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POLARIZATION PATTERNS OF HUMAN MONOCYTE-DERIVED MACROPHAGES IN RELATION TO ESTROGEN TREATMENT AND MENOPAUSAL STATUS

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RIASSUNTO

I fagociti mononucleari rispondono a stimoli ambientali acquisendo distinti fenotipi funzionali, definiti M1 (classico) o M2 (alternativo), che caratterizzano a loro volta differenti condizioni patologiche. Il 17β-estradiolo (E_2), il principale ormone del sistema riproduttivo femminile, media molteplici effetti sulla funzione immunitaria di monociti e macrofagi, agendo attraverso i recettori per gli estrogeni (ER). Ipotizziamo, dunque, che gli effetti dipendenti dagli estrogeni a carico del sistema monocito-macrofagico proteggano la donna in post-menopausa da disturbi cardiovascolari, ed, in particolare, che l'aggravarsi del rischio cardiometabolico nelle donne in post-menopausa sia associato ad uno *shift* della popolazione monocito-macrofagica verso un fenotipo infiammatorio. Pertanto, in questa tesi abbiamo investigato gli effetti dell'E_2 su macrofagi derivanti da monociti umani in condizioni basali (M0) ed in seguito ad attivazione polarizzata classica/M1 o alternativa/M2.

Partendo da sacche di *buffy coat* è stato possibile isolare i linfo-monociti tramite separazione in base alla densità degli emocomponenti, ed il differenziamento da monociti a macrofagi è avvenuto nell'arco di 7 giorni in assenza di agenti polarizzanti diversi dal siero. Abbiamo inizialmente dimostrato che i macrofagi ottenuti da differenziamento spontaneo polarizzano verso il fenotipo M1 ed M2 in seguito a 48h di stimolo rispettivamente con LPS/IFN-γ o IL-4/IL-13. Le cellule polarizzate sono caratterizzate da uno specifico profilo di espressione genica, la produzione di diverse citochine (TNFα, IL-1β, IL-10, CCL22) e l'espressione di specifici marcatori di superficie. In particolare, il fenotipo M1 è stato identificato come $CD68^+$, $CD68^+/CCR2^+$, $CD14^+/CD16^-/CD68^+$ o $CD80^+$, mentre il fenotipo M2 è stato definito come $CD163^+$, $CD206^+$, $CX3CR1^+$. È stato dimostrato che l'attivazione con LPS/IFN-γ è in grado di inibire significativamente il fenotipo M2. Analogamente a quanto osservato con il desametazone, scelto come farmaco di riferimento, E_2 previene l'effetto indotto da stimoli infiammatori sia sul fenotipo M2, sia sulla produzione di citochine. Successivamente, sapendo che gli effetti pro-infiammatori dei macrofagi giocano un ruolo importante nello sviluppo e la progressione delle patologie cardiovascolari, è stato valutato se la menopausa potesse alterare il rapporto M1/M2 di macrofagi ottenuti dal differenziamento spontaneo di monociti circolanti rispetto a quello osservato in età fertile. In condizioni basali, i macrofagi di donne in post-menopausa presentano un simile profilo M1/M2 rispetto ai macrofagi di donne in età fertile. Tuttavia, il trattamento con statine nelle donne in post-menopausa aumenta la frazione di cellule con fenotipo M2 e, allo stesso tempo, diminuisce la frazione di macrofagi M1. Inoltre, rispetto ai macrofagi di donne in pre-menopausa, i macrofagi di donne in post-menopausa mostrano una risposta simile alla polarizzazione con LPS/IFN-γ ma una perdita di risposta alla polarizzazione M2 (IL-4/IL-13), indicando una ridotta capacità di acquisire il fenotipo anti-infiammatorio e, probabilmente, di risolvere l'infiammazione. Nel tentativo di identificare un possibile biomarcatore che correllasse la menopausa al rischio cardiovascolare, è stato misurato il rapporto M1/M2 nei monociti circolanti nelle donne in pre- e post-menopausa ma la differenza non è risultata statisticamente significativa. In conclusione, gli estrogeni sono in grado di modulare i

fenotipi e le funzioni dei macrofagi umani e rappresentano un possibile intervento farmacologico per malattie su base infiammatoria. Le prospettive future includono lo studio della polarizzazione dei monociti-macrofagi nelle donne in relazione al ciclo mestruale e a malattie endocrine come la sindrome dell'ovaio policistico.

ABSTRACT

Mononuclear phagocytes respond to environmental cues with the acquisition of distinct functional phenotypes, M1 (classical) or M2 (alternative), which in turn are involved in different pathological conditions. 17β-estradiol (E_2), the major female sex hormone, is known to mediate profound effects on monocyte and macrophage immune function acting through estrogen receptors (ER). We hypothesized that estrogen-dependent effects on the monocyte/macrophage system protect postmenopausal women from cardiovascular disease. To test our hypothesis, we first investigated the effects of E_2 on human monocyte-derived macrophage subsets in resting state (M0) and after M1 or M2 polarized activation. Human monocytes were isolated from *buffy coats* by density gradient centrifugation and monocyte-to-macrophage differentiation occurred within 7 days in the absence of any stimulating factors other than serum. We demonstrated that spontaneously differentiated human macrophages polarized to M1/M2 phenotypes by 48h-stimulation with LPS/IFN-γ or IL-4/IL-13, respectively. Polarized macrophages showed specific gene expression profiles different cytokine production (TNFα, IL-1β, IL-10, CCL22) and surface markers. In particular, the M1 phenotype was characterized by flow cytometry as percentage of $CD68^+$, $CD68^+/CCR2^+$, $CD14^+/CD16^-/CD68^+$ or $CD80^+$ cells and the M2 phenotype was identified as $CD163^+$, $CD206^+$, $CX3CR1^+$ cells. We also demonstrated that M1 activation with LPS/IFN-γ down-regulated the M2 immunophenotype. Similarly to dexamethasone, used as a reference drug, E_2 promoted a M2 macrophage signature counteracting the negative regulation by pro-inflammatory stimuli of both M2 surface marker expression and cytokine production. Overall, these data suggest that differences in the functional status of macrophages are critical to investigate pharmacological macrophage targeting. Given that the pro-inflammatory activity of monocyte-macrophages plays a role in the development and progression of CVD, we subsequently investigated if an imbalance in the M1/M2 ratio of macrophages derived from peripheral blood monocytes menopausal women could be detected in relation to menopausal status. In the resting state, macrophages from post-menopausal women displayed similar M1/M2 phenotype with respect to macrophages from pre-menopausal women. However, among post-menopausal women, the M2 phenotype was enhanced and M1 was attenuated by ongoing statin therapy with respect to non statin-treated patients. Moreover, macrophages from post-menopausal women after polarized activation displayed similar M1 response but impaired alternative activation (M2) with respect to those from pre-menopausal women. In the attempt to identify a biomarker linking menopause to cardiovascular risk, the M1/M2 ratio in circulating monocytes from pre- and post-menopausal women was measured and found unchanged. In conclusion, estrogenic pathways modulate the phenotypes and function of human macrophages and represent a possible pharmacological intervention in inflammatory disease. Future perspectives include investigating monocyte-macrophage polarization in women in relation to menstrual cycle and endocrine disease such as polycystic ovary syndrome.

CONTENTS

INTRODUCTION

1.THE IMMUNE SYSTEM

The immune system is a network of cells, tissues and organs that work together to defend the body against attacks by “foreign” invaders: these are primarily microbes, i.e. tiny organisms such as bacteria, parasites and fungi that can cause infections. The inflammatory response usually has two components: an innate non adaptive response and an adaptive (acquired or specific) immunologic response (Delves and Roitt, 2000 a, b).

Innate immunity, also called natural immunity, consists of several body’s own mechanisms which are rapidly able to counteract pathogens invasion. The main components of the innate immunity are:

1. physical/chemical barriers such as cough reflex, enzymes in tears and skin oils, mucus, skin and stomach acid, that keep out harmful materials from entering the body thus forming the first line of defense in the immune response;
2. phagocytic cells, such as neutrophils and macrophages, and cells with cytotoxic activity, named natural killer (NK). The phagocytic cells use a combination of degrading enzymes, antimicrobial peptides and reactive oxygen species to kill the invading microorganisms. In addition, they release signaling molecules that trigger an inflammatory response and begin to marshal the forces of the adaptive immune system;
3. circulating proteins, such as those included in the complement system or other mediators of the immune response;
4. several proteins, such as cytokines and chemokines, which regulate and coordinate many functions performed by cells of innate immunity.

In addition to innate immunity, exposure to infectious agents initiate more complex mechanisms belonging to the so-called acquired immunity, whose power and defensive ability increases with each subsequent exposure to the same pathogen. The cumulative effects triggered by the immune system to defend the body against the etiologic agent is known as inflammation. In the long term, however, the lack of resolution and the chronicity of inflammatory responses may contribute to the development of a number of diseases including, among others, atherosclerosis (Moore et al., 2013) and obesity (Han et al., 2013).

2. THE MONOCYTE-MACROPHAGE SYSTEM

Monocytes, macrophages and dendritic cells (DCs) are part of the mononuclear phagocyte system (MPS), a body-wide specialized system of phagocytic cells. This system functions in the innate immune response, in support of the adaptive immune response and in the maintenance of tissue homeostasis.

Monocytes originate in the bone marrow from a common hematopoietic stem cell (HSC), which is the precursor of many different cell types including neutrophils, eosinophils, basophils, macrophages, DCs and mast cells. During monocyte development, myeloid progenitor cells (termed granulocyte/macrophage colony-forming units, GM-CFU) sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream (Fig. 1). Monocytes circulate for several days into the peripheral blood before entering tissues and replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes) and liver (Kupffer cells), for example.

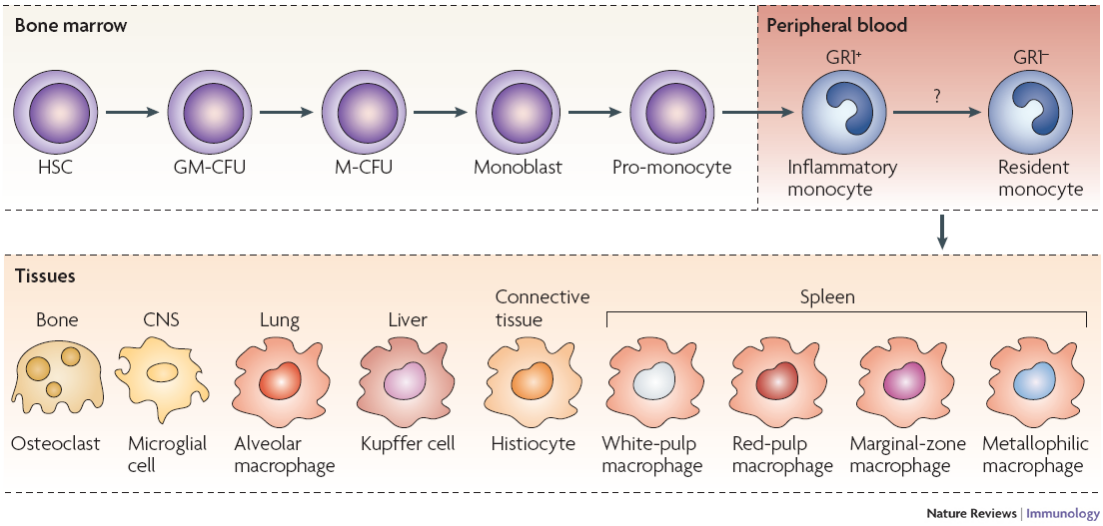

Fig. 1: Origins and differentiation of monocytes and macrophages (Mosser and Edwards, 2008).

The mononuclear phagocyte system represents a subgroup of leukocytes originally described as a population of bone marrow-derived myeloid cells that circulate in the blood as monocytes and populate tissues as macrophages in the steady state and during inflammation (Geissmann et al., 2010). The existence of proliferative precursors of mononuclear phagocytes, such as the monoblast and pro-monocyte, was initially proposed on the basis of morphological, cytochemical and adherence properties (van

Furth and Cohn, 1968). The subsequent discovery of DCs and the unexpected degree of heterogeneity in the mononuclear phagocyte system required a reevaluation of the development of monocytes and DCs from their progenitors. Studies have recently shown that monocytes, macrophages and DCs are developmentally related and share a common bone marrow–derived precursor called the 'monocyte-macrophage DC progenitor' (MDP) (Fogge et al., 2006). In the development of monocytes, the existence of a restricted progenitor downstream of the MDP has been suggested, although such a precursor has not been identified.

Monocytes, which circulate in the bloodstream, have long been considered the sole precursors of tissue macrophages. Nowadays, controversies exist regarding the ontogeny and self-renewal properties of tissue macrophages, which likely reflect the heterogeneity of these populations and the lack of distinct phenotypic markers able to identify unique subsets. Recently, it has become clear that macrophage populations in many tissues (such as liver and spleen) are established prenatally during embryonic development from progenitors derived from the yolk sac or fetal liver and are maintained independently from bone marrow-derived monocytes in the steady state (Davies et al., 2013). This concept was further supported by the observation that yolk sac–derived macrophages develop independently of the transcription factor c-Myb and thus are distinct from monocyte-derived macrophages. However, monocyte-derived macrophages can complement the prenatally established macrophage compartment, especially under conditions of tissue stress (such as irradiation and inflammation), which indicates a dual origin for macrophages under steady-state and inflammatory conditions (Hettinger et al., 2013). Thus, the model of the MPS needs to be extended to include not just monocytes as a major source of tissue macrophages but to highlight the prenatal origins of many populations, some of which involve cells arising from a distinct yolk-sac embryonic macrophage lineage (Davies et al., 2013).

2.1 Monocyte heterogeneity

Monocytes are a critical part of the mononuclear phagocyte system and are involved in many diseases with an inflammatory component, such as infection, cardiovascular disease, type-1 diabetes and cancer. Blood monocytes represent a large pool of scavenger and potential effector cells inside blood vessels in homeostasis as well as during inflammatory processes. In mammals, monocytes also represent accessory cells, which can link inflammation and the innate defense against microorganisms to the adaptive immune responses. Indeed, the best known function of monocytes is as a considerable systemic reservoir of myeloid precursors for the renewal of some tissue macrophages and antigen-presenting DCs (Auffray et al., 2009).

Several lines of evidence have indicated that the role of monocytes, both in the control of pathogens and in the pathophysiology of inflammation, can be attributed to different functional groups. Therefore, the issue of heterogeneity of monocytes becomes relevant for human health. Monocytes are equipped with a large array of scavenger receptors that recognize microorganisms but also lipids and dying cells, and stimulated monocytes can produce large quantities of effector molecules, such as reactive oxygen species (ROS), prostaglandins, cytokines including TNF-α, IL-1β, CXCL8, IL-6 and IL-10, VEGF and proteolytic enzymes involved in the defense against pathogen and in the pathogenesis of several inflammatory diseases, including arthritis and atherosclerosis. Although the heterogeneity of monocytes is not fully understood, one theory suggests that monocytes continue to grow and mature into the blood and tissues, and can be recruited at different stages during the maturation process. The state of maturation at the time they leave the blood stream can, in fact, define their function.

The existence of at least two phenotypically and functionally distinct monocyte subsets has been demonstrated in humans and mice, which suggests evolutionary conservation of monocyte heterogeneity (Gautier et al., 2009). Monocytes represent about 10% of leukocytes in human blood and 4% of leukocytes in mouse blood. Studies examining homing and differentiation of mouse monocytes *in vivo* have identified two major monocyte subpopulations based on their expression profile of the surface marker Ly6C (or GR-1), and chemokine receptors such as CCR2 (CCL2 chemokine receptor) and CX3CR1 (CX3C chemokine receptor 1, also known as fractalkine receptor; Hristov and Weber, 2011). Ly6C is a useful marker to distinguish different populations of murine monocytes

and macrophages: it belongs to a family of cell surface phosphatidylinositol-anchored glycoproteins expressed in varying degree in leukocytes, although its function remains unknown (Brancato and Albina, 2011). Inflammatory monocytes, characterized as Ly6C^{high} CX3CR1^{low}CCR2^{+} cells, migrate into sites of inflammation during the early phase of the response to injury. A second population of murine monocytes, often referred to as "resident" or "patrolling", are defined as Ly6C^{low} CX3CR1^{high}CCR2^{-} cells, which egress the circulation into wounds and other sites of inflammation to resolve the immune response (Hristov and Weber, 2011). In mice, classical and non-classical monocyte frequency is approximately 1:1 (Fig. 2).

In humans, monocytes were initially identified by their expression of large amounts of CD14 (a cluster of differentiation which is part of the lipopolysaccharide receptor. However, the subsequent identification of differential expression of antigenic markers showed that monocytes in human peripheral blood are heterogeneous, and this provided the first clues to the differential physiological activities of monocyte subsets. Differential expression of CD14 and CD16 (also known as FCγRIII) allowed human monocytes to be distinguished into two separate subsets, similar to monocyte classification in mice: as reported in Fig. 2, CD14^{hi}CD16^{-} cells, which are often called classical monocytes, represent 80% to 90% of circulating monocytes and express high levels of the chemokine receptor CCR2 and low levels of CX3CR1. Conversely, CD14^{low}CD16^{+} cells, or non-classical monocytes, have a CX3CR1^{high}/CCR2^{low} phenotype (Gordon and Taylor, 2005; Mantovani et al., 2009).

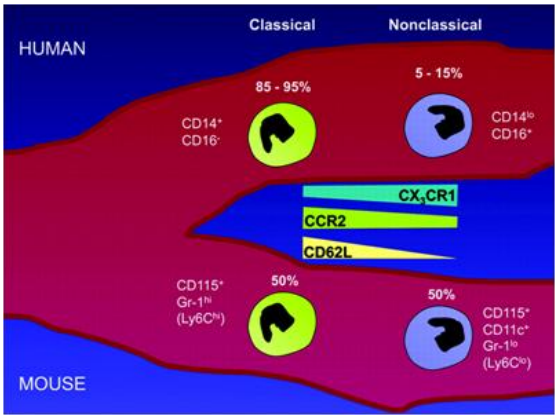

Fig. 2: Circulating monocyte heterogeneity. Peripheral blood circulating monocytes are present in two distinct populations, called "classical" and "non-classical" with different relative abundance (expressed in %) and characterized by different pattern of surface markers. This classification has been demonstrated both in mice (lower portion of the image) and in humans (upper portion). From Gautier et al. 2009.

2.2 Macrophage plasticity and polarization

Macrophages are one of the first lines of defense against bacterial pathogens. During bacterial infections, monocytes are recruited from the bloodstream into tissues, where they differentiate into macrophages. In response to tissue microenvironmental signals contributed by microbial components, the innate and adaptive immune systems, and damaged cells and tissues, macrophages become activated and acquire diverse phenotypes and functions. Since the discovery of macrophage activation and its heterogeneity, several classification schemes have been proposed, the most recent one defining the classical/pro-inflammatory phenotype (M1) and the non-classical/alternative/anti-inflammatory phenotype (M2). M1 and M2 macrophages are polarized by specific inducers, express a distinct combination of membrane receptors, cytokines, chemokines and other immune mediators, and have specialized functions in infection, resolution of inflammation, wound repairing, and tissue remodeling (Sica and Mantovani, 2012).

M1 polarization is typically induced by interferon (IFN)-γ and lipopolysaccharide (LPS). M1 macrophages are characterized by high interleukin (IL)-12 and IL-23 and low IL-10 expression, are efficient producers of effector molecules (reactive nitrogen and oxygen intermediates) and pro-inflammatory cytokines such as IL-6, IL-1β and tumor necrosis factor (TNF)-α, induce up-regulation of molecules associated with antigen presentation such as major histocompatibility complex (MHC) class II and the co-stimulatory molecules CD40, CD80 and CD86 (Fig. 3). Thus, M1 macrophages participate as inducers and effector cells in polarized Th1 responses, are microbicidal and mediate resistance against tumors (Mantovani et al., 2013).

By contrast, M2 polarization is induced by anti-inflammatory cytokines such as IL-4 and IL-13. Initial observations regarding the role of IL-4 in macrophage activation showed that this cytokine was able to inhibit the respiratory burst and the production of IL-1β and IL-8. It was also shown that IL-4 induced MHC class II expression and macrophage-macrophage fusion. Importantly, it was found that IL-13 induced both redundant and non-redundant effects of those of IL-4 in macrophages (McKenzie et al., 1993). With the finding by Stein et al. (1992) of mannose receptor (MR) up-regulation as a distinctive marker of IL-4–activated macrophages, together with the induction of MHC class II antigens, the concept of alternative activation was proposed, whereby IL-4, in an inflammatory focus, would

cause recruited macrophages to acquire an entirely different phagocytic receptor and secretory capability compared with macrophages classically activated by IFN-γ treatment or bacterial infection.

M2 macrophages share low levels of inflammatory cytokines such as IL-12 and IL-23, and high levels of the anti-inflammatory cytokine IL-10, with variable capacity to produce chemokines such as CCL17, CCL18 and CCL22, and generally have high levels of scavenger (SR), galactose-type and mannose receptors (MR) (Fig. 4). In general, M2 cells take part in polarized Th2 responses, parasite clearance, the dampening of inflammation, the promotion of tissue remodeling, angiogenesis, tumor progression and immunoregulation (Biswas and Mantovani, 2010).

M2 macrophages are heterogeneous and can be further classified into M2a, M2b, and M2c subsets (as summarized in Fig. 4). In fact, it has become clear that the phagocytic and secretory profile of macrophages could be further modified by many other self- and pathogen-derived signals. Beyond the activation induced by IL-4/IL-13 (M2a), the main stimuli associated with distinctive macrophage phenotypes include immune complexes (IC) recognized by the Fc receptor family (M2b), glucocorticoids (GC) recognized by the GR (glucocorticoid receptor) and IL-10 recognised by the IL-10R1 (M2c), among others (Mantovani et al., 2004).

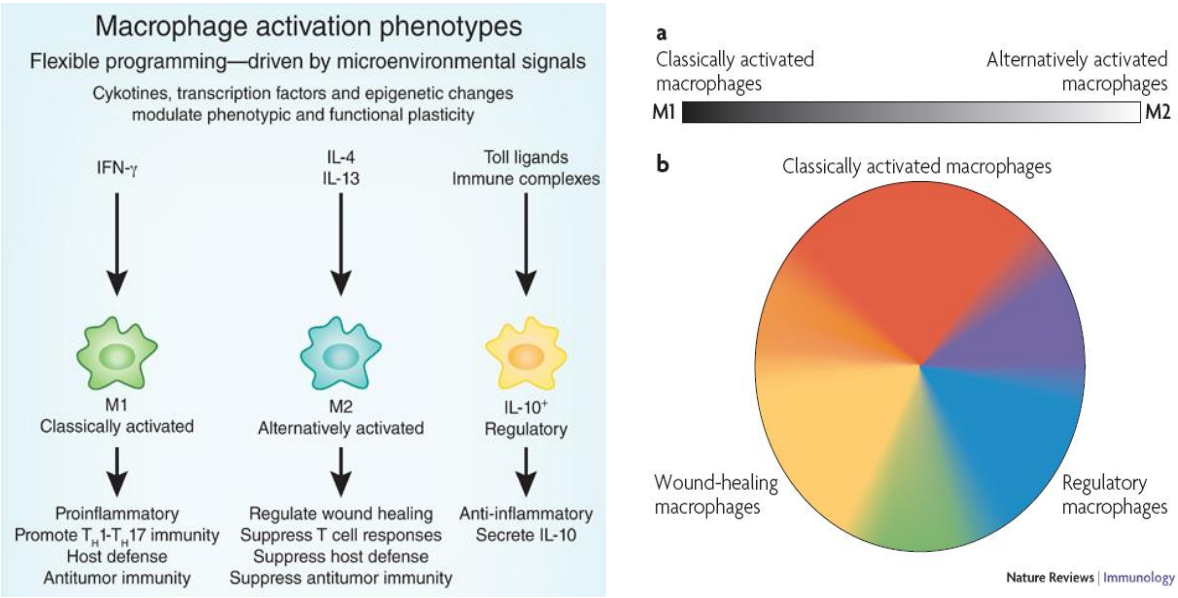

Fig. 3: Macrophage activation and polarization. The conventional M1/M2 pattern for macrophage classification represents two extremes of a ***continuum*** *of macrophage phenotypes and functions (Galli et al., 2011; Mosser and Edwards, 2010).*

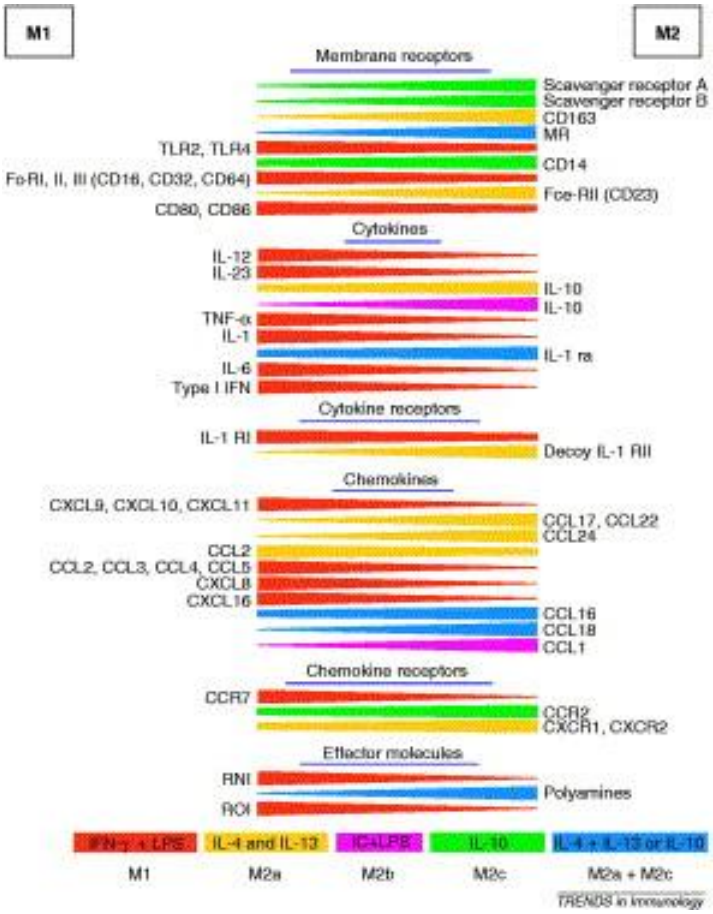

Fig. 4: M1 and M2 macrophage gene expression profile: the extremes of a continuum (Mantovani et al., 2004)

However, the common M1 and M2 classification is used to refer to the two extremes of a wide spectrum of activation states and functional phenotypes that macrophages can acquire thanks to their high plasticity, thus representing the extremes of a continuum of macrophage heterogeneity (Fig.3). Each specific functional phenotype is associated with the expression of a wide range of particular surface proteins that can be used for macrophage identification. For example, in agreement with literature data, M1 macrophages can be identify by their surface expression of the scavenger receptor CD68 (glycoprotein that binds LDL), CCR2, receptor for the chemokine CCL2 also known as MCP-1 (monocyte chemoattractant protein 1), and the co-stimulatory molecules CD80 and CD86; by contrast, M2 macrophages typically express CD163 (the scavenger receptor for the hemoglobin-haptoglobin complex), the mannose receptor CD206 (which recognizes pathogens that have mannose on their surface) and the chemokine-X3C receptor-1 (CX3CR1), also known as fractalkine receptor (Mantovani et al., 2010).

