DOCTOR OF SCIENCES

Study of the effects of cycling hypoxia on macrophages and on the interaction between macrophages and endothelial cells
role in the promotion of tumor inflammation

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Award date:
2021

Awarding institution:
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Study of the effects of cycling hypoxia on macrophages and on the interaction between macrophages and endothelial cells : role in the promotion of tumor inflammation

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Remerciements

Cette thèse n'aurait pu se faire sans l'aide de plusieurs personnes que je souhaite remercier.

Tout d'abord, je remercie les membres du jury : Olivier Feron, Fabrice Soncin, Catherine Lambert de Rouvroit, Carlos Graux et Jean-Pierre Gillet. Merci d'avoir pris le temps de lire le manuscrit, de vos remarques et améliorations apportées à ce manuscrit mais également pour les discussions fort enrichissantes, notamment durant la thèse privée et le comité d'accompagnement. Merci également à Olivier Feron pour la relecture critique du 1er et du 2e travail de cette thèse.

Je tiens à remercier fort chaleureusement mon promoteur de thèse, le Pr. Carine Michiels, qui m'a soutenu, même dans les moments difficiles. Merci pour votre encadrement tout au long de cette thèse, pour la correction des manuscrits, des idées scientifiques et de votre disponibilité, même suite à votre accession au poste de vice-rectrice à la recherche de l'UNamur. Merci beaucoup pour votre bonne humeur et votre positivité durant nos échanges. J'ai beaucoup appris à vos côtés.

Durant cette thèse, une collaboration entre l'UNamur/URBC et le SMMiL-E - une équipe de recherche située au sein de l'IRCL - a été réalisée. J'ai passé 3 mois au sein du SMMiL-E durant cette thèse, et je remercie tous les membres du SMMiL-E et de l'IRCL pour leur accueil fort chaleureux durant ces 3 mois. Je remercie tout particulièrement Fabrice Soncin et son équipe pour leur accueil et bonne humeur : Elise, Géraldine. Je remercie également Anthony qui interagit beaucoup avec le SMMiL-E pour son accueil et sa bonne humeur.

Je remercie fort chaleureusement Fabrice Soncin. Nous nous sommes tout d'abord côtoyés durant mon mémoire réalisé dans votre laboratoire à Lille, durant lequel j'ai beaucoup appris à vos côtés. Puis, durant cette thèse, vous avez été d'une aide précieuse, et nos discussions et vos conseils et idées ont permis de débloquer le projet à un moment où il était à l'arrêt. Merci pour votre implication avant, durant et après le séjour dans votre laboratoire. Merci également de votre temps passé à relire et améliorer le 2e travail de cette thèse.

Merci aux organismes financiers ayant permis à cette thèse de se dérouler. Merci tout particulièrement au Télévie et au FNRS pour m'avoir octroyé cette bourse de recherche et les frais de fonctionnements associés. Merci à ERASMUS, TRANSUNIV-Interreg et au FNRS pour le financement du séjour de 3 mois au sein du SMMiL-E. Merci également à NARILIS pour ses aides financières concernant les séminaires.

Je tiens à remercier les P.I et chercheur permanent présents au sein de l'URBC durant cette thèse : Thierry Arnould, Patsy Renard, Henri-François Renard, Florence Debacq-Chainiaux et Martine Raes. Merci à Thierry Arnould et Patsy Renard pour leur fonction de directeur du laboratoire durant ma thèse.

Je remercie également toute l'équipe technique du laboratoire, Maude, Marc, Antoine, Guy, Martine, Catherine, et Noëlle. Je remercie Marc pour sa bonne humeur quotidienne et son sens de l'humour. Merci à Catherine et Noëlle pour leur temps passé à prendre les photos de

mes IFs tout au long de ma thèse. Merci à Guy pour sa bonne humeur quotidienne et ses blagues. Merci à Maude et Antoine pour leur conseils techniques et leur temps alloué à la bonne marche du laboratoire.

Je remercie les membres de l'équipe TumHyp pour leur conseil et écoute durant les réunions d'équipe, ainsi que pour leur très bon gâteau apporté durant ces réunions. Je remercie François, Kathleen, Eleonore, Sophie, Géraldine, Jia-Wei, Christoph, ANK, Sébastien et Thomas. Je remercie également la « team macrophages », dans laquelle il y a eu beaucoup d'échanges de macrophages, avec beaucoup de simplicité. Merci à Géraldine de m'avoir formé aux macrophages et autres au début de ma thèse.

Je remercie également tous les copains du labo, Damien, Lola, Marino, Céline, Julie, Camille, Myriam, François, Géraldine, ANK, Sébastien, Joëlle, Maude, Alexis, Sophie. Je vous remercie pour tous les bons moments partagés ensemble, que ce soit au labo, ou en dehors. Le week-end dans le logement avec piscine et sauna, les sorties, « Beer-Hour » et les soirées ont été des super moments partagés avec vous. Je remercie les membres de mes 2 bureaux dans lesquels j'ai réalisé cette thèse, le bureau 5710 et 5715. Je remercie Kathleen, Eleonore, François, Joëlle, ANK et Sophie. Merci pour les discussions et rires partagés ensemble, ainsi qu'aux nombreuses aides de tous les jours apportés.

Enfin, je tiens à remercier Céline, ma famille et ma belle-famille. Un très grand merci à Céline pour tout ton soutien et ces très bons moments passés ensemble. Un énorme merci de m'avoir soutenu ainsi que pour ton aide tout au long de cette thèse et plus particulièrement dans la dernière ligne droite. Un grand merci pour ton écoute, tes conseils et tout ton soutien, particulièrement durant les moments difficiles. Merci d'avoir pris le temps de m'écouter et de m'avoir aidé dans les moments de découragements. Un tout grand merci à mon père, ma mère, Didier, Matthieu, et Simon, Amandine, Pierre, J, Jan et Céline pour votre soutien durant les moments difficiles, pour vos conseils et pour ces merveilleux moments passés ensemble, ainsi que pour les petits et grands bonheurs que vous me procurez.

Résumé

Deux types d'hypoxie impactent les tumeurs solides, l'hypoxie chronique et l'hypoxie cyclique. L'hypoxie chronique et l'hypoxie cyclique ont des causes différentes et induisent des effets différents dans les tumeurs. L'hypoxie chronique est caractérisée par une très faible oxygénation des cellules de la tumeur, et l'hypoxie cyclique à une alternance entre faible oxygénation (hypoxie) et réoxygénation de ces cellules. L'hypoxie cyclique promeut notamment une inflammation tumorale et la formation de métastases, deux caractéristiques associées à un mauvais pronostic. Les tumeurs sont composées de cellules cancéreuses, mais également de cellules non cancéreuses. Parmi ces dernières, les macrophages associés aux tumeurs représentent jusqu'à la moitié des cellules immunitaires infiltrant les tumeurs. Les macrophages sont très dépendants des conditions micro-environnementales. En fonction de ces conditions, ils peuvent être polarisés selon un axe de polarisation M1-M2, dans lequel les macrophages M1 sont pro-inflammatoires et les macrophages M2 anti-inflammatoires. De ce fait, ces leucocytes régulent fortement l'inflammation tumorale. Ils promeuvent également fortement la dissémination des cellules cancéreuses (appelée métastase), notamment via leur impact sur les vaisseaux sanguins tumoraux, et sur leur principale composante cellulaire, les cellules endothéliales. Les cellules endothéliales régulent fortement les métastases puisqu'ils constituent une barrière physique que les cellules cancéreuses doivent franchir à deux reprises afin d'envahir un second organe. En effet, les cellules cancéreuses doivent entrer dans les vaisseaux sanguins (par intravasation) puis en sortir (par extravasation) afin de se disséminer. De plus, les cellules endothéliales régulent fortement l'infiltration des cellules immunitaires au sein des tumeurs. Réciproquement, l'infiltration des monocytes dans les tumeurs secondaires augmente fortement les métastases. L'infiltration des cellules immunitaires et des cellules cancéreuses nécessite notamment leur adhérence sur les cellules endothéliales, suivi par leur extravasation. Les raisons de l'induction de l'inflammation par l'hypoxie cyclique sont encore inconnues, ainsi que l'effet de l'hypoxie cyclique sur les macrophages. Au cours de cette thèse, l'effet de l'hypoxie cyclique sur les macrophages murins et humains a été testé. Plus particulièrement, les macrophages M0 (non polarisé), M1 ou M2 ont été exposé à l'hypoxie cyclique, hypoxie chronique ou normoxie. L'impact de ces traitements sur le phénotype pro-inflammatoire des macrophages a été étudié. Ainsi, nous avons montré que l'hypoxie cyclique induit un phénotype pro-inflammatoire aux macrophages M0 et augmente le phénotype pro-inflammatoire des macrophages M1. L'effet de l'hypoxie cyclique est spécifique puisqu'il n'est pas observé avec l'hypoxie chronique. De plus, la voie de signalisation JNK/p65 est impliquée dans l'induction de ce phénotype dans les macrophages humains par l'hypoxie cyclique. Puisque les macrophages ont un fort impact sur les cellules endothéliales, l'effet des macrophages exposés à l'hypoxie cyclique sur le phénotype des cellules endothéliales a été étudié. Plus particulièrement, l'effet de ces macrophages sur le phénotype pro-inflammatoire des cellules endothéliales et sur leur capacité à permettre l'adhérence des monocytes et des cellules cancéreuses a été étudié. Nous avons montré que les macrophages exposés à l'hypoxie cyclique augmentent le phénotype pro-inflammatoire des cellules endothéliales, augmentent leur expression de molécules d'adhérence et augmentent la capacité des cellules endothéliales à permettre leur

adhérence pour les monocytes et les cellules cancéreuses. De façon intéressante, ces effets sont plus fortement induits par les macrophages exposés à l'hypoxie cyclique. Pour résumer, durant cette thèse, nous avons montré que l'hypoxie cyclique induit un phénotype pro-inflammatoire chez les macrophages M0 et M1 via la voie de signalisation JNK/p65. De plus, l'hypoxie cyclique potentialise l'impact des macrophages sur l'induction d'un phénotype pro-métastatique chez les cellules endothéliales. Ces résultats pourraient expliquer certains des mécanismes par lesquels l'hypoxie cyclique induit l'inflammation tumorale et favorise les métastases.

Abstract

Two types of hypoxia impact solid tumors, namely chronic hypoxia and cycling hypoxia. Chronic hypoxia and cycling hypoxia have different causes and induce different effects in tumors. Chronic hypoxia is characterized by a very low oxygenation of the cells in the tumor while cycling hypoxia is composed of cycles of low oxygenation (hypoxia) and reoxygenation of the cells in the tumor. Cycling hypoxia promotes, among other things, tumor inflammation and metastasis, both of which are associated with a poor prognosis. Tumors are composed of cancer cells, but also of non-malignant cells. Among the latter, tumor-associated macrophages account for up to half of the tumor-infiltrating immune cells. Macrophages are highly dependent on microenvironmental cues. Depending on these conditions, they can be polarized along an M1-M2 polarization axis, in which M1 macrophages are pro-inflammatory and M2 macrophages are anti-inflammatory. As a result, these cells strongly regulate tumor inflammation. They also strongly promote the dissemination of cancer cells (called metastasis), notably through their impact on tumor blood vessels, and on their main cellular component, the endothelial cells. Endothelial cells strongly regulate metastasis since they constitute a physical barrier that cancer cells must cross twice in order to invade a second organ. Indeed, cancer cells must enter the blood vessels (by intravasation) and then leave them (by extravasation) in order to disseminate. In addition, endothelial cells strongly regulate the infiltration of immune cells into tumors. Reciprocally, monocyte infiltration in secondary tumors strongly increases metastasis. The infiltration of immune cells and cancer cells requires, among other things, their adhesion to endothelial cells, followed by their extravasation. The reasons for the induction of inflammation by cycling hypoxia are still unknown, as is the effect of cycling hypoxia on macrophages. In this thesis, the effects of cycling hypoxia on murine and human macrophages were investigated. Specifically, M0 (unpolarized), M1 or M2 macrophages were exposed to cycling hypoxia, chronic hypoxia or normoxia. The impact of these treatments on the pro-inflammatory phenotype of macrophages was studied. Cycling hypoxia induced a pro-inflammatory phenotype in M0 macrophages and increased the pro-inflammatory phenotype of M1 macrophages. The effect of cycling hypoxia was specific as it was not observed with chronic hypoxia. Furthermore, the JNK/p65 signalling pathway was shown to be involved in the induction of this phenotype in human macrophages upon exposure to cycling hypoxia. Since macrophages have a strong impact on endothelial cells, the effect of macrophages exposed to cycling hypoxia on the phenotype of endothelial cells was studied. In particular, the effect of these macrophages on the pro-inflammatory phenotype of endothelial cells and on their ability to allow adhesion of monocytes and cancer cells was studied. We showed that macrophages exposed to cycling hypoxia increased the pro-inflammatory phenotype of endothelial cells, increased their expression of adhesion molecules and increased the ability of endothelial cells to allow monocytes and cancer cells to adhere to them. Interestingly, these effects were most strongly induced by macrophages exposed to cycling hypoxia. To summarize, during this thesis, we showed that cycling hypoxia induces a pro-inflammatory phenotype in M0 and M1 macrophages via JNK/p65 signaling pathway. Furthermore, cycling hypoxia potentiates the impact of macrophages on the induction of a pro-metastatic phenotype in endothelial cells.

These findings could explain some of the mechanisms by which cycling hypoxia induces tumor inflammation and metastasis.

Table of contents

Abbreviation list

ABCB1 : ATP-binding cassette B1

AP-1 : activator protein-1

Arg-1 : arginase 1

ATF : activating-transcription factor

BAFFR : B cell activation factor receptor

bFGF : basic fibroblast growth factor

BMDM : bone marrow-derived macrophage

CAC : colitis-associated cancer

CAFs : cancer-associated fibroblasts

CAM : chorioallantoic membrane

CCL : C-C motif chemokine ligand

chH : chronic hypoxia

COX2 : cyclooxygenase 2

CSCs : cancer stem cells

CSF1 : colony stimulating factor 1

CTCs : circulating tumor cells

CXCL : C-X-C motif chemokine ligand

cyH : cycling hypoxia

DSS : dextran sulfate sodium

ECs : endothelial cells

EGF : epidermal growth factor

EMT : epithelial to mesenchymal transition

EndMT : endothelial to mesenchymal transition

EPCs : endothelial progenitor cells

EPRI : electron paramagnetic resonance imaging

ERK : extracellular signal-regulated kinase

ESAM : endothelial cell selective adhesion molecule

ESL1 : E-selectin ligand 1

ETC : electron-transport chain

FasL : Fas ligand

FGF : fibroblast growth factor

GBM : glioblastoma

Gpx : glutathione peroxidase

GSK3 : glycogen synthase kinase 3

HIFs : hypoxia inducible factors

HUVECs : human umbilical vein endothelial cells

ICAM : intercellular adhesion molecule

IDO : indoleamine 2,3-dioxygenase

IFNγ : interferon γ

IFNγR : interferon γ receptor

IL- : interleukin-

IL4-RA : interleukin 4 receptor α chain

IL6-RA : interleukin 6 receptor α chain

iNOS : inducible nitric oxide synthase

IRF : interferon regulatory factor

IκBα : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α

JAMs : junctional adhesion molecules

JNK : c-jun N-terminal kinase

KD : knockdown

KO : knockout

L1CAM : L1 cell adhesion molecule

LCN2 : lipocalin 2

LFA1 : lymphocyte function-associated antigen 1

LLc : Lewis lung carcinoma

LM : leptomeningeal metastasis

LPS : lipopolysaccharide

LSECs : liver sinusoidal endothelial cells

LTβR : lymphotoxin β receptor

MAPK : mitogen-activated protein kinase

MAC1 : macrophage antigen 1

MMP9 : matrix metalloproteinase 9

MRI : magnetic resonance imaging

N : normoxia

NE : neutrophil elastase

NF-κB : nuclear factor κB

NOX : NADPH oxidase

NRF2 : nuclear factor erythroid 2-related factor 2

NRP : neuropilin

N-WASP : neural Wiskott–Aldrich syndrome protein

OSA : obstructive sleep apnea

PD-1 : programmed cell death protein 1

PD-L : programmed cell death ligand

PECAM1 : platelet endothelial cell adhesion molecule 1

PHDs : prolyl hydroxylase domain proteins

PI3K : phosphoinositide 3-kinase

PSGL1 : P-selectin ligand 1

pVHL : von Hippel-Lindau tumor suppressor protein

RCC : renal cell carcinoma

RNS : reactive nitrogen species

ROS : reactive oxygen species

SCID : severe combined immunodeficient

SLC25A1 : solute carrier family 25 member 1

SOD : superoxide dismutase

SPARC : secreted protein acidic and cysteine rich

ST6GALNAC5 : ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5

STAT : signal transducer and activator of transcription

TAMs : tumor-associated macrophages

TGF-β : transforming growth factor β

TIM3 : T cell immunoglobulin domain and mucin domain protein 3

TME : tumor microenvironment

TMEM : tumor microenvironment of metastasis

TNFR : tumor necrosis factor receptor

TNFα : tumor necrosis factor α

Tregs : T regulator lymphocytes

Trka : Tropomyosin receptor kinase A

uPA : urokinase-type plasminogen activator

uPAR : urokinase-type plasminogen activator receptor

VCAM1 : vascular adhesion molecule 1

VEGF : vascular endothelial growth factor

VLA4 : very late antigen 4

Foreword

Cancers are diseases which cause major problems to human health. According to world health organization, in 2016, cancers were the most frequently observed diseases worldwide (Mattiuzzi and Lippi, 2019). Furthermore, between 2000 and 2016, cancers were the second cause of mortality worldwide and the numbers of cancer-related deaths during these 15 past years increased (+28%). This is the 1st cause of mortality in most European country including France and Belgium (Bray et al., 2018).

There exist blood cancers (called leukemia) and solid tumors. The development of solid tumors can occur in almost all the tissues of the organism via the transformation of normal cells into cancer cells. During this transformation, normal cells acquire some characteristics to become cancerous such as unlimited proliferation, immortality and ability to disseminate in other regions of the body by a process called metastasis. Within tumors, non-cancer cells (also called stromal cells), blood vessels and physicochemical features are strongly involved and even sometimes needed in order to support tumor growth.

Among the stromal cells, immune cells highly regulate tumor growth. Among immune cells, macrophage is the type of immune cells which is the most frequent in almost all solid tumors (Gentles et al., 2015). Macrophages represent up to 50% of cancer immune infiltrate in solid tumors and the infiltration of these cells is associated with poor prognosis in most cancer types (Fridman et al., 2017). Macrophages are very plastic depending on microenvironmental cues. For example, macrophages can be polarized in either pro-inflammatory or anti-inflammatory macrophages depending on their environment. Macrophages are involved in several processes favoring tumor progression such as tumor-inflammation, tumor immunosuppression and the development of blood vessels from pre-existing one by a process called angiogenesis.

Blood vessels are also crucially involved in tumor progression. For example, tumors lacking blood vessels are often small in size (< 1-2 mm diameter), according to the tumor type, indicating the major involvement of these structures in the tumor progression. Indeed, an essential step in carcinogenesis of most solid tumors is the angiogenic switch. During this step, tumors change from an avascular phase to a vascular via the angiogenesis process. Tumor blood vessels are required for the supply of nutrients and O_2 to cancer cells and stromal cells thus allowing tumor growth. Blood vessels are composed of an endothelium which is a monolayer of endothelial cells which are in direct contact with the blood. Endothelial cells strongly regulate vascular biology, such as vascular dilation, thrombosis, angiogenesis and immune cell infiltration and activity. Tumor blood vessels are also involved in metastasis, notably via the regulation of cancer cell migration, intravasation and extravasation.

Macrophages and endothelial cells strongly interact within the tumor microenvironment (Delprat and Michiels, 2021). Endothelial cells regulate the infiltration of macrophages into tumor and their angiogenic phenotype. On the other hand, macrophages promote angiogenesis, lymphangiogenesis and change vascular phenotype into a pro-metastatic one.

The strong diminution in O_2 perfusion in tumor, called tumor hypoxia, is linked to poor prognosis. Two types of hypoxia occur in tumor, chronic and cycling hypoxia (Michiels et al.,

2016). Chronic hypoxia impacts malignant and stromal cells which are too far from blood vessels, mostly due to the fast proliferation of cancer cells. Cycling hypoxia occurs because of temporal occlusion occurring in tumor blood vessels, hence impacting cells which are located near to these tumor blood vessels. Cycling hypoxia corresponds to cycles of hypoxia and reoxygenation, whereas chronic hypoxia corresponds to a continuous period of hypoxia. Cycling hypoxia and chronic hypoxia activate some common but also some distinct signaling pathways in cancer and stromal cells. Cycling hypoxia promotes tumor angiogenesis, macrophage infiltration, tumor inflammation and metastasis. It also alters the communication between cells composing the tumor, such as between endothelial cells and cancer cells.

Since cycling hypoxia and macrophages regulate tumor inflammation, we wondered if the impact of cycling hypoxia on tumor inflammation could be due to the induction of a pro-inflammatory phenotype in macrophages. Furthermore, since cycling hypoxia, macrophages and endothelial cells regulate tumor inflammation and metastasis, the present thesis aimed to study the impact of cycling hypoxia on the communication between macrophages and endothelial cells. More particularly, the impacts of macrophages exposed to cycling hypoxia on endothelial cells pro-inflammatory and pro-metastatic phenotypes were studied.

In the first part of the introduction, a brief description of macrophage classification and polarization, as well as the impact of tumor-associated macrophages (TAMs) on tumor inflammation, metastasis, immune suppression will be described. Then, the role of blood vessels on tumor progression and metastasis and their impact on tumor immune system regulation will be the subject of the second part. The bi-directional dialog between monocytes/macrophages and vascular cells in the regulation of tumor progression will be addressed in the third part. Finally, the causes and impact of tumor hypoxia will be evoked in the fourth part of the introduction.

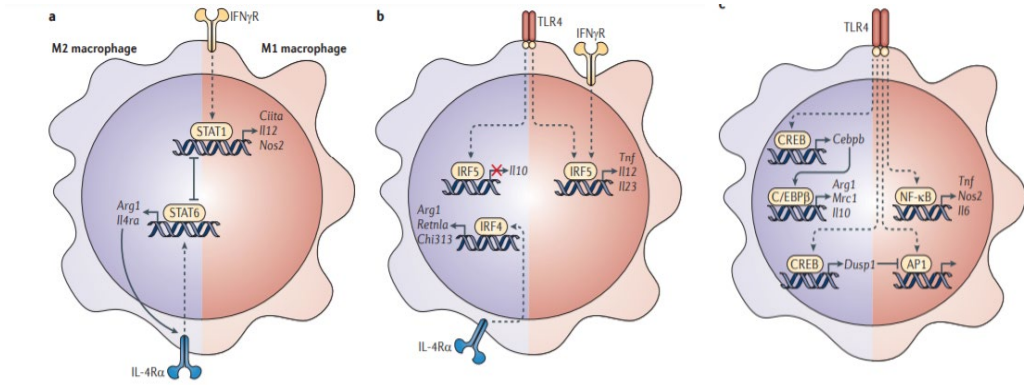

Fig. 1. Macrophage polarization signaling. Macrophages are polarized into M1 macrophages via IFNγR and TLR4 stimulation. IFNγR stimulation leads to the activation of STAT1 and IRF5, whereas TLR4 stimulation induces the activation of IRF5, NF-κB and AP1, leading to M1 polarization. Macrophages are polarized into M2 macrophages via IL-4R IL-10R and IL-6R stimulation. The stimulation of IL-4R triggers the activation of STAT6, whereas the stimulation of IL-6R and IL-10R induces the activation of STAT3, leading to M2 polarization (adapted from Lawrence and Natoli, 2011).

A. Introduction

1. Tumor-associated macrophages, cancer-related inflammation, metastasis and immunosurveillance/immunosuppression

Solid tumors are composed of cancer cells and stromal cells, which are all influenced by physicochemical features. Stromal cells correspond to non-cancer cells and comprise cancer-associated fibroblasts (CAFs), endothelial cells (ECs) and immune cells. The immune cells invading solid tumors are notably tumor-associated macrophages (TAMs), dendritic cells, neutrophils and T lymphocytes ($CD4^+$, $CD8^+$ and regulators). The infiltration of cytotoxic $CD8^+$ T cells in cancers is mostly associated with good prognosis, whereas macrophages infiltration is correlated with poor or good prognosis, according to the context (Bruni et al., 2020; Fridman et al., 2017). By a transcriptional analysis based on up to 18 000 human tumors, Gentles and Newman showed that monocytes/TAMs are the most represented immune cells in the tumor microenvironement (TME) of most cancer types and are associated to adverse outcomes in lung and brain tumors (Gentles et al., 2015).

1.1 Macrophage polarization

Macrophages are innate immune cells of the myeloid lineage. Macrophage phenotype is strongly dependent on microenvironmental cues. Macrophages are classified among the M1-M2 polarization axis, in which M1 and M2 macrophages are the two extremes (Lawrence and Natoli, 2011). This classification is oversimplified since macrophages with shared M1/M2 phenotype are often observed in tumors. Hence, there is a continuum of polarization among the M1-M2 polarization axis (Mori et al., 2015). M1 macrophages, also called “classically activated macrophages”, are pro-inflammatory macrophages, whereas M2 macrophages, also called “alternatively activated macrophages”, are anti-inflammatory macrophages. During the infection with pathogens, M1 macrophages are firstly recruited. They exert pathogen killing activity and ability to activate the adaptive immunity. M2 macrophages, after pathogen destruction, are critically involved in the resolution of inflammation, wound healing and clearance of apoptotic cells (Atri et al., 2018).

1.1.1 Signaling pathways regulating macrophage polarization

Macrophages are polarized into the M1 phenotype via the stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) and interferon γ (IFNγ) receptor (IFNγR) by IFNγ (Sica and Mantovani, 2012; Wang et al., 2014). The activation of IFNγR in macrophages induces the activation of the transcription factor signal transducer and activator of transcription (STAT) 1 and interferon regulatory factor 5 (IRF5), which notably induce the expression of IL-12, a M1 marker. LPS binding to TLR4 activates the transcription factors nuclear factor κB (NF-κB) (canonical) and activator protein-1 (AP-1), which are involved in pro-inflammatory cytokine expression (TNFα, IL-6, IL-1β and IL-8, among others). Macrophages are polarized into M2 macrophages mostly via IL-4, IL-13, IL-10 and IL-6. IL-4 and IL-13 induce STAT6 activation via the activation of IL-4 receptor. IL-10 and IL-6 induce the activation of STAT3 transcription factor via IL-10 receptor and IL-6 receptor activation. Macrophage polarization signaling is summarized in Fig. 1.

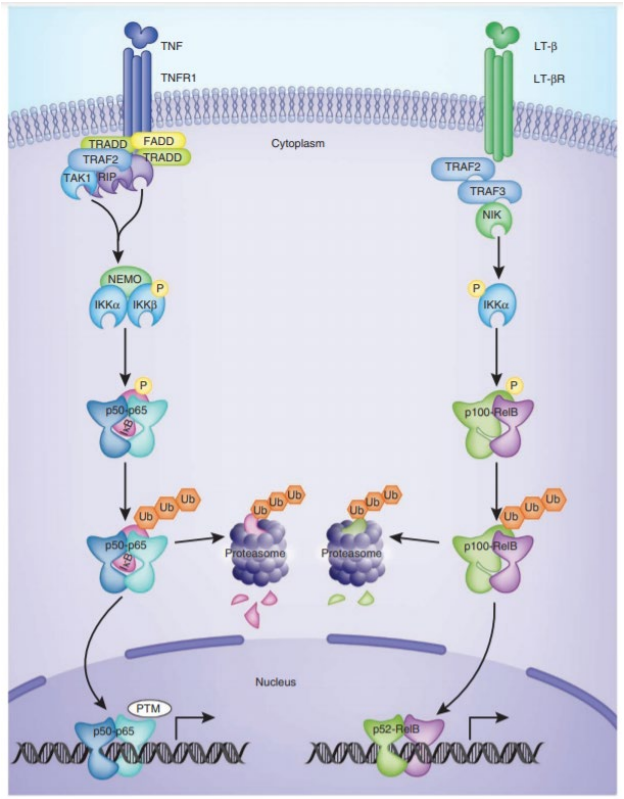

Fig. 2 : Canonical and non-canonical NF-κB signaling pathways. Left : canonical signaling. Right : non-canonical signaling. (Oeckinghaus et al., 2011)

In tumors, TAMs have a huge impact on tumor progression. Indeed, they regulate cancer-related inflammation, invasion/metastasis and the activity of immune cells against cancer cells. The origins of TAMs as well as their impact on blood vessels (angiogenesis, lymphangiogenesis) are discussed in the third chapter of this thesis and published in (Delprat and Michiels, 2021).

1.1.2 NF-κB

NF-κB signaling pathway has been discovered in 1986 by Sen and Baltimore (Sen and Baltimore, 1986). In mammals, 5 transcription factors belong to the NF-κB family, which all contain REL homology domain involved in DNA binding (Oeckinghaus et al., 2011; Sun, 2017). These members are p50, p52, p65 (also called RELA), RELB and c-REL. NF-κB activation can result from two distinct signaling pathways, the canonical and the non-canonical signalling pathways. These signaling pathways regulate various immune and inflammatory processes, as well as cell proliferation and survival. Accordingly, NF-κB is associated to several autoimmune diseases (Miraghazadeh and Cook, 2018), inflammatory disorders (Liu et al., 2017b), metabolic diseases (Baker et al., 2011) and cancers (Taniguchi and Karin, 2018).

The canonical pathway is composed of p65 and p50 transcription factors (Oeckinghaus et al., 2011; Sun, 2017; Viatour et al., 2005) (Fig. 2). In resting conditions, these transcription factors are sequestered in the cytoplasm by IκB proteins, such as nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (IκBα). The canonical pathway is induced upon cytokine receptor activation, such as TNF receptor (TNFR) 1, IL-1 receptor (IL-1R) and upon TLR4 activation by bacterial components (*e.g.* LPS). The activation of the receptors leads to the activation of a complex composed of 3 IKK kinases (IKKα, IKKβ and IKKγ), which then provokes the phosphorylation of IκBα on Ser 32 and Ser 36 by IKKβ. The phosphorylation of IκBα induces its ubiquitination, followed by its proteasomal degradation. The p65 and p50 dimer is then translocated into the nucleus where it binds to specific DNA elements, called κB enhancers of target genes.

The non-canonical pathway is composed of p52 and RELB transcription factors (Oeckinghaus et al., 2011; Sun, 2017). In resting conditions, RELB is associated with p100 in the cytoplasm. The non-canonical pathway is induced by TNFR superfamily activation, such as lymphotoxin β receptor (LTβR), CD40 and B cell activation factor receptor (BAFFR). The list of non-canonical pathway inducers is available in (Sun, 2017). The activation of these receptors leads to the phosphorylation and activation of IKKα, which then phosphorylates p100. Phosphorylated p100 is then ubiquitinylated and partially processed by the proteasome, leading to the formation of p52. The heterodimer p52/RELB is then translocated to the nucleus and regulates the transcription of several genes (Fig. 2).

1.1.2.1 NF-κB in macrophages

NF-κB signaling is a major regulator of the polarization of pro-inflammatory macrophages. This signaling is triggered by TLR4 activation by bacterial components, but also by cytokine receptor activation by pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and IL-1β. NF-κB then enhances the transcription of numerous pro-inflammatory genes -such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) - cytokines (such as

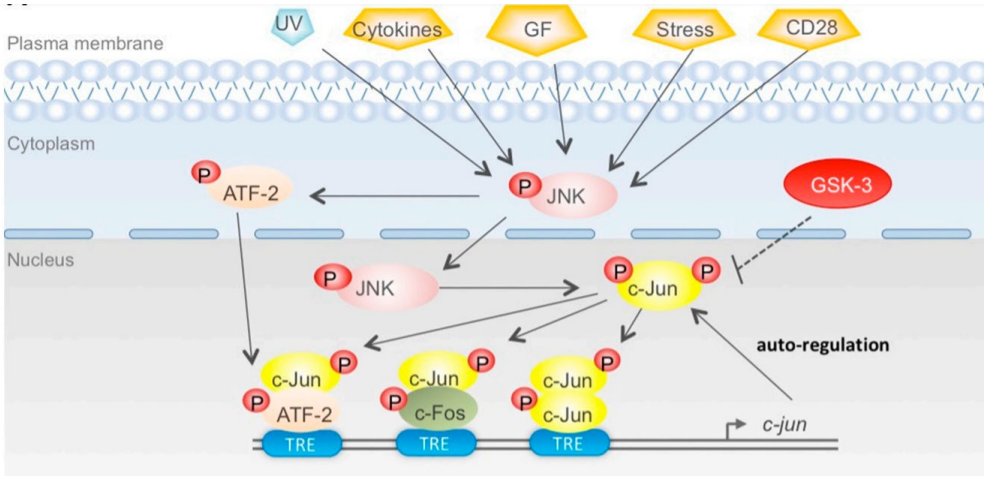

Fig. 3. c-jun/AP-1 transcription factor regulation. (Wirtz et al., 2011)

TNFα, IL-1β, IL-6 and IL-12) and chemokines (Hagemann et al., 2009; Mantovani et al., 2004; Wang et al., 2014).

In TAMs, NF-κB activation is linked to the development of several tumor types, via chronic inflammation promotion. Nonetheless, in later stage of cancer, TAM NF-κB activation is progressively inhibited, notably by p50 subunit overexpression and IL-10 autocrine signaling (Saccani et al., 2006; Sica and Bronte, 2007; Sica et al., 2000). This NF-κB inhibition is responsible for TAM insensitivity to pro-inflammatory cytokines and leads to TAM immunosuppressive phenotype. The re-activation of this NF-κB signaling in TAMs is hence a therapeutic target in cancers (Genard et al., 2017).

1.1.3 AP-1 transcription factors

AP-1 transcription factors are composed of activating-transcription factor (ATF), Jun, Fos, musculoaponeurotic fibrosarcoma (MAF) and Jun dimerization protein (JDP) transcription factor families. All these transcription factors contain a basic-leucine zipper (bZIP) domain needed for DNA-binding (basic) and dimerization (leucine zipper) (Eferl and Wagner, 2003; Papavassiliou and Musti, 2020). Among these transcription factor families, Jun and Fos families are the most represented and the most studied in mammals. Jun and Fos transcription factor families have very important physiological functions since several knockout (KO) of some of the transcription factors belonging to these families, such as c-jun, are lethal in mice. In tumors, these transcription factors are tumor promoters or tumor suppressors, according to the tissue and the transcription factor. AP-1 transcription factors regulate many cancer cell processes such as cancer cell proliferation, survival, apoptosis invasion, metastasis and angiogenesis. Furthermore, the implication of each AP-1 transcription in cancer cell processes is highly dependent on the tumor type. Members of the Jun family are able to homodimerize, whereas members from the Fos family only heterodimerize with a protein of the Jun family. c-jun (Jun family) and c-fos (Fos family) are tightly regulated, notably by their level of transcription, mRNA and protein stability as well as by post-translational modifications. The post-translational modifications regulating c-jun activity are reviewed in (Papavassiliou and Musti, 2020). The activation of c-jun and c-Fos are mostly regulated by c-jun N-terminal kinase (JNK) and glycogen-synthase kinase 3 (GSK3) (Fig. 3). JNKs phosphorylate c-jun which then induces the homodimerization of c-jun or heterodimerization of c-jun with c-fos, followed by activation of its transcriptional activity. JNKs also phosphorylate ATF2, inducing the heterodimerization of ATF2/c-jun, and subsequent activation. On the other hand, GSK3 phosphorylates c-jun at serine 243 and threonine 239 which inhibits the activity of c-jun. The regulation of c-jun activity is schematized in Fig. 3.

