentrations of IL-23 (10 ng/ml, 50 ng/ml and 100 ng/ml) with or without pro-inflammatory stimulus (lipopolysaccharide). After the treatments, the cells were detached using 1 × trypsin-EDTA, 100 µL of cell suspension was mixed with 100 µL trypan blue solution and cell counts were performed using a Neubauer chamber.

Transwell assay

Migration assay was performed following the procedure described by Buttari et al. 2014. We investigated the ability of M IFN and M IL-4 to migrate following exposure to rh-IL-23 using Transwell inserts with 8 µm pore size (Costar, Cambridge, MA, USA). All dilutions were carried

out in the migration medium RPMI 1640, and 600 µl of this was placed in each well to measure the migration. Inserts were loaded with 100 µl of cell suspension ($1x10^5$ cells/well), placed in the 24-well plates, and incubated in a humidified atmosphere of 5% CO_2 for 4 h at 37°C. To distinguish chemotaxis from chemokinesis, checkerboard assays were performed. Briefly, rh-IL-23 (50 ng/ml) was placed in the lower wells or in both upper and lower wells of the chemotaxis chamber to determine whether cells migrated with a positive gradient or no gradient of IL-23. According to the definition of chemotaxis and chemokinesis, cells migration towards a positive gradient represents chemotaxis, whereas random cell migration (no gradient) represents chemokinesis.

We used chemokine monocyte chemoattractant protein-1 (MCP-1)/CCL2 (R&D Systems, Minneapolis, Minnesota, USA) at 10 ng/ml and the bacterial products N-Formyl-Met-Leu-Phe (fMLF) (Sigma Aldrich, St. Louis, Missouri, USA) at 10 nM as positive control chemoattractants. Non-adherent macrophage cells migrated into the media in the lower chamber were counted by flow cytometry (60 s counts). Cells gated into the region of the large, granular cell population in the forward and side-scatter dot plot were counted as migrating cells. Results were expressed as migration indices that were calculated as follows: mean number of cells migrating in response to stimulus/mean number of cells migrating in response to control medium. Another counting method was then applied to evaluate adherent macrophages. Transwell membrane inserts were fixed with 4% paraformaldehyde (15 min at RT), lysed with 100% Methanol (15 min at RT), and stained with 0.2% crystal violet. The membranes were dipped into distilled water as many times as needed to remove the excess crystal violet. The dried membranes were viewed underneath an inverted microscope and the number of cells counted in different fields of view to get an average sum of cells that have migrated through the membrane towards the chemo-attractant and attached on the underside of the membrane.

Real-time PCR

The whole RNA was extracted from the control macrophage population and from the treated one by miRNeasy Micro kit (Qiagen, Hilden, Germany). The RNA was quantified using NanoDrop One/One C (Thermo Fisher Scientific, Waltham, MA, USA).

The cDNA was obtained using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA, USA) and quantitative real-time PCR (qPCR) was performed for

each sample in triplicate through an Applied Biosystems 7900HT Fast real-time PCR System (Applied Biosystem, Cheshire, UK) using the program SDS2.1.1 (Applied Biosystem, Foster City, CA, USA) and the Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). RT-PCR was used to study the panel of expression of genes in table (1). These primers were designed with the UCSC GENOME BROWSER (http://genome.cse.ucsc.edu/; University of California, Santa Cruz) and matched by BLASTn to the genome sequence to identify the primer locations with respect to the exons.

To analyse results a comparative threshold cycle (CT) method was applied. The amount of target, normalized to the endogenous reference of hGAPDH primers (ΔCT) and relative to the calibrator of untreated control (ΔΔCT), was calculated by the equation $2^{-\Delta\Delta CT}$.

GENE	Forward primer (5'-3')	Reverse primer (5'-3')	Accession numbers
hGAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC	NM_001357943.2
hIL-1β	GCTTATTACAGTGGCAATGAGG	GGTGGTCGGAGATTCGTAG	NM_000576.3
hIL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	NM_001371096.1
hTNF-α	ATCTTCTCGAACCCCGAGTGA	CGGTTCAGCCACTGGAGCT	NM_000594.4
hIL-10	GATGCCTTCAGCAGAGTGAA	GCAACCCAGGTAACCCTTAAA	NM_000572.3
hIL-17A	CTTGGAATCTCCACCGCAAT	CACGTTCCCATCAGCGTTG	NM_002190.3
hIL-22	TGGCAAAGAAGGGCTGTCAG	GCGGTGACCCTGGCATAGT	NM_021258.4
hCD86	CTGTAACTCCAGCTCTGCTCCGTA	GCCCATAAGTGTGCTCTGAAGTGA	NM_001206925.2
hCD206	CTGAATTGTACTGGTCTGTCCT	GCTTAGATGTGGTGCTGTGG	NM_002438.4
hSTAT-1	CAC CTA ACG TGC TGT GCG TA	GGT GAA CCT GCT CCA GGA AT	NM_139266.3
hSTAT-3	CATGCTGACCAACAATCCCA	TGGAGGAGAACTGCCAGCTC	NM_001384993.1
hRORc	TGCTGAGAAGGACAGGGAGCCAA	GGAAGAAGCCCTTGCACCCCTC	NM_001001523.2

Table 1.

Foward and reverse primers and accession numbers used for the Real-Time PCR analysis.

Cytokine production by immunoassay (TEST ELISA)

The production of cytokines was detected through precoated ELISA tests (Immunological Science, Rome, Italy). Pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-22 and IL-17A) and anti-inflammatory cytokine (IL-10) were analyzed in the cell culture medium after treatment according to the instructions of the manufacturer.

Immunofluorescence

$6x10^4$ macrophage cells were treated in chamber slides for 24h through the procedure previously described. After treatment, cells were fixed with 4% paraformaldehyde for 30 min, followed by 0,1% Triton X-100 in PBS to allow the permeabilisation. To reduce non-specific background staining, cells were treated with 0.1 M glycine in PBS for 20 min. In order to detect IL-17A and CD86, cells were incubated with the primary antibodies anti-IL-17A (abcam, Cambridge, UK) and anti-CD-86 (Santa Cruz Biotechnology, Dallas, Texas, USA) and, subsequently, with the secondary antibodies CF488A Goat Anti-Rabbit IgG (H+L) for IL-17A (BIOTIUM, San Francisco,USA) and CF594 Goat Anti-Mouse IgG (H+L) for CD86 (BIOTIUM; San Francisco, USA)

In the final stage of the experiment, cells were marked with DAPI to highlight the nucleus (Immunological Science, Roma, Italy). Images were then obtained through a Nikon Ni-E microscope (Nikon, Japan), equipped with the software "ImageJ" and the intensity of fluorescence was evaluated.

Flow cytometric analysis of macrophage phenotypes

A phenotypic analysis was carried out on all macrophage populations by means of a Gallios Flow Cytometer (Beckman Coulter) equipped with three lasers (488 nm, 638 nm and 405 nm), and results were analysed by using Fluorescence-activated cell sorting (FACS) and the software "Kaluza Analysis" (Beckman Coulter). The cells were treated with 1 μM Sytox Blue nucleic acid staining (Molecular Probes, Carlsband, CA, USA), trypan blue solution, and other chemicals (Sigma-Aldrich, Milan, Italy) in order to exclude dead cells.To determine macrophage phenotypic surface markers, macrophages (1×10^6) were stained with the following monoclonal antibodies (mAbs): phycoerythrin (PE)-CD86 and PE-CD204 mAbs (Miltenyi Biotec), for 30 min at 4°C.

Histological classification of plaques and immunohistochemical analysis

Some carotid atherosclerotic plaques and a healthy artery were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Since carotid endarterectomies involve a relatively large arterial area, we investigated several distant cross-sections for each plaque type.

Paraffin embedded samples were cut into sections of 5 μm, mounted on polarized slides and stained with hematoxylin-eosin for histological evaluation.

Plaques were divided into two groups (complicated and stable plaques), according to the histological classification of Stary (Stary, et al., 1995). Sections containing an inflammatory infiltrate were identified and consecutive serial sections were processed for a single-staining immunohistochemistry.

Single-staining immunohistochemistry

The tissue sections were deparaffinized and rehydrated, first with Xylene for 30 minutes, then with graduated doses of alcohol (100%, 90%, 75%) and, finally, dosed in distilled water. To reduce non-specific background staining due to endogenous peroxidase, sections were incubated with 3% hydrogen peroxide in methanol for 12 minutes and, to detect tissue antigens, sections were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave 400W oven with two cycles: one for 2 minutes and the other one for 3 minutes.

After rinsing the slides in PBS, endogenous avidin and biotin were blocked using Super block (UltraTek HRP-Anti Rabbit kit and UltraTek HRP- Anti Mouse kit, Scytek Laboratories, Logan, Utah, USA) at room temperature for 12 minutes.

Tissue sections were incubated with the primary antibody at 4°C in a humidified chamber overnight or at 37°C for 1 hour. The primary antibodies used are anti-IL-17A (abcam, Cambridge, UK), and anti-CD86 (Santa Cruz Biotechnology, Dallas, Texas, USA).

Then, the tissue sections were washed in PBS and incubated with horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies (UltraTek HRP-Anti-Mouse kit and UltraTek HRP-Anti-Rabbit kit, Scytek Laboratories, Logan, Utah, USA).

Tissue staining was visualized by Diaminobenzidine Tetrahydrochloride (DAB) Substrate Kit (Scytek Laboratories, Logan, Utah, USA), which produced a brown reaction.

The tissue sections were counterstained with haematoxylin, dehydrated, and mounted. As a negative control, the primary antibody was replaced with PBS.

Images were obtained with a Nikon Ni-E microscope (Nikon, Japan) and analysed by a NIS program for image analysis (Nikon, Japan). Sections were analysed in four areas, the same area was analysed for both markers (IL-17A, CD86).

Double-staining immunofluorescence of paraffin-embedded tissue section

The tissue sections were deparaffinized and rehydrated, first with Xylene for 30 minutes, then with graduated doses of alcohol (100%, 96%, 90%, 86% and 75%) and, finally, dosed in distilled water. To allow penetration of the antibody, sections were incubated with TritonX-100 0.1% for 5' and to detect tissue antigens, sections were immersed 10 mM citrate buffer (pH 6.0) and heated in autoclave at 121°C for 15 minutes. After rinsing in PBS, Glycine 0.1M BSA1% Tween 0.3% was used for 20' RT to block nonspecific binding. Tissue sections were incubated with the primary antibody at 4°C in a humidified chamber for 48 hours. The primary antibodies used were anti-IL-17A (abcam, Cambridge, UK), and anti-CD86 (Santa Cruz Biotechnology, Dallas, Texas, USA). Then, the tissue sections were washed in PBS and incubated with CF488A Goat Anti-Rabbit IgG (H+L) (1:200) and CF594 Goat Anti-Mouse IgG (H+L) (1:100) for 1 hour at RT. To decrease autofluorescence was used Vector® TrueVIEW® Autofluorescence Quenching Kit with DAPI. As a negative control, the primary antibody was replaced with PBS.
Images were obtained with a Nikon Ni-E microscope (Nikon, Japan) and analysed by a NIS program for image analysis (Nikon, Japan).

Statistical analysis

Average values and standard deviations (SD) were calculated for each variable under study. All statistical procedures were performed through the software "GraphPad Prism 8" (San Diego, CA, USA). Data were analysed using Student's t-test or one-way ANOVA with a Bonferroni post hoc test.

5) RESULT

Cytotoxicity assay

As previously stated, in the first part of the experiments the cytotoxicity of IL-23 was evaluated by the trypan exclusion assay.

M0, MIFN and MIL-10 were treated with different concentrations of IL-23 (10 ng/ml, 50 ng/ml and 100 ng/ml) without pro-inflammatory stimuli (LPS 10ng/ml).