3. ROLE OF M1 AND M2 MACROPHAGES IN CHRONIC INFLAMMATORY DISEASES

Macrophages undergo different functional states in response to various external stimuli, which can modify their immunophenotype thanks to their plasticity (Adamson and Leitinger, 2011; Sica et al., 2012). The dynamic balance between M1 and M2 phenotypes requires a fine regulation, otherwise the resulting loss of immune regulation contributes to the development and/or exacerbation of chronic inflammatory diseases, such as atherosclerosis (Pello et al., 2011), rheumatoid arthritis, obesity and insulin resistance. This occurs with combined deficits in the number and/or function of multiple types of regulatory cells leading to the inability to sustain M2 macrophages. Although what causes these immunoregulatory mechanisms to fail is unknown, inappropriate stimulation of pro-inflammatory cells could be one of the inciting events for chronic systemic inflammation (Han and Levings, 2013)

3.1 Atherosclerosis

Atherosclerosis is a progressive disease characterized by an inflammatory event in with monocyte-derived macrophages play a central role (Ross, 1999; Libby, 2012). Macrophages are involved in all key stages of atherosclerosis development including early inflammatory injury, development of fatty streaks and finally plaque rupture. In particular, the disease is characterized by intimal lesions of the vessel wall that can cause severe myocardial infarction, cerebral infarction and peripheral vascular disease. Interestingly, it has been hypothesized that a switch from M2 to M1 macrophage phenotype occurs during atherogenesis, similarly to what happens in obese subjects.

In healthy subjects, the endothelial monolayer in arteries resists prolonged contact with blood leukocytes, produces endogenous vasodilator molecules, combats thrombosis, favors fibrinolysis and expresses enzymes, such as superoxide dismutase, that can degrade reactive oxygen species. Laminar shear stress, as prevails in normal arteries, fosters these homeostatic endothelial functions. But endothelial cells become dysfunctional when exposed to disturbed flow, instead of laminar sheer stress, and to pro-atherogenic factors such as modified lipoprotein or pro-inflammatory cytokines (Libby et al., 2012).

The initial inflammatory response stimulates elevated expression of leukocyte adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in endothelial cells, which in turn increases monocyte attachment.

Once attached, chemokine interactions induce monocyte migration into the tunica intima and their differentiation into tissue resident macrophages. *In vivo* studies showed that atherosclerosis is virtually abolished in mice knockout for MCP-1, indicating that this protein is essential for the initial stages of the disease (Yadav, 2010).

Mature macrophages express scavenger receptors that attract oxidised low density lipoproteins (oxLDL) and lipids to produce foam cells and ultimately the "fatty streak" (Libby, 2012). In particular, oxLDL are internalized by M1 macrophages where they generate lipid peroxides, cholesterol esters, resulting in the formation of foam cells. Furthermore, macrophages within atheroma produce matrix-degrading proteases which solubilize extracellular matrix and render plaques instable and liable to rupture and thrombosis, thus initiating rupture of the fibrous cap (Libby 2012). Recent studies demonstrated that M1 macrophages dominate the rupture-prone shoulder regions of the plaque over M2 polarized cells, whereas M2 markers are readily detectable in stable plaques (Stöger er al., 2012). In addition, Khallou-Laschet et al. (2010) reported that M1 macrophages appear and prevail in lesions of aged ApoE KO mice and that lesion progression is correlated with the dominance of M1 over the M2 phenotype. The advanced atherosclerotic plaque contains a lipid core rich in cholesterol esters, cholesterol monohydrate crystals, and cellular debris. Some refer to this compartment of the plaque as the necrotic core (Fig. 5). The continue oxidation of LDL and the accumulation of M1 macrophages, which produce large amount of pro-inflammatory cytokines in the plaque, is the reason why atherosclerosis is considered an inflammatory disease and may explain the association with other disorders such as rheumatoid arthritis (Mosser and Edwards, 2008).

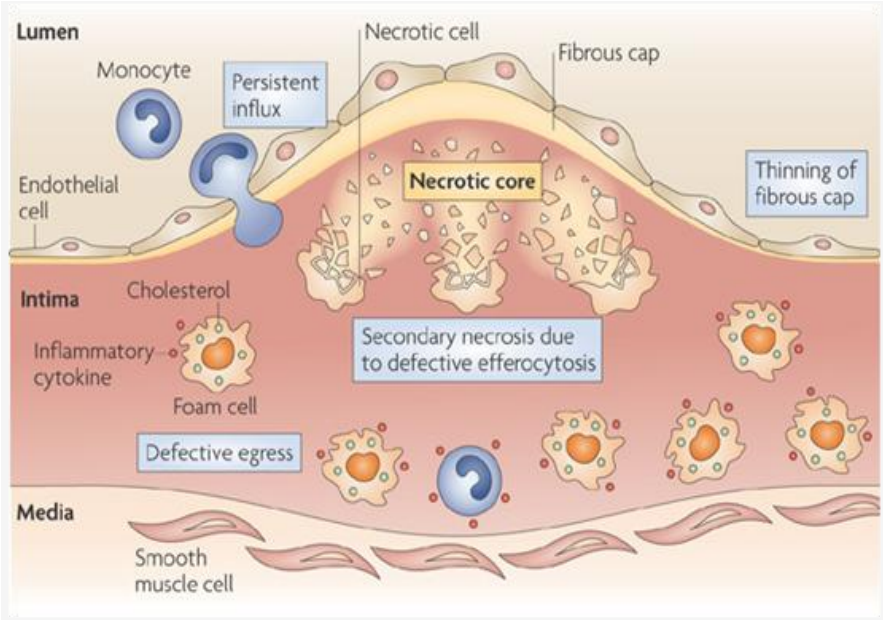

Fig.5: Role of macrophages in atherosclerosis development. Inflammatory cells, including lipid-laden macrophage foam cells, accumulate in the intima owing to the persistent influx of new monocytes. Moreover, apoptotic macrophages are not efficiently cleared by efferocytosis and so they undergo secondary necrosis. This process contributes to the formation of the necrotic core, which promotes plaque disruption, particularly thinning of the fibrous cap. If the process continues, the fibrous cap breaches, leading to luminal thrombosis and arterial occlusion (Tabas, 2010)

3.2 Obesity and type-2 diabetes

Obesity-associated adipose tissue inflammation appears to be caused by infiltration of inflammatory immune cells and a parallel loss or functional reprogramming of immunoregulatory cells. Together, these changes lead to a variety of positive feedback pathways that not only sustain chronic inflammation, but also contribute to the development of insulin resistance and type 2 diabetes. In parallel to the increase in pro-inflammatory cells in obese adipose tissue (AT), the steady-state high proportion of regulatory immune cells is also reduced.

In healthy subjects, AT macrophages produce relatively few pro-inflammatory cytokines, and express the enzyme arginase, which can inhibit the formation of nitric oxide. Therefore, AT-associated macrophages in non-obese subjects have a predominantly anti-inflammatory M2 phenotype and have been found to regulate important metabolic functions. M2 macrophages are induced by peroxisome proliferator activated receptor (PPAR)-γ signaling and maintain adipocyte function, insulin sensitivity and glucose tolerance, which can prevent the development of diet-induced obesity and type 2

diabetes (Murray, 2011). Both in mice and humans, AT macrophages accumulate with increasing body weight. Thus, as obesity progresses, AT-associated macrophages switch from the anti-inflammatory IL-10-producing M2 macrophages that normally occupy the lean AT, to pro-inflammatory M1 macrophages, with the NLRP3 inflammasome serving as the molecular switch by sensing obesity-associated danger signals . Normally, the predominant M2 phenotype is maintained by IL-4, with eosinophils and Th2 cells thought to be major source of this cytokine. Remarkably, 90% of IL-4-expressing cells in the AT are eosinophils, and evidence that AT M2 macrophages depend on IL-4/IL-13-expressing eosinophils suggests that these cells play an important role in sustaining alternative activation of macrophages in healthy AT (Han and Levings, 2013).

Obesity is also associated with extensive necrosis of adipocytes leading to the release of large amount of cytokines, especially TNF-α and IL-6 contributing to insulin resistance and thus promoting type 2 diabetes, and chemokines such as CCL2, which plays a role in recruiting additional macrophages in AT, thus propagating the state of inflammation (Fig. 6). In addition to macrophages, there is growing evidence for a role of other innate immune cells. For example, AT in obese mice is also infiltrated by $CD11c^{high}F4/80^{low}$ DCs, which have been shown to induce the differentiation of pro-inflammatory Th17 cells and promote further macrophage infiltration (Bertola et al., 2012). Not least, overexpression of procoagulant proteins by M1 macrophages could concur with the development of cardiovascular and atherogenic risk, which constitutes part of the metabolic syndrome associated with obesity (Mosser and Edwards, 2008).

A recent study on novel phenotypes of blood monocytes shows that type 2 diabetes is also associated with a marked reduction of the anti-inflammatory M2 phenotype. Fadini and colleagues (2013) found that type 2 diabetes is characterized by a marked reduction in M2 cells while M1 cells are unchanged compared with controls; as a result, the M1/M2 polarization ratio is increased in diabetes. As type 2 diabetes is considered a pro-inflammatory condition, it is striking that the monocyte polarization imbalance is attributable to a defect in anti-inflammatory rather than an excess of pro-inflammatory cells. This observation is in line, however, with the view that diabetes is a disease of impaired damage control (Schaper and Havekes, 2012), in which injury is worsened by defective repair.

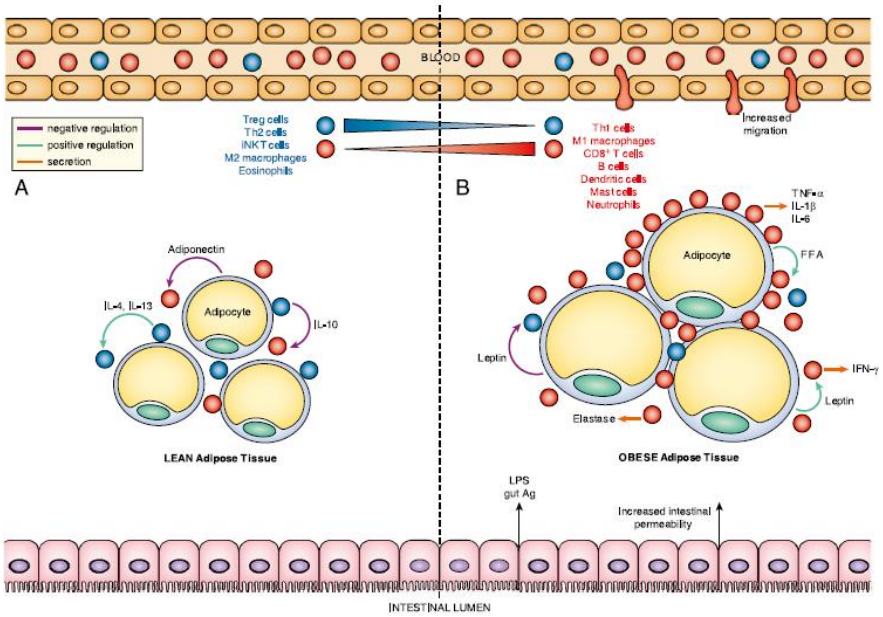

Fig. 6: The loss of immune regulation in obesity-associated AT inflammation. (A) Lean AT contains regulatory immune cells (blue) that suppress pro-inflammatory immune cells (red) and sustain alternative activation of macrophages via Th2-associated cytokines (IL-4/IL-13). Adipocytes in lean AT are of normal size. (B) In contrast, obese AT is infiltrated with pro-inflammatory immune cells that produce high amounts of inflammatory cytokines and chemokines. M1 macrophages accumulate in crown-like structures around hypertrophic adipocytes that have increased rate of lipolysis, and secrete free fatty acids (FFA) that can serve as endogenous danger signals to stimulate production of inflammatory cytokines, such as TNF-α (Han and Levings, 2013)

4 *In vitro* models of macrophage polarization

Macrophages, derived from monocyte precursors, undergo specific differentiation depending on the local tissue environment . The various macrophage functions are linked to the type of receptor interaction on the macrophage surface and, crucially, to the cytokine milieu in the macrophages resides in (Gordon andTaylor et al., 2005). While circulating monocytes can be obtained following different approaches, the isolation process of mononuclear cells from whole blood affects the subsequent differentiation method to obtain mature macrophages. Peripheral blood mononuclear cells (PBMCs) can be isolated from whole blood samples by density gradient centrifugation, for example using Ficoll-Paque™, an appropriate polymer which stratifies the diverse blood components according to their specific weight. Alternatively, purified monocytes can be obtained through magnetic cell sorting using specific CD14-labeled magnetic beads (Martinez et al., 2013). This method usally allows to obtain a 90% monocyte ($CD14^{+}$) pure suspension.

After density gradient isolation, the PBMC fraction is seeded and monocytes, in contrast to lymphocytes, spontaneously adhere to the culture plate within 1-2 h (Eligini et al., 2013; Toniolo et al., under submission). After isolation, the local milieu compels mononuclear phagocytes to express specialized and polarized functional properties. In most studies, high concentrations of cytokines (e.g. IFN-γ, IL-4, IL-10) and/or growth factors (e.g. GM-CSF, M-CSF) are added over the monocyte differentiation period in culture (Mantovani et al., 2004; Ambarus et al., 2012; Martinez et al., 2013; Jaguin et al., 2013), thereby steering the macrophage response. This aspect is even more relevant after $CD14^+$-magnetic beads purification (Bender et al., 2004), where the addition of exogenous growth factors and/or cytokines to the culture medium becomes necessary for survival of the high-purity isolated population but at the same time commits cells to specific differentiation pathways. Therefore, nature of polarizing agents and exposure time appear to be crucial, especially when testing the potential impact of pharmacological interventions.

Human monocyte-derived macrophages are able to spontaneously differentiate *in vitro* in the absence of growth factors other than those contained in animal or human serum (Cullen et al., 1998; Colli et al., 1999; Eligini et al., 2013). Spontaneous macrophage differentiation may be related to the relative abundance of Th2 cytokines secreted by $CD3^+$ lymphocytes early in culture before their detachment and/or by the fraction of lymphocytes which could be persistent in culture during the whole differentiation period (Eligini et al., 2013). It is conceivable that these dying cells determine at least in part the polarization state of differentiating monocytes. Again, polarizing growth factors such as M-CSF (Lacey et al., 2012) contained in FCS can influence macrophage growth and differentiation. In this regard, an early study provided evidence that two dominant MDM subsets, distinguishable by morphology (round- or spindle-shaped), co-exist in the same culture of blood-derived monocytes spontaneously differentiated in vitro in autologous serum (Eligini et al., 2013). In conclusion, this culture model yields a rather heterogeneous cell population that has not been exposed to additional exogenous polarizing factors over the course of monocyte differentiation while reflecting the features and plasticity of tissue macrophages. Upon differentiation, macrophages become resident and acquire specific functions according to the characteristics of tissue microenvironment that mould their behavior.

Therefore, developing gold standard differentiation/polarization protocols would be useful to compare data from different research groups and perform screens of macrophage activation-modifying pharmacological agents.

5. Pharmacological control of macrophage polarization

Specific macrophage-target therapies are now taking the first steps into clinical investigations. In addition, therapeutic approaches not originally designed as macrophage oriented or specific have been found to affect macrophage activation and polarization (Sica and Mantovani, 2012). Therefore, it is relevant to identify novel mechanisms for the pharmacological control of human macrophage polarization. For instance, while it is well established that glucocorticoids induce multiple effects on several immune cell types, glucocorticoid treatment of human monocytes does not cause a global suppression of monocytic effector functions but results in differentiation of a specific anti- inflammatory phenotype which seems to be involved in resolution of inflammation (Ehrchen et al., 2007). Similarly, Vallelian et al. (2010) found that glucocorticoid treatment in vitro (monocytes) and in patients on glucocorticoid-pulse therapy polarizes monocytes into a M2/alternatively activated phenotype with high Hb-scavenger receptor (CD163) expression and enhanced Hb clearance and detoxification. A positive regulation of CD163 following dexamethasone treatment was demonstrated in recent studies on human macrophages (Ambarus et al., 2012; Tang et al., 2013). Glucocorticoids are also considered to induce a particular M2 subset, called M2c (Li et al., 2012).

Opposite effects on macrophage polarization were observed on human monocyte-derived macrophages challenged with the immunosuppressant agent rapamycin after polarization. The presence of rapamycin induces apoptosis in M2 but not in M1 macrophages, and enhances M1 surface markers and pro-inflammatory cytokine production together with a reduction of typical M2 markers (Mercalli et al., 2013).

Statins are thought to reduce vascular inflammation through lipid-independent mechanism. Van dei Meij et al., (2013) evaluated the anti-inflammatory potency of simvastatin and atorvastatin in patients that were on statin therapy for at least 6 weeks. Both statins equally effectively and dose-dependently shifted macrophage polarization towards a M2 phenotype and reduced vascular wall NF-κB activity in abdominal aorta aneurysms samples, thus diminishing inflammatory responses.

PPARγ agonists (thiazolidindiones) have long been used in the treatment of diabetes. The evidence linking PPARγ to M2 polarization sheds fresh new light on their mode of action. It has been demonstrated that rosiglitazone markedly increases the number of macrophages in adipose tissue of obesity-induced mice. In particular, markers for classically activated macrophages including IL-18 are down-regulated, whereas markers characteristic for alternatively activated macrophages (arginase 1, IL-10) are up-regulated by rosiglitazone (Stienstra et al., 2008). Interestingly, a positive correlation between the expression of M2 markers and PPARγ has been shown in human atherosclerotic lesions. Moreover, PPARγ activation primes primary human monocytes into alternative M2 macrophages with anti-inflammatory properties (Bouhlel et al., 2007).

New insights come from microRNAs (miRNAs) studies. MiRNAs have recently emerged as a major class of gene expression regulators linked to most biological functions. In particular, the miRNA let-7c, which is overexpressed in M2 compared with M1 subset of **mouse** bone marrow-derived macrophages, plays an important role in regulating macrophage polarization (Banerjee et al., 2013).

Therefore, several drugs appear to have an impact on the functional status of macrophages; however, the extent to which their effect on macrophages explains their clinical efficacy remains to be defined. The identification of mechanisms and molecules associated with macrophage plasticity and polarized activation provides a basis for macrophage-centered diagnostic and therapeutic strategies.

6. 17β-ESTRADIOL AND ESTROGEN RECEPTORS

6.1 17β-estradiol

Estrogens are hormones of the female reproductive system that also mediate numerous biological functions in different types of tissues, even in male subjects. 17β-estradiol (E_2) modulates many physiological processes including the development and maturation of the reproductive system, but also exerts important actions in non-reproductive tissues, including the brain, the urogenital tract and the bone. In contrast, declining levels of estrogen in menopause are associated with several degenerative processes in various tissues, such as tumor progression or neurodegeneration, osteoporosis and atherosclerosis, thus leading to the progression of cardiovascular diseases.

E_2 is produced by the granulosa cells of the ovary, and to some degree by the adrenal cortex, adipose tissue and testicles, by aromatization of testosterone. Estrogen biosynthesis is initiated by the synthesis of the 19-carbon steroid hormone pregnenolone from cholesterol (Fig. 7). This compound is converted to testosterone and then to the estrogens estrone and 17β-estradiol. Estrogen biosynthesis is catalyzed by aromatase, a microsomal member of the cytochrome P450 superfamily that introduces the characteristic phenolic ring.

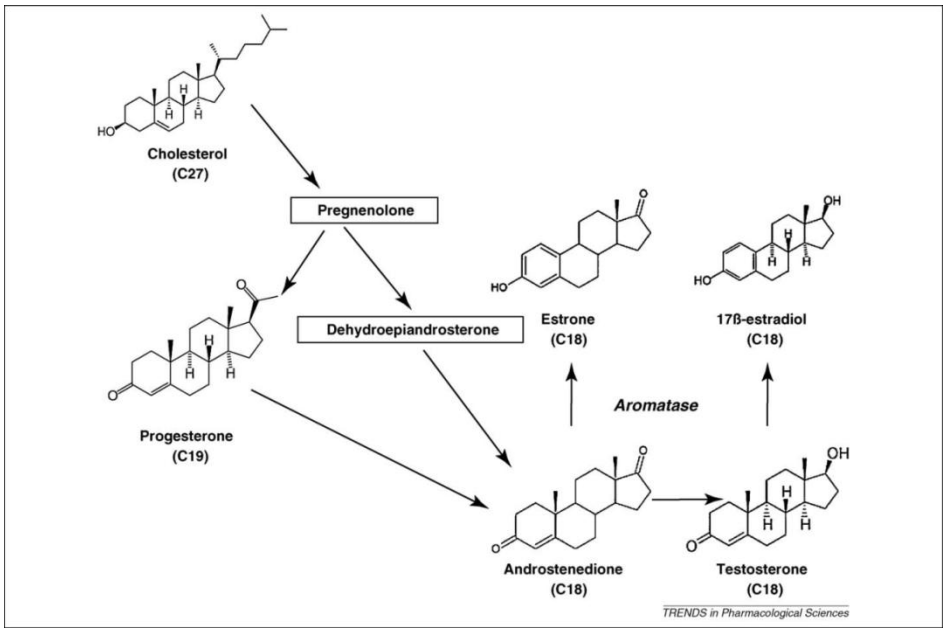

Fig 7: The metabolic pathway for estrogen (Cignarella et al., 2010)

Once synthesized, estrogen is secreted into the systemic circulation, where it binds in a reversible way to a sex-hormone-binding globulin (SHBG) or albumin, which conveys this lipophilic hormone in the blood; in fact, only a minimal part circulates as free molecule due to its high lipophilicity and reaches target tissues, where it exerts its genomic and non-genomic effects. Estradiol is then metabolized in the liver into less active estrone and estriol.

In women, circulating estrogen levels fluctuate according to the menstrual cycle phase and age (Ai-Min et al., 2003). In normally cycling adult women, the ovarian follicle secretes 70 to 500 µg E_2 per day, resulting in plasma estrogen levels ranging from 210 pmol/L in the early follicular phase and 720 pmol/L in the late follicular phase, to 490 pmol/L in the late luteal phase. The half-life of E_2 is ~3 hours, and much of it is converted into estrone (E_1) and estriol (E_3; Reslan and Khalil, 2012). During pregnancy, estrogen levels rise to 70 nM due to placental production, which increases near term and further with the onset of parturition; interestingly, estradiol concentrations in umbilical cord blood range from 2 to 150 nM, which exceed peak estradiol concentrations during the menstrual cycle (Giannoni et al., 2011). In the perimenopausal period, plasma estrogen levels decline to about 20% of the levels in the fertile period (Reslan and Khalil, 2012). After menopause E_2 concentrations fall to levels that are equivalent to those in males (0.04–0.21 nM). In particular, at menopause most of the ovarian production of sex hormones ceases, although some production of testosterone, androstenedione, dihydroepiandrostenedione (DHEA), estrone (derived peripherally into the adipose tissue by conversion from androstenedione) and estradiol has been shown 10 years after menopause; these hormones act locally as paracrine factors. Ovariectomy of post-menopausal women significantly decreases serum estrone and testosterone levels, revealing some remaining ovarian sex hormone production even after menopause (Fogle et al., 2007). Moreover, endothelial cells in both women and men may also be exposed to estrogens derived from the local conversion of testosterone or Δ^4-testosterone to E_2 by aromatase (Chambliss and Shaul, 2002).

6.2 Estrogen receptors

The biological actions of estrogen are mostly mediated by binding to specific intracellular and/or transmembrane receptors. The diverse effects of estrogens on distinct target tissues implicate a complex interplay of transcriptional, as well as nontranscriptional pathways. The mechanisms responsible for many genomic effects involve binding of estrogens to the nuclear estrogen receptors (ERs), which exist in two different isoforms, ERα and ERβ: nuclear ERs act as transcription factors that modulate gene expression by directly binding to DNA at specific estrogen response elements (EREs) (Bolego et al., 2006). Recently, a new membrane ER has been identified as a G-protein coupled receptor, termed GPER-1, which is able to trigger rapid intracellular responses (Nilsson et al., 2001).

6.2.1 Molecular structure. Estrogen receptors, encoded by their respective genes ESR1 and ESR2, belong to the steroid receptor superfamily and possess different sizes. Whereas ERα is comprised of 595 aminoacids, ERβ is comprised of 530 aminoacids. Their aminoacidic sequences are organized as described in Fig. 8: the ligand binding domain (LBD), which contains the activator factor-2 (AF-2), is located in the carboxyterminal region of the molecules, necessary for ligand binding; the DNA-binding domain, responsible for binding to specific DNA sequences (the estrogen response elements, EREs); and the transcriptional regulation domain (AF-1), which is highly immunoreactive and is located in the amino-terminal part of the receptor molecules. ERα and ERβ exhibit high homology in their DNA binding domain (97%), low homology (17%) in their AF-1 domain and partial homology (55%) in their ligand binding domain. Differences in AF-1 and AF-2 could allow drugs to be designed in order to recruit different cofactors to ERα and ERβ, thereby causing a different pattern of genes regulated. Various ERα and ERβ isoforms and splicing variants (hERβ1 long, hERβ1 short, hERβ2, hERβ4, hERβ5, hERα-46) have also been described (Kassi and Moutsatsou, 2010).

Fig. 8: Comparison of the structures and homology between ERα and ERβ. Human ERα contains 595 amino acids whereas ERβ contains 530 aminoacids. The DNA binding domains are nearly identical whereas the transcriptional regulation domain and LBD, which contains AF-1 and AF-2, respectively, have the least homology (Leitman et al., 2010).

The G-protein-coupled estrogen receptor-1 (GPER-1), a member of the G protein-coupled receptor superfamily, is characterized by the presence of 7 transmembrane helices and it is uniquely localized to the endoplasmic reticulum. GPER-1 is structurally unrelated to ERα and ERβ, but binds E_2 with high affinity and may be involved in estrogen signaling (Islander et al., 2011). Activation of GPER-1 by estrogen results in intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus. Thus, GPER-1 represents an intracellular transmembrane estrogen receptor that may contribute to normal estrogen physiology as well as pathophysiology (Haas et al., 2007; Bolego et al., 2006).

6.2.2 Molecular pathways. ERα and ERβ mediate their effects via different molecular pathways, as illustrated in Fig. 9. In the classical transcription pathway, following ligand binding, ER undergoes conformational changes and biochemical modifications that induce release of inhibitory proteins (heat shock proteins), receptor dimerization, and interaction with DNA. In fact, the nuclear ER acts as a transcription factor that modulates gene expression by directly binding to DNA at specific EREs (Fig. 9, pathway 1). In the non-classical transcription pathway, the estrogen/ER-complex starts transcription by binding to alternative transcription factors e.g. AP-1, SP-1, FoxA1 and NF-κB, which bind non-ERE sites (Fig. 9, pathway 2). As mentioned above, a rapid non-genomic pathway has recently been described that is mediated by membrane-associated ERs. GPER1 is a newly discovered G-protein-coupled receptor, and this receptor isoform has recently been suggested to be tightly coupled to estrogen membrane receptor signaling and may

thereby contribute to normal physiological as well as pathophysiological estrogenic effects. Some studies have indicated that ERα may also be membrane associated in some cells. Binding of these transmembrane receptors mediates several rapid cellular effects of estrogens, including activation of other transcription factors (TF) such as the mitogen-activated protein kinase (MAPK) signaling cascade and intracellular calcium mobilization, leading either to non-genomic signaling or altered transcriptional activity (Fig. 9, pathways 3 and 4). In addition, estrogen receptors can be activated through phosphorylation in the absence of estrogen by dopamine, insulin-like growth factor-1, epidermal growth factor and cyclic AMP (Bolego et al., 2006; Islander et al., 2010).

Different tissue distribution suggests that ERs mediate at least in part distinct biological functions. For example, ERα is the most abundant isoform expressed in ovary, prostate, bladder, lung and in the cardiovascular system. Moreover, within the same tissue, the expression of a receptor subtype can be limited to a specific cell type. For example, although the ovary expresses both nuclear ERs, ERα is expressed by the theca cells while ERβ is localized on granulosa (Barkhem et al., 2004).