1.1.3.1 c-jun/AP-1 in the regulation of macrophage phenotype

In macrophages, c-jun regulates M1 and M2 marker expression, according to the stimuli. Indeed, upon palmitic acid stimulation, c-jun is activated by TLR4. It is involved in the regulation of the expression of pro-inflammatory cytokines (TNFα, IL-6, IL-1β) and anti-inflammatory cytokine (IL-10) in Raw murine macrophages (Hu et al., 2020). Upon pro-inflammatory stimuli (LPS or IFNγ), c-jun promotes the expression of COX2 (M1 marker) and represses the expression of arginase-1 (Arg-1 ; a M2 marker) in Raw macrophages (Hannemann et al., 2017). Following stimulation of macrophages with LPS, c-jun slightly

Inflammation that causes insults or pathological conditions	Associated malignancy
Silica, asbestos, smoking-associated silicosis and bronchitis	Lung carcinoma
Pelvic inflammatory disease	Ovarian carcinoma
Chronic indwelling urinary catheter	Bladder carcinoma
TRYP1 mutation-associated pancreatitis and alcoholism-associated pancreatitis	Pancreatic carcinoma
UV irradiation-associated skin inflammation	Melanoma
Asbestos	Mesothelioma
Bile acids	Cholangiosarcoma and colorectal carcinoma
Gastric acid-associated Barrett's metaplasia and reflux oesophagitis	Oesophageal carcinoma
Gall bladder stone-associated cholecystitis	Gall bladder carcinoma
Lichen sclerosus (a skin condition)	Vulvar carcinoma
Inflammatory bowel disease	Colorectal carcinoma
Hashimoto's thyroiditis (an autoimmune disease of the thyroid) and Sjögren's syndrome (an autoimmune disease of exocrine glands)	Mucosa-associated lymphoid tissue lymphoma
Gingivitis (inflammation of the gum tissue) and lichen planus	Oral squamous cell carcinoma
Sialadenitis (inflammation of the salivary gland)	Salivary gland carcinoma

Table 1: Association of pre-existing inflammation with malignancies. (Elinav et al., 2013)

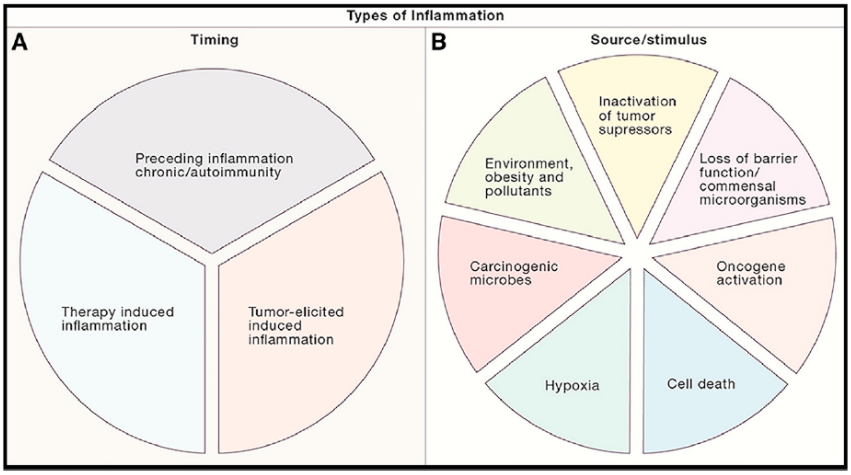

Fig. 4 : Source and timing of cancer-associated inflammation. A) Timing. Cancer-related inflammation can precede carcinogenesis in the cases of autoimmunity or infection, can be induced by malignant cells (tumor-elicited induced inflammation) or can be triggered by anti-cancer (therapy-induced inflammation) therapy. **B) Source.** Cancer-related inflammation is induced by external stimuli (environment, pollutants, microbes, infection …) or internal stimuli (inactivation of tumor suppressors, oncogene activation, cell death…). (Greten et al., 2019)

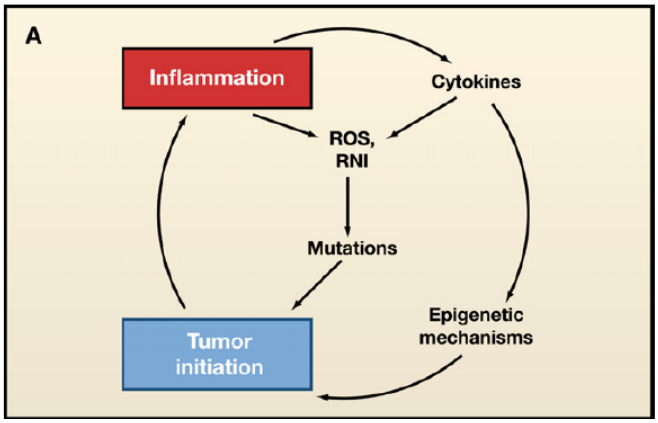

Fig. 5. Positive feedback-loop between chronic inflammation and tumor initiation. Chronic inflammation induces pro-inflammatory cytokines and ROS/RNI formation. Both cytokines and ROS/RNI induce DNA damage. When occurring in tumor suppressor or oncogenes, DNA mutations promote tumor initiation, hence sustaining cancer-related inflammation. ROS: reactive oxygen species ; RNI: reactive nitrogen intermediates. (adapted from Grivennikov et al., 2010)

increases TNFα expression, as well as it enhances C-X-C motif chemokine ligand (CXCL)2 expression in a synergistic manner with NF-κB (Kim et al., 2003; Liu et al., 2000). Furthermore, the main activator of c-jun, JNK, is strongly involved in a pro-inflammatory phenotype induction by IFNγ in bone marrow-derived macrophages and sustains the high-fat diet-induced macrophage inflammation in adipose tissue (Han et al., 2013). Nonetheless, upon LPS stimulation of peritoneal macrophages, the activation of c-jun by serine 63 and serine 73 phosphorylation is involved in the expression of M2 markers such as C-C motif chemokine ligand (CCL)2, CCL17 and CCL22 (Hefetz-Sela et al., 2014). Altogether, these results show that, upon pro-inflammatory stimuli, c-jun/AP-1 is involved in the induction of pro-inflammatory M1 markers also regulated by NF-κB. It is likely that c-jun is implicated in the complete M1 macrophage phenotype upon inflammatory stimuli by increasing the expression of several M1 markers while decreasing M2 marker expression (Arg-1), even if it also increases the expression of some M2 markers (IL-10, CCL2, CCL17 and CCL22).

1.2 Cancer-related inflammation

In 1863, Rudolf Virchow showed that tumors were infiltrated by leukocytes (Balkwill and Mantovani, 2001). He postulated that cancers arise from sites of inflammation. Since then, a lot of experiments have been performed about the impact of inflammation in the tumors. In 2009 and 2011, tumor inflammation was described as the 7th hallmark of cancer (Colotta et al., 2009; Hanahan and Weinberg, 2011). Cancer-related inflammation is involved in every step of cancer development, from tumor initiation to tumor progression and metastasis (Greten and Grivennikov, 2019). Indeed, a meta-analysis demonstrated that, in human, the daily intake of aspirin, an anti-inflammatory drug, significantly reduces the incidence of many cancers such as colorectal, oesophageal, gastric, biliary, and breast cancers (Rothwell et al., 2012). Furthermore, the daily intake of aspirin significantly reduces the metastasis appearance and the cancer-related deaths (Rothwell et al., 2011; Rothwell et al., 2012). Infection, chronic inflammation, or auto-immunity in an organ-site, precedes approximately 15 – 20% of cancers development in the same tissue (Greten and Grivennikov, 2019; Landskron et al., 2014) (Table 1). This inflammation induced by external stimuli is the cause of many cancer types (Landskron et al., 2014) (Table 1). Cancer-related inflammation can be initiated before and during cancer development by several stimuli (Fig. 4). Tumor inflammation sustains cancer initiation, genetic instability, mutation, cancer progression and cancer metastasis by several mechanisms which will be described here under. The impact of TAMs on these processes will also be detailed.

1.2.1 Inflammation, genetic instability, tumor initiation and tumor promotion

Acute Inflammation is a phenomenon in which an infection or an injury induces the recruitment of immune cells within the site of injury. These immune cells induce an inflammation which allows the resolution of the infection/injury. Then, inflammation is resolved. In cancer diseases, the acute inflammation and the immune system are involved in immunosurveillance, which means that the immune system allows cancer cell killing. Nonetheless, inflammation can persist and this is called chronic inflammation. It exists a positive feedback loop between chronic inflammation and genetic instability, leading to tumor initiation. Indeed, inflammation promotes genetic instability/mutations and vice-versa (Fig. 5). In case of chronic inflammation, immune cells, such as macrophages, produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) which cause DNA damage,

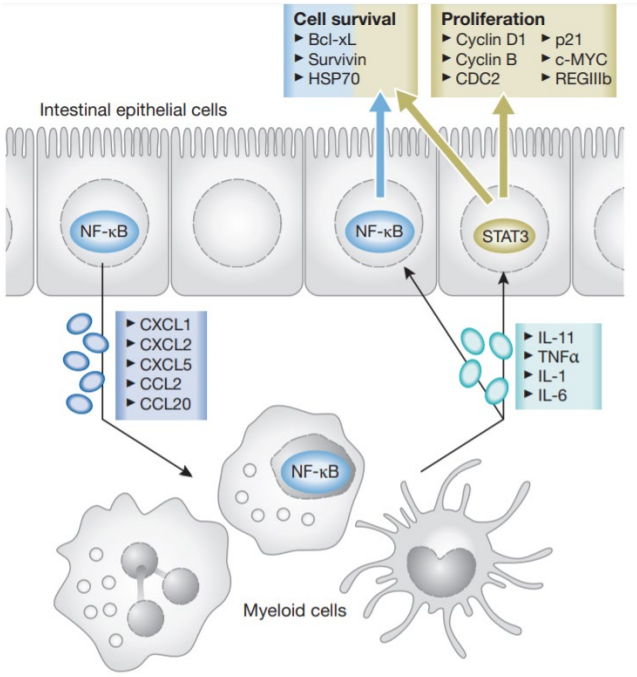

Fig. 6. NF-KB and STAT3 signaling induced in premalignant cells by cancer-related inflammation sustains their survival and proliferation thus allowing tumor promotion. Cancer-related inflammation is mostly initiated by macrophages, recruited via chemokines secreted by pre-malignant and malignant cells. (Bollrath and Greten, 2009)

mutations, DNA methylation and post-translational modifications of proteins (Canli et al., 2017; Colotta et al., 2009; Kay et al., 2019). These alterations can lead to the activation or inactivation of oncogenes and tumor suppressor genes, respectively, hence inducing malignant transformation. These mutations inactivating tumor suppressor genes favor tumor inflammation (Fig. 4). For example, the induction of inflammation in colon by dextran sodium sulfate (DSS) ingestion in mice triggers RNS formation and subsequent mutations. These mutations induce tumors in mice with altered DNA repair system (Meira et al., 2008). Furthermore, blockade of pro-inflammatory cytokine signaling or constitutive activation of the pro-inflammatory transcription factor NF-kB inhibits or promotes tumor initiation, respectively (Popivanova et al., 2008; Shaked et al., 2012). On the other hand, mutations in the tumor suppressor p53 gene potentiates NF-kB activation and its associated pro-inflammatory cytokine expression leading to/promoting chronic inflammation. Hence, mice with mutation in p53 gene are highly susceptible to inflammation-induced colon tumors (Cooks et al., 2013). To conclude, there is a feed-back loop between chronic inflammation and genetic instability which leads to tumor initiation.

Tumor promotion corresponds to the proliferation of tumor initiating cells into an established tumor (Greten and Grivennikov, 2019). Cancer-related inflammation is strongly involved in this process. This is mostly due to inflammation-induced NF-κB and STAT3 signaling pathways in cancer cells (Fig. 6). These signaling pathways are interconnected and promote respectively cancer cell survival and proliferation, thus sustaining tumor growth (Bollrath and Greten, 2009). For example, conditional inhibition of NF-kB signalling via ablation of IKKβ in intestinal epithelial cells markedly diminishes their expression of pro-survival genes leading to their apoptosis, hence decreasing tumor growth in colitis-associated cancer (CAC) (Greten et al., 2004). In the same manner, specific inhibition of NF-kB in hepatocytes inhibits cancer cell survival and tumor promotion of hepatitis-associated hepatocellular carcinoma (Pikarsky et al., 2004). Furthermore, STAT3 signaling pathway increases survival and proliferation of cancer cells during tumor promotion. Indeed, specific ablation of STAT3 in intestinal epithelial cells induces apoptosis during colitis-associated carcinogenesis, via an increase in the expression of pro-apoptotic proteins and a decrease in antiapoptotic factors, hence diminishing CAC promotion (Bollrath and Greten, 2009; Bollrath et al., 2009). Furthermore, STAT3 deletion in mice or specifically in pancreatic cells decreases cancer cell proliferation which markedly diminishes the promotion of pancreatic intra-epithelial neoplasia development associated with pancreatitis (Fukuda et al., 2011; Lesina et al., 2011).

The impact of macrophages on tumor initiation and promotion is mainly due to the production of reactive oxygen species (ROS) (including H_2O_2) and via the production and secretion of pro-inflammatory cytokines such as IL-6 and TNFα, which activate STAT3 and NF-kb signaling pathway in cancer cells (Greten and Grivennikov, 2019). In murine urethane-induced lung cancer, early depletion of macrophages (but not late depletion of macrophages), decreases the lung tumor incidence and size which is associated with a decrease in inflammation and in the occurrence of pre-cancerous lesions (Zaynagetdinov et al., 2011). Furthermore, the depletion of macrophages is associated with a strong diminution in NF-kB activity in developing tumors. IKKα knock-in mice develop spontaneous lung squamous cell carcinomas, and the depletion of macrophages in this murine model drastically prevents the formation of

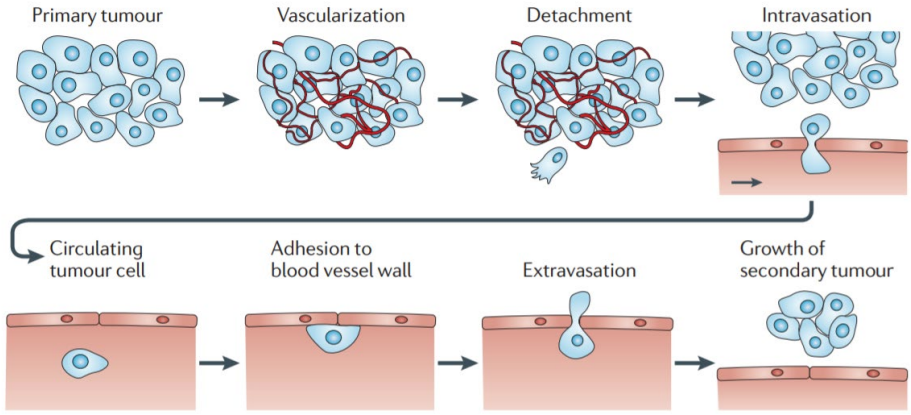

Fig. 7. Metastasis steps. (Wirtz et al., 2011)

these tumors (Xiao et al., 2013). The depletion of macrophages is also associated to a decrease in DNA damage as well as in the expression of inflammation and proliferation-associated genes. In mice developing spontaneous hepatocellular carcinoma (HCC) upon high-fat diet, macrophages are high TNFα producers, and the incidence and growth of HCC is diminished in TNF receptor 1 (TNFR1) KO mice (Nakagawa et al., 2014). Furthermore, TNFR1 is involved in NF-kB and STAT3 activation since the activation of these signaling is diminished by TNFR1 inhibition. The promotion of HCC initiation by macrophages depends on the activation of NF-kB and NADPH oxidase (NOX)1 in macrophages, since deletion of these genes specifically in myeloid cells decreases inflammation, STAT3 activation, HCC incidence and HCC growth (Liang et al., 2019; Maeda et al., 2005). Specific ablation of IKKβ (NF-kB signaling) in myeloid cells drastically diminishes the occurrence and growth of CAC (Greten et al., 2004).

The impact of macrophage-derived ROS on cancer development was postulated a long time ago. Indeed, macrophage are high producers of ROS, notably upon inflammation (Kay et al., 2019). Furthermore, ROS are strong DNA damage inducers, leading to mutations. Nonetheless, the evidence that macrophage-derived ROS are involved in tumor initiation has been only recently demonstrated *in vivo* (Canli et al., 2017). Indeed, in mice with specific deletion of glutathione peroxidase (Gpx)4 in macrophages, macrophages produce higher level of ROS (including H_2O_2) than in WT mice. In these mice, the appearance of tumors is markedly higher than in WT mice, even in the absence of carcinogen or pre-existing inflammation. Furthermore, there is a higher level of mutations in the tumor of Gpx4 KO mice than in WT mice. Nonetheless, it would be interesting to confirm the impact of macrophage-derived ROS by treating mice with antioxidant in this model.

In conclusion, macrophages are strongly involved in cancer-related inflammation, via pro-inflammatory cytokine and ROS production. The production of ROS by macrophages, by itself, induces tumor initiation and tumor growth, without pre-existing inflammation (Canli et al., 2017). Pro-inflammatory cytokine secretion by TAMs drives tumor initiation and support tumor promotion via the activation of STAT3 and NF-κB signaling in cancer cells (Bollrath and Greten, 2009).

1.2.2 Inflammation and tumor metastasis

Metastasis refers to the dissemination of cancer cells from a primary tumor site into a secondary tumor site. This process is responsible for up to 90% of cancer-related deaths. The impact of inflammation on this process is particularly important. Indeed, daily uptake of aspirin strongly reduces metastasis appearance in most cancers. Furthermore, the administration of anti-inflammatory drug before or after a surgery (surgeries enhance cancer related inflammation) drastically diminishes the occurrence of post-operation metastases (Greten and Grivennikov, 2019). Several steps are needed for metastasis formation. They include tumor vascularization (via angiogenesis), detachment of cancer cells from the primary site, intravasation, circulation and survival into the blood system, adhesion of cancer cells to the blood vessels followed by extravasation and subsequent growth to form a secondary tumor site (Fig. 7) (Wirtz et al., 2011). Cancer-related inflammation is involved in several steps -if not all- of cancer metastasis (Qian, 2017). Indeed, cancer related-inflammation triggers angiogenesis, migration and invasion of cancer cells, epithelial to mesenchymal transition

(EMT), extravasation and cancer cell seeding. EMT is observed in several physiological processes, including embryonic development, and in several pathologies, including cancer. EMT corresponds to a cellular transdifferentiation in which (cancer) cells lose their epithelial features and acquire mesenchymal characteristics (Chen et al., 2016b). During EMT, the ability of cell to migrate increases. This transition involves several transcription factors such as TWIST, SNAIL and SLUG (Puisieux et al., 2014). During metastasis, EMT in cancer cells increases their migration, invasion, intravasation and seeding in the secondary site (Suarez-Carmona et al., 2017). Biomarkers of EMT are observed in invasive and circulating cancer cells of most cancer types (Aiello and Kang, 2019). Pro-inflammatory cytokines induce and promote EMT in cancer cells among several tumor types, at least *in vitro* (Suarez-Carmona et al., 2017). For example, TNFα alone or in combination with other cytokines induces EMT in a huge panel of cancer cell lines. Furthermore, these cytokines are pro-metastatic *in vivo*, notably for lung metastasis (Solinas et al., 2010). Inflammation-induced recruitment of myeloid cells is also very important for metastasis (Hiratsuka et al., 2006; Qian et al., 2011; Wolf et al., 2012). In a murine melanoma model, ultraviolet-induced inflammation enhances angiogenesis, migration and adhesion of melanoma cells on blood vessels (Bald et al., 2014). Inflammation induced by LPS or tobacco smoke favors lung metastasis in murine tumor models (Luo et al., 2004; Murin et al., 2004). Injection of cancer cells into the circulation triggers inflammation-induced vascular adhesion molecule expression in liver endothelial cells (E-selectin and VCAM1), hence promoting their extravasation (Auguste et al., 2007). Altogether, these data strongly evidence that cancer-related inflammation is involved in several steps of tumor metastasis.

1.3 TAMs and metastasis

The impact of TAMs on metastasis is described in the chapter 3 of the thesis and was published in (Delprat and Michiels, 2021). In this review, we describe the impact of TAMs on the migration/invasion of cancer cells from the primary site into blood vessels via EGF/CSF1 paracrine loop, on their intravasation, extravasation and seeding in the secondary tumor site. In the following paragraph, other mechanisms by which TAMs modulate metastasis will be described.

TAMs secrete a lot of cytokines and chemokines, such as TNFα, IL-8, IL-6, transforming growth factor-β (TGF-β) and CCL18 which induce EMT *in vitro* in numerous cancer cell lines. These molecules increase cancer cell migration/invasion in the primary tumor site into blood vessels (Suarez-Carmona et al., 2017). *In vivo*, macrophage-derived CCL18 is able to induce EMT in humanized breast cancer mouse model (Su et al., 2014a; Su et al., 2014b). TAM-derived CCL18 initiates metastasis without affecting primary tumor size. It exists a paracrine loop between cancer cells and TAMs in which secretion of CCL18 by TAMs triggers GM-CSF secretion by breast cancer cells, which promote CCL18 secretion by TAMs. Another loop between cancer cells and macrophage which regulates cancer cells migration and invasion is through heregulin β1 (TAMs) and CXCL12 (cancer cells) (Boimel et al., 2012). ROS production by myeloid cells sustains the development of invasive properties in inflammation-induced CAC. These cancers are strongly more invasive when the production of ROS is increased specifically in myeloid cells for example via specific Gpx4 deletion (Canli et al., 2017). Nonetheless, it would be interesting to confirm the involvement of macrophage-derived ROS by using anti-oxidant in this model.

Phenotype or depletion	Immunodeficiency	Tumor susceptibility
RAG-2$^{-/-}$	T, B and NKT cells	MCA-induced sarcomas[49] Spontaneous intestinal neoplasia[49]
RAG-2$^{-/-}$ × STAT1$^{-/-}$ (RkSk)	T, B and NKT cells Insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas[49] Spontaneous intestinal and mammary neoplasia[49]
BALB/c SCID	T, B and NKT cells	MCA-induced sarcomas[100]
Perforin$^{-/-}$	Lack of perforin	MCA-induced sarcomas[42,45,46] Spontaneous disseminated lymphomas[43,47]
TCR Jα281$^{-/-}$	Subset of NKT cells	MCA-induced sarcomas[42,46,100]
Anti-asialo-GM1 antibody	NK cells and activated macrophages	MCA-induced sarcomas[100]
Anti-NK1.1 antibody	NK and NKT cells	MCA-induced sarcomas[46,100]
Anti-Thy1 antibody	T cells	MCA-induced sarcomas[46,100]
αβ T cell$^{-/-}$	αβ T cells	MCA-induced sarcomas[50]
γδ T cell$^{-/-}$	γδ T cells	MCA-induced sarcomas[50] DMBA/TPA-induced skin tumors[50]
STAT1$^{-/-}$	Insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas[41,49] Wider tumor spectrum in STAT1$^{-/-}$ × p53$^{-/-}$ (ref. 41)
IFNGR1 receptor$^{-/-}$	Insensitive to IFN-γ	MCA-induced sarcomas[41,49] Wider tumor spectrum in IFN-γ receptor$^{-/-}$ × p53$^{-/-}$ (ref. 41)
IFN-γ$^{-/-}$	Lack of IFN-γ	MCA-induced sarcomas[42] C57BL/6: Spontaneous disseminated lymphomas[43] BALB/c: Spontaneous lung adenocarcinoma[43]
Perforin$^{-/-}$ × IFN-γ$^{-/-}$	Lack of perforin and IFN-γ	MCA-induced sarcomas[42] Spontaneous disseminated lymphomas[43]
IL-12$^{-/-}$	Lack of IL-12	MCA-induced sarcomas[46]
WT + IL-12	Exogenous IL-12	Lower incidence of MCA-induced sarcomas[101]

Methylcholanthrene-treated wild-type (WT) mice were treated with IL-12 during tumor formation.

Table 2. Immunodeficient mice are more susceptible to carcinogen-induced or spontaneous tumor formation. (adapted from Dunn et al., 2002).

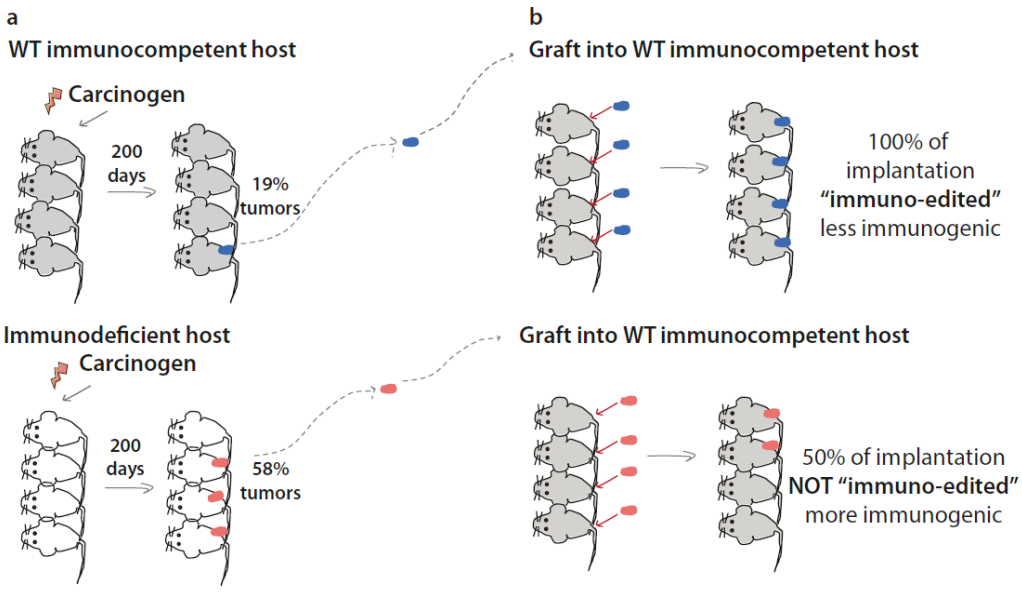

Fig. 8. Scheme of experiments performed by Shankaran et al., demonstrating the cancer immunoediting concept. Cancer incidence is higher in immunodeficient mice upon carcinogen exposure. Only 50% of tumors transferred from immunodeficient mice into immunocompetent mice take up whereas 100% of tumors transferred from immunocompetent mice into immunocompetent mice develop. This means that tumors from immunocompetent mice are « edited » by the immune system in allowing them to be less immunogenic. (Povoa and Fior, 2019; experiments performed in Shankaran et al., 2001).

TAMs are high producers of IL-1β in mouse breast cancers, and in patients, notably in patients with renal cell carcinoma (RCC). The secretion of IL-1β, (including by TAMs) supports the metastasis process, notably via the enhancement of systemic inflammation. The secretion of IL-1β triggers neutrophil activation which supports metastasis of breast cancer cells into lung in genetically engineered mouse models for breast cancer (Kersten et al., 2017; Wellenstein et al., 2019). *In vitro*, several cancer cells from renal cell carcinoma (RCC), Lewis lung carcinoma (LLc) and breast cancer enhance the expression of IL-1β by macrophages (Deng et al., 2019; Kersten et al., 2017; Kim et al., 2009; Wellenstein et al., 2019). IL-1β is also highly secreted by TAMs in patients with RCC (Chittezhath et al., 2014). In mouse model of RCC lacking IL-1 receptor (IL-1R), it is shown that IL-1R is crucial in the regulation of TAM pro-tumoral phenotype. In a model of leptomeningeal metastasis (LM), TAMs located in the cerebro spinal fluid support cancer cell metastasis, via the secretion of pro-inflammatory cytokine which induces lipocalin 2 (LCN2) expression in cancer cells (Chi et al., 2020). The ablation of LCN2 by shRNA in cancer cells in this model highly decreases LM formation.

In the secondary tumor site, TAMs are able to support the seeding of cancer cells. Upon the injection of LLc in the tail vein, cancer cells metastasize into the lung. Kim et al., with a mouse model of metastasis induced by injection of LLc into the tail vein, suggested that LLc cells induce the secretion of inflammatory cytokines via versican, and that the inflammation induced by TAMs in the lung stimulates tumor growth (Kim et al., 2009). Nonetheless, this model does not reproduce all the steps of metastasis formation. In metastatic sites, IL-35, an anti-inflammatory cytokine, which is secreted in high quantity by TAMs, is able to promote cancer cell metastatic colonization (Lee et al., 2018). This could be due to its ability to reverse the EMT, allowing cancer cell proliferation.

1.4 Cancer immunoediting, TAMs and tumor escape/immunosuppression

The development of cancers in human and other species is strongly inhibited by the immune system, through a process called cancer immunosurveillance (Povoa and Fior, 2019). The incidence of carcinogen-induced tumor is drastically increased in several models of immunodeficient mice (Dunn et al., 2006) (Table 2). Furthermore, in human, patients with primary immunodeficiencies have a higher risk to develop cancers than healthy subjects (Mortaz et al., 2016). Nonetheless, some examples show that immune system can also promote tumor growth. Indeed, woman receiving immunosuppressive therapy after organ transplantation have less risk to develop breast cancers (Stewart et al., 1995).

These conflicting results are explained by cancer immunoediting. This concept was postulated in 2002 (Dunn et al., 2002) and argues that cancers are edited by the immune system. Basically, cancer cells have high genomic instability and hence high rates of mutation. This phenomenon is responsible for a high heterogeneity between different cancer cells found within the same tumor. The immune system is able to kill cancer cells and hence puts cancer cells under strong selection pressure. Cancer cells that possess escape mechanisms from the immune system are specifically selected by the immune selection pressure. As a result, tumors become less and less immunogenic and develop immunosuppressive capabilities. The immunoediting concept was postulated after experiments performed in (Shankaran et al., 2001) which are summarized in Fig. 8. The development of carcinogen-induced tumor is

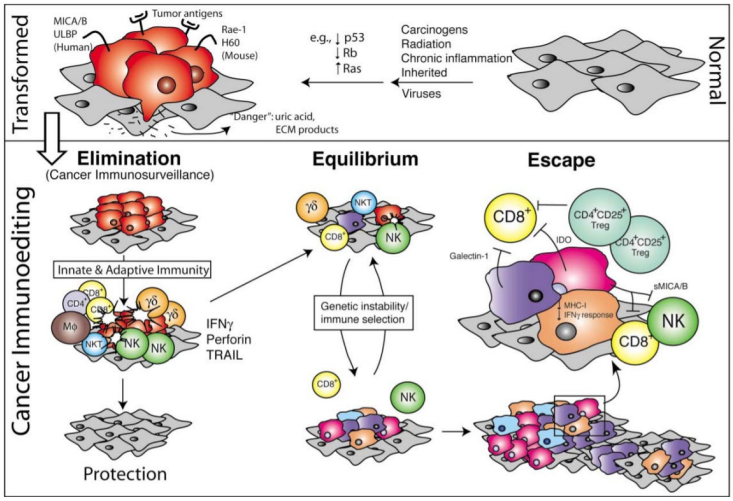

Fig. 9. Cancer immunoediting. Cancer immunoediting is composed of three phases, elimination, equilibrium and escape. When normal cells are transformed into cancer cells, they are detected and killed by the immune system during the elimination phase. Nonetheless, in some cases, cancer cells are not eradicated and enter into the equilibrium phase in which they are « edited » by the immune system, allowing them to become less immunogenic and enter in the escape phase. In the latter, tumor growth occurs and several mechanisms allow the tumor to escape from immunity. (Dunn et al., 2004)

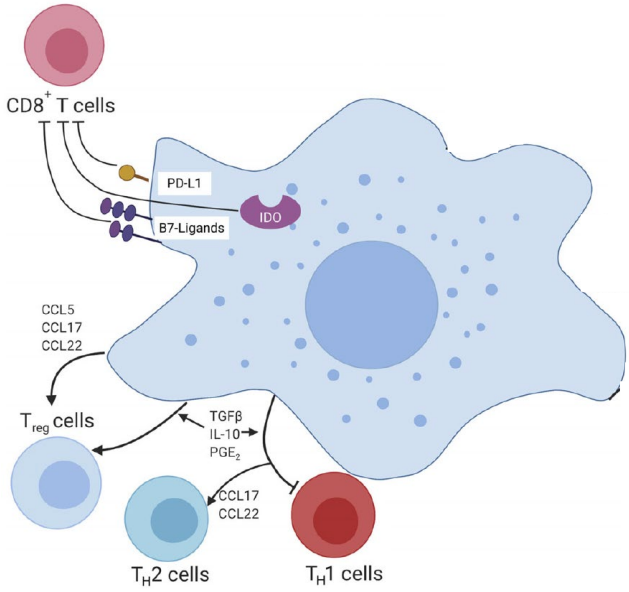

Fig. 10. Mechanisms of TAM-mediated immunosuppression. TAMs inhibit cytotoxic T cell function via immune checkpoint and amino acid starvation via IDO enzyme activity. TAMs promote the recruitment of Treg and Th2 cells via CCL17 and CCL22 chemokine secretion. The secretion of TGFβ and IL-10 by TAMs activates and inhibits Treg and Th1 cells, respectively. (adapted from Lopez-Yrigoyen et al., 2020)

strongly enhanced in immunodeficient RAG $2^{-/-}$ mice (mice lacking T cells, B cells and NKT cells). Furthermore, there is more spontaneous tumor formation in these mice. More interestingly, the tumors transplanted from WT mice into WT mice developed in 100% of recipient mice, whereas the tumor transplanted from RAG $2^{-/-}$ mice into WT mice developed in only 50% of recipient mice. This indicates that cancers are edited by immune system, which leads to tumors becoming less immunogenic.

Cancer immunoediting is composed of three phases, elimination, equilibrium and escape (Fig. 9) (Dunn et al., 2002; Dunn et al., 2006; Dunn et al., 2004). In the elimination phase, mutations in normal cells lead to the development of cancer cells, which are detected and can be eradicated by innate and adaptive immune cells. Nonetheless, in some cases, cancer cells are not eradicated by the immune system and some of them subsist and go to the equilibrium phase. In the latter phase, cancers are immunoedited and some mechanisms drive them to be less immunogenic in order to evade the immune system and enter in the escape phase. During the escape phase, tumors are growing and become detectable. They develop several immune escape mechanisms, such as diminution of immunogenicity, resistance to cell death, modification of immune cell recruitment, killing of immune cells, modification of immune cell phenotype leading to a pro-tumor phenotype and immunosuppression (Povoa and Fior, 2019)(De Sanctis et al., 2018). During cancer progression, TAMs are skewed from M1 phenotype into M2 phenotype by cancer cells and TME, and M2 TAMs play an important role in tumor escape (DeNardo and Ruffell, 2019; Mantovani et al., 2017; Noy and Pollard, 2014). Accordingly, M1/M2 macrophage ratio is lower in later stage than in early stage of colorectal cancer (Cui et al., 2013), pediatric classical Hodgkin lymphoma (Barros et al., 2015), oesophageal adenocarcinoma (Cao et al., 2015b), ovarian cancer (Vankerckhoven et al., 2020; Zhang et al., 2014b), bladder cancer (Takeuchi et al., 2016), and cervical cancer (Petrillo et al., 2015). Furthermore, several TME factors are involved in TAMs skewing into M2 phenotype. Indeed, TME-derived IL-4, IL-13, IL-10, lactic acid, cancer acidity and TGF-β induce the polarization of TAMs into a pro-tumoral immunosuppressive phenotype (DeNardo and Ruffell, 2019; Mantovani and Allavena, 2015; Mantovani et al., 2017; Ruffell et al., 2012; Zhang et al., 2016a). IL-4 and IL-13 mostly derive from type 2 t-helper cells (Th_2 cells), while IL-10 and TGF-β mostly derive from lymphocyte T-regulators (Tregs) and cancer cells.

Once polarized into an immunosuppressive phenotype, TAMs induce immunosuppression by several mechanisms such as induction of Tregs activity, inhibition of dendritic cell maturation, and inhibition of T cell activity (DeNardo and Ruffell, 2019; Lopez-Yrigoyen et al., 2020; Mantovani et al., 2017; Noy and Pollard, 2014)(Fig. 10). There is a feed-back loop between Tregs and TAMs which promotes their immunosuppressive phenotype and cancer immunosuppression via IL-10 and TGF-β secretion. $CD8^+$ cytotoxic T cells are inhibited by macrophages via amino acid starvation induced by indoleamine 2,3-dioxygenase (IDO) enzyme activity in TAMs (Munn et al., 1999; Yan et al., 2019), via the expression of immune checkpoint including B7 ligands (for example B7-H1), programmed cell death ligand (PD-L)1 and programmed cell death protein 1 (PD-1) (DeNardo and Ruffell, 2019; Lopez-Yrigoyen et al., 2020; Mantovani et al., 2017; Noy and Pollard, 2014). PD-1 expression in TAMs also decreases their phagocytosis ability (Gordon et al., 2017). Immunosuppressive TAMs are also responsible of immunosuppressive Th_2 cell and Treg recruitment via CCL17 and CCL22 (Lopez-

Yrigoyen et al., 2020). TAMs inhibit $CD8^+$ cytotoxic T cells via IL-10 dependent inhibition of IL-12 secretion by dendritic cells (DeNardo and Ruffell, 2019; Ruffell et al., 2014). In conclusion, TAMs are progressively skewed towards an immunosuppressive M2 phenotype by TME during cancer progression. These TAMs promote an immunosuppressive microenvironment in tumors by several mechanisms. Accordingly, a lot of clinical trials use the combination of immune modulators with TAM targeting agents (*e.g* CSF1-R antibody) in order to treat cancer patients (DeNardo and Ruffell, 2019).