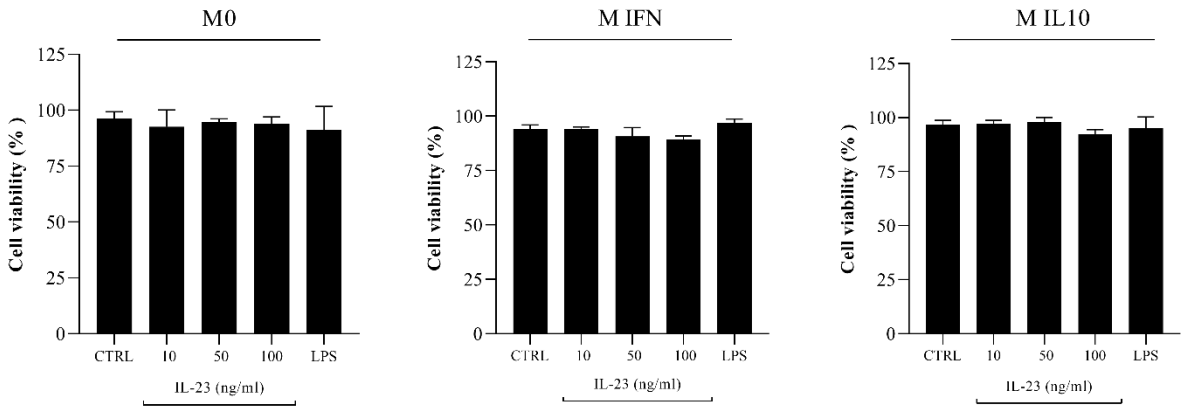


Figure 6

Dose–response curve of cell viability upon exposure to IL-23. The viability of M0, M IFN and M IL-10 cells, evaluated by trypan blue exclusion, was measured (average SD of 3 independent experiments).

Preliminary dose–response experiments were conducted to evaluate the toxicity of IL-23 (Fig. 6) in macrophage populations. None of the IL-23 concentrations affected cell viability. The 100 ng/ml concentration was used to investigate the change in the macrophages' phenotypes and functions.

Expression of cytokines in THP-1-derived macrophage populations by Real-Time PCR

The macrophage populations (M0, MIFN, MIL-4 and MIL-10) obtained from THP-1 cells treated with IL-23 (100ng/ml), with or without pro-inflammatory stimuli (LPS 10 or LPS 200ng/ml and IFNγ 25ng/ml) were examined for the expression of the pro-inflammatory cytokines IL-1β, IL-6

and TNF-α and of the anti-inflammatory cytokine IL-10 by qPCR to evaluate whether there was a change in the gene expression of these molecules.

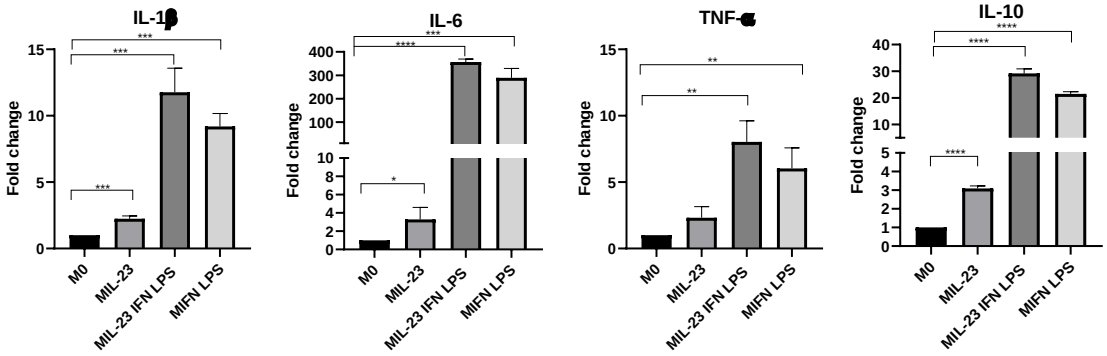


Figure 7

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in THP-1-derived M0 macrophages analysed by qPCR. Cells were treated with IL-23 (100ng/ml) in presence or absence of LPS (200 ng/ml) and IFN-γ (25 ng/ml) for 8 hours. Results are expressed as an average value in fold change vs M0 ± SD of 3 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0.

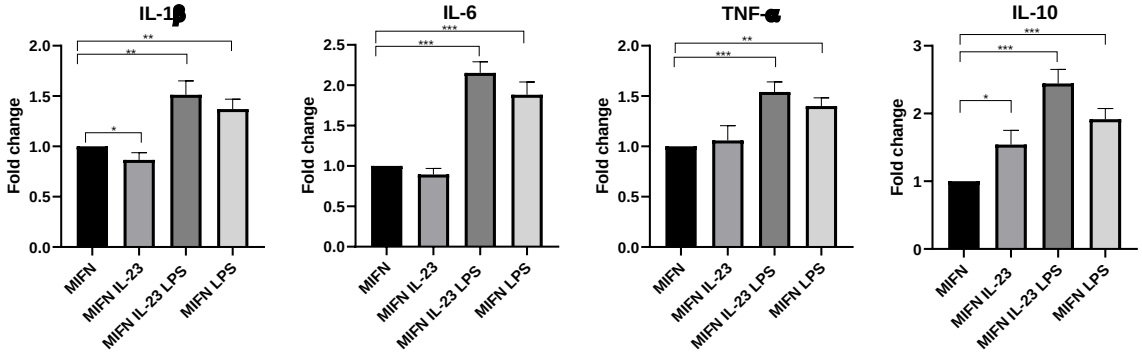


Figure 8

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in THP-1-derived M IFN macrophages analysed by qPCR. Cells were treated with IL-23 (100ng/ml) in presence or absence of LPS (10ng/ml) for 8 hours. Results are expressed as an average value in fold change vs M IFN ± SD of 3 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M IFN.

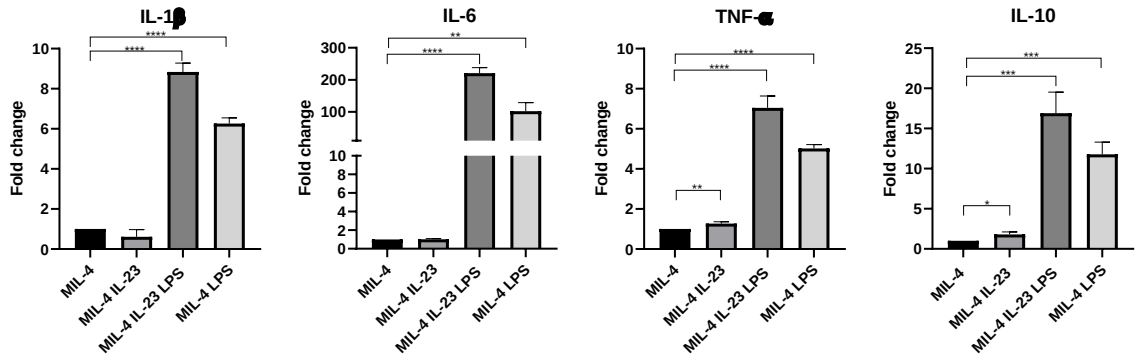


Figure 9

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in THP-1-derived M IL-4 macrophages analysed by qPCR. Cells were treated with IL-23 (100ng/ml) in presence or absence of LPS (10 ng/ml) for 8 hours. Results are expressed as an average value in fold change vs M IL-4 ± SD of 3 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M IL-4.

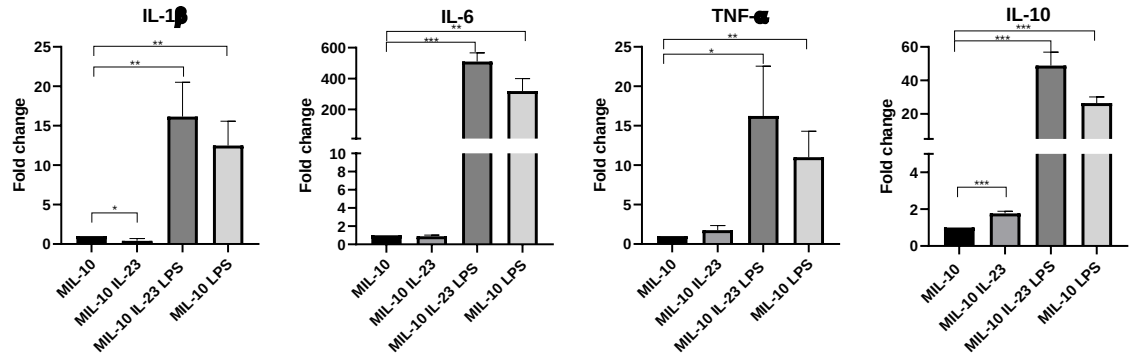


Figure 10

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in THP-1-derived M IL-10 macrophages analysed by qPCR. Cells were treated with IL-23 (100ng/ml) in presence or absence of LPS (10ng/ml) for 8 hours. Results are expressed as an average value in fold change vs M IL-10 ± SD of 3 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M IL-10.

The results presented in figure 7, show that IL-23 alone promotes a pro-inflammatory activation in M0 macrophages. In pro-inflammatory conditions, IL-23 enhances the expression of IL-1β, IL-6, TNF-α and IL-10 levels.

In M IFN macrophages, in figure 8, IL-23 alone induces a significant increase in the expression levels of IL-10, whereas it does not induce any change in the IL-1β, IL-6 and TNF-α expression

levels. In pro-inflammatory conditions, IL-23 significantly increases the IL-1β, IL-6, TNF-α and IL-10 expression levels.

In M IL-4 macrophages, in figure 9, IL-23 alone doesn't induce any change in the expression of the analysed cytokines. In pro-inflammatory conditions, IL-23 increases the IL-1β, IL-6, TNF-α and IL-10 expression levels.

Finally, in M IL-10 macrophages, in figure 10, IL-23 alone doesn't induce any change in the expression levels of IL-1β, IL-6 and TNF-α, whereas it induces a significant increase in the IL-10 expression levels. In pro-inflammatory conditions, IL-23 increases the IL-1β, IL-6 and TNF-α expression levels.

Migration

Macrophages have several physiological functions in order to maintain homeostasis, among these is chemotaxis. Macrophages are guided towards their target location within tissues by a broad range of chemoattractants. For this reason, we evaluated whether IL-23 can also intervene in the recruitment of monocyte-macrophage cells.

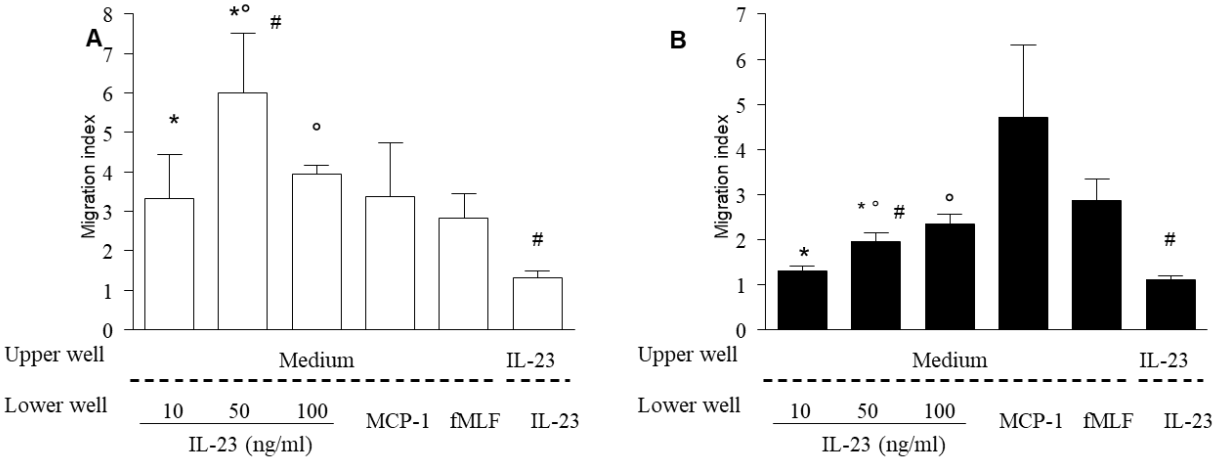


Figure 11

Figure 11 shows the results of the Boyden migration assay of macrophages polarized with IFN-γ (Fig A), and IL-4 (Fig. B).

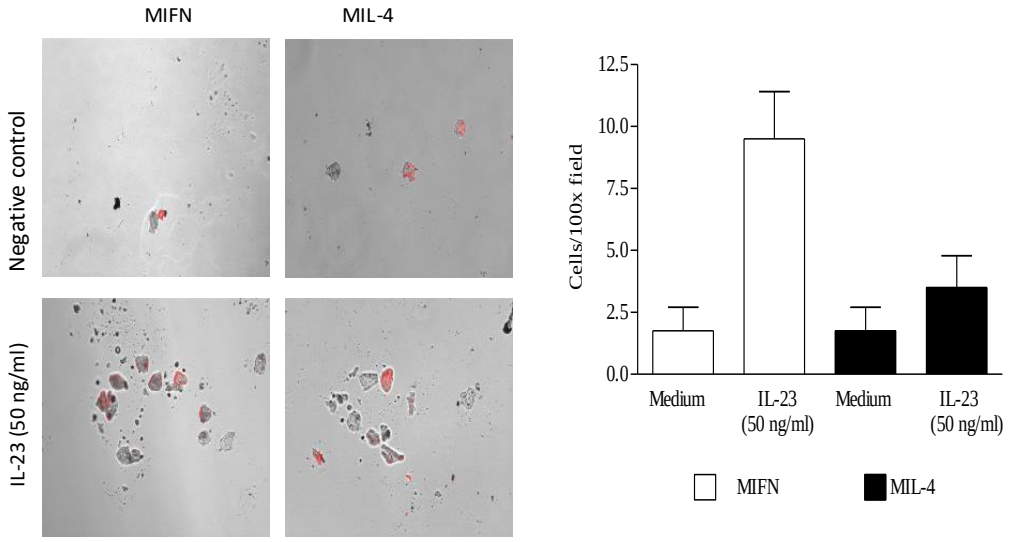


Figure 12

Another counting method was applied to evaluate adherent macrophages. In Figure 12, on the left, cells were stained with 0.2% crystal violet. Then the membranes were dipped into distilled water as many times as needed to remove the excess of crystal violet. The results on the right show the greater migratory capacity of M IFN when stimulated with IL-23 (50 ng/ml) compared to M IL-4.

