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Insight into the role of DUSP3-deficient macrophages in metastasis dissemination and sepsis

Submitted by
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Quelqu'un m'a dit un jour : « Faire une thèse c'est comme participer à un marathon. La course est longue et éprouvante mais l'arrivée est tellement gratifiante ». Cette thèse n'aurait jamais vu le jour sans la présence et le soutien de certaines personnes auxquelles j'aimerais exprimer ma reconnaissance.

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Abstract

DUSP3, or *Vaccinia* H1-Related (VHR), is a member of the atypical dual-specificity protein phosphatase family (A-DUSPs). This protein contains a 185 amino acid catalytic domain (Mr 21 kDa) and dephosphorylates both p-Tyr and p-Thr residues. The MAPKs ERK1/2, JNK and to a lesser extent p38 have been reported to be substrates of DUSP3. The phosphatase also dephosphorylates other proteins such as the transcription factor STAT5 and the tyrosine kinase receptors EGFR and ErbB2. DUSP3 is playing an important role in cell cycle regulation, in platelets activation and arterial thrombosis and in resistance to septic shock. DUSP3 could also act as tumour suppressor or as tumour promoter depending on the cell types and cancer models and DUSP3-deficiency also leads to a defective tumour-induced angiogenesis. Moreover, DUSP3 is highly expressed in macrophages. Macrophages are innate immune cells involved in numerous physiological processes maintaining homeostasis such as immune responses, debris clearance, thermoregulation and bone resorption. Therefore, disruption of these functions leads to severe diseases. In this work, we investigated the role of DUSP3 in two of these pathologies, metastasis dissemination and septic shock, using recently generated DUSP3-knockout mice.

In the first part of the work, we investigated the contribution of DUSP3 in the susceptibility to experimental metastasis. Using a Lewis Lung Carcinoma (LLC) experimental metastasis model, we observed that $DUSP3^{-/-}$ mice developed larger lung metastases than littermate controls. By creating bone marrow chimeric mice, we were able to transfer the phenotype to $DUSP3^{+/+}$ mice, indicating a role of hematopoietic cells in enhancing tumour cell dissemination to lung tissues. Interestingly, we found a higher percentage of tumour-promoting $Ly6C^{int}$ M2-like macrophages in $DUSP3^{-/-}$ LLC-bearing lung homogenates. This higher percentage of M2 macrophages is at least partially due to a better recruitment of these cells as confirmed by a higher percentage of $Ly6B^{hi}$ macrophages in $DUSP3^{-/-}$ lung homogenates and by a better migration of $DUSP3^{-/-}$ peritoneal macrophages and BMDMs under LLC-conditioned medium stimulation. These results suggest that DUSP3 plays a key role in metastatic growth through a mechanism involving the recruitment of M2-like macrophages towards LLC-bearing lungs.

In the second part of the work dedicated to the study of the role of DUSP3 in septic shock, we showed that the sepsis resistance previously reported in DUSP3$^{-/-}$ mice was gender dependant, female DUSP3$^{-/-}$ mice surviving to LPS- and CLP-induced septic shock while male mice did not. Using adoptive transfer experiments and ovariectomized mice we highlighted the role of female sex hormones in the phenotype. In ovariectomized and male mice the dominance of M2-like macrophages observed in female DUSP3$^{-/-}$ mice after LPS challenge was lost, confirming the role of these macrophages in sepsis tolerance. To understand molecular mechanisms involved, we stimulated *ex vivo* peritoneal macrophages with LPS. In DUSP3$^{-/-}$ female macrophages, we observed a hypo-phosphorylation of ERK 1/2, PI3K and Akt. Our results demonstrate that oestrogens modulate M2-like macrophage responses during endotoxemia in a DUSP3 dependent manner.

Taken together these two projects showed that DUSP3 activity is important to regulate macrophage polarization. Moreover, the study of the role of DUSP3 in sepsis highlighted a possible role of female sex hormones in this regulation.

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Abbreviations

A-DUSP	atypical-DUSP
bFGF	basic fibroblast growth factor
BM	bone marrow
BMDM	bone marrow derived macrophages
cIAP	inhibitor of cellular apoptotic protein
CLEC-2	C-type lectin receptor 2
CLP	cecal ligation and puncture
CLR	C-type lectin receptor
CSF1	colony-stimulating factor 1
CSF1R	colony-stimulating factor 1 receptor
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAMP	damage-associated molecular pattern
DC	dentritic cells
DIC	disseminated intravascular coagulation
DNA-PK	DNA dependent protein kinase
DUSP	dual specificity phosphatase
E2	oestrogen
ECM	extracellular matrix
EGF	epithelial growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal regulated kinase
FBS	foetal bovine serum
FGF	fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GPVI	glycoprotein VI
GSK3	glycogen synthase kinase 3
HMGB1	high motility group box 1
HnRNP	heterogeneous nuclear ribonucleoprotein
HSC	hematopoietic stem cells
ICAM	intercellular adhesion molecule

IFN	Interferon
IKK	inhibitor of kappa B kinase
IL	interleukin
IRAK	interleukin 1 receptor associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
KDM2A	Lysine-specific demethylase 2A
KGFR	keratinocyte growth factor receptor
LBP	LPS-binding protein
LLC	Lewis lung carcinoma
LPS	lipopolysaccharide
MAM	metastasis associated macrophage
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK Kinase Kinase
MDL-1	myeloid DAP-12 associated lectin
MET	mesenchymal-epithelial transition
MHC	major histocompatibility complex
MIF	migration inhibitory factor
MIP	macrophage inflammatory protein
MKP	MAPK kinase phosphatase
MMP	matrix metalloproteinase
MNK	MAPK interacting kinase
MSK	mitogen-and stress-activated protein kinase
mTORC	mechanistic target of rapamycin complex 1
MyD88	myeloid differentiation primary response gene 88
NET	neutrophils extracellular trap
NF-κB	nuclear factor kappa B
NK	natural killer
NLR	NOD-like receptor
NO	nitric oxide
NPM	nucleophosmin
NSCLC	non small cell lung cancer
NUCL	nucleolin

PAI-1	plasminogen activator inhibitor 1
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
PDGFR	platelet-derived growth factor receptor
PD-L1	programmed death-ligand 1
PI3K	phosphoinositide 3 kinase
PIP	phosphatidylinositol phosphate
PKC	protein kinase C
PlGF	placental growth factor
PP	protein phosphatase
PRR	pattern recognition receptor
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
RLR	RIG1-like receptor
RNAi	RNA interference
ROS	reactive oxygen species
RSK	ribosomal S6 kinases
SDF	stromal cell-derived factor
SP-1	specific protein 1
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
TAB	TAK1-binding protein
TAF-1	thrombin-activatable fibrinolysis inhibitor
TAK	TGF-β activated kinase
TAM	tumour associated macrophage
TCR	T-cell receptor
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF	transforming growth factor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TME	tumour microenvironment
TNF	tumour necrosis factor
Tpl-2	tumour-progression locus 2

TRAF	tumour necrosis factor receptor associated factor
TRAM	TRIF-related adaptor molecule
TREM-1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR-domain containing adaptor protein inducing IFN-β
TSC	tuberous sclerosis
Tyk2	tyrosine kinase 2
VCAM	vascular cell adhesion protein
VEGF	vascular endothelial growth factor
VH1	Vaccinia open reading frame H1
VHR	vaccinia-H1 related
VRK3	Vaccinia-related kinase 3
vWF	von Willebrand factor
ZAP-70	zeta-associated protein 70

Introduction

Introduction

1. Protein tyrosine phosphatase family

Many signalling pathways and physiological processes are regulated by reversible protein phosphorylations and dephosphorylations. This is achieved by a perfect balance between protein kinase and protein phosphatase activities. Based on their substrate specificity, protein phosphatases can be grouped in two main families: the protein serine/threonine phosphatase (PSTPs) family, dephosphorylating phospho-serine/threonine (pSer/pThr) residues and the protein tyrosine phosphatases (PTPs) family, hydrolysing phospho-tyrosine (pTyr) residues[1]. Based on the catalytic residues, the human PTPs are divided into 3 groups: the cysteine-based phosphatases, containing a common motif CxxxxR, the aspartic-based phosphatases and the histidine-based phosphatases. The cysteine-based phosphatases can further be grouped into several classes and subclasses (Figure 1.1)[2]:

- Class I is the most important group and contains 6 subclasses: the "classical" PTPs, the VH1-like PTPs which are dual specificity protein phosphatases, the SAC phosphoinositide phosphatases, the PTP-like phytases (PTPLPs), the INPP4 phosphatases and the TMEM55 phosphatases, the last two subclasses being inositol phosphatases.
- The low molecular protein tyrosine phosphatase (LMPTP) and SSU72 are the only members of the class II cysteine-based PTP.
- Class III contains the family of CDC25 proteins, rhodanese-like enzymes and tyrosine-threonine specific phosphatases.

Among the class I of cysteine-based PTPs, the dual specificity protein phosphatases (DUSPs) or VH1-like PTPs are the most common members. Unlike the "classical" PTPs that are strictly specific for pTyr residues, DUSPs are able to dephosphorylate both pTyr and pSer/pThr residues. This is achievable because DUSPs have a broader and shallower catalytic site than PTPs, allowing the binding of the shorter pSer/pThr amino-acids[3,4]. Therefore, DUSPs present a wide range of substrate specificities including: pTyr, pSer/pThr residues as well as phosphoinositides[5], mRNA[6] and glycans[7].

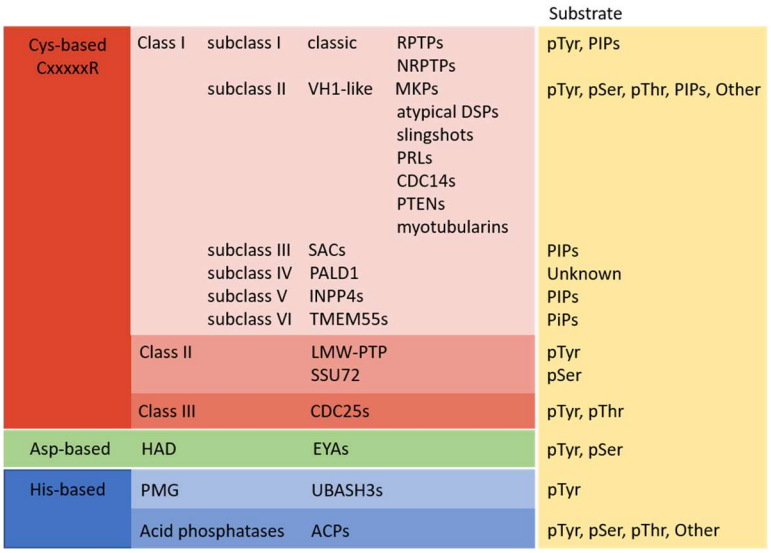

Figure 1.1. Protein tyrosine phosphatase classification. The PTPs can be divided in 3 families based on their catalytic amino acid: Cys-based PTPs, Asp-based PTP and His-based PTPs. Adapted from[2,8].

The DUSPs can be classified into 7 groups based on the sequence of their catalytic domain. The MAP kinase phosphatases (MKP, also called typical DUSPs) and the atypical-DUSPs (A-DUSPs) are part of the two major families and are evolutionary and structurally related. The MKPs dephosphorylate pTyr and pThr residues within the TXY activation motif of the MAP kinases[9]. Every MKP shows a substrate specificity preference for one or more MAP kinases, ERK, JNK or p38[10]. The MKPs are further classified into 3 subgroups based on sequence similarities, subcellular localization and functional properties[11,12]. All MKPs possess a N-terminal Cdc25/rhodanese-like domain or MAP kinase binding domain interacting with the common domain (CD) of MAP kinases. They also have a C-terminal catalytic domain and a variable linker region (Figure 1.2)[11]. On the contrary, A-DUSPs contain only the C-terminal phosphatase domain but some of them present specific N- or C-terminal regulatory motif (Figure 1.2)[12]. All A-DUSPs share similarities with VH1 (*Vaccinia* virus open reading frame H1) phosphatase present in *Vaccinia* virus, which was the first A-DUSP to be discovered in 1991[13]. Aside from dephosphorylating MAP kinases, it is known that A-DUSPs regulate a wide range of different substrates and present diverse physiological roles.

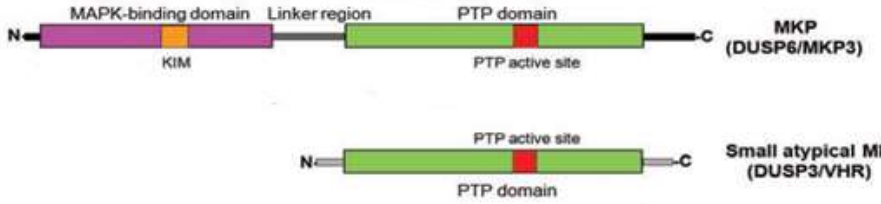

Figure 1.2. Schematic representation of domains of representative members of MKPs family (DUSP6/MKP3) and atypical DUSPs (DUSP3/VHR). Adaptated from [11].

Besides the MKPs and the A-DUSPs, the VH1-like PTP family contains 5 other groups: phosphatases of regenerating liver (PRL), slingshots, phosphatase and tensin homologues (PTENs), myotubularins and cell division cycle 14 homologues (Cdc14s)[8] (Figure 1.1).

2. DUSP3/VHR

In 1991, Ishibashi et al. discovered a novel protein tyrosine phosphatase which they called VHR (VH1-related phosphatase) because the protein shares sequence similarities with the *Vaccinia* virus-encoded phosphatase[14]. VHR, also called DUSP3, is a small protein of 185 amino acids encoded by *DUSP3* gene located on chromosome 17q21 in humans and on chromosome 11 in mice. DUSP3 lacks a binding domain and a signal-peptide consensus sequence or a transmembrane sequence. DUSP3 has an affinity for both pTyr and pThr residues and is therefore a dual specificity phosphatase. DUSP3 displays a preference for diphosphorylated residues over monophosphorylated residues[15]. Unlike the other DUSPs, DUSP3 preferentially dephosphorylates pTyr residues over pThr residues[16].

In their study, Ishibashi's team reported the epidermal growth factors receptor (EGFR), the keratinocyte growth factor receptor (KGFR), platelet-derived growth factor receptor (PDGFR) and the insulin receptor as potential substrates for DUSP3[14]. Since then, several other substrates have been proposed (Figure 1.3). The MAP kinases, ERK1/2, JNK and in a lesser extent p38 were described to be *in vitro* substrates of DUSP3[17–20]. STAT5 may undergo a tyrosine dephosphorylation by DUSP3 in HEK293 cells after stimulation with IFN-α and IFN-β, which leads to the inhibition of the transcription factor function[21]. In non-small cell lung cancer (NSCLC), ErbB2 is dephosphorylated by DUSP3[22]. Recently, DUSP3 was shown to be involved in PKC signalling pathway,

in HUVEC cells: the downregulation of DUSP3 using small interfering RNA (RNAi) led to a hyperphosphorylation of PKC both at basal level and with bFGF stimulation[23]. Moreover, Panico *et al*. reported that the proteins Nucleophosmin (NPM), Heterogeneous nuclear Ribonucleoprotein C1/C2 (HnRNP C1/C2) and Nucleolin (NUCL) are direct substrates of DUSP3, suggesting a novel role of DUSP3 in DNA damage repair[24,25].

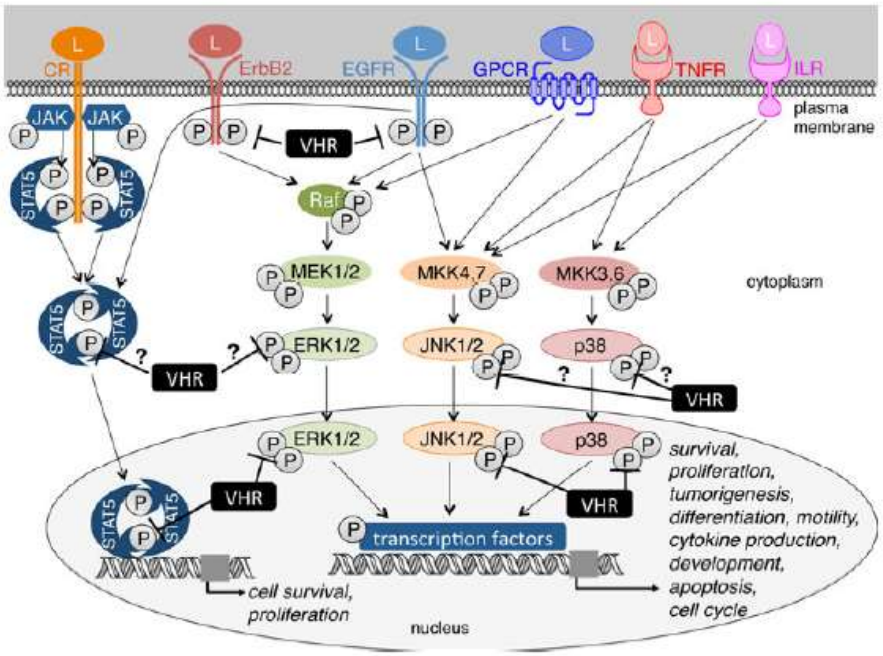

Figure 1.3. Reported potential physiological substrates of VHR (STAT5, ERK1/2, JNK1/2, p38, EGFR, ErbB2) and their involvement in different pathways (simplified, schematic)[26]. The question marks signify that it is unclear if VHR acts on the MAPKs ERK1/2, JNK1/2 and p38 also in the cytoplasm in addition to in the nucleus. L = hormones, growth factors, cytokines, interleukins, stress stimuli, lypopolysaccharide, mitogens, GPCR activation (corresponding to the receptors); ILR = interleukin receptor; TNFR = tumor necrosis factor receptor; GPCR = G-protein coupled receptor; CR = cytokine receptor; JAK = Janus kinase; Raf = rapidly accelerated fibrosarcoma.

In T cells, upon TCR stimulation by an antigen, the kinase ZAP-70 phosphorylates DUSP3 on Tyr138. The phosphorylation of this tyrosine residue is an essential event for the activation of DUSP3 and subsequent inhibition of ERK1/2 and JNK[27]. The dephosphorylation of STAT5 follows the same activation step: in HEK293 cells and upon IFN-α and IFN-β stimulation, the kinase Tyk2

phosphorylates DUSP3 at the Tyr138, which facilitates the liaison of DUSP3 through the SH2 domain of STAT5[21]. In neuronal cell line, rodent adult tissues and embryos, it has been described that the kinase VRK3 is another direct activator of DUSP3. This activation occurs through a direct interaction between the two proteins, which enhances the activity of DUSP3 and the dephosphorylation of ERK1/2 downstream target[28]. Similarly to other PTPs, DUSP3 may undergo negative regulation. Since the catalytic cysteine is susceptible to oxidation, DUSP3 is a target of reactive oxygen species (ROS) such as H_2O_2. The oxidation of DUSP3 by H_20_2 leads to a reversible inactivation of the phosphatase[29,30]. Recently, the dimerization of DUSP3 has been proposed as a novel negative regulatory mechanism since the formation of dimers results in a reduced catalytic activity of DUSP3[31]. Dimerization has been also suggested to help protecting the phosphatase from irreversible oxidation. In non-small cell lung cancer (NSCLC), DUSP3 was reported to be epigenetically repressed by an histone Lysine-specific Demethylase (KDM2A), which increases the activity of ERK 1/2 and contributes to tumour growth and invasiveness[32].

DUSP3 regulates the activity of ERK and JNK during the cell cycle. Indeed, the downregulation of DUSP3 in HeLa cells induces a prolonged activation of ERK and JNK and results in cell cycle arrest and cell senescence initiation[33]. The generation of DUSP3-KO mice facilitated the study of physiological roles of this phosphatase[23]. A non-expected novel function of DUSP3 was revealed in platelets and thrombosis. Indeed, Musumeci *et al*. showed that DUSP3 displays a key role in platelet activation and arterial thrombosis through a mechanism involving GPVI and CLEC-2 signalling pathways[34]. DUSP3 is also a key mediator of *in vivo* neo-vascularization by regulating the b-FGF-induced endothelial cell sprouting through the protein kinase C (PKC) signalling pathway[23]. Moreover, after subcutaneous injection of Lewis Lung Carcinoma cells (LLC), the $DUSP3^{-/-}$ mice presented a 30% decrease in the haemoglobin content of the tumours compared to WT mice. LLC tumours weight was also slightly, but significantly, reduced in the $DUSP3^{-/-}$ mice. This suggests that DUSP3-deficiency leads to a defective tumour-induced angiogenesis[23]. The role of DUSP3 in cancer has been reported in several other studies. In cervix carcinomas, the overexpression of DUSP3 would facilitate the cancer cell proliferation probably by preventing the activation of ERK and JNK[35]. An overexpression of DUSP3 was also discovered in prostate cancer, conferring to cancer cells a JNK-mediated resistance to apoptosis[36]. On the other hand, DUSP3 expression is

downregulated in several human cancers, such as breast cancer and NSCLC. In breast cancer, the expression of DUSP3 decreased upon overexpression of BRCA1-IRIS. This downregulation correlated with activation of cyclin D1 expression and provide a growth advantage to tumour cells[37]. Wang *et al*. showed the decrease in DUSP3 expression found in NSCLC improved ErbB signalling and this would cause cancer progression[22]. Moreover, as mentioned before, in another model of NSCLC, the repression of DUSP3 gene by KDM2A methylation was associated with tumorigenesis and metastasis[32]. This suggests that depending on the cell type/model used DUSP3 could act as tumour suppressor or as tumour promoter. Besides showing a role of DUSP3 in cancer, the generation of DUSP3-deficient mice revealed a role of DUSP3 in acute inflammation. The DUSP3-KO mice are protected from sepsis and septic shock through a mechanism involving M2-like macrophage polarization and a reduction of TNF-α production[38].

3. Innate immunity

Over the years the vertebrate's immune system has evolved in order to protect the host against attacks of pathogens. The immune system is an adaptive and dynamic network of organs, cells and molecules and is able to specifically recognize and eliminate microorganisms[39]. The immune response is classically divided in innate and adaptive immune responses.

Innate immunity is the most ancient defence system of multicellular organisms and is the first line of defence against microbial infection. Innate immune response also helps to maintain homeostasis[40]. Its activation occurs at early time of infection and induces inflammatory effective responses to neutralize the pathogens. Innate immunity also activates and regulates adaptive immune response[41] that is a unique and evolutionary feature of vertebrates[42]. Adaptive immunity consists of a specific response adjusted to each pathogen. The activation of this second line of defence is a late event and depends on the innate immune response. The specificity of adaptive immunity is based on the recognition of specific peptides and proteins by lymphocytes (B or T cells), the major players of adaptive immune response. This high specificity for antigens is allowed by the expression of specific receptors and determinants formed by rearrangement of germ line genes during maturation[39]. B cells are responsible for the humoral response mediated by the production and secretion of

antibodies. T cells, divided into $CD4^+$ and $CD8^+$ cells, are involved in cell-mediated immune responses. CD4+ T cells act as helper (T_h) and interact with CD8+ T cells and B cells. CD8+ cells are cytotoxic T lymphocytes. They mediate lysis of targeted cells and secretion of cytokines that recruit inflammatory cells[42]. Adaptive immunity also presents long-term immunological memory that helps to avoid reinfection[42].

Unlike adaptive immunity, innate immune responses are semi-specific. Innate immune cells are only able to recognize common molecular signature of potential pathogens known as pathogen-associated molecular pattern (PAMPs), such as polysaccharides, glycolipids, lipoproteins, nucleotides and nucleic acids, expressed on pathogens and not on host cells[43]. Discrimination between self and non-self and recognition of PAMPs is possible thanks to the expression of pattern recognition receptors (PRRs) by innate immune cells. The different families of PRRs, Toll-like receptors (TLRs)[44], C-type lectin receptors (CLRs)[43], NOD-like receptors (NLRs)[45,46], RIG-1-like receptors (RLRs)[47] recognise different PAMPs and consequently induce specific signalling pathways as well as soluble and cellular immune responses[48]. PRRs are products of germline genes and have genetically-determined specificity[43]. Innate immune cells can also detect a pathogen through the sensing of functional characteristics indicative of a pathogen's presence[41]. Recent evidences suggest that some innate immune cells (macrophages and NK cells) can develop memory-like responses, called "trained immunity", which confer host protection against reinfection by the same or a different pathogen. This innate immune memory has a shorter duration than adaptive immune memory and is mediated through epigenetic reprogramming[49,50].

3.1. Innate immune cells

In addition to physical and chemical barriers, innate immunity also includes several different types of immune cells: eosinophils, basophils, mast cells, neutrophils, dendritic cells (DC), natural killers (NK cells), monocytes and macrophages and innate lymphoid cells (ILCs). All these cells come from a common progenitor present in the bone marrow, the hematopoietic stem cells (HSC) and undergo different differentiation programs. Therefore, these immune cells play distinct roles in innate immune responses.

Neutrophils are short-lifetime cells circulating in the bloodstream. They are the

first cells arriving at the inflammation site. The main known functions of neutrophils are the elimination of pathogens by phagocytosis, the production of antimicrobial molecules and the maintenance of the tissue[39]. However, neutrophils are also regulators of innate and adaptive immunity. They produce cytokines and chemokines that activate and recruit immune cells[51]. In addition, they crosstalk with B and T cells modulating each other's recruitment and function at the inflammation site. They are able to dampen immune responses and promote the resolution of inflammation[52]. Basophils produce IL-4 and promote type II cytokine-mediated inflammation after exposition to parasites[53]. Basophils also contribute to antimicrobial activity[54]. Mast cells favour macrophage activation[55]. Moreover, mast cells and eosinophils possess anti-bacterial qualities[56,57]. NK cells exert cytotoxic activity against virus[58], bacteria and parasites and produce pro-inflammatory cytokines[59]. NK cells classically activate macrophages, monocytes and DCs[60]. DCs play a role in innate and adaptive immune responses, their main function being the antigen presentation to T cells[43].

3.2. Monocytes and macrophages

Traditionally, monocytes originate from myeloid precursors in bone marrow (Figure 1.4). Hematopoietic Stem Cells (HSCs) give rise to macrophage and DC precursors (MDP) able to differentiate into monocytes and DCs[61]. However monocytes and descendants arise from an intermediate population of precursors termed common monocyte progenitors (cMoP)[62]. Two main subsets of monocytes derive from cMoPs: Ly6C^{hi}CX$_3$CR1^{mid}CCR2^{+} (Ly6C^{hi}) and Ly6C^{low}CX$_3$CR1^{hi}CCR2^{-} (Ly6C^{low})[63]. The main lineage regulatory signalling pathway involves the colony stimulating factor 1 (CSF1) and its receptor CSF1R. Indeed, monocyte development and survival depend on CSF1 and IL-34[64–66]. Once in the circulation, Ly6C^{hi} monocytes patrol to pick up antigens and may rapidly be recruited to the site of inflammation where they differentiate into monocyte-derived macrophages or monocyte-derived DCs[67]. Ly6C^{hi} monocytes are therefore considered as classical monocytes and play a key role in immune responses against pathogen insults[68]. In steady-state conditions, circulating blood Ly6C^{hi} monocytes may differentiate into Ly6C^{low} monocytes and, probably, terminally become blood-resident macrophages[69,70]. Ly6C^{low} monocytes maintain endothelial integrity and coordinate intraluminal stress responses[71,72].

It has long been thought that tissue-resident macrophages originated from bone-marrow monocytes. However, although monocytes can give rise to tissue macrophages in certain conditions, emerging evidences suggest that most of the tissue macrophages have an embryonic origin (Figure 1.4)[73]. Tissue-macrophages can derive from yolk sac progenitors (for example, microglia or macrophages from skin, spleen, pancreas and liver, which are F4/80^{hi})[74,75] or from foetal liver progenitors (Langerhans cells have a mixed origin from yolk sac and foetal liver)[74]. In some tissues such as kidney or lungs, tissue-macrophages arise both from yolk sac progenitors (F4/80^{hi}) and adult bone marrow (F4/80^{low})[75]. Origins of tissue-macrophages imply that they are capable to maintain themselves by self-renewal[76].

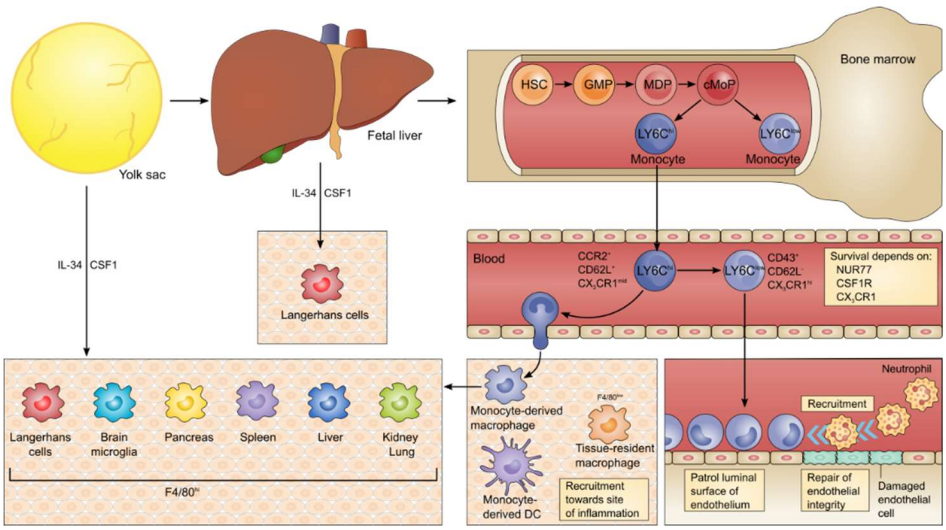

Figure 1.4. Origins of macrophages. Macrophages originate from three sources: yolk sac, fetal liver and bone marrow. The yolk sac gives rise to all F4/80^{hi} tissue resident macrophages. It seems that Langerhans cells originate from the fetal liver. These lineage developments depend on IL-34 and CFS1. In the bone marrow, progenitors differentiate to give Ly6C^{hi} circulating monocytes that will eventually differentiate into F4/80^{low} macrophages and DCs. Ly6C^{low} monocytes derive from Ly6C^{hi} monocytes and give rise to patrolling macrophages. Adapted from [67,77].

Thanks to the expression of PRRs, tissue macrophages function as sentinels. They are able to recognize and clear pathogens, foreign substances or dead cells by phagocytosis. Tissue macrophages also regulate immune responses. They act as antigen presenting cells and activate T_h cells[78]. If the action of tissue macrophages is not sufficient to contain microbial infection, inflammatory Ly6C^{hi} monocytes are recruited to the infected tissue, mainly

through the secretion of CCL2, CCL5 and granulocyte colony stimulating factor (G-CSF) to differentiate into monocyte-derived macrophages[78–80]. They produce pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, nitric oxide (NO) and ROS to activate antimicrobial defences. They also secrete IL-12 and IL-23 to regulate the differentiation and the expansion of T_h1 and T_h17 cells[77]. These macrophages rapidly undergo apoptosis or differentiate into regulatory macrophages to dampen the inflammation. Tissue macrophages also regulate resolution of inflammation by removing apoptotic cells and cell debris[81]. Moreover they provide trophic supports to fibroblasts or other cells involved in tissue repair[82,83].

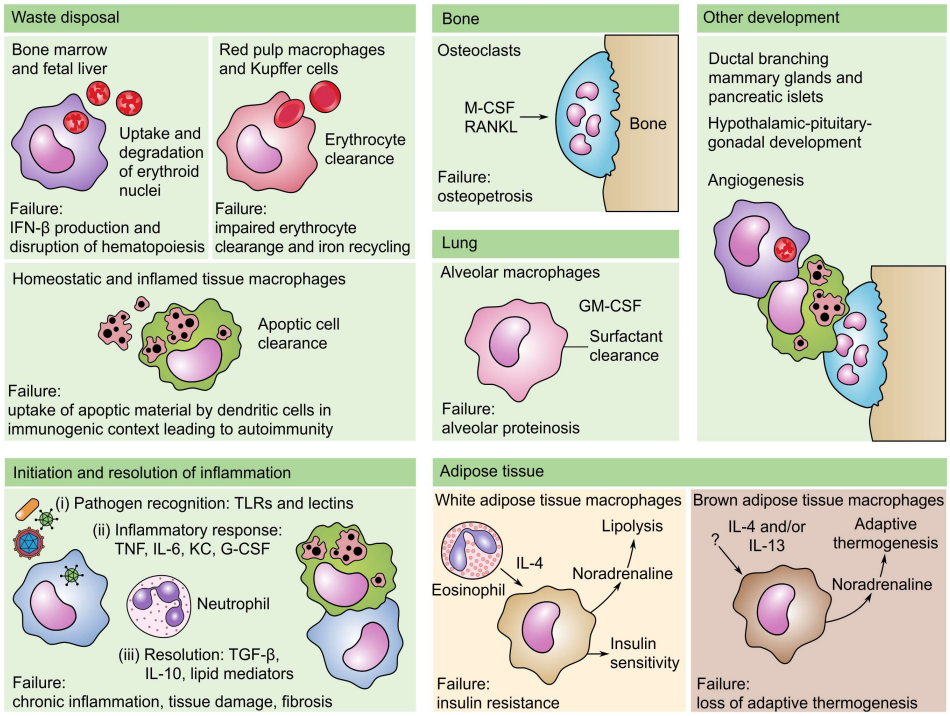

Figure 1.5. Functions of tissue-resident macrophages[84]. Tissue macrophages exert many roles. They are involved in erythrocyte degradation and apoptotic cells clearance. They also have a role in development (bone degradation and angiogenesis) and in metabolism regulation (insulin sensibility, thermogenesis in adipose tissue). They act as immune sentinels, initiate inflammatory responses, clear inflammatory debris and restore homeostatic tissue environments.

In addition to their role in pathogen defences and in inflammation resolution, tissue macrophages are involved in many other physiological processes (Figure 1.5). For example, tissue macrophages play a role in tissue clearance. They phagocyte apoptotic cells[85], expelled nuclei during erythropoiesis[86], aged

erythrocytes in the spleen[84] and activated lymphocytes that result from the germinal centre reaction[84]. They also recycle iron obtained from haemoglobin in spleen and liver[87]. During development, the presence of tissue macrophages is essential for tissue remodelling in bones, mammary glands, kidney and pancreas[88], but also for the control of stem cell functions[77], for the regulation of angiogenesis[89] and for brain development[90]. Participation of tissue macrophages in metabolism regulation has also been demonstrated. Tissue macrophages maintain insulin sensitivity in adipocytes[91] and coordinate homeostatic adaptations of white adipose tissue (WAT)[77]. They also facilitate metabolic adaptations of brown adipose tissue (BAT) and WAT to cold[92].

Macrophages are classically subdivided into two categories: classically activated macrophages and alternatively activated macrophages, also named M1 and M2 macrophages in non-pathogen-driven conditions, respectively. Classically activated macrophages respond to IFN-γ and TLRs and are pro-inflammatory macrophages with antimicrobial activities, secreting pro-inflammatory mediators and cytokines (IL-12, IL-1β, TNF-α, IL-6, IL-23). Alternatively activated macrophages suppress immune and inflammatory responses by secreting IL-10 and are involved in wound healing and tissue repair. They respond to the secretion of IL-4 and IL-13 (Table 1.1). However, because these stimuli are highly diverse, M2 macrophages subclasses emerged[93]. This subdivision is also based on transcriptional changes needed to achieve the polarization. Therefore, in addition to tissue macrophages and tumour associated macrophages, M2 macrophage classification comprises four other groups (Figure 1.6)[94]:

- M2a macrophages, also defined as alternative macrophages, respond to IL-4 and IL-13
- M2b macrophages or type II macrophages are activated in response to immune complexes and LPS
- M2c macrophages, also known as deactivated macrophages, are activated in response to glucocorticoids and TGF-β
- M2d macrophages are activated in response to adenosines and IL-6

	Classically activated Macrophages (M1)	Alternatively activated Macrophages (M2)
Factors of polarization	IFNγ ± TLR engagement of cytokines (TNF, GM-CSF)	IL-4 and IL-13 (IL-33 and IL-21)
Cytokine expression	IL-12, IL-1β, TNF-α, IL-6, IL-23	IL-10 ; anti-inflammatory cytokines
Chemokine expression	CXCL9, CXCL10	CCL17, CCL22, CCL24
Other molecules expression	Reactive nitrogen/oxygen intermediates (RNI/RNO)	Arginase 1 (ornithine and polyamine), mannose and galactose receptors
Functions	Anti-microbial activity (bacteria and viruses) Promotion of T_h1 and T_h17 responses Pro-inflammatory responses Anti-tumoural activity (cytotoxic and immune resistance)	Anti-parasite activity Immunoregulation: promotion of T_h2, T_{reg} Resolution of inflammation Pro-tumoural activity (M2-like) Phagocytosis Tissue repair and remodelling Angiogenesis

Table 1.1. Comparison of classically activated macrophages (M1 macrophages) vs alternatively activated macrophages (M2 macrophages).

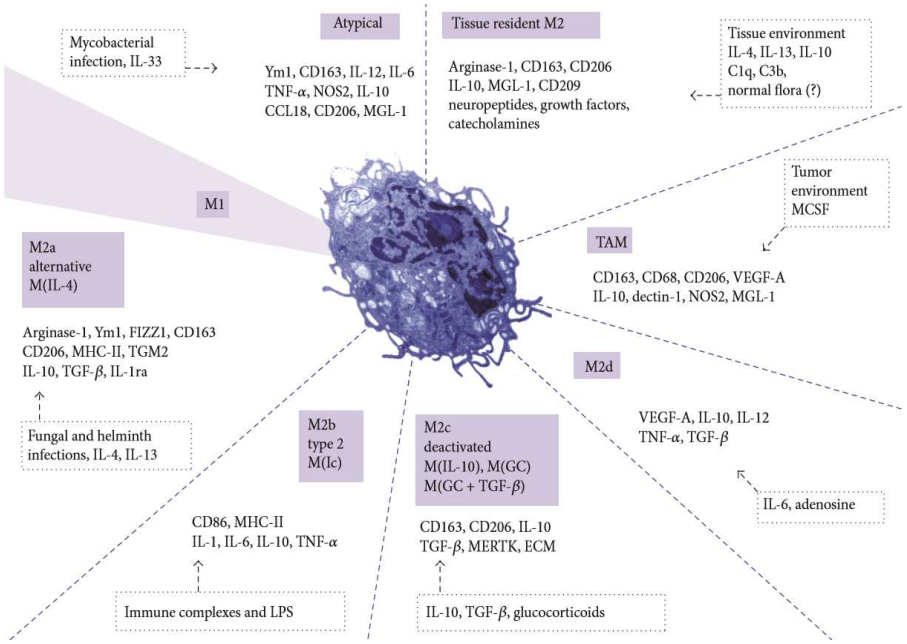

Figure 1.6. Macrophage activation states[94]. M2 macrophages population contains heterogeneous and functionally distinct subpopulations. Each subpoulation is characterized by a set of surface markers.