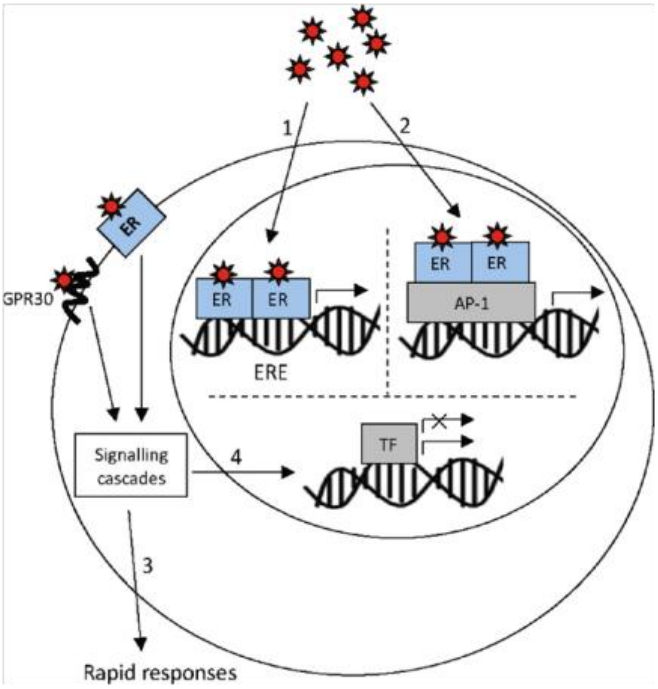

Fig. 9: Estrogen genomic and non-genomic transcription pathways (Islander et al.,2010)

7. ESTROGEN AND THE CARDIOVASCULAR SYSTEM

A number of studies in cellular, animal and human models have suggested a potential role of estrogen in cardiovascular protection. (Mendelsohn and Karas, 1999). In fact, the incidence of vascular disease is different between men and woman for the presence of various sex-related risk factors and different levels of circulating estrogen (Barrett-Connor, 1997). Coronary artery disease (CAD) accounts for one-third of all deaths in postmenopausal women. A potential role for estrogen in cardiovascular disease (CVD) protection has been long suggested by the observations that women have a reduced relative CVD risk as compared with men, but this benefit is lost after menopause, when circulating estrogen levels decrease dramatically (Bolego et al., 2006). The absence of circulating estrogen is not the only factor responsible for the increased cardiovascular risk in post-menopausal women: aging and an unbalanced ratio between androgens and estrogens are other factors that may contribute to an alteration in vascular physiology, thus to leading pro-atherogenic conditions (Mendelsohn and Karas, 2005).

Since the cloning of estrogen receptors (ERs) and generation of ER knockout animals, several mediators and mechanisms have been identified attesting a beneficial role for estrogen on the cardiovascular system in preclinical models (Bolego et al., 2006). However, which ER isoform is mainly responsible for cardiovascular protection is still controversial, as ERα and ERβ can exert distinct and sometimes opposite biologic functions (Gustafsson, 2003; Lindberg et al., 2003). In addition, polymorphisms for both isoforms have been associated with specific diseases: as an example, ERα polymorphisms are associated with coronary diseases, while ERβ polymorphisms correlate with left ventricle defects in post-menopausal women (Deroo and Korach, 2006).

Estrogen has been shown to slow down the development of atherosclerosis both in animal models and in humans acting on endothelial cells promoting vasodilatation and on vascular smooth muscle cells (VSMCs) by preventing cell proliferation (Knowlton and Lee, 2012). The generation of knockout (KO) mice lacking ERα or ERβ has provided insights into their specific role in the cardiovascular system. Overall, ERα appears to mediate most of the cardioprotective actions of estrogen including nongenomic vasodilation. As an example, estrogen treatment increases basal NO production in the aorta only in mice expressing functional ERα (Darblade et al., 2002) and the vasorelaxation to 17β-estradiol becomes more pronounced in ERβ-deficient mice (Nilsson et al., 2000). It is well

established that estrogen enhances NO production and vasorelaxation (Simoncini, 2003): in endothelial cells, E_2 activates endothelial NO synthase (eNOS) via the PI3-kinase Akt pathway leading to the production of NO, thus playing an important role in the regulation of vascular tone (Knowlton and Lee, 2012). As mentioned, E_2 is known to inhibit VSMC proliferation. Evidence demonstrates that E_2 (100 pM-10 nM) inhibits cell proliferation of VSMCs derived from atherosclerotic human arteries, positive for ERα but not for ERβ expression, with decreased levels of ERα being associated with progression of the atherosclerotic damage (Nakamura, 2004). Estrogen cardiovascular effects may also result from on direct actions on immune cells, key mediators of chronic inflammation associated with cardiovascular diseases such as atherosclerosis (Libby, 2012; Harkonen and Vaananen, 2006). This aspect will be discussed in more detail in section 10 below.

8. MENOPAUSE AND CARDIOVASCULAR RISK

Menopause is a physiological event of women's life that is the end of menstrual cycles and of the fertile period. Normally the age at which women reach menopause is between 50 and 52 years, as the world average (Fichera et al., 2013). The transition to menopause is a complex physiological process, often accompanied by the additional effects of ageing and social adjustment. Menopause results from reduced secretion of the ovarian hormones estrogen and progesterone, which take place as the finite store of ovarian follicles is depleted. Natural menopause is diagnosed after 12 months of amenorrhea not associated with a pathological cause. Menopause can also be induced by surgery, chemotherapy or radiation (Nelson, 2008). What follows, however, is the establishment of a state of hypoestrogenism, which potentially affects various organs and systems, such as the genitourinary system, the cardiovascular system, skeleton, skin, and brain, as well as the quality of life (varying degrees of vasomotor symptoms, vaginal atrophy, osteoporosis; Fichera et al., 2013).

Estrogenic deficiency of menopause is likely involved in the increased risk of osteoporosis, metabolic troubles and cardiovascular disease (CVD), a major cause of death in women from westernized societies (Wenger, 1997). Although CVD is more frequent in men than in women at any given age, aging women tend to outlive men, and the absolute number of women dying from CVD is equal or even higher (Fig. 10). Pre-menopausal women seem

to be protected against cardiovascular morbidity and mortality in comparison with men of similar age and post-menopausal women. Loss of ovarian function and subsequent deficiency of endogenous estrogens is believed to promote CVD and related death after menopause (Meyer et al., 2006).

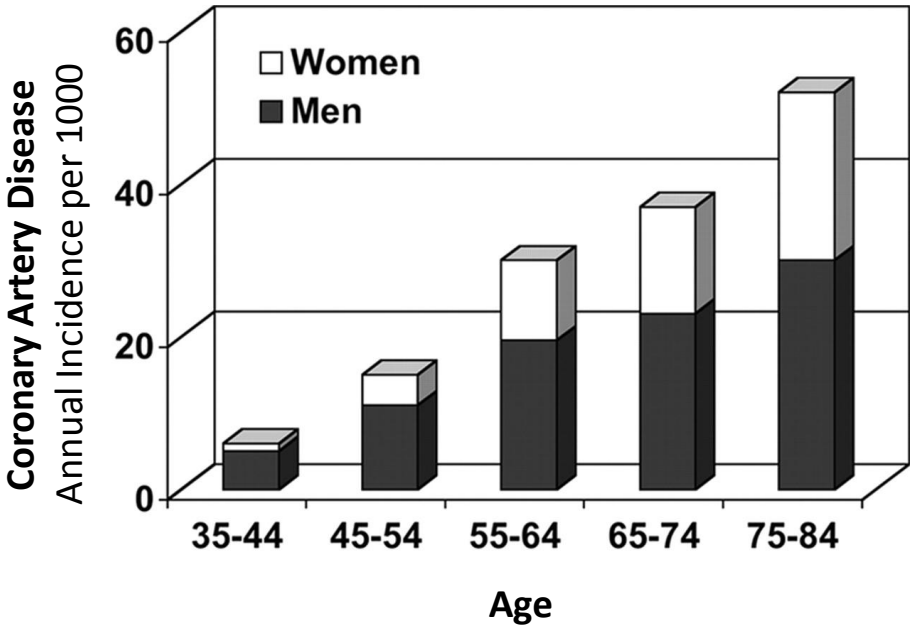

Fig. 10: Age-dependent incidence of coronary artery disease (CAD) in men and women (Framingham Heart Study). The prevalence of CAD in men is several times higher than that of age-matched pre-menopausal women, but these gender-based differences narrow after menopause, when the protection against vascular disease is gradually lost (Meyer et al., 2006)

Coronary heart disease (CHD) is widely perceived to be less of a public health problem for women than for men. However, CHD is the leading killer of women by 65 years of age. Mortality for CVD and CHD in particular has not decreased in the last 30 years in women as it has in men (Bassuk and Manson, 2010). The in-hospital mortality of an acute myocardial infarction (AMI) is higher in women than in men up to 70 years of age and survival after 6 months of AMI is lower in women (Ford and Capewell, 2007). Unfortunately, clinical trials on prevention and treatment of cardiovascular diseases have been conducted either exclusively in males or in populations with very low numbers of females. Many risk factors for CHD and strategy for preventing disease in men are also important for women; however, the magnitude of their effect may differ depending on sex (Mosca et al., 2011).

Smoking is associated with a 70% increase in CHD mortality. This risk is similar in men and women, or somewhat more pronounced in women. However, smoking rates are declining

more slowly for women than for men. Hypertension has an age-related increase and is more prominent in women. In the Women's Health Study (Martins et al., 2001), only systolic blood pressure predicts cardiovascular outcomes in women and isolated systolic hypertension, a marker of loss of large-artery elasticity, is more common in women than in men. Type 2 diabetes is also a coronary risk factor in women, increasing their risk of developing or dying from CHD by 3 to 7-fold, as compared with a 2 to 3-fold risk increase in men. Adverse cardiovascular profiles are more common among diabetic women than among men. Type 2 diabetes may be associated with greater endothelial dysfunction and inflammation in women than in men and with increase of androgen. With the menopause transition low density lipoprotein (LDL) cholesterol increases in women and small dense LDL particles, with greater susceptibility to oxidation, increase. Much of the seminal research on dyslipidemia and CHD has involved middle-aged men and none or very few women. However, in a meta-analysis of observational cohort studies of 86,000 women, high level of total and LDL-cholesterol strongly predicted CHD in women (Bassuk et al., 2010). High density lipoprotein (HDL) cholesterol level is associated with CHD in both young and old women. Women with HDL levels under 50 mg/dL experienced a doubling of risk of CHD mortality. Triglycerides may be particularly important coronary risk factors in women, especially in the presence of low HDL cholesterol levels: a meta-analysis found that hypertriglyceridemia was associated with significant risk increase of CHD of 37% and 14% in women and men, respectively (after adjustment for HDL cholesterol and other risk factors). Higher lipoprotein(a) quintiles are strongly associated with CHD mortality in women. Overall, taken together these factors represent a set of conditions leading to the development of atherosclerosis.

Additionally, menopause is itself an independent risk factor for the metabolic syndrome and its various components such as high blood pressure, abdominal adiposity, insulin resistance, and dyslipidemia (Cho et al., 2008). In the Study of Women's health Across the Nation (SWAN), the transition to menopause and the decreased estrogen levels were associated with changes in the common carotid intima-media thickness and adventitial thickness, which are indicative of the increased risk of CVD with menopause (Wildman et al., 2008) Thus, deficiency of endogenous estrogen may have significant role in the progression of atherosclerosis and increase CVD in post-menopausal women. Moreover, psychosocial factors such as depression, anxiety, and chronic psychosocial stress have

adverse effects on heart rate, blood pressure, visceral obesity, endothelial dysfunction, inflammatory activation and may raise CHD risk in women (Baggio et al., 2013).

These observations lead to the view that the estrogen deficit accompanying menopause plays a dominant role in the increased CVD risk and that this risk might be reversed by pharmacological interventions with exogenous hormones (Atsma et al., 2006).

8.1 Evolution of and controversies in menopausal hormone therapy

Experimental studies in animal models and vascular cells as well as early epidemiological data in post-menopausal women who are users and nonusers of menopausal hormone therapy (MHT) have suggested protective cardiovascular effects of estradiol. Post-menopausal hormone therapy (MHT) is used for the relief of menopausal symptoms, but the dosage has varied greatly throughout its existence (Taylor and Manson 2011). By the end of the 1990s, MHT was mainly used to prevent chronic diseases such as osteoporosis, coronary heart disease and dementia, and large prevention trials were undertaken in this context (Rozenberg et al., 2013).

Conjugated equine estrogens were first marketed in 1942 for the treatment of women with menopausal symptoms. Sales initially soared rapidly, but fell dramatically in the late 1970s when the link between estrogen use and endometrial cancer was clearly established (Smith et al., 1975). Subsequently observational studies published in the late 1980s and early 1990s almost shared the same opinion that using an MHT association of progestins and estrogens in post-menopausal women reduced the risk of CHD morbidity and mortality for both primary and secondary cardiovascular prevention (Grodstein et al., 1996). In addition, it was demonstrated that MHT had a preventive effect on loss of bone mass and on osteoporosis, thus leading MHT use to increase once again (Rozenberg et al., 1994). During the following decades, numerous clinical trials monitored the effects of MHT and presented conflicting results. In particular, the Heart and Estrogen/progestin Replacement Study (HERS) demonstrated that, after 4.1 and 6.8 years of follow-up, hormone therapy did not reduce the risk of cardiovascular events in women with CHD (Hulley et al., 1998; Herrington et al., 2000). Moreover, the Women's Health Initiative (WHI) trial was stopped early after an average 5.2-year follow-up among postmenopausal healthy women because women receiving hormone therapy had an increased risk of invasive breast cancer, along with evidence of some increase in CHD risk (Rossouw et al.,

2002). After few years of confusion about MHT and CVD, reevaluation of the results of previous randomized clinical trials such as HERS and the WHI has led to reconsideration of traditional MHT approaches and implementation of new strategies. Ongoing clinical trials such as Kronos Early Estrogen Protection Study (KEEPS; Miller et al., 2009) and Early Versus Late Intervention Trial with Estradiol (ELITE) will help clarify the benefits of MHT timing, and the possible differences in atherosclerosis effects between oral and transdermal estrogen treatment (Reslan and Khalil, 2013).

Trying to explain the reason of MHT failure in early trials, a number of factors have been proposed, including the lack of selectivity of the natural hormone 17β-estradiol for its different receptor isoforms, which can then lead to different and sometimes conflicting effects on the cardiovascular system, and the variable expression of the same receptor isoforms in relation to age and diseases already present (Murphy, 2011). With respect to female age, it was highlighted that women receiving MHT early after menopause had a significantly reduced risk of mortality, heart failure or myocardial infarction without any apparent increase in risk of cancer, venous thromboembolism, or stroke, suggesting that early initiation and prolonged MHT avoided increased collateral risks (Schierbeck et al., 2012).

In addition, it was investigated whether replacement therapy with natural or novel synthetic sex steroids could represent a therapeutic option for the treatment of atherosclerosis and its complications. Considering currently available data, it seems that an important distinction should be made between the treatment of climacteric symptoms in young, generally healthy, post-menopausal women and the prevention of chronic diseases in elderly women. As described in Fig.11, MHT seems to be beneficial and safe for post-menopausal symptomatic women aged <60 years (Schierbeck et al., 2012). Treatments with a high safety profile should be the preferred option, including low-dose MHT, estrogen-only therapy in women who have had a hysterectomy, and vaginal estrogen therapy for women with atrophic vaginitis. Non-androgenic progestin might have a reduced thrombotic and breast cancer risk, and transdermal estrogen could have a reduced thrombotic risk. Nevertheless, MHT should not be used for the prevention of chronic diseases in the elderly (>70 years old) owing to the increased risk of stroke and breast cancer in these patients (Rozenberg et al., 2013; Manson et al, 2013).

Key points

- Postmenopausal hormone therapy (PMHT) is indicated for the relief of menopausal symptoms in patients aged <60 years with climacteric symptoms
- Low doses of PMHT should be used when possible
- PMHT can be prescribed for a short period of time to treat osteoporosis when nonoestrogen therapies are unsuitable or in women who suffer simultaneously from climacteric symptoms and osteoporosis
- Use of a sequential progestin and a nonandrogenic progestin might be safer than use of continuous androgenic progestin
- Although data suggest that oestrogen therapy might prevent coronary heart disease, dementia and Alzheimer disease in young women, PMHT is not indicated for prevention of these conditions

Box 1 | Postmenopausal hormone treatment regimens

Oestrogen replacement therapy or oestrogen therapy

Includes an oestrogen only

Indicated in women who have had a hysterectomy or for vaginal therapy

Types: conjugated equine oestrogens, oestradiol, oestradiol valerate, ethinyl oestradiol, oestriol, ostradiol acetate, esterified oestrogen and oestropipate

Hormone replacement therapy or oestrogen and progesterone therapy

Includes an oestrogen and a progesterone

Sequential therapy

Oestrogen for 20–28 days followed by progesterone for 10–14 days

- Prescribed in late perimenopause or early postmenopause

Continuous therapy

- Prescribed in late menopause

Types

- Strong androgenic and/or glucocorticoid activity: medroxy progesterone acetate (oral), norethisterone acetate (orally or transdermal) or levonorgestrel (oral or intrauterine device)
- Low androgenic activity: didrogesterone (oral), micronized progesterone (oral or vaginal), trimegestone (oral) or norgestimate (oral)
- Antiandrogenic effect: cyproterone acetate (oral)
- Antimineralocorticoid activity: drospirenone (oral)

Tibolone

Has oestrogenic progestogenic and androgenic characteristics

Tissue-selective oestrogen complex

Conjugated equine oestrogen and bazedoxifene (a selective oestrogen-receptor modulator)

Fig. 11: Regimens and administration guidelines for MHT (Rozenberg et al., 2013)

9. ESTROGEN AND THE IMMUNE SYSTEM

The regulation of the immune response to infection or tissue damage is a complex interplay of multiple factors, but it has long been recognized that steroid hormones can exert powerful modulation effects at all levels of the innate and adaptive immune systems (Nadkarni and McArthur, 2013). As discussed previously, the mononuclear phagocytic system comprising monocytes, macrophages and dendritic cells together with polymorphonucleated subsets (neutrophils) are critical effectors and regulators of inflammation and the innate immune response. With regard to innate immunity, following exposure to infection or tissue damage, there is rapid mobilization and extravasation of neutrophils, effectively serving as the first arm of the immune response, but an excessive or inappropriate neutrophil accumulation, or a failure in their removal by macrophages, can induce significant damage to otherwise healthy tissue, which can lead to both acute and chronic inflammatory diseases.

A number of recent studies have focused on the role of estrogen in the regulation of neutrophil and monocyte recruitment in damaged or infected tissues. For example, estrogen has been shown to limit adhesion of both human and rodent neutrophils and monocytes to activated endothelial cell monolayers using both *in vitro* and *in vivo* models (Friedrich et al, 2006; Nadkarni, 2011), which directly limits cells transmigration and tissue infiltration. It has also been demonstrated that systemic administration of estrogen in ovariectomized animals reduces the expression of mediators of inflammation and infiltration of leukocytes in a model of vascular wall lesion (Miller et al., 2004) by inhibiting expression of vascular MCP-1, which results in decreased recruitment of monocytes to the vessel wall (Moore et al., 2013).

Another important feature that occurs during inflammation is that the vast majority of extravasated cells undergo apoptosis *in situ*, thus removal of these cells by monocytes and macrophages is of critical importance for inflammatory resolution. Phagocytic clearance of apoptotic neutrophils aids resolution not only through the removal of potential pro-inflammatory stimuli, but through the induction of an ‘alternatively activated’ phenotype in the phagocyte itself, accompanied by the induction of cytokines such as IL-10 and TGF-β (Savill et al., 2002). A recent study demonstrated the impact of ERα expression on murine macrophage function by showing that diminished ERα levels in

hematopoietic/myeloid cells impair metabolic homeostasis and accelerate atherosclerosis in female mice (Ribas et al., 2011).

Most interactions between leukocyte adhesion molecules and their endothelial receptors are induced by pro-inflammatory cytokines, and estrogens are known to affect the production of various pro-and anti-inflammatory cytokines. With regard to inflammatory cytokine production, the literature is discordant with E_2 enhancing or inhibiting secretion of tumor necrosis factor (TNF) and interleukin (IL)-1β likely related to the duration of estrogen exposure and experimental design, including cell models (Straub et al, 2007). For example, Calippe et al. (2010) observed that physiological levels of endogenous E_2 or exogenous administration of E_2 activate signaling pathways that promote inflammation in murine macrophages, while exposure to the hormone *in vivo* for a short time leads to a decreased IL-1β production (Calippe et al., 2008).

Estrogen has been also shown to enhance production (Xing et al., 2012) and prevent degradation of the endogenous NF-κB inhibitor IκB-α (Murphy et al., 2010). Inhibitory effects of E_2 on NF-κB activation were consistently found in several macrophage cell systems and are attributed to both genomic and non genomic mechanisms (Ghisletti et al., 2005; Murphy et al., 2010). Interestingly, E_2 strongly inhibits activation of the NF-κB pathway and inflammatory cytokine production by human cord blood mononuclear cells exposed to microbial products, suggesting that maternal hormones are physiological regulators of neonatal immune responses (Giannoni et al., 2011). Later in life, the production of cytokines by monocyte/macrophages is heavily influenced by the ovarian cycle and oral contraceptive use. An age-relationship of estrogen-monocyte/macrophage number and function has long been identified, which may have several implications for postmenopausal health. *In vitro* studies in human macrophages from older donors do not show significant effects of estrogens on the expression of pro-inflammatory mediators except an increase in C-reactive protein expression, which was positively correlated with the donors' plasma small-dense LDL concentration (Concoran et al., 2010).

Further studies reported that serum levels of IL-1, IL-6 and TNF-α are increased after menopause, when estrogen levels fall, and decreased by hormone therapy (Pfeilschifter, 2002). The ER isoform mediating estrogen effects on macrophage cytokine production was investigated in other studies. Expression of ERα is greater than ERβ in both

monocytes and macrophages, whereas macrophages express higher levels of ERα and lover levels of ERβ than monocytes (Murphy et al, 2009). A significant increase in lipopolysaccharide (LPS)-induced TNF release has been reported in ERα-deficient macrophages, suggesting that ERα, but not ERβ, mediates the inhibitory effects of endogenous estrogen on pro-inflammatory cytokine production in innate immune responses (Lambert et al., 2004). On the other hand, treatment with a selective GPER-1 agonist is able to inhibit, albeit at rather high concentrations, the production of TNF induced by LPS in human macrophages (Blasko et al., 2009). It has been suggested that this duality in the action of estrogen on monocytes/macrophages cytokine production may depend on many factors such as the stimulus triggering the inflammatory response (endogenous or exogenous antigens), the target organ, the concentration of estrogen present and different ER tissue expression (Straub, 2007).

It is also known that the balance in the immune system is tightly regulated by concerted interactions between antigen presenting cells, dendritic cells (DCs), T and B lymphocytes. Monocyte/macrophage functions are controlled by lymphocytes, and estrogen modulation of these pathways has been found in humans and animals. Lymphocytes are also targets for estrogens and express ERs: in particular, $CD8^+$ and regulatory T cells express ERs. The inhibitory effects of E_2 on T-cell activation are mediated through antigen-presenting cells, including monocyte-macrophages (Burger and Dayer, 2002). Dysregulation of the immune balance can lead to a variety of conditions including the failure to recognize self-antigens appropriately. Autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS) and systemic lupus erythematosus (SLE) can be used to highlight the distinct role estrogens can play on cells of the adaptive immune system (Whitacre, 2001). For example, women who suffer from MS and RA can undergo remission during pregnancy at a time when estrogen levels are high, but experience disease relapse post-partum (Ostensen and Villiger, 2007). On the other hand, women who suffer from SLE experience cyclical changes in their disease during the menstrual cycle, with a worsening of symptoms correlating with high estrogen levels during the cycle (Shabanova et al., 2008). This differential role of estrogen in autoimmune diseases could be dependent on whether the disease is B cell-mediated (such as SLE) or T cell-mediated (such as RA and MS).

A recent review on the role of estrogens on the monocyte-macrophage system in relation to cardiovascular disease has been published by our group (Bolego et al, 2013).

Overall, compared to the growing evidence on the existence of multiple phenotypes of monocytes and macrophages to which are linked specific functions (Auffray et al., 2009), little is known about estrogen effects and signaling pathways activated in these cell subsets.

AIM

The general objective of the present study was to evaluate the role of 17β-estradiol and menopausal status on human macrophage polarization.

The monocyte-macrophage system exists in at least two distinct phenotypes: classical/pro-inflammatory (M1) and alternative/anti-inflammatory (M2). Their activation state can be influenced by a variety of cytokines and microbial products that switch on distinct transcriptional networks, and an unbalanced M1/M2 ratio has been associated with several disease conditions. In particular, M1 macrophages are activated by microbial products and T helper-1 cytokines such as IFN-γ. They are associated with the production of additional inflammatory cytokines such as TNF-α and IL-1β and the induction of T cell immune response. By contrast, M2 macrophages are activated by the T helper-2 cytokines IL-4, IL-13, IL-18: alternative activation gives rise to tissue-remodeling cells, which release anti-inflammatory cytokines such as IL-10 and CCL22, and may play a role in the resolution of inflammation (Locati et al., 2013). However, the common M1/M2 classification is used to refer to the two extremes of a wide spectrum of activation states and functional phenotypes that macrophages can acquire thanks to their high plasticity.

17β-estradiol (E_2), the major female estrogen, modulates many physiological processes acting through specific estrogen receptors, which are expressed not only at the reproductive system level, but also in non-reproductive tissues including the heart, the vessel wall and immune cells. In particular, a potential role of estrogens in cardiovascular disease (CVD) protection has been long suggested by observational studies showing that premenopausal women have a reduced CVD risk compared with men, while this gender benefit is lost after menopause. However, the outcome of different clinical trials of menopausal hormone therapy has not been consistent (Reslan and Khalil, 2012; Schierbeck et al., 2012).

Given that pro-inflammatory activity of monocyte-macrophages plays a role in the development and progression of CVD, the central hypothesis of this project is that an imbalance in the M1/M2 phenotype of human macrophages is associated with differences in the inflammatory profile in relation to gender and menopausal status. Therefore, the general objective of this project was to determine the functional relationship between estrogenic pathways and human macrophage polarization *in vitro* and *ex vivo*. In particular, the specific aims were: (1) to evaluate the *in vitro* effects of 17β-

estradiol on human monocyte-derived macrophage phenotypes both under basal conditions and after polarized activation; (2) to assess the effect of estrogen status on activation phenotypes of monocyte-derived macrophages obtained from peripheral blood samples of women in their fertile age and after menopause, in an *ex vivo* model. To accomplish these aims, we developed experimental procedures to determine the activation phenotypes of monocytes (both from animal models and human subjects) and cultured human monocyte-derived macrophages. These procedures were instrumental to analyze cell morphology, gene expression profile of cytokines and other mediators as well as intracellular cytokine production, and to detect specific surface markers that characterize both M1 and M2 phenotypes (immunophenotyping). We then measured gene and protein expression of estrogen receptor (ER) isoforms in human monocyte-derived macrophages and subsequently investigated the effects of E_2 on macrophage immunophenotypes and intracellular cytokine production in resting state and after M1/M2 polarized activation. Finally, according to the hypothesis that estrogen-dependent effects on the monocyte-macrophage system protect post-menopausal women from cardiovascular disease, we characterized the phenotypes of macrophages differentiated from peripheral blood of pre- and post-menopausal women.

MATERIALS AND METHODS

1. ANIMALS

Wild-type male mice used throughout this study were obtained from the breeding of ERα and ERβ heterozygous mice, as previously described (Dupont S et al., 2000). Wild-type, *ERα*-/- strains with C57BL/6 background required for this project were purchased from Charles River Italia that serves as the exclusive, authorized commercial distributor and breeder of JAX® Mice. Mice were housed in the animal care facility of the Department of Pharmacological and Biomolecular Sciences of Milan, under the supervision of Dr. E. Vegeto. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Department of Pharmacological and Biomolecular Sciences of the University of Milan and were in accordance with the European legislation. The mice were kept under 22–23 °C on a 12h light/dark cycle for one week to adapt. Blood samples were collected by cardiac puncture: the blood volume in a mouse is approximately 2 mL (78-80 mL/kg).

2. HUMAN SUBJECTS

Human peripheral mononuclear cells (PBMCs) were isolated from buffy coats provided from the Immunotransfusional Centre of Padua University Hospital. The buffy coat is the fraction of an anticoagulated sample that contains most of the white blood cells and platelets following density gradient centrifugation of the blood. Blood samples from 20 healthy donors were processed.