Pictures of the migrated cells (purple stained) were taken using a microscope with a 10x objective (total magnification 100x). Results showed that the greatest effect was significantly found on MIFN cells exposed to a concentration of IL-23 equal to 50 ng/ml and the value was 50% higher than the effect produced by MCP-1. While in MIL-4 macrophages the chemotactic effect produced by IL-23 appears to be less than 50% compared to the effect produced by MCP-1. So IL-23 acts like a chemokine capable of inducing the chemotaxis of M IFN macrophages.

IL-23 added in increasing concentrations in the lower well of the Boyden chambers (10 to 100 ng / ml of IL-23) induced the migration of human monocyte-derived macrophages with a dose-response bell curve.

Detection of cytokines in human primary monocyte-derived M0, MIFN/LPS and MIL-4 supernatants through ELISA and qRT-PCR

Macrophages M0, MIFN and MIL-4 derived from mononuclear cells (PMCs) obtained from peripheral blood of healthy donors were treated with IL-23 (100ng/ml), with or without pro-inflammatory stimuli (LPS 10ng/ml or LPS 200ng/ml and IFN-γ 25 ng/ml) for 8 hours. We

studied the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the anti-inflammatory IL-10 through sandwich ELISA (DUO-set R&D system) and Real Time PCR.

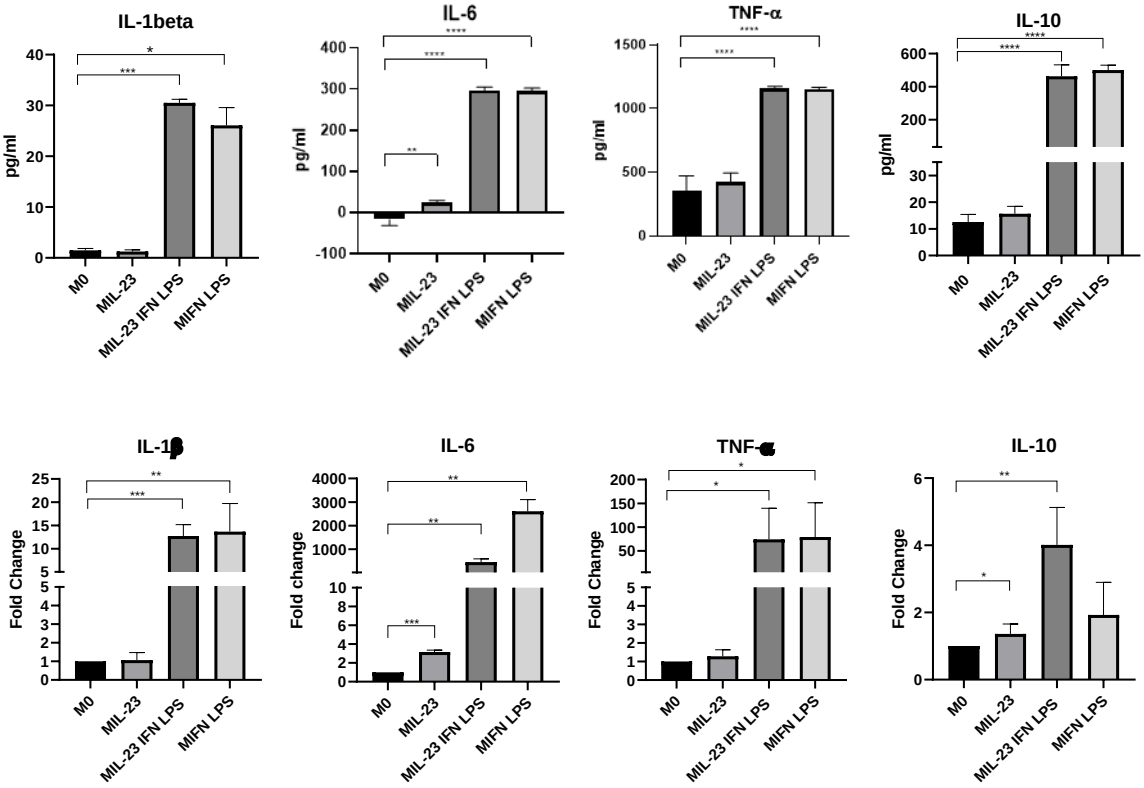


Figure 13

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in human monocyte-derived M0 macrophages analysed by ELISA test and qRT-PCR. Monocyte-derived M0 macrophages were treated with IL-23 (100ng/ml) in presence or absence of LPS (200ng/ml) and IFN-γ (25 ng/ml) for 8 hours. Results are expressed as an average value in pg/ml or fold change vs M0 ± SD of 4 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0.

In M0 macrophages, IL-23 alone induces the expression of IL-6 but doesn't induce any change in the expression of IL-1β, TNF-α and IL-10 levels.
In pro-inflammatory conditions IL-23 doesn't change the expression of IL-1β and TNF-α, but it increases the expression of IL-10 and reduces the expression of IL-6 levels.

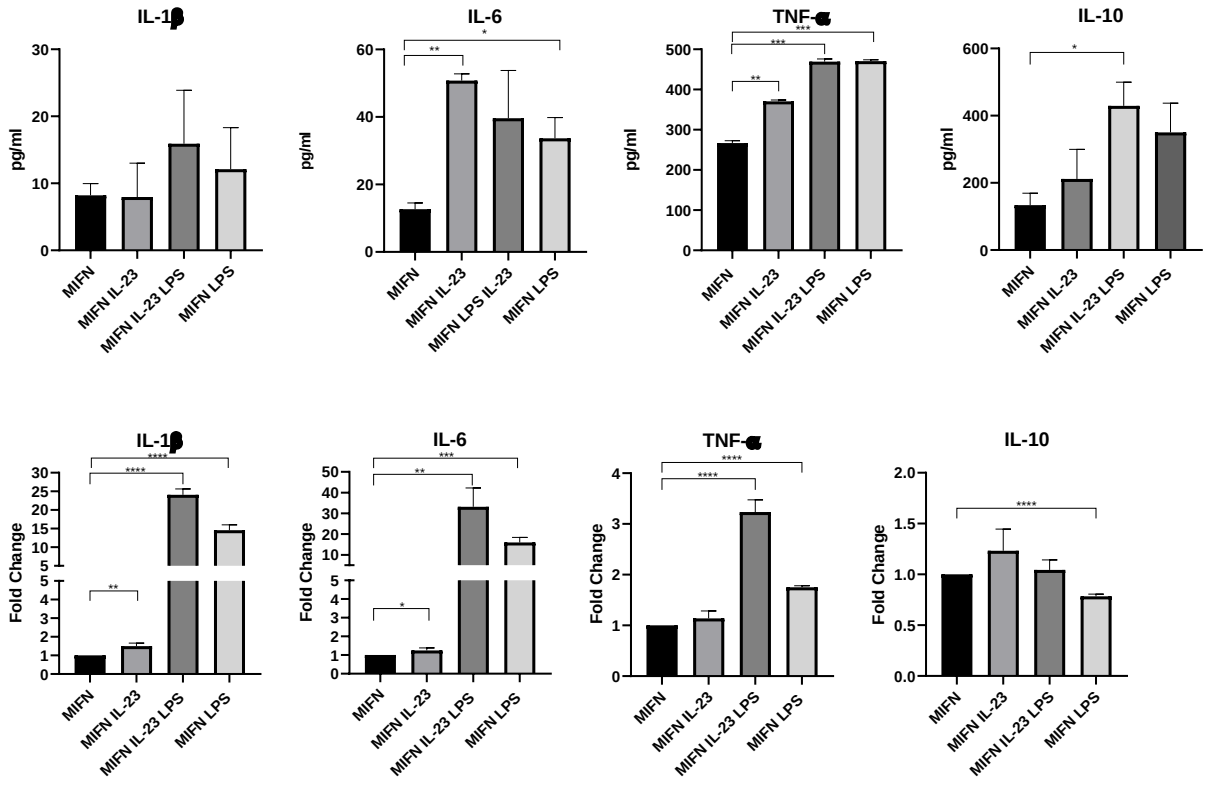


Figure 14

The charts in Figure 14 show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in human monocyte-derived M IFN macrophages analysed by ELISA test and qRT-PCR. Monocyte-derived M0 macrophages were treated with IL-23 (100ng/ml) in presence or absence of LPS (10 ng/ml) for 8 hours. Results are expressed as an average value in pg/ml or fold change vs M IFN ± SD of 4 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M IFN.

In M IFN macrophages, IL-23 alone induces a significant increase in the production of IL-6, whereas it does not induce any significant change in the expression and production of IL-1β, TNF-α and IL-10 levels. In pro-inflammatory conditions, IL-23 increases the IL-1β, IL-6, TNF-α and IL-10 expression levels.

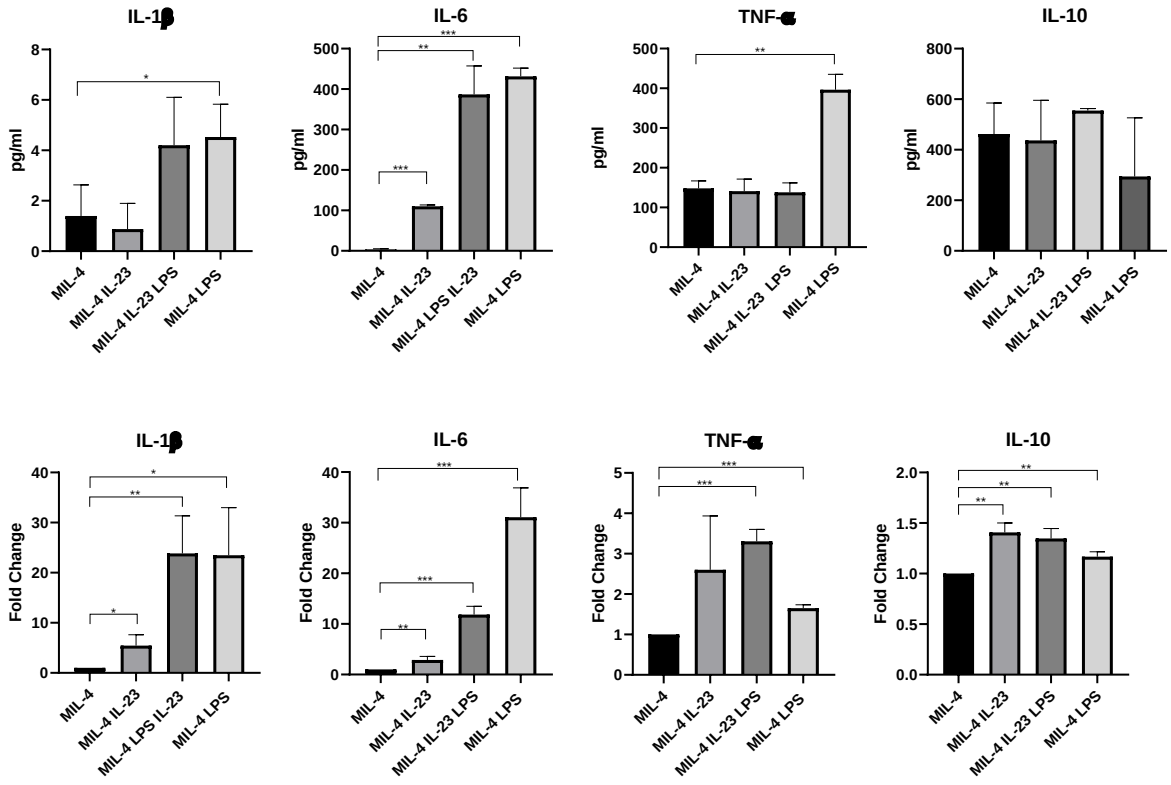


Figure 15

The charts above show the expression of the mRNAs levels of pro- and anti-inflammatory cytokines in human monocyte-derived M IL-4 macrophages analysed by ELISA test and qRT-PCR. Monocyte-derived M0 macrophages were treated with IL-23 (100ng/ml) in presence or absence of LPS (10 ng/ml) for 8 hours. Results are expressed as an average value in pg/ml or fold change vs M IL-4 ± SD of 4 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M IL-4.

