UNIVERSITÀ DEGLI STUDI DI MILANO

Dipartimento di Scienze Farmacologiche e Biomolecolari

Dottorato in Scienze Farmacologiche Sperimentali e Cliniche

XXIX ciclo

THE PHYSIOLOGICAL RESPONSE OF RESIDENT MACROPHAGES TO THE ESTROGEN SURGE

BIO/14

Tesi del dottorando:

Giovanna Luisa Pepe
Matricola: R10620

Tutor: Prof.ssa Elisabetta Vegeto

Coordinatore: Prof. Alberto Corsini

A.A 2015-2016

ABSTRACT

Abstract (English)

Macrophages are resident immune cells that play a key role in host defense against pathogenic infections and in inflammatory responses and in physiological tissue remodelling. They are able to respond to a variety of microenvironmental stimuli, adopting different activation states depending on the activating signal and context, in order to eliminate the pathogen and restore homeostasis.

Humans show strong sex differences in immunity to infection and autoimmunity, suggesting that sex hormones modulate immune responses. Indeed, estrogens regulate cells and pathways of the innate and adaptive immune systems, as well as immune cell development. Previous studies showed that 17β-estradiol (E2) is able to modulate the reactivity of macrophages during inflammation, down-regulating the expression of inflammatory genes. However, a full comprehension of the molecular details by which estrogens modulate macrophage activity is still unclear. To fill this gap we performed a transcriptomic analysis followed by biological assays of peritoneal macrophages isolated from female mice both with different endogenous estrogen levels and following short and long term estrogen administrations.

The bioinformatic data suggested that E2 modulates important biological processes in macrophage physiology; among these, proliferation and the induction of an anti-inflammatory and pro-resolution phenotype emerged as mostly significant. We thus confirmed this evidence by proving, through gene expression, FACS analyses and BrdU incorporation studies, that the proliferative index and macrophage cell number are induced by the estrogen surge. Furthermore, we demonstrated that hormone administration induces a dynamic activation process that evolves towards a pro-resolving phenotype through the synthesis of IL10. Moreover, we observed that this activity is also maintained during peritoneal inflammation.

By investigating the effects of E2 on microglia, a different macrophage population, my data highlighted the diversity in the E2 response of macrophages that reside in different tissues and the influence of the microenvironment on the hormone responsiveness and provided a list of E2 target genes that can be used as biomarkers for pharmacological and translational studies.

Altogether, these results deepen our understanding of the endocrine-immune interactions and allow future studies on their relevance for the pathogenesis and development of therapeutic strategies of inflammatory pathological conditions.

Abstract (Italian)

I macrofagi sono cellule immunitarie che risiedono in quasi tutti i tessuti dell'organismo e svolgono un ruolo chiave nella difesa dell'organismo contro le infezioni dovute a patogeni e nelle risposte infiammatorie e un'importante funzione nel rimodellamento tissutale fisiologico. Essi sono in grado di rispondere ad una varietà di stimoli microambientali, adottando diversi stati di attivazione a seconda del contesto e del segnale di attivazione, con il fine di eliminare l'agente patogeno e ripristinare l'omeostasi.

L'uomo mostra forti differenze legate al sesso nella risposta immunitaria alle infezioni e nell'autoimmunità, suggerendo che gli ormoni sessuali svolgono un importante ruolo nel modulare la risposta immunitaria. In effetti, gli estrogeni regolano diversi pathways del sistema immunitario innato e adattativo, nonché lo sviluppo delle cellule immunitarie. Studi precedenti hanno dimostrato che il 17β-estradiolo (E2) è in grado di modulare la reattività dei macrofagi durante l'infiammazione, down-regolando l'espressione di geni infiammatori. Tuttavia, non sono ancora del tutto chiari i dettagli molecolari attraverso cui gli estrogeni esercitano la loro attività anti-infiammatoria. Al fine di colmare questa lacuna, abbiamo effettuato un'analisi di trascrittomica, seguita da saggi biologici, dei macrofagi peritoneali isolati da topi femmina, sia in seguito a diversi livelli di estrogeni endogeni, sia in seguito a trattamento con estrogeni per breve e lungo termine.

L'analisi dei dati bioinformatici ha mostrato che E2 modula importanti processi biologici coinvolti nella fisiologia dei macrofagi; tra questi emergono come più significativi la proliferazione e l'induzione di un fenotipo anti-infiammatorio e pro-risolutivo. Abbiamo quindi confermato questi dati dimostrando, attraverso l'analisi di espressione genica, l'analisi citofluorimetrica e l'incorporazione di BrdU, che l'indice di proliferazione e il numero di macrofagi residenti aumentano in seguito ad un incremento dei livelli di estrogeno. Inoltre, abbiamo dimostrato che il trattamento con estrogeno induce nei macrofagi un processo di attivazione dinamico che evolve verso un fenotipo pro-risolutivo attraverso la sintesi di IL10. Inoltre questa azione dell'ormone sui macrofagi è mantenuta anche durante il corso di un'infiammazione peritoneale.

Al fine di estendere le nostre osservazioni ad una diversa popolazione di macrofagi, abbiamo analizzato la risposta della microglia al trattamento con estrogeni mediante l'analisi di espressione genica. I risultati hanno evidenziato la diversità nella risposta a

E2 dei macrofagi che risiedono nei diversi tessuti e l'influenza del microambiente sulla reattività dei macrofagi a E2, inoltre, forniscono un elenco di geni bersagli dell'estrogeno che possono essere utilizzati come biomarcatori per studi farmacologici e traslazionali.

Complessivamente, questi risultati approfondiscono la nostra conoscenza sulle interazioni del sistema endocrino-immunitario e permettono studi futuri sulla loro rilevanza nella patogenesi e lo sviluppo di strategie terapeutiche di condizioni patologiche infiammatorie.

Index

INTRODUCTION

1.1 Mechanisms of estrogen signaling

Estrogens are steroid hormones initially isolated and characterized in the late 1920ies by Adolf Butenandt (Nobel laureate for this discovery) and Edward A. Doisy [1]. They are present in all vertebrates and some invertebrates and are traditionally considered as the primary female sex hormones since they are produced by the ovary, released into the blood and play a key role in the control of female reproduction. In fact, estrogens most important role in women is to develop the secondary sexual characters of woman and establish and maintain reproductive functions, controlling uterine and mammary growth and function.

But, in addition to the effects on female reproductive functions, estrogens play important role in the regulation of skeletal homeostasis, lipid and carbohydrate metabolism, skin physiology, cardiovascular system, liver, central nervous system, immune system and tissue remodelling [2-6]. These effects are also due, at least in part, to extragonadal synthesis of estrogens; for this reason estrogens exert important functions not only in female but also in male physiology. Moreover, estrogens are been demonstrated to be crucial for early development, not only for primary and secondary sexual characteristics but also embryonal and fetal development of brain networks [7].

Estrogens action is mediated by the binding to their receptors; in spite of the importance and the wide role of estrogen signaling, there are only three known estrogen receptors (ER) that mediate hormone action: ERα, ERβ and G protein-coupled estrogen receptor 1 (GPER1 or GPR30). The complexity of estrogen action is further enlarged by the different mechanisms of hormone signaling, that is direct and indirect genomic signaling, ligand independent signaling and non genomic actions [8,9].

Considering the relevance of estrogen action on extragonadal tissues, that show both tissue- and cell-specific estrogen synthesis and signaling, estrogen treatments are currently under evaluation in clinical trials for several aging-related diseases.

1.1.1 Estrogens

They are three main forms of physiological estrogens in females: estrone (E_1), estradiol (E_2 or 17β-estradiol) and estriol (E_3) (Figure 1). Each of these forms represents different product that derives from cholesterol through a sequence of reactions of estrogen biosynthesis.

Estrone is the major estrogen in postmenopausal women; it is produced primarily in adipose tissue from adrenal dehydroepiandrosterone (DHEA), although little amounts are synthesized in ovaries and adrenal glands. Estrone is provided with mild estrogenic capacity compared to estradiol, as it weakly interacts with estrogen receptors. Estradiol is the major estrogen form present in premenopausal women; it is synthesized in ovaries, by placenta during pregnancy, and also in adrenal glands and through testosterone peripheral conversion at little amounts. Estradiol secretion into the circulation is regulated by pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and is characterized by a specific cyclical trend during menstrual cycle. Lastly, estriol plays an important role during pregnancy when it is produced in large quantities by the placenta and generated from E1 through 16α-hydroxylation.

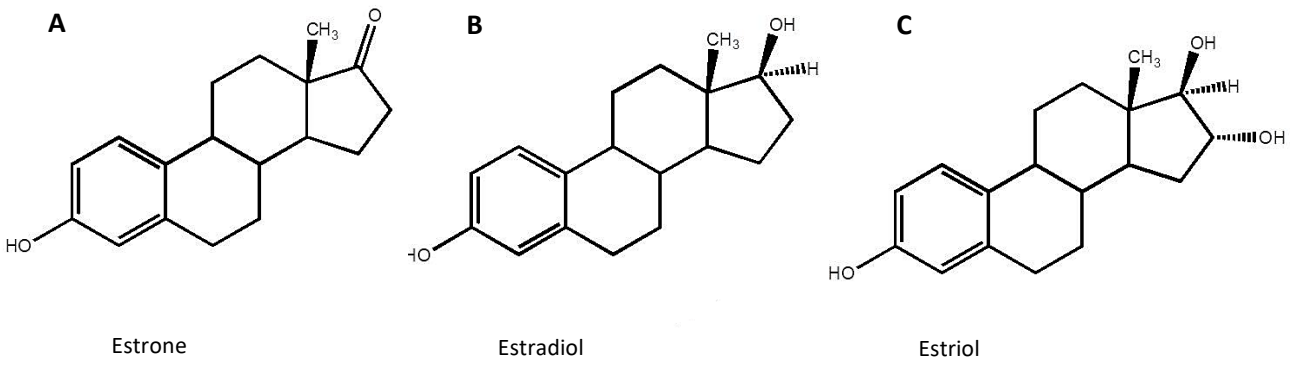


Figure 1. Chemical structure of physiological estrogens.
Chemical structure of A) estrone (E_1) B) estradiol (E_2) and C) estriol (E_3)

1.1.2 Estrogen synthesis

The synthesis of estrogen is different between reproductive and non reproductive women.

In reproductive women, ovaries are the main source of circulating estrogens, although during pregnancy, also placenta is able to produce significant amounts of hormone which is secreted into the circulation [2].

In the ovaries, the biosynthesis of estrogen is finely regulated by two hormones released from the pituitary gland, namely FSH and LH, which production is positively influenced by hypothalamic neurohormone, such as gonadotropin-releasing hormone (GnRH), and involved different cell types (Figure 2). In particular, thecal cells can produce androgens from progesterone but are unable to convert androgens into E_2. On the other hand, granulose cells are not able to produce androgens from progesterone, but can synthesize E_2 from androgens through the action of aromatase enzyme [10].

Thus, during folliculogenesis, thecal cells produce androgens following LH interaction with specific receptors. Then, following FSH stimulation, androgens are released, transported in granulose cells and converted in E_2; this latter is then released into the bloodstream to act on estrogen-responsive tissues.

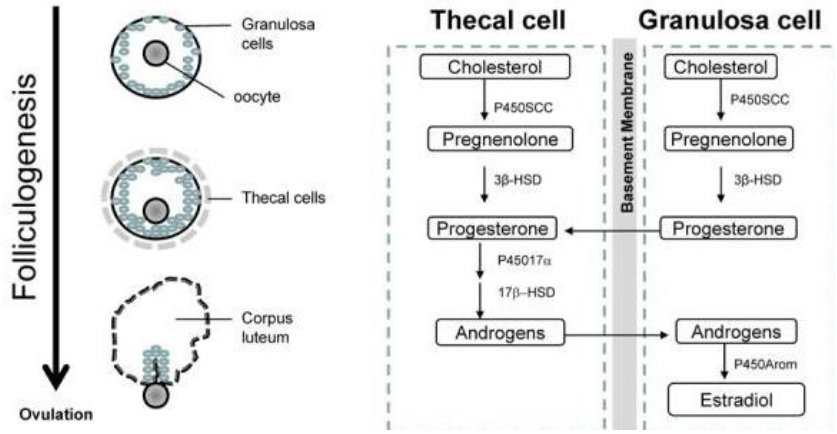


Figure 2. Cell-specific estrogen synthesis in the ovary during folliculogenesis. Figure taken from Cui *et al*., 2013 [11]. Estrogens synthesis starts with the production of pregnenolone from cholesterol, through the action of cytochrome P450 side chain cleavage enzyme (P450scc). Pregnenolone is then converted to progesterone by 3-beta-hydroxysteroid dehydrogenase (3β-HSD) in both thecal and granulose cells. Progesterone is converted to androgens by cytochrome P450 17α-hydroxylase (P45017α) and 17-beta-hydroxysteroid dehydrogenase (17β-HSD) in thecal cells during the follicular phase. The conversion of E2 is catalyzed by aromatase (P450Arom) in granulosa cells.

In nonreproductive women, instead, the main source of estrogens is represented by their synthesis in extragonadal tissues, such as breast, kidney, adipose tissue, skin and brain [12], and depend on the availability of C_{19} steroid precursors, that is testosterone, androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). The enzymatic reactions that lead to the production of estrogens from C_{19} steroid precursors are schematized in Figure 3.

In contrast to ovaries, estrogen produced in extragonadal sites is not released into the circulation, but acts locally as autocrine, paracrine or intracrine factor to control tissue homeostasis and functions [13].

Also men widely depend on local synthesis of E_2 in extragonadal sites; but it is also produced in male testes where control gonadal development and functions and regulate spermatogenesis [14].

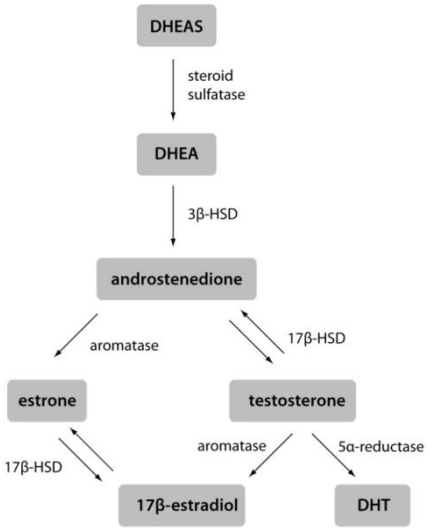


Figure 3. Schematic representation of enzymatic reactions of estrogen synthesis in extragonadal tissues. Figure taken from Vrtačnik *et al.*, 2014 [15]
Estrogens are synthesized from C19 steroid precursors through several enzymatic steps.
3β-hydroxysteroid dehydrogenase (3β-HSD); 17β- hydroxysteroid dehydrogenase (17β-HSD); dehydroepiandrosterone (DHEA); dehydroepiandrosterone sulfate (DHEAS); 5α-dihydrotestosterone (DHT).

Levels of circulating E_2 depend on reproductive status - reaching highest level during reproductive years and declining during postmenopausal period - and menstrual cycle, reaching the highest plasma concentration immediately before ovulation [16]. The main differences in estrogen synthesis as well as plasma E2 levels based on reproductive status and sex are summarized in Table 1.

	Premenopausal women	Postmenopausal women	Men
Source of circulating 17β-estradiol	ovaries (around 95%) adrenal cortex (around 5%)	extragonadal tissues (around 100%)	extragonadal tissues (around 100%)
Plasma levels of 17β-estradiol	0.11-2.20nM (depending on the phase of the cycle)	around 0.04 nM	around 0.10 nM
Source molecules for 17β-estradiol synthesis	blood-derived cholesterol and acetyl coenzyme A	testosterone, adronstenedione, DHEA and DHEAS	testosterone, adronstenedione, DHEA and DHEAS

Table 1 Differences in estrogen synthesis based on sex and menopause. Modified from Vrtačnik *et al*., 2014 [15].

Deactivation and circulating levels of E_2 are regulated by different mechanisms that involved estrogen metabolism, such as the conversion of E2 in less active forms (E1 or E3) [17] and, most of all, regulation of aromatase enzyme. Aromatase, a member of cytochrome P450 superfamily, catalyzes the last step of E2 synthesis and is broadly expressed in several tissues, such as gonads, liver, brain, adipose tissue and endometrium [18]. It is characterized by tissue-specific expression due to three main factors: alternative splicing, tissue-specific promoters and different transcription factors [19-21]. Moreover aromatase activity is also regulated by post-translational modifications such as phosphorylation [22].

1.1.3 Structure and function of estrogen receptors

Estrogen exerts their biological effects by binding their designated receptors (ERs), that are ERα, ERβ and GPR30 .

ERα and ERβ are members of nuclear receptors superfamily and act as ligand-activated transcription factors, modulating the transcription of specific genes that have an estrogen-responsive element (ERE) in their promoter. GPR30, instead, is a membrane receptor. While biological effects mediated by nuclear ERs take hours to days to develop, cell membrane ERs mediated a rapid intracellular response that takes seconds.

1.1.3.1 ERα and ERβ

These receptors are characterized by high sequence homology which it is reflected in a strong structural homology. ERα was the first estrogen receptor described, identified in 1986 by Chambon's laboratory [23]. After, a second ER, named ERβ, was identified in 1996 [24]. ERα and ERβ are not isoforms but different receptors encoded by two distinct genes located on different chromosomes, ESR1 on chromosome 6 (10 in mouse) and ESR2 on chromosome 14 (12 in mouse) respectively [25-29].

ERα and ERβ are expressed in several cell types, although each receptor has a tissue-specific distribution in the body. ERα is the receptor predominantly expressed in uterus, ovary, testes, breast, bone, liver, kidney, heart, pituitary gland and adipose tissue. ERβ is mainly expressed in ovary (granulose cells), prostate (epithelium), colon, vascular endothelium, lung and bladder [30]. In the brain, both receptors are broadly expressed by both neurons and glial cells.

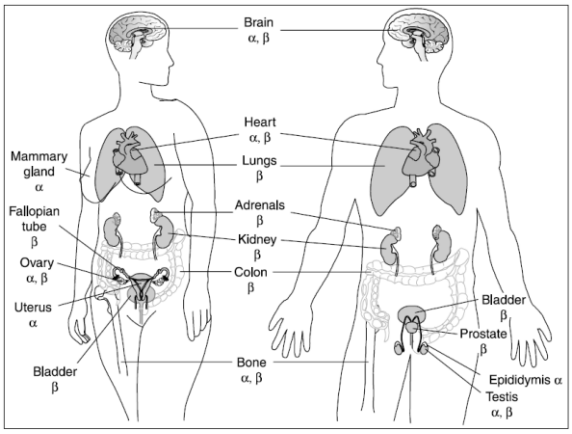


Figure 4. ERα and ERβ distribution throughout the body. Figure taken from Drummond and Fuller, 2010 [31].

Tissue distribution of ER correlates with their functions. In fact, ERα is mainly involved in the regulation of neuroendocrine and reproductive systems, instead, ERβ have an important role in the regulation of cognitive functions [30,32-33].

Nuclear ERs consist of five functional domains (Figure 5): A/B or N-terminal domain (NTD), C or DNA binding domain (DBD), D or hinge region, E region or Ligand binding domain (LBD) and F or C-terminal domain [34-38].

A/B or N-terminal domain (NTD). This region is the least conserved among the different members of the family of intracellular receptors. It contains Activation Function-1 (AF-1) domain, that is able to exercise its action in a ligand independent

way. The activation mediated by AF1 is very weak, but it synergizes with the AF2 domain present in region E [39]. Post-translational modifications, such as phosphorylation, of the A/B domain can dramatically affect the overall behavior of the receptor and represent a mechanism for the modulation of AF-1 functions [40].

C region or DNA binding domain (DBD). This is a highly conserved region involved in DNA binding. It is characterized by the presence of basic amino acids that facilitate the interaction with the phosphate groups of the DNA and contains two zinc fingers, each composed of four cysteine residues that chelate a single Zn^{2+} ion. These two domains recognize and bind specific DNA sequences, known generically as Hormone Responsive Elements (HREs) which in the specific case of the estrogen receptor are called EREs (Estrogen Responsive Elements). Further feature of the C region is the presence of the Proximal Box (P-box) consisting of three amino acids that confer DNA sequence recognition specificity to the receptor for HRE sequences and Distal Box (D-box) involved in receptor dimerization.

D or Hinge region. Flexible Region that links C domain to E and binds Hsp90 chaperone appointed to maintain the receptor in a metastable conformation capable of binding to the ligand. Moreover, this region contains a nuclear localization signal, it is involved in ER cellular compartmentalization and it is a site of post-translation modifications [41].

E region or Ligand binding domain (LBD). This domain has important role in ligand binding, receptor dimerization and in the binding with coactivators and co-repressors. Moreover, this region contains AF2 domain involved in the hormone-dependent transcriptional activity.

LBD consists of 11 alpha-helices (H1 and H3-H12) and 2 β-sheets that form a hydrophobic ligand-binding pocket closed to the C-terminal region [42].

Following receptor binding to an agonist, LBD changes its conformation, in particular, H12 helix rotates and forms a hydrophobic pocket leading to the exposure of a surface consisting of helixes 3, 4, 5 and 12 that allows coactivators recruitment and activation of the transcription. Conversely, estrogen antagonists are unable to induce a similar repositioning of H12, preventing coactivators recruitment and the activation of transcription.

F or C-terminal domain. It is a little conserved region with few known functions. Some data indicate its involvement in coactivators recruitment and receptor dimerization and stability [43-47].

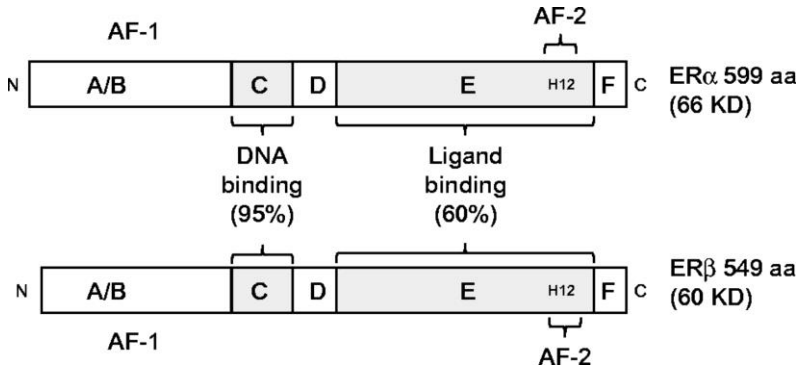


Figure 5. Structures of ERα and ERβ protein with functional domains. Figure taken from Hewitt *et al*. 2016 [48]. Structures of ERα and ERβ protein with functional domains. Estrogen receptors ERα and ERβ share a conserved domain structure. The A/B domain, at the amino terminus (N) of the protein contains AF-1. The C domain binds to DNA motifs called EREs. The D domain is called the hinge region, and contributes to DNA binding specificity and nuclear localization of the ERs. The E domain is called the ligand binding domain because it interacts with estrogen, through an arrangement of 11 α helices (H1, and H3 through H12). H12 in this region of the receptor is critical to mediating transcriptional activation via AF-2. At the carboxy terminus (C) is the F domain. The % homology shared between ERα and ERβ in the C and E domains is shown.

ERα and ERβ show high structural homology; both are formed from a single polypeptide chain consists of 599 aa for ERα and 549 aa for ERβ. However, sequence homology varies in the different domains and the less conserved regions appear to be those involved in transcription regulation. In fact, DBD and LBD have a homology of 95% and 60%, respectively, instead A/B and F regions have a homology of only 16% and 18%. This difference leads to hypothesize that the two receptors show a degree of specificity for the promoters of target genes.

Moreover, the two receptors differs also in dimerization process: while ERα acts preferentially in the homodimer form, the main active form for ERβ is the heterodimer with ERα and some evidence suggests that ERβ can exert an inhibitory effect [49].

1.1.3.2 GPER30

GPR30 is an orphan G protein coupled receptor, discovered by different independent groups as membrane estrogen receptor triggering rapid estrogen non-genomic signaling independent of ERα and ERβ [50]. Some data showed that higher concentration of E2 was needed to activate this receptor, due to the lower ligand affinity for this receptor compared to ERα and ERβ. GPR30 is expressed in ovaries, adrenal medulla, renal pelvis, and different brain regions [51, 52]. However, there are few studies about this receptor and conflicting information about its localization.

1.1.4 Mechanisms of estrogen action

Estrogens are able to mediate their function through their interaction with their receptors. Estrogen signaling can be divided into two main activation pathway: genomic and non-genomic signaling [53]. Genomic signaling can be divided into three pathways: direct genomic signaling, indirect genomic signaling and ligand-independent signaling.

1.1.4.1 Direct genomic signaling

This pathway is considered as the classical mechanism of estrogen signaling.
In the absence of ligand, the receptor is sequestered in the cytoplasm of the target cells, complexed with Heat shock proteins (Hsp)70, Hsp90 and Hsp56, whose function is to maintain the receptor in a conformation with high affinity for the hormone and to mask the nuclear localization sequence and the DNA binding domain [54].
The estrogen binding to the lipophilic cavity of the domain E of ERα or ERβ in the cytoplasm of target cells determine structural changes involving the reposition of helix-12 and the release of the Hsps that allow receptor dimerization (with homo or hetero dimers formation), translocation into the nucleus and the interaction with EREs located in the promoters of target genes.
The receptor-DNA binding in correspondence of ERE sites leads to a series of interactions with nuclear proteins, including co-regulators, and transcription factors, which allow the receptor-ligand complex to regulate the transcription of target genes; the link with co-activators facilitates, while the one with the corepressor inhibits the binding of the initiation transcription complex on the promoter of the target genes.
The co-activators such as p160 and P/CAF, and the proteins recruited, as CBP/p300, facilitate recruitment of the RNA polymerase II transcriptional machinery and the synthesis of the primary transcript. The co-repressors, instead, are proteins able to repress the transcriptional activity of nuclear receptors by binding to their LBD. Among the main co-repressors, the better characterized are NCoR (Nuclear Co-Repressor) and SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptors).
The modulated response is observed after a few hours, usually, by the interaction of estrogen with the receptor.

In this way, estrogen-ER complex acts as a transcriptional activator promoting gene expression and the response is usually observed after a few hours by the interaction of estrogen with the receptor [55].

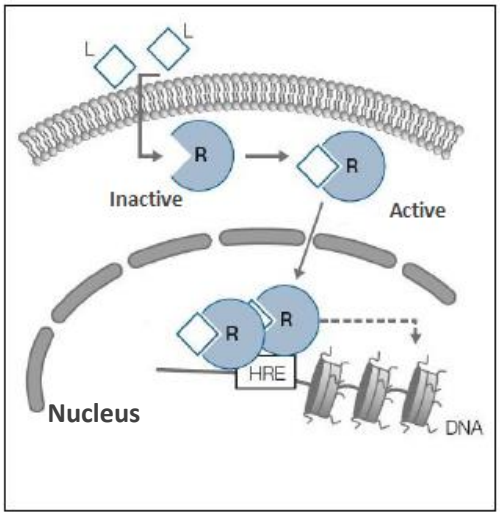


Figure 6. Direct genomic signaling. This pathway promotes target genes expression by binding the E_2-ER complex directly to the ERE [56].

1.1.4.2 Indirect genomic signaling

17β-estradiol can also modulate the expression of genes that have not EREs in their promoters. In fact, around one third of the estrogen responsive genes lack ERE-like sequences, for example, IL-6, TNF-α and MPC-1 genes don' t have ERE sequences in their promoters, but their transcription is inhibited by estrogen.

In the case of ERE-independent genomic signaling, ERs don't have a direct transcriptional effect, but its action is mediated by their binding with other classes of transcription factors at their respective response elements. This mode of action enables activation or repression of target genes expression. One of the best described examples includes interaction of estrogen-ER complex with FBJ murine osteosarcoma viral oncogene homolog (FOS) and jun proto-oncogene (JUN) proteins at the activator protein 1 (AP-1) binding sites in genes encoding insulin-like growth factor 1 (IGF1), collagenase, cyclin D1 (CCND1) and choline acetyltransferase [56-61]. The result depends on the ER subtype and type of the ligand.

Other transcription factors that facilitate estrogen signaling also include Sp1 transcription factor, nuclear factor kB (NFkB), CCAAT/enhancer binding protein β

(C/EBPβ) GATA binding protein 1 (GATA1) and signal transducer and activator of transcription 5 (STAT5) [56].

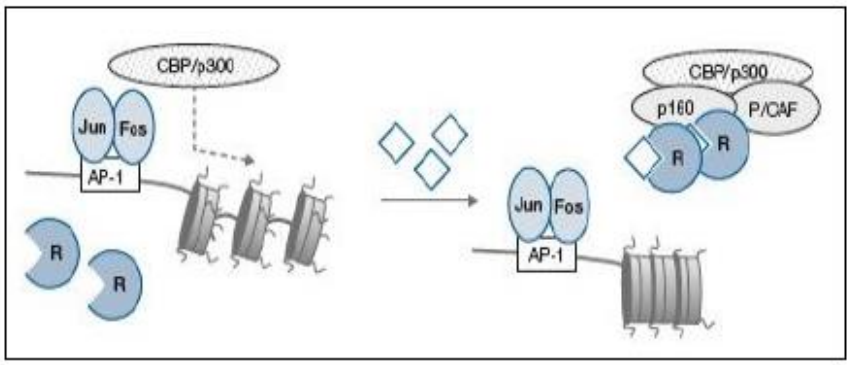


Figure 7. Indirect genomic signaling. E_2- activated ERs bind DNA trough protein-protein interactions with other classes of transcription factors at their respective response elements**.** [56].

1.1.4.3 Ligand independent signaling

ERs can be activated in the absence of the ligand. In fact, in the presence of particular extracellular signals, activated molecules are able to favor post-transcriptional modification, such as phosphorylation or acetylation, or phosphorylation of ERs associated coregulators allowing ligand-independent ER activation. These modifications are carried out by enzymes associated with membrane receptors, such as MAPK and JAK, and, when activated, generate a cascade of intracellular signals capable to regulate receptor activation.

Peptide growth factors, such as epidermal growth factor (EGF), insulin, IGF1 and transforming growth factor β (TGFβ), protein kinase A (PKA) or protein kinase C (PKC), neurotransmitters and cyclins are important group of estrogen-independent ER activators phosphorylating the receptor [62]. In fact, N terminal portion of ERs contains several serine residues that are target of phosphorylation. For example, phosphorylation of Ser 118 of ER is induced by EGF and depends on the activation of MAPK [63].

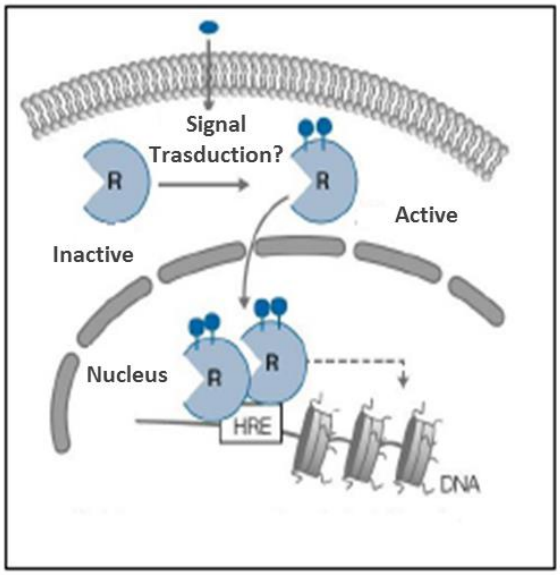


Figure 8. Ligand-independent signaling. This pathway causes ER activation and target gene transcription through phosphorylation of ERs on their associated coregulators **.** [56].

Classic genomic mechanism represents only a small part of to the complexity of estrogen signaling. Estrogen is able to modulate the expression of the same target gene through multiple mechanisms, both genomic and non-genomic. Moreover, the same promoter sequence can harbor both ERE as well as response elements associated with other transcription factors. The final result therefore depends on multiple factors including combination of transcription factors present on the gene promoter, expression levels and cellular localization of all three ERs, their various coregulators, and signaling components, as well as the nature of the stimuli. Since these variables can differ significantly among various cell types, it is possible that estrogens use distinct signaling pathways depending on the cellular context and in this way ensure very precise and cell-specific regulation of target gene expression.

1.1.4.4 Non genomic signaling

Estrogens are also able to modulate rapid biological effects (within seconds or minutes). Non-genomic estrogen signaling is mediated by a subset of membrane ER, e.g. GPR30 and variants of ERα and ERβ. Binding of estrogens to ERs located at the cell surface can cause mobilization of intracellular calcium, stimulation of adenylate cyclase activity and modulation of the cytoplasmic contents of cyclic adenosine monophosphate (cAMP), activation of the mitogen-activated protein kinase (MAPK) signaling pathway, modulation of the phosphoinositol 3-kinase (PI3K) signaling pathway and activation of membrane tyrosine kinase receptors [64-65].

For example, estrogen activates endothelial nitric oxide synthase (eNos), through MAPK and PI3K/Akt pathway [67-68]. In osteoblasts and osteoclasts, E2 rapidly activates the MAPK signaling involved in the proliferation and apoptosis of these cells, modulating bone homeostasis [69].

GPR30 instead is specifically associated with the stimulation of adenylate cyclase and activation of EGFR [66]. Molecular mechanisms underlying non-genomic estrogen signaling are different and numerous and may depend on a number of conditions, such as the availability of signal transduction molecules and downstream targets, suggesting a cell type-specific mechanism.

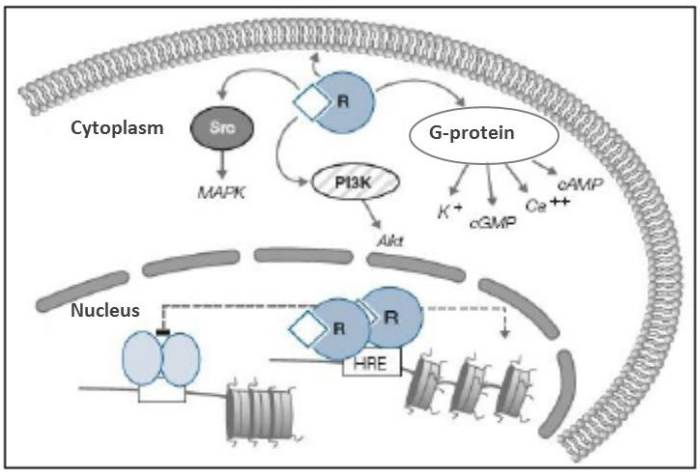


Figure 9. Non-genomic signaling. This pathway starts with the binding of E2 to the ERs located at the plasma membrane resulting in the activation of various protein-kinase cascades. [55].

1.2 Macrophages

Macrophages were originally identified in the late 19th century by Elie Metchnikoff [70], that described their phagocytic activity (Nobel Prize in Physiology or Medicine for the discovery of phagocytosis) [71].

Macrophages are a heterogeneous population of immune cells that populate all tissues and play key role in tissue integrity and homeostasis; they show a high phagocytic activity and are able to recognize and respond to tissue damage and infection [72-73].

Tissue macrophages show a strong anatomical and functional diversity as well as transcriptional difference recently revealed by a global transcriptome analysis of purified tissue macrophage populations by the Immunological Genome Project, highlighting their specialized role in different tissues.

In 1968 van Furth and Cohn showed that major populations of tissue macrophages were originated from blood monocytes [74] and classified macrophages as mononuclear phagocytic system (MPS); in this proposed model macrophages derived from blood monocytes developed from bone marrow precursors. This idea remained viable for over 40 years, although there was already evidence that the tissue macrophages were independent from circulating monocytes [74-76]. However, some recent evidence demonstrated that this definition is inadequate as macrophages have several origins during ontogeny and each of these different lineages persist into adulthood [77].

Functional classification of macrophages, instead, is based on inflammatory states and according to this criteria itis possible to group these cells into two main classes: the 'M1-M2 paradigm'.

Thus, macrophages are heterogeneous population of cells that constantly shift their phenotype in response to changes in environmental challenges and stimuli and they should be considered as different subtypes of cells according to their different origins.

1.2.1 Macrophage origin and functions

Evidence indicates that exist at least three lineages of macrophages that originate at different stages of development and persist in the adulthood.

The first lineage is based on MPS model and involves a series of progenitors in temporal succession. Primitive progenitors of macrophages originate from early and late erythro-myeloid progenitors (EMPs) generated in yolk sac from primitive ectoderm during primitive hematopoiesis at embryonic day 8. These EMPs can arise macrophages that not have a monocytic precursors. Subsequently, this primitive system is followed by definitive hematopoiesis in the fetal liver. Fetal liver monocytes are generated from EMPs derived from yolk sac or from hematopoietic stem cells (HSC) originated from hematogenic endothelium of the aortogonadal-mesonephros region of the embryo; these progenitors migrate to the fetal liver in two successive waves E9.5 (EMPs) and E10.5/E11 (HSCs) and generate circulating monocytes during embryogenesis. Following postnatal bone formation, hematopoiesis in the liver declines and starts in the bone marrow. Bone marrow hematopoiesis represent the source of circulating monocytes (resident, lymphocyte antigen 6c negative (Ly6c2) and inflammatory (Ly6c1) and from which some resident macrophages derived.

Second lineage of macrophages derives from yolk sac progenitors. Tissue resident populations of macrophages (F4/80 high) in skin, spleen, pancreas, liver, brain and lung originate from yolk sac progenitors, as demonstrating by studies using ablation of c-Myb-dependent bone marrow hematopoiesis and next transplantation with different bone marrow [78].

The third lineage arises from fetal liver; as occur for Langerhans cells that have a mixed origin from yolk sac and fetal liver [79].

The master regulator of macrophage lineage is macrophage colony-stimulating factor 1 receptor (CSF1R), a transmembrane tyrosine kinase receptor [80]. CSF1R is involved in differentiation of macrophages and its ablation lead to a severe depletion of macrophages in several tissues, such as brain, ovaries, bone and skin [81].

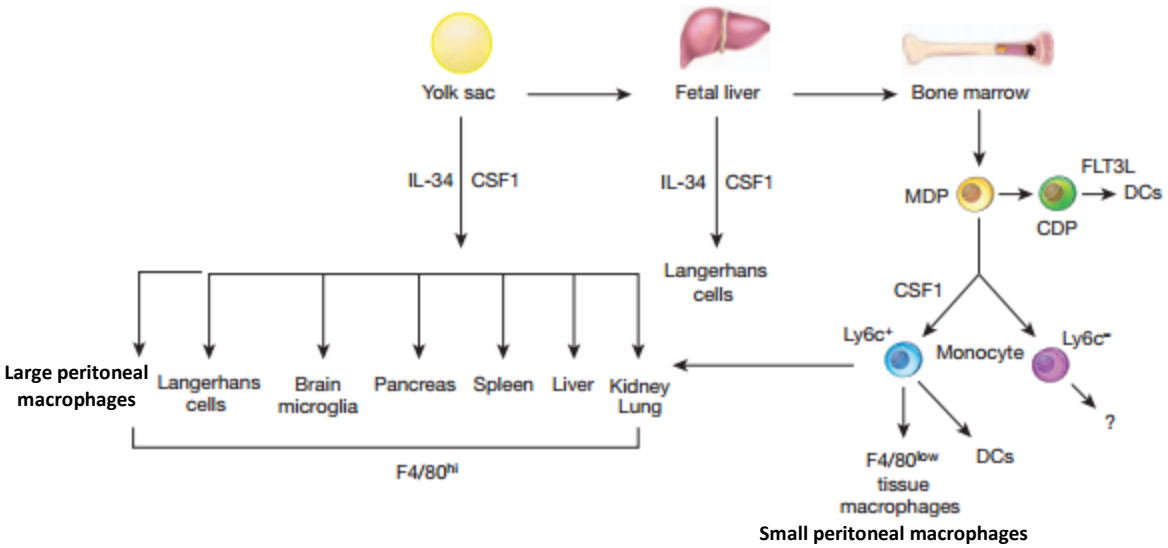


Figure 10. Macrophage lineages in mice Modified from Wynn *et al*., 2013 [73]
The mononuclear phagocytic system in adults derives from at least three sources. The first is the yolk sac, which produces progenitors that populate all tissues and that have progeny that persist throughout life as F4/80 bright resident macrophages. These lineages are mainly regulated by CSF1R and its ligands, IL- 34 and CSF1. The second is the fetal liver, and this is less well defined but seems to contribute to the production of adult Langerhans cells, perhaps through a progenitor that is derived from the yolk sac. The third lineage derives from the bone marrow (BM) to give circulating monocytes and their progeny F4/80low macrophages, and dendritic cells (DCs). In this case the Ly6c1 monocytes give rise to the classic Steinman dendritic cells under the regulation of FLT3, and these are continuously replenished. Other macrophages that are F4/80low also emanate from Ly6c1 monocytes, and in some cases—such as in kidney and lung—they co-exist with those derived from the yolk sac to give chimaeric organs. The exact role of the patrolling Ly6c– macrophages, and the contribution of fetal liver to adult tissue macrophages, remain unclear. CDP, committed dendritic cell progenitor; MDP, monocyte dendritic cell progenitor

Despite the diversity of tissue resident macrophages, they show some common functions such as phagocytosis and the secretion of pro- and anti- inflammatory cytokines with autocrine and paracrine functions. [82].

Macrophages play important role in several features of organism's biology; from development, homeostatic functions, such as iron processing ant clearance of lipoproteins, debris and dead cells, regulation of tissue metabolism, remodelling and repair [83-85], to immune response to pathogens, that involved immune surveillance, response to infection and the resolution of inflammation.

These cells participate in both the innate immune response, representing the first line defense of organism against external insults, and secondary or specific response; their primary function is to recognize and engulf pathogens, toxic substances and cellular debris, through pattern recognition, scavenger and phagocytic receptors [86-89].

The main functions of macrophages can be summarized in the following ones:

- **phagocytosis**, an important processes for the turnover of old or damaged cells (for example, erythrocytes), and especially for the immune response, since they are able to recognize, incorporate, and delete toxic elements;
- **secretion**, macrophages are able to produce and secrete several biologically active molecules such as cytokines (interleukins: IL-1, IL-12, INF-α, TNF), chemokines and proteolytic enzymes (such as plasminogen activators, elastase, collagenase).
- **antigen presentation to $CD4^+$ T lymphocytes,** macrophages are professional Antigen-Presenting- Cells (APC): they are specialized in antigen capture, processing and presentation by MHC Class II to other cells of the immune system.

1.2.2 Tissue resident macrophage populations

Macrophages populate the majority of tissues in the body and phenotypically distinct subsets of macrophages are often present in tissue microanatomical niches. Resident macrophages regulate tissue homeostasis acting as sentinels and responding to any changes in tissue environment.

Macrophages show specific phenotypic characteristics and express particular markers. For example, in mice, macrophages express the hematopoietic lineage marker CD45 but not markers of other immune cells, such as CD3 and CD20. They express the receptor for macrophage colony-stimulating factor (M-CSF or Csf1), the integrin CD11b, and two important scavengers of foreign antigens and apoptotic cells: Fcg receptor 1 (FcgRI) CD64 and the receptor tyrosine kinase MerTK [86].

However, it was observed a marked difference in gene expression profile of resident macrophages in the different tissues, suggesting a distinct transcriptional program for each population. For example, only spleen resident macrophages express Spi-C gene, essential in the recycling of iron from old red blood cells, or Gata6 is selectively expressed by peritoneal macrophages [90]. This transcriptional difference leads to a strong diversity and specialized functions of macrophages resident in the different tissues, that are summarized in Table 2. For example, in steady state condition, microglia regulates neuronal plasticity, alveolar macrophages, instead, engulf surfactant proteins preventing proteinase condition [92-93]. This diversity is regulated by different master controls in a tissue and niche-specific manner (Figure 11).

