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ABSTRACT

Macrophages function as control switches of the immune system, maintaining the balance between pro- and anti-inflammatory activities. Classic polarization (M1) and activation of macrophages into proinflammatory cells is characterized by generation of reactive oxygen and nitrogen species (ROS/RNS) and proinflammatory cytokines, including TNF-α, interleukin (IL)-6 and IL-1β. Alternative activation profile (M2) is characterized by secretion of anti-inflammatory cytokines, including IL-10. Bioactive food components such as polyphenols have recently gained attention for their anti-inflammatory properties. Cocoa, a polyphenols-rich food, has many beneficial effects on human health, including anti-inflammatory effects.

In this study, we investigated the hypothesis that cocoa polyphenol extract affects macrophage phenotype favoring an alternative M2 anti-inflammatory state.

Cocoa polyphenols were extracted from roasted cocoa beans from Ghana, West Africa. Macrophages deriving from THP-1 cells were cultured at a starting density of 2-3 10^5 cells/mL for 24 h. Cells were then activated for an additional 12 h with either LPS (1μg/ml) and INF-γ (20ng/ml) for M1 activation or IL-4 (20 ng/ml) for M2 activation. Specific cytokines were detected and quantified by ELISA assay. Cellular metabolism was evaluated through mitochondrial oxygen consumption by polarographic assay and total and glycolytic ATP levels were measured using a luciferin–luciferase reaction system.

In vitro, cocoa polyphenolic extract attenuated inflammation with significantly decreased macrophage response to M1 activation. This was evidenced by a significant decrease of proinflammatory cytokines secretion (TNF-α, IL-6, IL-1β) after stimulation with LPS 1μg/ml and INF-γ 20ng/ml. Data showed a significant increase of O_2 consumption by mitochondrial complexes (I, II+III) and a higher production of ATP trough oxidative phosphorylation in M1 macrophages after treatment with cocoa polyphenols.

This study indicates that cocoa polyphenolic extract suppresses inflammation in macrophage inflammatory phenotype and influences macrophage metabolism, promoting oxidative pathways and also macrophage polarization toward the M2 alternative phenotype.

INTRODUCTION

Chapter 1

Macrophages: biology and their role in diseases.

1.1. Origin and fate of blood monocytes.

Monocytes are white blood cells characterized by an irregular nucleus and light blue cytoplasm. They develop in bone marrow from progenitor cells and from here they go into blood and then into various tissues, where they develop into macrophages. It has been demonstrated in the mouse that blood monocytes can return to the bone marrow (Varol et al. 2007) and that the spleen forms a reservoir from which these blood cells can be mobilized (Swirski *et al.* 2009). About their fate, monocytes do not contribuite to the microglia of the brain in the mouse (Ginhoux *et al.* 2010), while they can replenish macrophages in tissues like kidney and lung (Schulz et al. 2012) or they can differentiate into dendritic cells. However, monocytes under homeostatic conditions circulate in blood for 1-3 days (van Furth and Cohn 1968; Whitelaw 1972). Under patologic conditions blood monocytes are attracted by chemokines to the site of infection and infiammation and activated into macrophages. Macrophages derived from monocytes are crucial to innate immune defense and they are involved in tissue remodeling and repair. Not only blood monocytes are the progenitors for tissue macrophages, but themselves may contribute to defense against infection, phagocytosing and destroying microbes that enter the blood.

Human blood monocytes are distinguished into three functionally distinct subpopulations: classical, $CD14^{++}$ $CD16^{-}$; intermediate, $CD14^{++}$ $CD16^{+}$ and non- classical $CD14^{+}$ $CD16^{++}$ (Ziegler-Heibrock *et al.* 2010). The existence of monocyte subpopulations is also evident in mouse and other species like non-human primates, rats and pigs.

The human cell lines of monocytes which are most widely used are: U937, THP-1 and Mono Mac 6. U937 was the first cell line to be described (Sundstrom and Nilsson 1976); it is an immature cell type with no or little expression of the monocyte marker CD14. THP-1 is more mature and can be further differentiated with vitamin D3 (Tsuchiya *et al.* 1980). Mono Mac 6 expresses CD14, responds rapidly to lipopolysaccharide and can be treated with vitamin D for further maturation. These cell lines constitute useful models for molecular studies. None of these cell lines expresses CD16 and

therefore they cannot serve as a model for non-classical or intermediate monocytes.

Several cells lines representing monocytes and macrophages derive from mouse, like: WEHI-3B that represent bone marrow monocytes (Leenen *et al.* 1986, 1990); Pu5 line (Ralph *et al.* 1974) which are similar to the non-classical blood monocyte or RAW 264.7 (Rascke *et al.* 1978), that are more mature beyond the level of the blood monocyte because they are representative of tissue macrophages.

Blood monocytes are extremely sensitive cells and any manipulation can alter their properties. Therefore the favored strategy for the study of monocytes involves minimal processing and no-touch isolation. Activation of the monocytes can be performed with the typical ficoll-hypaque technique, antibodies against cells surface molecules or stimulation by LPS and other microbial products.

1.2 Monocyte functions and their involvement in diseases.

Monocytes are able to engulf apoptotic cells or microbes. They have a multitude of receptors involved in phagocytosis like receptors for the Fc-portion of immunoglobulin, complement receptors, scavenger receptors and lectins (Underhill and Ozinsky 2002). Phagocytosis of apoptotic cells is important to development and repair and it is a silent process. By contrast, phagocytosis of microbes goes along with cytokine production and antigen presentation; these processes are crucial tasks of tissue macrophages, but blood monocytes can also execute all of these steps and thereby may contribute to immune defense against infection.

Blood monocytes can produce a broad array of pro- and anti-inflammatory cytokines (TNF and IL-10) and chemokines, like CCL5 and CXCL10. Production of these mediators changes among monocyte subpopulations and this can be explained by differential expression of cells surface receptors and intracellular signaling molecules. About cytokines production, the CD16-positive cells were shown to produce more TNF in response to LPS (Belge *et al.* 2002; Kanai *et al.* 2007; Mikilajczyk *et al.* 2009; Dimitrov *et al.* 2013; Balboa *et al.* 2013; Hearps *et al.* 2012a). Higher TNF production by non-classical monocytes was also reported for cells stimulated with tumor microvesicles (Baj-Krzyworzeka *et al.* 2010).

Monocytes in man are among the few cell types, which constitutively express high levels of MHC class II and these cells therefore can support T cell activation, proliferation and production of cytokines driven by antigen.

Monocytes express chemokine receptors on the cell surface which are target G-protein 7-transmembrane receptors (Murphy *et al.* 2000) and their activation leads to migration of leukocytes, including monocytes, from blood into tissue. Migration of monocytes governed by chemokines suggest that these blood cells reside in the marginal pool, i.e., they localize to vascular endothelium (Steppich *et al.* 2000) and they are ready for rapidly emigrating into tissue in case of an inflammatory signal.

When monocytes go into tissue then they mature into macrophages with different phenotypic and functional properties, that depend on the type of tissue. The classical and the non-classical monocytes in man may be committed to become unique types of macrophages; this has not been demonstrated as yet for intermediate monocytes.

Many studies have tried to simulated the process of monocyte-to-macrophage differentiation in vitroputting monocytes into culture without or with addition of different cytokines. In such system monocytes can develop into large macrophages in few days. Culture in the presence of INF-gamma plus LPS generates classically activated macrophages with pro-inflammatory features (M1) while culture in the presence of IL-4 or IL-13 will generate alternatively activated macrophages (M2) (Gordon 2003).

Number of monocytes in blood is related to age and gender. Lower absolute numbers for total monocytes and for CD16-positive (intermediate and non-classical types) monocytes have been reported for females (Heimbeck *et al.* 2010; Hearps *et al.* 2012a). Also, CD16-positive monocytes increase with age (Seidler *et al.* 2010; Nyugen *et al.* 2010) and they have linked to increased levels of TNF production (Hearps *et al.* 2012a). Number of monocytes in blood can also increase or reduce during disease. An increase of monocytes can indicate that the total pool of these cells in the body expands. In alternative the increase can also be due to an increased influx from compartments like bone marrow, spleen or the marginal pool or a reduced efflux (reduced emigration, reduced apoptosis). The same deliberations apply to decreases of monocytes. Therefore the increase and decrease always have to be interpreted with caution within the pathophysiological context.

Many studies report an increase of CD16-positivemonocytes in cancer (Subimerb *et al.* 2010, Feng *et al.* 2011), but their role in this process is unclear. One attractive possibility for their expansion, however, is that their induction is secondary to cancer and due to tumor-derived factors, a concept supported by the finding that these cells decrease with tumor resection (Subimerb *et al.* 2010).

The CD16-positive monocytes are also increased in:sepsis, that is the most dramatic form of infection,severe bacterial infections, hemolytic uremic syndrome (Fernandez *et al.* 2005) and post cardiac surgery (Fingerle-Rowson *et al.* 1998a). In these conditions monocytes were shown to produce high levels of cytokines (Shalova *et al.* 2012). This indicates that the non classical-monocytes may contribute to the cytokine storm seen in the early phase of sepsis. Volk *et al.* (1996) have shown that the increase of blood monocytes in sepsis goes along with a pronounced down-regulation of HLA-DR cell surface expression on blood monocytes. The down-regulation of HLA-DR on monocytes can be detected in many other conditions like severe burn (Venet *et al.* 2007). This parameter correlates with higher mortality (Wu *et al.* 2010) and it has potential as a predictor of clinical outcome in patients with sepsis, trauma, and immunosuppressive therapy (Docke *et al.* 2005).

Therefore, blood monocytes can respond to infection, but they also can be targeted by microbes, expecially viruses. Each virus is able to infect a specific monocyte type: Merkel cell polyomavirus, implicated in a neuroendocrine skin carcinoma called Merkel cell carcinoma, was detected exclusively in the classical and not in the non-classical monocytes (Mertz *et al.* 2010), while Hepatitis C virus was found in CD16-positive but not in CD16-negative monocytes (Coquillard and Patterson 2009). In the last infection the selective tropism of virus was attributed to the higher expression of the CD81-HCV receptor on CD16-positive monocytes.

The infection of monocytes by these viruses can result in further spreading in the body once the monocytes migrate into the different tissues.

Autoimmune diseases are characterized by increases of blood monocytes. In rheumatoid arthritis CD16-positive monocytes are expanded (Baeten *et al.* 2000, Cairns *et al.* 2002, Kawanaka *et al.* 2002) and these cells were linked to increased development of Th17 cells (Rossol *et al.* 2012). In immune thrombocytopenia CD16-positive monocytes are also increased and their number changes with the decrease of the platelet count (Zhong *et al.* 2012).

About liver disease, in acute liver failure the intermediate monocytes increase and the non-classical monocytes strongly decrease (Abeles *et al.* 2012). One explanation for the decrease of non-classical monocytes in blood is that these cells may migrate into severely damaged liver tissue. Also in acute liver failure a pronounced down-regulation of HLA-DR expression on CD14-positive blood monocytes has been noted and this was associated with poor prognosis

(Antoniades *et al.* 2006). In fibrosis of the liver the CD16-positive monocytes are increased in blood (Zimmermann *et al.* 2010) and there also is a strong increase of CD16-positive macrophages in liver tissue as shown by immunohistology. This accumulation may be due in part to increased transmigration of the CD16-positive monocytes across hepatic sinusoidal endothelial cells (Aspinall *et al.* 2010).

Monocytes are crucial to atherosclerosis. Oxidated LDL attract monocytes into arterial vessel wall in a process involving up-regulation of endothelial adhesion molecules and chemokines. Once arrived in the lesion monocytes can develop into macrophages and can become lipid laden foam cells. The local macrophages can produce cytokines and chemokines, which orchestrate the local inflammatory process. The plaque is a dynamic structure and involves egress and continuous influx of monocytes, such that blockade of influx will lead to resolution of lesion (Potteaux *et al.* 2011).

The number of total monocytes has no predictive value for cardiovascular events in atherosclerosis patients (Wheeler *et al.* 2004). However, an increased number of CD16-positive cells was found to be associated with increased CRP (Huang *et al.* 2012). Hence this subset is associated with a main risk factor for cardiovascular events in atherosclerosis (Ridker 2007). Then the intermediate monocytes are predictors of cardiovascular events in at risk dialysis patients (Heine *et al.* 2008). CD16-positive monocytes were shown to positively correlate with vulnerable plaques in coronary heart disease patients (Kashiwagi *et al.* 2010). Also these cells were shown to be linked to intima media thickness and to obesity (Poitou *et al.* 2011). Rogacev *et al.* (2012) demonstrated that it is the intermediate $CD14^{++}$ $CD16^{+}$ monocytes, which are predictive for cardiovascular events in such patients.

Patients affected by kidney disease show increased numbers of CD16-positive monocytes because they have a state of cronic microinflammation.

In patients with immunodeficiency higher number of CD-16 postive monocytes were noted (Amoras *et al.* 2007). The expansion of these cells may reflect an inflammatory reaction in patients, who with their antibody defect may have smouldering bacterial infection. The moderate antibody defect in patients with immunodeficiency may lead to a weaker signal such that only intermediate monocytes increase. By contrast, the almost complete antibody defect may lead to more pronounced bacterial infections and thereby can provide a stronger signal leading to further differentiation and increase of non-classical monocytes. Also in HIV infection an increase of CD-16 positive

monocytes has been noted; this may be influenced by the infection of the CD16-positive monocytes by the HIV virus.

1.3 Origin and diversity of macrophages.

Tissue macrophages were classically viewed as terminally differentiated cells derived from circulating monocytes that originate in the bone marrow (**fig.1**).

Some evidences recently suggest that tissue macrophages to originate prior to birth and maintained in the adulthood, independent of the contribution from monocytes (Wynn *et al.* 2013; Yona *et al.* 2013). In the mouse at least three distinct lineages of mononuclear phagocytes have been described: the first one derives from the yolk sac (YS) and gives rise to the tissue-resident populations of macrophages (defined as F4/80high) in skin, spleen, pancreas, liver brain and lung; the second population originates from fetal liver and gives rise to Langerhans cells, with a minor contribution from YS; and the third one originates from the bone marrow and gives rise to circulating monocytes that differentiate into the F4/80+ macrophage population within the tissues, e.g., lamina propria macrophages (Schulz *et al.* 2012; Wynn *et al.* 2013; Ginhoux *et al.* 2010; Hoeffel *et al.* 2012). In some organs like kidney and lung, co-existence of macrophages derived from YS as well as circulating monocytes has been proposed. In the same vein, two distinct populations of Kupffer cells (KCs) have been identified: the first one, radiosensitive, bone marrow-derived, rapidly replaced from hematopoietic precursors upon reconstitution following irradiation reconstitution and a second one, radioresistant, termed "sessile" because of the absence of rapid turnover and the missing capacity for local recruitment. Only the first KC population was found to take part into inflammatory responses (Klein *et al.* 2007). Interestingly in the case of lung, a recent paper demonstrated fetal monocytes to give rise to alveolar macrophages (Guilliams *et al.* 2013). However, further studies will be required to clarify the relative contributions of YS and fetal liver in the origin of different tissue-resident macrophages. Colony stimulating factor-1 (CSF-1) and its receptor (CSF-1R) is a major cytokine regulating the differentiation of macrophages (Chitu and Stanley 2006; Hamilton and Achuthan 2013). Accordingly, genetic ablation of CSF-1 or its receptor, CSF-1R results in the loss of macrophages in many tissues, such as skin, brain, bone, testis, and ovary. However compared to the CSF-1 deficiency, genetic ablation of CSF-1R resulted in a more severe phenotype, characterized by a complete lack of microglia, suggesting a role for another ligand of the CSF-1R (Erblich *et al.* 2011). This was identified as IL-34 (Wang *et al.* 2012; Lin *et al.* 2008; Wei *et al.* 2010). Indeed, genetic

ablation of IL-34 had resulted in loss of microglia and Langerhans cells (Wang *et al.* 2012). A recent study on the transcriptional factors responsible for macrophage differentiation revealed an interesting dichotomy with YS-derived murine tissue-resident macrophages (such as microglia) to be dependent on CSF-1R and the transcription factor PU.1 but not Myb, while the bone marrow-derived macrophages being dependent Myb (Schulz *et al.* 2012). Other factors like GM-CSF has been shown to be critical to differentiation of alveolar macrophages (Guilliams *et al.* 2013) and the compensatory role of VEGF in osteoclast development (Niida *et al.* 1999).

Under pathological situation like inflammation, blood monocytes are a key source of inflammatory macrophages and inflammatory DCs. However, in murine models of Type 2 inflammation, macrophage accumulation was found to be maintained by local self-renewal, independently of replacement by circulating monocytes or other putative precursors (Jenkins *et al.* 2011; Liddiard *et al.* 2011; Davies *et al.* 2011). Interestingly, in the liver, KCs have also been suggested to be maintained by local proliferation, following hepatectomy (Widmann and Fahimi 1975). However, further studies are necessary to ascertain the relative role of monocyte-derived and local expansion of macrophages in other tissue compartments upon pathological settings. Lastly, considering the emerging differences between murine and human macrophages differ a lot from each other (Martinez *et al.* 2013), it will be interesting to explore the existence of multiple origins and the functional significance of distinct lineages of macrophages in human settings.

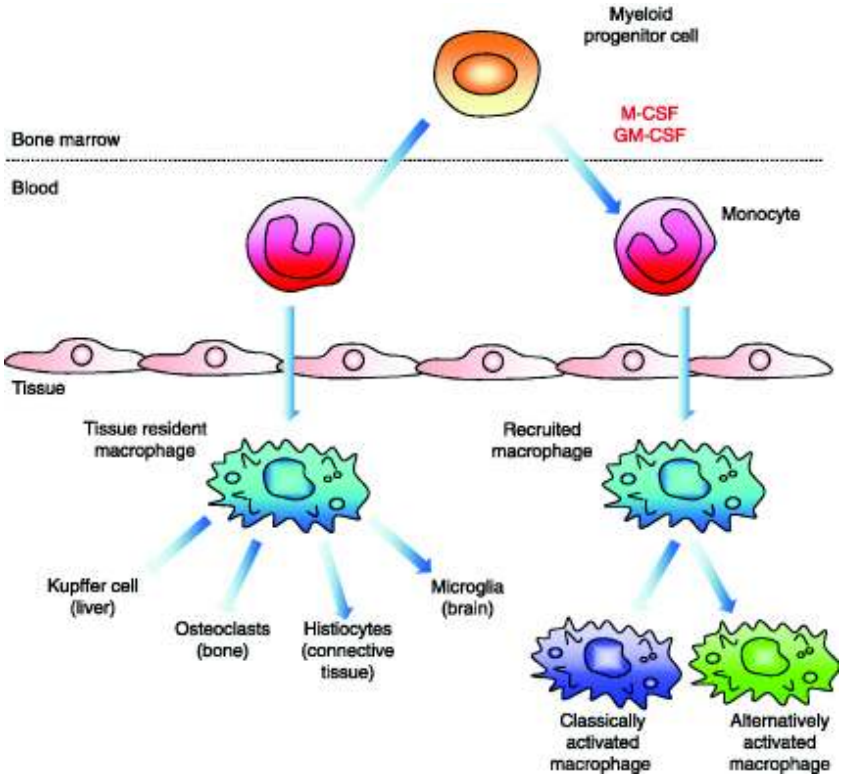


Fig. 1 - Development and differentiation of macrophages. The Figure is adapted from Rickard and Young 2009 and it shows that bone marrow-derived monocytes enter peripheral blood and circulate for several days in an inert state before entering tissues and differentiating into tissue resident macrophages. These include histiocytes (connective tissue), microglial cells (nervous system), Kupffer cells (liver) and osteoclasts (bone). Macrophages are recruited to tissues to restore resident populations or respond to diverse inflammatory and immune stimuli. The acquired phenotype of recruited macrophages reflects signals from the microenvironment in which they reside. These macrophages are broadly classified as classically or alternatively activated macrophages.

M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor.

1.4 Macrophage biology and classification.

Macrophages are the key components of innate defense against pathogens. These cells along with the dendritic cells play a pivotal role in initiating, orientating, and modulating many aspects of the adaptive response. In addition to their role as innate effector cells, macrophages and neutrophils also represent a major source of humoral, fluid phase pattern recognition molecules (Bottazzi *et al.* 2010; Deban *et al.* 2010). Since they can be considered as functional ancestors of antibodies, the production of these soluble mediators by phagocytes bridges the gap between the cellular and the humoral arm of innate immunity (Bottazzi *et al.* 2010).

Macrophages are mononuclear cells capable of phagocytosis. They are distributed throughout mammalian organs and their morphology varies depending on their state of activity. Peritoneal macrophages in Giemsa-stained measure 10 to 30 μm in diameter. The cytoplasm contains vacuoles and is slightly basophilic. The nucleus is ovoid and measure 6 to 12 μm in diameter. By phase contrast microscopy, peritoneal macrophages contain light gray diffuse cytoplasm with dark gray rod-shaped mitochondria. Granules and vacuoles are seen depending on the physiological state of the cell.

Various types of stimuli may cause "macrophage activation". Examples are stimuli which accompany phagocytosis of bacteria. Activated macrophages are metabolically highly active and contain organelles such as lysosomes rich in hydrolytic enzymes. Following infection with pathogens, the cytoplasmic membrane of mice macrophages are smoother and had fewer protuberances and invaginations. Indeed such macrophages contain many free ribosomes but very few profiles of endoplasmic reticula. The mitochondria are smaller, more numerous and contained more cristae. The cytoplasm of activated macrophages is less dense and appear to be highly dehydrated. It contains fewer vescicles and many Golgi bodies. Alveolar macrophages are morphologically distinct from peritoneal macrophages. With light and electron microscopy it was found that the nuclei of alveolar macrophages are round or slightly ovoid and nucleoli are seen more often than in peritoneal macrophages (Leake and Heise 1967).