In M IL-4 macrophages, IL-23 alone induces an increase in the production and expression of IL-6, whereas it does not induce any change in the expression and production of IL-1β, TNF-α and IL-10 . In pro-inflammatory conditions, IL-23 doesn't change the expression of IL-1β, IL-6, TNF-α and IL-10.

Results obtained by Real-Time PCR and ELISA assay confirmed the previous results in all the subpopulations examined. However, treatment with IL-23 alone was observed to induce a slight increase in the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the M0 subpopulation. This observation and the work on the murine model by Hou and colleagues (Hou Y. et al, 2018), which highlight that IL-23 induces the production of IL-17A, IL-17F, IL-22 and IFN-γ in peritoneal macrophages, brought us to the study of the IL-23/IL-17 axis in M0

macrophages which were more responsive to further stimuli. The basal production of the cytokines IL-17, IL-22 and IL-10 in all M0, M IFN and M IL-4 subpopulations were then analysed by ELISA Tests.

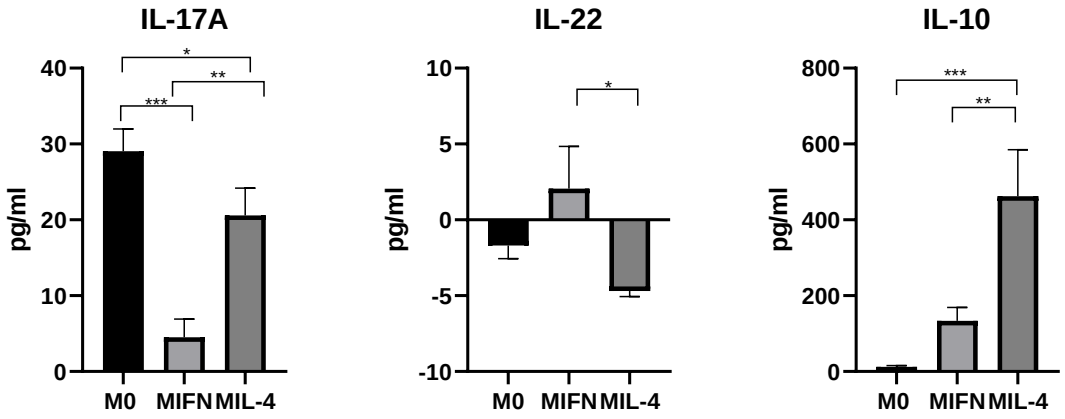


Figure 16

The charts in Figure 16 show the level of IL-17A, IL-22 and IL-10 proteins in human monocyte-derived M0 macrophages, M IFN and M IL-4, evaluated through ELISA Tests. Results are expressed as an average value in pg/ml ± SD of 4 independent experiments. Significance was determined by One-way anova: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Results show that all macrophage populations produce IL-17. However, M0 macrophages produce higher levels of this cytokine. Moreover, M IFN macrophages produce IL-22 while MIL-4 macrophages produce IL-10.

Following the observation of the murine model work by Hou and colleagues (Hou Y. et al, 2018), and based on the results obtained in the baseline population M0, M IFN and M IL-4, we decided to carry out further experiments on the primary monocyte-derived M0 macrophages only. Such experiments were performed through ELISA Tests and qRT-PCR following IL-23 treatment in presence and absence of LPS and IFN pro-inflammatory stimulus. Specifically, the cytokines produced in the IL-23/IL-17 axis were analysed.

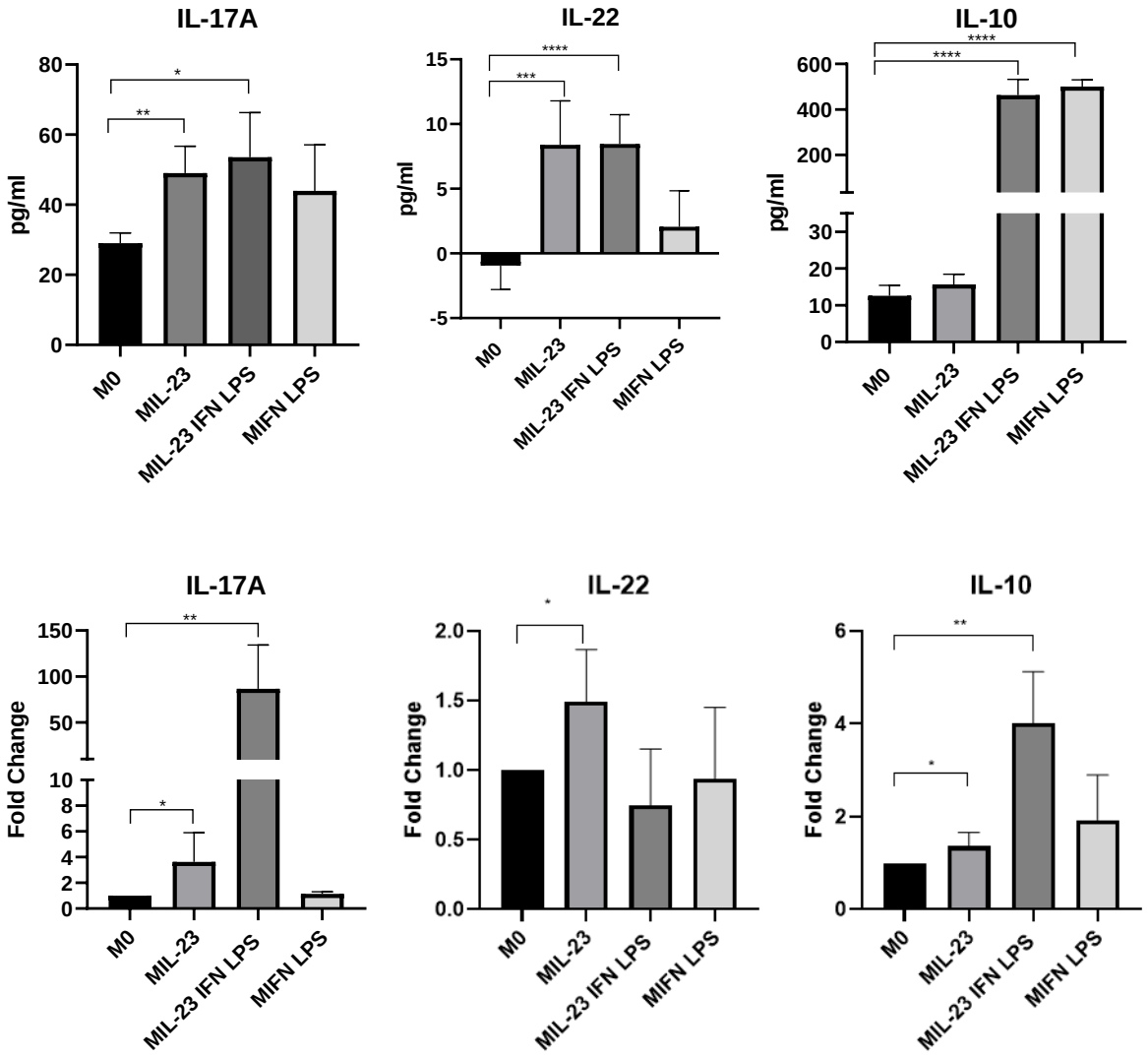


Figure 17

Levels of proteins and expression of mRNAs of IL-17A, IL-22 and IL-10 cytokines in M0 macrophages analysed by ELISA test and qRT-PCR. Monocyte-derived M0 macrophages were treated with IL-23 (100ng/ml) in presence or absence of LPS (200 ng/ml) and IFN-γ (25 ng/ml) for 8 hours. Results are expressed as an average value in pg/ml or fold change vs M0 ± SD of 4 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0.

Results show that IL-23 alone significantly increases the expression and the production of IL-17A and IL-22 levels in M0 macrophages and, in pro-inflammatory conditions IL-23 enhances the levels of IL-17A and IL-10.

At the same time, the transcription factors involved in the IL-23/IL-17 axis were evaluated by qRT-PCR.

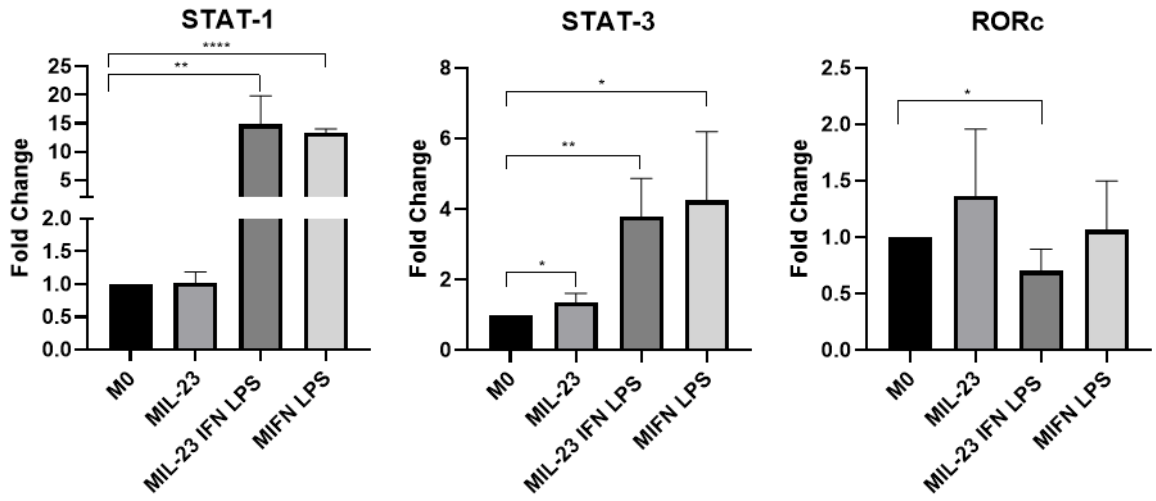


Figure 18

The charts above show the levels of expression of mRNAs of STAT-1, STAT-3 and RORc transcription factors in M0 macrophages analysed by qRT-PCR. Monocyte-derived M0 macrophages were treated with IL-23 (100ng/ml) in presence or absence of LPS (200 ng/ml) and IFN-γ (25 ng/ml) for 8 hours. Results are expressed as an average value in fold change vs M0 ± SD of 4 independent experiments. Significance was determined by Unpaired T test: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0.

Results show that IL-23 alone increases the STAT3 and RORc expression levels in M0 macrophages.

Analysis of surface markers in M0 macrophages by flow cytometry analysis and qRT-PCR

M1 macrophages express the CD86 marker on their surface, while M2 macrophages express the CD206 surface marker.

Studying their expression and their presence allows to classify the phenotype of macrophages produced following the stimulation with IL-23.

The monocyte-derived M0 macrophage population, after treatment with IL-23 (100ng/ml), with or without pro-inflammatory stimuli with LPS (200ng/ml) and IFN (25ng/ml) were evaluated by flow cytometry analysis and qRT-PCR.

Results show that IL-23 alone induces a significant increase of the CD86 surface marker in M0 macrophages. However, it is also possible to observe a slight increase in the expression of the CD206 surface marker.