Tissue	[a]Cell type	[b]Functions & notes	Phenotypic markers ([c]Tissue-selective transcriptional regulators)
Adipose tissue	'Adipose-associated macrophages'	Involved in control of insulin sensitivity[96] and adaptive thermogenesis[99].	F4/80$^+$, CD45$^+$ (white and brown adipose tissue)[99] (PPARγ[96])
Blood	Ly-6C^{lo} monocytes	These monocytes function analogously as "intravascular housekeepers" clearing endothelial cell debris[115].	CX3CR1$^+$, Ly-6C^{lo}, F4/80$^+$, CSF1R$^+$[115] (Nr4a1[115])
Bone	Osteoclasts	Multinucleated cells formed by fusion that resorb bone by disruption of the mineralized matrix[36].	Calcitonin receptor$^+$ (multinucleate)[117]
	Bone marrow macrophages	Supporting erythropoeisis[87, 88]. Maintenance of hematopoietic stem cells in stem cell niches[116]. An independent self-renewing population[30].	CD169$^+$, F4/80$^+$, ER-HR3$^+$[118]
Central nervous system	Microglia	Promotes neuronal survival, front-line immune-surveillance cell, removal of dead neurons, synaptic remodelling[76, 119]. Derived from yolk sac and maintained and during inflammation independently of the bone marrow[15, 26, 65].	F4/80$^+$, CD11b$^+$, CD45^{lo}[120]
	Perivascular macrophages	Immune surveillance.	F4/80$^+$, CD11b$^+$, CD163$^+$, CD45^{hi}[120]
	Meningeal macrophages	Immune surveillance[120].	F4/80$^+$, CD11b$^+$, CD45^{hi}[120]
Gastrointestinal tract	Intestinal macrophages	Maintenance of intestinal homeostasis and regulation of immune responses to commensals[23, 121].	CX3CR1^{hi}, F4/80$^+$, CD11b$^+$, CD11c$^+$, CD64$^+$[121]
Liver	Kupffer cells (sessile)	Clearance of microorganisms and cell debris from the blood. Clearance of aged erythrocytes[91, 122]. Pre-natal origins[22]. Maintained in the adult independently of the bone marrow[17]	F4/80^{hi}, CD11b^{lo}, CD169$^+$, CD68$^+$, Galectin-3$^+$[123] [d]CD80$^{lo/-}$[122] (PPARδ[98])
	Motile liver macrophages	Immune surveilance[122]	F4/80$^+$, CD11b$^+$, CD80^{hi}[122]
Lung	Alveolar macrophages	Immune-surveillance of the lung for inhaled pathogens[51], homeostatic regulation of tissue function[72, 124], for example clearance of surfactant. Prenatal origins[22]. Maintained in adult and during inflammation independently of the bone marrow[30, 125].	F4/80^{lo}, CD11b^{lo}, CD11c^{hi}, CD68$^+$, Siglec F$^+$, MARCO$^+$, CD206$^+$, Dectin-1$^+$[127], Galectin-3$^+$[123] (PPARγ[72])
	Interstitial macrophages	Regulates DC maturation/activation[126].	F4/80$^+$, CD11c$^-$, CD68$^+$, MHCII$^+$[126]
Serosal tissues	Peritoneal macrophages: F4/80^{hi} majority	Immune surveillance and regulation of homeostatic environment[50, 128]. Apoptotic cell clearance[80]. Pre-natal origins[22]. Maintained in adult and during inflammation independently of the bone marrow[22, 29].	F4/80^{hi}, CD11b^{hi}, [d]Tim4$^+$[83] MHCII^{lo}
	F4/80^{lo}	Immune surveillance[129].	F4/80^{lo}, CD11b$^+$, Tim4$^-$, MHCII^{hi}, CD11c$^{+/-}$ (This population is most likely heterogeneous, mixed with dendritic cells)
	Pleural macrophages: F4/80^{hi} majority	Maintained in adult and can expand during T_H2 inflammation independently of the bone marrow[41]	F4/80^{hi}, CD11b^{hi}, dTim4$^+$
	F4/80^{lo}		F4/80^{lo}, CD11b$^+$, Tim4$^-$ (Dendritic cell-macrophage content undetermined, unpublished phenotypic observations)
Skin	Dermal macrophages	Immune surveillance[130].	F4/80$^+$, CD11b$^+$, CD11c^{lo}, CD206$^+$, MHCII^{lo}, CD169$^+$ (In the deep dermis)[123], Dectin-1$^+$, CD301$^+$[132], Dectin-2$^+$[133]
	Langerhans cells	Interaction with T lymphocytes[131]. Derived from yolk sac and/or fetal liver and maintained independently of the bone marrow[14, 16].	F4/80$^+$, CD11b$^+$, CD11c$^+$, Langerin$^+$[14] (Id2[134], Runx3[135])
Spleen	Marginal zone macrophages	Immune surveillance of the circulation[102].	CD68$^+$, CD209b$^+$, MARCO$^+$, Dectin-2$^+$[133], Tim4$^+$[109] (LXRα[109])
	Metallophilic macrophages	Immune surveillance[102].	CD68$^+$, CD169$^+$[102] (LXRα[109])
	Red-pulp macrophages	Erythrocyte clearance and iron metabolism[103]. Pre-natal origins[17, 22]. Maintained in adult independently of the bone marrow[30].	F4/80$^+$, CD206$^+$, Dectin-2$^+$[133] (Spi-C[103])
	White pulp (tingible body) macrophages	Clearance of apoptotic cells resulting during the germinal center reaction[100].	CD68$^+$[102]

Table 2. Distinct locations and functions of tissue macrophages. Table from Davies *et al*., 2013 [91].

aThis table represents a simplification and marked heterogeneity is evident in many tissues (for example, bone marrow, peritoneum, lung and liver) highlighted through fate mapping studies and phenotypic variation.

bOrigin indicated only where experimentally established.

cSelect examples of tissue selective transcriptional regulators involved in cellular development or function are indicated.

dMarker is expressed by the majority of the indicated cells. Subsets are only listed where distinct anatomical localization, function or origins are reported, and not those that are simply defined by variation in select receptor/antigen expression.

Surrounding microenvironment itself is crucial to modulate macrophage functions and phenotype, providing tissue-specific signals influencing the expression of several genes regardless of origin and orchestrating the differentiation [94]. In fact, tissue local production of cytokine and substances are involved in the regulation, maintenance and functional specialization of macrophages: Csf1 is important for survival and proliferation of macrophages in several tissues, instead, IL-34 (whose signaling is mediated by Csf1r), Csf2 (or GM-CSF) and oxysterols regulate macrophage functions in specific tissues (Figure 11), although the role of these cytokines must to be better investigated *in vivo* [95].

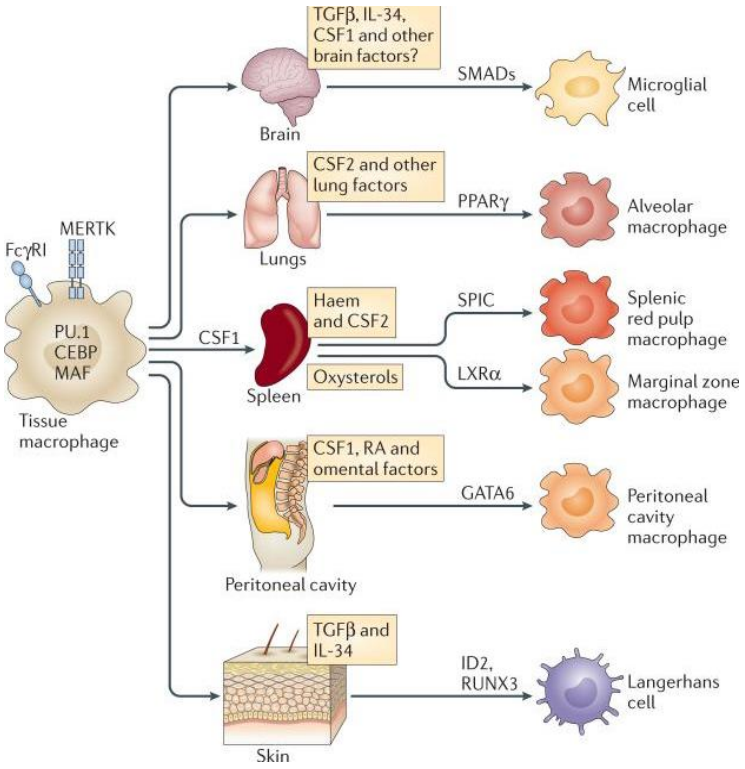


Figure 11. Tissue microenvironment determines macrophage differentiation. Taken from Lavin *et al*., 2015 [96] Macrophages in all tissues are characterized by expression of the cell surface marker, such as FcγRI (also known as CD64), tyrosine-protein kinase MER (MERTK) and the transcription factors PU.1. In the tissues, macrophage identity and functions are shaped by cytokines and metabolites that are produced in the local environment and drive specific transcription factor expression.

1.2.2.1 Peritoneal macrophages

The peritoneal cavity (PerC) is a single fund, in which coexist a variety of immune cells, such as eosinophils, neutrophils, B cells, dendritic cells and macrophages.

Peritoneal macrophages are the best studied population of macrophages. In fact, most of the current information about macrophage biology, function, specialization and development come from studies performed using peritoneal macrophages as a cell source. However, only recently it has been demonstrated the presence of two different subpopulations of resident macrophages in PerC that are functionally and phenotypically heterogeneous [97].

On the basis of their size, these subpopulations are defined as Large Peritoneal Macrophages (LPMs), that constitute the most abundant population of macrophages present in the peritoneal cavity, about 90%, and Small Peritoneal Macrophages (SPMs).

Both subpopulations show phagocytic activity *in vivo* and express macrophage typical surface markers, such as F4/80 and CD11b, although they show different expression levels of these markers. In fact, LPMs express higher levels of F4/80 and CD11b while SPMs show F4/80^{low}CD11b^{low} phenotype (Table 3). Analysis of microscopy and flow cytometry demonstrated that SPMs and LPMs are characterized by different morphology and phenotype. Indeed LPMs display classical morphology with prominent vacuolization and abundant cytoplasm, whereas SPMs have a polarized morphology with dendrites similar to DCs [99]. Moreover, the analysis of a complex panel of cell surface molecules demonstrated that they differ in the expression of a wide variety of other surface molecules (Table 3), such as Gr-1 and MHC-II, and several studies demonstrated different response patterns following treatment with classical macrophage stimuli (such as lipopolysaccharide).

This difference in quantitative expression of a series of surface molecule between the two subtypes of peritoneal macrophages or the lack of expression by SPMs or LPMs of some surface markers allows to distinguish these two populations in qualitative ways. In fact, LPMs express Gr-1 and AA4.1 while SPM not. In contrast, SPMs express MHC-II while LPMs express very low levels of this marker (Table 3).

Surface molecule	LPMs	SPMs
F4/80	+++	+
CD11b	+++	+
CD11c	+	−
MHC-II	+	++
GR1	+	−
Ly6C	−	−
c-kit	−	−
CD62L	−	++
Dectin-1	+	++
DC-Sign	−	++
TLR4	++	+
CD80	++	+
CD86	+++	+
CD40	++	+
12/15-LOX	+	−
TIM4	+	−

Table 3. Different expression profile of surface markers between SPMs and LPMs. Taken from Cassado *et al*., 2013 [98].

LPMs and SPMs are also functionally distinct; although both populations show phagocytic activity *in vivo*, SPMs seem to be characterized by a more efficient phagocytosis. Furthermore, it has been demonstrated that LPMs produce more NO in response to LPS *in vitro* compared to SPMs, although *in vivo* were obtained quite different results [97].

The two populations differ also for the origin and development (Figure 12): in homeostatic conditions LPMs derive from proliferation *in situ* regardless hematopoiesis, SPMs, instead, are produced from circulating monocytes. Factors arising from the microenvironment play an essential role in promoting phenotypic development of peritoneal macrophages, an example of this mechanism is represented by RA-derived factor that promotes the omentum expression of GATA-6 transcription factor selectively expressed by LPMs, determining their location and function. Instead, the factors that regulate the SPMs pool in the steady state are still unclear.

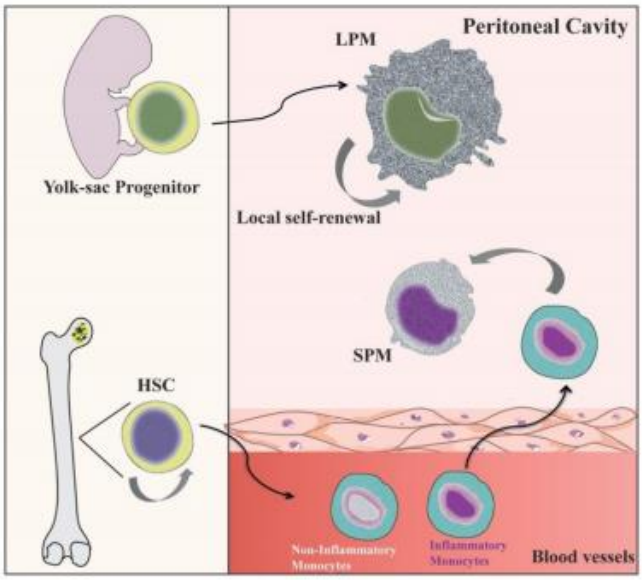


Figure 12. Origin of peritoneal macrophages. Taken from Cassado *et al*., 2015 [98]
SPMs are produced by the cell hematopoietic stem cell (HSC) in bone marrow (BM) for differentiation of circulating inflammatory monocytes. LPMs, instead, originate in the fetal yolk sac and undergo to proliferation *in situ*.

In the steady condition, peritoneal cavity includes a wide variety of immune cells and the two subpopulations of macrophages represent about 30-35 percentage of total cells. However the presence of an inflammatory or infectious stimulation leads to a dramatic change in the number and in the percentage of each subpopulation. Modifications in the composition of the peritoneal cavity include the disappearance of LPMs, the increase in the number of SPMs and a massive recruitment of circulating monocytes.

These changes occur during hypersensitivity reactions and during the process of acute inflammation; the increase in the number of SPMs and monocytes is to be correlated with the renewal and improvement of the immune conditions of the peritoneal cavity.

In conclusion, we can say that the SPMs and their precursor (circulating inflammatory monocyte) are the main population present in stimulated peritoneal cavity, in fact this subpopulation has a pro-inflammatory functional profile while LPMs seem have a role in the maintenance of the peritoneal cavity in physiological conditions [98].

1.2.2.2 Microglia

Microglia are myeloid cells that populate the parenchyma of the central nervous system (CNS), representing 5%-12% of the total number of cell depending on the brain region [100-101] and were firstly identified in 1919 [102-103].

Microglia derive from yolk sac progenitors that colonize the neuroepithelium in early stage of embryogenesis [104], acquiring their definitive characteristics, in terms of numbers and phenotype, immediately after birth; although a peak of microglia proliferation has been demonstrated at early postnatal stages [105-106].

Microglia population is maintained locally by self-renewal, without the contribution of bone-marrow-derived progenitors [104, 107-108], even though the mechanisms that regulate microglia proliferation in steady state are unclear. Some studies reported the involvement of transcription factor PU.1, CSF1 and IL-34 in the regulation of microglia proliferative pathway [109].

Microglia physiological activity includes most of the biological properties that are typical for peripheral macrophages, although their developmental origin and anatomical distribution allows these cells to perform distinctive immune and neuromodulatory functions in the CNS. Through their physical and biochemical interactions with neurons, microglia are able to sense and remodel neuronal activity, support neurogenesis, and maintain CNS homeostasis. Microglia constantly scan the microenvironment to detect and remove neurotoxic substances or inflammatory mediators. Microglia phagocytic activity is essential during development (pruning microglia), removing supernumerary synapses in some neuronal pathways [110] (Figure 13).

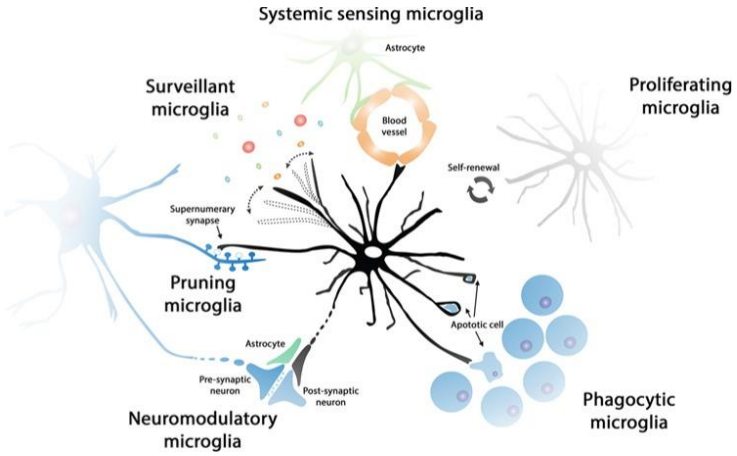


Figure 13. Functions of microglia in the healthy brain. Taken from Gomez-Nicola and Perry., 2015 [111]
The population of microglial cells is maintained by self-renewal. Surveillant microglia sense and remodel neuronal activity, support neurogenesis, and maintain CNS homeostasis.

1.2.3 Macrophage self-renewal

Although it was considered that the tissue macrophages were not able to divide and renew themselves, several recent studies have shown that it is possible to maintain a homeostatic number of tissue resident macrophage population, which undergoes to local proliferation, without the contribution of circulating monocytes or other hematopoietic precursors. This phenomenon ensures an adequate number of cells in the tissue, even if it has been demonstrated that following a severe depletion of tissue macrophages, monocytes are able to repopulate in a short time the deficient area.

An exception is represented by intestinal macrophages that originate from bone marrow precursors and are constantly supplied by circulating monocytes [112-113].

During inflammation, inflammatory macrophages derive from circulating monocytes derived from bone marrow that are recruited to injury site. These cells show a short half-life and not proliferate. However some studies demonstrated that both resident macrophages and monocyte derivated cells are able to self-renew in response to IL-4 or during helminth infection [119, 122] and in zymosan peritonitis model [114], leading to an increased number of inflammatory macrophages. Furthermore, it has been demonstrated that lesional macrophages that derived from bone marrow precursors proliferate in a murine model of atherosclerosis and this mechanism involved the scavenger receptor SR-A [115].

It has been suggested that while the common functions of macrophages, such as phagocytosis, may also be implemented by monocytes that migrate in the tissue, the more specific functions, for example, the antigen presentation, appear to be possessed exclusively by those cells that are renewed locally [94].

Different soluble factors regulate proliferative pathway of macrophages (Figure 14).

The main factor that regulates the macrophage proliferation in the tissues is the growth factor CSF1, which acts binding its receptor CSF1R. CSF1R signaling is essential in several aspects of macrophage biology [116] and is mediated by CSF1 but, also, by IL-34. CSF1R signaling is involved both in homeostatic macrophage maintenance and proliferation [112, 117-119] and in macrophage inflammatory renewal [114]. CSF2, instead, plays an important role in lung alveolar macrophage homeostasis and self-renewal [120-121]. Recently some studies have shown that even interleukin 4 (IL-4) is able to promote the proliferation of these cells, through a mechanism independent of CSF1. [119,122].

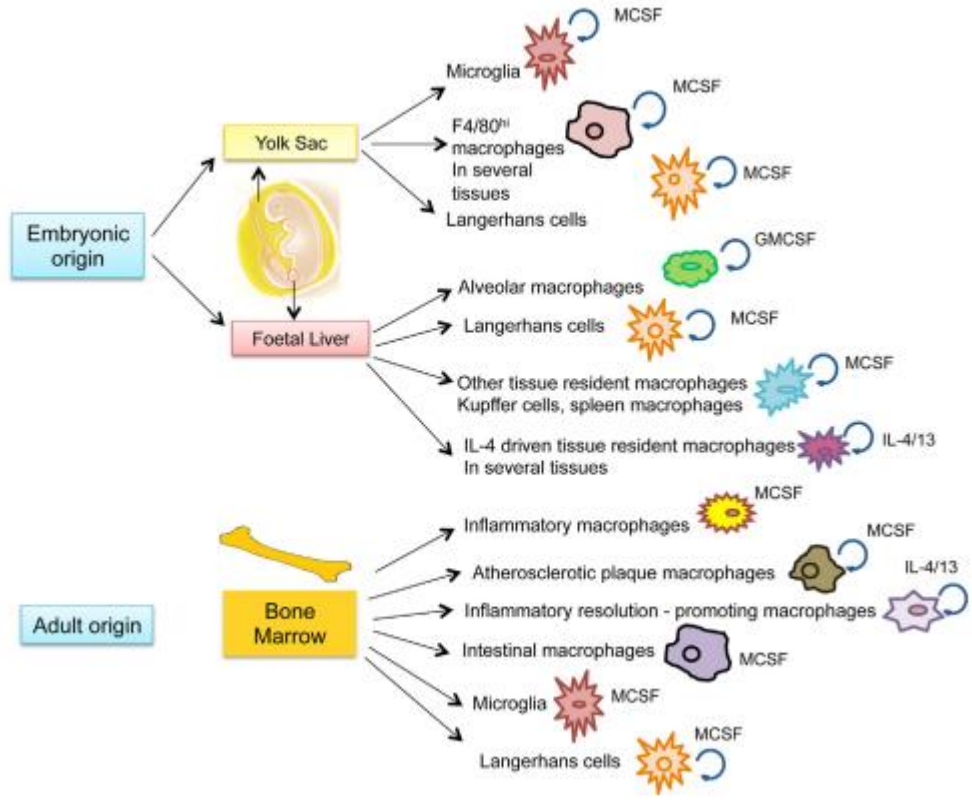


Figure 14. Soluble factors that modulate tissue resident macrophages. Taken from Fejer *et al.*, 2015 [123]
Embryonic macrophages from the yolk sac and fetal liver self-renew in homeostatic conditions and are driven by M-CSF or GM-CSF. Inflammatory macrophages of embryonic origin also self-renew

1.2.3.1 Macrophage proliferation induced by CSF1

An essential regulator of macrophage homeostasis is CSF1 (macrophage colony-stimulating-factor), so defined since it is able to promote *in vitro* the growth of macrophages derived from bone marrow progenitor cells [124] . It is the main factor involved in the survival, proliferation, differentiation of all mononuclear phagocytic cells, thus, also dendritic cells, osteoclasts and microglia in addition to macrophage cells [125].

There are three major isoforms of CSF1 protein, that derived from alternative transcripts: one secreted as proteoglycan is the predominant form, one secreted as glycoprotein and one anchored on the surface of cells that, however, can be released through a proteolytic cleavage [125].

CSF1 is produced locally in stroma tissue, although during inflammation can be produced by macrophages themselves and acts by binding to its receptor CSF1R or FMS (c-fms), that is a member of the family of tyrosine kinase present in macrophages.

Recently it has been observed that the activation of CSF1R receptor requires also a second ligand: interleukin 34 (IL-34). Computational studies showed that IL-34 is able to bind different region of the receptor [126]; in fact Chihara *et al*., have identified a monoclonal antibody able to bind to CSF1R receptor blocking the binding of CSF1, but not of IL-34 [127]. The binding of IL-34 in the extracellular domain of the receptor induces the dimerization and autophosphorylation of the intracellular part of the receptor, that induces a cascade of intracellular signals, with activation of protein kinase PI3K and MEK, able to regulate the production, the survival, and the functions of macrophages.
CSF1 exerts its functions on monocyte-macrophage cells both in bone marrow and in the peripheral tissues. In the bone marrow CSF1 is able to promote the differentiation of myeloid precursors in monocytes, mostly cooperating with other factors such as GM-CSF, IL-3, INF-γ. In other tissues, it controls proliferation, differentiation and survival of tissue macrophages, regulating the density of tissue resident population in the steady state, but, also, in response to an acute infection [125].

1.2.3.2 Macrophage proliferation induced by IL-4

IL-4 is a multifunctional cytokine that acts as a potent regulator of immunity; it is secreted primarily from CD4+ T lymphocytes belonging to the Th2 subpopulation, mast cells, eosinophils and basophils. It has been discovered initially by Howard and Paul, as a "co-mitogen" for B lymphocytes, later it was discovered its importance in the survival of leukocytes both in physiological and pathological conditions and in cell differentiation of naïve T cells stimulated by antigen, leading to produce by themselves these and other cytokines such as IL-5, IL-10, IL-13. Moreover, IL-4 has important role in the regulation immunity mediated by Th2 cells. It is also involved in the switching of IgE in B cells [128], tissue repair and alternatively activation of macrophages [129]. The inflammatory process that takes place in response to helminth infections or in allergy leads to the recruitment of various cells able to produce IL-4 including Th2 cells.

Recently it has been demonstrated that, during parasitic infections, there is a vast increase in resident macrophage number in infected tissues, which is due to a proliferation *in situ* of resident macrophages without the contribution of circulating monocytes. This local proliferation of these cells is regulated by IL-4 produced mostly from Th2 cells. This is demonstrated by the fact that following a IL-4 intraperitoneal injection in mice there is an increase in the local proliferation of macrophage cells for more than four days, and this effect is comparable to the one which occurs during infection with the parasite [130]. In addition macrophages that are locally generated are characterized by an alternative phenotype (M2).

Moreover, it was estimated that following IL-4 administration, this local proliferation of resident macrophages occurs in all tissues, including liver [130], lung and spleen [119].

The mechanism by which this occurs is independent of CSF1R and depends on IL-4 receptor (IL-4Rα) that through PI3K/Akt signaling regulates the proliferation as well as the alternative activation of macrophages. In fact this does not occur in mice lacking IL-4Rα.

1.2.3 Macrophage activation

Macrophages are characterized by high plasticity: in fact they are able to take part in different processes such as inflammation, defense against pathogens and tissue repair. This is due to their peculiarities to change their morphology and their functional characteristics in response to various types of stimuli, both endogenous and from the external environment.

Under normal conditions, macrophages are in a quiescent state, but they can undergo to activation as a result of appropriate molecular signals from surrounding microenvironment. These signals are typically produced by innate immune cells and lead to long-term alterations in macrophages [130]. Furthermore, macrophages themselves can also produce some factors that influence their own physiology.

According to the current classification, macrophages are functional grouped in two main phenotypes (Figure 15): the "classical" activation (M1), and the "alternative" activation (M2) [131, 239]. However these are two extremes of a series of phenotype that macrophages can assume.

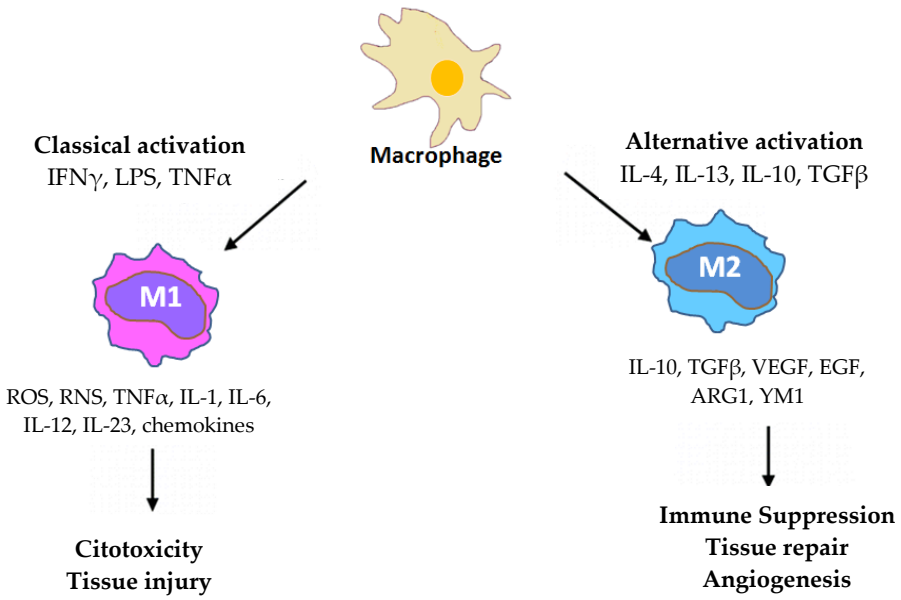


Figure 15. Macrophages polarization and functions.
Schematic representation of M1 and M2 macrophage polarization phenotypes and functions.

1.2.3.1 M1 macrophage activation

M1 macrophages are involved in the initial phase of the inflammatory response and provide to the elimination of invading microorganisms through production of reactive oxygen intermediates (such as O2- OH-), nitrogen (as NO-, NO2-, ONOO-), cytokines (IL-1 beta, TNF alpha, IL-6) and chemokines (CXCL11). Since this was the first antimicrobial mechanism of macrophages recognized, it was called classical macrophage activation or M1 [132].

Since there isn't a discrimination of microbial targets and host tissue, M1 macrophages can lead to tissue damage as a side effect of their defense activity. Thus, although it is an essential event for the organism integrity and protection from external attacks, the inflammatory response requires a strict regulation in order to eliminate the dangerous agent without causing damage to the body itself.

The classical activation of macrophages is induced by the recognition of molecules associated with the presence of pathogenic microorganisms, such as the bacterial lipopolysaccharide (LPS), component of the cell wall of the gram-negative bacteria, and TLR4 ligand or peptidoglycan. Even helper Th1 cells, CD8 + cytotoxic T cells (Tc1) and Natural Killer (NK) cells [133] can activate macrophages through the production of interferon-γ (IFN-γ), as well as molecules associated to a damage of the cells, such as intracellular proteins or nucleic acids.

M1 macrophages have increased capacity for presenting the antigen, elevated synthesis of pro-inflammatory molecules such as interleukin IL-1, IL-6, IL-12, IL-1β, tumor necrosis factor alpha (TNF) and toxic mediators such as nitric oxide (NO); moreover, they have an increased phagocytic activity mediated by the complement.

Through this activation, macrophages acquire a bactericidal capacity, especially against intracellular pathogens [134].

In general, the classically activated macrophages are more commonly associated with disease characterized by low-grade of inflammation, such as atherosclerosis and type 2 diabetes (T2D) [135] and are involved in the immunopathology that occurs during several autoimmune diseases, such as rheumatoid arthritis [136] and inflammatory bowel disease [137]. The silencing of genes that promote the classical activation, such as the gene encoding TLR4, leads to an improvement of these diseases in some murine models.

1.2.3.2 M2 macrophage activation

The alternative activation of macrophages, or M2, usually occurs in a second time compared to the M1 state during inflammation process, and exerts anti-inflammatory effects leading to tissue repair.

M2 macrophages are able to block the action of M1 macrophages and produce important factors that are involved in the repair and remodelling of damaged tissue and promote vascularization [139], regulating angiogenesis. They are also involved in the regulation of immune response and tumor progression [140].

The molecules able to induce M2 macrophage polarization are various and include glucocorticoids, cytokines produced mainly by Th2 such as IL-4 and IL-13, IL-33, IL-21 or by transforming growth factor β1 (TGFβ1). Also CSFs seem to be important in promoting M2 phenotype [141-142].

The M2 phenotype, promoted by Th2 is also essential to control parasite infection, including helminthes, protozoa and fungi, as well as contributing to the states allergic and increasing susceptibility to other pathogens.

These macrophages produce various proteins involved in the repair, healing, angiogenesis, cell proliferation, which may be used as markers for identify their presence. Among the markers used for their identification there are: Arginasi1 (Arg1), YM1 (or Chi3l3), Fizz1 (or RELM-α), mannose receptor (MR), VEGF and IL-10.

The classification of macrophages in M1 and M2 groups was further extended by Mantovani and collaborators; thus, on the basis of the different signals that activate macrophages, alternative polarized macrophages can be classified into subgroups with specific phenotype and functions, called M2a, M2b, and M2c [134, 143].

The M2a phenotype can be produced *in vitro* by exposing macrophages to IL-4 or IL-13, that acting through the common IL-4Rα receptor increase the expression of CD206, arginase and TGF-β [145-147]. M2a macrophage state has important role in allergy and is involved in the killing and encapsulation of parasites.

The M2b phenotype is produced, instead by exposure of a combination of IgG immune complexes and LPS. Unlike the other M2 phenotypes, characterized by low levels of pro-inflammatory cytokines (IL-1, TNF and IL-6), M2b macrophages produce many inflammatory cytokines and toxic molecules along with high levels of IL-10 and low levels of IL-12 [148-150]. Despite this, the M2b cells protect mice from LPS toxicity [146, 149] and promote Th2 differentiation and production of humoral antibodies.

In vitro exposure to IL-10 or glucocorticoids induces in macrophages the M2c phenotype, which it is characterized by the production of high levels of IL-10 and low levels of IL-12, as well as by an increase on the cell surface expression of scavenger receptor CD163 [151-153]. M2c phenotype promotes the "switching" of macrophages M1 towards M2 and is induced by adenosine A2A receptor. It is characterized by the production of high levels of IL-10 and VEGF, a growth factor of vascular endothelium, that is able to promote angiogenesis. [134].

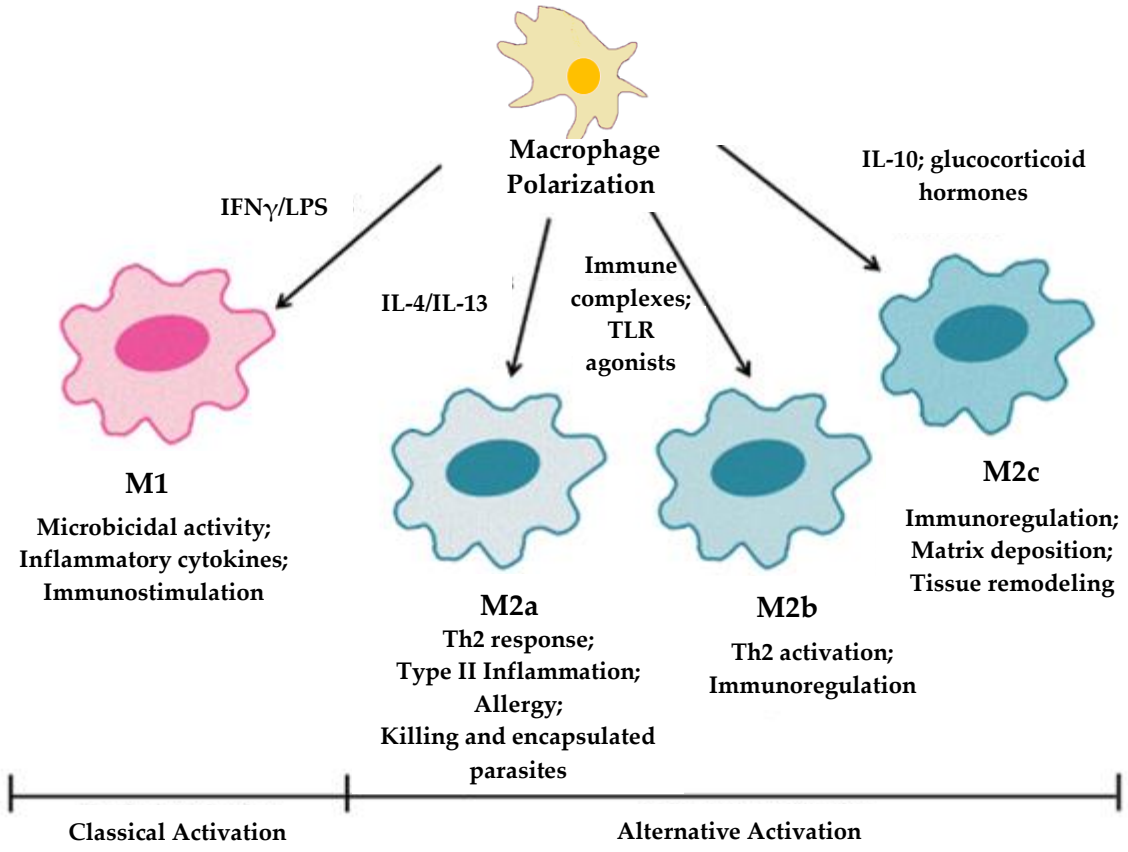


Figure 16. Macrophage polarization. Modified from Zanluqui *et al*., 2015 [154]
Macrophage micro-environment stimuli define differential macrophage polarization via classical activation (M1) or alternative activation (M2). Pathogen-derived LPS alone or in combination with IFN-γ leads to classical activation of M1 macrophages, which improves microbicidal activity and secretion of pro-inflammatory mediators. According to the host-parasite microenvironment, alternative macrophage activation could be subdivided into three subpopulations. M2a differentiation is promoted by IL-4 or IL-3, and this subpopulation is associated with Th2 response, allergy process, internalization, and parasite killing. M2b is related to the presence of immune complexes, TLR or IL-1R agonists, and promotion of immunoregulation. Glucocorticoids and IL-10 secretion lead to differentiation into M2c, which also induces immunoregulation, tissue remodeling, and repair.

M2 macrophage actions seem favorable for the protection and restoring a damage in the body. Moreover, macrophage alternative activation is generally associated with the protection against diseases in which the classical activation is pathogenic. However, the activation of alternatively polarization can be deleterious in other pathologies, in particular cancer [138]. In fact, they are involved in the complex mechanism that leads to the development and growth of tumors, and, in particular, in the invasion and metastasis of the tumor: these macrophages are defined as TAMs (tumor associated macrophages) [145].

The relationship between the activation of macrophages phenotypes observed *in vitro* and the functional states of macrophages in pathological condition *in vivo* is a topic of debate in progress.

1.3 Estrogen action on macrophages in physiology

The influence of estrogen on immune and inflammatory processes initially came to light after a series of clinical observations. Humans show strong sex differences in immunity to infection and autoimmunity, suggesting that sex hormones modulate immune responses.

In fact, it is widely accepted that females have a more efficient immune system than males [155-157] and this difference in the inflammatory response may explain the significantly lower incidence of infections and resultant mortality in women than in men. This more enhanced inflammatory response in women represents an advantage in infection and sepsis, but, on the other hand, is a disadvantage in immune response against self, as demonstrated by their higher incidence of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis [158-162] (Figure 17). Moreover females more rapidly reject skin allografts than males, but suffer lower rates of some tumors, such as colorectal [163] , renal cell [164] and liver carcinomas [165].

In accordance to this proofs, estrogens have been shown to be protective in several diseases characterized by a relevant inflammatory component, such as atherosclerosis, wound inflammation and asthma. Indeed, epidemiological and immunological evidence has been shown that menopause, menstrual cycle and pregnancy are important influencing factors in the etiology and course of chronic inflammatory diseases [166-169].

Particularly, the onset of menopause, when the levels of circulating estrogens rapidly decline, is characterized by an increase of the incidence in women of diseases characterized by a relevant inflammatory component (Figure 17). Instead, hormone replace therapy (HRT) in postmenopausal women have been demonstrated beneficial effects in these conditions, such as osteoarthritis, multiple sclerosis, atherosclerosis and neurodegenerative diseases [170-174].

Moreover also polymorphisms of estrogen receptor genes affect the incidence of different inflammatory pathological conditions [175-178].

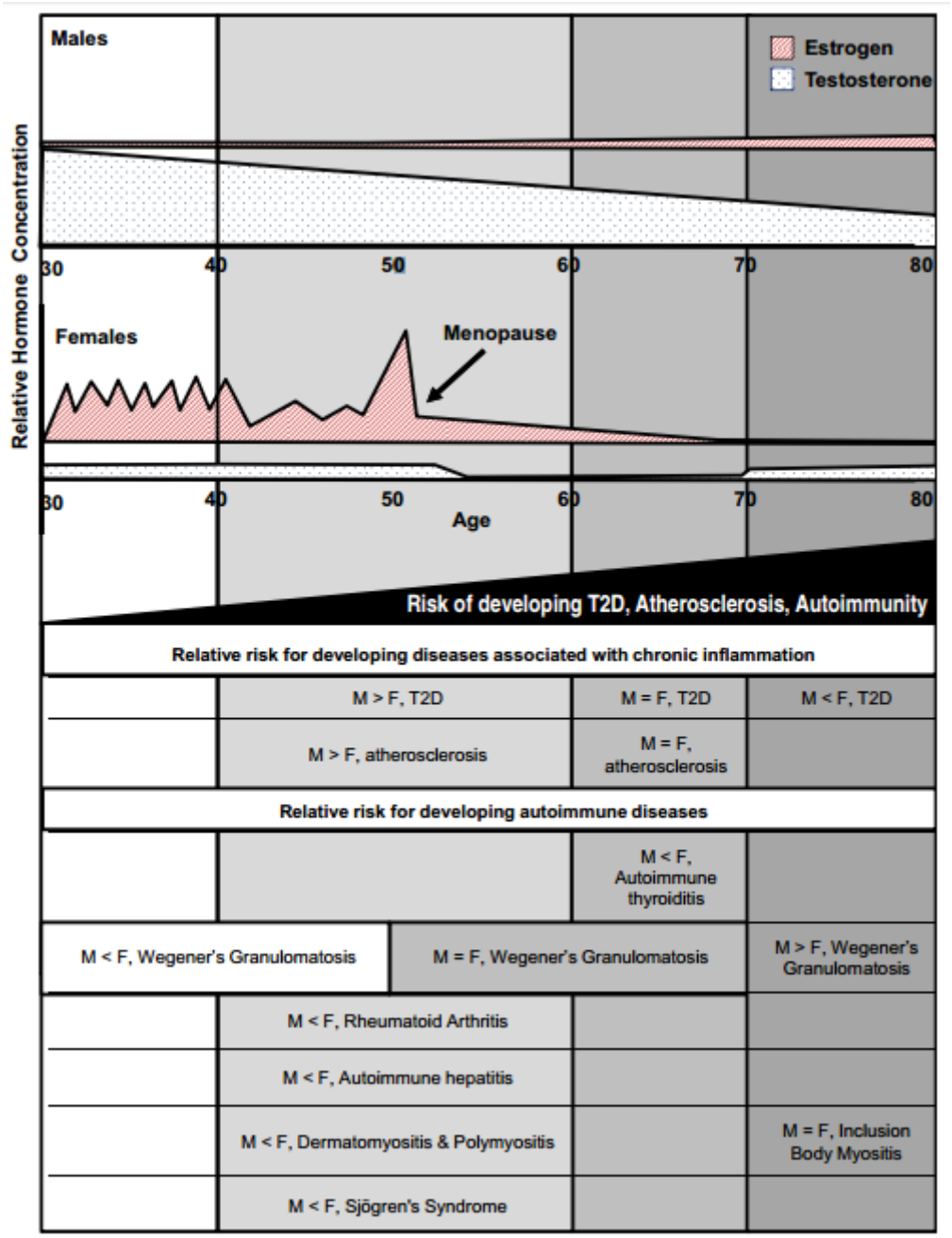


Figure 17. Correlations between the relative levels of estrogen and testosterone and the incidence of diseases associated with chronic inflammation. Taken from Gubbels., 2015 [179]

Type 2 diabetes, atherosclerosis, and autoimmunity in men and women between the ages of 30 and 80 years. White and shaded columns demarcate periods in the lifespan during which chronic inflammatory and autoimmune diseases manifest clinically. A comparison of the incidence of each disease in men and women for each time period and disease is indicated.

1.3.1 Estrogen as inflammatory regulator

Many cells of the immune system are target of estrogen action, that through ERs and membrane receptor (GPR30) binding modulate development and functions of innate immune cells.

Some studies have reported that ER mRNAs and proteins are expressed by hematopoietic progenitors and mature immune cells and have determined their levels of expression (Table 4) (as reported in the review of Kowats, 2015 [180]).

These studies reported that B lymphocytes express highest levels of Esr1 RNA, whereas lymphocytes CD4+ and CD8+, natural killer cells and dendritic cells (DC) have intermediate levels of expression [181-183]. Monocytes, instead, are those that have the lowest levels of RNA expression of Esr1, even if dendritic cells that derived from monocytes have increased levels of expression of Esr1 and Esr2, suggesting that ERs are induced during activation of myeloid cells [185].

As regards the Th1 and Th2 lymphocytes, they do not have differences in RNA expression of ERs, as well as there are no differences in T cells in normal conditions. This suggests that the effects of estrogen in these cells do not depend on differences in expression of ER receptor. However, evidence demonstrated that naïve T-cells are more sensitive to the effect of estrogen [184].

Regarding the expression levels of Esr2, coding for ERβ, these are very high in B cells and dendritic cells, and are very low in all other cell types.

Bone-marrow derived macrophages and peritoneal macrophages express Esr1 but not or very little Esr2 [186-187].

Interestingly, there are no differences in the expression of ESR1 and ESR2 mRNA in immune cells between men and women or in pre and postmenopausal women, suggesting that it is not the change in the number of receptors that lead to alterations in immune function and the consequent pathologies associated with menopause, even in women in postmenopausal there is an increase in the number of ER in monocytes, as compared to monocytes of premenopausal women, that is associated to an altered responsiveness to estrogen [184]. It would therefore the different sensitivity of the receptor to the hormone to influence its final response.

	Human		Murine	
Cell type	**ESR1 (Erα)**	**ESR2 (Erβ)**	**Esr1 (Erα)**	**Esr2 (Erβ)**
B cell	yes (+++)[b]	yes (+++)	yes	yes
$CD4^+$ T cell	yes (++)	yes (++)	yes	c
$CD8^+$ T cell	yes (++)	yes (++)		
NK cell	yes (++)	yes (++)	yes	yes
Plasmacytoid DC	yes (++)	yes (+++)	yes	
Monocyte	yes (+)	yes (+)		
Monocyte-derived DC	yes (++)	yes (+)		
BM-derived DC			yes	yes
Splenic DC			yes	no
Inflammatory DC CNS				yes
Peritoneal Macrophages			yes	no
BM-derived Macrophages			yes	no
Hematopoietic stem cell	yes	yes	yes	no
Myeloid progenitor			yes	no

Table 4. Expression of estrogen receptors by immune cells. Modified from Kowats S., 2015 [180]
[a]"Yes" indicates either RNA or protein expression, depending on the study. "No" indicates that the RNA or protein was queried but not found. [b]Plus (+) marks indicate relative amounts of RNA determined using quantitative. [c]Empty cells indicate cell types for which actual data showing ER expression was not readily found in literature sear

A lot of works demonstrated that E2 and ER signaling regulate inflammatory pathways of immune cells, specially dendritic cells and macrophages. Particularly, these studies showed that low levels of estrogen promote the induction of type I IFN and suggest that ERα and IFNs interact to modulate immune response [188-189].