Macrophages are ancient cells in metazoan phylogeny. In adult mammals, they are found in all tissues where they display great anatomical and functional diversity (**fig.2**). In tissues, they are organized in defined patterns with each cell occupying its own territory, a type of tissue within a tissue. Although several attempts have been made to classify macrophages, the most successful

definition is the mononuclear phagocytic system (MPS), which encompasses these highly phagocytic cells (professional phagocytes) and their bone marrow progenitors. In the MPS schema, adult tissue macrophages are defined as end cells of the mononuclear phagocytic lineage derived from circulating monocytes that originate in the bone marrow. However, this definition is inadequate as macrophages have several origins during ontogeny and each of these different lineages persist into adulthood (Gautier *et al.* 2012). Other functional classifications of macrophages have included binary classifications that refer to inflammatory states. These include the activated macrophage and alternatively activated macrophage (AAM) categories, and the derivative M1 and M2 categories for these types of macrophage in the non-pathogen-driven condition (Gordon 2003; Sica and Mantovani 2012). These two states are defined by responses to the cytokine interferon-γ (IFN-γ) and activation of Toll-like receptors (TLRs), and to interleukin-4 (IL-4) and IL-13, respectively. Although this classification is a useful heuristic that may reflect extreme states, such as that of activated macrophages during immune responses mediated by T helper cells that express IFN-γ (TH1) or of AAMs during parasitic infections, such binary classifications cannot represent the complex *in vivo* environment for most macrophage types, in which numerous other cytokines and growth factors interact to define the final differentiated state. Indeed, transcriptional profiling of resident macrophages by the Immunological Genome Project show that these populations have high transcriptional diversity with minimal overlap, suggesting that there are many unique classes of macrophages.

Macrophages have roles in almost every aspect of an organism's biology; from development, homeostasis and repair, to immune responses to pathogens. Resident macrophages regulate tissue homeostasis by acting as sentinels and responding to changes in physiology as well as challenges from outside. During these homeostatic adaptations, macrophages of different phenotypes can also be recruited from the monocyte reservoirs of blood, spleen and bone marrow (Geissmann *et al.* 2010), and perhaps from resident tissue progenitors or through local proliferation (Jenkins *et al.* 2011; Schulz *et al.* 2012). Unfortunately, in many cases these homeostatic and reparative functions can be subverted by continuous insult, resulting in a causal association of macrophages with disease states, such as fibrosis, obesity and cancer. Thus, macrophages are an incredibly diverse set of cells that constantly shift their functional state to new metastable states in response to changes in tissue physiology or environmental challenges. They should not even be considered as one

cell type but should be subdivided into different functional subsets according to their different origins.

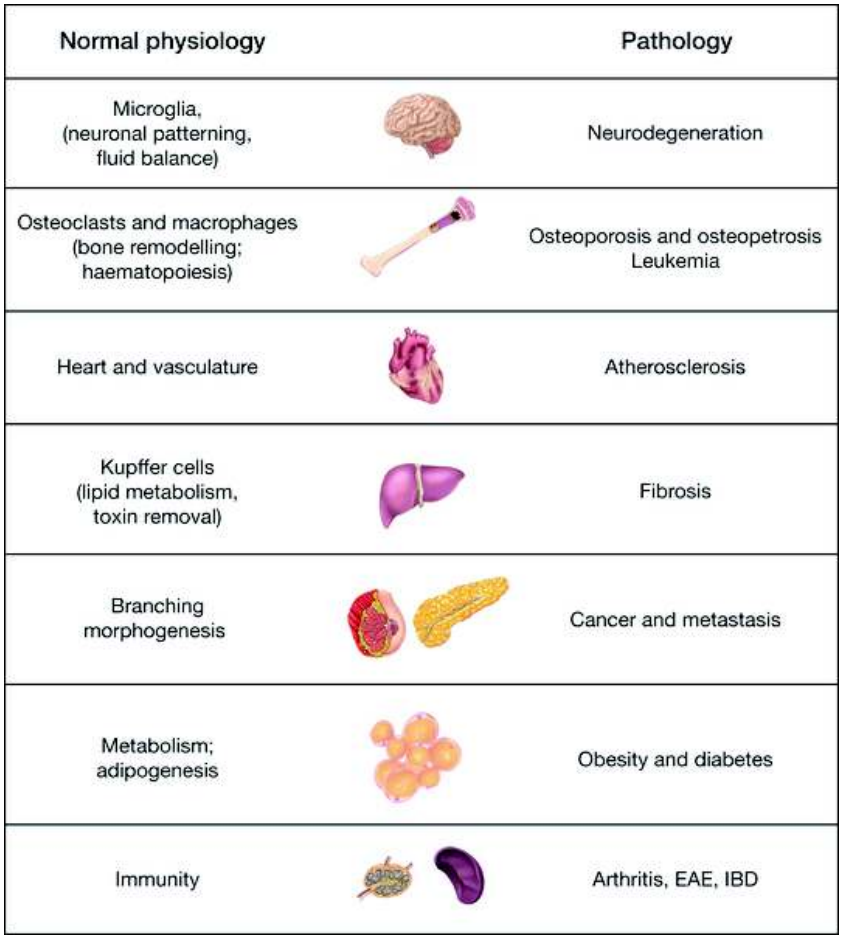


Fig. 2 - Macrophages in various tissues during normal physiology or pathology. Macrophages have many developmental roles in shaping the architecture of various tisuues, such as brain, bone and mammary gland tissues. After development of the organism, macrophages modulate homeostasis and normal physiology through their regulation of diverse activities, including metabolism and neural connectivity, and by detecting damage. However, these trophic and regulatory roles often subverted by continuous insult, and macrophages contribute to many diseases that are often associated with ageing. The figure is adapted from Wynn *et al.* 2013.

(EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease).

1.5 Macrophages in development.

Macrophages participate in the maintenance of tissue integrity and homoeostasis. To do so, macrophages would need to be able to discriminate self from non-self, sense tissue damage and recognize invading pathogens, an insight that led to the concept of innate immunity for which Metchnikoff was awarded the Nobel prize. The inherent properties of macrophages, which include sensing inside from out, motility throughout the organism, phagocytosis and degradation, were later sequestered to instruct the acquired immune system as it evolved to more efficiently deal with changing pathogenic challenges. This enhanced sophistication of the immune system probably resulted in the evolution of dendritic cells as specialized mononuclear phagocytes to interface with the acquired immune system. Indeed, in mammals, dendritic cells seem to be focused on initiating tissue immune responses, whereas tissue macrophages seem to be focused on homeostasis and tissue integrity (Gordon and Taylor 2005).

Emphasis on the immunological and repair aspects of macrophage function has overshadowed their importance in the development of many tissues; for example, studies of $Csf1^{op/op}$ mice, which lack many macrophage populations, have revealed a cluster of developmental abnormalities (Pollard 2009). Most notable among these is the development of osteopetrosis, which is caused by the loss of bone-reabsorbing macrophages known as osteoclasts. This phenotype, which is also observed in Sfpi1- null mice, is axiomatic for the roles of macrophages in development, in that cell fate decisions are unchanged but the tissue remodelling and expression of growth factors is lost. Specifically, although bone formation is intact in Csf1- or Spi1-null mice, the bones are not sculpted to form the cavities in which haematopoiesis commences. Consequently, the functional integrity of the bones, in terms of load bearing and haematopoiesis, is compromised. $Csf1^{op/op}$ mice survive to adulthood because of extra-medullary haematopoiesis in the spleen and liver, and as mice age, osteoclastogenesis is rescued by compensatory expression of VEGF and therefore bone marrow haematopoiesis commences (Niida *et al.* 1999).

Remodelling deficiencies in the absence of macrophages have also been noted in several other tissues, including the mammary gland, kidney and pancreas, suggesting a general requirement for macrophages in tissue patterning and branching morphogenesis (Niida *et al.* 1999; Stefater *et al.* 2011). In the mammary gland, the best studied of these tissues, macrophages are recruited to the growing ductal structure and their loss results in a slower rate of outgrowth and

limited branching, phenotypes that are reiterated during the mammary growth caused by pregnancy. This stems partly from the failure to remodel the extracellular matrix during the outgrowth of the ductal structures. However, recent studies have also implicated macrophages in maintaining the viability and function of mammary stem cells, which reside at the tip of the duct known as the terminal end bud and are responsible for the outgrowth of this structure (Gyorki *et al.* 2009). In stem cell biology similar roles for macrophages have been suggested in the maintenance of intestinal integrity and its regeneration after damage (Pull *et al.* 2005), whereas a subpopulation of macrophages in the haematopoietic niche regulates the dynamics of haematopoietic stem cell release and differentiation (Chow *et al.*, 2011). Furthermore, in regenerating livers, macrophages specify hepatic progenitor fate through the expression of WNT ligands and antagonism of Notch signaling (Boulter *et al.* 2012). Macrophage control of stem cell function is clearly an emerging and important research area.

As 'professional' phagocytes (macrophages were originally defined by their exceptional phagocytic ability), macrophages perform critical functions in the remodelling of tissues, both during development and in the adult animal; for example, during erythropoiesis, maturing erythroblasts are surrounded by macrophages that ingest the extruded erythrocyte nuclei. Remarkably, this function of macrophages is critical because in its absence, erythropoiesis is blocked and lethality ensues (Kawane *et al.* 2001). Macrophages also make decisions about haematopoietic egress from the bone marrow through engulfing cells that do not express the CD47 ligand (Jaiswal *et al.* 2009). They also maintain the haematopoietic steady state through engulfment of neutrophils and erythrocytes in the spleen and liver, and the failure of this activity results in neutropenia, splenomegaly and reduced body weight (Gordy *et al.* 2011). Phagocytosis, particularly of apoptotic cells, is clearly central to macrophage function and this is emphasized by the build-up in macrophage-depleted mice of such cells during development; for example, during the resolution of the inter-digit areas during limb formation (Dai *et al.* 2002). However, there is no apparent consequence to this phenomenon, as less-efficient 'non-professional' phagocytes clear excess apoptotic cells. Despite this, macrophages have evolved to 'eat' cells, and to suppress inflammation and autoimmunity in response to self-antigens that may arise during homeostasis (Savill *et al.* 2002).

Macrophages also regulate angiogenesis through a number of mechanisms. This has been most extensively studied in the eye during its development. Early in the postnatal period, during regression of the

hyaloid vasculature, macrophages identify and instruct vascular endothelial cells to undergo apoptosis if these cells do not receive a counterbalancing signal from pericytes to survive. WNT7B that is synthesized by macrophages delivers this cell-death signal to the vascular endothelial cells, and in the absence of either WNT7B or macrophages there is vascular over-growth (Rao *et al.* 2011). WNT secretion is also required later in retinal vasculature development but in this case macrophage synthesized WNT5A and WNT11, a non-canonical WNT, induces expression of soluble VEGF receptor 1 (VEGFR1) through an autocrine mechanism that titrates VEGF and thereby reduces vascular complexity so that the vascular system is appropriately patterned (Stefater *et al.* 2011). Furthermore, at other times of ocular development, macrophages regulate vascular complexity. In this circumstance, macrophage synthesized VEGFC reinforces Notch signaling (Tammela *et al.* 2010). In addition, during angiogenesis in the hindbrain, macrophages enhance the anastomosis of tip and stalk cells to give functional vessels (Fantin *et al.* 2010). These macrophage functions are not restricted to the vascular arm of the circulatory system, as they also have roles in lymphangiogenesis during development (Gordon *et al.* 2010), and in adults they have a notable role in maintaining fluid balance through their synthesis of VEGFC (Machnik *et al.* 2009).

Brain development is also influenced by macrophages. These macrophages called microglia depend on CSF1R signalling for their presence. In the absence of this signalling there are no microglia, and the brains of these mice have substantial structural defects as they mature. Both CSF1 and IL-34 are expressed by neurons in a mutually restricted pattern of expression, and IL-34 is the major factor for microglial differentiation and viability (Nandi *et al.* 2012). The disruption of architecture in the brain of the Csf1r- null mouse, together with well-documented deficiencies in neuronal processing regulating olfaction and the reproductive axis in the hypothalamus in Csf1-null mice, strongly suggests that microglia are involved in the development of neuronal circuitry and the maintenance of brain structure1. Indeed, microglia have been shown to promote neuron viability, modulate neuronal activity (Li *et al.* 2012) and prune synapses during development (Paolicelli *et al.* 2011), as well as express a range of neuronal growth and survival factors, including NGF19. This conjecture is supported by the finding that hypomorphic mutation in CSF1R in humans is responsible for hereditary diffuse leukoencephalopathy with spheroids that results from loss of myelin sheaves and axonal destruction. These trophic activities of microglia are also consistent with macrophages having roles in neuroprotection after injury, as defined in a variety of models. These effects include

the promotion of survival and proliferation of retinal progenitor cells, and the regeneration of adult sensory neurons (London *et al.* 2011; Kigerl *et al.* 2009; Salegio *et al.* 2011). However, caution needs to be exercised in attributing all of the phenotypes observed in the brains of Csf1r-mutant mice or humans to the loss of microglia, as Csf1r expression has been reported on neuronal stem cells and their development *in vivo* is regulated by CSF1R. Nevertheless, it seems likely that microglia have important roles in the development of neuronal circuitry, though their effects on the proliferation, survival and connectivity of neurons, through their effects on myelination, or by modulating angiogenesis and fluid balance in the brain.

The examples given above indicate a few of the roles for macrophages in normal development and these are likely to expand with further study. Phenotypically in mice, macrophages are $CD11b^+$, $CD68^+$ $CSF1R^+$ $F4/80^+$ and phagocytic and their activities are through the temporal and spatial delivery of developmentally important molecules such VEGFs and WNTs as well as proteases. These developmental roles of macrophages are recapitulated in repair as described below but are also intimately involved in chronic conditions that lead to pathologies as well as the development and progression of malignancies.

1.6 Macrophages in metabolic homeostasis.

Mammalian metabolic organs, such as the liver, pancreas and adipose tissue, are composed of parenchymal and stromal cells, including macrophages, which function together to maintain metabolic homeostasis (Hotamisligil *et al.* 2006). By regulating this interaction, mammals are able to make marked adaptations to changes in their environment and in nutrient availability. For example, during bacterial infection, innate activation of macrophages results in secretion of pro-inflammatory cytokines, such as TNF-a, IL-6 and IL-1b, which collectively promote peripheral insulin resistance to decrease nutrient storage (Chawla *et al.*, 2011; Odegaard and Chawla, 2013). This metabolic adaptation is necessary for mounting an effective defence against bacterial and viral pathogens because nearly all activated immune cells preferentially use glycolysis to fuel their functions in host defence. However, this adaptive strategy of nutrient re-allocation becomes maladaptive in the setting of diet-induced obesity, a state that is characterized by chronic low-grade macrophage-mediated inflammation (Olefsky and Glass 2010). Although our current knowledge in this area is primarily derived from studies in obese insulin-resistant mice, it is likely that tissue-resident macrophages also participate in facilitating metabolic adaptations in healthy animals.

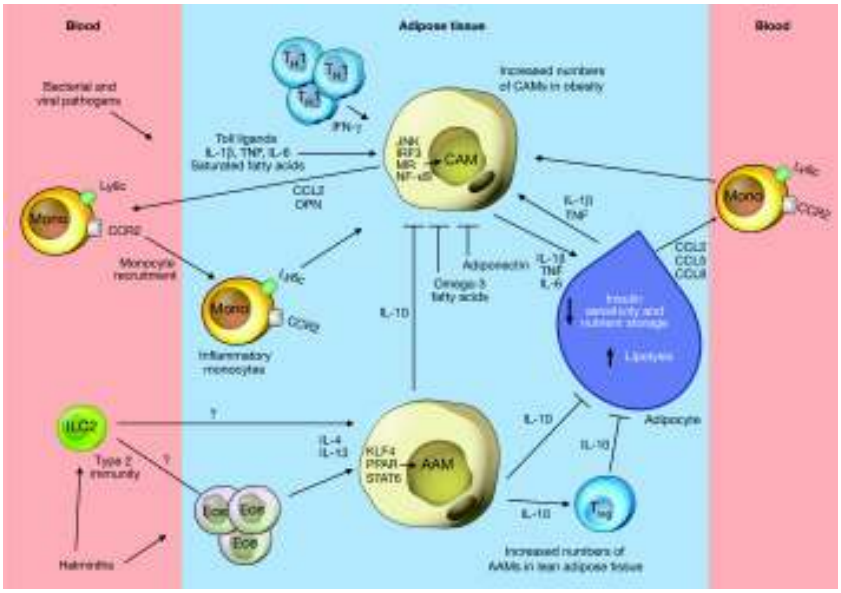


Fig.3 - Activated and alternatively activated macrophages differentially regulate insulin sensitivity in obesity. In lean healthy animals, adipose tissue macrophages produce anti-inflammatory cytokines, such as IL-10, IL-4 and IL-13, which are important for insulin sensitivity. While, during obesity, higher number of monocytes are recruited and derived macrophages, in contrast to normal resident ones, express an inflammatory phenotype, characterized by the production of TNF-α, IL-6 and IL-1β. These inflammatory macrophages decrease insulin sensitivity, facilitating the storage of excess nutrients. The enlarging white adipose tissues in turn release chemokines to recruit additional inflammatory monocytes that exacerbate the process. This mechanism is also enhanced during bacterial and viral infections, so essential nutrients are diverted to lymphocytes, which must use glycolysis to enhance their activation at times of stress. The figure is adapted from Wynn *et al.*, 2013.

(CAM, classically activated macrophage. Eos, eosinophils; ILC2, type 2 innate lymphoid cells; Mono, monocytes).

1.7 Macrophages in white adipose tissue.

White adipose tissue (WAT) is not only the principal site for long-term storage of nutrients but also regulates systemic metabolism through the release of hormones called adipokines (Rosen and Spiegelman 2006). These metabolic functions of WAT are primarily performed by adipocytes with trophic support provided by stromal cells, including macrophages. Thus, macrophage representation in WAT, both in terms of numbers and their activation state, reflects the metabolic health of adipocytes. For example, in lean healthy animals, adipose tissue macrophages comprise 10–15% of stromal cells and express the canonical markers ($Arg1^+$, $CD206^+$, $CD301^+$) of AAMs (Lumeng *et al.* 2007). In contrast, macrophage content increases to 45–60% during obesity (Weisberg *et al.* 2003; Xu *et al.* 2003), resulting from increased recruitment of $Ly6C^{hi}$ monocytes that differentiate into inflammatory macrophages, as judged by their expression of *NOS2, TNFα* (also known as TNF) and *Itgax*. Although these macrophages contribute to the development of insulin resistance in adipocytes, recent studies suggest that these cells also participate in remodelling of the enlarging WAT, functions that facilitate the storage of excess nutrients in adipocytes (Sun *et al.* 2011). This suggests that two macrophage subsets coordinate homeostatic adaptations in adipocytes of lean and obese animals.

In healthy animals, AAMs are critical for maintaining insulin sensitivity in adipocytes. This trophic effect of AAMs is partly mediated by secretion of IL-10, which potentiates insulin action in adipocytes. These observations led various groups to focus on cell-intrinsic and cell-extrinsic mechanisms that control alternative activation of adipose tissue macrophages. For cell-intrinsic factors, transcription factors downstream of IL-4 and IL-13 signalling, such as PPAR-γ, PPAR-δ and KLF4, were found to be required for the maintenance of AAMs in WAT and metabolic homeostasis (Kang *et al.* 2008; Liao *et al.* 2011; Odegaard *et al.* 2007; Odegaard *et al.* 2008). The dominant cell-extrinsic factors regulating maturation of AAMs in lean WAT are the type 2 cytokines IL-4 and IL-13. Absence of eosinophils, which constitute the major cell type capable of IL-4 secretion in WAT (Wu *et al.* 2011), impairs alternative activation of adipose tissue macrophages and makes mice susceptible to obesity-induced insulin resistance. Together, these reports have established that homeostatic functions performed by AAMs in WAT are required for metabolic adaptations to excessive nutrient intake.

Although adipocytes in lean animals can easily accommodate acute changes in energy intake, chronic increase in energy intake places adipocytes under considerable metabolic stress. Consequently, the

enlarging WAT releases chemokines, such as CC-chemokine ligand 2 (CCL2), CCL5 and CCL8, to recruit $Ly6C^{hi}$ inflammatory monocytes into the WAT (Weisberg *et al.* 2006), where these cells differentiate into $CD11c^{+}$ macrophages and form 'crown-like structures' around dead adipocytes. As these $CD11c^{+}$ macrophages phagocytize dead adipocytes and become lipid engorged, they initiate expression of inflammatory cytokines, such as TNF-α and IL-6, which promote insulin resistance in the surrounding adipocytes (Cinti *et al.* 2005). Presumably, this initial decrease in adipocyte insulin sensitivity is an adaptation to limit nutrient storage. However, in the setting of unabated increase in caloric intake, this adaptive response becomes maladaptive, contributing to pathogenesis of obesity-induced systemic insulin resistance.

1.8 Macrophages in brown adipose tissue.

In mammals, brown adipose tissue (BAT) is the primary thermogenic organ that is activated by exposure to environmental cold (Lowell and Spiegelman 2000). For decades, it had been thought that hypothalamic sensing of cold triggers an increase in sympathetic nerve activity to stimulate the BAT program of adaptive thermogenesis. However, recent work has demonstrated that resident macrophages are required to facilitate the metabolic adaptations of BAT and WAT to cold. Specifically, exposure to cold temperatures results in alternative activation of BAT and WAT macrophages, which are required for induction of thermogenic genes in BAT and lipolysis of stored triglycerides in WAT (Nguyen *et al.* 2011). Accordingly, mice lacking AAMs are unable to mobilize fatty acids from WAT to maximally support BAT thermogenesis, which is necessary for the maintenance of core body temperature in cold environments. These supportive functions of macrophages are mediated by their secretion of norepinephrine, which surprisingly accounts for approximately 50% of the catecholamine content of BAT and WAT in the cold. Thus, cold-induced alternative activation of BAT and WAT macrophages provides an example of how resident macrophages provide trophic support to facilitate the function of tissue parenchymal cells, in this case the white and brown adipocytes.