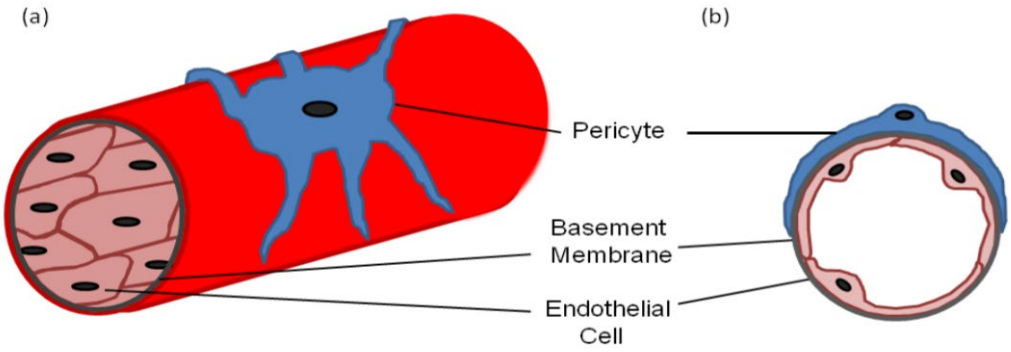

Fig. 11. (a) Longitudinal section of blood vessel. (b) Cross-sectional section of blood vessels. (Mills et al., 2013)

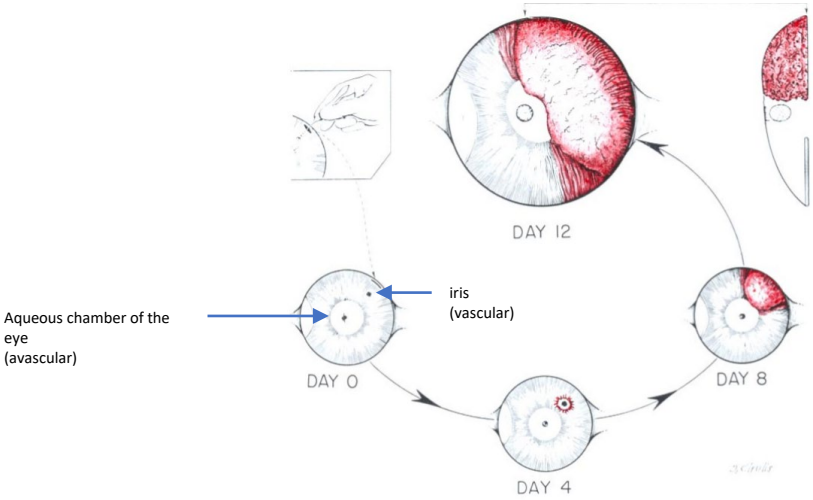

Fig. 12. Experiments performed in Gimbrone et al., 1972. Tumors implanted in avascular area grow very slowly and are small in size, whereas tumors implanted in vascular area grow slowly during avascular phase, and fast as the tumor becomes vascularized. (adapted from Gimbrone et al., 1972)

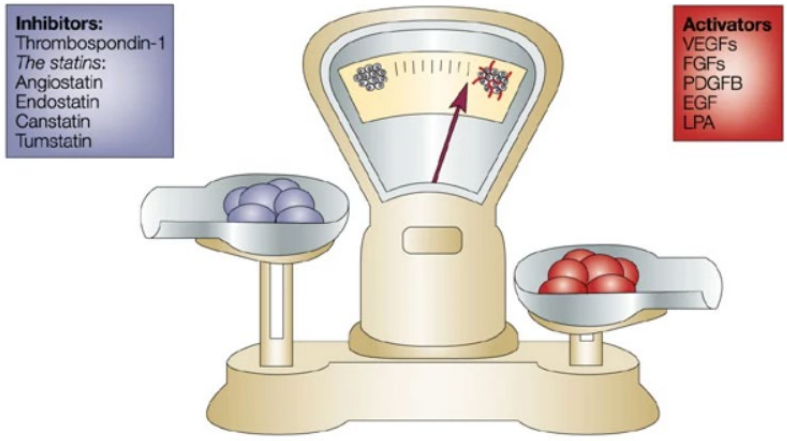

Fig. 13. The angiogenic switch occurs when the balance between pro-angiogenic factors and anti-angiogenic factors is in favor of angiogenesis. (Bergers and Benjamain, 2003)

2. Tumor blood vessels

2.1 Blood vessel structure

Blood vessels are channels which supply oxygen and nutrients to almost all organs of the body (some tissues are unvascularized such as cartilage, epidermis and the lens and cornea of the eye). Blood vessels first comprise an endothelium composed of a monolayer of endothelial cells (ECs). ECs are in direct contact with the blood and based on a basement membrane (Mills et al., 2013). ECs are surrounded by mural cells (such as pericytes and/or vascular smooth muscle cells) in contact with the basement membrane. Pericytes are involved in the regulation of blood flow and vessel permeability (Mills et al., 2013). ECs regulate blood vessel permeability, blood coagulation, vascular tone, blood vessel formation from pre-existing one (by a process called angiogenesis) and regulate leukocyte extravasation during inflammation (Michiels, 2003). The structure of a blood vessel is depicted in Fig. 11.

Dysfunctioning of blood vessel and/or ECs is at least in part responsible for plethora of diseases such as rheumatoid arthritis, atherosclerosis, multiple sclerosis and cancers. The involvement of blood vessels and ECs in cancers will be described hereunder. In particular, their roles in tumor growth, metastasis and in the regulation of antitumor immunity will be detailed.

2.2 Angiogenic switch

Blood vessel formation, by a process called angiogenesis, is strongly involved in tumorigenesis and tumor growth. This was firstly proposed in 1972 (Gimbrone et al., 1972) (Fig. 12). Cancer cells implanted in areas in which the formation of blood vessels is impossible (aqueous chamber of the eye), leads to the formation of tumor of less than 2 or 3 mm^3 with slow tumor growth. On the other hand, the implantation of cancer cells in areas permissive for angiogenesis (such as iris) leads to a slow tumor growth during an avascular phase followed by a rapid tumor growth during the vascular phase. The transition from the avascular phase to the vascular phase is called the angiogenic switch (Baeriswyl and Christofori, 2009). The induction of angiogenesis occurs during the transition from pre-malignant to malignant transition in several transgenic murine cancer models and in several human cancers (Baeriswyl and Christofori, 2009; Menakuru et al., 2008).

The angiogenic switch occurs when the balance between pro-angiogenic factors and anti-angiogenic factors is in favor of angiogenesis (Bergers and Benjamin, 2003) (Fig. 13). Among angiogenic factors, vascular endothelial growth factor A (VEGFA) is likely the most important one involved in the angiogenic switch. Indeed, in RIP1-Tag2 murine pancreatic tumor, specific deletion of VEGFA in β-cell or chemical inhibition of VEGFA drastically reduces the number of angiogenic islets observed (Bergers et al., 2000; Inoue et al., 2002). Proteases are also involved in the angiogenic switch, and the most important one is matrix metalloproteinase (MMP) 9, likely via increasing angiogenic factor bioavailability. The genetical deletion of MMP9 and its chemical inhibition strongly decrease the onset of angiogenesis in the RIP1-Tag2 model (Bergers et al., 2000). Other proteases, such as cathepsin B and S, and other angiogenic factors, such as fibroblast growth factor (FGF); are also involved in the onset of angiogenesis during tumorigenesis, but to a lesser extent (Baeriswyl and Christofori, 2009).

Some stromal cells, such as neutrophils, TAMs and endothelial progenitor cells (EPCs) also favor the angiogenic switch (Baeriswyl and Christofori, 2009). This was evidenced by experiments depleting these cells.

2.3 Blood vessels and metastasis

As explained above, several steps are needed to allow cancer cell metastasis (Fig. 7). Blood vessels are involved in some of them, including cancer cell migration, intravasation and extravasation.

2.3.1 Cancer cell migration

In the chapter 3, we described some of the mechanisms by which ECs (and TAMs) induce cancer cell migration towards blood vessels. Basically, paracrine loop between ECs (hepatocyte growth factor) and cancer cells (c-Met receptor), and between macrophages, ECs (endothelin) and cancer cells (endothelin receptor) promote the migration of cancer cells toward blood vessels (Delprat and Michiels, 2021). Another paracrine loop is described in the next paragraph.

Tumor EC phenotype can be modified by the TME to a more pro-metastatic phenotype. Indeed, tumor ECs from highly metastatic melanoma tumors are more potent inducers of cancer cell migration toward blood vessels than tumor ECs from low metastatic tumors *ex vivo* (Maishi et al., 2016).. In highly metastatic melanoma, ECs promote cancer cell migration towards blood vessels via biglycan secretion. Biglycan induces the activation of TLR2 and TLR4 in cancer cells since the migration of cancer cells promoted by ECs is abolished by biglycan shRNA in ECs and by anti-TLR2 or anti-TLR4 antibodies. In conclusion, tumor ECs in highly metastatic tumors are skewed towards a pro-metastatic phenotype by the TME, and several paracrine loops between tumor ECs and cancer cells are involved in cancer cell migration toward blood vessels.

2.3.2 Cancer cell intravasation

Cancer cell intravasation requires at least 3 steps which are cancer cell migration/invasion, adhesion onto endothelium and transendothelial migration. Hence, it is difficult to discriminate between cancer cell migration and intravasation since some molecules which have an impact on migration/invasion will likely also increase the number of intravasated cancer cells (Chiang et al., 2016). Nonetheless, some *in vivo* and *in vitro* models allow for the study of intravasation process, even if this is technically challenging. *In vivo*, this is possible to use intravital high-resolution imaging to directly visualize cancer cell intravasation process in murine and zebrafish cancer models (Harney et al., 2015; Stoletov et al., 2007), or to quantify the number of circulating tumor cells (CTCs). The latter is particularly relevant when the primary tumor size and microvascular density are similar between the different conditions studied. Another *in vivo* model is to transplant cancer cells into chorioallantoic membrane (CAM) of the chick embryos, and to analyze the ability of cancer cells to enter into the vascular network. Since the chick embryos are immunodeficient, the immune rejection of cancer cells is prevented (Conn et al., 2008). *In vitro*, 3D models have been setup in order to study the intravasation of cancer cells (Zervantonakis et al., 2012), whereas there exist also intravasation studies using cancer cell transendothelial migration within boyden chambers

(Chiang et al., 2016). In conclusion, although intravasation process is challenging to study, a lot of methods allow the study of cancer cell intravasation.

Intravasation and extravasation are different mechanisms since cancer cells cross the ECs from opposite side, and since ECs have an apico-basal polarity. Indeed, EC adhesion molecules and EC-EC junction are distributed in a well-defined organization. Nonetheless, cancer cell intravasation and extravasation are both promoted by EC permeability. The most prominent factor inducing EC permeability is VEGF, which enhances both cancer cell intravasation and extravasation as well as metastasis (Delprat and Michiels, 2021; Garcia-Roman and Zentella-Dehesa, 2013; Harney et al., 2015; Weis et al., 2004). Another mechanism inducing endothelial permeability is a process called endothelial to mesenchymal transition (EndMT), which is characterized by a loss of endothelial features, markers, apico-basal polarity and tight junction and an increase in mesenchymal marker expression (Delprat and Michiels, 2021; Platel et al., 2019). A specific ablation of endoglin in ECs induces EndMT both *in vitro* and *in vivo* in murine tumors. This drastically increases endothelial permeability and cancer cell transendothelial migration *in vitro* and the occurrence of metastases of LLc cancer cells injected subcutaneously *in vivo* (Anderberg et al., 2013).

Stromal cells such as TAMs, CAFs, neutrophils and ECs strongly support cancer cell intravasation. TAMs strongly favor cancer cell intravasation in breast cancers, in areas called tumor microenvironment of metastasis (TMEM). The impact and mechanisms of TAMs mediated intravasation is developed in the chapter 3 of this thesis (Delprat and Michiels, 2021) and well-reviewed in (Borriello et al., 2020). Neutrophils also enhance the intravasation of cancer cells, via protease secretion. Neutrophils are high producers of MMP9 and neutrophil elastase (NE) within tumors. NE is a serine protease mostly secreted by neutrophils that regulates a lot of processes such as inflammation and cancer progression (Huang et al., 2020). The co-injection of cancer cells with neutrophils or NE strongly increases the intravasation in CAM model, and this enhancement of intravasation is abolished by elastase inhibitor (Deryugina et al., 2020). Consistently, lung cancer spontaneous metastasis of orthotopically injected head and neck carcinoma cells is strongly diminished in NE KO mice, without affecting primary tumor growth. The expression of MMP9 by neutrophils also promotes cancer cell intravasation in CAM model. Indeed, anti-IL8 treatment decreases the number of infiltrated neutrophils as well as the level of intravasation and the latter is rescued by the injection of recombinant MMP9 (Bekes et al., 2011). CAFs also sustain cancer cell intravasation notably via CXCL12 secretion. Specific deletion of CXCL12 in fibroblasts strongly diminishes the formation of lung metastasis in MMTV-PyMT breast cancer model and in orthotopically induced breast cancer model but does not affect the formation of metastasis upon injection of cancer cells into mice tail vein (Ahirwar et al., 2018). *In vitro*, CAF-derived CXCL12 strongly increases cancer cell migration, invasion and intravasation. Accordingly, *in vitro*, knockdown (KD) of CXCR4 - the CXCL12 receptor- in breast cancer cells or treatment of ECs with CXCL12 antibody decreases cancer cell intravasation (Jin et al., 2012). ECs also regulate cancer cell intravasation and metastasis. Indeed, specific deletion of slit2 in ECs strongly decreases lung metastasis induction in several murine cancer models and the number of CTCs as well. *In vitro*, slit2 expression in ECs is enhanced by highly metastatic cells via TLR3 activation in ECs, and endothelial slit2 promotes cancer cell migration and transendothelial migration (Tavora et al.,

2020). Endothelial P-selectin strongly regulates cancer cell transendothelial migration *in vitro* since this process is decreased with P-selectin KD in ECs (Zhang et al., 2017). Consistently, CD24 (ligand of P-selectin) expression in cancer cells sustains cancer cell transendothelial migration *in vitro*. Furthermore, chemical inhibition of P-selectin decreases the intravasation of renal cell carcinoma (RCC) cell in the CAM model. In conclusion, several stromal cells strongly regulate cancer cell intravasation in the primary tumor.

The intravasation process also relies on cancer cell invadopodia formation, NOTCH signaling and protease secretion. Invadopodia are cell protrusions with high proteolytic activity, which allow extracellular matrix break and intravasation (Eddy et al., 2017). Invadopodia formation is strongly stimulated by TAMs in TMEM of breast cancers (Borriello et al., 2020; Delprat and Michiels, 2021). Invadopodia formation in cancer cells depends on neural Wiskott–Aldrich syndrome protein (N-WASP), which is involved in cytoskeleton reorganization. Indeed, N-WASP KD decreases invadopodia formation in breast cancer cells both *in vitro* and *in vivo.* Consistently, the number of CTCs, level of intravasation and lung metastasis *in vivo* are strongly impaired in N-WASP KD breast cancer cells (Gligorijevic et al., 2012). The expression of MMP1, MMP17, urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) in cancer cells are linked to an enhanced intravasation occurrence. MMP1 or MMP17 inhibition in cancer cells decreases the vascular leakage, cancer cell intravasation and metastasis occurrence in CAM and spontaneous murine model of metastasis, respectively (Chabottaux et al., 2009). Furthermore, transfection of MMP1 siRNA in cancer cells impairs transendothelial migration and the induction of EC permeability *in vitro*. Inhibition of uPA or uPAR expression impairs the intravasation of cancer cells in CAM model, although this could be due to an effect on their invasive properties (Conn et al., 2009; Kim et al., 1998). The KD of NOTCH signaling in colon cancer cells strongly impairs their ability to metastasize to the lung, and impairs their ability to perform transendothelial migration *in vitro* (Sonoshita et al., 2011). In conclusion, cancer cell invadopodia and cancer cell-derived proteases are strongly involved in cancer cell invasion and intravasation, and NOTCH signaling in cancer cells is involved in cancer cell intravasation and metastasis.

2.3.3 Extravasation

2.3.3.1 Leukocyte extravasation

The extravasation of cancer cells shares common mechanisms with leukocyte extravasation (Strell and Entschladen, 2008). The process of leukocyte extravasation requires several steps (Vestweber, 2015). First, leukocytes are attracted to inflamed/damaged tissue via tissue-derived chemokines and via EC selectin which is recognized by the leukocytes. Then, leukocyte rolling on ECs is followed by the adhesion/firm arrest of the leukocytes on ECs, and then the leukocyte performs transendothelial migration mostly via paracellular diapedesis, even if transcellular diapedesis also occurs. For every step, the expression and interaction of several extracellular molecules on ECs and leukocytes are required. The interaction of extracellular E-selectin expressed on ECs and E-selectin ligand expressed on leukocytes such as P-selectin ligand 1 (PSGL1) CD44 and E-selectin ligand 1 (ESL1) is involved in leukocyte rolling. The firm arrest is mediated by endothelial adhesion molecule interaction with leukocyte integrins. Intercellular adhesion molecule (ICAM)1 and ICAM2 on ECs interact with lymphocyte function-associated antigen 1 (LFA1 ; also named αLβ2 integrin) and macrophage antigen 1 (MAC1; also

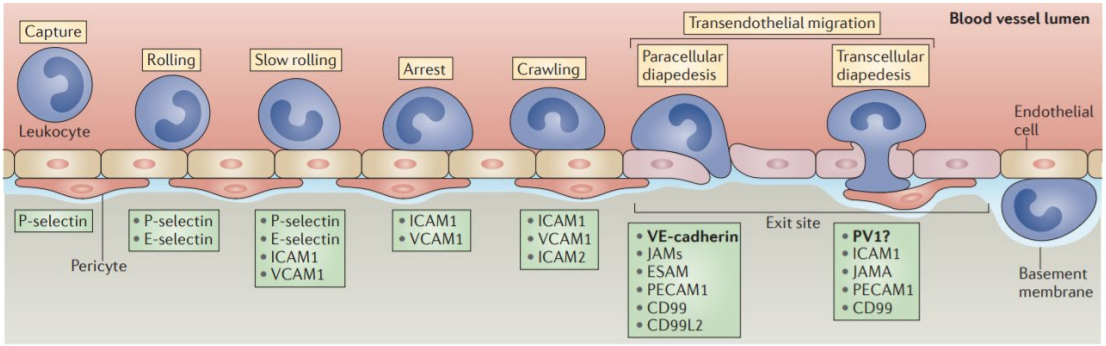

Fig. 14. **Leukocyte extravasation and EC molecules involved in this process**. (adapted from Vestweber et al., 2015)

named αMβ2 integrin), to sustain leukocyte firm adhesion. Furthermore, vascular adhesion molecule 1 (VCAM1) on ECs is also involved in leukocyte firm adhesion, via its interaction with very late antigen 4 (VLA4; also named α4β1 integrin). Then, leukocytes are crawling on ECs in order to find extravasation site, which are mainly located at EC-EC junction. ICAM1, ICAM2 and VCAM1 on ECs and LFA1, MAC1 and VLA4 on leukocytes are involved in leukocyte crawling. Then leukocyte transendothelial migration occurs. This process mostly occurs at EC-EC junction and involves a large set of EC and leukocyte extracellular proteins. During the paracellular diapedesis, EC-leukocyte interaction leads to intracellular signaling in ECs which triggers EC-EC junction weakening which allows leukocyte transmigration. More precisely, in early diapedesis, the leukocyte destabilizes the adherens junction of EC which induces the formation of a gap in which the leukocyte inserts a pseudopodium (Heemskerk et al., 2016). Then, during mid-diapedesis, RhoA activation in EC induces EC pore confinement via the formation of a ring of F-actin and actomyosin contractility. This pore confinement is very important since it ensures that leukocyte extravasation occurs without inducing endothelium permeability at the site of leukocyte extravasation. The pore is then closed by the persistence of the actomyosin contractility (Heemskerk et al., 2016). The paracellular diapedesis mainly involves junctional adhesion molecules (JAMs ; expressed on ECs), CD99 (expressed on ECs and leukocytes), platelet endothelial cell adhesion molecule (PECAM1 ; expressed on leukocyte and ECs), endothelial cell selective adhesion molecule (ESAM ; expressed on ECs) and VE-cadherin (expressed on ECs). The transcellular diapedesis phenomenon is less well known and occurs predominantly in areas in which EC-EC junctions are very tight, such as in blood-brain-barrier (Filippi, 2016; Wettschureck et al., 2019). However, a majority of adhesion molecules on ECs and on leukocytes are involved in both paracellular and transcellular diapedesis processes whereas VE-cadherin is only involved in the paracellular diapedesis. Leukocyte extravasation process is schematized in Fig. 14.

2.3.3.2 Cancer cell extravasation

Cancer cell extravasation is a critical phenomenon involved in cancer metastasis. This process is less well known that leukocyte extravasation. Nonetheless, it is known that, in order to extravasate, cancer cells need to adhere to ECs. Furthermore, cancer cells and premetastatic niche can induce EC permeability, hence promoting cancer cell extravasation.

2.3.3.2.1 EC molecules mediating cancer cell adhesion

During extravasation, cancer cells adhere to ECs. Some EC molecules are involved in cancer cell adhesion onto tumor ECs. The most well characterized is E-selectin and several cancer cells express E-selectin ligands such as various forms of CD44 (CD44v8,CD44v4, hematopoietic cell E/L selectin ligand) sialyl lewis X, Mac-2 binding protein and carcinoembryonic antigen (CEA) (Burdick et al., 2006; Dimitroff et al., 2004; Hanley et al., 2006; Ingersoll et al., 2009; Jassam et al., 2017; Shirure et al., 2012; Zen et al., 2008; Zhang et al., 2014c). The cancer cell EC interaction promotes cancer cell transendothelial migration *in vitro*. For example, the overexpression of CD44v8 in melanoma cells promote cancer cell transendothelial migration *in vitro* (Zen et al., 2008). Furthermore, CD44v4 KD in MDA-MB-231 diminishes their adhesion and transendothelial migration (Zhang et al., 2014c). Several evidences show that E-selectin expression on ECs is strongly involved in cancer cell extravasation and subsequent metastasis *in vivo*. The injection of melanoma cells into the tail vein of WT mice exclusively induces lung

tumors, whereas, when melanoma cells are injected into transgenic mice overexpressing E-Selectin, liver metastases are observed (Biancone et al., 1996). Hence, expression of E-selectin is able to redirect the organ in which cancer cells metastasize. Accordingly, the occurrence of lung metastases in severe combined immunodeficient (SCID) mice E- and P-selectin KO is strongly diminished without affecting primary tumor growth (Hiratsuka et al., 2011; Kohler et al., 2010). Consistently, the occurrence of lung metastases is diminished in E-selectin KO mice in an experimental metastasis model. Treatment of mice with E-selectin antibody diminishes the occurrence of lung metastases after injection of cancer cells into the tail vein (Hiratsuka et al., 2011). Furthermore, during adhesion of cancer cells, E-selectin is activated via ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathway. The activation of E-selectin also increases transendothelial permeability and mediates cancer cell diapedesis *in vitro* (Tremblay et al., 2006; Tremblay et al., 2008).

EC ICAM1 is also involved in cancer cell adhesion to ECs *in vitro*. This has been observed with several cancer cell lines (Benedicto et al., 2019; Benedicto et al., 2017; Laurent et al., 2014; Park et al., 2009; Regimbald et al., 1996). For example, treatment of EC with anti-ICAM1 antibody decreases the adhesion of breast cancer cells to ECs (Regimbald et al., 1996). Furthermore, pretreatment of liver sinusoidal ECs (LSECs) with anti-ICAM1 antibody decreases their adhesiveness for colon cancer cells and the transmigration of these cancer cells through the EC monolayer (Benedicto et al., 2019) (Benedicto et al., 2019). Some evidence suggest the implication of ICAM1 in liver metastasis (Benedicto et al., 2019; Benedicto et al., 2017). Pretreatment of mice with ICAM1 siRNA decreases the retention of cancer cells within the liver (Benedicto et al., 2019).

EC VCAM1 is also involved in cancer cell adhesion. There is an interaction between integrin VLA4 integrin with EC VCAM1, which mediates *in vitro* transendothelial migration (Klemke et al., 2007; Taichman et al., 1991). *In vivo*, in mice, TNFα treatment strongly promotes experimental lung metastasis. Indeed, the effect of TNFα on this process is strongly diminished and abolished by anti-VCAM1 antibody or by pretreatment of cancer cell with VLA4 antibody, respectively (Okahara et al., 1994). Furthermore, pretreatment of mice with anti-VCAM1 antibody strongly diminishes the induction of lung metastases induced by melanoma cell injection (Wieland et al., 2017). Furthermore, activation of endothelial NOTCH1 promotes melanoma cancer cell extravasation in lung, and this effect is totally abolished by VCAM1 antibody.

Homophilic interaction of JAM-C is also involved in cancer cell-EC interaction *in vitro* (Santoso et al., 2005). Furthermore, the adhesion of cancer cells in CHO cells is enhanced by the overexpression of JAM-C in CHO cells and is inhibited by anti-JAM-C antibody. Similarly, transendothelial migration of melanoma cells is inhibited by anti-JAM-C antibody (Ghislin et al., 2011). Consistently, the treatment of melanoma cells or ECs with JAM-C siRNA decreases transendothelial migration *in vitro* (Langer et al., 2011). Furthermore, the deletion of JAM-C specifically in ECs or the injection of soluble JAM-C in mice strongly decreases the occurrence of lung metastases when melanoma cells are injected into the tail vein. Hence, JAM-C ad EC-JAM-C are involved in pulmonary metastasis occurrence (Langer et al., 2011). *In vitro*, melanoma cells adhere on ECs via JAM-C (on melanoma) and JAM-B (on ECs) interaction.

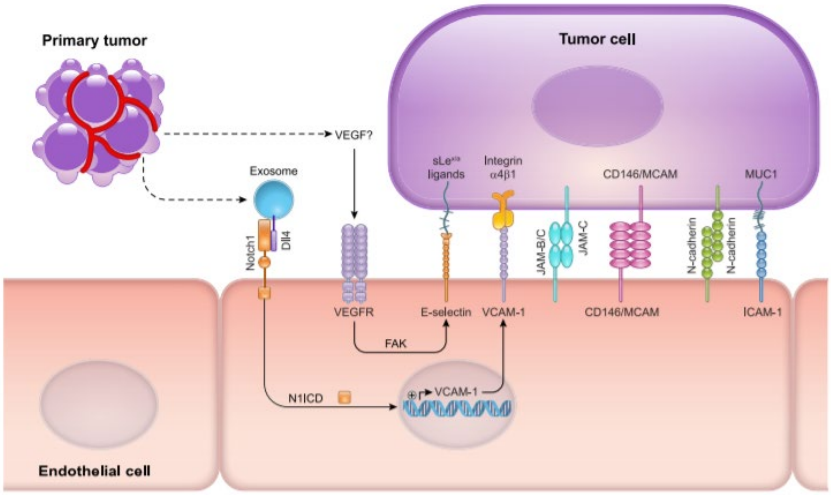

Fig. 15. EC and cancer cell ligands involved in cancer cell adhesion and transendothelial migration. (Wettschureck et al., 2019)

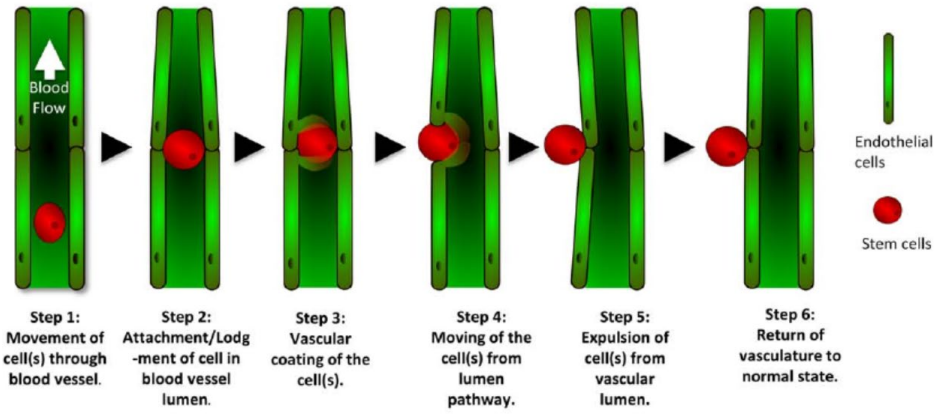

Fig. 16. Cell extravasation via the angiopellosis process . (Allen et al., 2017)

Moreover, the occurrence of pulmonary metastasis is strongly decreased in JAM-B deficient mice in an experimental metastasis model (Arcangeli et al., 2012).

The interaction of EC integrin α5 with RCC cell neuropilin (NRP)2 sustains cancer cell adhesion (Cao et al., 2013). Indeed, the KD of NRP2 in cancer cells impairs lung metastasis, without affecting primary tumor growth. The expression of CD146 by cancer cells is involved in cancer cell transendothelial migration induced by VEGF *in vitro*. Accordingly, the deletion of CD146 drastically decreases the occurrence of experimental metastases, while it does not affect primary tumor growth (Jouve et al., 2015). L1 cell adhesion molecule (L1CAM) expression in ECs also sustains adhesion and transendothelial migration of pancreatic cancer cells *in vitro*. Inhibition of L1CAM in ECs or its binding partner neuropilin 1 in cancer cells impairs cancer cell adhesion and transendothelial migration (Issa et al., 2009).

2.3.3.2.2 Cancer cell proteins modulating their adhesion to ECs and EC permeability

Cancer cells also express proteins involved in their interaction with ECs and their subsequent extravasation. The expression of N-cadherin in melanoma cells is involved in the transendothelial migration of melanoma cells *in vitro* (Qi et al., 2005). Furthermore, N-cadherin overexpression in MCF-7 cells strongly enhances cancer cell adhesion on human umbilical vein endothelial cell (HUVEC) (Hazan et al., 2000). Some data suggest that the homophylic N-cadherin interaction between cancer cells and ECs mediates cancer cell extravasation (Reid et al., 2017). The expression of integrin β1, dimerized with several α integrin subunits, also strongly supports cancer cell extravasation and metastasis. KD of integrin β1 in several cancer cell types impairs their transendothelial migration *in vitro*, extravasation and experimental metastasis formation *in vivo* (Chen et al., 2016a; Reymond et al., 2012). Integrin α3β1 and α6β1 specifically interact with laminin-5 and this interaction mediates *in vitro* cancer cell transendothelial migration (Chen et al., 2016a; Wang et al., 2004). The expression of ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (ST6GALNAC5) in breast cancer cell mediates breast cancer cell metastasis to the brain (Bos et al., 2009). ST6GALNAC5 KD and overexpression respectively decreases and increases transendothelial migration through blood brain barrier ECs in an *in vitro* model. Furthermore, treatment of breast cancer cells with ST6GALNAC5 shRNA strongly impairs the occurrence of brain cancer metastasis. The formation of invadopodia by cancer cells is involved in intravasation and extravasation (Leong et al., 2014). Additionally, vascular permeability induced by cancer cells also enhances cancer cell extravasation and metastasis. Cancer cells secrete VEGF, angiopoietin4, CCL2 or secreted protein acidic and cysteine rich (SPARC) to induce vascular permeability which then favors their extravasation (Wettschureck et al., 2019). EC necroptosis induced by cancer cells also promotes cancer cell extravasation and metastasis (Strilic et al., 2016).

In conclusion, several cancer cell and EC proteins mediate cancer cell-EC interaction. These proteins are depicted in Fig. 15. Moreover, several mechanisms lead to an increase in EC permeability which supports cancer cell extravasation.

2.3.3.2.3 Angiopellosis

A new mechanism of cancer cell extravasation, called angiopellosis has been discovered very recently (Allen et al., 2019; Follain et al., 2018) (Fig. 16). Stem cells also extravasate via

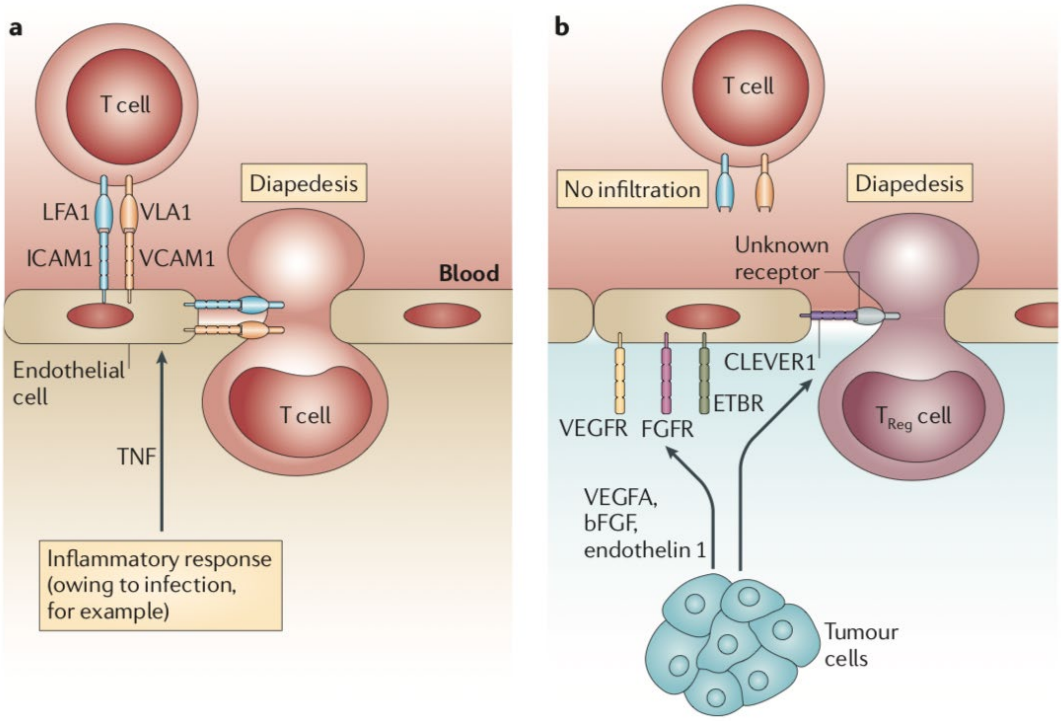

Fig. 17. Tumor EC anergy. A) The stimulation of endothelial cells with pro-inflammatory cytokines increases their expression of adhesion molecules such as ICAM1 and VCAM1, hence promoting the recruitment of leukocytes. B) In tumor, endothelin-1, VEGF and bFGF induce EC anergy. In this state, ECs are unresponsive to pro-inflammatory cytokines, and hence the recruitment of leukocytes in the tumor is diminished. Furthermore, the infiltration of immunosuppressive CLEVER1$^+$ Tregs and TAMs is specifically induced by homophilic CLEVER1 interaction in ECs. **(**Motz and Coukos., 2011)

angiopellosis. For example, in zebrafish model, about 50% of stem cells extravasate by this mechanism, while the other 50% extravasate by diapedesis (Allen et al., 2017). Extravasation by angiopellosis is a process which is much slower than diapedesis, and it is the main mechanism of cancer cell extravasation in a zebrafish model (Allen et al., 2019). This type of extravasation requires a strong endothelial activity/remodelling which is sustained by the blood flow (Allen et al., 2019; Follain et al., 2018). By a combination of experiments performed in zebrafish model and *in vitro,* Follain et al., showed that the adhesion of CTCs on ECs is inhibited by high blood flow, whereas the EC remodelling allowing the extravasation is sustained by high blood flow (Follain et al., 2018). For example, the adhesion of CTCs predominantly occurs in the arterio-venous junction of the caudal plexus in zebrafish, an area in which the blood flow is the slowest. Consistently, *in vitro*, the adhesion of cancer cells on HUVEC is higher in low blood flow perfusion vs high blood flow. The extravasation of cancer cells by angiopellosis is strongly reduced by a reduced blood flow (via lidocain treatment) in zebrafish model. *In vitro,* EC remodelling is promoted by flow perfusion. Accordingly, cancer cell extravasation is higher under blood flow vs static conditions. Furthermore, Follain et al., performed *in vitro* experiments highly suggesting that cancer cell extravasation occurs mostly via diapedesis in static conditions whereas it mostly occurs via angiopellosis under flow conditions. Nonetheless, little is known about the molecular mechanisms allowing the extravasation of cancer cells by angiopellosis and it would hence be interesting to investigate this further.

2.4 Regulation of tumor immune system by tumor endothelial cells

Tumor immune system is affected by tumor ECs by at least two mechanisms, which are the regulation of their infiltration and the direct regulation of immune cells activity.

2.4.1 Regulation of immune cell infiltration by tumor ECs

During inflammation, ECs are activated by pro-inflammatory cytokines such as IL-1β and TNFα, which trigger the expression of adhesion molecules. In tumors, the stimulation of ECs with growth factors such as FGF2, endothelin-1 and VEGF impairs the expression of adhesion molecules by ECs upon pro-inflammatory cytokine stimulation. The unresponsiveness of ECs to pro-inflammatory stimuli is called EC anergy. This is responsible for a decrease in leukocyte infiltration in the tumor and hence a decrease in antitumor immunity. Additionally, ECs are more permissive to immunosuppressive cells. For example, Clever 1 expression allows the infiltration of TAMs and Tregs which display immunosuppressive activities. In conclusion, tumor ECs regulate the infiltration of leukocytes in tumor, and are permissive for immunosuppressive immune cells (Motz and Coukos, 2011) (Fig. 17).

2.4.2 Regulation of immune cell activation by tumor ECs

Tumor ECs express numerous factors which are involved in immunosuppression such as Fas ligand (FasL), PD-L1, PD-L2, IDO, T cell immunoglobulin domain and mucin domain protein 3 (TIM3). They also secrete immunosuppressive factors such as IL-6, IL-10 and TGF-β (De Sanctis et al., 2018; Motz and Coukos, 2011). FasL is overexpressed in ECs of solid tumors and is involved in lymphocyte T $CD8^+$ killing, without affecting Treg viability (Motz et al., 2014). Tumor ECs regulate $CD8^+$ T cell activity in a PD-1/PD-L1 dependent manner and induce an immunosuppressive phenotype in $CD4^+$ T cells (Taguchi et al., 2020). Endothelial IDO1

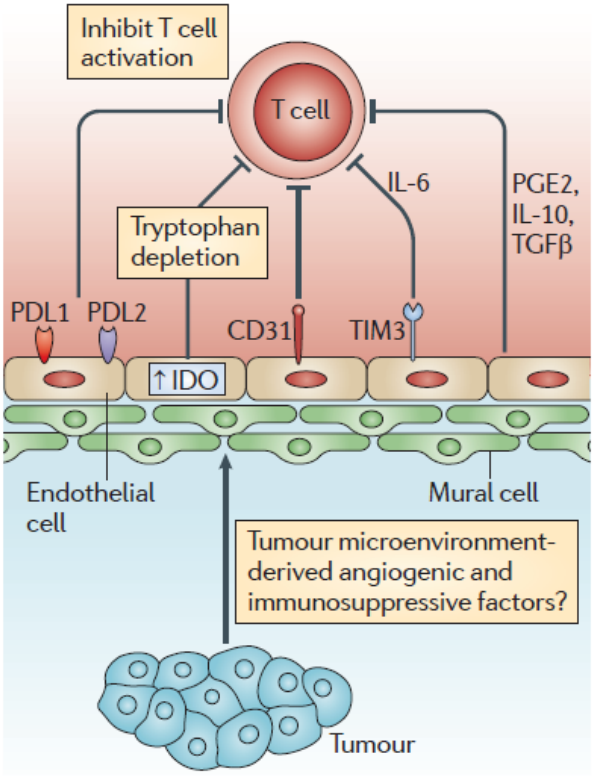

Fig. 18. Mechanisms involved in T cell activity inhibition and T cell immunosuppressive phenotype induction by tumor ECs. (Motz and Coukos., 2011)

expression is induced by CD40 immunotherapy and promotes immunosuppression (Georganaki et al., 2020). EC-derived IL-6 shifts CD4+ phenotype towards Treg and Th17 response (Taflin et al., 2011). In lymphoma, ECs that express TIM3 inhibit $CD4^+$ T cell activation (Huang et al., 2010). The production of immunosuppressive factors such as PGE2, IL-10, IL-6 and VEGF is strongly enhanced in tumor-ECs compared to ECs from normal tissues (Mulligan et al., 2010; Mulligan and Young, 2010). Additionally, ECs skew TAMs from M1 into M2 phenotype (Delprat and Michiels, 2021). The effect of tumor ECs on T cells is depicted in Fig. 18.