Furthermore, E2 affects cytokine production, but its effects depend on cell types, condition in the milieu and estrogen concentration. Some works showed that estrogen induces the production of pro-inflammatory cytokines in response to TLR ligand activation in DCs and macrophages [190-191]. However a lot of studies demonstrated that estrogen at higher physiological or supra-physiological levels induces an anti-inflammatory response that attenuates inflammation [192-194]. In addition, estrogen leads to the inhibition of nuclear factor kappaB (NF-κB) signaling through different mechanisms such as the displacement of coactivators, enhancing IκBα levels, the inhibition of NF-κB nuclear translocation and the repression of Iκbkg [195-200].

Moreover ER signaling modulates also hematopoietic progenitors and their proliferation, regulating number and type of immune cells [201].

1.3.2 Effect of estrogen on macrophages

Estrogen modulates through autocrine and paracrine mechanisms various activities of macrophages, such as cell differentiation and maturation and regulation of proinflammatory cytokines and/or production of immunosuppressive and growth factors.

The mechanisms involved in the regulation of inflammatory response mediated by estrogens are different; the ERs activation in macrophages leads to the regulation of gene transcription and the modulation of non-genomic pathways [202].

The activation by estrogen of ERs, is able to reduce the synthesis of pro-inflammatory molecules [200, 203], control mitochondrial respiratory and mitosis, induce the degradation of damaged proteins by proteasome [204], reduce monocytes apoptosis and activate hypoxic genes [205].

The main mechanism mediated by ERs involves the modulation of NF-κB pathway and regulates the transcription of cytokines and other inflammatory mediators [155, 200]. In fact, in presence of powerful inflammatory stimuli (such as LPS), estrogen blocks NF-κB nuclear translocation and modulating p65 activity; this effect is mediated by PI3K signaling [200]. Pro-inflammatory cytokines that are inhibited by estrogen through this mechanism are, for example, IL-6, IL-8, TNFα, MCP-1 and IL-1β. Some *in vitro* studies showed that estrogen, on the contrary, stimulates the secretion of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β [169]. However, as previously cited, the effects of estrogen on macrophage cytokine production is strongly dependent on hormone concentration, microenvironment and the presence or absence of inflammatory stimuli.

In addition to regulate cytokines expression, estrogen modulates macrophage functions, regulating other important inflammatory factors. In fact, estrogen at proestrus to pregnancy levels inhibits NO formation in mouse macrophages (RAW 264.7) [206] and the reactive oxygen species (ROS) production in primary rat microglia and microglial cell line (N9) [207].

Also adhesion molecules play key role in inflammation, attracting inflammatory cells to inflamed sites. It has been demonstrated that E2 at proestrus to pregnancy levels is able to inhibit monocyte adhesion to endothelial cells in an *in vitro* model of the vasculature [208-209]. However, low levels of estrogen can produce different effect: some studies showed that E2 is able to induce the expression of adhesion molecules [210-211].

Apoptosis, instead, in inflammatory condition has both beneficial effects, inhibiting autoaggresive cells, and deleterious results, hampering repair process and tissue

regeneration. It has been demonstrated an antiapoptotic action induce by estrogen in murine microglial cells (N9), confirmed *in vivo* [212-213]. Although estrogen promote antiapoptotic effect in immune cells, it is able to have proapoptic effect in osteoclasts [214].

Furthermore, estrogen at proestrus to pregnancy levels promotes angiogenesis and increases the production of vascular endothelial growth factor (VEGF) in different cell types, among which macrophages [206, 215-216].

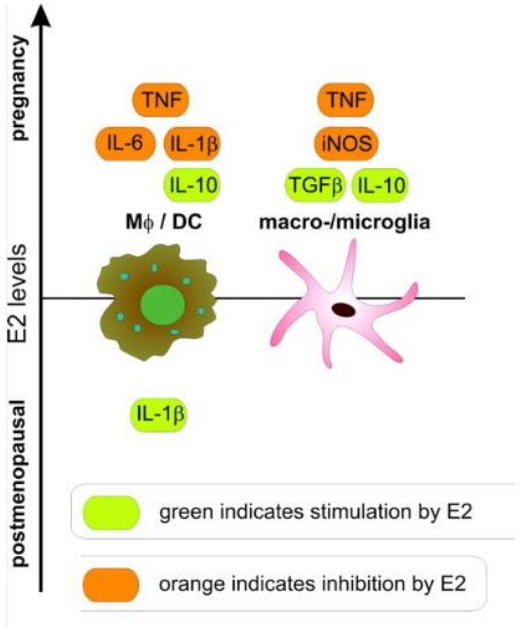


Figure 18. Estrogen pro and anti-inflammatory effect on different cell type. Modified from Straub R., 2007 [169]
On the y-axis, the concentration of estrogens is given. Depending on the concentration of estrogens, factors in green boxes are stimulated, and factors in orange boxes are inhibited by estrogens. DC, Dendritic cell; MΦ, monocyte/macrophage

In addition to classic nuclear receptors ERs, also membrane receptor GPR-30 present in macrophages seems to modulate the anti-inflammatory effects of estrogen. An *in vitro* study has shown that short treatments with 17β-estradiol are able to reduce the expression of TLR4 in a short time, attenuating the response of cells to this inflammatory stimulus [217].

1.3.2.1 Estrogen action on M2 macrophage activation

Despite the action of estrogen on M1 macrophage activation is well documented, as previously described, the capacity of estrogen to modulate M2 macrophage polarization is still little known and most of the experiments on this topic are performed *in vitro*.

It has been shown that 17-βestradiol regulates phagocytic activity of microglia under hypoxic conditions and promotes the switch from an M1 toward an M2 phenotype of primary rat microglial cultures and BV2 cells [218]. Moreover estrogen is able to modulate microglia activation, down-regulating the expression of COX2, iNOS and NO production in BV2 cells following LPS stimulation [219] and transient hypoxia [220].

The effect of estrogen on M2 macrophage polarization is evident in the animal model of wound healing. Some studies have demonstrated in a murine incisional model of wound healing in ovariectomized animals that, while ovariectomy impairs macrophage alternative activation, that is essential to resolve inflammation, estrogen treatment is able to reverse this effect. Indeed, estrogen promotes macrophage switch toward M2 phenotype, driving wound repair, angiogenesis and remodelling [221] and this hormone action is mediated by ERα [222].

Moreover, a recent study performed in our laboratory showed in an *in vitro* model that estrogen, following a proinflammatory stimulus, such as LPS, decreases the duration of proinflammatory phase and accelerates its progression to the resolution phase. This occurs because estrogen modifies intrinsic and extrinsic programs in macrophages, promoting a rapid transition of these cells from a pro-inflammatory phenotype to immunomodulatory M2c state, also called "acquired deactivation" (AD) [141]. Estrogens exerts this effect through the regulation of STAT3 and SOCS3 pathway and promote inflammation progression, by regulating the production of IL-10 that is the main marker of macrophages with M2c polarization. M2c macrophages secrete some factors, such as VEGF or TGFβ, that lead damage repair, restructuration and remodeling of the connective tissue, angiogenesis and restore tissue homeostasis [223].

1.3.2.2 Macrophage ERαKO

The studies performed using cell-specific ablation of ERα in monocyte/macrophage cell lineage have led to a more comprehensive knowledge of the role of estrogen in these cells.

A study demonstrated that E2, thorough ERα activation, enhances *in vivo* proinflammatory response of peritoneal macrophages following TLR4 activation, increasing the expression of IL-1β, IL-6, IL-12p40 and NO synthase production, and this mechanism is due, at least in part, to the inhibition of PI3K/Akt pathway [191].

Another work investigated the role of estrogen in obesity-induced insulin resistance and showed that E2 is essential for the repression of inflammation, maintenance of macrophage metabolism and macrophage phagocytic ability. In fact, ERα macrophage deletion promotes different aspects of metabolic syndrome, leading to glucose intolerance, insulin resistance, tissue inflammation and obesity [187]. Moreover, ERα deletion accelerates atherosclerotic lesion development in LDL receptor-KO female mice, this is probably due to a diminished expression in ERαKO macrophages of Tgm2 (protective agent against atherosclerotic lesions), ApoE and Abca1, which exert atheroprotective action, a reduction of fatty acid oxidation rates and an increase of inflammatory gene expression [187].

Few studies showed that estrogen action mediated by ERα is important to promote macrophage alternative activation in some experimental animal model [187, 222].

Altogether these studies suggest that estrogen induces a more efficient inflammatory response in macrophages in order to accelerate the resolution phase.

This evidence indicates that molecular players of estrogen signaling in macrophages may represent important pharmaceutical targets to modulate inflammation in several pathological conditions.

1.4 Relevance of estrogen action on macrophages in pathology

During tissue development, macrophages play a key role in shaping and remodelling the architecture of many of them, such as brain, gland, bone and mammary tissues. After development, macrophages maintain important roles in these tissues, modulating homeostasis and tissue physiology, regulating several tissue features, including metabolism and neural connectivity, and detecting damage. Since their trophic and regulatory functions are often subverted by chronic inflammatory insults, they are involved in many diseases often related with aging (Figure 19).

On the other hand, estrogen play a key role in pathology characterized by a relevant inflammatory component, modulating macrophage inflammatory response and functions, as previously described.

Thus, I focused my attention on some pathologies where estrogen-macrophage interplay may be crucial for disease development progression and where the modulation of estrogen signaling in macrophages might be a therapeutic strategy to develop new drugs. Particularly, I focused on diseases, such as endometriosis and cancer, specially ovarian cancer, where peritoneal macrophages are involved in the pathogenesis, and neurodegenerative diseases, where microglia play important role.

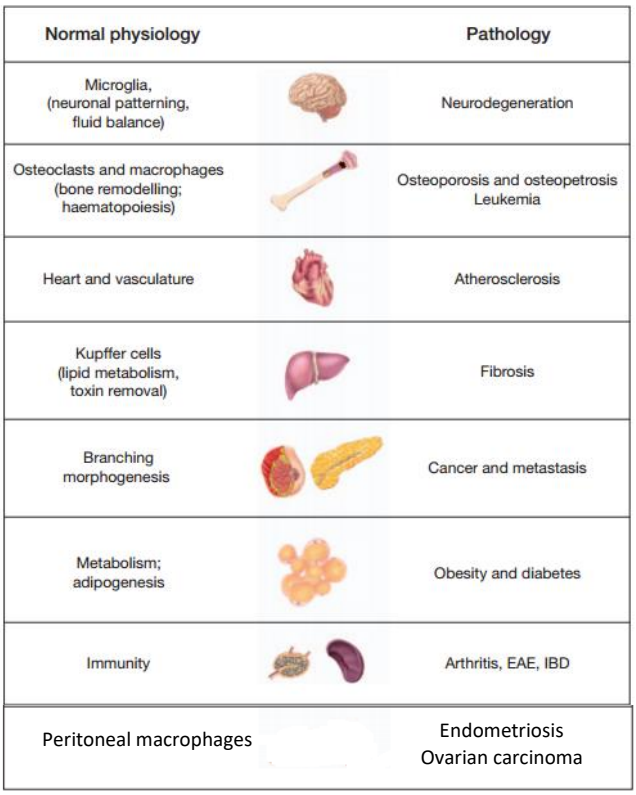


Figure 19. Macrophage biology in development, homeostasis and disease. Figure modified from Wynn T.A. *et al.*, 2013 [225]. Diseases in which are involved the different tissue resident macrophages.

1.4.1 Endometriosis

Endometriosis is a gynecologic disease that affects women's fertility. It is a chronic inflammatory condition driven by estrogen and progesterone hormones characterized by endometrial cells that colonize and grow in peritoneal cavity, but also in other abdomen organs, such as gut or bladder. These endometrial cells produce high quantities of chemotactic and angiogenic factors that recruit macrophage and other immune cells and promote neovascularization [226-228]

Several mechanisms are involved in this process, such as anatomical and mechanical changes, immune and inflammatory factors, ovarian reserve alterations.

It was been observed a negative role of M2 macrophages in endometriosis, in which they seem to be crucial in promoting and developing its characteristic injuries [229].

Estrogen, instead, plays important role in endometriosis. Indeed, the ectopic growth and persistence of endometrial tissue in pelvic area are supported by estrogen, as well as biological changes and clinical events associated to the disease [230]. Moreover estrogen strongly affect inflammation [226-228]. Further evidence of estrogen-macrophages interplay is brought by *in vivo* model of peritoneal macrophages depletion leading to tissue growth and development reduction of the ectopic lesion [231]. Moreover, immunological changes can be detected; patients' peritoneal liquid is characterized by high level of pro-inflammatory cytokines, such as TNF-α [232-233], IL-6 [234], IL-12 [235] , IL-8, IL-10 [232] and other inflammation mediators and cells, in particular activated macrophages [236-238]. Endometrial macrophages appear to be also involved in red blood cells and cellular endometrial debris elimination through phagocytosis and in promotion of angiogenesis through trophic factors production, in agreement with M2 phenotype [131, 239]. Indeed , in *in vivo* murine model of the pathology, as well as in sick women, peritoneal macrophages express high levels of CD206 and CD163, typical M2 markers. These cells release growth factors, such as VEGF-α and HGF, in response to ovaric steroids. In particular, HGF production is inhibited by estrogen antagonist administration. Mitogenic effect of HGF is explicated by interaction with c-MET receptor on peritoneal macrophages, promoting their proliferation and the development of endometrial pathology [241]. Moreover, through the release of chemotactic factors such as MIF, these macrophages lead to their accumulation in the peritoneal cavity, with consequent exacerbation of inflammation [242]. Thus, macrophages ability of remodeling and regenerate tissues is responsible of their involvement in pathologies characterized by persistent tissue reshaping [243-244].

Local hypoxia and iron overload might be triggering factors of macrophages alternative activation, although the precise mechanism is still unclear. In conclusion, the purpose of alternative peritoneal macrophages activation is to counteract inflammation in the peritoneal environment, but, in some circumstances, it can lead to ectopic endometrial tissue growth and vascularization [229].

1.4.2 Cancer

Estrogens play a critical role in the development of tumors in several tissues, such as breast, prostate and endometrial tissue [245-248] and is crucial in disease progression.
On the other hand, macrophages plenty populate tumors [249] . Originally was thought that macrophages had anti-tumor activity, but clinical and experimental evidence showed that macrophages have tumorigenic effects, promoting tumor initiation, progression and metastasis [250]. During persistent infection, macrophages increase the production of inflammatory cytokines, such as IFN-γ, TNF-α and IL-6, sustaining a state of chronic inflammation that seems to be crucial for tumor initiation and promotion [251]. Once tumors become established, macrophage differentiate as tumor-associated macrophages (TAMs) switching from an immunologically active phenotype towards an immunosuppressive phenotype capable of supporting tumor progression and malignancy [250]. TAMs are involved in tumor-cell migration, invasion and in the angiogenic response essential for tumor growth [250,252,253]. These events lead to the formation of metastasis, promoting tumor cells escape into the circulatory or lymphatic system. Some evidence suggests that macrophages acquire these activities in response to CSF1, IL-4 and IL-13, that are present at high levels in tumor microenvironment, leading to tumor-cell invasion and intravasation in mammary cancer [254].
Ovarian carcinoma is sustained by E2 production by stromal cells and is characterized by the dissemination of cancer cells into the whole peritoneum. Some evidence indicates that ovarian tumor progression is orchestrated by the interplay between tumor cells and peritoneal macrophages, as well as other immune cells. It has been recently shown that ovarian tumor-derived TGF-β2 and CCL2 stimulate normal peritoneal macrophages to acquire features of TAMs [255]. TAMs promote tumor progression by suppressing adaptive immunity and producing proangiogenic factors, such as Vegfα [256-257].

In this scenario estrogen might support tumor progression and growth, promoting macrophage alternative activation and angiogenesis. In fact, in the high-grade serous ovarian cancer (HGSC) E2 promotes tumor growth, inducing alternative activation of macrophages and increasing their infiltration in the tumor area [].

However, interestingly, a study on hepatocellular carcinoma (HCC), a malignant tumor that affects mainly males, showed that the 17β-estradiol inhibits development of malignant cells, through the control of macrophages polarization by blocking the alternative activation [258]. In particular, the hormone explicates its repressive action on ATP5J factor(ATPase-coupling factor 6) by interaction with ERβ. ATP5J is a component of ATPasi complex, an enzyme that allows ATP synthesis from ADP and inorganic phosphate (Pi), by exploiting proton-driving force. In this way JACK1-STAT6 signaling, responsible of alternative activation, is inhibited [258].

4.3 Neurodegenerative diseases

Several evidence shows that neuroinflammation mediated by microglia is a critical contributor to the pathogenesis of neurodegenerative diseases [259-261].

Microglia persistently activated and their production of inflammatory cytokines, ROS and immune mediators lead to the maintenance of inflammatory milieu in different degenerative disease, such as Alzheimer's Disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's Disease (PD) [259-260, 262], and contribute to neuronal death.

Moreover it has been demonstrated a marked expansion of M1 activated microglia, without the contribution of bone-marrow-derived cells, during the course of these diseases, particularly in ALS, PD and Huntington's disease (HD) and AD, especially around plaque of amyloid β (Aβ) [109. 263-265]. This local proliferation of activated cells leads to a detrimental contribution of microglia to the progression of the pathology [109, 266].

On the other hand, it has been demonstrated that M2 microglia ameliorates several aspects of neurodegenerative diseases in different animal models. For example, M2 microglia showed beneficial role in AD mouse model [267-269], maybe also increasing the clearance and the degradation of Aβ, and potentially had protective role in the incidence and progression of MS, since M2 polarized cells reduce the severity of the pathology in an experimental model of autoimmune encephalitis (EAE) [270-272].

Estrogens, instead, exert anti-inflammatory and neuroprotective effects in CNS in acute and chronic brain diseases [218]. Several studies have demonstrated a neuroprotective function of E2 in animal model of neurodegenerative diseases, traumatic brain injury and ischemic stroke [168, 273-278]. This protective effect of estrogen is due, at least in part, to its ability to modulate microglia activation, inhibiting M1 macrophage phenotype and promoting a M2 pro-resolving state [219-220, 279].

AIM OF THE STUDY

Despite the evidence of an important role of estrogen in inflammation and its relevance in the regulation of physiology and pathology, the molecular and biological details of estrogen action in macrophages are still poorly understood.

Thus, the aim of this study was to investigate the effects induced by estrogen alone on resident macrophages in healthy mice. To this purpose, we obtained peritoneal macrophages exposed to physiological and pharmacological surge of estrogen *in vivo*.

This comprehensive study allowed us to identify the molecular targets and the biological role of the estrogen-macrophage interplay in female physiology.

METHODS

3.1 Animal models and treatments

C57BL/6 female mice at 4 months of age were supplied by Charles River Laboratories; ERαKO female mice were obtained from P. Chambon, IGBMC, Strasbourg, France [305]. Animals were allowed to food and water access ad libitum and kept in temperature-controlled facilities on a 12-hour light and dark cycle. Animals were housed in the animal care facility of the Department of Pharmacological and Biomolecular Sciences at the University of Milan. The phase of the reproductive cycle in female mice was assessed by blind analysis of vaginal smears mounted on glass microscope slides and stained with May-Grünwald-Giemsa method (MGG Quick Stain Kit; Bio-Optica) according to the manufacturer's protocol. 17β-estradiol (E2; Sigma-Aldrich) was administered by a 100 μL s.c. injection of 5 μg/kg E2 dissolved in corn oil by o/n stirring in the dark at room temperature in order to obtain physiologic plasma levels of E2 [306]. Since previous laboratory investigation demonstrated that s.c. injection of corn oil did not significantly affect macrophage parameters and expression of gene analyzed; control ME animals were processed immediately after their identification in ME phase in order to not have an influence of the fluctuating levels of endogenous hormones during estrous cycle. Ovariectomy (ovx) or sham surgery was performed under mild anesthesia obtained by s.c. injection of 50 μl solution of ketamine (93.6 mg/kg, Ketavet 100; Intervet) and xylazine (7.2 mg/kg, Rompun; Bayer). At the specified time points, animals were euthanized by intraperitoneal (i.p.) injection of lethal ketamine and xylazine solution (150 and 12 mg/kg, respectively). Animal groups (n=3) for the RNA sequencing experiment were given an estrogen-free diet (AIN93M; Mucedola) 2 weeks before and throughout the experiment. Animal investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institute of Health, and in accordance with the European Guidelines for Animal Care and Use of Experimental Animals. Experiments were approved by the Italian Ministry of Research and University and by the Ethical Committee of the University.

3.1.1 Zymosan peritonitis model

For the zymosan peritonitis model, 4 weeks after sham/ovx surgery mice were injected i.p. with 1 mg of Zymosan (Sigma-Aldrich) in 1 ml of 0.9% NaCl, while control mice received physiological solution alone. Animals were sacrificed after 12, 36 and 60 h. Ovx mice were treated with veh or E2 by repeated injections given 3 h before zymosan and 16 h before sacrifice at 36 and 60 h. The numerosity of each control or treated group was n=3. Macroscopic and weight analyses of uterine tissue were used to confirm the success of ovx and E2 treatments.

3.1.2 BrdU injection

For *in vivo* BrdU labelling experiments, mice were injected i.p. with 30 ul of a 10 mg/ml solution of BrdU (Sigma-Aldrich) dissolved in 0.9% NaCl. Animals were sacrificed 2 h after BrdU injection (n=4-6)

3.1.3 Intracerebroventricular injection of IL-4

Intracerebroventricular (icv) injections were made as previously described [307]. Briefly, mice were deeply anesthetized with a subcutaneous injection of a mixture of ketamine and xylazine (78 and 6 mg/kg, respectively) and positioned on a specific stand for the surgical operation. Injections in the third cerebral ventricle (icv) were performed according to specific stereotaxic coordinates (bregma, −0.25 mm; lateral, 1 mm; depth, 2.25 mm), as previously described [307]. Interleukin-4 was injected in 2.5 μl of 0.9% NaCl using a 26S-gage Hamilton Syringe; 250 ng were injected to assess RNA levels. Infusions were made at a rate of 0.1 μl in 3 s. The needle was kept in place for 30 s after the injection and then removed slowly. Animals injected with the same volume of vehicle (0.9% NaCl) alone were used as controls. The skin incision was closed with a suture and animals were allowed to recover for 24 hours before sacrifice by a lethal ketamine and xylazine solution (150 and 12 mg/kg, respectively). For RNA quantification, the right frontal cortex, contralateral to the injection site, were collected, immediately frozen on dry ice, and stored at −80°C until RNA preparation. The numerosity of each control or treated group was n=12.

3.2 Peritoneal and bone-marrow-derived macrophages (BMDMs)

Peritoneal cells were recovered by peritoneal lavage. Briefly, 5 ml of pre-chilled 0.9% NaCl were injected into the peritoneal cavity using a 21G needle, cell suspension centrifuged and dissolved in PBS + 0.5%BSA. After counting, peritoneal cells were isolated either by incubation with anti-CD11b antibody loaded MicroBeads (Miltenyi Biotec) for RNA sequencing, gene expression validations and proliferative index analyses, or by anti-CD11b, F4/80 and Ly6C antibodies for cytometry phenotyping or macrophage sorting for gene expression analyses. Briefly, 10^7 peritoneal cells were suspended in 90 μL PBS + 0.5% BSA, and 10 μL CD11b MicroBeads were added to the cell suspension and incubated for 15 min at 4°C. After washing, cells were resuspended in 500 uL PBS + 0.5% BSA and applied to LS Miltenyi columns (Miltenyi Biotec) for the magnetic separation procedure. After 3 washing steps, CD11b-positive cells were eluted from the columns and counted. Cells were either stored in TRIzol reagent (Invitrogen) for gene expression studies or fixed for flow cytometry analyses or plated at the concentration of 1×10^6 cells/ml in RPMI medium w/o phenol red with 10% dextran coated charcoal-FBS for *in vitro* assay .

To prepare BMDMs, bone marrow from the tibia and femur was flushed with RPMI (Life Technology-Invitrogen) using a 21 gauge needle. Cells were centrifuged at 1200 rpm for 5 min at 10°C, seeded in flask cell culture T75 in DMEM+GlutaMAX (Life Technology-Invitrogen) supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate and incubated o/n. On the next day, the supernatant was collected, seeded at the concentration of 5-8 x 10^6 cells/dish and grown for 6 days in DMEM+GlutaMAX containing 20% endotoxin-free FBS, 30% L929-cell conditioned media, 1% penicillin and streptomycin, and 1% Na pyruvate. After 6 days BMDMs were harvested with Accutase (Merck-Millipore) and plated at the concentration of 5×10^5 cells/ml. On the next day, RPMI medium w/o phenol red with 10% dextran coated charcoal-FBS was added and cells were treated for 24 h with vehicle or E2 at the specified concentrations, with hormone being added in the last 3 hours of incubation for the 3 h E2 treatment.

3.3 Microglia isolation

After 24 h of E2 treatment, brains were dissected and washed in Hank's Balanced Salt Solution (HBSS; Life Technologies); after removing the meninges. Enzymatic cell dissociation was performed using Neural Tissue Dissociation Kit P (Miltenyi Biotec), following a modified version of the protocol supplied by the manufacturer. Briefly, after enzymatic digestion with papain, samples were dissociated mechanically, homogenized, and filtered through a 40-μm cell strainer. After extensive washes in HBSS, myelin was removed by centrifuging the dissociated brain cells, which had previously been suspended in 10 ml of cold 0.9 M sucrose solution, at 850 g and 4°C for 10 min without braking. Floating myelin and the supernatant were discarded and cells were processed for microglia magnetic sorting by incubating with CD11b MicroBeads (diluted 1:10 in PBS + 0.05% BSA; Miltenyi Biotec) for 15 min at 4°C; after washings, cells were suspended in 500 μl of PBS + 0.05% BSA and applied to a magnetic column to purify CD11b+ cells, namely microglia. CD11b+ cells were then processed for RNA preparation.

3.4 Flow cytometry analysis

To assess cell proliferation index, CD11b-positive cells obtained from each animal were divided in 3 aliquots; 2 aliquots of $2.5x10^5$ cells each were used to detect BrdU and Ki67 by flow cytometry, the remaining aliquot was used for gene expression. Cells were suspended in 2 ml of pre-chilled ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA; pH 7.3). For Ki67 staining, cells were fixed in 4% paraformaldehyde for 15 min, extensively washed with 125 mM glycine in PBS and permeabilized o/n in PBS containing 0,5% Triton X-100 and 1% BSA, at 4°C. Cells were incubated with rabbit anti-mouse Ki67 antibody conjugated with eFluor660 (eBioscence-Affymetrix) diluted 1:100 in Incubation Solution (PBS containing 0.5% Triton X-100 and 0.05% BSA) at room temperature for 1 h. After extensive washes in PBS, cells were analyzed with a flow cytometry system (BD FACS Calibur). For BrdU staining, cells were fixed and permeabilized in 70% EtOH for 30 min at 4°C and DNA was denaturated with 2 N HCl/0.5% Triton X-100 and incubated 30 min at room temperature. Cells were washed with 0.1 M sodium tetraborate (pH 8.5) and incubated with rat anti-mouse BrdU antibody (AbD Serotec) diluted 1:100 in Incubation Solution (PBS containing 0.05% Tween-20 and 1% BSA). After washes in PBS/1%BSA, cells were incubated with Alexa488-conjugated goat anti-rat secondary antibody (1:200 in incubation solution; Molecular Probes) for 1 h at room temperature. Cells were extensively washed with PBS, resuspended in PI solution (H2O containing 10% NP40, 1 mg/ml RNase A and 5 μg/ml PI stock; Sigma-Aldrich). Samples were analyzed using FACSCalibur and analyzed with FlowJo version 9 software (Tree Star). Animals with no pulse of BrdU were used for gating strategy to evaluate nonspecific signals.

In the sham/ovx animals and zymosan-induced peritonitis model immunofluorescent staining of peritoneal cells was performed with V450 anti-mouse CD45, PerCP-Cy5.5 anti- mouse CD11b, FITC anti-mouse Ly6C, PE-Cy7 anti-mouse Ly6G, PE anti-mouse CD11c, PE anti-mouse CD19 (BD Biosciences), AlexaFluor 647 anti-mouse F4/80 (AbD Serotec) and PE anti-mouse CCR3 (R&D Systems). Samples were analyzed using FACS Canto II and DIVA software (BD Biosciences); gating strategies are reported in Supplementary Figure 1. Resolution indices of zymosan-induced peritonitis were calculated as previously described [308]. Ly6G-negative CD11b-positive and F4/80-positive macrophages were sorted using sorter Aria (FACS Aria, Becton Dickinson Biosciences) and resuspended in TRIzol reagent for gene expression analyses.

3.5 RNA preparation and analysis

Total RNA was purified using RNeasy minikit protocol (Qiagen), according to the manufacturer's instructions, including a step with deoxyribonuclease incubation. For real time PCR, 1 μg RNA (250 ng in peritonitis model and 50 ng in isolated microglia) was used for cDNA preparation using 8 U/μl of Moloney murine leukemia virus reverse transcriptase (Promega) in a final volume of 25 μl; the reaction was performed at 37°C for 1 h, and the enzyme inactivated at 75°C for 5 min. Control reactions without the addition of the reverse transcription enzyme were performed (data not shown). A 1:4 cDNA dilution was amplified using GoTaq®qPCR Master Mix technology (Promega) according to the manufacturer's protocol. The PCR was carried out in triplicate on a 96-well plate using 7900HT fast real time PCR system (Applied Biosystems) with the following thermal profile: 2 min at 95°C; 40 cycles, 15 sec at 95°C, 1 min at 60°C. Primer sequences are reported in Supplemental Table 10. Data were analyzed using the 2-ΔΔCt method. For RNA sequencing, RNA Quality Control was performed on all RNA samples with an electrophoretic run on a Bioanalyzer instrument using the RNA 6000 Nano Kit (Agilent). RNA Integrity Number was determined for every sample and all the samples were considered suitable for processing based on the RNA integrity (RIN > 8). RNA concentration was estimated through spectrophotometric measurement using a Nanoquant Infinite M200 instrument (Tecan). Sequencing libraries were prepared using the TruSeq™ RNA Sample Preparation Kit (Illumina) using 1.8 ug of total RNA as input. Polyadenylated transcripts were purified using poly-T oligo-attached magnetic beads. PolyA RNA was fragmented at 94°C for 8 min and retrotranscribed using random hexamers. Multiple indexing adapters were ligated to the ends of the cDNA and the amount of DNA in the library was amplified with 10 PCR cycles. Final libraries were validated and quantified with the DNA1000 kit on the Agilent Bioanalyzer Instrument. Pooled libraries were sequenced on the Illumina Genome Analyzer IIx producing an average of 13 M reads per library.

3.6 Bioinformatics analysis

BaseCall files were converted to FastQ files using Casava 1.8.2. Sequencing reads were aligned to the mouse genome (mm10) using TopHat v.2.0.9. Transcripts were reconstructed and quantified using Cufflinks v2.1.1 and differential expression analysis was performed using CuffDiff [309]. CuffDiff uses the test statistics T = E[log(y)]/Var[log(y)], where y is the ratio of the normalized counts between two conditions. A t-test is used to calculate the P value for Differential Expression [310]. A threshold of 0.05 was applied to False Discovery Rate (FDR) adjusted p values in order to select the differentially expressed genes (DEGs) to use in downstream analysis; we also included genes with a log2 fold-change (lgFC) > 1 either showing one anomalous triplicate FPKM value and an FPKM average value between 1 and 2. Heat map of DEGs was made with Genesis software using triplicates mean, after normalization and log2 transformation. Cluster analysis was performed with the Genesis software tool using k-means clustering function (k=8) in order to identify group of genes with a similar regulation at 3 and 24 h of treatment [311]; genes with lgFC > +/-0.40 were selected. In each cluster of genes, the regulatory sequences in 20 Kb around the transcription start site were analyzed using iRegulon Cytoscape App and candidate transcription factors were predicted [312]. Overrepresentation analysis (ORA) on DEGs lists was performed using the Functional Annotation Tool in DAVID website [313]. The lists of DEGs at 3 and 24 h of estradiol treatment were used as input gene list and the mouse genome was used as background list. Biological processes, molecular functions and KEGG pathways were investigated focusing on enriched terms with a Benjamini adjusted p-value less than 0.05. A Protein-Protein Interaction Network of the differentially expressed genes has been created using STRING [280].

3.7 Statistical analysis

Unless otherwise stated, statistical significance was carried out with the GraphPad Prism version 5.02 for Windows by 1-way or 2-way ANOVA followed by Bonferroni post hoc test or unpaired t test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *versus* veh, and "n" indicates biological replicates.

Based on studies previously conducted by our group, we assessed a "signal / noise" ratio of approximately 1.2. Establishing a significance level of 5% and a potency equal to 80%, the minimum number of subjects for each experimental group more suitable to achieve the objectives of this project turns out to be greater than or equal to 12 [144]. For this reason we chose n=12 for icv experiments.

Instead for zymosan (n=3), BrdU (n=4-6) and RNA-Seq (n=3) studies, we have not followed this rule, but we used fewer animals for each experimental groups, because they are initial and pilot experiments that need further experiments to improve statistical significance.

RESULTS

Most of the information about the effects of estrogen on macrophages in literature is related to the estrogen ability to modify their response to immunological stimuli, such as LPS or IL-4, and show that estrogen treatment is able to induce a more efficient inflammatory response in macrophages in these models. Then, to deeper understand the physiological response of macrophages to estrogen alone, in our laboratory was previously performed a study of genome-wide gene expression analysis of macrophages exposed to estrogen. Since our previous studies carried out in laboratory demonstrated that estrogen receptors (ERα and Gpr30) expression is decreased in primary macrophages cultures maintained *in vitro* compared to *ex vivo* macrophages, mouse *in vivo* treatment was chosen in order to better resemble physiological conditions. Because estrogens are endogenous hormones fluctuating during the estrous cycle, for this study we used female mice selected through the analysis of vaginal smears in metaestrous (ME) phase, a phase of estrous cycle with low endogen levels of estrogen. These animals were then injected with a physiological dose of 17β-estradiol (5μg/Kg). This approach provides an experimental asset to more faithfully mimic the mechanisms induced by the physiological estrogen surge in intact animals.

Since macrophage activation is a dynamic process that leads macrophages to progressively adapt and modify their phenotype, we performed a time-course experiment, evaluating the effects on macrophage gene expression after short (3 hours) or long times (24 hours) from hormone treatment.

Furthermore, female mice identified in estrous phase, which immediately follows endogenous hormone peak production, were used to evaluate estrogen physiological effect (Figure 1).

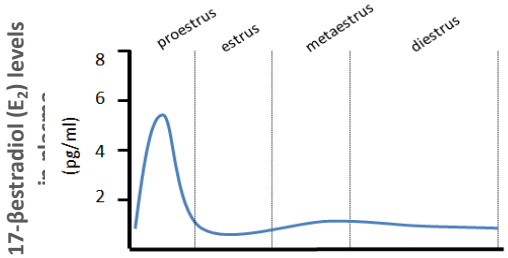


Figure 1. Plasmatic estrogen levels in female mice. Figure modified from Della Torre *et al*., 2016 [281]
17β-estradiol levels measured in the plasma of female mice during the estrous cycle, that in mice last four days.

Experimental groups and schematic representation of the study is shown in Figure 2. Briefly, following the peritoneal lavage, resident macrophages were isolated through magnetic immunosorting using antibody raised against cd11b loaded with magnetic beads. RNA was extracted from sorted macrophage cells and analyzed by RNA sequencing.

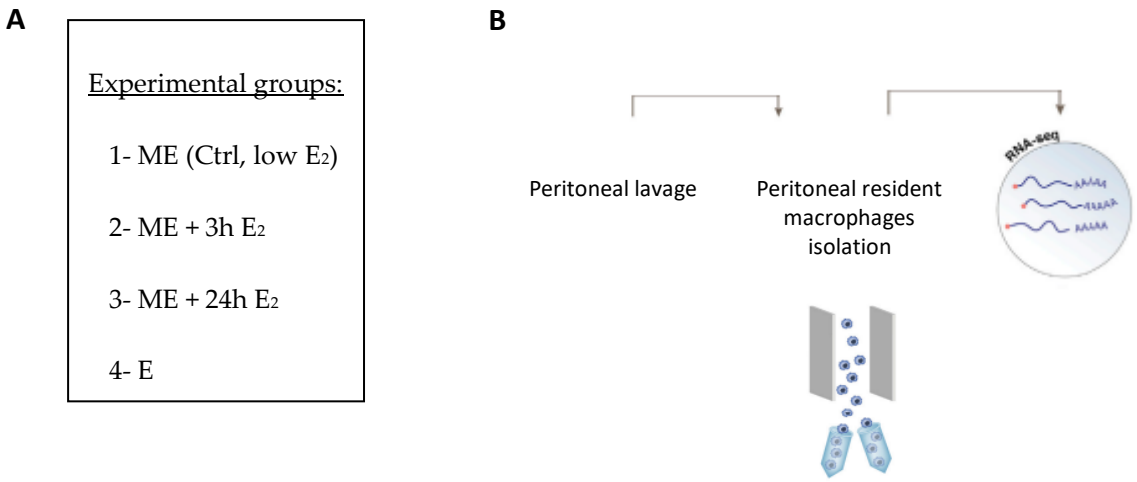


Figure 2. Genome-wide gene expression analysis of estrogen action on resident peritoneal macrophages. Figure modified from Lavin *et al*., 2014 [282] Schematic representation of experimental groups (A) and experimental design (B).

4.1 Bioinformatic analysis of macrophage genes modulated by estrogen

Firstly I performed bioinformatics analyses of the results obtained from RNA sequencing experiment, in order to evaluate the biological relevance of estrogen action on macrophages. In particular I focused on the differentially expressed genes (DEGs) analysis between the following groups,:

_ female at metestrous (ME) *vs* female at metestrous after 3 hours from E2 injection (ME_3hE2)

_ female at metestrous (ME) *vs* female at metestrous after 24 hours from E2 injection (ME_24hE2)

4.1.1 Differentially expressed genes (DEGs) in macrophages following E2 treatment

Our data show that short and long term hormonal treatments significantly affect expression levels of 569 transcripts in total. In particular, as shown in Figure 3A estrogen regulates 238 genes following 3 hours of treatment; of these ones, 114 genes are up-regulated and 124 are down-regulated. Instead, 24h hormone treatment modulates the expression of 441 DEGs, 217 up-regulated and 224 down-regulated. Moreover, 110 DEGs are regulated both 3 and 24 hours by estrogen treatment: 46 up-regulated, 63 down-regulated and only one gene (Itgb3) with opposite trend between the two experimental comparisons.

Heatmap in Figure 3B shows the graphical representation of the 569 differentially expressed genes in the 3 experimental groups: ME, ME_3hE2 and ME_24hE2.

Grouping up- or down-regulated DEG according to onset and duration of hormone action, we identified different expression profile that we can cluster into 4 groups by their expression pattern across experimental groups: cluster I) includes the early and transient expressed genes, induced or inhibited only at 3h of E2 treatment; cluster II) includes early and persistent responsive genes, similarly modulated at 3 and 24h; cluster III) gathers early and progressively DEGs, regulated at 3 h and furthermore at 24 h treatment; cluster VI) includes late expressed genes, up- or down-regulated only at 24h (Figure 3C). The differentially expressed genes (DEGs) list is shown in Supplementary Table 1. DEGs ranking is based on both their cluster belonging to and their estrogen responsiveness (LogFC).

Analysis of clusters kinetics showed a significant fraction of DEGs (35%; clusters I+II) behaving as early responsive genes, since it's already modulated at 3 h after E2

administration. Only a minor fraction of them returns to control levels after 24 h treatment (16%; cluster I), while the majority of all DEGs are modulated at this late time point (86%; clusters II+III+IV). Progressively responsive genes represent the highest number of regulated genes (37%; Cluster III), while genes regulated only at late time point constitute a high proportion of genes (28%; cluster IV) (Figure 1B).

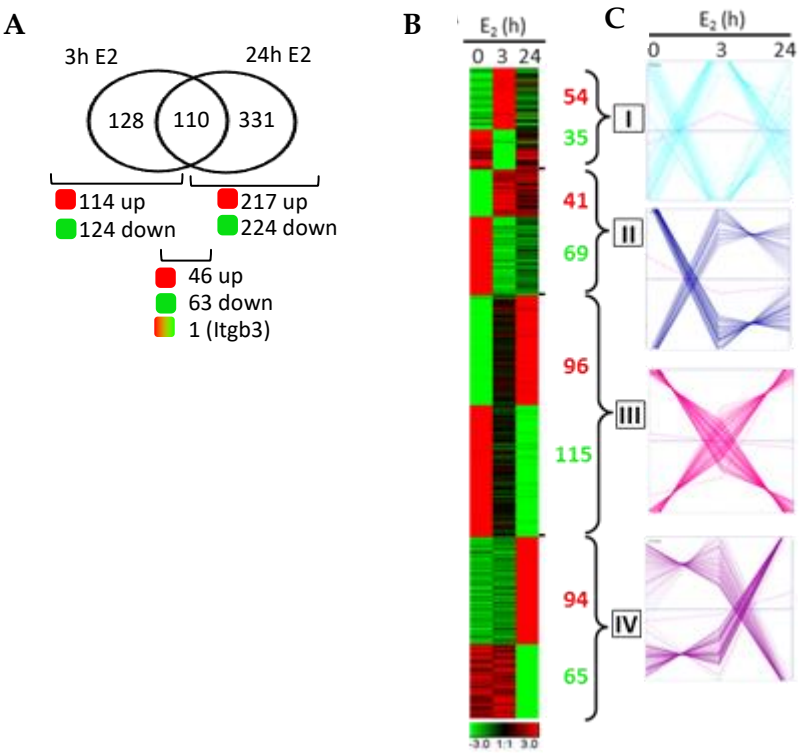


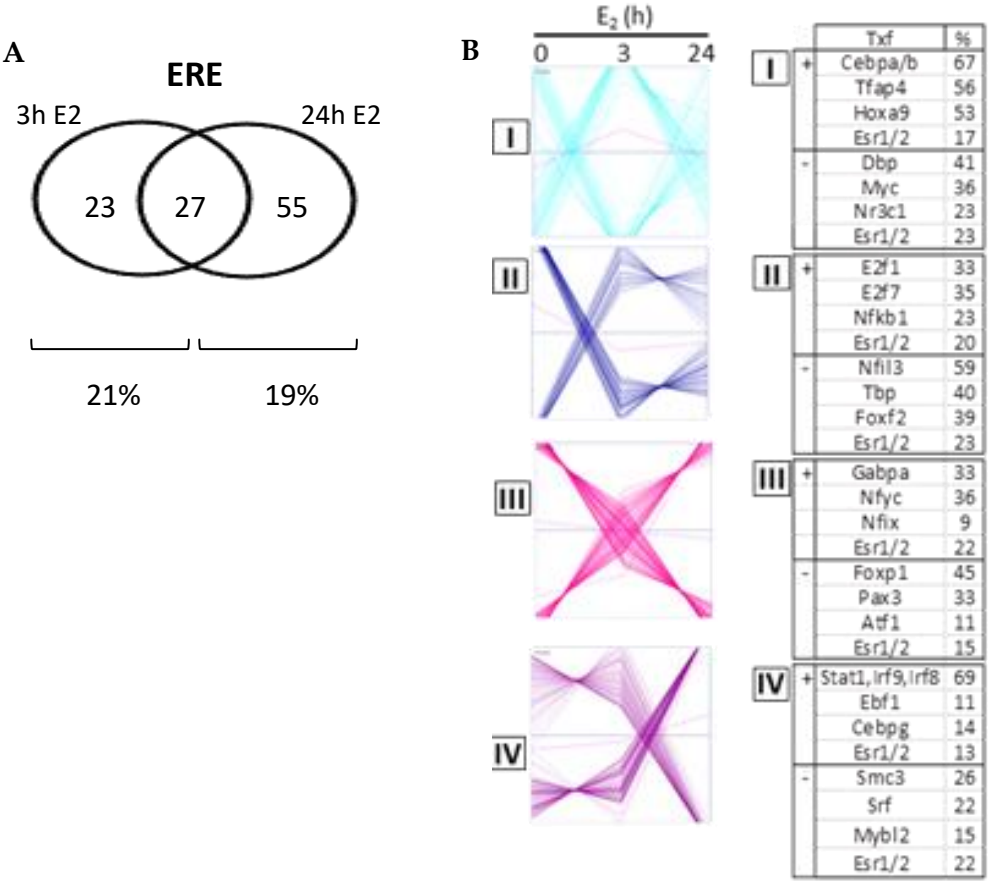
Figure 3. Gene clustering by temporal expression profile in response to *in vivo* E2 administration.
RNA sequencing analysis was performed on non-elicited peritoneal macrophages obtained from metaestrous (ME) female mice after *in vivo* treatment with vehicle (ME) or with E2 for 3h (3hE2) or 24 h (24hE2).
(A) Venn diagrams representing the overlap and the number of macrophage DEGs regulated by E2 after 3 and 24 hours of treatment. The number of up (red) and down (green)-regulated genes in each comparison are shown. (B) Heat map representing the expression profile of DEGs in the 3 experimental groups (red, high relative expression; black, mean expression; green, low relative expression). (C) DEGs were grouped in 8 clusters, according to the net expression profile calculated by k-means clustering. The clusters with the same absolute trend among the 3 experimental groups were merged. Relative expression levels for each gene in the different experimental groups are shown in relation to the mean expression values (magenta line).
The number of genes resulting in clusters a-d) are shown, together with the number of up (red) or down (green)-regulated genes.