1.9 Macrophages in liver and pancreas.

Liver integrates nutrient, hormonal and environmental signals to maintain glucose and lipid homeostasis in mammals. Over the past few years, evidence has emerged that Kupffer cells, the resident macrophages of liver, facilitate the metabolic adaptations of hepatocytes during increased caloric intake. During obesity, an imbalance between the uptake, synthesis and oxidation of fatty acids

results in increased lipid storage in hepatocytes, a key factor in the development of hepatic insulin resistance (Samuel and Shulman 2012). Interestingly, Kupffer cells directly participate in this process by regulating the oxidation of fatty acids in hepatocytes. An early insight into this process came from studies that identified PPAR-δ as an important regulator of the IL-4- and IL-13-driven program of alternative macrophage activation. These studies revealed that loss of PPAR-δ in myeloid cells specifically impaired alternative activation of Kupffer cells, resulting in hepatic steatosis and insulin resistance. A similar phenotype was observed when Kupffer cells were depleted in rodents using gadolinium chloride or clodronate-containing liposomes (Huang *et al.* 2010). Although the precise factors elaborated by Kupffer cells are still not known, co-culture studies suggest that Kupffer-cell-derived factors work in a trans-acting manner to maintain hepatic lipid homeostasis.

Pancreas functions as an endocrine and exocrine gland in mammals. Recent findings suggest that, analogous to obesity-induced WAT inflammation, high-fat feeding induces the infiltration of macrophages into the insulin-producing islets (Eguchi *et al.* 2012; Ehses *et al.* 2007). In this case, the increased intake of dietary lipids results in beta-cell dysfunction, which induces the expression of chemokines, such as CCL2 and CXCL1, to recruit inflammatory monocytes or macrophages into the islets. Consequently, the secretion of IL-1β and TNF-α by the infiltrating macrophages augments beta-cell dysfunction, resulting in impaired insulin secretion and hyperglycaemia in obese mice. Although these reports have elucidated the pathogenic role of macrophages in beta-cell dysfunction, in the future it will be important to determine whether macrophages also participate in the physiological regulation of beta-cell biology as they do during development and pregnancy.

1.10 Macrophages in disease

When tissues are damaged following infection or injury, inflammatory monocytes ($Ly6c^+$ in mice) are recruited from the circulation and differentiate into macrophages as they migrate into the affected tissues. These recruited macrophages often show a pro-inflammatory phenotype in the early stages of a wound-healing response. They secrete a variety of inflammatory mediators, including TNF-α, IL-1 and nitric oxide, which activate anti-microbial defence mechanisms, including oxidative processes that contribute to the killing of invading organisms. They also produce IL-12 and IL-23, which direct the differentiation and expansion of anti-microbial T_H1 and T_H17 cells (T helper cells that express IFN-γ and IL-17) that help to drive inflammatory responses forward. Although these inflammatory

macrophages are initially beneficial because they facilitate the clearance of invading organisms, they also trigger substantial collateral tissue damage because of the toxic activity of reactive oxygen and nitrogen species and of T_H1 and T_H17 cells (Nathan and Ding 2010). Indeed, if the inflammatory macrophage response is not quickly controlled, it can become pathogenic and contribute to disease progression, as is seen in many chronic inflammatory and autoimmune diseases (Sindrilaru *et al.* 2011; Krausgruber *et al.* 2011). To counteract the tissue-damaging potential of the inflammatory macrophage response, macrophages undergo apoptosis or switch into an anti-inflammatory or suppressive phenotype that dampens the pro-inflammatory response while facilitating wound healing. These regulatory macrophages often produce ligands associated with development, such as WNT ligands, that are essential for tissue repair (Ahn *et al.* 2010). It is becoming increasingly clear that the mechanisms that regulate the transformation of inflammatory macrophages into an anti-inflammatory cell or suppressive macrophages back into a pro-inflammatory phenotype has a major impact on the progression and resolution of many chronic diseases.

1.10.1 Cancer.

Tumours are abundantly populated by macrophages. Although macrophages were originally thought to be part of an anti-tumour response, clinical and experimental data indicate that in the large majority of cases macrophages promote tumour initiation, progression and metastasis (Qian and Pollard 2010). In response to persistent infections or chronic irritation, macrophages synthesize inflammatory cytokines, IFN-γ, TNF-α and IL-6, which engage other immune cells to sustain the chronic inflammation that seems to be causal in tumour initiation and promotion (Balkwill and Mantovani 20120). The tumour-inducing activities are multifactorial; for example, through the production of inflammatory cytokines, such as IFN-γ in skin cancer that is induced by exposure to ultraviolet light (Zaidi *et al.* 2011) and TNF-α in carcinogen-induced cancer, through the generation of a mutagenic environment (Deng *et al.* 2010) or through alterations of the microbiome (Arthur *et al.* 2012). However, once tumours become established they cause differentiation so that the tumour-associated macrophages (TAMs) change from an immunologically active state to adopt a trophic immunosuppressive phenotype that promotes tumour progression and malignancy (they become "tumour-educated").

In established tumours, TAMs stimulate tumour-cell migration, invasion and intravasation, as well as the angiogenic response required for tumour growth (De Nardo *et al.* 2010; Hanahan and Coussens 2012). These events are required for tumour cells to become

metastatic, as they facilitate their escape into the circulatory or lymphatic system. Evidence from autochthonous models of breast cancer suggests that the macrophages take on these activities in response to CSF1, IL-4 and IL-13 encountered in the tumour microenvironment. For example, IL-4-mediated differentiation results in a reciprocal paracrine dialogue between CSF1 and EGF, synthesized by tumour cells and TAMs, respectively, that promotes tumour-cell invasion and intravasation in mammary cancer (Condeelis and Pollard 2006). In mammary cancers, this loop is initiated by CXCL12 in the polyoma virus middle T (PyMT) model or heregulin (also known as pro-neuregulin-1, membrane-bound isoform) in the HER2/Neu model. In human xenograft models, CCL18 is also required for tumour-cell invasion and metastasis, because it has a role in triggering integrin clustering (Chen *et al.* 2011). TAMs also remodel the tumour microenvironment through the expression of proteases such as matrix metalloproteinases (MMPs), cathepsins and urokinase plasminogen activator, and matrix remodelling enzymes such as lysyl oxidase and SPARC (Mason and Joyce 2011). The proteases, such as cathepsin B, MMP2, MMP7 and MMP9, cleave extracellular matrix and thereby provide conduits for the tumour cells and release growth factors such as heparin-binding EGF (HB-EGF) and EGF mimics that foster tumour-cell invasion and metastasis (Kessenbrock *et al.* 2010).

Macrophages have an important role in tumour angiogenesis as they regulate the marked increase in vascular density, known as the angiogenic switch, that is required for the transition to the malignant state (Lin *et al.* 2006). These angiogenic TAMs are characterized by the expression of the angiopoietin receptor TIE2, which is also expressed in macrophages during development (Mazzieri *et al.* 2011; Murdoch *et al.* 2008). Ablation of this specific population inhibits tumour angiogenesis and thus tumour growth and metastasis in a variety of models. TAMs secrete many angiogenic molecules, including VEGF family members TNF-α, IL-1β, IL-8, PDGF and FGF (Stockmann *et al.* 2008). Of these, myeloid-derived VEGF is required for the angiogenic switch but other aspects of angiogenesis can be independent of VEGF and involve the secreted protein Bv8 (also known as prokineticin 2 PROK2) (Shojaei F *et al.*, 2007). Angiogenic macrophages can be recruited to the tumours by hypoxia (Du R *et al.*, 2008) but also by growth factors such as CSF1 and VEGF (Lin EY *et al.*, 2009).

Tumours have a proclivity to metastasize to particular sites, and this phenotype is partially defined by macrophages. Data suggest that the tumour-produced fragments of ECM molecules or exosomes prepare

these sites, known as pre-metastatic niches, to be receptive to the circulating tumour cells through recruitment of myeloid cells characterized by CD11b and VEGFR1 positivity (Psaila and Lyden 2009; Peinado *et al.* 2012). These niches are tumour-type-dependent and the fate of the tumour cells can be reprogrammed to a different tissue by the transfer of tumour-conditioned serum to a naive mouse strain. These niches are also dependent on coagulation as this is necessary for recruitment of the myeloid cells that have recently been more precisely defined as $F4/80^+$ monocytes (or $F4/80^+$ macrophages) (Gil-Bernabè *et al.* 2012). At lung metastatic sites, mini-clots form that enable the arrest of tumour cells that then produce CCL2 to recruit $CCR2^+$ $Ly6c^+$ inflammatory monocytes that rapidly develop into $Ly6c^-$ metastasis-associated macrophages (MAMs) (Qian *et al.* 2011). These monocytes and MAMs promote tumour-cell extravasation, partly through their expression of VEGF, which induces local vascular permeability. MAMs that are intimately associated with the tumour cells also promote their viability through clustering of tumour-cell-expressed VECAM1 that interlocks with the MAM expressed counter receptor integrin α4. MAMs also promote subsequent growth of the metastatic cells and, importantly, ablation of these cells after the metastases are established inhibits metastatic growth.

In mice, these individual pro-tumoral functions are carried out by different subpopulations, although they all express canonical markers such as CD11b, F4/80 and CSF1R. This view is consistent with recent profiling of immune cells in various tumour types in mice and humans that indicates that there are differences in the extent of macrophage infiltration and in phenotype (Coussens and Pollard 2011). For example, detailed phenotypic profiling in human hepatocellular carcinoma shows various macrophage subtypes defined by specific location that have both pro- and anti-tumoral properties through their engagement of the acquired immune system, although overall the balance is tilted towards pro-tumoral functions (Wu and Xheng 2012). Transcriptional profiling of TAM subpopulations in mice suggest they more closely resemble embryonic macrophages than inflammatory ones, as they have higher expression of developmentally relevant molecules, such as those of the WNT pathway. This strongly suggests that the trophic roles of macrophages found during development, in metabolism and in the maintenance of homeostasis, are subverted by tumours to enhance their growth, invasion and complexity. However, transcriptional control of these different phenotypes is only just being revealed, particularly in *in vivo* contexts. Many studies have analysed macrophage responses to LPS signalling through nuclear factor-kB (NF-kB), but this results in 'activated' macrophages that are mainly

involved in antibacterial responses and are likely to be anti-tumoral. In contrast, in their trophic and immunosuppressive functions, TAMs are shaped by IL-10 and IL-4 or IL-13 that signal to STAT3 and STAT6, respectively (Hagermann *et al.* 2008). The PARP proteins and KLF4 also co-operate to induce a pattern of gene expression associated with their tumour-promoting phenotype. In macrophages, CSF1R also signals to a wide range of transcriptional factors, including MYC and FOS. MYC signalling has been shown to be important for pro-tumoral phenotypes100. CSF1R expression is regulated in turn by ETS2 transcription factors, and genetic ablation of this factor in macrophages in PyMT tumours recapitulates the loss of CSF1 in tumours, as angiogenesis is inhibited and tumour growth decreases101. To study the interaction of these factors and other regulatory molecules such as microRNAs and epigenetic controls will require sophisticated genomic analyses that will help to differentiate the regulation of the multiple subsets.

1.10.2 Inflammatory disease.

Macrophages have important roles in many chronic diseases, including atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis and fibrosis (Hansson and Hermansson, 2011; Kamada *et al.* 2008; Libby *et al.* 2011). Their contributions to these diseases vary greatly in different stages of disease and are controlled by many factors. For example, allergic asthma is a complex chronic inflammatory disease of the lung defined by airway inflammation, airway obstruction, airway hyper-responsiveness and pathological lung remodelling. The inflam- matory response is characterized by the recruitment of T_H2 lymphocytes, mast cells, eosinophils and macrophages to the lung, and by elevated expression of allergen-specific immunoglobulin-E (IgE) in the serum. It has been suggested that the chronicity of type 2 cytokine-mediated airway inflammation that is characteristic of allergic asthma is explained by the presence of a macrophage-like antigen-presenting cell population that persists in the airway lumen (Julia *et al.* 2002). Pulmonary macrophages produce a variety of factors that directly stimulate airway smooth-muscle contractility and degradation of the ECM that contributes to pathological airway remodelling. Airway macrophages from some asthmatics are bathed in type-2-associated cytokines, including IL-4, IL-13 and IL-33, causing their differentiation, which has been implicated in the pathogenesis of asthma. These macrophages in turn promote the production of type 2 cytokines by pulmonary CD4 T lymphocytes, and produce a variety of cytokines and chemokines that regulate the recruitment of eosinophils, T_H2 cells and basophils to the lung, suggesting a viscous cycle that worsens disease. Adoptive

transfer studies have shown that the severity of allergen-induced disease is exacerbated by IL-4R^{+} macrophages (Ford *et al.* 2012), whereas protection from allergic airway disease is associated with a reduction in IL-4R^{+} macrophages in some studies (Moreira *et al.* 2010). Increased numbers of IL-4R^{+} macrophages have also been reported in the lungs of asthmatic patients that have reduced lung function (Melgert *et al.* 2012). Nevertheless, studies conducted with LysM^{cre} IL-4Rα$^{-/lox}$ mice in which Cre-mediated recombination results in deletion of the IL-4Rα chain in the myeloid cell lineage identified no substantial role for IL-4Rα-activated macrophages in ovalbumin- and house-dust-mite-induced allergic airway disease (Nieuwenhuizen *et al.* 2012).

Macrophages have also been implicated in the pathogenesis of a variety of autoimmune disease, including rheumatoid arthritis, multiple sclerosis and inflammatory bowel diseases. In these diseases, macrophages are an important source of many of the key inflammatory cytokines that have been identified as drivers of autoimmune inflammation, including IL-12, IL-18, IL-23 and TNF-α (Murray and Wynn 2011). Macrophage-derived IL-23 promotes end-stage joint autoimmune inflammation in mice. TNF-α also functions as an important driver of chronic polyarthritis, whereas IFN-γ- and TNF-α-dependent arthritis in mice has been attributed to macrophages and dendritic cells that produce IL-18 and IL-12. The pathogenesis of chronic demyelinating diseases of the central nervous system (CNS) has also been attributed to macrophages that display a pro-inflammatory phenotype. These inflammatory macrophages contribute to axon demyelination in experimental autoimmune encephalomyelitis in mice, a frequently used model of multiple sclerosis. Consequently, novel therapeutic strategies that target specific myeloid cell populations could help to ameliorate pathogenic inflammation in the CNS (Ponomarev *et al.* 2011). The pathogenesis of inflammatory bowel disease is also tightly regulated by inflammatory macrophages. A subset of TLR2^{+} CCR2^{+} CX3CR1^{int} Ly6c^{hi} GR1^{+} macrophages has been shown to promote colonic inflammation by producing TNF-α (Platt *et al.* 2010). A recent study showed that inflammatory mediators produced in the colon convert homeostatic anti-inflammatory macrophages into pro-inflammatory dendritic-cell-like cells that are capable of producing large quantities of IL-12, IL-23, iNOS and TNF-α (Rivollier *et al.* 2012). CD14^{+} macrophages that produce IL-23 and TNF-α have also been identified in Crohn's disease patients. Thus, macrophages and dendritic cells are key producers of many of the cytokines that have been implicated in the pathogenesis of inflammatory bowel disease.

Although there is substantial evidence to support the idea that inflammatory macrophages have roles in autoimmune inflammation, many studies have also reported suppressive roles for macrophages. For example, macrophages that produce reactive oxygen species can protect mice from arthritis by inhibiting T-cell activation (Gelderman *et al.* 2007). Pro-inflammatory cytokines that are produced by activated macrophages have also been shown to protect mice from Crohn's disease by facilitating the clearance of pathogenic commensal bacteria from the mucosal lining of the bowel (Smith *et al.* 2009). Recruited monocytes and resident tissue macrophages are also thought to maintain homeostasis in the intestine by clearing apoptotic cells and debris, promoting epithelial repair, antagonizing pro-inflammatory macrophages, and by producing the suppressive cytokine IL-10, which is critical for the maintenance of FOXP3 expression in colonic regulatory T cells (Treg cells) (Smith *et al.* 2009; Murai *et al.* 2009). Macrophages also protect rodents from demyelinating diseases of the CNS by promoting T-cell apoptosis and by expressing the anti-inflammatory cytokines TGF-β1 and IL-10. The inhibitory receptor CD200 (also known as OX2), which is also expressed on anti-inflammatory macrophages, has been shown to prevent the onset of experimental autoimmune encephalomyelitis in mice (Hoek *et al.* 2000). A unique population of monocyte-derived macrophages also reduces inflammation resulting from spinal cord injury, providing further evidence of a protective role for macrophages in the CNS (Schecter *et al.* 2009). Together, these observations show how changes in macrophage differentiation in the local environment can have a decisive role in the pathogenesis of a wide variety of autoimmune and inflammatory diseases.

1.10.3 Fibrosis.

Although macrophages phagocytose and clear apoptotic cells as a part of their normal homeostatic function in tissues, when they encounter invading organisms or necrotic debris after injury, they become activated by endogenous dangers signals and pathogen-associated molecular patterns. These activated macrophages produce anti-microbial mediators, like reactive oxygen and nitrogen species and proteinases, that help to kill invading pathogens and thus assist in the restoration of tissue homeostasis. However, they also produce a variety of inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6 and CCL2 that help to drive inflammatory and anti-microbial responses forward. This exacerbates tissue injury and in some cases leads to aberrant wound healing and ultimately fibrosis (scarring) if the response is not adequately controlled, as has been demonstrated by the selective depletion of macrophages at various stages of the wound-

healing response (Duffield *et al.* 2005). Therefore, in recent years research has focused on elucidating the mechanisms that suppress inflammation and prevent the development of fibrosis. Although most wound-healing responses are self-limiting once the tissue-damaging irritant is removed, in many chronic fibrotic diseases the irritant is either unknown or cannot be eliminated easily (Wynn and Ramalingam 2012). In this situation, it is crucial that the dominant macrophage population converts from one exhibiting a pro-inflammatory phenotype to one exhibiting anti-inflammatory, suppressive or regulatory characteristics so that collateral tissue damage is kept at a minimum. A variety of mediators and mechanisms have been shown to regulate this conversion, including the cytokines IL-4 and IL-13, Fcc receptor and TLR signalling, the purine nucleoside adenosine and A2A receptor signalling, prostaglandins, Treg cells, and B1 B cells (Wynn and Ramalingam 2012; Mosser and Edwards 2008). Each of these mediators has been shown to activate distinct populations of macrophages with suppressive or regulatory characteristics. These "regulatory" macrophages express a variety of soluble mediators, signalling intermediates and cell-surface receptors, including IL-10, arginase 1, IKKα, MMP13, maresins, CD200, RELMα and PD-L2, which have all been shown to decrease the magnitude and/or duration of inflammatory responses, and in some cases to contribute to the resolution of fibrosis. They also produce a variety of soluble mediators, including CSF1, insulin-like growth factor 1, and VEGF, that promote wound healing (Thomas *et al.* 2011). Consequently, in addition to promoting fibrosis, macrophages are intimately involved in the recovery phase of fibrosis by inducing ECM degradation, phagocytizing apoptotic myofibroblasts and cellular debris, and by dampening the immune response that contributes to tissue injury. Therefore, current fibrosis research is focused on characterizing these regulatory macrophage populations and devising therapeutics strategies that can exploit their anti-inflammatory, anti-fibrotic and wound-healing properties.

Chapter 2

Polarized activation of macrophages.

2.1 Macrophage activation and polarization.

Phenotypic and functional plasticity is a key feature of macrophages (Mantovani *et al.* 2002, 2004; Martinez *et al.* 2009; Sica and Mantovani 2012; Mosser and Edwards 2008; Gordon and Taylor 2005; Biswas and Mantovani 2010). Under physiological conditions, macrophages modulate their morphological and functional aspects in response to their tissue microenvironment and give rise to distinct resident populations, such as Kupffer cells in the liver, alveolar macrophages in lungs, lamina propria macrophages in the gut, and microglia in the brain. Under pathological stimuli, such as microbes or tissue damage, macrophages activate their effector functions, namely antimicrobial and antitumoral activities. However, far from being only a transient increase in their effector functions, it is now clear that macrophage "activation" is characterized by several distinct aspects which give rise to macrophage phenotypes with distinct and specific roles (Mackaness 1969; Adams and Hamilton 1984; Evans and Alexander 1972). In this regard, the discovery of an IL-4 mediated "alternative" form of macrophage activation (Stein *et al.* 1992) has shed new light on the complexity of macrophage polarization. It is now widely accepted that, mirroring the Th1–Th2 paradigm and under the crucial influence of soluble mediators, namely IFN-γ or IL-4/IL-13, macrophages can be polarized toward a "classical" M1 or "alternative" M2 phenotype, respectively. However, M1 and M2 phenotypes are two extremes of a spectrum of functional states which make up the complexity of macrophage plasticity (Biswas and Mantovani 2010; Mantovani *et al.* 2002; Mosser and Edwards 2008; Sica and Bronte 2007; Sica and Mantovani 2012).