As all these macrophage subclasses differ in receptor expression, cytokine production and chemokine repertoires (summarized in Figure 1.6)[94], M2 subsets exert distinct functions. M2a macrophages are involved into type-II inflammation, Th2 responses, parasite infections and allergy. M2b macrophages exert immunomodulatatory activities and are part of Th2 responses. Tissue remodelling, matrix deposition and immunoregulation are achieved by M2c macrophages[93,95]. As for M2d macrophages they are involved in angiogenesis[96]. However this classification is not fixed and many subsets of macrophages show an intermediate state of differentiation. Atypical macrophages, displaying both M1 and M2 associated genes and transcription patterns may also be found in tissue[94]. Moreover, macrophage polarization is reversible since exposure of M2 macrophages to M1 signals, and vice-versa, can induce “re-polarization” of already differentiated macrophages. This reflects the high plasticity of macrophages[97–100].

Several mechanisms influence the polarization of macrophages into different subsets (Figure 1.7).

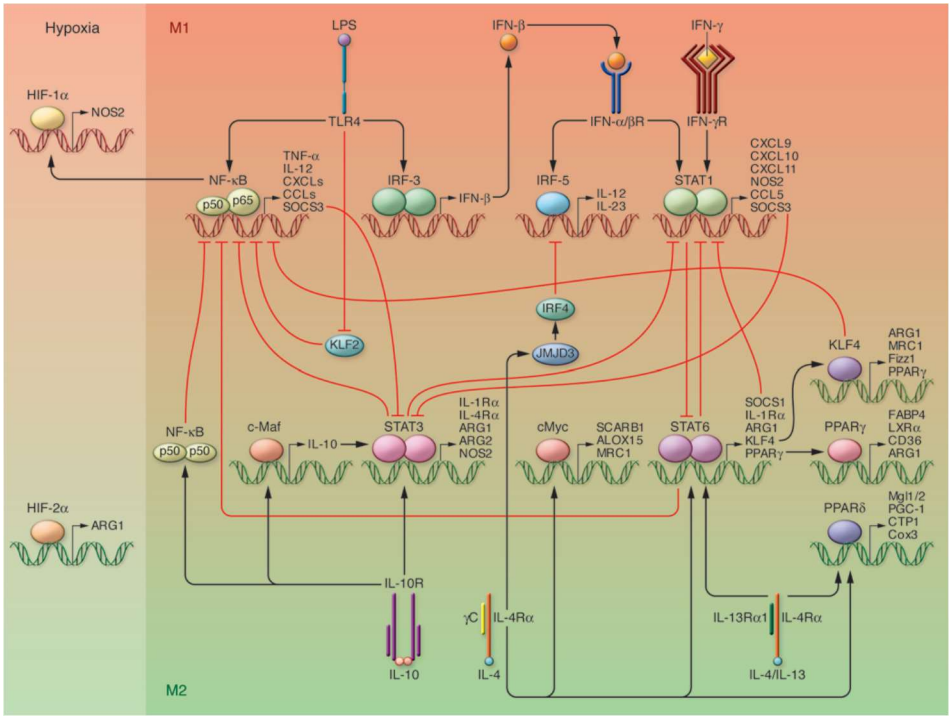

Figure 1.7. Mechanisms of macrophage polarization[97]. IRF/STAT/SOCS signalling is the major pathway influencing macrophage polarization. A predominance of STAT1 activation promotes M1 polarization while a predominance of activated STAT6 and STAT3 reflects a M2 polarization. PPARγ and PPARδ also control the expression of specific genes associated with alternative activation of macrophages. Together with STAT6, KLF4 influence M2 polarization and suppress the NF-κB/HIF-1α-dependent transcription. HIF-1α and HIF-2α are involved in specific gene expression of M1 and M2 macrophages, respectively.

IFNs and TLR4 signalling induce the activation of STAT1 and upregulate IRF5 and IRF3, respectively, leading to M1 polarization and inducing the secretion of pro-inflammatory cytokine such as IL-12, IL-23 and TNF[97,101,102]. On the other hand, IL-4 and IL-13, involved in M2 polarization, induce the activation of STAT6, essential to activate M2 genes such as mannose receptor, resistin-like α (fizz1) and chitinase3-like 3 (Ym1)[97,102]. IL-10 activates STAT3, leading to the expression of genes specific to M2-like macrophages such as Arginase or IL-1Rα and IL-4Rα[97,103]. STAT signalling is regulated by members of the SOCS family. Indeed, IFNγ and TLR4 upregulate SOCS3 and therefore inhibit STAT3 activation[104]. Similarly, IL-4 upregulate SOCS1, leading to the inhibition of STAT1[105]. Besides IRF/STAT signalling pathways, macrophage polarization is also regulated by transcription factors. For example, PPARγ[106] and PPARδ[107] control the expression of genes associated with M2 phenotype and oxidative

metabolism. KL4, together with STAT6, inhibits M1-like gene expression and activates M2-specific genes[108]. The hypoxia-inducible factors HIF-1α and HIF-2β also play a role in macrophage polarization. In M1 macrophages, HIF-1α regulates NOS2 expression. In M2 macrophages, HIF-2α induces arginase 1 expression[109]. Other important actors in macrophage polarization are miRNAs. For example, miR-223 has been found to be upregulated after LPS stimulation and down regulated following IL-4 treatment[110]. miR-155 is known to target the transcriptional factor C/EBP-β, able to regulate arginase 1 and to be involved in the regulation of Akt-dependent M1/M2 macrophage polarization[111,112]. miR-155 can also inhibit IL-13-dependent M2 polarization by targeting the IL-13Rα1[113].

The primary functions of macrophages and innate immune system is to protect the organism against pathogens and injuries and to assure homeostasis. However, when dysregulated, it can lead to serious and severe pathologies. Two examples of such inappropriate responses are cancer and sepsis.

4. Cancer and metastasis

"Cancer is the uncontrolled growth of cells, which can invade and spread to distant sites of the body" (WHO). The World health organization (WHO) estimated that cancer is the cause of 13% of all deaths worldwide and 90% of these deaths are due to metastasis development.

4.1. Cancer and metastasis dissemination

Cancer is an evolutive disease. Normal cells progressively turn into cancer cells as a consequence of acquisition and accumulation of some hallmarks. According to Hanahan and Weinberg, the acquisition of these new properties is facilitated by the genetic instability of cancer cells, leading to numerous mutations and the inflammatory state of premalignant and malignant lesions, which allow the release of growth factors or proangiogenic molecules by the recruited immune cells. Combined, these two enabling characteristics promote the emergence of cancer cells hallmarks:

- sustained proliferative signalling
- evasion from growth suppressor activity
- resistance to cell death

- potential of replicative immortality
- induction of angiogenesis
- ability to actively invade surrounding tissue and metastasize
- capacity to reprogram the energy metabolism of the cell
- evasion from the immune surveillance[114].

As the primary tumour grows, there is a lack of oxygen and nutrients. To counterbalance this deficiency there is production and secretion of pro-angiogenic molecules to form new vascularization inside the tumour[115,116]. This process is called angiogenic switch and is regulated by hypoxia, mechanic stress but also by the release of vascular endodelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PlGF) and angiopoietins by cancer cells or cells from the tumour microenvironment[117,118]. Eventually, tumour cells will invade the basement membrane and enter in the bloodstream through intravasation. To do so, cancer cells have to detach from the extracellular matrix (ECM) and survive. This is enabled by the epithelial-mesenchymal transition (EMT), a mechanism by which epithelial cells switch from polarized and organized cells to undifferentiated and isolated cells (mesenchymal-like cells). During EMT, cancer cells undergo changes in properties such as cell-to-cell and cell-to-matrix adhesion, cell polarity, migration and invasion[119,120]. Cancer cells also acquire stem-cell properties[121]. Once in the bloodstream, cancer cells are cloaking by platelets. This protects the cancer cells from shear forces and immune clearance by NK cells[122,123]. The interaction between cancer cells and platelets also renders cancer cells more adhesive and help to form embolisms in the microvasculature[124] where extravasation to the surrounding parenchyma will occur, probably by paracellular route[125]. Cancer cells will also undergo a new differentiation program, the mesenchymal-epithelial transition (MET), by which they will revert into a non-invasive state[126]. To proliferate and grow, the metastatic cells have to survive in the new environment and develop a new vasculature[127] (Figure 1.8).

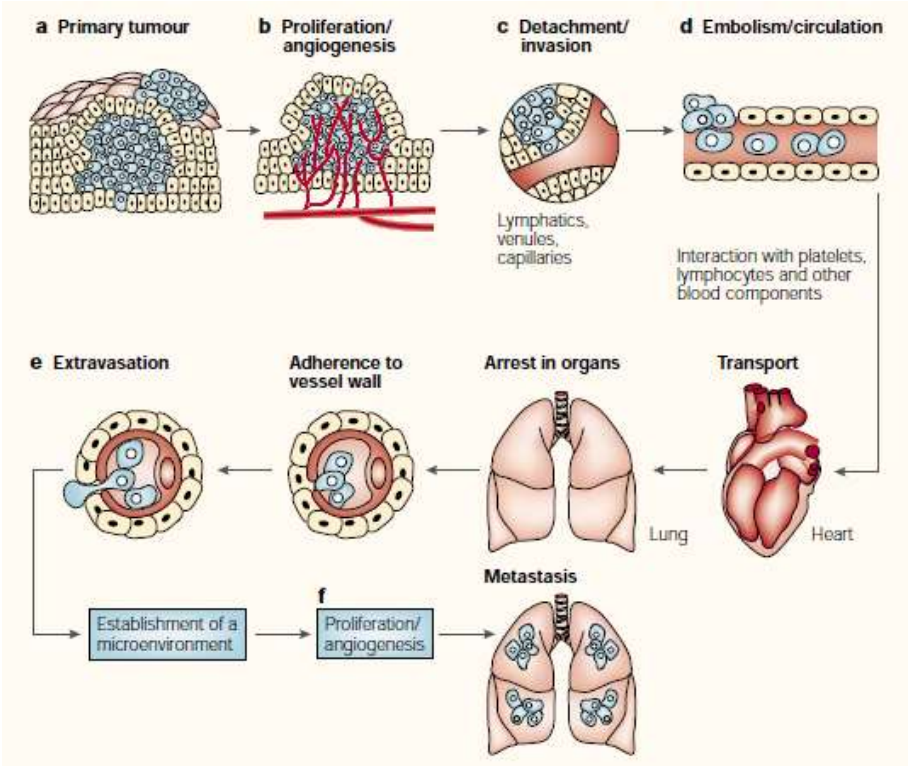

Figure 1.8. Invasion-metastasis cascade (**a-b**). When oxygen and nutrients lack, the primary tumour induces angiogenesis (**c**). At some point, tumour cells extravasate, enter in the circulation (**d**), home and extravasate at distant sites (**e-f**). Cancer cells growth into micrometastasis, induce angiogenesis and adapt to their microenvironment to form macrometastasis[127].

The invasion-metastasis cascade is an inefficient process since less than 0,01% of cells will develop metastasis[127]. But what are the rate-limiting steps of the cascade? It is known that the initial steps (intravasation, survival in bloodstream and extravasation) are highly efficient. On the opposite, very few cells survive from following steps (lodging, survival, extravasation and growth) and form micrometastasis. Few micrometastasis will turn into macrometastasis[128,129]. The local microenvironment is mainly responsible for this inefficiency.

4.2. Tumour microenvironment

Cancer cells only constitute a portion of the primary tumour. Various other stroma cell types are also present: endothelial cells, pericytes, fibroblasts, bone marrow-derived cells (macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs)). Together these cells constitute the tumour microenvironment (TME) (Figure 1.9)[130].

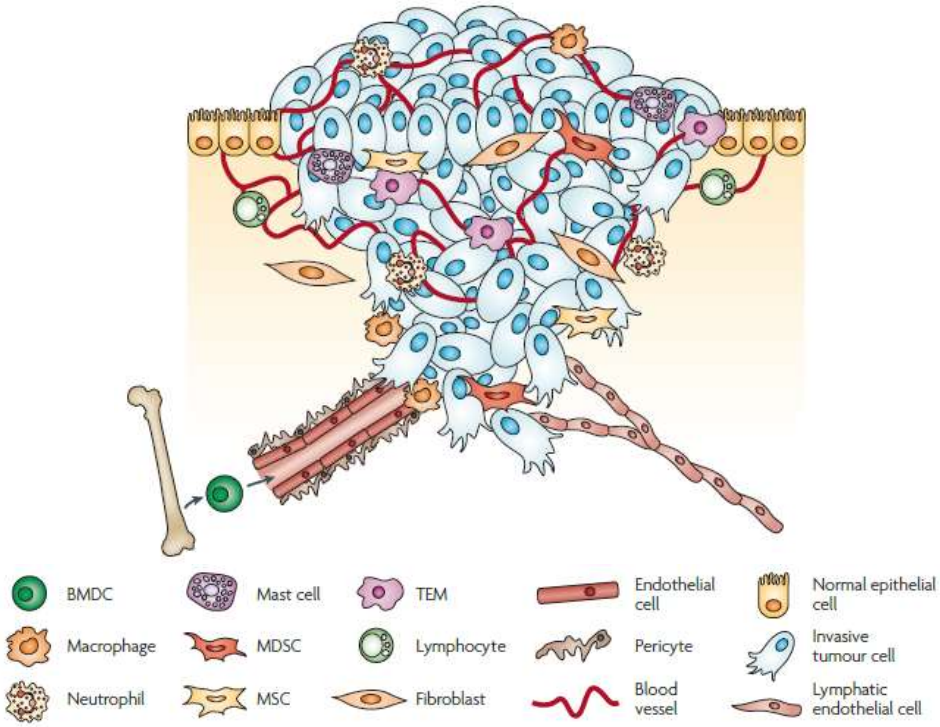

Figure 1.9. Composition of the primary tumour microenvironment (TME). TME is composed of tumour cells, endothelial and epithelial cells, pericytes, fibroblasts, macrophages, neutrophils, mast cells, MDSCs, MSCs, lymphocytes[130].

Initially, the TME protects against tumorigenesis. The injection of teratocarcinoma in mouse blastocyte showed that normal microenvironment can not only restrict tumorigenesis but also reprogram malignant cells[131]. The extracellular matrix (ECM) serves as a physical scaffold that maintains homeostasis and provides survival and differentiation signals. Integrity of ECM helps prevent neoplastic transformation[132,133]. Immune surveillance is another mechanism use to maintain homeostasis. By patrolling, immune cells are able to recognize and clear abnormal cells. Stromal fibroblasts may modulate

malignant progression of transformed epithelial cells. When co-cultured with normal fibroblasts, the growth of initiated prostatic epithelial cells was reduced[134] and in another model of prostate carcinoma, presence of normal mesenchyme was associated with a loss of tumorigenesis[135]. However, during malignancy progression, cancer cells develop several ploys to influence and circumvent the inhibitory effects of the TME. These modifications are mediated by cell-cell interactions, secretion of growth factors, cytokines and chemokines, ECM proteins, proteases and protease inhibitors. This leads to the activation and/or recruitment of bone marrow derived and stromal cells. Because of these changes, the TME is becoming part of the tumour machinery by enhancing angiogenesis, proliferation, survival and invasion of tumour cells.

TME also influences metastatic dissemination and homing. Although metastasis location is in part governed by vasculature anatomy (circulating cancer cells follow blood flow and are forced to arrest in capillaries due to mechanical forces such as size of the cells, size of the capillaries, blood pressure and deformability of the cells)[129,136], this is not sufficient to explain all sites of metastasis. Metastasis dissemination depends also on specific tissue tropism, as first conceptualized by Paget and his "seed and soil" theory[137]. Nowadays, tissue tropism is defined as the interaction, the compatibility and the adaptation of cancer cells and local environment[138]. Primary tumours are able to prime targeted distant sites of metastasis by secreting numerous growth factors and chemokines such as VEGFA, PlGF, TGFβ and TNF and render the microenvironment suitable for the engraftment of metastatic cells (pre-metastatic niche)[139,140]. The secretion of these factors triggers a cascade of events allowing the recruitment of stromal and bone marrow-derived cells as well as the remodelling of local tissue facilitating homing of metastatic cells and micrometastasis formation[140,141]. Moreover, circulating metastatic cells may condition their own environment to form a metastatic niche thanks to the collaboration of stromal cells and the recruitment of myeloid cells[141]. Tissue tropism is also influenced by chemokine secretion at metastasis sites. In breast cancer for example, CCL21 and SDF1 secretion induced chemotaxis and invasion of CCR7- and CXCR4-expressing tumour cells[142,130].

After homing, solitary cells or micrometastasis may not be able to develop into macrometastasis, probably because they encountered a non-permissive microenvironment. They are defined as cells in a dormant state. Different

mechanisms have been brought forward to explain cell dormancy. Solitary cells might enter in G0-G1 phases of the cell cycle (cellular dormancy). The rate of proliferation may be counterbalanced by apoptosis within the micrometastasis, possibly because they fail to induce the angiogenic switch (angiogenic dormancy). It is also possible that immune surveillance prevents disseminated cells to proliferate. In any case, to grow in macrometastasis, dormant cells have to undergo systemic changes to counteract the negative effect of the local environment[143–145].

4.3. Tumour-associated macrophages

Macrophages are the most frequent immune cells found in the tumour site. In most cancers, macrophage's presence is associated with poor patient prognosis[146,147] although some exceptions exist[148]. Macrophages found inside the tumour are called tumour-associated macrophages (TAMs) and are mostly of M2-like phenotype.

TAMs originate from blood Ly6C^{hi} monocytes recruited at the tumour site mostly by cancer cells. The major drivers of Ly6C^{hi} monocytes recruitment and differentiation into TAMs are CSF1 and IL-34, the two ligands of CSF1R expressed on Ly6C^{hi} monocytes[149]. CCL2 is also involved in the recruitment of inflammatory Ly6C^{hi} monocytes in primary tumours but also at metastasis sites[150], where monocytes differentiate into metastasis-associated macrophages (MAM). Monocytes recruitment and differentiation is also mediated through VEGF[151,152]. Once inside the tumour, Ly6C^{hi} monocytes differentiate into different subsets of macrophages depending on their location and thus perform various functions[153] (Figure 1.10). TAMs involvement in tumorigenesis goes from initiation to metastasis development including invasion and migration of cancer cells and immune regulation[154] (Figure 1.10).

Persistent irritation or chronic inflammation due to various stimuli from systemic or local environment (such as pathogen infection, inflammatory conditions, exposure to irritants…) is known to be one initiating cause of cancer[155,156]. Macrophages take a major place in regulating inflammation and consequently are playing a role in cancer initiation. The production and release of reactive nitrogen and oxygen species confer a suitable environment for the apparition of mutations in epithelial cells and the expression of growth factors

IL-6 and TNF-α, EGF, TGF-β promote the proliferation and survival of these mutated cells[157].

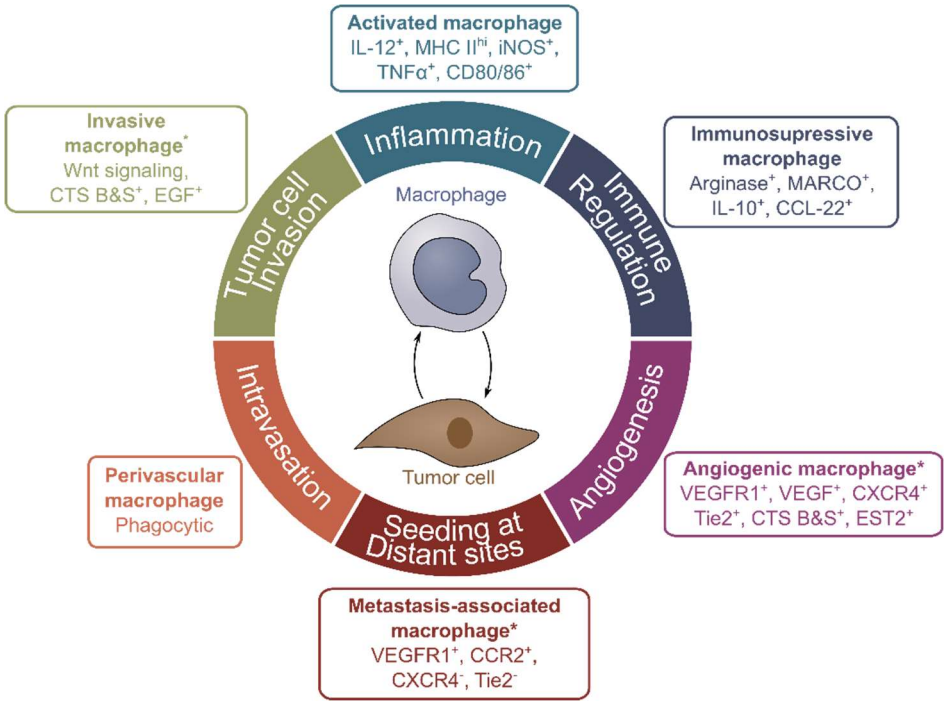

Figure 1.10. Macrophage phenotypes and tumorigenesis[157]. Microenvironment educate macrophages to differentiate into pro-tumour subtypes. Each of these macrophage subpopulations function to promote tumour development by regulating inflammation, immune regulation, angiogenesis, invasion, intravasation and seeding.

Growing-primary tumour needs more nutrients and oxygen. This is provided by angiogenesis triggering, called "angiogenic switch". TAMs are important players in the regulation of this process. Hypoxia recruits a specific subset of TAMs, Tie2-macrophages at the tumour site[158] and the low oxygen tension increase expression of HIF1 and HIF2 transcription factors. This induce the production of VEGF, bFGF, CXCL8 and glycolytic enzymes such as MMP9 and enhance endothelial cell survival, proliferation and motility as well as tissue remodelling[158–161]. The degradation of the ECM by MMP-9 also releases VEGF from extracellular depots[154].

TAMs also contribute to tumour cells invasion and migration via a paracrine loop involving EGF and CSF1[162,163]. Tumour cells synthesize CFS1 which in turn induce the expression of EGF by macrophages and cause the migration of both

type of cells and the cluster of tumour cells around blood vessels. The CSF1/EGF-dependant co-migration may be triggered by heregulin β1 and CXCL12/SDF-1[164]. Tumour cells and ECM interaction and thus migration are also enhanced by osteonectin[165]. TAMs produce proteases such as cathepsins, MMPs and serine proteases that mediate remodelling and destruction of the ECM allowing tumour cells to escape into the circulation to metastasize[99,166].

Monocytes and macrophages play an important role in metastasis by both preparing the site and promoting extravasation survival and persistent growth of metastatic cells[167]. Primary tumours prepare the pre-metastatic niche by sending signals to recruit monocytes/macrophages and render the site more suitable for homing[157]. Moreover these macrophages are able to establish concentration gradients of growth factors that coordinate tumour cell movements[168]. At metastatic site, the metastasis-associated macrophages (MAM) promote extravasation by expressing VEGF causing vascular permeability[169]. They also facilitate MET and tumour growth by inhibiting TGF-β signalling[170].

In addition to the lack of cytotoxic activity, TAMs inhibit T cell activity and promote T_{reg} responses. This is rendered possible thanks to the secretion of IL-10[171], TGF-β[149], CCL22[172] and CCL20[173] and the expression of cell surface receptors such as CTLA-4[167] and PD-L1[174] by TAMs. TAMs also suppress T cell activity through the depletion of L-arginine in the TME[175].

5. Sepsis

Systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock have long been considered as a continuum rather than distinct clinical entities, sepsis being defined as SIRS with suspected or confirmed infection. Severe sepsis was defined as sepsis with organ dysfunction, evidence of hypoperfusion or hypotension and septic shock was characterized as persistent sepsis-induced hypotension. However, in 2016, new consensus definitions were established: sepsis is now defined as a “life-threatening organ dysfunction caused by a dysregulated host response to infection”. Sepsis arises when the local body response to pathogens becomes systemic and injures its own tissues and organs. In line, septic shock is “the subset of sepsis in which underlying circulatory and cellular or metabolic abnormalities are profound

enough to increase mortality substantially". Patients with septic shock present sepsis-induced hypotension[176,177].

Sepsis originates from the penetration of a pathogen into the bloodstream due to a breach of integrity of the host barrier. It can arise from breaches constituted by indwelling urinary catheters, intravenous cannulas or an endotracheal tube. Insect bites, thorn pricks, or minor skin abrasions can also lead to sepsis. Both infectious and non infectious causes trigger sepsis. As for non infectious origins, we can cite: pancreatitis, tissue ischemia, trauma and surgical tissue injury, burns, thromboembolism, vasculitis, drug reactions (including neuroleptic malignant syndrome) and autoimmune and neoplastic processes. Concerning infectious causes, both the site of infection and the infectious agent have to be taken into account: the most common site of infection are lungs (64%), followed by the abdomen (20%), the bloodstream (15%) and renal or genitourinary tract (14%). 62% of the patient are infected by Gram- bacteria (Pseudomonas and Escherichia coli), while Gram+ bacteria (Staphylococcus aureus) and fungi cause 47% and 19% of the infection, respectively[176,178].

Sepsis is the primary cause of death from an infection with an incidence ranging between 0,4/1000 to 1/1000 of the population[176]. Sepsis is shaped by pathogen characteristics but also by host factors such as age, race, comorbidities, environment and sex[177]. Indeed, women have a lower incidence of sepsis and this difference may be explained by the role of sex hormones on innate and adaptive immune responses and the cardiovascular response to cytokine signalling[179].

5.1. Physiopathology of sepsis

As mentioned above, when an infection occurs, the host immune responses recognize specific patterns (PAMPs) expressed by the pathogen thanks to PRRs present on innate immune cells and endothelial cells. This recognition triggers a signalling cascade and leads to secretion and production of cytokines, chemokines and other factors that cause local vasodilation, increased vascular permeability, recruitment of leukocytes at the site of infection and a local coagulopathy[180]. All these phenomena aim to fight the pathogen. However, the immune system may be overwhelmed by the magnitude of the infection and systemic infection and inflammation ensue[181]. The systemic pro-inflammatory

state leads to large scale tissue-damage and severe organ dysfunction (Figure 1.11). For example, the release of TNF-α that activates NO synthase, increases the level of NO and leads to relaxation of smooth muscle cells and to vasodilation[182]. In local inflammation response, this helps to bacterial clearance by allowing the arrest of leukocytes and clotting factors. However a diffuse vascular smooth muscle cell relaxation contributes to hypotension, tissue hypoxia and mitochondrial shutdown[183]. Inflammatory cytokines, histamine, serotine, bradykinine, thrombin, ROS secretion and complement activation dysregulate endothelial cell integrity and increase vascular permeability. In sepsis this is followed by oedema and loss of circulating volume and cause tachycardia and hypoperfusion[184].

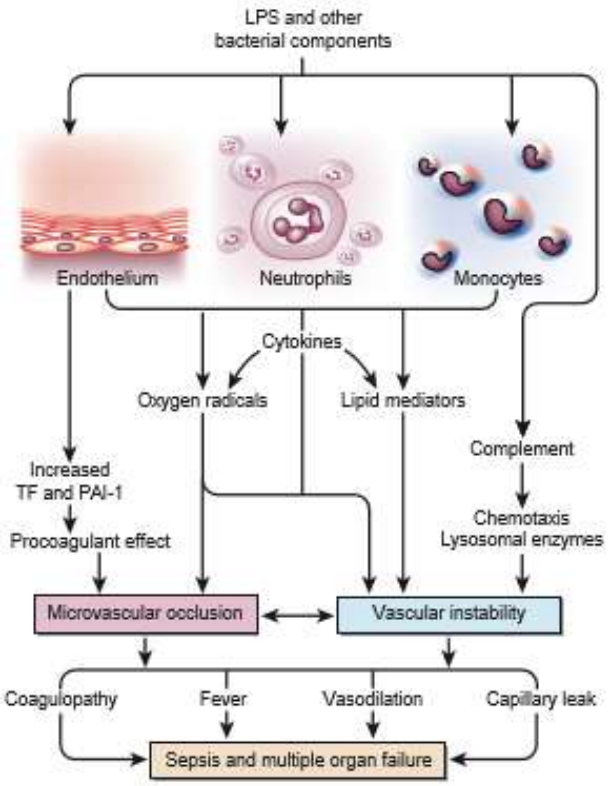

Figure 1.11. Pathogenesis in septic shock. Bacterial components activate immune responses, endothelial cells and the complement system. This triggers a cascade of events leading to microvascular occlusion, vascular instability, sepsis and multi-organ failure[185].

As part of innate immune responses, the complement is also activated during infection and plays an important role during early phase of sepsis. The

complement cascade produces high amounts of anaphylatoxins C3a and C5a that have pro-inflammatory effects. C5a is a central mediator of sepsis since it regulates TLR-4-mediated responses of phagocytes[186], triggers degranulation and the release of cytokines such as HMGB1 and the macrophage migration-inhibitory factor (MIF)[187,188]. C5a also contributes to smooth muscle cell contraction, recruitment of PMCs, monocytes and vascular permeability[189,190]. Moreover, C5a is involved in immunoparalysis[191], multi-organ failure[192] and favours coagulopathy[193]. Indeed complement system and coagulation pathways are interconnected. Activation of the complement activates platelets, induces modification of membrane phospholipids and the expression of tissue factor (TF) by endothelial cells and expression of TF and PAI by leukocytes[194,195]. Complement also influences indirectly the coagulation through the action of the pro-inflammatory cytokines TNF-α, IL-6 and high-mobility group box 1 (HMGB1)[196]. TNF-α and IL-6 as well as bacterial products and endothelial cell damages induce expression of TF on monocytes/macrophages and endothelial cells[197]. TF is the initiator of the extrinsic coagulation cascade that leads to the transformation of prothrombin into thrombin and to the conversion of fibrinogen into fibrin. Cross-linked fibrin clot results in the formation of microvascular thrombi[185]. In sepsis, this pro-coagulant state is emphasized by a high concentration of plasminogen activator inhibitor type I (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAF1)[198] but also by a decrease of anticoagulant factors such as anti-thrombin III, activated protein C and tissue factor pathway inhibitor (TFPI)[199,200]. Altogether these events contribute to the development of an impaired fibrinolysis and of disseminated intravascular coagulation (DIC), respectively[196]. In addition to its anti-coagulant effects, activated protein C is also an anti-apoptotic and anti-inflammatory factor[196]. It has been shown that HGMB1 is in part responsible for its decrease[201]. The development of DIC during late sepsis leads to consumption of platelets and clotting factors, bleeding, organ dysfunction and death[176].

The activation of endothelium increases the expression of adhesion molecules such as E-selectin, ICAM and VCAM, which facilitates arrest and infiltration of leukocytes[184]. Among these molecules, von Willebrand factor (vWF) is also overexpressed. The expression of vWF by endothelial cells supports the binding of platelets[202]. Platelets have pro-thrombotic effects and enhance the expression of TF on endothelial cells and monocytes[203]. The coagulation is

further amplified by the release of neutrophil extracellular traps (NETs) by neutrophils. NETs show procoagulant activity by activating platelets and provide a scaffold for thrombus formation. NETs are responsible for endothelial cell death[204,205].

In addition to thrombi formation, thrombin contributes to endothelial dysfunction through the binding to proteases-activated receptors (PARs)[205]. It has also been reported that thrombin can activate complement system by acting as a C5 convertase[206]. Moreover coagulation and inflammation are interconnected. Activated thrombin promotes the activation of several signalling pathways such as TNF, IL-1β and IL-6 and the generation of C5a. In turn, these cytokines can stimulate coagulation[196,207].

When the inflammation becomes critical, the host responses attempt to restore an immunological equilibrium. For example, lymphocytes display T_{h2}-type responses instead of T_{h1}-types and release counter-inflammatory molecules such as soluble TNF-α receptors, IL-1 receptor antagonists (IL-1RA), IL-1 receptor type II, IL-10, IL-4 and by inactivating the complement system[208]. Another observed downregulation of immunity is the sepsis-induced apoptosis of immune cells. The number of B cells, T cells and dendritic cells decreases resulting in an increase of suppressive activities and a T_{h2} polarization. This also leads to a decrease of antigen presentation, cytotoxic responses, macrophage activation and antibody production[209,210]. This can result in an immunosuppressive state and in the inability for the host to combat infection[180,211].

In sepsis, primary infection is rarely the cause of death. The major cause of death in sepsis patients is multi-organ failure due to tissue hypo-perfusion and hypoxia caused by microvascular occlusion, vascular instability and oedema. Moreover, the molecules released by cellular infiltrate directly damage tissue. Immunosuppression may also be responsible for secondary infections and the death of the patient[211].

5.2. Macrophages in sepsis

Macrophages express the TLR receptors that are specialized in the recognition of specific patterns expressed by infectious agents. TLRs are a family of 11 specific transmembrane receptors. TLRs 1, 2, 4, 5 and 6 recognise bacterial

products whereas TLRs 3, 7 and 8 detect viral particles. TLR9 is involved in bacterial detection as well as in viral recognition[212]. In sepsis, TLR4 and TLR2 play an important role. TLR4 recognises lipopolysaccharides (LPS, also known as endotoxin) of Gram-negative bacteria, the main source of infection in sepsis. On the other hand, TLR2 detects cell-wall structures from Gram-positive bacteria, such as peptidoglycan and lipoteichoic acid or exotoxins (for example TSST-1)[181]. In addition to TLRs, macrophages express other receptors involved in the response to LPS. TREM-1 and MDL-1 are responsible for monocyte activation and inflammatory responses[213,214]. Moreover, NOD-1 and NOD-2, members of the NLR family, are able to bind LPS and therefore are involved in LPS response[46].

Mediators	Effects
Cytokines :	Activate neutrophils, lymphocytes and vascular endothelium; upregulate cellular adhesion molecules; induce prostaglandins, nitric oxide synthase and acute phase proteins; induce fever.
IL-1, IL-6, IL-12, IL-15,	
IL-18, TNF-α, MIF, HMGB1	
IL-10	IL-10 is predominantly a negative regulator of these effects
Chemokines:	
IL-8, MIP-1α, MIP-1β,	Mobilize and activate inflammatory cells (neutrophils) ;
MCP-1, MCP-3	Activate macrophages
Lipid mediators:	
Platelets-activating factor, prostaglandins, leukotrienes, thromboxane, tissue factor	Activate vascular endothelium; regulate vascular tone; activate extrinsic coagulation cascade
Oxygen radicals:	
Superoxide and hydroxyl radicals; nitric oxide	Antimicrobial properties; regulation of vascular tone; Macrophage apoptosis

Table 1.2. Macrophage products implicated in the pathogenesis of sepsis[185].

The activation of TLRs leads to specific intracellular signalisation pathways and to production and release of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α, IL-15, IL-12, IL-18, HMGB1 and MIF), chemokines (IL-8, MIP1-α and β, MCP-1 and MCP-3), lipids mediators (prostaglandins, leukotriene, thromboxane and tissue factor) and oxygen radicals (NO, ROS)[185]. These pro-inflammatory molecules induce a range of effects that help to fight the invaders but also contribute to sepsis initiation (summarized in Table 1.2). For example, the cytokines activate innate and adaptive immunity as well as vascular endothelium, increase the expression of adhesion molecules and chemokines by endothelial cells and induce hepatic acute phase proteins (complement or fibrinogen)[176]. They also cause the release of NETs[204] and microparticles[215] as well as the upregulation of tissue factor[216]. The chemokines recruit and activate inflammatory cells such as neutrophils and macrophages. The lipids mediators and the oxygen radicals activate endothelium and the coagulation cascade, regulate vascular tone and exert antimicrobial activities[185].

Vascular endothelium plays an important role in the progression of sepsis. The interaction between macrophages/monocytes and endothelial cells is a key step in this expansion. Monocytes are attracted to the endothelium by IL-8, MCP-1 and MIP-1 during acute inflammation. Adherent monocytes express TF and contribute to start coagulation at the site of infection[205]. TF expression on circulating monocytes further propagates coagulation at distant site and favours systemic coagulation, leading to DIC[196]. Recruited monocytes activate inducible nitric oxide synthase creating an increase in NO efflux and inducing vasodilatation, gaps in endothelium and loss of endothelial barrier function[217]. These effects contribute to an increase of vascular permeability and hypotension in sepsis. HMGB1, mainly secreted by macrophages, also contributes to endothelial permeability and induces the production of inflammatory cytokines by endothelial cells[218,219]. Tissue macrophages also secrete VEGF-A, CSF-1, IL-1β and TNF-α and therefore support endothelial cell growth and the expression of P-selectin, E-selectin, ICAM1 and VCAM[220].

From early to late stages of sepsis, macrophages undergo a M1/M2 polarization shift. This is a protective mechanism to avoid damages due to an overwhelmed inflammation. M2-like macrophages are important for tissue repair and reestablishment of endothelial barrier[220] but also for restraining inflammation by engulfing death cells and eliminating debris and residual

PAMPs and DAMPs[176]. This shift corresponds to a state called endotoxin tolerance characterized by a reduced antigen presenting capacity and a down-regulation of major histocompatibility complex II (MHC-II) and its co-stimulatory molecules[221–223]. Furthermore, the production of IL-6 and TNF-α by macrophages is reduced. On the opposite, the secretion of IL-10, soluble TNF-α receptor and IL-1 receptor antagonist is increased[211,224]. However, despite an apparent "immunoparalysis", endotoxin tolerant macrophages show an increase energy metabolism and enhanced phagocytosis[223,225]. Endotoxin tolerance seems to be mediated by up-regulation of IRAK-M, a major negative regulator of TLR signalling[209,223,224].

5.2.1. TLR4 signalling

The sensing of LPS is dependent on the LPS-binding proteins (LBP) which form together the LPS-LBP complex and signal through TLR4[226]. TLR4 is a type I transmembrane protein that consists of an extracellular domain (leucine-rich repeat domain LRR) and a cytoplasmic domain containing a Toll/IL-1R homology (TIR) domain, necessary for signalling[44]. Upon TLR4 stimulation, two main intracellular signalling pathways are activated: the MyD88-dependent signalling pathway acting via NF-κB and leading to production of pro-inflammatory cytokines and the MyD88-independent signalling pathway acting via type 1 interferons and leading to the expression of interferon-inducible genes[227].