Selected experiments were carried out with blood samples taken from 5 pre-menopausal (Pre-MW) and 8 post-menopausal (Post-MW) women. The protocol was approved by the local ethical committee and conducted in accordance with the Declaration of Helsinki as revised in 2000. Partecipant subject were recruited at the Division of Metabolic diseases of the University Hospital of Padua, in collaboration with Dr. GP. Fadini's and Prof. A. Avogaro's laboratory. All consecutive patients were deemed eligible, pending provision of informed consent and meeting inclusion/exclusion criteria (Table 1).

Inclusion criteria	Exclusion criteria
Age: 25-75 Pre-menopause: females during the follicular phase of the menstrual cycle Post-menopause: females with > 12 months of consecutive amenorrhea	Chronic inflammatory conditions such as autoimmune disease or inflammatory bowel disease Abuse of alcohol or recreational drugs Smoking (current or within the last year) Current or recent (within 1 month) chronic intake of medication likely interfering with study endpoints such as menopausal hormone therapy, insulin, oral hypoglycemic agents, anabolic steroids, glucocorticosteroids, non-steroidal anti-inflammatory drugs, oral contraceptives, warfarin, antibiotics, probiotics Pregnancy or breastfeeding Recent cardiovascular events Recent surgeries

Table 1: Inclusion and exclusion criteria of the study population

3. CELL CULTURE

PBMCs were isolated by density gradient centrifugation using a Ficoll-Paque solution, an hydrophilic polysaccharide commonly used in biology laboratories to separate blood to its components (density 1.077±0.001). Ficoll-Paque was placed at the bottom of a conical tube, and blood was then slowly layered above it in a 20:15ml ratio. After being centrifuged at 600g for 30', different layers were visible in the conical tube, from top to bottom: plasma and other constituents, a white layer of mono-nuclear cells called buffy coat (PBMC/MNC), Ficoll-Paque, and erythrocytes & granulocytes (FIG). PBMCs were harvested using a sterile Pasteur pipette and transferred to a new centrifuge tube. To reduce platelet contamination, cells were washed twice with PBS+5mM EDTA at 300g for 15'. Cells were then seeded at $1x10^6$/ml in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine (Lonza, Switzerland), 100 U/ml penicillin and 100 µg/mL streptomycin (Invitrogen, France). After 2h, non-adherent cells were removed by repeated washing and the remaining adherent fraction was cultured over 7 days at 37°C and 5% CO_2 in the presence of 10% fetal bovine serum (FBS, Euroclone, Italy). The medium was not replaced throughout the culture period and no further exogenous agent

was added in order to allow spontaneous monocyte differentiation into resting macrophages (M0).

Cell morphology was monitored during the whole differentiation transition from monocyte to macrophages as well as at the end of polarization protocols, and images were recorded using a phase contrast Nikon Eclipse Ti-S microscope (20x or 40x magnification).

4. PROTOCOLS OF POLARIZED ACTIVATION

Cells were seeded at the density of $15*10^6$ cells in 100mm dishes and $6*10^6$ cells in 60mm dishes. After removing the culture medium at day 7 of differentiation, M0 macrophages were polarized toward M1 phenotype by incubation for 48h with lipopolysaccharide (LPS; 1 µg/ml, Sigma, Italy and IFN-γ; 10 ng/ml). M2 polarization was obtained by adding IL-4 (20 ng/ml) and IL-13 (5 ng/ml) (PeproTech, UK) for 48h. In selected experiments M0 cells were challenged overnight with either 100nM dexamethasone or 100nM 17β-estradiol (E_2; Sigma, Italy) before polarized activation. Finally, pulse-chase experiments were performed by polarizing the cells toward M1/M2 immuno-phenotypes for 48h as described above and incubating for further 72h in the absence of any activating agent.

After polarization, macrophages were harvested by gently scraping culture plates with 1ml PBS containing 5 mM EDTA and 2% FBS for qPCR and Western blot analysis. Additional cell samples were collected in a round bottom tube for flow cytometry for phenotype characterization and cytokine detection, and analyzed using a FacsCanto II flow cytometer (BD Biosciences).

5. FLOW CYTOMETRY

5.1-Mouse

5.1.1-Characterization of murine monocyte immune-phenotypes

Identification of monocyte subsets of blood from 10 male ERα-knockout and 7 wild-type mice was performed using multiparameter flow cytometry, in collaboration with Dr. GP. Fadini's and Prof. A. Avogaro's laboratory at Padua University Hospital.

Mouse monocyte M1 and M2 subsets were defined as $Ly6C^{high}CCR2^{+}$ and $Ly6C^{Low}CX3CR1^{+}$, respectively. For analysis of classical (M1) monocytes, cells were stained with PE-anti-CCR2 monoclonal antibody (mAb, 10µl/$1*10^6$ cells), whereas for analysis of non-classical

(M2) monocytes cells were incubated with FITC-anti-CX3CR1 mAb (20µl/1*10^6 cells). The analysis was performed by selectively gating the monocyte population defined by APC-anti-Ly6C mAb staining (5µl/1*10^6 cells).

5.2-Human

5.2.1-Evaluation of leukocyte populations in a whole blood sample

We first analyzed the different leukocyte populations (lymphocytes, monocytes, granulocytes) in a sample of whole blood collected from the buffy coat used for monocyte separation. Blood was diluted 1:2 with saline buffer (0.9% NaCl) and then incubated with 2 ml of lysis buffer 1 (0.2% NaCl w/v) for 10‘ at room temperature to lyse the erythrocytes, followed immediately by the addition of lysis buffer 1 (1.6% NaCl, 0.2% sucrose) for further 10’ incubation. The sample was centrifuged at 300*g* for 5’ at room temperature, and the white cells pellet was washed twice in PBS/EDTA, suspended in 500 µl of PBS containing 2% FBS and analyzed by flow cytometry (Beckman Coulter Epics XL). A two-dimensional dot plot hystogram displayed a light scatter plot of white blood cells passing through a flow cytometer. The X-axis shows forward light scatter (FS) indicative of cell size, whereas the y-axis shows side light scatter (SS) indicative of cell granularity. The correct voltage settings of these two parameters permitted to discriminate the free PBMCs populations.

5.2.2 Identification and characterisation of monocyte subsets

Identification of monocyte subsets was performed using multiparameter flow cytometry. For analysis of classical and non-classical monocytes, cells were stained with a FITC or phycoerythrin (PE) anti-CD14 monoclonal antibody (mAb) (BD Bioscience) and a FITC- or PE-Cy5 anti-CD16 mAb (Beckman Coulter). The analysis was performed according to standardied gating strategy (Ziegler-Heitbrock et al., 2010). For more definite monocyte subsets, we stained cells with FITC anti-CD68 mAb (Dako) and PE or AlexaFluor-647 anti-CCR2 mAb (R&D Systems) for identification of M1 cells and with FITC anti-CX3CR1 (Biolegend), PE anti-CD163 (BD) and allophycocyanin (APC) anti-CD206 (BD) mAbs to identificate M2 subset. M1 cells were defined as $CD68^+CCR2^+$ cells and M2 cells were defined as $CX3CR1^+CD163^+/CD206^+$. The relative frequency of these monocyte subsets were expressed as the percentage of the total monocyte gate.

5.2.3 Characterization of human macrophage phenotypes by flow cytometry

Human macrophages were harvested by gently scraping culture plates with PBS containing 5 mM EDTA, collected in round-bottom tubes for immunolabeling (BD Biosciences) and resuspended at $1x10^6$/ml in 100 µl PBS with 2% FBS to block F_c receptors. Purity in these cultures was first assessed by staining cells with PE-anti-CD14 (20µl/$1*10^6$ cells), which is a well-known circulating monocyte marker, also expressed on mature macrophage surface; furthermore, we excluded lymphocyte contamination by staining cells with PeCy7-labeled antibody against CD3 (5µl/$1*10^6$ cells), an antigen bound to the membranes of all mature T-cells.

In order to characterize the phenotypes of spontaneously human monocyte-derived macrophages, we analyzed specific surface marker expression of resting, M1 and M2 polarized immune-phenotypes.

Macrophages were stained for 30 minutes in the dark with fluorochrome-tagged monoclonal antibodies against surface CD68-FITC (10µl/$1*10^6$ cells) and CCR2-PE (10µl/$1*10^5$ cells)to typify the M1 phenotype, and against CD206-FITC (20µl/$1*10^6$ cells), CD163-PE (20µl/$1*10^6$ cells) and CX3CR1-PerCP (10µl/$1*10^5$ cells) to characterize the M2 phenotype. In selected experiments specific staining of CD80-PE (20µl/$1*10^6$ cells) in combination with CD68-FITC was performed to further characterize M1 profile. Moreover, the established monocyte markers CD14 and CD16 were measured in subset experiments by staining cells with anti-CD14-PE, CD16-PeCy7 (5µl/$1*10^6$ cells) together with CD68-FITC after M1 polarization with LPS/IFNγ for 48h. The last panel of M1/M2 markers was selected based on a recent characterization performed in human monocytes (Fadini et al, 2013).

After incubation with specific antibodies, samples were washed and suspended in 250µl PBS/EDTA, and 10,000 events/sample for each tube were recorded in a FacsCanto II flow cytometer (BD Biosciences). Data were analyzed using the FacsDiva software (BD Biosciences). Isotype-matched controls were used as a reference .

Expression of each surface antigen and its modulation after stimulus was evaluated individually or in combination with other markers, in order to explore how polarization can affect macrophage phenotypes by down-regulating or enhancing surface protein expression or localization within the cells. During analysis, typically less than 2% positive cells were allowed beyond the statistical marker in appropriate controls.

5.2.4 Intracellular cytokine production

Intracellular cytokine production was evaluated using flow cytometry in monocytes (A) and monocyte-derived macrophages (B).

- A. Anti-coagulated blood samples from buffy coats of different donors (200µl/tube) were stimulated with IFNγ (10ng/ml) and/or LPS (1µg/ml) for 4h in the presence of monensin (10µM) added 45' before stimulation. Monensin is a protein transport inhibitor commonly used to enhance intracellular cytokine staining signals by blocking transport from the Golgi towards the extracellular environment. Samples were maintained in water bath at 37°C under gentle shaking. At the end of incubation, whole blood aliquots were put on ice for 5' to block protease activity and stained with antibody against CD14 for the identification of monocytes during flow cytometry analysis. Before cytokine detection, erythrocytes were lysed by adding 0.2% NaCl for 10' at room temperature, followed immediately by 1.6% NaCl and 0.2% sucrose for another 10'. Samples were then centrifuged (300g, 5'), supernatants were discarded and PBMCs were subsequently processed for immunostaining.
- B. After spontaneous differentiation, macrophages were stimulated with either IFNγ (10ng/ml) and/or LPS (1µg/ml) or with IL-4 (20 ng/ml) and IL-13 (5 ng/ml), for 6 to 48h as indicated for each cytokine. Cells were pretreated with brefeldin (10µM) to block cytokine secretion. At the end of stimulus, cells were harvested by gently scraping culture plates with 1ml PBS containing 5 mM EDTA and 2% FBS. Cells were collected in a round bottom tube for flow cytometry processed for immunostaining.

Where indicated, whole blood samples or monocyte-derived macrophages were incubated with 100nM 17β-estradiol or 100nM dexamethasone overnight before stimulation.

PBMCs or macrophage pellets were fixed with 100µl 4% paraformaldehyde solution and permeabilized adding 2ml of Perm Buffer solution (1x, eBioscience) containing 0.1% saponin and 0.009% sodium azide. Cells were centrifuged twice and incubated in 100µl of Perm Buffer solution in the presence of PE-anti-TNFα, FITC-anti-IL-1β,PE-anti-IL-10 or PE-anti-CCL22 (5µl/1*10^6 cells) for 20' in the dark. Subsequently, cells were washed, resuspended in 250µl PBS/EDTA and 10,000-50,000 events/sample for each tube were

recorded. In particular, to evaluate the cytokines specifically produced by monocytes in whole blood samples the monocyte subset was analyzed by gating $CD14^+$ cells.

6. GENE EXPRESSION ANALYSIS

Total RNA was isolated from about $6x10^5$ cells. Cells were washed once in PBS and RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Switzerland). cDNA was generated from 200 ng total RNA (nanodrop Thermo Scientific, Reinach, Switzerland) using RevertAid Reverse Transcriptase (Fermentas) and random primers according to the manufacturer's instructions. The relative quantification of the genes of interest was measured by real-time quantitative PCR (Q-PCR) performed using Max SYBR Green PCR Master Mix for 40 cycles of denaturation (15 sec, 95°C), annealing (30 sec, 60°C) and extension (30 sec, 72°C) on a CFX96 Real-Time PCR Detection System thermocycler (Biorad, Milan, Italy). Oligonucleotide primers were designed using the online tool for Real-Time PCR Blast and obtained from Invitrogen. The primer sequences are shown in Table 2. Results were normalized using the housekeeping gene 18S and the ΔΔ cycle threshold method, and are expressed as relative fold of stimulated over control group, used as calibrator.

	Forward	Reverse
TNF-α	TCCTTCAGACACCCTCAACC	AGGCCCCAGTTTGAATTCTT
IL-1β	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA
IL-10	TGCAAAACCAAACCACAAGA	TCTCGGAGATCTCGAAGCAT
ERα	TCCACCTGCATTTCCTTTCC	TGGGAACATGGCAGCATTTA

Table 2: qPCR primer sequences for TNF-α, IL-1β, IL10 and ERα. The primers were designed using the online tool for Real-Time PCR Blast.

7. WESTERN BLOT

For macrophage protein extraction, cells were seeded at $6*10^6$ cells/60mm dish. After polarization, cells were washed once with PBS and M0, M1 and M2 macrophages were collected in 150µl lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 25mM NaF, 0.5% Na desoxycholate, 10% SDS, 1mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and Complete® protease inhibitors from Roche). Each sample comprised 3 pooled plates and was stored

at -20°C until further analysis. Protein concentration was determined by protein assay using Lowry protocol and reagents (Lowry et al., 1951). Protein lysates were heated at 100°C for 5' to denature proteins and 40 µg of each sample were loaded onto a 10% SDS/polyacrylamide gel and run at 120V for 1h. Cell proteins were then transferred to PVDF membrane at 100V for 1h at room temperature, using a 25mM Tris, 192nM glycine and 20% methanol buffer. The membrane was blocked with 5% milk in TBS (1M Tris HCl pH 7.4, 5M NaCl, 0.1% Tween 20) for 1h at room temperature, washed, and then incubated with specific primary antibodies against ERα, ERβ and GADPH (Table 3). Immunolabeled proteins were detected by using appropriate HRP-conjugated secondary antibodies, and immunoreactive bands were revealed using enhanced chemiluminescence (ECL) detection. Band intensities were normalized to GADPH. The intensity of each blot was measured with the densitometry program ImageJ.

	Primary antibody		**Secondary antibody**	
Protein	**Origin**	**Dilution**	**Origin**	**Dilution**
ERβ	Mouse monoclonal	1:1000	Anti-mouse	1:5000
ERα	Rabbit monoclonal	1:1000	Anti-rabbit	1:5000
GADPH	Rabbit monoclonal	1:1000	Anti-rabbit	1:5000

Table 3: Antibodies used for Western blot analysis

8. IMMUNOCYTOCHEMISTRY

Estrogen receptors (ER) expression was assessed by immunocytochemistry. Cells cultured in 24-well plates were fixed with cooled 95% ethanol for 30' at room temperature, permeabilized using 0.1% Triton X-100 for 1 min, and treated with 0.3% hydrogen peroxide for 30'. Non-specific reactive sites were blocked using PBS with 2% FBS. Macrophages were incubated with rabbit or mouse monoclonal antibody toward human ERα (1:50 in ADS) and ERβ (1:50 in ADS), respectively, for 2h at room temperature. After washing, cells were stained with anti-mouse or anti-rabbit biotinylated secondary antibody (1:1000 in ADS) for 30' and subsequently incubated with streptavidin (ABC kit, Vector, UK). The reaction developed by adding a peroxidase substrate kit (Vector). Images were captured using Nikon Eclipse Ti-S microscope at 20 and 40x magnifications and a Digital Sight DS-Fi1 camera.

9. STATISTICAL ANALYSIS

Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA). Data were expressed as mean ± SEM. ANOVA followed by Bonferroni post-hoc test were used for comparison between samples. A P value < 0.05 was considered to be statistically significant.

RESULTS

1. IMMUNOPHENOTYPES OF CIRCULATING MONOCYTES FROM ERKOα MICE

In order to evaluate a possible contribution of estrogenic pathways in modulating monocyte immunophenotypes, we first analyzed monocyte subsets in both wild type (WT) and ERα-knockout (ERKOα) mice. In particular, we determined the percentages of classical ($Ly6C^{high}CCR2^{+}$) and non-classical ($Ly6C^{low}CX3CR1^{+}$) monocytes in 7 WT and 10 ERKOα. The analysis was performed by selectively gating the monocyte population defined by APC-anti-Ly6C ($Ly6C^{high}$ green circle; $Ly6C^{low}$ red circle; Fig. 1). In WT animals, the fraction of classical (M1) and non-classical (M2) monocytes was 8.1±2.2% (Fig. 1B) and 49.4±9.0% (Fig. 1C), respectively; in ERKOα mice we found 23.6±5.9% Fig. 1B) M1 and 41.5±7.6% (Fig. 1C) M2 cells, indicating a significant increase in pro-inflammatory and a slight decrease of anti-inflammatory cells in ERKOα mice. In particular, the M1/M2 ratio calculated for each group was 0.26±0.1 for WT and 0.84±0.2 for ERKOα ($p<0.05$; Fig. 1A), suggesting an imbalance ratio between M1 and M2 circulating monocytes towards the pro-inflammatory subset after genetic deletion of ERα.

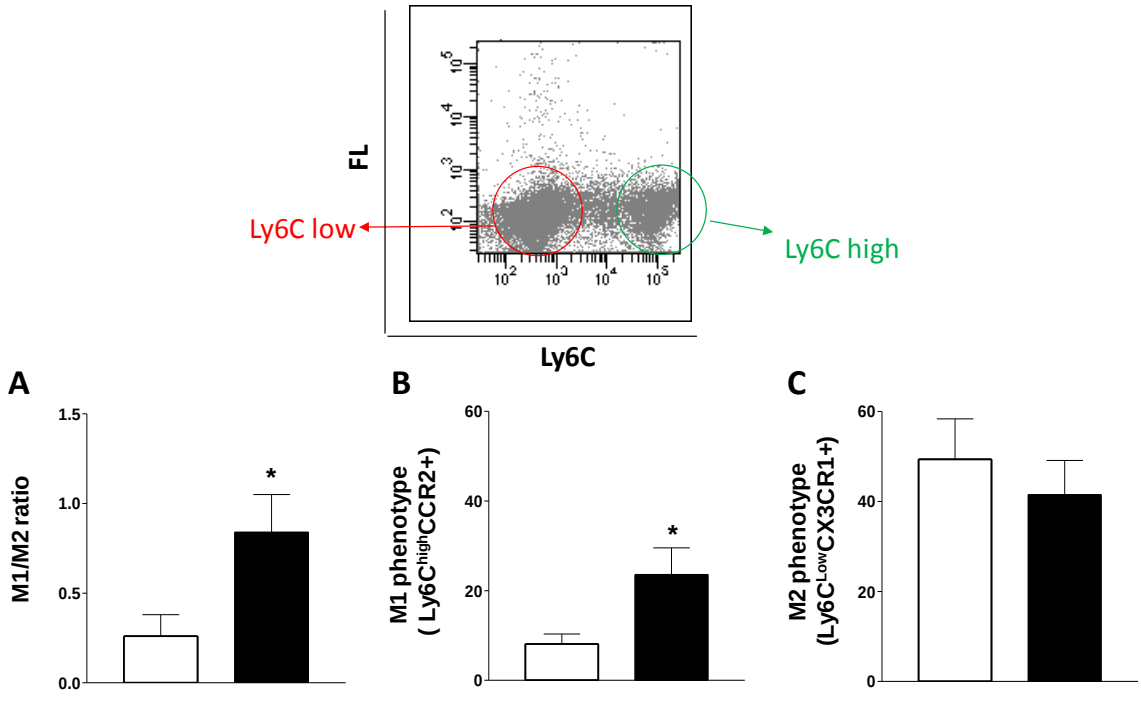

*Fig. 1: Analysis of mouse monocyte subsets. A representative plot of $Ly6C^{high}$ (green circle) and $Ly6C^{low}$ (red circle) monocytes is shown. Circulating monocytes from wild tipe (WT, white bars) and ERα-knockout (ERKOα, black bars) mice were further classified as classical M1 ($Ly6C^{high}CCR2^{+}$) and non-classical M2 ($Ly6C^{low}CX3CR1^{+}$) positive cells. Graphs represent the media (±SEM) of 7 WT and 10 ERKOα mice in separate experiments. *p<0.05 vs WT.*

2. HUMAN MONOCYTE-DERIVED MACROPHAGE CULTURES

2.1 Leukocyte distribution in human peripheral blood samples

We initially evaluated leukocyte populations in human blood samples by flow cytometry. Fig. 2 shows a two-dimensional dot plot histogram displaying a light scatter plot of white blood cells obtained from buffy coats after erythrocyte lysis passing through a flow cytometer. In particular, the x-axis shows forward light scatter (FS) indicative of cell size, whereas the y-axis shows side light scatter (SS), indicative of cell granularity. The correct voltage settings of these two parameters allow large and very granular cells (granulocytes) to be differentiated from the smaller, less granular cells (monocytes) and, furthermore, from the smallest lymphocytes. In particular, in the representative plot shown below, monocytes represented nearly 10% of total leukocytes in human blood, as reported previously (Auffray et al., 2009).

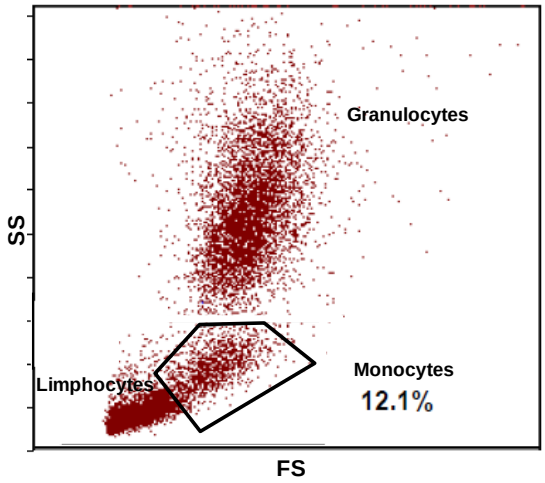

Fig. 2: The three main components of human leukocytes (lymphocytes, monocytes and granulocytes) are represented on the dot plot following flow cytometry. The three distinct populations can be distinguished basing on their size and cell complexity (granularity).

2.2 Characterization of human monocyte-derived macrophages

Blood samples from buffy coats were stratified on a Ficoll-Paque solution and the lympho-monocyte fraction was separated by density gradient centrifugation. After plating, non-adherent lymphocytes were removed, and morphology and size of adherent monocytes were observed during the differentiation period. Cell growth and differentiation were monitored from day 2 to 15. We observed that at day 2 monocytes were small and round, while at day 6 and 7 of culture monocyte-derived macrophages showed, as expected, a larger cytoplasm volume than that of monocytes, possibly due to an increase in cytoplasmatic organelles size (Fig. 3), which further increased till day 15.

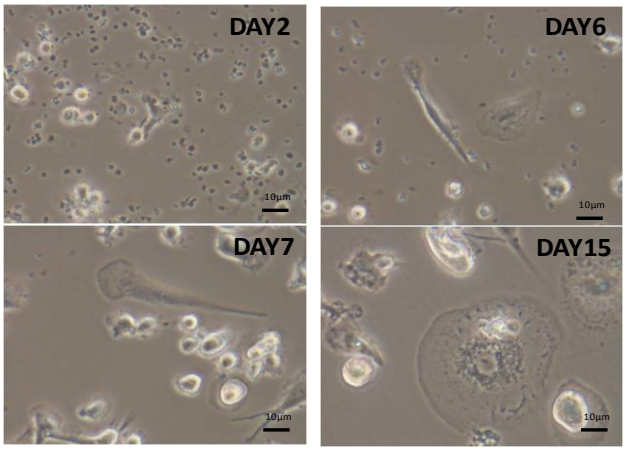

Fig. 3: Morphological changes of monocytes during differentiation toward macrophages. 40x magnification, Nikon Eclipse Ti microscope.

In order to determine the extent of lymphocyte contamination, the culture was stained with antibodies against CD14 (for monocytes-macrophages) and CD3 (for lymphocytes) surface markers. In particular, we found that after monocyte plating, at day 1 of culture, about 40% of adherent cells were $CD3^+$, while during the differentiation period the lymphocyte fraction declined progressively until day 8, when only about 10-15% of cells were $CD3^+$. Thus, at the end of the differentiation period, a minor fraction of $CD3^+$ cells was detectable in our macrophage culture consistent with previous studies using a similar differentiation protocol (Eligini et al., 2013). Representative panels of a single experiment in Fig. 4 show 77.1% of $CD14^+$cells (blue events) and 16.8% of $CD3^+$ cells (green events) at the end of the differentiation period.

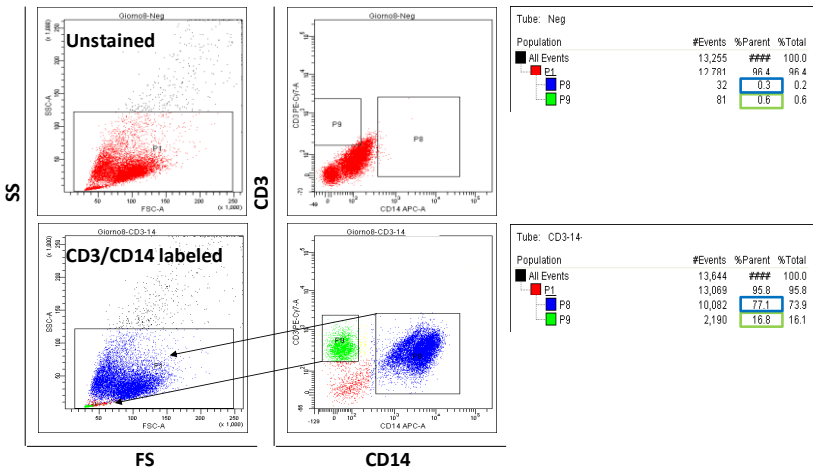

Fig. 4: Representative flow cytometry dot plots of surface CD3 and CD14 expression in a 7-day culture of monocyte-derived. Cells were stained with PeCy7-anti CD3 antibody for the lymphocyte population (green events) and PE-anti CD14 antibody for the macrophage population (blue events). Back-gating strategy allowed to localize these populations within the FS/SS plot (lower left panel).

At the end of differentiation, resting macrophages (M0) showed two dominant and distinct morphotypes as observed by phase contrast microscopy (Fig. 5A): spindle/elongated and round-“fried-egg” shaped, which routinely occurred in resting cultures consistent with recent studies (Eligini et al., 2012). Following 48h of activated polarization with LPS/IFNγ to induce the M1 phenotype or with IL-4/IL-13 to obtain the M2 phenotype, the cell morphology changed: in fact, M1-polarized macrophages were enriched in the long and spindle-shaped morphotype (Fig. 5B), while M2 polarized macrophages were largely round-shaped (Fig. 5C). These observations suggest that polarized activation affected macrophage morphology.