The concept of plasticity of mononuclear phagocytes became more complex when an alternative form of macrophage activation induced by IL-4 was found (Stein *et al.* 1992). Indeed, the Th1 cytokine, IFN-γ alone or together with microbial stimuli (e.g., LPS) or inflammatory cytokines (e.g., TNF and GM-CSF) was the first soluble mediator found to activate classical effector functions of macrophages (**Fig. 4**). IL-4 and IL-13 were subsequently found to be responsible for an "alternative" (M2) form of macrophage activation (Gordon 2003) (**Fig. 4**). The term M1 and M2 was initially proposed to describe macrophage populations that showed distinct nitrogen metabolism pathways (NO versus arginine) upon LPS or IFNγ stimulation, depending on whether they were derived from Th1 mice strains (e.g., C57/BL6) or Th2 mice strains (e.g., Balb/c) (Mills *et al.* 2000). This

concept was extended and further developed by Mantovani and colleagues to propose a general scheme for macrophage polarization, wherein M1 state represented the classi- cally activated macrophages, whereas the M2 state included the alternatively activated macrophages (Mantovani *et al.* 2002). It is now known that other mediators besides IL-4 and IL-13 can also drive M2 polarization. For example, IL-33, a cytokine of the IL-1 family (Hazlett *et al.* 2010; Kurowska-Stolarska *et al.* 2009), amplifies IL-13-induced polarization of alveolar macrophages to an M2 phenotype which is responsible for lung eosinophilia and inflammation (Kurowska-Stolarska *et al.* 2009). Similarly, IL-21 is another Th2-associated cytokine that is shown to promote M2 activation of macrophages (Pesce *et al.* 2006). CSF-1 and IL-34 have also been suggested to polarize macrophages to a M2 phenotype (Foucher *et al.* 2013; Martinez *et al.* 2006). Indeed, a study of the LPS response of GM-CSF and CSF-1 derived bone marrow macrophages showed the former to induce more IL-12 and IL-23 resembling an M1 state while the latter induced more of IL-10 but no IL-12/23, suggesting a M2 polarization (Fleetwood *et al.* 2007). Activin A was found to be one of the molecules responsible for the M1 polarization of GM-CSF-derived macrophages (Sierra-Filardi *et al.* 2011).

In addition, among the various activation states that characterize the macrophage complexity is an "M2-like" state, which shares some but not all the functional aspects of M2 cells (Biswas and Mantovani 2010). Various stimuli, such as immunoglobulin complexes, glucocorticoids, transforming growth factor-β (TGF-β), and IL-10, give rise to M2-like functional phenotypes that exhibit properties similar to IL-4 or IL-13-activated macrophages (e.g., high expression of man- nose receptor, IL-10, and angiogenic factors) (Mantovani *et al.* 2004). In addition, many *in vivo* conditions were found to be characterized by the appearance of M2-like macrophages, such as in the placenta and embryo, helminth or *Listeria* infection, obesity, and cancer (Auffray *et al.* 2007; Gustafsson *et al.* 2008; Odegaard *et al.* 2007; Rae *et al.* 2007; Raes *et al.* 2005).

From the functional point of view, M1 cells play a pivotal role in polarized Th1 responses and mediate resistance against intracellular parasites and tumors. Infact, these cells produce high levels of IL-12 and IL-23, as well as effector molecules (e.g., reactive nitrogen and oxygen intermediates, RNI, ROI) and inflammatory cytokines (IL-1β, TNF, IL-6), but low levels of the immunoregulatory cytokine IL-10. On the contrary, the various forms of M2 macrophages generally express high levels of IL-10, low levels of IL-12 and IL-23, and display variable capacity to produce inflammatory cytokines. M2 cells

are generally characterized by high expression of scavenger, mannose, and galactose-type receptors as well as Arginase-1, which is responsible for the production of ornithine and polyamines. M2 cells also express low levels of IL-1β and caspase I, high levels of IL-1ra and decoy type II receptors (Dinarello 2005). In terms of function, M2 cells are mainly involved in polarized Th2 responses, such as parasite clearance (Noel *et al.* 2004). In addition, these cells along with M2-like macrophages display immunoregulatory properties, promote tissue remodeling, angiogenesis, and tumor progression (Wynn 2004; Mantovani *et al.* 2013; Biswas and Mantovani 2010).

Chemokine production and chemokine receptor expression are also differentially represented by M1- and M2-polarized macrophages. M1 macrophages express typical Th1 cell-attracting chemokines such as CXCL9 and CXCL10, while M2 macrophages express the chemokines CCL17, CCL22 and CCL24 (Mantovani 2008; Martinez *et al.* 2006; Medzhitov and Horng 2009). Chemokines themselves can also influence macrophage polarization such as CCL2 promoting an M2-like phenotype while CXCL4 inducing a unique macrophage phenotype with a mixture of both M1 and M2 characteristics (Gleissner *et al.* 2010; Roca *et al.* 2009). Finally, cellular metabolism of iron, folate, and glucose is also differentially regulated between M1- and M2-polarized macrophages (Puig-Kroger *et al.* 2009; Recalcati *et al.* 2010; Rodriguez-Prados *et al.* 2010; Biswas and Mantovani 2012) (**Fig. 4**). In this regard, the expression of the protein metabolism enzyme, Transglutaminase 2 has been found to be a conserved M2 characteristics in human and mouse macrophages (Martinez *et al.* 2013).

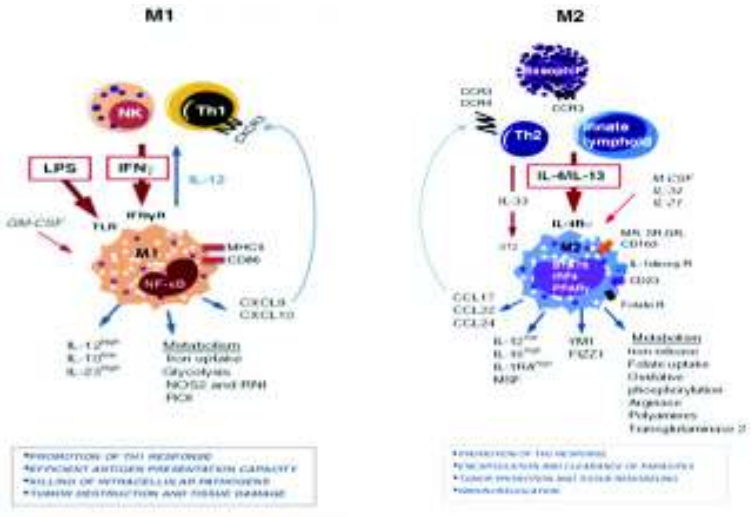


Fig. 4 - M1 and M2 phenotypes. The figure is adapted from Biswas and Mantovani (2014) and shows the salient features of the M1 and M2 polarized macrophages, which represent extremes of a continuum in a universe of activation states. For two phenotypes key genes, metabolic features, transcription factors and functional properties associated with them are indicated. The principal stimuli responsible for each polarizing state are indicated by *red box*. Crosstalk between polarized macrophage and lymphocyte subsets is also shown.

2.2 Transcriptional Regulation of Macrophage Polarization.

Over the last few years, considerable progress has been made in toward characterizing the transcription factors, epigenetic mechanisms, and post-transcriptional events regulating macrophage polarization (Natoli and Lawrence data?). IFN-γ was the first cytokine identified to induce M1 polarization (Nathan *et al.* 1983). Binding of IFN-γ to its receptor induces JAK 1/2-mediated phosphorylation and dimerization of Signal transducer and activator of transcription 1 (STAT1) (Shuai *et al.* 1993), which in turn engages responsive elements in the promoters of M1 phenotype related genes such as iNOS, IL-12, and CXCL10 (Darnell *et al.* 1994). STATs are also involved in LPS-mediated M1 polarization through TLR4 activation. In response to LPS, the TRIF-dependent TLR4 pathway triggers IFN Regulatory Factor 3 (IRF3) activation which induces the expression of IFN-β. IFN-β in turn through the IFNAR triggers STAT1 and STAT2 phosphorylation. The STAT1/STAT2 heterodimer also recruits IFN-Recognition Factor 9 (IRF9) as part of a complex that binds the IFN-response gene elements (Stark *et al.* 1998). Another member of the IRF family, namely IRF5, whose stabil- ity is regulated through the interaction with the COP9 signalosome (Korczeniewska and Barnes 2012), was found to be activated in M1 macrophages. IRF5 activation

in M1 cells regulates the expression of IL-12, IL-23, and TNF, thereby controlling Th1 and Th17 responses (Krausgruber *et al.* 2011). Expression of M1 macrophage- associated genes is also promoted by the Notch-RBP-J axis, which induces the syn- thesis of IRF8 by selectively augmenting IRAK2-dependent signaling via TLR4 (Xu *et al.* 2012). JNK activation is also required for pro-inflammatory macrophage activation and lack of JNK in macrophages protected mice against insulin resistance (Han *et al.* 2013).

While M1-promoting signals activate STAT1, IL-4 and IL-13 skew macrophages toward the M2 phenotype via STAT6 (Sica and Bronte 2007). Indeed, binding of IL4 or IL13 to their receptors promote the phosphorylation and dimerization of STAT6, which in turn recruits IRF4 and triggers the transcription of M2-associated genes, such as mannose receptor (*Mrc1*), resistin-like α (*Retnla*, *Fizz1*), chitinase 3-like 3 (*Chi3l3*, *Ym1*) (Gordon and Martinez 2010; Junttila *et al.* 2008; Pauleau *et al.* 2004) as well as the inhibition of many inflammatory genes (Ohmori and Hamilton 1998). Another member of the same family, STAT3 is the main transcription factor regulated by IL-10 and induces the expression of several genes related to the M2-like phenotype (*Il10*, *Tgfb1*, *Mrc1*) (Lang *et al.* 2002; Gordon 2003; Mantovani *et al.* 2002). Moreover, among the M2-promoting transcription factors, STAT5 also finds a place due to its activation in response to IL-3 (Kuroda *et al.* 2009).

Members of the suppressor of cytokine signaling (SOCS) family block JAK/ STAT pathway by negative feedback in macrophages. In fact, IL-4 and IL-13 up- regulate SOCS 1 and 2, thus inhibiting STAT1 pathway and interfering with M1 polarization. On the contrary, IFN-γ in concert with TLR stimulation, up-regulates SOCS3, which in turn inhibits STAT3, resulting in the dampening of M2 polariza- tion (Spence *et al.* 2013; Whyte *et al.* 2011). A SOCS3-dependent pathway has also been found to be involved in RBP-J-mediated Notch signaling regulating macro- phage polarization (Wang *et al.* 2010b).

IL-4-STAT6 pathway modulates various transcription factors promoting M2 polarization of macrophages. For instance, the nuclear receptor peroxisome proliferator-activated-γ (PPARγ) is constitutively expressed at low levels in macro- phages but once induced by IL4-STAT6, it inhibits STAT, NF-κB, and AP-1, thus inhibiting M1 response (Odegaard *et al.* 2007; Ricote *et al.* 1998; Szanto *et al.* 2010). PPARγ activity similarly promotes a M2 phenotype in tissues and its expres- sion is also induced by IL4-STAT6 pathway (Odegaard *et al.* 2008; Kang *et al.* 2008). Of relevance, STAT6 synergizes with Krüppel-like factor 4 (KLF4) (Liao *et al.* 2011; Cao *et al.* 2010). Indeed, IL4 induces STAT6 phosphorylation to promote KLF4 gene

expression. KLF4 in turn synergizes with STAT6 to promote M2 gene expression (*Arg-1*, *Mrc1*, *Fizz1*, *PPARg*) and inhibits M1 genes (*TNFa*, *COX-2*, *CCL5*, *iNOS*) preventing NF-κB activation through the sequestration of the necessary co-activators. Thus, KLF4 functions as a point of no return in M1 versus M2 polarization: in the absence of KLF4 M1 polarization is facilitated and M2 polar- ization is impaired (Liao *et al.* 2011). In parallel to KLF4, KLF2 impairs macrophage activation by inhibiting the NF-κB/HIF-1α functions, even though the lack of KLF2 is not associated to defects in M2 marker expression (Mahabeleshwar *et al.* 2011).

Downstream of IL-4 signaling, human macrophages also activate c-Myc, which modulates the expression of genes associated with M2 activation (*Scarb1*, *Alox15*, and *Mrc1*), as well as STAT6 and PPARγ activation (Pello *et al.* 2012b). Accordingly, in an *in vivo* model, the myeloid-specific c-Myc deletion resulted in a delayed maturation of tumor-associated macrophages (TAMs) and a reduction of their pro-tumoral functions (reduced expression of VEGF, MMP9, and HIF1α) that was associated with impaired tissue remodeling, angiogenesis, and reduced tumor growth (Pello *et al.* 2012a).

NF-κB is a key transcriptional regulator of both M1 and M2 polarization. LPS and TLR-induced NF-κB activation play a piv- otal role in the expression of many inflammatory genes and orchestration of M1 polarization (Bonizzi and Karin 2004). Indeed, many M1 genes present a NF-κB binding site in their promoter region, including iNOS, CCL2, COX2, and CCL5 (Huang *et al.* 2009). In contrast, NF-κB activation also triggers a genetic program necessary for resolution of inflammation and M2 skewing of tumor-associated macrophages (Lawrence and Gilroy 2007; Hagemann *et al.* 2008). In this regard, nuclear accumulation of p50 NF-κB homodimers was observed in both TAMs and LPS-tolerant macrophages, suggesting a role of this transcriptional repressor in promoting M2 phenotype and impairing M1 polarization (Porta *et al.* 2009; Saccani *et al.* 2006). Thus, depending on the temporal framework, the stimuli involved and the relative amounts of different NF-κB homo- or heterodimers, this master transcription factor can drive macrophage polarization to distinct and contrasting outcomes. The nuclear receptor NR4A1 (Nur77) which is expressed in macrophages and within atherosclerotic lesions was recently found to inhibit the activation of the p65 NF-κB in macrophages, thus acting as a negative regulator of M1 polarization (Hanna *et al.* 2012).

Hypoxia Inducible Factors (HIFs) play important roles in macrophage polarization. HIF-1α and HIF-2α were found to be differentially expressed in M1- versus M2-polarized macrophages (Takeda *et al.*

2010). Moreover, Th1 cytokines promote HIF-1α activity via NF-κB and mediate transcription of iNOS (M1-associated gene); Th2 cytokines promote HIF-2α activation, which limits NO production by inducing arginase 1 (M2 associated gene) (Takeda *et al.* 2010). Earlier study with myeloid cell-specific HIF1α knockout also indicated its contribution to the inflammatory and bactericidal response of macrophages (Cramer *et al.* 2003). However, myeloid cell-specific HIF2α knockout has revealed its dominant role in macrophage inflammatory response to M1 stimuli and macrophage migration into tumors (Imtiyaz *et al.* 2010). Further studies considering the temporal interaction of the HIF isoforms may shed further light on their differential contribution in macro- phage activation and polarization.

2.3 Epigenetic Regulation of Macrophage Polarization.

Epigenetic changes define modifications in DNA that do not alter the genetic sequence but regulate the expression of encoded information in a context-specific way. They usually occurs post-translationally and comprises modifications of histones, such as methylation, acetylation, and phosphorylation, that set the "histone code," aimed at controlling the "availability" of chromatin for selected transcription factors, thus regulating the final rate of the expression of a target gene (Ivashkiv 2012). Recent evidence suggests that macrophage polarization may be controlled by different chromatin states of relevant gene loci (Medzhitov and Horng 2009; Smale 2010; Natoli *et al.* 2011). As a general point of view, the gene loci involved in macrophage polarization may present three transcriptional states: (a) a repressed state, characterized by "repressive" histone marks, namely trimethylation of histone 3 lysine 9 (H3K9me3), lysine 27 (H3K27me3), and lysine 79 (H3K79me3); (b) an intermediate transcriptional state, characterized by the simultaneous presence of activating (H3K4me3, H3K9,14-Ac) and repressing histone marks (H3K9me3 and H3K27me3); and (c) an active transcriptional state, characterized by open chroma- tin configuration and active histone marks, such as trimethylation of histone 3 lysine 4 (H3K4me3) (Medzhitov and Horng 2009; Wei *et al.* 2009; Barski *et al.* 2007).

Epigenetic remodeling modulates macrophage activation, differentiation, and polarization. As an example, a reduced global DNA methylation is associated with myeloid commitment from hematopoietic stem cells, in comparison with that occur- ring during lymphoid commitment (Takeuch and Akira 2011). During macrophage differentiation, some pivotal transcription factors such as PU.1 and CCAAT/enhancer binding protein (C/EBP)α may directly bind and open the regulatory regions of several M1 genes induced in response to TLR ligands (e.g., TNF, IL-1β, IL-6, IL-12p40, CXCL10)

(Ghisletti *et al.* 2010; Jin *et al.* 2011). During M1 macrophage polariza- tion, IFNγ-induced STAT1 activation mediates chromatin opening (Chen and Ivashkiv 2010). In resting macrophages, inflammatory gene transcription is "silenced" by repressive elements, such as repressors (e.g., BCL6) or receptors that sequester activating factors (histone deacetylases and demethylases). TLR activation results in dissociation of repressors from relevant gene loci and in activation of demethylases, such as Jumonji JMJD3, JMJD2d, PHF2, and AOF1, that eliminate negative histone marks, thus making chromatin accessible to the transcription machinery (De Santa *et al.* 2009; Stender *et al.* 2012; Zhu *et al.* 2012). Among these enzymes, TLR engagement induces the expression of the H3K27 demethylases JMJD3 that is involved in fine-tuning the expression of a set of genes skewing macrophages toward M1 polar- ization (De Santa *et al.* 2009). However, in vivo evidence showed that JMJD3 is dispensable for M1 polarization, but is fundamental for M2 macrophage polarization to helminth infection, through the induction of IRF4, required for M2 polarization, as mentioned before (Satoh *et al.* 2010). Noteworthy, M2 polarization in response to IL-4 seems to be independent of JMJD3, suggesting that macrophage polarization in one direction in response to distinct stimuli may follow distinct ways (Satoh *et al.* 2010). IL-4-induced M2 polarization is counteracted by HDAC3, which deacetylates enhancers of IL-4 responsive genes (Mullican *et al.* 2011).

Histone methylation is also controlled by oxygen availability. In macrophages, hypoxia impairs Jumonji histone demethylases activity, thus favoring the prevalence of repressive marks, (H3K9me2/me3), and the consequent inhibition of transcription of chemokine (Ccl2) and chemokine receptor (Ccr1 and Ccr5) genes (Tausendschon *et al.* 2011).

Epigenetic regulation consists of a further level of modulation of cellular functions and phenotypes. Thus, proteins that interact with post-translationally modified histones may represent an intriguing molecular target for new therapeutic strategies. To this regard, the bromodomain and extracellular domain (BET) family proteins recognize acetylated histones, thus promoting transcription by RNA polymerase II (Jang *et al.* 2005; Yang *et al.* 2005). A synthetic compound (I-BET) which inhibit the interaction between BET proteins and acetylated histones has been found to repress the expression of genes involved in M1 polarization in LPS-activated macrophages, thus providing a new potential tool against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis (Nicodeme *et al.* 2010). Compounds that inhibit JMJD3 and

related demethylases were found to reduce the LPS-induced inflammatory cytokines production by human primary macrophages (Kruidenier *et al.* 2012).

Glucocorticoids (GCs) are one of the most potent anti-inflammatory drugs that interact with homodimeric nuclear receptors (GRs), which regulate transcription of target genes by binding to glucocorticoid response elements (GREs) (Glass and Saijo 2010). It was recently found that following TLR4 engagement by LPS in mac- rophages, GR cistrome is dramatically remodeled to an expanded inflammatory cistrone, that includes both GR-induced and -repressed genes (Uhlenhaut *et al.* 2013). Interestingly, negative GR enhancers selectively use the co-repressor GRIP1, interfere with the IRF3 activity, and present reduced histone acetylation, thus suggesting a role for the epigenetic regulation and chromatin status in driving the transcriptional effect on GR controlled target genes, beyond the GR itself (Uhlenhaut *et al.* 2013).

2.4 Micro-RNA regulation of macrophage activation and polarization.

Micro-RNA (miRNAs) are small (20–22 nucleotides) non-coding RNAs which bind to the 3′ untranslated regions (UTRs) of target genes, thus repressing mRNA trans- lation and/or inducing degradation of target gene transcripts, resulting in the inhibition of the target gene expression (Bartel 2009). A huge number of miRNAs have been identified and each miRNA may control several mRNA transcripts: this post-transcriptional mechanism of gene expression regulation is emerging as a major player in modulating a number of biological processes.

An intense experimental effort has been made in identifying miRNAs that are differentially expressed in polarized macrophages and recent evidence describe a role for these non-coding sequences in regulating the differential gene expression profiles in macrophages during inflammation and tumorigenesis. In particular, both in human and murine monocytes and macro- phages, TLRs signaling has been associated to the regulation of miRNAs, thus activating multiple targeting strategies that modulate expression of key molecules and fine-tune pro- and anti-inflammatory processes. The best characterized pro-inflammatory miRNA is miR-155, which is strongly induced by LPS or Type I interferons in both mouse and human monocytes and macrophages (O'Connell *et al.* 2007). The well-established pro-inflammatory function of this molecule is due to its ability in increasing the TNF transcript stability (Bala *et al.* 2011), promoting antiviral immunity through SOCS1 down-regulation (Wang *et al.* 2010a), and favoring atherosclerosis by targeting BCL6 (Nazari-

Jahantigh *et al.* 2012). In contrast, miR-146a, the first miRNA shown to be induced by TLRs activation in macrophages, can itself regulate TLR signaling pathway by targeting key signaling molecules such as IRAK-1 and TRAF6 (Taganov *et al.* 2006), thus acting as negative regulator of inflammation (Jurkin *et al.* 2010) and in endotoxin tolerance (Nahid *et al.* 2009). TLRs engagement induces others miRNAs, namely, miR-9 (Bazzoni *et al.* 2009), miR-21 (Sheedy *et al.* 2010), and miR-147 (Liu *et al.* 2009), all of which have been demonstrated to operate a feedback control of the NF-κB-dependent response, by directly fine-tuning the expression of key members of the NF-κB family (miR-9) or down-regulating the translation of the pro-inflammatory tumor suppressor, programmed cell death 4—PDCD4, an inhibitor of IL-10 production (miR-21). MiR-125a/b play a dual role in the control of inflammatory circuitries, as they reduce the TNF transcript stability (Tili *et al.* 2007), but at the same time, also sustain inflammation enhancing NF-κB signaling by targeting the NF-κB negative regulator, TNFα Induced Protein 3 (TNFAIP3, A20) (Kim *et al.* 2012), and IRF4, thus resulting in an enhanced macrophage activation (Chaudhuri *et al.* 2011).