In the MyD88-dependent pathway, the binding of LPS to TLR4 and the co-receptor MD2 is followed by the recruitment of TIRAP and its interaction with PIP_2[228]. TIRAP facilitates the recruitment and the binding of MyD88 to TLR4 through TIR-TIR interactions[229]. IRAK-4 interacts with MyD88 via its death domain (DD) and in turn activates and binds other members of the IRAK family such as IRAK1 and IRAK2[230]. The MyD88-IRAK complex recruits ubiquitin ligase TRAF6 and cIAP1/2, to form a larger complex[48]. TRAF6 catalyses the formation of a polyubiquitin chain allowing the recruitment of a complex created by the association of TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins (TAB), the TAK1/TAB complex[227]. This complex activates the IKK complex and this results in the phosphorylation of IκB-α, the NF-κB inhibitory proteins. The phosphorylated IκB-α is ubiquitinated and degraded. This leads to the release of NF-κB and allows it to translocate into the nucleus to trigger the production of pro-inflammatory cytokines[231]. The TAK1/TAB complex is also responsible

for the activation of MAPKs ERK 1/2, JNK 1/2 and p38 and the subsequent production of growth factors and cytokines such as TNF, IL-10 CXCL1, CXCL2, IL-1, IL-12, cyclooxygenase 2 (COX2)[48] (Figure 1.12).

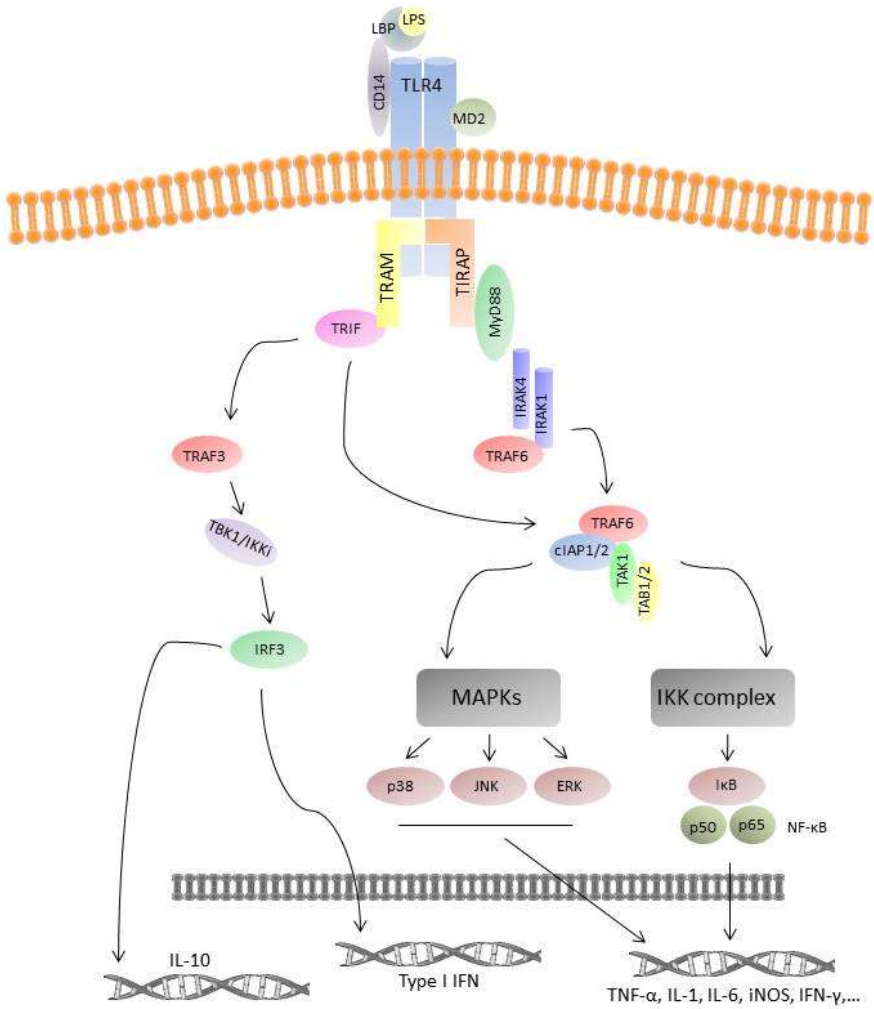

Figure 1.12. TLR4 signalling pathway. The LPS/LBP complex interacts with CD14 and TLR4 on the surface of macrophages. The interaction leads to the activation of TLR4 that signals through MyD88 and the kinase IRAK. IRAK, in turn, interacts with TRAF6, which leads to the activation of NF-κB and MAPK signalling pathways and the subsequent activation of genes under the control of these two pathways. Adapted from[227].

In MyD88-independent signalling, TRAM is required for the recruitment and activation of TRIF. TRIF next interacts with TRAF3 or TRAF6[232,233]. The association of TRIF with TRAF6 is an event common also to the MyD88-dependent signalling pathway[227]. TRAF3, however, activates TBK1 and IKKi which, in turn, phosphorylate IRF3[234]. This phosphorylation allows the translocation of IRF3 to the nucleus and the initiation of type I interferons and IL-10[235,236] (Figure 1.12).

5.2.2. MAPK pathways

MAPK pathways respond to various stimuli such as hormones, growth factors, cytokines, PAMPs, DAMPs and environmental stresses. They orchestrate numerous effects on cell physiology among which we can cite gene transcription, protein biosynthesis, cell cycle control, apoptosis and cell differentiation[237]. In mammals, three MAPKs families have been identified: the extracellular signal regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and p38. These MAPKs are activated by simultaneous phosphorylations of Tyr and Thr residues, catalysed by dual-specificity MAPK kinases (MAPKK). MAPKK are themselves activated by Ser and Thr residue phosphorylations mediated by MAPK kinase kinases (MAPKKK)[238]. Considering the diversity of their effects, MAPKs have many different substrates including transcription factors, protein kinases and phosphatases and other functional proteins[239]. MAPKs are inactivated by dephosphorylations, catalysed by dual-specificity phosphatases, the MKPs, among others[240]. For brevity, in this introduction, we will focus on ERKs signalling pathways.

ERK1 and ERK2 were the first mammalian MAPKs to be identified. Upon stimulation, Ras recruits and activates the MAPKKK Raf. In turn, Raf activates two ERKs specific MAPKK, MEK 1 and MEK 2 which phosphorylate the Tyr and Thr residues of ERK 1 and ERK 2, triggering their activation[241]. Once activated, ERK 1/2 activate numerous substrates including protein kinases families such as ribosomal S6 kinases (RSKs), MAPK interacting kinases (MNKs), mitogen- and stress- activated protein kinase 1 and 2 (MSK1/2) and the transcription factors c-Fos, Elk1 and SP-1 (Figure 1.13.A)[239]. Consequently this signalling cascade regulates many cellular functions such as cell adhesion, cell cycle progression cell migration and survival, differentiation, metabolism, proliferation and transcription[239].

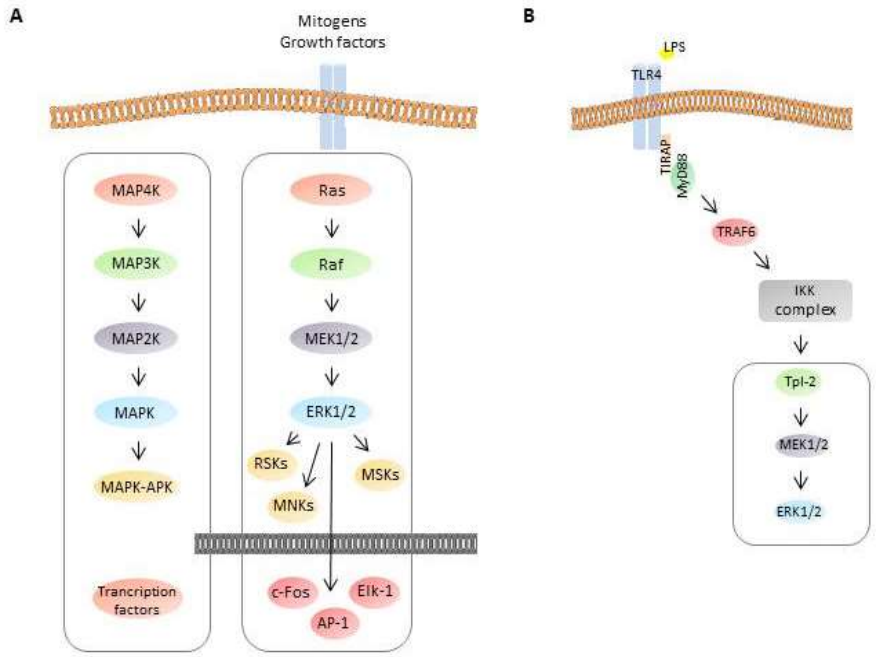

Figure 1.13. ERK signalling pathways. **(A)** ERK signalling pathway can be activated by mitogens and growth factors. Following receptor stimulation, Ras is recruited and activates Raf. Raf, in turn, phosphorylates and activates MEK 1 and MEK 2 triggering the activation of ERK and the regulation of many cellular functions. **(B)** ERK signalling pathway is also activated following TLR4 stimulation by LPS. TLR4 activation leads to activation of IKK complex and Tpl-2. Tpl-2, in turn, activates MEK 1 and MEK2. Adaptated from[237,239].

ERK can also be activated independently of Ras by TNF, PAMPs such as LPS and DAMPs[237]. Following LPS activation, Tpl-2 is activated under the influence of the IKK complex. Tpl-2 is then able to activate MEK 1/1 and consequently ERK1/2 (Figure 1.13.B). It has been demonstrated that in $Tpl2^{-/-}$ mice, the disruption of Tpl-2 was associated with an impaired activation of ERK and a decreased LPS-stimulated macrophage TNF release[242]. Tpl-2 and ERK are also playing a role in the differentiation of polarized T_h cells[243].

5.2.3. PI3K/Akt signalling and implications in TLR4 signalling

Type 1a PI3K contains a regulatory subunit p85 and a catalytic subunit p110[227].

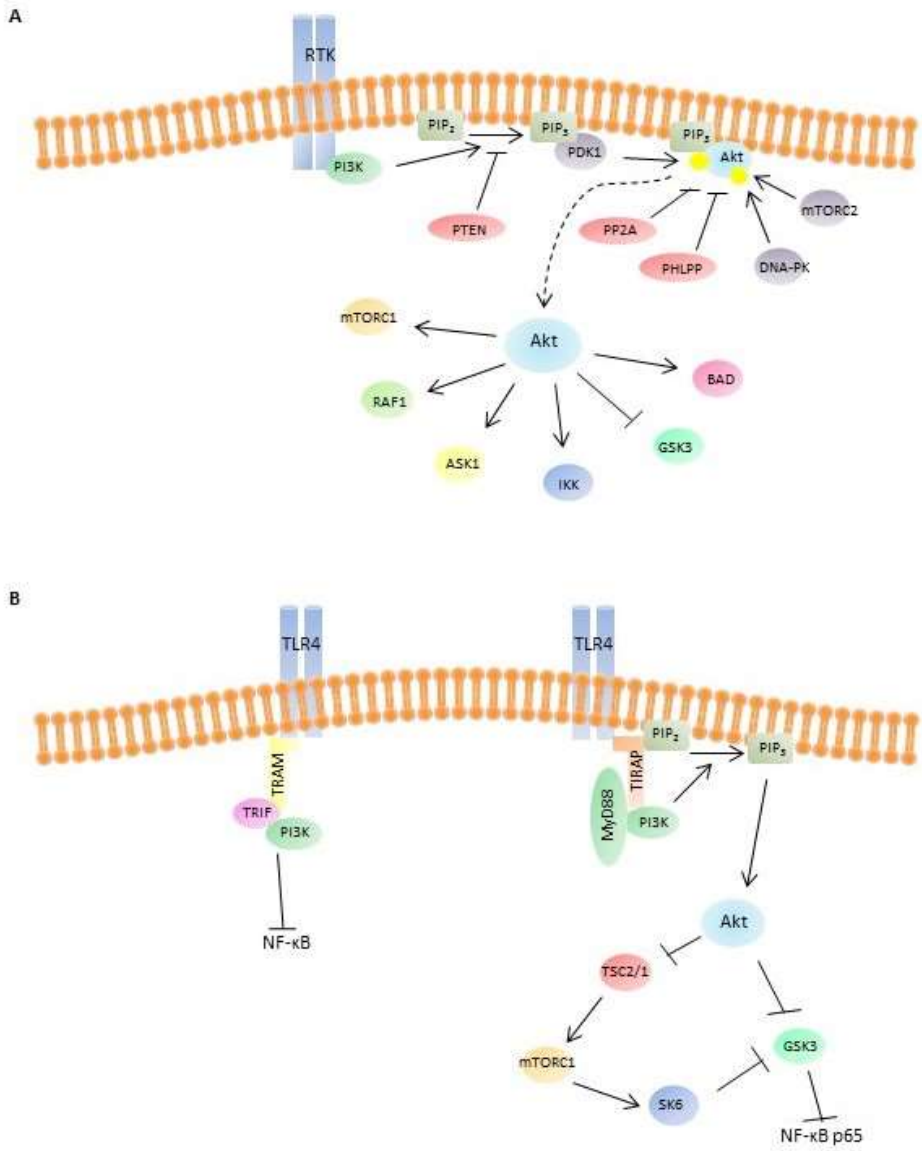

Figure 1.14. PI3K/Akt signalling pathway. **(A)** The activation of PI3K leads to the conversion of PIP_2 into PIP_3 and to the subsequent recruitment of Akt. Akt is then activated by phosphorylation by mTORC2 and DNA-PK. The conversion of PIP2 into PIP3 is inhibited by the phosphatase PTEN. PP2A and PHLPP inhibit Akt activation. Once activated Akt can act on numerous substrates such as mTORC1, BAD, GSK, RAF1, …. **(B)** PI3K/Akt influence TLR4 signalling. The interaction of TIRAP with PIP3 allows the recruitment of MyD88/PI3K complex and the activation of Akt. Akt directly or indirectly inactivates GSK3 leading to the inhibition of NF-κb p65 subunit. PI3K also interact with TRIF and this interaction leads to a decrease of NF-κB activities.

The activation of PI3K by specific receptors triggers the conversion of phosphatidylinositol (3,4)-bisphosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) allowing the recruitment of signalling molecules such as Akt/PKB through their plekstrin homology domain[227]. The conversion of PIP_2 into PIP_3 is inhibited by PTEN[244]. Akt is fully activated by the phosphorylation of Thr308 by PDK1 and of Ser473 by mTORC2 or DNA-PK. The phosphorylation of Thr308 leads to a partial activation of Akt, sufficient to activate mTORC1 and promote protein synthesis and cellular proliferation. Partially-activated Akt can also inactivate TSC1-2 and PRAS40, which, in turn, inhibit mTORC1[245]. The fully-activated Akt can phosphorylate GSK3-α and GSK3-β. This double phosphorylation inactivates GSK3 and results in increased nuclear levels of CREB. Akt possesses numerous substrates such as FOXO proteins, BAD, caspase 9, *etc* and exerts many functions including angiogenesis, metabolism, growth, proliferation, survival, protein synthesis, transcription, and apoptosis[245]. The Akt dephosphorylation of Thr308 by PP2A[246] and of Ser473 by PHLPP1/2[247] inhibits Akt signalling (Figure 1.14.A).

Upon stimulation of TLR4 with LPS, TIRAP is recruited and interacts with PIP_2 allowing the subsequent attraction of MyD88. PI3K constitutively binds to MyD88 through its regulatory subunit p85. PI3K is then activated through phosphorylation by Src kinase[248]. It is thought that PI3K is a negative regulator of TLR signalling and acts early during the signalisation[249]. The activation of PI3K and Akt leads to the phosphorylation of GSK3 and its inactivation[250]. This results in an enhanced transcriptional activity of CREB and a reduced activity of the transcription factor NF-κB p65. This also ensue an increased production of IL-10 and a drop in pro-inflammatory cytokines expression (IL-12, TNF-α)[251]. Akt can also indirectly inactivate GSK3. The activation of Akt leads to the inhibition of the tuberous sclerosis complex (TSC2/1 complex) and to the activation of mTORC1. mTORC1 in turn phosphorylates and activates S6K, known to affect the phosphorylation of GSK3[227,252]. The negative regulation of TLR4-MyD88-dependant pathway limits the production of IL-12 and TNF-α and the expression of nitric oxide synthase resulting in the reduction of excessive inflammation and the control of T_{h1} vs T_{h2} responses[249]. However, PI3K can also act on the MyD88-independent pathway. PI3K interacts with the adaptor molecule TRIF and interferes with the ability of TRIF to conduct an optimal NF-κB binding and transcriptional activity. This results in a decrease in IFN-β production[253] (Figure 1.14.B). Additionally, it should be noted that, following

LPS stimulation PI3K is able to exert pro-inflammatory effects using alternative pathways independent of TLR4-MyD88[254]. In TLR4-deficient mice, innate resistance to LPS was reduced following inhibition of PI3K signalling. This higher mortality was associated with an increase in tissue superoxide, IL-1β and IL-2[254].

5.3. Gender differences in sepsis

Women have a heightened immune response which leads to an increased incidence of autoimmune diseases compared to men. On the other hand, it confers to women a higher protection against infection and sepsis[255]. Indeed, women younger than 50 years show a lower incidence of severe sepsis and a better survival compared to age-matched men[256,257]. The effects of oestrogens (E_2) on immune responses are complex and depend on the cell type and the concentration of such hormones. In brief, E_2 stimulate the production of antibodies from B cells[258,259] but induce a decrease of B cell precursors in the bone marrow[260]. E_2 also downregulate T cell-dependent immunity due to a shift from T_{h1} to T_{h2} responses[255]. At high concentrations, E_2 show anti-inflammatory responses by decreasing the release of TNF, IL-6, IL-1β, MCP-1, iNOS and MMP and by increasing the production of IL-4, IL-10, TGF-β and TIMP[255]. Oestrogens also have inhibitory effects on the expression of various adhesion molecules such as E-selectin, VCAM and ICAM[261,262]. An anti-apoptotic effect of E_2 on immune cells has also been shown[255].

In LPS-stimulated macrophages, E_2 treatment inhibited the production and release of IL-6, TNF and NO[263,264]. The decrease of TNF and IL-6 is associated with a reduced NF-κB activity. Indeed by preventing nuclear translocation, E_2 blocks LPS-induced DNA binding and transcriptional activity of NF-κB p65 subunit and c-Rel, respectively[265]. E_2 also affects neutrophils and monocytes migration at inflamed sites and inhibits expression of MCP-1[255]. Furthermore the expression of CCR2 and CXCR3 on monocytes is downregulated by E_2[266]. Moreover, treatment with E_2 showed effects on leukocytes recruitment (reduced leukocyte-endothelial cell interactions and improved functional capillary density) during sepsis[267].

In addition of having anti-inflammatory properties, E_2 influence other cell types and functions during sepsis. In a model of endotoxemia, female showed less myocardial dysfunction compared to male mice. The authors assign this effect

to an attenuated RAC1 activation by oestrogens, reducing expression of TNF-α and potentially detrimental myocardial effects[268]. E_2 also protect against other organ dysfunction such lung, liver and small intestine by reducing the level of proinflammatory cytokines and thus maintaining tissue homeostasis[269,270].

Objectives and specific aims

Objectives and specific aims

DUSP3 is an ubiquitously expressed atypical dual specificity phosphatase. Although some *in vitro* and *in vivo* substrates have been identified, the physiological role of this phosphatase remains unclear. In a previous study, we reported that DUSP3 was highly expressed in monocytes and macrophages[38], suggesting that DUSP3 plays an important role in innate immunity and inflammation.

Objective 1: investigation of the role of DUSP3 in metastasis formation.

DUSP3 has been reported to play contradictory roles as tumour suppressor or as tumour promoter depending on the cell type/model used[26]. Moreover, using DUSP3$^{-/-}$ mice, we recently reported that DUSP3 deficiency leads to a defective tumour-induced angiogenesis[23,271].

Considering the important role of macrophages in cancer development, the first objective of the presented work was to study the contribution of DUSP3 to the susceptibility of metastasis development, more particularly the role of the myeloid cells in metastasis formation and the function of the phosphatase in these cells. To do so, we used two experimental metastasis models, namely the Lewis Lung Carcinoma (LLC) and B16 melanoma cell models. We investigated the involvement of myeloid cells, more particularly macrophages, using different *in vivo* and *in vitro* approaches.

Objective 2: investigation of the role of DUSP3 in gender-dependent responses to septic shock.

In a previous report, we showed that DUSP3$^{-/-}$ female mice were protected from sepsis and septic shock through a mechanism involving M2-like macrophage polarization[38]. Considering the role of sex hormones in regulating immune responses[255] and the reduced survival of men to septic shock[256], the second objective of this work was to investigate the role of DUSP3 in gender-dependent resistance to LPS- induced endotoxemia and polymicrobial infection CLP-induced septic shock.

The first specific aim of this part of the work was to determine the involvement of female sex hormones in the DUSP3 deletion-induced endotoxemia and septic shock observed resistance. To do so, we challenged male and ovariectomized (OVX) female mice with lethal doses of LPS. We next examined the influence of female sex hormones on macrophage polarization.

The second specific aim of this part of the work was to investigate the molecular mechanisms associated with the observed phenotype and the role of DUSP3 in macrophages. To do so, we stimulated *ex vivo* peritoneal macrophages of female, male and OVX mice with LPS and explored the effect of DUSP3-deficiency on TLR4 induced-signalling pathways.

RESULTS

Results

1. Context of the research

In the laboratory, the research focus on studying the role of the dual phosphatase DUSP3 in diverse biological processes such as cervix carcinoma, platelets aggregation and thrombosis, metabolic syndrome, hepatocarcinoma, angiogenesis and sepsis. The results presented in this work are along the same lines of the last two projects.

DUSP3 and metastasis formation

In a previous work studying angiogenesis, we found that DUSP3 was a key mediator of neovascularization by affecting at least the b-FGF-induced endothelial cell sprouting most probably via the PKC pathway. We also found that DUSP3-deficiency prevented neo-vascularisation of Matrigel plugs and LLC xenograft tumours suggesting that DUSP3 plays an important and non-redundant function in tumour-induced angiogenesis[23].

Considering the crucial role of angiogenesis in metastasis dissemination, the next logical step was to assess the role of DUSP3 in metastasis formation. The first part of the present work try to answer this question. In the following pages, we demonstrate that DUSP3 acts as an anti-metastatic agent by regulating the migration monocytes/macrophages to the site of metastasis. Indeed, we observed:

- bigger LLC-lung tumours in $DUSP3^{-/-}$ mice compared to $DUSP3^{+/+}$ mice
- a higher percentage of M2-like macrophages in lung tumours of $DUSP3^{-/-}$ mice
- a better migration of $DUSP3^{-/-}$ BMDM compared to $DUSP3^{+/+}$ BMDM in presence of LLC-conditioned medium. This was strengthened by a higher percentage of $Ly6B^{hi}$ M2-like macrophages in $DUSP3^{-/-}$ lung tumour.
- a reduction of LLC-lung metastasis in $DUSP3^{-/-}$ mice after depletion of macrophages.

DUSP3 in sepsis

The first results published on the role of DUSP3 in septic shock identified DUSP3 as a key and nonredundant regulator of the innate immune response to lethal inflammatory shock induced by endotoxin or polymicrobial infection, we showed that it acts by a mechanism involving M2-like macrophages. Moreover, we reported that DUSP3 is required for the production of TNF and, therefore, contributes to inflammatory responses[38].

However, in that previous work, we circumvented the gender problematic by using only female mice to conduct our research. The goal of the second part of this work is to include this variable in the study of the role of DUSP3 in sepsis. We demonstrated that DUSP3 play an important role in macrophage alternative activation and sexual dimorphism in innate immune response to infection. The presented data suggest that DUSP3 inhibition, combined to oestrogen administration, may lead to protection from sepsis and septic shock. Indeed, we observed:

- DUSP3$^{-/-}$ female mice, but not male mice, survived to LPS challenge and polymicrobial-induced septic shock.
- DUSP3$^{-/-}$ sham-operated mice survived to LPS challenge while DUSP3$^{-/-}$ OVX mice did not
- the mortality of male and OVX mice was associated with a decrease of the M2-like macrophage percentage in the peritoneal cavity
- the survival of DUSP3$^{-/-}$ female mice was associated with a hypo-phosphorylation of ERK and PI3K/Akt

Common lines of the two projects

Even though metastasis dissemination and septic shock are not related processes, they share common characteristics. The most important one is that inflammation and innate immune cells are crucial for the development of both diseases. Considering the demonstrated role of DUSP3, particularly in macrophages, studying both metastasis dissemination and sepsis was interesting to deepen our knowledge on DUSP3 function in these cells.

DUSP3-deficient macrophages and experimental metastasis

2. Dusp3 deletion promotes experimental lung tumour metastasis in a macrophage dependent manner

2.1. Abstract

Background

The *Vaccinia*-H1 Related (VHR) dual specificity phosphatase or DUSP3 is a small phosphatase dephosphorylating both tyrosine and serine/threonine phosphorylated residues. DUSP3 plays an important role in cell cycle regulation and its expression is altered in several human cancers. We have generated DUSP3 full-knockout mice ($DUSP3^{-/-}$) and found that these mice are healthy with no spontaneous phenotype. However, DUSP3 deficiency prevented neo-angiogenesis and b-FGF-induced microvessel outgrowth. Considering the importance of angiogenesis in metastasis formation, our study aimed to investigate the role of DUSP3 in tumour cell dissemination

Results

Using a Lewis Lung Carcinoma (LLC) experimental metastasis model, we observed that $DUSP3^{-/-}$ mice developed larger lung metastases than littermate controls. By creating bone marrow chimeric mice, we were able to transfer the phenotype to $DUSP3^{+/+}$ mice, indicating a role of hematopoietic cells in enhancing tumour cell dissemination to lung tissues. Interestingly, we found a higher percentage of tumour-promoting $Ly6C^{int}$ M2-like macrophages in $DUSP3^{-/-}$ LLC-bearing lung homogenates. This higher percentage of M2 macrophages is at least partially due to a better recruitment of these cells as confirmed by a higher percentage of $Ly6B^{hi}$ macrophages in $DUSP3^{-/-}$ lung homogenates and by a better migration of $DUSP3^{-/-}$ peritoneal macrophages and bone marrow differentiated macrophages (BMDM) under LLC-conditioned medium stimulation.

Conclusion

The phosphatase DUSP3 plays a key role in metastatic growth through a mechanism involving the recruitment of M2-like macrophages towards LLC-bearing lungs.

Key words: DUSP3/VHR, dual specificity phosphatases, microenvironment, metastasis, macrophages

2.2. Introduction

DUSP3, or *Vaccinia* H1-Related (VHR), is a member of the atypical dual-specificity protein phosphatase family (A-DUSPs). This protein contains a 185 amino acids (Mr 21 kDa) catalytic domain and lacks targeting or docking domains[14]. Its shallow and broad catalytic pocket allows DUSP3 to dephosphorylate both p-tyrosine (p-Tyr) and p-threonine (p-Thr) residues[272]. The MAPKs ERK1/2, JNK and to a lesser extend p38 have been reported as substrates of DUSP3[17–20]. The phosphatase also dephosphorylates other proteins such as the transcription factor STAT5[21] and tyrosine kinase receptors EGFR and ErbB2[22]. DUSP3 expression is regulated during cell cycle progression. Indeed its downregulation using RNA interference contributed to arrest HeLa cells in G1/S and G2/M phases and triggered their senescence. This was correlated with the hyper-phosphorylation of ERK1/2 and JNK[33]. Consequently, it is not surprising that overexpression of DUSP3 was found in human cervix carcinomas[35] and prostate cancer[36]. However, DUSP3 is also downregulated in other cancers such as breast cancer[37] and non-small cell lung carcinoma (NSCLC)[22,32], indicating contradictory and complex roles of DUSP3 in cancer development.

Recently, we generated full knock-out DUSP3-deficient ($DUSP3^{-/-}$) mice by homologous recombination. These mice are viable, healthy and fertile, with no spontaneous phenotype. However, in these mice, DUSP3 deficiency prevented neo-angiogenesis and b-FGF-induced microvessel outgrowth[23].

In solid tumours, host cells such as endothelial cells, fibroblasts and immune cells represent a major part of cell populations within the tumour[130]. Macrophages are the most frequent immune cells in the tumour and their presence is mostly correlated with poor prognosis for the patient[146,147]. These macrophages are called tumour-associated macrophages (TAM) and regulate many, if not all stages of tumour progression from initiation of tumour development to metastatic dissemination including invasion, migration and extravasation processes[273]. Indeed TAM control the induction of angiogenesis, extracellular matrix remodelling, the stimulation of cancer cell proliferation and metastasis and the inhibition of anti-tumour immune responses[154]. TAM subpopulations are very heterogeneous and have different phenotypes. Hence, they can execute multiple and different functions related to tumour growth and reside in distinct areas within the tumour[274].

TAMs are shaped by factors secreted by tumour cells or by the tumour microenvironment to become immunosuppressive macrophages and exert pro-tumour responses. Indeed in addition to their trophic functions, TAMs are usually unable to lyse tumour cells, present tumour-associated antigens to T cells and express immune-stimulatory cytokines to stimulate the proliferation of anti-tumour functions of T cells and natural killer (NK) cells *in vitro*. Therefore, it has been proposed that TAMs are polarized into a M2-like phenotype (or alternatively activated-like macrophages)[275]. The molecular mechanisms responsible for this polarization are, however, poorly understood.

In this study, using a Lewis Lung carcinoma (LLC)-experimental metastasis model and DUSP3-deficient mice, we reported that the phosphatase DUSP3 is a key player in metastatic growth, modulating the recruitment of M2-like macrophages towards LLC-bearing lungs.

2.3. Materials and Methods

Antibodies and reagents

Purified anti-CD16/CD32 (FcγIII/II receptor) (2.4G2), PE-anti-Ly6G (1A8), APC-Cy7 anti-Ly6G (1A8), V450-anti-CD45.2 (A20), V500-anti-I-A/I-E (MHC-II) (M5/114.15.2), APC-anti-CD11c (HL3), APC-Cy7 anti-CD11c (HL3), PE anti-Siglec-F (E50-2440), PE-Cy7-streptavidin and Biotin anti-IgG2B (RG/11.1) were from BD Biosciences (BD Biosciences, San Jose, CA). APC-anti-F4/80 (BM8) and PerCp-Cy5-anti-CD11b (M1/70) were from eBiosciences (eBioscience, San Diego, CA). Alexa 647-anti-CD206 (C068C2) and PE-Cy7-anti F4/80 (BM8) were from Biolegend (Biolegend, San Diego, CA). Alexa647 anti-Ly6b.2 (7/4) and Alexa 488-anti-Ly6G (ER-MP20) were from AbD Serotec (AbD Serotec, Kidlington, UK). Anti-VHR (DUSP3) (sc8889) antibody was from Santa Cruz (Santa Cruz, Dallas, Texas). Anti-GAPDH antibody was from Sigma (Sigma-Aldrich, Diegem, Belgium). HRP-conjugated anti-goat, anti-mouse and anti-rabbit were used as secondary antibodies and were from Amersham Biosciences (Amersham Biosciences, GlattBrugg, Switzerland). Collagenase I and DNaseI were from Roche (Roche, Basel, Switzerland)

Mice and Ethic statement

C57BL/6 (CD45.2)-DUSP3$^{-/-}$ mice were generated as previously reported[23]. These mice were backcrossed with C57BL/6-CD45.2 mice (Charles River) to generate heterozygotes that were mated to generate DUSP3$^{+/+}$ and DUSP3$^{-/-}$ littermate colonies used for experimentation. Age matched female DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice were used in all the experiments. Mice were kept in ventilated cages under 12-hour dark/12-hour light cycle in an SPF animal facility and received food and water and libitum. Health status was evaluated every 3 months and mice were always found free of specific pathogens.

All mice experiments and procedures were carried out following the guidelines of and in agreement with the animal ethics committee of the University of Liège.

Tumour metastasis model

LLC cells (1x10^6 cells) or B16 cells (1x10^6 cells) were inoculated to 8-12 weeks old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice on day 0 via tail vein injection. Tumour development was examined by xenogen imaging at 7 and 14 days after cells

injection and mice were sacrificed on day 14. Lungs were photographed and weighted before fixation for 2h in 4% paraformaldehyde or preparation of single-cell suspensions.

Xenogen imaging

The LLC or B16 tumour progression was monitored using Imaging System Xenogen IVIS 200 (Advanced Molecular Vision, Caliper Lifesciences, Waltham, MA, United States). 100 µl of luciferin potassium salt (30mg/ml) was intraperitoneally injected in tumour bearing $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice. After 12 minutes, the photons emitted by the luciferase activity were detected. The bioluminescence was quantified using the Living Image Software (Caliper Life Sciences, Waltham, MA, United States) and by delimitation of region of interest (ROI) around the lungs.

Mice irradiation and bone marrow transplantation

10 to 12 weeks old donor mice were sacrificed by cervical dislocation. Tibias and femurs were collected, separated and cleaned in sterile PBS on ice. Bone marrow cells (BMs) were flushed using a 25-gauge needle and 1 mL syringe filled with cold PBS. BM cell suspensions were passed through 70 µm nylon cell strainer (BD biosciences, San Jose, CA, United States) and centrifuged 10 min at 1200 rpm at 4°C. Single cells were then counted and resuspended in PBS at a concentration of $10x10^6$ cells/150 µL on ice. Cells were immediately transplanted to 6 weeks old lethally irradiated (849,1 cGy) recipient mice, via intravenous (i.v) injection. The efficiency of the transplantation was assessed by Western blot using anti-DUSP3. Anti-GAPDH immunoblot was used for normalization.

Macrophage depletion

150 µL of clodronate-liposome or empty-liposome (ClodronateLiposomes, Haarlem, The Netherlands) were injected intravenously and intraperitoneally in $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice, 2 days prior to LLC injection. The intraperitoneal injections were repeated every other day during 14 days. The efficiency of the depletion was assessed by flow cytometry on peritoneal washes and lung cell suspensions.

Isolation of Bone Marrow Derived Macrophages

Bone marrow cells were aseptically flushed out from femurs and tibias of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice on ice, using a 25-gauge needle and FBS-free RPMI. The cell suspensions were filtered using a 100 µm cell strainer and centrifuged 10 min at 1200 rpm at 4°C. 25x10^6 cells were then plated in 12 mL of RPMI supplemented with 10% heat-inactivated FBS, 10% L929-conditionned medium, streptomycin (100µg/mL) and penicillin (100U/mL) for 7 days. The cells were washed and the medium changed every other day. The macrophage differentiation was assessed by a F4/80-CD11b flow cytometry staining.

Isolation of peritoneal macrophages

Resident peritoneal macrophages (PMs) were selected by adherence to tissue culture plastic dishes in culture conditions at a cell density of 1 3 106 cells /mL in complete RPMI 1640 medium. After 2h, cells were gently rinsed twice with FBS/RPMI 1640 and used for experiments.

Cell migration

The bone marrow derived macrophage (BMDM) migration was assessed using 5 µm polycarbonate transwells (Corning, Lowell, MA). The membrane was equilibrated for 1h at 37°C with 600 µL and 100 µL of DMEM in the lower chamber and in the upper chamber, respectively. 3x10^5 cells were plated in 100 µL of medium in the upper chamber while 600 µL of DMEM, LLC- or B16-conditionned-medium was loaded in the lower chamber. The cells were incubated for 18h at 37°C. The cells migrated to the lower chamber were recovered and counted using the Millipore ScepterTM cell counter after gating on live cells using the Scepter Software 1.2 (Millipore, Overijse, Belgium). Migration index was calculated as number of cells transmigrated in the presence of chemokine per number of cells transmigrated in the absence of the chemokine multiplied by 100.

Lung histology

Lungs were fixed in 4% paraformaldehyde; paraffin embedded, cut in 5-µm sections, and stained with haematoxylin and eosin. 5 randomly selected sections per mouse lungs were analysed. Tumour areas were quantified using Nanozoomer Digital Pathology Image (Hamamatsu, Japan).

Cell culture

Lung Lewis carcinoma cells, stably transfected with luciferase gene (LL/2-luc-M38, LLC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100U/mL), streptomycin (100ug/mL) and neomycin (G418, 0,02mg/mL) (life technologies, Carlsbad, CA, United States). The B16 melanoma cell line (B16-F10-luc-G5, B16), stably transfected with luciferase gene, was maintained in culture in RPMI (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated FBS and penicillin (100U/mL), streptomycin (100ug/mL) and zeocyn (0,2mg/mL) (life technologies, Carlsbad, CA, United states). LLC and B16 cells were purchased at Xenogen/Caliper.

Western blot

Cells were lysed using RIPA buffer (50 mMTris-HCl (pH = 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM orthovanadate, complete protease inhibitor cocktail tablets EDTA free and 1 mM phenylmethylsulfonyl fluoride) on ice during 20 minutes. Lysates were next clarified by centrifugation at 21.000 g during 20 min at 4°C. The resulting supernatants were collected and protein concentrations were determined using the colorimetric Bradford reagent (Bio-Rad, Nazareth, Belgium). Samples were next denaturated at 95°C in Laemmli buffer. Samples were then run on SDSPAGE gel and transferred to Hybond-nitrocellulose membranes. To block the non-specific binding sites, membranes were incubated for one hour in Tris-buffered saline-Tween 20 containing 5% of non-fat milk. The membranes were next immunoblotted with anti-DUSP3 antibody. Membranes were next stripped, blocked and immunoblotted with anti-GAPDH, antibody for normalization. Immunoreactivity was then revealed using HRP conjugated secondary antibodies. The blots were developed by enhanced chemiluminescence (Amersham, Gent, Belgium) according to the manufacturer's instructions.

Preparation of single-cell suspensions from lungs

Lungs were perfused with 5mL PBS through the right ventricle, then dissected and shopped into small pieces before digestion for 1h at 37°C in 4mL of HBSS 1x containing 1 mg/mL collagenase A, 0.05 mg/mL DNaseI and

5% FBS. Mechanical stress was applied to the cells by flushing them through 18 gauge-needle. The cells were then passed through a 70 µm nylon cell strainer, centrifuged for 7 min at 1400 rpm at 4°C and the red blood cells were lysed. Isolated cells were directly stained for flow cytometry analysis.