4.1.2 Bioinformatic transcriptional analysis of DEGs

After I performed bioinformatic analysis to identify the main transcriptional factors involved in the regulation of DEGs expression in order to gain insight into the mechanisms of hormone responsiveness and individuate common regulatory networks.

To analyze ER binding sites, I referred to database of Bourdeau et al. [283] which identifies consensus EREs in mouse genome located up to approximately 10 kb from transcriptional start sites, through a genome-wide screening. Our data show cross-interference among different clusters, as this regulatory element is enriched with similar frequency of DEGs enhancers/promoters in all fours clusters (Figure 1C and Supplemental Table 1). Thus, these analyses support the hypothesis that early and late effects induced by estrogen on macrophage transcriptome are mediated by direct ERE-mediated mechanisms as well as by the ordered engagement of distinct sets of transcription factors that allow the specific temporal profile of gene regulation observed in response to estrogen.

Then, a motif enrichment analysis was conducted to search for transcriptional regulatory elements in the promoters of macrophage DEGs. Each cluster includes unique transcription factors that are involved either in the up- or down-regulation effects, with up-regulated genes in cluster I showing a high abundance of binding sites for C/EBP transcription factors, an enrichment for E2f binding sites in cluster II, and for Irf and Stat families of transcription factors in cluster IV (Figure 1C). In parallel, specific transcriptional regulators are involved also in the down-regulation of DEGs in a cluster-specific manner



		Txf	%
I	+	Cebpa/b	67
		Tfap4	56
		Hoxa9	53
		Esr1/2	17
	-	Dbp	41
		Myc	36
		Nr3c1	23
		Esr1/2	23
II	+	E2f1	33
		E2f7	35
		Nfkb1	23
		Esr1/2	20
	-	Nfil3	59
		Tbp	40
		Foxf2	39
		Esr1/2	23
III	+	Gabpa	33
		Nfyc	36
		Nfix	9
		Esr1/2	22
	-	Foxp1	45
		Pax3	33
		Atf1	11
		Esr1/2	15
IV	+	Stat1, Irf9, Irf8	69
		Ebf1	11
		Cebpg	14
		Esr1/2	13
	-	Smc3	26
		Srf	22
		Mybl2	15
		Esr1/2	22

Figure 4. Transcriptional analysis of macrophage DEGs promoters.
A) Venn diagrams representing the number of macrophage DEGs with at least one estrogen responsive element (ERE) in their promoters in each comparison. The percentages of DEGs with ERE on total DEGs for each comparison are shown. B) Transcription factors (Txf) that bind putative binding sites in up (+) or down (-)-regulated genes are listed for each cluster; the column on the right shows the number of genes with Txf binding sites in their promoters reported as the percentage of the total number of up (+) or down (-)-regulated genes in each cluster. Esr1/2: binding sites for estrogen receptors

Taken together, these data show that the *in vivo* administration of a physiological dose of E_2 results in the regulation of expression of a distinct set of genes in macrophages, an effect which is evident shortly after hormone administration and progresses with time through a balanced combination of positive and negative distinct or overlapping pathways.

4.1.3 Biological pathways regulated by 17β-estradiol in resident macrophages

In order to translate comprehensive genomic information into functional relevance, we performed functional annotation analyses using Gene Ontology term enrichment to search for significant terms related with biological programs. Enrichment of specific biologic categories (Supplemental Table 2) showed that, in analogy with gene expression, the time-course evaluation of estrogen action identifies biological programs that are either uniquely or commonly regulated as early or late responses to estrogen in macrophages (Figure 5A). Genes showing early and transient response to estrogen resulted enriched in specific gene ontologies including transcription factors, apoptosis, stress response, and protein folding, while the ontology related with lipid metabolism was enriched as a late responsive pathway. However, three functional programs clearly emerged as persistent estrogen-responsive pathways in macrophages: these relate with cell cycle, immune response, and wound healing. As shown in Figure 5B, a search for functional interactions using the String database resulted in a high number of connections among proteins encoded by genes involved in the Cell cycle, Immune response and Wound healing processes. The first category appears to be supported by the coordinated regulation of a core of highly interconnected genes, while the last two categories share a high number of proteins modulated by estrogen and are known to converge on the alternative polarization of macrophages (Figure 5B). Interestingly, some of the main transcription factors shown in Figure 1C are known to be involved in cell cycle progression, including C/EBPs, E2fs and Myc, or in the control of inflammation and macrophage polarization, including NF-kB, FOXP1 and Gabpa 16, further supporting the functional implications of estrogen action on macrophages and providing a first hint on the underlying molecular mechanisms. Altogether, these results show that estrogen induces in macrophages specific biological pathways related to distinct functional responses supported by an highly interconnected network of hormone-responsive genes (Figure 5B).

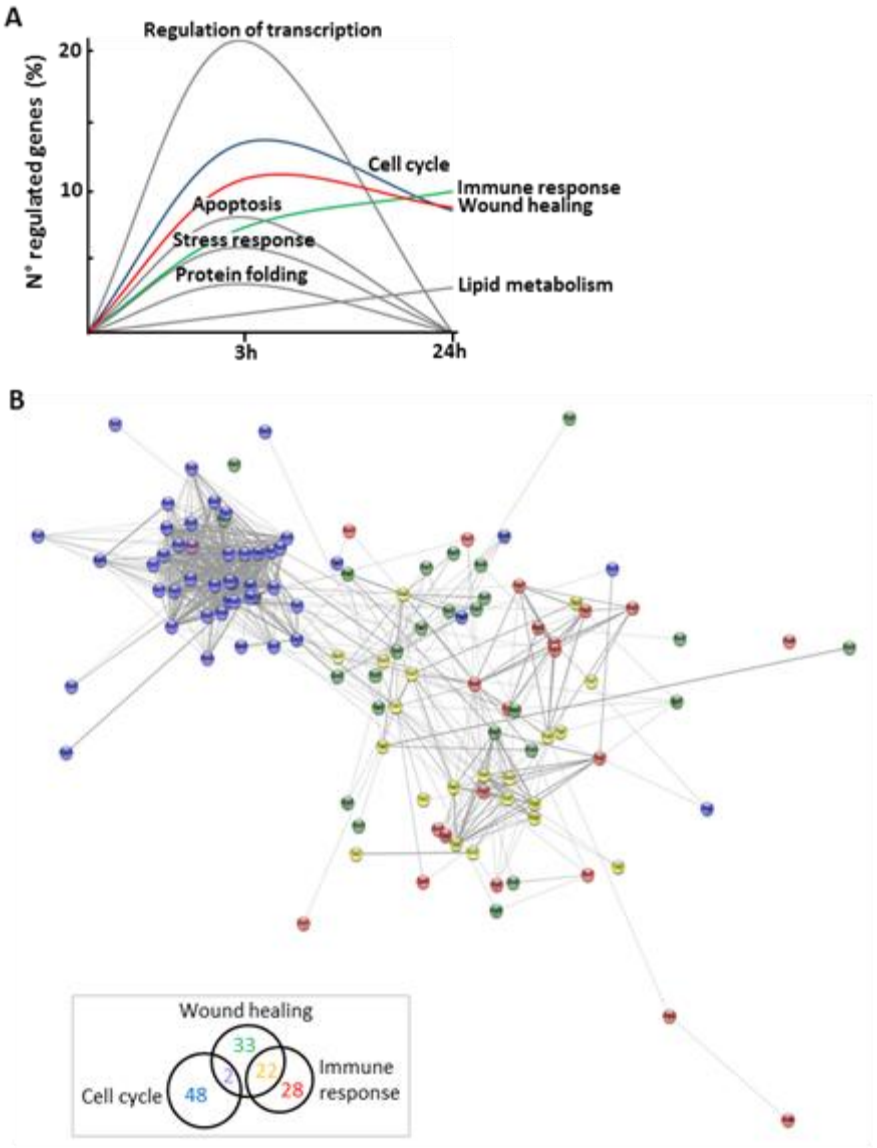


Figure 5. Biological pathways regulated by E2 in macrophages.
A) The DEGs list from RNA sequencing analysis was submitted to the DAVID database for functional annotation. GO ontologies significantly modulated following 3 or 24 h of estrogen treatment are represented, with the number of genes regulated in each ontology reported as % of all regulated genes at each treatment. Immune response includes Immune response, Chemotaxis and Endocytosis; Wound healing includes Response to wounding, Hemopoietic/lymphoid organ development and Blood vessel development; Lipid metabolism includes Positive regulation of lipid metabolic process, Cholesterol metabolic process and Lipid localization. Ontology gene lists are reported in Supplementary Table 2. **B)** String diagram of the interactions among regulated genes. Genes of the Cell cycle (blue), Immune response (green) and Wound healing (red) are evidenced, with commonly regulated genes resulting in purple (Cell cycle + Immune response) and yellow (Immune response + Wound healing). The Venn diagram in the insert shows the numbers of unique or common regulated genes.

In conclusion, these analyses revealed that E2 modulates the expression of genes that are involved in biological processes in a dynamic manner, as some pathways are modulated earlier and others later in estrogen action. In particular, our data suggested that estrogen could induce macrophage proliferation as well as trigger a phenotypic adaptation of resident macrophages as a physiological response to hormone signal.

4. 2 Estrogen is a physiological trigger of peritoneal macrophage cell cycle

Cell replication and polarization consistently emerged as hormone-responsive programs and were thus selected for deeper investigation. First we focused our attention on the study of the estrogen action on peritoneal macrophage proliferation. To this purpose, we performed a set of experiments to extend our observation by further modifying hormonal treatments and, more importantly, to evaluate the effects of changes in endogenous estrogen.

Thus, for this study, we carried out a time course experiment, treating ME female animals with E_2 for 3, 24 and 48 hours. Female mice at ME were used as control, instead females in estrous phase were added to evaluate peritoneal macrophages physiological response to the endogenous increase in estrogen levels.

Peritoneal macrophages extracted from these experimental groups were analyzed to assess estrogen action on macrophage proliferation through gene expression analysis and BrdU incorporation and Ki67 protein expression.

4.2.1 Cell cycle genes

First, the expression of genes associated to active replication, such as Ki67 and Ube2c, and cell cycle, such as Ccnb2 and Cdk1 (G1/S phase) has been analyzed through RealTime-PCR semi-quantitative analysis. Ct values of genes analyzed are shown in Supplementary Table 3A and 3B and are referred to two different independent experiments.

As shown in Figure 6, all these genes were induced by the exogenous treatment with estrogen, as early as 3 h after injection. Particularly, for all genes except Ki67, the highest induction was reached after 3 h of estrogen treatment and was followed by a substantial decrease at later time-points. Importantly, also the physiological increase in

endogenous sexual hormones is able to induce the expression of cell cycle and replication genes, as demonstrated by the significant increase in mRNA levels of these genes in estrous phase compared to metaestrous group.

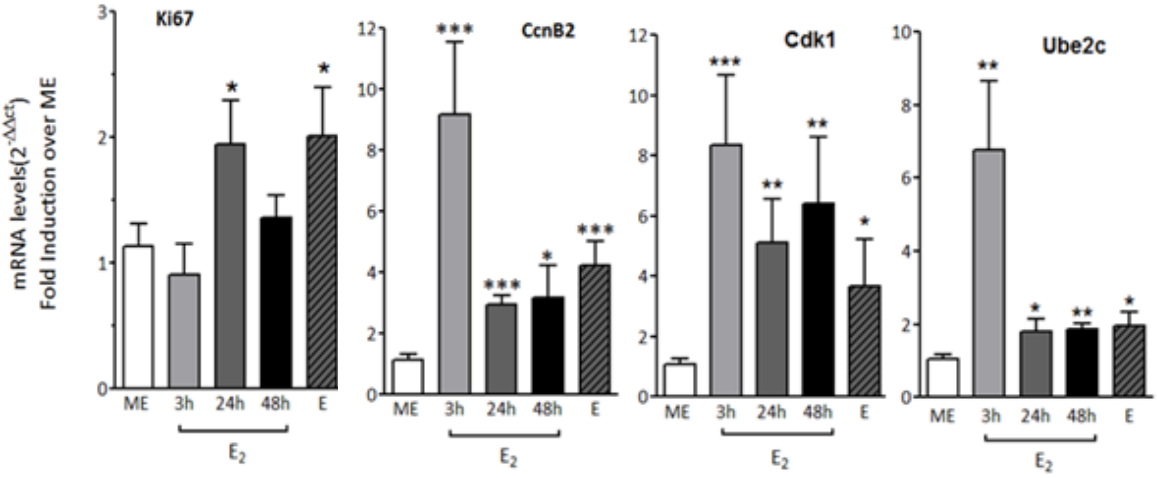


Figure 6. Validation of E2 target cell cycle genes in peritoneal macrophages *in vivo*.
Peritoneal macrophages were isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 3, 24 and 48 h. Females in the estrous phase (E) were also investigated. Real time PCR was performed to detect the mRNAs coding for genes related with cell cycle phases and active replication (CcnB2, Cdk1, Ube2c; Ki67). Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the control group (ME). Bars represent mean values ± SEM (n = 5-10). Student's unpaired t-test, *p<0.05 ; **p<0.01; ***p<0.001 *versus* ME.

4.2.2 Macrophage proliferation

As previously described in the introduction, recently it has been demonstrated that peritoneal macrophages, in analogy with macrophages that reside in other tissues, such as brain or liver, are maintained by local self-renewal without the contribution of the infiltration and differentiation of circulating monocytes. Considering the strong effect of estrogen on macrophage cell cycle gene expression, we speculate that estrogen can act as a proliferative signal for resident peritoneal macrophages.

So, in order to have more biological proof of the effect of estrogen on macrophage proliferation, we evaluate Ki67 protein expression and BrdU incorporation by FACS analyses of macrophages sorted from ME female mice untreated or treated with estrogen for 24 and 48 hours and female mice in E, as previously described.

According with the results of gene expression, a significant increase in the fraction of Ki67+ peritoneal macrophages was obtained from animals treated with E_2 (Figure 7A and B) and, more importantly, also in female mice at the estrous phase (Figure 7B).

Even more evident was the effect of E2 on DNA synthesis (Figure 7C and D), which we measured by BrdU incorporation. BrdU is a nucleotide that is incorporated in the DNA during cell replication; we injected BrdU in the peritoneum of mice 2h before their sacrifice and analyzed its presence in the nucleus of *ex vivo* sorted macrophages by FACS using an antibody raised against BrdU. E2 significantly increased the percentage of cells that were duplicating their DNA following 24 h of treatment. However BrdU incorporation was not increased in the other experimental groups, suggesting that the effect on DNA duplication was restricted to a specific time window of E2 action encompassing the first 24 h after treatment without being detected in the estrous phase or at a later time point (48 h; Figure 7D), when the increased number of cells progressing in the cell cycle (assessed by Ki67 expression in Fig. 7B) is probably too small to allow the detection of cells within the S-phase. Of note, the amplitude of estrogen action on BrdU incorporation is similar to that reported for IL-4, the most well-characterized signal inducing local expansion of peritoneal macrophages in inflammatory conditions [174].

Moreover, these results lead us to hypothesize that the number of peritoneal macrophages is increased following estrogen treatment. To this purpose, number of macrophages extracted from the experimental groups previously described was assessed by evaluating the number of cd11b+ cells isolated from each sample through magnetic immunosorting. Figure 7E shows the percentage of cd11b+ cells on total number of cells isolated from peritoneum. As expected, the number of cd11b+ cells was significantly increased following 24 h of estrogen treatment and, more importantly, also in E phase (Figure 7E; Supplementary Table 4). However, there was no significant increase in the number of macrophages at the later time point (48 h), suggesting that in response to estrogen macrophages proliferate and subsequently undergo apoptosis or migrate in other tissues.

These results clearly show that estrogen is a physiological trigger of macrophage proliferation, leading a 8% of cells to replicate and proliferate.

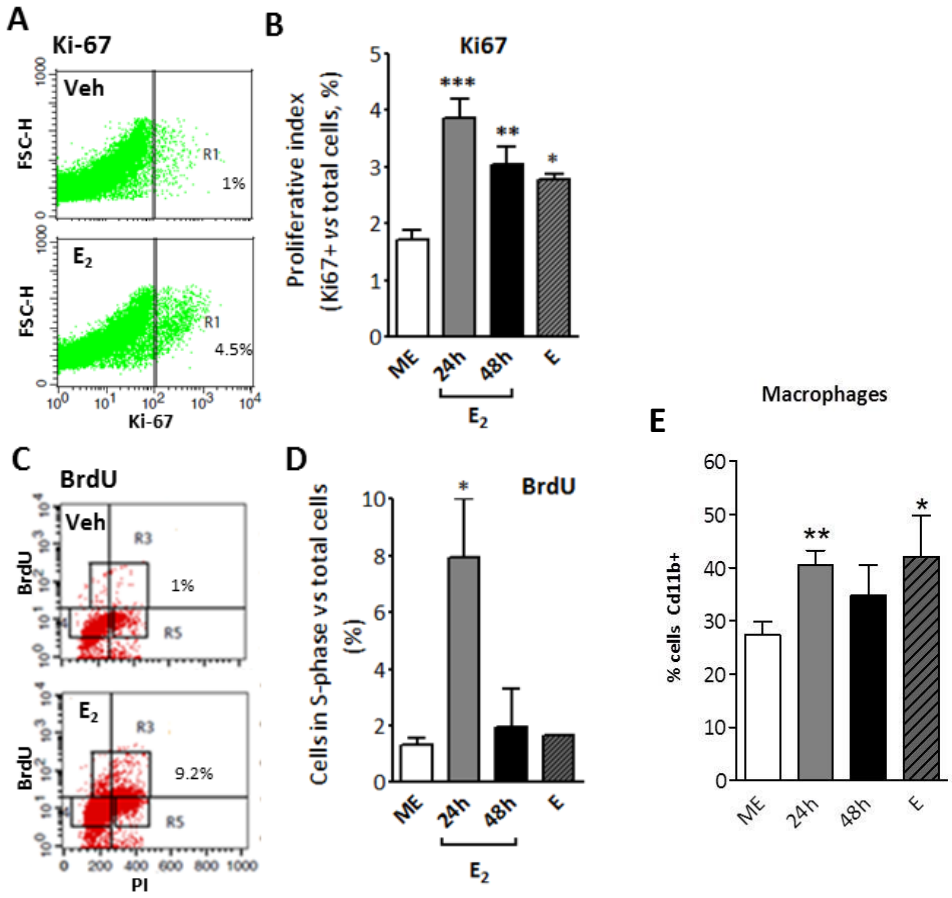


Figure 7. Estrogen induces macrophage proliferation.

(A-D), Proliferation was analyzed by evaluating Ki67 expression and BrdU incorporation by peritoneal macrophages from female mice at ME or 24 and 48 h after E2 treatment. Females in the estrus phase (E) were also analyzed. Mice were injected i.p. with BrdU 2 h prior to analysis. Representative dot plots depicting gating schemes for Ki67 and BrdU analysis are shown in panels A and C, respectively. Doublets were removed based on FL2 scatter width (FL2-W)/FL2 scatter area (FL2-A). Bar charts show the percentage of Ki67 (B) and BrdU (D) positive macrophages. Data are representative of three independent experiments. Graphs represent the mean ± SEM of 4-9 mice per group. (E), Bar charts represent the percentage of cd11b$^+$ cells isolated from the peritoneal lavage fluid. Results are expressed as the mean ± SEM (n=4-9).

Student's unpaired t-test, *p < 0.05; °; **p<0.01; ***p<0.001 versus ME.

.

4.2.3 Ovariectomy affects macrophage population

From these data we reasoned that, if estrogen is involved in peritoneal macrophage self-renewal, we would expect that the number of peritoneal macrophages was reduced following estrogen deprivation. To this aim, we analyzed the number of macrophages in female mice that underwent ovariectomy, in order to eliminate the endogenous production of sex hormones. Cell peritoneal populations, in particular, macrophages, neutrophils, monocytes and eosinophils, were analyzed by FACS analysis evaluating the expression of specific markers. Gating strategy is shown in Supplementary Figure 1. Our gene expression and flow cytometry data also confirmed that increased estrogen

levels do not change the expression of leukocyte markers such as CD11b and F4/80 (Supplemental Table 1 and Supplemental Figure 2)

As expected, ovx animals showed a significant reduction in the number of resident peritoneal macrophages as compared to sham-operated animals, and this effect was completely reverted by estrogen replacement (Figure 8). While other leukocyte populations remained unchanged (Figure 8), indicating a specific action of estrogen on macrophage proliferation.

Taken together, these observations demonstrate that estrogen acts as a proliferative signal for resident peritoneal macrophages

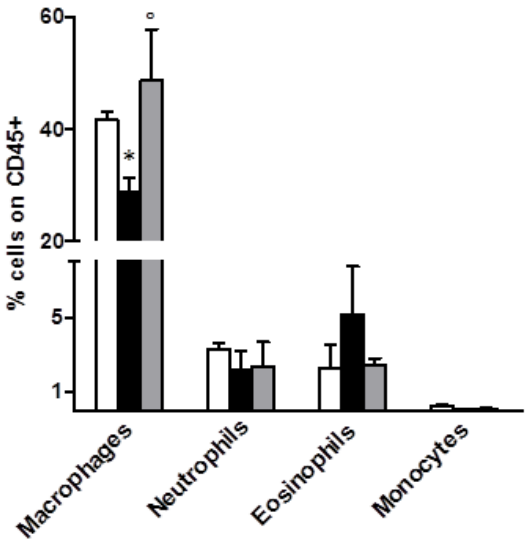


Figure 8. Long-term exposure to exogenous E2 induces macrophage proliferation in ovx animal

Ovariectomized female mice were treated with vehicle (ovx, black bars) or E2 (grey bars) for 60 h. Sham-operated mice treated with vehicle were used as controls (open bars). The peritoneal lavage fluid was analyzed by flow cytometry to evaluate the percentage of macrophages and other immune cells, as indicated. Results are expressed as the mean ± SEM (n=3). Student's unpaired t-test, *$p < 0.05$ versus sham; °$p < 0.05$ versus ovx.

4.3 Estrogen is a physiological trigger of M2 peritoneal macrophage polarization

In addition to the cell cycle ontology, our bioinformatics analyses suggested other ontologies to be responsive to estrogen: macrophage immune polarization and wound healing. These two functional categories are associated with the alternative polarization of macrophages, a process involved in the resolution of inflammation and in tissue repair.

Thus, we performed a set of experiments, in order to deeper understand the effect of estrogen on macrophage alternative polarization.

4.3.1 M2 macrophage polarization genes

First, We evaluated the expression of genes involved in M2 polarization, such as Arg1 Ym1 and Vegfa, following estrogen administration. The expression of these genes has been analyzed, through RealTime-PCR semi-quantitative analysis, on peritoneal macrophages isolated from female mice at ME or following E2 treatments for 3, 24 and 48 h and female in E, as previously described. Ct values of genes analyzed are shown in Supplementary Table 5A and 5B and are referred to two different independent experiments.

As shown in Figure 9, estrogen significantly increases the expression of these genes already after 3 hours of estrogen treatment, where was reached the highest induction. Moreover, for all these genes the effect was still observed at extended time points. Remarkably, also the endogenous increase in estrogen levels, that is present in the females in the estrous phase, resulted in significant higher levels of the mRNA coding for Arg1 and Vegfα.

These data suggested that estrogen was able to trigger a macrophage phenotype that resembled the alternative polarization induced by immune mediators, like IL-4.

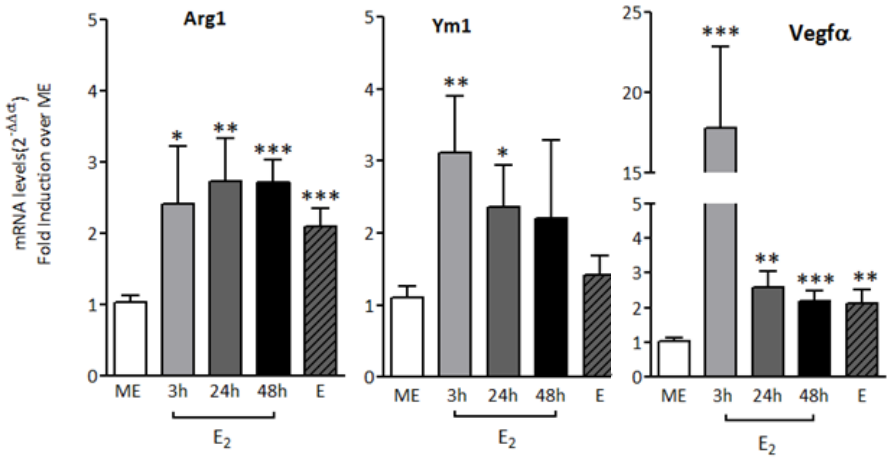


Figure 9. Validation of E2 target M2 polarization genes in peritoneal macrophages *in vivo*.
Peritoneal macrophages were isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 3, 24 and 48 h. Females in the estrous phase (E) were also investigated. Real time PCR was performed to detect the mRNAs coding for genes related with M2 polarization (Arg1, Ym1, Vegfα).
Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle group (ME). Bars represent mean values ± SEM (n = 5-10). Student's unpaired t-test, *p<0.05 ; **p<0.01; ***p<0.001 *versus* ME.

4.3.2 Macrophage phenotypic adaptation following E2 action

It is known that the induction of one phenotypic state can change with time and progress towards distinct phenotypes that allows tissue remodeling and resolution of inflammation. We thus asked whether also estrogen was able to operate this phenotypic conversion.

In order to evaluate macrophage polarization as a dynamic response to estrogen, ovariectomized mice were repeatedly administered with estrogen for prolonged periods of time (36 and 60 hours) and macrophages were sorted from the peritoneum. The expression of genes associated with activation state were analyzed by RealTime-PCR.

As shown in Figure 10, ovariectomy did not alter the basal expression of Arg1, Tgm2, and Vegfα mRNAs. However, as expected, estrogen induced a phenotypic adaptation of macrophages, as demonstrated by the expression of selected M2 genes, such as Arg1, Tgm2 and Vegfa, that are significantly increased after 36 h and return to basal levels after 60 h E2 treatment, suggesting a reduced estrogen responsiveness at this late time point or a phenotypic transition of macrophages to the resolution phase.

In fact, at this longer period of time, estrogen induced the expression of IL-10, a key mediator in the resolution phase of inflammation as shown by the significant increase in animals treated for 60 h with estrogen as compared to sham or ovx groups.

Moreover, according to our previous data, a significant induction of Ki67 expression, was observed in estrogen treated animals.

These results demonstrated that macrophage response to estrogen dynamically changes the expression programs to support the alternative polarization of resident macrophages and its progression towards a pro-resolution state.

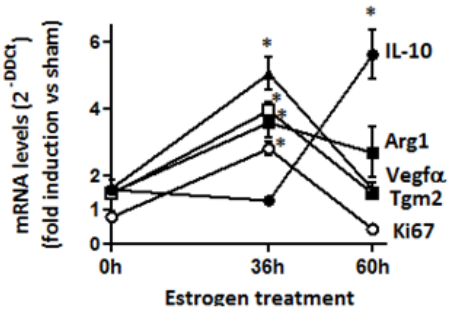


Figure 10. Estrogen long exposure induce macrophage phenotypic adaptation.
Peritoneal lavage fluid was collected from sham-operated (not shown), ovariectomized (ovx, shown as time 0) or ovx mice treated with E2 for 36 or 60 h. Macrophages were immunosorted and analyzed by real time PCR for gene expression. Data show the expression levels of genes related with proliferation (Ki67, open circles), alternative activation (Arg1, filled squares; Tgm2, open squares) and wound healing (Vegfα, triangles; IL-10, filled circles). Values were calculated using the 2^{-ddCt} method and normalized for the value of the sham group used as reference (=1) for each gene. Results are expressed as the mean ± SEM (n=3). Student's unpaired t-test, *$p<0.05$ versus ovx.

4.3.3 Estrogen promotes macrophage alternative polarization in an *in vivo* model of self-resolving inflammation

In order to better understand the functional relevance of estrogen action on macrophage, we performed an experiment using an acute inflammatory animal model that is peritonitis induced by zymosan injection.

Zymosan is a polysaccharide component of the yeast wall that induce activation of the immune cells, characterized by the activation of inflammatory response followed by the resolution of inflammation. The first phase of inflammation is driven by M1 macrophage, mostly derived from monocyte recruitment following zymosan injection. The resolution phase, instead, is driven by the different subtypes of M2 macrophages that lead to tissue repair and restore homeostasis.

For this experiment, we used ovariectomised animals to block estrogen endogenous production; these animals were treated with vehicle or E2 immediately before zymosan injection and after 24 and 48 h. Sham-operated animal were used as control.

At different time points (12, 36 and 60 hours), we evaluated cell populations recruited in the peritoneum trough FACS analysis and extracted RNA specifically from sorted macrophages for gene expression analysis. Moreover, secreted factors involved in macrophage polarization, such as Tnfα (M1) and Vegfα and IL-10 (M2) was measured in peritoneal fluid of these experimental groups, through ELISA assay.

Facs analyses in sham experimental group show the typical alteration of cell populations in zymosan animal model during the progression of inflammation, with reduction of macrophage number following their increasing in time to resolve inflammation (triangles, Figure 11C) and, on the contrary, the increase of neutrophils (PMN) that decrease during disease. Gating strategy for the detection of the different peritoneal cell populations is shown in Supplementary Figure 1.

The absence of estrogen didn't affect the number of PMN, monocytes and macrophages (open cycle, Figure 11A, B and C). But, also in this model estrogen treatment increased the number of resident peritoneal macrophages following 60 h of estrogen administration. Moreover estrogen treatment didn't affect the number of neutrophils and monocytes indicating a direct effect of estrogen on resident macrophages, not due to an increase of monocytes recruitment.

Gene expression analysis clearly shows an effect of estrogen on M2 polarization (Figure 11 E-H). In fact, estrogen potentiates Arg1 and Vegfa after 36 h treatment and their expression is reduced e after 60 h. In agreement with this anti-inflammatory effect of

estrogen, TNFα expression, an M1 gene, is increased by hormone loss and strongly reduced by estrogen treatment. Moreover IL-10 expression is significantly higher in animals treated for 60 h with estrogen as compared to sham or ovx groups.

Analysis of protein expression of Vegfα, IL-10 and TNFα in peritoneal fluid of experimental samples partially confirm gene expression results (Figure 11 H-L). In fact, even if Vegfa is not increased following estrogen treatment, maybe due to the high biological difference in the 3 replicates or to the sensibility of the assay, TNFα is significantly increased in ovx groups and reduced following estrogen treatment. According to gene expression, also IL-10 is increased by estrogen following 60 hours of treatment, although not in significantly way (Figure 11I, Supplementary Table 6A,B,C).

These results demonstrated that during local inflammation estrogen dynamically with time induces programs to support an increase in number of resident macrophages and their alternative polarization to sustain the resolution phase, in agreement with our previous data.

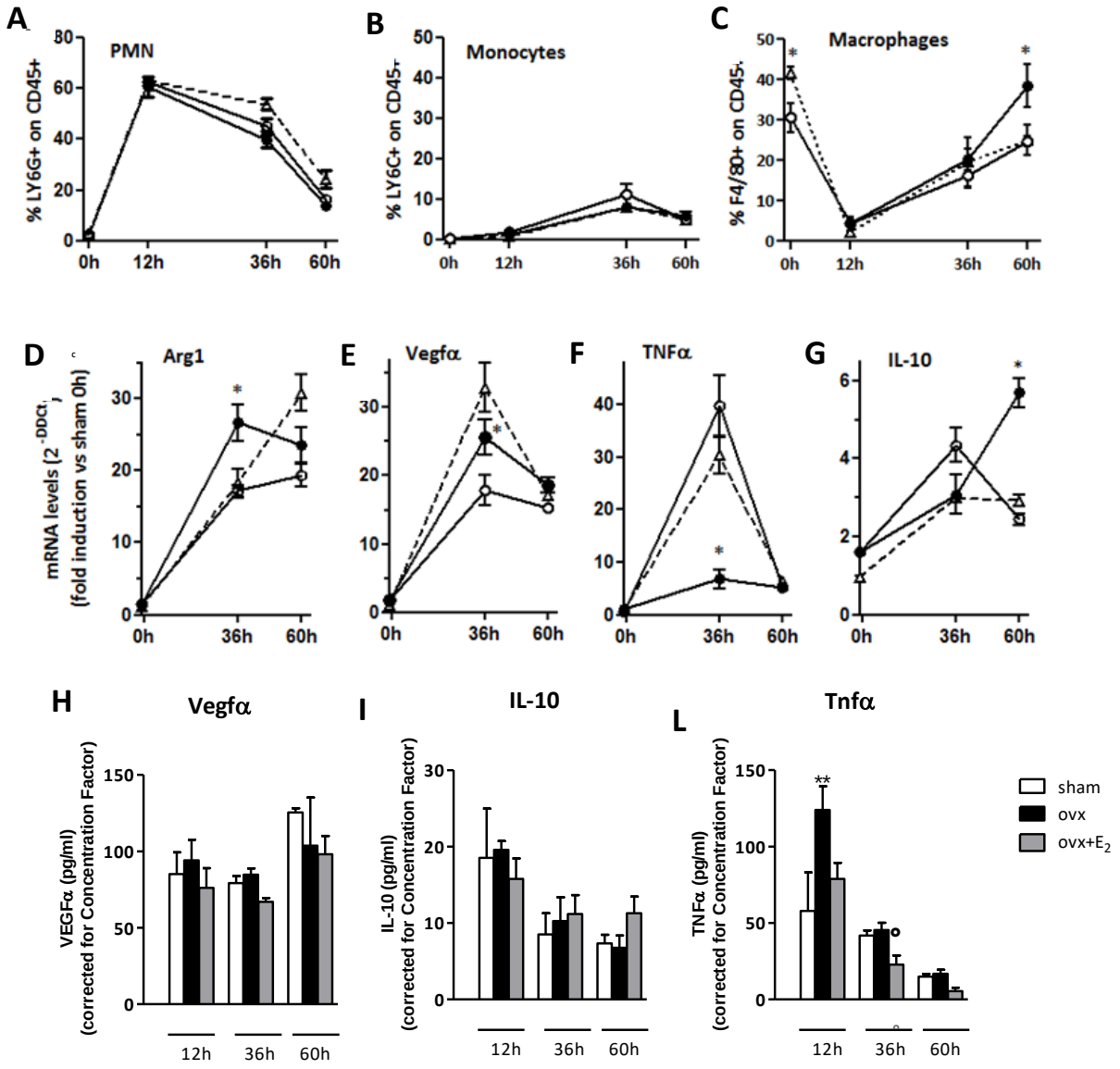


Figure 11. Estrogen induces resident macrophage polarization.

(B-H) Sham-operated (triangles), ovariectomized (open circles), or ovariectomized and treated with E2 (filled circles) animals were injected with zymosan and the number of peritoneal macrophages, polymorphonuclear cells (PMN) and monocytes was analyzed by flow cytometry at different time points (panels B-D, respectively). Immunosorted macrophages were analyzed by real time PCR for the expression of genes, as indicated (panels E-H). Values of the sham group were used as reference (=1) and are not shown; ovx values are shown at time 0. Data were calculated using the 2^{-ddCt} method and normalized for the value of the sham group for each gene. Results are expressed as the mean ± SEM (n=3). Student's unpaired t-test, *p<0.05 versus ovx.

(H-L) Protein concentration of Vegfα, IL10 and TNFα was evaluated in peritoneal fluid.

Results are expressed as the mean ± SEM (n=3). Student's unpaired t-test, **p<0.01 versus sham; ° p<0.05 versus ovx.

4.4 Molecular mechanism of estrogen action on macrophages

We believed it was necessary to develop a model of macrophages in culture, in order to investigate the mechanism of estrogen action on macrophages and study in future the drug activity on this pathway. To this purpose, bone marrow derived macrophages (BMDMs) are a useful model that are a widely used and easy availability, although there may be some differences due to the *in vitro* system. We thus prepared primary cultures of BMDMs, treated with estrogen and analyzed target gene expression in comparison with peritoneal macrophages. Ct values obtained from RealTime PCR analysis are reported in Supplementary Table 7.

As shown in Figure 12A, transcripts coding for Angtpl-4 and VEGFα, an ERE-positive genes (Supplementary Table 1), were modulated by increasing concentrations of estrogen in isolated macrophages. Interestingly, levels of transcript coding for Arg1, a cluster II gene with no ERE in its promoter/enhancer region, were also significantly increased by estrogen treatment, though the overall effect of estrogen was significantly lower.

Since effect of estrogen in isolated macrophages was similar between the two different populations of macrophages and to that observed *in vivo* (Supplemental Table 1), we used primary cultures of BMDMs for further analysis, using ERα-KO animals and assessed whether the observed effects are dependent on this specific receptor .

Ablation of ERα gene resulted in loss of estrogen action in macrophages, as estrogen-induced changes in the mRNA levels coding for Vegfα, Angtpl4 and Arg1 observed in wild-type cells were not detected in ERα-KO macrophages (Figure 12B).

These results demonstrate that estrogen may directly act on macrophages to modulate gene expression and this effect is mediated by the activation of ERα signaling and allow further studies of SERMs activity on these cells.

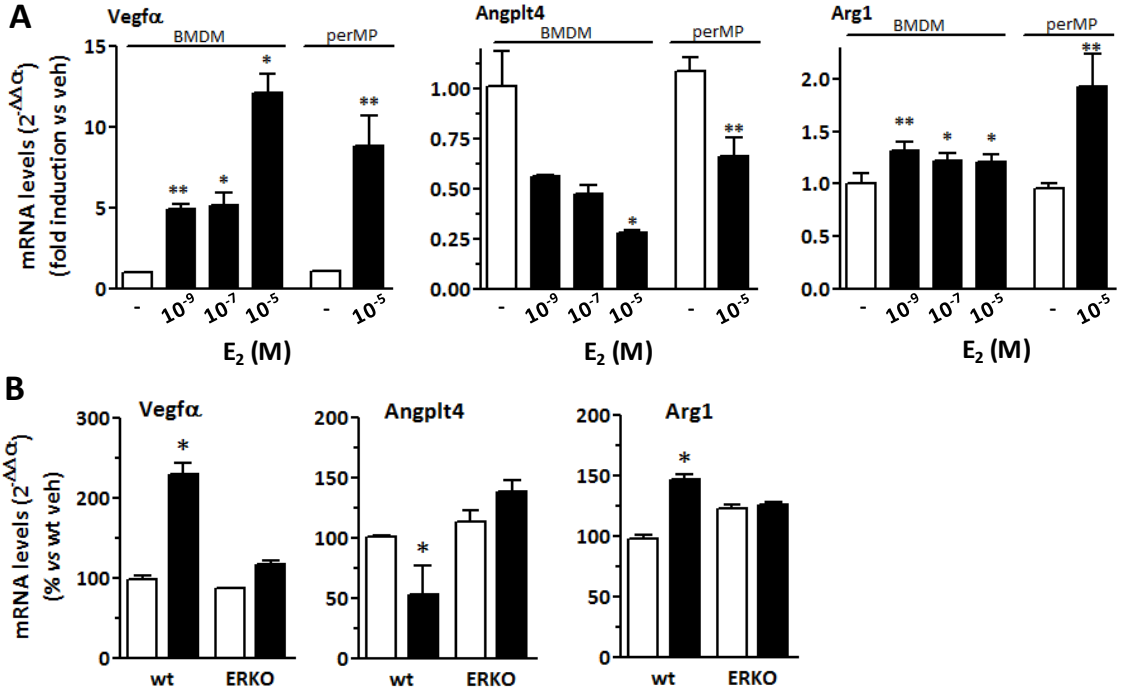


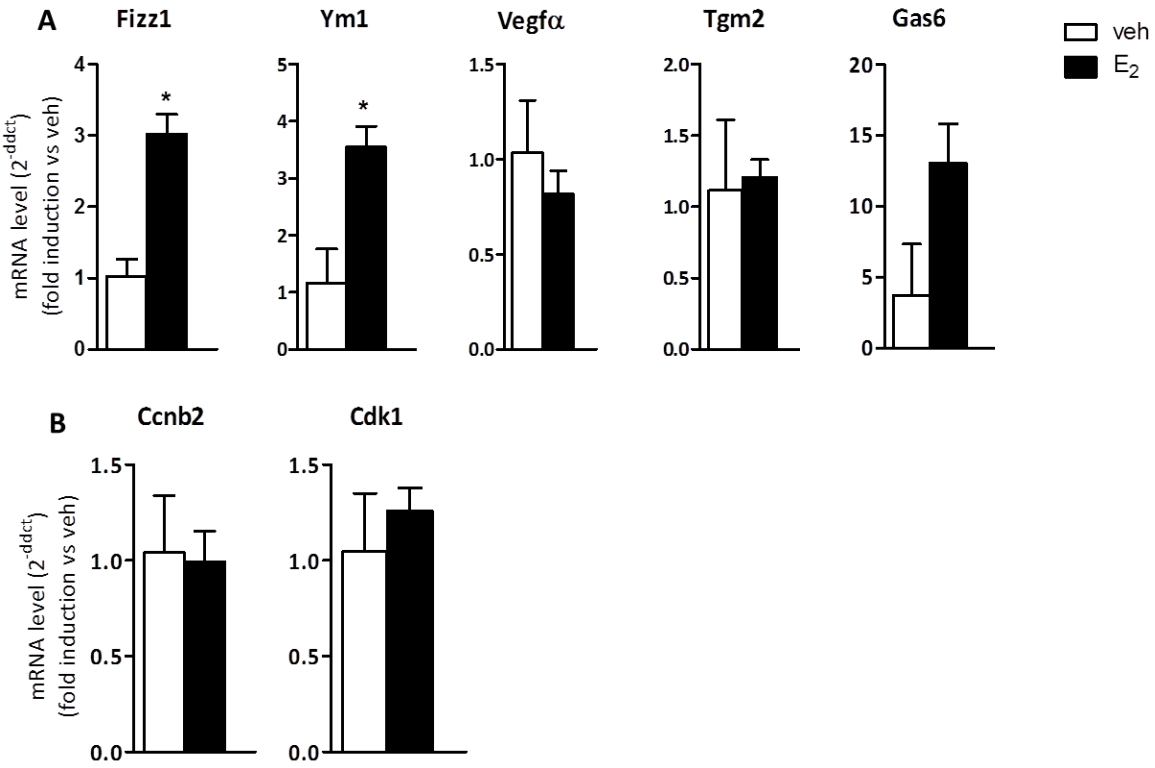
Figure 12. Regulation of target gene expression by E_2 in isolated macrophages.
BMDM were grown in culture and assayed for gene expression following E2 administration. (A) BMDM cells were treated with vehicle (veh, open bars) or increasing concentrations of E2 (10^{-9}, 10^{-7} and 10^{-5} M, filled bars) for 3 h. Real time PCR was used to analyze the mRNA levels coding for Vegfα, Angtpl4 and Arg1. (B) Comparison of estrogen action in BMDM cells from WT and ERα-KO (ERKO) mice. The mRNA levels coding for Vegfα, Angtpl4 and Arg1 were analyzed 24 h after the addition of vehicle (veh, open bars) or 10^{-7} M E2 (filled bars). Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle group. Bars represent mean values ± SEM (n=2). Student's unpaired t-test, *p<0.05; **p<0.01 versus veh.

4.5 Microglia response to the estrogen surge

Macrophages are widely distributed in almost all tissues of the body where they perform innate immune reactions; however, it has been shown that the microenvironment is also important for shaping macrophage metabolism and reactivity. Leading to resident macrophages with specific functions and responsiveness to signals. We thus were interested in extending our observations and evaluating the degree of conservation of estrogen signaling in different tissue resident macrophages. We chose microglia cells for our investigations, since some studies, also conducted in our laboratory, reported that these cells are responsive to estrogen which modifies cell activation leading to a reduced M1 in favor of an M2 activation state [223, 307]. Moreover, we also set up the experimental procedures to evaluate microglia M2 polarization following IL4 injection (Pepe *et al.*, 2014 [314]). Since microglia M1 activation is a common feature of different neurodegenerative diseases and it contributes to tissue damage and neuronal death, induction of M2 microglia polarization by estrogen might counteract chronic inflammation and slow disease progression. However, the molecular targets of estrogen action in microglia are still poorly understood. In order to find biomarkers of estrogen action in microglia we thus decided to evaluate the microglia expression of some of the genes validated in my previous experiments in response to in vivo estrogen administration..