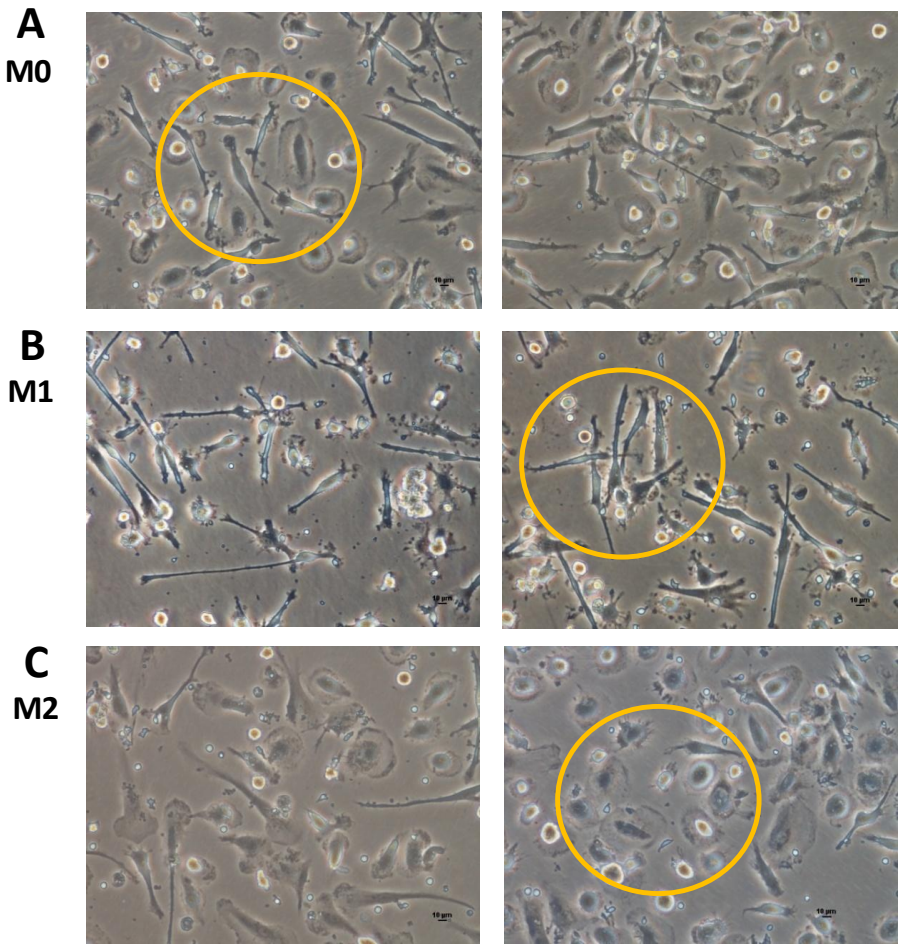

Fig. 5: Phase contrast images of macrophages differentiated from monocytes, in resting state (M0, A) and after activation with LPS/IFNγ (M1, B) or IL-4/IL-13 (M2, C) for 48h. Circles indicate the spontaneously differentiated macrophage heterogeneous population (A) and enrichment for the two main spindle-shaped (B) and round (C) morphotypes. Representative images were captured from macrophages obtained from the same donor. 20x magnification, Nikon Eclipse Ti microscope.

We further analyzed resting monocyte-derived macrophages by flow cytometry, recording 10,000 (Fig. 6A), 30,000 (Fig. 6B) and 50,000 (Fig. 6C) events of the same sample. We detected two distinct populations distributed in the FS/SS plot based on their size and granularity (i.e. intracellular complexity). In particular, as described in Fig. 6A, all macrophages were found in the same SS range, thus reflecting similar granularity, but were differently distributed on FS, hence with increasing size. The two macrophage morphotypes were already detectable when recording 10,000 events and appeared to match the two morphotypes as detected using phase contrast microscopy (Fig. 5).

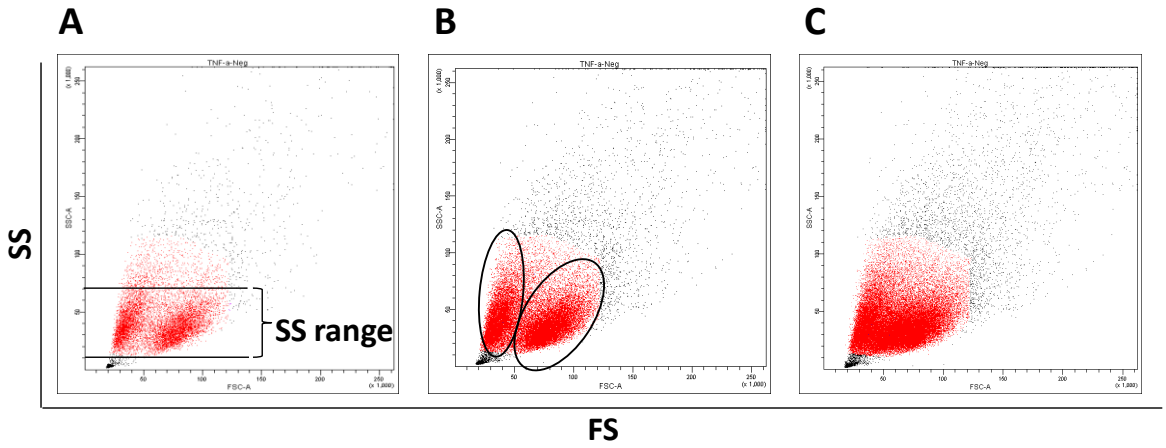

Fig. 6: Flow cytometry dot plots showing forward scatter (FS) and side scatter (SS) of spontaneously differentiated macrophages in resting state. Representative panels report 10,000 (A), 30,000 (B) and 50,000 (C) events recorded from the same sample.

In order to confirm that the M1 and M2 immunophenotypes correlated with the two distinct cell population observed in the FS/SS plots above, experiments were performed by incubating cells with a FITC-anti-CD68 antibody to identify M1 macrophages, and a PE-anti-CD163 antibody to identify M2 macrophages, both in resting state (M0) and after polarization with either LPS/IFNγ (M1) or IL-4/IL-13 (M2) for 48h. In the resting state (M0) the two distinct populations as described above could be clearly distinguished. Representative panels in Fig. 7 show subsets of $CD68^+$ (blue) and $CD163^+$ (green) cells. These results suggest that $CD68^+$ cells (left) corresponded to the spindle-elongated sub-population, while the $CD163^+$ subset (right) corresponded to the round-shaped macrophages, in agreement with the morphological changes observed by phase contrast microscopy in Fig. 5B and C. After polarization with LPS/IFNγ, the percentage of $CD68^+$ cells increased, while that of $CD163^+$ cells decreased; conversely, following IL-4/IL-13 treatment, the percentage of $CD163^+$ cells tended to increase.

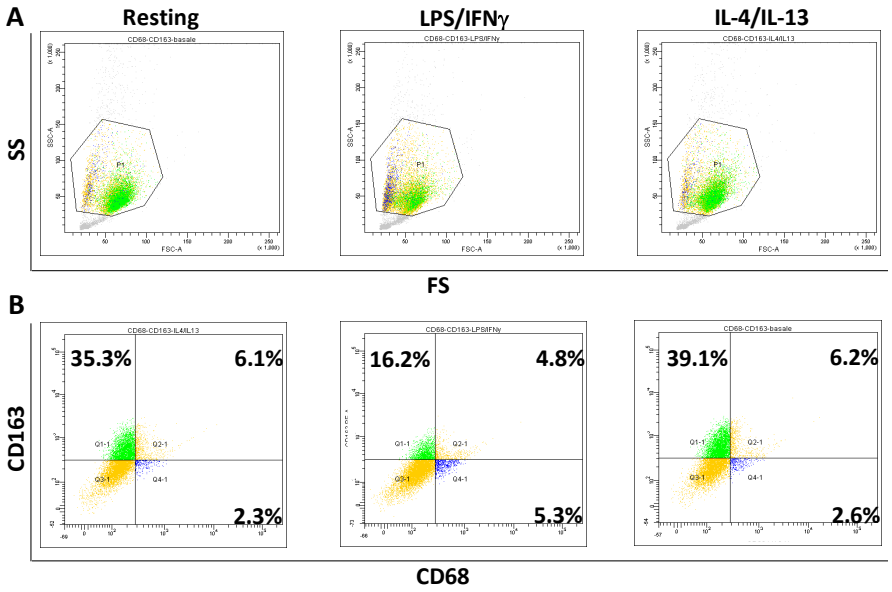

Fig. 7: Flow cytometry FS/SS (lane A) and fluorescence quantification dot plots (lane B) of CD68$^+$ and CD163$^+$ cells in M0 (left), M1 (middle) and M2 (right) macrophages. Cells were cultured for 7 days in RPMI1640 with 10% FBS and incubated with fresh medium or stimulated with LPS/IFNγ (M1) and IL-4/IL-13 (M2) for 48h. Blue and green events represent CD68$^+$ or CD163$^+$ macrophages, respectively. Images from one representative experiment are shown.

3. IMMUNOPHENOTYPING OF DIFFERENTIATED MACROPHAGE

Human monocyte-derived macrophage immunophenotypes were further characterized by analyzing specific surface markers expression of cells cultured without stimuli other than the growth medium (M0) or in the presence of a pro- (LPS/IFNγ) or anti-inflammatory (IL-4/IL-13) microenvironment.

3.1 Characterization of the M1/pro-inflammatory macrophage subset

We then analyzed by flow cytometry the fraction of M1 macrophages as defined by either positive single-staining with an antibody against CD68 or by specific double-staining of CD68$^+$/CCR2$^+$ cells. Graphs in Fig. 8 show that, under resting conditions, 10.9±2.4% of M0 macrophages were CD68$^+$ and 10.3±2.5% were CD68$^+$/CCR2$^+$ (see also the representative flow cytometry analysis in Fig. 8A). Following incubation with LPS/IFNγ for 48h, the amount of CD68$^+$ macrophages increased (19.1±2.9 vs 10.9±2.4%, $p<0.05$; n=9, Fig. 8B; see also Fig. 8A, red squares) but no significant modulation of CD68$^+$/CCR2$^+$ cells was observed, compared with resting macrophages (12.9±3.4 vs. 10.3±2.5 %; n=9, Fig. 8C). M2 polarized activation with IL-4/IL-13 did not significantly affect the fraction of CD68$^+$ and CD68$^+$/CCR2$^+$ cells compared with resting macrophages (14.7±6.6 vs. 10.9±2.4% for CD68$^+$cells and 13.3±6.1 vs 10.3±2.5% for CD68$^+$/CCR2$^+$ cells, respectively, n=9).

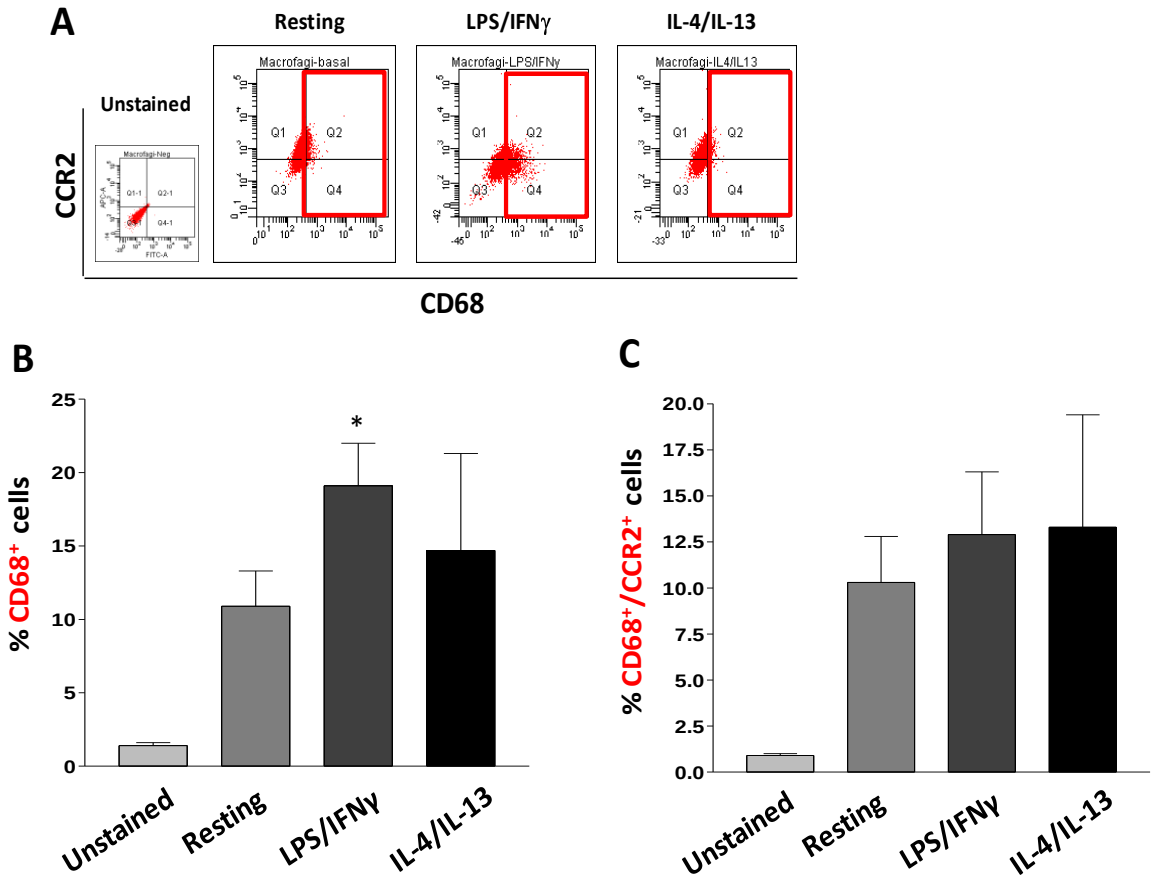

*Fig. 8: Percentage of M1 macrophages as defined by $CD68^+$ and $CCR2^+$ cells. Cells were differentiated from monocytes for 7 days in RPMI1640 + 10% FBS and thereafter activated with LPS/IFNγ or IL-4/IL-13 for 48h. Fluorescence quantification dot plots (A) are representative of a single experiment: red squares highlight $CD68^+$ cells and their modulation after M1 and M2 polarization. Bars represent the mean (±SEM) of $CD68^+$ (panel B) and $CD68^+/CCR2^+$ (panel C) cells from 9 independent experiments. *p<0.05 vs resting.*

We further characterized M1 monocyte-derived macrophages by quantifying the standard monocyte markers cluster of differentiation (CD)14 and CD16 together with CD68. Based on CD14 and CD16 expression, the standard monocyte nomenclature distinguishes M1/pro-inflammatory ($CD14^+$ $CD16^-$) and M2/anti-inflammatory ($CD14^+CD16^+$) monocytes (Fadini et al., 2013). We found that the percentage of $CD14^+/CD16^-/CD68^+$ macrophages was about three-fold higher in M1 compared with M0 macrophages (22.9±5.8 vs 8.6±1.4%, n=3; p<0.07, Fig. 9). Overall, these data indicate that M1 cells share characteristics with pro-inflammatory circulating monocytes and can be defined as either $CD68^+/CCR2^+$ or $CD68^+CD14^+CD16^-$ cells.

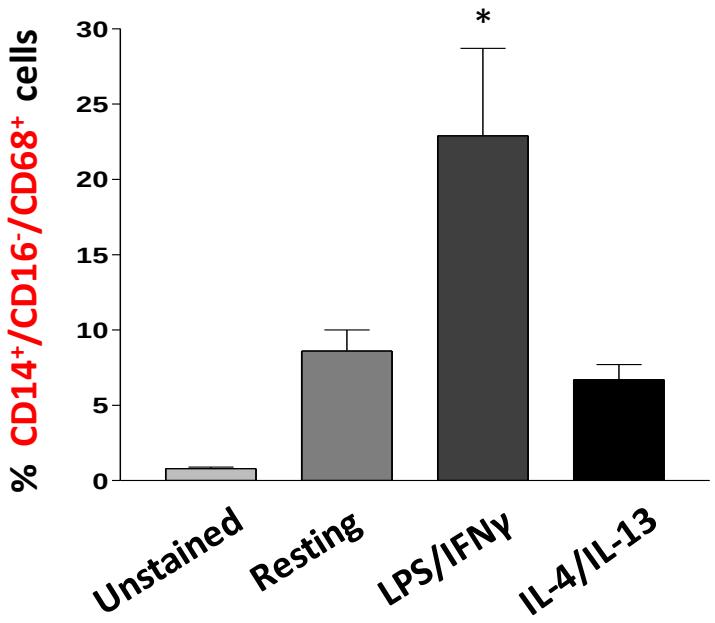

*Fig. 9 Percentage of M1 macrophages as defined by $CD14^+/CD16^-/CD68^+$ cells. Macrophages under resting conditions and after incubation with LPS/IFNγ or IL-4/IL-13 for 48h were stained with PE-anti CD14, PeCy7-anti CD16 and FITC-anti CD68 specific antibodies. Data are expressed as mean (±SEM) of 3 independent experiments. *p<0.07 vs resting.*

In a second set of experiments we evaluated the percentage of macrophages expressing another marker of the M1/pro-inflammatory phenotype, CD80, a co-stimulatory antigen that supports T-cell activation. After polarization with LPS/IFNγ, the fraction of $CD80^+$ cells significantly increased compared with resting macrophages (86.8±4.3 vs 60.3±9.1%; *p<0.05, n=4. Fig. 10), while the percentage of $CD80^+$ cells was similar to that observed in resting state following polarization with IL-4/IL-13 (58.3±5.3 vs 60.3±9.1%; n=4).

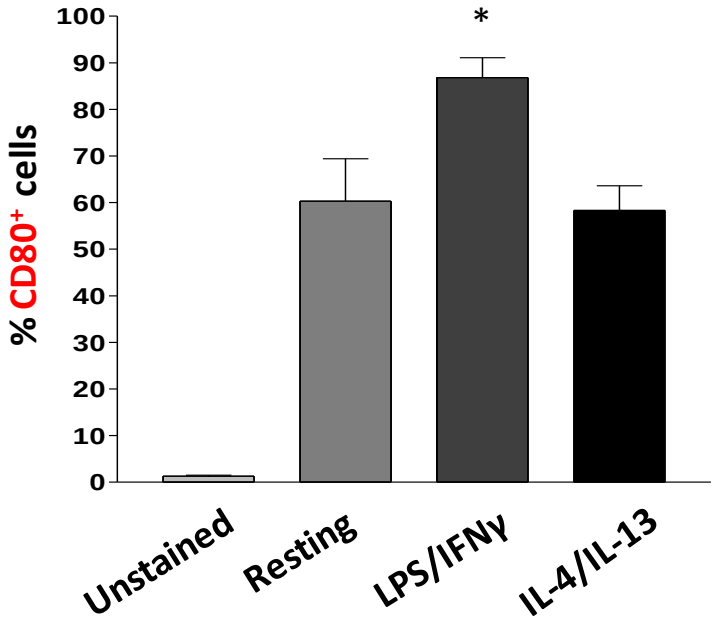

*Fig. 10: Percentage of M1 macrophages as defined by $CD80^+$ cells. Macrophages under resting conditions and after incubation with LPS/IFNγ or IL-4/IL-13 for 48h were stained with PE-anti CD80 specific antibody. Bars represent the mean (±SEM) of 4 independent experiments. *p<0.05 vs resting.*

3.2 Characterization of the M2/anti-inflammatory macrophage subset

We next analyzed by flow cytometry the M2/anti-inflammatory subset as defined by $CD163^+$, $CD206^+$ and/or $CX3CR1^+$ cells. In resting state, 60.8±4.2% of cultured macrophages were $CD206^+$, 46.7±5.8% were $CD163^+$ and 31.9±10.6% were $CX3CR1^+$. After polarization with LPS/IFNγ, the percentages of $CD206^+$ and $CD163^+$ as well as $CX3CR1^+$ cells were significantly decreased compared with resting macrophages (34.6±4.5 vs 60.8±4.2% $CD206^+$, Fig. 11B; 17.3±4.8 vs 46.7±5.8% $CD163^+$, $p<0.005$, n=18, Fig. 11C; 31.9±10.6 vs 17.6±7.7% $CX3CR1^+$, n=11, Fig. 11D). Similarly, when evaluating the double-stained $CD206^+/CD163^+$ as well as the triple-stained $CD206^+/CD163^+/CX3CR1^+$ cell subpopulations, LPS/IFNγ treatment decreased the abundance of both subsets with respect to resting macrophages (20.9±6.4 vs 42.8±8.8% $CD206^+CD163^+$, $p<0.05$, n=11, Fig. 12A; 14.3±5.7 vs 29.2±10.4% $CD206^+CD163^+CX3CR1^+$ cells, n=11, Fig. 12B).

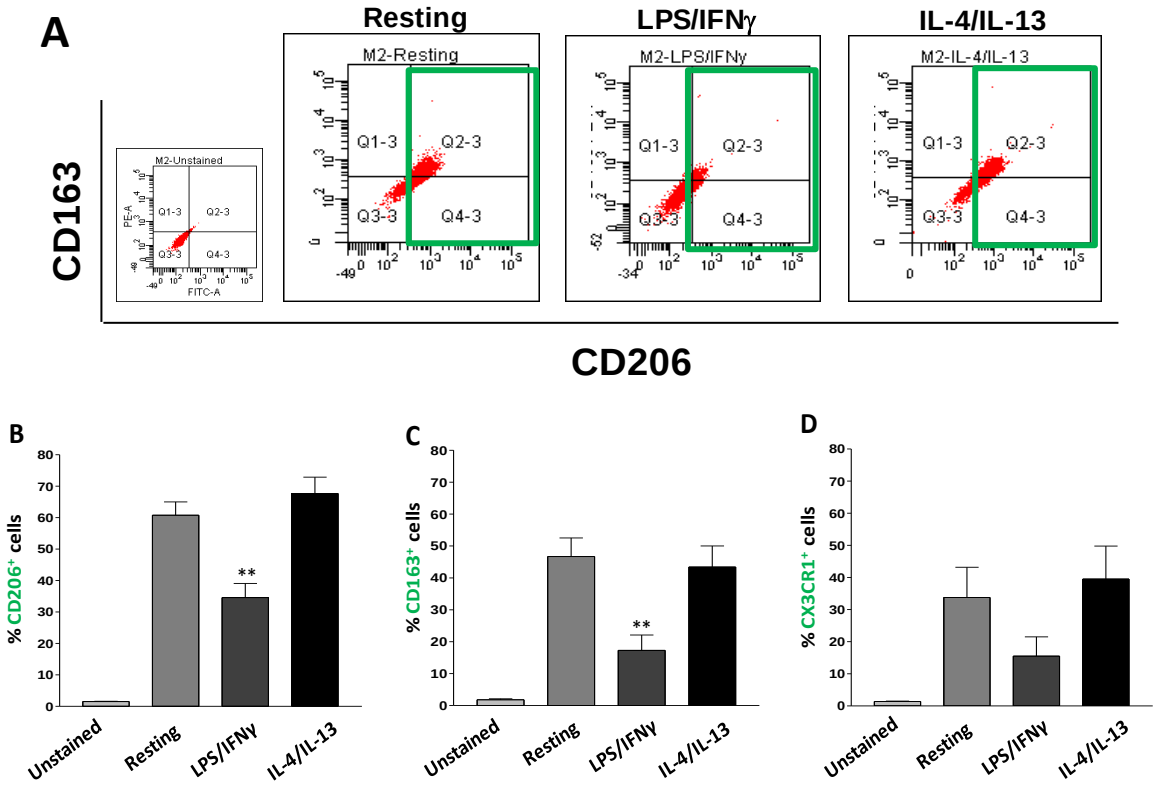

*Fig. 11: Quantification of M2 macrophage markers as percentage of $CD206^+$, $CD163^+$ and $CX3CR1^+$ cells. Spontaneously differentiated macrophages were polarized as described above in the legend to Fig. 8 and stained with FITC-anti-CD206, PE-anti-CD163 and PerCP-anti-CX3CR1 specific antibodies. Fluorescence quantification dot plots (A) are representative of a single experiment: green squares highlight $CD206^+$ cells. Bars represent the mean (±SEM) of $CD206^+$ (panel B), $CD163^+$(panel C) and $CX3CR1^+$ (panel D) cells of 18 (B, C) and 11 (D) independent experiments. *p<0.005 vs resting.*

When polarizing with IL-4/IL-13 for 48h, the percentage of cells specifically stained for each marker was not different from that in resting cells (Fig. 11A, B and C). Similar results were obtained for the percentage of $CD206^+/CD163^+$ (Fig. 12A) as well as $CD206^+/CD163^+/CX3CR1^+$ cells (Fig. 12B).

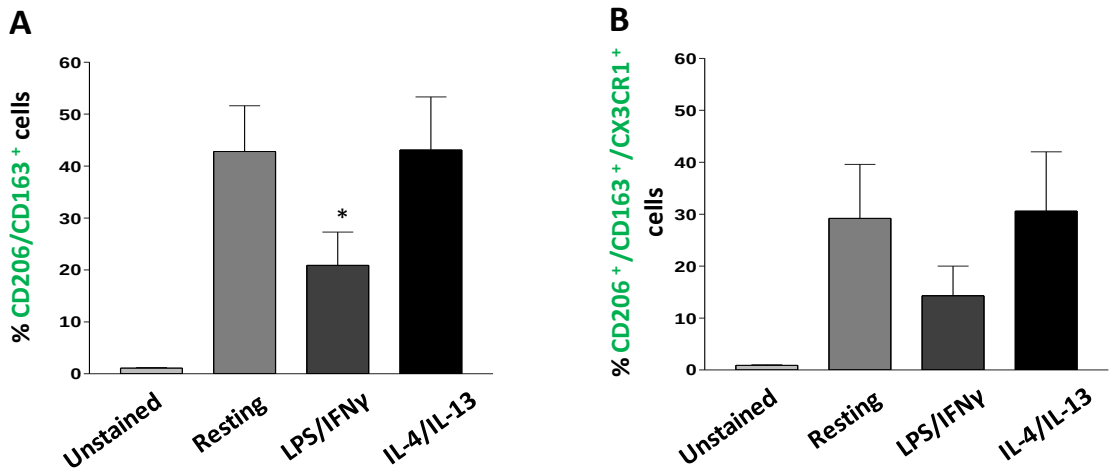

*Fig. 12: Percentage of M2 macrophage subsets identified as $CD206^+CD163^+$ cells (panel A) and $CD206^+CD163^+CX3CR1^+$ cells (panel B) under basal conditions and after 48h polarization with either LPS/IFNγ or IL-4/IL-13. Bars represent the mean (±SEM) of 11 independent experiments. *p<0.06 vs resting.*

The M2/anti-inflammatory phenotype kinetics was further analyzed in a set of pulse-chase experiments. The fractions of $CD206^+$, $CD163^+$ and $CX3CR1^+$ cells were measured after 48h polarization (*Pulse*) in the presence or absence of LPS/IFNγ or IL-4/IL-13, and after further 72h (*Chase*) in the absence of any activating agent. In the resting state, the percentage of resting $CD206^+$, $CD163^+$ as well as $CX3CR1^+$ cells was slightly decreased following chase (further 72h incubation) when compared with percentages at pulse end (29.5±6.9 vs 58.2±12.3% $CD206^+$ cells, Fig. 13A; 28.9±9.5 vs 40.5±18.6% $CD163^+$ cells, Fig.13B; 18.7±9.3 vs 31.7±21.1% $CX3CR1^+$ cells, Fig 13C, n=4). After M1 polarization (Pulse), expression of M2 markers was significantly lower with respect to that observed in resting macrophages (36.5±10.3 vs 58.2±12.3% $CD206^+$ cells, $p<0.05$, Fig. 13A; 8.1±3.7 vs 40.5±18.6% $CD163^+$ cells, $p<0.05$, Fig. 13B; 5.2±4.2 vs 31.7±21.1% $CX3CR1^+$ cells, Fig. 13C, n=4) and further decreased after further 72h in the absence of any activating agent (Chase) (18.4±8.4 vs 29.5±6.9% $CD206^+$cells, Fig. 13A; 1.7±0.7 vs 28.9±9.5% $CD163^+$cells, $p<0.05$, Fig. 13B; 0.5±0.1 vs 18.7±9.3% $CX3CR1^+$cells, Fig. 13C, n=4). Moreover, $CD206^+$ cells doubled after chase in cells that had undergone M2 (IL-4/IL-13) polarization with

respect to unpolarized cells (74.2±7.5 vs 29.5±6.9% CD206$^+$ cells, p<0.05, n=4, Fig. 13A, black bars). By contrast, no significant changes in CD163$^+$ as well as in CX3CR1$^+$ cell subsets were observed using this experimental design.