Considering their purpose in modulating TLR-mediated cell activation, anti- inflammatory stimuli may also act through miRNA induction or repression. To this regard, IL-10 has recently been show to directly induce miR-187 in TLR4-activated monocytes: miR-187 directly targets TNF mRNA and indirectly decreases IL-6 and IL-12p40 expression, through the down-modulation of IκBζ, a positive transcriptional regulator of these two cytokines (Rossato *et al.* 2012). In addition, our group recently identified miR-146b as a second IL-10-dependent miRNA and demonstrated its ability in dampening the production of inflammatory mediators by mul- tiple targeting of components of the TLR signaling pathway (Curtale *et al.* 2013).

A clear role of miRNAs is emerging in macrophage polarization. To this regard, miR-125 and miR-29b interfere with M2 activation by targeting IRF4 and sustain M1 activation via targeting the NF-κB negative regulator A20, respectively (Chaudhuri *et al.* 2011; Graff *et al.* 2012). In the same direction, miR-155 targets the IL-13 receptor α chain, thus interfering with M2 polarization, and C/EBPβ, a major transcription factor controlling the expression of M2 markers such as Arg1 and Chi3l3 (He *et al.* 2009), thus resulting in macrophage skewing toward the M1 phenotype.

In contrast, two intronic miRNAs are co-regulated together with their host M2 genes and thus their expression increases in response to alternative activation. MiR- 378 is hosted in the first intron of the PPARγ gene and acts as a negative regulator as it targets the IL-4

signal transducer Akt1 (Ruckerl *et al.* 2012). MiR-511 is hosted in the fifth intron of the mannose receptor 1 gene (also known as CD206): it is highly expressed in M2 macrophages and TAMs and is shown to down-regulate the pro-tumoral genetic program of TAMs, inhibiting tumor growth (Squadrito *et al.* 2012). Quite recently, miRNA let-7c has been found to be involved in promoting murine M2 polarization, by targeting the negative regulator of TLR4-mediated inflammatory response C/EBP-δ and by regulating bactericidal and phagocytic activities of murine macrophages (Banerjee *et al.* 2013). Conversely, murine *in vitro* and *in vivo* evidence indicate that miR-19a-3p inhibits the M2 phenotype of macro- phages, by targeting the protooncogene Fra-1, thus repressing its downstream genes VEGF, STAT3, and pSTAT3. As a result, miR-19a-3p was found to inhibit in vivo breast cancer progression and metastasis (Yang *et al.* 2013). Finally, a recent report identified a set of miRNAs specifically expressed in distinct subsets of M2 and M1 human monocyte-derived macrophages and showed their influence on cytokine profile (Graff *et al.* 2012). However, seven of the eight identified miRNAs were passenger strands, that usually are not included in the silencing complex, but are quickly degraded (Bartel 2004): the real functional significance for these miRNAs in macrophage polarization needs to be further clarified.

Plasticity is a well-known characteristic of the monocyte–macrophage lineage. Within the tissue microenvironment, the complex integration of tissue-specific signals, microbial factors, and soluble mediators determines genetic re-programming, phenotypic changes, and differential activation of these cells. The pathologic consequences of macrophage polarization imply that specific targeting of polarized macrophage subsets (or activation states) can be considered as the final goal for therapeutic intervention. It is emerging that the therapeutic effect of some current drugs that were not specifically designed to target macrophages, such as PPARγ inhibitors, statins, zolendronic acid, and glucocorticoid hormones, are likely to act by targeting cells of the monocyte–macrophage lineage. For instance, the clinically approved anticancer agent Trabectedin has recently been found to be effective because of its major effect in inducing depletion of TAMs (Germano *et al.* 2013). Further investigations of mechanisms and molecules involved in polarized activation of macrophages may facilitate the finding of novel diagnostic and therapeutic tools aimed at modulating the multifaced functions of mononuclear phagocytes.

New transcriptomic studies on macrophages revealed a spectrum model of macrophage activation states extending the current M1

versus M2-polarization model (Xu *et al.* 2014). Simultaneously, an effort has been made by the community to suggest an uniform nomenclature for macrophage activation (Murray *et al.* 2014).

2.5 Polarized M1-M2 macrophages in pathology.

Consistent with concept of polarization, the functional phenotypes expressed by macrophages *in vivo* or *ex vivo* in pathological conditions such as parasite infections, allergic reactions, and tumors reflect many aspects of polarized M1 and M2 macrophages (**Fig.5**). However, macrophages with overlapping M1–M2 characteristics and shifts in their polarization states in course of pathological setting have also been noted suggesting the plasticity of these cells (Biswas and Mantovani 2010).

In several pathologies classically activated M1 cells are implicated in initiating and sustaining inflammation (arthritis, steatosis and viral infection), while M2 cells are associated with resolution or smoldering chronic inflammation (fibrosis, wound healing and atopic dermatitis) (Martinez *et al.*, 2009).

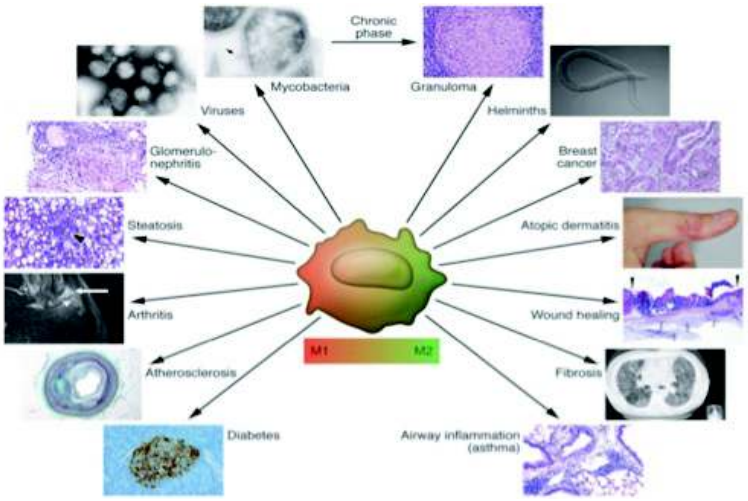


Fig. 5 - Schematic representation of macrophage plasticity and polarization in pathology. Dynamic changes occur over time with evolution of pathology: for istance, a switch from M1 to M2 macrophage polarization characterizes the transition from early to chronic phases of infection. Moreover, mixed phenotypes or populations with different phenotypes can coexist. The picture is adapted from Sica and Mantovani (2014).

During wound healing M1-polarized macrophages mediate tissue damage and initiate inflammatory responses (Gordon and Martinez 2010; Biswas SK and Mantovani A, 2010). During the early stages of the repair response after wounding the skin, infiltrating macrophages have an M2 phenotype and their depletion inhibits the formation of a highly vascularized, cellular granulation tissue and of scar tissues (Lucas *et al.* 2010).

In a peritoneal model of inflammation, resolution phase macrophages expressed a unique mixed M1-M2 phenotype, and cAMP was essential to restrain M1 activation (Bystrom *et al.* 2008).

In a murine model of hind limb ischemia, haplodeficiency of the oxygen sensor prolyl-hydroxylase PHD2 induced the canonical NF-kB pathway in macrophages, which promoted their M2 polarization and proarteriogenic phenotype (Takeda et al 2011). This observation may suggest a functional link between oxygen deprivation, as occurring in wounds and tumors, and progressive induction of proarteriogenic M2 macrophages.

Macrophage activation has been found in autoimmune and inflammatory diseases and in particular in lupus nephritis. In a murine model of SLE, proinflammatory activation of macrophages was sustained by Notch signaling. Conversely, the acute phase protein serum amyloid P (SAP) skewed macrophages toward an antinflammatory M2-like phenotype, thus alleviating lupus nephritis (Zhang *et al.* 2011).

M1 macrophages characterize infection with Listeria monocytogenes, Salmonella typhi and salmonella typhimurium, as well as the early phases of infection with Mycobacterium tuberculosis, Mycobacterium ulcerans and Mycobacterium avium (Shaughnessy and Swanson 2010; Chacon-Salinas *et al.* 2005; Kiszewski *et al.* 2006). The M1-M2 switch observed during the transition from acute to chronic infection may provide protection against overwhelming uncontrolled inflammation; however, a phenotype switch can also favor pathogens that have envolved strategies to interfere with M1-associated killing (Benoit *et al.* 2008; Pathak *et al.* 2007).

Polarized activation of macrophages has been associated with virus infection nad this polarization may be important in containing and limiting tissue damage. In particular, during virus-induced bronchiolitis, M2 macrophages activation reduces inflammation and epithelial damage in lungs (Shirey *et al.* 2010).

Allergy is associated with M2 polarization of macrophages (Kim *et al.* 2010; Melgert *et al.* 2011). Asthma is associated with tissue

remodeling, including collagen deposition and goblet cell hyperplasia. IL-4 driven M2 polarization is likely to play a key role as an orchestrator of these processes (Wynn 2003).

Allergy represents a paradigm for IL-4/IL-13-driven type 2 inflammation. Moreover, a Th1-associated cytokine, IL-18, has also been implicated in allergic inflammation (Tsutsui *et al.* 2004). It is therefore perhaps not surprising that mixed phenotype macrophages have been observed (Moreira *et al.* 2011).

Classically activated M1-polarized macrophages have the potential to exhibit antitumor activity and to elicit tumor tissue disruption (Mantovani *et al.* 2002). At least in some model of carcinogenesis in the mouse, progression is associated with a phenotype switch from M1 to M2 (Zaynagetdinov *et al.* 2011). Th1-driven macrophage activation was found to mediate elimination of senescent hepatocytes, which drive subsequent carcinogenesis (Kang *et al.* 2011). It is therefore likely that classically activated M1-macrophages contribute to the T cell- mediated elimination and equilibrium phases during tumor progression (Schreiber *et al.* 2011). At later stages of progression in mice and humans, macrophages generally have an M2-like phenotype with low IL-12 expression, high IL-10 expression, and low tumoricidal activity and promotion of tissue remodeling and angiogenesis. M2 macrophages are generally associated with poor prognosis, as shown in glioma, cholangiocarcinoma and breast carcinoma (Steidl *et al.* 2010; Chen *et al.* 2011). However, macrophages with various functional states can coexist in the same tumor (Movahedi *et al.* 2010).

Macrophages from obese mice and humans are polarized toward an M1 phenotype, with upregulation of TNF and iNOS. In contrast"lean" macrophages express high levels of M2 genes, including IL-10 and Arginasi 1 (Hevener *et al.* 2007; Lumeng *et al.* 2007). Weight loss is associated with a shift back to an M2-like phenotype. However, evidence indicates that macrophages in obese patients is diverse, with M2 population coexisting with M1 cells (Shaul *et al.* 2010). Nguyen *et al.* have recently demonstrated that adaptation to lower temperatures is associated with polarization of brown adipose tissue (BAT) and white adipose tissue (WAT) macrophages to the alternative state M2 (Nguyen *et al.* 2011). In response to cold, IL-4-driven M2 macrophages release noradrenaline in BAT and WAT, which coordinates fatty acid mobilization and energy expenditure. Although further work is needed to dissect the diversity and dynamics of macrophages in obesity, M2 cells in nonobese individuals are likely involved in maintaining adipose tissue homestasis, preventing inflammation, and promoting insulin sensitivity. In contrast, M1

macrophages drive obesity-associated inflammation and insulin resistance (Lumeng *et al.* 2007). The role of macrophages in obesity and associated disorders underlines a homeostatic function of macrophages in metabolism as cells capable of reorienting their own metabolic activity and as orchestrators of general metabolism.

2.6 Terapeutic target.

Reorienting and reshaping deranged macrophage polarization is the holy grail of macrophage therapeutic targeting (Mantovani and Sica 2010). Polarized phenotype are reversible *in vitro* and *in vivo* (Hagemann *et al.* 2008; Sica *et al.* 2000; Duluc *et al.* 2009). In proof of concept and in a large clinical study in ovarian cancer patients, INF-γ was found to activate macrophage tumoricidal activity, inducing a phenotype switch with unequivocal evidence of clinical response (Allavena *et al.* 1990). In a model of pancreatic ductal adenocarcinoma, CD40 agonist antibodies promoted a remarkable antitumor effect and induced high expression of M1 markers in macrophages (Beatty *et al.* 2011).

Modulation of macrophage function is an off-target effect for a number of diverse therapeutic agents. PPARγ agonist, called thiazolidinediones, have long been used in the treatment of diabetes. The evidence linking PPARγ to M2 polarization and hence to the homeostatic role of adipose tissue macrophages sheds fresh new light on their mode of action. Preclinical evidence suggests that PPARγ promotes M2-like polarization and homeostatic function in adipose tissue macrophages and that alteration of this function is a key pathogenic feature in diabetes (Stienstra *et al.* 2008; Charo 2007). Other therapeutic strategies that have been reported to affect macrophage polarization include zoledronic acid, an agent used for preventing recurrence of breast cancer bone metastasis, statins, trabectedin and TRL ligands (e.g., imiquimod and CpG), but also bioactive compounds naturally present in many fruits and vegetables, such as polyphenols. Aharoni *et al.* have recently demonstrated that pomegranate polyphenols dose-dependently attenuated macrophage response to M1 proinflammatory activation in J774.A1 macrophage-like cell line. This was evidenced by a significant decrease in TNFa and IL-6 secretion in response to stimulation by IFN-γ and Lipopolysaccharide (LPS). In addition, pomegranate juice dose-dependently promoted the macrophages toward a M2 anti-inflammatory phenotype, as determined by a significant increase in the spontaneous secretion of IL-10 (Aharoni *et al.* 2015).

Therapeutic macrophage targeting is in its infancy. Selected clinically approved therapeutic strategies, such as use of PPARγ inhibitors,

statins, zolendronic acid, and preventive activities such as weight loss may 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.

Chapter 3

Cocoa: antioxidant and immunomodulator.

1.2. Nutritional informations.

Cocoa is a product derived from the beans of the Theobroma cacao plant,tropical evergreentree (family Malvaceae, formerly Sterculiaceae), whose scientific name means "food of the gods" in Greek. Native to lowland rainforests of the Amazon and Orinoco river basins, cacao is grown commercially in the New World tropics as well as western Africa and tropical Asia. Its seeds, called cocoa beans, are processed into cocoa powder, cocoa butter, and chocolate.

Cocoa has been consumed since 600 BC, first by ancient civilisations, such as the Mayans and Aztecs (Hurst *et al.* 2002). Cocoa consumption in Europe dates from the 16th century when Hernàn Cortès introduced it to the Iberian Peninsula; from there its use spread rapidly to Western Europe. Cocoa powder is a rich source of fibre (26 – 40 %), proteins (15 – 20 %), carbohydrates (about 15 %) and lipids (10 – 24 %; most, 10 – 12 %), and it contains minerals (mainly Mg, Ca and K) and vitamins (A, E, B and folic acid) (**Table 1**).

NUTRIENT (Macronutrients and micronutrients)	**CONTENT** (for 100g)
Energy (kJ)	836,8
Energy (kcal)	200
Proteins (g)	21
Carbohydrate (g)	16
Starch	13
Sugar	3
Lipids (g)	10,4
Saturated	6,5
Monounsaturated	3,6
Polyunsaturated	0,3
Fibre (g)	32,7
Na (g)	0,02
K (g)	4,2
Ca (g)	150
P (g)	700
Fe (g)	25
Mg (g)	550
Zn (g)	7
Cu (g)	4
Vitamin A (Retinol) (mg)	<0,2
Vitamin E (Tocopherol) (mg)	2,4
Vitamin B_1 (mg)	0,2
Vitamin B_2 (mg)	0,4
Niacin (mg)	0,6

Table 1 - Cocoa powder: nutritional information per 100g. The table is adapted from Ramiro-Puig and Castell (2009).

Cocoa has become a subject of increasing interest because of its high content of polyphenolic antioxidants, particularly flavonoids. Cocoa powder is reported to contain up to 70 mg polyphenols/g (expressed as catechin) (Vinson *et al.* 1999). A serving size portion of certain cocoa-derived products provides more phenolic antioxidants than beverages and fruits such as tea and blueberries, traditionally considered high in antioxidants (Lee *et al.* 2003; Vinson *et al.* 2006). Cocoa mainly contains the monomers (2)-epicatechin and catechin, and various

polymers derived from these monomers, known as procyanidins (**Figs 6; 7**). Monomer content ranges from 0,20 to 3,50mg/g, depending on the type of product, with epicatechin content being higher than (+)-catechin in most cocoa products (Gu *et al.* 2006). Procyanidins are the major flavonoids in cocoa and chocolate products, with reported levels ranging from 2,16 to 48,70 mg/g. Methylxanthines have also been identified in cocoa powder, and account for 0,5–2% of the DM (Pura Naik 2001).

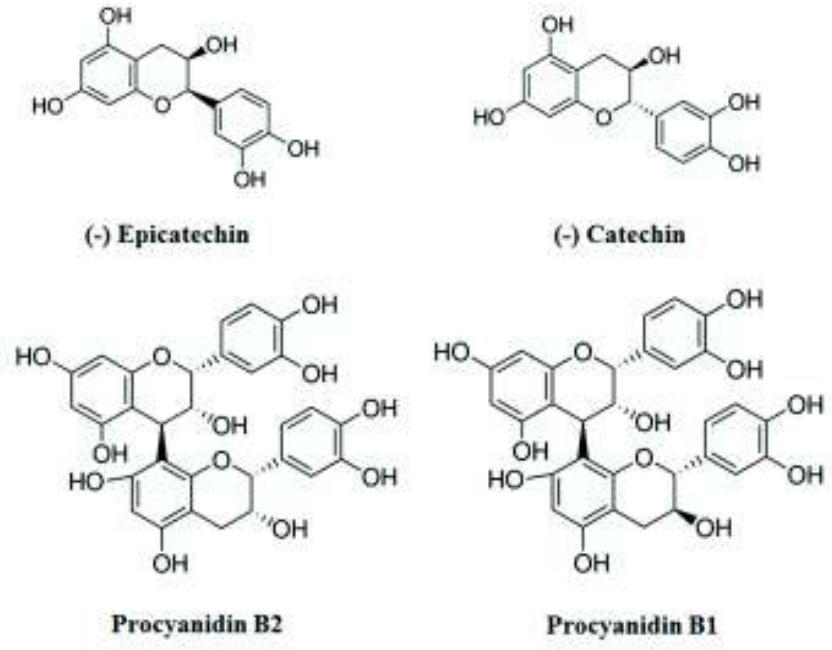
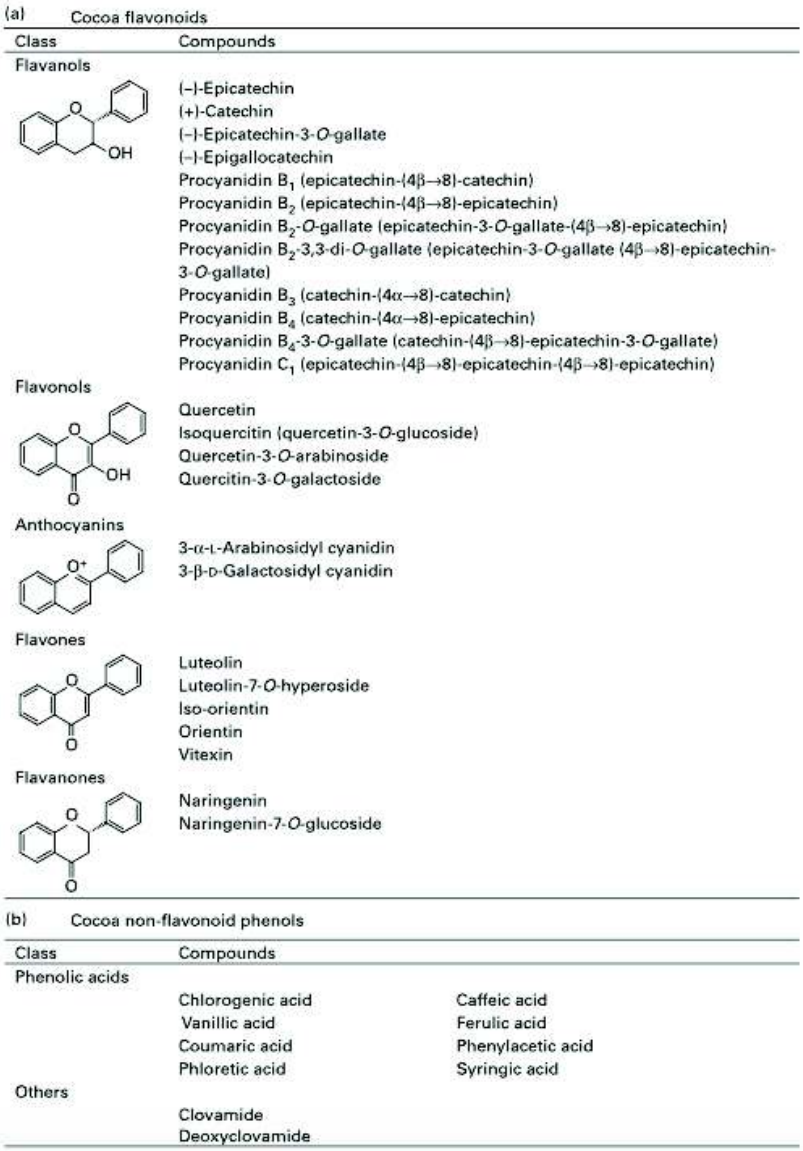


Fig. 6 - Chemical structure of the main cocoa flavonoids. The figure is adapted from Martìn *et al.* 2016).