To this purpose, female mice identified in ME were treated with physiological dose of E2 or veh for 24 h. Isolated microglia was analyzed for the gene expression of those genes regulated by the hormone, found trough genome wide analysis previously performed (Supplementary Table 1). Ct values obtained from RealTime PCR analysis are reported in Supplementary Table 8. As shown in Figure 13, estrogen significantly induce the expression of some M2 markers, such as Fizz1 and Ym1, but no Vegf*α* and Tgm2. Instead, estrogen modulates Gas6 expression as well as in peritoneal macrophages (Supplemental Table 1). Instead, no effect on cell cycle genes was observed in E2 treated samples.

These data show that in response to E2 microglia acquire a M2-like phenotype, although inducing a different panel of M2 markers as compared to peripheral macrophages. However, differently to peritoneal macrophages, microglia are not able to proliferate in response to E2.



13. Validation of E2 target genes in microglia *in vivo*.

Microglia was isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 24 h. Real time PCR was performed to detect the mRNAs coding for genes related with (**A**) M2 polarization (Fizz1, Ym1, Vegfα, Gas6) and (**B**) cell cycle (Ccnb2 and Cdk1).

Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the control group (ME). Bars represent mean values ± SEM (n = 2-5). Student's unpaired t-test, *p<0.05 versus ME.

4.5.1 Microglia and macrophage response to IL-4

Microglia local proliferation is a new concept in neurology and neuroinflammation, however it has not yet been studied whether microglia is able to proliferate following stimulation with IL-4, the typical M2 activation signal, which instead is known to induce the proliferation of peripheral macrophages, such as peritoneal cells [119, 130].

Since E2 previous results showed that microglia are not able to proliferate after E2 treatment, differently from what happens for peritoneal macrophages, we tried to understand if microglia was able to proliferate in response to another M2 signal.

Thus, we evaluated proliferative response of microglia following IL-4, which is known to induce macrophage proliferation in the periphery.

To this purpose, we used icv injection of IL-4, a model that we recently set up in our laboratory in order to study M2 microglia polarization in mice (Pepe *et al*. [314]).

Our results show that IL-4 central injection is not able to induce cell cycle genes expression in the brain, while, as expected, IL-4 is able to induce macrophage proliferation in the peritoneum, as demonstrated by the significant increase in mRNAs coding for genes related to the cell cycle, such as Ccnb2, Cdk1 and Ki67 (Figure 14A). In microglia IL-4 is able to efficiently induce the M2 phenotype, as demonstrated by the increase in Arg1. (Ct values obtained from RealTime PCR analysis are reported in Supplementary Table 9).

These results indicate that IL-4, the prototypical M2 signals, similarly to E2, is not associated with the proliferation of microglia cells, in contrast to its activity on peritoneal macrophages.

Taken together these results demonstrate that estrogen has a different effect on microglia as compared to that exerted on peripheral macrophages (peritoneal macrophages and BMDMs) (Supplementary Table 1 and Figure 6,9,12), suggesting that microenvironment crucially affect macrophage responsiveness.

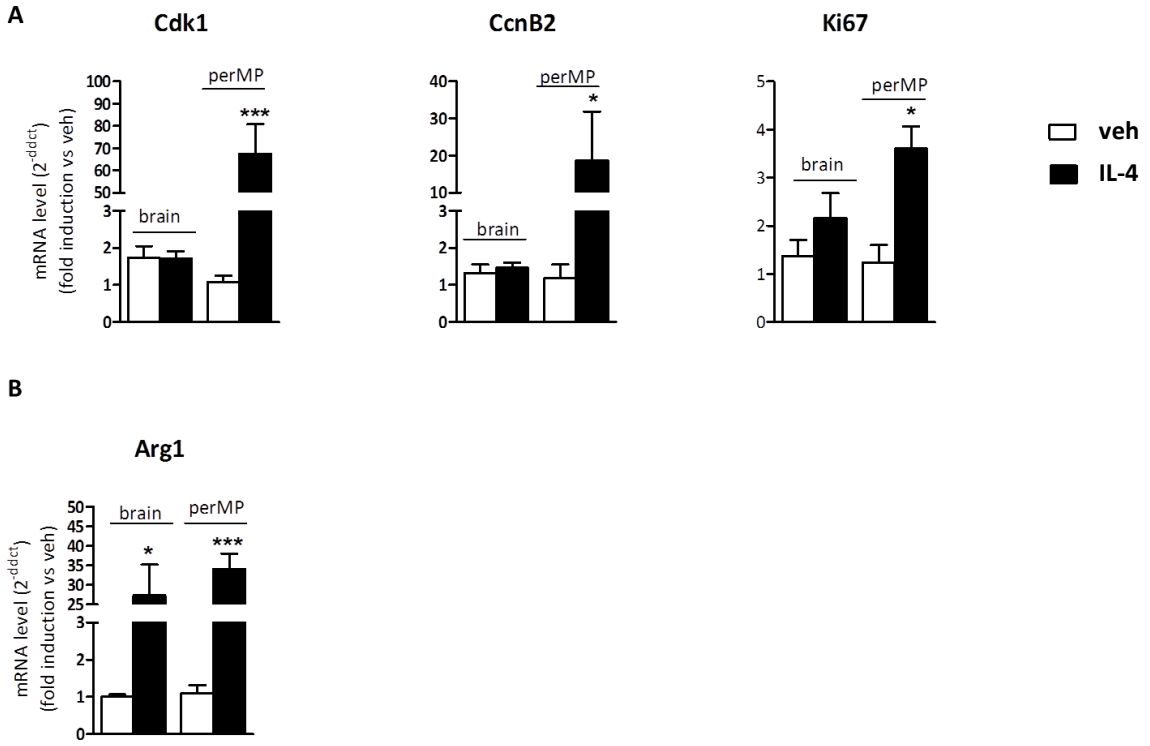


Figure 14. Regulation of cell cycle genes by IL-4 in peritoneal macrophages and brain microglia *in vivo*.
Animals were treated for 24h with IL-4 or veh through icv injection for brain analysis or i.p. for peritoneal macrophages analysis. Real time PCR was performed to detect the mRNAs coding for genes related with (**A**) cell cycle(Cdk1, Ccnb2 and Ki67) (**B**) M2 polarization (Arg1) in brain and peritoneal macrophages.
Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle group (ME). Bars represent mean values ± SEM (n = 2 for peritoneal macrophages, n = 12 for brain). Student's unpaired t-test, *p<0.05 ; **p<0.01; ***p<0.001 versus ME.

DISCUSSION

A combination of mechanisms, including direct genomic and epigenetic effects as well as interactions with transcription factors, co-regulators, cytoplasmic signaling pathways and intercellular communications, have been shown to support the effects of estrogen on the immune system [133, 284-285]. This integrated analysis of the *in vivo* response of macrophages to estrogen has revealed the existence of a physiological endocrine-immune crosstalk that links estrogen action with proliferation of resident macrophages and their immunophenotypic adaptation, thus contributing relevant insights to our understanding of estrogen impacts on the regulation of the inflammatory response. The experimental conditions and transcriptomic approach adopted have led to a detailed temporal prospective of this endocrine-immune interplay and have identified a comprehensive list of molecular mediators involved.

The biological pathways most prominently modulated included macrophage proliferation, immune response, transcription factor expression, amino acid utilization, and energy metabolism, and on a whole indicated a key role for estrogen as a physiologic immunoregulatory factor for macrophage biology. Though the identification of the molecular mechanisms underlining the genomic events here reported require future investigation, our study reveals the identity and temporal responsiveness of a comprehensive set of novel estrogen-responsive genes (Supplemental Tables 1 and 2) that represent useful biological indicators of hormone signaling in macrophages.

It has recently been demonstrated that peritoneal macrophages are maintained by local self-renewal, with a low level of proliferation throughout life regulated by growth factors and immune mediators including CSF-1 and IL-4 [114, 118-119, 286-288]. Functional annotation of DEGs suggested that estrogen also acts as a proliferative signal for resident macrophages. From a physiological point of view, our results provide a novel view of estrogen action in peritoneal homeostasis, in which macrophages are stimulated by estrogen to adapt and develop specific immune regulatory functions, possibly to cope with estrogen-induced changes in the environment. The nature of these concomitant events may include ovulation, with the need of increased macrophage number and activation in order to handle the wound healing response associated with follicle rupture and the induction of an immunotolerant environment which either favors egg fertilization and further implantation or eliminates dead cells and tissue debris resulting from luteal degradation. The advantage for estrogen to induce resident

macrophage proliferation instead of monocytes recruitment is unclear. As the estrogen surge occurs every 4-5 days in mice and it is perpetuated throughout the fertile life of females, it is possible that such circumstances direct the request of resident macrophages towards the expansion of local cells, a process that is energetically more favorable than the constant increase of hematopoietic precursors and their migration into the peritoneum [97]. Interestingly, our data also show that genes related with lipid metabolism are modulated by the hormone. Although not addressed in the present study, this evidence represents an additional indication of the metabolic adaptation of macrophages induced by estrogen which might serve the role of directing cholesterol and phospholipid metabolism towards the formation of membranes required for cell proliferation.

Macrophage proliferation has been observed in selected murine and human tumors [289-290], including peritoneal neoplasia [291-292] and in Th2 inflammatory conditions [130], with variable involvement of IL-4 and CSF-1 depending on the specific experimental conditions [293]. Though a deep investigation of the underlying molecular mechanisms was precluded as estrogen proven to be unable to support macrophage proliferation *in vitro*, similar to what observed for IL-4, in our experimental conditions a significant induction of CSF-1 or IL-4 was not observed, arguing against the hypothesis that the estrogen effect is mediated by these mediators. Indirect mechanisms involving other peritoneal cells able to respond to the hormone cannot be excluded [284, 186], however we observed that EREs are present in the promoter of several cell cycle-related genes identified in the present study, including Chaf1a, CcnB2 and Wee1 [283], suggesting a direct binding of estrogen to the promoter of proliferation-related genes in macrophages.

This study also provides the first demonstration that *in vivo* estrogen induces a phenotype resembling alternative macrophage activation that further converts towards a pro-resolving phenotype, as shown by changes induced by hormone replacement on polarization gene expression culminating with the induction of a key immunosuppressive cytokine typically expressed by macrophages during the resolution phase of inflammation, namely IL-10. Importantly, results obtained in the zymosan peritonitis model reveal that these mechanisms are maintained during an acute inflammation of the peritoneum. These results are in agreement with previous studies which suggested that estrogen is involved in macrophage activation and

polarization in experimental models of inflammation [191, 221, 294] or in isolated macrophages/cell lines challenged with specific immune stimuli [223, 295-296]. Our study provides a major advancement to this knowledge as it proves estrogen to be per se a physiological regulator of the number and reactivity of resident macrophages in intact healthy animals, in the absence of confounding factors such as recruited circulating monocytes achieved in wound healing or in different models of peritonitis.

An abundant number of peritoneal macrophages showing a dysregulated polarization have also been involved in endometriosis, a pathological condition caused by the ectopic growth of endometrial cells in the peritoneum, [229, 297-298]. In particular, estrogen is known to drive detrimental effects on endometrial lesion development and progression through its well-recognized activity on endometrial cells [299-300]. Macrophages are involved in growth, sustainment and vascularization of endometriosis lesions, and their ability to switch from inflammatory to anti-inflammatory and resolving macrophages is altered in endometriosis [229, 301-302]. In this respect, the present description of the estrogen-macrophage signaling has intriguing implications for our understanding of endometriosis pathogenesis and therapeutic approaches, and candidate macrophage estrogen-responsive genes identified in this study as potential biomarkers in this clinical setting. Furthermore, the expression of the progesterone receptor is either absent or very limited in peritoneal macrophages, being below the detection limits in our assay. On the contrary, the progesterone receptor is highly expressed in endometrial cells and used as target of current therapeutic interventions for endometriosis that use progesterone analogues to block ER activity. The lack of progesterone receptor in peritoneal macrophages leaves estrogen action unopposed by progestin drugs in these immune cells, with possible reduction in therapeutic efficacy. Although the mechanisms provided in the present work suggest detrimental effect on certain pathologies, such as endometriosis, these events might be involved in the powerful suppressive influence exerted by estrogen in other inflammatory pathologies, such as those affecting the CNS or the lung [303-304].

Several neurodegenerative diseases are associated with an increased inflammation due to a chronic or unrestrained M1 activation of microglial cells that produce a wide array of chemokines, cytokines, reactive oxygen species that results in neurotoxicity. Together with activation, an increased proliferation of activated microglia has also been observed in association with acute CNS injury and chronic degenerative diseases. It is

hypothesized that dysregulated proliferation of microglia plays a detrimental role on neural cells in some neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis, as demonstrated by experimental approaches in which microglia proliferation has been blocked. Thus, targeting microglia proliferation may offer a new therapeutic strategy to selectively modulate disease outcome. On the other hand, an increased number of microglia with pro-resolving phenotype might have beneficial effects on disease progression; however, the ability of microglia to undergo alternative activation and proliferate in response to M2 signals in brain is not yet understood. In this scenario, our data showed that microglia, differently from peripheral macrophages, are not able to undergo proliferation in response to estrogen or IL-4, although these signals are able to induce a M2-like phenotype. We do not know the explanation of this phenomenon, but we propose two hypothesis. The first one is that microglia express peculiar transcriptional regulators which are different from those expressed by peripheral macrophages and are engaged in the E2 response only in microglia cells. A second hypothesis envisions that the different microenvironment signals instruct microglia to respond differently to M2 agents, causing proliferation to be dependent upon additional brain specific brain specific factors.

Our results suggest that the modulation of estrogen signaling in macrophages might represent an important therapeutic strategy to develop new drugs for pathologies where estrogen-macrophage interplay may be crucial for disease development and progression, such as endometriosis, cancer and neurodegenerative diseases, and provide a list of E2 target genes that can be used as biomarkers for pharmacological and translational studies. This can lead to the design of more selective anti-inflammatory agents for pathologies characterized by chronic inflammation, such as neurodegenerative diseases, that, instead to act only blocking inflammatory response, improve the resolution phase of inflammation, or to develop of selective modulators of estrogen signaling to limit M2 macrophage deleterious effects in pathologies like endometriosis and tumors.

In conclusion, this study represents a key advancement for the understanding of the impact of estrogen on macrophage physiology and identifies novel mechanisms and underlying molecular players of potential pathophysiological relevance.

Future goals will be to deeper study the mechanisms activated by ERs, by which estrogen modulate macrophage physiology, particularly proliferation and polarization, for example the activation of PI3K/Akt signaling or Gpr30 involvement, in order to identify novel drug targets and leading to the design of more selective anti-inflammatory agents. In light of the wide use of estrogenic drugs in therapeutic settings, it will also be interesting to understand the pharmacological activity of SERMs in macrophages, that is, whether these molecules act as agonists or antagonists of estrogen receptors, leading to predict a modulatory activity on inflammation also for this drugs.
It will also be interesting to translate the data obtained in human pathology and study estrogen role in pathologies in which macrophage proliferation and polarization participate to disease progression, such as endometriosis and tumor.

ACKNOWLEDGMENTS

I would like to express my gratitude to Prof. Elisabetta Vegeto who conceived the study, guided my work and gave me the opportunity to grow and broaden my knowledge.
I want to specially thank Prof. Adriana Maggi who contributed to my professional growth.
I thank Alessandro Villa for his help in the development of new techniques and his valuable advice and Giorgia Calderazzi for her contribution.
I want to thank Prof. Massimo Locati and Cristina Barlassina and their team, who contributed to this research, particularly Tiziana Renzi who performed facs analysis of zymosan experiments, Lorenzo Drufuca who performed ELISA assay, Matteo Massara for his experimental advice and Daniele Braga who performed RNA sequencing and bioinformatics analyses.
Finally, I thank Clara Meda and Monica Rebecchi for technical assistance and all students, particularly Chiara Sgaragli, Stefania Locatelli, Elisa Alessandrini, Federica Mornata, Francesca Moro and Marta Frigeni, for their contribution in this research, by carrying out some analysis.

This work was supported by grants from the European Union's Seventh Framework Program (FP7/2007-2013; grant no. 278850 INMiND; grant no. 281608 TIMER), the CARIPLO Foundation (grant no. 2011–0591) and University of Milan that funded my PhD fellowship.

BIBLIOGRAPHY

1. Tata, J.R. One hundred years of hormones. *EMBO reports* **6**, 490–496 (2005).
2. Hall, J.E. Guyton and Hall textbook of medical physiology. 12th ed.Philadelphia, PA. *Saunders/Elsevier* (2011).
3. Nilsson, S. & Gustafsson, J. Estrogen receptors: therapies targeted to receptor subtypes. *Clin Pharmacol Ther* **89**, 44-55, doi:10.1038/clpt.2010.226 (2011).
4. Brinton, R.D. Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: recent insights and remaining challenges. *Learn Mem* **8**, 121-133 (2001).
5. Lee, S.J. & McEwen, B.S. Neurotrophic and neuroprotective actions of estrogens and their therapeutic implications. *Annu. Rev. Pharmacol. Toxicol.* **41**, 569-591 (2001).
6. Maggi, A., Ciana, P., Belcredito, S. & Vegeto, E. Estrogen in the nervous system: mechanisms and nonreproductive functions. *Annu. Rev. Physiol.* **66**, 291-313 (2004).
7. Rao, M.L. & Kolsch, H. Effects of estrogen on brain development and neuroprotection--implications for negative symptoms in schizophrenia. *Psychoneuroendocrinology* **28**(Suppl2), 83-96 (2003).
8. Bjornstrom, L. & Sjoberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol*. **19**, 833–842 (2005).
9. Nilsson, S., Mäkelä, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M. & Gustafsson, J. Mechanisms of estrogen action. *Physiol Rev* **81**, 1535-1565 (2001).
10. Hillier, S.G., *et al*. Follicular oestrogen synthesis: the `two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol* **100**, 51–54 (1994).
11. Cui, J., Shen, Y. & Li, R. Estrogen synthesis and signaling pathways during ageing: from periphery to brain. *Trend Mol Med* **19**(3), 197–209 (2013).
12. Simpson, E.R. Source of estrogen and their importance. *J Steroid Biochem Mol Biol* **86**, 225-230, doi:10.1016/S0960-0760(03)00360-1 (2003).
13. Labrie, F. Extragonadal synthesis of sex steroids: intracrinology. *Ann Endocrinol* (Paris) **64**, 95-107 (2003).
14. Carreau, S., *et al*. Role of estrogens in spermatogenesis. *Front Biosci* (Elite Ed) **4**, 1–11 (2012).
15. Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S. & Marc, J. The many faces of estrogen signaling.*Biochem Med* (Zagreb) **24**(3), 329-42, doi: 10.11613/BM.2014.035 (2014)
16. Stricker, R., *et al*. Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol, and progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT analyzer. *Clin Chem Lab Med* **44**, 883–887 (2006).
17. Birkhauser, M. Treatment of pain in estrogen deficiency. *Arch Gynecol Obstet* **259**(Suppl 1), S74–79 (1996).
18. Santen, R.J., *et al*. History of aromatase: saga of an important biological mediator and therapeutic target. *Endocr Rev* **30**, 343–375 (2009).

19. Simpson, E.R., *et al*. Aromatase expression in health and disease. *Recent Prog Horm Res* **52**,185–213 (1997).
20. Toda, K., *et al*. Transcriptional regulation of the human aromatase cytochrome P450 gene expression in human placental cells. *J Steroid Biochem Mol Biol* **53**, 181–190 (1995).
21. Harada, N., *et al*. Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc *Natl Acad Sci* USA **90**, 11312–11316 (1993).
22. Charlier, T.D., *et al*. Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology* **152**, 4199–4210 (2011).
23. Green, S. & Chambon, P. Estradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. *Nature* **325**(6099), 75-8 (1987).
24. Mosselman, S., Polman, J. & Dijkema, R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* **392**(1), 49-53 (1996).
25. Greene, G.L., *et al*. Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**, 1150–1154 (1986).
26. Green, S., *et al*. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320**, 134–139 (1986).
27. Kuiper, G.G., *et al*. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci* USA **93**, 5925–5930 (1996).
28. Gosden, J.R., *et al*. Localization of the human oestrogen receptor gene to chromosome 6q24----q27 by in situ hybridization. *Cytogenet Cell Genet* **43**, 218–220 (1986).
29. Enmark, E., *et al*. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* **82**, 4258–4265 (1997).
30. Couse, J.F. & Korach, K.S. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20**, 358–417 (1999).
31. Drummond, A.E. & Fuller, P.J. The importance of ERbeta signaling in the ovary. *J Endocrinol* **205**(1), 15-23, doi:10.1677/JOE-09-0379 (2010).
32. Christian, C.A., *et al*. Classical estrogen receptor alpha signaling mediates negative and positive feedback on gonadotropin-releasing hormone neuron firing. *Endocrinology* **149**, 5328–5334 (2008).
33. Walf, A.A., *et al*. Estradiol or diarylpropionitrile administration to wild type, but not estrogen receptor beta knockout, mice enhances performance in the object recognition and object placement tasks. *Neurobiol Learn Mem* **89**, 513–521 (2008).
34. Laudet, V. & Gronemeyer, H. The Nuclear Receptor. *FactsBook* Cambridge, MA, USA: Academic Press (2001).
35. Aagaard, M.M., Siersbaek, R. & Mandrup, S. Molecular basis for gene-specific transactivation by nuclear receptors. *Biochimica et Biophysic Acta* **1812**, 824–835, doi:10.1016/j.bbadis.2010.12.018 (2011).

36. Hilser, V.J, & Thompson, E.B. Structural dynamics, intrinsic disorder, and allostery in nuclear receptors as transcription factors. *Journal of Biological Chemistry* **286**, 39675–39682, doi:10.1074/jbc.R111.278929 (2011).
37. Brelivet, Y., Rochel, N. & Moras, D. Structural analysis of nuclear receptors: From isolated domains to integral proteins. *Molecular and Cellular Endocrinology* **348**, 466–473, doi:10.1016/j.mce.2011.08.015 (2012).
38. Helsen, C., Kerkhofd, S., Clinckemalie, L., Spans, L., Laurent, M., Boonen, S, Vanderschueren D, & Claessens, F., Structural basis for nuclear hormone receptor DNA binding. *Molecular and Cellular Endocrinology* **348**, 411–417, doi:10.1016/j.mce.2011.07.025 (2012).
39. Wärnmark, A., Treuter, E., Wright, A.P. & Gustafsson, J.A. Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. *Mol Endocrinol* **17**(10), 1901-9 (2003).
40. Le Romancer, M., Poulard, C., Cohen, P., Sentis, S., Renoir, J.M. & Corbo, L. Cracking the estrogen receptor's posttranslational code in breast tumors. *Endocrine Reviews* **32**, 597–622, doi:10.1210/er.2010-0016 (2011).
41. Kim, H.M, Yu, Y.& Cheng, Y. Structure characterization of the 26S proteasome. *Biochimica et Biophysica Acta* **1809** 67–79, doi:10.1016/j.bbagrm.2010.08.008 (2011).
42. Huang, P.C., Chandra, V. & Rastinejad, F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. *Annual Review of Physiology* **72**, 247–272, doi:10.1146/annurev-physiol-021909-135917 (2010).
43. Katzenellenbogen, B.S., Choi, I.H., Delage-Mourroux R., Ediger, T.R., Martini, P.G., Montano, M., Sun, J., Weis, K., & Katzenellenbogen, J.A. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. *Journal of Steroid Biochemistry and Molecular Biology* **74**, 279–285, doi:10.1016/S0960-0760(00)00104-7 (2000).
44. Koide, A., Zhao, C., Naganuma, M., Abrams, J., Deighton-Collins, S., Skafar, D.F., & Koide, S. Identification of regions within the F domain of the human estrogen receptor α that are important for modulatin transactivation and protein-protein interactions. *Molecular Endocrinology* **21**, 829-842, doi:10.1210/me.2006-0203 (2007).
45. Yang, J., Singleton, D.W., Shaughnessy, E.A., & Khan, S.A. The F-domain of estrogen receptor-α inhibits ligand induced receptor dimerization. *Molecular and Cellular Endocrinology* **295**, 94–100, doi:10.1016/j.mce.2008.08.001 (2008).
46. Kumar, R., Zakharov, M.N., Khan, S.H., Miki, R., Jang, H., Toraldo, G., Singh, H., Bhasin, S. & Jasuja, R. The dynamic structure of the estrogen receptor. *Journal of Amino Acids* **2011**, 812540, doi:10.4061/2011/812540 (2011).
47. Arao, Y., Hamilton, K.J., Coons, L.A. & Korach, K.S. Estrogen receptor α L543A, L544A mutation changes antagonists to agonists which correlates with the ligand binding domain dimerization associated with DNA binding activity. *Journal of Biological Chemistry* **288**, 21105-21116, doi:10.1074/jbc.M113.463455 (2013).
48. Hewitt, S.C., Winuthayanon, W. & Korach, K.S. What's new in estrogen receptor action in the female reproductive tract. *J Mol Endocrinol* **56**(2), R55-71, doi:10.1530/JME-15-0254 (2016).

49. Pettersson, K. & Gustafsson, J.A. Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **63**, 165-92, doi:10.1146/annurev.physiol.63.1.165 (2001).
50. Thomas, P., Pang, Y., Filardo, E.J. & Dong, J. Identity of an estrogen membrane receptor coupled to a G protein in human breat cancer cells. *Endocrinology* **146**(2), 624–632, doi:10.1210/en.2004-1064 (2005).
51. Levin, E.R. Plasma membrane estrogen receptors. *Trends Endocrinol Metab* **20**(10), 477–482, doi:10.1016/j.tem.2009.06.009 (2009).
52. Revankar, C.M., Cimino, D.F., Sklar, L.A. Arterburn, J.B. & Prossnitz, E.R. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**, 1625–1630, doi:10.1126/science.1106943 (2005).
53. Bourque, M., Dluzen, D.E. & di Paolo, T. Neuroprotective actions of sex steroids in Parkinson's disease. *Front neuroendocrinol* **30**(2), 142-57, doi:10.1016/j.yfrne.2009.04.014 (2009).
54. Smith, D.F & Toft, D.O. Steroid receptors and their importance. *Mol Endocrinol* **7**, 4-11 (1993).
55. Lodish, H., Berk, A., Kaiser, C.A., Krieger, M., Scott, M.P., Bretscher A., *et al*. Molecular cell biology. 6th ed. New York: WH Freeman and Company (2008).
56. Clementi, F. & Fumagalli, G. Farmacologia generale e molecolare. Section: "I recettori intracellulari" Maggi, A. & Vegeto E. *UTET* (2001).
57. Kurebayashi, S., Miyashita, Y., Hirose, T., Kasayama, S., Akira, S. & Kishimoto, T. Characterization of mechanisms of interleukin-6 gene repression by estrogen receptor. *J Steroid Biochem Mol Biol* **60**, 11-7 (1997).
58. Galien, R. & Garcia, T. Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-κB site. *Nucleic Acids Research*, **25,** 2424-9 (1997).
59. Ray, A., Prefontaine, K.E. & Ray, P. Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem* **269**(17), 12940-6 (1994).
60. Ray, P., Ghosh, S.K., Zhang, D.H. & Ray, A. Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor. *FEBS Lett* **409**(1), 79-85 (1997).
61. Philips, A., Chalbos, D. & Rochefort, H. Estradiol increases and anti-estrogens antagonize the growth factor-induced activator protein-1 activity in MCF7 breast cancer cells without affecting c-fos and c-jun synthesis. *J. Biol. Chem*. **268**(19), 14103-8 (1993).
62. Maggiolini, M., donzé, O., Jeannin, E., Andò, S. & Picard, D. Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha. *Cancer Res* **59**(19), 4864-9 (1999).
63. Kato S., H. Endoh, Y., Masuhiro, T., Kitamoto, S., Uchiyama and *et al*. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**, 1491-4 (1995).

64. Björnström, L., & Sjöberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* **19**, 833-42, doi: 10.1210/me.2004-0486 (2005).
65. Ajj, H., Chesnel, A., Pinel, S., Plenat, F., Flament, S. & Dumond, H. An alkylphenol mix promotes seminoma derived cell proliferation through an Eralpha36-mediated mechanism. *Plos One* **8**, e61758, doi:10.1371/journal.pone.0061758 (2013).
66. Filardo, E.J. & Thomas, P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* **153**, 2953-62, doi:10.1210/en.2012-1061 (2012).
67. Mendelsohn, M.E. Mechanisms of estrogen action in the cardiovascular system. *J. Steroid Biochem.* **74**, 337-343 (2000).
68. Simoncini, T., Moghadam, A.H., Brazil, D.P., Ley, K., Chin, W.W. & Liao, J.K. Interaction of estrogen receptor with the regulatory subunit of phosphatidylinositol -3-OH kinase. *Nature* **407**, 538-41 (2000).
69. Kousteni, S., Bellido, T., Plotkin, L.I., O'Brien, C. A. *et al.* Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* **104**, 719–730 (2001).
70. Metchnikoff, E. Leçons sur la pathologie comparée de l'inflammation. Paris. *Masson* (1982).
71. Kauffman, S.H. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat Immunol* **9**, 705-712 (2008).
72. Gordon, S. & Taylor, P.R. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964 (2005).
73. Wynn, T.A., Chawla, A. & Pollard, J.W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455 (2013).
74. Van Furth, R.& Cohn, Z.A. The origin and kinetics of mononuclear phagocytes. *J Exp Med* **128**, 415-435 (1968).
75. Volkman, A., Chang, N.C., Strausbauch, P.H., & Morahan, P.S. Differential effects of chronic monocyte depletion on macrophage populations. *Lab Invest* **49**, 291– 298 (1983).
76. Sawyer, R.T., Strausbauch, P.H., & Volkman, A. Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. *Lab Invest* **46**, 165– 170 (1982).
77. Gautier, E. L. *et al*. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature Immunol* **13**, 1118–1128 (2012).
78. Ginhoux, F. *et al*. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841–845 (2010).
79. Hoeffel, G. *et al*. Adult Langerhans cells derive predominantly from embryonic fetal livermonocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* **209**, 1167–1181 (2012).

80. Hume, D. A. Macrophages as APC and the dendritic cell myth. *J Immunol* **181**, 5829–5835 (2008).
81. Chitu, V. & Stanley, E. R. Colony-stimulating factor-1 in immunity and inflammation. *Curr Opin Immunol* **18**, 39–48 (2006).
82. David, M.M. & Justin P.E. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8**(12), 958–969 (2008).
83. Murray, P.J. & Wynn, T.A. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* **11**, 723-737 (2011).
84. Hussell, T. & Bell, T.J. Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol* **14**, 81-93 (2014).
85. Kohyama, M *et al*. Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature* **457**, 318-321 (2009).
86. Gautier, E.L. *et al.* Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* **13**, 1118-1128 (2012).
87. Zagorska, A., Traves, P.G., Lew, E.D., Dransfield, I. & Lemke, G. Diversification of TAM receptor tyrosine kinase function. *Nat Immunol* **15**, 920-928 (2014).
88. Canton, J., Neculai, D. & Grinstein, S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol* **13**, 621-634 (2013).
89. Kawai, T., Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-384 (2010).
90. Gautier, E.L. *et al.* Gata6 regulates aspartoacylase expression in resident peritoneal macrophages and controls their survival. *J Exp Med* **211** No 8: 1525-1531 (2014).
91. Davies, L.C., Jenkins, S.J., Allen, J.E & Taylor, P.R. Tissue-resident macrophages. *Nat Immunol* **14**(10), 986-995, doi:10.1038/ni.2705 (2013).
92. Dranoff, G., Crawford, A.D., Sadelain, M., Ream, B., Rashid, A., Bronson, R.T., Dickersin, G.R., Bachurski, C.J., Mark, E.L., Whitsett, J.A., *et al*. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science* **264**, 713–716 (1994).
93. Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., Deswarte, K., Malissen, B., Hammad, H. & Lambrecht, B.N. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GMCSF. *J Exp Med* **210**, 1977-1992 (2013).
94. Epelman, S., Lavine, K.J., Beaudin, A.E., Sojka, D.K., Carrero, J.A., Calderon, B., Brija, T., Gautier, E.L., Ivanov, S., Satpathy, A.T., *et al*. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity* **40**, 91–104 (2014).
95. Spann, N.J. & Glass, C.K. Sterols and oxysterols in immune cells function. *Nat Immunol* **14**, 893-900 (2013).
96. Lavin, Y., Mortha, A., Rahman, A. & Merad, M. Regulation of macrophage development and function in peripheral tissues. *Nat Rev Immunol* **15**(12), 731-744 (2015).

97. Ghosn, E.E., Cassado, A.A., Govoni, G.R., Fukuhara, T., Yang, Y., Monack, D.M., Bortoluci, K.R., Almeida, S.R., Herzenberg, L.A. & Herzenberg, L.A. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *PNAS* **107**(6), 2568-2573, doi:10.1073/pnas.0915000107 (2010).
98. Cassado, A.A., D'Império Lima, M.R. & Bortoluci, K.R. Revisiting mouse peritoneal macrophages: heterogeneity, development, and function. *Front Immunol* **19**, 6:225 (2015).
99. Cassado, A.A., de Albuquerque, J.A., Sardinha, L.R., Buzzo, C.L., Faustino, L., Nascimento, R., *et al*. Cellular renewal and improvement of local cell effector activity in peritoneal cavity in response to infectious stimuli. *PLoS One* **6**(7), e22141.10.1371/journal.pone.0022141 (2011).
100. Soulet, D. & Rivest, S. Microglia. *Curr Biol* **18**, R505-R508, doi:10.1016/j.cub.2008.04.047 (2008).
101. Lawson, L.J., Perry, V.H., Dri, P. & Gordon S.. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39**(1), 151–70 (1990).
102. Tremblay, M.È., Lecours, C., Samson, L., Sànchez-Zafra, V., & Sierra, A. From the Cajal alumni Achùcarro and Rio-Hortega to the rediscovery of never-resting mciroglia. *Front Neuroanat* **9**, 45, doi: 10.3389/fnana.2015.00045 (2015).
103. Tremblay, M.È., Stevens, B., Sierra, A., Wake, H., Bessis, A., & Nimmerjahn, A. The role of microglia in the healthy brain. *J Neurosci* **31**, 16064-16069, doi:10.1523/JNEUROSCI.4158-11.2011 (2011).
104. Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., *et al*. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**(6005), 841–5 (2010).
105. Alliot, F., Godin, I. & Pessac, B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain Res Dev Brain Res* **117**(2), 145–52 (1999).
106. Tambuyzer, B.R., Ponsaerts, P. & Nouwen, E.J. Microglia: gatekeepers of central nervous system immunology. *J Leukoc Biol* **85**(3), 352–70 (2009).
107. Lawson, L.J., Perry, V.H. & Gordon, S. Turnover of resident microglia in the normal adult mouse brain. *Neuroscience* **48**(2), 405–15 (1992).
108. Prinz, M. & Mildner, A. Microglia in the CNS: immigrants from another world. *Glia* **59**(2), 177–87 (2011).
109. Gomez-Nicola, D., Fransen, N.L., Suzzi, S. & Perry, V.H. Regulation of microglial proliferation during chronic neurodegeneration. *J Neurosci* **33**(6), 2481–93 (2013).
110. Saijo, K. & Glass, C.K. Microglial cell origin and phenotypes in health and disease. *Nat Rev Immunol* **11**, 775–787, doi: 10.1038/nri3086 (2011).
111. Gomez-Nicola, D. & Perry, V.H. Microglial Dynamics and Role in the Healthy and Diseased Brain: A Paradigm of Functional Plasticity *Neuroscientist* **21**(2), 169–184, doi: 10.1177/1073858414530512 (2015).
112. Yona, S., Kim, K.W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M. & Misharin, A. Fate mapping reveals origins and

dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**, 79 (2013).

113. Bain, C., Scott, C., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., Guilliams, M., Malissen, B., Agace, W. & Mowat, A.M. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C^{hi} monocyte precursors. *Mucosal Immunol* **6**, 498 (2013).
114. Davies, L.C., Rosas, M., Jenkins, S.J., Liao, C.-T., Scurr, M.J., Brombacher, F., Fraser, D.J., Allen, J.E., Jones, S.A., Taylor, P.R. Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation. *Nat Commun* **4**, 1886 (2013).
115. Robbins, C.S., Hilgendorf, I., Weber, G.F., Theurl, I., Iwamoto, Y., Figueiredo, J.-L., Gorbatov, R., Sukhova, G.K., Gerhardt, L.M. & Smyth, D. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med* **19**, 1166 (2013).
116. Hume, D.A. & MacDonald, K.P. Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. *Blood* **119**, 1810 (2012).
117. Schulz, C., Perdiguero, E.G., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W. & Pollard, J.W. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* **336**, 86 (2012).
118. Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M.B., Leboeuf, M., Becker, C.D., See, P., Price, J. & Lucas, D. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* **38**, 792 (2013).
119. Jenkins, S.J., Ruckerl, D., Thomas, G.D., Hewitson, J.P., Duncan, S., Brombacher, F., Maizels, R.M., Hume, D.A. & Allen, J.E. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med* **210**, 2477 (2013).
120. Dranoff, G., Crawford, A.D., Sadelain, M., Ream, B., Rashid, A., Bronson, R.T., Dickersin, G.R., Bachurski, C.J., Mark, E.L., Whitsett, J.A. Involvement of granulocytemacrophage colony-stimulating factor in pulmonary homeostasis. *Science* **264**, 713 (1994).
121. Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., Deswarte, K., Malissen, B., Hammad, H. & Lambrecht, B.N. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med* **210**, 1977 (2013).
122. Jenkins, S. J. *et al*. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **332**, 1284-1288, doi:10.1126/science.1204351 (2011).
123. Fejer, G., Sharma, S. & Gyory, I. Self-renewing macrophages - a new line of enquiries in mononuclear phagocytes. *Immunobiology* **220**(2), 169-74, doi: 10.1016/j.imbio.2014.11.005 (2015).

124. Hume, D.A. & MacDonald P.A. Therapeutic applications of macrophage colony stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. *Blood* **119**(8), 1810-20 (2012).
125. Chitu, V. & Stanley, E.R. Colony-stimulating factor-1 in immunity and inflammation. *CurrOpinImmunol* **18**(1), 39-48 (2006).
126. Garceau, V., Smith J, Paton IR, *et al.* Pivotal advance: avian colony-stimulating factor 1 (CSF-1),interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. *J Leukoc Biol* **87**(5), 753- 764 (2010).
127. Chihara, T., Suzu, S., Hassan, R., *et al*. IL-34 andM-CSF share the receptor Fms but are not identical in biological activity and signal activation". *Cell Death Differ* **17**(12), 1917-1927 (2010).
128. Geha, R.S., Jabara, H.H. & Brodeur, S.R. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* **3**, 721-732 (2003).
129. Gadani, S.P., Cronk, J.C., Norris, G.T. & Kipnis, J. Interleukin-4: a cytokine to remember. *J Immunol* **189**(9), 4213-4219 (2012).
130. Jenkins, S.J., Ruckerl D., Cook P.C., Jones L., Finkelman F.D., Rooijen N., MacDonald, A.S & Allen J.E. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **332**, 1284 (2011).
131. Gordon S. The macrophage: past, present and future. *Eur J Immunol* **37**, S9-S17 (2007).
132. Martinez, F.O., Helming L. & Gordon, S. Alternative activation of macrophages: an immunologic functional perspective. *Annuv Rev Immunol* **27**, 451-83 (2009).
133. Nathan, C.F., Murray, H.W., Wiebe, M.E. & Rubin BY. Identification of interferonγ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med* **158**, 670-89 (1983).
134. Ferrante, C.J. & Leibovich, S.J. Regulation of macrophage polarization and wound healing. *Advances in wound care* **1**(1), 10-16 (2012).
135. Odegaard, J.I. & Chawla, A. Alternative macrophage activation and metabolism. *Annu Rev Pathol* **6**, 275-97 (2011).
136. Szekanecz, Z. & Koch, A.E. Macrophages and their products in rheumatoid arthritis. *Curr Opin Rheumatol* **19**, 289-295 (2007).
137. Zhang, X. & Mosser, D.M. Macrophage activation by endogenous danger signals. *J Pathol* **214**, 161-178 (2008).
138. Sica, A. & Mantovani, A. Macrophage fusion cuisine. *Blood* **114**(21), 4609-10, doi:10.1182/blood-2009-09-242800 (2009).
139. Sica, A & Bronte, V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* **117**(5), 1155-66 (2007).
140. Biswaset, S.K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol* **11**, 889–896 (2010).
141. Hamilton, J.A. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* **8**, 533-544 (2008).

142. Lacey, D.C., Achuthan, A., Fleetwood, A.J., *et al*. Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *J Immunol* **188**, 5752-5765 (2012).
143. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**, 549-555 (2002).
144. Ruxton, G. & Colegrave, N. Experimental design for the life sciences. *Oxford: Oxford University Press* 3th edition (2011).
145. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* **3**, 2335 (2003).
146. Louis, C.A., Mody, V., Henry, W.L., Reichner, J.S. & Albina, J.E. Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. *Am J Physiol* **276**, R237-R242 (1999).
147. Song, E., Ouyang, N., Hörbelt, M., Antus, B., Wang, M. & Exton, M.S. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol* **204**, 19-28 (2000).
148. Mosser, D.M. The many faces of macrophage activation. *J Leukoc Biol* **73**, 209-212 (2003).
149. Anderson, C.F., Gerber, J.S. & Mosser, D.M. Modulating macrophage function with IgG immune complexes. *J Endotoxin Res* **8**, 477-481 (2002).
150. Stout, R.D., Jiang, C., Matta, B., Tietzel, I., Watkins, SK. & Suttles, J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* **175**, 342-349 (2005).
151. Mosser, D.M & Karp, C.L. Receptor mediated subversion of macrophage cytokine production by intracellular pathogens. *Curr Opin Immunol* **11**, 406-411 1999.
152. Buechler, C., Ritter, M., Orsó, E., Langmann, T., Klucken, J. & Schmitz, G. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* **67**, 97-103 (2000).
153. Ambarus, C.A., Krausz, S., van Eijk, M., Hamann, J., Radstake, TR., Reedquist, KA., Tak, P.P. & Baeten, D.L. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. *J Immunol Methods* **375**, 196-206 (2012).
154. Zanluqui, N.G., Wowk, P.F. & Pinge-Filho, P. Macrophage Polarization in Chagas Disease. *J Clin Cell Immunol* **6**, 2 doi:10.4172/2155-9899.1000317 (2015).
155. Härkönen, P.L. & Väänänen, H.K.V. Monocyte–Macrophage System as a Target for Estrogen and Selective Estrogen Receptor Modulators. *Ann N.Y. Acad Sci* **1089**, 218-227 (2006).
156. Grossman, C.J. Interaction between the gonadal steroids and the immune system. *Science* **227**(4684), 257-61 (1985).
157. Olsen, N.J. & Kovacs, W.J. Gonadal steroids and immunity. *Endocr Rev* **17**(4), 369-84 (1996).

158. Offner, P.J., Moore, E.E & Biffl, W.L. Male gender is a risk factor for major infections after surgery. *Arch Surg* **134**(9), 935-938 (1999).
159. Verthelyi, D. Sex hormones as immunomodulators in health and disease. *Int Immunopharmacol* **1**(6), 983-993 (2001).
160. Talal, N. Sex steroid hormones and systemic lupus erythematosus. *Arthritis Rheum* **24**(8), 1054-6 (1981).
161. Bijlsma, J.W. & Van den Brink, H.R. Estrogens and rheumatoid arthritis. *Am J Reprod Immunol* **28**(3-4), 231-4 (1992).
162. Cutolo, M. & Wilder, R.L. Different roles for androgens and estrogens in the susceptibility to autoimmune rheumatic diseases. *Rheum Dis Clin North Am* **26**(4), 825-39 (2000).
163. McCashland, T.M., Brand, R., Lyden, E. & de Garmo, P.P. Gender differences in colorectal polyps and tumors. *Am J Gastroenterol* **96**(3), 882-886 (2001).
164. Woldrich, J.M., Mallin, W., Ritchey, J., Carrol., P.R. & Kane C.J. Sex differences in renal cell cancer presentation and survival: an analysis of the National Cancer Database, 1993-2004. *J Urol* **179**(5), 1709-1713 (2008).
165. Bosch, F.X., Ribes, J., Diaz, M. & Cléries, R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* **127**(5 Suppl. 1), S5-S16 (2004).
166. Matsuda, J., Vanier, M.T., Saito, Y., Suzuki, K. & Kunihiko, S. Dramatic phenotypic improvement during pregnancy in a genetic leukodystrophy: estrogen appears to be a critical factor. *Hum Mol Genet* **10**, 2709–2715 (2001).
167. Islander, U., Jochems, C., Lagerquist, M.K., Forsblad-d'Elia, H. and Carlsten, H. Estrogens in rheumatoid arthritis; the immune system and bone. *Mol Cel Endocrinol* **335**, 14-29 (2011).
168. Spence, R.D. & Voskuhl, R.R. Neuroprotective effects of estrogens and androgens in CNS inflammation and neurodegeneration. *Front Endocrinol* **33**, 105-115 (2012).
169. Straub, R.H. The Complex Role of Estrogens in Inflammation. *Endocr Rev* **28**(5), 521-7 (2007).
170. Richette, P., Corvol, M. & Bardin, T. Estrogens, cartilage, and osteoarthritis. *Joint Boin Spine* **70**(4), 257-262 (2003).
171. Gold, S.M. & Voskuhl, R.R. Estrogen treatment in multiple sclerosis. *J Neurol Sci* **286**(1-2), 99-103 (2009).
172. Adams, M.R., Kaplan, J.R., Manuck, S.B., Koritnik, D.R., Parks, J.S., Wolfe, M.S. & Clarkson, T.B. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkey. Lack of an effect of added progesterone. *Arteriosclerosis* **10**(6), 1051-1057 (1990).
173. Adams, M.R., Register, T.C., Golden, D.L., Wagner, J.D. & Williams, J.K. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscler Thromb Vasc Biol* **17**(1), 217-221 (1997).
174. Yaffe, K., Sawaya, G., Lieberburg, I. *et al*. Estrogen Therapy in Postmenopausal Women Effects on Cognitive Function and Dementia. JAMA **279**(9), 688-695. doi:10.1001/jama.279.9.688 (1998).