Flow cytometry and phenotyping

For surface cell staining, $5x10^5$ to$1x10^6$ cells were incubated for 15 minutes with anti-CD16/CD32 (FcγIII/II receptor) using 0.5µg/100µL concentration to block non-specific interactions, prior to labelling for 30 minutes with specific antibodies. All stainings were performed on ice in PBS followed by one washing in PBS. Cells were next analysed on FACS Canto II (Becton Dickson, San Jose, CA, United States). Analysis was done using Flowjo (Flowjo, Ashland, Or, United States)

BMBM CFSE proliferation assay

BMDMs proliferation was performed using the CellTrace CFSE proliferation kit (ThermoFisher) following the instructions of the manufacturer. Briefly, cells were washed twice with pre-warmed PBS. 5 µM of CFSE was added and cells were incubated for 15 min at 37°C. Labelling was stopped by removing the medium. Cells were washed twice with pre-warmed PBS and then stimulated with 2 mL of LLC-CM for 24h or 48h. CFSE intensity was assessed by flow cytometry (FACSCanto II, BD Biosciences). Data were analysed using FlowJo software (Tree Star Inc)

LLC migration assay

LLC cells migration was assessed using 5 µm polycarbonate transwells (Corning). The membrane was equilibrated for 1h at 37°C using RPMI medium. $1x10^5$ cells were plated in 100 µL of the medium in the upper chamber while 600 µL of RPMI, $DUSP3^{+/+}$ BMDMs- or $DUSP3^{-/-}$ BMDM-conditionned medium was loaded in the lower chamber. The cells were incubated for 18h at 37°C. The cells that migrated to the lower chamber were recovered and counted using the Millipore ScepterTM cell counter after gating on live cells using the Scepter Software 1.2 (Millipore). Migration index was calculated as a ratio of the number of trans-migrating cells in the presence and absence of conditioned medium multiplied by 100.

LLC proliferation assay

$2x10^4$ LLC-luciferase positive cells were plated in 48-well plates, washed twice with pre-warmed PBS and incubated with 2 mLof RMPI, $DUSP3^{+/+}$ or $DUSP3^{-/-}$ BMDM-conditioned medium for 8h, 16h or 24h at 37°C. Cells were then incubated with 30 mg/mL of luciferine potassium salt (Promega) for 12 minutes and the photons emitted by the luciferase activity were detected for each time point. The bioluminescence was quantified using the Living Image Software (Caliper Life Sciences).

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA, United States). The student T-test was applied to determine the difference between two experimental conditions. All the results are presented as mean ± SEM. The results were statistically significant when p-value <0,05. *p<0,05; **p<0,01; ***p<0,001.

2.4. Results

DUSP3 deletion accelerates experimental LLC metastatic growth

We have previously shown that DUSP3 plays an important role in tumour neo-vascularization[23]. We showed that matrigel plugs and LLC subcutaneous tumours were less vascularized in $DUSP3^{-/-}$ mice compared to $DUSP3^{+/+}$ littermates. To investigate more in depth the roles of DUSP3 in experimental metastasis formation, we intravenously injected $1x10^6$ LLC-luciferase (LLC) cells into age and sex-matched $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice. LLC metastatic growth was followed *in vivo* by LLC cell luminescence signal quantification using the *in vivo* imaging system IVIS 200. Remarkably, the incidence of LLC lung metastasis was significantly higher in $DUSP3^{-/-}$ compared to $DUSP3^{+/+}$ mice both at day 7 and day 14 after LLC injection (Figure 2.1.A and 2.1.B). At the time of sacrifice (day 14 after LLC injection), the $DUSP3^{-/-}$ metastatic lung weight was significantly increased compared to $DUSP3^{+/+}$ mice. Photographs of the lungs showed a major metastatic development in $DUSP3^{-/-}$ lungs while only few nodules were visible in $DUSP3^{+/+}$ mice (Figure 2.2.A and 2.2.B). Haematoxylin-eosin staining of lung sections and tumour area quantification confirmed that $DUSP3^{-/-}$ lung tumours were significantly larger than in $DUSP3^{+/+}$ lungs (Figure 2.2.C and 2.2.D).

To verify whether the marked increase of LLC growth in $DUSP3^{-/-}$ mice was tumour model-dependent, we challenged $DUSP3^{+/+}$ and $DUSP3^{-/-}$ with metastatic melanoma B16-F10-luciferase (B16) cells and monitored metastasis growth using IVIS 200. Interestingly, there was no significant difference in the number and frequency of B16 metastatic foci between $DUSP3^{+/+}$ and $DUSP3^{-/-}$ mice (Figure 2.3.A and 2.3.B). This was supported by the weight of B16-bearing $DUSP3^{+/+}$ and $DUSP3^{-/-}$ lungs and haematoxylin-eosin staining (Figure 2.4). The lack of difference in tumour development after B16 intravenous injection suggests that the accelerated metastasis growth in $DUSP3^{-/-}$ is LLC-dependant.

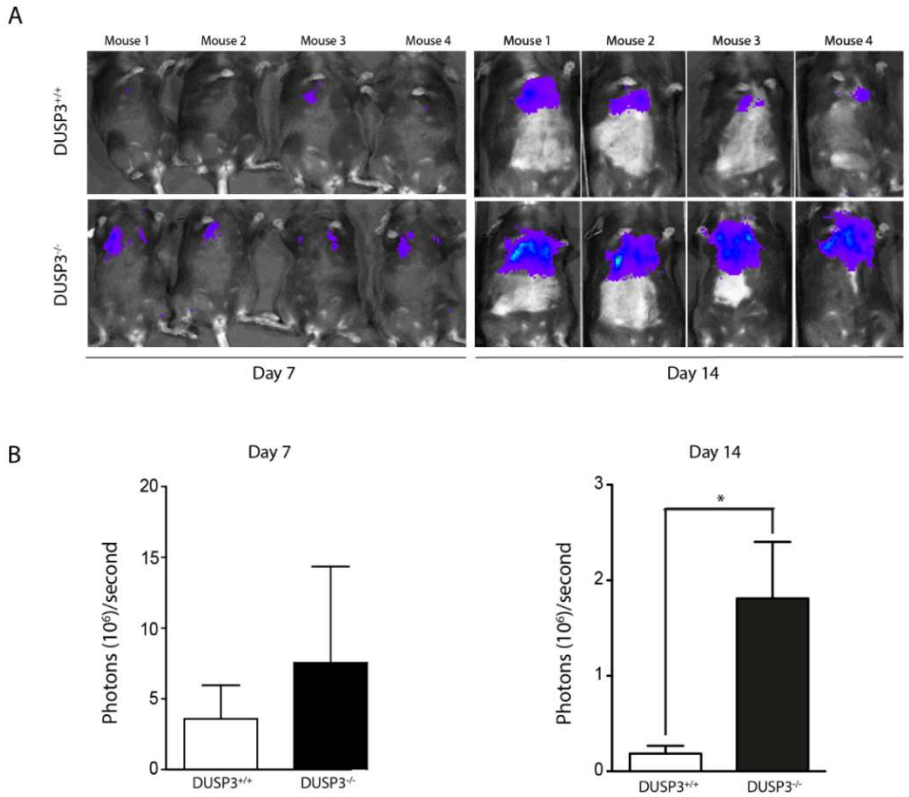

Figure 2.1. DUSP3 deletion accelerates experimental LLC metastasis growth. LLC tumour growths were monitored by xenogen bioluminescence imaging. Tumours were established by i.v. injection of 10^6 LLC-Luc+ cells to DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. **(A)** Representative xenogen imaging results at day 7 and day 14 after LLC injection. **(B)** Quantification of xenogen bioluminescence imaging data shown in A at day 7 and day 14 after LLC injection. *, $p < 0,05$. n= 10 for each experimental group. Results are shown as mean ± SEM

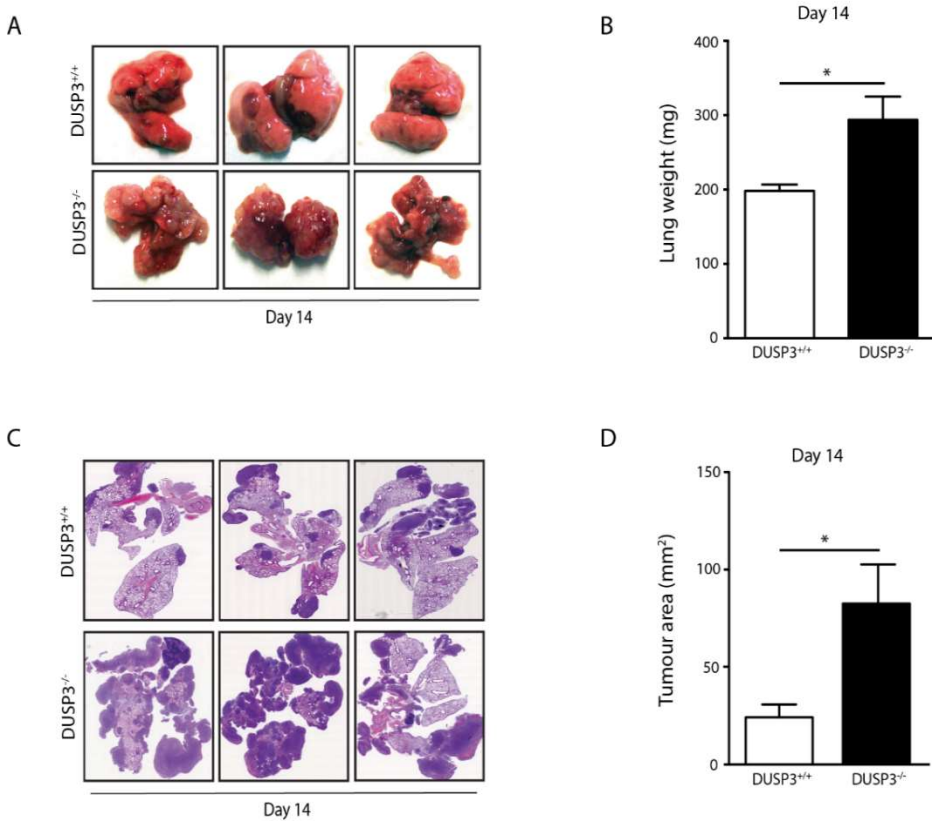

Figure 2.2. DUSP3 deletion accelerates experimental LLC metastasis growth. **(A)** Representative lung macroscopic view. **(B)** Comparison of lung weights from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice**. (C)** Hematoxylin eosin staining of lung sections from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice**. (D)** Comparison of tumour areas from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. *, $p < 0,05$. n= 10 for each experimental group. Results are shown as mean ± SEM.

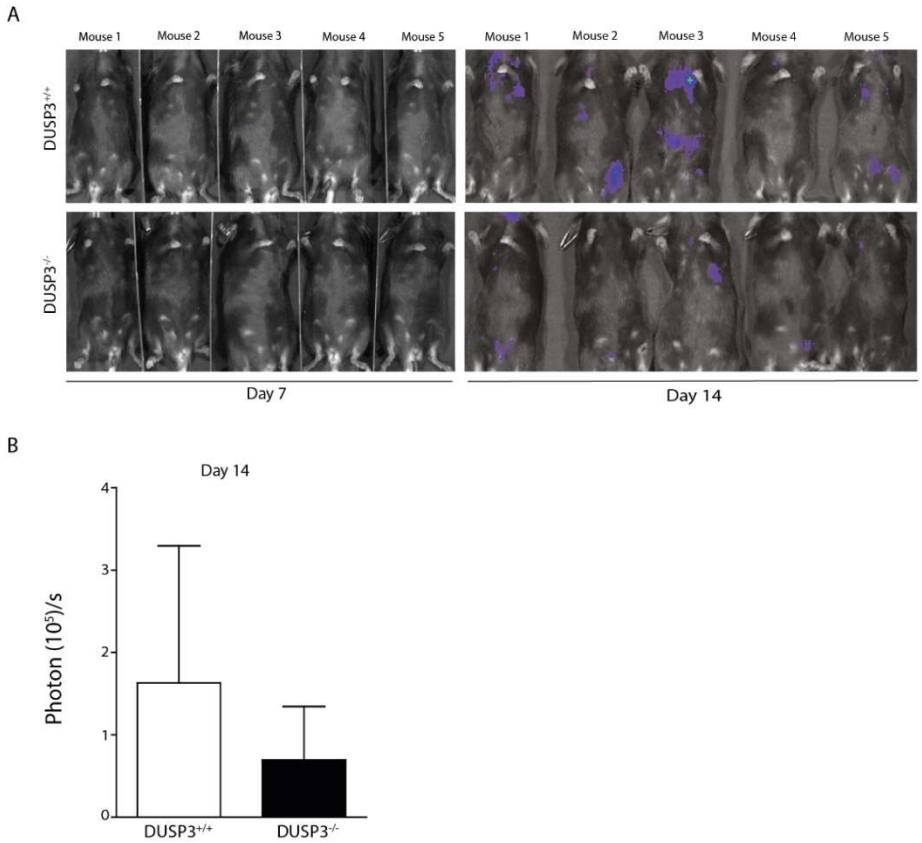

Figure 2.3. DUSP3 deletion does not impact experimental B16 metastasis growth. B16 tumour growths were monitored by xenogen bioluminescence imaging. Tumours were established by i.v. injection of 10^6 B16-Luc+ cells to DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. **(A)** Representative xenogen imaging results at day 7 and day 14 after B16 injection and **(B)** quantitative xenogen bioluminescence imaging data (day 14). n= 10 for each experimental group. Results are shown as mean ± SEM.

LLC metastasis growth in DUSP3$^{-/-}$ mice is driven by myeloid cells

Lung carcinomas are highly infiltrated with heterogeneous myeloid cell populations; among which some display a more tumour-promoting phenotype than others, although their precursors seem to be the same[276]. In a previous study, we showed that DUSP3 deletion in mice was associated with a polarization of macrophages towards the M2-like phenotype[38].

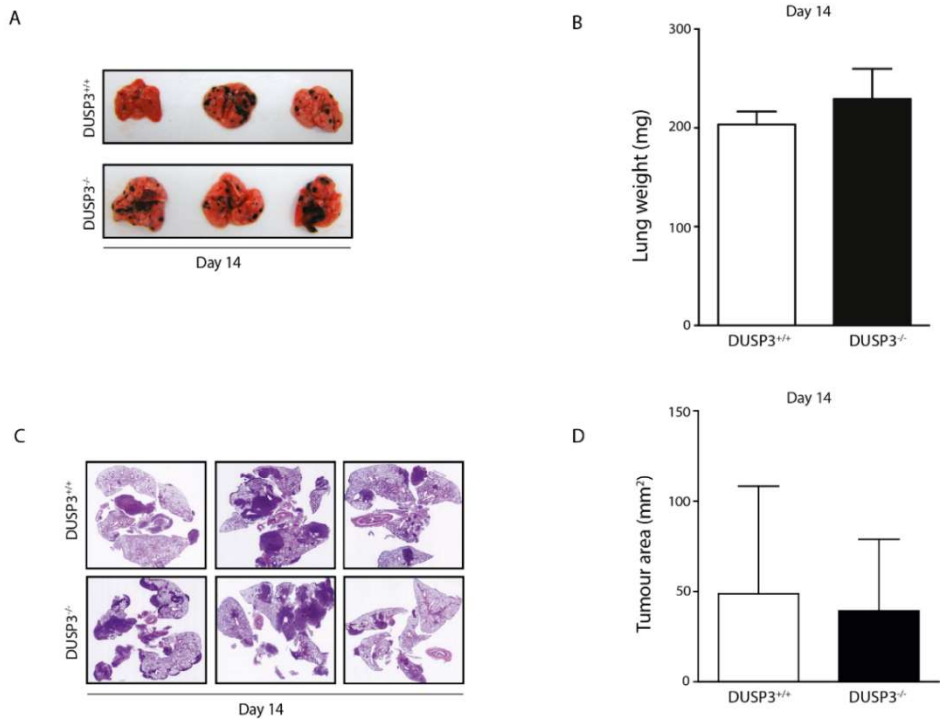

Figure 2.4. DUSP3 deletion does not impact experimental B16 metastasis growth **(A)** Representative lung macroscopic view and **(B)** comparison of lung weights from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice**. (C)** Hematoxylin eosin staining of lung sections from each experimental group**. (D)** Comparison of tumour areas from each group. n= 10 for each experimental group. Results are shown as mean ± SEM.

Since this cell population has been shown to play an important role in tumour promotion, we hypothesized that M2 macrophages might be key actors in the enhanced LLC tumour cell dissemination observed in the LLC experimental model. Since DUSP3 knockout mice were generated using the standard homologous recombination method[23], we first generated chimeric mice by bone marrow (BM) transplantation of bone marrow cells from DUSP3$^{-/-}$-C57BL/6-CD45.2 mice to lethally irradiated DUSP3$^{+/+}$-C57BL/6-CD45.1 mice (DUSP3$^{-/-}$→DUSP3$^{+/+}$ mice). Successful hemato-lymphoid reconstitution was verified by flow cytometry for the recipient mice 3-4 weeks after transplantation (data not shown) and by anti-DUSP3 immunoblot of spleen lysates at the day of sacrifice (Figure 2.5.A). As a control, DUSP3$^{+/+}$-C57BL/6-CD45.1 mice were transplanted with DUSP3$^{+/+}$-C57BL/6-CD45.2 BM cells (DUSP3$^{+/+}$→DUSP3$^{+/+}$ mice). The obtained chimeric mice were then i.v. challenged with 1x10^6 LLC cells. Lung metastasis development was monitored for 2 weeks after tumour cell injection, by measuring luciferase activity of

tumour cells. Interestingly, DUSP3$^{+/+}$ mice adoptively transferred with DUSP3$^{-/-}$ bone marrow cells (DUSP3$^{-/-}$→DUSP3$^{+/+}$ mice) displayed an increased luciferase activity in lungs compared to DUSP3$^{+/+}$→DUSP3$^{+/+}$ transferred mice (Figure 2.5.B and 2.5.C). A significant correlation was measured between DUSP3 protein expression and the LLC bioluminescence (Figure 2.5.D). Moreover lung weight and lung tumour area were significantly higher in DUSP3$^{-/-}$→DUSP3$^{+/+}$ mice compared to DUSP3$^{+/+}$→DUSP3$^{+/+}$ mice (Figure 2.6.A-C) suggesting that DUSP3-deficient hematopoietic cells contribute to enhance LLC tumour aggressiveness.

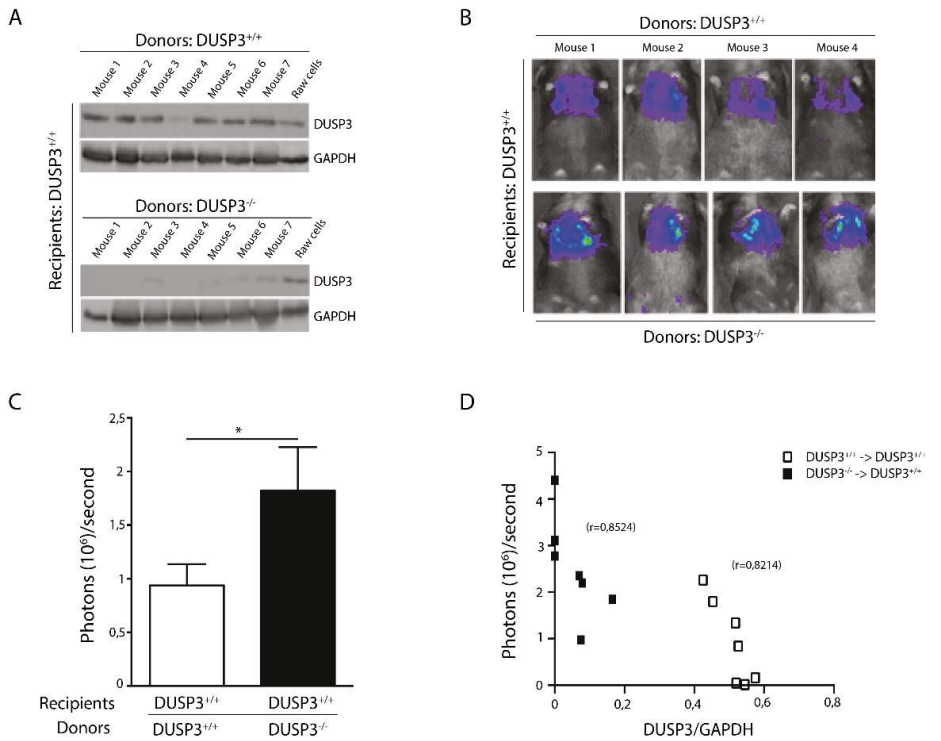

Figure 2.5. DUSP3$^{-/-}$ bone marrow transfer into DUSP3+/+ mice enhances metastasis growth. Tumours were established by injection (i.v.) of 10^6 LLC-Luc+ cells to DUSP3$^{+/+}$ or DUSP3$^{-/-}$ BM-transplanted irradiated DUSP3$^{+/+}$ mice. **(A)** Western blot analysis for DUSP3 expression from spleen lysates of transplanted mice. (**B**) Representative xenogen bioluminescence imaging results. **(C)** Quantitative imaging data (day 14). **(D)** Correlation between size of tumours (quantified as photons/second) and DUSP3 expression. *, $p < 0,05$. n= 10 for each experimental group. Results are shown as mean ± SEM.

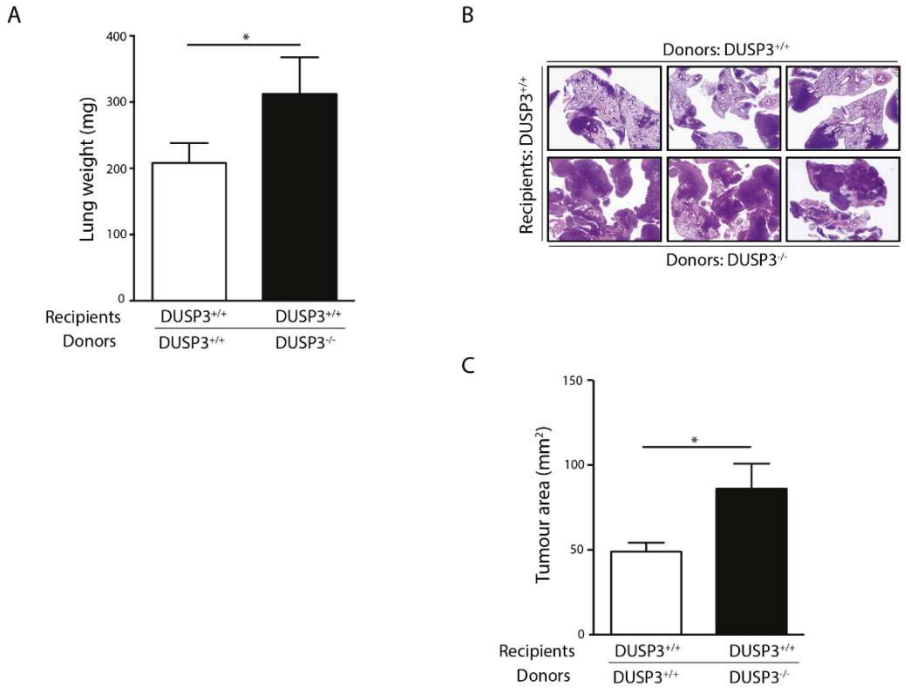

Figure 2.6. DUSP3$^{-/-}$ bone marrow transfer into DUSP3$^{+/+}$ mice enhances metastasis growth **(A)** Comparison of lung weights of DUSP3$^{+/+}$ or DUSP3$^{-/-}$ BM-transplanted irradiated DUSP3$^{+/+}$ mice. **(B)** Hematoxylin-eosin staining of lung section from DUSP3$^{+/+}$ or DUSP3$^{-/-}$ BM-transplanted irradiated DUSP3$^{+/+}$ mice. **(C)** Comparison of tumour areas from DUSP3$^{+/+}$ or DUSP3$^{-/-}$ BM-transplanted irradiated DUSP3$^{+/+}$ mice. *, $p < 0,05$. n= 10 for each experimental group. Results are shown as mean ± SEM.

To further assess which hematopoietic cell populations could be involved in the increased tumour aggressiveness, we analysed, in first instance, by flow cytometry, the myeloid cell subsets present in established DUSP3$^{+/+}$ and DUSP3$^{-/-}$ lung homogenates bearing LLC tumours. The gating strategy is described in the Figure 2.7. Briefly, after gating on CD45^{+} and CD11b^{+} cells to select myeloid cells, neutrophils, eosinophils and alveolar macrophages were discriminated based on Ly6G and Siglec-F surface markers. Macrophages/monocytes were considered Ly6G^{-}siglecF^{-} and with various expression of Ly6C and MHC-II as previously reported[153].

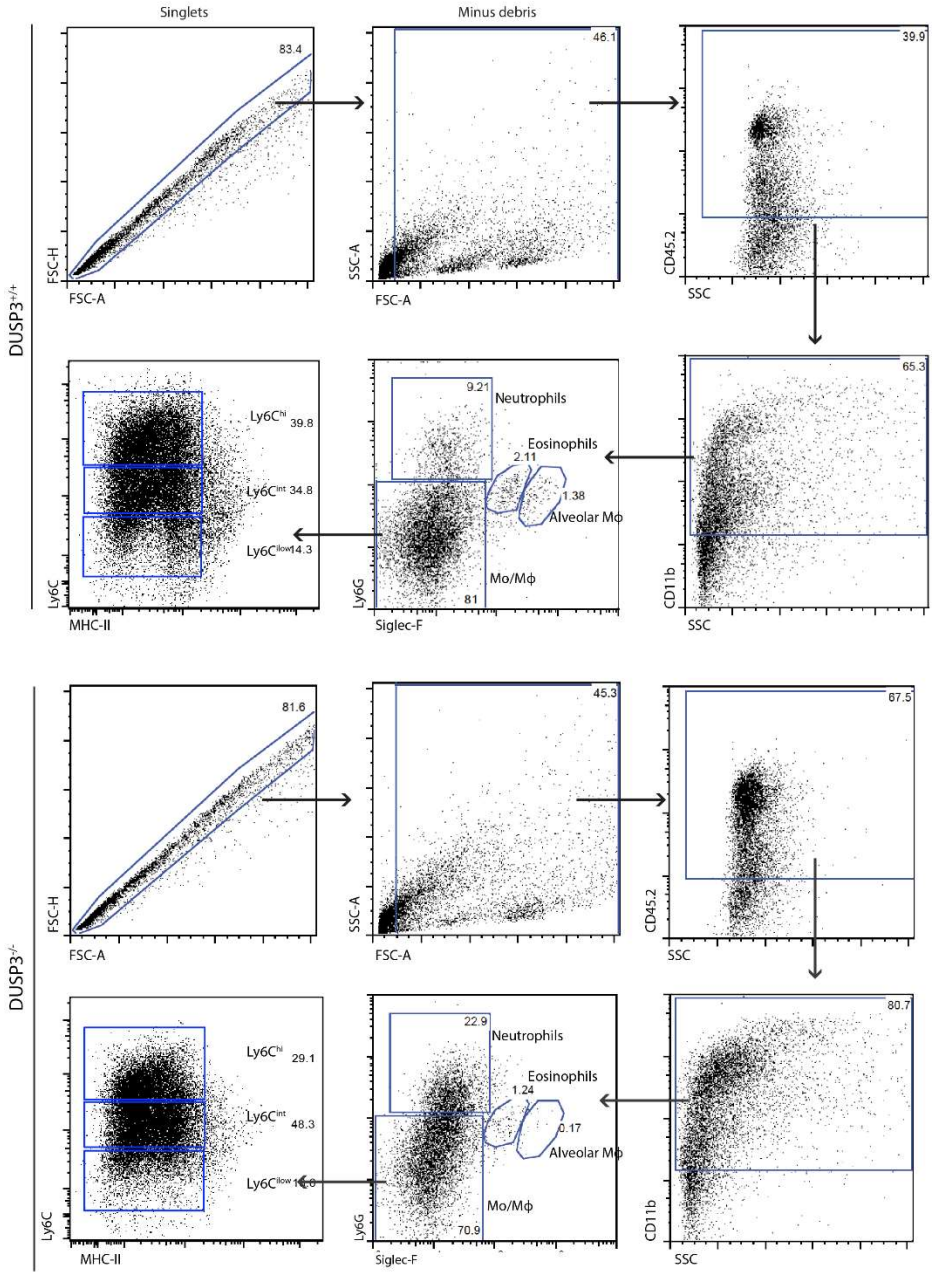

Figure 2.7. Gating strategy used to identify immune cell populations in DUSP3$^{+/+}$ and DUPS3$^{-/-}$ LLC-bearing lungs. Cells were isolated from enzymatically digested mice lungs and, after exclusion of doublets and debris, immune cells were identified by CD45.2 and CD11b staining. A sequential gating strategy was then employed to first identify populations expressing specific markers: alveolar macrophages (alveolar MΦ) (Siglec-F^{+} Ly6G^{-}), eosinophils (Siglec-F^{int} Ly6G^{-}), neutrophils (Ly6G^{+} Siglec-F^{-}). Distinct populations of Ly6G^{-} Siglec-F^{-} monocytes/macrophages (Mo/MΦ) were further identified based on their expression of Ly6C and MHC-II.

The percentage of CD45.2 and CD11b positive cells was higher in DUSP3$^{-/-}$ lung homogenates compared to DUSP3$^{+/+}$ (Figure 2.8.A and 2.8.B).

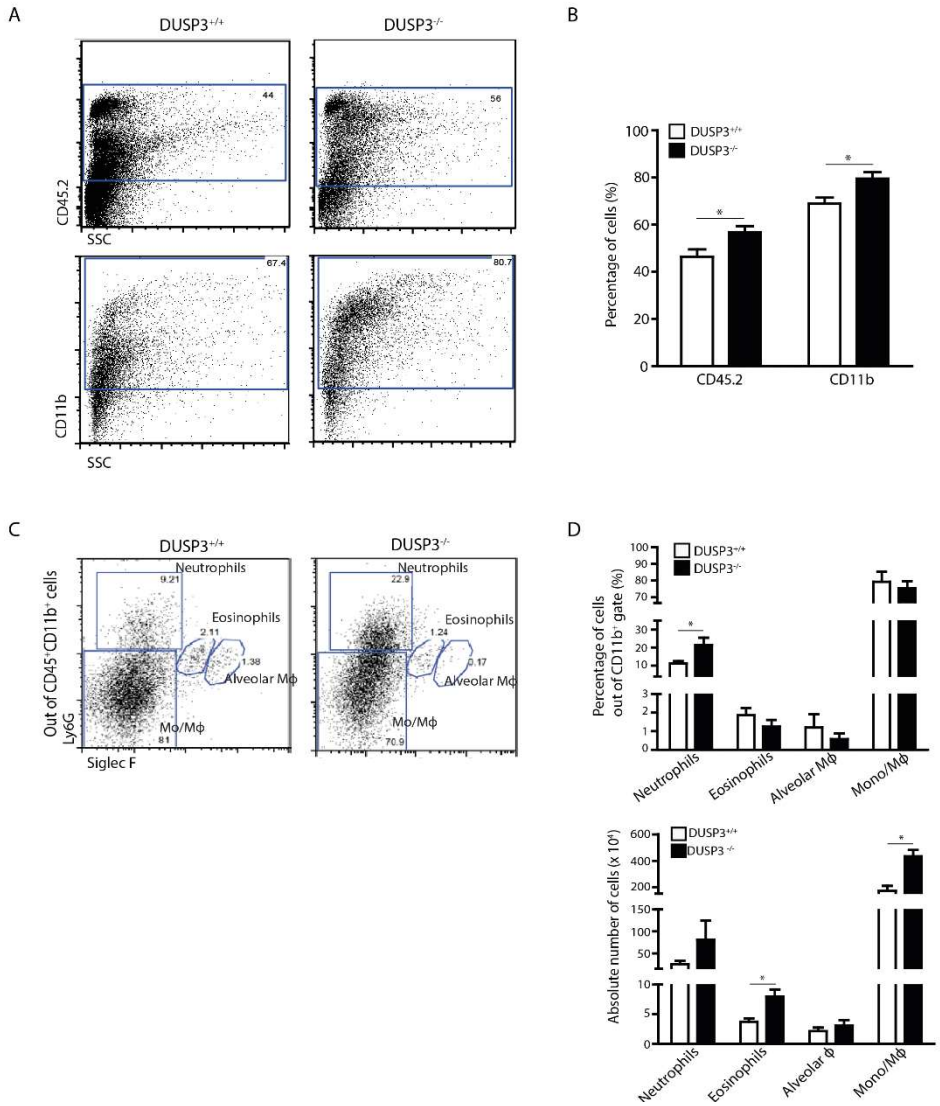

Figure 2.8. LLC metastasis growth in DUSP3$^{-/-}$ mice is driven by myeloid cells. Immune cell population phenotyping in LLC tumour-bearing lungs by flow cytometry. **(A)** Representative gating on CD45.2 and CD11b positive cells. **(B)** Percentage of CD45.2 and CD11b positive cells. **(C)** Representative gating strategy on neutrophils, eosinophils, alveolar macrophages and monocytes/macrophages. **(D)** Percentages of neutrophils, eosinophils, alveolar macrophages and monocytes/macrophages in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ lungs. **(E)** Absolute number of neutrophils, eosinophils, alveolar macrophages and monocytes/macrophages in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ lungs. *, $p < 0,05$. N=5 for each experimental group. Results are shown as mean ± SEM

The percentage of neutrophils was significantly higher in DUSP3$^{-/-}$ lungs compared to DUSP3$^{+/+}$. On the contrary, the percentage of eosinophils, alveolar macrophages and monocytes remained unchanged in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ lungs (Figure 2.8.C and 2.8.D). However, the absolute number of eosinophils and monocytes/macrophages were significantly higher in DUSP3$^{-/-}$ lungs compared to DUSP3$^{+/+}$. The number of neutrophils and alveolar macrophages was slightly but not significantly elevated in DUSP3$^{-/-}$ compared to DUSP3$^{+/+}$ lungs (Figure 2.8.D).

Since DUSP3 deletion in mice was also associated with a macrophage polarization to the M2-like phenotype[38], we further characterized the different monocyte/macrophage subpopulations within LLC-bearing lungs. Based on the expression of the Ly6C surface marker, we could distinguish 3 populations of monocytes/macrophages: Ly6C^{low}, Ly6C^{int} and Ly6C^{hi} (Figure 2.9.A). The percentage of Ly6C^{int} cells was significantly increased in DUSP3$^{-/-}$ LLC-lungs. No difference was observed for Ly6C^{low} macrophages and a slight but not statistically significant decrease of Ly6C^{hi} monocytes was observed in LLC-lung homogenates from DUSP3$^{-/-}$ compared to DUSP3$^{+/+}$ mice (Figure 2.9.B). These data suggest a continuous recruitment of new monocytes/macrophages to the metastatic site. Therefore, we analysed the surface expression of Ly6B on lung monocyte/macrophages populations, which is a well-known marker of newly recruited neutrophils and macrophages[277] (Figure 2.9.C). In DUSP3$^{+/+}$ metastatic lungs, only 10% of macrophages were Ly6B^{hi}, while about 20% and 65% of cells were Ly6B^{int} and Ly6B^{low}, respectively. On the opposite, the percentage of Ly6B^{hi} and Ly6B^{int} macrophages were significantly increased in DUSP3$^{-/-}$ mice with more than 20% and 30% among the MHC-II positive cells, respectively. The DUSP3$^{+/+}$ Ly6B^{low} cells were significantly decreased compared to DUSP3$^{+/+}$ cells (Figure 2.9.D). The expression of F4/80, Ly6C and CD206 on these populations confirmed the identity of M2-like macrophages (Figure 2.9.E). As a control, Ly6B expression was measured on B16-tumour bearing lungs but no difference was found between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ M2-like macrophages (Figure 2.10.A and 2.10.B).

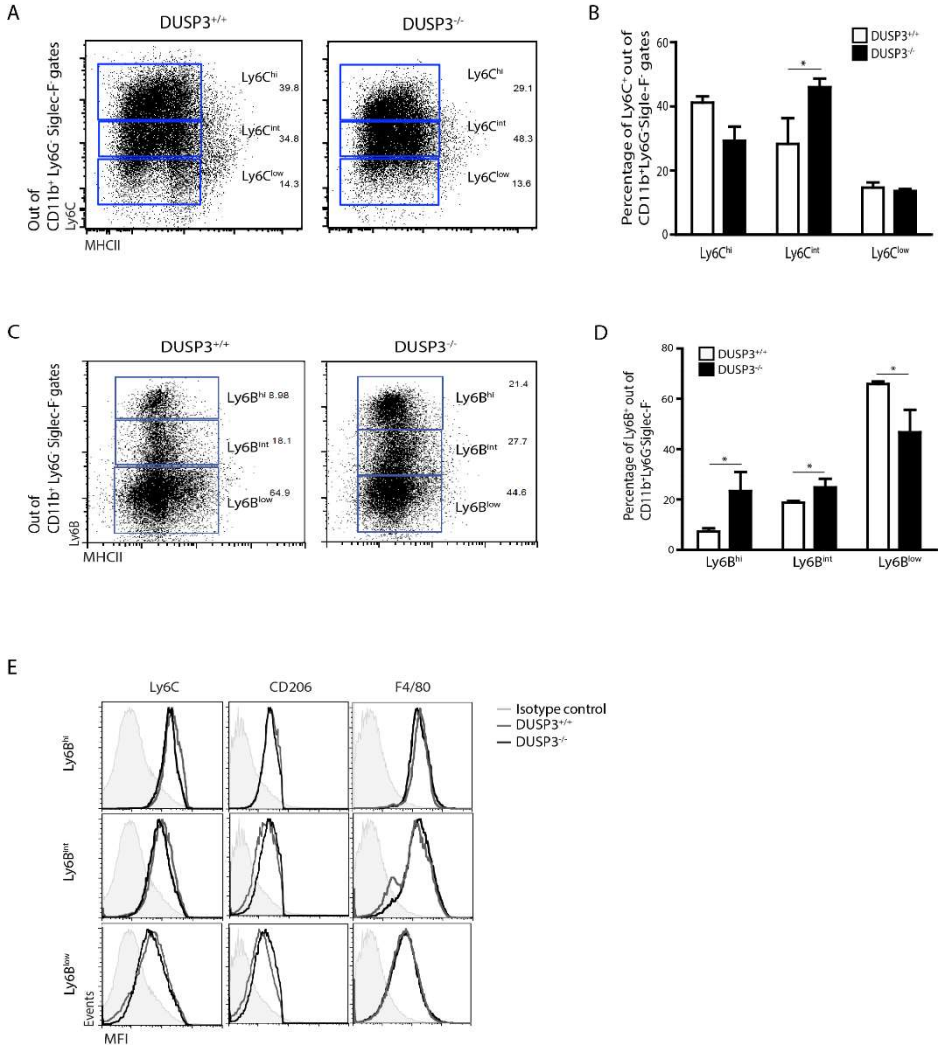

Figure 2.9. Macrophages accelerate LLC lung tumour progression in DUSP3$^{-/-}$ mice. **(A)** Monocytes/macrophages phenotype characterization in LLC tumours: gating strategy and percentage of distinct Ly6C^{+} populations **(B)**. Comparison **(C)** and percentage **(D)** of Ly6B^{hi}, Ly6B^{int} and Ly6B^{low} macrophages in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. **(E)** Expression of specific macrophages markers in distinct Ly6B^{+} populations. Alveolar Φ = alveolar macrophages; Mo/MΦ = monocytes/macrophages. *, $p < 0,05$. n= 5 for each experimental group. Results are shown as mean ± SEM

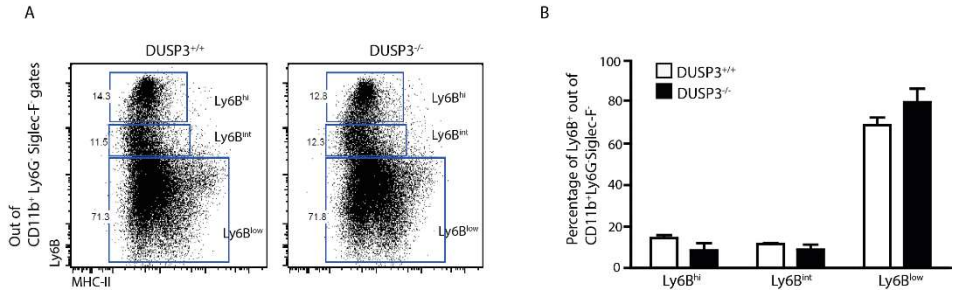

Figure 2.10. Ly6B expression of macrophages from B16-tumour bearing lungs. Comparison **(A)** and percentage **(B)** of Ly6B^{hi}, Ly6B^{int} and Ly6B^{low} macrophages in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. n= 5 for each experimental group. Results are shown as mean ± SEM.