(a) Cocoa flavonoids

Class	Compounds
Flavanols	(−)-Epicatechin (+)-Catechin (−)-Epicatechin-3-*O*-gallate (−)-Epigallocatechin Procyanidin B_1 (epicatechin-(4β→8)-catechin) Procyanidin B_2 (epicatechin-(4β→8)-epicatechin) Procyanidin B_2-*O*-gallate (epicatechin-3-*O*-gallate-(4β→8)-epicatechin) Procyanidin B_2-3,3-di-*O*-gallate (epicatechin-3-*O*-gallate (4β→8)-epicatechin-3-*O*-gallate) Procyanidin B_3 (catechin-(4α→8)-catechin) Procyanidin B_4 (catechin-(4α→8)-epicatechin) Procyanidin B_4-3-*O*-gallate (catechin-(4β→8)-epicatechin-3-*O*-gallate) Procyanidin C_1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin)
Flavonols	Quercetin Isoquercitin (quercetin-3-*O*-glucoside) Quercetin-3-*O*-arabinoside Quercitin-3-*O*-galactoside
Anthocyanins	3-α-L-Arabinosidyl cyanidin 3-β-D-Galactosidyl cyanidin
Flavones	Luteolin Luteolin-7-*O*-hyperoside Iso-orientin Orientin Vitexin
Flavanones	Naringenin Naringenin-7-*O*-glucoside

(b) Cocoa non-flavonoid phenols

Class	Compounds	
Phenolic acids	Chlorogenic acid	Caffeic acid
	Vanillic acid	Ferulic acid
	Coumaric acid	Phenylacetic acid
	Phloretic acid	Syringic acid
Others	Clovamide	
	Deoxyclovamide	

Fi. 7 - Flavonoids and non-flavonoid phenols contained in cocoa. This schematic rappresentation is adapted from Ramiro-Puig and Castell 2009.

3.2 Bioavailability of cocoa flavonoids.

The biological effects of flavonoids depend on the bioavailability of the compound. The various manners and rates in which flavonoids are absorbed have been recently reviewed (Hackman *et al.* 2008). Flavan-3-ols show no changes after 40min in the human stomach, indicating that flavonols and procyanidins are stable in the harsh environment of the digestive system (Rios *et al.* 2002). Gut flavonoid absorption basically depends on the chemical structure of the individual type. Monomeric flavonoids and certain dimeric and trimeric procyanidins are absorbed in the small intestine and are rapidly detected in the plasma (Baba *et al.* 2000; Deprez *et al.* 2001; Holt *et al.* 2002; Tsang *et al.* 2005). Certain monomers are better absorbed than others (Baba *et al.* 2001). For example, epicatechin was the main flavonoid detected in the plasma after intake of a cocoa beverage containing equal amounts of catechin and epicatechin. Absorption of (2)-epicatechin in humans is relatively efficient, and the plasma concentration of its primary metabolite, (2)-epicatechin glucuronide, is about 600 nmol/l at 2 h after the consumption of a cocoa beverage containing 54,4 mg (2)-epicatechin. Intestinal absorption of epicatechin and catechin has also been demonstrated in rats (Chang *et al.* 2005; Zuo *et al.* 2006).

Short procyanidins (dimers and trimers) are absorbed in the small intestine and rapidly detected in the plasma (Baba *et al.* 2000) and urine (Tsang *et al.* 2005), whereas large procyanidins are less efficiently absorbed, but may have an important local function in the gut, neutralising oxidants and carcinogenic compounds.

Moreover, flavonoids can also be metabolised by colon microflora to phenolic acids, which are then absorbed (Manach *et al.* 2004; Gu *et al.* 2007).

The influence of the food matrix on flavonoid absorption has been an issue of discussion in the past few years, particularly after the publication of a study reporting decreased absorption of cocoa flavonoids owing to an interaction with milk proteins, which are present in most cocoa-derived pro- ducts (Serefini *et al.* 2003). Conversely, other studies in human subjects found no significant effects of milk on epicatechin absorption and the total amount of metabolites excreted after ingestion of a cocoa beverage (Keogh *et al.* 2007; Roura *et al.* 2007; Roura *et al.* 2008). Nonetheless, differences in the excreted metabolite profile have recently been described, suggesting that milk components have an effect on cocoa metabolism (Roura *et al.* 2008). Polysaccharides also seem to enhance flavonoid absorption, although the mechanism causing this effect remains

uncertain (Schramm *et al.* 2003).

Data on the distribution of flavonoid metabolites in tissues after cocoa intake are limited, even in experimental studies. Absorbed flavonoids are widely distributed and can be detected in many organs, including lymphoid tissues, at a concentration of nmol/g tissue (Manach *et al.* 2004; de Boer *et al.* 2005). Levels of flavan-3-ol metabolites have been found in rat liver and kidney over a 24 h period following flavonoid ingestion (Tsang *et al.* 2005). In a study in rats and pigs, multiple tissues were found to accumulate quercetin metabolites, with high levels in rat lung and little accumulation in brain, spleen and white fat (Boer *et al.* 2005).

3.3 Cocoa as antioxidant.

Cocoa has a potent antioxidant capacity as compared with other products, a quality related to its flavonoid content. Procyanidins account for the highest percentage of antioxidants in cocoa products. A serving of dark chocolate (40g) provides about 517mg procyanidins with an antioxidant capacity of 9100 Trolox equivalents (TE), and a glass of homemade cocoa milk supplies 108mg procyanidins and 3200 TE.

Flavonoids act as antioxidants by directly neutralising free radicals, chelating metals (Fe^{2+} and Cu^{+}) that enhance highly aggressive reactive oxygen species, inhibiting enzymes responsible for reactive oxygen species production (xanthine oxidase) and up-regulating or protecting antioxidant defences (Cotelle 2001). Epicatechin and catechin are very effective in chelating Fe (Morel *et al.* 1993) and neutralising several types of radicals such as peroxyl, peroxynitrite, superoxide and 1,1 diphenyl-2-picryl-hydrazyl (Hatano *et al.* 2002; Yilmaz and Toledo 2004; Pollard *et al.* 2006). Epicatechin and catechin are more highly active on alkyl (ROO) peroxyl radicals than the well-recognised antioxidants L-ascorbate and β-carotene (Nakao *et al.* 2003). In addition, epicatechin can regenerate α-tocopherol from its corresponding radical (Pazos *et al.* 2007). Cocoa procyanidins also scavenge radicals, such as peroxynitrites, with activity that is proportional to the number of monomeric units they contain (Arteel and Sies 1999; Counet and Collin 2003). Even though quercetin is present at a smaller percentage, it may also contribute to cocoa's antioxidant activity by neutralising radicals and chelating metal ions (Formica and Regelson 1995; Lamuela-Raventos *et al.* 2001). Despite these well- defined antioxidant characteristics, however, flavonoids can become pro-oxidants under certain conditions, such as high flavonoid concentrations and the presence of redox-active metals (Nijveldt *et al.* 2001). Lastly, other compounds present in cocoa,

particularly methylxanthines (0,5–2% of cocoa powder), can also contribute to its antioxidant properties (Azam *et al.* 2003).

Several *in vitro* studies have demonstrated the antioxidant capacity of cocoa flavonoids and their metabolites (Spencer *et al.* 2001; Natsume *et al.* 2004). Epicatechin, catechin and procyanidin B2 reduce oxidant induced erythrocyte haemolysis in a dose-dependent manner (Zhu *et al.* 2002; Zhu *et al.* 2005). Cocoa procyanidins protect intestinal Caco-2 cell monolayers from the loss of integrity induced by a lipophilic oxidant (Erlejman *et al.* 2006). In addition, cocoa polyphenolic extract inhibits superoxide anion formation and xanthine oxidase activity in stimulated myelocytic leukaemia HL-60 cells (Lee *et al.* 2006).

Going beyond these *in vitro* assays, a small number of studies have investigated the effects of cocoa *in vivo*. Because it is difficult to isolate large amounts of cocoa polyphenols, almost all *in vivo* studies are performed using whole cocoa powder. Cocoa intake increases total antioxidant capacity and decreases lipid oxidation products in murine plasma and human plasma from healthy subjects (Wang *et al.* 2000; Lecumberri *et al.* 2006). In these studies, the enhancement of antioxidant capacity was greatest 1–2 h after cocoa administration and gradually decreased thereafter to reach baseline levels at about 6 h post ingestion. Plasma antioxidant capacity was not enhanced in blood collected more than 6 h after cocoa intake (Orozco *et al.* 2003; Engler *et al.* 2004; Ramiro-Puig *et al.* 2007), probably because of the short plasma half-life of flavonoids and their uptake in cells.

A cocoa-enriched diet increases the antioxidant capacity of cell tissues to varying degrees, with the activity in thymus > spleen >liver (Ramiro-Puig *et al.* 2007), an effect that can be attributed to differing levels of flavonoid accumulation (de Boer *et al.* 2005). Cocoa boosts catalase and superoxide dismutase activity in rat thymus, but not in spleen and liver (Ramiro-Puig *et al.* 2007). Although the exact mechanism remains to be established, enhancement of superoxide dismutase and catalase activity by cocoa could be due to direct neutralisation of enzyme substrates (O_2^- and H_2O_2, respectively) or to up-regulation of antioxidant enzyme expression. Cocoa phenols could have an important role in these actions, since they are potent antioxidants able to induce enzymes such as superoxide dismutase and glutathione peroxidase (Yeh and Yen 2006).

Cocoa also improves antioxidant defences in experimentally induced oxidative stress. For example, the long-term intake of a cocoa enriched diet containing 0,3% polyphenols reduced lipid peroxidation in the plasma and liver of hypercholesterolaemic rats (Mateos *et al.* 2005). The consumption of 100 g chocolate containing 0,2 %

polyphenols for 2 weeks counteracted oxidative stress in soccer players, as was shown by reductions in plasma malondialdehyde and α-tocopherol increases (Fraga *et al.* 2005).

A recent review of the impact of cocoa on the cardiovascular system has compiled all the interventional studies in human subjects published over the last 10 years. In these trials, cocoa intake was seen to reduce the risk of CVD by a combination of several effects, including improvements in antioxidant status (shown by decreases in oxidative stress biomarkers, such as thiobarbituric acid-reactive species and LDL oxidation), vasodilation, and inhibition of platelet activation and aggregation (Cooper *et al.* 2008).

3.4 Cocoa as immunomodulator.

Due to its activity as an antioxidant, cocoa consumption has been reported to be beneficial for cardiovascular health, brain function and cancer prevention. Furthermore, cocoa influences the immune system, in particular the inflammatory innate response and the systemic and intestinal immune response (Perez-Cano *et al.* 2013).

3.5 *In vitro* effects of cocoa on immune cells.

In vitro studies have demonstrated the regulatory effects of cocoa on the secretion of inflammatory mediators from macrophages and other leucocytes. Ono *et al.* (2003) and Ramiro *et al.* (2005) found that flavonoid-rich cocoa extract decreases the secretion of TNF-α, monocyte chemoattractant protein-1 (MCP-1) and NO by lipopolysaccharide-stimulated macrophages. The *in vitro* anti-inflammatory behaviour of cocoa differs from that of individual flavonoids. The influence of cocoa on macrophages is much stronger when cocoa is added before lipopolysaccharide stimulation, whereas when macrophages are treated with epicatechin alone, a stronger effect is achieved with addition after stimulation. This can be attributed to partial oxidation of epicatechin by reactive oxygen species produced during the prestimulation period, and to the effect of cocoa procyanidins, which can be hydrolysed along the pre-stimulation period to monomeric and dimeric compounds that are taken up by macrophages (Mackenzie *et al.* 2004). The inhibitory effect of whole cocoa extract on MCP-1 secretion is higher than that of epicatechin, but lower than that of isoquercitrin (Ramiro *et al.* 2005). These findings illustrate the differing potency of the flavonoids for modulating macrophage function, and suggest possible synergism or an influence of the wide spectrum of compounds present in whole cocoa, such as other polyphenols and methylxanthines (Pura Naik *et al.* 2001).

A recent study by Kenny *et al.* (2007) investigated the effect of two different fractions of purified cocoa flavonoids on lipopolysaccharide-stimulated human peripheral blood mononuclear cells (PBMC). The short-chain flavonol fraction (including monomers to pentamers), and particularly the long-chain fraction (including hexamers to decamers), enhanced the secretion of TNF-α, IL-1, IL-6 and IL-10 from stimulated human PBMC. The differences between these results and those described by Ono *et al.* (2003) and Ramiro *et al.* (2005) may be attributable to differences in the compounds tested and the cells used, or to the experimental design. Kenny *et al.* (2007) tested purified cocoa flavonoid fractions, whereas Ramiro *et al.* (2005) used whole cocoa extract, which contained flavonoids and other immunomodulatory compounds. Moreover, the study by Kenny *et al.* (2007) was performed on PBMC including several immune cell types that can interact, whereas Ramiro *et al.* (2005) used a macrophage cell line, which may not have had the same susceptibility to cocoa compounds. As mentioned above, flavonoids can act as antioxidants or oxidants, depending on certain conditions (for example, a high flavonoid concentration induces an oxidant environment); therefore, it is reasonable to suggest that oligomeric flavonoids could induce an oxidant state in PBMC that would lead to cell activation and production of inflammatory mediators. These results do not reflect what occurs *in vivo*, however, when tissue cells are under the effect of flavonoid metabolites and/or different concentrations of these compounds.

The effect of cocoa flavonoids on adaptive immunity has been investigated using lymphocyte cultures. In phorbol myristate acetate-stimulated lymphocytes, cocoa extract reduces lymphocyte proliferation by down-regulating IL-2 secretion and IL-2 receptor expression (Sanbongi *et al.* 1997; Mao *et al.* 1999; Ramiro *et al.* 2005). In keeping with the results obtained in macrophages, the inhibitory effect of whole cocoa extract on lymphocytes is higher than that of epicatechin (Ramiro *et al.* 2005). In addition, cocoa procyanidins decrease IL-2 in phytohaemagglutinin-stimulated PBMC, demonstrating that flavonoids are the main compounds responsible for the regulation of lymphocyte activation. Moreover, it has been reported that epicatechin, catechin and dimeric procyanidins, the flavonoid forms usually found in plasma, reduce IL-2 secretion by phorbol myristate acetate-stimulated Jurkat T cells (Mackenzie *et al.* 2004). Although the exact mechanism is still unclear, cocoa flavonoids have been shown to inhibit IL-2 at the transcriptional level. Moreover, given the regulatory effect of IL-2 on its receptor, IL-2 receptor down-regulation could also be a consequence of the IL-2 decrease.

Considering the effects of cocoa on cytokines attributed to effector T helper (Th)1 and Th2 cells, cocoa extract has been found to slightly increase *in vitro* IL-4 secretion and, therefore, Th2-response (Ramiro *et al.* 2005). In this setting, cocoa produces a less pronounced effect than epicatechin. The effects of cocoa flavonoids on cytokine secretion seem to be related to the degree of polymerisation: short-chain cocoa procyanidins increase IL-4 and IL-5, whereas long-chain procyanidins reduce both these Th2 cytokines (Mao *et al.* 2000; Mao *et al.* 2002).

The mechanism by which cocoa exerts its opposing effects on Th1/Th2 cytokines remains to be established. It is likely that cocoa differentially modulates transcription factor activation: signal transducer and activator of transcription-4 (STAT4), involved in IL-2 expression, and STAT6, the main IL-4 inducer (Mowen *et al.* 2004). Cytokine interactions should also be taken into account; the increase in Th2 cytokines, which are known to be potent down-regulators of Th1, could contribute to IL-2 inhibition.

Cocoa procyanidins promote homeostatic levels of transforming growth factor-b (TGF-b) in PBMC. TGF-b is enhanced in non-stimulated immune cells, whereas it is decreased in stimulated cells (Mao *et al.* 2003). Although TGF-b is generally considered a regulatory cytokine that helps to maintain an appropriate Th1/Th2 balance in certain situations (for example, in advanced CVD), it can act as a pro-inflammatory mediator through the induction of immune cell recruitment and activation (Redondo *et al.* 2007; Wahl 2007).

In summary, cocoa down-regulates both macrophage and lymphocyte activation *in vitro*. Given their powerful antioxidant activity, flavonoids seem to be the perfect candidates for immune regulation; nonetheless, studies showing opposite effects among different cocoa flavonoid fractions suggest that other compounds may contribute to cocoa's immune effects. In any case, as the profile of flavonoids absorbed *in vivo* differs from that present in crude cocoa extract, the physiological relevance of these data is limited.

3.6 *In vivo* effects of cocoa on the immune system.

A few studies have gone farther than *in vitro* assays: to investigate the *in vivo* influence of cocoa on lymphoid organs and immune cell functionality.

The effect of long-term intake (3 weeks) on rat thymus has been recently reported (Ramiro-Puig Urpi-Sarda *et al.* 2007). In young rats, an isoenergetic diet containing 10 % cocoa promotes the progression

of immature thymocytes (double negative T cell receptor (DN TCR) $\alpha\beta^{low}$ and double positive (DP) TCR$\alpha\beta^{low}$ cells) towards more mature T cell stages ($CD4^+$ $CD8^-$ TCR$\alpha\beta^{high}$ cells) (Ramiro *et al.* 2005). Thymus cell differentiation is triggered by complex signalling cascades, most of which are sensitive to changes in the redox environment (Valko *et al.* 2007). As was mentioned previously, long-term cocoa intake increases the thymic antioxidant status, as has been shown by enhanced superoxide dismutase and catalase activities (Ramiro *et al.* 2005). These activities might stimulate a slight shift towards a mildly oxidising environment that would favour lymphocyte maturation. In addition, high cocoa intake may promote the differentiation of other immune cell subsets, such as B cells, T cells, myeloid cells, natural killer cells and dendritic cells (Bhandoola and Sambandam 2006).

The influence of cocoa intake is not only restricted to T cell maturation in the thymus, but also affects lymphocyte composition and function in other immune tissues. High cocoa intake (10%) for 3 weeks increases the percentage of B cells and decreases the percentage of Th cells in the spleen of young rats (Ramiro-Puig *et al.* 2007). In addition, the composition of gut-associated lymphoid tissue, the first line of immune cells to oral challenge and diet (Calder and Kew 2002), is also influenced by cocoa intake (Ramiro-Puig *et al.* 2008). Peyer's patches and mesenteric lymph nodes are gut-associated lymphoid tissue compartments that show changes in lymphocyte composition in young rats fed 10% cocoa during 3 weeks. In Peyer's patches, cocoa intake reduces the TCR$\alpha\beta^+$T cell percentage (mainly the Th subset) and increases B and TCR$\gamma\delta^+$T cell percentages. Similarly, in mesenteric lymph nodes, high cocoa intake decreases the Th percentage and raises the percentage of gd T cells (Ramiro-Puig *et al.* 2008).

One of the most important findings of these studies is that a diet containing 10 % cocoa fed to rats increases γδ T cell percentages in gut-associated lymphoid tissue (Ramiro-Puig *et al.* 2008). These results are consistent with the effects of apple polyphenol intake in healthy mice (Akiyama *et al.* 2005). Intestinal γδ T lymphocytes are mainly involved in innate immunity, where they have a noteworthy role, with participation in oral tolerance, mucosal tissue repair, and immunity against viral antigens and tumour cells (Boismenu 2000; Hannine and Harrison 2000; Born *et al.* 2006). In murine models of food allergy, apple polyphenols prevented the development of oral sensitisation, and this inhibition correlated with a rise in the intestinal γδ T cell population (Ramiro-Puig *et al.* 2008). Taken together, these results suggest that certain diets rich in flavonoids from cocoa or other

sources may increase γδ T cell functionality. This finding could be especially important during childhood when the immune system is maturing (Perez-Cano *et al.* 2005).

On the other hand, a diet containing 10% cocoa during 3 weeks seems to produce a relative reduction of Th cells in secondary lymphoid tissues (Ramiro-Puig *et al.* 2007; 2008). This finding appears to contrast with the impact on thymic tissue, in which cocoa intake promotes Th maturation (Ramiro-Puig 2007). These contradictory findings may result from a reduction in thymic Th mobility or an increase in maturation speed, leading to a short life of these lymphocytes. The effects of cocoa on Th cell percentage also suggest an influence on the proliferation rate of these cells. *In vitro* studies have shown that cocoa inhibits Th activation (Ramiro *et al.* 2005); this might explain the smaller percentage of Th cells in lymphoid organs. However, research in the proliferative response and secretion of IL-2 (the main cytokine involved in Th proliferation) in the spleen and mesenteric lymph nodes of rats fed high doses of cocoa has not shown any reduction (Ramiro-Puig *et al.* 2007; Ramiro-Puig *et al.* 2008). Because the results of these studies were expressed as relative percentages, it is conceivable that the decrease in Th percentage in spleen and gut-associated lymphoid tissue may be due to an increase in the absolute number of other lymphocytes, such as B cells. Nonetheless, the ability of these cells to secret antibodies is down-regulated in rats fed a high-cocoa diet, as was reflected by lower plasma IgG, IgM and IgA levels (Ramiro-Puig *et al.* 2007) and gut secretory IgA and secretory IgM (Ramiro-Puig *et al.* 2008). This effect cannot be directly related to a decrease of Ig-secreting cells; instead, it could be the result of down-regulation of B cell differentiation caused by the decrease in Th2 cytokines, including IL-4 (Ramiro-Puig *et al.* 2007; Ramiro-Puig *et al.* 2008).

A decrease in IL-4 secretion was detected in the spleen and mesenteric lymph nodes of rats fed a 10% cocoa diet (Ramiro-Puig *et al.* 2007; Ramiro-Puig *et al.* 2008). These results suggest that intake of high doses of cocoa in young rats can favour the Th1 response, in contrast to what has been seen in*in vitro* studies. The reason for these contradictory results may reside in the differing compounds that reach lymphocytes in in vitro studies, in which cells are directly incubated with cocoa, and in vivo studies, in which cells take up absorbed and metabolised cocoa derivatives. Moreover, *in vitro* studies use a polyphenol-concentrated cocoa extract, whereas cocoa powder contains other compounds with immunomodulatory properties, such as fibre and lipids (Schley and Field 2002).