175. Lehtimäki, T., Kunnas, T.A., Mattila, M., Perola, M., Penttilä, T., Koivula, T. & Karhumen, P.J. Coronary artery wall atherosclerosis in relation to estrogen receptor 1 gene polymorphism: an autopsy study. *J Mol Med* **80**(3), 176-180 (2002).
176. Ashworth, J.J., Smyth, J.V., Pendleton, N., Horan, M., Payton, A., Worthington, J., Ollier, W.E. & Ashcroft, G.S. The dinucleotide (CA) repeat polymorphism of estrogen receptor beta but not the dinucleotide (TA) repeat polymorphism of estrogen receptor alpha is associated with venous ulceration. *J Steroid Biochem Mol Biol* **97**(3), 266-270 (2005).
177. Di Paolo, G. & Kim, T.W. Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat Rev Neurosci* **12**(5), 284-296 (2011).
178. Liu, C.C., Kanekiyo, T., Xu, H. & Bu, G. Apoliprotein E and Alzheimer disease: risk, mechanism and therapy. *Nat Rev Neurol* **9**(2), 106-118 (2013).
179. Gubbels Bupp M.R. Sex, the aging immune system, and chronic disease. *Cellular Immunology* **294**(2), 102-110, doi: 10.1016/j.cellimm.2015.02.002 (2015).
180. Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol* **294**(2), 63-9, doi: 10.1016/j.cellimm.2015.01.018 (2015).
181. Grimaldi, C.M., Cleary, J., Dagtas, A.S., Moussai, D. & Diamond, B. Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest* **109**, 1625-133 (2002).
182. Curran, E.M., Berghaus, L.J., Vernetti, N.J., Saporita, A.J., Lubahn, D.B. & Estes, D.M. Natural killer cells express estrogen receptor-alpha and estrogen receptor-beta and can respond to estrogen via a nonestrogen receptor-alpha-mediated pathway. *Cell Immunol* **214**, 12–20 (2001).
183. Lelu, K., Laffont, S., Delpy, L., Paulet, P.E., Perinat, T., Tschanz, S.A., Pelletier, L., Engelhardt, B. & Guery, J.C. Estrogen receptor alpha signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J Immunol* 187, 2386–2393 (2011).
184. Phiel, K.L., Henderson, R.A., Adelman, S.J. & Elloso, M.M. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett **97**(1), 107-13 (2005).
185. Du, S., Sandoval, F., Trinh, P., Umeda, E. & Voskuhl, R. Estrogen receptor-beta ligand treatment modulates dendritic cells in the target organ during autoimmune demyelinating disease. *Eur J Immunol* **41**, 140–150 (2011).
186. Lambert, K.C., Curran, E.M., Judy, B.M., Lubahn, D.B. & Estes, D.M. Estrogen receptor-alpha deficiency promotes increased TNF-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. *J Leukoc Biol* **75**, 1166–1172 (2004).
187. Ribas, V., Drew, B.G., Le, J.A., Soleymani, T., Daraei, P., Sitz, D., Mohammad, L., Henstridge, D.C., Febbraio, M.A., Hewitt, S.C., Korach, K.S., Bensinger, S.J. & Hevener, A.L. Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *Proc Natl Acad Sci U S A* **108**, 16457–16462 (2011).

188. Panchanathan, R., Shen, H., Zhang, X., Ho, S.M., Choubey, D. Mutually positive regulatory feedback loop between interferons and estrogen receptor-alpha in mice: implications for sex bias in autoimmunity. *PLoS One* **5**, e10868 (2010).
189. Smith, S., Ni Gabhann, J., McCarthy, E., Coffey, B., Mahony, R., Byrne, J.C., Stacey, K., Ball, E., Bell, A., Cunnane, G., Doran, M.F., Molloy, E.S., Lee, R.Z., Harvey, B., Kearns, G. & Jefferies, C.A. Estrogen receptor alpha regulates tripartite motif-containing protein 21 expression, contributing to dysregulated cytokine production in systemic lupus erythematosus. *Arthritis Rheumatol* **66**, 163–172 (2014).
190. Cunningham, M.A., Naga, O.S., Eudaly, J.G., Scott, J.L. & Gilkeson, G.S. Estrogen receptor alpha modulates Toll-like receptor signaling in murine lupus. *Clin Immunol* **144**, 1–12 (2012).
191. Calippe, B., Douin-Echinard, V., Delpy, L., Laffargue, M., Lelu, K., Krust, A., Pipy, B., Bayard, F., Arnal, J.F., Guery, J.C. & Gourdy, P. 17Beta-estradiol promotes TLR4-triggered proinflammatory mediator production through direct estrogen receptor alpha signaling in macrophages in vivo. *J Immunol* **185**, 1169–1176 (2010).
192. Relloso, M., Aragoneses-Fenoll, L., Lasarte, S., Bourgeois, C., Romera, G., Kuchler, K., Corbi, A.L., Munoz-Fernandez, M.A., Nombela, C., Rodriguez-Fernandez, J.L. & Diez-Orejas, R. Estradiol impairs the Th17 immune response against Candida albicans. *J Leukoc Biol* **91**, 159–165 (2012).
193. Robinson, D.P., Lorenzo, M.E., Jian, W. & Klein, S.L. Elevated 17beta-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses. *PLoS Pathog* **7**, e1002149 (2011).
194. Robinson, D.P., Hall, O.J., Nilles, T.L., Bream, J.H. & Klein, S.L. 17beta-estradiol protects females against influenza by recruiting neutrophils and increasing virus-specific CD8 T cell responses in the lungs. *J Virol* **88**, 4711–4720 (2014).
195. Nettles, K.W., Gil, G., Nowak, J., Metivier, R., Sharma, V.B. & Greene, G.L. CBP is a dosage-dependent regulator of nuclear factor-kappaB suppression by the estrogen receptor. *Mol Endocrinol* **22**, 263–272 (2008).
196. Xing, D., Oparil, S., Yu, H., Gong, K., Feng, W., Black, J., Chen, Y.F. & Nozell, S. Estrogen modulates NFkappaB signaling by enhancing IkappaBalpha levels and blocking p65 binding at the promoters of inflammatory genes via estrogen receptor-beta. *PLoS One* **7**, e36890 (2012).
197. Dai, R., Phillips, R.A. & Ahmed, S.A. Despite inhibition of nuclear localization of NF-kappa B p65, cRel, and RelB, 17-beta estradiol up-regulates NF-kappa B signaling in mouse splenocytes: the potential role of Bcl-3. *J Immunol* **179**, 1776–1783 (2007).
198. Dai, R., Phillips, R.A., Karpuzoglu, E., Khan, D. & Ahmed, S.A. Estrogen regulates transcription factors STAT-1 and NF-kappaB to promote inducible nitric oxide synthase and inflammatory responses. *J Immunol* **183**, 6998–7005 (2009).
199. Lasarte, S., Elsner, D., Sanchez-Elsner, T., Fernandez-Pineda, A., Lopez-Fernandez, L.A., Corbi, A.L., Munoz-Fernandez, M.A. & Relloso, M. Estradiol

downregulates NF-kappab translocation by Ikbkg transcriptional repression in dendritic cells. *Genes Immun* **14**, 462–469 (2013).

200. Ghisletti, S., Meda, C., Maggi, A. & Vegeto, E. 17beta-estradiol inhibits inflammatory gene expression by controlling Nf-kappaB intracellular localization. *Mol Cell Biol* **25**(8), 2957-68 (2005).
201. Illing, A., Liu, P., Ostermay, S., Schilling, A., de Haan, G., Krust, A., Amling, M., Chambon, P., Schinke, T. & Tuckermann, J.P. Estradiol increases hematopoietic stem and progenitor cells independent of its actions on bone. *Haematologica* **97**, 1131–1135 (2012).
202. Moggs, J.G & Orphanides, G. Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Rep* **2**(9), 775-81 (2001).
203. Lang, T.J. Estrogen as an immunomodulatory. *Clin Immunol* 113(3), 224-30 (2004).
204. Reed, J.L., Dimayuga, F.O., Davies, L.M., Keller, J.N. & Bruce-Keller, A.J. Estrogen increases proteasome activity in murine microglial cells. *Neurosci Lett* **367**(1), 60-5 (2004).
205. Vegeto, E., Pollio, G., Pellicciari, C. & Maggi, A. *FASEB J* **13**(8), 793-803 (1999).
206. Tomaszewska, A., Guevara, I., Wilczok, T. & Dembinska-Kiec, A. 17β-Estradiol- and lipopolysaccharide-induced changes in nitric oxide, tumor necrosis factor-α and vascular endothelial growth factor release from RAW 264.7 macrophages. *Gynecol Obstet Invest* **56**, 152–159 (2003).
207. Bruce-Keller, A.J., Keeling, J.L., Keller, J.N., Huang, F.F., Camondola, S., Mattson, M.P. Antiinflammatory effects of estrogen on microglial activation. *Endocrinology* **141**, 3646–3656 (2000).
208. Mikkola, T.S. & St Clair, R.W. Estradiol reduces basal and cytokine induced monocyte adhesion to endothelial cells. *Maturitas* **41**, 313–319 doi: 10.1210/er.2007-0001#sthash.gCFfCp0K.dpuf (2002).
209. Friedrich, E.B., Clever, Y.P., Wassmann, S., Hess, C. & Nickenig, G. 17β-Estradiol inhibits monocyte adhesion via down-regulation of Rac1 GTPase. *J Mol Cell Cardiol* **40**, 87–95 (2006).
210. Chantakru, S., Wang, W.C., van Den, H.M., Bashar, S., Simpson, A., Chen, Q., Croy, B.A. & Evans, S.S. Coordinate regulation of lymphocyte-endothelial interactions by pregnancy-associated hormones. *J Immunol* **171**, 4011–4019 (2003).
211. Bender, J.R., Cleman, M.W., Cabin, H.S. & Remetz, M.S. Modulation of circulating cellular adhesion molecules in postmenopausal women with coronary artery disease. *J Am Coll Cardiol* **31**, 1555–1560 (1998).
212. Dimayuga, F.O., Reed, J.L., Carnero, G.A., Wang, C., Dimayuga, E.R., Dimayuga, V.M., Perger, A., Wilson, M.E., Keller, J.N. & Bruce-Keller, A.J. Estrogen and brain inflammation: effects on microglial expression of MHC, costimulatory molecules and cytokines. *J Neuroimmunol* **161**, 123–136 (2005).
213. Billig, H., Furuta, I. & Hsueh, A.J. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* **133**, 2204–2212 (1993).

214. Sunyer, T., Lewis, J., Collin-Osdoby, P. & Osdoby, P. Estrogen's bone-protective effects may involve differential IL-1 receptor regulation in human osteoclast-like cells. *J Clin Invest* **103**, 1409–1418 (1999).
215. Losordo, D.W. & Isner, J.M. Estrogen and angiogenesis: a review. *Arterioscler Thromb Vasc Biol* **21**, 6–12 (2001).
216. Rubanyi, G.M., Johns, A. & Kauser, K. Effect of estrogen on endothelial function and angiogenesis. *Vascul Pharmacol* **38**, 89–98 (2002).
217. Rettew, J.A, McCall, S.H. 4th & Marriott, I. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. *Mol Cell Endocrinol* **328**(1-2), 87-92, doi: 10.1016/j.mce.2010.07.017 (2010).
218. Habib, P. & Beyer, C. Regulation of brain microglia by female gonadal steroids. *J Steroid Biochem Mol Biol* 00047–00048 (2014).
219. Baker, A.E., Brautigam, V.M. & Watters, J.J. Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. *Endocrinology* **145**, 5021–5032 (2004).
220. Habib, P., Slowik, A., Zendedel, A., Johann, S., Dang, J. & Beyer, C. Regulation of hypoxia-induced inflammatory responses and M1-M2 phenotype switch of primary rat microglia by Sex steroids. *J Mol Neurosci* **52**, 277–285 (2014).
221. Routley, C.E. & Ashcroft, G.S. Effect of estrogen and progesterone on macrophage activation during wound healing. *Wound Rep Reg* **17**, 42-50, doi:10.1111/j.1524-475X.2008.00440.x (2009).
222. Campbell, L., Emmerson, E., Williams, H., Saville, C.R., Krust, A., Chambon, P., Mace, K.A. & Hardman M. Estrogen receptor-alpha promotes alternative macrophage activation during cutaneous repair. *Journal of Investigative Dermatology* **134**, 2447-2457, doi:10.1038/jid.2014.175 (2014).
223. Villa, A., Rizzi, N., Vegeto, E., Ciana, P. & Maggi, A. Estrogen accelerates the resolution of inflammation in macrophagic cells. *Scientific reports* **5**, 15224 (2015).
224. Davies, L.C., Jenkins, S.J., Allen, J.E. & Taylor, P.R. Tissue resident macrophages. *Nat Immunol* **14**(10), 986–995 (2013).
225. Wynn, T.A., Chawla, A. & Pollard, J.W. Macrophage biology in development, homeostasis and disease. *Nature* **496**(7446), 445-55, doi: 10.1038/nature12034 (2013).
226. Nothnick, W.B. Treating endometriosis as an autoimmune disease. *Fertil Steril* **76**, 223–231 (2001).
227. Gleicher, N. & Pratt, D. Abnormal (auto)immunity and endometriosis. *Int J Gynaecol Obstet* **40**Suppl, S21—S27 (1993).
228. Giudice, L.C. & Kao, L.C. Endometriosis. *Lancet* **364**, 1789 –1799 (2004).
229. Bacci, M., Capobianco, A., Monno, A., Cottone, L., Di Puppo, F., Camisa, B., Mariani, M., Brignole, C., Ponzoni, M., Ferrari, S. *et al*. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *The American journal of pathology* **175**, 547-556 (2009).

230. Hong, M., Hua, Y.Q, Yu, L., Sun, X.Y., Li, X.D. & Zhu, Q. Effect of TCTS on experimental endometriosis of rats. *Zhongguo Zhong Yao Za Zhi* **28**(1), 69-72 (2003).
231. Haber, E., Danenberg, H.D., Koroukhov, N., Ron-El, R., Golomb, G. & Schachter, M. Peritoneal macrophage depletion by liposomal bisphosphonate attenuates endometriosis in the rat model. *Hum Reprod* **24**(2), 398-407 (2009).
232. Rana, N., Braun, D.P., House, R., Gebel, H., Rotman, C. & Dmowski, W.P. Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. *Fertil Steril* **65**(5), 925-30 (1996).
233. Eisermann, J., Gast, M.J., Pineda, J., Odem, R.R & Collins, J.L. Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. *Fertil Steril* **50**(4), 573-9 (1988).
234. Schroder, W., Gaetje, R. & Baumann, R. Interleukin-6 and soluble interleukin-6 in peritoneal fluid and serum of patients with endometriosis. *Clin Exp Obstet Gynecol* **23**(1), 10-4 (1996).
235. Wu, M.Y., Ho, H.N., Chen, S.U., Chao, K.H., Chen, C.D. & Yang, Y.S. Increase in the production of interleukin-6, interleukin-10, and interleukin-12 by lipopolysaccharide-stimulated peritoneal macrophages from women with endometriosis. *Am J Reprod Immunol* **41**, 106-11 (1999).
236. Halme, J., Becker, S., Hammond, M.G., Raj, M.H. & Raj, S. Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* **145**, 333-7 (1983).
237. Hill, J.A & Anderson, D.J. Lymphocyte activity in the presence of peritoneal fluid from fertile women and infertile women with and without endometriosis. *Am J Obstet Gynecol* **161**, 861-4 (1989).
238. Olive, D.L., Haney, A.F. & Weinberg, J.B. The nature of the intraperitoneal exudate associated with infertility: peritoneal fluid and serum lysozyme activity. *Fertil Steril* **48**(5), 802-6 (1987).
239. Martinez, F.O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Front Biosci* **13**, 453-61 (2008).
240. Ciucci, A., Zannoni, G.F., Buttarelli, M., Lisi, L., Travaglia, D., Martinelli, E., Scambia, G. & Gallo, D. Multiple direct and indirect mechanism drive estrogen-induced tumor growth in high grade serous ovarian cancers. *Oncotarget* **7**, 8155-71, doi: 10.18632/oncotarget.6943 (2016).
241. Khan, K.N., Masuzaki, H., Fujishita, A., Kitajima, M., Sekine, I., Matsuyama, T. & Ishimaru, T. Estrogen and progesterone receptor expression in macrophages and regulation of hepatocyte growth factor by ovarian steroids in women with endometriosis. *Hum Reprod* **20**, 2004-13 (2005).
242. Akoum, A., Lemay, A. & Maheux, R. Estradiol and interleukin-1beta exert a synergistic stimulatory effect on the expression of the chemokine regulated upon activation, normal T cell expressed, and secreted in endometriotic cells. *J Clin Endocrinol Metab* **87**(12), 5785-92 (2002).

243. Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev* **222**, 155-61, doi: 10.111/j.1600-065X.2008.00607.x (2008).
244. Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-84 (2007).
245. Tworoger, S.S., Zhang, X., Eliassen, A.H. *et al*. Inclusion of endogenous hormone levels in risk prediction models of postmenopausal breast cancer. *J Clin Oncol* **32**, 3111–7 (2014).
246. Subramanian, A., Salhab, M. & Mokbel, K. Oestrogen producing enzymes and mammary carcinogenesis: a review. *Breast Cancer Res Treat* **111**, 191–202 (2008).
247. Bonknoff, H. & Berges, R. The evolving role of oestrogens and their receptors in the development and progression of prostate cancer. *Eur Urol* **55**, 533-42 (2009).
248. Zhou, L., Cai, B., Bao, W. *et al*. Crosstalk between estrogen receptor and mitogen-activated protein kinase signaling in the development and progression of endometrial cancer. *Int J Gynecol Cancer* **21**, 1357–65 (2011).
249. Sica, A. & Mantovani, A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* **122**, 787–795 (2012).
250. Qian, B.Z. & Pollard, J.W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39–51 (2010).
251. Balkwill, F.R. & Mantovani, A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol* **22**, 33–40 (2012).
252. DeNardo, D.G., Andreu, P. & Coussens, L.M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev* **29**, 309–316 (2010).
253. Hanahan, D. & Coussens, L.M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* **21**, 309–322 (2012).
254. Condeelis, J. & Pollard, J.W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263–266 (2006).
255. Ko, S.Y., Ladanyi, A., Lengyel, E. & Naora, H. Expression of the homeobox gene *HOXA9* in ovarian cancer induces peritoneal macrophages to acquire an M2 tumor-promoting phenotype. *Am J Pathol* **184**, 271–81 doi: 10.1016/j.ajpath.2013.09.017 (2014).
256. O'Sullivan, C., Lewis, C.E., Harris, A.L. & McGee, J.O. Secretion of epidermal growth factor by macrophages associated with breast carcinoma. *Lancet* **342**, 148–9 doi: 10.1016/0140-6736(93)91348-P (1993).
257. Leek, R.D. & Harris, A.L. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia* **7**, 177–89, doi: 10.1023/A:1020304003704 (2002).
258. Yang, W., Lu, Y. Xu, Y., Xu, L., Zheng, W., Wu, Y., Li, L. & Shen, P. Estrogen represses hepatocellular carcinoma (HCC) growth via inhibiting alternative activation of tumor-associated macrophages (TAMs). *J Biol Chem* **287**, 40140-9, doi: 10.1074/jbc.M112.348763 (2012).
259. Heneka, M.T., Carson, M.J., El Khoury, J., Landreth, G.E., Brosseron, F., Feinstein, D.L., *et al*. Neuroinflammation in Alzheimer's disease. *Lancet Neurol* **14**, 388-345 (2015).

260. Tang, Y. & Le, W. Differential role of M1 and M2 microglia in neurodegenerative diseases. *Mol Neurobiol* **53**, 1181-94(2015)
261. Herrera, A.J., Espinosa-Oliva, A.M., Carrillo-Jimenez, A., Oliva-Martin, M.J. Garcia-Revilla, J., *et al*. Relevance of chronic stress and the two faces of microglia in Parkinson's disease. *Front Cell Neurosci* **9**, 312 (2015).
262. Mesci, P., Zaidi, S., Lobsiger, C.S., Millecamps, S., Escartin, C., Seilhean, D., *et al*. System xC - is a mediator of microglia function and its deletion slows symptoms in amyotrophic lateral sclerosis mice. *Brain* **138**, 53-68. (2015).
263. Bolmont, T., Haiss, F., Eicke, D., Radde, R., Mathis, C.A., Klunk, W.E., *et al*. Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. *J Neurosci* **28**(16), 4283–92 (2008).
264. Frautschy, S.A., Yang, F., Irrizarry, M., Hyman, B., Saido, T.C., Hsiao, K., *et al*. Microglial response to amyloid plaques in APPsw transgenic mice. *Am J Pathol* **152**(1), 307–17 (1998).
265. McGeer, P.L., Itagaki, S., Boyes, B.E. & McGeer, E.G. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* **38**(8):1285–91 (1988).
266. Beers, D.R., Henkel, J.S., Xiao, Q., Zhao, W., Wang, J., Yen, A.A., *et al*. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci* U S A **103**(43), 16021–6 (2006).
267. Kiyota, T., Okuyama, S., Swan, R.J., Jacobsen, M.T., Gendelman, H.E. & Ikezu, T. CNS expression of anti-inflammatory cytokine interleukin-4 attenuates Alzheimer's disease-like pathogenesis in APP+PS1 bigenic mice. *FASEB J* **24**, 3093–3102 2010.
268. Shimizu, E., Kawahara, K., Kajizono, M., Sawada, M. & Nakayama, H. IL- 4-induced selective clearance of oligomeric beta-amyloid peptide(1-42) by rat primary type 2 microglia. *J Immunol* **181**, 6503–6513 (2008).
269. Yamamoto, M., Kiyota, T., Walsh, S.M., Liu, J., Kipnis, J. & Ikezu, T. Cytokine-mediated inhibition of fibrillar amyloid-beta peptide degradation by human mononuclear phagocytes. *J Immunol* **181**, 3877–3886 (2008).
270. Falcone, M., Rajan, A.J., Bloom, B.R. & Brosnan, C.F. A critical role for IL-4 in regulating disease severity in experimental allergic encephalomyelitis as demonstrated in IL-4-deficient C57BL/6 mice and BALB/c mice. *J Immunol* **160**, 4822–4830 (1998).
271. Mikita, J., Dubourdieu-Cassagno, N., Deloire, M.S., Vekris, A., Biran, M., Raffard, G., Brochet, B., Canron, M.H., Franconi, J.M., Boiziau, C., & Petry, K.G. Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult Scler* **17**, 2–15 (2011).
272. Qin, H., Yeh, W.I., De Sarno, P., Holdbrooks, A.T., Liu, Y., Muldowney, M.T., Reynolds, S.L., Yanagisawa, L.L., Fox, T.H., Park, K., *et al*. Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation. *Proc Natl Acad Sci USA* **109**, 5004–5009 (2012).

273. Dang, J., Mitkari, B., Kipp, M. & Beyer, C. Gonadal steroids prevent cell damage and stimulate behavioral recovery after transient middle cerebral artery occlusion in male and female rats. *Brain Behav Immun* **25**, 715–726 (2011).
274. Kipp, M., Berger, K., Clarner, T., Dang, J. & Beyer, C. Sex steroids control neuroinflammatory processes in the brain: relevance for acute ischaemia and degenerative demyelination. *J Neuroendocrinol* **24**, 62–70 (2012).
275. Spence, R.D., Wisdom, A.J., Cao, Y., Hill, H.M., Mongerson, C.R.L., Stapornkul, B., Itoh, N., Sofroniew, M.V. & Voskuhl, R.R. Estrogen mediates neuroprotection and anti-inflammatory effects during EAE through ERa signaling on astrocytes but not through ERb signaling on astrocytes or neurons. *J Neurosci* **33**, 10924–10933 (2013).
276. Spence, S., Fitzsimons, A., Boyd, C.R., Kessler, J., Fitzgerald, D., Elliott, J., Gabhann, J.N., Smith, S., Sica, A., Hams, E., Saunders, S.P., Jefferies, C.A., Fallon, P.G., McAuley, D.F., Kissenpfennig, A. & Johnston, J.A. Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization. *Immunity* **38**, 66–78 (2013).
277. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* **176**, 287–292 (1992).
278. Ulbrich, C., Zendedel, A., Habib, P., Kipp, M., Beyer, C., Dang, J. Long-term cerebral cortex protection and behavioral stabilization by gonadal steroid hormones after transient focal hypoxia. *J Steroid Biochem Mol Biol* **131**, 10–16 (2012).
279. Habib, P., Dreymueller, D., Ludwig, A., Beyer, C. & Dang, J. Sex steroid hormonemediated functional regulation of microglia-like BV-2 cells during hypoxia. *J Steroid Biochem Mol Biol* **138**, 195–205 (2013).
280. von Mering, C. *et al*. STRING: known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic acids research* **33**, D433-437, doi: 10.1093/nar/gki005 (2005).
281. Della Torre, S. *et al*. An essential role for liver ERα in coupling hepatic metabolism to the reproduction cycle. *Cell Rep* **15**(2), 360-371, doi:10.1016/j.celrep.2016.03.019 (2016).
282. Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S. & Amit, I. Tissue-resident macrophage enhancer landscapes are shaped by local microenvironment. *Cell* **159**, 1312-1326, doi:10.1016/j.cell.2014.11.018 (2014).
283. Bourdeau, V. *et al*. Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Molecular endocrinology* **18**, 1411-1427, doi:10.1210/me.2003-0441 (2004).
284. Salem, M.L. Estrogen, a double-edged sword: modulation of TH1- and TH2-mediated inflammations by differential regulation of TH1/TH2 cytokine production. Current drug targets. *Inflammation and allergy* **3**, 97-104 (2004).

285. Nadkarni, S. & McArthur, S. Oestrogen and immunomodulation: new mechanisms that impact on peripheral and central immunity. *Current opinion in pharmacology* **13**, 576-581, doi:10.1016/j.coph.2013.05.007 (2013).
286. Yona, S. et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**, 79-91, doi: 10.1016/j.immuni.2012.12.001 (2013).
287. Cain, D. W. *et al*. Identification of a tissue-specific, C/EBPbeta-dependent pathway of differentiation for murine peritoneal macrophages. *Journal of immunology* **191**, 4665-4675, doi:10.4049/jimmunol.1300581 (2013).
288. Jenkins, S. J. *et al*. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *The Journal of experimental medicine* **210**, 2477-2491, doi:10.1084/jem.20121999 (2013).
289. Bottazzi, B. *et al*. A paracrine circuit in the regulation of the proliferation of macrophages infiltrating murine sarcomas. *Journal of immunology* **144**, 2409-2412 (1990).
290. Campbell, M. J. *et al*. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast cancer research and treatment* **128**, 703-711, doi:10.1007/s10549-010-1154-y (2011).
291. Tymoszuk, P. *et al*. In situ proliferation contributes to accumulation of tumor-associated macrophages in spontaneous mammary tumors. *European journal of immunology* **44**, 2247-2262, doi:10.1002/eji.201344304 (2014).
292. Kawamura, K., Komohara, Y., Takaishi, K., Katabuchi, H. & Takeya, M. Detection of M2 macrophages and colony-stimulating factor 1 expression in serous and mucinous ovarian epithelial tumors. *Pathology international* **59**, 300-305, doi:10.1111/j.1440-1827.2009.02369.x (2009)
293. Sieweke, M.H. & Allen, J.E. Beyond stem cells: self-renewal of differentiated macrophages. *Science* **342**, 1242974, doi:10.1126/science.1242974 (2013).
294. Scotland, R.S., Stables, M.J., Madalli, S., Watson, P., and Gilroy, D.W. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female. *Blood* **118**, 5918-27 (2011).
295. Calippe, B., Douin-Echinard, V., Laffargue, M., Laurell, H., Rana-Poussine, V., Pipy, B., Guery, J.C., Bayard, F., Arnal, J.F. & Gourdy, P. Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: involvement of the phosphatidylinositol 3-kinase pathway. *Journal of immunology* **180**, 7980-7988 (2008).
296. Toniolo, A., Fadini, G.P., Tedesco, S., Cappellari, R., Vegeto, E., Maggi, A., Avogaro, A., Bolego, C. & Cignarella, A. Alternative activation of human macrophages is rescued by estrogen treatment in vitro and impaired by menopausal status. *The Journal of clinical endocrinology and metabolism* 100, E50-58 (2015).
297. Espinosa, I., Catasus, L., E, D.A., Mozos, A., Pedrola, N., Bertolo, C., Ferrer, I., Zannoni, G.F., West, R.B., van de Rijn, M., *et al*. Stromal signatures in endometrioid endometrial carcinomas. *Modern Pathol* **27**, 631-639 (2014).

298. Capobianco, A. & Rovere-Querini, P. Endometriosis, a disease of the macrophage. *Front Immunol* **4**, 9 (2013).
299. Burns, K.A., Rodriguez, K.F., Hewitt, S.C., Janardhan, K.S., Young, S.L. & Korach, K.S. Role of estrogen receptor signaling required for endometriosis-like lesion establishment in a mouse model. *Endocrinology* **153**, 3960-3971 (2012).
300. Han, S.J., Jung, S.Y., Wu, S.P., Hawkins, S.M., Park, M.J., Kyo, S., Qin, J., Lydon, J.P., Tsai, S.Y., Tsai, M.J., *et al*. Estrogen Receptor beta Modulates Apoptosis Complexes and the Inflammasome to Drive the Pathogenesis of Endometriosis. *Cell* **163**, 960-974 (2015).
301. Han, S.J. & O'Malley, B.W. The dynamics of nuclear receptors and nuclear receptor coregulators in the pathogenesis of endometriosis. *Human reproduction update* **20**, 467-484 (2014).
302. Lin, Y.J., Lai, M.D., Lei, H.Y. & Wing, L.Y. Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model. *Endocrinology* **147**, 1278-1286 (2006).
303. Vegeto, E., Cuzzocrea, S., Crisafulli, C., Mazzon, E., Sala, A., Krust, A. & Maggi, A. Estrogen receptor-alpha as a drug target candidate for preventing lung inflammation. *Endocrinology* **151**, 174-184 (2010).
304. Bebo, B.F., Jr., Fyfe-Johnson, A., Adlard, K., Beam, A.G., Vandenbark, A.A. & Offner, H. Lowdose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. *Journal of Immunology* **166**, 2080-2089 (2001).
305. Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P. & Mark, M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127**, 4277-4291 (2000).
306. Shults, C.L., Pinceti, E., Rao, Y.S. & Pak, T.R. Aging and Loss of Circulating 17beta-Estradiol Alters the Alternative Splicing of ERbeta in the Female Rat Brain. *Endocrinology* **156**, 4187-4199, doi:10.1210/en.2015-1514 (2015).
307. Vegeto, E., Belcredito, S., Etteri, S., Ghisletti, S., Brusadelli, A., Meda, C., Krust, A., Dupont, S., Ciana, P., Chambon, P. & Maggi, A. Estrogen receptor-α mediates the brain antiinflammatory activity of estradiol. *Proc Natl Acad Sci USA* **100**, 9614–9619. doi: 10.1073/pnas.1531957100 (2003).
308. Schwab, J.M., Chiang, N., Arita, M. & Serhan, C.N. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447**, 869-874 (2007).
309. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L. & Pachter, L. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature protocols* **7**, 562-578 (2012).
310. Rapaport, F., Khanin, R., Liang, Y., Pirun, M., Krek, A., Zumbo, P., Mason, C.E., Socci, N.D. & Betel, D. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. *Genome biology* **14**, R95 (2013).
311. Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. *Bioinformatics* **18**, 207-208 (2002).

312. Janky, R., Verfaillie, A., Imrichova, H., Van de Sande, B., Standaert, L., Christiaens, V., Hulselmans, G., Herten, K., Naval Sanchez, M., Potier, D., *et al*. iRegulon: from a gene list to a gene regulatory network using large motif and track collections. *PLoS computational biology* 10:e1003731 (2014).
313. Huang da, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4**, 44-57 (2009).
314. Pepe, G., Calderazzi, G., De Maglie, M., Villa, A.M. & Vegeto, E. Heterogeneous induction of microglia M2a phenotype by central administration of interleukin-4. *J Neuroinflammation* **11**, 211, doi: 10.1186/s12974-014-0211-6 (2014).

SUPPLEMENTARY MATERIALS

Supplementary Table 1. Differentially regulated genes (DEGs) in macrophages following 3 and 24 h of estrogen administration to metaestrous female mice are listed according to lgFC; cluster and ontologies associations and the number of ERE in the promoter/enhancer are reported.

GENE	logFC		Cluster	ERE	Ontologies
	3h	24h			
Vegfa*	3.22		I	1	IR; WH; TX; AP
H2-M3	1.97		I		IR
Lbx2§	1.80		I		TX
Rpl39l*	1.56		I		
Hgd	1.53		I		
Fam187b*	1.47		I		
Prelid2*	1.43		I		
Ubxn6*	1.41		I		
Gsg1*	1.40		I		
Cdk1	1.39		I		CC; SR
Ofcc1*	1.32		I		
Rad51ap1§	1.27		I		SR
Ckap2l	1.22		I		
Kif22	1.18		I		SR
Oaf*	1.17		I		
Mxd3*	1.16		I		TX
Itga7*	1.15		I		
Spc25*	1.15		I		CC
Cd46*	1.13		I		
Trappc1§	1.13		I		
Egr3	1.12		I		TX
Cenph*	1.09		I		CC
Depdc1b*	1.08		I		
Il1b*	1.08		I		IR; WH; LM
Plekhf1*	1.07		I		AP
Chaf1b*	1.07		I		CC; TX; SR
Cdca5§	1.06		I		CC
Pole*	1.04		I	1	SR
Lpl	1.03		I	3	
Tgfbr3	1.03		I		WH; TX
Fam101b§	1.01		I		
Cdca7	0.99		I		TX
Sox7	0.97		I	1	TX
Gmnn	0.95		I		CC
Plod3	0.95		I		
Asf1b	0.90		I		TX
Cxcr7	0.83		I		
Lonrf3	0.83		I		
Loxl2	0.81		I		
Nlrp3	0.79		I		IR; WH; TX; AP
Tipin	0.76		I		SR; CC
Nr4a1	0.76		I	1	TX; AP
Hes1	0.71		I		TX
Tacc3	0.70		I		CC; WH
Itgb3	0.69	-0.63	I		
Fabp7	0.67		I	1	
Slpi	0.65		I	1	
Cdkn1a	0.63		I	1	CC; AP; SR
Id2	0.63		I		WH; TX
Klf4	0.62		I		TX
Rab27a	0.52		I		IR; WH; AP
Lrrk2	0.51		I		
Cdc42ep4	0.47		I	1	
Ccr2	0.44		I		IR; WH
Fhit*	-2.17		I		
Ccl5	-2.14		I		IR; WH
Ms4a4b	-2.12		I		
Il7r*	-1.89		I	1	IR; WH
Cldn20§	-1.59		I		
Lat*	-1.56		I		IR; WH
Il2rb	-1.45		I	2	AP
Slc18a2§	-1.43		I		
Mylpf*	-1.42		I	2	
Gm20735*	-1.36		I		
Serpine1*	-1.27		I	1	
Dnajb13§	-1.24		I		PF
Oas1g*	-1.24		I		IR
Gm14393§	-1.17		I		
Dhrs13*	-1.16		I		
Sec16b	-1.15		I		
Bri3*	-1.13		I		
Per1	-1.13		I	1	TX
Ifitm1§	-1.10		I		
Tcf7	-1.09		I	1	TX; AP
Ly6c, Ly6i*	-1.09		I	1	
Lck*	-1.04		I		

Tmem220[*]	-1.02		I		
Cyp11a1[§]	-1.01		I		
Lyve1	-0.81		I		
Usp18	-0.80		I		
Dnajb1	-0.68		I		PF
Per3	-0.63		I		TX
Tbc1d9	-0.61		I		
Ccr7	-0.57		I		IR
Frmd4b	-0.57		I		
Ltb	-0.51		I	1	IR; WH; AP
Pou2f1	-0.49		I		TX
Manf	-0.47		I		
March1	-0.44		I		
Chaf1a[**]	1.55	1.34	II	1	CC; TX; SR
S100a5[§,**]	1.37	1.45	II	1	
Zfp580[*,**]	1.32	1.03	II		TX
Bub1b	1.3	1.38	II		CC
Prc1	1.22	1.23	II		CC
Cx3cr1[§,**]	1.20	1.13	II	1	IR; AP
Ccr6	1.16	1.29	II		
Cenpk[*,**]	1.14	1.20	II		TX
Nusap1	1.08	1.19	II		CC
Serpinb2	1.03	1.00	II		
Ube2c[*,**]	1.00	1.07	II		CC
Fam217b	0.95	0.95	II		
Slc16a3	0.92	0.59	II		
Ldlr	0.82	0.47	II		IR; LM
Mcm5	0.82	0.78	II		TX
Uhrf1	0.77	0.81	II		CC; TX; SR
Lig1	0.76	0.83	II		CC; SR
Ptgir	0.74	0.50	II		
Hk2	0.72	0.60	II		
Incenp	0.67	0.60	II	2	CC
Mapk6	0.64	0.51	II		CC
Adam8	0.63	0.61	II		
E2f3	0.60	<0.4	II		CC; TX
Bcl3	0.60	<0.4	II		IR; WH; TX; AP; SR
Fam171a1	0.59	<0.4	II		
Slc9a9	0.59	<0.4	II		
Cd40	0.58	<0.4	II		
Cdkn2d	0.57	<0.4	II		CC
Arf2	0.57	0.45	II		
Dram1	0.57	<0.4	II		
Ripk3	0.56	0.57	II		AP
Kremen1	0.54	<0.4	II		
Abcg1	0.54	<0.4	II	1	LM; TX
Padi4	0.53	<0.4	II	1	TX
5430435G22Rik/Rab7b	0.51	<0.4	II		
Arhgef10l	0.50	<0.4	II		
Steap3	0.49	0.57	II		CC
Plscr1	0.46	<0.4	II	1	WH
B430306N03Rik/Treml6	0.45	0.46	II	2	
Dmpk	0.44	<0.4	II		
Mcm2	<0.4	0.53	II		CC
Mrgprb2[*,**]	-5.04	-2.44	II		
Cpa3[*,**,^]	-4.57	-1.90	II		
Mcpt4[*,**,^]	-3.91	-1.65	II		
Cma1[*,**,^]	-3.80	-1.87	II		
Tpsb2[*,**,^]	-3.72	-1.83	II		
Mrgprb1[*,**]	-3.57	-1.21	II		
Kit[*,**,^]	-2.90	-1.43	II	2	
Angptl4	-2.76	-1.51	II	2	LM; AP; SR
Slc6a4[*,**]	-2.50	-2.32	II	1	
Pdk4	-2.13	-2.46	II	2	
Ly6k	-1.86	-1.74	II		
Cxcr4	-1.32	-0.83	II		WH
Hsph1	-1.29	-1.28	II		PF
Wee1	-1.23	-1.13	II	2	CC
Dgke	-1.10	-0.74	II		
Mbd1	-1.04	-1.09	II	1	TX
Jdp2	-0.96	-0.48°	II		TX
Klf9	-0.92	-0.65	II		TX
Fam46c	-0.91	-0.74°	II		
Gas5,Mir5117	-0.90	-0.95	II		
Per2	-0.85	-0.81	II	2	TX
Stip1	-0.77	-0.58	II	1	
Cpt1a	-0.77	-0.56	II		
Cep85	-0.77	-0.91	II		
Pik3r5	-0.76	-0.79	II		
Hsp90aa1	-0.74	<0.4	II		PF
Ddit4	-0.74	-0.62	II		
Pgm2l1	-0.73	-0.55	II		
Herpud1	-0.72	-0.56	II	1	
Trim65	-0.70	<0.4	II		
Sfn	-0.69	<0.4	II		
Sik1	-0.68	-0.54	II		CC
Dnaja1	-0.68	-0.62	II	1	PF
Cacna1e	-0.68	<0.4	II		
Mll1	-0.67	-0.60	II		WH; TX
Tagap	-0.67	-0.56	II		
Oas2	-0.66	<0.4	II		IR

Trim47	-0.66	-0.73	II		
Stxbp3a	-0.66	-0.71	II		
Trim7	-0.63	-0.56°	II		
Nr1d2	-0.62	<0.4	II		TX
Erdr1	-0.60	-0.50	II		
Tef	-0.60	<0.4	II		TX
Ino80d	-0.60	-0.54	II		
Rusc2	-0.59	-0.66	II		
Slc37a2	-0.59	-0.54	II		
Jmy	-0.58	<0.4	II		CC; TX; AP; SR
Rnf169	-0.57	<0.4	II		
Fkbp4	-0.56	<0.4	II	1	
Tsc22d3	-0.56	<0.4	II		TX; AP
Atxn1	-0.56	<0.4	II		TX
Lmo7	-0.51	<0.4	II		
Tfb2m	-0.51	<0.4	II		TX
Rabgap1l	-0.51	<0.4	II		
Ctns	-0.51	-0.53	II	1	
Phf15	-0.50	<0.4	II		
Epas1	-0.50	-0.56	II	1	WH; TX; SR
Mlxip	-0.50	-0.44	II		TX
Slc16a10	-0.48	<0.4	II		
Ypel2	-0.47	-0.49	II		
Ptpn22	-0.46	<0.4	II		WH
Cacybp	-0.45	<0.4	II	1	
Ubfd1	-0.44	<0.4	II		
Glcci1	-0.44	-0.5	II	1	
Il16	-0.43	<0.4	II	1	IR; TX
Rc3h1	-0.43	<0.4	II		
Ahsa1	-0.43	<0.4	II		PF
Crebbp	<0.4	-0.5	II		
Diras2	<0.4	-0.41	II		
Bex6**		4.46	III		
Spp1*,**	1.51	3.96	III		AP
Arg1	0.81	2.81	III		WH
Trem1*	1.72	2.09	III	2	
Nuf2*,**	1.41	1.86	III		CC
Birc5*	1.26	1.78	III		CC; AP
Ccnb2	1.38	1.76	III	1	CC; WH
Kifc1		1.72	III		CC
Gins2§	1.34	1.71	III		
Spc24§	1.09	1.70	III		CC
Fignl1*	1.18	1.47	III	1	
Ccnb1		1.45	III		CC
Ccnf		1.45	III		CC
Top2a	1.14	1.43	III	1	TX

Mcm10*,**	1.07	1.41	III		
Camkk1		1.40	III	1	
Gm16907**		1.39	III		
Cdca3		1.36	III		CC
Mag§§		1.35	III		
Kif11		1.29	III		CC
Rn45s	0.59	1.26	III		
Slc35g2§§		1.25	III		
Mki67	0.94	1.24	III		CC
Stmn1	0.89	1.24	III		CC
Timeless**		1.19	III	1	CC
Tpx2		1.16	III		CC
Fxn§§		1.15	III		
Slc7a8	0.85	1.13	III		
Smim5§§		1.09	III		
Gm10814**		1.08	III		
Dtl**		1.07	III		
Mefv§§		1.07	III		WH
Cdc6*		1.04	III		CC
Ccr5	0.5	1.02	III		IR; WH
Kif23		1.02	III		
Syce2**		1.01	III		CC
Echdc3**		1.01	III		
Lair1	0.54	0.99	III		
Dnajb5		0.98	III	1	
Rrm2		0.95	III	2	
Tgm2		0.94	III	1	WH
Slfn9		0.94	III		
Marco	0.46	0.93	III	1	
Fcgr4	0.45	0.91	III		
Tgm1		0.91	III	1	
Aurkb		0.86	III		CC
Arhgap19		0.86	III		
Emp1		0.86	III		
Layn		0.83	III		
Idi1		0.82	III		LM
Guca1a		0.79	III	1	
Stxbp1		0.78	III	1	IR
Cd300lf		0.78	III		IR
Dab2		0.77	III	1	IR
Il21r		0.77	III		
Ckb		0.76	III		
Cwc27		0.74	III		
Gm6682		0.74	III		
Dusp16		0.73	III		
Arl4c		0.70	III		