A potential explanation for the higher presence of recently migrated Ly6B^{hi} cells in DUSP3$^{-/-}$ mice could be that soluble factors secreted by LLC enhance the migration of these cells. We therefore performed an *in vitro* migration assay of DUSP3$^{-/-}$ and DUSP3$^{+/+}$ peritoneal macrophages and bone marrow-derived macrophages (BMDM) in response to LLC-conditioned medium (LLC-CM). After 18h migration, both DUSP3 deficient-peritoneal macrophages and BMDMs migrated significantly better in response to LLC-CM compared to DUSP3$^{+/+}$ derived macrophages (Figure 2.11.A and 2.11.B). As a control, we performed the same *in vitro* migration assay in response to B16-conditioned medium (B16-CM). On the contrary to LLC-CM, B16-CM did not influence differently the migration of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages and BMDMs (Figure 2.11.C and 2.11.D).

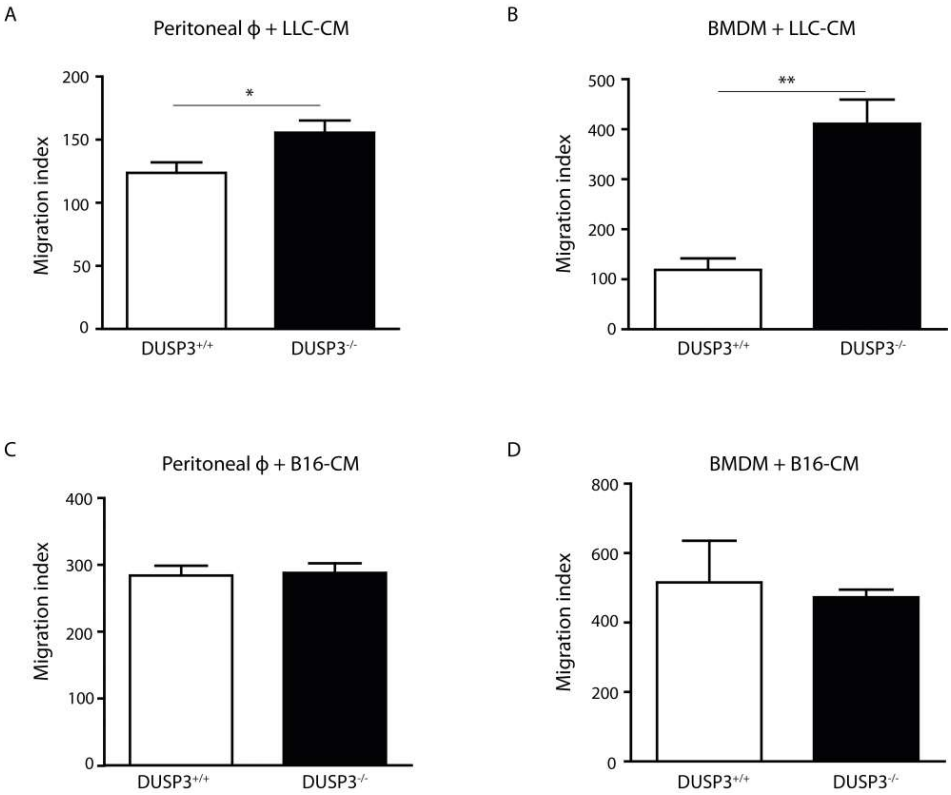

Figure 2.11. DUSP3$^{-/-}$ macrophage migration is enhanced in response to LLC-conditioned medium. *In vitro* migration assay of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages **(A)** and BMDMs **(B)** in presence of LLC-CM. Migration of DUSP3$^{+/+}$ or DUSP3$^{-/-}$ peritoneal macrophages **(C)** and BMDMs **(D)** in presence of B16-CM. BMDM = Bone Marrow-Differentiated Macrophages; CM = conditionned-medium. *, $p < 0,05$ and **, $p < 0.01$. n= 3 for each experimental condition. Results are shown as mean ± SEM.

Macrophage depletion reduces LLC tumour growth in DUSP3$^{-/-}$ mice

Together, the obtained data suggest that myeloid cells, and in particular macrophages, accelerate LLC lung tumour progression by a mechanism involving the phosphatase DUSP3. To confirm this finding, we chemically depleted macrophages in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice, using clodronate-liposomes. Mice were first intravenously and intraperitoneally injected with clodronate-liposomes or with empty-liposomes as control. 48 hours after the first injection of liposomes, mice were intravenously injected with $1x10^6$ LLC cells, after which the intraperitoneal injection of clodronate-liposomes was repeated every other day and up to two weeks after LLC challenge. Finally, the

lung metastasis formation was monitored, using in vivo imaging. The efficiency of macrophage depletion was verified at the day of sacrifice by comparing the presence of residual macrophages in both peritoneal exudates and lung cell suspensions of empty-liposome versus clodronate-liposome injected mice. In peritoneal exudates, CD11b^{+}-F4/80^{+} macrophages completely disappeared after clodronate-liposome injection. In lung homogenates, one population of Ly6B^{hi} macrophages was significantly reduced after clodronate-liposome injection compared to control conditions (Figure 2.12).

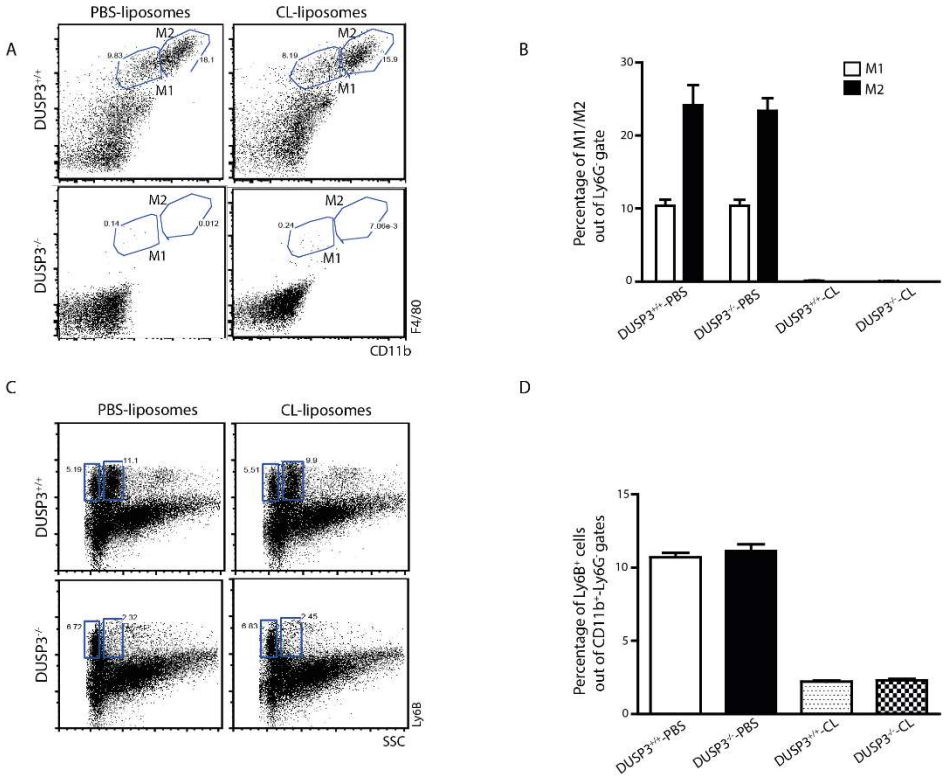

Figure 2.12. Efficiency of specific macrophage depletion using clodronate-liposomes. **(A)** Gating strategy and **(B)** percentages of M1-like and M2-like macrophages of peritoneal cavity for each condition. **(C)** Gating strategy and **(D)** percentage of Ly6B^{+} cells in LLC-bearing lung cell suspension from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. PBS = Empty-liposomes; CL = clodronate liposomes.

Compared to vehicle liposome-treated mice, elimination of macrophages decreased significantly LLC metastatic dissemination in both DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice as demonstrated by the decreased bioluminescence (Figure 2.13.A and 2.13.B), weight (Figure 2.14.A) and tumor area in lungs (Figure 2.14.B and 2.14.C). Interestingly, in the absence of macrophages, no significant difference could be observed in LLC metastasis growth between DUSP3$^{-/-}$ and DUSP3$^{+/+}$ mice (Figure 2.13 and 2.14.). These data indicate that DUSP3-deficiency in monocytes and macrophages accelerates metastatic growth.

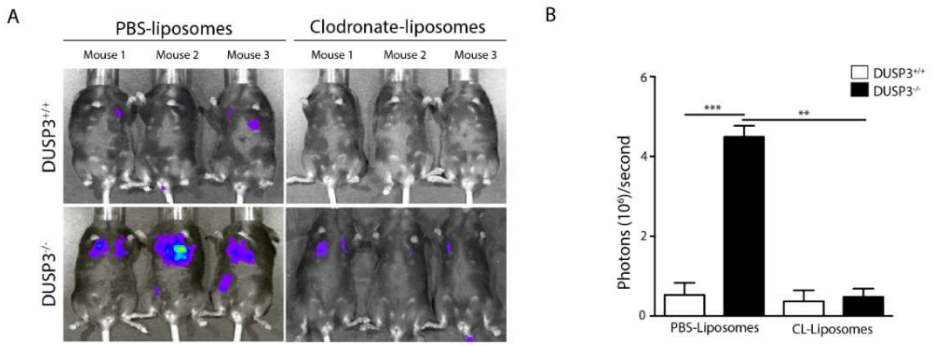

Figure 2.13. Macrophage depletion reduces LLC tumour growth in DUSP3$^{-/-}$ mice. Tumours were established by i.v. injection of 10^6 LLC-Luc+ cells to clodronate-liposomes-depleted mice. **(A)** Representative xenogen imaging results and **(B)** quantification of the xenogen bioluminescence imaging data shown in A at day 14 after LLC injection. **, $p < 0.01$ and ***, $p < 0{,}001$. n= 6 for each experimental group. Results are shown as mean ± SEM.

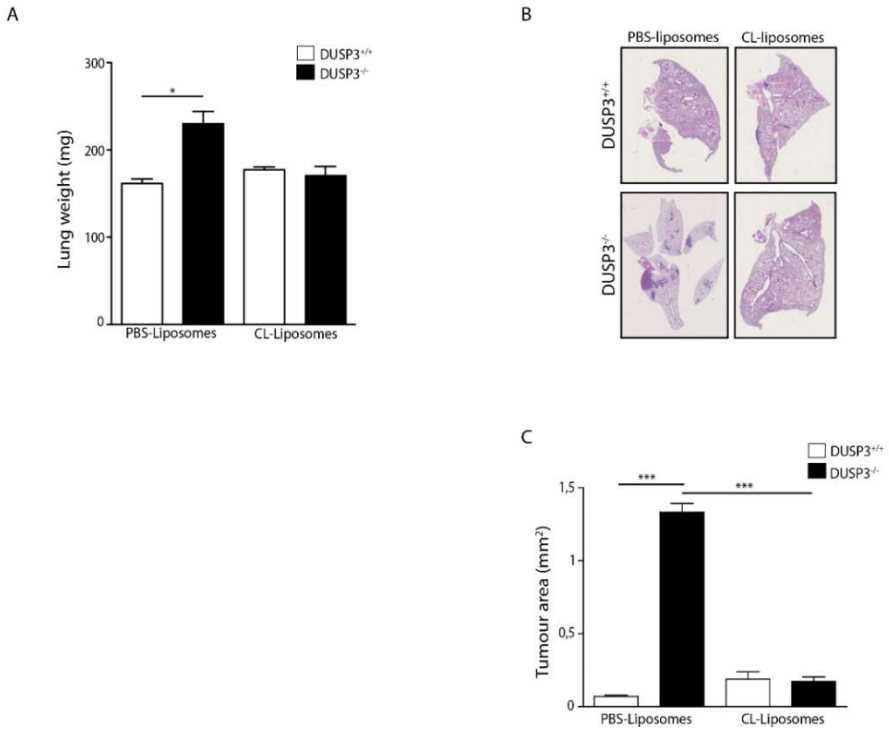

Figure 2.14. Macrophage depletion reduces LLC tumour growth in DUSP3$^{-/-}$ mice. Tumours were established by i.v. injection of 10^6 LLC-Luc+ cells to clodronate-liposomes-depleted mice **(A)** Comparison of lung weights. **(B)** Representative hematoxylin eosin staining of lung sections from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice injected with PBS-lipsomes or clodronate-liposomes**. (C)** Comparison of tumour areas from each group. *, $p < 0,05$; **, $p < 0.01$ and ***, $p < 0,001$. n= 6 for each experimental group. Results are shown as mean ± SEM.

2.5. Discussion and conclusion

Metastasis is the primary cause of death in cancer patients. The mechanisms underlying metastasis development are however not yet completely understood. Several DUSPs have been associated with metastasis formation and their expression/activity correlates with poor clinical outcome[278,279]. Most scientific studies investigating the roles of DUSPs in metastasis formation focused on tumour themselves, while only few studies analysed the roles of DUSPs in the tumour microenvironment[271]. Using a recently developed DUSP3 full-knockout mouse strain and a model of experimental LLC-metastasis, shortcutting primary tumour growth and intravasation processes, we were able to report that DUSP3 deficiency favours LLC-induced M2-like macrophage recruitment at the tumour site, thus enhancing pulmonary metastasis formation. This study highlights a new role for DUSP3 in the susceptibility to develop lung metastasis.

In the present work, we were able to demonstrate that LLC-lung metastasis formation is enhanced in DUSP3$^{-/-}$ mice compared to DUSP3$^{+/+}$ littermates. This phenomenon was however specific to LLC cells since no difference of B16 metastatic dissemination was observed between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. Several hypotheses can be drawn to explain this observation. Indeed, studies have reported that a reduced vascular permeability led to a decrease in metastasis after LLC and B16 i.v. injection[280,281]. Although vascular permeability is significantly enhanced in DUSP3$^{-/-}$ mice when compared with DUSP3$^{+/+}$ mice (unpublished observations), the fact that B16-induced metastasis was not influenced by DUSP3 deletion rules out the possible involvement of vascular permeability in the observed phenotype. The second plausible premise could be that LLC-metastatic cells respond differently to the DUSP3$^{-/-}$ tumour microenvironment. This differential regulation of experimental LLC and B16 metastasis formation has been previously shown in Nrf2-deficient mice. Indeed Nrf2-deficient mice developed lung metastasis faster compared to control mice upon LLC but not B16 cells i.v. injection[282]. In the case of LLC metastasis, tumour formation was associated with a higher recruitment of immune cells (MDSC). The authors concluded that Nrf2 facilitates appropriate immune responses against LLC cells and therefore plays an anti-metastatic role and that the phenotype was restricted to the lung microenvironment[282]. The differential involvement and regulation of immune cells was further confirmed by the fact that the recruitment of

myeloid-derived suppressor cells and dendritic cells are different in LLC and B16 experimental metastasis[283]. In our model, the role of immune cells was clearly demonstrated by the use of bone marrow chimeric mice. The transplantation of DUSP3$^{-/-}$ bone marrow cells into irradiated DUSP3$^{+/+}$ mice was sufficient to transfer the metastatic phenotype since DUSP3$^{-/-}$ -> DUSP3$^{+/+}$ mice developed significantly larger lung metastasis compared to control mice, demonstrating that the hematopoietic compartment is responsible for the increased LLC metastasis in DUSP3$^{-/-}$ mice.

We recently reported that DUSP3 plays an important role in innate immunity and most precisely in macrophages[38]. Tumour-associated macrophages (TAM) display in general M2-like properties, although a high diversity exists[284]. Based on this knowledge, macrophages could play a role in the present LLC metastasis model by creating a tumour-sustaining environment. Specific elimination of macrophages using clodronate-liposomes decreased LLC-metastasis in both DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. More importantly, macrophage depletion abolished the difference of lung tumour growth between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. Moreover, in this experiment, we did not observe any decrease of neutrophil percentage, as verified by flow cytometry staining. This experiment rules out the involvement of neutrophils in the observed phenotype, despite the fact that a higher percentage of these cells was found in LLC-bearing lungs from DUSP3$^{-/-}$ mice. It would be interesting to investigate the significance of such increase and the exact role of neutrophils in LLC-metastasis formation in a DUSP3-dependent manner.

In a subcutaneous model of LLC tumour growth, Mohavedi et al. showed that distinct subsets of monocytes/macrophages existed inside the tumour. They characterized the different monocyte/macrophage populations based on the expression of CD11b, Ly6C and MHC-II surface markers[153]. We performed a similar analysis on tumour-bearing lung cell suspensions of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice. Phenotypically, DUSP3$^{+/+}$ and DUSP3$^{-/-}$ monocytes/macrophages did not differ from each other. Each population equally expressed M1 or M2 markers. However, using the additional marker Ly6B, known to be a marker of newly recruited immune cells[277,285], we found a higher percentage of Ly6B^{hi} M2-like macrophages in DUSP3$^{-/-}$ pulmonary cell suspensions compared to DUSP3$^{+/+}$ control mice. The DUSP3$^{-/-}$ M2-like macrophages are more easily recruited to LLC tumour-conditioned medium

than DUSP3$^{+/+}$ macrophages, possibly explaining the higher number monocytes/macrophages found in DUSP3$^{-/-}$ LLC-bearing lungs. Proliferation assays, using CFSE labelling, performed on BMDMs stimulated with LLC-CM showed no difference between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ BMDMs. This result excludes the idea that the higher number of macrophages found in DUSP3$^{-/-}$ lung homogenates could be due to higher macrophage proliferation and strengthens the involvement of cell migration (Figure 2.15.A and 2.15.B). The enhanced recruitment of DUSP3$^{-/-}$ macrophages is further confirmed by an *in vitro* migration assay. Hence, upon stimulation with LLC-conditioned medium, DUSP3$^{-/-}$ BMDMs migrated faster than DUSP3$^{+/+}$ BMDMs. This effect could not be observed upon stimulation with B16-conditioned medium. These results are in line with *in vivo* observations on B16 lung metastasis. Indeed, the percentage of macrophages was identical in B16-bearing lungs of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice and the percentage of Ly6B^{hi} M2-like macrophages was equal if not lower in DUSP3$^{-/-}$ mice compared to control mice. This further proves that there is a higher infiltration of M2-like macrophages in DUSP3$^{-/-}$ mice after LLC i.v. injection and that they are responsible for the enhanced lung metastasis formation even if they don't influence LLC proliferation or migration (Figure 2.15.C and 2.15.D).

One explanation for the higher recruitment of macrophages under LLC but not B16 influence would be that the profiles of cytokines and chemokines secreted by LLC or B16 differ. For example, tumour cells are the major source of CCL2 in Lewis lung carcinomas while B16 cells express low level of CCL2[286]. Cytokines that are strong monocyte/macrophage chemottractants such as CSF-1, TGF-β or CCL7 may thus also be differentially expressed between those two cell lines, which would explain the differential recruitment of macrophages to the metastatic site of LLC and B16 cells. It is likely that one cytokine or the combination of some cytokines secreted by LLC and not B16 may favour the recruitment of DUSP3$^{-/-}$ M2-like macrophages which would explain the differential recruitment of macrophages to the metastatic site of LLC versus B16 cells. Consequently it would be worthwhile to further investigate the precise signalling pathway implicating DUSP3 in the enhanced LLC tumour cell dissemination to lung tissues and the exact molecular role of DUSP3 in this event

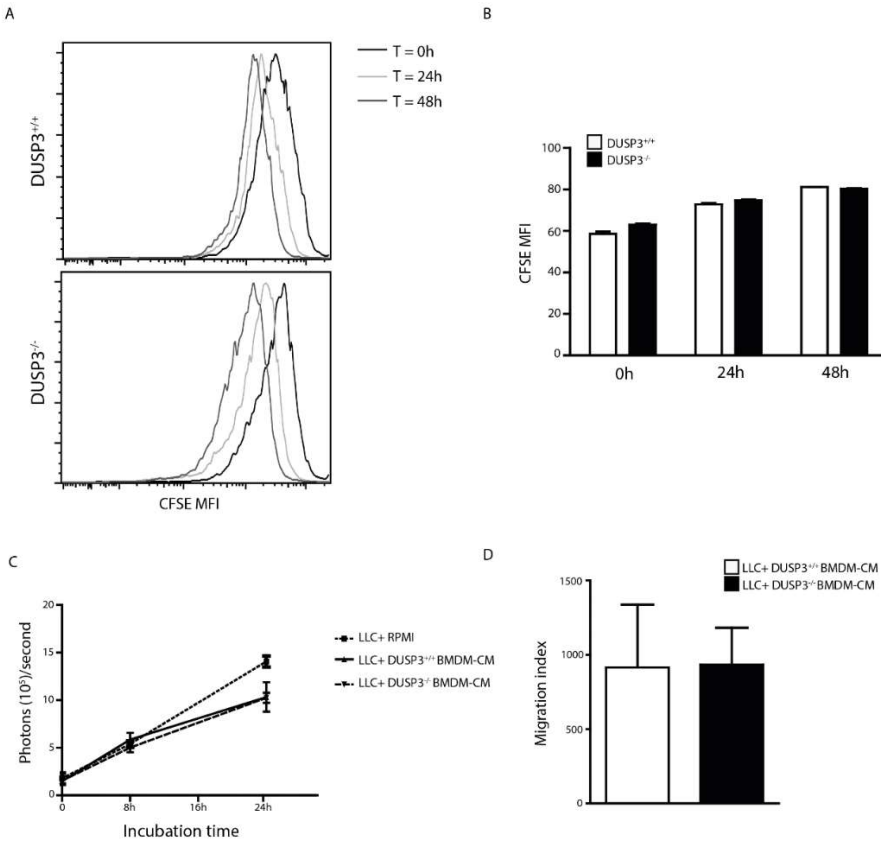

Figure 2.15. Macrophage proliferation, LLC proliferation and migration. Macrophages proliferation in response to LLC-conditioned medium **(A)** CFSE was incorporated into BMDMs from DUSP3$^{-/-}$ and DUSP3$^{+/+}$ mice. Cells were next cultured for 24h and 48h in presence of LLC-conditioned medium. Fluorescence intensity of CFSE was recorded at basal level (T0) and 24h and 48h after addition of the LLC-CM. One mean CFSE fluorescent intensity histogram for each mouse genotype is shown in (**A**). **(B)** Mean fluorescence intensity of CFSE collected from 3 different mice from each genotype. Data are presented as mean ± SEM. **(C)** LLC cells proliferation in presence of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ BMDM-conditioned medium. Quantitative xenogen bioluminescence of LLC at time 0 and at 8, 16 and 24h after incubation with DUSP3$^{+/+}$ and DUSP3$^{-/-}$ conditioned media. Data are presented as mean ± SEM. **(D)** LLC cells migration after incubation for 18 hours in the presence of DUSP3$^{+/+}$ or DUSP3$^{-/-}$ BMDM-conditioned medium. Data were collected from 3 independent experiments and are presented as a mean ± SEM. BMDM = Bone Marrow-Differentiated Macrophages.

Recently, we demonstrated that DUSP3 deficiency prevented neo-angiogenesis and b-FGF-induced microvessel outgrowth. Since angiogenesis has been regarded as essential for tumour growth and metastasis, our presented results seem contradictory to these previous observations. However, studies of many

human tumours suggest that tumours can grow and metastasize without angiogenesis. Indeed, several reports showed evidence for tumour resistance and adaptation to anti-angiogenic therapy leading to more metastasis[287,288]. Moreover, low doses of angiogenesis inhibitors resulted in vessel normalization, well oxygenated tumours and increased effectiveness of chemotherapy[289]. On the other hand, growing evidences indicate that tumour cells may use alternative mechanisms for blood supply. For example, vessel co-option, a procedure of hijacking the blood vessels in surrounding normal tissue, was reported in vascularized tissues such as brain, lung, and liver[290]. Vessel co-option may occur in tumours independently of sprouting angiogenesis and there is increasing evidence supporting the use of this alternative blood supply in metastasis and resistance to anti-angiogenic therapy[291,292]. Although the impact of vessel co-option in our model is still to be demonstrated, we can assume that metastasis in our tumour model is not dependent on angiogenesis.

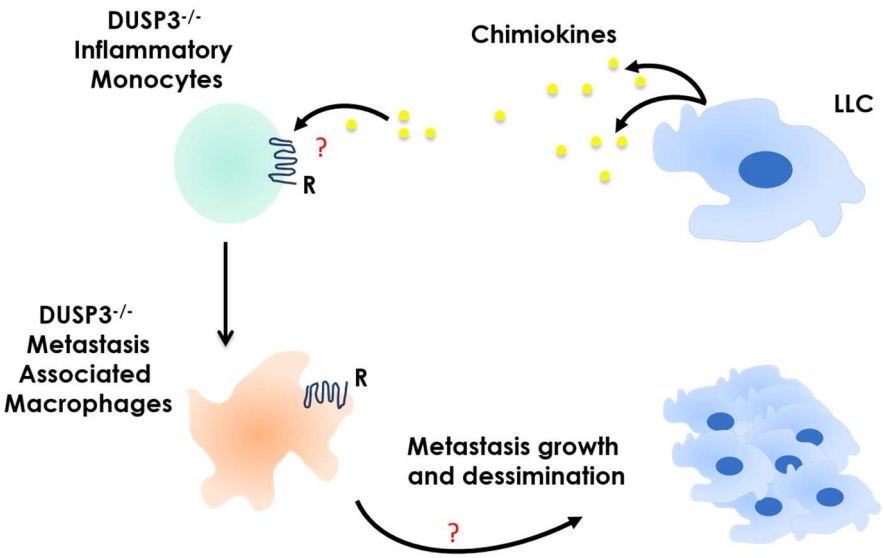

Figure 2.16. DUSP3 acts as an anti-metastatic agent by regulating the migration monocytes/macrophages to the site of metastasis. LLC cells secrete specific chimiokines to recruit DUSP3$^{-/-}$ monocytes. These monocytes differentiate into metastasis-associated macrophages and help the metastasis to grow.

In this study, we provide evidences for an unexpected role of DUSP3 in cancer metastasis. In DUSP3-deficient mice, the enhanced LLC-lung metastasis involves a better recruitment/migration of M2-like macrophages. The presence of M2-like macrophages in turn favours metastasis growth (Figure 2.16). In conclusion, we show that DUSP3 acts as an anti-metastatic agent by regulating the migration monocytes/macrophages to the site of metastasis.

DUSP3-deficient macrophages and septic shock

3. DUSP3 deletion protects female, but not male mice, from LPS-induced endotoxemia and polymicrobial-induced septic shock

3.1. Abstract

DUSP3, is a small dual specificity phosphatase. Only few substrates are known and the physiological role of this phosphatase is poorly known. Using DUSP3-deficient C57/BL6 mice, we recently reported that DUSP3 deficiency confers resistance to endotoxin- and polymicrobial-induced endotoxemia and septic shock. We showed that this protection was macrophage-dependant. In this work, we further investigate the role of DUSP3 in sepsis tolerance and show that the resistance is gender dependent. Using adoptive transfer experiments and ovariectomized mice, we highlighted the role of female sex hormones in the phenotype. Indeed, in ovariectomized and male mice the dominance of M2-like macrophages observed in DUSP3$^{-/-}$ female mice was lost confirming the role of this macrophage subset in sepsis tolerance. In DUSP3$^{-/-}$ female peritoneal macrophages stimulated *ex vivo* by LPS, ERK 1 and 2, PI3K and Akt were hypophosphorylated. Our results demonstrate that mice female hormones modulate M2-like responses during endotoxemia in a DUSP3 dependent manner.

Key words: DUSP3, sepsis, endotoxemia, LPS, female sex hormones, macrophages

3.2. Introduction

Sepsis and septic shock are complex clinical syndromes that arise when the local body response to pathogens becomes systemic and injures its own tissues and organs[177]. When infection occurs, bacterial components such as LPS are recognized by the host and inflammation is initiated. TLR4 pathway is activated and triggers the release of cytokines, chemokines and nitric oxide (NO)[185,196]. Systemic release of pro-inflammatory cytokine causes a large-scale of cellular and tissue injuries, leading to microvascular disruptions, severe organ dysfunctions and eventually to death[181]. Sepsis occurrence and outcome depend on pathogen characteristics but also on risk factor such as age or sex[177]. Indeed, women are better protected against infection and sepsis. Women younger than 50 years show a lower incidence of severe sepsis and a better survival compared to age-matched men. This may be explained by the influence of female sex hormones on the immune system responses[179].

DUSP3, or *Vaccinia*-H1-related (VHR), is an atypical dual specificity phosphatase of 21kDa. The phosphatase contains one catalytic domain but lacks a binding domain[14]. DUSP3 broader catalytic site allows the protein to dephosphorylate both phospho-Tyr and phospho-Thr residues[272]. The MAPK ERK 1/2 and JNK were the first substrates of DUSP3 to be described[17,18,20]. Other substrates such as the tyrosine receptors EGFR and ErbB2[22] and the transcription factor STAT5[21] were also reported. DUSP3 physiological functions started to be elucidated thanks to the knockout mouse we have generated. Studies from our laboratory using DUSP3-KO mice showed that DUSP3 plays an important role in platelets biology, in monocytes and macrophages and in endothelial cells[23,34,38]. In platelets, DUSP3 plays an important role in arterial thrombosis and platelet activation through GPVI and CLEC-2 signalling[34]. DUSP3 plays also an important role in endothelial cells and angiogenesis and seems to play a pro-angiogenic function[271]. Surprisingly, this function was not correlated with reduced tumour or metastatic growth. In an experimental metastasis model using Lewis lung carcinoma cells (LLC), we found that DUSP3 plays a rather anti-tumour role since DUSP3-KO mice were more sensitive to LLC metastatic growth when compared to WT littermates. This enhanced tumour growth in DUSP3$^{-/-}$ mice was associated with higher recruitment of M2-like macrophages (Vandereyken et al, under revision). Previous studies from our laboratory and others showed that DUSP3 was downregulated in some human cancers and upregulated in others (reviewed in[26,271]). Further

studies are required to better understand the role of this phosphatase in cancer biology.

DUSP3 plays also an important role in immune cell functions. In T cells, DUSP3 is activated by the kinase ZAP-70 after TCR triggering. This activation, through tyrosine phosphorylation of DUSP3 residue Tyr138, allows targeting of the MAPK ERK1/2 and the activation of its downstream signalling pathway[27]. Moreover, in Jurkat leukemia T cells, DUSP3 targets ERK and JNK, but not p38. Together, these data suggest that DUSP3 controls T cell physiological functions at least partially through the MAPKs ERK and JNK[20]. In innate immune cells, we recently showed that DUSP3 is the most highly expressed atypical DUSPs in monocytes. This was also true in mice[38]. These finding suggested to us that DUSP3 could play an important role in innate immune responses. Indeed, using DUSP3-KO mice, we found that DUSP3 deletion conferred resistance of female mice to LPS-induced endotoxemia and to polymicrobial infection-induced septic shock. This protection was macrophage dependent since a higher percentage of M2-like macrophage subsets was found in DUSP3$^{-/-}$ mice. Moreover, the resistance was also associated with a hypo-phosphorylation of the serine/threonine kinases ERK1 and ERK2 and a subsequent decreased in TNF-α production[38].

In this study, we report that DUSP3 deletion does not protect male mice from LPS-induced endotoxemia and CLP-induced septic shock and that this protection is female sex hormone dependent. Furthermore, we report that sepsis resistance was associated with a higher percentage of M2-like macrophages in peritoneal cavity of DUSP3$^{-/-}$ female mice but not with a decreased of pro-inflammatory cytokines production. We also showed that sepsis resistance in females but not in males nor in ovariectomized females was associated with a decrease in ERK1/2 and PI3K and Akt activation.

3.3. Materials and methods

Mice and ethic statement

C57BL/6 (CD45.2)-DUSP3$^{-/-}$ mice were generated by homologous recombination as previously reported[23]. These mice were backcrossed with C57BL/6-CD45.2 mice (Charles River) to generate heterozygotes that were mated to generate DUSP3$^{+/+}$ and DUSP3$^{-/-}$ littermate colonies used for experimentation. Age matched male and female DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice were used in all the experiments. Mice were kept in ventilated cages under 12-hours dark/12-hours light cycle in an SPF animal facility and received food and water and libitum. Health status was evaluated every 3 months and mice were always found free of specific pathogens.

All mouse experiments and procedures were approved by the animal ethics committees of the Universities of Ghent and Liege and were carried out according to their guidelines.

Cecal ligation and puncture and in vivo challenge with LPS

Cecal ligation and puncture (CLP) was performed as previously described[293]. For LPS challenge, mice were i.p. injected with LPS (6mg/kg). Body temperature was monitored using a rectal thermometer at various times after LPS injection and after CLP. Death of mice was recorded and the data were analysed for statistical significance of differences between groups.

Mice irradiation and bone marrow transplantation

CD45.2 donor mice of 10–12 weeks old were killed by cervical dislocation. Tibia and fibula were collected and BM cells were flushed with PBS. BM cells ($10x10^6$) were immediately i.v. injected to CD45.1 lethally irradiated (866, 3cGy) 6-8 weeks old recipient mice. 4 weeks later, transplantation efficiency was evaluated on the basis of the ratio of CD45.2 to CD45.1 cells in the blood of transplanted mice.

Female ovariectomy

4 weeks old females were anesthetized using ketamine/xylazine (150mg/kg and 20mg/kg). A vertical incision of 2-3cm was performed in the middle of the back. 1cm lateral of the midline, another incision of 2-3mm was performed in the fascia. Adipose tissue surrounding ovary was pulled out and ovary was

removed after clamping. The same operation was realized for contralateral ovary. The incision in fascia was closed with stitches and the skin incision with clips. Shame operated mice were used as a control. All above procedures were applied to these mice except the removal of ovaries.

Antibodies and reagents

The following materials were from Cell Signalling Technology Inc. anti-phosho-AKT (Ser473), anti-AKT, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK, anti-phospho-PI3K p85 (Tyr458)/p55 (Tyr199), anti-PI3K p85, anti-phospho-GSK3α/β (Ser21/9) Anti-GSK3α/β was from Santa Cruz. Anti-GAPDH antibody was from Sigma. HRP-conjugated anti-goat antibody was from Dako. HRP-conjugated anti-mouse antibody was from GE healthcare. HRP-conjugated anti-rabbit antibody was from Merck Millipore. APC-anti-CD45.1 (A20) and PerCp-Cy5.5-anti-CD45.2 (104) were from BD Biosciences. APC–anti-F4/80 and PerCP–Cy5.5–anti-CD11b were from eBiosciences. LPS from Escherichia coli serotype O111:B4 was from Sigma and was diluted in pyrogen-free PBS.

Animal blood sampling and plasma preparation

Peripheral blood was drawn in EDTA-coated tubes (BD Microtainer K2E tubes; BD Biosciences) by puncturing the heart with 26G needle. Centrifugation was performed twice at 800g for 15 min at RT. Plasma samples were separated in sterile Eppendorf tubes, aliquoted in small volumes, and stored at -80°C until used.

Meso Scale Discovery electrochemiluminescence assay

MSD assay was performed according to manufacturer's instructions (Mesoscale Discovery). Briefly, plasmas were diluted 15 and 15000 times for TNF-α and IL-6 respectively. Samples were loaded on the 96 well plates, incubated 2h at RT and washed. Detection antibodies were added for 2h at RT. Signal detection was measured within 15 minutes after read buffer addition using MSD instrument.

Isolation and stimulation of thioglycollate elicited peritoneal macrophages

Peritoneal washes were performed 4 days after intraperiteonal injection of 1mL of 4% thioglycollate broth (Sigma). 5 mL of PBS-EDTA 0.6 mM were

injected twice in the peritoneal cavity using an 18G needle and then collected. Peritoneal macrophages were selected by adherence to tissue culture plastic dishes in complete RPMI 1640 medium. Peritoneal macrophages were stimulated with LPS 1µg/mL during 15, 30 or 60 minutes.

Phenotyping and flow cytometry.

Peritoneal washes were centrifuged 10 min at 350g and the pellet was resuspended in PBS. For surface cell staining, cells were incubated for 15 min with anti-CD16/CD32 (Fcγ III/IIR) before labelling for 30 min with specific antibodies for 30 min at 4°C. Cells were then washed and fixed with 1% paraformaldehyde solution. Cells were next analysed on FACSCanto II (Becton Dickson) using FlowJo (Tree Star).

Protein extraction and Western blot

For Western blot experiments, cells were stimulated for the indicated time points and lysed using RIPA buffer (50 mMTris-HCl (pH = 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM orthovanadate, complete protease inhibitor cocktail tablets EDTA free and 1 mM phenylmethylsulfonyl fluoride) on ice during 20 min. Lysates were next clarified by centrifugation at 19000g during 20 min at 4°C. The resulting supernatants were collected and protein concentrations were determined using the colorimetric Bradford reagent (Bio-Rad). Proteins were next denatured at 95°C in Laemmli buffer (40% glycerol ; 8% SDS 5% ; 20% B-mercaptoethanol ; 20% Tris-HCl 0.5 M pH6,8 ; 0.05% bromophenol blue and water) during 5 min.

Denatured samples were run on 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. To block the non-specific binding sites, membranes were incubated for one hour at room temperature in Tris-buffered saline-Tween 20 containing 5% of non-fat milk or 3% BSA (bovine serum albumin). Membranes were incubated overnight with primary antibody at 4°C. Membranes were next washed thrice in Tris-buffered saline-Tween and incubated with HRP-conjugated secondary antibody during one hour at room temperature. The blots were developed by enhanced chemiluminescence (ECL kit, Amersham) according to the manufacturer's instructions.