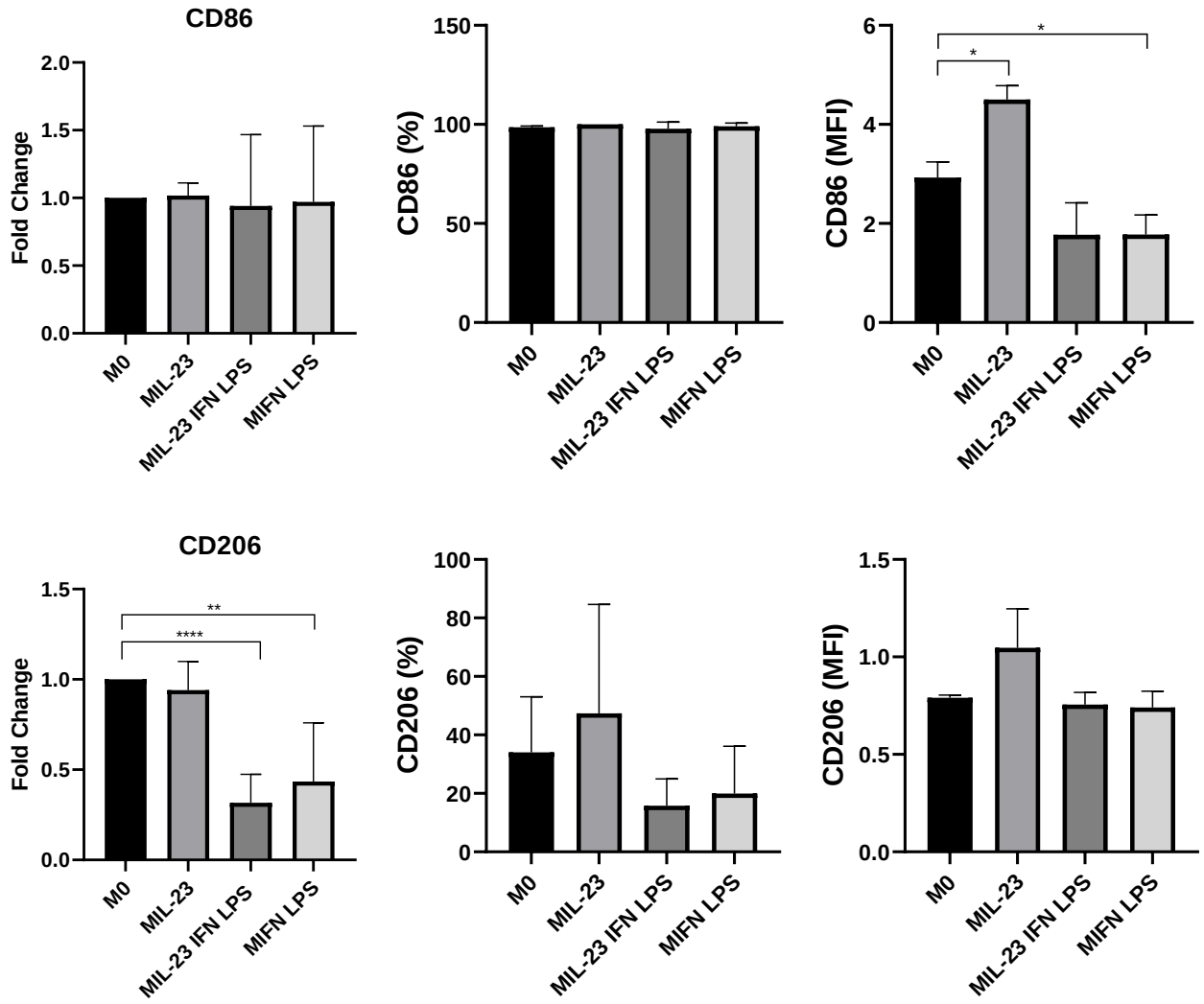
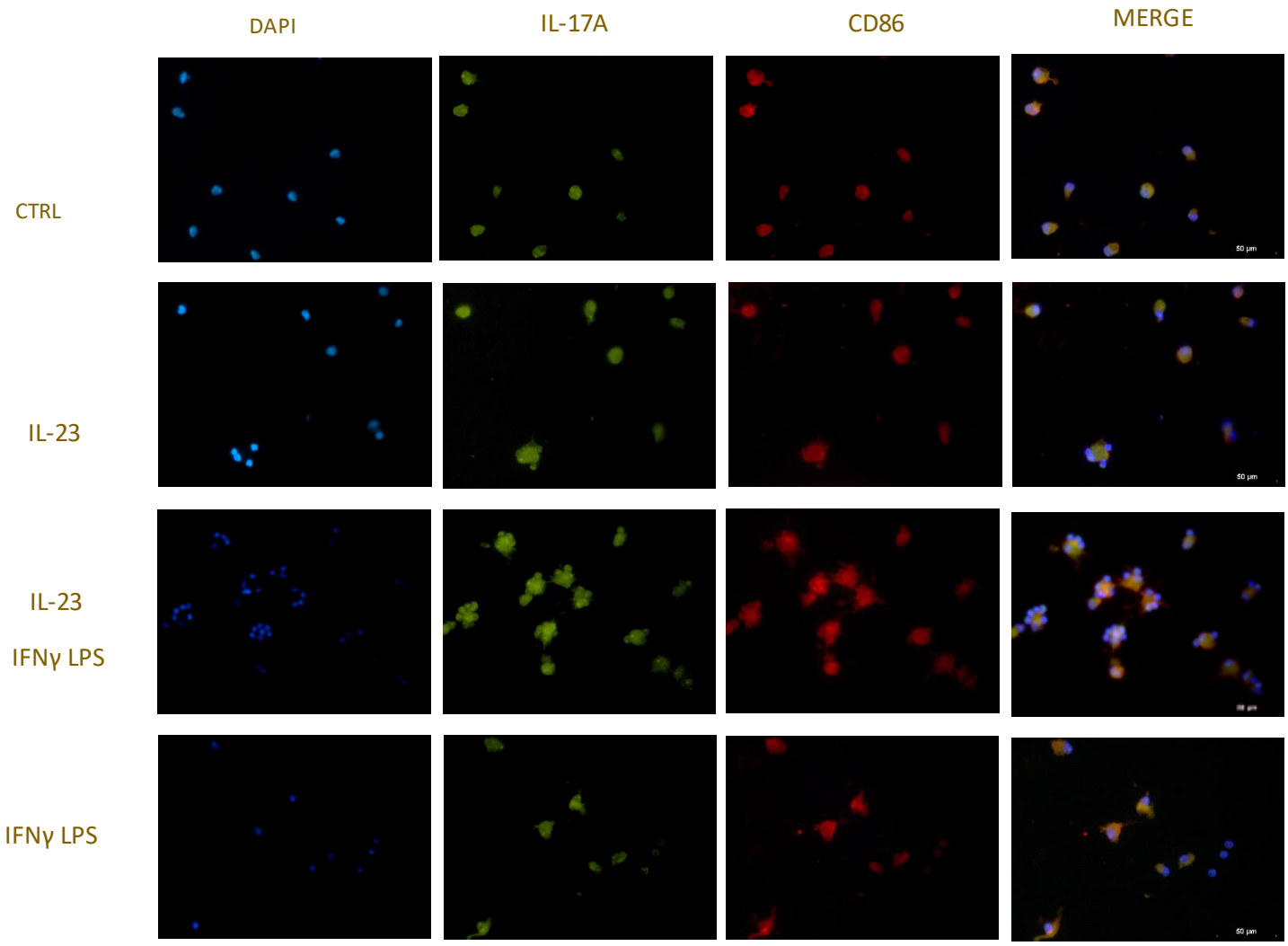


Figure 19

On the left of Figure 19, the levels of expression of CD86 and CD206 are expressed as an average value of fold change vs M0 ± SD of 4 independent experiments. Significance was determined by Unpaired Ttest analysis; *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0. On the right, the flow cytometric analysis of the M1 marker CD86 and M2 marker CD206. Histograms show the percentages of positive cells (%) and the Mean Fluorescence Intensity (MFI). Results are expressed as an average value ± SD of 4 independent experiments. Significance was determined by Unpaired Ttest analysis; *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs M0.

Immunofluorescence staining

To confirm previous results, the amount of IL-17A produced was evaluated by immunofluorescence. Macrophages were treated with IL-23 (100ng/ml) with or without inflammatory stimuli (LPS 200ng/ml and IFNγ 25ng/ml) for 24 hours and analysed for the production of IL-17A and CD-86.



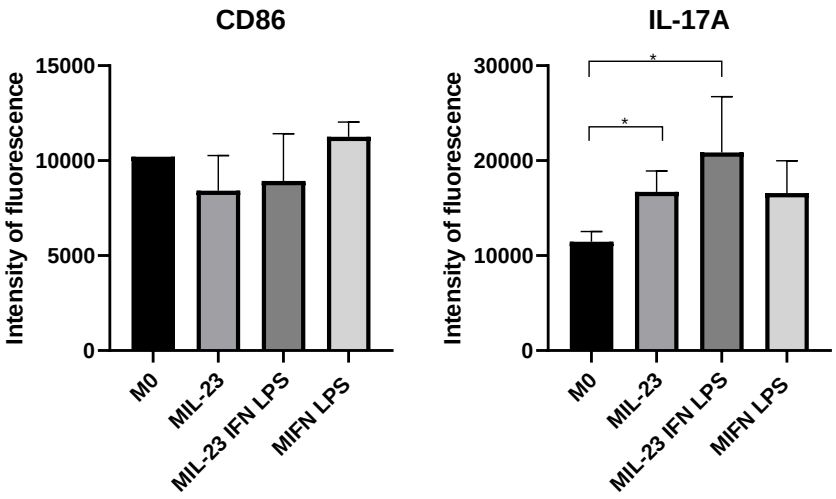


Figure 20

The analysis using double stain immunofluorescence confirmed the previous results and that macrophages produce IL-17A. Macrophages treated with IL-23 increase the production of IL-17A and, in presence of pro-inflammatory stimuli, there is an increase in the release of this cytokine.

Results show that all human macrophages derived from primary monocytes (M0) produce IL-17A at a basal level but, after treatment with IL-23, in presence or absence of pro-inflammatory condition, there is an increase in its production.

We wondered if the cells analysed were influenced by the presence of lymphocytes present in the peripheral blood. To address this question, we carried out a further analysis by immunofluorescence using double staining. The presence of CD86 simultaneously with the presence of IL-17A was analysed.

Different distribution of IL-17A and CD86 macrophages within atherosclerotic lesions

To analyse the presence of IL-17A-producing macrophages, we carried out immunohistochemistry analyses. The histopathological analysis of the plaques was performed according to Stary's histological classification (Stary H.R. et al., 1995). Results showed that the presence or absence of inflammatory infiltrates was evident near the atheroma (Stary H.R., et al., 1995).

Results showed that in complicated plaques there is a higher presence of IL-17A and CD86 than in the stable plaques. Moreover, the absence of CD86 and IL-17A macrophages can be noticed in healthy arteries.

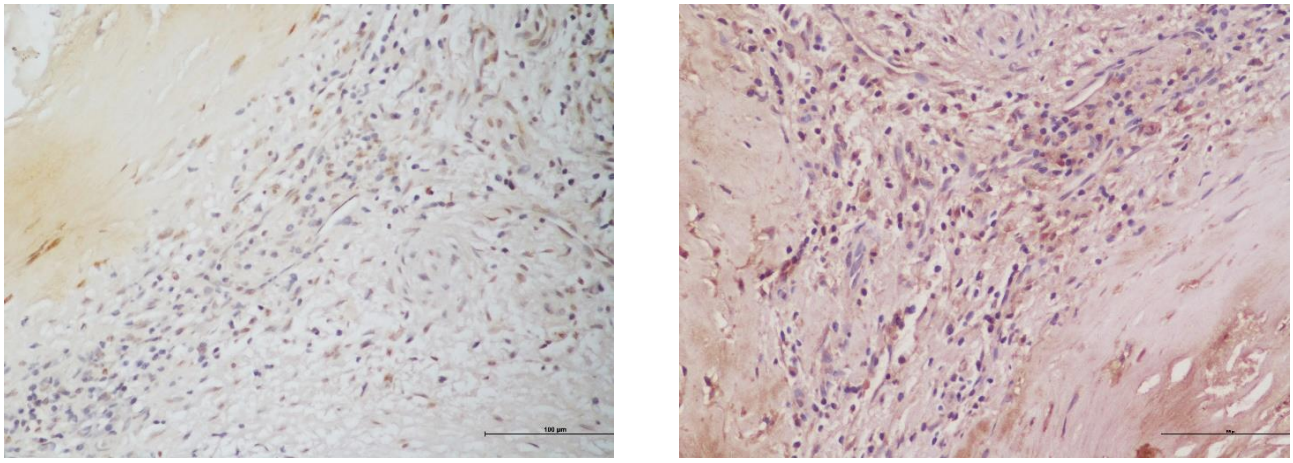


Figure 21

Representative images of immunohistochemistry analysis of CD86 and IL-17A markers on atherosclerotic lesions (complicated plaque) of human carotid arteries are based on immunoperoxidase reactivity. Focusing on the infiltrate of the shoulder, the picture located on the left shows a marked expression of CD86, whereas the right one shows a distinct expression of IL-17A magnification x20.