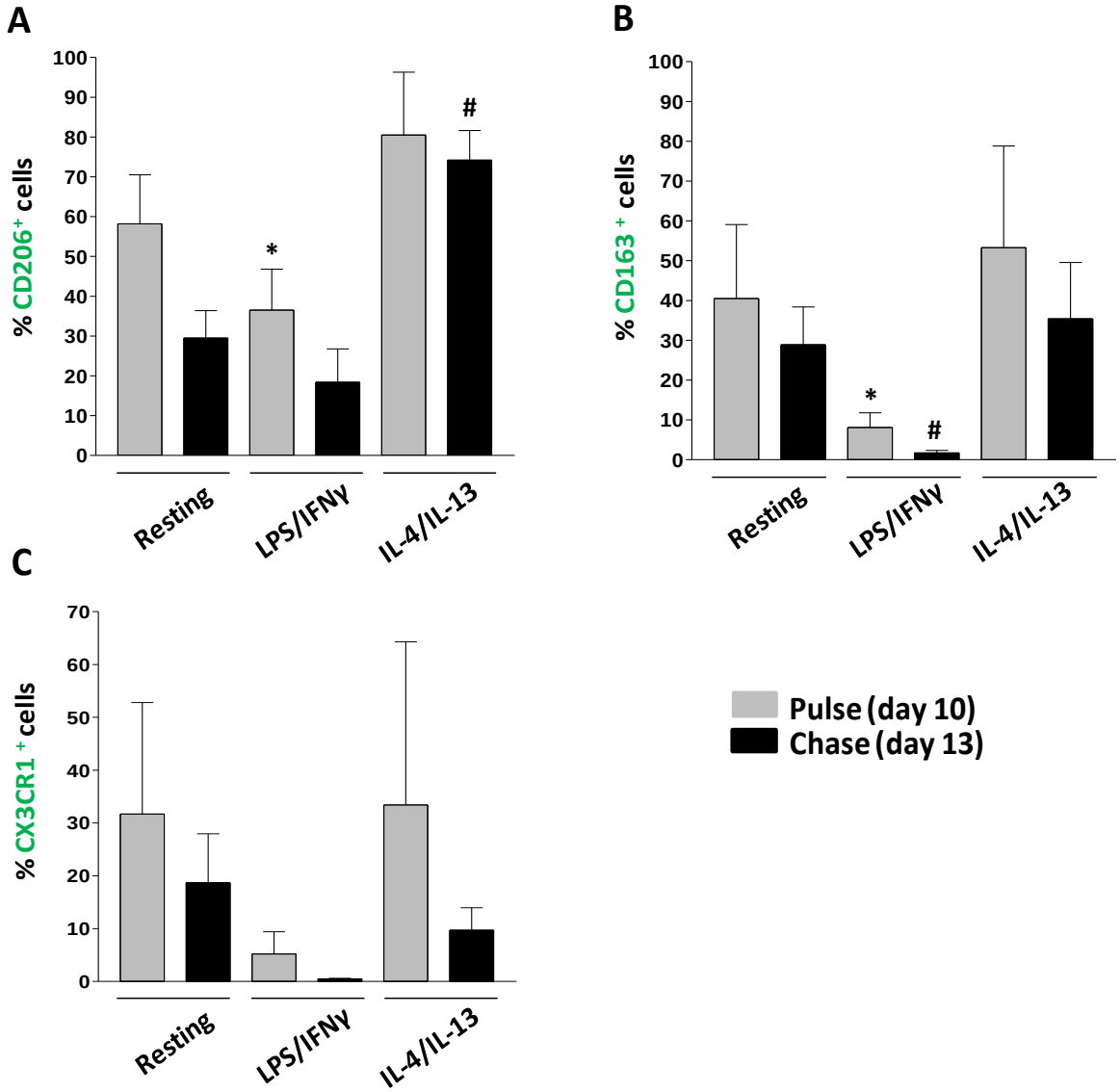

*Fig. 13: Percentage of CD206$^+$, CD163$^+$ and CX3CR1$^+$ cells in pulse-chase experiments. Spontaneously differentiated macrophages were polarized for 48h as described in the legend to Fig. 8 (Pulse; grey bars). At this time point cells from all groups were washed and incubated for another 3 days in RPMI in the absence of polarizing agents. At the end of the latter incubation the percentages of cells expressing M2 markers were determined again (Chase; black bars). Data are expressed as mean (±SEM) of 4 independent experiments. *p<0.05 vs. Pulse; # p<0.05 vs. Resting Chase).*

3.3 Gene expression profile of target cytokines

We further characterized the immunophenotypes obtained after either M1 or M2 polarization by performing qRT-PCR analysis for TNF-α, IL-1β and IL-10 mRNA levels. In another set of experiments (see paragraph 4.3 below) we analyzed the intracellular accumulation of the same cytokines by flow cytometry. After 6h stimulation with LPS/IFNγ macrophages showed higher mRNA levels of the genes encoding for TNF-α (57.5±10.4 fold change, $p<0.005$) and IL-1β (9.0±1.9 fold change, $p<0.005$) compared with M0 (Fig. 14). The relative increase in mRNA levels after 48h was more marked for IL-1β (408.9±92.9 fold change, $p<0.005$) than for TNF-α (25.2±7.4 fold change, $p<0.05$). Conversely, mRNA levels for TNF-α rose more sharply after 6h and decreased after 48h (57.5±10.3 fold change at 6h vs 25.2±7.4 fold change at 48h). Macrophages stimulated for 6 and 48h with IL-4/IL-13 to enhance the M2 phenotype displayed comparable mRNA levels of genes encoding both TNF-α and IL-1β with respect to those of resting macrophages. The anti-inflammatory cytokine IL-10 mRNA was unexpectedly more abundant in cells treated with LPS/IFNγ (M1) than in cells treated with IL-4/IL-13 (M2) at both time points (49.3±3.9 (M1) vs 6.6±3.7 (M2) fold change at 6 h, $p<0.005$; 21.2±8.4 (M1) vs 1.2±0.4 (M2) fold change at 48 h, respectively; $p<0.05$). In particular, IL-10 mRNA peaked after 6h incubation with LPS/IFNγ (Fig. 14).

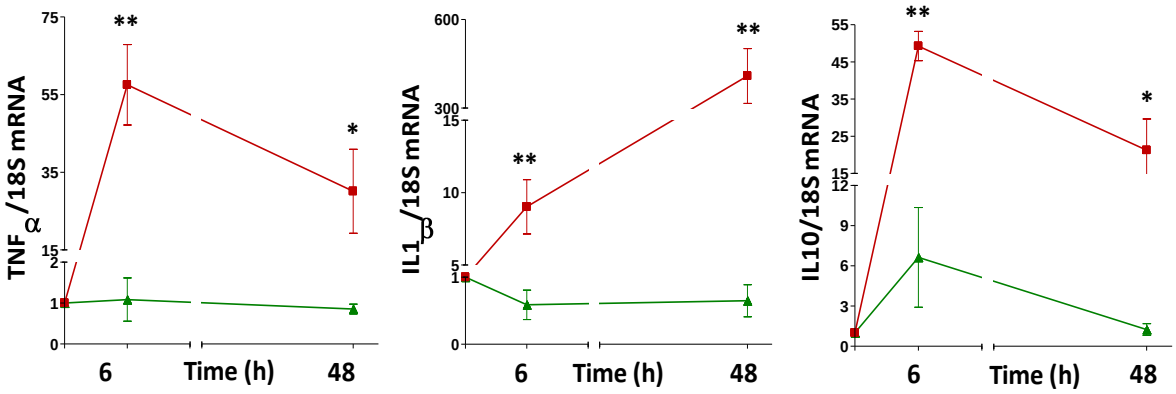

*Fig. 14: Expression profiles of M1 and M2 phenotypic markers. Macrophages were differentiated from monocytes for 7 days in RPMI1640 + 10% FBS and thereafter incubated with fresh medium (Resting) or activated with LPS/IFNγ (M1, red lines) or IL-4/IL-13 (M2, green lines) for 6 or 48h. TNF-α, IL-1β and IL-10 mRNA levels were measured by Q-PCR and normalized to 18S. The mRNA level of selected genes in resting macrophages was assigned a value of 1, and mRNA levels following activation were shown relative to resting. Data are shown as mean ±SEM of 6 independent experiments. *p<0.05 and **p<0.005 vs resting.*

4. EFFECTS OF ESTROGEN ON HUMAN MONOCYTES AND MACROPHAGE POLARIZATION AND FUNCTION

4.1 ER expression in macrophage immunophenotypes

Few data are available on the modulation of macrophage phenotype by estrogen/ER pathways. We therefore evaluated whether estrogens may influence the M1/M2 balance in human macrophages. Firstly, we assessed the expression profile of ER isoforms in spontaneously differentiated monocyte-derived macrophages by immunocytochemistry and Western blot analysis. Resting macrophages expressed both ER isoforms (Fig. 15). In particular, ERα and ERβ were localized in the nucleus and cytoplasm of both spindle- and round-shaped cells.

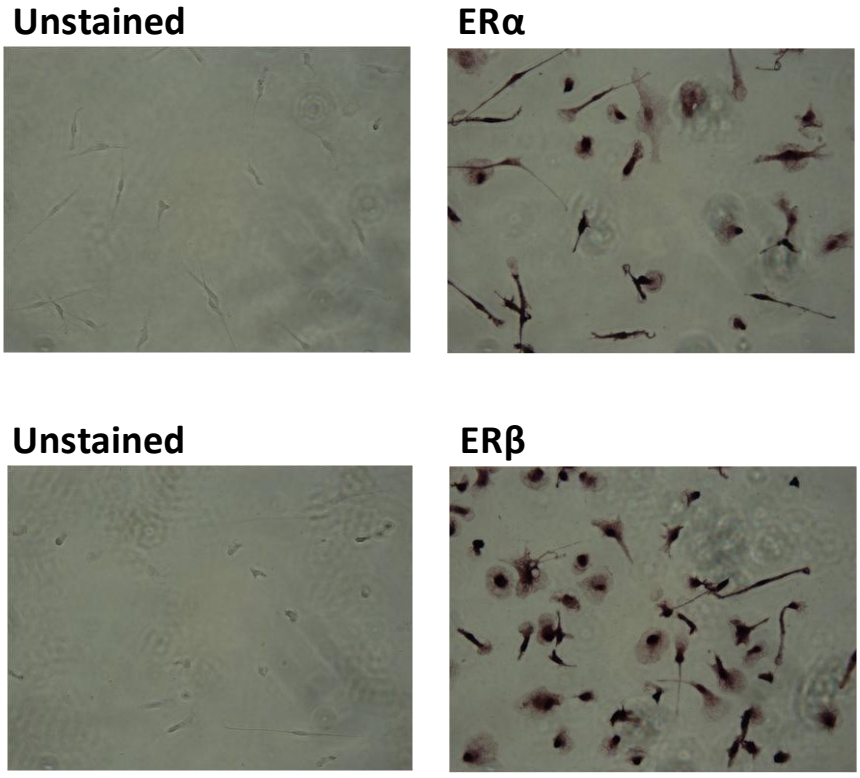

Fig. 15: Representative immunocytochemistry staining of estrogen receptor (ER) isoforms in resting macrophages. After 7 days of spontaneous differentiation from human monocytes, cells were incubated with antibodies against ERα and ERβ or vehicle. Positive staining was revealed using diaminobenzidine (DAB) which is reduced by peroxidase in the presence of hydrogen peroxide forming a brown precipitate. 20x magnification, Nikon Eclipse Ti microscope.

We then assessed how polarized activation toward M1 or M2 immunophenotypes could modulate ER expression profiles. After 48h incubation with LPS/IFNγ (M1), ERα expression was significantly down-regulated by 23% compared with resting (M0) macrophages, while incubation with IL-4/IL-13 (M2) did not alter the protein level with respect to M0 (Fig.

16A). By contrast, ERβ expression was comparable in all macrophage subsets (Fig. 16B). Consistently, we also observed that M1 macrophages showed lower mRNA levels for ERα gene after 48h polarization with respect to M0 macrophages (0.5±0.1 fold change, p<0.005, Fig. 16C). Conversely, M2 macrophages showed increased ERα gene expression after 6h polarization with respect to M0 macrophages (1.78±0.2 fold change, p<0.005, Fig. 16C).

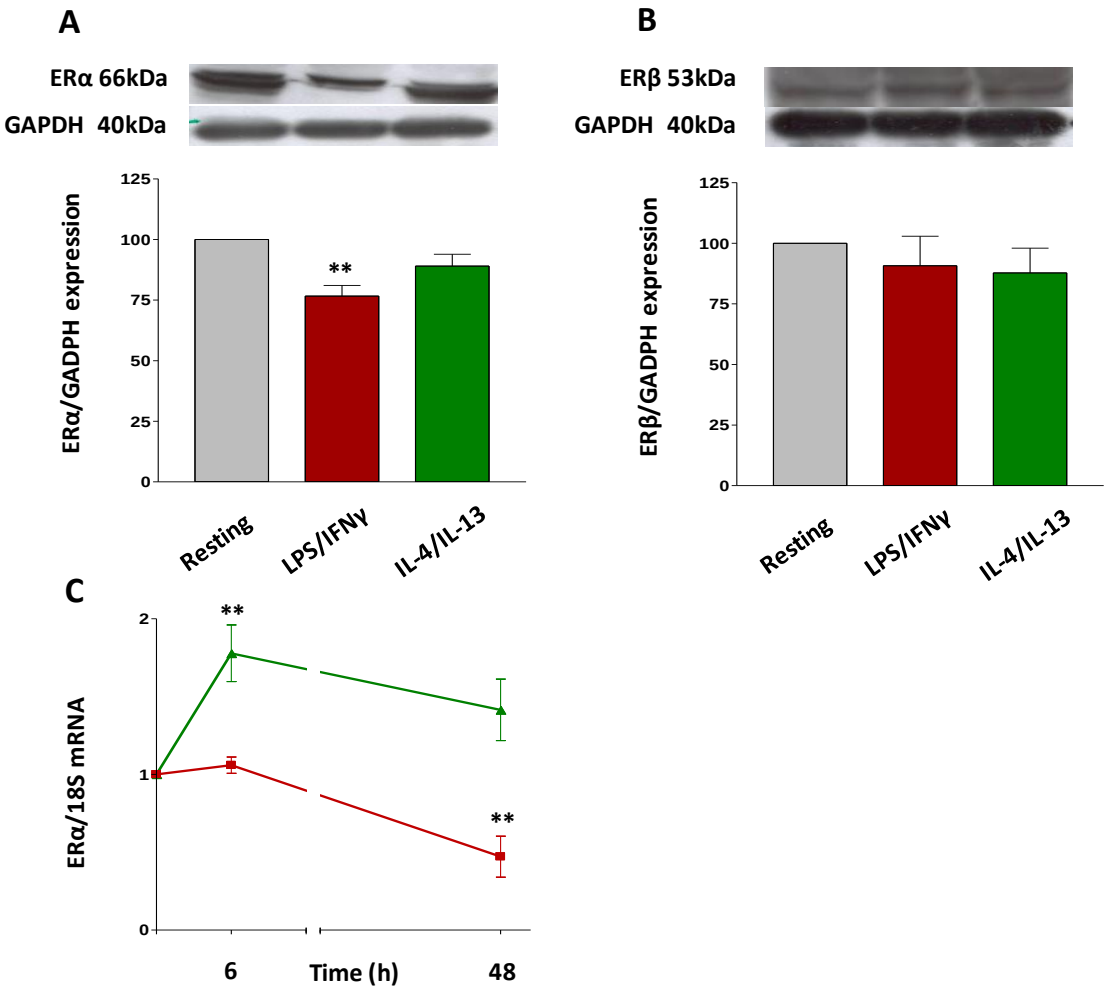

*Fig. 16: Estrogen receptor (ER) expression in spontaneously differentiated macrophages. Results from a representative immunoblot analysis of total protein extracts from Resting, M1 and M2 macrophages show a significative down-regulation of ERα (panel A), but not of ERβ (panel B) after 48h LPS/IFNγ incubation. GADPH served as loading control. Bars represent the mean (±SEM) of 6 independent experiments. *p<0.005 vs resting. (C). ERα mRNA levels of LPS/IFNγ-(red) and IL-4/IL-13-(green) stimulated macrophages were measured by Q-PCR and normalized to 18S. Results are presented relative to M0, to which we assigned a value of 1. Data are shown as mean ±SEM of 6 independent experiments. **p<0.005 vs resting.*

4.2 Effects of dexamethasone and 17β-estradiol on macrophage polarization

In a separate set of experiments, we evaluated the effect of dexamethasone (Dexa) and 17β-estradiol (E_2) on macrophage immunophenotypes after polarization. Cells were pre-treated with dexa or E_2 (both 100nM) overnight and subsequently incubated with fresh medium to obtain resting macrophages (M0) or with medium in the presence of either LPS/IFNγ or IL-4/IL-13 to obtain macrophages polarized to M1 and M2 phenotypes, respectively. Under basal conditions and after polarization, glucocorticoid treatment significantly increased the percentages of both $CD163^+$ and $CD206^+/CD163^+$ cells (M2) with respect to untreated macrophages (M0; Table 2). Treatment with Dexa also prevented the decrease in M2 macrophages, as measured taking into account the fraction of $CD163^+$ and $CD206^+$/CD163 cells, induced by pro-inflammatory activation (LPS/IFNγ).

	% $CD163^+$ cells	% $CD206^+CD163^+$ cells
Unstained	1.5±0.1	1.0±0.1
Resting	49.6±9.5	37.1±7.1
Dexa	83.3±5.5#	70.3±7.5 #
LPS/IFNγ	23.4±8.3*	20.8±4.6*
Dexa + LPS/IFNγ	64.4±10.#	40.0±7.9#
IL-4/IL-13	50.7±11.2	46.3±7.5
Dexa + IL-4/IL-13	78.8±8.8#	73.5±10.2#

*Table 2: Effect of dexamethasone on macrophage polarization. Macrophages were pre-treated overnight with dexamethasone (Dexa; 100nM) and incubated with medium in the presence or absence of LPS/IFNγ or IL-4/IL-13 for 48h. The percentage of $CD163^+$ or $CD206^+/CD163^+$ cells was measured by flow cytometry. Data represent media (±SEM) of 10 and 12 independent experiments, respectively. *p<0.05 vs resting, #p<0.05 vs untreated.*

Fig. 17 reports the effect of E_2 on macrophages polarization. A significant decrease in the fraction of $CD206^+/CD163^+$ cells was observed after incubation with LPS/IFNγ compared with resting cells (13.7± 3.1 vs 39.2± 5.2%, $p<0.005$, n=12, Fig. 17). Treatment of macrophages with E_2 prevented the effect on M2 markers induced by LPS/IFNγ: in fact, the fraction of $CD206^+CD163^+$ cells was 27.6±3.8% in the presence of E_2 *vs* 13.7±3.1% in untreated macrophages ($p<0.05$, n=12, Fig. 17). However, E_2 treatment did not modify the percentage of $CD206^+CD163^+$ cells compared with M0 (resting) and M2-polarized macrophages (35.2±36.6 vs 39.2±5.2% and 37.6±7.5 vs 39.6±6.1%, respectively; n=12).

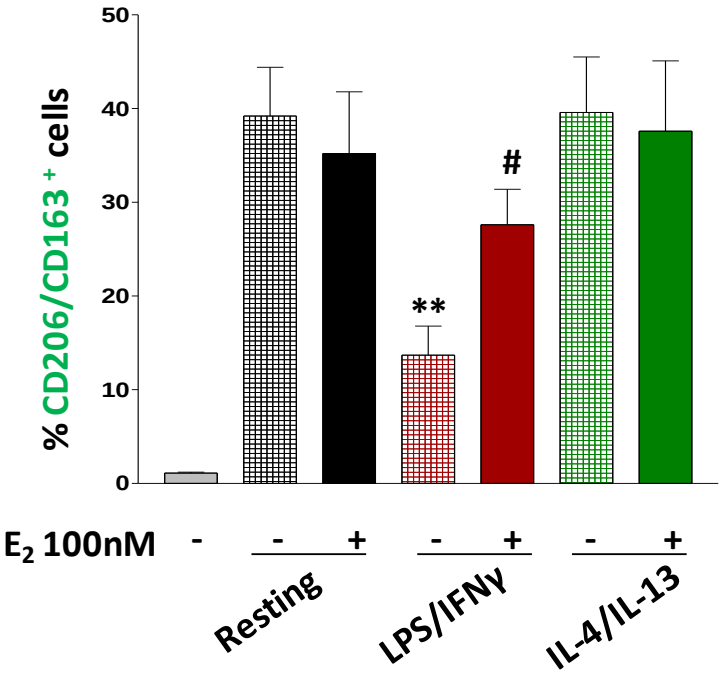

*Fig. 17: Effect of E_2 on the $CD206^+/CD163^+$ cell subset. M0 (resting), M1 (LPS/IFNγ) and M2 (IL-4/IL-13) macrophages were pre-treated overnight with 100nM 17β-estradiol. Cells were then stained with FITC-anti-CD206 and PE-anti-CD163 specific antibodies. Bars indicate the percentage of double-positive cells and represent the mean (±SEM) of 12 independent experiments. **p<0.005 vs resting, #p<0.05 vs untreated.*

4.3 Effects of 17β estradiol on cytokine production in circulating monocytes and cultured macrophages

Once demonstrated the effect on macrophage immunophenotypes, we moved on to investigate the functional effect of 17β-estradiol on circulating monocytes and cultured macrophages in terms of amounts of cytokines produced after incubation with pro-inflammatory stimulus (LPS/IFNγ). In all experiments Dexa was used as a positive control. Monocytes were defined by their positive staining with specific FITC- or PE-anti CD14 antibody. In the representative experiment reported in Fig. 18B, monocytes were 13.7% of total PBMCs. The analysis of intracellular cytokine expression in $CD14^+$ monocytes revealed 1.5% of $CD14^+/TNF\text{-}\alpha^+$ cells under basal conditions and 23.8% of $CD14^+/TNF\text{-}\alpha^+$ cells after stimulation with LPS/IFNγ for 4h (Fig. 18C and D). Data in Fig. 18E and F indicate that pro-inflammatory (LPS/IFNγ) activation enhanced the fraction of human peripheral blood monocytes expressing both TNF-α and IL-1β with respect to untreated cells (39.4±6.5 vs 4.5±1.5% $CD14^+/TNF\text{-}\alpha^+$ cells, $p<0.005$; and 46.6±12.6 vs 9.2±3.4% $CD14^+/IL\text{-}1\beta^+$ cells, $n=6$, $p<0.05$). After overnight pre-treatment with E_2, the fraction of TNF-α-

expressing monocytes was unchanged (43.4±7.6 vs 39.4±6.5% $CD14^+/TNF\text{-}\alpha^+$ cells), while expression of cell-bound IL-1β declined by 20% without reaching statistical significance (37.6±15.5 vs 46.6±12.6% $CD14^+/IL\text{-}1\beta^+$ cells). For comparison, overnight pre-treatment with Dexa appeared to decrease both TNF-α and IL-1β intracellular accumulation in LPS/IFNγ-stimulated monocytes (21.5±4.6 vs 39.4±6.5% $CD14^+/TNF\text{-}\alpha^+$ cells and 24.1±14.4 vs 46.6±12.6% $CD14^+/IL\text{-}1\beta^+$ cells, respectively, n=6; Fig. 18E and F).

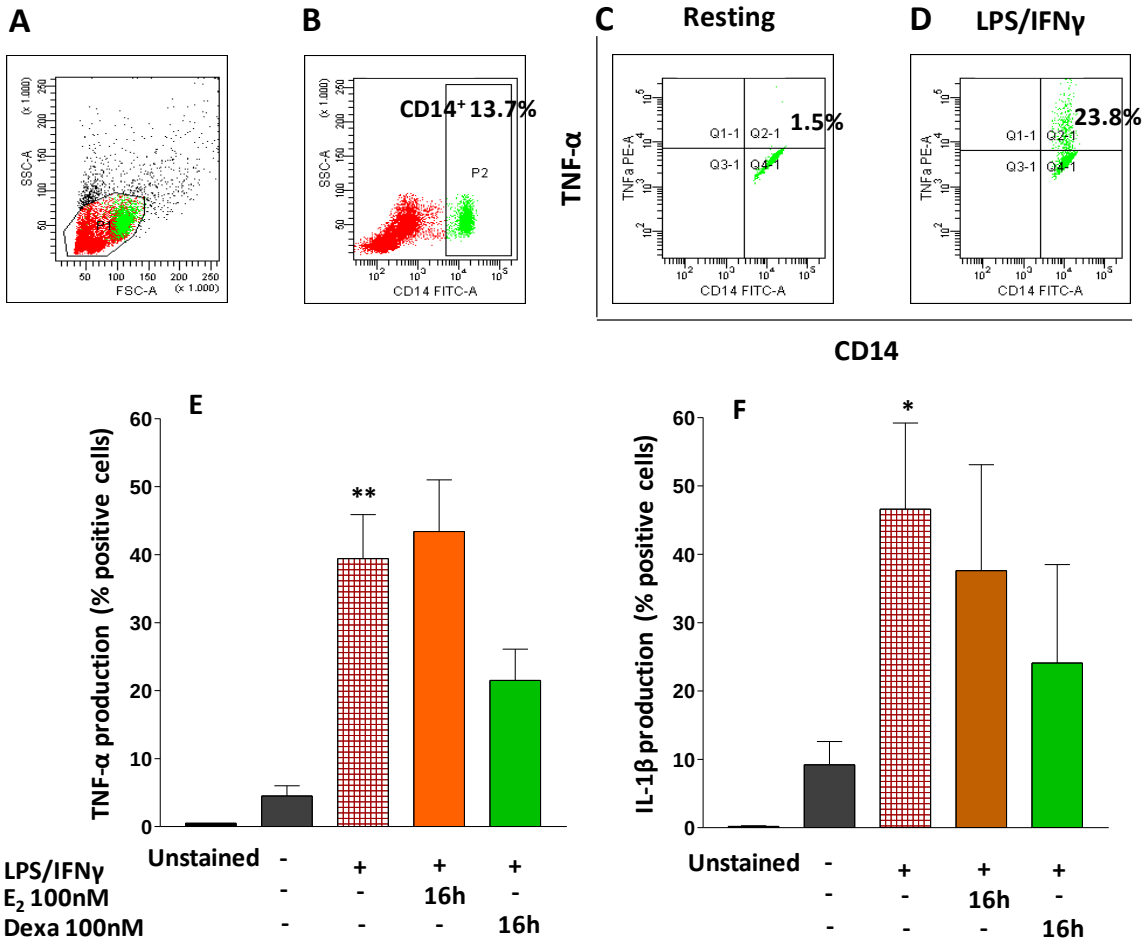

Fig. 18: Effect of E_2 and Dexa on cytokine production in human monocytes. ***A.*** *Forward/side scatter plots of human PBMCs.* ***B****. Gating of CD14+ monocytes (green dots). Prior to flow cytometry analysis erythrocytes were lysed and CD14-labeled and fixed/permeabilized PBMCs were stained with PE-conjugated anti-TNF-α or anti-IL-1β specific antibodies. Representative flow cytometry analyses of cell-bound TNF-α are shown in resting monocytes (****C****) and after incubation with LPS/IFNγ for 4h (****D****) in the presence of monensin (10 µM) to block cytokine secretion. Bar graphs represent the mean (±SEM) for $CD14^+/TNF\text{-}\alpha^+$ (****E****) and $CD14^+/IL\text{-}1\beta^+$ (****F****) cells in the presence or absence of 100 nm E_2 or Dexa from 6 independent experiments. **p<0.005, *p<0.05 vs resting.*

We also measured the percentage of monocyte-derived macrophages expressing intracellular TNF-α, IL-1β and IL-10 in and investigated whether E_2 or dexamethasone pre-treatment could influence this process. As shown in Fig. 19, a small fraction of TNF-α^+ and IL-$1\beta^+$ cells (2.5±0.6% and 1.9±0.6%, respectively) was detected under basal conditions. The percentage of IL-10^+ cells under resting conditions was slightly higher (5.9±2.3%). Following 6-h incubation with LPS/IFNγ, the fraction of cells staining for cell-bound TNF-α and IL-1β was significantly increased (19.5±3.1 vs 2.5±0.6% TNF-α^+ cells vs unstimulated, $p<0.005$, n=8; 14.1±5.6 vs 1.9±0.6% IL-$1\beta^+$ cells vs unstimulated, $p<0.05$, n=5). Unexpectedly but in line with our findings with mRNA levels (Fig. 13), the percentage of IL-10^+ cells significantly rose after 24h LPS stimulation (15.0±1.4 vs 5.9±2.3%, $p<0.05$ vs unstimulated, n=6) but did not change after IL-4/IL-13 stimulation with respect to unstimulated cells (5.9±1.2 vs 5.9±2.3%).