Statistical analysis

The student t-test was used to assess statistical differences between different groups. Survival differences after LPS challenge and CLP were analysed by Kaplan-Meier analysis with log rank test. Results were considered as significant if p-value < 0.05. Results are presented as mean $\pm$ SEM. Prism software (GraphPad) was used to perform statistical analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.4. Results

DUSP3$^{-/-}$ female, but not male, mice are resistant to LPS-induced endotoxemia and to CLP-induced septic shock

In a previous study, we showed that DUSP3 deletion protected mice from LPS-induced endotoxemia and polymicrobial-induced septic shock[38]. Only females were used in the first study. To investigate whether the protection observed is a general feature of DUSP3 deletion or restricted to a specific gender, we challenged DUSP3-KO males with a lethal dose of LPS (i.p. injection of 6 mg/kg) and compared survival of DUSP3$^{-/-}$ male to female mice and to WT littermate controls. Body temperature was also monitored. As expected and previously reported, 90% of DUSP3$^{-/-}$ female mice were resistant to LPS while only 5% of DUSP3$^{+/+}$ female mice survived the challenge[38].

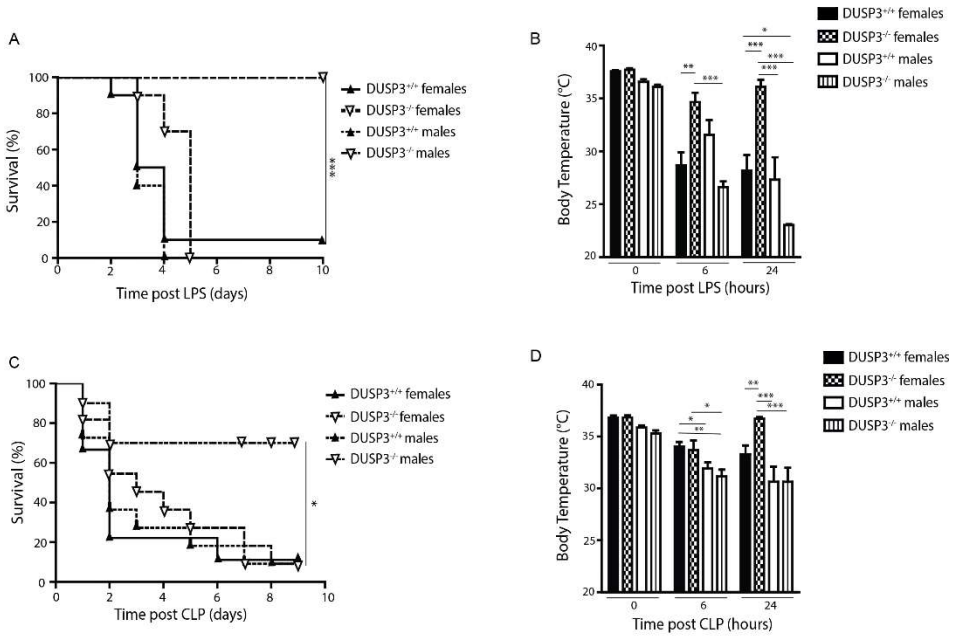

Figure 3.1. DUSP3$^{-/-}$ female mice, but not male mice, are resistant to LPS- and CLP-induced septic shock. **(A)** DUSP3$^{+/+}$ male (n = 12) and female (n = 17), DUSP3$^{-/-}$ male (n = 13) and female (n = 19) mice were i.p injected with 6 mg/kg of LPS. Percent survival was assessed twice a day for 10 days. **(B)** Body temperature of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice before, 6 h, and 24 h after LPS injection. **(C)** DUSP3$^{+/+}$ male (n = 10) and female (n = 11) and DUSP3$^{-/-}$ male (n = 9) and female (n = 11) mice were subjected to CLP (one puncture with 21-gauge needle). Survival was documented twice a day for 7 days. Survival data were compared using Kaplan–Meir with log-rank test. **(D)** Body temperature of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice before, 6 h, and 24 h after CLP. *p < 0.05, ***p <0,001.

Interestingly, DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice were equally sensitive to LPS-induced death (Figure 3.1.A). Body temperature of all groups of mice, but not DUSP3$^{-/-}$ females, decreased after LPS injection. 24h later, almost all DUSP3$^{-/-}$ females recovered while the other groups remained hypothermic (Figure 3.1.B). These results were further confirmed in the cecal ligation and puncture (CLP) model performed on DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male and female mice. As expected, only 10% of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice and DUSP3$^{+/+}$ female mice were still alive by the end of the experiment whereas 70% of DUSP3$^{-/-}$ female mice survived (Figure 3.1.C.). The body temperature of each group dropped after surgery and only DUSP3$^{-/-}$ female mice recovered (Figure 3.1.D). These results indicate a gender specific response to septic shock in DUSP3$^{-/-}$ mice.

DUSP3 deletion does not impact the percentage of M2-like macrophages nor TNF and IL-6 level in male mice after LPS-induced endotoxemia

We previously reported that increased survival of DUSP3$^{-/-}$ female mice after LPS and CLP was associated with a higher percentage of M2-like macrophages in the peritoneal cavity of these mice compared to DUSP3$^{+/+}$ female mice and with a subsequent decrease of the pro-inflammatory cytokine TNF[38]. We phenotyped DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages from male and female mice challenged with LPS based on the characterization previously reported by Ghosn et al[294]. M1 macrophages are F4/80^{int}CD11b^{int}Ly6G^{-}CD11c^{-} MHCII^{+}CCR2^{+}, whereas M2-like macrophages are F4/80^{hi}CD11b^{hi}Ly6G^{-}CD11c^{-} MHCII^{low}CCR2^{+} (Figure 3.2.A and data not shown). We confirmed previous findings showing that the percentage of M2-like macrophages was higher in the peritoneal cavity of DUSP3$^{-/-}$ female mice compared to littermate controls 2h and 24h after LPS injection (Figure 3.2.A and 3.2.B). Interestingly, we observed that the percentage of M2-like macrophages in male mice was slightly lower compared to DUSP3$^{-/-}$ female mice 2h after LPS challenge. This difference was exacerbated at 24h after LPS injection. There was not significant difference for the percentage of M2-like macrophages between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice. Similarly, there was no difference in the percentage of M1-like macrophages at 2h and 24h after LPS injection between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ female mice. However we noticed a slight increase in the percentage of M1-like macrophages in males compared to female mice 2h

after LPS injection. This difference was accentuated, though not significant, at 24h (Figure 3.2.A and 3.2.B).

We previously reported that DUSP3$^{-/-}$ female survival to LPS was associated with decreased systemic TNF level compared to DUSP3$^{+/+}$ mice[38]. Therefore, we wanted to know whether the susceptibility of DUSP3$^{-/-}$ males to LPS-induced death could be linked to differential expression of TNF and/or IL6. We measured and compared plasma levels of these two pro-inflammatory cytokines after LPS challenge in all group of mice, using MSD assay. For TNF, there was no difference between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ males. However and as previously reported[38] there was a significantly decrease of this cytokine in DUSP3$^{-/-}$ females compared to DUSP3$^{+/+}$ female mice 2h after LPS challenge (Figure 3.2.C). Compared to DUSP3$^{+/+}$ mice, DUSP3$^{-/-}$ mice of both genders had a slight, but not significant decrease of IL6 2h after LPS injection (Figure 3.2.D).

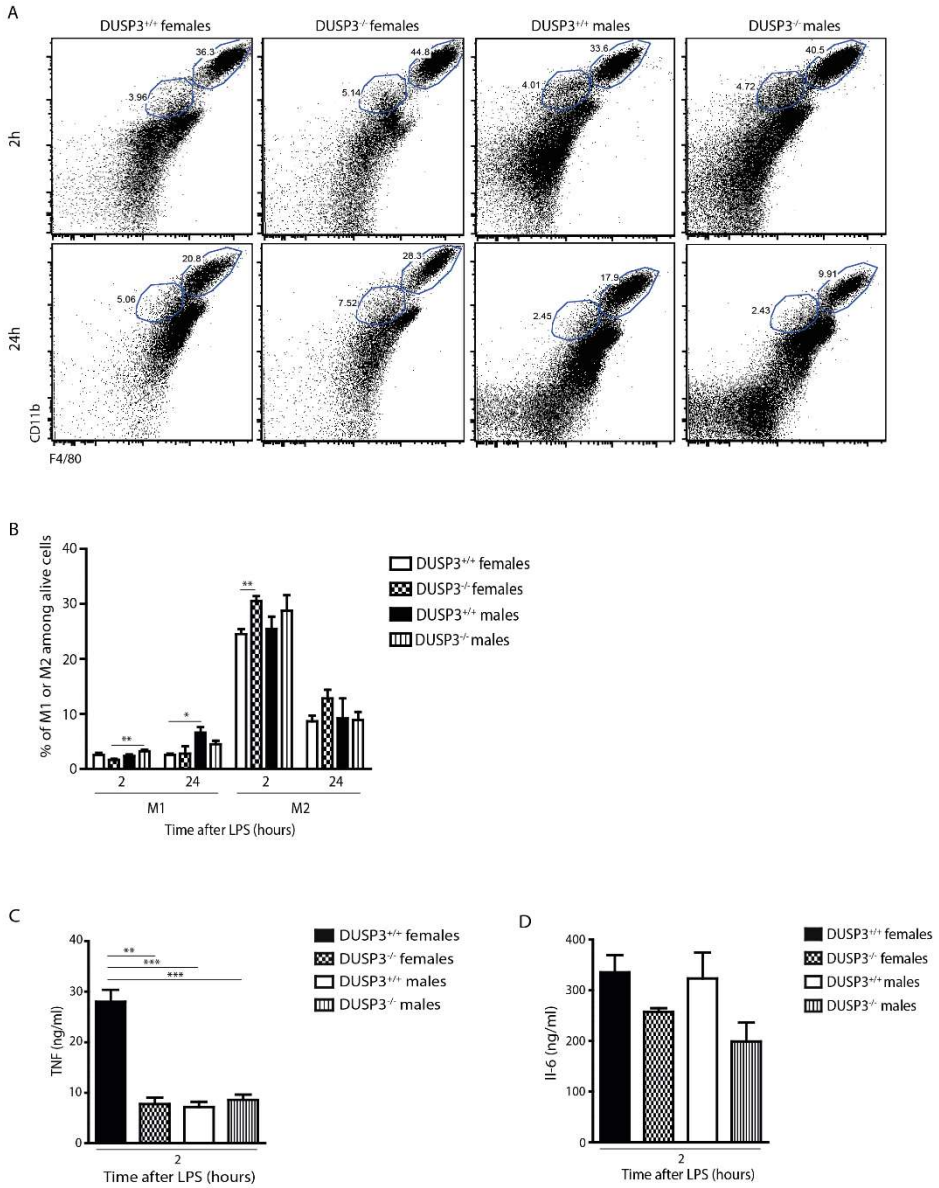

Figure 3.2. The percentage of M2-like macrophages is decreased in male mice after LPS-induced endotoxemia. **(A)** Peritoneal cells harvested from LPS-challenged DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male and female mice were analyzed by flow cytometry for CD11b and F4/80 to discriminate between M1-like macrophages (F4/80^{int}CD11b^{int}) and M2-like macrophages (F4/80^{high}CD11b^{high}). **(B)** Percentage of M1-like and M2-like macrophages out of total live cells. TNF **(C)** and IL-6 **(D)** concentrations from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male and female after LPS challenge (6mg/ml) were determined using MSD assays. Plasmas were collected 2h after LPS challenge. Results are presented as mean ± SEM. n = 5 mice per group/time point. *p < 0.05, **p < 0.01, ***p <0,001

DUSP3$^{-/-}$ female bone marrow cells rescue DUSP3$^{+/+}$ female, but not male mice from LPS-induced lethality

The previous data suggest an involvement of M2-like macrophages in the gender specific response to LPS-induced lethality in DUSP3$^{-/-}$ mice. To study the role of DUSP3$^{-/-}$ myeloid cell population in the observed phenotype, we created chimeric mice by adoptive transfer experiment. 10x10^6 bone marrow cells (BM) from DUSP3$^{-/-}$ C57BL/6-CD45.2 female mice were intravenously injected into lethally irradiated DUSP3$^{+/+}$ C57BL/6-CD45.1 recipient male and female mice (DUSP3$^{-/-}$->M-DUSP3$^{+/+}$ and DUSP3$^{-/-}$->F-DUSP$^{+/+}$, respectively). As a control, DUSP3$^{+/+}$ female BM were transplanted into lethally irradiated DUSP3$^{+/+}$ male or female mice (DUSP3$^{+/+}$->M-DUSP3$^{+/+}$ and DUSP3$^{+/+}$->F-DUSP3$^{+/+}$, respectively).

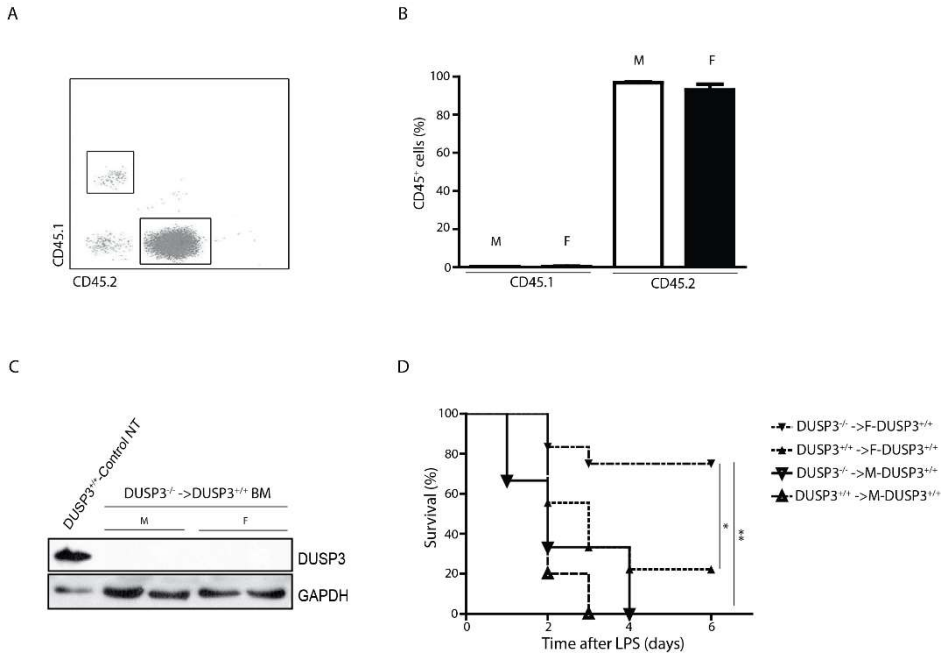

Figure 3.3. DUSP3$^{-/-}$ female bone marrow cells rescue DUSP3$^{+/+}$ female, but not male mice from LPS-induced lethality. **(A)** Representative dot plot of CD45.1 and CD45.2 immune cells in BM transplanted mice. **(B)** Percentage of CD45.1 and CD45.2 immune cells in all transplanted mice. Data are presented as mean ± SEM. n = 10 for each experimental group. **(C)** Western blot was performed on peritoneal cells from transplanted mice using anti-DUSP3 antibody. Anti-GAPDH was used as a loading control. **(D)** Transplanted mice survival after LPS i.p. injection (6mg/ml). Percent survival was assessed twice a day for 10 days. Survival data were compared using Kaplan–Meir with log-rank test. n = 10 in each experimental group. ***p <0,001

Successful hemato-lymphoid reconstitution was verified by flow cytometry 3 to 4 weeks after the transplantation. 95% of peripheral blood cells were CD45.2 positive (Figure 3.3.A and 3.3.B). Moreover, in recipient mice, the expression of DUSP3 in peritoneal macrophages was completely abolished in the recipient mice transplanted with DUSP3$^{-/-}$ BM cell suspension, as showed by DUSP3 immunoblotting (Figure 3.3.C). 4 weeks after BM transplantation, 6 mg/kg of LPS were i.p. injected into recipient mice and survival was monitored during 8 days (Figure 3.3.D). Interestingly, more than 70% of the chimeric DUSP3$^{-/-}$->F-DUSP3$^{+/+}$ mice survived up to the end of the experiment compared to 9% of DUSP3$^{+/+}$->F-DUSP3$^{+/+}$ mice. On the other hand, all DUSP3$^{-/-}$->M-DUSP3$^{+/+}$ and DUSP3$^{+/+}$->M-DUSP3$^{+/+}$ mice died within 4 days after LPS injection (Figure 3.3.D). These data suggest that female sex hormones are required for LPS-induced septic shock survival.

Ovariectomized DUSP3$^{-/-}$ mice are sensitive to LPS-induced death

Male and female sex hormone receptors have been identified on immune cells suggesting direct effects of androgen and oestrogen on these cells[295]. Sexual steroid hormones have been recognized to influence numerous immune pathophysiological processes[296]. To elucidate the effect of female hormones, we ovariectomized 4 weeks old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice (OVX mice). As controls, another group of 4 weeks old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ were sham operated. To assess the ovariectomy's efficiency, we checked the presence and the size of the uterus. Successful ovariectomized mice were deprived of normal uterus development whereas sham operated mice presented a normally developed uterus (Figure 3.4.C). 6 weeks after surgery, sham and OVX mice were challenge with 6mg/kg of LPS and survival and temperature were monitored (Figure 3.4.A and 3.4.B). Ovariectomy impaired the observed endotoxemia resistance of DUSP3$^{-/-}$ mice, whereas sham operated DUSP3$^{-/-}$ mice were still fully protected from endotoxin-induced death. These results demonstrate that female sex hormones are involved in the resistance of DUSP3$^{-/-}$ female mice to LPS-induced lethality.

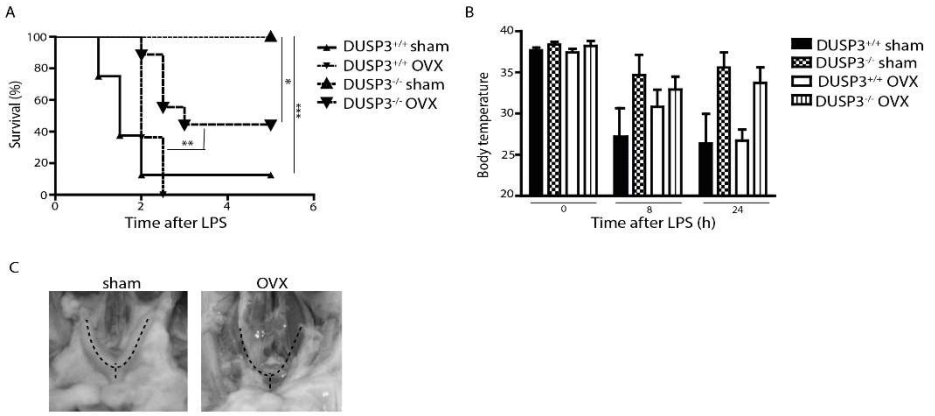

Figure 3.4. Ovariectomized DUSP3$^{-/-}$ mice are sensitive to LPS lethality. **(A)** DUSP3$^{+/+}$ sham (n= 8) or OVX (n = 11) and DUSP3$^{-/-}$ sham (n = 9) or OVX (n = 9) mice were i.p injected with 6 mg/kg LPS. Percent survival was assessed twice a day for 5 days. **(B)** Body temperature of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice before, 8 h, and 24 h after LPS injection. **(C)** Macroscopic picture of uterus after sham surgery or ovariectomy. Results are presented as mean ± SEM. n = 5 mice per group/time point. *p < 0.05, **p < 0.01, ***p <0,001

Loss of resistance of DUSP3$^{-/-}$ OVX mice to LPS-induced endotoxemia is associated with decreased M2-like macrophages but not with increased TNF levels.

The BM adoptive transfer experiments together with mice OVX showed that female sex hormones were involved in the resistance of DUSP3$^{-/-}$ females to LPS-induced death. It is possible therefore that these hormones could influence hematopoietic cells phenotype and response to inflammation. To investigate this hypothesis we phenotyped DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages from sham and OVX mice after LPS challenge based on the characterisation described in[294] (Figure 3.5.A). 2h after LPS injection, the percentage of M1-like macrophages (F4/80^{int}CD11b^{int}) was higher in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ OVX mice compared to DUSP3$^{-/-}$ sham mice. The difference was maintained at 24h, although not significant (Figure 3.5.B).

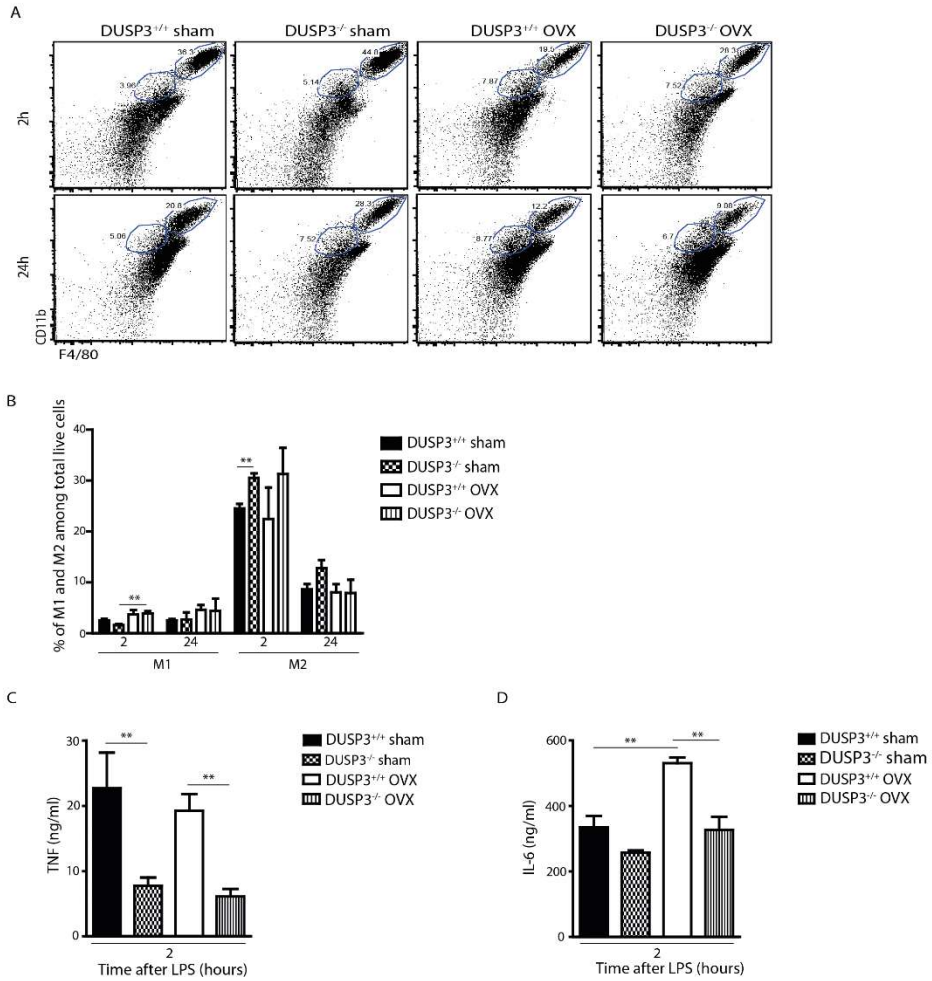

Figure 3.5. Ovariectomized DUSP3$^{-/-}$ mice are sensitive to LPS lethality **(A)** Peritoneal cells harvested from LPS-challenged DUSP3$^{+/+}$ sham and OVX and DUSP3$^{-/-}$ sham and OVX mice were analyzed by flow cytometry for CD11b and F4/80 to discriminate between M1-like macrophages (F4/80^{int}CD11b^{int}) and M2-like macrophage (F4/80^{high}CD11b^{high}). **(B)** Percentage of M1-like and M2-like macrophages out of total live cells. TNF **(C)** and IL-6 **(D)** levels from DUSP3$^{+/+}$ and DUSP3$^{-/-}$ sham and OVX after LPS challenge (6 mg/mL) were determined using MSD assays. Plasmas were collected 2h after LPS challenge. Results are presented as mean ± SEM. n = 5 mice per group/time point. *p < 0.05, **p < 0.01, ***p <0,001

The percentage of M2-like macrophages was equal in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ OVX mice compared to DUSP3$^{+/+}$ and DUSP3$^{-/-}$ sham mice 2h after LPS injection. However 24h after LPS challenge, the percentage of M2-like macrophages in the peritoneal cavity of OVX mice decreased, but did not reach statistical significance when compared to DUSP3$^{-/-}$ sham mice (Figure 3.5.B).

These data suggest that M2-like macrophages are likely to be involved in the resistance to LPS-induced endotoxemia

We next measured the level of the pro-inflammatory cytokines TNF and IL-6 in plasma 2h after LPS challenge. As previously reported[38], the level of TNF and IL-6 decreased in OVX DUSP3$^{-/-}$ mice compared to DUSP3$^{+/+}$ sham operated mice 2h after LPS injection. Interestingly, ovariectomy of mice did not impact TNF nor IL-6 level (Figure 3.5.C and 3.5.D). Together with the data obtained for males, these results suggest that M2-like macrophages, but not TNF, are involved in DUSP3-induced resistance to LPS-induced endotoxemia.

DUSP3-deficiency affects ERK 1/2 and PI3K/Akt phosphorylation in female, but not in male mice macrophages.

We have previously reported that DUSP3 deficiency leads to a decrease in ERK 1/2 phosphorylation following LPS stimulation of peritoneal macrophages[38]. Therefore we verified if the alteration of ERK 1/2 phosphorylation is associated with the gender-specific resistance to septic shock. We stimulated DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages from sham or OVX mice with LPS (1 µg/mL) at different time point and probed ERK 1/2 with phospho-specific antibody. As expected, ERK phosphorylation decreased in DUSP3$^{-/-}$ sham peritoneal macrophages at all time points compared to DUSP3$^{+/+}$ macrophages. Interestingly, in OVX mice, LPS stimulation of peritoneal macrophages led to an equal activation of ERK, as demonstrated by its phosphorylation level in DUSP3$^{+/+}$ and DUSP3$^{-/-}$. There was no difference of ERK phosphorylation in male mice from both genotypes (Figure 3.6.A and 3.6.B).

The observed hypo-phosphorylation of ERK 1/2 in DUSP3$^{-/-}$ sham mice suggests that DUSP3 is targeting either ERK 1/2 kinase or one of ERK1/2 phosphatases. Therefore we analysed MAPKK MEK 1/2 activation following *ex vivo* LPS stimulation (1 µg/mL) of peritoneal macrophages. MEK 1/2 kinetic phosphorylation was equal between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ sham mice of both gender (Figure 3.6.C and 3.6.D). These data suggest that DUSP3 does not target MEK 1/2 but more probably one of ERK 1/2 phosphatase*s.*

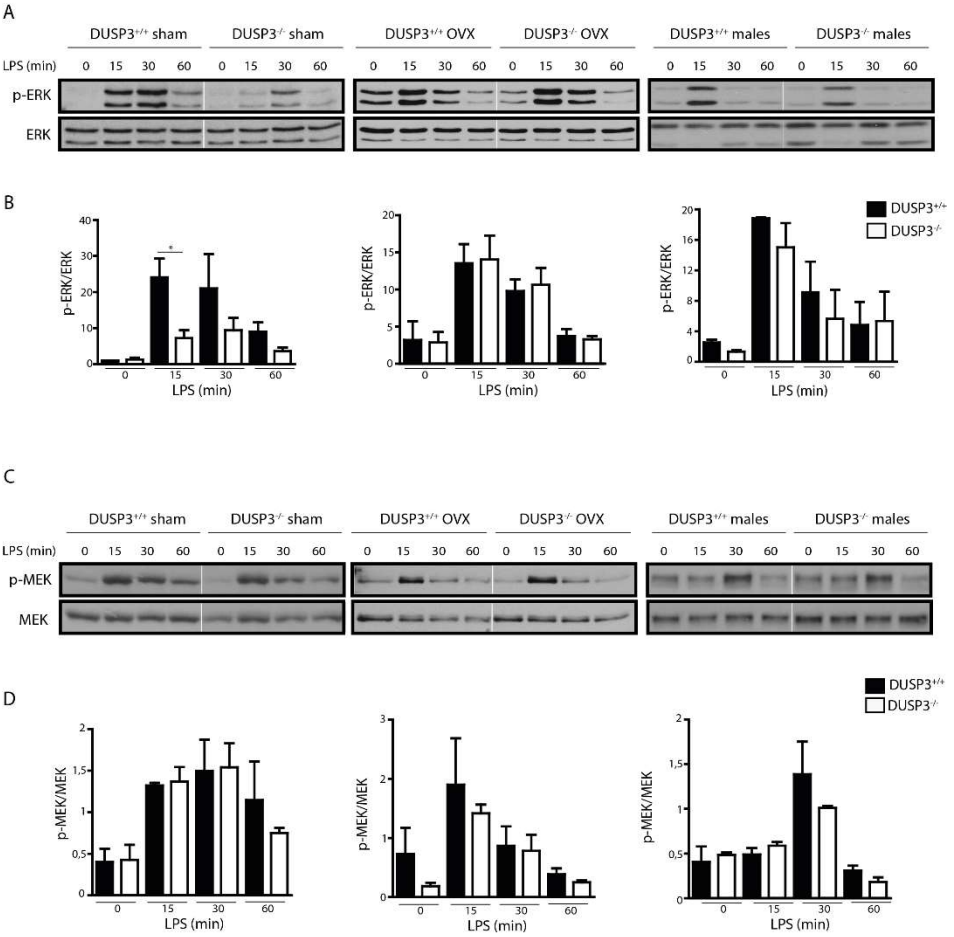

Figure 3.6. DUSP3-deficiency affects ERK 1/2 phosphorylation in female mice macrophages but not in males. Peritoneal macrophages isolated from 12-weeks-old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ female, male and OVX mice were stimulated *ex vivo* with 1 mg/ml LPS for the indicated time points. **(A)** Western blots were performed using anti–phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 as a loading control. Representative blots are shown for each detected (phospho)protein. Densitometry quantifications of phosphor-ERK1/2 and ERK1/2 were performed. **(B)** Anti–phospho- MEK1/2 (Ser217/221) and anti-MEK1/2, as loading control and densitometry quantifications of phospho-MEK and MEK. Results are presented as a ratio of phospho-ERK/ERK and phospho-MEK/MEK from four independent experiments. Data are shown as mean ± SEM. *p , 0.05.

The PI3K/Akt pathway is another important pathway activated after TLR4 triggering[248]. We therefore investigated whether DUSP3 deletion could impact this pathway after activation with LPS and whether the kinetic and magnitude of this activation could be gender dependent. PI3K and Akt activations were

evaluated using phospho-specific antibodies and Western blot after *ex vivo* LPS stimulation (1 µg/mL) of peritoneal macrophages at different time points.

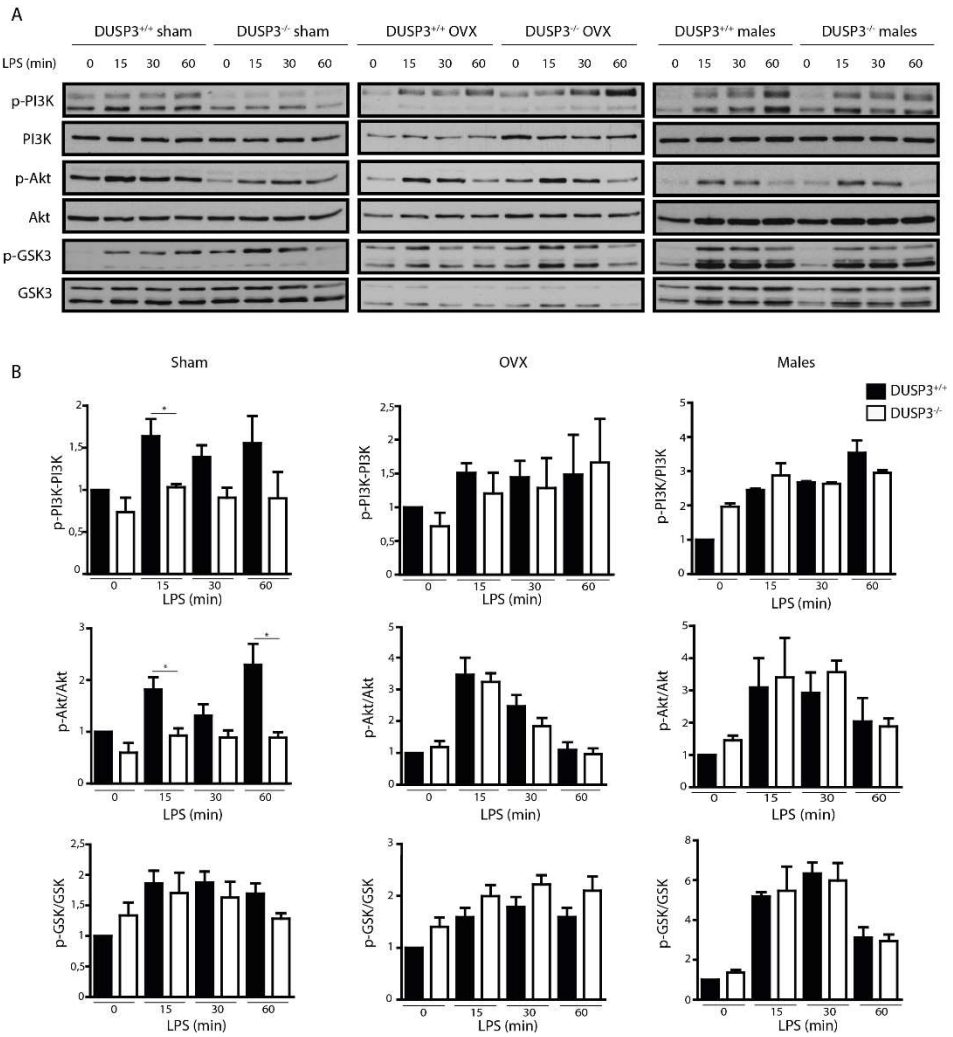

Figure 3.7. DUSP3-deficiency affects PI3K/Akt pathway in female, but not in male mice macrophages. Peritoneal macrophages isolated from 12-weeks-old DUSP3$^{+/+}$ and DUSP3$^{-/-}$ female, male and OVX mice were stimulated *ex vivo* with 1 mg/ml LPS for the indicated time points. **(A)** Western blots were performed using anti–phospho-PI3K (p85 Tyr 458/ p55 Tyr199), anti-phospho-Akt (Ser473), anti-phospho-GSK3α/β (Ser21/9) and anti-PI3K, anti-Akt and anti-GSK3α/β as loading controls. **(B)** Densitometry quantifications of phospho-PI3K, phospho-Akt, phospho-GSK3α/β, PI3K, Akt and GSK3α/β. Results are presented as a ratio of phospho-PI3K/PI3K, phospho-Akt/Akt and phospho-GSK3α/β/GSK3α/β from four independent experiments. Data are shown as mean ± SEM. *p , 0.05.

Interestingly, PI3K and Akt activations were decreased in DUSP3$^{-/-}$ sham peritoneal macrophages compared to DUSP3$^{+/+}$ peritoneal macrophages at all time points. This difference was abolished in OVX mice since the phosphorylation level of PI3K and Akt were remained equal between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ peritoneal macrophages. The activation of GSK3 downstream target of Akt, was however not affected by DUSP3 deficiency neither in sham nor OVX mice (Figure 3.7.A and 3.7.B.). There was no difference in PI3K and Akt activations in male peritoneal macrophages after LPS stimulation. PI3K and Akt were equally activated at all time points in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ LPS-stimulated peritoneal macrophages. Again GSK3 activation was not affected by DUSP3 deficiency (Figure 3.7.A and 3.7.B). These data suggest that DUSP3 also affects PI3K and Akt activation in a gender dependent manner.

3.5. Discussion and conclusion

It is well recognized that immune responses to infection are gender dependent. Indeed stronger immune responses confer to women protection against infection and sepsis[255]. Several epidemiological studies have been performed and showed a greater incidence of sepsis in males compared to females[297]. Consequently, compared to males, there are less female hospitalizations associated with infections. In addition, male sex, and presence of comorbidities were commonly reported independent predictors of post-acute mortality in sepsis survivors[298]. Interestingly, many of the differences between males and females in response to infections become apparent at puberty[299]. In line with this, women younger than 50 years show lower incidence of severe sepsis and better survival compared to age-matched men[256]. Altogether, these observations suggest a role for sexual hormones in the protection from severe infections and sepsis. This hypothesis has been supported by the finding that receptors for reproductive hormones are present in a variety of immune cells types[300]. On the other hand, oestrogens have been demonstrated to increase resistance to several bacterial infections whereas the removal of endogenous oestrogens have been shown, for example, to markedly increase the severity of *Mycobacterium avium* infections, an effect that can be reversed after 17β-oestra-diol replacement[301,302]. The role of female reproductive hormones in susceptibility to acute infection and sepsis is still however poorely understood.

In the present study, we report that DUSP3 deletion confers resistance to LPS-induced lethality and to polymicrobial-induced septic shock in female mice but not in males. We demonstrated that this protection is female sexual hormones and monocytes/macrophages dependent. Indeed, ovariectomy induced a loss of resistance and DUSP3$^{-/-}$ monocytes transfer to WT females was sufficient to transfer the resistance to WT recipient mice[38]. This protection was, however, not due to decreased TNF production as suggested by our previous study[38]. To our knowledge, this is the first report demonstrating a signalling molecule-induced synergic immunoprotective effect of monocytes/macrophages and female sexual hormones against sepsis.

The observed resistance to LPS-induced septic shock of DUSP3$^{-/-}$ female mice was associated with a dominance of M2-like macrophages. DUSP3-deficient mice ovariectomy induced a loss of resistance to LPS-induced death with no

difference in M2-like macrophage percentage between control groups and OVX-DUSP3$^{-/-}$. Together with the fact that the percentage of M2-like macrophages was also equal in both DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male mice suggest that female sex hormones may influence macrophage alternative activation. Our observations are in line with studies showing that oestrogens influence numerous immunological processes, among which monocytes and macrophages physiological functions[303]. Indeed, ovarian sex hormones modulate monocyte adhesion and chemotaxis, TLR expression, cytokines production as well as phagocytosis activity[304]. Moreover several evidences suggest that oestrogens also influence macrophage polarization. ER-α knockout mice undergo a decrease of alternative activated macrophages[304]. ER-α-deficient macrophages are indeed refractory to IL-4-induced alternative activation as demonstrated by a decrease of IL-4R and STAT6 phosphorylation in these cells[305]. Oestrogens have also been reported to increase the expression of the transcription factor IRF4 (*interferon regulatory factor-4*) involved in alternative activation of macrophages[306]. Using transcriptomic assay, we did not observe differences in IL4 or IL4R expression levels between DUSP3$^{-/-}$males and female neither at basal level nor after LPS challenge (data not shown). On the other hand, TNF production does not seem to play a role in the observed phenotype since ovariectomy of DUSP3$^{-/-}$ mice did not influence the level of this pro-inflammatory cytokine, although mice succumb to endotoxemia. These data were rather surprising wince sex steroids are known to regulate pro- and anti-inflammatory cytokine levels released by macrophages. On the other hand, female sex hormones are known to negatively regulate TNF production[307], one of the most important in sepsis[308,309]. The change of TNF production upon DUSP3 deletion in female mice should be therefore considered as an independent phenomenon not related to DUSP3$^{-/-}$ mice survival to sepsis.