3 Review : a bi-directional dialog between vascular cells and monocytes/macrophages regulates tumor progression

Cancer and Metastasis Reviews
https://doi.org/10.1007/s10555-021-09958-2

A bi-directional dialog between vascular cells and monocytes/macrophages regulates tumor progression

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Received: 3 December 2020 / Accepted: 23 February 2021

Abstract
Cancer progression largely depends on tumor blood vessels as well on immune cell infiltration. In various tumors, vascular cells, namely endothelial cells (ECs) and pericytes, strongly regulate leukocyte infiltration into tumors and immune cell activation, hence the immune response to cancers. Recently, a lot of compelling studies unraveled the molecular mechanisms by which tumor vascular cells regulate monocyte and tumor-associated macrophage (TAM) recruitment and phenotype, and consequently tumor progression. Reciprocally, TAMs and monocytes strongly modulate tumor blood vessel and tumor lymphatic vessel formation by exerting pro-angiogenic and lymphangiogenic effects, respectively. Finally, the interaction between monocytes/TAMs and vascular cells is also impacting several steps of the spread of cancer cells throughout the body, a process called metastasis. In this review, the impact of the bi-directional dialog between blood vascular cells and monocytes/TAMs in the regulation of tumor progression is discussed. All together, these data led to the design of combinations of anti-angiogenic and immunotherapy targeting TAMs/monocyte whose effects are briefly discussed in the last part of this review.

Keywords Cancer · Endothelial cell · Pericyte · Monocyte/macrophage · Angiogenesis · Metastasis

1 Introduction

1.1 Tumor-associated macrophages

1.1.1 TAMs in the tumor microenvironment

Tumor-associated macrophages (TAMs) are major tumor microenvironment (TME) cells and represent an important part of the cancer immune infiltrate. TAM infiltration and TAM numbers are correlated with poor prognosis in a majority of cancer types [1]. TAMs play an important role in cancer development notably via the promotion of tumor growth, tumor inflammation, angiogenesis, lymphangiogenesis, metastasis, immunosuppression, and chemotherapeutic resistance [2–5]. Macrophages are classified as pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages. This classification is oversimplified since TAMs can express both M1 and M2 markers, and hence, TAMs are classified onto a M1 and M2 polarization axis in which M1 and M2 macrophages are the two extremes. Basically, and based on *in vitro* experiments, M1 macrophages are polarized with pro-inflammatory cytokines and bacterial molecules such as interferon γ and lipopolysaccharides. M1 macrophages express high levels of pro-inflammatory cytokines (e.g., IL-12, tumor necrosis factor α (TNFα), and IL-6) and intracellular host response genes (e.g., CD80 and IFIT1) [6, 7]. M2 macrophages are divided into at least three subsets called M2a, M2b, and M2c. This M2 classification in three subsets was firstly proposed in [6]. M2a are activated by IL-4 and/or IL-13 and express high levels of CD206, CD163, and fibronectin [6, 7]. M2b are induced by Toll-like receptors ligands and immune complex activation, whereas M2c are activated by IL-10. Interestingly, macrophage M2 polarization is also induced by the TME [8–14]. Nonetheless, the three classes of M2 macrophages share common features such as IL-12^{low} and IL-10^{high} and arginase-1 (Arg-1)^{high}, whereas M1 macrophages are IL-12^{high}, IL-23^{high}, and IL-10^{low}. In the TME, CD163 and CD206 are commonly used to identify macrophages from the M2 population, whereas CD86 is a common M1 marker.

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Published online: 30 March 2021

1.1.2 Origins of TAMs

There exist at least two origins of TAMs. TAMs can originate either from tissue-resident macrophages (TRMs) or from blood vessel inflammatory monocytes (IMs) CCR2$^+$, which are recruited via CCL2 chemotaxis [15–17]. TRMs are present in healthy tissues, hence before cancer initiation [15]. TRMs arise from embryonic progenitor–derived macrophages (e.g., brain macrophages also called microglia) or from blood monocytes (e.g., intestine or dermis). Furthermore, TRMs are able to self-maintain without adult blood monocyte contribution. Although TRMs are known for a while, the implication of TRMs in cancers has only recently been investigated, mostly in murine tumor models. For example, TRMs promote pancreatic ductal adenocarcinoma (PDAC) progression [18]. Indeed, colony-stimulating factor 1 (CSF1) antibodies combined with clodronate liposome followed by 10 days of blood monocyte recovery induce an almost complete TRM depletion without affecting circulating monocyte. In these conditions, tumor burden and high-grade carcinoma development are drastically reduced [18]. Nonetheless, monocyte-derived macrophages represent the major macrophage population in a majority of murine cancer types, such as breast, lung, brain, and hepatocellular carcinoma [15]. Monocytes are classified into 3 subsets in humans and in mice, according to marker expression [19, 20]. There are IMs (CD16$^-$/CD14$^+$/CX$_3$CR1^{lo} in human, Ly6C^{high}/CD43^{lo}/CX3CR1^{lo} in mouse), non-classical monocytes (or patrolling, CX3CR1^{high}, CD14^{lo}, CD16$^+$ in human and Ly6C^{lo}/CD43^{high}/CX3CR1^{high} in mouse), and intermediate monocytes (CX3CR1^{high}, CD14$^+$, CD16$^+$ in human, Ly6C^{int}CD43^{hi}CX3CR1^{hi} in mouse). Numerous murine studies showed that IMs are the major source of TAMs in tumors, such as mammary tumors and their associated lung metastases, hepatocellular carcinoma, orthotopic Lewis lung carcinoma (LLc), and PDAC [19]. Furthermore, IMs display pro-tumoral functions such as angiogenesis and metastasis promotion [19]. Non-classical and intermediate monocytes have pro and anti-tumoral functions. Indeed, human intermediate and non-classical CD16$^+$ monocytes promote angiogenesis *in vitro* [21] and murine Ly6G^{lo} patrolling monocytes are immunosuppressive in vivo [22, 23], whereas murine patrolling monocytes prevent breast to lung metastasis in murine PyMT breast cancer model [24]. Another type of monocyte classification exists, based on the receptor tyrosine kinase Tie2 expression. Indeed, recently, Tie2-expressing monocytes (TEMs) have been discovered by De Palma and colleagues [25, 26]. Before these studies, only ECs were thought to express the angiopoietin (1–4) receptor Tie2 [25]. Nowadays, some cell types have been discovered to express Tie2: endothelial cells (ECs), TEMs, a subset of TAMs, pericyte precursors of mesenchymal origin, a subset of hematopoietic stem cells, and some cancer cell lines [26–28]. Two studies showed that Tie2 is expressed mainly by intermediate monocyte (CD14$^+$ CD16$^+$), whereas one study shows that Tie2 is also expressed in non-classical monocyte (CD14^{dim} CD16$^+$). Hence, Tie2 is expressed mostly but not exclusively in CD16$^+$ monocytes and to a lesser extent in CD16$^-$ monocytes [29].

1.2 Tumor blood vessels

1.2.1 The onset of angiogenesis or the "angiogenic switch"

During cancer development, the transition from an avascular tumor to a vascularized tumor, called the "angiogenic switch" is a critical step [30, 31]. This switch occurs when the balance between pro-angiogenic factors (e.g., vascular endothelial growth factor (VEGF)-A) and anti-angiogenic factors (e.g., statins) shifts towards angiogenesis [30]. This switch appears during the progression from hyperplasia to neoplasia and coincides with malignant transition in PyMT and RIP1-Tag2 mice models. It is needed for malignant tumor progression [32, 33]. Immune cells such as TAMs and neutrophils are involved in this process. For example, in the PyMT murine breast cancer model, high TAM infiltration precedes the onset of angiogenesis. Furthermore, vasculature development is observed earlier in this model when macrophage infiltration is induced with CSF1 transgenic overexpression specifically in mammary tissues [32]. In the Rip1-Tag2 mouse pancreatic tumor model, neutrophil ablation with anti-Gr1 antibody strongly diminishes tumor vessel development [34].

1.2.2 Lymphatic vasculature and lymphangiogenesis

Lymphatic vasculature is critically involved in fluid homeostasis regulation, immune cell dissemination/surveillance, and lipid reabsorption. Absence or non-functional lymphatic system causes lymphedema, a disease characterized by huge swelling and repeated skin infections.

Lymphangiogenesis is defined as the formation of new lymphatic vessels from existing ones. It occurs during embryonic development and during tumor growth. It is correlated with a bad prognosis in cancer [35]. Lymphatic vessel hyaluronic receptor 1 (LYVE 1), podoplanin, and prospero homeobox 1 (prox1) are lymphatic EC (LEC) markers. Mechanistically, VEGF-C and VEGF-D are the two main lymphangiogenic factors which promote lymphangiogenesis by activating LECs VEGF receptor 3 (VEGFR-3) [5]. Lymphatic vasculature is critically involved in the metastatic spread of cancer cells into lymph nodes and finally to distant organs [36–39]. Lymphatic vessel density and lymph node status (i.e., the presence or the absence of cancer cells) is associated with poor prognosis and metastasis in several cancers [35]. The link between VEGF-C, VEGF-D, lymphatic vessel density, lymph node metastasis, and prognosis is extensively reviewed in [40].

1.2.3 Tumor blood vessels and immune system: endothelial anergy

During cancer progression, the immune system is progressively modified by the TME in a process called immunoediting. This process is composed of three phases, namely elimination, equilibrium, and escape. In the two first phases, the immune system is able to kill cancer cells notably via $CD8^+$ T cells and natural killer (NK) cells. During these stages, TAMs belong mostly to M1 phenotype and are able to kill cancer cells and to activate the immune system. For example, in early-stage human lung tumors, TAMs mostly share both M1 and M2 markers and are able to activate T cell function, and hence are anti-tumoral [41]. In pancreatic pre-cancerous lesions, in gastrointestinal stromal tumors, in ovarian cancer, and in bladder cancer, TAMs mostly belong to the M1 phenotype and are progressively skewed toward the M2 phenotype during disease progression [42–44]. In later stages, TAMs display mostly M2 phenotype and are protumoral and immunosuppressive.

Tumor blood vessels constitute a barrier regulating immune cell recruitment from blood into tumor via extravasation. The regulation of immune cell extravasation into tumor through blood vessels is then crucial in the regulation of tumor progression. This process is highly regulated and is composed of several steps. First, there is leukocyte rolling followed by the arrest and firm adhesion to ECs. Then, leukocytes transmigrate through ECs to extravasate and infiltrate the tissue. This process requires adhesion molecules expressed by ECs such as E-selectin (rolling), ICAM1 and VCAM1 (firm arrest), and VE-cadherin and CD31 (transendothelial migration) [45]. The expression of these proteins is tightly regulated and promoted by inflammatory stimuli such as TNFα. Tumor vessels are modified by TME to induce endothelial anergy, notably via VEGF [46]. In this state, tumor endothelial cells are unresponsive to pro-inflammatory stimuli such as TNFα and hence do not promote anymore leukocyte extravasation [47]. This anergy is crucial in tumor growth promotion, likely more importantly during the elimination and equilibrium phases, because the immune system is anti-tumoral. For example, the overexpression of EGF-like domain–containing protein 7 (Egfl7) in cancer cells, an endothelial activation repressor [48], promotes tumor growth and development by preventing leukocyte infiltration via endothelial E-selectin and ICAM1 and VCAM1 adhesion molecule repression [49].

Tumor blood and lymphatic vessels also modulate the immune system (this is well reviewed in [46]). Indeed, lymphatic ECs (LECs) and ECs both express program death-ligand 1 (PD-L1), which inhibits T cell function [50, 51]. Furthermore, ECs can induce T cell apoptosis by Fas ligand expression [52]. Tumor ECs are modified by the TME. Indeed, IL-6 and IL-10 secretion from lung tumor ECs is strongly increased. Normal lung ECs induce strong NK cell activation, whereas this ability is strongly reduced in ECs from lung tumors [53]. Furthermore, IL-6 and IL-10 cytokines are involved in macrophage polarization towards M2 phenotype and hence promote tumor growth [54, 55].

To recapitulate, tumor blood vessels regulate immune cell infiltration as well as their activation in tumors. In this review, the impact of vascular cells (ECs and pericytes) on monocyte and TAM recruitment into tumors will be discussed. Furthermore, the impact of vascular cells on monocyte and TAM angiogenic phenotype and polarization will also be described. Reciprocally, the impact of TAMs, TEMs, and classical and non-classical monocytes on blood vessels will be emphasized. Their impact on angiogenesis, lymphangiogenesis, and metastasis will be detailed.

2 Effects of vascular and perivascular cells on macrophages (related to Fig. 1)

2.1 Monocyte and macrophage recruitment by ECs and pericytes (related to Fig. 1a)

TAM recruitment in cancer is involved in the angiogenic switch induction; promotes tumor growth, metastasis, vessel "abnormalization"; and is associated with a bad prognosis in most cancer types. Indeed, macrophage depletion by different ways has a negative impact on these features. TAMs are recruited by different chemokines and cytokines such as chemokine (C-C motif) ligand 2 (CCL2), CCL5, CCL7, angiopoietin-2 (Ang-2), CSF1, VEGF, IL-33, semaphorin 3D, endothelial monocyte–activating polypeptide-II (EMAP-II), endothelin (ET)-1 and 2, stromal cell–derived factor 1α (SDF1α/CXCL12), eotaxin, and oncostatin which are secreted by cancer cells, stromal cells, and perivascular and vascular cells. This is extensively reviewed in [69, 70]. TAMs are classified not only according to their marker expression into M1 or M2 phenotype, but also according to their tumor localization into migratory TAMs or perivascular TAMs [4, 17]. Here, we will focus on the effects of ECs and perivascular cells on TAM and monocyte recruitment as well as on their localization within the tumor.

2.1.1 EC-derived angiopoietin-2 (Ang-2)

Ang-2 is mainly released by ECs in tumors, but in some cases, Ang-2 is also expressed by cancer cells [71]. Ang-2 is stored in Weibel-Palade bodies in ECs [72, 73], and its expression and release from EC are regulated by CTHRC1/ERK/AP-1 signaling and by neuroligin 2 [74, 75]. *In vitro*, EC-derived Ang-2 induces chemotaxis of $Tie2^+$ macrophages and monocytes. THP-1 $Tie2^+$ monocytes but not $Tie2^-$ migrate towards Ang-2 in the Boyden chamber model [76]. U937 monocytes exposed to Kaposi's sarcoma EC conditioned media migrate towards the conditioned medium compartment. This

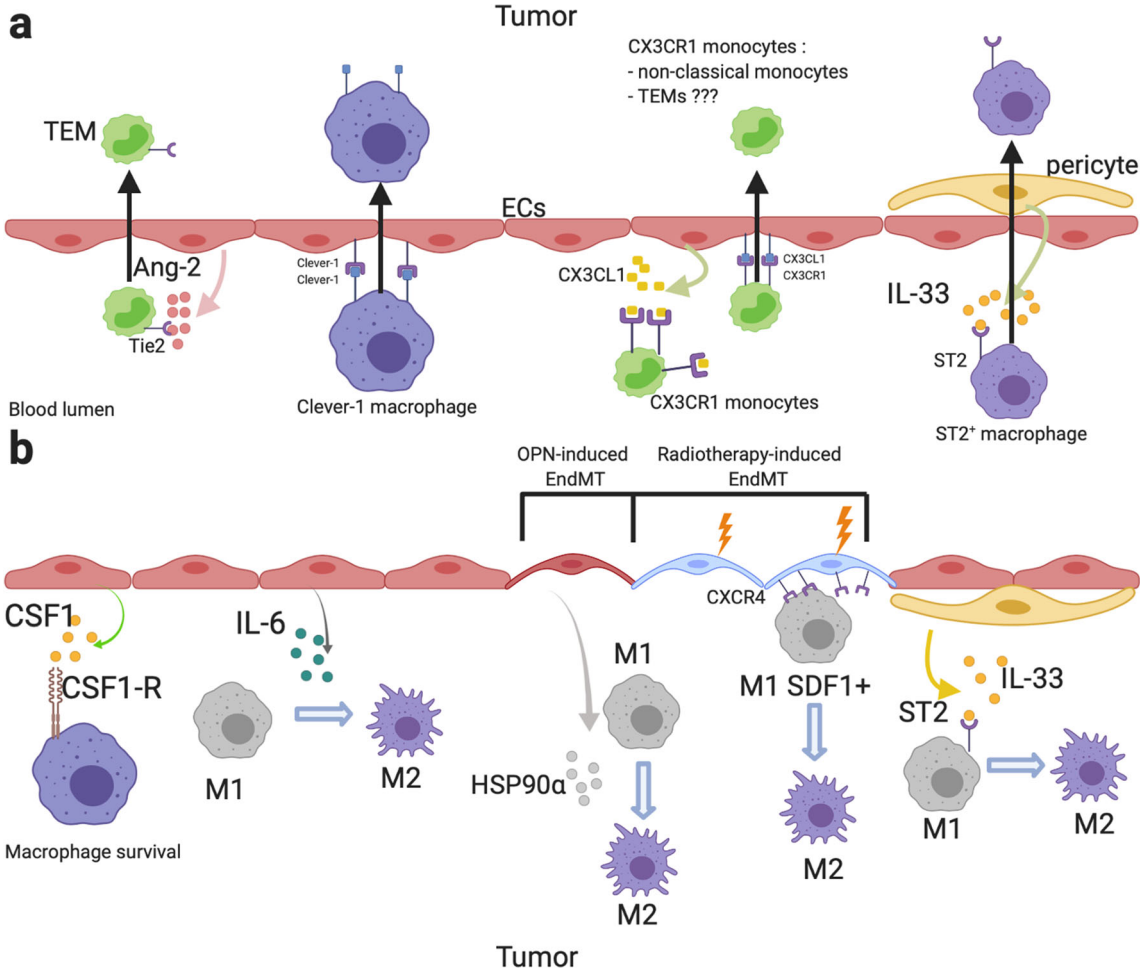

Fig. 1 Effects of blood vessels on monocyte/macrophage recruitment and polarization. **a** Effects of ECs and pericytes on monocyte/macrophage recruitment. ECs secrete high dose of Ang-2 which induces TEM recruitment in tumor [56–59]. Furthermore, Ang-2 promotes angiogenic phenotype in TEMs and in Tie2-expressing macrophages [58]. Homophilic interaction between Clever-1 in ECs and TAMs induces TAM infiltration [60]. ECs secrete CX3CL1 which induces CX3CR1-expressing monocyte (non-classical monocyte) chemotaxis toward ECs [21, 61]. CX3CL1/CX3CR1 interaction induces non-classical monocyte recruitment via VEGF-A-dependent CX3CL1 shedding [21, 62, 63]. IL-33 secreted by pericytes promotes TAM recruitment via the IL-33 receptor ST2 activation [64]. **b** Effects of ECs and pericytes on TAM survival and polarization. CSF1 promotes TAM survival in the TME via CSF1R activation [65]. ECs are high IL-6 producer. EC-derived IL-6 induces TAM M2 polarization [12]. Osteopontin-induced EndMT promotes M2 TAMs polarization via HSP90α secretion [66]. Radiotherapy-induced EndMT induces CXCR4 expression in ECs, which promotes SDF1α-expressing TAM M2 polarization [67]. IL-33 secreted by pericytes promotes M2 polarization in a ST2-dependent manner [68]. This figure was created with BioRender.com

migration is abolished with anti-Ang-2 antibody or with Ang-2 shRNA in ECs [56]. Hence, Ang-2 expression and release by EC are tightly regulated and promote TEM migration *in vitro*.

In vivo, Ang-2 induces Tie2^{+} TAM and TEM infiltration by stimulating the expression of their Ang-2 receptor Tie2. Indeed, Ang-2 induces macrophage and TEM infiltration that is correlated with metastasis in murine MDA-MB-231-induced breast cancer, in pancreatic cancers, in lung cancer, in Kaposi's sarcoma, in glioblastomas (GBMs), and in gliomas [56–58, 74, 77]. Indeed, specific EC Ang-2 overexpression increases macrophage and TEM infiltration in murine GBM and LLc lung tumor models [57, 58]. Ang-2 inhibition diminishes TAM and/or TEM infiltration in Kaposi's sarcoma and breast cancer murine models [56, 59]. Nonetheless, in MMTV-PyMT breast cancer and Rip1-Tag2 pancreatic cancer, Ang-2 inhibition does not modify macrophage or TEM infiltration but rather inhibits their perivascular localization [78]. Ang-2 blockade induces SDF1α overexpression in the MMTV-PyMT model, which can counterbalance the effects of Ang-2 blockade on TAM and TEM infiltration. Ang-2 induces EC ICAM1 and VCAM1 expression that hence increases monocyte and TAM adhesion on EC [77]. Moreover, Ang-2 increases vessel permeability, angiogenesis, and CCL2 expression in ECs that also leads to C-C chemokine receptor type 2 (CCR2)$^{+}$ monocyte and TAM infiltration [77, 79]. Hence, in tumors, Ang-2 is an important EC-secreted protein that is involved in macrophage and monocyte Tie2^{+}

infiltration and perivascular localization. Moreover, Ang-2-induced EC CCL2 overexpression induces $CCR2^+$ IM recruitment.

In line with the fact that Ang-2 induces TAM and TEM infiltration, Ang-2 expression is correlated with microvascular density and associated with poor prognosis in several cancers [71]. Furthermore, Ang-2 is overexpressed in tumor tissues compared to normal tissues [71]. Ang-2 expression is increased by anti-VEGF therapies in tumor but not in normal tissues [76, 80–82]. This Ang-2 overexpression leads to therapy failure by increasing TEM and TAM infiltration. This TAM recruitment induced by anti-VEGF therapy is blocked by the addition of Ang-2 antibody or soluble Tie2 [76]. This bitherapy has been tested in phase I in human cancer patients and showed acceptable safety and encouraging antitumor activity [83]. To summarize, Ang-2 is involved in tumor resistance against VEGF therapy and anti-Ang-2/VEGF combination shows encouraging results in pre-clinical and clinical studies.

2.1.2 Pericytes and perivascular cancer-associated fibroblasts in TAM recruitment

Pericytes and perivascular cancer-associated fibroblasts (CAFs) are involved in TAM recruitment and their perivascular localization. Platelet-derived growth factor (PDGF)-BB secretion by cancer cells induces IL-33 expression and secretion by pericytes and CAFs via a PDGF receptor β (PDGFRβ)–dependent mechanism. IL-33 further stimulates macrophage migration *in vitro* and TAM infiltration *in vivo* via the IL-33 receptor ST2–dependent mechanism [64]. Indeed, pericytes exposed to PDGF-BB *in vitro* or *in vivo* in lung tumor model with LLc overexpressing PDGF-BB overexpress IL-33 and this overexpression is abolished by anti-PDGFRβ antibodies. IL33-induced RAW cell migration is abolished by ST2 RAW siRNA. *In vivo*, TAM infiltration is increased in tumors overexpressing PDGF-BB. This increase is abolished in mice $IL\text{-}33^{-/-}$, $ST2^{-/-}$ or with ST2 soluble factors. These IL-33 recruited TAMs are also involved in tumor growth and in cancer cell stemness via prostaglandin 2 secretion [84]. Milk fat globule-epidermal growth factor 8 (MFG-E8), expressed mostly by pericytes in melanoma tumors, is also involved in TAM infiltration by an unknown mechanism which would be interesting to clarify [85]. Consistently, high MFG-E8 expression is associated with high TAM infiltration in bladder cancer [86]. As said above, TAMs are also classified according to their tumor localization in which there are migratory TAMs and perivascular TAMs [4, 17]. In fact, there is a unidirectional mechanism by which a newly recruited monocyte will differentiate in migratory TAMs which then will be recruited to blood vessels and hence become perivascular [17]. Indeed, in the mammary PyMT model, newly tumor-infiltrated blood CCR2+ monocytes are recruited by cancer cell– and stromal cell–derived CCL2. Then, monocytes differentiate into migratory TAMs and C-X-C chemokine receptor type 4 (CXCR4) expression by TAMs is then promoted by tumor-derived transforming growth factor-β (TGF-β). These migratory TAMs are then recruited near to the blood vessel by SDF1α-derived perivascular CAFs [17]. In summary, perivascular cells are involved in TAM recruitment via IL-33 secretion and in TAM perivascular localization via SDF1α secretion.

2.1.3 Monocyte/TAM recruitment via direct interactions with ECs

Whereas most endothelial-leukocyte adhesion molecules are shared between all leukocyte types [46], monocytes and TAMs are also specifically recruited by tumor ECs [46]. Clever-1/stabilin-1^+ is a scavenger receptor and an adhesion molecule regulating macrophage and T regulator lymphocyte transendothelial migration as well as tumor infiltration [60, 87]. Indeed, Clever-1 overexpressing ECs are involved in Clever-1^+ monocyte/macrophage and Treg recruitment [60]. Indeed, Clever-1 deletion in mice or specifically in macrophages or in ECs leads to a diminished TAM recruitment, without affecting lymphocyte $CD4^+$ or $CD8^+$ recruitment [60]. Apoptosis signal-regulating kinase 1 (ASK1), a factor involved in the regulation of EC activation, is involved in TAM recruitment in tumor, without affecting lymphocyte $CD3^+$ recruitment [88, 89]. In non-inflammatory conditions, ASK1 is consistently degraded via suppressor of cytokine signaling 1 (SOCS1) by the proteasome and pushes ECs in an inactivated state. In inflammatory condition, ASK1 is stabilized and stimulates EC activation via JNK/p38MAPK activation [89]. EC ASK1 expression induces macrophage infiltration into tumors without affecting lymphocyte recruitment [88]. TAM infiltration in tumors is decreased in ASK1 KO mice or with ASK1 inhibition specifically in EC (via SOCS1 overexpression specifically in EC) or with ASK1 inhibitor. This TAM infiltration prevention by ASK1 inhibition leads to a decrease in tumor growth and in metastasis and to an increased survival in mice [88]. Nonetheless, the mechanism by which ASK1 leads to specific TAM infiltration remains unclear and it would be interesting to be investigated. That could be either by chemotactic factor over-secretion specifically inducing TAM infiltration (e.g., CCL2, Ang-2) or via a direct contact between TAMs and ECs inducing TAM transmigration (e.g., via Clever-1 interaction). *In vitro*, TAM transmigration is impaired across EC ASK1–specific inhibition, but the lymphocyte transmigration has not been investigated [88]. All these data demonstrate that homophylic interaction between EC and macrophage Clever-1/stabilin-1 is involved in TAM recruitment into tumor without affecting $CD4^+$ or $CD8^+$ lymphocyte recruitment.

2.1.4 CX3CL1/CX3CR1 axis in non-classical monocyte recruitment

Chemokine (C-X3-C motif) ligand 1 (CX3CL1) expression in ECs is involved in the CX3CL1 receptor (CX3CR1)–dependent recruitment of immune cells, such as NK cells, $CD8^+$ T cell, and CX3CR1 non-classical monocytes [90]. CX3CL1 expression in EC specifically regulates CX3CR1-expressing monocyte recruitment into tumors without affecting IM recruitment. This process may be also involved in TEM recruitment since around 50% of TEMs express CX3CR1 [29]. CX3CL1 exists as membrane bound and soluble forms. Soluble CX3CL1 is involved in CX3CR1 monocyte chemotaxis, whereas membrane bound is involved in their adhesion to ECs [61, 91]. Indeed, soluble CX3CL1 induces human peripheral blood mononuclear cell–derived monocyte migration, more effectively than CCL5 [91]. $CX3CL1^+$ monocytes adhere to HEK293 overexpressing membrane bound CX3CR1 but not to WT HEK293. The membrane-bound CX3CL1 promotes human non-classical monocyte crawling and adhesion on endothelium via CX3CR1 activation on non-classical monocytes [21]. The subsequent monocyte transmigration is promoted by angiogenic factors such as VEGF-A. VEGF-A involvement in non-classical monocyte transmigration is due to VEGF-A-induced a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10) and ADAM17 activity stimulation [62, 63], which subsequently promotes CX3CR1 monocyte transmigration via CX3CL1 shedding [92] and hence non-classical monocyte transmigration. Consistently with these results, *in vitro* transendothelial migration and *in vivo* infiltration of non-classical monocytes into tumors are critically lower in non-angiogenic tumors, whereas they are increased in angiogenic tumors [21]. Indeed, human non-classical monocytes are recruited mostly in DLD1 or HCT116 tumor expressing high level of VEGF-A, whereas they are less recruited in SKBR1 tumor expressing low level of VEGF-A. Furthermore, treatment of DLD1 tumors with anti-VEGF-A antibody bevacizumab reduces the human $CD16^+$ monocyte recruitment. Nonetheless, the DC101 anti-VEGFR2 antibody increases $Ly6C^{lo}$ monocyte infiltration into orthotopic murine colorectal tumors. Furthermore, non-classical monocytes require CX3CR1 to infiltrate tumors since $Ly6C^{lo}$ monocytes infiltration in murine orthotopic colorectal tumors is abolished in CX3CR1 KO mice [22]. These data suggest that the interaction between CX3CL1 (EC) and CX3CR1 (non-classical monocyte) promotes non-classical monocyte recruitment into tumor. This recruitment is enhanced in angiogenic tumors and it would be interesting to investigate if this process is involved in TEM infiltration since 50% of TEMs express CX3CR1.

2.2 Impact of blood vessel cells on macrophage polarization and angiogenic phenotype (related to Fig. 1b)

2.2.1 ECs promote M2 polarization and angiogenic phenotype

ECs are involved in macrophage survival, proliferation, M2-polarization, and angiogenic phenotype acquisition in malignant and non-malignant tissues [12, 54, 65]. The impact of ECs on macrophage survival has been demonstrated by co-culture experiments. The macrophage survival and expansion are mediated by direct contact between ECs and macrophages since macrophage colony formation is observed with direct co-cultures but not with transwell assays. CSF1-membrane bound (EC) and CSF1 receptor (CSF1R) (macrophage) juxtacrine interaction is involved in macrophage survival and expansion, since a CSF1 exclusive inhibitor inhibits macrophage survival and expansion [65]. This survival/proliferation induced by ECs in macrophages is likely due to mechanistic target of rapamycin (mTOR) activation in macrophage since mTOR inhibition with rapamycin inhibits CSF1+IL-6-induced macrophage proliferation [12]. Furthermore, ECs induce M2 polarization *in vitro* and *in vivo*, notably via IL-6 secretion [12, 54]. Indeed, the macrophage-EC co-cultures increase M2 marker expression such as Tie2 and decrease M1 marker expression such as major histocompatibility complex II (MHCII) [65]. Furthermore, EC conditioned media induce M2 polarization associated with the enhanced expression of CD206 or Arg-1, which is reduced by anti-IL-6 antibody [12]. This M2 polarization is enhanced in pre-incubated ECs with GBM cells which seems that this EC-induced M2 polarization is amplified by the TME. In human and murine GBM, alternatively activated TAMs are localized proximately to ECs, which are a major source of IL-6. Indeed, *in vivo*, specific inducible deletion of IL-6 in ECs reveals that ECs are the major source of IL-6 in murine GBM [12]. Furthermore, IL-6 expression is highly detected in ECs cytoplasm of newly formed vessels in human GBM [93]. Specific inducible deletion of IL-6 in ECs strongly decreases M2 macrophage population and slightly increases M1 population, decreases tumor growth, and enhances mice survival [12]. In summary, ECs are involved in macrophage survival and expansion via CSF1-CSF1R juxtacrine loop, and in macrophage M2 polarization, notably via IL-6 secretion *in vivo*, at least in murine GBM.

ECs induce angiogenic phenotype in macrophages associated with an increase in Tie2 or VEGF-A expression and macrophages co-cultivated with ECs increase murine prostate tumor growth and angiogenesis, when these macrophages are co-injected with cancer cells in mice [65]. EC-derived Ang-2 is not only a chemoattractant for

TEMs. Indeed, Ang-2 also promotes M2 polarization and angiogenic profile in TEMs by increasing the expression of M2 markers (IL-10 and MRC1) and of angiogenesis-related gene (cathepsin B and thymidine phosphorylase) expression [58]. Furthermore, *in vivo*, Ang-2 and Ang-2 + VEGF inhibitions shift macrophage population from M2 towards M1. Anti-Ang-2 increases M1/M2 intermediate macrophage population in murine GBM. Anti-Ang-2 combined with an anti-VEGF increases M1 proportion among total leukocytes in the PyMT model and increases M1 population and decreases M2 population among total macrophages [81, 94]. Hence, Ang-2 is involved in macrophage and TEM M2 polarization and promotes their angiogenesis phenotype.

2.2.2 Endothelial-to-mesenchymal transition (EndMT) promotes macrophage M2 polarization

Endothelial-to-mesenchymal transition (EndMT) is defined as a phenotypic change in ECs characterized by a loss of endothelial features, markers (e.g., CD31), cellular tight junctions, apico-basal polarity, and the acquisition of mesenchymal features and markers such as fibroblast specific protein-1 and α-smooth muscle actin (α-SMA) [95]. EndMT is a source of up to 40% of CAFs; can be induced by radiotherapy, TGFβ-1, or osteopontin; and has an impact on tumorigenesis, metastatic extravasation, and therapy resistance [67, 95–98]. Radiotherapy-induced EndMT is mediated via p53 activation in ECs, whereas it is mediated via transcription factor 12 (TCF12) in osteopontin-induced EndMT since p53 siRNA and TCF12 shRNA inhibit radiotherapy-induced EndMT and osteopontin-induced EndMT, respectively. Furthermore, ECs undergoing EndMT with osteopontin or radiotherapy induce M2 polarization and inhibit M1 polarization [66, 67]. This is mediated via heat shock protein 90 α (HSP90α) secretion by osteopontin-induced EndMT, whereas it is mediated via CXCR4/SDF1α signaling in radiotherapy-induced EndMT [66, 67]. *In vitro*, osteopontin-induced EndMT conditioned media induce THP-1-derived macrophage M2 polarization which is blocked by anti-HSP90α antibody. On the other hand, bone marrow–derived macrophages (BMDMs) co-cultivated with irradiated tumor ECs display an increased $CD206^+$ M2 macrophage proportion (in total $F4/80^+$ macrophage population) compared with non-irradiated ECs. This effect is abolished in BMDMs co-cultivated with tumor ECs from EC-p53 KO mice. Furthermore, *in vivo*, subcutaneous co-injection of osteopontin-induced EndMT cells with Panc02 pancreatic cancer cells drastically enhances M2 macrophage population and tumor growth (compared with Panc02 injected alone or injected with ECs). These changes are strongly reduced with intravenously injected anti-HSP90α antibody [66]. Irradiation induces CXCR4 expression in ECs both *in vitro* and *in vivo*. This effect is abolished with p53 siRNA and in EC-p53 KO mice. The irradiation-induced CXCR4 expression induces macrophage $SDF1\alpha^+$ recruitment and M2 polarization *in vivo* since this is inhibited with CXCR4 antagonist [67]. Consistently with these results, in human PDAC, there is a correlation between EndMT numbers and M2 macrophage infiltration. Furthermore, M2 macrophages are located close to EndMT cells [66]. All together, these data evidence that ECs undergoing osteopontin- or radiotherapy-induced EndMT induce macrophage M2 polarization in murine tumors via HSP90α secretion and CXCR4/SDF1α signaling, respectively.

2.2.3 Pericytes and perivascular mesenchymal stem cells (MSCs) induce macrophage M2 polarization

Perivascular cells regulate macrophage polarization in melanoma and pancreatic cancers. In melanoma, pericytes and MSCs influence macrophage polarization notably via MFG-E8 secretion [99]. MFG-E8, also called lactadherin, is a secreted integrin-binding protein which is overexpressed in several tumor types compared to normal tissues [100]. MFG-E8 promotes cancer progression, cancer chemoresistance, and tumor angiogenesis and is associated with poor prognosis in human melanoma. Pericytes and perivascular MSCs are the major sources of MFG-E8 secretion in melanoma tumors [85, 99]. MFG-E8 is involved in macrophage M2 reprograming since macrophage incubation with MFG-E8 induces IL-10, TGF-β, and VEGF-A secretion, and increases the proportion of $CD206^+$ macrophages [101]. MFG-E8 released by apoptotic ECs or MSCs is also involved in M2 polarization [85, 101]. Indeed, *in vitro,* RAW macrophages co-cultivated with MSCs display higher M2 marker expression, which is not observed in macrophages co-cultivated with MSC MFG-E8 KO. Nonetheless, the way by which MFG-E8 induces M2 polarization still needs to be investigated. *In vivo*, MFG-E8 enhances tumor angiogenesis and tumor growth. Furthermore, higher vascularization is observed in MFG-E8 WT mice compared to MFG-E8 KO [99]. This angiogenesis enhancement is likely due to MFG-E8-induced macrophage M2 polarization. In pancreatic cancers, pericytes and CAFs are the main cells responsible for IL-33 secretion in the TME [68]. IL-33 causes M2 polarization and matrix metalloprotease-9 (MMP-9) expression in TAMs, which are mediated by the IL-33 receptor ST2 activation. MMP-9 and M2 polarization induce cancer cell intravasation and metastasis *in vivo* [68]. Furthermore, IL-33 induces TAM prostaglandin-2 secretion which enhances cancer stemness and tumor growth [84]. To conclude, perivascular cells induce TAMs M2 and pro-angiogenic phenotype via MFG-E8 and IL-33 secretion, which impacts tumor growth.