Compared with untreated cells, overnight pre-treatment with either E_2 or Dexa significantly decreased the percentage of both TNF-α^+ cells (12.3±1.6% E_2, 6.0±2.6% Dexa vs 19.5±3.1% untreated $p<0.05$, n=8, Fig. 19A) and IL-10^+ cells (8.8±0.9% E_2, 4.0±1.3% Dexa vs 15.9±1.3% untreated, $p<0.005$, n=6; Fig. 19C). However, E_2 did not affect IL-1β production in cultured macrophages after LPS/IFNγ treatment (37.6±15.5 vs 46.6±12.6% in the presence or absence of E_2, respectively, n=5; Fig. 19B), whereas treatment with dexamethasone reduced the percentage of IL-$1\beta^+$ cells (3.3±0.6 vs 14.1±5.6% compared with untreated; $p<0.05$, n=5).

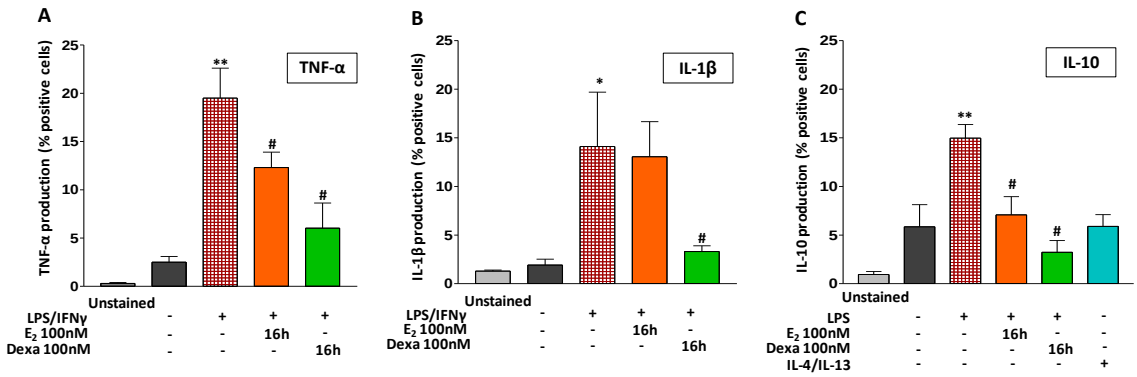

*Fig. 19: Effect of E_2 on macrophage cytokine expression. After differentiation, macrophages were pre-treated with E_2 or dexamethasone (Dexa) for 16h, then incubated with LPS/IFNγ for 6h, or LPS for 24h, at 37°C in the presence of brefeldin (10μM) to block cytokine secretion. Prior to flow cytometry analysis fixed/permeabilized cells were stained with PE-anti-TNF-α (panel A), FITC-anti IL-1β (panel B) and PE-anti IL-10 (panel C) specific antibodies. Bar graphs represent the mean (±SEM) of 8 (A), 5 (B) and 6 (C) independent experiments. *p<0.05, **p<0.005 vs resting, #p<0.05 vs untreated.*

Finally, we assessed the percentage of $CCL22^+$ cells in spontaneously differentiated macrophages under basal conditions and after IL-4/IL-13 polarization at different time points (6-48 h) in the presence or absence of E_2 or dexamethasone (dexa). As depicted in Fig. 20, fractions of $CCL22^+$ cells were 9.5±3.4% (panel A), 6.2±1.0% (panel B) and 8.0±2.5% (panel C) at 6, 24, and 48h after differentiation, respectively. Incubation with IL-4/IL-13 for 6 and 24h did not significantly affect the $CCL22^+$ cell subset compared with resting macrophages (11.1±3.2 vs 9.5±3.4% after 6h (A) and 7.4±1.8 vs 6.2±1.0% after 24h (B), respectively, n=3), whereas longer (48h) IL-4/IL-13 polarization led to a significant increase in the relative amount of $CCL22^+$ cells compared with resting macrophages (17.8±3.8 vs 8.0±2.5%, $p<0.05$, n=5, Fig. 20C). E_2 treatment did not affect IL-4/IL-13-induced CCL22 accumulation in cultured macrophages (16.9±3.6 vs 17.8±3.8%, n=5). The percentage of $CCL22^+$ cells was also unchanged with respect to resting after 48h treatment with E_2 alone (3.4±2.1 vs 8.0±2.5%, n=5, Fig. 20C). Similarly, overnight pre-treatment with Dexa (Fig. 20C) did not change the percentage of CCL22-expressing cells with respect to resting macrophages (9.9±4.6 vs 8.0±2.5% $CCL22^+$ cells, n=5); on the contrary, after 48h incubation with Dexa alone, the $CCL22^+$ subpopulation was significantly decreased with respect to resting (0.5±0.3 vs 8.0±2.5%) and to untreated cells (0.5±0.3 vs 17.8±3.8% $p<0.05$, n=5).

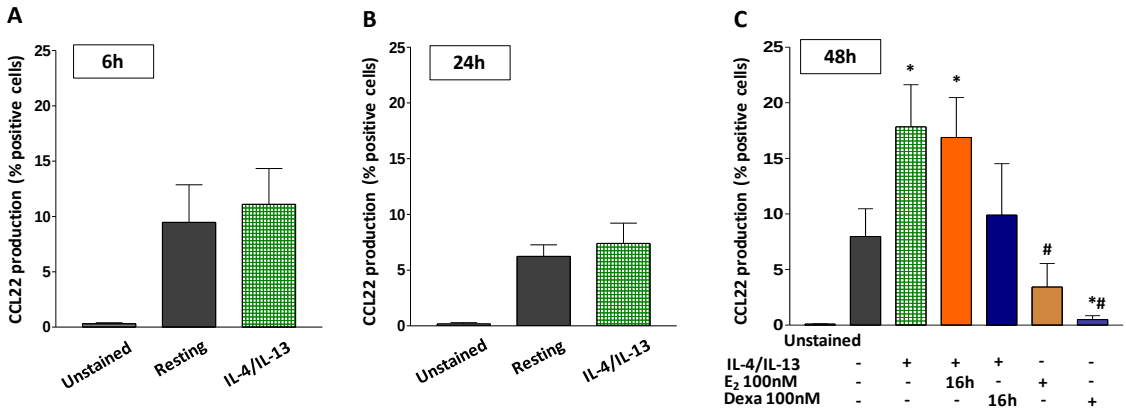

*Fig. 20: Percentage of $CCL22^+$ cells under basal conditions and after 6 (A), 24 (B) and 48h (C) polarization with IL-4/IL-13. Where indicated (panel C) after differentiation, macrophages were incubated for 48h or overnight pre-treated (16h) with E_2 or dexamethasone. Bar graphs represent the mean (±SEM) of 3 (A, B) and 5 (C) independent experiments. *p<0.05 vs resting, #p<0.05 vs untreated.*

5. MENOPAUSAL STATUS AND MACROPHAGE POLARIZATION

Our final specific aim was to compare phenotypes of monocyte-derived macrophages differentiated from blood samples of women in pre- (Pre-MW) and post-menopause (Post-MW). We evaluated the percentage of M2 ($CD206^+CD163^+CX3CR1^+$) and M1 ($CD80^+CCR2^+$) cells first under basal conditions, and then after 48h polarization with LPS/IFNγ (M1) or IL-4/IL-13 (M2).

5.1 Macrophage immunophenotypes in the resting state

As depicted in Fig. 21A, macrophages from both Pre-MW and Post-MW unexpectedly displayed a comparable M1 profile under basal conditions. In fact, the percentage of $CD80^+CCR2^+$ cells was 38.3±11.6% for Pre-MW (green circles, n=5, Fig. 21A) and 39.4±8.4% for Post-MW (black circles, n=8, Fig. 21A). We further analyzed these data after stratification by statin therapy and observed that the subgroup of statin-treated Post-MW (black triangles, n=5, Fig. 21B) showed lower fractions of $CD80^+CCR2^+$ macrophages compared with Post-MW not on pharmacological therapy with statins (26.8±9.4 vs 46.9±11.6%, n=3, Fig. 21B).

Just as unexpectedly, Post-MW displayed a percentage of M2 macrophages comparable to that of Pre-MW. Indeed, the $CD206^+CD163^+CX3CR1^+$ subset accounted for 17.7±6.6% (red circles, n=8, Fig. 21C) and 11.5±4.1% (green circles, n=6, Fig. 21C) of events in Post- and Pre-MW, respectively. Again, we analyzed data stratifying Post-MW by ongoing statin therapy. Post-MW treated with statins (red triangle, n=5, Fig. 21D) showed a higher fraction of $CD206^+CD163^+CX3CR1^+$ macrophages compared to Post-MW not on statin therapy (32.3±14.8 vs 8.8±1.7%, $p<0.07$, n=3, red squares, Fig. 21D). This is consistent with a recent study using a variety of experimental designs showing that statins enhance M2-like polarization (van der Meij et al., 2013).

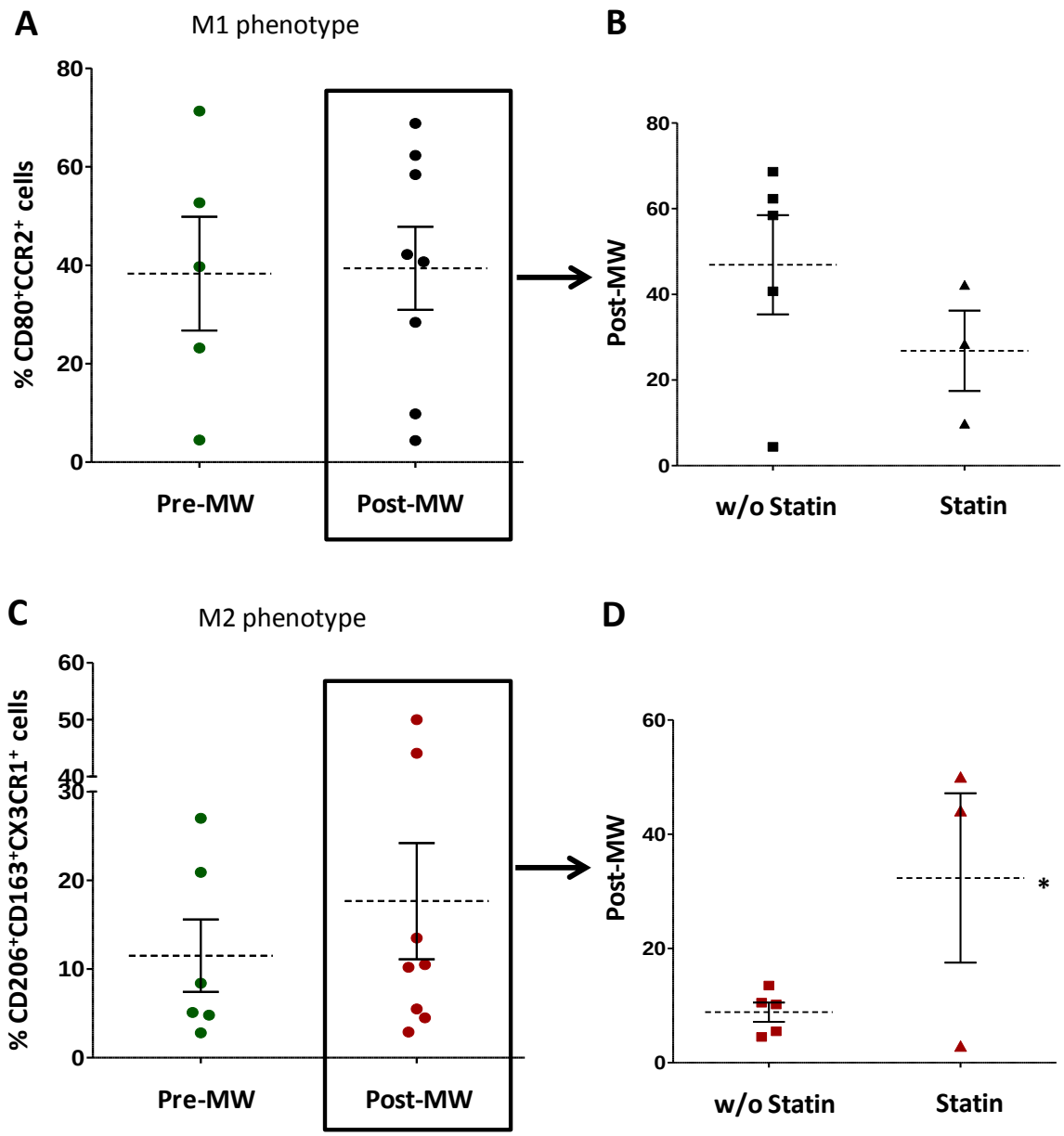

*Fig. 21: Percentage of CD80$^+$CCR2$^+$(M1) and CD206$^+$CD163$^+$CX3CR1$^+$(M2) cells in resting macrophages differentiated from monocyted isolated from blood of fertile (Pre-MW) and post-menopausal women (Post-MW). Resting macrophages were incubated with antibodies specific for markers of both immunophenotypes as indicated. Results from individual women are shown. Green circles (A, C) were used for Pre-MW, black symbols were used for M1 in Post-MW and red symbols were used for M2 in Post-MW (A to D). Panels B and D represent data for Post-MW with (squares) or w/o (triangles) statin therapy, respectively. Dashed lines in scatter plots indicate mean values (±SEM) within each group. *p<0.05 vs w/o statin.*

When comparing the mean M1/M2 ratio of Pre- and Post-MW under basal conditions (Table 3), no significant differences were observed between groups (8.0±4.8 vs 5.4±2.1). The M1/M2 ratio was also unchanged when comparing Pre-MW macrophage immunophenotypes with the subgroup of Post-MW not on statin treatment. Within the

Post-MW group, no differences in the M1/M2 ratio was detected according to statin treatment.

	n	M1/M2 (mean±SEM)
Pre-MW	5	8.0±4.8
Post-MW	8	5.4±2.1
w/o statin therapy	5	5.5±2.6
with statin therapy	3	5.2±4.4

*Table 3: Mean of M1/M2 ratio between donors of each group***.**

5.2 Macrophage immunophenotypes after polarized activation

Next, monocyte-derived macrophages from Pre-MW and Post-MW were incubated in the absence (M0) or presence of either LPS/IFNγ (M1) or IL-4/IL-13 (M2) for 48h. Subsequently, immunophenotyes were determined as described above in 5.1. Bar graphs in Fig. 22 represent the response to polarization as indicated in terms of cell subsets displaying the M1 or M2 phenotype and as average of % intra-individual change after polarized activation. After LPS/IFNγ polarization, a trend to increased percentage of $CD80^{+}CCR2^{+}$ cells was observed in both Pre-MW and Post-MW macrophages, which reached statistical significance in the latter group (Pre-MW: 65.7±14.2 vs 38.3±11.6%, n=5, Figure 22A; Post-MW: 65.1±9.3 vs 39.4±8.4%, $p<0.05$, n=8, Figure 22C). Additional analysis of % intra-individual changes following the M1 polarization protocol showed a 105±39% increase within the into Pre-MW group and a 113±40% increase within the Post-MW group, suggesting a similar enhancement of the M1 macrophage immunophenotype in response to external pro-inflammatory stimuli. After M2 polarization, the fraction of macrophages from Pre-MW expressing the M2 phenotypic markers CD206, CD163 and CX3CR1 was unchanges with respect to that measured in the resting/M0 state (14.5±4.1 vs 11.5±4.1%, n=6), while the percentage of M2 cells from the Post-MW group was slightly decreased (12.8±2.2 vs 17.7±6.6%, n=8; Fig. 22D). However, the % intra-individual change in response to M2 polarization was found to be 55±25% for Pre-MW (Fig. 22B) and 14±21% for Post-MW (Fig. 22C), suggesting an impaired plasticity of macrophages from Post-MW in acquiring the M2 phenotype compared with macrophages from Pre-MW.

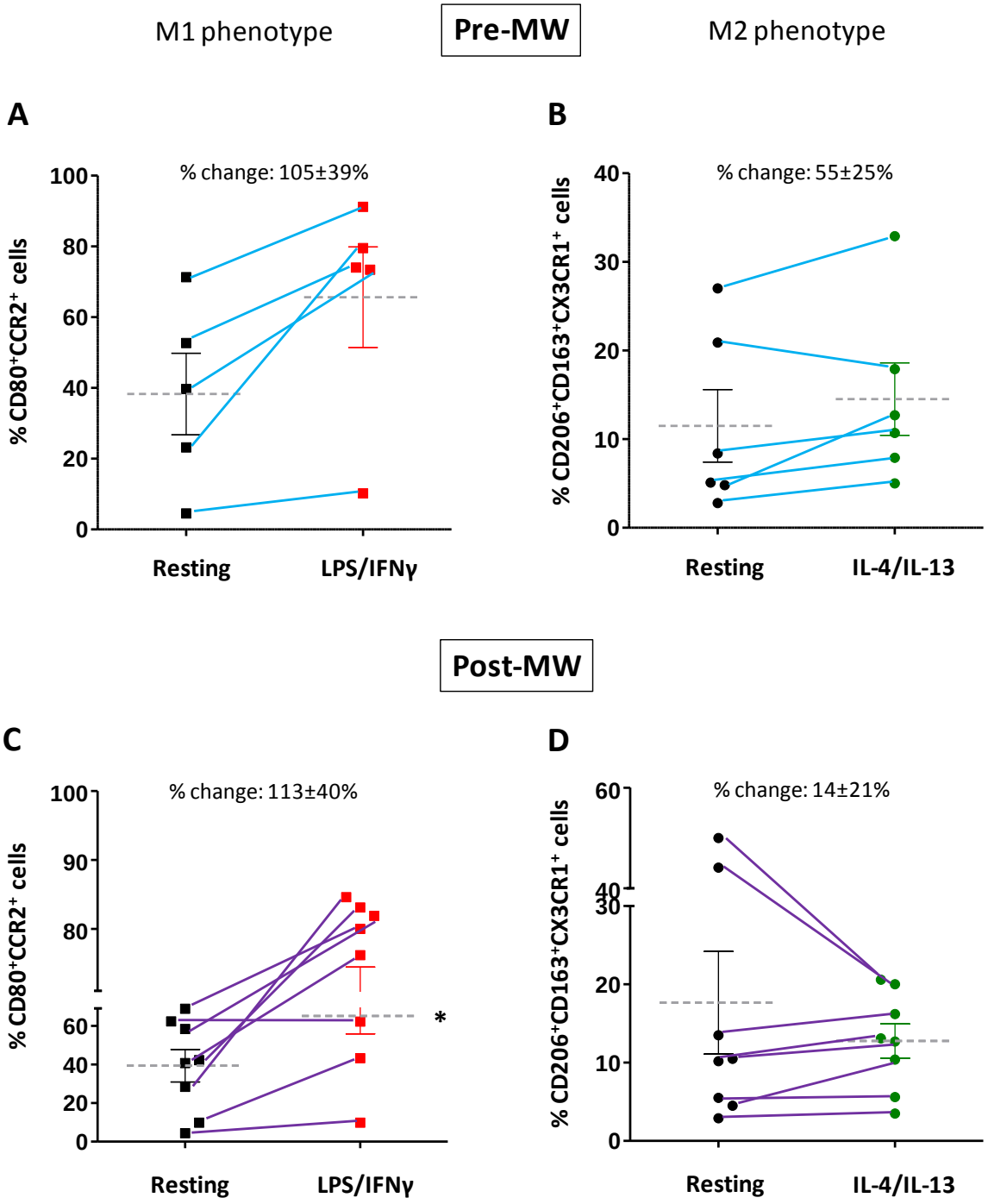

*Fig. 22: Modulation of macrophage immunophenotypes in response to polarized activation. Cells were differentiated from peripheral blood monocytes from Pre- and Post-MW for 7 days in RPMI1640 + 10% FBS and thereafter activated with LPS/IFNγ (M1) or IL-4/IL-13 (M2) for 48h. Results from individual donors are shown. Blue and purple lines link the intra-individual response to pro-inflammatory (LPS/IFNγ) and anti-inflammatory (IL-4/IL-13) cytokine stimulation of macrophages from Pre-MW [n=5 (M1) and 6 (M2)] and Post-MW (n=8). Dashed lines in scatter plots indicate mean values (±SEM) within each group. *p<0.05 vs resting macrophages.*

5.3 Immunophenotypes of circulating monocytes from pre- and post-menopausal women

We finally analyzed circulating monocyte subsets in Pre-MW and Post-MW. In particular, we defined the percentage of classical (M1, $CD14^+/CD16^-$) monocytes as $CD68^+CCR2^+$ cells and the percentage of non-classical (M2, $CD14^+/CD16^+$) monocytes as $CD206^+CD163^+CX3CR1^+$ cells. The fraction of specific immunophenotypes along with the mean M1/M2 ratio in relation to menopausal status were measured in this set of donors (Fig. 23). No significant differences between Pre- and Post-MW in the M1/M2 ratio were observed (6.5±2.3 Pre-MW vs 4.4±3.8 Post-MW), consistent with the finding obtained in cultures of resting macrophages derived from circulating monocytes of Pre- and Post-MW.

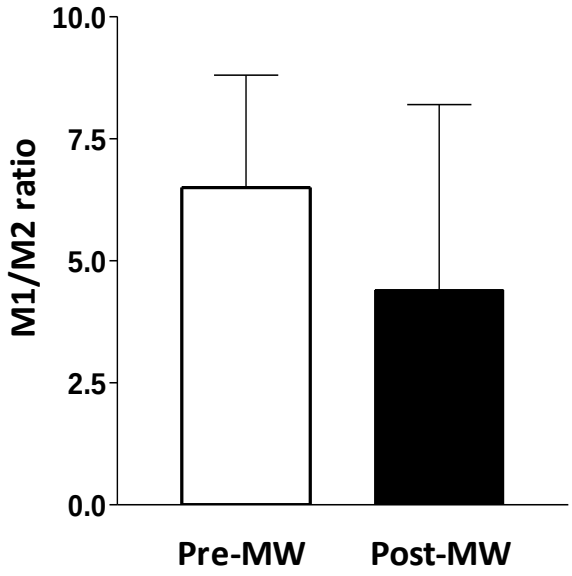

Fig. 23: *Analysis of human monocyte subsets from Pre- and Post-menopausal women (MW). Human circulating monocytes from Pre- (white bar; n=13) and Post-MW (black bar; n=32) were classified as classical M1 ($CD68^+/CCR2^+$) and non-classical M2 ($CD206^+/CD163^+/CX3CR1^+$) subsets. The bar graph represents the mean (±SEM) M1/M2 ratio of donors within each group.*

DISCUSSION

Pre-menopausal women appear to be protected against cardiovascular morbidity and mortality in comparison with men of similar age and with post-menopausal women. Loss of ovarian function and subsequent deficiency of endogenous estrogens is believed to promote cardiovascular diseases (CVD) after menopause (Vitale et al., 2009; Stock and Redberg, 2012).

Hormone replacement therapy (HRT) for post-menopausal women has been subject of much discussion and speculation since the 1960s. Before 2002 the effects of HRT were believed to be beneficial, as numerous studies demonstrated a statistically significant reduction in CVD, osteoporosis and colon cancer in women taking HRT. However, some clinical studies did not support the use of HRT due to increased risk of breast cancer and coronary heart disease (Roussouw et al., 2002 per WHI; Hulley et al., 1998). These conflicting results led to the "timing hypothesis", i.e. the idea that the variability in cardiovascular outcome as shown in early trials can be accounted for by differences in time since menopause at the initiation of hormone therapy (Mendelshon and Karas, 2005). This hypothesis was tested in a recent randomized clinical trial investigated the long term effect of hormone therapy on cardiovascular outcomes in recently post-menopausal women, by synthetic 17β-estradiol administration. The authors found a significantly decreased risk of the composite endpoint of death, heart failure, or myocardial infarction when HRT was started early in post-menopause. Importantly, early initiation and prolonged HRT avoided an increased risk of breast cancer or stroke (Schierbeck et al., 2012). Current findings also suggest caution when considering hormone therapy treatment in older age groups, even in the presence of persistent vasomotor symptoms, given the high risk of CHD and other outcomes associated with hormone therapy use in this setting (Manson et al., 2013). Thus, it appears that the beneficial effects of estrogen on the cardiovascular system as described in epidemiological and preclinical studies can be also observed in the clinical setting in specific subgroups of patients.

Several mechanisms have been proposed to explain the protective effects of estrogens on the cardiovascular system (Mendelshon and Karas, 1999). As an example, experimental studies suggest that estrogen regulates production of nitric oxide (NO) through genomic and non-genomic mechanisms mostly mediated by ERα, and therefore prevents

endothelial dysfunction and vascular inflammation (Cignarella et al., 2010). Moreover, estrogen has a key action in the vasculature by inhibiting VSMC proliferation and decreasing adhesion molecule expression and monocyte adhesion to the vessel wall. In fact, it is becoming increasingly clear that estrogens exert widespread effects upon the immune system and in particular the monocye-macrophage system, for example by attenuating the release of pro-inflammatory mediators (Bolego et al, 2013; Nakdarni and McArthur, 2013), which in turn may prevent atherosclerosis development and progression (Knowlton and Lee, 2012).

Mononuclear phagocytes are an essential element in the orchestration and expression of innate immunity and adaptive immune responses. Recently, it has been demonstrated that, in response to signals derived from microbes, damaged tissues or activated lymphocytes, monocyte/macrophages undergo a reprogramming which leads to the emergence of a spectrum of distinct functional phenotypes (Sica and Mantovani, 2012). Mirroring the Th1/Th2 nomenclature, macrophages undergo two different polarization states: the classically activated M1 phenotype (in response to LPS, IFNγ, TNF-α) and the alternatively activated M2 phenotype (in response to IL-4, IL-13, GC or IL-10; Mantovani et al., 2013). The existence of at least two phenotypically and functionally distinct macrophages as well as circulating monocyte subsets has been demonstrated in both humans and mice (Mosser and Edwards, 2008; Mantovani et al., 2009). This is of great interest because an unbalanced M1/M2 ratio can be observed in metabolic and chronic inflammatory diseases, which contribute to the development and progression of CVD. To date, there are few studies which describe the role of estrogens on macrophage polarization and, in particular, no evidence can be found on macrophages obtained from human subjects. A recent work demonstrated the impact of ERα expression on murine macrophage function by showing that diminished ERα levels in hematopoietic/myeloid cells impair metabolic homeostasis and accelerate atherosclerosis in female mice (Ribas et al., 2011).

Our preliminary data on circulating monocytes obtained from ERα-knockout and wild-type mice demonstrated that genetic deletion of ERα is associated with an unbalanced M1/M2 ratio due to a significant increase in the M1 phenotype, along with a minor change in the anti-inflammatory monocyte subset, suggesting that ER-mediated pathways

govern monocyte distribution and could therefore be involved in the development of inflammatory phenotype. We hypothesized that a similar pattern occurs in post-menopausal women compared with women in their fertile age, in that loss of ovarian function could negatively affect the monocyte-macrophage system and therefore expose post-menopausal women to increased CVD risk.

In order to evaluate the effect of 17β-estradiol on macrophage polarization, the first step was to set up a cellular model of human monocyte-derived macrophage. A variety of protocols to isolate blood circulating monocytes and promote monocyte-to-macrophage differentiation have been described. In particular, macrophages can be obtained by purifying monocytes either through magnetic cell sorting using specific CD14-labeled magnetic beads (Martinez et al., 2013) or by density gradient centrifugation with monocytes that undergo separation from lymphocytes by adherence to an appropriate support for 2 h. Based on previous studies from our group and others (Cullen et al, 1998; Eligini et al, 2012), monocytes were separated from lymphocytes following adherence to culture dishes and differentiated to macrophages after 7 days in culture. After spontaneous differentiation, we obtained about 80-90% pure macrophages in the cell population along with 10-15% $CD3^+$ cells, most likely lymphocytes (Eligini et al., 2012). Moreover, at the end of the differentiation period, resting macrophages (M0) showed mainly two dominant and distinct morphotypes: spindle/elongated and round/“fried-egg” shaped, which routinely occurred in resting cultures. The presence of two distinct macrophage subsets was confirmed by flow cytometry as a differential distribution on the forward scatter (FS) parameter, which is indicative of the cell size. In line with our results, Eligini and colleagues (2012) recently demonstrated the presence of two dominant monocyte-derived macrophages morphotypes under basal conditions. In particular, they highlighted the presence of about 50% spindle-shaped cells that display an M1-like phenotype, and about 50% of round-shaped cells that display an M2-like phenotype utilizing laser capture microscopy followed by RT-PCR for characterization of specific immunophenotypes.