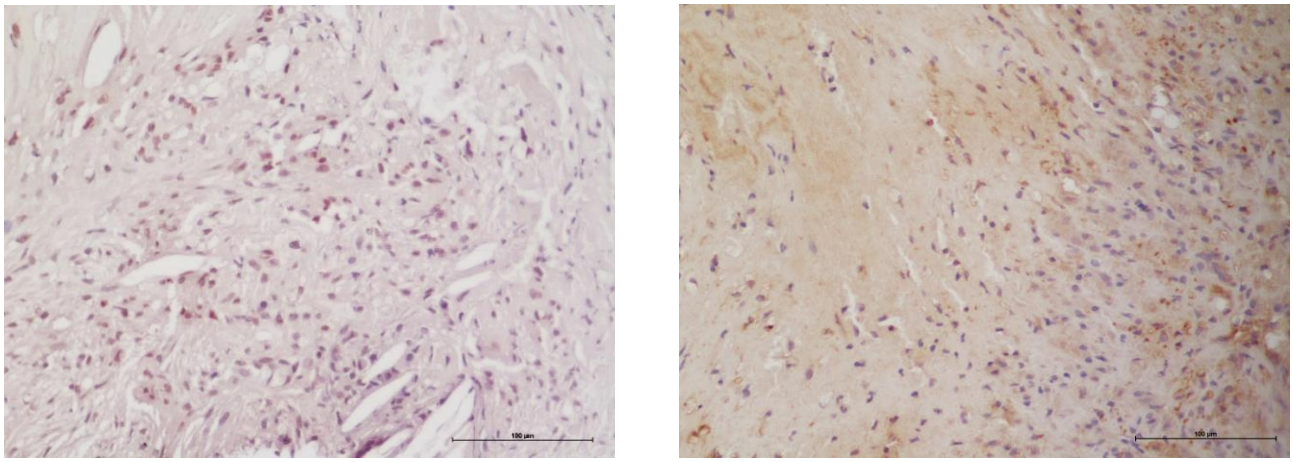


Figure 22

Representative images of immunohistochemistry analysis of CD86 and IL-17A markers on atherosclerotic lesions (complicated plaque) of human carotid arteries are based on immunoperoxidase reactivity. Focusing on the infiltrate of the shoulder, the picture located on the left shows a marked expression of CD86, whereas the right one shows a distinct expression of IL-17A magnification x20.

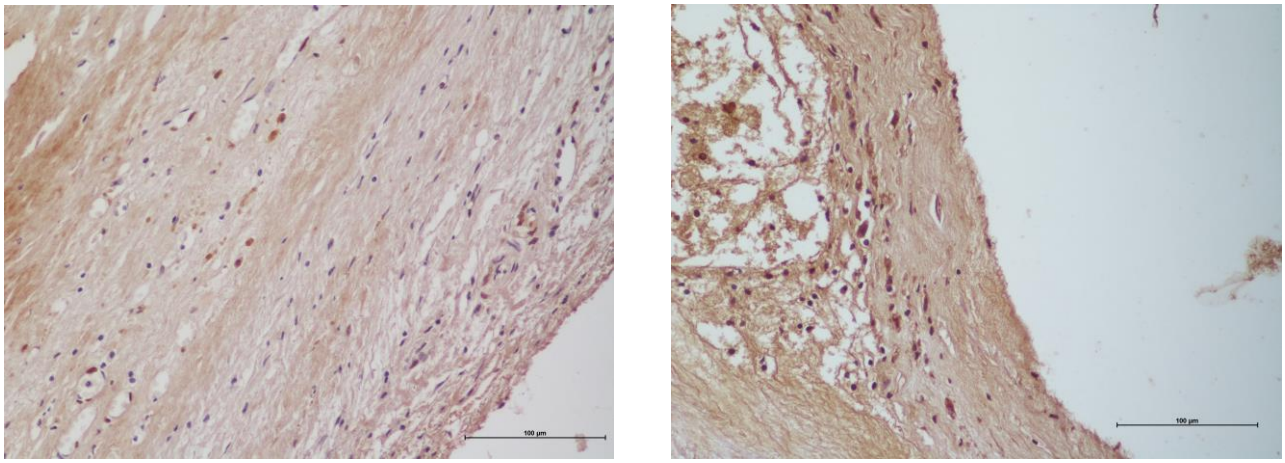


Figure 23

Representative images of immunohistochemistry analysis of CD86 and IL-17A markers on atherosclerotic lesions (stable plaque) of human carotid arteries are based on immunoperoxidase reactivity. Focusing on the infiltrate of the shoulder, the picture located on the left shows a marked expression of CD86, whereas the right one shows a distinct expression of IL-17A magnification x20.

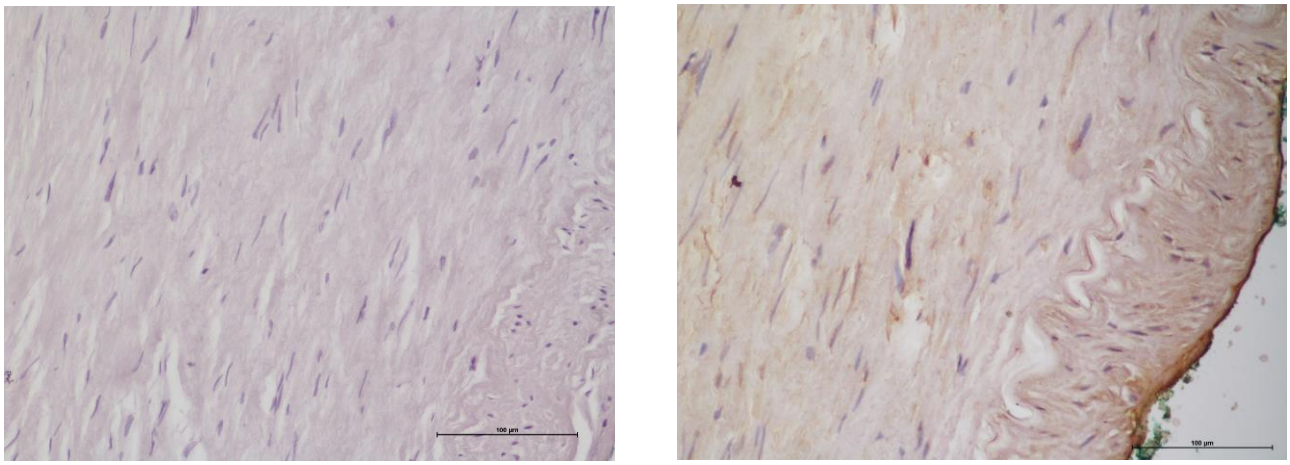


Figure 24

Representative images of immunohistochemistry analysis of CD86 and IL-17A markers on human healthy arteries are based on immunoperoxidase reactivity. The picture located on the left shows a marked expression of CD86, whereas the right one shows a distinct expression of IL-17A Microscopic magnification x20.

Double-staining immunofluorescence of paraffin-embedded tissue section

Expression and co-localization of CD86 and IL-17A were analysed by double-staining immunofluorescence of paraffin-embedded tissue sections. Human carotid atherosclerotic plaques and human healthy arteries were analysed to verify the presence of CD86 and IL-17A. The immunofluorescence analysis confirms the presence of IL-17A into CD86 positive macrophages in atherosclerotic plaques, at the same time the absence of macrophages in the healthy artery.

Pearson's coefficient of colocalization was determined to be 0.738, indicating a strong colocalization of IL17A and CD86 associated reactivity in macrophages.

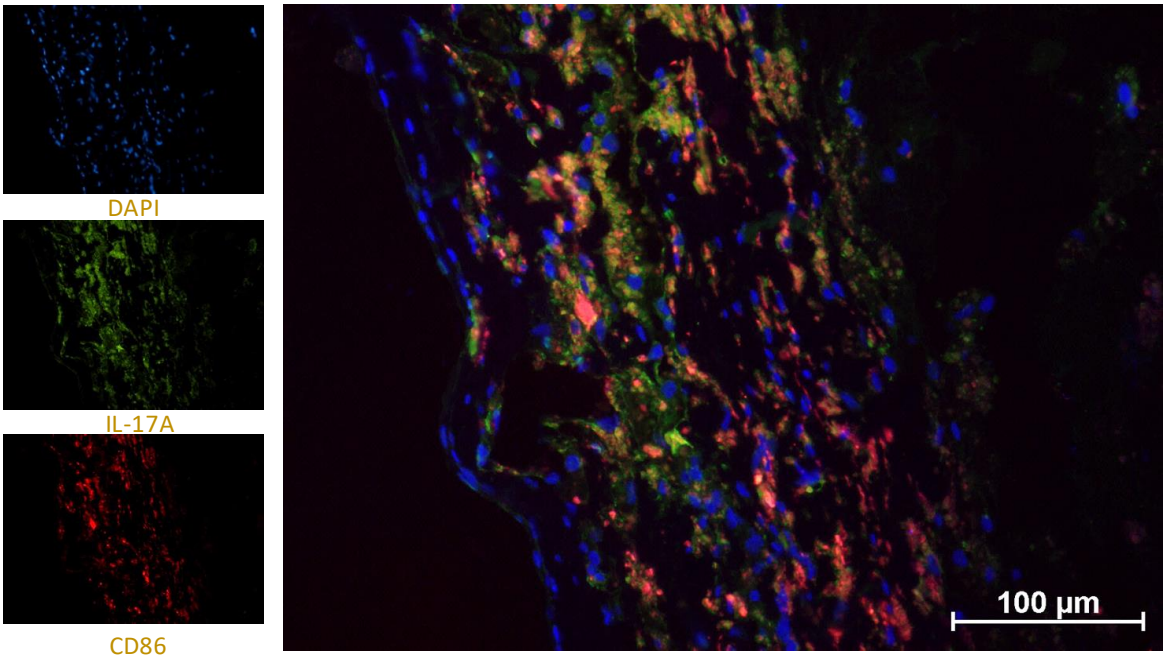


Figure 25

Representative images of immunofluorescence analysis showing CD86 (stained in red) and IL-17A (stained in green) and nuclei counterstained with DAPI (blue) from shoulder region of complicated plaque derived from human carotid artery. The pictures confirm the presence of IL-17A positive macrophages within atherosclerotic plaques at 20× magnification.

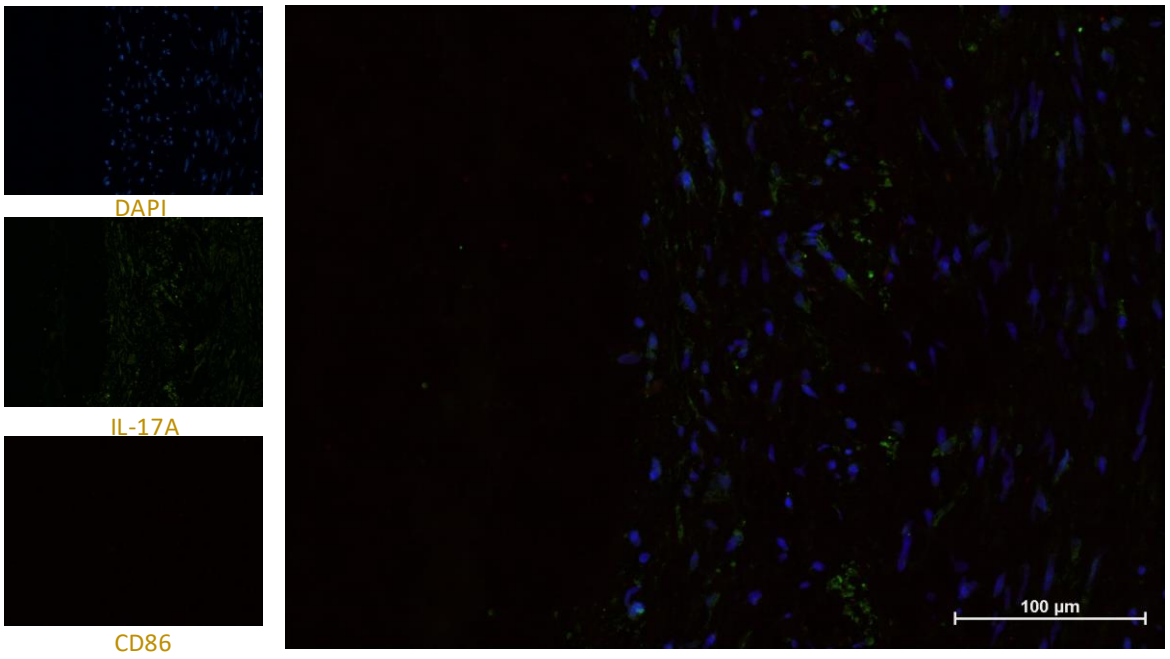


Figure 26

Representative images of immunofluorescence analysis showing CD86 (stained in red), IL-17A (stained in green) and nuclei counterstained with DAPI (blue) on a healthy artery. The pictures confirm the absence of IL-17A positive macrophages in a healthy artery at 20× magnification.