3 Effects of TAMs and monocytes on tumor blood vessels

3.1 Angiogenesis and TAMs (related to Fig. 2)

Angiogenesis refers to the formation of new blood vessels from pre-existing ones [124]. Tumor blood vessels are critical in regulating tumor growth via oxygen supply and in supporting metastasis via cancer cell dissemination. Microvessel density corresponds to the small blood vessel density in a tumor and hence is the reflection and a way to assess tumor angiogenesis [125]. It is well described that microvessel density correlates with angiogenic factors, metastasis risk, and prognosis in a huge panel of solid tumors [125, 126]. TAMs are important regulators of tumor angiogenesis [5]. Correlation between TAMs, microvessel density, and poor prognosis is observed in a lot of solid tumors. TAMs are involved in tumor blood vessel development and in the angiogenic switch [32, 127]. Indeed, in the early stage of tumor development, the vessel network development is observed several weeks earlier in CSF1-overexpressing mice than that in WT mice. TAMs promote tumor angiogenesis

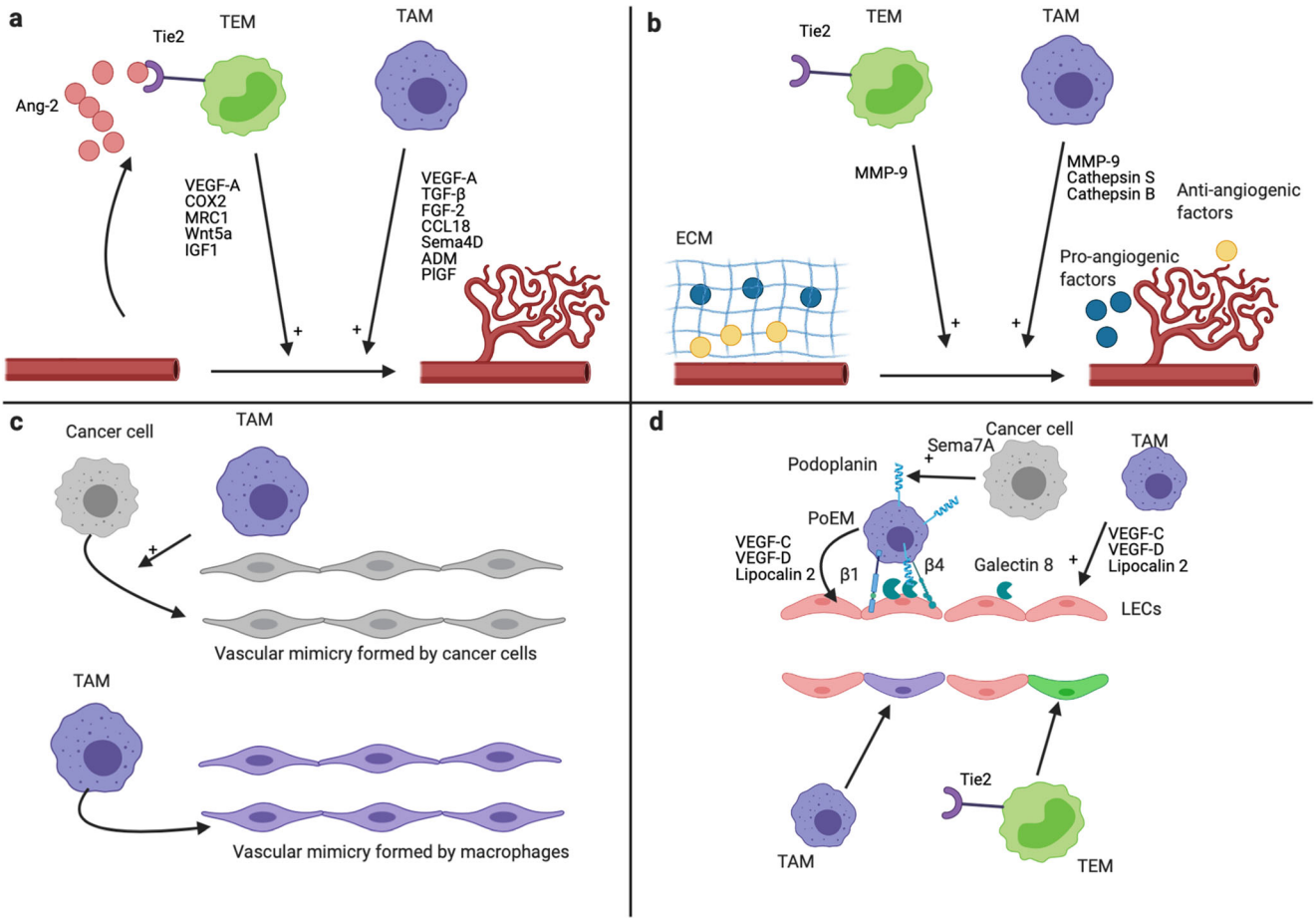

Fig. 2 Mechanisms of tumor angiogenesis and lymphangiogenesis promotion by TAMs and TEMs. Effects of TAMs and TEMs on angiogenesis (**a**, **b**, **c**) and lymphangiogenesis (**d**). **a** TAMs and TEMs promote tumor angiogenesis via secreted factors [5]. EC-derived Ang-2 enhances the pro-angiogenic phenotype of TEMs [58, 102]. **b** TAMs and TEMs promote tumor angiogenesis via the secretion of protease. TAMs secrete MMP-9, cathepsin B, and cathepsin S, whereas TEMs secrete high level of MMP-9 [58, 103–105]. MMP-9 increases VEGF-A bioavailability via ECM degradation [106, 107]. Cathepsin S is involved in the degradation of anti-angiogenic proteins and in the formation of pro-angiogenic peptides via ECM degradation [108]. The promotion of angiogenesis by cathepsin B occurs via the induction of VEGF expression by cancer cells [109, 110]. All together, these proteases lead to an increase and a decrease of pro-angiogenic factor and anti-angiogenic factor in the TME, respectively, which promote tumor angiogenesis. **c** Upper panel: Vascular mimicry structures are perfused non-endothelial channels. They are formed by cancer cells in several cancer types, and promote tumor growth, metastasis, and angiogenesis [111, 112]. TAMs promote, at least *in vitro*, the formation of vascular mimicry channels by cancer cells [113, 114]. Lower panel: TAMs can directly form vascular mimicry structures in tumors [115]. **d** Upper panel: TAMs promote tumor lymphangiogenesis via the secretion of VEGF-C, VEGF-D, and LCN2 [116–118]. Furthermore, podoplanin-expressing macrophages (PoEMs) are able to interact with tumor LECs and are strongly involved in the promotion of tumor lymphangiogenesis [119–121]. This interaction is dependent on GAL8 (LECs), podoplanin, and β1 and β4 integrins (PoEMs) [119, 121]. The secretion of Semaphorin 7A by cancer cells promote the expression of podoplanin by TAMs [120]. Lower panel: TEMs and a subset of TAMs (called M-LECP) are able to integrate into pre-existing lymphatics, which promotes tumor lymphangiogenesis [122, 123]. This figure was created with BioRender.com

by pro-angiogenic factor secretion, protease secretion, and transdifferentiating themselves into vessel-like structures in a process called "vascular mimicry."

3.1.1 Pro-angiogenic factor secretion

Once in the tumor, TAMs secrete pro-angiogenic factors such as VEGF-A, TGF-β, fibroblast growth factor-2 (FGF-2), CCL18, semaphorin 4D (Sema4D), adrenomedullin (ADM), and placental growth factor (PlGF) [128–133]. Macrophage pro-angiogenic phenotype is regulated by hypoxia and lactate. Indeed, *in vitro*, conditioned media from macrophages exposed to lactate or hypoxia have higher angiogenic capacity than conditioned media from macrophages exposed to normoxia, as shown in rat corneal angiogenesis assays [134]. Hypoxia and lactate induce VEGF-A expression in macrophages via hypoxia-inducible factor-1α (HIF-1α), since this is abolished in macrophage from HIF-1α KO mice [135, 136]. It is strongly suggested in [136] that tumor-derived lactate induces TAM M2 phenotype and promotes their angiogenic phenotype. Very recently, it was shown that the expression of the lactate transporter MCT1 by macrophages is strongly involved in lactate uptake and oxidation by macrophages and in lactate-induced macrophage M2 polarization and VEGF secretion [137]. Furthermore, *in vitro*, HIF-1α and HIF-2α stability in macrophage is regulated by PI3K/Akt signaling, since HIF-1α, HIF-2α, and VEGF induction by hypoxia is strongly inhibited with PI3K inhibitors or AKT siRNA [138]. *In vivo*, TAM angiogenic phenotype and microvessel density are reduced in tumors exposed to PI3K inhibitor or in p110$\gamma^{-/-}$ (a subunit of PI3K) mice. In tumors, TAMs are major VEGF producers and are located mostly in avascular and hypoxic areas [70, 136, 139]. In breast cancer, VEGF-A and TGF-β expression and secretion in TAMs are also regulated by cancer cells, notably via macrophage Fra-1 activation [132]. *In vitro*, Fra-1, VEGF-A, and TGF-β expression in macrophages from Balb/c mouse peritoneum co-cultivated with 4T1 breast cancer cells is enhanced, whereas Fra-1 siRNA diminish the enhanced VEGF-A and TGF-β expression. *In vivo*, co-injection of 4T1 and RAW macrophages subjected to Fra-1 knockdown in Balb/c mice induces tumor with less VEGF-A and TGF-β expression and with lower microvessel density than in 4T1 and RAW WT co-injected tumors [132]. FGF-2 expression and secretion in TAMs are regulated by the long non-coding RNA MALAT1. *In vitro*, MALAT1 knockdown in TAMs inhibits FGF-2 expression and secretion. MALAT1 siRNA diminishes the vascular structure formation induced by TAMs conditioned media in HUVECs and is reversed in TAMs overexpressing FGF-2 [133]. Sema4D expression and CCL18 expression in TAMs are correlated with microvascular density and these two proteins are mainly produced by TAMs [129]. *In vitro*, CCL18 induces EC tube formation via the CCL18 receptor PITPNM3 activation since this CCL18-induced tube formation is decreased in si-PITPNM3 HUVECs. Microvascular density in tumor xenografts treated with CCL18 is higher than that in the control. High angiogenesis inhibition is observed in Sema4D KO mice. The injection of WT TAMs in sema4D mice enhances angiogenesis to the same extent as that in WT mice, whereas the injection of sema4D KO TAMs does not [128]. *In vitro*, ADM secretion by macrophages is enhanced by melanoma cancer cells. TAMs promote angiogenesis via ADM secretion in ECs since these TAM conditioned media–induced angiogenesis is abolished by anti ADM. *In vivo*, colocalization between $CD68^{+}$ RAW macrophages and ADM indicates that TAMs are a source of ADM in this melanoma murine model [131].

3.1.2 Protease secretion

TAMs also promote angiogenesis via the secretion of proteases such as cathepsins (S and B) and MMPs such as MMP-9. *In vitro*, cathepsin S and B secretion by macrophages is stimulated by the combination of M2 polarization cytokines such as IL-4, IL-10, and IL-6. This occurs in an inositol-requiring enzyme 1α (IRE1α)–dependent manner since this secretion stimulation is abolished with both IRE1α inhibitor and siRNA [140]. *In vivo*, TAMs promote angiogenesis in PDAC murine tumor model via cathepsin B and S secretion. Indeed, Rip1-Tag2 tumors inoculated with BMDMs from cathepsin B and S KO mice have a lower average vessel density than Rip1-Tag2 tumors inoculated with BMDMs from WT mice [103]. Furthermore, cathepsin S promotes angiogenesis in pancreatic Rip1-Tag2 tumors via matrix protease activity leading to an increase in pro-angiogenic factor release and in anti-angiogenic factor degradation [108]. Cathepsin B angiogenesis regulation is not fully understood but cathepsin B down-regulation in multiple models leads to angiogenesis inhibition. VEGF secretion by cancer cells and in tumor is regulated by cathepsin via an unknown mechanism and could explain the positive impact of cathepsin B on tumor angiogenesis [103, 140–144]. Indeed, cathepsin B inhibition or overexpression in GBM cell lines respectively decreases or increases VEGF secretion by these cells. Furthermore, VEGF protein level is higher in breast tumor from mouse PyMT overexpressing cathepsin B than that in tumor from PyMT WT mice [109, 110, 141]. In Rip1-Tag2 pancreatic tumors, MMP-9 is involved in the angiogenic switch by the VEGF-A bioavailability enhancement [106, 107]. *In vitro*, M2 macrophages secrete high levels of MMP-9 and low levels of tissue inhibitor of metalloproteinase (TIMP)1, a MMP inhibitor, whereas M1 macrophages secrete both MMP-9 and TIMP1 [145]. Hence, cancer cells by skewing TAMs toward M2 phenotype promotes MMP-9 activity. Accordingly, M2 macrophages favor angiogenesis *in vivo* in a MMP-9-dependent manner since this ability is decreased in MMP-9 KO macrophages [145]. MMP-9

expression in macrophages is regulated by the M2 polarization marker cyclooxygenase 2 which is activated notably by MMP-1/3 and IL-6 [146, 147]. *In vivo*, tumor angiogenesis is strongly inhibited in MMP-9 KO mice or by chemical compounds inhibiting MMP-9 [104, 106]. In tumors, MMP-9 is strongly expressed in immune cells [148], mostly by neutrophils [145]. Although neutrophils constitute the major source, TAMs are also high MMP-9 producers, as shown in human colon cancer and in murine cervical cancer [104, 149]. MMP-9 expression and activity in tumors and in TAMs increase during tumor progression of the Rip1-Tag2 cancer model [104].

3.1.3 Vascular mimicry

Vascular mimicry, also called vasculogenic mimicry, refers to vascular channels formed by non-endothelial cells (mostly cancer cells). These structures were firstly described by Maniotis et al., in 1999 [150]. They showed that highly invasive melanoma cells, which notably are expressing high level of tie1, were able to form vascular perfused channels both *in vitro* and *in vivo*. Nowadays, it is known that vascular mimicry networks are also observed in numerous cancer types [111, 112, 151]. These vascular mimicry channels are perfused and connected to the general circulation. They are known to increase tumor growth and to be associated with poor prognosis and metastasis in patients [111, 112].

In vitro, M2 macrophages induce vascular mimicry in glioma and GBM cells [113, 114]. The macrophage-induced vascular mimicry in gliomas cells is dependent on IL-6 and COX2 induction in gliomas and GBM cells, respectively. Indeed, IL-6 expression inhibition in glioma cells and COX2 inhibition in GBM cells abolish the impact of macrophages on vascular mimicry formation by these cells. This is consistent with the fact that, in GBM patients, vascular mimicry positive areas display high TAMs infiltration. Furthermore, in glioma patients, vascular mimicry density is correlated with the quantity of M2 macrophages. In uveal melanoma, there are more macrophages in tumors having vascular mimicry than in those without vascular mimicry [152]. The correlation between macrophages and vascular mimicry appearance in tumors may be due to hypoxia since hypoxia promotes the formation of vascular mimicry as well as the infiltration of macrophages [69, 70, 153]. It would hence be interesting to investigate if TAMs induce vascular mimicry formation by cancer cells *in vivo*.

TAMs can also form vascular mimicry structures *in vitro* and *in vivo* in melanoma tumor model, in multiple myeloma, in human anaplastic thyroid carcinoma, in human meningioma, and benign melanoma tumor [115, 154, 155]. *In vitro*, exposure of multiple myeloma macrophages to VEGF and FGF-2 induces a capillary-like network associated with increased EC marker expression (factor VIII–related antigen, VE-cadherin, and VEGFR2) [154]. In the murine melanoma tumor model, these channels are functional, perfused, and connected to the vasculature since dextran is detected in these structures upon its injection in the tail vein [115]. Hypoxia is a key factor involved in vascular mimicry formation since less vascular mimicry channels are formed with HIF-1α KO macrophages [115]. Consistently with these results, in human anaplastic thyroid carcinoma, cancer cells that are closed to these macrophage channels are not necrotic or hypoxic, even at long distance from blood vessels [155]. This indicates that these channels are perfused or at least lead to tumor cell oxygenation. Additionally, vascular mimicry is observed in human malignant meningioma and benign melanoma tumors [115]. The functional significance of these TAM-derived vascular mimicry structures for tumor growth, angiogenesis, metastasis as well as for prognosis is thus worth investigating.

3.1.4 Communication with pericytes

The effects of TAMs on blood vessel angiogenesis rely on communication not only with ECs but also with pericytes. This communication between macrophages and pericytes occurs notably via Notch signaling and PDGFB-PDGFRβ-induced pericyte migration and periostin expression [156, 157]. *In vitro*, HUVEC cells co-cultivated with macrophages or pericytes enhance the formation of microvessels. Furthermore, the triple co-culture of macrophages, pericytes, and ECs is synergic in promoting angiogenesis. Notch signaling is involved in this process since Notch inhibition in each cell type inhibits angiogenesis [157]. *In vitro*, the secretion of PDGF-BB by macrophages induces pericyte PDGFRβ$^+$ migration and secretion of VEGF-A and pro-angiogenic extracellular matrix component (ECM) periostin by pericytes which enhance angiogenesis [156]. The expression and secretion of PDGF-BB by macrophages are promoted by IL-4 and IL-13 but not by IL-10 [158]. The induction of PDGF-BB expression by IL-4 is mediated at least by PI3Kγ since this induction is diminished in macrophages from p110γ KO mice [159]. Accordingly, PDGF-BB expression and secretion are higher in M2 macrophages compared to M1 macrophages [158, 160]. PDGF-BB expression and secretion in macrophages are also stimulated by cancer cells. *In vitro*, macrophages exposed to U87 GBM cancer cells show higher PDGF-BB expression. This induction occurs via cat eye syndrome critical region protein 1 (CECR1) induction since it is abolished in siRNA CECR1-treated macrophages [156]. Furthermore, the stimulation of TAMs with CECR1 induces PDGF-BB expression in TAMs. Consistently with this, CECR1 expression in GBM is highly produced by TAMs and is correlated with human GBM microvascular density [156, 161]. *In vivo*, in the early steps of murine brain tumors, macrophages are involved in pericyte-endothelial interaction and thereby in tumor angiogenesis. Indeed, neural glial

antigen 2 (NG2) KO specifically in macrophages strongly decreases macrophage recruitment during the beginning of murine brain tumors. Then, macrophage recruitment returns to the same level as in WT tumors in the later stages of tumor growth [162]. Interestingly, macrophage recruitment is correlated with the level of tumor blood vessel covered with pericyte and tumor angiogenesis, indicating that macrophages are most likely involved in these processes. Indeed, 10 days after the development of NG2 macrophage KO tumor, macrophage infiltration is reduced by 90% compared to WT tumors. This decrease in macrophage infiltration is associated with a lower pericyte coverage of tumor blood vessels. After 16-day tumor development, macrophage infiltration and pericyte coverage of tumor blood vessel are comparable in NG2 macrophages KO mice and in WT mice. In conclusion, TAM communication with pericytes promotes angiogenesis *in vitro*, via Notch signaling and secretion of PDGF-BB which induces pericyte recruitment. In the early steps of murine brain tumors, TAMs promote angiogenesis and the pericyte coverage of tumor blood vessels. Hence, it would then be interesting to investigate the impact of TAM-derived PDGF-BB and Notch signaling involvement in the regulation of tumor angiogenesis and pericyte recruitment in the early steps of other cancer types.

3.2 TAMs promote tumor lymphangiogenesis (related to Fig. 2)

Tumor lymphatic vessels are formed via tumor lymphangiogenesis process and are involved in the spread of cancer cells. In tumors, VEGF-C and VEGF-D are the main factors involved in tumor lymphangiogenesis, via VEGFR3 activation in LECs. In murine tumor models, overexpression of VEGF-C/D increases tumor lymphangiogenesis. Accordingly, VEGF-C/D inhibition or VEGFR3 inhibition decreases lymph node metastasis [163]. In the TME, TAMs are major VEGF-C and VEGF-D producers [5, 116, 117]. There exists a correlation between lymphatic vessel density and VEGF-C/D production by TAMs. Furthermore, there is a correlation between TAM density, lymphatic vessel density, and lymph node metastasis in several cancers (reviewed in [39]). Recently, TAM-derived lipocalin 2 (LCN2) was observed to induce lymphangiogenesis [118, 164]. *In vitro*, TAM-derived LCN2 induces LEC proliferation, which is abolished in TAMs transfected with LCN2 siRNA. LCN2 induces lymphangiogenesis via VEGF-C expression induction in LECs, which induces VEGFR3 activation in LECs. *In vivo*, LCN2 is involved in tumor lymphangiogenesis and its associated metastases since there are less lung metastases and lower lymphatic vessel density in PyMT LCN2 KO mice than those in WT mice. Consistently with these results, LCN2 expression is correlated with lymph node metastasis in human breast and colorectal cancers [165, 166].

Other mechanisms by which TAMs promote lymphangiogenesis are by their abilities to become perilymphatic and to integrate into pre-existing lymphatics [39, 122]. These mechanisms occur in a subset of TAMs, called myeloid-lymphatic endothelial cell progenitors (M-LECP) [167]. These cells co-express macrophage markers such as CD68 (human) or CD11b (mouse) and lymphatic markers such as podoplanin and LYVE 1. These TAMs colocalize around lymphatic structures and compose macrophage-derived lymphatic structures which thereby promote lymphangiogenesis [39, 119–121]. Indeed, TAMs can transdifferentiate into LEC progenitors and acquire LEC markers such as LYVE 1 and podoplanin in murine and human tumors [39, 167, 168]. The adhesion between TAMs and LECs depends on podoplanin expression in TAMs and galectin 8 (GAL8) expression in LECs [119]. Podoplanin expression in TAMs is induced by semaphorin 7A both *in vitro* and *in vivo* during tumorigenesis and during physiological postpartum mammary gland involution [120]. Semaphorin 7A is also involved in macrophage motility, chemotaxis towards lymphatics, and TAM incorporation in lymphatics during lymphangiogenesis *in vitro* [120]. Podoplanin-expressing macrophages (PoEMs) are located near lymphatic vessels in murine breast cancer. The perilymphatic localization of PoEMs is mediated by interaction with GAL8-expressing LECs [119]. Indeed, GAL8-specific deletion in LECs or GAL8 pharmacological inhibition impairs PoEM perilymphatic localization *in vivo*. Furthermore, this interaction between PoEMs and GAL8 induces TAMs β1 integrin clustering which is needed for TAM-LEC adhesion. Another team showed that TAM location around lymphatic structures is also dependent on TAMs β4 integrin interaction with laminin 5 in murine triple-negative breast cancer [121]. Finally, PoEMs secrete high amounts of MMPs (and VEGF-C and VEGF-D) which increases VEGF-C and VEGF-D bioavailability and hence promotes lymphangiogenesis [119]. In conclusion, TAMs favor tumor lymphangiogenesis and their subsequent lymph node metastasis, either by secreted factors (VEGF-C, VEGF-D, and LCN2) or by integration of a subset of TAMs, called M-LECP, into lymphatic vessels.

3.3 Angiogenesis and lymphangiogenesis promotion by Tie2-expressing monocytes (TEMs) (related to Fig. 2)

In vitro, TEMs secrete more pro-angiogenic factors such as VEGF-A, TNFα, cyclooxygenase 2, MRC1, and Wnt5a than $Tie2^{-}$ monocytes. They are a major source of MMP-9 [58, 105]. These TEMs are recruited into tumors by EC-derived Ang-2 (see above) [56–58, 74, 77]. Furthermore, these Ang-2-activated TEMs secrete higher levels of insulin-like growth factor 1 (IGF1), cathepsin B, and thymidine phosphorylase and are more pro-angiogenic *in vitro* [58, 102]. TEMs are also

pro-angiogenic *in vivo*. For example, the co-injection of glioma or ovarian cancer cells with TEMs in mice induces more vascularized tumors compared to injection of tumor cells alone or of tumor cells co-injected with $CD11b^+$ myeloid cells without TEMs [102, 123, 169]. Ang-2-induced TEM IGF1 secretion induces angiogenesis and tumor growth via an IGF1 receptor–dependent activation of ECs [102]. Indeed, Ang-2-treated TEMs are more pro-angiogenic *in vitro* and *in vivo* and this increase is abolished by anti-IGF1 antibodies. Consistently with these results, the proportion of TEMs amongst total tissue TAMs is correlated with total tumor microvascular density in human ovarian, renal cell carcinoma, hepatocellular carcinoma, and non-small cell lung cancer, but not in colorectal cancer [29, 102, 170, 171]. High TEM infiltration or high number of circulating TEMs is correlated with poor prognosis in ovarian cancer and in hepatocellular carcinoma, respectively, whereas it is surprisingly correlated with good prognosis in hilar cholangiocarcinoma [102, 172–175]. These TEMs are also found in hypoxic and tumor areas enriched in small immature non-pericytic blood vessels [123, 176]. Less TAM-expressing Tie2 infiltration in murine GBM tumors is observed in HIF-1 KO mice [176]. Interestingly, in murine and human breast cancers, TEMs express lymphatic markers (e.g., LYVE 1, VEGFR-3, and podoplanin) and lymphangiogenic factors (VEGF-C and VEGF-D) and are associated with lymphatic structures [123]. These isolated breast cancer TEMs induce lymphangiogenesis both *in vitro* and *in vivo* (as shown with corneal vascularization assays) by Tie2- and VEGFR-1-dependent mechanism. Indeed, TEMs isolated from breast cancer induce lymphangiogenesis. This process is slightly inhibited by Tie2 or VEGFR inhibitors while it is abolished by the combination of both inhibitors. Interestingly, TEMs are involved in chemotherapy relapse and vessel reconstruction after chemotherapy [177]. Indeed, in murine fibrosarcoma tumors, chemotherapy (doxorubicin) firstly decreases vessel density and tumor volume which is followed by a strong increase in tumor growth and vessel density (tumor relapse). These features are correlated with TEM accumulation in the tumors. Vessel density and tumor growth promotion by doxorubicin are strongly diminished in mice with Tie2 deletion specifically in the myeloid cells. In summary, TEMs promote angiogenesis and lymphangiogenesis in several cancer types and are involved in chemotherapy relapse of murine fibrosarcoma tumors [177].

3.4 TAMs promote metastasis (related to Fig. 3)

Metastasis process is defined as the dissemination of cancer cells from a primary tumor site into a secondary site [187]. This process is responsible for up to 90% of cancer deaths [188]. It is composed of different steps including cancer cell migration/invasion through ECM, cancer cell intravasation, cancer cell circulation and survival into the blood, cancer cell extravasation, and metastasis formation. The effects of perivascular TAMs on blood vessels are involved in cancer cell migration/invasion, intravasation, extravasation, and metastatic formation. TAM deletion in 3 different ways ($CD11b^+$ TAM deletion, CSF1R mice KO, or clodronate liposomes) and monocyte recruitment inhibition into the lung by CCL2 blockade all inhibit metastatic spread from primary murine PyMT mammary tumor to lungs [16, 184].

3.4.1 TAMs and tumor ECs promote cancer cell migration toward blood vessels

Cancer cell migration toward blood vessel is enhanced by a paracrine loop between TAMs and cancer cells, ECs and cancer cells, and the three cell types. Indeed, cancer cell migration is enhanced by perivascular TAMs involving a paracrine loop of TAMs CSF1 secretion and epidermal growth factor (EGF) secretion by cancer cells. Indeed, the inhibition of either EGF or CSF1 results in strong cancer cell migration diminution [178]. The migration of cancer cells toward blood vessels is also stimulated both *in vitro* and *in vivo* with EC-derived HGF which promotes cancer cell migration in a c-Met receptor–dependent manner [179]. Breast cancer cell motility towards HUVEC conditioned medium is impaired by cancer cell c-Met knockdown or by HUVECs HGF knockdown. Furthermore, cancer cell migration towards blood vessel is impaired by c-Met inhibition *in vivo* in breast murine cancer. A paracrine loop between TAMs, ECs, and cancer cells is involved in breast cancer cell migration/invasion toward blood vessels. Indeed, macrophage conditioned media induce ET secretion by HUVECs and ET receptor activation in cancer cells. These effects create cancer cell chemotaxis toward blood vessels which is blocked by ET-1 blocking antibody both *in vitro* and *in vivo* [180]. This paracrine loop is also responsible for tumor cell transendothelial migration and for metastasis.

3.4.2 TAMs promote cancer cell intravasation in areas called tumor microenvironment of metastasis

In breast cancer, cancer cell intravasation is enhanced in areas called tumor microenvironment of metastasis (TMEM) which is composed of one TAM, one cancer cell overexpressing the invasive isoform of "mammalian enabled" protein ($Mena^{INV}$; an actin regulatory protein), and one EC, all three in direct contact [189]. Mechanistically, direct contact of macrophages with breast cancer cells induces Notch1-dependent $Mena^{INV}$ expression in breast cancer cells [190]. Then, this interaction induces Rhoa GTPase–mediated invadopodia which helps cancer cells to break ECM during transendothelial migration [181]. Furthermore, VEGF-A released by $Tie2^{high}$ TAMs enhances local and transient vascular leakiness and hence cancer

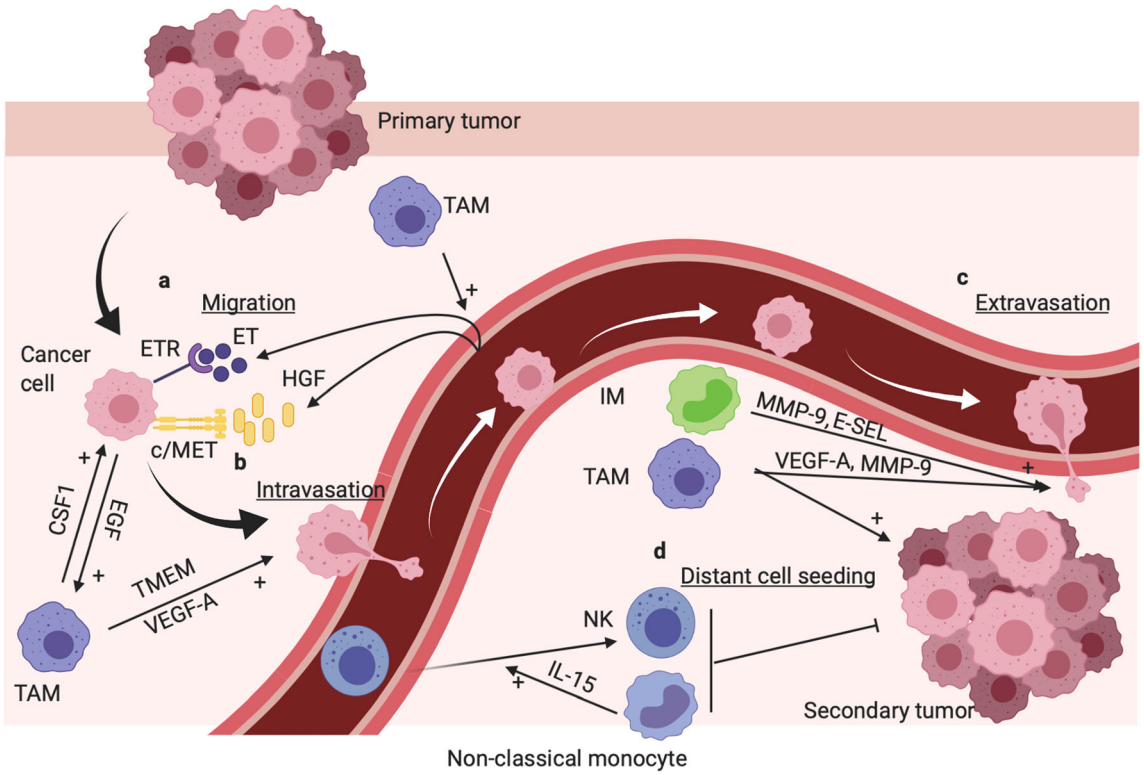

Fig. 3 TAMs and inflammatory monocytes (IMs) promote tumor metastasis, whereas murine non-classical monocytes prevent metastasis. **a** TAMs promote cancer cell migration from primary tumor site towards blood vessel via CSF1 (TAMs) EGF (cancer cell) paracrine loop [178]. TAMs promote endothelin secretion (ET) by ECs; moreover, ET and HGF secretion by ECs induce cancer cell chemotaxis toward blood vessel via ET receptor and c-Met receptor activation, respectively [179, 180]. **b** In breast cancer, specific area called TMEM composed of a cancer cell, a TAM, and an EC promote cancer cell intravasation. Basically, TAMs induce invadopodia formation in the cancer cell which is involved in ECM breaking during EC transendothelial migration [181]. Furthermore, Tie2^{high} TAM-derived VEGF-A promote transient and local vascular leakage which favor cancer cell transendothelial migration [182]. **c** TAMs and IMs promote cancer cell extravasation notably via MMP-9 and VEGF-A-dependent vascular leakage [16, 183]. **d** TAMs and IMs promote metastasis and distant cancer cell seeding [16, 184]. Murine non-classical monocytes prevent distant cell seeding, notably via IL-15-induced NK cell recruitment [24, 185, 186]. This figure was created with BioRender.com

cell transendothelial migration *in vitro* and *in vivo* [182]. TAM-derived TNF-α also enhances cancer cell migration toward ECs, endothelial permeability, and cancer cell intravasation in 3D fibrosarcoma and breast cancer models [191]. TAM-derived IL-1β enhances cancer cell adhesion and transendothelial migration throughout blood and lymphatic cells *in vitro* [192]. TMEM structures are observed in early tumor lesions from breast cancer of MMTV-PyMT and MMTV-HER2 mice [193]. Furthermore, in human breast cancer, TMEMs are restricted to the blood vessels (not seen in lymphatic vessels) and a high number of TMEMs are associated with increased risk of distant metastasis and correlated with breast cancer grade [194–196]. In conclusion, TAMs, as a crucial part of TMEMs, are involved in breast cancer cell intravasation and thereby involved in breast cancer metastasis. Nonetheless, since this effect of macrophages on cancer cell intravasation is restricted to breast cancer, it would be interesting to investigate if macrophages could promote cancer cell intravasation or if TMEM structures are observed in other cancer types.

3.4.3 TAMs promote cancer cell extravasation, cancer cell seeding, and distant metastasis

The extravasation step is enhanced by TAMs and monocytes, notably via blood vessel permeabilization [16, 183]. Blood vessel permeabilization is mostly promoted by TAM and monocyte VEGF-A secretion and monocyte-induced endothelial retraction in an E-selectin-dependent manner. In a 3D transmigration assay, cancer cell transmigration is diminished by 5-fold in the absence of macrophages. Interestingly, the effects of TAMs are inhibited by CCL2 blocking antibody and totally ablated in VEGF-A KO TAMs. TAM-secreted VEGF-A also enhances vascular permeability [16]. VEGF-A-induced vascular permeability is mediated by tyrosine phosphatase density-enhanced phosphatase-1 (DEP-1)–dependent Src kinase activation, which then mediates VE-cadherin uncoupling, thereby creating endothelial gaps [197, 198]. *In vivo*, VEGF-A-induced tumor vascular permeability is diminished in DEP1 KO mice or with Src inhibitor. Indeed, there is less Evans blue diffusion in healthy and tumor tissues

(Miles assay) upon its injection in the tail vein of DEP-1 KO mice or in Src inhibitor–treated mice than that in WT mice or untreated mice, respectively [197, 198]. TAM-derived VEGF-A-induced vascular leakiness is a key factor involved in distant seeding of cancer cells and metastatic spread [16]. Indeed, VEGF-A deletion specifically in monocytes inhibits the efficiency of mammary cancer cell seeding in the lung, without affecting monocyte recruitment into the secondary site. Furthermore, SRC KO and DEP-1 KO mice have less metastatic spread than WT mice [198]. Vascular leakage is also enhanced by monocyte-derived MMP-9 and via monocyte-induced EC retraction in an E-selectin-dependent manner [183, 199]. In 3D *in vitro* model, monocyte MMP-9 secretion induces EC tight junction zonula occludens-1 (ZO-1) and occludin disruption, thus enhancing cancer cell extravasation [183]. Accordingly, in murine breast cancers, monocyte/macrophages are major MMP-9 producers and have a strong impact on cancer cell extravasation since MMP-9 expression and cancer extravasation are strongly reduced in tumor mice ablated of $CCR2^{+}$ inflammatory monocytes. Moreover, in co-culture experiments, monocytes promote EC permeability and VE-cadherin dephosphorylation, which sustains cancer cell extravasation. This is dependent on monocyte interaction with EC E-selectin since this is not observed with monocytes lacking E-selectin ligands or with ECs from E-selectin KO mice. Cancer cell injection into mice induces lung vessel permeability, which depends on monocytes since this is not observed in mice depleted of monocytes. Furthermore, upon extravasation, TAMs are involved in cancer cell invasion and seeding in the ECM. Indeed, in 3D *in vitro* model, cancer cell invasion and migration are enhanced by pre-invaded macrophages [183]. Furthermore, *in vivo*, breast cancer cell pulmonary seeding is blocked by three different methods of macrophage depletion and by monocyte recruitment inhibition by CCL2 blockade [16, 184]. In conclusion, TAMs and monocytes are strong factors involved in the promotion of cancer cell extravasation, cancer cell seeding, and thereby distant metastasis.