The effect of a diet containing 10% cocoa on the ovalbumin-specific immune response has also been investigated (Perez-Berezo *et al.* 2008). A cocoa diet started at weaning and maintained throughout the study down-modulated ovalbumin-specific antibody levels of IgG1 (the main subclass associated with the Th2 immune response in rats), IgG2a, IgG2c and IgM isotypes, but led to higher levels of anti-ovalbumin IgG2b anti- bodies (the subclass linked to the Th1 response). Spleen cells from cocoa-fed animals have shown decreased IL-4 secretion (main Th2 cytokine), and lymph node cells from the same rats displayed increased interferon γ secretion (main Th1 cytokine). Therefore, a cocoa diet attenuates antibody synthesis, and this may be attributable to specific down-regulation of the Th2 immune response. Because IL-4 also induces IgE up-regulation and increases intestinal permeability (Colgan *et al.* 1994), IL-4 down-regulation together with the γδ T cell increase induced by cocoa diet may be beneficial to promote intestinal innate immunity and to reduce certain states of hypersensitivity, such as food allergies, conditions characterised by an immune response against innocuous food antigens and a high IgE response.

In conclusion, a high cocoa intake modulates immune cell function in rats and affects both the intestinal and systemic compartments. Flavonoids seem to be the best candidates as the source of these immune effects; however, other compounds present in cocoa, such as fibre and lipids, should also be taken into account in future studies. To date, evidence of cocoa's immunoregulatory activity has been documented in experimental animal studies. It is difficult to extrapolate these results to human consumption because of the differences in metabolism. However, if flavonoid content were responsible for the immunomodulatory effects, a useful goal for the future could be the design of cocoa formulations with a higher flavonoid content, as has been recently reported by Tomas-Barberan FA *et al.*, 2007.

3.7 Mechanism of action on immune system.

The exact mechanism by which cocoa modulates innate and acquired immune functions remains unclear. As was indicated above, cocoa is a rich source of flavonoid antioxidants, which might promote changes in redox-sensitive signalling pathways involved in the expression of many genes and, consequently, in several cell functions, such as the immune response.

In macrophages and lymphocytes, cocoa compounds can target transcription factors, such as NF-kB which is redox-sensitive and triggers expression of over 100 genes, many of them involved in the

immune response (Pantano *et al.*, 2006). NF-kB is found in the cytoplasm of non-stimulated cells bound to kB inhibitor proteins (IkB). Upon cellular stimulation, IkB is phosphorylated by the serine-specific kinase, inhibitor of kB kinase (IKK), allowing NF-kB to translocate to the nucleus where it is reduced to initiate transcription of cellular genes (Nakamura *et al.* 1997). Other redox-sensitive kinases, including mitogen extracellular signal-regulated kinase kinase 1 (MEKK-1), protein kinase B (PKB or AKT)/phosphatidylinositol 3-kinase (PI-3-K) and c-Jun N-terminal kinase (JNK), can affect NF-kB activation. Monomeric flavonoids present in cocoa, such as epicatechin, catechin and quercetin, are known to inhibit the NF-kB pathway and decrease TNF-α and NO production in stimulated macrophages (Park *et al.* 2000; Comalada *et al.* 2005). Mackenzie *et al.* (2004) shed light on the regulatory role of cocoa flavonoids on the NF-kB pathway. Epicatechin, catechin and B dimeric procyanidins can act at different stages of NF-kB activation: at early stages, accumulated flavonoids in the cytosol regulate oxidant levels and reduce IKK phosphorylation, and at later stages, flavonoids – mainly dimeric procyanidins, which penetrate the nuclei – selectively prevent NF-kB binding to its consensus sequence (Mackenzie *et al.* 2004). More recently, Kang *et al.* (2008) showed that cocoa procyanidins inhibit the kinase activity of mitogen extracellular signal-regulated kinase 1 (MEK1), thus attenuating activation of NF-kB and activator protein-1 (AP- 1). These results support an inhibitory effect of cocoa on cyto- kine production by interacting with NF-kB activation. In addition, apart from the effects on NF-kB, cocoa flavonoids may also have an influence on other transcription factors involved in cytokine production, such as AP-1 (Kang *et al.* 2008) and signal transducer and activator of transcription-4 (STAT4) (Muthian and Bright, 2004).

This evidence is in keeping with the statement that the anti-oxidant properties of cocoa are responsible for its immunoregulatory role, but it also shows that certain cocoa flavonoids can directly interact with cell signalling and gene expression factors. Therefore, antioxidant-independent mechanisms must also be considered to better understand the effects of cocoa *in vivo*. In addition, future mechanistic studies should look into the specific metabolites of cocoa that interact with cells *in vivo* and determine the physiological concentrations of these metabolites after normal cocoa intake.

There is an increasing interest in food compounds that can promote health and reduce the risk of disease. Because of its antioxidant activity, mainly attributed to flavonoids, cocoa is currently attracting considerable attention in this regard. The health benefits of cocoa in reducing cardiovascular risk are emerging. In addition, the influence

of whole cocoa and cocoa flavonoids on the immune system is gaining recognition. Cocoa has been shown to have an effect on innate and acquired immune function. Various *in vitro* studies have attributed down-regulation of the inflammatory response to cocoa compounds. However, more *in vivo* approaches investigating this anti-inflammatory effect are needed to estimate the true impact of cocoa in this respect.

Cocoa has shown regulatory effects on the acquired immune response in both *in vitro* and *in vivo* experiments. In rats, high cocoa intake modulates intestinal and systemic immune cell functionality. Because immune cell function is controlled by redox-sensitive pathways, flavonoids, which are potent antioxidant compounds, seem to be the best candidates as the source of cocoa's beneficial effects. In addition, there is some evidence that certain cocoa flavonoids can directly interact with cell signalling and gene expression factors. Further research is needed to shed light on the interactions between cocoa and cell physiology, contributing thus to the body of knowledge of the effects of food compounds on health.

Aim of study

Macrophages are essential components of innate immunity and play a central role in inflammation and host defense. Moreover, these cells fulfill homeostatic functions beyond defense, including tissue remodeling in ontogenesis and orchestration of metabolic functions.

Macrophages are characterized by considerable diversity and plasticity. In tissues they can acquire two distinct phenotypes called M1 or M2, in response to specific environmental cues, such as cytokines, glucocorticoid hormones, microbial products and molecules released from apoptotic cells. This process is known as macrophage polarization.

The M1 phenotype is stimulated by TRL ligands (lypopolysaccharides, LPS) and INF-γ and it is characterized by the expression of high levels of proinflammatory cytokines (IL-12), high production of reactive nitrogen and oxygen intermediates, promotion of Th1 response, and strong microbicial and tumoricidal activity. In contrast, M2 macrophages are stimulated by IL-4/IL-13 and they are considered to be involved in parasite containment and promotion of tissue remodeling and tumore progression and to have immunoregulatory functions. Moreover, M2 cells are characterized by efficient phagocytic activity, high expression of scavenging molecules, the expression of mannose and galactose receptors and production of ornithine and polyamines through the arginase pathway. M1 and M2 macrophages are also distinct in metabolism, glycolytic and oxidative respectively. Recent studies have shown that hypoxia inducible factor-1α (HIF-1α) involved in glycolytic pathways supports proinflammatory classical activation of macrophages, M1 (Cramer *et al.*, 2003), while STAT 6 and the coactivator protein PGC-1β enhance the metabolic, biogenic and anti-inflammatory programs of M2 alternative phenotype (Divya V *et al.*, 2006).

The phenotype of polarized M1-M2 macrophages can be reversed *in vitro* and *in vivo*. Moreover, pathology is frequently associated with dynamic changes in macrophage activation, with classicaly activated M1 cells implicated in initiating and sustaining inflammation and M2 cells associated with resolution or smoldering chronic inflammation.

It was still not well definedmolecular basis underlying macrophage polarization, including signaling pathways, transcription factors and epigenetic regulation. However, only a few limited studies focused on the ability of different molecules to influence macrophage polarization.

Recent attention has focused on the beneficial effects of bioactive food components, such as the polyphenols. Cocoa is a food particularly rich in polyphenols. Main phenols contained in cocoa are flavonoids, mainly the monomers(-)-epicatechin and catechin, and various polymers derived from these monomers, known as procyanidins. Cocoa flavonoids are very potent antioxidants. In addition to the beneficial effects on oxidative stress, cocoa has been shown to have an effect on innate and acquired immune function. Various *in vitro* studies have attributed down-regulation of the inflammatory response to cocoa polyphenols. In particular, it was demonstrated that cocoa treatment reducethe production of inflammatory molecules, such as cytokines (TNF-α, IL-6 and IL-1β), ROS and NO, in LPS-stimulated macrophages. About the mechanism by which cocoa flavonoidsmodulate immune function, they can directly interact with cell signaling and gene expression factors, such as NF-kB and signal transducer and activator of transcription-4, STAT4, but further research is needed to shed light on the interactions between cocoa and cell physiology.

Although these studies document that cocoa has anti-inflammatory effects, a study on the direct effect of cocoa polyphenols on macrophage inflammatory phenotype has never been conducted.

In this study, we investigated the hypothesis that polyphenolic cocoa extract affect macrophage phenotype favoring an alternative M2 anti-inflammatory state.

Chapter 4

Materials and methods.

4.1 Preparation of cocoa polyphenolic extract.

Cocoa beans originated from Ghana, West Africa. To remove lipids, cocoa beans (3 g) were ground to a powder with quartz sand, then placed in 10 ml of hexan (apolar solvent) and centrifugated for 5 minutes at 800 xg and 4°C. Hexane extraction was repeated for three times. After non-fat cocoa grain was dissolved in 10 ml of methanol/water solution, 70:30 (v/v), and polyphenols were extracted by three centrifugations at 800 xg and 4°C for 5 minutes. Cocoa polyphenolic extract was filtered, evaporated until dry in a rotary evaporator and dissolved in methanol/water/acetic acid solution, *70:28:2 (v/v/v).*

4.2 Determination of phenolic content.

Phenolic content of cocoa extract was estimated by Folin- Ciocalteu method, which was adapted from Singleton *et al.* (1965). The diluted aqueous solution of extract (20 µl) was mixed with water (1,58 ml) and Folin-Ciocalteu reagent (100 µl). The mixture was allowed to stand at room temperature for 8 min before adding sodium carbonate solution (1,89 M, 300 µl). Two hours later, samples were dispensed in a 96-well cell culture plate (Greiner Bio-one) and the absorbance was measured at 765 nm using a multifunctional microplate reader (Tecan infinite 200 PRO). Cocoa extract was diluted 1:40 and analyzed in triplicate. Values are expressed as g gallic acid equivalents (GAE) l^{-1}.

4.3Determination of total antioxidant capacity.

Total antioxidant capacity of cocoa extract was measured using TEAC assay (Gil *et al.* 2000) with some modifications. The radical cation $ABTS^{\bullet+}$ was generated dissolving in phosphate buffer (5mM NaH_2PO_4-H_2O and 5mM Na_2HPO_4-$2H_2O$, pH 7.4) ABTS powder and potassium persulfate ($K_2S_2O_8$) at a final concentration of 7 mM and 2.5mM, respectively. This solution was allowed to react for 12 hours at room temperature and in the dark. The next day the $ABTS^{\bullet+}$ solution was diluted in phosphate buffer to an absorbance of 0.70 ± 0.05 at 734 nm. A volume of 10 µL of cocoa extract were mixed with 200 µL of $ABTS^{\bullet+}$ solution in a multiwell-96 and the absorbance was recorded at 734 nm for 90 seconds. TEAC values were calculated from the Trolox standard curve (60-300 µM).

4.4 Cell line culture and treatment.

THP-1 cells were cultured at 37°C in 5% CO_2in RPMI 1640 medium supplemented with 10% fetal bovine serum FBS, 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

THP-1 cells were differentiated into macrophages by treatment with 100 ng/mL phorbol-12-myristate-13-acetate (PMA) for 48-72 hours and then cultured for another 24 hours with either LPS (1 µg/ml)+INF-γ 20 ng/ml to generate M1-polarized cells, or with IL-4 (20 ng/ml) to generate M2-polarized cells (Lopez-Castejòn *et al.* 2011), in the absence or presence of cocoa polyphenolic extract (100 µM GAE). The concentration for cocoa polyphenols was optimized based on the literature and preliminary MTT results.

4.5 MTT assay.

THP-1 cells were seeded and differentiated into macrophages in a 96-well plate at concentration of 1,5 x 10^5 cells per well. After 24 h treatment with 0,1-100 µM of cocoa polyphenolic extract, MTT solution (0,5 mg/ml, 100 µL) was added to cells in each well and the plate was incubated at 37°C + 5% CO_2 for about three hours, until MTT formazan crystals were visible in the culture liquid. Then MTT solution was removed and DMSO (70 µl/well) was added for dissolving the formazan crystals. The plate was read at 565 nm using Tecan infinite 200 PRO.

4.6 ELISA analysis.

The levels of cell-released TNF-α, IL-6, -1β, -12 and -10 were measured in the collected incubation medium and determined using an ELISA Ready-Set-Go kit (eBioscience), following manufacturer's instructions. Optical density was determined using the microplate reader, Tecan infinite 200 PRO.

4.7 Evaluation of endogenous and substrate-supported respiration in intact and in digitonin-permeabilized cells.

Mitochondrial oxygen consumption was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments, Norfolk, UK), (Piccoli *et al.* 2008). Measurements of respiration rates by endogenous substrates were carried out in intact exponentially growing cells; culture medium was changed 1 day before the assays. Cells were trypsinized, centrifuged

and resuspended at 2×10^6 cells/ml in TD Buffer (0.137M NaCl, 5mM KCl, 0.7mM Na_2HPO4, 25mM Tris–HCl, pH7.4). An aliquot of cell suspension was used for counting and protein determination and cells were then transferred to the polarographic chamber. After slope measurement, the coupled endogenous respiration was inhibited by 0.5 µg/ml oligomycin followed by 30 µM dinitrophenol (DNP). For the measurement of respiration rates by exogenous substrates in digitonin-permeabilized cells, after full uncoupling of the endogenous respiration of intact cells with 30 µM DNP, digitonin was added directly into the oxygraphic chamber at the optimal concentration of 30 µg/10^6 cells. After 2 min, respiratory substrates and inhibitors were added at the following concentrations: pyruvate (5 mM)/ malate (2,5 mM) for Complex I, succinate (5 mM) in the presence of 200 nM rotenone for Complex II+Complex III, in the presence of 13 nM antimycin A, -driven respiration, respectively. All rates of oxygen consumption were normalized to cellular protein content.

4.8 ATP Measurement.

Cellular ATP levels under basal conditions were measured by a luminometre (Tecan infinite 200 PRO) using a luciferin–luciferase reaction system with the ATPlite kit (Perkin Elmer, Waltham, MA), according to the manufacturer's instructions. THP-1 cells were harvested from Petri dishes with 0.05% trypsin, 0.02% EDTA, pelleted by centrifugation at 500 ×g and then washed in phosphate-buffered saline, pH 7.4 (PBS). For each assay 60000 cells in 96-well plate were used. For the evaluation of ATP content under strict glycolytic conditions, M1 and M2 were incubated for 5h at 37°C in the presence of antimycin A (1 mmol/L), a specific inhibitor of complex III and rotenone (1 mmol/L) which is a specific inhibitor of complex I. An ATP calibration curve was made using titrated ATP solutions following the manifacturer's instructions. An aliquot of cell lysate was used for protein content determination according to the Bradford method, using bovine serum albumin as standard.

Chapter 5

Results and discussion.

5.1 Chemical characterization of cocoa polyphenolic extract.

5.1.1 Total phenolic content (TPC) and antioxidant capacity.

Table 2 shows the total phenolic content (TPC) and the antioxidant capacity of cocoa polyphenolic extract determined by Folin-Ciocalteu and TEAC assays, respectively. The results, expressed as mg gallic acid equivalent (GAE) l^{-1} and mM Trolox equivalent (TE)/ g fresh mass, suggest that the total phenolic content reflects the antioxidant activity of cocoa extract. However, cocoa extract showed high values of total polyphenols and antioxidant activity, as reported in literature.

Beans weight (g)	Extraction solvent (v/v)	Yield (mg polyphenols/g dry mass)	Polyphenols content (mg GAE l^{-1})	Antioxidant capacity (mM TE/g fresh mass)
3	**Me OH/ H_2O (70:20)**	**170**	**8,40**	**158**

Table 2 - Chemical properties of cocoa extract. (for description see in the text).

5.1.2 HPLC-MS characterization.

Through HPLC-MS (qualitative) analysis (**figs. 8, 9**) chemical composition of cocoa extract was determined. The results suggest that cocoa extract contains flavonoids, in the form of (-)-epicatechin, catechin and procyanidins, phenolic acids and antocyanins, as reported in leterature (Borchers AT *et al.*, 2000; Sanchez-Rabaneda F. *et al.*, 2003).

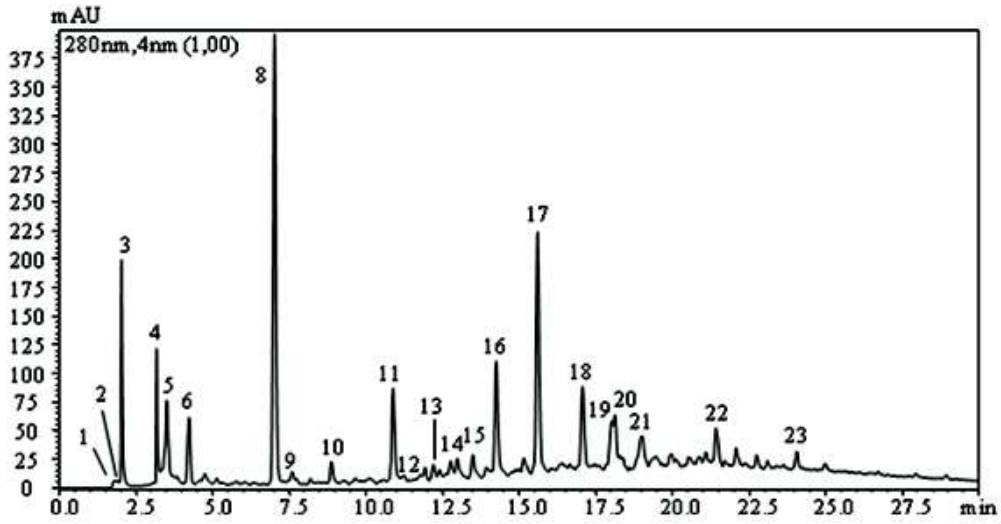


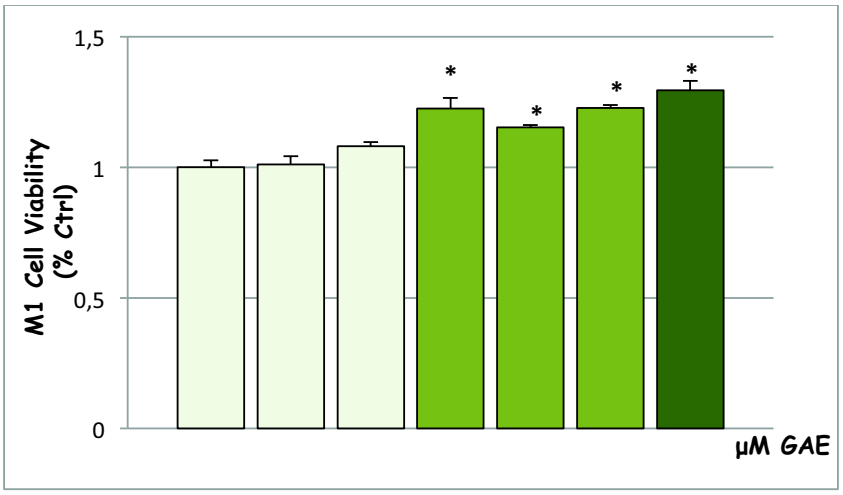
Fig. 8 - HPLC-PDA (280nm) of cocoa extract. The cromatogram shows the phenolic compounds (about 23) present in the cocoa extract, identified by LC-MS/MS.

Peak No	Compound	UV	t_R	m/z $[M-H]^-$
1	teobromin	272	1.7	181
2	caffein	272	2.0	195
3	unknown	210, 270	2.2	387, 453
4	chrysophanol-hexoside	210, 274	3.3	415, 253
5	quinic acid	298, 320	3.6	191
6	vanillic acid derivative	220, 275	4.7	282
7	unknown	296	5.1	449
8	4-β-D-glucopyranosyloxy-5-dihydroxyprenyl caffeate	260, 275	7.5	441
9	protocatechuic acid	228, 260, 294	8.0	153
10	unknown	467	9.2	467
11	cinnamic acid derivative	213, 301, 320	11.2	294
12	catechin-3-O-glucoside	278	11.4	451, 289
13	catechin derivate	278	12.0	497, 451
14	catechin sulphonic acid	274	12.6	369, 289
15	unknown	294, 298, 307	13.6	407, 305
16	procyanidin B dimer	278	14.4	577
17	procyanidin B dimer	278	15.5	577
18	procyanidin trimer	278	17	865
19	procyanidin tetramer	278	17.5	1153
20	clovamide	320	17.9	358
21	catechin derivative	278	18.6	720
22	procyanidin B dimer	278	21.5	577
23	dideoxyclovamide	320	23.8	326, 282

Fig. 9 - Peak identification by HPLC-PDA-MS of cocoa extract.