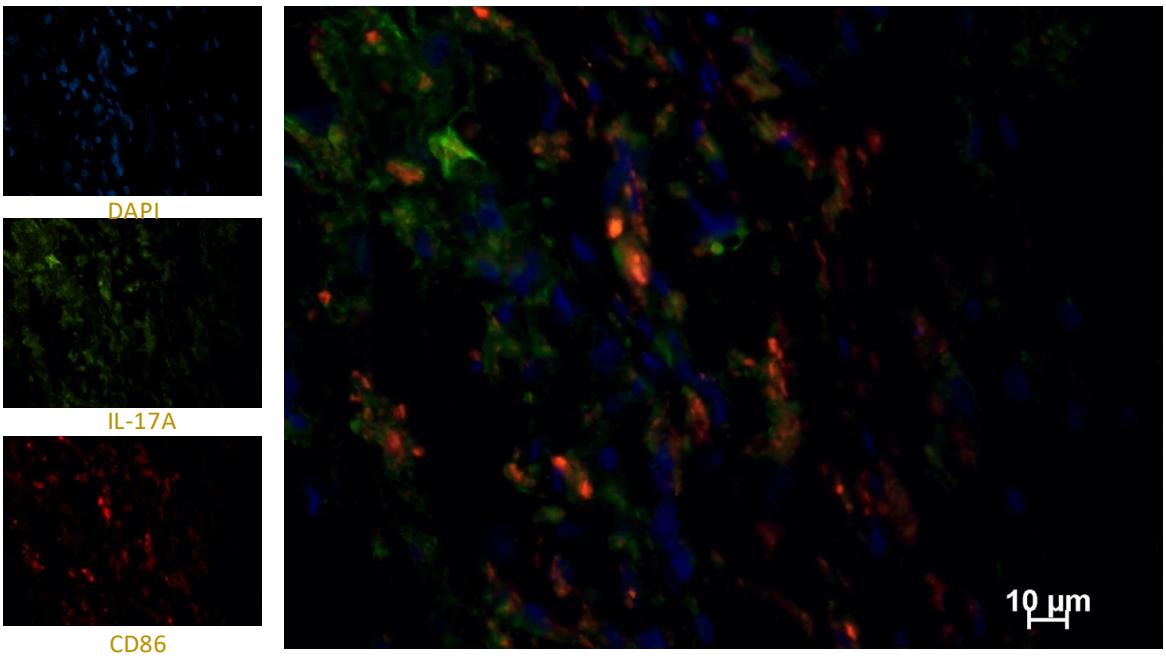


Figure 27

Representative images of immunofluorescence analysis showing CD86 (stained in red) IL-17A (stained in green) and nuclei counterstained with DAPI (blue) from shoulder region of complicated plaque obtained from human carotid artery. The pictures confirm the presence of IL-17A positive macrophages within atherosclerotic plaques at higher magnification images (40×).

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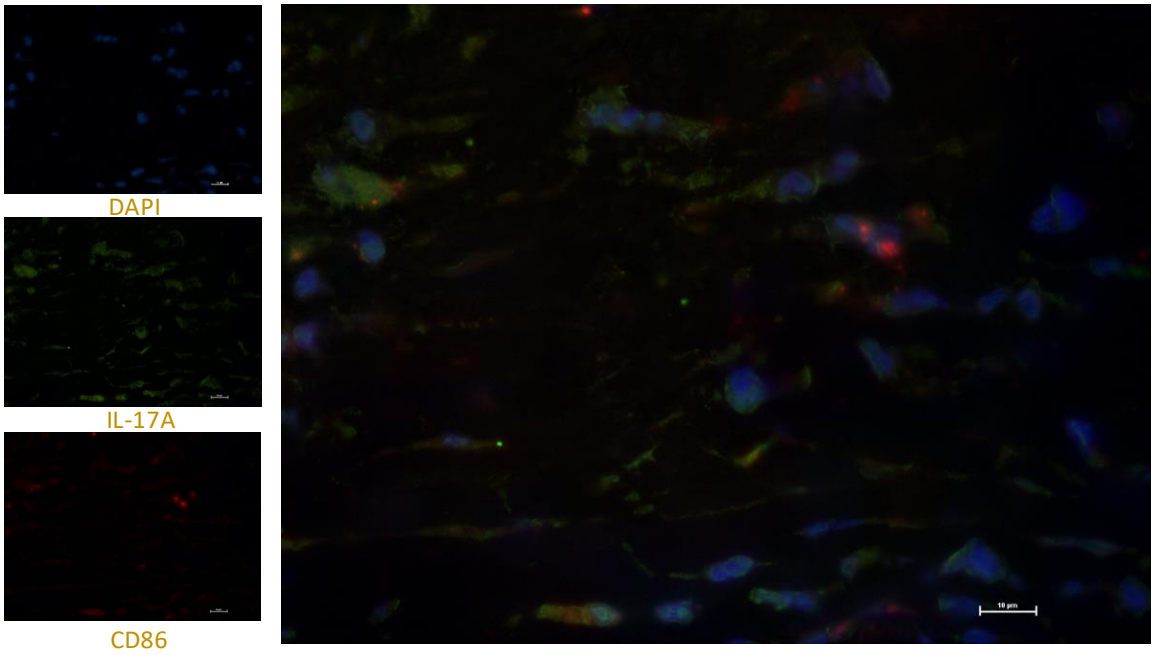

Figure 28

Representative images of immunofluorescence analysis showing CD86 (stained in red) IL-17A (stained in green) and nuclei counterstained with DAPI (blue) from shoulder region of complicated plaque of human carotid artery. The pictures confirm the presence of IL-17A positive macrophages within atherosclerotic plaques at higher magnification images (60×).

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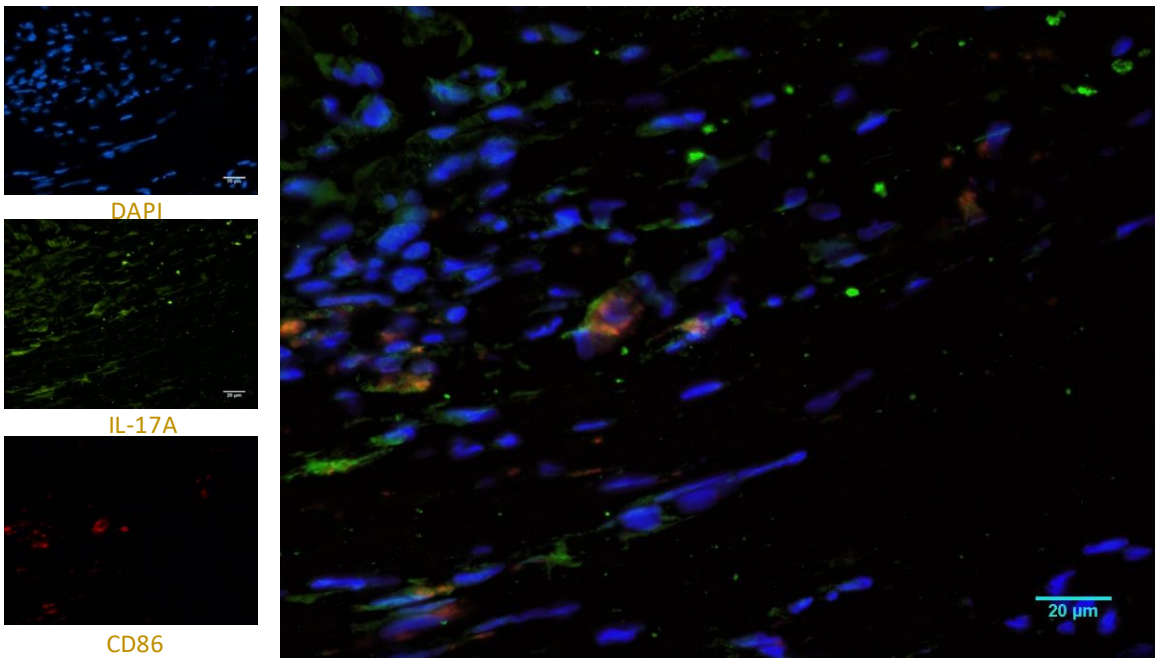

Figure 29

Representative images of immunofluorescence analysis showing CD86 (stained in red) IL-17A (stained in green) and nuclei counterstained with DAPI (blue) from shoulder region of complicated plaque of human carotid artery. The pictures confirm the presence of IL-17A positive macrophages within atherosclerotic plaques at higher magnification images (40×).

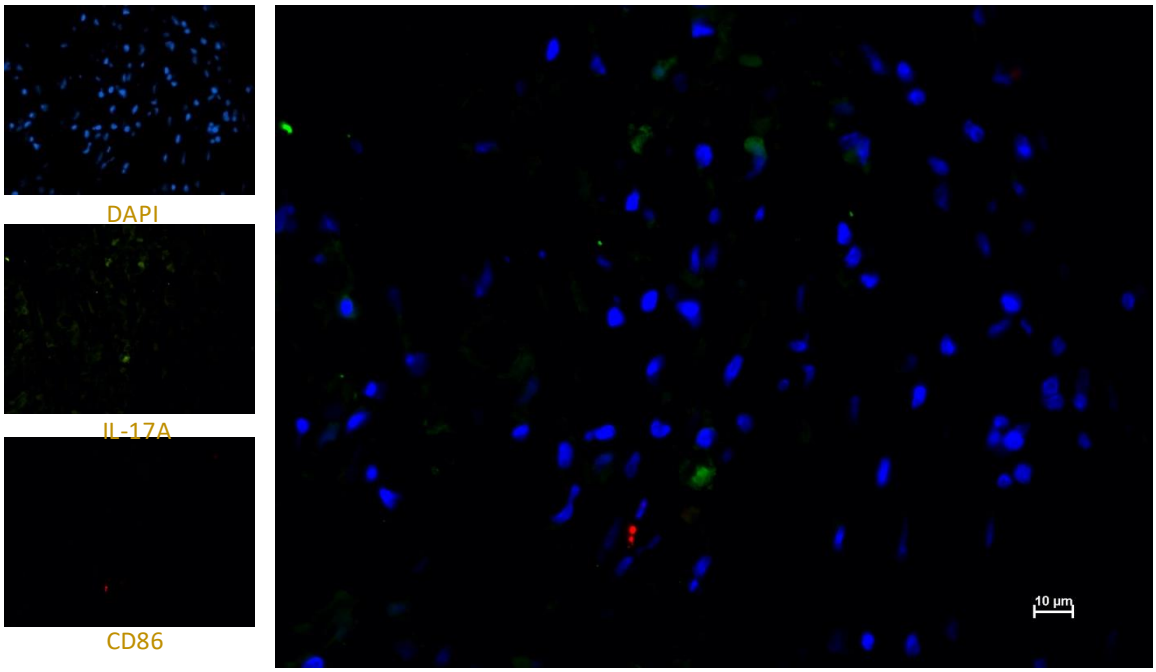

Figure 30

Representative images of immunofluorescence analysis showing CD86 (stained in red) IL-17A (stained in green) and nuclei counterstained with DAPI (blue) on a healthy artery. The pictures confirm the absence of IL-17A positive macrophages in a healthy artery at 40× magnification.

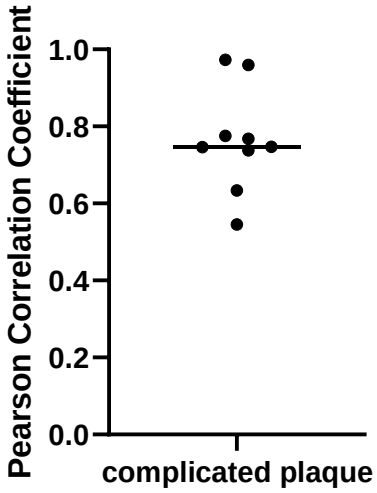

Figure 31

Pearson's Correlation Coefficient between IL-17A and CD86 in the human atherosclerotic plaques with $n \geq 3$ sections.