3.4.4 Murine non-classical monocytes prevent lung metastasis

Murine non-classical monocyte ($CX3CR1^{high}/Ly6C^{lo}$) differentiation and survival depend on the orphan nuclear receptor Nr4a1, and hence, Nr4a1 KO mice have drastically less non-classical monocytes without affecting IM or macrophage population. Non-classical monocytes prevent lung metastasis formation in the PyMT breast cancer murine model or induced by LLc or B16-F10 cancer cells injected intravenously [24, 185, 186, 200]. Indeed, more lung metastases are observed in non-classical monocyte–depleted Nr4a1 KO mice upon cancer cells injected intravenously, and this is counteracted by $Ly6C^{lo}$ monocyte injection [24]. Furthermore, PyMT mice transplanted with bone marrow from Nr4a1 KO mice show drastically more lung metastases than mice transplanted with WT bone marrow, without affecting primary tumor growth [24]. Upon cancer cell injection, non-classical monocytes interact with cancer cells in a CX3CR1-dependent manner, infiltrate the lung, engulf cancer cell material, and promote NK cell recruitment. These processes are responsible for the inhibition of lung metastases [24]. Non-classical monocyte infiltration into the lungs depends on Kindlin-3 since specific Kindlin-3 deletion in non-classical monocytes diminished their lung infiltration after cancer cell injection. This diminution is associated with an increase in lung metastases [200]. The interactions between cancer cells and non-classical monocytes and subsequent cancer cell material engulfment by non-classical monocytes depend on CX3CR1 expression in non-classical monocytes since these processes are decreased in CX3CR1 KO mice [24]. The NK cell recruitment is induced by non-classical monocytes via IL-15 secretion. Indeed, B16F-10 primary melanoma tumors induce NK cell recruitment into the lungs which is abolished with non-classical monocyte depletion or IL-15 inhibition [186]. Non-classical monocytes are high IL-15 producers, and this secretion is enhanced by primary tumors [186]. Moreover, non-classical monocytes enhance NK cell activation, notably by increasing their stimulatory receptor expression and by diminishing their inhibitory receptor expression [185]. Non-classical monocytes prevent lung metastases also via targeting exosomal content from primary tumors [201, 202]. Non-classical monocyte infiltration in lungs is enhanced by BAG6-presenting exosomes and by non-metastatic A375 melanoma cell line exosomes [201, 202].

In conclusion, several murine studies showed that murine non-classical monocytes are involved in the prevention of metastasis. Since some differences are observed between human and mouse monocytes [203, 204], it would be very interesting to confirm/correlate the results with studies performed with human monocytes.

3.4.5 TAMs promote tumor vessel abnormalization

Tumor blood vessels are abnormal, which means that they have higher permeability, less pericytes, and poor architectural network, functionality, and perfusion enhancing tumor hypoxia and acidosis. Furthermore, the decrease in blood perfusion observed in abnormal tumor vessels is responsible for the decrease in the delivery of chemotherapeutic drugs within the tumor. Tumor vessel "abnormalization" is the process by which blood vessels become abnormal. TAM promotion of vessel "abnormalization" is involved in metastasis notably by promoting cancer cell intravasation and extravasation. M2 TAMs and VEGF and PlGF secretion by TAMs are involved in the tumor blood vessel abnormalization. This abnormalization is characterized by a decrease in pericyte-covered vessels and vessel perfusion, associated with an

increase in EC gaps and tumor hypoxia [130, 205]. The deletion of VEGF specifically in myeloid cells (i.e., TAMs and neutrophils) decreases tumor angiogenesis and promotes vascular normalization characterized by an increase in pericyte coverage associated with a decrease in vessel permeability. Furthermore, histidin-rich glycoprotein (HRG) drastically reduces hepatocellular carcinoma (HCC) metastasis via the inhibition of M2 TAM polarization and PlGF expression in TAMs. HRG has no effect on TAM-depleted tumors or on tumors with PlGF KO TAMs. Tumor blood vessel abnormalization and metastasis are markedly inhibited in mice with PlGF KO TAMs [130]. Interestingly, the blood vessel normalization is proposed as an emerging concept in antiangiogenic therapy since 2005 [206]. In conclusion, TAMs are strongly involved in angiogenesis induction and promotion. Blood vessels whose creation is induced by TAMs are abnormal, notably because of TAM-derived VEGF and PlGF which decrease the coverage of tumor blood vessel with pericytes, and thus promoting tumor vessel permeability and tumor metastasis.

Interestingly, the pro-metastatic activity of TAMs in hypoxia areas is regulated by metabolism. Indeed, the glycolysis and glucose uptake by TAMs are regulated by DNA damage responses 1 (REDD1). REDD1 deletion specifically in TAMs enhances the glucose uptake as well as glycolysis in TAMs. This effect induces glucose competition with ECs leading to vessel normalization and metastasis inhibition [207]. Indeed, REDD1 deletion in TAMs increases glycolytic metabolism in TAMs *in vitro*, inhibits metastasis in multiple mouse tumor models, and induces vessel normalization characterized by an increase in tumor blood vessel pericyte coverage and tumor perfusion. These effects of REDD1 deletion depend on the increase in glycolytic metabolism in TAMs since they are abolished when the increase in TAM glycolytic metabolism is abolished with the glycolytic activator PFKB3 deletion [207]. In conclusion, glucose competition between tumor ECs and TAMs regulates blood vessel features. High glucose consumption by TAMs reduces glucose availability to ECs and allows the formation of a mature and poorly metastatic vascular network. On the other hand, low glucose consumption by TAMs allows high glucose uptake by ECs which allows the formation of immature, abnormal, and pro-metastatic leaky vessels.

4 Discussion

There are strong reciprocal interactions between tumor monocytes/macrophages and tumor blood/lymphatic vessels. TAMs and TEMs are involved in angiogenesis, in lymphangiogenesis, and in multiple metastasis steps, whereas blood vessels are involved in the recruitment of monocytes/macrophages/TEMs into tumors and in macrophage polarization into M2 pro-tumoral phenotype. In the last few years, many discoveries have been made about the effects of blood vessels on the polarization of macrophages, although research is still needed. IMs promote tumor growth and metastasis. Conversely, murine non-classical monocytes prevent lung metastasis, whereas human non-classical monocytes promote angiogenesis *in vitro*. Since human and murine monocytes have functional differences, it would be interesting to better understand how these monocytes prevent metastasis and to confirm that human non-classical monocytes have similar effects on tumor metastasis. Furthermore, it would be interesting to know the impact of non-classical monocytes on tumor angiogenesis *in vivo* and to better understand mechanisms regulating their infiltration into tumors. The improvement of the knowledge of the physiology of tumor blood vessels and TAMs led to the development of several therapies. Some therapy targets only TAMs with the aims to diminish TAM survival and TAM recruitment (CCL2/CCR2 or CSF1/CSF1R inhibition) or to induce a reprogramming of TAMs from M2 phenotype towards M1 phenotype [208, 209]. On the other hand, some therapies target only tumor blood vessels and aim to inhibit angiogenesis, to improve endothelial junctional integrity, to improve tumor perfusion, or to promote vascular normalization [210]. More recently, a lot of researches have been performed about the combination of anti-angiogenic drugs and immunotherapies, and some of them are currently in clinical trials (reviewed in [211, 212]). Anti-angiogenic therapies have beneficial effects on immunotherapy, and inversely. More related to this review, the combination of Ang-2 and VEGF inhibition induces the normalization of the tumor vasculature and promotes TAM reprogramming from M2 toward M1 phenotype and hence increases the M1/M2 ratio and the overall survival in sarcoma and GBM murine models [81, 213, 214]. More recently, the dual Ang-2/VEGF inhibition has been combined with CD40 or PD-1 immune therapies and showed strong synergistic effects in terms of tumor growth, overall survival, and immune cell activation in several murine tumor models [94, 215]. Interestingly, the combination of Ang-2/VEGF with PD-L1 or CD40 immunotherapies are currently in clinical trials (NCT01688206; NCT02665416). In conclusion, the improved knowledge in tumor-associated monocyte/macrophage and tumor blood vessels leads to the development of new promising and innovative therapeutic strategies which could enhance patient overall survival. Nonetheless, research on this topic is still needed in order to improve patient outcome and to diminish adverse effects of the treatments.

Abbreviations *ADAM,* a disintegrin and metalloproteinase domain–containing protein; *ADM,* adrenomedullin; *Ang-2,* angiopoietin-2; *AP-1,* activator protein-1; *Arg-1,* arginase-1; *ASK1,* apoptosis signal-regulating kinase 1; *BMDM,* bone marrow–derived macrophages; *CAF,* cancer-associated fibroblast; *CCL,* chemokine (C-C motif) ligand; *CCR2,* C-C

chemokine receptor type 2; *CD,* cluster of differentiation; *CSF1,* colony-stimulating factor 1; *CSF1R,* CSF1 receptor; *CTHRC1,* collagen triple helix repeat containing 1; *CX3CL1,* chemokine (C-X3-C motif) ligand 1; *CX3CR1,* CX3CL1 receptor; *CXCR4,* C-X-C chemokine receptor type 4; *EC,* endothelial cell; *ECM,* extracellular matrix; *Egfl7,* EGF-like domain–containing protein 7; *EMAP-II,* endothelial monocyte–activating polypeptide-II; *EndMT,* endothelial-to-mesenchymal transition; *ERK,* extracellular signal–regulated kinase; *ET,* endothelin; *FGF-2,* fibroblast growth factor-2; *GAL8,* galectin 8; *GBM,* glioblastoma; *HIF-1α,* hypoxia-inducible factor-1α; *HIF-2α,* hypoxia-inducible factor-2α; *HRG,* histidin-rich glycoprotein; *HSP90α,* heat shock protein 90 α; *ICAM1,* intercellular adhesion molecule 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IGF1,* insulin-like growth factor 1; *IL,* interleukin; *IM,* inflammatory monocyte; *IRE1α,* inositol-requiring enzyme 1α; *JNK,* c-Jun N-terminal kinases; *KO,* knockout; *LCN2,* lipocalin 2; *LEC,* lymphatic endothelial cell; *LLc,* Lewis lung carcinoma; *LYVE 1,* lymphatic vessel hyaluronic receptor 1; *MCT1,* monocarboxylate transporter 1; *Mena^{INV},* invasive isoform of mammalian enabled protein; *MFG-E8,* milk fat globule-epidermal growth factor 8; *MHCII,* major histocompatibility complex class II; *M-LECP,* myeloid-lymphatic endothelial cell progenitors; *MMP,* matrix metalloprotease; *MSC,* mesenchymal stem cell; *mTOR,* mechanistic target of rapamycin; *NG2,* neural glial antigen 2; *NK,* natural killer; *p38MAPK,* p38 mitogen–activated protein kinase; *PDAC,* pancreatic ductal adenocarcinoma; *PDGF,* platelet-derived growth factor; *PDGFRβ,* PDGF receptor β; *PD-1,* programmed cell death 1; *PD-L1,* programmed death-ligand 1; *PI3K,* phosphoinositide 3-kinase; *PlGF,* placental growth factor; *PoEM,* podoplanin-expressing macrophage; *REDD1,* regulated in development and DNA damage responses 1; *SDF1α,* stromal cell–derived factor 1α; *Sema4D,* semaphoring 4D; *SOCS1,* suppressor of cytokine signaling 1; *TAM,* tumor-associated macrophage; *TCF12,* transcription factor 12; *TEM,* Tie2-expressing monocyte; *TGF-β,* transforming growth factor β; *TIMP,* tissue inhibitor of metalloproteinase; *TME,* tumor microenvironment; *TMEM,* tumor microenvironment of metastasis; *TNFα,* tumor necrosis factor α; *TRM,* tissue-resident macrophage; *VCAM1,* vascular cell adhesion molecule 1; *VEGF,* vascular endothelial growth factor; *VEGFR,* VEGF receptor; *WT,* wild-type; *ZO-1,* zonula occudens-1

Author contribution V.D wrote the whole manuscript and designed the figures. C.M supervised the entire work and critically revised the paper.

Funding Victor Delprat is financed by a Televie grant (FNRS- National Funds for Scientific Research, Belgium).

Declarations

Conflict of interest The authors declare no competing interests.

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Box 1 : Immunotherapy using CD40 agonists in cancer

CD40 is mostly expressed at the cell surface of antigen-presenting cells such as dendritic cells and macrophages (Beatty et al., 2017). CD40 ligand (CD154) is mostly expressed at the cell surface of $CD4^+$ T cells. The interaction between CD40 and CD154 induces antigen presenting cell and T cell activation (Beatty et al., 2017). In several murine tumor models, the use of CD40 agonistic antibody induces T cell and antigen presenting cell activation (including macrophages), which decreases tumor growth (Choi et al., 2020). Some preclinical studies investigated the combination of CD40 with anti-angiogenic or checkpoint inhibitors and showed interesting effects (Choi et al., 2020; Delprat and Michiels, 2021). In clinic, several CD40 agonistic antibodies have been tested in phase 1 in patients with lymphoma and melanoma. Furthermore, the combination of CD40 agonist (APX005M) with gemcitabine and nab-paclitaxel with or without nivolumab showed interesting results in patients with pancreatic cancer (Choi et al., 2020). APX005M is currently in phase 2 clinical trial, with or without combination with other therapies in non-small cell lung cancer and metastatic melanoma (Choi et al., 2020).

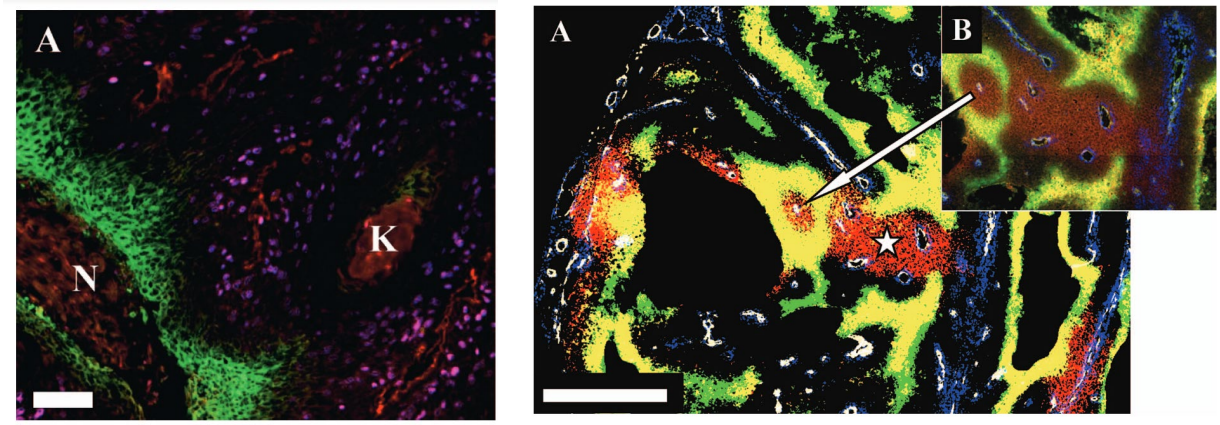

Fig. 19. Tumor staining suggesting that cyH occurs in tumors. Left : patient biopsie of human head and neck cancer, showing hypoxic areas (in green) localized near to blood vessels N (in red). This suggests that the tumor blood vessel N was not perfused during the staining . Right : human mucoepidermoid carcinoma xenograft, in which two hypoxic markers were injected at 2h interval. These hypoxic markers stain differently, the first one in green (pimonidazole) and the second one in red (CCI—103F). Regions stained in yellow were hypoxic during the first and the second injection. Regions in red were in hypoxia only during the injection of the second hypoxic marker but not duiring the first hypoxic marker injection, suggesting an intermittent oxygenation in these red areas. (adapted from Ljungkvist et al., 2007)

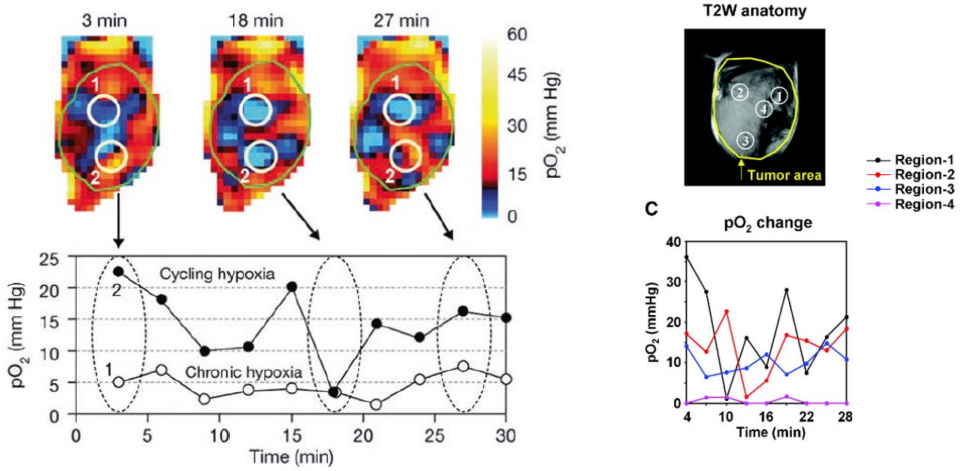

Fig. 20. Oxygenation levels in tumors assessed by magnetic resonance imaging (MRI) and electron paramagnetic resonance imaging (EPRI). Left : tumor oxygenation assessed by EPRI in SCCVII murine tumors. Right : tumor oxygenation assessed by MRI in HT29 murine tumors. Left and right : in both tumor types, there are areas of cycling hypoxia, areas of chronic hypoxia and areas of normoxia. (adapted from Rickard et al., 2019 and Yasui et al., 2010)

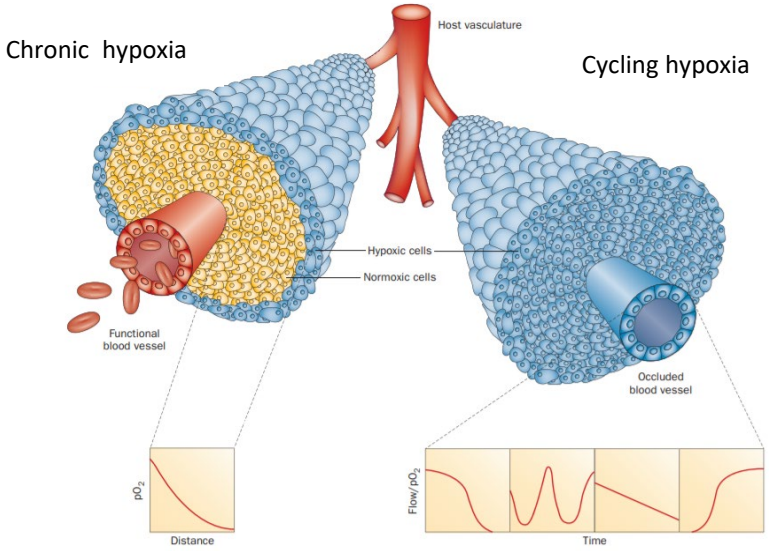

Fig. 21. Chronic and cycling hypoxia. In tumors, chronic hypoxia impacts cells which are too far from functional blood vessels. Cycling hypoxia impacts cells localized around a blood vessel which is intermittently perfused. **(**adapted from Horsman et al., 2012)

4 Tumor cycling hypoxia

4.1 Causes and evidence of cycling hypoxia in solid tumors

In 1979, Brown suggested that cancer cells are exposed to cycles of hypoxia and reoxygenation in solid tumors (Brown, 1979). Since then, much work has been done to demonstrate that cycling hypoxia (cyH) actually occurs in cancers, and to determine the causes of this phenomenon (Michiels et al., 2016). By the co-staining of hypoxic areas (e.g via pimonidazole) and vascular cells (CD31 staining), a lot of studies performed in murine and human tumors showed that some cancer cells which are located adjacently to blood vessels are exposed to hypoxia, suggesting that these blood vessels are not or intermittently perfused (Ljungkvist et al., 2007). An example of such a staining is shown in Fig. 19. Consistently, the injection of 2 hypoxic markers (with different colors) at different time points, showed that some areas in murine tumors are intermittently hypoxic (Ljungkvist et al., 2007) (Fig. 19). More directly, several experiments performed using laser Doppler technology on murine tumors, human tumor xenografts and human tumors confirmed the intermittent perfusion of some blood vessels in solid tumors (Chaplin and Hill, 1995; Hill and Chaplin, 1996; Hill et al., 1996; Pigott et al., 1996). Interestingly, these authors observed a correlation between blood vessel perfusion and the saturation in O_2. More recently, experiments using magnetic resonance imaging (MRI) and electron paramagnetic resonance imaging (EPRI) allowed the non-invasive observation of oxygenation in tumor bearing-mice and in human patients (Matsumoto et al., 2010; Panek et al., 2017; Rickard et al., 2019; Yasui et al., 2010) (Fig. 20). These experiments allowed the observation that, in a same tumor, there are areas exposed constantly to hypoxia (called chronic hypoxia), areas with cycles of hypoxia and reoxygenation (called cycling hypoxia) and areas of normoxia/physioxia (fig. 20).

CyH impacts stromal and cancer cells surrounding intermittently occluded tumor blood vessels, whereas chronic hypoxia (chH) impacts cells which are too far from blood vessels (>100 µm), mostly due to cancer cell high proliferation rate (Fig. 21) (Horsman et al., 2012). Cycles of hypoxia/reoxygenation frequency are comprised between 0.5 to 5 cycles/hour (Bader et al., 2020; Dewhirst and Birer, 2016). Additionally, hypoxia/reoxygenation cycles with an estimated frequency calculated in days is also observed, which is hypothesized to be due to vascular remodelling (Bader et al., 2020). Angiogenesis occurring in tumors leads to the formation of tortuous blood vessels with an anarchical architecture of vascular network (also called abnormal blood vessels), which is the cause of an instability of red cell flux (Michiels et al., 2016). Furthermore, temporary occlusions of tumor blood vessels by cancer cells, leukocytes or platelets causes cyH. The compression of blood vessels by highly proliferative cancer cells is also an explanation for cyH. The normalization of abnormal blood vessels, by low dose of anti-angiogenic sunitinib drastically diminishes the occurrence of cyH in squamous cell carcinoma murine tumors (Matsumoto et al., 2011).

It is suggested that cyH in tumors is also caused by obstructive sleep apnea (OSA), even if this has not been demonstrated yet (Almendros and Gozal, 2018; Bader et al., 2020; Cao et al., 2015a; Kukwa et al., 2015). OSA is a sleep disorder caused by upper airway obstruction leading to cessation of breathing several times an hour during the sleep. The frequency of cycles of

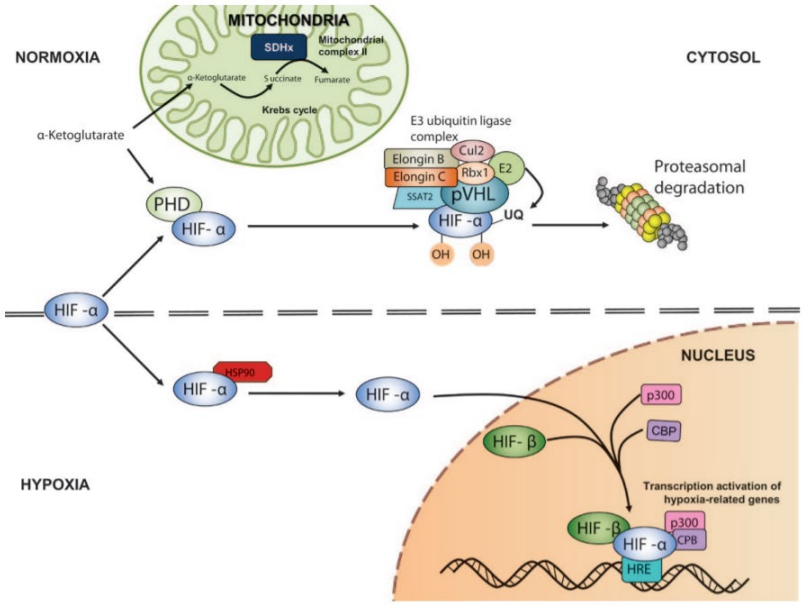

Fig. 22. HIF signaling pathway regulation by oxygenation. Under normoxia, HIFα is hydroxylated by PHDs at specific proline residues. Hydroxylated HIFα is recognized by E3 ubiquitin ligase complex and is then degraded by the proteasome. Under hypoxia, HIFα is stabilized and dimerizes with HIFβ, recruits co-activator and triggers transcription of hypoxia-related genes (Jochmanová et al., 2013)

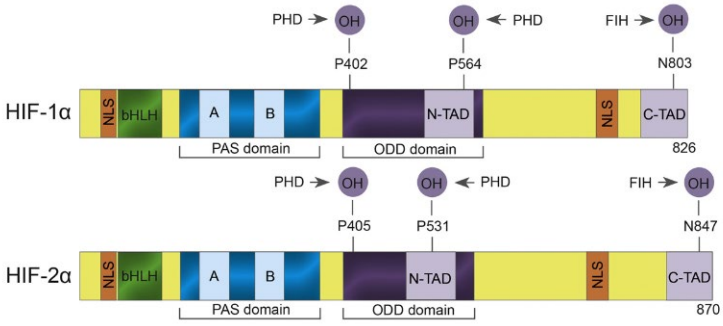

Fig. 23. Structure of HIF1α and HIF2α with the localization of the hydroxylated proline residues. (Wigerup et al., 2016)

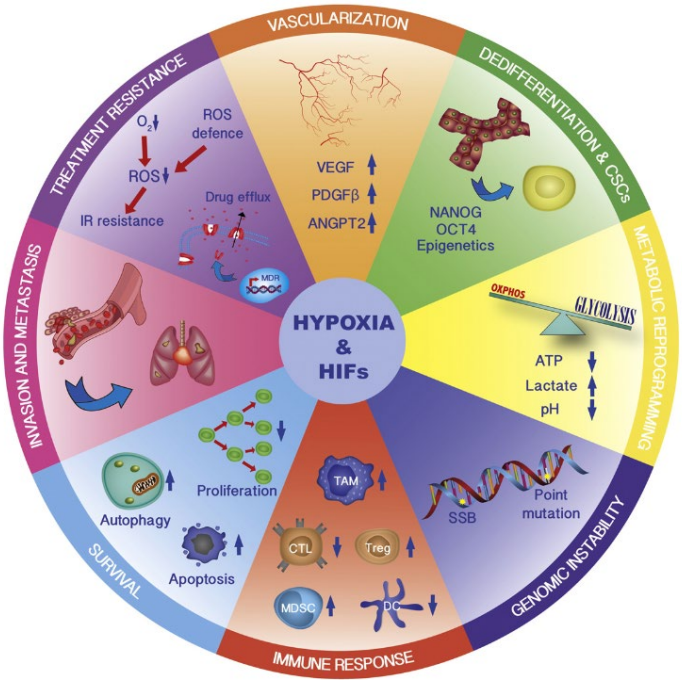

Fig. 24. Hypoxia and HIFs favor several hallmarks of cancer. (Wigerup et al., 2016)

hypoxia/reoxygenation is strongly higher than cyH caused by intermittent blood vessel perfusion.

In conclusion, cyH is characterized by cycles of hypoxia/reoxygenation of a frequency of 0.5 to 5 cycles an hour. Additionally, it can occur in a slower frequency, due to vascular remodelling or to a faster frequency due to OSA.

4.2 Cellular adaptations to (cycling) hypoxia

4.2.1 Hypoxia-inducible factors

Cellular adaptation to hypoxia (0.5%-2% O_2) is a crucial phenomenon (Lee et al., 2020). Notably, Nobel prize 2019 awarded 3 researchers – namely Gregg Semenza, Peter Ratcliffe and William Kaelin – for their research on the cellular adaptation to hypoxia by hypoxia-inducible factors (HIFs) signalling (Moslehi and Rathmell, 2020). HIFs are heterodimer transcription factors, composed of a α subunit and a β subunit. The β subunit is constitutively expressed, whereas the α proteins are only stabilized during hypoxia. There are 3 HIF α subunits, HIF1α, HIF2α and HIF3α. HIF1α and HIF2α are the most important and studied HIFα isoforms. Under normoxia, HIFα isoforms are hydroxylated at 2 specific prolyl residues by prolyl hydroxylase domain proteins (PHDs). In order to be processed, this hydroxylation reaction needs O_2 and α-ketoglutarate and leads to succinate release (a metabolite involved in the Krebs cycle). The hydroxyprolyl residues on HIFα proteins are recognized by von Hippel-Lindau tumor suppressor protein (pVHL). The α proteins are then polyubiquitinated by E3 ubiquitin ligase complex called pVHL–elongin BC-CUL2 or VHL complex. Once ubiquitinylated, HIFα is targeted for proteasomal degradation. Structures and hydroxylated prolyl residues in HIF1α and HIF2α are shown in Fig. 22 (Wigerup et al., 2016) On the other hand, under hypoxia, HIFα isoforms are not hydroxylated by PHD proteins and hence are stabilized. HIFα translocates to the nucleus and heterodimerizes with HIFβ to form HIF transcription factor. This transcription factor binds to hypoxia response elements of targets genes, which induces their transcription (Jochmanova et al., 2013; Lee et al., 2020). HIF regulation by hypoxia and normoxia is depicted in Fig. 23 (Jochmanova et al., 2013).. HIF transcription factors regulate the expression of a huge set of genes involved in several hallmarks of cancer, such as angiogenesis, invasion/metastasis, survival, genomic instability, metabolic reprogramming and immunosuppression (Fig. 24).

Several studies performed *in vitro* compared the impact of chH and cyH on the induction of HIF1α and HIF2α (Bader et al., 2020). Most of these studies showed that HIF1α accumulation and transcriptional activity is induced more strongly upon exposure to cyH. This effect relies on the overactivation of nuclear factor erythroid 2-related factor 2 (NRF2) and the overproduction of ROS induced by cyH (vs chH). Mitochondrial ROS are well described HIF1α stabilizer (Diebold and Chandel, 2016; Thomas and Ashcroft, 2019). For example, the expression and activity of HIF1α are strongly enhanced in HEK293 cells incubated with H_2O_2, whereas the activation of HIF1α under hypoxia is abolished by catalase – an enzyme which degrades H_2O_2 into H_2O and O_2 (Chandel et al., 2000). Nonetheless, each study which shows that cyH induces more strongly ROS production and HIF1α activity reoxygenated cells upon exposure to 21% O_2, which is not physiological and more likely to promote ROS production. It would hence be interesting to confirm the results with a physiological reoxygenation (e.g 7%

O_2). CyH induces more strongly HIF1α stabilization than chH in A549 lung cancer cells. NRF2 siRNA in A549 cells abolished the difference of HIF1α expression between chH and cyH conditions (Malec et al., 2010). On the other hand, HIF2α is degraded under cyH whereas it is stabilized under chH (Bader et al., 2020). In conclusion, cyH induces more strongly the activation of HIF1α *in vitro* than chH, whereas cyH decreases the expression of HIF2α *in vitro* and *in vivo*.

4.2.2 Production of reactive oxygen species

ROS are involved in multiple cellular processes, such as cell proliferation, cellular senescence, differentiation and apoptosis (Davalli et al., 2016; Zhang et al., 2016b). Accordingly, ROS regulate the activation of several signaling pathways, such as NRF2, PI3K-AKT, MAPK, NF-κB, AP-1 and HIF1 (Ansari et al., 2020; Zhang et al., 2016b). The involvement in these cellular processes depends on the level of ROS in the cell, and hence, the global level of ROS is tightly regulated by enzymes such as superoxide dismutase (SOD) and catalase (Wang et al., 2013a). ROS are produced by mitochondrial electron-transport chain (ETC), membrane-bound NADPH oxidase (NOX) complex and by endoplasmic reticulum (ER). Surprisingly, hypoxia promotes the production of ROS in cells, via the complex III of the mitochondrial electron transport chain, although the level of O_2 is very low in hypoxia (Bader et al., 2020; Guzy et al., 2005). The production of ROS in cells and *in vivo* is also induced by cyH, during the reoxygenation periods notably, and to a stronger extent than chH (Michiels et al., 2016). The production of ROS by cyH strongly involves NOX4 and to a lesser extent NRF2. Cell incubation in cyH strongly promotes the expression of NOX4 and the production of ROS in glioblastoma cell lines (Hsieh et al., 2012a; Hsieh et al., 2011; Hsieh et al., 2012b) . Accordingly, in murine glioblastoma, the expression of NOX4 is enhanced in cyH areas and in mice exposed to cyH (Hsieh et al., 2011; Hsieh et al., 2012b). Furthermore, the expression of Nox4 is enhanced in the kidney of rats exposed to cyH (Lu et al., 2017). The overproduction of ROS induced by cyH in glioblastoma cell lines is abolished with nox4 siRNA/shRNA (Hsieh et al., 2011; Hsieh et al., 2012b). Consistently, the production of ROS in murine glioblastoma xenograft induced by cyH is abolished with conditional KD of Nox4 (Hsieh et al., 2011). In conclusion, cyH induces an overproduction of ROS to a stronger extent than chH, notably via NOX4.

4.2.3 NF-κB activation

Several reports evidenced that cyH induces NF-κB activation. The exposure of HeLa cells to hypoxia/reoxygenation induces NF-κB DNA binding and transcriptional activity, whereas hypoxia alone does not induce NF-κB activation (Rupec and Baeuerle, 1995). Exposure of primary human umbilical vein endothelial cells (HUVEC) to cyH promotes TNFα-induced NF-κB activation (Tellier et al., 2015). In model with a single hypoxia/reoxygenation cycle, the induction of NF-κB transcriptional activity mostly depend on the phosphorylation of IκBα on tyrosine 42 (and not on Ser 32/36 residues) (Fan et al., 2003; Imbert et al., 1996). The phosphorylation of IκBα on tyrosine 42 impairs its association with p65 but does not induce IκBα proteasomal degradation (Imbert et al., 1996).

ROS overproduction induced by cyH is potentially involved in the activation of NF-κB by cyH. Indeed, the overexpression of two enzymes involved in ROS degradation -namely catalase and glutathione peroxidase 1 (gpx1) - reduces the activation of NF-κB and the phosphorylation of

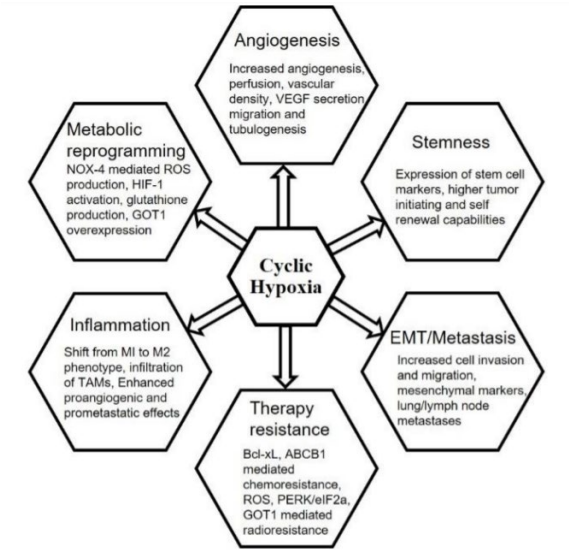

Fig. 25. Cycling hypoxia and cancer. Cycling hypoxia favors ROS production, angiogenesis, stemness, metastasis, therapy resistance and inflammation. ROS production, metastasis and radiotherapy resistance are more potently increased by cyH than chH. (Saxena et al., 2019)

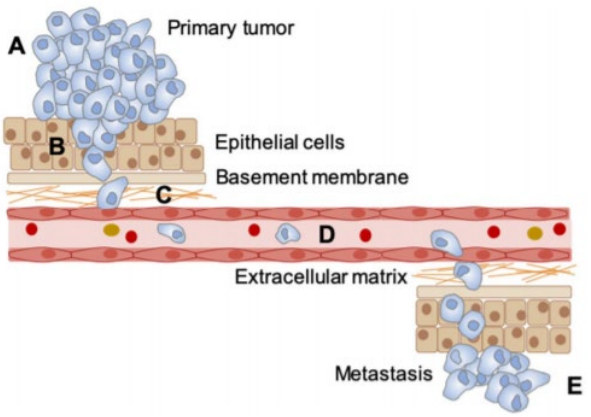

Fig. 26. Mechanisms by which cyH favors cancer metastasis. CyH induces EMT/stemness (A), increases cancer cell migration/invasion, modulates ECM matrix (C), increases cancer cell survival in the blood (D), cancer cell seeding in secondary tumor site (E) (Bader et al., 2020)

IκBα induced by hypoxia/reoxygenation (Fan et al., 2003). Furthermore, treatment of glioblastoma cells with the antioxidant tempol impairs the activation of NF-κB induced by cyH (Chen et al., 2015). The induction of ROS by cyH mostly depends on NOX4 in glioblastoma cells. The KD of NOX4 in glioblastoma cells reduces the activation of NF-κB by cyH (Hsieh et al., 2012a). The activation of p38 MAPK is also likely involved in cyH-induced NF-κB since p38 MAPK KD in ECs impairs the activation of NF-κB by cyH (Li et al., 2014; Ryan et al., 2007).

4.2.4 AP-1 activation

As said above, AP-1 are dimeric transcription factors, mostly composed of the heterodimerization of fos and jun family. Yuan et al. showed that the exposition of PC 12 cells to cyH -but not chH- enhances c-fos mRNA expression (Yuan et al., 2004). Furthermore, cyH induces AP-1 transcriptional activity, which is abolished by c-fos siRNA, showing that the enhanced expression of c-fos is responsible of the enhanced AP-1 transcriptional activity induced by cyH. More intriguingly, treatment of cells with SOD - an enzyme which degrades $O_2.^-$ - abolished the effect of cyH on c-fos mRNA expression and AP-1 transcriptional activity, suggesting that ROS are involved in the effects observed in cyH. Accordingly, another study showed that treatment of cells with ROS - notably H_2O_2 - enhances c-fos and c-jun mRNA expression and protein abundance, and strongly induces AP-1 transcriptional activity (Janssen et al., 1997).

4.3 Cycling hypoxia and cancer progression

ChH and cyH increase several features of cancer such as metastasis, radioresistance and chemoresistance, and cyH increases some of these features to a higher extent than chH (Fig. 25) (Saxena and Jolly, 2019).

4.3.1 Metastasis

CyH strongly promotes spontaneous cancer metastasis, and in some experiments to a higher extent than chH (Bader et al., 2020). The impact of cyH on experimental metastasis is weaker than on spontaneous metastasis and differs according to the model. The exposition of murine fibrosarcoma tumors to cyH strongly enhances the number of lung micrometastases compared to N, whereas chH has no impact (Cairns et al., 2001). CyH strongly promotes spontaneous metastasis and slightly promotes experimental metastasis of murine melanoma tumors (Almendros and Gozal, 2018; Almendros et al., 2013; Li et al., 2018; Rofstad et al., 2010). The effect of cyH on experimental metastasis depends on the induction of oxidative stress, since its effect is completely abolished with the antioxidant tempol treatment (Li et al., 2018). CyH enhances the number of lymph node metastases in a model of orthotopically injected human cervical carcinoma in mice (Cairns and Hill, 2004). The appearance of metastases in the lungs of murine LLc tumors is strongly enhanced by cyH since 60% of mice exposed to cyH develop node metastasis, whereas only 20% of mice exposed to normoxia develop node metastasis (Zhang et al., 2014a). Furthermore, the number of node metastases is higher in cyH-exposed mice.