How does DUSP3 regulate macrophage alternative activation in a female sexual hormone dependent manner is a complex question to answer. The molecular mechanisms involved are probably linked to the observed decrease of ERK1/2 and Akt/PI3K activations. Upon *ex vivo* LPS stimulation, DUSP3$^{-/-}$ female peritoneal macrophages showed a hypo-phosphorylation of both ERK1/2 and Akt when compared DUSP3$^{+/+}$ female macrophages. These differences were not observed in macrophages from OVX DUSP3$^{-/-}$mice. Together, these data suggest that, under inflammation conditions, female sex

hormones control macrophage polarization through DUSP3-ERK1/2-Akt signalling pathway axis. ERK1/2 has been previously reported to play a role in macrophage polarization through mTOR signalling pathway[310]. Indeed, ERK1/2 phosphorylates and dissociates the tuberous sclerosis protein (TSC) complex leading to its inactivation and subsequent activation of mTOR[310], constitutive activation of which leads to decreased IL-4-induced M2 polarization in TSC-deficient mice[310,311]. The role of sex hormone has not been investigated in these studies. In our model, it would be interesting to investigate whether the observed ERK1/2 hypo-phosphorylation found in DUSP3$^{-/-}$ female peritoneal macrophages could lead to TCS activation and consequently to M2 polarization. On the other hand, it has been reported that, upon TLR4 stimulation, PI3K engagement is followed by Akt and mTORC1 activation due to TSC inactivation by Akt[312]. This may lead to M1 macrophages polarization[312,313]. Similarly to ERK hypo-phosphorylation, decreased PI3K/Akt activation may lead to TSC activation and shifts macrophages polarization towards a M2 phenotype.

Another important question raised by our study is how does DUSP3 deletion lead to hypo-activation of the ERK1/2 and Akt signalling molecules. Decreased phosphorylation of these kinases clearly suggests that they are not directly targeted by DUSP3. The observed hypo-phosphorylation could be due to hyper-activation of specific ERK1/2 and PI3K/Akt yet unknown phosphatase. Indeed, preliminary data from our laboratory show that pervanadate (non-specific protein tyrosine phosphatases inhibitor) treatment of LPS-stimulated peritoneal macrophages restores ERK1/2 phosphorylation while okadaic acid (inhibitor of Ser/Thr PP1/PP2A), at low and high concentration, did not (Figure 3.8). Further investigations using, among others, phosphoproteomics approaches are required to confirm this hypothesis and identify the specific substrate(s) for DUSP3 and assess the exact role of this phosphatase in TLR4 signalling under the influence of female sex hormones.

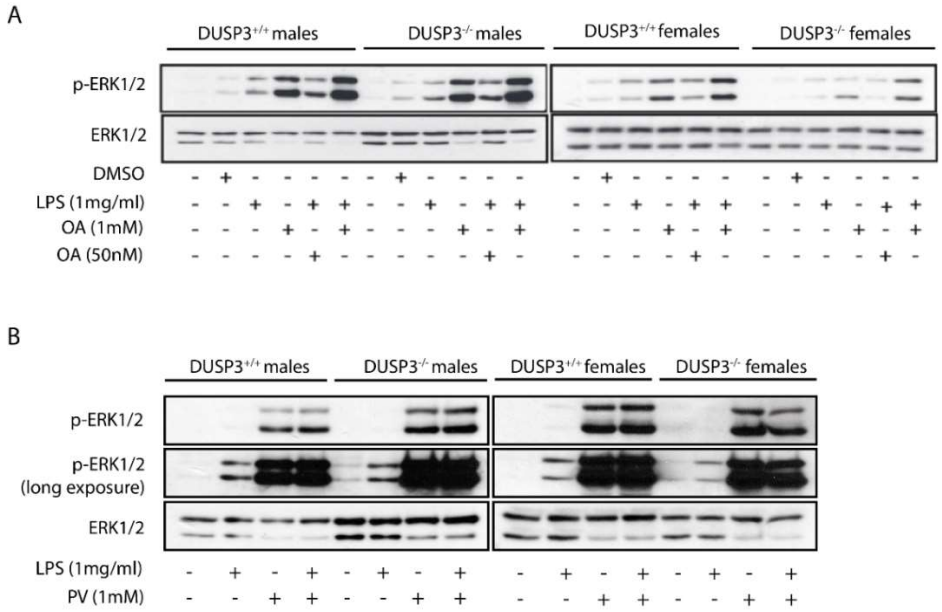

Figure 3.8. Effect of OA and PV on ERK phosphorylation in DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice peritoneal exudate. Cells from peritoneal cavity of DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice were pre-incubated with two different concentration of OA (50nM and 1µM). **(A)** or With PV (1mM) **(B)** for 30 minutes at 37°C. Cells were then activated with LPS (1µg/ml) for 15 minutes at 37°C. As a control condition, cells were incubated with DMSO (vehicle of the OA) at final concentration of 0.1%. Cells were lysed and equal amount of proteins were loaded on SDS- PAGE. Western blot analysis was performed using anti-p-ERK1/2 and anti-ERK1/2 as loading controls.

In summary, we identified DUSP3 dual-specificity phosphatase as a new key signalling molecule playing an important role in macrophage alternative activation and sexual dimorphism in innate immune response to infection. Our data suggest that DUSP3 inhibition, combined to oestrogen administration, may lead to protection from sepsis and septic shock (Figure 3.9).

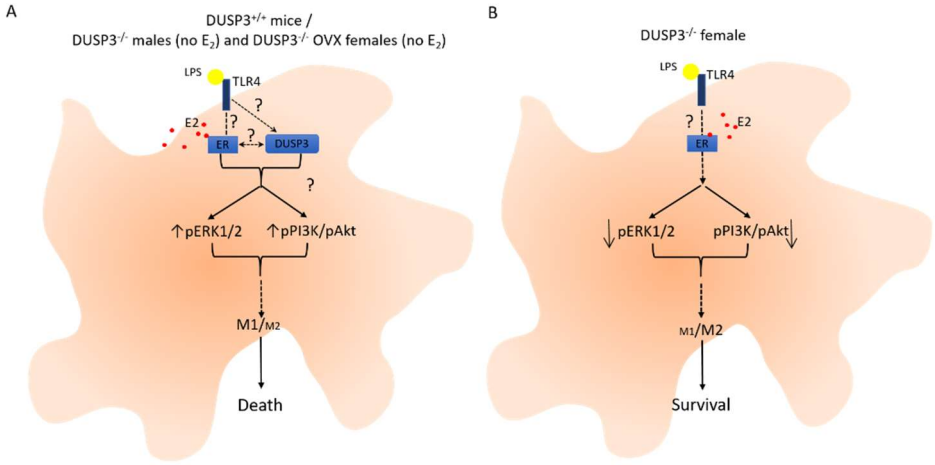

Figure 3.9. DUSP3 inhibition, combined to oestrogen administration, may lead to protection from sepsis and septic shock. **(A)** In DUSP3$^{+/+}$ females or DUSP3$^{-/-}$ males, TLR4 stimulation by LPS leads to the phosphorylation of ERK1/2 and PI3K/Akt and to proinflammatory cytokine production. The ratio M1/M2 macrophages is also in favour of M1 macrophages and these events eventually lead to the death of the mice. **(B)** In DUSP3$^{-/-}$ females, DUSP3 deficiency and the presence of female sex hormones trigger a hypo-phosphorylation of ERK1/2 and PI3K/Akt and a higher percentage of M2-like macrophages, leading to the survival of the mice.

Conclusions and perspectives

Conclusions and perspectives

The physiological roles of DUSPs in innate immunity and particularly in monocytes and macrophages are not completely understood yet. Indeed, most of the reported studies focused on MKPs since they regulate MAPKs, known to play important roles in immune responses. Several MKPs have been reported to regulate, negatively or positively, macrophage functions such as production of cytokines, chemokines or other inflammatory mediators. In addition, MKPs also affect immune cells survival[240,314]. However, little is known about the role of other DUSPs in immune responses and even less about the function of DUSP3 in innate immunity. We recently showed that DUSP3 is highly expressed in monocytes and macrophages, suggesting an important role of the phosphatase in these immune cells[38]. Macrophages exert numerous physiological functions, such as immune responses, tissue repair, waste-disposal and bone resorption[84]. Therefore, a dysregulation of macrophage activities may lead to homeostasis breakdown and diseases. The goal of the work presented here was to evaluate the role of DUSP3 in two mouse disease models, more particularly metastasis development and sepsis.

This study was conducted using full knockout DUSP3$^{-/-}$ mice. Although interesting and useful, full knockout models present some limitations that may interfere with future research objectives. In DUSP3-deficient mice, every cell types lack DUSP3. Therefore, it is difficult to evaluate the contribution of specific cell types in a given phenotype. The creation of cell type specific conditional knockout mice would be necessary. In addition , in our case, as expected DUSP3 may act as a phosphatase and dephosphorylate its substrates but it may also act as a scaffold to regulate substrate activities[21]. Therefore, to investigate more deeply molecular interactions of DUSP3 in present or future projects it would be interesting to create knock-in mice in which the endogenous DUSP3 is substituted to inactive mutants such as the D92A or C124S.

Despite these limitations, we were able to highlight a new role of DUSP3 in macrophages. In LLC experimental metastasis model, the enhanced lung metastasis observed in DUSP3-deficient mice was explained, at least partially, by a better recruitment of M2-like monocytes/macrophages to tumour site, creating a more permissive microenvironment than in WT mice. This stronger

presence of DUSP3$^{-/-}$ M2-like monocytes/macrophages at inflammation site was also found in the endotoxemia- and polymicrobial-induced septic shock models. This M2-like monocyte/macrophage presence conferred a survival advantage to DUSP3-deficient mice after LPS challenge and CLP. Indeed M2-like macrophages are important for tissue repair, restoration of endothelial barrier[220] and for restraining inflammation by engulfing death cells and eliminating debris[176]. Taken together, the results of these two projects strongly suggest that DUSP3 is involved in monocytes/macrophage's fate, most probably by governing their polarization. It is also possible that DUSP3 could influence M2-like monocyte/macrophage proliferation and/or migration. However, this latter hypothesis was rapidly abandoned since macrophage recruitment was equal between DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice (data not shown). The other alternative could be that DUSP3$^{-/-}$ M2-like macrophages proliferate better compared to DUSP3$^{+/+}$ macrophages. It has been shown that, besides the classical mode of inflammation during which large number of macrophages are recruited, alternative macrophages are also able to proliferate *in situ*[315]. It would be interesting to investigate the hypothesis using Ki67 staining or BrDU incorporation. Depletion of peripheral blood monocytes, precursors of M2-like macrophages, by i.v. injections of clodronate-liposomes prior to LPS challenge would also allow us to discriminate between macrophage recruitment and proliferation. Indeed, if local macrophage proliferation plays a role, the number of M2-like macrophages in peritoneal cavity should not be affected by the depletion. On the contrary, if these macrophages are recruited, their number should decrease following clodronate-induced macrophage depletion. However, we also observed that M2-like peritoneal macrophage percentage was higher in DUSP3$^{-/-}$ mice compared to DUSP3$^{+/+}$ mice at basal level[38]. Although subtle, these findings suggest that DUSP3 is involved in macrophage polarization. This new perspective, let us hypothesize that, in the experimental metastasis model, M2-like monocytes/macrophages, most abundant in DUSP3$^{-/-}$ mice, would be more sensitive to certain chemokines secreted by LLC than M1-like macrophages, explaining the better recruitment and the higher percentage of M2-like monocytes/macrophages at tumour site. Using qPCR we showed that LLC cells expressed high levels of CSF-1, CCL2, CCL7 and TGF-b (data not shown), chemokines known to strongly attract monocytes and macrophages[316–319]. It is also possible that LLC cells express/secrete other chemokines, as important for monocyte/macrophage recruitment as these four cytokines. A microarray analysis of LLC cells would be necessary to

identify all these chemokines. To identify the chemokine(s) involved in M2-like recruitment, migration assays in presence of these chemokines alone, or in combination, should be performed. We could also carry out migration tests in presence of LLC-CM supplemented with the identified chemokine inhibitors. The fact that DUSP3$^{-/-}$ mice didn't develop bigger B16-lung metastasis than DUSP3$^{+/+}$ mice may be explained by a reduced expression of potent monocyte/macrophage chemottractants. Another way to prove the involvement of a precise cytokine would be to inject transduced B16 cells, expressing this cytokine, into DUSP3$^{-/-}$ mice and compared lung metastasis to DUSP3$^{+/+}$ mice.

In line with the involvement of DUSP3 in macrophage polarization and considering that M2 macrophages may be polarized in several subpopulations, it would be interesting to further explore which subpopulation of M2 macrophages is associated with DUSP3$^{-/-}$ mice survival to endotoxemia-induced septic shock. It is known that TLR ligands and immune complexes induce M2b polarization. M2b macrophages are characterized by the expression of MHC-II and CD86 but also by the production and secretion of high level of IL-10, IL-1, IL-6 and TNF[93,94]. The presence of M2b macrophages is associated with a susceptibility to sepsis[320,321]. Probably due, at least in part, to the fact that they are involved in immunoregulation and T_{h2} activation, the presence of T_{h2} cells being associated with an impaired survival[322]. These observations, in conflict with our observed survival of DUSP3$^{-/-}$ mice, indicate that another subpopulation of M2 macrophages is probably involved in the phenotype. M2a and M2c macrophages are good candidates since they present more anti-inflammatory properties than M2b macrophages. Moreover, they are involved in clearing debris and tissue remodelling[93,94]. Therefore, it would be interesting to further phenotype DUSP3$^{-/-}$ peritoneal macrophages by assessing the expression of Arg1, Fizz1 or Ym1 and MerTK, markers of M2a and M2c, respectively.

Another important point highlighted by the "sepsis project" is the link between female sex hormones, DUSP3 activity and macrophage polarization. It is likely that oestrogens are involved in the observed phenotype. However, oestrogens are not the only hormones to be depleted after ovariectomy and one way to confirm their involvement would be to inject oestrogens to OVX mice and challenge them with LPS. If oestrogens protect against LPS-induced

death, DUSP3$^{-/-}$ OVX mice should survive. The effects of oestrogens on immune cells are cell type- and dose-dependent. Indeed, at physiological concentrations, oestrogens stimulate the production of antibodies from B cells and downregulate T cell-dependent immunity. In addition, oestrogens decrease the production of inflammatory cytokines such as TNF, IL-6 and IL-1β[255]. However, at supraphysiological concentrations, oestrogens may have opposite effects and lead to more sensitized innate immune cells and to a rapid inflammatory response after LPS challenge. This may result in greater sepsis severity[323]. In light of this, it would be interesting to measure the level of oestrogens in naïve DUSP3$^{+/+}$ and DUSP3$^{-/-}$ mice but also after LPS challenge as high level of oestrogens is a predictor of death in septic shock[302]. It has also been reported that oestrogens may play a role in macrophage polarization. ER-α-KO mice undergo a decrease of alternative activated macrophages[304]. ER-α-deficient macrophages are refractory to IL-4-induced alternative activation as demonstrated by a decrease of IL-4R and STAT6 phosphorylation in these ER-α deficient macrophages[305]. Oestrogens have also been reported to increase the expression of interferon regulatory factor-4 transcription factor (IRF4), involved in alternative activation of macrophages[306]. Therefore, it would be interesting to check ER-α, and to a lesser extent ER-β, expression on DUSP3$^{+/+}$ and DUSP3$^{-/-}$ macrophages at basal and after endotoxemia challenge. The link between DUSP3, female sex hormones and macrophage polarization is further strengthened by data showing the engagement of DUSP3 in ERK and PI3K signalling pathways, known to influence numerous physiological processes such as macrophage polarization[313]. Consequently, it is likely that female sex hormones and DUSP3 act hand in hand to regulate macrophage polarization. This interaction between female sex hormones and DUSP3 is less obvious in the case of LLC experimental metastasis model. Indeed, in LLC lung metastasis formation, although DUSP3$^{-/-}$ females developed bigger LLC-lung metastasis than males, the difference was not significant (data not shown). Although less pronounced than in the “sepsis project”, it may still suggest a possible role of female sexual hormones in the phenotype.

Despite the discovery of this new role of DUSP3, several aspects of the work remain unclear and deserve to be investigated more in depth. For example, the exact molecular role of DUSP3 is still not known. Although the involvement of DUSP3 in ERK and PI3K/Akt signalling pathways has been identified, the physiological substrate(s) of the phosphatase is (are) still unknown. However,

findings from the "sepsis project" may allow us to narrow the possibilities. The hypo-phosphorylation of ERK 1/2 and PI3K found in female DUSP3-deficient peritoneal macrophages after LPS challenge and the equal activation level of the MAPKK MEK suggest that DUSP3 activates a phosphatase or inactivates a kinase. This hypothesis is strengthened by the fact that the activation defect of DUSP3$^{-/-}$ platelets has also been associated with Syk hypo-phosphorylation[34]. Since DUSP3, as a phosphatase, may act on several targets, which also have many substrates and since these substrates are often involved in many physiological processes, it is likely that the range of action of DUSP3 is broader than previously expected. Although challenging, phosphoproteomic analysis on peritoneal macrophages from LPS challenged-DUSP3$^{+/+}$ and DUSP3$^{-/-}$ male and sham and OVX female mice would give hints to follow in the search of new DUSP3 substrates. Potential DUSP3 targets may also be found in literature. Indeed, many phosphatases are able to dephosphorylate ERK ½, namely PP2A[324], PP1[325], Shp2[326], HePTP[327], PTP-SL[328], STEP[328] but also DUSP2, DUSP4, DUSP5, DUSP6, DUSP7 and DUSP9[10]. Based on results obtained from experiments using specific phosphatase inhibitors (okadaic acid and pervanadate, data not shown), we can already narrow the potential candidates. Indeed, since okadaic acid, an inhibitor of serine/threonine phosphatases, failed to restore ERK phosphorylation in DUSP3$^{-/-}$ female mice, the serine/threonine phosphatase PP2A may be excluded. Moreover, the observed difference in ERK 1/2 phosphorylation rapidly occurs after LPS stimulation (15 minutes), suggesting that an early LPS-Induced DUSP is targeting ERK. Consequently, because their inductions require a longer LPS stimulation than 15 minutes, DUSP2, DUSP4 and DUSP5 may also be excluded from potential candidates. PI3K can be activated by several proteins such as the GTPase Ras, for example[245]. GTPase proteins are activated by the exchange of a GDP by GTP by a guanine nucleotide releasing protein (GNRP) such as SOS, bond to the adaptor protein Grb2. The serine/threonine phosphorylation of SOS results in disassociation of the Grb2-SOS complex thereby limiting Ras activation[329]. Consequently, it is possible that DUSP3-deficiency leads to a stable phosphorylation of SOS followed by a subsequent decreased activation of Ras and PI3K. Another activator of PI3K is Src kinase[248]. To be active and to phosphotylate its substrates, Src has to undergo a dephosphorylation of Tyr527[330]. This may result in a hypo-phosphorylation of PI3K. It is worth noting that Src family kinases are also activators of the kinase Syk[331]. Ideally, experiments should be performed on peritoneal macrophages *in vivo* or *ex*

vivo stimulated with LPS. However, due to reduced number of peritoneal macrophages that could be challenged, another possibility would be to perform experiments using cell lines transfected with RNAi targeting DUSP3 or immortalized BMDMs.

Another aspect of the work that has not been addressed is the role played by female sex hormones in DUSP3 dependent-macrophage polarization. According to our observations and to literature, the general consensus is that M2-macrophage polarization is enhanced by the action of oestrogens[332]. However, the molecular mechanisms are not known. Oestrogens exert their effect through the binding of their receptors ERα and ERβ[333]. In addition to their transcription factor activities, ERs also interact with cytoplasmic and nuclear proteins, interfering in many signalling pathways[333]. Moreover, it has been reported that oestrogens also bind the membrane-bound G-protein coupled receptor GPR30[334]. Consequently, the range of action of oestrogens is wide. However, if we look at molecular mechanisms influencing macrophage polarization[112,332], several hypotheses can be formulated, although non exhaustive. Oestrogens may influence the balance between STAT1 and STAT3/STAT6 activation; a predominance of STAT1 leading to M1 polarization while a predominance of STAT3/STAT6 activation increases the alternative activation of macrophages. To do so, oestrogens would positively influence IL-4/IL-13 or IL-10 signalling or act on SOCS1 or SOCS3, protein inhibiting STAT1 and STAT3, respectively.

Although we discovered a new role of DUSP3 in macrophage polarization, several big challenges remain in understanding the role of DUSP3 and oestrogens in macrophages polarization. Answering these questions would help in the comprehension of the pathophysiology of several diseases, such as asthma, cardiovascular diseases, auto-immunity or even some cancers, known for their sex hormone-dependent incidence and the involvement of macrophages.

References

References

1. Xun Li, Matthias Wilmanns, J. T. and M. K. Elucidating Human Phosphatase-Substrate Networks. *Sci. Signal.* **6,** (2013).
2. Alonso, A. & Pulido, R. The extended human PTPome: A growing tyrosine phosphatase familyl. *FEBS J.* **283,** 1404–1429 (2016).
3. Denu, J. M. & Dixon, J. E. Protein tyrosine phosphatases : mechanisms of catalysis and regulation. *Curr. Opin. Chem. Biol.* 633–641 (1998).
4. Yuvaniyama, J., Denu, J. M., Dixon, J. E. & Saper, M. A. Crystal structure of the dual specificity protein phosphatase VHR. *Sciences (New. York).* **272,** 1328–1331 (1996).
5. Maehama, T., Taylor, G. S. & Dixon, J. E. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem* **70,** 247–279 (2001).
6. Deshpande, T., Takagi, T., Hao, L., Buratowski, S. & Charbonneau, H. Human PIR1 of the protein-tyrosine phosphatase superfamily has RNA 5'-triphosphatase and diphosphatase activities. *J Biol Chem* **274,** 16590–16594 (1999).
7. Tagliabracci, V. S. *et al.* Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 19262–19266 (2007).
8. Alonso, A. *et al.* Protein tyrosine phosphatases in the Human Genome. **117,** 699–711 (2004).
9. Farooq, A. & Zhou, M.-M. Structure and regulation of MAPK phosphatases. *Cell. Signal.* **16,** 769–79 (2004).
10. Patterson, K. I., Brummer, T., O'brien, P. M. & Daly, R. J. Dual-specificity phosphatases: critical regulators with diverse cellular targets. *Biochem. J.* **418,** 475–489 (2009).
11. Nunes-Xavier, C. *et al.* Dual-specificity MAP kinase phosphatases as targets of cancer treatment. *Anticancer. Agents Med. Chem.* **11,** 109–32 (2011).
12. Huang, C.-Y. & Tan, T.-H. DUSPs, to MAP kinases and beyond. *Cell Biosci.* **2,** 24 (2012).
13. Guan K. L, Broyles S.S, D. J. E. A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature* **350,** 359–362 (1991).
14. Ishibashi, T., Bottaro, D. P., Chan, a, Miki, T. & Aaronson, S. a. Expression cloning of a human dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12170–4 (1992).
15. Denu, J. M. *et al.* The purification and characterization of a human dual-specific protein tyrosine phosphatase. *The Journal of biological chemistry* **270,** 3796–3803 (1995).
16. Schumacher, M. A., Todd, J. L., Rice, A. E., Tanner, K. G. & Denu, J. M. Structural basis for the recognition of a bisphosphorylated MAP kinase

peptide by human VHR protein phosphatase. *Biochemistry* **41,** 3009–3017 (2002).

17. Todd, J. L., Rigas, J. D., Rafty, L. A. & Denu, J. M. Dual-specificity protein tyrosine phosphatase VHR down-regulates c-Jun N-terminal kinase (JNK). *Oncogene* (2002). doi:10.1038/sj/onc/1205344
18. Todd, J. L., Tanner, K. G. & Denu, J. M. Extracellular Regulated Kinases (ERK) 1 and ERK2 Are Authentic Substrates for the Dual-specificity Protein-tyrosine Phosphatase. **274,** 13271–13280 (1999).
19. Nakamura, K. *et al.* A novel low-molecular-mass dual-specificity phosphatase, LDP-2, with a naturally occurring substitution that affects substrate specificity. *J Biochem* **132,** 463–470 (2002).
20. Alonso, a, Saxena, M., Williams, S. & Mustelin, T. Inhibitory role for dual specificity phosphatase VHR in T cell antigen receptor and CD28-induced Erk and Jnk activation. *J. Biol. Chem.* **276,** 4766–71 (2001).
21. Hoyt, R. *et al.* Cutting edge: selective tyrosine dephosphorylation of interferon-activated nuclear STAT5 by the VHR phosphatase. *J. Immunol.* **179,** 3402–6 (2007).
22. Wang, J.-Y. *et al.* Vaccinia H1-related phosphatase is a phosphatase of ErbB receptors and is down-regulated in non-small cell lung cancer. *J. Biol. Chem.* **286,** 10177–84 (2011).
23. Amand, M. *et al.* DUSP3/VHR is a pro-angiogenic atypical dual-specificity phosphatase. *Mol. Cancer* **13,** 108 (2014).
24. Panico, K. & Forti, F. L. Proteomic, cellular, and network analyses reveal new DUSP3 interactions with nucleolar proteins in hela cells. *J. Proteome Res.* **12,** 5851–5866 (2013).
25. Forti, F. L. Combined experimental and bioinformatics analysis for the prediction and identification of VHR/DUSP3 nuclear targets related to DNA damage and repair. *Integr Biol* **7,** 73–89 (2014).
26. Pavic, K., Duan, G. & Köhn, M. VHR/DUSP3 phosphatase: Structure, function and regulation. *FEBS J.* **282,** 1871–1890 (2015).
27. Alonso, A. *et al.* Tyrosine phosphorylation of VHR phosphatase by ZAP-70. *Nat. Immunol.* **4,** 44–8 (2003).
28. Kang, T.-H. & Kim, K.-T. Negative regulation of ERK activity by VRK3-mediated activation of VHR phosphatase. *Nat. Cell Biol.* **8,** 863–9 (2006).
29. Denu, J. M. & Tanner, K. G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: Evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37,** 5633–5642 (1998).
30. Wentworth, C. C., Alam, A., Jones, R. M., Nusrat, A. & Neish, A. S. Enteric commensal bacteria induce extracellular signal-regulated kinase pathway signaling via formyl peptide receptor-dependent redox modulation of dual specific phosphatase. *J. Biol. Chem.* **286,** 38448–

38455 (2011).
31. Pavic, K. *et al.* Unnatural amino acid mutagenesis reveals dimerization as a negative regulatory mechanism of VHR's phosphatase activity. *ACS Chem. Biol.* **9,** 1451–1459 (2014).
32. Wagner, K. W. *et al.* Kdm2a promotes lung tumorigenesis by epigenetically enhancing erk1/2 signaling. *J. Clin. Invest.* **123,** 5231–5246 (2013).
33. Rahmouni, S. *et al.* Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. *Nat. Cell Biol.* **8,** 524–531 (2006).
34. Musumeci, L. *et al.* Dual-specificity phosphatase 3 deficiency or inhibition limits platelet activation and arterial thrombosis. *Circulation* **131,** 656–668 (2015).
35. Henkens, R. *et al.* Cervix carcinoma is associated with an up-regulation and nuclear localization of the dual-specificity protein phosphatase VHR. *BMC Cancer* **8,** 147 (2008).
36. Arnoldussen, Y. J. *et al.* The mitogen-activated protein kinase phosphatase vaccinia H1-related protein inhibits apoptosis in prostate cancer cells and is overexpressed in prostate cancer. *Cancer Res.* **68,** 9255–64 (2008).
37. Hao, L. & ElShamy, W. M. BRCA1-IRIS activates cyclin D1 expression in breast cancer cells by downregulating the JNK phosphatase DUSP3/VHR. *Int. J. Cancer* **121,** 39–46 (2007).
38. Singh, P. *et al.* DUSP3 Genetic Deletion Confers M2-like Macrophage-Dependent Tolerance to Septic Shock. *J. Immunol.* **194,** 4951–4962 (2015).
39. Kindt Thomas, Goldsby Richard, Osborne Barbara. *immunology*. (2008).
40. Riera Romo, M., Pérez-Martinez, D. & Castillo Ferrer, C. Innate immunity in vertebrates: An overview. *Immunology* 125–139 (2016). doi:10.1111/imm.12597
41. Iwasaki, A. & Medzhitov, R. Control of adaptive immunity by the innate immune system. *Nat. Immunol.* **16,** 343–353 (2015).
42. Wherry, E. J. & Masopust, D. *Adaptive Immunity*. *Pattern Recognition* (Elsevier). doi:10.1016/B978-0-12-800964-2.00005-7
43. Biron, C. A. *Innate Immunity*. *Viral Pathogenesis* (Elsevier, 2016). doi:10.1016/B978-0-12-800964-2.00004-5
44. Medzhitov R, Preston-Hurlburt P & Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388,** 394–397 (1997).
45. Ogura, Y. *et al.* Nod2, a Nod1/Apaf-1 Family Member That Is Restricted to Monocytes and Activates NF-kB. *J. Biol. Chem.* **276,** 4812–4818 (2001).
46. Inohara, N., Ogura, Y., Chen, F. F., Muto, A. & Nunez, G. Human Nod1 Confers Responsiveness to Bacterial Lipopolysaccharides. *J. Biol. Chem.*

276, 2551–2554 (2001).

47. Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5,** 730–737 (2004).
48. Dixit, N. K. Signaling in innate immunity and inflammation. *Cold Spring HArb Perspect Biol* **4,** 1–19 (2012).
49. Netea, M. G. Training innate immunity: The changing concept of immunological memory in innate host defence. *Eur. J. Clin. Invest.* **43,** 881–884 (2013).
50. Netea, M. G., Latz, E., Kingston, H. G., Mills, L. A. & Neill, J. O. Innate immune memory: a paradigm shift in understanding host defense. *Nat. Immunol.* **16,** 675–679 (2015).
51. Mócsai, A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J. Exp. Med.* **210,** 1283–99 (2013).
52. Mantovani, A., Cassatella, M. a, Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* **11,** 519–531 (2011).
53. Min, B. *et al.* Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* **200,** 507–17 (2004).
54. Yousefi, S. *et al.* Basophils exhibit antibacterial activity through extracellular trap formation. *Allergy Eur. J. Allergy Clin. Immunol.* **70,** 1184–1188 (2015).
55. Pamoate, P. Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. *Proc. Natl. Acad. Sci.* 4–7 (2012). doi:10.1073/pnas.1112268109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1112268109
56. Svensson, L. & Wennerås, C. Human eosinophils selectively recognize and become activated by bacteria belonging to different taxonomic groups. *Microbes Infect.* **7,** 720–728 (2005).
57. Sandig, H. & Bulfone-Paus, S. TLR signaling in mast cells: Common and unique features. *Front. Immunol.* **3,** 1–13 (2012).
58. Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B. & Lanier, L. L. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296,** 1323–1326 (2002).
59. Newman, K. C. & Riley, E. M. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* **7,** 279–91 (2007).
60. Rivera, A., Siracusa, M. C., Yap, G. S. & Gause, W. C. Innate cell communication kick-starts pathogen-specific immunity. *Nat. Immunol.* **17,** 356–363 (2016).
61. Fogg D, Sibon C, Miled C, Jung S, Aucouturier P, Dan R. Littman, Ana Cumano, F. G. A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells. *Science (80-.).* **311,** 83–88 (2006).

62. Hettinger, J. *et al.* Origin of monocytes and macrophages in a committed progenitor. *Nat. Immunol.* **14,** 821–830 (2013).
63. Geissmann, F., Jung, S. & Littman, D. R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19,** 71–82 (2003).
64. Cecchini, M. G. *et al.* Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* **120,** 1357–72 (1994).
65. Dai, X. *et al.* Targeted disruption of the mouse CSF-1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primititive progenitor cell frequencies and reproductive defects. *Blood* **99,** 111–120 (2002).
66. Wei, S. *et al.* Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J. Leukoc. Biol.* **88,** 495–505 (2010).
67. Ginhoux, F. & Jung, S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* **14 VN-r,** 392–404 (2014).
68. Serbina, N. V, Jia, T., Hohl, T. M. & Pamer, E. G. monocyte-mediated defense against microbial pathogens. *New York* 421–452 (2010). doi:10.1146/annurev.immunol.26.021607.090326.Monocyte-Mediated
69. Sunderkotter, C. *et al.* Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response. *J. Immunol.* **172,** 4410–4417 (2004).
70. Tacke, F. & Randolph, G. J. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* **211,** 609–618 (2006).
71. Cedric Auffray, Darin Fogg, Meriem Garfa, Gaelle Elain, Olivier Join-Lambert, Samer Kayal, Sabine Sarnacki, Ana Cumano, Gregoire Lauvau, F. G. Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior. *Science (80-.).* **317,** 666–670 (2007).
72. Carlin, L. M. *et al.* Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. *Cell* **153,** 362–375 (2013).
73. Randolph, G. J. A macrophage revolution-and beyond. *Immunol. Rev.* **262,** 5–8 (2014).
74. Ginhoux, F. *et al.* Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science (80-.).* **330,** 841–845 (2010).
75. Schulz, C. *et al.* A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science (80-.).* **336,** 2–7 (2012).
76. Epelman, S., Lavine, K. J. & Randolph, G. J. Origin and Functions of Tissue Macrophages. *Immunity* **41,** 21–35 (2014).

77. Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* **496,** 445–55 (2013).
78. Murray, P. J. & Wynn, T. a. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **11,** 723–37 (2011).
79. Serbina, N. V & Pamer, E. G. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* **7,** 311–7 (2006).
80. Tsou, C. L. *et al.* Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* **117,** 902–909 (2007).
81. Lucas, T. *et al.* Differential roles of macrophages in diverse phases of skin repair. *J. Immunol.* **184,** 3964–77 (2010).
82. Heredia, J. E. *et al.* Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell* **153,** 376–388 (2013).
83. Duffield, J. S., Lupher, M., Thannickal, V. J. & Wynn, T. A. Host responses in tissue repair and fibrosis. *Annu Rev Pathol* **8,** 241–276 (2013).
84. Davies, L. C., Jenkins, S. J., Allen, J. E. & Taylor, P. R. Tissue-resident macrophages. *Nat. Immunol.* **14,** 986–995 (2013).
85. Kobayashi, N. *et al.* TIM-1 and TIM-4 Glycoproteins Bind Phosphatidylserine and Mediate Uptake of Apoptotic Cells. *Immunity* **27,** 927–940 (2007).
86. Yoshida, H. *et al.* Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. *Nature* **437,** 754–758 (2005).
87. Ganz, T. Macrophages and systemic iron homeostasis. *J. Innate Immun.* **4,** 446–453 (2012).
88. Pollard, J. W. Trophic macrophages in development and disease. *Nat. Rev. Immunol.* **9,** 259–270 (2009).
89. Rao, S. *et al.* Obligatory participation of macrophages in an angiopoietin 2-mediated cell death switch. *Development* **134,** 4449–4458 (2007).
90. Erblich, B., Zhu, L., Etgen, A. M., Dobrenis, K. & Pollard, J. W. Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. *PLoS One* **6,** (2011).
91. Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117,** 175–184 (2007).
92. Nguyen, K. D. *et al.* Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* **480,** 104–108 (2011).
93. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25,** 677–86 (2004).

94. Roszer, T. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediat. Inflamm.* **2015,** 1–16 (2015).
95. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* **6,** 13 (2014).
96. Ferrante, C. J. *et al.* The adenosine-dependent angiogenic switch of macrophages to an M2-like phenotype is independent of interleukin-4 receptor alpha (IL-4R??) signaling. *Inflammation* **36,** 921–931 (2013).
97. Sica A and Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J. Clin. Invest.* **122,** 787–795 (2012).
98. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* **3,** 23–35 (2003).
99. Mantovani, A. Macrophages, Neutrophils, and Cancer: A Double Edged Sword. *New J. Sci.* **2014,** 1–14 (2014).
100. Sica, A., Erreni, M., Allavena, P. & Porta, C. Macrophage polarization in pathology. *Cell. Mol. Life Sci.* **72,** 4111–4126 (2015).
101. Udalova, I. A. *et al.* IRF5 promotes inflammatory macrophage polarization and Th1 / Th17 response To cite this version : *Nat. Immunol.* **12,** 231–238 (2011).
102. Sica, A. & Bronte, V. Altered macrophage differentiation and immune dysfunction in tumor development. *J. Clin. Invest.* **117,** 1155–1166 (2007).
103. Lang, R., Patel, D., Morris, J. J., Rutschman, R. L. & Murray, P. J. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol.* **169,** 2253–2263 (2002).
104. Liu, Y. *et al.* Unique expression of suppressor of cytokine signaling 3 is essential for classical macrophage activation in rodents in vitro and in vivo. *J. Immunol.* **180,** 6270–6278 (2008).
105. Whyte, C. S. *et al.* Suppressor of cytokine signaling (SOCS)1 is a key determinant of differential macrophage activation and function. *J. Leukoc. Biol.* **90,** 845–854 (2011).
106. Odegaard, J. *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447,** 1116–1120 (2007).
107. Justin I. Odegaard, Roberto R. Ricardo-Gonzalez, Alex Red Eagle, D. V., Christine R. Morel, Matthew H. Goforth, Vidya Subramanian, Lata Mukundan, A. & W. Ferrante, and A. C. Alternative (M2) activation of Kupffer cells by PPARδ ameliorates obesity-induced insulin resistance. *Cell Metab* **7,** 496–507 (2008).
108. Liao, X., Sharma, N. & Kapadia, F. Krüppel-like factor 4 regulates macrophage polarization. *J. Clin. Invest.* **121,** 2736–2749 (2011).
109. Takeda, N. *et al.* Differential activation and antagonistic function of HIF-?? isoforms in macrophages are essential for NO homeostasis. *Genes*

Dev. **24,** 491–501 (2010).
110. Zhuang, G. *et al.* A novel regulator of macrophage activation: MiR-223 in obesity-associated adipose tissue inflammation. *Circulation* **125,** 2892–2903 (2012).
111. Cai, X. *et al.* Re-polarization of tumor-associated macrophages to pro-inflammatory M 1 macrophages. *J. Mol. Cell Biol.* 1–3 (2012). doi:10.1093/jmcb/mjs044
112. Wang, N., Liang, H. & Zen, K. Molecular mechanisms that influence the macrophage M1-M2 polarization balance. *Front. Immunol.* **5,** 1–9 (2014).
113. Martinez-Nunez, R. T., Louafi, F. & Sanchez-Elsner, T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor ??1 (IL13R??1). *J. Biol. Chem.* **286,** 1786–1794 (2011).
114. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).
115. Bergers, G. & Benjamin, L. E. Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* **3,** 401–410 (2003).
116. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353–364 (1996).
117. Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407,** 249–257 (2000).
118. Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69,** 4–10 (2005).
119. Thiery, J. P., Acloque, H., Huang, R. Y. J. & Nieto, M. A. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* **139,** 871–890 (2009).
120. Guan, X. Cancer metastases: Challenges and opportunities. *Acta Pharm. Sin. B* **5,** 402–418 (2015).
121. Mani, S. a *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133,** 704–715 (2008).
122. Uppal, A., Wightman, S. C., Ganai, S., Weichselbaum, R. R. & An, G. Investigation of the essential role of platelet-tumor cell interactions in metastasis progression using an agent-based model. *Theor. Biol. Med. Model.* **11,** 17 (2014).
123. Palumbo, J. S. *et al.* Platelets and fibrin (ogen) increase metastatic potential by impeding natural killer cell – mediated elimination of tumor cells. *Blood J.* **105,** 178–185 (2005).
124. Takagi, S. *et al.* Platelets Promote Tumor Growth and Metastasis via Direct Interaction between Aggrus/Podoplanin and CLEC-2. *PLoS One* **8,** 1–11 (2013).
125. Strell, C. & Entschladen, F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun. Signal.* **6,** 10 (2008).