6) DISCUSSION

Atherosclerosis is a chronic inflammatory immune-mediated disease of the arterial wall. Macrophage cells, namely a type of innate immune cells, play an important role in the progression and regression of such pathology. As mentioned above, macrophages are heterogeneous and their phenotype and functions are regulated by the surrounding micro-environment. Indeed, thanks to their high plasticity, they can produce a specific response based on the stimuli, polarizing in the direction of "classically activated" M1 or "alternatively activated" M2 (the latter have further subtypes M2a, M2b, M2c) (Shapouri-Moghaddam A. et al., 2018; Rojas J. Et al., 2015). Cytokines are soluble mediators which are relevant in the polarization process of macrophages. The study of cytokines and their role in the pathogenesis of atherosclerosis has suggested that designing new drugs, specifically acting on the cytokines, could modify the course of the disease. In particular, the role of the IL-23 cytokine in the pathogenesis of atherosclerosis is still not clear, although Abbas A. et al., 2015 have demonstrated the increase of IL-23 and IL-23R in human atherosclerotic plaques (Abbas A. et al., 2015). Investigating the activity of this cytokine at the level of atherosclerotic lesions is the main aim of this thesis. As a first step, the toxicity of IL-23 was evaluated. Initial results on its cytotoxicity showed that none of the concentrations of IL-23 tested induced toxicity. In subsequent analyses, the role of IL-23 in the polarisation of M0, M1, M2a and M2c macrophages was evaluated. Specifically, the expression of pro-inflammatory and anti-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IL-10, in macrophages derived from THP-1 cell line models was analysed by Real-Time PCR. Our results showed that IL-23 promotes the induction of the expression of pro-inflammatory cytokines (IL-1ß, IL-6 and TNF-α) in the M0 population only. This suggests that IL-23 activates M0 population, not yet polarized and, hence, more responsive to stimuli. A study by Cua et al., highlighted that IL-23, unlike IL-12, induces the mRNA expression of IL-1β and TNF-α (Cua D.J. et al., 2003), in a murine model of EAE, confirming the pro-inflammatory activity of IL-23. This pro-inflammatory activity may be due to the activation of the NF-KB signaling pathway. Indeed, several studies have demonstrated that the interaction of IL-23 with its receptor activates NF-KB in other cell types (Li X. et al., 2010; Ju J.H. et al., 2008; Stato K. et al., 2006; Sutton C. et al., 2006). As a consequence, NF-KB induces the expression of cytokines, chemokines, adhesion molecules and anti-apoptotic factors and is a central mediator of NLRP3 inflammasome activation. This transcription factor plays an important role in various inflammatory responses and promotes transcription of pro-inflammatory

cytokines such as PGE2-induced TNF-α or IL-1β which are released after the activation of the NLRP3 inflammasome. IL-23 did not induce any variation in the expression of pro-inflammatory cytokines in the other macrophage subpopulations examined since they are already differentiated and this stimulus alone is probably not sufficient to affect their polarization. However, our results highlighted that IL-23 induces an increase in the expression of IL-10 in all populations examined. Indeed, Sun R. et al., 2020 showed that IL-23 directly activates macrophages and induces the expression of pro-inflammatory cytokines typical of the M1 phenotype as well as anti-inflammatory cytokines such as IL-10 and TGF-β, suggesting the induction of a mixed phenotype (Sun R. et al., 2020). The production of IL-10, in particular, could be due to an activation of STAT3. Indeed, although, the molecular mechanisms regulating the expression of IL-10 remain unknown, a study carried out on cord blood CD4+ and CD8+ T cells showed that IL-23 induces the production of IL-10 and IL-17 in naïve T cells. The same study also observed that IL-23 induces an up-regulation of IL-10 and that this mechanism was mediated by STAT3 (Vanden Eijnden S. et al., 2005). According to results obtained in pro-inflammatory conditions (in presence of LPS or LPS and IFN-γ), IL-23 increases the expression of IL-1β, IL-6 and TNF-α and IL-10 levels in all macrophage phenotypes, suggesting that IL-23 has a synergistic action with LPS by increasing inflammation.

Not the same results were obtained in monocyte derived macrophages coming from healthy donors. Indeed, IL-23 induced the expression of mRNA and release of IL-6 in all subpopulations examined. As already known, IL-23 drives the differentiation of Th17 lymphocytes which, in turn, produce IL-6 and the same release has also been confirmed in macrophages and dendritic cells (Iwakura Y. et al., H. 2006). Such cytokine which is regulated by various transcription factors, including AP1, C/EBP and the nuclear factor (NF)-κB (Xiao W. et., al 2004), is known as "hormone of the immune system" since it has the ability to induce systemic changes in the affected endothelium by modulating the expression of ICAM-1, VCAM-1 and E selectin and, consequently, to enhance the adhesion of leukocytes to the endothelium (Hirano T. et.al, 1998). While IL-23 increased the expression of IL-1β, IL-6, TNF-α and IL-10 in the M1 macrophage phenotype in pro-inflammatory conditions, as previously seen, in the other subpopulations this synergism was not observed. The variation in the results obtained by testing human monocyte derived macrophages compared to the ones regarding the THP-1 derived macrophages may be attributable to the use of circulating monocytes derived from healthy donors who may show

individual variability. It should also be noted that, although the THP-1 cell line model is a reproducible model of human macrophages, there is evidence that the expression of several cytokines may be different (Tedesco S. et al., 2018). Chronic inflammation is essential for the development and progression of atherosclerosis, and the migration of monocytes and macrophages to sites of inflammation appear to play an important role in the development of this disease. Macrophages are guided to target sites within tissues by a variety of chemoattractants. The results obtained from the migration assays highlighted that IL-23 appeared to induce migration in macrophages and this effect was greater in M IFN macrophages than in M IL-4 macrophages, suggesting a chemotactic function in M IFN macrophages. It is widely known that IL-23 binds with its membrane receptor IL-23R which is expressed into macrophages (Mezghiche I. et al., 2023; Leitner M. at al.,2021; Liu Z. et al., 2011) and that the macrophages are localized in the atherosclerotic plaque (Abbas A. et al., 2015), hence producing the migration of the cells. Moreover, studies have shown that IL-23 drives the migration of Tγδ17 and Th17 cells and that this process occurs through the activation of ROCK. IL-23 phosphorylates myosin regulatory light chain (RLC) following the catalytic activity of JAK2 and ROCK (Álvarez-Salamero C., et al., 2020). IFN-γ, unlike LPS, induces the expression of IL-23R while LPS remarkably reduces the expression of IL-12 β1 on macrophage cells, thus suggesting the possibility that IFN-γ polarized macrophages are more responsive to IL-23, since they own an higher number of receptors (Parham C. et al., 2002).

The role of IL-23 in inducing the production of IL-17A, IL-17F, IL-22 and IFN-γ in peritoneal macrophages was highlighted (Hou Y. et al, 2018). Consequently, we focused on the IL-23 pathway and, specifically, on the IL-23/IL-17 axis. We analysed the production of IL-17A, IL-22 and IL-10 in M IFN, M IL-4 and M0 macrophages by ELISA tests. We showed that all ~~the~~ macrophages surprisingly produced IL-17A. However, M0 macrophages represented the major source of production. As is known, M IL-4 macrophages are the major producers of IL-10, although there are M IFN macrophages that produce IL-10, although in smaller quantities. Studies have shown that low doses of LPS induce an increase in IL-10 expression (Matsumara N et al., 2012; Ruma A. et al., 2006). This slight production has been known for some time now and numerous researches have shown that IL-10 is produced by the stimulation of TLRs. This anti-inflammatory cytokine plays an important role in balancing the inflammatory response and avoiding the establishment of a chronic infection. Its production, mediated by the p38 pathway,

occurs shortly after an LPS stimulus and, at the same time, its degradation is highly regulated (Ernest O. et al., 2019; Teixeira-Coelho M. et al., 2014).

Based on these results, our focus was redirected towards M0 macrophages. The expression and production of cytokines involved in the IL-23/IL-17 axis were then analysed in monocyte-derived macrophages from healthy donors. Results showed that IL-23 induces an increase in the expression of IL-17A in M0 macrophages, thus suggesting that IL-23 binds with its receptor present in macrophages, although to minor extent compared to other immune cells, and that this interaction activates the JAK2/STAT3 signaling pathway. IL-17A was discovered in the Th population, such as Th17, but is also produced by other types of cells: γδ T cells, CD8+ cytotoxic T cells, innate tissue-specific cells, innate lymphoid cells (ILC) and myeloid cells. The ability of IL-23 to induce IL-17 production in macrophages has also been observed in studies showing the involvement of IL-17 in allergic asthma (Song C. et al., 2008). Furthermore, our results showed that IL-23, in association with LPS and IFN-γ, potentiates the expression and release of IL-17A and IL-10 suggesting that IL-23 has a synergistic activity with LPS. These data were also confirmed by immunofluorescence analysis which confirmed the production of IL-17A in the M0 population and that the treatment with IL-23 increased its production. The immunofluorescence analysis, by double staining, did not only allow to confirm the production of IL-17A but also to verify if a lymphocyte contamination had occurred. The interaction of IL-23 with its receptor activates JAK2 which, in turn, activates and phosphorylates STAT3, inducing the migration of the same transcription factor to the nucleus and the upregulation of the expression of RORγ-t which is a key factor in the production of IL-17A. Indeed, our results confirmed that IL-23 induces a slight expression of STAT3 and the expression of RORc while it does not induce the expression of STAT1.

M1 and M2 macrophages markers have been described in depth (Chistiakov D.A. et al., 2019; Buechler C., et al., 2000; Högger P., et al., 1998, Stöger J.L., et al., 2012). M1 macrophages express surface markers such as CD86, while M2 macrophages express surface markers such as CD206 (Gröger M., et al., 2000; Law SKA., et al., 1993). The surface marker analysis showed that IL-23 induced an unclear phenotype in M0 macrophages. Indeed, the stimulus with IL-23 increased both M1 and M2 markers. However, the surface marker CD86 appeared to be prevalent. Double treatment with LPS and IFN-γ down-regulated the expression of CD86 markers and this effect could be due to an excessively strong stimulation coming from these two molecules.

Immunohistochemistry analyzed the presence of macrophages in stable or complicated plaques. In the case of healthy arteries, the majority of macrophages are found in the adventitia while the intima, the site where atheromatous plaques develop, hosts a smaller proportion of all macrophages and this presence decreases with age. Previous studies have shown that M1 macrophages dominate the region of the shoulder which is prone to plaque rupture and that there is a greater presence of activated M2 macrophages in the vascular adventitial tissue (Lin P, et al., 2021; Stöger J.L. et al., 2012). Taleb et al. (2019) have demonstrated the presence of IL-17A not only in the media but also in the adventitia of normal and atherosclerotic vessels (Taleb S. et al., 2019). In addition, a study by Erbel and collaborators demonstrated that IL-17A blocking prevented the progression of atherosclerosis in murine models by increasing plaque stability (Erbel C. et al., 2011).

Our results also confirmed the presence of IL-17A in atherosclerotic plaques, especially in the complicated ones. Moreover, by carrying out double-staining immunofluorescence on paraffin-embedded tissue sections we also highlighted that some macrophages stain positive for IL-17A.

7) CONCLUSION

Our study deals with the role of IL-23 in human atherosclerotic plaques, mainly focusing on macrophage subpopulations. Our results showed that IL-23 promotes the induction of the pro-inflammatory cytokines (IL-1ß, IL-6 and TNF-α) in the M0 macrophage population only, suggesting a pro-inflammatory activity. IL-23 did not induce any variation in the expression of pro-inflammatory cytokines in the other subpopulations examined since they are already differentiated and, probably, this stimulus alone is not sufficient to polarize them. But, at the same time, IL-23 induced an increase in the expression of IL-10 in all populations studied. In pro-inflammatory conditions (LPS), IL-23 increased the expression of IL-1β, IL-6 and TNF-α and IL-10 in all macrophage phenotypes, suggesting that IL-23 had a synergistic action with LPS by increasing inflammation. Analyzing macrophages derived from monocytes coming from healthy donors, IL-23 induced the expression and release of IL-6 in all subpopulations examined. In pro-inflammatory conditions, IL-23 increased the expression of IL-1β, IL-6, TNF-α and IL-10 in M IFN macrophages while, in the other subpopulations, this synergism was not observed. At the same time, IL-23 appeared to induce migration in M IFN macrophages, suggesting a chemotactic function. When focusing on the IL-23/IL-17 axis, we noticed that M0 macrophages surprisingly produced IL-17A and M IFN macrophages were the only ones that produced IL-22. However, IL-23 induced an increase in the expression and consequent production of IL-17A, IL-22 and IL-10 in M0 macrophages and the association with LPS and IFN-γ potentiated the expression and release of IL-17A and IL-10, suggesting that LPS had a synergistic action with IL-23. However, observing the phenotype induced by IL-23, the data showed that the stimulus with IL-23 increased both M1 and M2 markers. However, the surface marker CD86 appeared to be prevalent. Immunohistochemistry confirmed the presence of IL-17A within atherosclerotic plaques but also highlighted that some macrophages produce IL-17A.

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