Srxn1		0.67	III		
Dhcr24		0.65	III	2	LM
Sel1l3		0.65	III		
Kpna2		0.64	III	1	
Kank2	0.51	0.64	III		
Msr1		0.62	III		IR; LM
Fcrl5		0.62	III		
Pgk1		0.61	III		
Fmnl3		0.60	III		
Dpysl3		0.60	III		
Pkm		0.60	III		
Lbp		0.60	III		IR; WH; LM
Cd300ld		0.59	III		IR
Jam2		0.59	III		
Pla2g4a		0.58	III	1	
Fus		0.57	III		
Fgr		0.57	III		
C1ra		0.57	III		IR; WH
Cd22		0.56	III	1	
Phyh		0.56	III		
Pdk1		0.56	III		
Rfx5		0.54	III		
Rhoh		0.53	III		
Lars		0.53	III		
Tlr1		0.51	III	1	IR; WH
Nin		0.50	III		
Itga9		0.50	III	1	
Ifi30,Pik3r2		0.50	III		
Lrrc25		0.49	III		
Myo1e		0.49	III		WH
Rhoc		0.47	III		
Impdh1		0.46	III		
Tpi1		0.46	III		
Ap2m1		0.46	III	1	
Ldha		0.45	III		
Jarid2		0.44	III		
Serpine2		-2.66	III	1	
Rasl10a[*,**]	-1.19	-2.55	III		
Gm16701		-2.44	III		
Fkbp5	-1.35	-2.37	III	2	PF
Zbtb16	-1.09	-2.32	III		WH; TX; AP
Hspb1	-1.33	-2.29	III		
Slc15a2		-2.19	III		
Klf15[*,**]	-1.34	-2.05	III		TX
Tcf23[**]		-1.91	III		
Zfp811	-0.87	-1.89	III		TX

Rfx2	-0.55	-1.64	III		TX
Tppp	-0.51	-1.61	III		
Cyp26a1	-0.96	-1.58	III		
Plekha6	-0.56	-1.54	III		
Gpr114[**]		-1.53	III		
Serf1[**]		-1.50	III	1	
Map3k6	-0.72	-1.49	III	1	
Shank3		-1.46	III	1	
Ralgds	-0.74	-1.45	III		
Lims2		-1.44	III		
Prtg		-1.39	III		
Krt7		-1.36	III		CC
Eps8l1		-1.35	III		
Apoc1	-0.54	-1.29	III		LM
Fam222a[**]		-1.28	III		
Thbd	-0.6	-1.26	III		WH
Ryr2		-1.24	III		
Tacc2		-1.18	III		CC
Klf11	-0.68	-1.18	III		WH; TX
Fcer1a[^]		-1.17	III		
Tmem150b[**]		-1.12	III		
Il6[**]		-1.10	III		WH
Orm2,Orm3[§§]		-1.09	III		WH
Plin2	-0.78	-1.05	III		LM
Il1rl1	-0.64	-1.05	III	2	IR
Ccdc15[§§]		-1.04	III		
Frat2		-1.01	III		
Slc10a6		-0.98	III		
Tnfsf8		-0.93	III		IR
Tnfrsf8		-0.92	III		
Cpm		-0.92	III		
Ier3		-0.91	III		
Cd93	-0.53	-0.90	III		
Ccm2l		-0.89	III		
Banp	-0.64	-0.89	III		CC
Stxbp3b		-0.88	III		
Sgms1	-0.6	-0.84	III		WH; AP
Rai14		-0.84	III		
Glul		-0.80	III		
Hilpda		-0.80	III		
Sult1a1		-0.78	III		
Engase	-0.5	-0.77	III		
Hist1h2bc		-0.76	III		
Nfkbiz		-0.76	III		WH
Zfp97		-0.75	III		
Elovl5		-0.75	III		

Mst1r		-0.73	III	2	
Snta1		-0.73	III	1	
Nrip1		-0.71	III	1	LM
Irf2bp2		-0.71	III		
Zkscan3		-0.69	III		
Mdm1		-0.69	III		
Cdan1		-0.69	III		
Tns1		-0.68	III		
Epha2		-0.68	III	1	WH
Igf1r		-0.68	III		
Dusp6		-0.68	III		
Slc27a1		-0.67	III	1	LM
Prkar2b	-0.49	-0.66	III	1	
Snhg11		-0.66	III		
Chordc1		-0.66	III	1	
Dapk1		-0.64	III		
Dhrs3		-0.63	III		
Wdr45		-0.62	III		
Cd300a		-0.62	III		IR
Sh3bgrl2		-0.61	III		
Ulk1		-0.61	III	1	IR
Notch1		-0.61	III		WH
Edil3		-0.60	III		
Zbtb44		-0.58	III		
Mrvi1		-0.57	III		
Zfp36l1		-0.57	III	1	WH
Cds2		-0.56	III		
Klhl24		-0.56	III		
Cnst		-0.55	III		
Dennd4c		-0.55	III		
Efcab4a,Pnpla2		-0.55	III		
Dnase1l2,E4f1		-0.55	III		CC
Net1		-0.54	III		
2210018M11Rik/Emsy		-0.53	III		
Sorbs3		-0.53	III		
Pcmtd2		-0.53	III		
Ppl		-0.52	III	2	
Hspa12a		-0.51	III		
Arrdc3		-0.51	III		
Zmiz1		-0.51	III		WH
Fam20c		-0.51	III		
Fam46a		-0.50	III		
Mob3b		-0.50	III		
Pgap1		-0.49	III		
Rfwd2		-0.48	III		
Ubn2		-0.48	III		
Bach1		-0.48	III		
Ube2h		-0.48	III		
Jag1		-0.47	III	1	
Wnt2		-0.47	III		WH
Clec10a		-0.47	III		
Pnpla7		-0.46	III		
Dapp1		-0.46	III		
Pan3		-0.46	III		
Rassf3		-0.45	III		
Heca		-0.44	III		
Slc25a37		-0.43	III		
Zscan26		-0.43	III		
Dusp11		-0.42	III		
Ccl12**		4.10	IV		IR; WH
Upp1**		3.30	IV		
Rab3il1		2.31	IV		
Chi3l3		2.16	IV	1	WH
AA467197/Nmes1**		2.05	IV		
Vcan		2.00	IV		
3110057O12Rik/Abhd18		1.95	IV		
Rarres2§§		1.75	IV		
Gas6		1.68	IV	1	
Ckap2**		1.67	IV		CC
Vpreb3**		1.66	IV	2	
Siglec1		1.60	IV		
Aif1		1.55	IV	2	
Ccl22**		1.53	IV		IR; WH
Gm9920§§		1.50	IV		
Ucp1§§		1.40	IV		
Isg15		1.37	IV	1	
Chst11		1.37	IV		
Lars2		1.36	IV		
Rnf128		1.33	IV		
Eme1**		1.32	IV		
Ifit3		1.31	IV		
Nxpe5		1.29	IV		
Il2ra		1.27	IV		
Cmss1		1.23	IV		
Fdps		1.19	IV	2	LM
Fcgr1		1.17	IV		IR; WH
Sfxn5		1.13	IV		
Papss2		1.12	IV		WH
Trem2		1.09	IV		
Ngfr		1.08	IV		WH
Gatm§§		1.04	IV		WH
Rsad2		1.03	IV		IR

Soat2		0.97	IV		LM
Mvb12b		0.95	IV		
Nme4		0.95	IV		
Tmem8		0.95	IV	1	
Fam213b		0.94	IV		
P2ry6		0.94	IV		
Folr2		0.93	IV		
Irf7		0.87	IV		IR
Cbr2		0.86	IV		
Ifi27l2a		0.86	IV		
Slfn1		0.85	IV	1	CC
Nrp1		0.74	IV		WH
Fpr2		0.72	IV		IR
Oas3		0.72	IV		IR
Cpne2		0.71	IV		
Pram1		0.70	IV		
Cd163		0.68	IV		WH
Pam		0.68	IV		
Nlrc3		0.67	IV		
Ms4a6d		0.66	IV		
Dhx58		0.64	IV		IR
Ddx60		0.64	IV		
Fcrl1		0.63	IV		
Epb4.1l1		0.62	IV		
Tpcn2		0.52	IV		
Lgals1		0.51	IV	3	
Lman1		0.49	IV		
Lrrc59		0.49	IV		
F13a1		0.49	IV		WH
Mrc1		0.49	IV		IR
Etv5		0.49	IV	1	
Rtp4		0.48	IV		
Icam1		0.48	IV	1	IR
Cd38		0.46	IV		
Nme1		0.46	IV		IR
Ncbp1		0.45	IV		
Stat1		0.45	IV		
Ostc		0.44	IV		
Rgs1		-2.44	IV		
Mylk3[§§]		-1.88	IV		
Abca6		-1.84	IV	1	
Spa17[**]		-1.72	IV		
Hhipl1		-1.71	IV		
Ccl3		-1.66	IV		IR; WH
Nupr1l[§§]		-1.66	IV		

Cplx2		0.85	IV		IR
Ms4a6c		0.83	IV		
Mlkl		0.82	IV		
Ppapdc1b		0.82	IV		
Adap2		0.80	IV		
Galk1		0.77	IV		
Glrx		0.76	IV		
H2-Eb2		0.76	IV		IR
Marcksl1		0.76	IV		
Zbp1		0.75	IV		
Tnfrsf11a		0.61	IV		
Rbm3		0.61	IV		
Dhx29		0.57	IV		
Pik3ap1		0.56	IV		
Rhobtb1		0.55	IV		
Slfn5		0.55	IV		
Ctsc		0.54	IV		
Ckap4		0.54	IV		
Acp2		0.53	IV	2	
Lpin2		0.52	IV		
Plcb1		0.52	IV		
Dck		0.52	IV		
Atrip,Trex1		0.52	IV		
Dusp1		-1.49	IV		CC
Fos		-1.34	IV		
Xlr3b[**]		-1.26	IV		
Nr1d1		-1.19	IV	2	
Rab44		-1.15	IV		
Gm15471[**]		-1.15	IV		
Cxcl2		-1.1	IV	2	IR; WH
Cables1		-1.07	IV		CC
Hgf		-1.03	IV	1	
Gfod1		-1.01	IV		
Art3[**]		-1.01	IV		
Zfp72[**]		-1.00	IV		
Egr1		-1.00	IV	1	
Tppp3		-0.99	IV		
Adamtsl4		-0.95	IV		
Fosb		-0.93	IV	1	
Nt5e		-0.91	IV		
Zfp36		-0.87	IV	2	WH
Ccrl2		-0.87	IV		
Tox2		-0.86	IV		
Rgs2		-0.85	IV		CC

Ppap2a		-0.84	IV	1	
Cyp2ab1		-0.84	IV		
Insr		-0.81	IV		
Nedd4		-0.79	IV		
Tmem62		-0.76	IV		
Aqp9		-0.75	IV		
Hr		-0.74	IV	1	
Fabp4		-0.72	IV		
Jun		-0.69	IV		
Garnl3		-0.65	IV		
Ocln		-0.65	IV		
Abca9		-0.64	IV		
Rbpms		-0.62	IV		
Cd14		-0.60	IV	2	IR; WH
Fgfr1		-0.60	IV		WH
Hist1h1c		-0.60	IV		
Slc22a17		-0.60	IV		
Tln2		-0.60	IV		
Cav1		-0.58	IV		IR; WH; LM
Plcb4		-0.58	IV		
Nfia		-0.57	IV		
Egln3		-0.57	IV		
Ston2		-0.56	IV		IR
Tgfb2		-0.53	IV	2	IR; WH
Calcoco1		-0.52	IV		
Myadm		-0.51	IV		
Pi16		-0.51	IV		
Icam2		-0.50	IV	1	
Gata6		-0.50	IV	1	
Csf3r		-0.50	IV		IR
Cask		-0.47	IV	1	
Lama3		-0.46	IV		
Parvb		-0.46	IV		
Fcrls		-0.46	IV		
Slc9a3r2		-0.46	IV		
Junb		-0.45	IV		WH
Mgst1		-0.43	IV		

AP, apoptosis; CC, cell cycle; IR, immune response; LM, lipid metabolism; PF, protein folding; SR, stress response; TX, transcription factors; WH, wound healing. *DEGS excluding anomalous value at 3h, * *DEGS excluding anomalous value at 24h; § DEGS with FPKM < 2 and >1 at 3h; §§ DEGS with FPKM < 2 and >1 at 24h; ^ genes excluded from GO analysis; ° not a DEG value.

Supplementary Table 2 . Functional annotation analysis on the differentially expressed genes (DEGs) lists from 3 and 24 h estrogen treatment was performed using Gene Ontology.

Ontologies		Genes regulated at 3h	Genes regulated at 24h	Grouped categories
GO:0007049	Cell cycle	**CHAF1A, NUF2, CDK1, CCNB2, BUB1B, BIRC5, PRC1, SPC25, SPC24, CENPH, NUSAP1, CHAF1B, CDCA5, UBE2C, GMNN, MKI67, STMN1, UHRF1, TIPIN, LIG1, TACC3, INCENP, MAPK6, CDKN1A, E2F3, CDKN2D, STEAP3**, WEE1, SIK1, BANP, JMY	**NUF2, BIRC5, CCNB2, KIFC1, SPC24, CKAP2, CCNF, CCNB1, BUB1B, CDCA3, CHAF1A, KIF11, MKI67, STMN1, PRC1, NUSAP1, TIMELESS, TPX2, UBE2C, CDC6, SYCE2, AURKB, UHRF1, SLFN1, LIG1, INCENP, STEAP3, MCM2, MAPK6**, DUSP1, KRT7, TACC2, WEE1, CABLES1, BANP, E4F1, SIK1	**Cell cycle (CC)**
GO:0006955	Immune response	**VEGFA, H2-M3, CX3CR1, IL1B, NLRP3, BCL3, RAB27A, CCR5, CCR2**, CCL5, IL7R, LAT, OAS1G, OAS2, IL1RL1, CCR7, LTB	**CCL12, CCL22, FCGR1, CX3CR1, RSAD2, CCR5, IRF7, CPLX2, CD300LF, H2-EB2, OAS3, DHX58, LBP, CD300LD, C1RA, TLR1, ICAM1**, CCL3, IL1RL1, CXCL2, IL16, TNFSF8, CD300A, CD14	**Immune response (IR)**
GO:0006935	Chemotaxis		**CCL12, CCL22, FPR2, LBP**, CCL3, CXCL2, TGFB2, CX3CR1, CSF3R	
GO:0006897	Endocytosis		**FCGR1, STXBP1, DAB2, MSR1, LBP, MRC1, LDLR, NME1**, ULK1, CAV1, STON2	
GO:0009611	Response to wounding	**IL1B, ARG1, NLRP3, RAB27A, CCR5, CCR2**, CCL5, LAT, SGMS1, THBD	**CCL12, ARG1, CHI3L3, CCL22, FCGR1, , PAPSS2, MEFV, GATM, CCR5, NGFR, CD163, LBP, C1RA,TLR1, F13A1**, CCL3, THBD, IL6, CXCL2, ORM2, ORM3, SGMS1, NFKBIZ, CD14, TGFB2	**Wound healing (WH)**
GO:0048534	Hemopoietic/ lymphoid organ development	**VEGFA, CCNB2, TGFBR3, TACC3, ID2, BCL3, PLSCR1, CCR2**, IL7R, ZBTB16, KLF11, MLL1, LTB, EPAS1, PTPN22		
GO:0001568	Blood vessel development		**TGM2, NRP1, MYO1E**, CXCR4, EPHA2, FGFR1, NOTCH1, CAV1, EPAS1, ZFP36L1, ZMIZ1, TGFB2, WNT2, JUNB	
GO:0045834	Positive regulation of lipid metabolic process	**IL1B, ABCG1**, ANGPTL4		**Lipid metabolism (LM)**
GO:0008203	Cholesterol metabolic process		**FDPS, SOAT2, IDI1, LDLR**, APOC1, DHCR24	
GO:0010876	Lipid localization		**MSR1, LBP, LDLR**, APOC1, PLIN2, SLC27A1, CAV1, NRIP1	
GO:0006355	Regulation of transcription	**VEGFA, LBX2, CHAF1A, ZFP580, MXD3, CENPK, TOP2A, EGR3, CHAF1B, TGFBR3, CDCA7, SOX7, ASF1B, MCM5, NLRP3, UHRF1, NR4A1, HES1, ID2, KLF4, E2F3, BCL3, ABCG1, PADI4**, KLF15, PER1, ZBTB16, TCF7, MBD1, JDP2, KLF9, ZFP811, PER2, KLF11, MLL1, PER3, NR1D2, TEF, JMY, TFB2M, TSC22D3, ATXN1, RFX2, EPAS1, MLXIP, POU2F1, IL16		**Regulation fo transcription (TX)**
GO:0042981	Regulation of apoptosis	**VEGFA, SPP1, BIRC5, CX3CR1, PLEKHF1, NLRP3, NR4A1,CDKN1A, BCL3, RIPK3, RAB27A**, ANGPTL4, IL2RB, ZBTB16, TCF7, SGMS1, JMY, TSC22D3, LTB		**Apoptosis (AP)**
GO:0006457	Protein folding	FKBP5, HSPH1, DNAJB13, HSP90AA1, DNAJA1, DNAJB1, FKBP4, AHSA1		**Protein folding (PF)**
GO:0033554	Cellular response to stress	**CHAF1A, CDK1, RAD51AP1, KIF22, CHAF1B, POLE, UHRF1, TIPIN, LIG1, CDKN1A, BCL3**, ANGPTL4, JMY, EPAS1		**Stress Response (SR)**

Genes in bold, up-regulated genes; genes in normal typing, down- regulated genes. Cognate biological categories are grouped as: cell cycle (CC); immune response (IR); wound healing (WH); lipid metabolism (LM); transcription factors (TX); apoptosis (AP); protein folding (PF); stress response (SR). P-value <0.05.

Supplementary Table 3. Cell cycle genes expression. Ct values and 2^{-ddCt} calculation.
Peritoneal macrophages were isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 3, 24 and 48 h. Females in the estrous phase (E) were also investigated. Real time PCR of two independent experiment (A and B) was performed to detect the mRNAs coding for genes related with cell cycle phases and active replication (CcnB2, Cdk1, Ube2c; Ki67).

Gene expression was normalized on 36B4 expression and data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the control group (ME).

A.

		36B4			CDK1					CCNB2					KI67					UBE2C				
		Ct	media	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct		media	dCT	Media ddCt	ddCT	2-ddct
ME	1	18.90	18.98	32.34	32.13	13.15	12.15	1.00	**0.50**	33.48	33.20	14.21	13.12	1.10	**0.47**	32.80	32.44	13.46	12.04	1.42	**0.37**	30.82	30.62	11.64	10.97	0.67	**0.63**
		19.06		31.92						32.92						32.08						30.42					
	2	19.39	19.36	32.06	32.19	12.83		0.68	**0.62**	32.42	33.71	14.35		1.24	**0.42**	31.92	32.34	12.98		0.94	**0.52**	31.37	31.17	11.81		0.84	**0.56**
		19.33		32.32						35.01						32.77						30.97					
	3	19.08	18.99	30.14	30.20	11.21		-0.94	**1.91**	30.89	31.14	12.15		-0.97	**1.96**	30.24	30.30	11.31		-0.73	**1.66**	29.29	29.27	10.28		-0.69	**1.61**
		18.90		30.25						31.38						30.35						29.25					
	4	18.82	19.47	28.91	28.90	9.43		-2.72	**6.57**	29.57	29.56	10.10		-3.02	**8.12**	28.39	28.50	9.04		-3.00	**8.01**	27.90	27.81	8.35		-2.63	**6.18**
		20.12		28.88						29.56						28.62						27.73					
	5	18.67	19.00	31.82	31.82	12.82		0.67	**0.63**	32.42	32.37	13.37		0.25	**0.84**	31.49	31.35	12.35		0.31	**0.80**	29.92	30.01	11.01		0.03	**0.98**
		19.33		31.82						32.32						31.22						30.09					
	6	19.02	18.67	31.08	31.16	12.14		-0.01	**1.01**	31.45	32.20	13.17		0.06	**0.96**	30.50	30.34	11.32		-0.72	**1.65**	29.82	29.79	10.77		-0.21	**1.16**
		19.38		31.24						32.94						30.18						29.76					
	7	19.48	19.48	30.94	31.06	11.58		-0.57	**1.48**	31.85	31.63	12.15		-0.96	**1.95**	30.90	30.84	11.36		-0.68	**1.60**	31.47	30.67	11.19		0.22	**0.86**
		19.48		31.18						31.42						30.77						30.67					
	8	19.37	19.19	31.27	31.00	11.81		-0.34	**1.26**	31.62	32.05	12.86		-0.26	**1.20**	31.52	31.43	12.23		0.19	**0.87**	30.36	30.17	10.98		0.00	**1.00**
		19.02		30.73						32.48						31.33						29.98					
	9	18.84	19.05	30.96	31.34	12.29		0.14	**0.91**	31.87	31.81	12.75		-0.37	**1.29**	30.97	31.14	12.08		0.05	**0.97**	29.92	29.69	10.63		-0.34	**1.27**
		19.27		31.73						31.74						31.31						29.45					
	10	19.75	19.52	30.97	31.01	11.50		-0.65	**1.57**	32.62	32.55	13.03		-0.08	**1.06**	30.59	30.77	11.25		-0.79	**1.73**	29.94	29.97	10.45		-0.52	**1.43**
		19.28		31.06						32.49						30.94						30.01					
ME + 3h E_2	29	21.15	21.51	31.66	31.32	9.81		-2.34	**5.06**	31.96	31.98	10.47		-2.65	**6.27**	33.89	34.27	12.75		0.72	**0.61**	30.64	30.67	9.16		-1.81	**3.52**
		21.87		30.97						32.00						34.64						30.71					
	30	21.29	21.34	31.39	31.14	9.80		-2.34	**5.07**	31.50	31.51	10.17		-2.94	**7.70**	32.24	32.90	11.56		-0.48	**1.39**	29.94	30.03	8.69		-2.28	**4.87**
		21.39		30.89						31.52						33.55						30.11					
	31	21.27	21.27	29.55	29.60	8.33		-3.82	**14.09**	29.67	29.86	8.59		-4.53	**23.05**	33.72	33.78	12.50		0.47	**0.72**	28.61	28.66	7.39		-3.59	**12.03**
		21.27		29.65						30.06						33.83						28.70					
	32	19.44	19.40	27.64	27.75	8.35		-3.80	**13.89**	28.48	28.50	9.10		-4.02	**16.20**	27.24	27.26	7.85		-4.18	**18.17**	27.00	26.99	7.59		-3.39	**10.46**
		19.37		27.87						28.52						27.27						26.98					
	33	19.63	19.58	29.69	29.82	10.24		-1.90	**3.74**	29.93	29.99	10.42		-2.70	**6.51**	29.61	29.50	9.92		-2.12	**4.33**	28.79	28.99	9.42		-1.56	**2.95**
		19.52		29.95						30.05						29.39						29.19					
ME + 24h E_2	34	19.01	19.06	30.33	30.26	11.19		-0.95	**1.93**	31.09	31.08	12.02		-1.10	**2.14**	30.22	30.41	11.34		-0.69	**1.62**	29.86	29.52	10.45		-0.52	**1.43**
		19.12		30.19						31.08						30.60						29.18					
	35	36.33	36.20	36.06																							
	36	21.82	21.89	29.68	29.71	7.81		-4.33	**20.14**	29.86	29.73	7.83		-5.28	**38.92**	34.31	34.60	12.70		0.66	**0.63**	29.90	29.92	8.02		-2.95	**7.75**
		21.97		29.74						29.60						34.88						29.93					
	37	18.86	18.93	29.56	29.65	10.71		-1.43	**2.70**	30.65	30.72	11.79		-1.33	**2.52**	29.73	29.83	10.90		-1.14	**2.21**	28.92	28.85	9.92		-1.06	**2.08**
		19.01		29.73						30.79						29.94						28.78					
ESTROUS	38	22.11	22.28	30.19	30.03	7.75		-4.40	**21.06**	29.59	29.64	7.36		-5.76	**54.06**	33.09	33.30	11.02		-1.02	**2.03**	29.42	29.24	6.95		-4.02	**16.23**
		22.45		29.87						29.69						33.51						29.05					
	39	19.29	19.29	29.55	30.09	10.80		-1.35	**2.54**	30.26	30.17	10.89		-2.23	**4.69**	30.33	30.32	11.03		-1.01	**2.01**	29.35	29.39	10.11		-0.87	**1.82**
		19.29		30.63						30.09						30.31						29.44					
	40	18.99	19.02	29.95	29.93	10.91		-1.24	**2.36**	29.81	30.01	10.99		-2.13	**4.37**	29.33	29.50	10.48		-1.56	**2.95**	28.91	28.96	9.94		-1.04	**2.05**
		19.05		29.91						30.22						29.67						29.01					
	41	19.69	19.84	31.57	31.38	11.54		-0.60	**1.52**	32.22	31.99	12.15		-0.97	**1.96**	31.49	31.83	11.99		-0.04	**1.03**	30.73	30.73	10.89		-0.08	**1.06**
		19.99		31.19						31.76						32.18						undetermined					

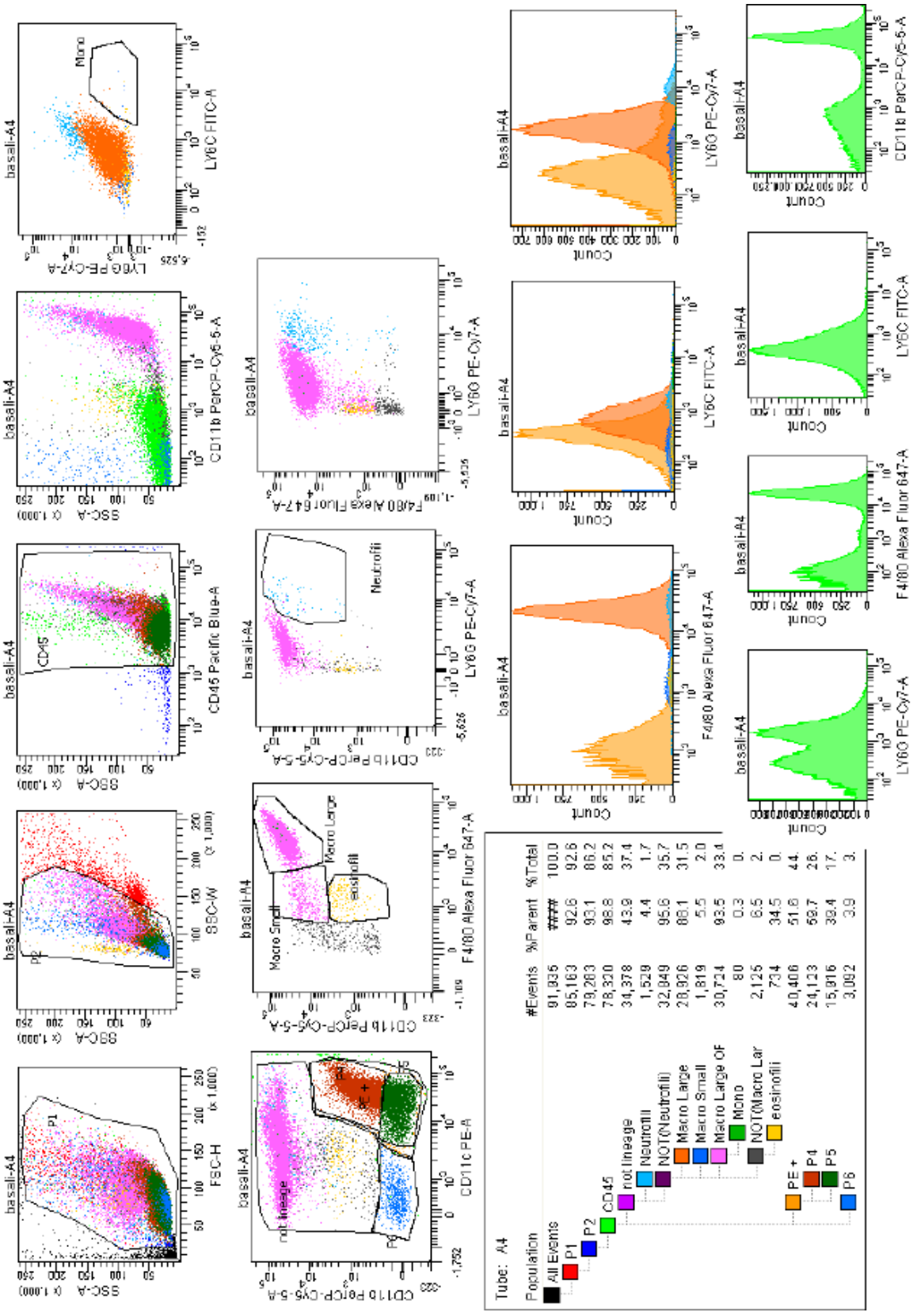
B.

			36B4		CCNB2						CDK1						KI67						UBE2C				
			Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}	Media	dCt	Media ctr	ddCt	2^{-ddCt}
MITO	VEH ME	6.1	15.30	15.18	27.48	27.23	12.05	12.16	-0.12	**1.09**	26.06	26.06	10.88	11.00	-0.12	**1.09**	27.98	27.99	12.81	13.47	-0.67	**1.59**	25.21	10.03	9.98	0.05	**0.96**
			15.06		26.97						27.97						28.00										
		6.2	15.77	15.87	28.04	27.93	12.06		-0.11	**1.08**	Undetermined	26.11	10.24		-0.75	**1.69**	29.71	29.91	14.04		0.57	**0.67**	25.09	9.22		-0.75	**1.69**
			15.96		27.82						26.11						30.12										
		6.3	16.25	15.85	25.10	25.39	9.54		-2.63	**6.18**	24.85	25.13	9.28		-1.72	**3.29**	27.97	27.82	11.97		-1.51	**2.84**	24.99	9.13		-0.84	**1.79**
			15.46		25.67						25.41						27.67										
		6.4	14.23	14.50	25.03	25.13	10.63		-1.53	**2.89**	25.52	25.10	10.60		-0.40	**1.32**	25.66	26.47	11.97		-1.50	**2.83**	25.05	10.55		0.58	**0.67**
			14.77		25.23						24.68						27.28										
		6.5	17.13	17.23	32.88	31.16	13.92		1.76	**0.30**	29.44	29.50	12.27		1.27	**0.41**	33.33	32.31	15.07		1.60	**0.33**	27.33	10.10		0.13	**0.92**
			17.33		29.43						29.56						31.28										
	ME+E2 24H	7.1	15.12	15.22	23.77	23.57	8.35		-3.81	**14.03**	22.72	22.93	7.71		-3.28	**9.74**	27.52	27.86	12.64		-0.83	**1.78**	22.72	7.50		-2.47	**5.55**
			15.31		23.37						23.14						28.20										
		7.2	15.76	15.67	25.13	25.01	9.34		-2.82	**7.06**	Undetermined	24.40	8.73		-2.27	**4.81**	27.41	27.62	11.95		-1.52	**2.87**	24.35	8.68		-1.29	**2.45**
			15.57		24.89						24.40						27.83										
	ME+E2 48H	8.1	14.88	15.06	24.43	24.45	9.39		-2.78	**6.85**	24.04	24.11	9.05		-1.95	**3.85**	27.81	27.60	12.55		-0.93	**1.90**	24.34	9.29		-0.69	**1.61**
			15.23		24.46						24.18						27.40										
		8.2*	20.17	20.23	28.21	27.94	7.71		-4.45	**21.93**	32.56	32.56	12.33		1.33	**0.40**	26.48	27.68	7.45		-6.03	**65.26**	26.98	6.74		-3.23	**9.39**
			20.29		27.68						Undetermined						28.88										
		8.3	18.80	18.81	29.73	29.40	10.59		-1.57	**2.97**	29.09	29.07	10.27		-0.73	**1.66**	31.69	32.53	13.73		0.26	**0.84**	28.42	9.62		-0.36	**1.28**
			18.81		29.07						29.06						33.38										
	Maschi	10.3	15.18	15.22	26.27	26.27	11.05		-1.12	**2.17**	24.76	25.15	9.94		-1.06	**2.09**	27.18	27.20	11.98		-1.49	**2.81**					
			15.25		Undetermined						25.54						27.22										
		10.4*	16.42	16.60	27.14	27.41	10.54		-1.63	**3.09**	31.63	31.63	15.03		4.04	**0.06**	27.36	27.40	10.81		-2.67	**6.36**					
			16.78		27.68						Undetermined						27.45										
WT	VEH ME	1.1	15.61	15.79	26.62	26.52	10.73	12.51	-1.43	**2.70**	25.31	25.32	9.53	12.90	-3.37	**10.32**	27.15	27.20	11.41	13.41	-2.00	**4.00**	25.31	9.52	11.01	-1.49	**2.80**
			15.96		26.42						25.33						27.25										
		1.2	16.55	16.16	28.26	28.03	11.87		-0.29	**1.22**	29.57	29.03	12.87		-0.03	**1.02**	21.58	29.73	13.57		0.15	**0.90**	27.17	11.01		0.00	**1.00**
			15.77		27.81						28.49						29.73										
		1.3	16.36	16.31	23.96	24.17	7.85		-4.31	**19.84**	24.43	24.36	8.05		-4.86	**28.96**	27.52	27.38	11.07		-2.34	**5.08**	24.14	7.82		-3.18	**9.09**
			16.26		24.38						24.28						27.25										
		1.4	18.55	18.51	31.83	31.66	13.16		0.99	**0.50**	31.17	31.44	12.93		0.03	**0.98**	32.10	31.77	13.26		-0.15	**1.11**	29.51	11.01		0.00	**1.00**
			18.46		31.49						31.71						31.43										
	ME+E2 24H	3.1	15.61	15.70	26.88	26.83	11.13		-1.38	**2.60**	25.89	25.82	10.12		-2.78	**6.88**	27.41	27.45	11.75		-1.66	**3.17**	25.15	9.45		-1.56	**2.95**
			15.80		26.79						25.75						27.49										
		3.2	15.97	15.86	26.60	26.47	10.61		-1.90	**3.74**	25.46	25.46	9.60		-3.30	**9.88**	28.96	28.99	13.13		-0.28	**1.21**	26.34	10.48		-0.53	**1.44**
			15.75		26.35						26.66						29.03										
		3.3	17.21	17.34	28.48	28.01	10.66		-1.85	**3.61**	28.18	28.17	10.83		-2.08	**4.22**	29.77	30.19	12.84		-0.57	**1.49**	28.17	10.83		-0.18	**1.13**
			17.48		27.53						28.16						30.61										
	ME+E2 48H	4.1	14.89	15.09	27.03	26.83	11.74		-0.78	**1.71**	25.78	26.03	10.94		-1.97	**3.91**	27.64	27.82	12.72		-0.69	**1.61**	25.27	10.18		-0.83	**1.78**
			15.30		26.63						26.29						28.00										
		4.2	15.45	15.43	25.57	25.36	9.94		-2.58	**5.97**	24.60	24.63	9.20		-3.70	**12.97**	26.55	27.10	11.67		-1.74	**3.35**	24.04	8.61		-2.40	**5.27**
			15.41		25.16						24.66						27.64										
		4.3*	17.22	17.31	27.33	28.00	10.69		-1.82	**3.53**	28.27	28.30	10.99		-1.91	**3.77**	30.11	30.20	12.89		-0.53	**1.44**	27.63	10.32		-0.69	**1.61**
			17.40		28.67						28.33						30.29										
		4.4	18.89	18.82	30.01	30.77	11.95		-0.57	**1.48**	29.43	29.42	10.59		-2.31	**4.95**	30.75	32.19	13.37		-0.05	**1.03**	28.71	9.89		-1.12	**2.17**
			18.75		31.53						29.40						33.64										
	ESTRO	11.2	15.06	15.07	24.41	25.04	9.98		-2.54	**5.80**	24.99	24.91	9.84		-3.06	**8.33**	25.48	25.51	10.44		-2.97	**7.85**	24.54	9.48		-1.53	**2.89**
			15.07		25.67						24.83						25.53										

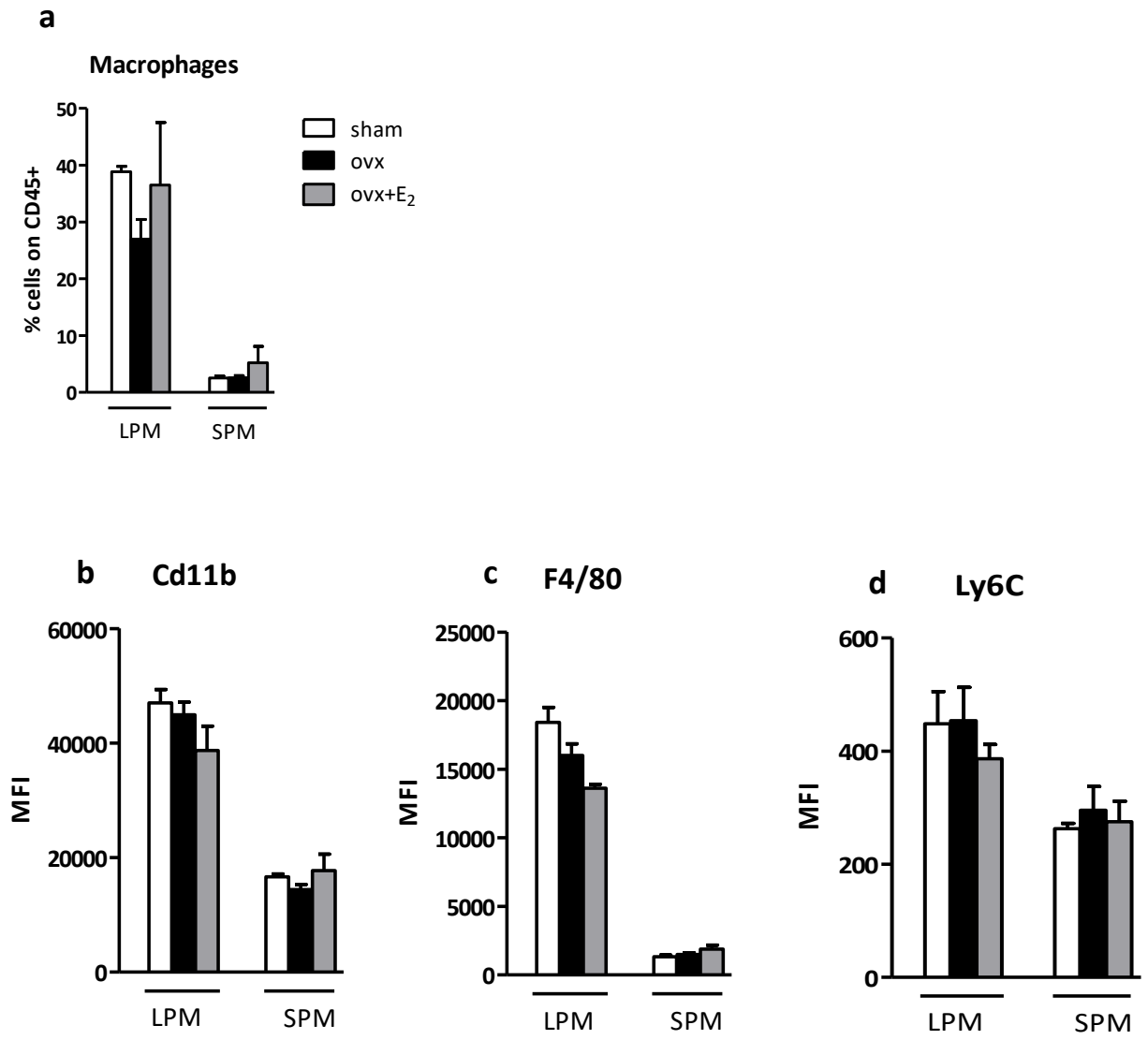
Supplementary Table 4. % of cd11b^{+} peritoneal cells isolated from peritoneal lavage.
Peritoneal macrophages were isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 24 and 48 h. Females in the estrous phase (E) were also investigated.
The number of cd11b+ cells isolated from each sample through magnetic immunosorting was calculated as % on the total number isolated from peritoneum.

		Peritoneal Cells		Peritoneal Cd11b+ Cells	
Experimental group	Sample	Cells/ml (x10^6)	Tot (x3ml)	Cells/ml (x10^6)	%
ME	6.2	1.93	5.79	1.67	28.84
	6.3	1.37	4.11	1.53	37.23
	6.4	3.33	9.99	2.41	24.12
	1.1	2.18	6.54	1.94	29.66
	1.2	0.99	2.97	0.66	22.22
	1.3	1.26	3.78	0.85	22.49
	MEDIA				**27.43**
ME + E2 24h	7.1	1.71	5.13	1.88	36.65
	7.2	1.79	5.37	1.97	36.69
	3.1	1.46	4.38	1.61	36.76
	3.2	0.77	2.31	0.95	41.13
	3.3	0.85	2.55	1.30	50.98
	MEDIA				**40.44**
ME + E2 48h	8.1	2.10	6.30	2.89	45.87
	8.2	1.41	4.23	0.855	20.21
	4.1	3.20	9.60	2.67	27.81
	4.2	1.43	4.29	2.14	49.88
	4.3	1.77	5.31	1.6	30.13
	MEDIA				**38.43**
ESTRO	11.1	1.35	4.05	1.39	34.32
	11.2	1.60	4.80	2.39	49.79
	MEDIA				**42.06**

Supplementary Figure 1. Flow cytometry gating strategy of peritoneal macrophages.
Gating strategy for the identification by FACS analysis of neutrophils (CD45+ , CD11c- ,CD19- ,CCR3- , CD11b+ , LY6G+), eosinophils (CD45+ , CD11c- ,CD19- ,CCR3- , CD11blow, LY6G- ,F4/80low), monocytes (CD45+ , CD11c- ,CD19- ,CCR3- , CD11b+ , LY6G- ,F4/80+ , LY6C+), small peritoneal macrophages (SPM; CD45+ , CD11c- ,CD19- ,CCR3- , CD11b+ , LY6G- ,F4/80low) and large peritoneal macrophages (LPM; CD45+ , CD11c- , CD19- , CCR3- , CD11b+ , LY6G- , F4/80hi). One representative example for peritoneal cells from an unstimulated animal is shown.



Supplementary Figure 2. Flow cytometry analysis of peritoneal macrophages.
Large and small peritoneal macrophages (LPM and SPM, respectively) were identified by FACS analysis in the peritoneal lavage of sham (open bars), ovx (black bars) and ovx+E2 (60 h treatment; grey bars) animals as described in Supplementary Figure 1. The percentage of LPM and SPM with respect to the total number of CD45+ cells is reported in panel a, the expression level of CD11b, F4/80 and Ly6C are reported as Mean Fluorescence Intensity (MFI) in panels b to d, respectively. Results are expressed as the mean ± SEM (n=3). *p < 0.05 versus sham.



Supplementary Table 5. M2 genes expression. Ct values and 2^{-ddCt} calculation.
Peritoneal macrophages were isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 3, 24 and 48 h. Females in the estrous phase (E) were also investigated. Real time PCR of two independent experiment (A and B) was performed to detect the mRNAs coding for genes related with M2 macrophage polarization (Arg1, Ym1,Vegfα).
Gene expression was normalized on 36B4 expression and data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the control group (ME).

A.