5.2 Cocoa effect on viability and inflammation.

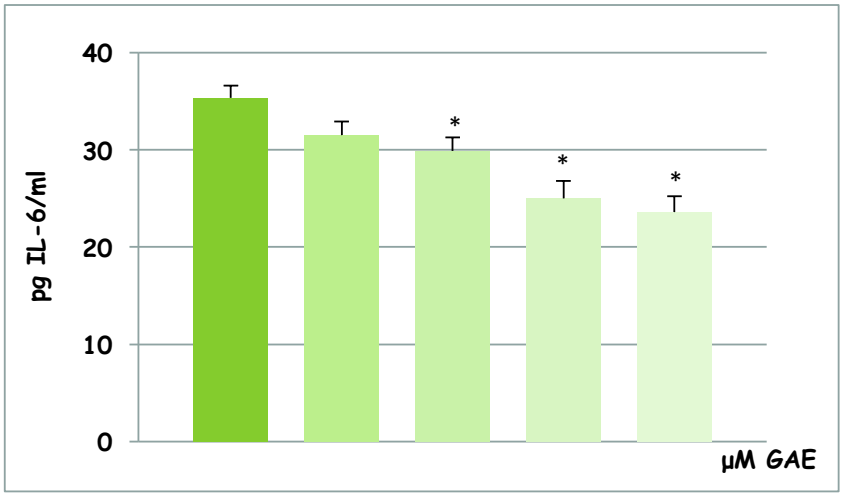
Cocoa extract (0,1-100 µM GAE) was tested on macrophages, after differentiation and polarization, as described above (par. 4.4). The effect of cocoa polyphenols on cell viability and inflammation in inflammatory macrophages was investigated through quantification of various cytokines determined by ELISA assay. MTT data, expressed as % control, suggest that cocoa extract did not cause toxicity in macrophages, rather it increased cell viability at higher concentrations, from 5 up to 100 µM, significantly ($p<0,05$) (**fig.10**). In particular, cocoa extract 100 µM increased cell vitality of about 30% compared to control ($p>0,05$).

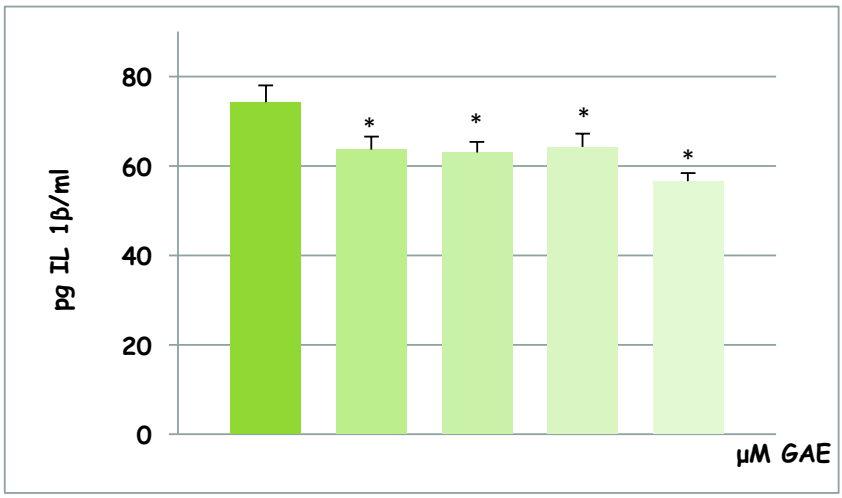


***p<0,05 vs Ctrl**

Fig. 10 - MTT results. After treatment with higher extract concentrations (5-100µM) cell viability increases significantly, p value < 0,05 determined by T-test.

ELISA quantification of IL-6 and -1β suggests that cocoa polyphenolic extract (5-100 µM) decreased the production of these proinflammatory cytokines in a dose-dependent manner and significantly($p<0,05$) in M1 macrophages (fig.11). However, the results, expressed as pg standard ml^{-1}, show that cocoa extract at 100 µM reduced cytokine secretion of the same percentage by which it increased cell viability (about 30%), compared to control.





***p<0,05 vs Ctrl**

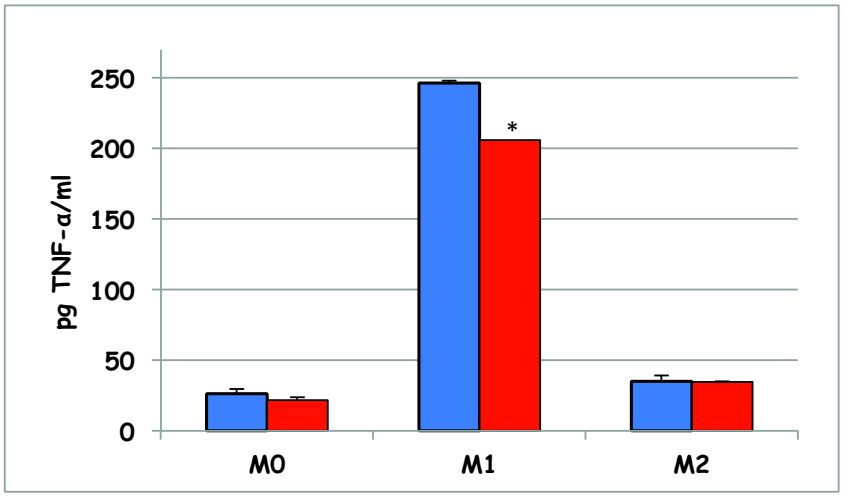
In summary, cocoa extract at 100 µM induced: an increase of cell vitality and a down-regulation of production of IL-6 and -1β, in M1 phenotype (anti-inflammatory effect).

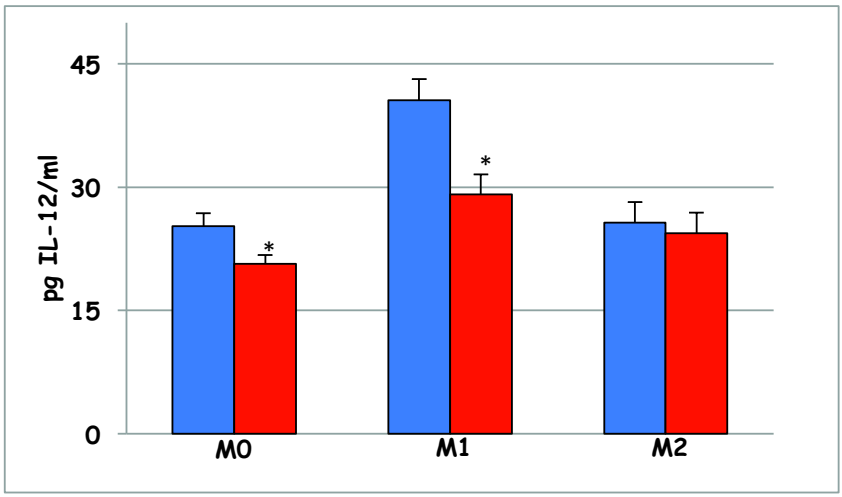
5.3 Effect of cocoa polyphenolic extract on macrophage phenotypes.

Levels of TNF-α, IL-12 and -10 were determined by ELISA assay in the two macrophage phenotypes, M1 and M2, after 24-treatment with cocoa extract 100 µM. The results reported in **fig. 12a, b, c** suggest that cocoa polyphenols reduced the production of proinflammatory cytokines, TNF-α (20%) and IL-12 (30%), in M1 phenotype (rappresented in red) compared to control (untreated M1) (data are significative, p value < 0,05). Cocoa extract also increased the secretion of IL-10 (47%), which is an anti-inflammatory cytokine and for this M2 marker, in M1 cells significantly.In addition, after cocoa treatment M1 macrophages showed the same levels of IL-12 and IL-10 present in M2 phenotype. So, after cocoa treatment M1

macrophages are comparable to M2 cells for cytokines production. Cocoa extract not had effect on cytokine secretion in M0 and M2 macrophages.

Taken altogether, these results indicate that cocoa extract reduces inflammation in M1 cells, but also promotes macrophage polarization toward the M2 alternative phenotype.





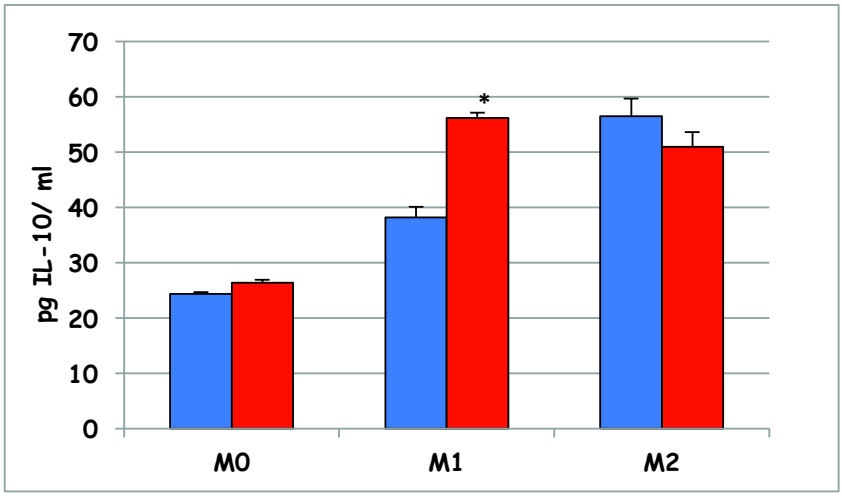


Fig.12 - Cocoa effect on TNF-α, IL-12 and IL-10 production in the different macrophage phenotypes (M0, M1 and M2). Untreated cells are reported in blu, treated ones (100 μM) in red; *= $p < 0,05$.

5.4 Effect of cocoa extract on macrophage metabolism.

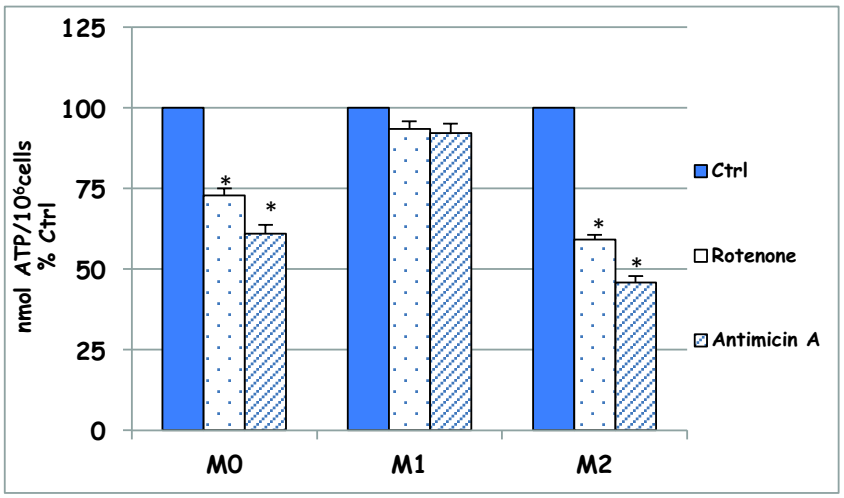
Macrophage metabolism was evaluated through measurement of total cellular ATP levels and of oxygen consumption by mitochondrial complexes (I and II+III) in the different macrophage phenotypes, after treatment with cocoa polyphenolic extract (100 μM).

5.4.1 Effect on total cellular levels of ATP.

Measurement of ATP level was performed in M1 and M2 cells which were incubated for 5h at 37°C in the presence of Rotenone(1 mmol l^{-1}) and Antymicin A (1 mmol l^{-1}) for blocking ATP production by oxidative phosphorylation. In particular, Rotenone is a specific inhibitor of NADH dehydrogenase or complex I, which is the first protein in the electron transport chain, while Antymicin A is a specific inhibitor of cytochrome c reductase or complex III,which represents the second protonic pomp which contributes to generate electrochemical gradient across mithocondrial membrane used for the synthesis of ATP by oxidative phosphorylation.

ATP results, expressed as nmol/10^6 cells and reports as % Ctrl, suggest that ATP levels not changed in M1 macrophages, in the presence of Rotenone and Antymicin A, so M1 phenotype has a glycolytic metabolism, mainly (**fig. 13**). On the other hand, ATP levels reduced in M2 cells in the presence of mithocondrial inibitors significantly ($p < 0,05$). This result suggest that an aliquot of cellular ATP was produced by oxidative phosphorylation, therefore M2 phenotype has a more oxidative metabolism (fig.13).

After identifying M1 and M2 metabolism, effect of cocoa polyphenolic extract on ATP levels was estimated in polarized cells. Data indicates that M1 macrophages produced more ATP through oxidative phosphorylation after cocoa treatment (**fig.14**). This reduction of glycolytic ATP in M1 cells suggest that this phenotype acquires more oxidative metabolism in the presence of cocoa polyphenols, like M2 phenotype. However, cocoa extract not influenced ATP levels in M0 and M2 macrophages.



***p<0,05 vs Ctrl**

Fig.13 - Measurement of ATP levels in polarized macrophages.

Figure shows that M1 phenotype has a glycolytic metabolism, while M2 activation has an oxidative metabolism.

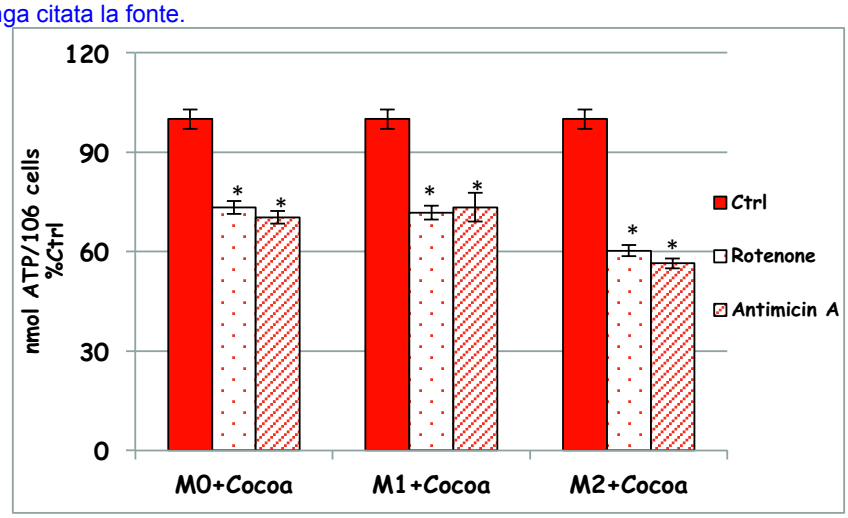


Fig.14 - Effect of cocoa extract on M1/M2 phenotype through metabolic switch. M1 macrophages show higher production of ATP through oxidative phosphorylation after cocoa treatment (* $p < 0,05$).

5.4.2 Effect on activity of mithocondrial complexes.

Measurement of oxygen consumption was used for evaluating functionality of mitochondrial complexes. In particular, activity of complex I, Rotenone-sensitive, and complex II+III, Antimycin A-sensitive, were estimated in M1 and M2 phenotypes, through polarografic assay. The results, expressed as nmol O_2 / mg total proteins, suggest that there was lower oxygen consumption by mithocondrial complexes in M1 macrophages (**fig.15**). Therefore M1 cells have lower respiratory capacity and high levels of glicolitic ATP. Data also show that mithocondrial complexes consumed more oxygen in M2 macrophages, indicating that these cells have a good respiratory capacity (**fig.15**).

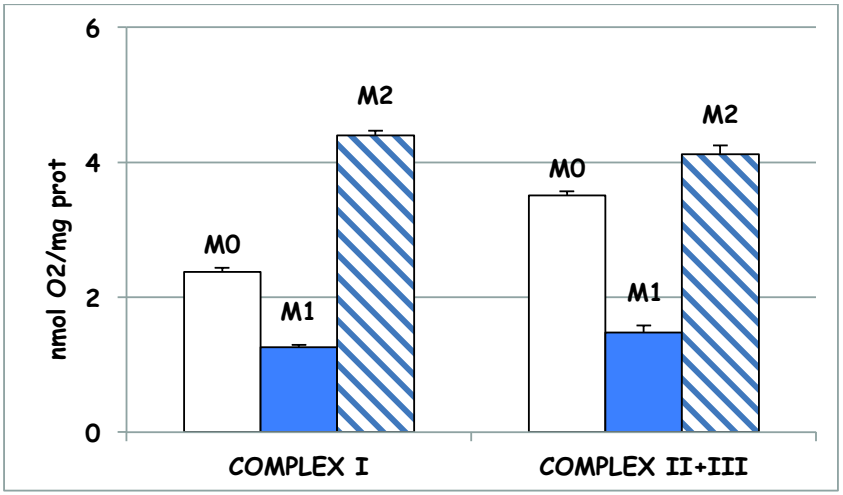


Fig.15 - Polarografic assay. In M1 macrophages: lower consumption of oxygen by the mitochondrial complexes, which suggest a low respiratory capacity. In M2 cells: higher consumption of oxygen by the mitochondrial complexes which reflect a good respiratory capacity.

Successively, polarografic assay was performed for estimating mithocondrial functionality in macrophage phenotypes after treatment with cocoa extract. The results suggest that cocoa induced a significative increase of oxygen consumption by mithocondrial complexes (I and II+III) in M1 macrophages, indicating a more oxidative metabolism like phenotype M2 (fig. 16). In particular, oxygen consumption by complex I was similar in M1 and M2 cells in the presence of cocoa.This suggest that cocoa flavonoids, due to their antioxidant activity, act at the level of complex I mainly, which is redox-sensitive. Cocoa treatment had no effect on M0 and M2 macrophages.

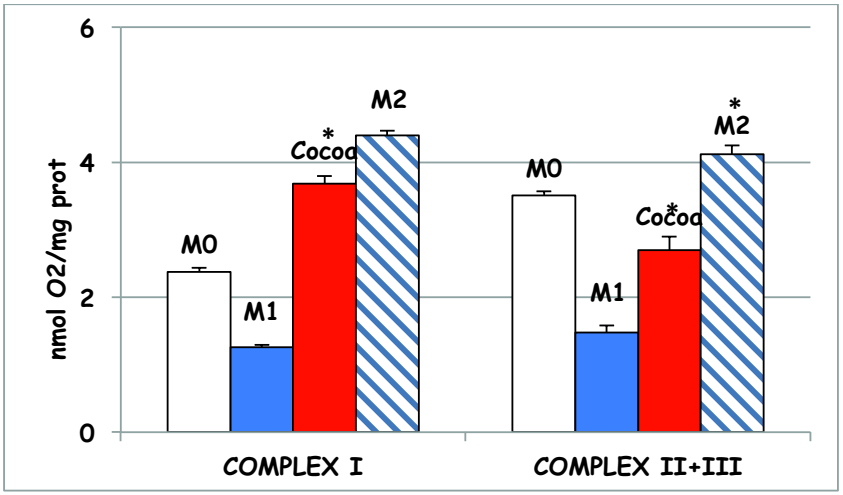


Fig. 16 - Effect on mithocondrial functionality. In M1 phenotype there is a significant (*p<0,05) increase of O_2 consumption by mitochondrial complex (I, II+III) after treatment with cocoa polyphenols, indicating a more oxidative metabolism like M2 cell type.

Taken altogether, experimental data indicate that cocoa polyphenolic extract is able:

- to reduce inflammatory response in M1 macrophage, favoring secretion of anti-inflammatory cytokines;
- to influence macrophage metabolism, favoring more oxidative pathways;
- to induce a phenotypic switch in polarized macrophages, favoring antinflammatory or alternative M2-state.

Conclusions and future prospectives.

This study demonstrates novel findings on the anti-inflammatory role of cocoa and its polyphenols, *in vitro* in a model of THP-1 derived macrophages.

We show, for the first time, that cocoa extract dramatically inhibited the production and secretion of the proinflammatory cytokines TNFa, IL-6, IL-1β and IL-12 in INFγ/LPS-stimulated macrophages. More interesting however, was the finding that cocoa polyphenols stimulated production of the anti- inflammatory cytokine IL-10 in inflammatory macrophages, suggesting that cocoa promotes a shift toward an alternative M2 macrophage phenotype.

Our results also demonstrated that cocoa extract influence macrophage metabolism, increasing ATP production through oxidative phosphorylation and O_2 consumption by mitochondrial complexes (I, II+III) in M1 macrophages after treatment with cocoa polyphenols. Therefore,cocoa polyphenolic extract not also suppresses inflammation in macrophage inflammatory phenotyp, but also changes macrophage metabolism, promoting oxidative pathways. We can asserted that cocoa extract induce a phenotypic switch in macrophage polarization favoring a M2 anti-inflammatory state.

The main phenolic coumponds present in cocoa extract belong to flavonoids group, which are powerful antioxidants with anti-inflammatory and immune system benefits. We demonstrate in this study that cocoa flavonoids, when present together as in the cocoa extract, have a notable anti-inflammatory effect in polarized macrophages, which may over that of one single compound due to synergistic interactions among different compounds, as previously demonstratedfor other foods, e.g. pomegranate juice (Saar Aharoni *et al.* 2014).

The concentration of polyphenols used in our *in vitro* experiments is comparable to the *in vivo* concentration after oral ingestion of a food rich in polyphenols (Yinghui *et al.* 2010). Cocoa flavonoids are water soluble and have a good bioavailability. The compounds appear maximally in the plasma at 30 minutes after oral ingestion and decrease gradually after 2-3 hours. This indicates that flavonoids are absorbed rapidly from cocoa and are rapidly eliminated from plasma (Baba *et al.* 2002). However, plasma concentration of cocoa flavonoids correlate well with the dose of chocolate. Beneficial effects of cocoa on human health are associated with 40-80 g of dark chocolate daily. Monomeric flavonoids (epicatechin and catechin) are

absorbed as such in the small intestine, but polymeric procyanidins may be degraded by intestinal and colonic micrloflora followed either by absorption of the metabolites or excretion in the feces (Maleyki, 2008).

The anti-inflammatory action of cocoa polyphenols *in vitro*, which are demonstrated in this study, is to bioavailability of flavonoids present in cocoa, but the extact mechanism by which enter into the cells and molecular pathways which are involved are unclear. There is some evidence that certain cocoa flavonoids can directly interact with cell signalling and gene expression factors, which regulate expression of many cytokine genes. Further research is needed to shed light on the interactions between cocoa and cell physiology, contributing thus to the body of knowledge of the effects of food compounds on health.

In summary, our results have shown the anti-inflammatory and metabolic effects of cocoa and its polyphenols on polarized macrophages, indicating polarizing ability of cocoa towards the M2 phenotype. For using cocoa as dietary supplementation o in the prevention of pathologies, more work is needed to better evaluate the effects of cocoa polyphenolic extract on primary cell lines and/or *in vivo* on experimental animal models and to identify pathways or molecular signals involved in the M1/M2 metabolic switch, induced by the cocoa extract.

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