126. Eriksson, M., Taskinen, M. & Leppä, S. Mitogen Activated Protein Kinase-Dependent Activation of c-Jun and c-Fos is required for Neuronal differentiation but not for Growth and Stress Reposne in PC12 cells. *J. Cell. Physiol.* **207,** 12–22 (2006).
127. Fidler, I. J. Hypothesis Revisited. **3,** 1–6 (2003).
128. Luzzi, K. J. *et al.* Multistep Nature of Metastatic Inefficiency. *Am. J. Pathol.* **153,** 865–873 (1998).
129. Chambers, A. F., Groom, A. C. & MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* **2,** 563–72 (2002).
130. Joyce, J. a & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* **9,** 239–52 (2009).
131. Mintz, B. & Illmensee, K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3585–9 (1975).
132. Weaver, V. M. *et al.* Reversion of the malignant phenotype of human breast cells in three- dimensional culture and in vivo by integrin blocking antibodies. *J. Cell Biol.* **137,** 231–245 (1997).
133. Nelson, C. M. & Bissell, M. J. Of Extracellular Matrix, Scaffolds, and Signaling: Tissue Architecture Regulates Development, Homeostasis, and Cance. *Annu Rev Cell Dev Biol* **22,** 287–309 (2006).
134. Olumi, A. F. *et al.* Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* **59,** 5002–5011 (1999).
135. Hayashi, N. & Cunha, G. R. Mesenchyme-induced changes in the neoplastic characteristics of the Dunning prostatic adenocarcinoma. *Cancer Res.* **51,** 4924–4930 (1991).
136. J, E. *Neoplastic diseases*.
137. S, P. the distribution of secondary growths in cancer of the breast. *Lancet* **1,** 571–573 (1889).
138. Gupta, G. P. & Massagué, J. Cancer Metastasis: Building a Framework. *Cell* **127,** 679–695 (2006).
139. Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche Rosandra. *Nature* **438,** 820–827 (2005).
140. Hiratsuka, S., Watanabe, A., Aburatani, H. & Maru, Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat. Cell Biol.* **8,** 1369–75 (2006).
141. Psaila, B. & Lyden, D. The metastatic niche: adapting the foreign soil. *Nat. Rev. Cancer* **9,** 285–93 (2009).
142. Müller, a *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410,** 50–6 (2001).
143. Irmisch, A. & Huelsken, J. Metastasis: New insights into organ-specific

extravasation and metastatic niches. *Exp. Cell Res.* **319,** 1604–1610 (2013).
144. Aguirre-Ghiso, J. a. Models, mechanisms and clinical evidence for cancer dormancy. *Nat. Rev. Cancer* **7,** 834–846 (2008).
145. Quail, D. & Joyce, J. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19,** 1423–1437 (2013).
146. Ryder, M., Ghossein, R. A., Ricarte-Filho, J. C. M., Knauf, J. A. & Fagin, J. A. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. *Endocr. Relat. Cancer* **15,** 1069–1074 (2008).
147. Zhu, X.-D. *et al.* High Expression of Macrophage Colony-Stimulating Factor-1 Receptor in Peritumoral Liver Tissue Is Associated with Poor Outcome in Hepatocellular Carcinoma After Curative Resection. *J. Clin. Oncol.* **26,** 2707–2716 (2008).
148. Kim, D.-W. *et al.* High tumour islet macrophage infiltration correlates with improved patient survival but not with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer. *Br. J. Cancer* **98,** 1118–24 (2008).
149. Biswas, S. K., Allavena, P. & Mantovani, A. Tumor-associated macrophages: Functional diversity, clinical significance, and open questions. *Semin. Immunopathol.* **35,** 585–600 (2013).
150. Qian, B. *et al.* CCL2 recruits inflammtatory monocytes to facilitate breast tumour metastasis. **475,** 222–225 (2012).
151. Linde, N. *et al.* Vascular Endothelial Growth Factor Induced Skin Carcinogenesis depends on Recruitment and Alternative Activation of Macrophages. *J. Pathol.* **1,** 17–28 (2012).
152. Lin, E. Y. *et al.* Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages. *Mol. Oncol.* **1,** 288–302 (2007).
153. Movahedi, K. *et al.* Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* **70,** 5728–5739 (2010).
154. Qian, B.-Z. & Pollard, J. W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141,** 39–51 (2010).
155. Balkwill, F., Charles, K. A. & Mantovani, A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7,** 211–217 (2005).
156. Balkwill, F. R. & Mantovani, A. Cancer-related inflammation: Common themes and therapeutic opportunities. *Semin. Cancer Biol.* **22,** 33–40 (2012).
157. Qian, B. Z. & Pollard, J. W. Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell* **141,** 39–51 (2010).
158. Murdoch, C., Muthana, M., Coffelt, S. B. & Lewis, C. E. The role of

myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* **8,** 618–631 (2008).

159. Huang, S. *et al.* Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. *J. Natl. Cancer Inst.* **94,** 1134–1142 (2002).
160. Granata, F. *et al.* Production of vascular endothelial growth factors from human lung macrophages induced by group IIA and group X secreted phospholipases A2. *J. Immunol.* **184,** 5232–41 (2010).
161. Wang, R. *et al.* Tumor-associated macrophages provide a suitable microenvironment for non-small lung cancer invasion and progression. *Lung Cancer* **74,** 188–196 (2011).
162. Goswami, S. *et al.* Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res.* **65,** 5278–83 (2005).
163. Wyckoff, J. *et al.* A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* **64,** 7022–7029 (2004).
164. Hernandez, L. *et al.* The EGF/CSF-1 paracrine invasion loop can be triggered by heregulin beta1 and CXCL12. *Cancer Res.* **69,** 3221–7 (2009).
165. Sangaletti, S. *et al.* Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer Res.* **68,** 9050–9059 (2008).
166. Gocheva, V. *et al.* IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev.* **24,** 241–255 (2010).
167. Noy, R. & Pollard, J. W. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* **41,** 49–61 (2014).
168. Hanahan, D. & Coussens, L. M. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell* **21,** 309–322 (2012).
169. S, F. *et al.* VCAM-1 and VAP-1 recruit myeloid cells that promote pulmonary metastasis in mice. *Blood* **121,** 3289–3297 (2013).
170. Gao, D. *et al.* Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer Res.* **72,** 1384–1394 (2012).
171. Sica, A. *et al.* Autocrine Production of IL-10 Mediates Defective IL-12 Production and NF- B Activation in Tumor-Associated Macrophages. *J. Immunol.* **164,** 762–767 (2000).
172. Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **10,** 942–949 (2004).
173. Liu, J. *et al.* Tumor-associated macrophages recruit CCR6+ regulatory T

cells and promote the development of colorectal cancer via enhancing CCL20 production in mice. *PLoS One* **6,** 1–13 (2011).

174. Kuang, D.-M. *et al.* Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J. Exp. Med.* **206,** 1327–1337 (2009).
175. Chang, C., Liao, J. C., Kuo, L. & Cytotoxicity, O. T. Macrophage Arginase Promotes Tumor Cell Growth and Suppresses Nitric Oxide-mediated Tumor Cytotoxicity Macrophage Arginase Promotes Tumor Cell Growth and Suppresses Nitric. 1100–1106 (2001).
176. Gotts, J. E. & Matthay, M. A. Sepsis: pathophysiology and clinical management. *Bmj* i1585 (2016). doi:10.1136/bmj.i1585
177. Singer, M. *et al.* The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *Jama* **315,** 801–10 (2016).
178. Lever, A. & Mackenzie, I. Sepsis: definition, epidemiology, and diagnosis. *Bmj* **335,** 879–883 (2007).
179. Angele, M. K., Pratschke, S., Hubbard, W. J. & Chaudry, I. H. Gender differences in sepsis: cardiovascular and immunological aspects. *Virulence* **5,** 12–9 (2014).
180. Li, J., Carr, B., Goyal, M. & Gaieski, D. F. Sepsis: the inflammatory foundation of pathophysiology and therapy. *Hosp. Pract. (1995)* **39,** 99–112 (2011).
181. Seeley, E. J., Matthay, M. a. & Wolters, P. J. Inflection points in sepsis biology: from local defense to systemic organ injury. *AJP Lung Cell. Mol. Physiol.* **303,** L355–L363 (2012).
182. Fernandes, D. & Assreuy, J. Nitric oxide and vascular reactivity in sepsis. *Shock* **30 Suppl 1,** 10–3 (2008).
183. Brown, K. A., Brown, G. A., Lewis, S. M., Beale, R. & Treacher, D. F. Targeting cytokines as a treatment for patients with sepsis: A lost cause or a strategy still worthy of pursuit? *Int. Immunopharmacol.* **36,** 291–299 (2016).
184. Lopez-Bojorquez, L. N. *et al.* Molecular Mechanisms Involved in the Pathogenesis of Septic Shock. *Arch. Med. Res.* **35,** 465–479 (2004).
185. Cohen, J. The immunopathogenesis of sepsis. *Nature* **420,** 885–891 (2002).
186. Hawlisch, H. *et al.* C5a negatively regulates toll-like receptor 4-induced immune responses. *Immunity* **22,** 415–426 (2005).
187. Rittirsch, D. *et al.* Functional roles for C5a receptors in sepsis. *Nat. Med.* **14,** 551–557 (2008).
188. Riedemann, N. C. *et al.* Regulatory role of C5a on macrophage migration inhibitory factor release from neutrophils. *J. Immunol.* **173,** 1355–1359 (2004).
189. Ward, P. a. The dark side of C5a in sepsis. *Nat. Rev. Immunol.* **4,** 133–42 (2004).

190. Pomara, C. *et al.* A Pathophysiological Insight into Sepsis and Its Correlation with Postmortem Diagnosis. *Mediators Inflamm.* **2016,** (2016).
191. Huber-Lang, M. S. *et al.* Complement-Induced Impairment of Innate Immunity During Sepsis. *J. Immunol.* **169,** 3223–3231 (2002).
192. Huber-Lang, M. *et al.* Role of C5a in multiorgan failure during sepsis. *J. Immunol.* **166,** 1193–1199 (2001).
193. Laudes, I. J. *et al.* Anti-c5a ameliorates coagulation/fibrinolytic protein changes in a rat model of sepsis. *Am. J. Pathol.* **160,** 1867–1875 (2002).
194. Wojta, J. *et al.* C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils. *Blood* **100,** 517–523 (2002).
195. Muhlfelder, T. W. *et al.* C5 chemotactic fragment induces leukocyte production of tissue factor activity. A link between complement and coagulation. *J. Clin. Invest.* **63,** 147–150 (1979).
196. Rittirsch, D., Flierl, M. A. & Ward, P. A. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol* **8,** 776–787 (2009).
197. Maugeri, N. *et al.* Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation. *J. Thromb. Haemost.* **4,** 1323–1330 (2006).
198. Zeerleder, S., Schroeder, V., Erik, H. C., Peter, K. H. & A, W. W. TAF1 and PAI-1 levels in human sepsis. *Thromb. Res.* **118,** 205–212 (2006).
199. Danese, S., Vetrano, S., Zhang, L., Poplis, V. A. & Castellino, F. J. The protein C pathway in tissue inflammation and injury: pathogenic role and therapeutic implications. *Inflammation* **115,** 1121–1130 (2010).
200. Levi, M. & Van Der Poll, T. Endothelial injury in sepsis. *Intensive Care Med.* **39,** 1839–1842 (2013).
201. Ito, T. *et al.* High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. *J. Thromb. Haemost.* **5,** 109–16 (2007).
202. Bockmeyer, C. L. *et al.* Inflammation-associated ADAMTS13 deficiency promotes formation of ultra-large von Willebrand factor. *Haematologica* **93,** 137–140 (2008).
203. Furie, B. & Furie, B. C. Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. *Trends Mol. Med.* **10,** 171–178 (2004).
204. Martinod, K., Wagner, D. D., Martinod, K. & Wagner, D. D. Thrombosis : tangled up in NETs. **123,** 2768–2776 (2014).
205. Opal, S. M. & van der Poll, T. Endothelial barrier dysfunction in septic shock. *J. Intern. Med.* **277,** 277–293 (2015).
206. Huber-Lang, M. *et al.* Generation of C5a in the absence of C3: a new complement activation pathway. *Nat. Med.* **12,** 682–687 (2006).
207. JM Stouthard, M Levi, C E Hack, C H Veenhof, H A Romijn, H P Sauerwein, T. van der P. Interleukin-6 stimulates coagulation, not

fibinolysis, in humans. *Thromb. Haemost.* **76,** (1996).
208. Gogos, C. A., Drosou, E., Bassaris, H. P. & Skoutelis, A. Pro-versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J. Infect. Dis.* **181,** 176–180 (2000).
209. Hotchkiss, R. S. *et al.* Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans. *J. Immunol.* **166,** 6952–63 (2001).
210. Hotchkiss, R. S. *et al.* Depletion of dendritic cells, but not macrophages, in patients with sepsis. *J. Immunol.* **168,** 2493–2500 (2002).
211. Hotchkiss, R. S., Monneret, G. & Payen, D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat. Rev. Immunol.* **13,** 862–74 (2013).
212. Tsujimoto, H. *et al.* Role of Toll-Like Receptors in the Development of Sepsis. *Shock* **29,** 1 (2007).
213. Bouchon, a, Dietrich, J. & Colonna, M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* **164,** 4991–4995 (2000).
214. Bouchon, a, Facchetti, F., Weigand, M. a & Colonna, M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* **410,** 1103–1107 (2001).
215. Meziani, F., Delabranche, X., Asfar, P. & Toti, F. Bench-to-bedside review: circulating microparticles--a new player in sepsis? *Crit Care* **14,** 236 (2010).
216. Engelmann, B. & Massberg, S. Thrombosis as an intravascular effector of innate immunity. *Nat. Rev. Immunol.* **13,** 34–45 (2013).
217. Dinarello, C. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* **117,** 3720–3733 (2015).
218. Lotze, M. T. & Tracey, K. J. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* **5,** 331–42 (2005).
219. Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418,** 191–195 (2002).
220. Baer, C., Squadrito, M. L., Iruela-Arispe, M. L. & De Palma, M. Reciprocal interactions between endothelial cells and macrophages in angiogenic vascular niches. *Exp. Cell Res.* **319,** 1626–1634 (2013).
221. Cavaillon, J.-M. & Adib-Conquy, M. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Crit. Care* **10,** 233 (2006).
222. Porta, C. *et al.* Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kB. **2,** 2–7 (2009).

223. Shalova, I. N. *et al.* Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1a. *Immunity* **42,** 484–498 (2015).
224. López-Collazo, E. & del Fresno, C. Pathophysiology of endotoxin tolerance: mechanisms and clinical consequences. *Crit. Care* **17,** 242 (2013).
225. Motwani, M. P. & Gilroy, D. W. Macrophage development and polarization in chronic inflammation. *Semin. Immunol.* **27,** 257–266 (2015).
226. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249,** 1431–3 (1990).
227. Brown, J., Wang, H., Hajishengallis, G. N. & Martin, M. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *J Dent Res* **90,** 417–427 (2011).
228. Kagan, J. C. & Medzhitov, R. Phosphoinositide-Mediated Adaptor Recruitment Controls Toll-like Receptor Signaling. *Cell* **125,** 943–955 (2006).
229. Fitzgerald, K. A., Palsson-mcdermott, E. M., Bowie, A. G., Jefferies, C. A. & Mansell, A. S. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. **413,** (2001).
230. Li, S., Strelow, A., Fontana, E. J. & Wesche, H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5567–5572 (2002).
231. Ghosh, S., May, M. J. & Kopp, E. B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–60 (1998).
232. Yamamoto, M., Sato, S. & Hemmi, H. Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. *Science (80-.).* **301,** 640–643 (2003).
233. Yamamoto, M. *et al.* TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* **4,** 1144–50 (2003).
234. Doyle, S. *et al.* IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17,** 251–263 (2002).
235. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. & Goeddel, D. V. TRAF6 is a signal transducer for interleukin-1. *Nature* **383,** 443–446 (1996).
236. Häcker, H. *et al.* Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* **439,** 204–207 (2006).
237. Kyriakis, J. M. & Avruch, J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol. Rev.* **92,** 689–737 (2012).

238. Tidyman, W. E. & Rauen, K. a. *Mutational and functional analysis in human Ras/MAP kinase genetic syndromes. Methods in molecular biology (Clifton, N.J.)* **661,** (2010).
239. Roskoski, R. ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacol. Res.* **66,** 105–143 (2012).
240. Liu, Y. S., Shepherd, E. G. & Nelin, L. D. MAPK phosphatases - regulating the immune response. *Nat. Rev. Immunol.* **7,** 202–212 (2007).
241. McKay, M. M. & Morrison, D. K. Integrating signals from RTKs to ERK/MAPK. *Oncogene* **26,** 3113–21 (2007).
242. Dumitru, C. D. *et al.* TNF-alpha Induction by LPS iks Regulated Posttranscriptionally via a Tpl2 / ERK-Dependent Pathway. *Cell* **103,** 1071–1083 (2000).
243. Watford, W. T. *et al.* Ablation of Tumor Progression Locus 2 Promotes a Type 2 Th Cell Response in Ovalbumin-Immunized Mice. *J. Immunol.* **184,** 105–113 (2010).
244. Stambolic, V. *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor repressor PTEN. *Cell* **95,** 29 (1998).
245. Hemmings, B. A. & Restuccia, D. F. PI3K-PKB / Akt Pathway. *Cold Spring Harb Perspect Biol* 1–4 (2012). doi:10.1101/cshperspect.a011189
246. Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X. & Han, J. Activation and phosphorylation of a pleckstrin homology domain protein phosphatase inhibitors containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. **93,** 5699–5704 (1996).
247. Brognard, J., Sierecki, E., Gao, T. & Newton, A. C. PHLPP and a Second Isoform, PHLPP2, Differentially Attenuate the Amplitude of Akt Signaling by Regulating Distinct Akt Isoforms. *Mol. Cell* **25,** 917–931 (2007).
248. Laird, M. H. W. *et al.* TLR4/MyD88/PI3K interactions regulate TLR4 signaling. *J. Leukoc. Biol.* **85,** 966–77 (2009).
249. Fukao, T. & Koyasu, S. PI3K and negative regulation of TLR signaling. *Trends Immunol.* **24,** 358–363 (2003).
250. Martin, M. *et al.* Role of the Phosphatidylinositol 3 Kinase-Akt Pathway in the Regulation of IL-10 and IL-12 by Porphyromonas gingivalis Lipopolysaccharide. *J. Immunol.* **171,** 717–725 (2003).
251. Martin, M., Rehani, K., Jope, R. S. & Michalek, S. M. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* **6,** 777–784 (2005).
252. Weichhart, T. *et al.* The TSC-mTOR Signaling Pathway Regulates the Innate Inflammatory Response. *Immunity* **29,** 565–577 (2008).
253. Aksoy, E. *et al.* Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-kB activation and IFN-beta synthesis downstream of Toll-like receptor 3 and 4. *Eur. J. Immunol.* **35,** 2200–2209 (2005).

254. Yang, J. C.-S. *et al.* Inhibition of the phosphoinositide 3-kinase pathway decreases innate resistance to lipopolysaccharide toxicity in TLR4 deficient mice. *J. Biomed. Sci.* **21,** 20 (2014).
255. Straub, R. H. The complex role of estrogens in inflammation. *Endocr. Rev.* **28,** 521–574 (2007).
256. Wichmann, M. W., Inthorn, D., Andress, H. J. & Schildberg, F. W. Incidence and mortality of severe sepsis in surgical intensive care patients: the influence of patient gender on disease process and outcome. *Intensive Care Med* **26,** 167–172 (2000).
257. Frink, M. *et al.* Influence of Sex and Age on Mods and Cytokines After Multiple Injuries. *Shock* **27,** 151–156 (2007).
258. Paavonen, B. Y. T., Andersson, L. C. & Adlercreutz, H. Regulation of Estradiol Enhances Human B cell Maturation via Inhibition of Suppressor T Cells in Pokeweed Mitogen-stimulated cultures. *J. Exp. MEd* **154,** 1935–1945 (1981).
259. Kanda, N. & Tamaki, K. Estrogen enhances immunoglobulin production by human PBMCs. *J. Allergy Clin. Immunol.* **103,** 282–288 (1999).
260. Medina, K. L. & Kincade, P. W. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5382–5386 (1994).
261. Mukherjee, T. K., Nathan, L., Dinh, H., Reddy, S. T. & Chaudhuri, G. 17-Epiestriol, an estrogen metabolite, is more potent than estradiol in inhibiting vascular cell adhesion molecule 1 (VCAM-1) mRNA expression. *J. Biol. Chem.* **278,** 11746–11752 (2003).
262. Caulin-Glaser T., Watson C.A., Pardi R., B. J. R. Effects of 17beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression. **98,** 36–42 (1996).
263. Tomaszewska A, Guevara I, Wilczok T, D.-K. A. 17beta-estradiol- and lipopolysaccharide-induced changes in nitric oxide, tumor necrosis factor-alpha and vascular endothelial growth factor release from RAW 264.7 macrophages. *Gynecol Obs. Invest* **56,** 152–159 (2003).
264. Kramer, P. R., Kramer, S. F. & Guan, G. 17beta-estradiol regulates cytokine release through modulation of CD16 expression in monocytes and monocyte-derived macrophages. *Arthritis Rheum.* **50,** 1967–1975 (2004).
265. Ghisletti, S., Meda, C., Maggi, A. & Vegeto, E. 17beta-Estradiol Inhibits Inflammatory Gene Expression by Controlling NF- κ B Intracellular Localization. *Mol. Cell. Biol.* **25,** 2957–2968 (2005).
266. Janis, K. *et al.* Estrogen decreases expression of chemokine receptors, and suppresses chemokine bioactivity in murine monocytes. *Am. J. Reprod. Immunol.* **51,** 22–31 (2004).
267. Sharawy, N. *et al.* Estradiol receptors agonists induced effects in rat intestinal microcirculation during sepsis. *Microvasc. Res.* **85,** 118–127

(2013).
268. Zhu, H., Shan, L. & Peng, T. Rac1 mediates sex difference in cardiac tumor necrosis factor-alpha expression via NADPH oxidase-ERK1/2/p38 MAPK pathway in endotoxemia. *J. Mol. Cell. Cardiol.* **47,** 264–274 (2009).
269. Weniger, M., D'Haese, J. G., Angele, M. K. & Chaudry, I. H. Potential therapeutic targets for sepsis in women. *Expert Opin. Ther. Targets* **19,** 1531–1543 (2015).
270. Yu, H.-P. *et al.* Salutary effects of estrogen receptor-beta agonist on lung injury after trauma-hemorrhage. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290,** L1004-9 (2006).
271. Amand Mathieu, Erpicum Charlotte, Gilles Christine, Noel Agnes, R. S. functional analysis of dual specificity phosphatases in angiogenesis. *Methods Mol Biol* **1447,** 331–349 (2016).
272. Yuvaniyama, J., Denu, J. M., Dixon, J. E. & Saper, M. A. Crystal structure of the dual specificity protein phosphatase VHR. *Science (80-.).* **272,** 1328–1331 (1996).
273. Galdiero, M. R. *et al.* Tumor associated macrophages and neutrophils in cancer. *Immunobiology* **218,** 1402–1410 (2013).
274. Lewis, C. E. & Pollard, J. W. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* **66,** 605–612 (2006).
275. 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).
276. Laoui, D. *et al.* Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. *Cancer Res.* **74,** 24–30 (2014).
277. Rosas, M., Thomas, B., Stacey, M., Gordon, S. & Taylor, P. R. The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *J Leukoc Biol* **88,** 169–180 (2010).
278. Saigusa, S. *et al.* Decreased expression of DUSP4 is associated with liver and lung metastases in colorectal cancer. *Med. Oncol.* **30,** (2013).
279. Wu, S. *et al.* Decreased expression of dual-specificity phosphatase 9 is associated with poor prognosis in clear cell renal cell carcinoma. *BMC Cancer* **11,** 413 (2011).
280. Galaup, A. *et al.* Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 18721–18726 (2006).
281. Criscuoli, M. L., Nguyen, M. & Eliceiri, B. P. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability Tumor metastasis but not tumor growth is dependent on Src-mediated

vascular permeability. *Vascular* **105,** 1508–1514 (2008).
282. Satoh, H. *et al.* Nrf2-deficiency creates a responsive microenvironment for metastasis to the lung. *Carcinogenesis* **31,** 1833–1843 (2010).
283. Zhong, H. *et al.* Origin and pharmacological modulation of tumor-associated regulatory dendritic cells. *Int. J. Cancer* **134,** 2633–2645 (2014).
284. Mantovani, A. & Sica, A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr. Opin. Immunol.* **22,** 231–237 (2010).
285. Pérez-Rial, S. *et al.* Role of Recently Migrated Monocytes in Cigarette Smoke-Induced Lung Inflammation in Different Strain of Mice. *PLoS One* **8,** 1–9 (2013).
286. Yoshimura, T., Liu, M., Chen, X., Li, L. & Wang, J. M. Crosstalk between tumor cells and macrophages in stroma renders tumor cells as the primary source of MCP-1/CCL2 in Lewis lung carcinoma. *Front. Immunol.* **6,** 1–10 (2015).
287. Sounni, N. E. *et al.* Blocking lipid synthesis overcomes tumor regrowth and metastasis after antiangiogenic therapy withdrawal. *Cell Metab.* **20,** 280–294 (2014).
288. Ebos, J. M. L. *et al.* Accelerated Metastasis after Short-Term Treatment with a Potent Inhibitor of Tumor Angiogenesis. *Cancer Cell* **15,** 232–239 (2009).
289. Wong, P. P. *et al.* Dual-action combination therapy enhances angiogenesis while reducing tumor growth and spread. *Cancer Cell* **27,** 123–137 (2015).
290. Donnem, T. *et al.* Vessel co-option in primary human tumors and metastases: An obstacle to effective anti-angiogenic treatment? *Cancer Med.* **2,** 427–436 (2013).
291. Frentzas, S. *et al.* Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. *Nat. Med.* **22,** 1294–1302 (2016).
292. Kuczynski, E. A. *et al.* Co-option of liver vessels and not sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. *J. Natl. Cancer Inst.* **108,** 1–13 (2016).
293. Rittirsch, D., Huber-lang, M. S., Flierl, M. a & Ward, P. a. Immunodesign of experimental sepsis by cecal lingation and puncture. *Nat protoc* **4,** 31–36 (2009).
294. Ghosn, E. E. B. *et al.* Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 2568–73 (2010).
295. Klein, S. L. & Roberts, C. W. *Sex hormones and immunity to infection*. *Most* (2010). doi:10.1007/978-3-642-02155-8
296. Verthelyi, D. Sex hormones as immunomodulators in health and

disease. *Int. Immunopharmacol.* **1,** 983–993 (2001).
297. De La Rica, A. S., Gilsanz, F. & Maseda, E. Epidemiologic trends of sepsis in western countries. *Ann. Transl. Med.* **4,** 325–325 (2016).
298. Shankar-Hari, M. *et al.* Evidence for a causal link between sepsis and long-term mortality: a systematic review of epidemiologic studies. *Crit. Care* **20,** 101 (2016).
299. Beery, T. . Sex differences in infection and sepsis. *Crit Care Nurs Clin North Am.* **15,** 55–62 (2003).
300. Angele, MK, Schwacha MG, Ayala A, C. I. Effect of gender and sex hormones on immune responses following shock. *Shock* **14,** 81–90 (2000).
301. Tsuyuguchi, K. *et al.* Effect of oestrogen on Mycobacterium avium complex pulmonary infection in mice. *Clin Exp Immunol* **123,** 428–434 (2001).
302. Leone, M., Textoris, J., Capo, C. & Mege, J. Sex Hormones and Bacterial Infections. *Culture* **15,** 100–0 (2012).
303. Fairweather, D. & Cihakova, D. Alternatively activated macrophages in infection and autoimmunity. *J. Autoimmun.* **33,** 222–230 (2009).
304. Bolego, C., Cignarella, A., Staels, B. & Chinetti-Gbaguidi, G. Macrophage function and polarization in cardiovascular disease a role of estrogen signaling? *Arterioscler. Thromb. Vasc. Biol.* **33,** 1127–1134 (2013).
305. Ribas, V. *et al.* Myeloid-specific estrogen receptor deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *Proc. Natl. Acad. Sci.* **109,** 645–645 (2012).
306. Carreras E, Turner S, Frank MB, Knowlton N, Osban J, Centola M, Park CG, Simmons A, Alberola-lla J, K. S. Estrogen receptor signaling promotes dendritic cell differentiation by increasing expression of the transcription factor IRF4. *Blood* **115,** 238–246 (2010).
307. Angele, M. K. *et al.* Sex steroids regulate pro- and anti-inflammatory cytokine release by macrophages after trauma-hemorrhage. *Am. J. Physiol.* **277,** C35–C42 (1999).
308. Srivastava, S. *et al.* Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD. *J. Clin. Invest.* **104,** 503–513 (1999).
309. Ray, P., Ghosh, S. K., Zhang, D.-H. & Ray, A. Repression of interleukin-6 gene expression by 17β-estradiol: *FEBS Lett.* **409,** 79–85 (1997).
310. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P. P. Phosphorylation and functional inactivation of TSC2 by ERK: Implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121,** 179–193 (2005).
311. Byles, V., Covarrubias, A. J., Ben-sahra, I. & Lamming, D. W. The TSC-mTOR pathway regulates macrophages polarization. *Nat. Commun.* **4,** (2013).

312. Inoki, K., Li, Y., Zhu, T., Wu, J. & Guan, K.-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* **4,** 648–57 (2002).
313. Gard, T., Hoge, E. A. & Kerr, C. Control of macrophage metabolism and activation by mTOR and Akt signaling. *Semin. Immunol.* **27,** 286–296 (2015).
314. Jeffrey, K. L., Camps, M., Rommel, C. & Mackay, C. R. Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. *Nat. Rev. Drug Discov.* **6,** 391–403 (2007).
315. Jenkins, S. J. *et al.* Local Macrophage Proliferation, Rather than Recruitment from the Blood, Is a Signature of TH2 Inflammation. *Sciences (New. York).* **32,** 1284–1288 (2011).
316. Lin, E. Y., Nguyen, A. V, Russell, R. G. & Pollard, J. W. Colony-stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. *J. Exp. Med.* **193,** 727–739 (2001).
317. Lu, B. *et al.* Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187,** 601–8 (1998).
318. Van Damme, J., Proost, P. & Lenaerts, J. Structural and Functional Identification of Two Human, Tumor-derived Monocyte Chemotactic Proteins (MCP-2 and MCP-3) Belonging to the Chemokine Family. *J. Exp. Med.* **176,** 59–65 (1992).
319. Kim, J. *et al.* Transforming growth factor- □ 1 regulates macrophage migration via RhoA. *Blood* **108,** 1821–1829 (2006).
320. Nishiguchi, T. *et al.* Macrophage polarization and MRSA infection in burned mice. *Immunol Cell Biol* 1–36 (2016). doi:10.1038/icb.2016.84
321. Tsuchimoto, Y. *et al.* M2b Monocytes Provoke Bacterial Pneumonia and Gut Bacteria-Associated Sepsis in Alcoholics. *J. Immunol.* **195,** 5169–5177 (2015).
322. Hotchkiss R, K. I. The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* 1–19 (2003).
323. Rettew, J. A., Huet, Y. M. & Marriott, I. Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. *Endocrinology* **150,** 3877–3884 (2009).
324. Silverstein, A. M., Barrow, C. a, Davis, A. J. & Mumby, M. C. Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4221–4226 (2002).
325. Mitsuhashi, S. *et al.* Usage of tautomycetin, a novel inhibitor of protein phosphatase 1 (PP1), reveals that PP1 is a positive regulator of Raf-1 in vivo. *J. Biol. Chem.* **278,** 82–88 (2003).
326. Deb, T. *et al.* A Common Requirement for the Catalytic Activity and Both SH2 Domains of SHP-2 in Mitogen-activated Protein (MAP) Kinase

Activation by the ErbB Family of Receptors. *J Biol Chem* **273,** 16643–16646 (1998).

327. Gronda, M. *et al.* Hematopoietic Protein Tyrosine Phosphatase Suppresses Extracellular Stimulus-Regulated Kinase Activation Hematopoietic Protein Tyrosine Phosphatase Suppresses Extracellular Stimulus-Regulated Kinase Activation. **21,** 6851–6858 (2001).
328. Pulido, R., Zúñiga, Á. & Ullrich, A. PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J.* **17,** 7337–7350 (1998).
329. Chen, D., Waters, S. B., Holt, K. H. & Pessin, J. E. SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways. *J. Biol. Chem.* **271,** 6328–6332 (1996).
330. Frame, M. C. Src in cancer: Deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta - Rev. Cancer* **1602,** 114–130 (2002).
331. Kurosaki, B. T. *et al.* Brief Det~nitive Report. **179,** 0–4 (1994).
332. Keselman, A. & Heller, N. Estrogen signaling modulates allergic inflammation and contributes to sex differences in asthma. *Front. Immunol.* **6,** (2015).
333. Levin, E. R. Cell localization, physiology, and non genomic actions of estrogen receptors. *J. Appl. Physiol.* **89,** 1860–1867 (2002).
334. Rettew, J. A., McCall, S. H. & Marriott, I. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. *Mol. Cell. Endocrinol.* **328,** 87–92 (2010).

Annexe

List of publications

Serkan Ismail Göktuna, Kateryna Shostak, Tieu-Lan Chau, Lukas C. Heukamp, Benoit Hennuy, Hong Quan Duong, Aurélie Ladang, Pierre Close, Iva Klevernic, Fabrice Olivier, Alexandra Florin, Grégory Ehx, Frédéric Baron, **Maud Vandereyken**, Souad Rahmouni, Lars Vereecke, Geert van Loo, Reinhard Büttner, Florian R. Greten and Alain Chariot. **The pro-survival IKK-related kinase IKKε integrates LPS and IL-17A signaling cascades to promote Wnt-dependent tumor development in the intestine.** *Cancer research,* 2016

Singh, P.*, Dejager L.*, Amand M.*, Theatre E., **Vandereyken M**., Zurashvili T., Singh M., Mack M., Timmermans S., Musumeci L., Dejardin E., Mustelin T., Van Ginderachter J. A., Moutschen M., Oury C., Libert C., Rahmouni, S. (2015, April 15). **DUSP3 genetic deletion confers M2-like–macrophage-dependent tolerance to septic shock**. *Journal of Immunology*, 2015

Musumeci L., Kuijpers M., Gilio K., Hego A., Theatre E., Maurissen L., **Vandereyken M.,** Diogo C., LECUT C., Guilmain W., Bobkova E., Eble J., Dahl R., Drion P., Rascon J., Mostofi Y., Yuan H., Sergienko E., Chung T., Thiry, M., Senis Y., MOUTSCHEN M., Mustelin T., Lancellotti P., Heemskerk J., Tautz L., Oury C., Rahmouni S. **DUSP3 Phosphatase Deficiency or Inhibition Limit Platelet Activation and Arterial Thrombosis**. *Circulation, 131*(7),656-68, 2015

Amand Mathieu, Erpicum Charlotte, Bajou Khalid, Cerignoli Fabio, Blacher Silvia, Martin Maud, Dequiedt Franck, Drion Pierre, Singh Pratibha, Tinatin Zurashvili, **Vandereyken Maud**, Musumeci Lucia, Mustelin Tomas, Moutschen Michel, Gilles Christine, Noel Agnes and Rahmouni Souad. **DUSP3/VHR is a pro-angiogenic atypical dual-specificity phosphatase** (*Molecular Cancer* 2014, **13**:108**)**

Under revision

Maud Vandereyken, Eva Van Overmeire, Mathieu Amand, Natacha Rocks, Céline Delierneux, Pratibha Singh, Maneesh Singh, Carolie Wathieu, Tinatin Zurashvili, Michel Moutschen, Agnès Noël, Christine Gilles, Didier Cataldo, Cécile Oury, Jo Van Ginderachter and Souad Rahmouni. **Dusp3 deletion promotes lung tumour metastasis in macrophages dependent manner.** Accepted with major revisions in Molecular Cancer

Maud Vandereyken, Caroline Wathieu, Prathiba Sing, Tinatin Zurashvilli, Lien Dejager, Mathieu Amand, Lucia Musumeci, Maneesh Sing, Cecile Oury, Michel Moutschen, Claude Libert and Souad Rahmouni. **Dual-specificity phosphatase 3 deletion protects female, but not male mice, from LPS-induced endotoxemia and polymicrobial-induced septic shock.** Submitted in Journal of Immunology

Delierneux, Céline; Donis, Nathalie; Servais, Laurence; Wéra, Odile; **Vandereyken, Maud**; Musumeci, Lucia; Lecut, Christelle; Rahmouni, Souad; Schneider, Eble, Johannes; Lancellotti, Patrizio; Oury, Cécile. **Targeting of C-type lectin-like receptor 2 or P2Y12 for the prevention of platelet activation by immunotherapeutic CpG oligodeoxynucleotides**. Accepted with major revisions in Journal of Thrombosis and Haemostasis.