To investigate macrophage polarization in vitro, starting from purified monocytes, specific growth factors such as granulocyte/monocyte-colony stimulating factor (GM-CSF), monocyte (M)-CSF or selected cytokines (i.e. IL-4, IL-10, IFNγ or TNFα; Ambarus et al.,

2012) are usually added to the culture medium, which in turn drives monocyte differentiation into a homogeneous macrophage phenotype (Beyer et al., 2012; Bender et al., 2003). As an alternative, the lymphocyte fraction can be added back to the monocyte culture to obtain a macrophage population reflecting a more "physiological" status. As opposed to many other studies, we exposed the macrophages to M1- and M2-polarizing agents only after these cells have spontaneously differentiated into macrophages and not during the differentiation step from monocyte to macrophages. On one hand, during inflammation monocytes will enter inflamed tissues and will be exposed to TLR-ligands or cytokines during their differentiation. However, tissue-resident macrophages, which have recently been shown to be particularly long-lived (Schulz et al, 2012; Yona et al, 2012), will in a lot of cases be exposed to these agents only after their differentiation. As a potential future research question, it would be very interesting to compare a similar cytokine exposure to monocytes and to differentiated macrophages, for example, 3 days of exposure during differentiation and then also 3 days of exposure but after differentiation. Along the same lines it would be interesting to sort spontaneously differentiated M2 cells and then expose them to M1-triggers to see if they can switch from phenotype or not. And one could then do the same with M2 cells that were exposed to IL-4/IL-13 during their differentiation to see if a monocyte that got exposed to IL-4 during its differentiation is more locked in an M2 state for example.

Therefore, we specifically polarized monocyte-derived macrophages with LPS/IFN-γ to induce the M1 phenotype, or with IL-4/IL-13 to obtain the M2 phenotype (48h incubation after the differentiation period in both cases). We observed that macrophage polarized activation toward M1 or M2 immunophenotypes were accompanied by specific changes in cell morphology compared with resting state. In particular, we observed that M1 macrophages were enriched in the long, spindle-shaped morphotype, while M2 macrophages were largely round-shaped. M1 and M2 phenotypes can be identified by a wide range of surface markers (cluster of differentiation, as well as cytokine and chemokine receptors) which are distinct between human and mouse (Auffray at al., 2009; Mosser and Edwards, 2008; Mantovani et al., 2009) Unfortunately, a gold standard classification of phenotype markers is still lacking. In particular, the most commonly surface markers used to identify classical M1 macrophages are CD68, the T-cell co-stimulatory molecules CD80 and CD86, and the monocyte chemoattractant protein-1

receptor CCR2, while the mannose scavenger receptor CD206 together with CD163 (haemoglobin-heptoglobin receptor) and CX3CR1 (fractalkine receptor) are often used to identify the M2 subset. More recent studies used additional surface markers to better distinguish macrophage subsets. In particular, Beyer et al. (2012) investigated novel M1-associated (CD120b, TLR2, SLAMF7) as well as M2-associated (CD1a, CD1b, CD93, CD226) cell surface markers, by combined transcriptome analysis. Ambarus and colleagues (2012) validated specific phenotypic markers for *in vitro* polarized human macrophages, such as CD80 and CD64 for IFNγ-induced M1 macrophages and CD200R for IL-4-induced M2a macrophages. Martinez et al (2013) performed extensive analysis of mouse and human alternative macrophage activation and found a set of highly conserved genes that were regulated by IL-4 including transglutaminase (TGM)-2. Taken together, these findings suggest that any selection of phenotypic markers is certainly non-exhaustive and strictly dependent to the differentiation protocol as well as the polarizing agents used to induce M1/M2 immunophenotypes. We have chosen CD68, CCR2 and/or CD80 as possible M1 markers and CD206, CD163, CX3CR1 as markers of the M2 immunophenotype.

CD68 expression has long been used as a macrophage marker, independent from their activation state (Holness and Simmons 1993). Indeed, we demonstrated by immuncytochemistry and flow cytometry analysis that CD68 is expressed both in cytoplasmic granules and on the cell membrane of all macrophage subsets. In particular, the amount of CD68 localized on the plasma membrane was significantly increased after pro-inflammatory stimuli (LPS/IFNγ, 48 h) compared with resting cells. Therefore, CD68 could be regarded as a M1 polarization marker under our experimental conditions. CCR2, also known as the receptor for MCP-1/CCL2, is widely accepted as an M1 macrophage marker (Mantovani et al., 2009). Interestingly, it is also expressed on circulating monocytes and has long been used to identify classical/M1 Ly6C^{high} CX3CR1^{low}CCR2^{+} monocytes (Fadini et al., 2013). We found that the number of CCR2^{+} macrophage was already high in the resting state (M0) and did not further increase after LPS/IFNγ treatment, which limited CCR2 relevance to our model. Notably, Zhou and colleagues (1999) reported that *in vitro* treatment of mouse peritoneal macrophages with LPS resulted in a dramatic decrease in CCR2 mRNA levels, and *in vivo* administration of LPS completely abolished macrophage recruitment presumably through down-regulation of CCR2 on monocytes.

In order to better characterize the M1 phenotype, in further experiments, we looked at other possible M1 markers. In a separate set of experiments we further characterized M1 monocyte-derived macrophages by quantifying the standard monocyte cluster of differentiation (CD)14 and CD16 together with CD68. This approach relates to the standard monocyte nomenclature, which distinguishg M1/pro-inflammatory ($CD14^+CD16^-$) and M2/anti-inflammatory ($CD14^+CD16^+$) monocytes (Fadini et al., 2013, Gautier et al., 2009). We found that the percentage of M1 macrophages, defined as $CD14^+/CD16^-/CD68^+$ cells, significantly increased (about three-fold) after M1 polarization. This may reflect a physiological activation of monocytes toward a pro-inflammatory macrophage subset, which could be further able to modulate its phenotype and function in response to specific microenvironments or events including pathogen invasion or any condition requiring activation of the immune system. Finally, in the attempt to find additional markers which could be more representative for the M1 phenotype, we looked at expression of CD80, a co-regulatory receptor expressed on the surface of antigen presenting cells (Dakappagari et al., 2012). Macrophage subpopulations have been reported to express high levels of CD80 and CD86; these macrophages can present antigens to T cells, thus promoting the inflammatory immune response (Mosser and Edwards, 2008). Recently, Jaguin et al. (2013) demonstrated that expression of CD80, which is a strong membrane marker of M1 polarization, significantly increased after 24h LPS/IFNγ polarization of M-CSF-differentiated human macrophages. In addition, Stöger et al (2012) demonstrated the prevalence of the M1 macrophage expressing CD86 in atherosclerotic plaques prone to rupture. We observed a significantly increase in the fraction of $CD80^+$ spontaneously differentiated macrophages after M1 polarization compared with resting macrophages. These results are in line with those of Ambarus and colleagues (2012), who recently demonstrated the up-regulation of CD80 in human macrophages treated with IFNγ or TNFα for 4 or 7 days starting at the beginning of the maturation protocol. Overall, these data suggest that 48h polarization with LPS/IFNγ of spontaneously differentiated macrophages is able to enhance the pro-inflammatory macrophage immunophenotype, specifically by increasing the fraction of $CD68^+$, $CD14^+/CD16^-/CD68^+$ and $CD80^+$ cells.

We also characterized the M2 immunophenotype and demonstrated a large amount of $CD206^+$, $CD163^+$ and $CX3CR1^+$ cells in resting conditions. Interestingly, after LPS/IFNγ

polarization, the percentage of the M2 immunophenotype, independently from the marker used for M2 characterization, significantly decreased. Importantly, these results demonstrated that pro-inflammatory stimuli are able to enhance the M1 macrophage subset, as well as to attenuate the M2 phenotype. This indicates that a pro-inflammatory microenvironment causes an M2-to-M1 immunophenotype switch and confirms the plasticity of these cells, which are able to adapt their phenotype and in turn their functions according to specific microenvironmental signals. A study on adipose tissue macrophages in a mildly obese mouse model showed that the M1 subset increased concomitantly with a reduction in M2 macrophages, due to enhanced secretion of chemoattractant molecules and pro-inflammatory cytokines (Lumeng et al., 2007). To the best of our knowledge, however, similar findings were not reported before in previous studies in cultured human macrophages and deserve further investigation.

In our experimental model, after 2 days polarization of spontaneously differentiated macrophages with IL-4/IL-13, the percentage of M2 cells was comparable to that observed in M0/resting macrophages. Therefore, it is possible that the polarization toward a more established anti-inflammatory (M2) phenotype needs more than 48 h incubation. Bouhlel et al. (2007) found that the M2 macrophage marker CD206 was strongly induced by 7 days incubation with IL-4. Accordingly, we also demonstrated that after 7 days incubation with IL-4 together with IL-13, the fraction of $CD206^+$ cells significantly increased, while the fraction of $CX3CR1^+$ cells as well as $CD163^+$ cell did not significantly change / was comparable to M0 (Toniolo et al., unpublished). Another possible explanation for the lack of IL-4/IL-13 effect on M2 immunophenotype relates to the already high subset of cells expressing M2 markers (CD206, CD163 and CX3CR1) under resting condition. Indeed, in a series of Pulse-Chase experiments, we analyzed the M2 immunophenotype first after 48h challenge in the presence or absence of either IL-4/IL-13 or LPS/IFNγ (Pulse) and then after further 72h without any stimulus (Chase). Under basal conditions, we found that all M2 selected surface markers appeared to decrease after further 72h in culture in the absence of polarizing agents. In particular, under these experimental conditions we detected a significant increase in the $CD206^+$ (but not $CD163^+$ nor $CX3CR1^+$) subset of M2 macrophages with respect to resting (M0) macrophages. These results do not support the idea that FBS has itself a polarizing effects as it contains growth factors. However, a decrease in M2 macrophages as identified by $CD206^+$ cells

may be likely due to surface marker expression turnover according to macrophage time in culture. Taken together, these data suggest that the nature of polarizing agents and exposure time appear to be crucial to investigate human macrophage immunophenotype.

A last key point was to address whether the percentages of M1 and M2 cells reflect "mixed activated" cells expressing varying levels of the different M1 or M2 markers or whether there is a true heterogeneity with subpopulations that remained more M1 oriented and others that are more M2 oriented. In this regards, we characterized spontaneously differentiated macrophages by combining a selected M1 and a selected M2 marker, in the presence or absence of polarizing stimuli. In preliminary experiments, macrophages with different shapes (see results paragraph 2.2) were either $CD68^+$ (2.3%) or $CD163^+$ (35.3%) or expressed both markers ($CD68^+/CD163^+$: 6.4%). In the presence of LPS/IFNγ, the fraction of $CD68^+$ elongated-shaped macrophages more than doubled (5.3%) while the fraction of round-shaped $CD163^+$ macrophages decreased (16.2%) compared with resting. Overall, this data suggests that in our experimental model we have a true heterogeneous population of human monocyte-derived macrophages.

We further defined macrophages subsets after polarization in terms of gene expression and cytokine production. Several genes were previously identified as M1 or M2 markers (Martinez et al., 2006; Mantovani et al., 2013). The pro-inflammatory cytokines TNFα, IL-6 and IL-1β, are critical mediators of the acute phase response, and are potently induced after LPS or IFNγ treatment. We observed a higher mRNA expression of TNFα and IL-1β in M1, than in unpolarized (M0) or M2 macrophages. In particular the mRNA for these cytokines was differently time-regulated where TNF-α mRNA peaked at 6h while IL-1β reached the highest expression level after 48h. It is possible that macrophages respond to pro-inflammatory stimuli producing TNF-α that in turn induces IL-1β gene expression, thus fortifying the concept that macrophage activation encompasses a quantifiable and temporally dynamic process (Barish et al. 2005). In agreement with mRNA expression data, we found increased TNF-α and IL-1β intracellular protein accumulation after incubation with LPS/IFNγ in monocyte-derived macrophages. Similar data were obtained in human monocytes identified as $CD14^+$ cells. IL-10 was previously described as a key marker for the M2 immunophenotype (Mantovani et al., 2013) as well as an M2 polarizing agent (Mantovani et al., 2004). Several studies are consistent in defining M2

anti-inflammatory macrophages as cells releasing high amounts of this cytokine (Li et al., 2012; Mantovani et al., 2013). In addition, differences in IL-10 expression in lean(higher expression) compared with obese (lower expression) mouse adipose tissue macrophages (ATMs) have been found, implying an important role of this cytokine as a homeostatic regulator in insulin sensivity. Unexpectedly, we found that IL-10 mRNA levels were significantly increased in M1 (LPS/IFNγ) but not in M2 (IL-4/IL-13) polarized cells, both after 6 and 48h. Just as unexpectedly, we found that 24h-stimulated M1 but not M2 macrophages secrete higher levels of IL-10 compared with resting macrophages as detected by flow cytometry, in contrast to data reported by Verreck et al. (2006). Nevertheless, several studies are consistent with our findings, demonstrating either increased IL-10 release into the culture medium after macrophage LPS stimulation (Chanteux et al., 2007), LPS-induced IL-10 intracellular accumulation in PBMCs (Muris et al., 2012) and increased IL-10 mRNA levels in human M-CSF-generated macrophages after M1 polarization (Jaguin et al., 2013). We hypothesize that the LPS/IFNγ stimulus is able to induce a typical anti-inflammatory cytokine, such as IL-10, which probably triggers M2 macrophage polarization via more complex and long-term feedback mechanisms. In fact, the rapid peak (6h) in IL-10 mRNA levels suggests a possible role of this LPS-responsive cytokine in a M1-to-M2 transition involved in the resolution of acute inflammatory events (Couper et al., 2008).

Finally, we looked at the production of CCL22 in order to correlate the modulation of M2 macrophage immunophenotype with its function. Monocyte-derived macrophages, together with dendritic cells, appear to be the most relevant producers of CCL22, also known as macrophage derived chemokine (MDC; Vulcano et al., 2001). We found that the fraction of $CCL22^{+}$ spontaneously differentiated macrophages polarized with IL-4/IL-13 (M2) for either 6 or 24h was comparable to that in M0, but was markedly increased after 48h polarization. In agreement with our data, IL-4 and IL-13 have been reported to induce CCL22 production in monocytes, monocyte-derived macrophages NK cells and dendritic cells (Bonecchi et al., 1998; Vulcano et al., 2001). Moreover, CCL22 expression and secretion was found to be increased in M2 when compared with M0 and M1 macrophages (Jaguin et al., 2013).

The second specific aim of this thesis was to evaluate the impact of 17β-estradiol (E_2) on macrophage immunophenotypes and function. So far few studies addressed if and how specific pharmacological agents affect human macrophage immunophenotypes (Bouhlel et al., 2007, Van der Meij et al., 2013). In particular, the effects of glucocorticoids were explored in mouse bone marrow-derived macrophages (Yang et al., 2012) in human circulating monocytes (Ehrchen et al., 2007; Vallelian et al., 2010). Recently, dexamethasone was shown to induce expression of specific human macrophage phenotypic markers according to activation protocol and treatment duration including CD163 (Ambarus et al., 2012; Tang et al., 2013). Glucocorticoids have been also found to induce a particular mouse macrophages M2 subset, called M2c (Li et al., 2012). Hence, it is of relevance to understand if and how drugs endowed with an anti-inflammatory potential such as dexamethasone and estrogens (Bolego et al, 2013) modulate human macrophage polarization. Although attention has been paid mostly to glucocorticoids given their widespread clinical use, it is becoming increasingly clear that sex steroid hormones, and in particular the main female sex steroid estrogen, are key players in the immune response (Nadkarni and McArthur, 2013, Scotland et al., 2011, Bolego et al, 2013). To our knowledge, no studies addressed whether estrogens affect macrophage polarization in humans. This could be of great relevance to find new potential gender-specific pathophysiological and/or protective mechanisms in inflammatory disease.

Using dexamethasone for the first time in spontaneously differentiated macrophages as a model drug to explore phenotype modulation, we found that dexamethasone treatment in M0 or activated M2 macrophages enhanced $CD163^+$ as well as $CD206^+/CD163^+$ macrophage subsets, consistently with findings from different experimental models (Ambarus et al., 2012; Heasman et al., 2004, Tang et al. 2013). Similarly, treatment with E_2 significantly modulated the M2 macrophage immunophenotype. In particular, E_2 treatment was able to prevent LPS/IFNγ-induced down-regulation of M2 markers thereby inducing its anti-inflammatory action. Recently, hematopoietic/myeloid-specific deletion of ERα has been shown to impact on macrophage function in female mice. In particular, ERα is required for macrophage IL-4 responsiveness and ERα-deficient macrophages are refractory to IL-4-induced alternative (M2) activation (Ribas et al., 2011). Moreover, Scotland et al. (2011) examined the mechanisms which regulate sex differences in immune cell phenotypes in mice and demonstrated that ovarian hormones regulate

macrophage phenotype, function and numbers, but have no significant impact on T-lymphocyte populations in females.

E_2 exerts its genomic and nongenomic effects binding both to the nuclear estrogen receptors (ERs), which exist in two different isoforms, ERα and ERβ (Bolego et al., 2006) and to a newly identified membrane G protein-coupled receptor, termed GPER1, which is able to trigger rapid intracellular responses (Nilsson et al., 2011). Different ER tissue and cellular distribution suggests that ERs mediate at least in part distinct biological functions. It has been demonstrated that both monocytes and macrophages express ERα and ERβ, and treatment with 17β-estradiol modulates ERα expression at both mRNA and protein levels in macrophages. In addition, the expression of ERα is greater than ERβ in both monocytes and macrophages (Murphy et al., 2009). However, to our knowledge no data have been published on the expression of ER isoforms in human polarized macrophages. We found that both isoforms were expressed in spontaneously differentiated macrophages under basal condition (M0). Interestingly, we demonstrated that both ERα mRNA and protein amounts were significantly lower in M1 compared with M0 macrophages, in agreement with several *in vitro* and *ex vivo* studies supporting a beneficial role for ERα in cardiovascular protection (Cignarella et al., 2010, Cignarella et al., 2001). One could speculate that reduced ERα expression after LPS stimulation is associated with an impaired macrophage function. As the endogenous ER agonist 17β-estradiol has the same binding affinity for ERα, ERβ and GPER-1 in tissues and since each specific receptor isoform differentially contributes to the biological actions of the female hormone (Bolego et al., 2006), understanding which ER isoform is responsible for the effect of 17β-estradiol on macrophage polarization would be important to design pharmacological agents reproducing estrogenic effects on the monocyte-macrophage system. Thus, future experiments will explore the effect of the selective synthetic ligands PPT (ERα agonist), DPN (ERβ agonist) and G1 (GPER-1 agonist) on macrophage immunophenotype modulation.

In order to evaluate whether the female sex hormone E_2 affects human monocyte-macrophage functions along with immunophenotypes, we invesigated the effect of E_2 on cytokine production by both circulating monocytes and cultured monocyte-derived macrophages, again using dexamethasone as a positive control. With regard to tumor necrosis factor (TNF) and interleukin (IL)-1β production, conflicting data are reported with

E_2 enhancing or inhibiting these inflammatory cytokine secretion by both human monocytes or macrophages (Bolego et al, 2013). It is possible that the experimental design and/or the duration of estrogen exposure could lead to different results (Straub et al, 2007). For example, Calippe et al. (2010) observed that physiological levels of endogenous E_2 or exogenous administration of E_2 activate signaling pathways that promote inflammation in murine macrophages, while exposure to the hormone *in vivo* for a short time leads to a decreased IL-1β production (Calippe et al., 2008). In addition, a significant increase in LPS-induced TNF release has been reported in ERα deficient macrophages, suggesting that ERα, but not ERβ, mediates the inhibitory effects of endogenous estrogen on pro-inflammatory cytokine production in innate immune responses (Lambert et al., 2004). Interestingly, E_2 strongly inhibits activation of the NF-κB pathway and inflammatory cytokine production by human cord blood mononuclear cells exposed to microbial products, suggesting that maternal hormones are physiological regulators of neonatal immune response. Later in life, the production of cytokines by monocyte/macrophages is heavily influenced by the ovarian cycle and oral contraceptive use (Campesi et al., 2012).

We found that E_2 significantly reduced the amount of intracellular TNF-α accumulation induced by exposure to LPS/IFNγ for 6h in monocyte-derived macrophages, but did not alter IL-1β production. By contrast, estrogen treatment did not affect the fraction of circulating human monocytes expressing TNF-α and IL-1β in response to LPS/IFNγ. We cannot exclude that pro-inflammatory cytokine production as well as the effect of E_2 could change at different time points. It is important to highlight that we noticed a large variability between donors in macrophage cytokine production in response to LPS. It would be also of interest to combine our results on cell-bound cytokine accumulation with assays of cytokines released into the medium. Inter-individual variability in cell function has to be always taken into account, particularly with the monocyte-macrophage system, which is very sensitive to manipulation and the microenvironment. We then evaluated whether E_2 treatment affected M2 macrophage function, in particular by increasing the release of anti-inflammatory cytokines or chemokines such as CCL22 and IL-10. Unexpectedly, both E_2 and dexamethasone treatment decreased basal secretion and did not significantly modify intracellular CCL22 accumulation after IL-4/IL-13 (M2) polarization. Moreover, similarly to dexamethasone, E_2 down-regulated LPS-induced IL-10

production. Conversely, the amount of intracellular IL-10 after M2 polarization was neither different with respect to resting cells nor affected by E_2 treatment. In line with our results, Vulcano et al. (2001) reported that inhibition of LPS-induced dendritic cell maturation by dexamethasone resulted in a reduced production of CCL22. Overall, our data are in agreement with recent studies indicating that estrogen attenuates the release of pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 from both neutrophils and macrophages, confirming the marked anti-inflammatory action of the hormone (Nadkarni and McArthur, 2013). Interestingly, 17β-estradiol counteracts the effects of LPS on cytokine production, which in turn could affect macrophage polarization.

Taken together, the results of the specific aim 1 and 2 provided the basis to tackle the central hypothesis of this project that estrogen could affect monocyte-macrophage immunophenotypes, thus possibly protecting pre-menopausal women from cardiovascular disease. To this end, we characterized macrophage immunophenotypes obtained from pre- and post-menopausal women (MW). Declining levels of estrogen in menopause are associated with several degenerative processes in various tissues, such as atherosclerosis (Harkonen and Vaananen, 2006), thus leading to the progression of cardiovascular diseases. Estrogen has been shown to slow down the development of atherosclerosis both in animal models and humans. It has been suggested that this process is mainly due to its inhibitory effect on the accumulation of lipid-laden macrophage foam cells in the vessel wall. In addition, it has been demonstrated that systemic administration of estrogen in ovariectomized animals reduces the expression of mediators of inflammation and infiltration of leukocytes in a model of lesion of the vascular wall (Miller et al., 2004) by inhibiting the expression of vascular monocyte chemoattractant protein-1 (MCP-1), which results in decreased recruitment of monocytes to the vessel wall (Moore et al., 2013). Exploring estrogen effects on the monocyte-macrophage system including macrophage polarization appears to be relevant because (1) an unbalanced M1/M2 ratio can be observed in metabolic diseases such as obesity and atherosclerosis, and (2) loss of ovarian function plays a role in the progression of atherosclerosis and increased CVD in Post-MW. M1 and M2 macrophages both characterize diverse stages of human atherosclerotic plaque development, but localize to distinct morphological features of the lesions. In particular, M1 macrophages dominate the rupture-prone shoulder regions of the plaque, while M2 cells are detectable in the

stable and hemorrhagic plaque regions (Stöger et al., 2012), suggesting that atherosclerotic lesion progression is correlated with the dominance of M1 over the M2 phenotype (Khallou-Laschet et al., 2010).

Unexpectedly, we found that both the M1 ($CD80^+CCR2^+$) and the M2 ($CD206^+CD163^+CX3CR1^+$) macrophage subpopulations were comparable between Pre- and Post-MW under basal conditions. Based on our preliminary data on circulating monocytes from ERα-knockout and wild-type mice, in which the genetic deletion of ERα leads to an unbalanced M1/M2 ratio due to enhanced M1 phenotype, we anticipated a similar pattern in cells from women with different circulating estrogen levels as related to menopausal status. By contrast, the mean M1/M2 ratio in resting macrophages displayed no significant differences between Pre- and Post-MW. It is widely known that statins exert anti-inflammatory effects on the vasculature through mechanisms that appear to be independent of their lipid-lowering effect (Jain and Ridker, 2005). Moreover, Van der Meij and colleagues (2013) recently demonstrated that selected statins effectively shift macrophage polarization towards an M2 phenotype. We therefore stratified individual Post-MW donors by ongoing statin therapy, and found that cultured macrophages from statin-treated donors simultaneously displayed decreased M1 and enhanced M2 phenotype under basal conditions. Yet the M1/M2 ratio between Pre- and Post-MW remained unchanged irrespective of statin treatment. Our clinical investigation of *ex vivo* macrophage characterization is in its early stages, so the number of study participants is quite small (i.e. 5 Pre-MW and 8 Post-MW) and we need to recruit more donors to strengthen the present results. The lack of differences in the M1/M2 ratio in the resting state for the current data set may be also interpreted considering the particular phase of Pre-MW menstrual cycle upon blood collection, because data on plasma 17β-estradiol levels for each donor are not yet available. For these reasons, we are currently investigating how fluctuations of hormonal levels during the menstrual cycle modulate the immune response, by recruiting Pre-MW during the early follicular phase, the ovulation phase and the luteal phase. This more focused approach may contribute to identify estrogen-dependent differences in macrophage phenotypes compared with those of Post-MW.

Finally, the polarized activation of macrophages from Pre-MW and Post-MW was investigated. The M1 and M2 phenotypes were enhanced in response to *in vitro* polarization with either LPS/IFNγ or IL-4/IL-13, respectively (mean % change after M1 and M2 polarization was 105±39% and 55±25%, respectively). These results reflect remarkable macrophage plasticity in the Pre-MW group. Interestingly, while LPS/IFNγ polarization modulated M1 macrophage phenotype in Post-MW similarly to what observed for Pre-MW (% change= 113±40%), the M2 phenotype was essentially unchanged following IL-4/IL-13 polarization (% change= 14±21%), suggesting an impaired potential for resolving inflammatory responses in the macrophages from older women.

A recent investigation from our research group focused on human monocyte polarization status in type 2 diabetes and showed that the M1/M2 polarization ratio was increased in monocytes from type 2 diabetic patients compared with healthy controls (Fadini et al, 2013). Based on these findings, in the attempt to identify a possible biomarker correlating menopausal status with cardiovascular risk (Satoh et al, 2010), we also assessed the percentage of both M1 and M2 phenotypes (defined as $CD68^+/CCR2^+$ or $CD163^+/CD206^+/CX3CR1^+$ cells, respectively) in monocytes from Pre-and Post-MW. We found no differences in M1/M2 phenotype ratio in monocytes from Pre-and Post-MW, consistent with the finding obtained in cultures of resting macrophages derived from the same donor groups.

To sum up, we found that exogenous estrogen treatment affected macrophage polarization and function by preventing LPS effects on M2 immunophenotype and cytokine production. In addition, macrophages from post-menopausal women appeared to be less responsive to M2 polarized activation. Future experiments taking into account hormonal fluctuations during the menstrual cycle will contribute to better understand the role of female hormones on the monocyte-macrophage system and will pave the way to new research on the involvement of monocyte-macrophage polarization in pathological conditions such as polycystic ovary syndrome or autoimmune diseases, whose severity and progression correlate with circulating endogenous estrogen levels (Whitacre, 2001).

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