The impact of cyH on cancer cell metastatic phenotype has been studied in several works (Bader et al., 2020)(Fig. 26). CyH induces an EMT and/or cancer stem cell (CSC) phenotype in cancer cells and promotes their migration/invasion ability. Furthermore, cancer cells pre-exposed to cyH have a greater ability to form metastasis upon injection in murine tail vein, a

characteristic of CSCs. *In vitro*, cyH induces a CSC-like phenotype in gastric, pancreatic, neuroblastoma and breast cancer cells (Bhaskara et al., 2012; Chen et al., 2018; Louie et al., 2010; Miao et al., 2014; Zhu et al., 2014). Furthermore, cyH enhances the tumor-initiating ability of breast cancer cells *in vivo,* a characteristic of CSCs (Chen et al., 2018; Louie et al., 2010). *In vitro*, pancreatic, medulloblastoma and breast cancer cells exposed to cyH undergo EMT, and this EMT depends on HIF1α induction for pancreatic and medulloblastoma cancer cells (Gupta et al., 2011; Zhu et al., 2014). *In vitro*, cyH enhances migration/invasion capacities of gastric, pancreatic and breast cancer cells, and this effect involves HIF1α activation for breast and pancreatic cancer cells (Liu et al., 2017a; Miao et al., 2014; Zhu et al., 2017; Zhu et al., 2014). MMTV breast cancer cells and hepatocarcinoma cells exposed to cyH have stronger ability to form lungs metastasis than cells exposed to normoxia or chH, when injected in the tail veins of mice (Chen et al., 2018; Daneau et al., 2010). The metastatic ability of hepatocarcinoma cancer cells exposed to cyH is strongly inhibited by COX2 inhibition, meaning that the induction of COX2 in cancer cells by cyH is involved in their enhanced ability to metastasize (Daneau et al., 2010). *In vitro*, the exposure of breast cancer cells to cyH induces the expression of breast to lung metastasis-associated genes (Chen et al., 2018). Furthermore, cyH specifically induces the expression of pro-inflammatory cytokines in breast cancer cells. This means that cyH promotes the ability of cancer cells to metastasis. In a fate-mapping model allowing the discrimination between cancer cells which have been exposed or not to tumor hypoxia, Godet et al. demonstrated that breast cancer cells exposed to hypoxia in the primary tumor are more potent to become CTCs and to induce spontaneous metastases (Godet et al., 2019). Hence, cyH could promote metastasis via the induction of a pro-metastatic phenotype in cancer cells. Overall, these results show that cyH promotes spontaneous and experimental metastasis, notably by shifting cancer cell phenotype towards a pro-metastatic phenotype. Nonetheless, little is known about the impact of cyH on stromal cells, and if the impact of cyH on stromal cells could modulate the development of metastases.

4.3.2 Tumor inflammation

Tumor inflammation is a hallmark of cancer. It is involved in several processes in tumors such as genomic instability, metastasis and cancer development (Greten and Grivennikov, 2019). *In vitro*, cyH promotes the activation of NF-κB, the master transcription factor regulating inflammatory phenotype, in several cell type (Bader et al., 2020; Michiels et al., 2016). In EA.hy926 and HUVEC ECs, cyH amplifies the pro-inflammatory phenotype induced by TNFα (Tellier et al., 2015). Notably, cyH enhances TNFα-induced expression and secretion of IL-6 and IL-8 in a NF-κB-dependent manner in these cells. Furthermore, short-term exposure (6h) of ECs to cyH enhances TNFα-induced ICAM1 expression, and long-term exposure of ECs (>48h) to cyH strongly increases ICAM1 and VCAM1 protein abundance (Sun et al., 2019; Tellier et al., 2015). Accordingly, short-term and long-term exposure of ECs to cyH enhances TNFα-induced or basal (without TNFα) monocyte binding to ECs, respectively. *In vitro*, cyH specifically induces IL-6, TNFα, CXCL1 and CXCL2 secretion, and both cyH and chH induce granulocyte macrophage-colony stimulating factor (GM-CSF) in PyMT breast cancer cells (Chen et al., 2018). Furthermore, cyH specifically induces COX2 mRNA expression and protein abundance in ECs and cancer cells (Daneau et al., 2010). *In vivo*, cyH strongly increases tumor inflammation in LLc tumor bearing mice, characterized by an enhanced IL-6, COX2, CXCL2 and

keratinocyte chemoattractant (KC; a pro-inflammatory chemokine) (Tellier et al., 2015). Accordingly, cyH increases TNFα and IL-6 expression, and p65 protein abundance in melanoma metastasis by a ROS-dependent manner (Li et al., 2018). Furthermore, the infiltration of CD11b myeloid cells in LLc tumor-bearing mice is strongly enhanced by cyH exposure (Tellier et al., 2015). Overall, these results show that cyH promotes pro-inflammatory phenotype in cancer cells and ECs *in vitro* as well as tumor inflammation *in vivo*.

4.3.3 Cancer development

Several epidemiologic studies showed that patients with severe OSA have higher risk to develop cancers (Cao et al., 2015a). This could be partly explained by the fact that patients with OSA are mostly obese, and that obesity increases risk to develop cancer (Kolb et al., 2016). Furthermore, two murine studies showed that the exposure of mice to cyH increases spontaneous tumorigenesis and CAC development (Gallego-Martin et al., 2017; Yoon et al., 2019). In the first one, mice exposed during 3 months to 8h severe cyH (cycle of 40s hypoxia 7.5 % O_2 followed by 80s room air) developed significantly more lung tumors and any type of tumors than mice exposed to room air (Gallego-Martin et al., 2017). This increase in cancer development is dependent on the severity of cyH since mice exposed to mild cyH (cycle of 40s hypoxia 12 % O_2 followed by 80s) do not develop more tumors than those exposed to room air. In the second study, the exposure of mice to cyH (vs normoxia) strongly increased the induction of CAC (with tumor size >2mm but not with tumor size < 2mm) (Yoon et al., 2019). CAC of mice exposed to cyH had more oxidative stress, and the impact of cyH on CAC development was strongly reduced by antioxidant. Overall, these data suggest that cyH favors cancer development although the underlying mechanisms are not known.

4.3.4 Resistance to therapy

4.3.4.1 Radioresistance

Radiotherapy is a widely used cancer treatment (Baskar et al., 2012). Tumor hypoxia is associated with poor outcome following radiotherapy in most cancer types (Horsman et al., 2012). Radiotherapy induces DNA damage which lead to cell death if they are not repaired. Tumor hypoxic cells are less radiosensitive than those oxygenated (Deschner and Gray, 1959; Gray et al., 1953). Radiation causes more DNA damage in the presence of oxygen, and DNA repair mechanisms ae more effective under hypoxia (Moeller et al., 2007). Furthermore, hypoxia modifies cancer cell phenotype leading to their radioresistance (Moeller et al., 2007). Interestingly, cyH induces a better radioresistance than chH for cancer cells and stromal cells (such as ECs) (Bader et al., 2020; Michiels et al., 2016). There are two main reasons which can explain this phenomenon. First, cyH induces a stronger activation of HIF1α than chH, and HIF1α is involved in radioresistance (Bader et al., 2020). Secondly, cyH increases the resistance of cells to ionizing radiation independently of HIF1α, notably by rendering them resistant to ROS (Saxena and Jolly, 2019). The formation of ROS by radiotherapy is a cause of cell death via the induction of DNA damage in treated cells.

CyH increases radioresistance of U87 glioma cells to a stronger extent than chH (Hsieh et al., 2010). Interestingly, HIF1α siRNA abolished the increased radioresistance induced by cyH (and chH). Indeed, radioresistance of U87 glioma cells with HIF1α KD is the same in normoxic, chH and cyH. The same phenomenon is observed in A549 cells but not in H446 cells, indicating that

HIF1α-dependent radioprotection induced by cyH is cell type dependent (Liu et al., 2010). More interestingly, cyH confers radioresistance to ECs in a HIF1α-dependent manner (Martinive et al., 2006). Radiotherapy leads to an increase in cytokine secretion leading to a protection of the vascular network, in an HIF1α-dependent manner (Moeller et al., 2004). Furthermore, HIF1α blockade radiosensitizes tumor vasculature (Moeller et al., 2005). Hence, HIF1α is involved in radioresistance induced by cyH. This involvement is cell type-dependent, and since HIF1α regulates several signalling pathways, it would be interesting to investigate more precisely how cyH-induced HIF1α leads to this radioresistance.

Some radioresistance mechanisms promoted by cyH are independent of HIF1α. Notably, the exposure of cancer cells to cyH renders them more tolerant to ROS-inducing treatment, such as ionizing radiation (Hlouschek et al., 2018; Matschke et al., 2016; Saxena and Jolly, 2019). Indeed, the production of ROS by ionizing radiation is strongly diminished by the pre-exposure of NIH-460 lung adenocarcinoma cells to cyH (Matschke et al., 2016). This protection relies on glutaminolysis stimulation by cyH, since an inhibitor of glutaminolysis abolishes the radioprotection induced by cyH (Matschke et al., 2016). Cancer cells can become addicted to glutamine, and hence sensitive to glutamine starvation (Wise and Thompson, 2010). Another study showed that cyH induces solute carrier family 25 member (SLC25A1) expression, and SLC25A1 is involved in DNA damage repair after ionizing radiation (Hlouschek et al., 2018). Overall, these data suggest that cyH also confers radioresistance independently of HIF1α, notably via rendering them resistant to ROS.

4.3.4.2 Chemoresistance

Hypoxic tumors are less sensitive to chemotherapy. Hypoxic tumors have a reduced drug perfusion and hypoxia confers chemoresistance to cancer cells, notably via increasing drug efflux, apoptosis resistance and by decreasing DNA damage (Jing et al., 2019; Manoochehri Khoshinani et al., 2016). These cellular features conferring cancer cell resistance relies on HIF1α and acidic environment-induced by tumor hypoxia (Jing et al., 2019).

When compared with chH, cyH induces a stronger chemoresistance than chH (Bader et al., 2020). The chemoresistance induced by cyH depends on increased drug efflux, and on the enhanced survival protein expression and decreased pro-apoptotic protein expression. *In vitro*, cyH enhances the expression of ATP-binding cassette B1 (ABCB1) expression to a stronger extent then chH (Chou et al., 2012). Consistently, ABCB1 expression in glioblastoma (GBM) is mostly observed in cyH areas. CyH-induced ABCB1 is responsible of a decreased sensibility of GBM cells to doxorubicin and BCNU drugs. GBM cells exposed to cyH *in vivo* are less sensitive to chemotherapeutic drugs than chH and N-exposed GBM cells. *In vitro*, cyH induces the pro-survival protein expression in GBM and medulloblastoma cells and decreases the expression of pro-apoptotic protein Bax in medulloblastoma cells (Chen et al., 2015; Gupta et al., 2011). CyH-induced BcL-xL confers GBM cell chemoresistance to temozolomide (TMZ). BcL-xL is induced by cyH via NF-κB and HIF1α activation, which are themselves activated by ROS. Consistently, the antioxidant tempol abolishes the induction of BcL-xL by cyH and improves the effect of TMZ in term of mice survival *in vivo*. Interestingly, a study showed that prolonged treatment of cancer cells with cyH (50 cycles of hypoxia/reoxygenation during 6

days) durably improves chemoresistance of MCF10A to etoposide, even after 8 days resting in normoxia (Verduzco et al., 2015).

4.3.5 Angiogenesis

Tumor chH and cyH hypoxia strongly increases tumor vascularization (Saxena and Jolly, 2019; Schito, 2019). It is not known if chH and cyH have different potential to promotes tumoral angiogenesis. *In vitro*, cyH increases ECs migration and tubule formation, notably via HIF1α activation (Martinive et al., 2006; Toffoli et al., 2009b). *In vivo*, cyH treatment increases vascularization in A-07 human melanoma xenografts and in subcutaneous RCC (Gaustad et al., 2013; Rofstad et al., 2010; Vilaseca et al., 2017). VEGFA secretion is increased by cyH in A-07 human melanoma xenografts (Rofstad et al., 2010). *In vitro*, chH induces a stronger pro-angiogenic phenotype to liver cancer cells, whereas there is no difference between chH and cyH in pro-angiogenic phenotype induction in breast CSCs (Alhawarat et al., 2019). Hence, cyH promotes tumor vascularization and it would be interesting to know the mechanism involved in this process and to compare the effect of chH and cyH in tumoral angiogenesis.

B. Results

5 Part I

5.1 Cycling hypoxia and macrophage phenotype

5.1.1 Context

Tumor inflammation is a hallmark of cancer, which is involved in every stage of the disease (Greten and Grivennikov, 2019). Indeed, tumor inflammation can triggers cancer development and enhances metastasis. Accordingly, this is a marker of poor prognosis in human colon cancer patient (Tellier et al., 2015). Furthermore, chronic inflammation in tumors leads to the development of an immunosuppressive microenvironment, hence favoring tumor growth and dissemination of cancer cells (Greten and Grivennikov, 2019).

TAMs are the most abundant immune cells in the TME of solid tumors (Gentles et al., 2015). These cells are very plastic and their phenotypes strongly depends on the microenvironmental cues, and are classified among the M1 (pro-inflammatory phenotype) M2 axis (anti-inflammatory) (Mantovani et al., 2017). Due to this plasticity, these cells strongly regulate tumor inflammation (Mantovani et al., 2008). These cells belong mostly to M1 phenotype in the early stage of many cancer, whereas their phenotype is progressively skewed towards an immunosuppressive M2 phenotype, during the process of cancer immunoediting (Mantovani et al., 2017). Although M1 macrophages are mostly associated to a good prognosis in human cancer, they also are strongly involved in the development of inflammation-associated cancer development (Bruni et al., 2020; Fridman et al., 2017; Greten and Grivennikov, 2019). M2 macrophages strongly promotes an immunosuppressive phenotype and are hence associated to a poor prognosis in most cancer types (Bruni et al., 2020; Fridman et al., 2017).

In the lab, it was previously shown that cyH promotes EC inflammation *in vitro* and tumor inflammation *in vivo* in tumor-bearing mice (Tellier et al., 2015). More particularly, cyH enhances the impact of TNFα in the induction of pro-inflammatory phenotype in EC. CyH also increases *in vitro* the ability of EC to bind monocytes, a characteristic of EC activation (Sun et al., 2019; Tellier et al., 2015). Furthermore, *in vivo*, cyH strongly induces tumor inflammation, notably characterized by the increase in IL-6, KC and CXCL2 expression and by an increase in myeloid cells infiltration (Tellier et al., 2015). Accordingly, another team showed that cyH promotes inflammation in experimental metastasis model, characterized by an increase of TNFα and IL-6 expression, and an increase of the abundance of p65/NF-κB (Li et al., 2018). The latter effects were specific to cyH since they were not observed under chH or normoxia.

All these results together show that cyH promotes tumor inflammation, that TAMs strongly regulate tumor inflammation and that TAMs are very sensitive to external stimuli. Indeed, TAMs can either have a pro-inflammatory phenotype (M1 macrophages) or have an anti-inflammatory phenotype (M2 macrophages). Furthermore, cyH promotes pro-inflammatory phenotype in ECs and triggers NF-κB activation in several cell types. Nonetheless, the impact of cyH on macrophage phenotype is still not well known. All these results led us to ask about the effect of cyH on M0, M1 and M2 macrophages. More particularly, we hypothesized that cyH could induces or promotes a pro-inflammatory phenotype in macrophages. This is

particularly important since the impact of cyH on inflammatory phenotype in macrophages *in vitro* could be involved in the effect of cyH in tumor inflammation *in vivo*.

In order to responds to this question, the impact of normoxia, chH and cyH on THP-1-derived macrophages and bone marrow-derived macrophages (BMDM) phenotype was studied. The effect of cyH on the activation of signalling pathway involved in M1 polarization was assessed.

5.1.2 Experimental model

5.1.2.1 THP-1-derived macrophages model

THP-1 monocytes were isolated from the blood of young patients with acute myeloid leukemia. This is an immortalized cell line of blood monocytes, which confers some advantages such as this is easy to use, great availability and genetic homogeneity allowing to decreases variability (Chanput et al., 2014). These THP-1 monocytes were differentiated in macrophages and left unpolarised (M0 macrophages), or polarized into M1 macrophages or M2 macrophages, according to the protocol set up in the laboratory (Genin et al., 2015). This protocol is widely used in the literature since this article has been cited more than 400 times since his publication, based on google scholar website. Basically, THP-1 monocytes are polarized with 150nm phorbol 12-myristate 13-acetate (PMA) during 24h and left without PMA during 24h in order to have M0 macrophages. PMA treatment changes the morphology of monocytes and renders them adherent to the plastic. Furthermore, PMA induces an increases of macrophages markers expression (*e.g*. CD68) and a decreases of monocyte markers (*e.g.* CD14). M0 macrophages are polarized or not into M1 macrophages - by incubating them with 10 pg/mL LPS and 20 ng/mL IFNγ during 24h - or into M2 macrophages - by incubating them with 20 ng/mL IL-4 and 20 ng/mL IL-13 -. M1 macrophages strongly express and secrete pro-inflammatory markers (*e.g.* TNFα) and host defense markers (*e.g* CD80). There are at least three types of M2 macrophages *in vitro*, namely M2a, M2b and M2c. The polarization with IL-4 and IL-13 leads to the formation of M2a macrophages notably characterized by an increase in CD206 cell surface expression. Morphology of M0, M1 and M2 macrophages is different and pictures of these cells are represented in Fig. 27.

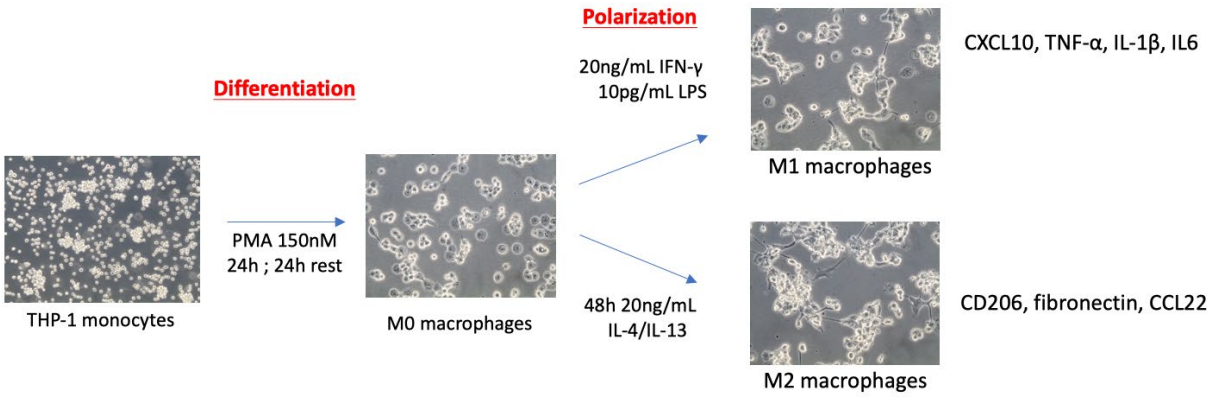

Fig. 27: Differentiation and polarization protocol of THP-1 monocytes into M0, M1 and M2 macrophages

5.1.2.2 Murine bone marrow-derived macrophages model

In order to obtain murine bone marrow-derived macrophages, monocytes from the bone marrow of C57BL/6 mice (6-8 weeks old) were differentiated and polarized into M0, M1 and M2 macrophages. Incubation of monocytes with 10% L-929 mouse fibroblast medium during 144h. Then, macrophages were left unpolarized (M0 macrophages), or polarized in M1 macrophages by 24h incubation with 10 ng/mL LPS and 20 ng/mL IFNγ, or polarized in M2 macrophages via 24h incubation with 20 ng/mL IL-4 and 20 ng/mL IL-13.

5.1.2.3 Incubation of macrophages in normoxia, chronic hypoxia and cycling hypoxia

Once polarized, M0, M1 and M2 macrophages were incubated under normoxia, chH or cyH during 6h. CyH corresponded to 4 cycles of 1h hypoxia (1% O_2) and 30 min reoxygenation (21% O_2). ChH and normoxia corresponded to 6h incubation in 1% O_2 or in 21% O_2. Additionally, for ELISA experiments, cells were incubated 16h more under room air to let the time to macrophages to secrete their cytokines. The protocol for hypoxia experiments is depicted in Fig. 28.

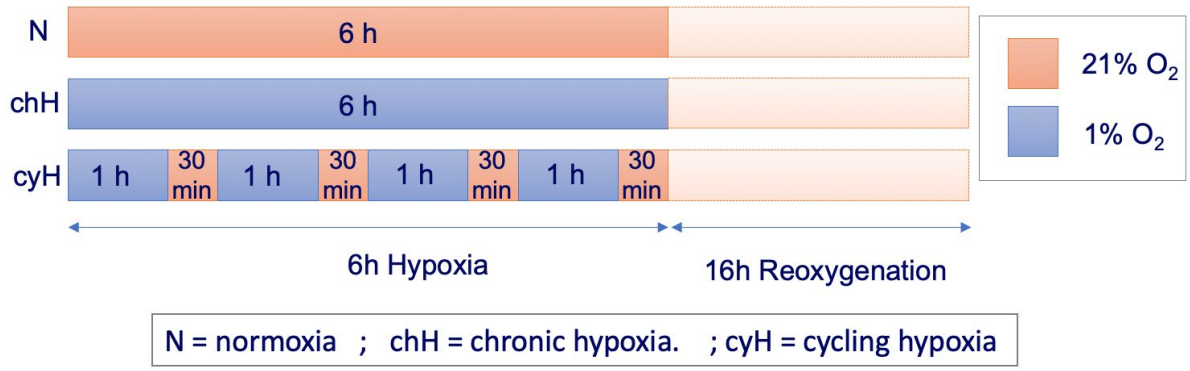

Fig. 28: Incubation of macrophages in normoxia, chH and cyH used in the study.

This frequency of hypoxia/reoxygenation is of 0,67 cycles an hour, which is in great accordance with the literature showing a frequency of 0.5 to 5 cycles an hour in tumors. Furthermore, this is based on a study which studied cyH in fibrosarcoma-bearing mice.

5.1.3 Discussion about the model used

The incubation of macrophages in N, chH and cyH was performed via a homemade pressurizer. The cyH model used in this study was used in several other studies performed notably on ECs. For example, the incubation of ECs in this model of cyH amplifies the TNFα-induced pro-inflammatory phenotype, induces EC activation, increases EC migration, increases ECs radiotherapy resistance and ROS production (Martinive et al., 2006; Tellier et al., 2015; Toffoli et al., 2009a; Toffoli et al., 2009b). Hence, the model of cyH used in (4 cycles of 1h hypoxia 30 min reoxygenation) the present work was very robust and mastered by the lab. Furthermore, as discussed in the section 5.1.2.3, the frequency of hypoxia/reoxygenation performed in this work (0.67 cycles an hour) was consistent with those observed *in vivo* (0.5 to 5 cycles an hour).

Several limitations of the model must be discussed. Some of them have already been discussed in the present article such as the fact that macrophages were reoxygenated at 21%

O_2. Here, another limitation of the model is that cancer cells and TME strongly modifies macrophage phenotype, and that macrophages were incubated in the absence of cancer cells. More particularly, cancer cells promote macrophage M2 phenotype and increases the immunosuppressive properties of macrophages. Hence, the present results have to be taken with precaution. It would be moreover very interesting to confirm the present results either *in vivo* or in human cancer.

In the present study, the secretion of proteins by macrophages was analyzed after the incubation of macrophages 6h in N, chH and cyH followed by 16h of reoxygenation in order to allow time for the cells to secrete their proteins. The main limitation of this is that the period of 16h was not in chH or in cyH. Furthermore, it could be argued that macrophages exposed to chH have one cycle of 6h hypoxia followed by 16h reoxygenation and were hence exposed to cyH. Nonetheless, in the literature, it was shown that the incubation of ECs 6h in N, chH and cyH followed by 16h reoxygenation increased the effect of cyH on the secretion of IL-6 and IL-8 by EA.hy926 and the effect of cyH on IL-6 and IL-8 secretion was similar at 6h or 6h + 16h reoxygenation in HUVEC (Tellier et al., 2015). Hence, the effect of cyH was either increased or similar between 6h and 6h + 16h reoxygenation, at least in ECs. It would hence be very likely, although it has not been tested, that the same phenomenon would also occur in macrophages, and that the study of protein secretion in macrophages at the timing of 6h + 16h reoxygenation is appropriate.

5.1.4 Research article: “Cycling hypoxia promotes a pro-inflammatory phenotype in macrophages via JNK/p65 signaling pathway”

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Cycling hypoxia promotes a pro-inflammatory phenotype in macrophages via JNK/p65 signaling pathway

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Cycling hypoxia (cyH), also called intermittent hypoxia, occurs in solid tumors and affects different cell types in the tumor microenvironment and in particular the tumor-associated macrophages (TAMs). As cyH and TAMs both favor tumor progression, we investigated whether cyH could drive the pro-tumoral phenotype of macrophages. Here, the effects of cyH on human THP-1 macrophages and murine bone marrow-derived macrophages (BMDM), either unpolarized M0, or polarized in M1 or M2 phenotype were studied. In M0 macrophages, cyH induced a pro-inflammatory phenotype characterized by an increase in TNFα and IL-8/MIP-2 secretion. CyH amplified the pro-inflammatory phenotype of M1 macrophages evidenced by an increased pro-inflammatory cytokine secretion and pro-inflammatory gene expression. Furthermore, cyH increased c-jun activation in human M0 macrophages and highly increased c-jun and NF-κB activation in M1 macrophages. C-jun and p65 are implicated in the effects of cyH on M0 and M1 macrophages since inhibition of their activation prevented the cyH pro-inflammatory effects. In conclusion, we demonstrated that cyH induces or amplifies a pro-inflammatory phenotype in M0 and M1 macrophages by activating JNK/p65 signaling pathway. These results highlight a specific role of cyH in the amplification of tumor-related inflammation by modulating the inflammatory phenotype of macrophages.

Tumors are complex tissues composed of multiple cell types interacting and influencing each other, namely malignant cells and stromal cells like endothelial cells, immune cells and fibroblasts[1]. The intricate combination of tumor microenvironment composition and environmental factors strongly determines tumor outcome[2]. A major factor altering the tumor microenvironment is the presence of low oxygen tension called hypoxia, that is a common feature of malignant tumors[3]. Two types of hypoxia can be distinguished: chronic and cycling hypoxia (cyH). Chronic hypoxia (chH) is associated to the limited oxygen distribution in a tissue; it is mainly the result of uncontrolled proliferation of O_2-consuming cancer cells and the O_2 diffusion gradient from blood capillaries[4,5]. In contrast, cyH, also called intermittent hypoxia, is related to the irregular erythrocyte flux circulating in the anarchical tumor blood network characterized by the presence of temporary occlusions[6–8]. The instability of blood flow leads to periods of hypoxia followed by periods of reoxygenation, occurring over hours through a clear pattern of periodicity[9]. We previously demonstrated that cyH amplifies the endothelial inflammatory response induced by TNFα notably through an overactivation of NF-κB. Moreover, we showed that cyH enhances the overall tumor inflammation characterized by a global increase in inflammatory gene expression and by an increase in intratumor leukocyte infiltration in tumor-bearing mice[10]. Inflammation is indeed described to be mutagenic and to favor proliferation and survival of malignant cells, angiogenesis, metastasis, corruption of the adaptive immune system and resistance to treatments[11]. Tumor-promoting inflammation has been designated as a new enabling characteristic for cancer, contributing to the acquisition of multiple hallmark capabilities[12]. Inflammation is firstly designed to fight disorders like infections or transformed cells. In a normal tissue, inflammation is resolved when

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disorders are eliminated. However, for malignant tumors that evade immune system, chronic inflammation persists[13].

All the cell types present in the tumor microenvironment could participate in the tumor-related inflammation but a major role is assigned to immune cells[12]. Macrophages constitute the main leukocytic infiltrate in tumors and are referred as tumor-associated macrophages (TAMs). Their extensive infiltration correlates with poor patient prognosis in more than 80% of analyzed cancers[14]. As these cells display a remarkable plasticity, their phenotype highly varies in function of environmental cues. Macrophages have been classified along a continuum of functional states where M1 and M2 are the two extreme polarization phenotypes[15,16]. M1 macrophage polarization refers to the classical activation in response to TLR ligands (such as LPS) and IFNγ whereas M2 polarization constitutes an alternative activation of macrophages induced by IL-4 and/or IL-13[17]. M1 macrophages are pro-inflammatory as they are characterized by the secretion of high amounts of pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6). They play major roles in host defense by phagocytosing and killing pathogens, by releasing cytotoxic components like NO and by activating the adaptive immune system through antigen presentation and T cell activation. M2 macrophages are anti-inflammatory characterized by the secretion of anti-inflammatory cytokines (e.g. CCL22, IL-10, CCL-18). They induce the resolution of inflammation (e.g. via an increased expression of MRC-1 also named CD206) and tissue repair (e.g. via the increased production of fibronectin[18]). By receiving particular local signals, different subpopulations of macrophages reside in the same tumor, changing according to their localization and along the time of tumor progression[19,20].

We postulated that cyH could modulate macrophage phenotype towards a phenotype that could promote tumor inflammation. In this study, we investigated the effects of cyH and chH on the polarization of human THP-1 macrophages and murine bone marrow-derived macrophages (BMDM). Results showed that cyH induces, on its own, a pro-inflammatory phenotype in unpolarized human and murine macrophages and reinforces the pro-inflammatory phenotype of human and murine M1 macrophages through the activation of the JNK/p65 signaling pathway.

Material and Methods

Cell culture and hypoxia incubation. Human monocytic THP-1 cells were maintained, until 12–13 passages, in RPMI medium 1640 with L-glutamine (#11875-093, Gibco) supplemented with 10 mM HEPES (#15630-056, Gibco), 1 mM pyruvate (#11360-039, Gibco), 0.05 mM β-mercaptoethanol (#31350-010, Gibco) and 2.5 g/l D-glucose (Merck), and containing 10% of heat decomplemented FBS. THP-1 monocytes were seeded at 800,000 cells/well in 6-well plates (Costar) and directly differentiated into macrophages by 24 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA, # P8139, Sigma) followed by 24 h rest period in complete RPMI medium without PMA. At the end of 48 h, THP-1 macrophages were used as M0 macrophages or were polarized into M1 or M2 macrophages. For M1 polarization, macrophages were incubated for 24 h with 10 pg/ml LPS (#L8630, Sigma) and 20 ng/ml rhIFNγ (R&D Systems). For M2 polarization, macrophages were incubated for 48 h with 20 ng/ml rhIL-4 (R&D Systems) and 20 ng/ml rhIL-13 (R&D Systems).

Murine bone marrow-derived macrophages (BMDM) were obtained from the differentiation of monocytes recovered from femur and tibia bone marrow of male C57BL6 mice, 6–8 weeks old. The local ethic committee of the university of Namur (Commission d'éthique en expérimentation animale; CEEXPANI) approved the procedure according to the animal care regulation (agreement number 14 229, University of Namur). All experiments were performed in accordance with their relevant guidelines and regulations. Bone marrow cells were firstly transferred in 100 mm dishes in DMEM high glucose containing 4.5 g/l D-glucose, L-glutamine and sodium pyruvate (#11995, Gibco) + 10% heat decomplemented low endotoxin FBS (HIS-LE FBS, #F7524, Sigma). 24 h later, non-adhering cells comprising monocytes were harvested and then monocyte differentiation into macrophages was launched for 6 days by adding 10% of conditioned media of L-929 mouse fibroblasts (L-929 CM), enriched in M-CSF. Conditioned medium was generated by seeding 500,000 L-929 cells in T75 flask in the presence of 20 ml of DMEM high glucose + 10% heat decomplemented low endotoxin FBS. After 6 days, 20 ml of L-929 conditioned medium/T75 flask were collected, filtered (0.2 µm) and stored at −20 °C. Macrophage differentiation medium (DMEM high glucose + 10% HIS-LE FBS + 10% L-929 CM) was replaced at the third day and at the fifth day of the differentiation process. 6 days after launching the differentiation, macrophages were detached by trypsinization and by the use of a cell scraper and were then seeded at 750,000 cells/6-well (Greiner) for M0 and at 500,000 cells/well for M1 and M2, in macrophage differentiation medium. 24 h after seeding, murine macrophages were used as M0 or were polarized for 24 h in M1 with 10 ng/ml LPS (#L8630, Sigma) and 20 ng/ml rmIFNγ (R&D Systems) or for 24 h in M2 with 20 ng/ml rmIL-4 (R&D Systems) and 20 ng/ml rmIL-13 (R&D Systems).

Chronic and cycling hypoxia exposure. Macrophages were incubated in CO_2 independent medium supplemented with 4 mM L-glutamine (Sigma) and 3.75 g/l D-glucose (Merck). Normoxic cells (N) were incubated in the same conditions but in normal atmosphere (21% O_2). For chronic hypoxia (chH), cells were exposed to a continued period of 6 h under 1% O_2. For cycling hypoxia (cyH), cells were exposed to four consecutive cycles of 1 h hypoxia (1% O_2) followed by 30 min reoxygenation (air, 21% O_2) (6 h). In order to expose cells to hypoxia, a homemade pressurized incubator was used. N_2 gas was injected (and air contained in the incubator was rejected) into this incubator until the 1% O_2 99% N_2 concentration was reached. The O_2 concentration was measured with an Eppendorf electrode.

NF-κB-pathway and JNK inhibition. The NF-κB-pathway inhibitor (Bay11-7082; S2913, Selleckchem) or JNK inhibitor (SP600125; Sigma Aldrich) were added to the THP-1 macrophages at 10 µM or 30 µM 1 h or 2 h before the hypoxia experiments in the CO_2 independent medium, respectively. Then, the macrophages were exposed to N, chH or cyH during 6 h. The efficiency of the inhibition of p65 nuclear translocation was analyzed by immunofluorescence and the efficiency of the phosphorylation of c-jun inhibition was confirmed by western

blotting (Supplementary Figs. 1A and 2A). The absence of toxicity of Bay11-7082 and SP600125 were confirmed by western blotting for cleaved PARP and MTT assay, respectively (Supplementary Figs. 1B and 2B).

Immunofluorescence labeling. Immunofluorescence labeling was performed as described before[21,22]. Briefly, cells were fixed 10 min in 4% paraformaldehyde in PBS. Cells were washed with PBS, then permeabilized with 0.1% Triton X100 in PBS during 5 min. Cells were blocked with 2% BSA in PBS 30 min and incubated O/N with primary antibody at 4 °C (CST #8242; p65; 1:400 diluted in PBS BSA 2%). Cells were rinsed 30 min in PBS BSA 2% and incubated with secondary antibody (Alexa Fluor 488-conjugated anti-rabbit IgG antibody; Molecular Probes, #A11034). Cells were then incubated with TOPRO-3 to stain the nucleus. The coverslips were mounted on Mowiol (Sigma) and the pictures taken with confocal microscope (SP5, Leica).

MTT assay. 150 000 THP-1 cells were differentiated in macrophages in 24-well plate as described before. Cells were incubated 8 h with SP600125 at 10, 20, 50 or 100 μM in 500 μL of CO_2 independent medium during 8 h. Then, 500 μL of MTT solution (2.5 mg/mL in PBS; Sigma; #M2128) were added and cells were incubated 2 h at 37 °C. Media were removed and cells were incubated 1 h at 37 °C in 1 ml of lysis Buffer ((SDS 30%/N,N-dimethyl-formamide 2:1 pH 4.7), with 70 rpm agitation. Absorbance was then measured at 570 nm.

RT-qPCR. After the incubation, total RNA was extracted from cells using the QIAcube system with RNeasy Mini kit (THP-1) or Micro kit (BMDM) and DNase digest protocol (QIAGEN). mRNA in 2 μg of total RNA was reverse transcribed by using Transcriptor First Strand cDNA synthesis kit (#4379012001, Roche). A sample processed without reverse transcriptase enzyme was used as negative control for qPCR analyses. The sequences of qPCR forward and reverse primers are available in Supplementary Table S1. Amplification reaction assays contained SYBRGreen PCR Master Mix (#4309155, Applied Biosystem) and primers (IDT, 300 nM). RPS9 was used as the reference gene for normalization and mRNA abundance was quantified using the threshold cycle method.

ELISA. For cytokine secretion analysis, human or murine macrophages were seeded and polarized in 24-well plates at 150,000 cells/well. For N, chH, cyH incubation, 750 μl of CO_2 independent medium + 4 mM L-glutamine + 3.75 g/l D-glucose were added per well. For murine BMDM, incubation medium was supplemented with 5% L-929 conditioned-medium. Cytokine concentrations in conditioned media were assayed using specific ELISA kits (Quantikine, R&D Systems) according to supplier's recommendations. Cytokine concentrations (pg/ml) were normalized by total protein concentrations (μg/ml) determined by the Folin Method after cell lysis with 200 μl 0.5 N NaOH/well.