		36B4		Arg1					YM1					VEGFα				
		Ct	media	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct	Ct	media	dCT	Media ddCt	ddCT	2-ddct
ME	1	18.90	18.98	25.36	25.46	6.47	6.33	0.14	**0.91**	27.961	28.07	9.09	9.57	-0.48	**1.40**	31.20	31.97	12.99	12.34	0.64	**0.64**
		19.06		25.55						28.186						32.74					
	2	19.39	19.36	26.52	26.52	7.16		0.83	**0.56**	28.345	28.37	9.01		-0.56	**1.47**	35.62	31.71	12.35		0.00	**1.00**
		19.33		26.52						28.402						31.71					
	3	19.08	18.99	25.16	25.09	6.10		-0.22	**1.17**	28.911	29.10	10.11		0.54	**0.69**	32.33	31.48	12.49		0.15	**0.90**
		18.90		25.02						29.292						30.64					
	4	18.82	19.47	25.96	25.46	6.00		-0.33	**1.26**	28.207	28.35	8.88		-0.69	**1.61**	32.05	31.58	12.11		-0.24	**1.18**
		20.12		24.97						28.498						31.10					
	5	18.67	19.00	25.28	25.34	6.34		0.01	**0.99**	30.009	29.72	10.72		1.15	**0.45**	31.82	31.33	12.33		-0.02	**1.01**
		19.33		25.40						29.435						30.84					
	6	18.67	19.02	24.90	25.38	6.36		0.03	**0.98**	28.809	29.02	10.00		0.43	**0.74**	32.14	30.85	11.82		-0.52	**1.44**
		19.38		25.87						29.237						30.85					
	7	19.47	19.48	25.60	25.67	6.19		-0.14	**1.10**	27.899	28.08	8.60		-0.97	**1.96**	30.78	31.15	11.67		-0.67	**1.60**
		19.48		25.73						28.268						31.52					
	8	19.37	19.19	24.72	24.77	5.58		-0.75	**1.68**	29.749	29.48	10.29		0.71	**0.61**	31.56	31.49	12.30		-0.04	**1.03**
		19.02		24.83						29.212						31.43					
	9	18.84	19.05	25.47	25.37	6.31		-0.02	**1.01**	28.366	28.45	9.40		-0.18	**1.13**	31.88	32.07	13.01		0.67	**0.63**
		19.27		25.26						28.539						32.26					
	10	19.75	19.52	26.36	26.30	6.78		0.45	**0.73**	29.342	29.15	9.63		0.06	**0.96**	32.01	31.90	12.38		0.03	**0.98**
		19.28		26.23						28.958						31.78					
ME + 3h E_2	29	21.15	21.51	25.79	25.96	4.44		-1.89	**3.70**	29.254	29.23	7.72		-1.85	**3.61**	30.74	31.03	9.52		-2.83	**7.11**
		21.87		26.12						29.215						31.32					
	30	21.29	21.34	27.59	27.45	6.12		-0.21	**1.16**	29.57	29.09	7.75		-1.82	**3.54**	29.75	29.69	8.36		-3.99	**15.87**
		21.39		27.32						28.601						29.64					
	31	21.27	21.27	26.90	27.26	5.99		-0.34	**1.27**	28.772	28.69	7.42		-2.16	**4.46**	30.82	30.82	9.54		-2.80	**6.96**
		21.27		27.61						28.604						32.63					
	32	19.44	19.40	23.37	23.42	4.01		-2.32	**4.98**	22.593	22.69	3.28		-6.29	**78.34**	27.04	26.95	7.54		-4.80	**27.90**
		19.37		23.46						22.778						26.85					
	33	19.63	19.58	25.87	25.88	6.30		-0.03	**1.02**	29.411	29.37	9.80		0.22	**0.86**	26.95	26.95	7.38		-4.96	**31.21**
		19.52		25.89						29.337						26.96					
Met + 24h E_2	34	19.01	19.06	24.56	24.88	5.81		-0.52	**1.43**	28.01	28.12	9.06		-0.51	**1.43**	30.84	31.41	12.35		0.00	**1.00**
		19.12		25.19						28.238						31.98					
	35	36.33	36.20	Undetermined	39.70					39.633	39.68										
		36.06		39.70						39.727											
	36	21.82	21.89	28.61	28.71	6.81		0.48	**0.72**	27.151	27.21	5.31		-4.26	**19.15**	32.72	32.72	10.82		-1.52	**2.87**
		21.97		28.81						27.268						37.69					
	37	18.86	18.93	20.54	20.56	1.63		-4.70	**26.02**	26.442	26.49	7.56		-2.02	**4.05**	29.52	29.04	10.11		-2.24	**4.72**
		19.01		20.58						26.542						28.56					
Estrous	38	22.11	22.28	27.35	27.31	5.03		-1.30	**2.46**	29.026	28.84	6.56		-3.02	**8.08**	33.61	33.61	11.32		-1.02	**2.03**
		22.45		27.27						28.656						33.60					
	39	19.29	19.29	24.50	24.40	5.11		-1.22	**2.32**	28.382	28.68	9.39		-0.18	**1.13**	30.11	29.97	10.69		-1.66	**3.15**
		19.29		24.30						28.982						29.84					
	40	18.99	19.02	25.13	25.13	6.10		-0.23	**1.17**	28.617	28.60	9.58		0.01	**1.00**	32.32	31.22	12.20		-0.15	**1.11**
		19.05		25.12						28.587						31.22					
	41	19.69	19.84	25.06	25.11	5.28		-1.05	**2.08**	28.386	28.35	8.51		-1.06	**2.09**	30.78	31.08	11.25		-1.10	**2.14**
		19.99		25.17						28.314						31.39					

B.

			36B4		ARG1						YM1						VEGFα					
			Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
MITO	VEH ME	6.1	15.30	15.18	21.34	21.70	6.51	6.61	-0.09	**1.07**	25.52	25.31	10.13	9.29	0.84	**0.56**	23.63	23.80	8.62	8.75	-0.13	**1.09**
			15.06		22.05						25.10						23.97					
		6.2	15.77	15.87	22.92	23.19	7.32		0.71	**0.61**	24.05	24.46	8.59		-0.71	**1.63**	24.92	25.11	9.24		0.49	**0.71**
			15.96		23.45						24.86						25.31					
		6.3	16.25	15.85	23.29	23.06	7.20		0.60	**0.66**	24.86	24.93	9.07		-0.22	**1.16**	24.18	24.23	8.38		-0.37	**1.29**
			15.46		22.82						24.99						24.28					
		6.4	14.23	14.50	18.99	18.86	4.36		-2.25	**4.75**	24.03	24.25	9.75		0.46	**0.73**	23.73	23.64	9.15		0.40	**0.76**
			14.77		18.73						24.48						23.56					
		6.5	17.13	17.23	22.70	22.63	5.40		-1.21	**2.31**	26.73	26.62	9.38		0.09	**0.94**						
			17.33		22.56						26.51											
	ME+E2 24H	7.1	15.12	15.22	19.66	19.48	4.26		-2.35	**5.09**	23.39	23.81	8.59		-0.70	**1.63**	25.92	25.95	10.73		1.99	**0.25**
			15.31		19.30						24.23						25.99					
		7.2	15.76	15.67	20.53	20.60	4.94		-1.67	**3.18**	25.80	25.77	10.10		0.81	**0.57**	22.93	23.35	7.68		-1.07	**2.09**
			15.57		20.68						25.74						23.76					
	ME+E2 48H	8.1	14.88	15.06	20.34	20.44	5.38		-1.23	**2.34**	24.11	24.40	9.34		0.05	**0.97**	22.74	22.82	7.77		-0.98	**1.97**
			15.23		20.54						24.68						22.91					
		8.2*	20.17	20.23	25.44	25.12	4.88		-1.72	**3.31**	26.72	26.83	6.60		-2.70	**6.48**	27.43	27.31	7.08		-1.66	**3.17**
			20.29		24.79						26.94						27.20					
		8.3	18.80	18.81	24.18	24.22	5.41		-1.20	**2.29**	29.48	29.03	10.23		0.93	**0.52**						
			18.81		24.25						28.58											
	ESTRO	11.1	17.57	17.89	22.99	23.18	5.29		-1.32	**2.50**	27.54	27.44	9.55		0.25	**0.84**	27.84	27.98	10.09		1.34	**0.39**
			18.22		23.38						27.35						28.12					
WT	VEH ME	1.1	15.61	15.79	21.16	21.69	5.90	6.23	-0.33	**1.26**	25.93	25.92	10.13	9.52	0.61	**0.65**	25.48	25.54	9.76	9.76	0.00	**1.00**
			15.96		22.22						25.90						25.60					
		1.2	16.55	16.16	22.51	22.58	6.42		0.19	**0.88**	26.72	26.65	10.49		0.97	**0.51**						
			15.77		22.65						26.58											
		1.3	16.36	16.31	24.25	24.39	8.08		1.85	**0.28**	23.21	23.27	6.96		-2.56	**5.92**						
			16.26		24.54						23.33											
		1.4	18.55	18.51	24.84	24.88	6.37		0.14	**0.91**	26.65	26.44	7.94		-1.58	**2.99**						
			18.46		24.91						26.24											
	ME+E2 24H	3.1	15.61	15.70	20.10	20.13	4.43		-1.80	**3.49**	23.52	23.52	7.81		-1.71	**3.26**	25.33	24.95	9.24		-0.51	**1.43**
			15.80		20.17						23.51						24.57					
		3.2	15.97	15.86	21.65	21.67	5.81		-0.42	**1.34**	23.11823	23.25	7.39		-2.13	**4.39**	24.00	24.05	8.19		-1.57	**2.96**
			15.75		21.69						23.38008						24.11					
		3.3	17.21	17.34	22.35	22.67	5.33		-0.91	**1.87**	26.74509	26.56	9.22		-0.30	**1.24**	25.55	25.53	8.18		-1.57	**2.98**
			17.48		22.99						26.37556						25.50					
	ME+E2 48H	4.1	14.89	15.09	20.44	20.31	5.22		-1.02	**2.02**	20.85	20.78	5.68		-3.84	**14.31**	24.21	24.40	9.30		-0.45	**1.37**
			15.30		20.18						20.70						24.59					
		4.2	15.45	15.43	19.60	19.79	4.37		-1.87	**3.65**	20.11	20.31	4.89		-4.63	**24.81**	23.78	23.88	8.45		-1.30	**2.47**
			15.41		19.99						20.52						23.97					
		4.3*	17.22	17.31	22.63	22.61	5.30		-0.93	**1.91**	26.44605	26.40	9.09		-0.43	**1.34**	26.19	26.09	8.78		-0.98	**1.97**
			17.40		22.58						26.35791						25.98					
		4.4	18.89	18.82	24.44	24.47	5.64		-0.59	**1.50**	27.37	27.58	8.75		-0.77	**1.70**						
			18.75		24.49						27.79											
	ESTRO	11.2	15.06	15.07	22.14	22.51	7.45		1.21	**0.43**	23.45024	23.58	8.51		-1.01	**2.01**	25.98	26.27	11.21		1.45	**0.37**
			15.07		22.88						23.70526						26.56					

Supplementary Table 6. Protein expression of Vegfα (A), IL-10 (B), TNFα (C) in the peritoneal fluid.
Sham-operated (A1-A13 representative samples), ovariectomized (B1-B12 representative samples), or ovariectomized and treated with E2 (C1-C15 representative samples) animals were injected with zymosan (zymo) or veh(physiological). Protein concentration of Vegfα (A), IL10 (B) and TNFα (C) was evaluated in peritoneal fluid through ELISA assay.

A.

STIMULUS	TIME POINT (?)	ID	Raw (pg/ml)	CF	Real (pg/ml)	Media (pg/ml)	V lavaggio	Totale (pg)	Media (pg)
Zymo	12h	A1	250.46	4.44	56.35	42.53	2	112.71	85.07
Zymo		A2	161.8	5.00	32.36		2	64.72	
Zymo		A3	194.42	5.00	38.88		2	77.77	
Physiological		A4	145.28	5.13	28.33	28.33	2	56.66	56.66
Zymo	36h	A5	221.38	5.00	44.28	39.61	2	88.55	79.22
Zymo		A6	187.48	5.00	37.50		2	74.99	
Zymo		A7	190.05	5.13	37.06		2	74.12	
Physiological		A8	162.41	5.26	30.86	30.86	2	61.72	61.72
Zymo	60h	A9	338.95	5.41	62.71	62.74	2	125.41	125.49
Zymo		A10	326.64	5.41	60.43		2	120.86	
Zymo		A11	361.66	5.56	65.10		2	130.20	
Physiological		A12	197.5	5.26	37.53	33.32	2	75.05	66.64
Physiological		A13	157.39	5.41	29.12		2	58.23	
Zymo	12h	B1	219.32	5.41	40.57	47.07	2	81.15	94.14
Zymo		B2	318.88	5.26	60.59		2	121.17	
Zymo		B3	210.76	5.26	40.04		2	80.09	
Physiological		B4	168.96	5.26	32.10	32.10	2	64.20	64.20
Zymo	36h	B5	209.77	5.41	38.81	42.37	2	77.61	84.75
Zymo		B6	235.1	5.13	45.84		2	91.69	
Zymo		B7	217.78	5.13	42.47		2	84.93	
Physiological		B8	151.17	5.13	29.48	29.48	2	58.96	58.96
Zymo	60h	B9	257.17	5.13	50.15	51.86	2	100.30	103.72
Zymo		B10	127.64	5.00	25.53		2	51.06	
Zymo		B11	399.51	5.00	79.90		2	159.80	
Physiological		B12	156.63	5.41	28.98	28.98	2	57.95	57.95
Zymo	12h	C1	153.82	5.26	29.23	38.04	2	58.45	76.07
Zymo		C2	195.63	5.71	34.24		2	68.47	
Zymo		C3	266.57	5.26	50.65		2	101.30	
Physiological		C4	155.81	5.88	26.49	24.12	2	52.98	48.24
Physiological		C5	117.58	5.41	21.75		2	43.50	
Zymo	36h	C6	177.66	5.41	32.87	33.51	2	65.73	67.02
Zymo		C7	219.22	6.90	31.79		2	63.57	
Zymo		C8	188.86	5.26	35.88		2	71.77	
Physiological		C9	127.54	5.00	25.51	28.30	2	51.02	56.61
Physiological		C10	159.48	5.13	31.10		2	62.20	
Zymo	60h	C11	265.97	5.56	47.87	49.03	2	95.75	98.06
Zymo		C12	300.01	5.00	60.00		2	120.00	
Zymo		C13	150.82	3.85	39.21		2	78.43	
Physiological		C14	158.31	5.13	30.87	29.19	2	61.74	58.39
Physiological		C15	137.58	5.00	27.52		2	55.03	

B.

STIMULUS	TIME POINT (?)	ID	Raw (pg/ml)	CF	Real (pg/ml)	Media (pg/ml)	V lavaggio	Totale (pg)	Media (pg)
Zymo	12h	A1	11.72	4.44	2.64	7.05	2	5.27	14.10
Zymo		A2	30.15	5.00	6.03		2	12.06	
Zymo		A3	62.39	5.00	12.48		2	24.96	
Physiological		A4	19.06	5.13	3.72	3.72	2	7.43	7.43
Zymo	36h	A5	31.68	5.00	6.34	4.25	2	12.67	8.50
Zymo		A6	24.04	5.00	4.81		2	9.62	
Zymo		A7	8.26	5.13	1.61		2	3.22	
Physiological		A8	24.61	5.26	4.68	4.68	2	9.35	9.35
Zymo	60h	A9	21.58	5.41	3.99	3.66	2	7.98	7.33
Zymo		A10	24.03	5.41	4.45		2	8.89	
Zymo		A11	14.18	5.56	2.55		2	5.10	
Physiological		A12	19.31	5.26	3.67	2.78	2	7.34	5.56
Physiological		A13	10.24	5.41	1.89		2	3.79	
Zymo	12h	B1	55.99	5.41	10.36	12.05	2	20.72	24.10
Zymo		B2	87.19	5.26	16.57		2	33.13	
Zymo		B3	48.52	5.26	9.22		2	18.44	
Physiological		B4	7.66	5.26	1.46	1.46	2	2.91	2.91
Zymo	36h	B5	36.69	5.41	6.79	5.13	2	13.58	10.25
Zymo		B6	10.36	5.13	2.02		2	4.04	
Zymo		B7	33.71	5.13	6.57		2	13.15	
Physiological		B8	13.94	5.13	2.72	2.72	2	5.44	5.44
Zymo	60h	B9	21.8	5.13	4.25	3.38	2	8.50	6.76
Zymo		B10	8.96	5.00	1.79		2	3.58	
Zymo		B11	20.52	5.00	4.10		2	8.21	
Physiological		B12	11.26	5.41	2.08	2.08	2	4.17	4.17
Zymo	12h	C1	30.07	5.26	5.71	7.89	2	11.43	15.78
Zymo		C2	43.71	5.71	7.65		2	15.30	
Zymo		C3	54.23	5.26	10.30		2	20.61	
Physiological		C4	13.41	5.88	2.28	2.33	2	4.56	4.66
Physiological		C5	12.86	5.41	2.38		2	4.76	
Zymo	36h	C6	43.05	5.41	7.96	5.59	2	15.93	11.18
Zymo		C7	26.04	6.90	3.78		2	7.55	
Zymo		C8	26.46	5.26	5.03		2	10.05	
Physiological		C9	19.67	5.00	3.93	3.11	2	7.87	6.23
Physiological		C10	11.76	5.13	2.29		2	4.59	
Zymo	60h	C11	10.86	5.56	1.95	4.41	2	3.91	8.83
Zymo		C12	33.59	5.00	6.72		2	13.44	
Zymo		C13	17.58	3.85	4.57		2	9.14	
Physiological		C14	13.79	5.13	2.69	2.41	2	5.38	4.82
Physiological		C15	10.64	5.00	2.13		2	4.26	

C.

STIMULUS	TIME POINT (?)	ID	Raw (pg/ml)	CF	Real (pg/ml)	Media (pg/ml)	V lavaggio	Totale (pg)	Media (pg)
Zymo	12h	A1	26.56	4.44	5.98	28.92	2	11.95	57.84
Zymo		A2	156.18	5.00	31.24		2	62.47	
Zymo		A3	247.73	5.00	49.55		2	99.09	
Physiological		A4	1.29	5.13	0.25	0.25	2	0.50	0.50
Zymo	36h	A5	96.18	5.00	19.24	#VALORE!	2	38.47	41.80
Zymo		A6	112.84	5.00	22.57		2	45.14	
Zymo		A7	-	5.13	#VALORE!		2	#VALORE!	
Physiological		A8	5.94	5.26	1.13	1.13	2	2.26	2.26
Zymo	60h	A9	47.79	5.41	8.84	7.46	2	17.68	14.93
Zymo		A10	41.69	5.41	7.71		2	15.43	
Zymo		A11	32.42	5.56	5.84		2	11.67	
Physiological		A12	10.73	5.26	2.04	2.28	2	4.08	4.56
Physiological		A13	13.61	5.41	2.52		2	5.04	
Zymo	12h	B1	300.86	5.41	55.66	61.97	2	111.32	123.93
Zymo		B2	407.46	5.26	77.42		2	154.83	
Zymo		B3	278.01	5.26	52.82		2	105.64	
Physiological		B4	17.12	5.26	3.25	3.25	2	6.51	6.51
Zymo	36h	B5	146.61	5.41	27.12	22.69	2	54.25	45.39
Zymo		B6	113.1	5.13	22.05		2	44.11	
Zymo		B7	96.94	5.13	18.90		2	37.81	
Physiological		B8	-	5.13	#VALORE!	#VALORE!	2	#VALORE!	#VALORE!
Zymo	60h	B9	31.25	5.13	6.09	8.40	2	12.19	16.80
Zymo		B10	42.01	5.00	8.40		2	16.80	
Zymo		B11	53.53	5.00	10.71		2	21.41	
Physiological		B12	-	5.41	#VALORE!	#VALORE!	2	#VALORE!	#VALORE!
Zymo	12h	C1	163.08	5.26	30.99	39.43	2	61.97	78.86
Zymo		C2	219	5.71	38.33		2	76.65	
Zymo		C3	257.82	5.26	48.99		2	97.97	
Physiological		C4	13.84	5.88	2.35	1.73	2	4.71	3.45
Physiological		C5	5.94	5.41	1.10		2	2.20	
Zymo	36h	C6	33.12	5.41	6.13	11.41	2	12.25	22.82
Zymo		C7	113.01	6.90	16.39		2	32.77	
Zymo		C8	61.63	5.26	11.71		2	23.42	
Physiological		C9	16.06	5.00	3.21	2.58	2	6.42	5.15
Physiological		C10	9.94	5.13	1.94		2	3.88	
Zymo	60h	C11	8.78	5.56	1.58	11.54	2	3.16	23.08
Zymo		C12	146.03	5.00	29.21		2	58.41	
Zymo		C13	14.74	3.85	3.83		2	7.66	
Physiological		C14	1.45	5.13	0.28	0.56	2	0.57	1.13
Physiological		C15	4.23	5.00	0.85		2	1.69	

Supplementary Table 7. Target gene expression by E_2 in isolated macrophages. Ct values and 2^{-ddCt} calculation. BMDM (A) or peritoneal macrophages (B) were grown in culture and assayed for gene expression following E2 administration. (A) BMDM cells were treated with vehicle (veh, open bars) or increasing concentrations of E2 (10^{-9}, 10^{-7} and 10^{-5} M, filled bars) for 3 h. Real time PCR was used to analyze the mRNA levels coding for Vegfα, Angtpl4 and Arg1. (C) Comparison of estrogen action in BMDM cells from WT and ERα-KO (ERKO) mice. The mRNA levels coding for Vegfα, Angtpl4 and Arg1 were analyzed 24 h after the addition of vehicle (veh, open bars) or 10^{-7} M E2 (filled bars). Gene expression was normalized on 36B4 expression and data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle group.

A.

	36B4		Vegfα				Arg1				Angptl4			
	Ct	media Ct	Ct	media Ct	dCT	media dCT ctrl	ddCT	2-ddCT	Ct	media Ct	dCT	media	ddCT	2-ddCT	Ct	media Ct	dCT	media dCT ctrl	ddCT	2-ddCT
9 veh 3h	15.63	15.51	25.46	25.40	9.89	**9.89**	0.00	**1.00**	29.87	29.86	14.35	**14.49**	-0.14	**1.10**	21.96	22.26	6.75	**7.13**	-0.38	**1.30**
	15.40		25.34						29.85						22.56					
10 veh 3h	14.89	15.36	25.39	25.51	9.88		0.00	**1.00**	29.91	29.99	14.64		0.14	**0.91**	23.77	22.87	7.51		0.38	**0.77**
	15.82		25.62						30.08						21.97					
11 E2 10^{-9}M 3h	15.33	15.43	23.04	23.13	7.70		-2.19	**4.56**	30.01	29.44	14.01		-0.48	**1.40**	23.29	23.28	7.84		0.71	**0.61**
	15.53		23.23						28.88						23.26					
12 E2 10^{-9}M 3h	15.33	15.39	22.96	22.89	7.50		-2.38	**5.22**	29.61	29.57	14.19		-0.31	**1.24**	23.38	23.22	7.84		0.71	**0.61**
	15.44		22.82						29.54						23.06					
13 E2 10^{-7}M 3h	15.43	15.37	22.87	23.13	7.76		-2.13	**4.36**	29.35	29.64	14.28		-0.22	**1.16**	23.67	23.57	8.21		1.08	**0.47**
	15.30		23.38						29.94						23.47					
14 E2 10^{-7}M 3h	15.47	15.21	22.55	22.54	7.33		-2.56	**5.90**	29.19	29.34	14.13		-0.37	**1.29**	23.21	23.15	7.94		0.81	**0.57**
	14.95		22.52						29.48						23.09					
15 E2 10^{-5}M 3h	15.09	15.25	21.66	21.68	6.43		-3.46	**10.98**	29.56	29.38	14.14		-0.36	**1.28**	23.86	24.01	8.77		1.64	**0.32**
	15.40		21.70						29.21						24.16					
16 E2 10^{-5}M 3h	15.49	15.60	21.73	21.76	6.16		-3.73	**13.27**	29.91	29.91	14.31		-0.18	**1.14**	24.52	24.51	8.91		1.78	**0.29**
	15.71		21.79						29.91						24.51					

B.

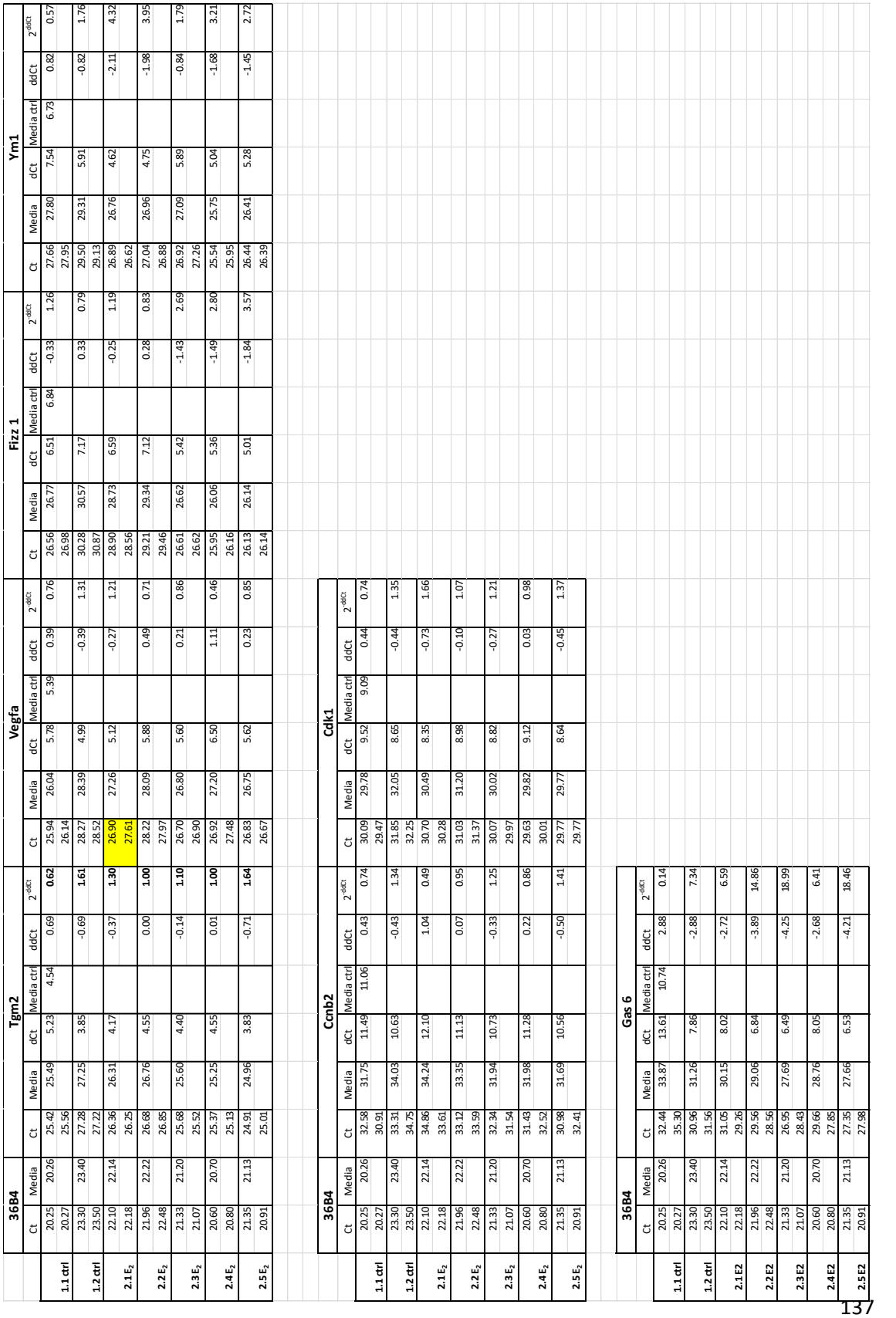
	36B4		Arg1						Vegfα						Angptl4					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1 RPMI+0.01% EtOH 3h	16.40	16.49	22.70	22.79	6.31	6.31	0.00	**1.00**	26.01	26.33	9.85	9.85	0.00	**1.00**	22.98	22.99	6.51	6.51	0.00	**1.00**
	16.58		22.89						26.66						23.01					
2 E_2 10^{-5} M 3h	16.92	16.92	21.92	21.91	4.99		-1.32	**2.49**	22.76	22.89	5.97		-3.88	**14.68**	24.46	24.58	7.66		1.16	**0.45**
	16.92		21.90						23.02						24.70					

	36B4		Arg 1						VEGFα						Angptl4					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1 RPMI+0.01% EtOH 3h	17.80	17.91	25.47	25.60	7.69	7.69	0.00	**1.00**	27.09	27.25	9.34	9.34	9.34	**0.00**	25.22	25.20	7.29	7.29	0.00	**1.00**
	18.01		25.72						27.40						25.17					
2 E_2 10^{-5} M 3h	18.37	18.34	24.72	24.73	6.39		-1.30	**2.46**	23.82	23.82	5.49		-3.85	**14.46**	26.29	26.48	8.14		0.85	**0.55**
	18.31		24.73						23.83						26.67					

C.

	36B4		ARG1						VEGFa						Angptl4					
	Ct	media Ct	Ct	media Ct	dCT	media dCT ctrl	ddCT	2-ddCT	Ct	media Ct	dCT	media dCT ctrl	ddCT	2-ddCT	Ct	media Ct	dCT	media dCT ctrl	ddCT	2-ddCT
9 veh 24h	15.78	15.77	31.87	32.26	16.48	16.16	0.33	**0.80**	27.20	27.10	11.33	11.69	-0.36	**1.28**	17.33	25.29	9.52	10.47	-0.95	**1.93**
	15.76		32.64						27.01						33.26					
10 veh 24h	15.62	15.63	30.77	31.46	15.83		-0.33	**1.25**	27.72	27.69	12.06		0.36	**0.78**	26.93	27.05	11.42		0.95	**0.52**
	15.65		32.15						27.66						27.17					
5 E2 10 $^{-7}$	16.28	16.34	31.53	31.84	15.50		-0.66	**1.58**	27.04	26.88	10.54		-1.15	**2.22**	27.93	28.16	11.82		1.35	**0.39**
	16.41		32.15						26.73						28.40					
6 E2 10 $^{-7}$	16.37	16.34	31.97	32.05	15.71		-0.45	**1.36**	26.55	26.69	10.35		-1.34	**2.54**	27.61	28.09	11.75		1.28	**0.41**
	16.31		32.13						26.83						28.57					
33 veh 24h	16.49	16.34	30.22	30.56	14.22		-1.94	**3.84**	28.73	28.72	12.38		0.68	**0.62**	26.95	27.32	10.97		0.50	**0.70**
	16.20		30.90						28.71						27.68					
34 veh 24h	16.26	16.33	32.01	31.62	15.29		-0.87	**1.83**	29.63	29.15	12.82		1.13	**0.46**	27.78	27.74	11.41		0.94	**0.52**
	16.40		31.22						28.68						27.69					
29 E2 10 $^{-7}$	16.41	16.27	31.79	32.02	15.75		-0.40	**1.32**	29.60	28.78	12.51		0.81	**0.57**	26.82	26.92	10.65		0.18	**0.88**
	16.13		32.26						27.96						27.03					
30 E2 10 $^{-7}$	16.14	16.15	30.91	30.58	14.43		-1.73	**3.32**	28.40	28.20	12.05		0.35	**0.78**	27.43	26.94	10.79		0.32	**0.80**
	16.17		30.24						28.00						26.44					

Supplementary Table 8. Expression of E2 target genes in microglia *in vivo*. Ct values and 2^{-ddCt} calculation.
Microglia was isolated from female mice at the metaestrous phase (ME) of the estrous cycle treated with E_2 for 24 h. Real time PCR was performed to detect the mRNAs coding for genes related with M2 polarization (Fizz1, Ym1, Vegf*α*, Gas6) and cell cycle (Ccnb2 and Cdk1).
Gene expression was normalized on 36B4 expression and data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the control group (ME).



	36B4		Tgm2					Vegfa					Fizz 1					Ym1				
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1.1 ctrl	20.25	20.26	25.42	25.49	5.23	4.54	0.69	**0.62**	25.94	26.04	5.78	5.39	0.39	0.76	26.56	26.77	6.51	6.84	-0.33	1.26	27.66	27.80	7.54	6.73	0.82	0.57
	20.27		25.56						26.14						26.98						27.95					
1.2 ctrl	23.30	23.40	27.28	27.25	3.85		-0.69	**1.61**	28.27	28.39	4.99		-0.39	1.31	30.28	30.57	7.17		0.33	0.79	29.50	29.31	5.91		-0.82	1.76
	23.50		27.22						28.52						30.87						29.13					
2.1 E_2	22.10	22.14	26.36	26.31	4.17		-0.37	**1.30**	26.90	27.26	5.12		-0.27	1.21	28.90	28.73	6.59		-0.25	1.19	26.89	26.76	4.62		-2.11	4.32
	22.18		26.25						27.61						28.56						26.62					
2.2 E_2	21.96	22.22	26.68	26.76	4.55		0.00	**1.00**	28.22	28.09	5.88		0.49	0.71	29.21	29.34	7.12		0.28	0.83	27.04	26.96	4.75		-1.98	3.95
	22.48		26.85						27.97						29.46						26.88					
2.3 E_2	21.33	21.20	25.68	25.60	4.40		-0.14	**1.10**	26.70	26.80	5.60		0.21	0.86	26.61	26.62	5.42		-1.43	2.69	26.92	27.09	5.89		-0.84	1.79
	21.07		25.52						26.90						26.62						27.26					
2.4 E_2	20.60	20.70	25.37	25.25	4.55		0.01	**1.00**	26.92	27.20	6.50		1.11	0.46	25.95	26.06	5.36		-1.49	2.80	25.54	25.75	5.04		-1.68	3.21
	20.80		25.13						27.48						26.16						25.95					
2.5 E_2	21.35	21.13	24.91	24.96	3.83		-0.71	**1.64**	26.83	26.75	5.62		0.23	0.85	26.13	26.14	5.01		-1.84	3.57	26.44	26.41	5.28		-1.45	2.72
	20.91		25.01						26.67						26.14						26.39					

	36B4		Ccnb2					Cdk1					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1.1 ctrl	20.25	20.26	32.58	31.75	11.49	11.06	0.43	0.74	30.09	29.78	9.52	9.09	0.44	0.74
	20.27		30.91						29.47					
1.2 ctrl	23.30	23.40	33.31	34.03	10.63		-0.43	1.34	31.85	32.05	8.65		-0.44	1.35
	23.50		34.75						32.25					
2.1 E_2	22.10	22.14	34.86	34.24	12.10		1.04	0.49	30.70	30.49	8.35		-0.73	1.66
	22.18		33.61						30.28					
2.2 E_2	21.96	22.22	33.12	33.35	11.13		0.07	0.95	31.03	31.20	8.98		-0.10	1.07
	22.48		33.59						31.37					
2.3 E_2	21.33	21.20	32.34	31.94	10.73		-0.33	1.25	30.07	30.02	8.82		-0.27	1.21
	21.07		31.54						29.97					
2.4 E_2	20.60	20.70	31.43	31.98	11.28		0.22	0.86	29.63	29.82	9.12		0.03	0.98
	20.80		32.52						30.01					
2.5 E_2	21.35	21.13	30.98	31.69	10.56		-0.50	1.41	29.77	29.77	8.64		-0.45	1.37
	20.91		32.41						29.77					

	36B4		Gas 6					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1.1 ctrl	20.25	20.26	32.44	33.87	13.61	10.74	2.88	0.14
	20.27		35.30					
1.2 ctrl	23.30	23.40	30.96	31.26	7.86		-2.88	7.34
	23.50		31.56					
2.1 E2	22.10	22.14	31.05	30.15	8.02		-2.72	6.59
	22.18		29.26					
2.2 E2	21.96	22.22	29.56	29.06	6.84		-3.89	14.86
	22.48		28.56					
2.3 E2	21.33	21.20	26.95	27.69	6.49		-4.25	18.99
	21.07		28.43					
2.4 E2	20.60	20.70	29.66	28.76	8.05		-2.68	6.41
	20.80		27.85					
2.5 E2	21.35	21.13	27.35	27.66	6.53		-4.21	18.46
	20.91		27.98					

Supplementary Table 9 . Cell cycle genes expression following IL-4 treatment in peritoneal macrophages and brain microglia *in vivo*. Ct values and 2^{-ddCt} calculation.

Animals were treated for 24h with IL-4 or veh through icv injection for brain analysis (**A**; some representative samples) or i.p. for peritoneal macrophages analysis (**B**). Real time PCR was performed to detect the mRNAs coding for genes related with cell cycle(Cdk1, Ccnb2 and Ki67) and M2 polarization (Arg1) in brain (**A**) and peritoneal macrophages (**B**). Gene expression was normalized on 36B4 expression and data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle group (ME).

A.

	36B4		KI67						CCNB2						CDK1					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1 veh	18.16	18.06	31.77	31.37	13.31	13.53	-0.21	**1.16**	27.58	27.43	9.37	9.44	-0.06	**1.04**	27.51	27.64	9.58	9.89	-0.30	**1.23**
	17.96		30.9771						27.29						27.78					
2 veh	17.85	17.91	30.70	31.53	13.63		0.10	**0.93**	26.94	27.11	9.21		-0.23	**1.17**	27.11	27.07	9.16		-0.72	**1.65**
	17.96		32.3656						27.29						27.03					
3 veh	17.89	17.93	30.13	30.43	12.50		-1.02	**2.03**	26.50	26.54	8.61		-0.82	**1.77**	26.58	26.60	8.67		-1.21	**2.32**
	17.97		30.7311						26.58						26.62					
4 IL4	16.93	17.04	28.27	28.50	11.46		-2.06	**4.17**	25.80	25.78	8.75		-0.69	**1.61**	26.15	26.15	9.11		-0.78	**1.71**
	17.14		28.7342						25.76						26.14					
5 IL4	17.11	17.38	30.44	30.63	13.25		-0.28	**1.21**	26.31	26.21	8.83		-0.61	**1.52**	26.96	26.86	9.48		-0.41	**1.33**
	17.65		30.8122						26.12						26.76					
6 IL4	17.68	17.76	30.77	30.40	12.64		-0.88	**1.85**	26.46	26.29	8.53		-0.91	**1.87**	27.19	27.34	9.58		-0.31	**1.24**
	17.84		30.0308						26.12						27.48					
7 IL4	17.58	17.41	30.07	29.96	12.55		-0.97	**1.96**	26.74	26.46	9.05		-0.39	**1.31**	25.94	26.14	8.73		-1.16	**2.23**
	17.24		29.8633						26.17						26.34					
8 IL4	17.78	17.50	30.50	30.31	12.81		-0.72	**1.64**	26.88	26.88	9.38		-0.06	**1.04**	26.52	26.34	8.84		-1.04	**2.06**
	17.22		30.1165						Undeterm						26.17					

	36B4		ARG1					
	Ct	Media	Ct	Media	dCt	Media ctrl	ddCt	2^{-ddCt}
1 veh	18.16	18.06	30.12	30.07	12.01	12.04	-0.03	**1.02**
	17.96		30.01					
2 veh	17.85	17.91	30.03	29.97	12.07		0.03	**0.98**
	17.96		29.91					
3 veh	17.89	17.93						
	17.97							
4 IL4	16.93	17.04	29.50	29.59	12.56		0.52	)
	17.14		29.69					
5 IL4	17.11	17.38	25.21	25.35	7.97		-4.07	**16.78**
	17.65		25.48					
6 IL4	17.68	17.76	24.40	24.47	6.71		-5.32	**40.04**
	17.84		24.55					
7 IL4	17.58	17.41						
	17.24							
8 IL4	17.78	17.50						
	17.22							

B.

		36B4		CCNB2						CDK1						KI67					
		Ct	Media	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}
VEH ME	6.1	15.30	15.18	27.48	27.23	12.05	12.16	-0.12	**1.09**	26.06	26.06	10.88	11.00	-0.12	**1.09**	27.98	27.99	12.81	13.47	-0.67	**1.59**
		15.06		26.97						27.97						28.00					
	6.2	15.77	15.87	28.04	27.93	12.06		-0.11	**1.08**	Undetermined	26.11	10.24		-0.75	**1.69**	29.71	29.91	14.04		0.57	**0.67**
		15.96		27.82						26.11						30.12					
	6.3	16.25	15.85	25.10	25.39	9.54		-2.63	**6.18**	24.85	25.13	9.28		-1.72	**3.29**	27.97	27.82	11.97		-1.51	**2.84**
		15.46		25.67						25.41						27.67					
	6.4	14.23	14.50	25.03	25.13	10.63		-1.53	**2.89**	25.52	25.10	10.60		-0.40	**1.32**	25.66	26.47	11.97		-1.50	**2.83**
		14.77		25.23						24.68						27.28					
	6.5	17.13	17.23	32.88	31.16	13.92		1.76	**0.30**	29.44	29.50	12.27		1.27	**0.41**	33.33	32.31	15.07		1.60	**0.33**
		17.33		29.43						29.56						31.28					
IL-4 24H	9.1	15.15	15.00	21.96	22.17	7.17		-5.00	**31.91**	19.59	19.66	4.66		-6.34	**80.82**	26.92	26.45	11.45		-2.02	**4.07**
		14.86		22.38						19.74						25.98					
VEH ME	1.1	15.61	15.79	26.62	26.52	10.73	12.51	-1.43	**2.70**	25.31	25.32	9.53	12.90	-3.37	**10.32**	27.15	27.20	11.41	13.41	-2.00	**4.00**
		15.96		26.42						25.33						27.25					
	1.2	16.55	16.16	28.26	28.03	11.87		-0.29	**1.22**	29.57	29.03	12.87		-0.03	**1.02**	21.58	29.73	13.57		0.15	**0.90**
		15.77		27.81						28.49						29.73					
	1.3	16.36	16.31	23.96	24.17	7.85		-4.31	**19.84**	24.43	24.36	8.05		-4.86	**28.96**	27.52	27.38	11.07		-2.34	**5.08**
		16.26		24.38						24.28						27.25					
	1.4	18.55	18.51	31.83	31.66	13.16		0.99	**0.50**	31.17	31.44	12.93		0.03	**0.98**	32.10	31.77	13.26		-0.15	**1.11**
		18.46		31.49						31.71						31.43					
IL-4 24H	5.1*	17.21	17.26	27.59	27.27	10.01		-2.51	**5.69**	24.33	24.40	7.13		-5.77	**54.48**	28.81	29.02	11.76		-1.65	**3.14**
		17.31		26.94						24.46						29.23					

		36B4		ARG1					
		Ct	Media	Ct	Media	dCt	Media ctr	ddCt	2^{-ddCt}
VEH ME	6.1	15.30	15.18	21.34	21.70	6.51	6.61	-0.09	**1.07**
		15.06		22.05					
	6.2	15.77	15.87	22.92	23.19	7.32		0.71	**0.61**
		15.96		23.45					
	6.3	16.25	15.85	23.29	23.06	7.20		0.60	**0.66**
		15.46		22.82					
	6.4	14.23	14.50	18.99	18.86	4.36		-2.25	**4.75**
		14.77		18.73					
	6.5	17.13	17.23	22.70	22.63	5.40		-1.21	**2.31**
		17.33		22.56					
IL-4 24H	9.1	15.15	15.00	15.68	16.70	1.70		-4.91	**30.02**
		14.86		17.72					
VEH ME	1.1	15.61	15.79	21.16	21.69	5.90	6.23	-0.33	**1.26**
		15.96		22.22					
	1.2	16.55	16.16	22.51	22.58	6.42		0.19	**0.88**
		15.77		22.65					
	1.3	16.36	16.31	24.25	24.39	8.08		1.85	**0.28**
		16.26		24.54					
	1.4	18.55	18.51	24.84	24.88	6.37		0.14	**0.91**
		18.46		24.91					
IL-4 24H	5.1*	17.21	17.26	18.50	18.25	0.98		-5.25	**38.02**
		17.31		18.00					

Supplementary Table 10. Oligonucleotides used in real time PCR assays.

Gene	Forward sequence	Reverse sequence
Vegfα	5'-AGCAGAAGTCCCATGAAGTGA-3'	5'-ATGTCCACCAGGGTCTCAAT-3'
Arg1	5'-GAATCTGCATGGGCAACCT-3'	5'-ACACGATGTCTTTGGCAGATAT-3'
Ym1	5′-GAAGGAGCCACTGAGGTCTG-3′	5′-GAGCCACTGAGCC TTCAAC-3′
Tgm2	5'-GGCCACTTCATCCTGCTCTA-3'	5'-TCCAAGGCACACTCTTGATG-3′
Il10	5'-GGTTGCCAAGCCTTATCGGA-3'	5'-ACCTGCTCCACTGCCTTGCT-3′
Fizz1	5'-GGAACTTCTTGCCAATCCAGC-3'	5'-AAGCCACAAGCACACCCAGT-3'
Gas6	5'-CTTAGCCAGGATGACCGGAG-3'	5'-AGCACAGTGTGAGAAGACTCG-3'
Tnfα	5'-CCTATGTCTCAGCCTCTTCTC-3'	5'-CTCTTGCTTATCCCCTCTTCC-3'
CcnB2	5'-CCGACGGTGTCCAGTGATTT-3'	5'-CTGAGGTTTCTTCGCCACCT-3'
Cdk1	5'-ACACGAGGTAGTGACGCTGT-3'	5'-TCAATCTCTGAGTCGCCGTG-3'
Ube2c	5′-ATAGCCCTTTGAACACACACG-3′	5′-TGGAGACCTGCTTTGAATAGG-3′
Ki67	5'-AGAGCTAACTTGCGCTGACT-3'	5'-TCAATACTCCTTCCAAACAGGCA-3'
Angptl4	5'-ATGACTTCAGATGGAGGCTGG-3'	5'-AATTGGCTTCCTCGGTTCCC-3'
36B4	5′-GGCGACCTGGAAGTCCAACT-3	5′-CCATCAGCACCACGGCCTTC-3′