Western blot analysis. Total protein extraction from macrophages plated in 6-well plates was performed using a lysis buffer containing 40 mM Tris pH 7.5, 150 mM KCl, 1 mM EDTA, 1% Triton-X-100, PIC (Protease Inhibitor Cocktail, Roche), PIB 25x (Phosphatase Inhibitor Buffer, 25 mM Na_3VO_4, 250 mM PNPP, 250 mM β-glycerophosphate, 125 mM NaF). Cell lysate was recovered and centrifuged for 5 min at 15,700 g and 4 °C to pellet cell debris. The supernatant was collected and stored at −70 °C before western blotting. 20 μg of proteins were separated on 10% SDS-PAGE gels and transferred onto a low fluorescence background PVDF blotting membrane (Millipore). Quantitative LI-COR technology was used for western blot analyses (Odyssey Infrared Imaging System v3.0.16, LI-COR, Biosciences). Membranes were blocked with Odyssey blocking buffer diluted 1:2 in PBS for 1 h at RT. Primary antibodies diluted in Odyssey Blocking buffer-Tween 0.1% were incubated overnight at 4 °C, then membranes were washed with PBS-Tween 0.1%, and finally incubated with secondary antibodies diluted 10,000 x for 1 h at RT. Membranes were washed with PBS-Tween 0.1%, and then with PBS, and finally dried before scanning. Loading control was assessed with α-tubulin or β-actin according to the molecular weight of the protein of interest. Antibodies used are listed in Supplementary Table S2.

Statistical analysis. Data are reported as mean ± 1 SEM. Statistical analyses were performed using SigmaPlot Software. When normality tests failed, statistical analyses were performed on square root- or log-transformed data. Corresponding statistical tests are outlined in figure captions.

Results

Cycling hypoxia induces a pro-inflammatory phenotype in human M0 macrophages and amplifies the pro-inflammatory phenotype displayed by human M1 macrophages. We firstly examined the impact of 4 cycles of 1 h hypoxia/30 min reoxygenation (cyH) on the mRNA expression of M1 and M2 markers in human THP-1 macrophages either unpolarized (M0) or polarized into M1 or M2 phenotype. The effects of cyH were compared to chronic hypoxia (chH) or normoxia (N). We separated the study of M1 markers in two categories with those contributing to inflammation and those playing a role in the intracellular host defense response. For pro-inflammatory M1 markers (Fig. 1A), in M0 macrophages, cyH significantly increased the mRNA expression of TNFα, IL-1β and IL-8. In M1 macrophages, cyH increased TNFα, IL-8 and PTGS2 mRNA expression. M2 macrophages were less affected by cyH compared to M0 and M1 macrophages, as only an increase in IL-8 mRNA expression was observed. For intracellular host defense response M1 markers (Fig. 1B), chH and cyH decreased HLA-DR (antigen presentation to T cells[23]) and CD80 (T cell activation[24]) mRNA expression in M1 macrophages, and cyH did not affect their expression neither in M0 nor in M2 macrophages. Moreover, cyH (but not chH) highly decreased IFIT1 (inhibition of viral replication[25]) mRNA expression in M1 macrophages while it left unaltered its expression in M0 or M2 macrophages. Regarding M2 markers (Supplementary Fig. 3), cyH did not alter their mRNA expression in M2 macrophages and did not induce their expression neither in M0 nor in M1 macrophages.

In order to check whether these cyH–induced mRNA expression changes could lead to an effective pro-inflammatory phenotype induced in M0 macrophages or amplified in M1 macrophages, we investigated the

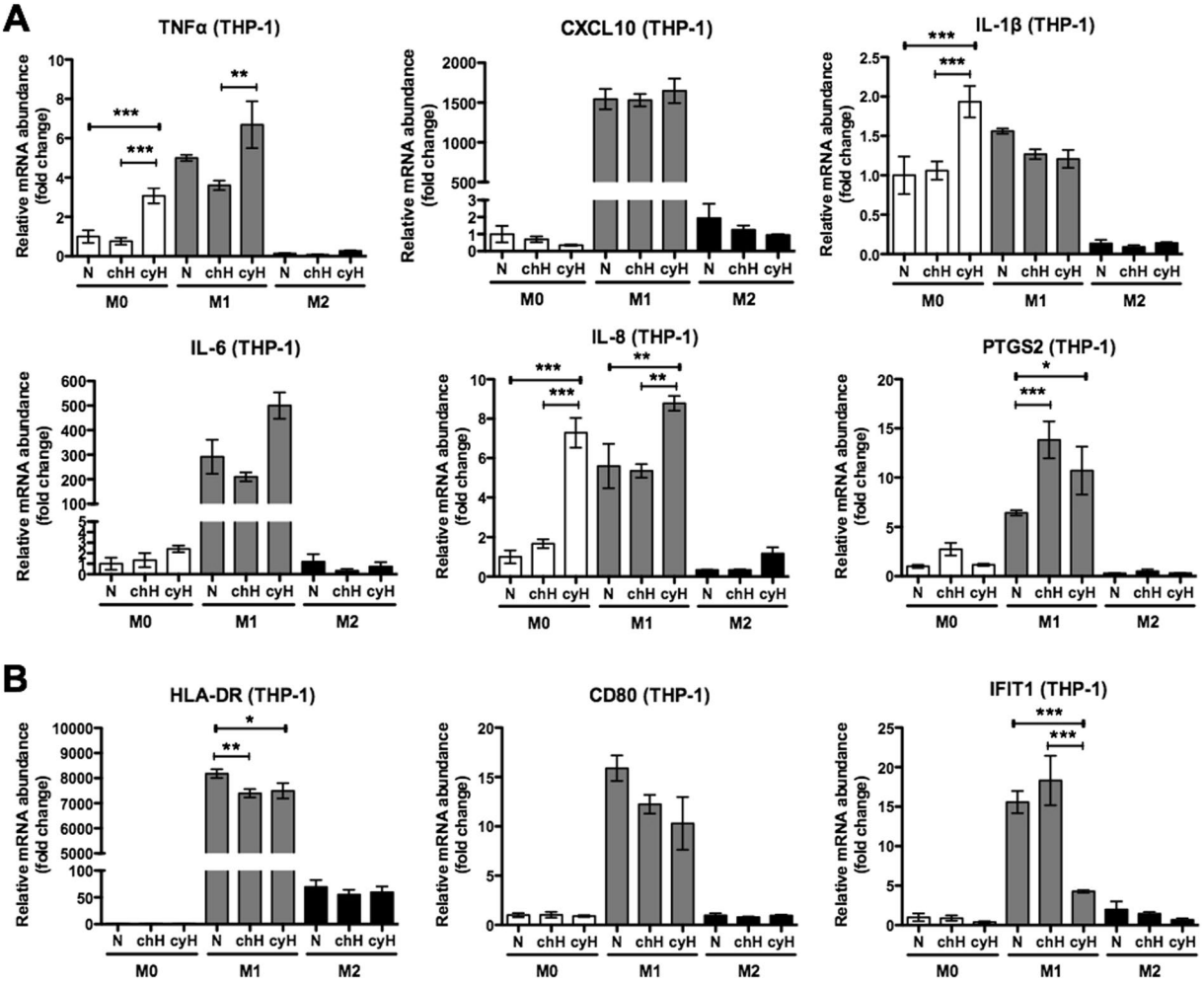

Figure 1. Effects of cycling hypoxia on the mRNA expression of M1 markers in human M0, M1 and M2 macrophages. THP-1 M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. mRNA expression of pro-inflammatory (**A**) and intracellular host defense response (**B**) M1 markers was evaluated directly after the incubation by RT-qPCR (n = 3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

secretion of pro-inflammatory cytokines. Cytokine concentrations were determined by ELISA after 16 h reoxygenation following the 6 h incubation under N, chH or cyH (6 h + 16hR) (Fig. 2). Firstly, it has to be noted that in comparison to M0 macrophages, M1 macrophages secreted higher amounts of TNFα, IL-8, IL-6 and IL-1β while M2 macrophages secreted lesser amounts of IL-8 and IL-1β and did not secrete TNFα and IL-6. cyH highly increased the secretion of TNFα by M0 macrophages to nearly reach the level detected for M1 macrophages under normoxia (N) (Fig. 2A). It also increased TNFα secretion by M1 macrophages. Moreover, cyH highly increased IL-8 secretion by all types of macrophages (Fig. 2B). In contrast, cyH did not increase IL-6 secretion by M1 macrophages (Fig. 2C) and significantly decreased IL-1β secretion by M1 macrophages (Fig. 2D).

Then, we examined whether a concomitant cyH exposure at the beginning of THP-1 macrophage polarization process could modulate the intensity of M1 and M2 polarization (Supplementary Fig. 4). Therefore, M0 macrophages were simultaneously exposed to 6 h of N, chH or cyH and to M1 or M2 polarization molecules and the polarization protocol was ended as usual before measurements of mRNA expression for M1 (Supplementary Fig. 4A,B) and M2 markers (Supplementary Fig. 4C). Results showed that this co-stimulation increased not only the mRNA expression of TNFα and PTGS2 in M1 macrophages but also significantly decreased the mRNA expression of CD206 and CCL22 in M2 macrophages, indicating that cyH is also able to partially impair the M2 polarization process.

Cycling hypoxia induces a pro-inflammatory phenotype in murine M0 macrophages and amplifies the pro-inflammatory phenotype displayed by murine M1 macrophages. In contrast to THP-1 macrophages for which the protocol of polarization was previously well optimized by our team[22], M1 and M2 polarization of murine bone marrow derived macrophages (BMDM) required fine tuning[26]. For M1 polarization, M0 macrophages were incubated for 24 h with LPS (1 ng/ml or 10 ng/ml) in combination with 20 ng/ml IFNγ. For M2 polarization, M0 macrophages were incubated with 20 ng/ml IL-4 and 20 ng/ml IL-13

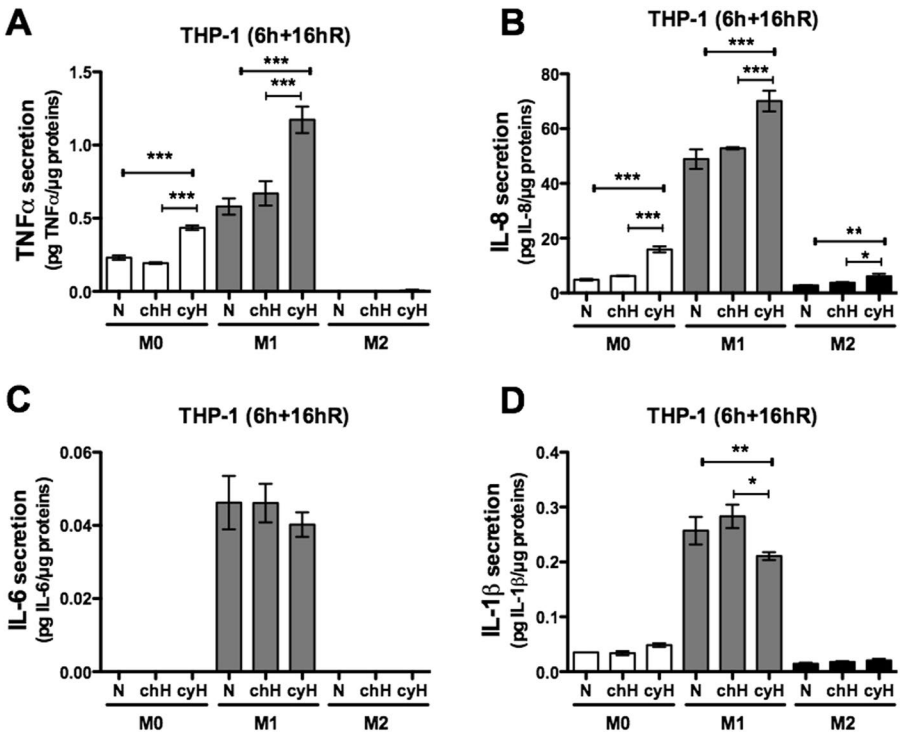

Figure 2. Effects of cycling hypoxia on the secretion of pro-inflammatory cytokines by human THP-1 M0, M1 and M2 macrophages. THP-1 M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. Conditioned media were harvested 16 h of reoxygenation after the 6 h of incubation (6 h + 16hR). Concentration of TNFα (**A**), IL-8 (**B**), IL-6 (**C**) and IL-1β (**D**) was assayed by ELISA (n = 3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

for either 24 h or 48 h. M0 macrophages were placed in the same medium as the one used for M1 and M2 but without the polarization cocktail, and were used as control cells for polarization validation. We then evaluated the mRNA expression of M1 and M2 markers (Supplementary Fig. 5). In M1 polarized macrophages but not in M2, we observed an increase in the mRNA expression of the six pro-inflammatory cytokine coding genes (Supplementary Fig. 5A), of the three pro-inflammatory enzyme coding genes (Supplementary Fig. 5B) and of the three intracellular host defense response genes (Supplementary Fig. 5C). Of note, all M1 marker gene transcripts in M1 polarized macrophages reached higher expression levels in the presence of 10 ng/ml LPS (vs. 1 ng/ml). As this concentration is not cytotoxic for murine BMDM (data not shown), we used 10 ng/ml LPS in combination with 20 ng/ml IFNγ for M1 polarization in the next experiments. In M2 polarized macrophages, we observed an increase in the mRNA expression of MRC-1 and Arg-1 at both times of polarization but to a higher extent after 24 h (vs. 48 h) (Supplementary Fig. 5D). The 24 h timing was thus chosen for further experiments of M2 polarization from BMDM.

After validating M1 and M2 polarization, we studied the effects of cyH on the mRNA expression of polarization markers in murine BMDM. The effects of cyH for M0, M1 and M2 macrophages are presented on different graphs. For the pro-inflammatory cytokines (Fig. 3A), cyH exerted a higher effect on M0 macrophages compared to M1 and M2 macrophages as we observed a highly significant increase in TNFα, MIP-2 and KC mRNA expression. In M1 macrophages, an increase in MIP-2 and KC mRNA expression was detected but to a lower extent than in M0 macrophages. However, a high increase in CXCL10 mRNA expression by cyH in M1 macrophages was evidenced. In M2 macrophages, cyH only increased MIP-2 and KC mRNA expression.

For the pro-inflammatory enzymes (Fig. 3B), cyH increased the mRNA expression of genes encoding the enzymes PTGS2, iNOS and Arg-2 in M1 macrophages. Moreover, a high increase in iNOS mRNA level was induced by chronic hypoxia in M0 and M2 macrophages. cyH increased Arg-2 mRNA expression in M0 and in M1 macrophages. cyH had no effect on the mRNA expression of these enzymes in M2 macrophages.

Regarding the intracellular host defense response markers (Supplementary Fig. 6), cyH decreased MARCO and IFIT1 mRNA expression in M0 macrophages but increased IFIT1 mRNA expression in M1 macrophages.

Then, we studied the effects of cyH on the mRNA expression of the two M2 markers (Supplementary Fig. 7). CyH slightly induced the mRNA expression of Arg-1 in M2 macrophages and induced the mRNA expression of MRC-1 in M0 macrophages. Interestingly, chH increased Arg-1 mRNA expression in M1 and M2 macrophages and, to a very high extent, in M0 macrophages.

Then, we investigated the effects of cyH on the secretion of pro-inflammatory cytokines. Therefore, murine M0, M1 and M2 BMDM were exposed for 6 h to N, chH or cyH and then conditioned media were harvested after 16 h reoxygenation (6 h + 16hR). Results showed that M1 macrophages secreted more TNFα than M0

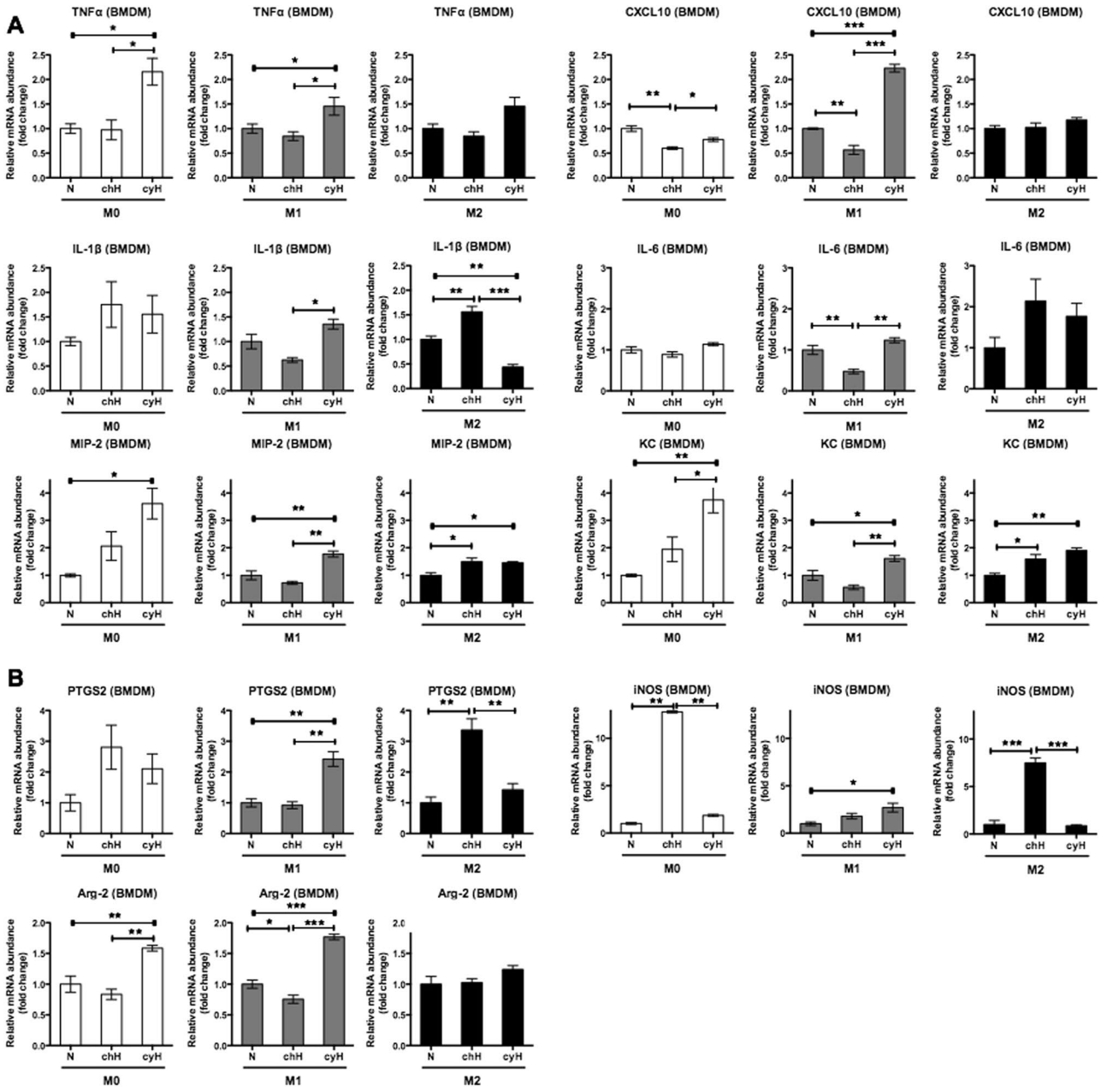

Figure 3. Effects of cycling hypoxia on the mRNA expression of M1 markers in murine M0, M1 and M2 macrophages. M0, M1 and M2 macrophages (BMDM) were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. mRNA expression of pro-inflammatory cytokines (**A**) and pro-inflammatory enzymes (**B**) was evaluated directly after the incubation by RT-qPCR (n = 3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

macrophages while M2 macrophages secreted less TNFα. cyH increased TNFα secretion by M0 macrophages but not by M1 macrophages (Fig. 4A). M0 macrophages secreted much higher amounts of MIP-2 than M1 and M2, and MIP-2 secretion by M0 macrophages was increased when exposed to cyH (Fig. 4B).

Cycling hypoxia differentially activates STAT1, NF-κB and c-jun transcription factors in macrophages as function of polarization and tissue origin. Since most of the effects of cyH were related to an enhancement of the inflammation associated to the M1 phenotype, we next analyzed the effects of cyH on the major transcription factors described to be involved in M1 polarization, namely STAT1, NF-κB, AP-1 and IRF5[27]. To this aim, we studied, by western blotting, the phosphorylated (active) forms of STAT1, p65 (one of the subunits of NF-κB) and c-jun (one of the subunits of AP-1). For IRF-5, we studied the total abundance of the protein (and not a post-translational modification) as variations in the total abundance directly reflect stimulation.

For human THP-1 macrophages (Fig. 5), total protein extracts were recovered from M0, M1 and M2 macrophages exposed to N, chH or cyH after 1h30 (A), 3 h (B) and 6 h (C). Results showed that phosphorylated

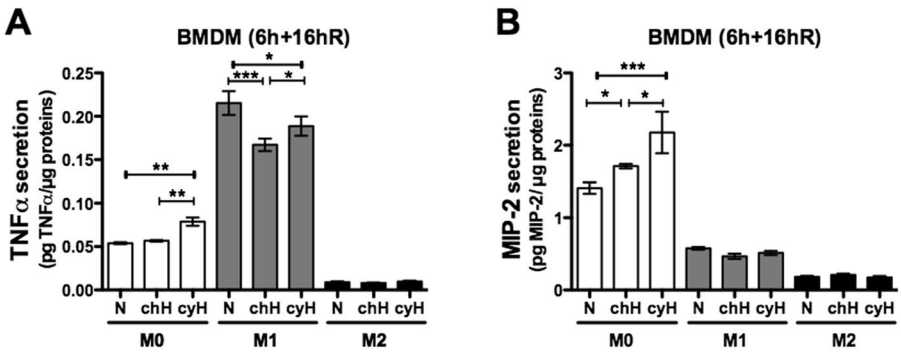

Figure 4. Effects of cycling hypoxia on the secretion of pro-inflammatory cytokines by murine macrophages. M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. Conditioned media were harvested 16 h of reoxygenation after the 6 h of incubation (6 h + 16hR). Concentration of TNFα (**A**) and MIP-2 (**B**) was assayed by ELISA (n = 3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

STAT1 (Tyr701) was only detected in M1 macrophages and that the level of phosphorylated p65 (Ser536), phosphorylated c-jun (Ser63) and total IRF5 was higher in M1 than in M0 and in M2 macrophages. In M0 macrophages, cyH increased the abundance of P-c-jun (Fig. 5F). In M1 macrophages, cyH increased the abundance of P-p65 (Fig. 5E), of P-c-jun (Fig. 5F) and to a lesser extent, of P-STAT1 (Fig. 5D). In M2 macrophages, a trend to an increase in the abundance of P-p65 and P-c-jun was observed in response to cyH.

For murine BMDM (Fig. 6 and Supplementary Fig. 8), total protein extracts were recovered from M0, M1 and M2 after 1h30 (A), 3 h (B) and 6 h (C). Phosphorylated STAT1 was only present in M1 macrophages and the level of phosphorylated p65, phosphorylated c-jun and IRF5 was higher in M1 macrophages compared to M0 or M2 macrophages. In M0, M1 and M2 macrophages, no significant change in the level of the phosphorylated form of p65, c-jun nor in the total abundance of IRF5 was observed under cyH in comparison to N (Supplementary Fig. 8). In contrast, in M1 macrophages, cyH increased the abundance of P-STAT1 (Fig. 6D) while chH significantly decreased it.

NF-κB and c-jun activation is implicated in the induction by cycling hypoxia of a pro-inflammatory phenotype in human M0 macrophages and in the amplification of the pro-inflammatory phenotype in human M1 macrophages. In order to examine whether the effects of cyH on THP-1 M0 and M1 macrophages were due to NF-κB or c-jun activation, M0 and M1 macrophages were pre-incubated with the NF-κB-pathway inhibitor Bay11-7082 or with the JNK inhibitor SP600125 before exposure to N, chH or cyH. The efficiency and inocuity of NF-κB-pathway and JNK inhibitors were validated (Supplementary Figs. 1 and 2). The increase in TNFα and IL-6 expression induced by cyH in M1 macrophages was completely abolished by the inhibition of NF-κB or c-jun (Fig. 7A,B). Furthermore, the increased expression of IL-8 induced by cyH were slightly inhibited by SP600125 in M1 macrophages and abolished in M0 macrophages. The increase in IL-1β expression in M0 macrophages induced by cyH was partly inhibited in the presence of Bay11-7082 and totally abolished in the presence of SP600125 (Fig. 7A,B). These results indicate that NF-κB and c-jun are implicated in the pro-inflammatory effects induced by cyH in THP-1 M0 and M1 macrophages.

Cycling hypoxia induced a c-jun/p65 signaling pathway in THP-1 M0 and M1 macrophages. Because p65 and c-jun inhibition were both able to abolish the pro-inflammatory effects of cyH on THP-1 M0 and M1 macrophages, we wondered if the activation of p65 could influence the activation of c-jun, and vice-versa. M0 and M1 THP-1 macrophages were incubated with Bay11-7082 or SP600125 under N, chH or cyH during 1h30 (M1) or 4h30 (M0). The phosphorylation of p65 and c-jun was then analyzed by western blotting (Fig. 8). As expected, the phosphorylation of p65 and c-jun was inhibited in M0 and M1 macrophages by Bay11-7082 and SP600125, respectively. In M0 macrophages, JNK inhibition did not influence p65 phosphorylation level (Fig. 8A). In M1 macrophages, JNK inhibition decreased p65 phosphorylation in macrophages exposed to cyH and to a lesser extent to macrophages exposed to N or chH (Fig. 8B). In both types of macrophages, the phosphorylation of c-jun was strongly increased by Bay11-7082. All these results are summarized and indicated that a c-jun/p65 signaling pathway is activated in M0 and M1 macrophages exposed to cyH (Fig. 8C).

Discussion

Inflammation is one of the hallmarks of cancer associated with bad prognosis[12]. Previously, we showed that cyH enhanced the pro-inflammatory phenotype of endothelial cells *in vitro* and enhanced tumor inflammation *in vivo*[10]. Numerous studies have evidenced the critical role of immune cells in tumor progression and inflammation. TAMs display a remarkable plasticity to environmental cues. Indeed, in the early state of tumor, TAMs display mostly monocyte/M1 phenotype, whereas at later stages, TAMs mostly belong to the M2 phenotype[28–30]. While M1 macrophages are pro-inflammatory, they have anti-tumoral effects, associated with cytotoxicity towards cancer cells, and immune-stimulatory functions[30]. On the other hand, while M2 macrophages are anti-inflammatory, they have pro-tumoral effects due to immune-suppression and angiogenesis induction[31]. Since TAMs are key cells

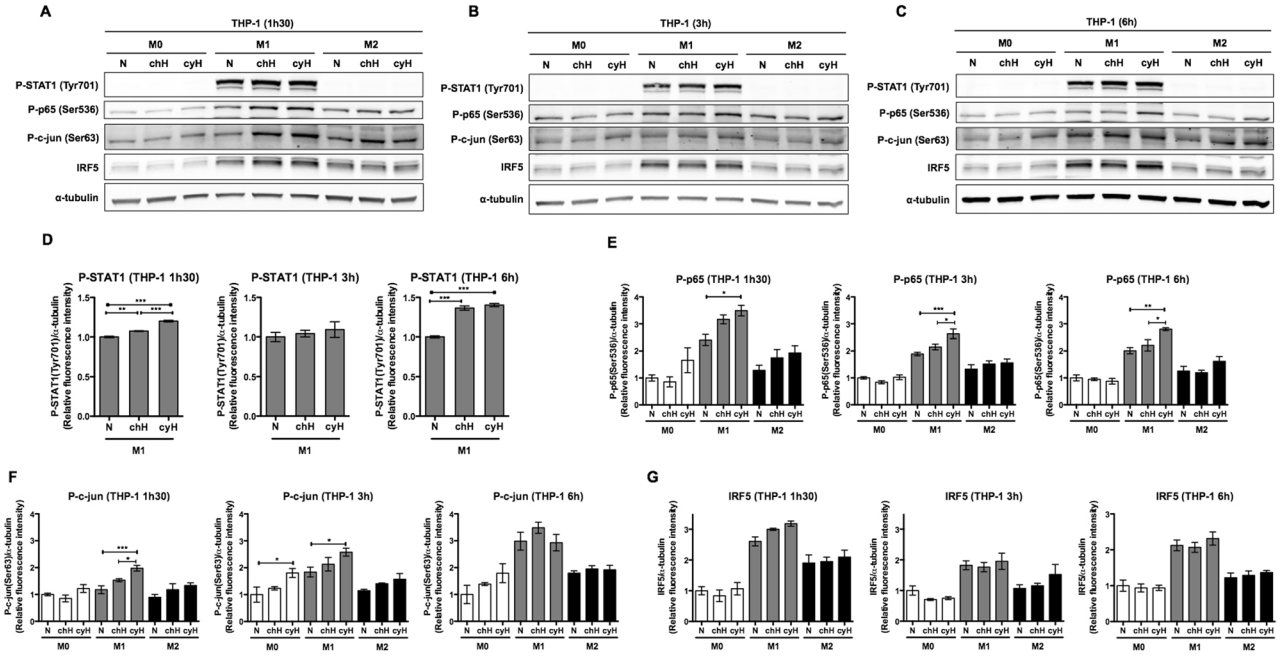

Figure 5. Effects of cycling hypoxia on the abundance of the phosphorylated form of STAT1, p65 and c-jun as well as on the abundance of IRF5 in human M0, M1 and M2 macrophages. THP-1 M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 1h30 (**A**), for 3 h (**B**) or for 6 h (**C**), and then total protein extraction was performed. Abundance of the phosphorylated form of STAT1 (Tyr701), p65 (Ser536) and c-jun (Ser63) as well as the total abundance of IRF5 was detected by western blotting (n = 3). α-tubulin was used as loading control. Fluorescence intensity of each immunoblotted protein was quantified and normalized for α-tubulin (**D–G**). Statistical analysis was performed by two-way ANOVA (or one-way ANOVA for P-STAT1 in M1 macrophages) and Holm-Sidak test as post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

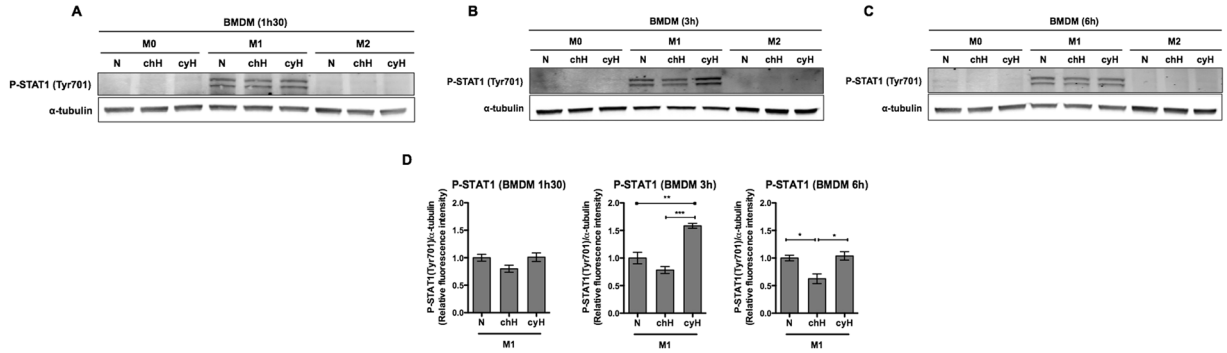

Figure 6. Effects of cycling hypoxia on the abundance of the phosphorylated form of STAT1 in murine M0, M1 and M2 macrophages. M0, M1 and M2 macrophages (BMDM) were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 1h30 (**A**), for 3 h (**B**) or for 6 h (**C**), and then total protein extraction was performed. Abundance of the phosphorylated form of STAT1 (Tyr701) was detected by western blotting (n = 3). α-tubulin was used as loading control. Fluorescence intensity of the phosphorylated form of STAT1 was quantified and normalized for α-tubulin (**D**). Statistical analysis was performed by one-way ANOVA and Holm-Sidak test as post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

involved in the control of tumor inflammation and progression, we investigated the role of cyH on the phenotype of human and murine M0, M1 and M2 macrophages.

In human THP-1 M0 macrophages, cyH promoted a pro-inflammatory phenotype characterized by an increase in TNFα and IL-8 secretion but did not induce the functions related to intracellular host defense. CyH thus favors a shift of unpolarized M0 macrophages towards a M1-like phenotype that is incomplete and possibly not fully functional. In M1 macrophages, cyH amplified the pro-inflammatory phenotype evidenced by an increase in TNFα and IL-8 secretion but partially decreased the expression of intracellular host defense genes, HLA-DR, CD80 and IFIT1. Interestingly, the decrease in the expression of the intracellular host defense genes

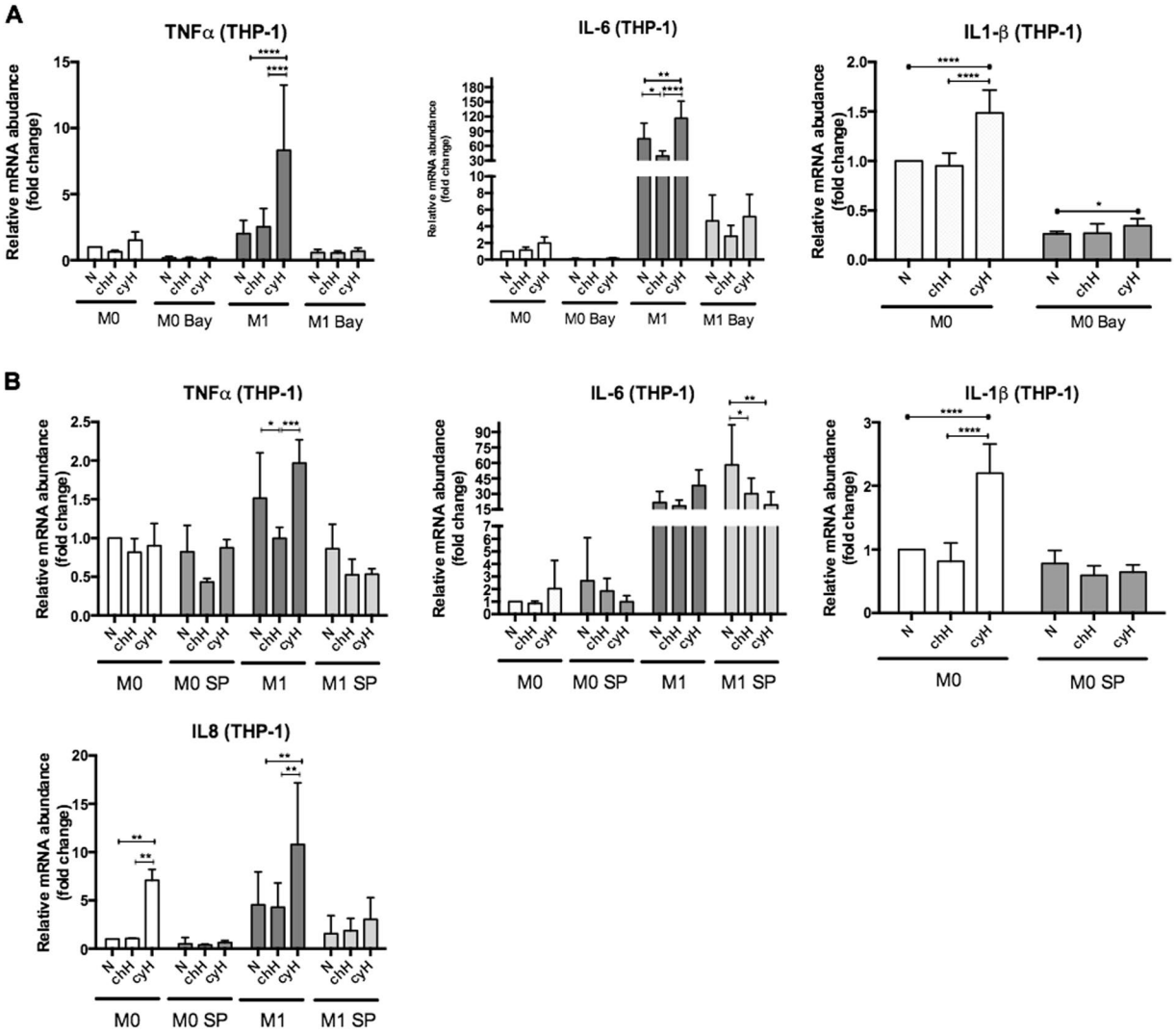

Figure 7. Effects of NF-κB and c-jun inhibition on cycling hypoxia induced pro-inflammatory phenotype in human THP-1 M0 and M1 macrophages. THP-1 M0 and M1 macrophages were incubated with Bay11-7082 (10 μM) or SP600125 (30 μM) for 1 h or 2 h, respectively. Then, THP-1 M0 and M1 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. The mRNA expression of TNFα, IL-6, IL1-β and IL-8 was analyzed by RT-qPCR (n = 4, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

has already been observed in a mouse tumor model of sleep apnea, a phenomenon associated with intermittent hypoxia[32]. Furthermore, hypoxia has already been described to inhibit the ability of macrophages for phagocytosis and for T cell activation via the reduction of CD80 expression[33]. Moreover, cyH increased the mRNA expression of PTGS2, the gene encoding cyclooxygenase 2 (COX2). COX2-derived PGE2 is known to inhibit the functions of T cells[34,35] and infiltration of COX2-expressing macrophages is associated with tumor neovascularization and tumor growth[36]. Altogether our data suggest that in response to cyH, THP-1 M1 macrophages reveal a decreased potential to trigger an adaptive immune response toward cancer cells together with a stimulation of angiogenesis.

In murine BMDM, cyH also induced a pro-inflammatory phenotype in unpolarized M0 macrophages characterized by an enhanced secretion of TNFα and MIP-2, and decreased the basal level of the intracellular host defense response. In murine M1 macrophages, cyH amplified the pro-inflammatory phenotype especially by increasing the expression of pro-inflammatory enzyme genes while it did not alter the intracellular host defense response. The major effects of cyH evidenced in murine macrophages were observed in M0 macrophages. Unlike THP-1 M1 macrophages, murine M1 macrophages did not appear to be highly sensitive to cyH. This could be explained by the higher concentration of LPS used for the murine M1 polarization.

Our results demonstrated that M1-associated genes were not all induced or amplified by cyH, suggesting that cyH specifically modulated some aspects of the phenotype of macrophages. This modulation depends on transcription factors activated under cyH exposure. We focused on the major transcription factors described to orientate the M1 polarization. In murine BMDM, cyH mainly increased STAT1 activation in M1 macrophages.

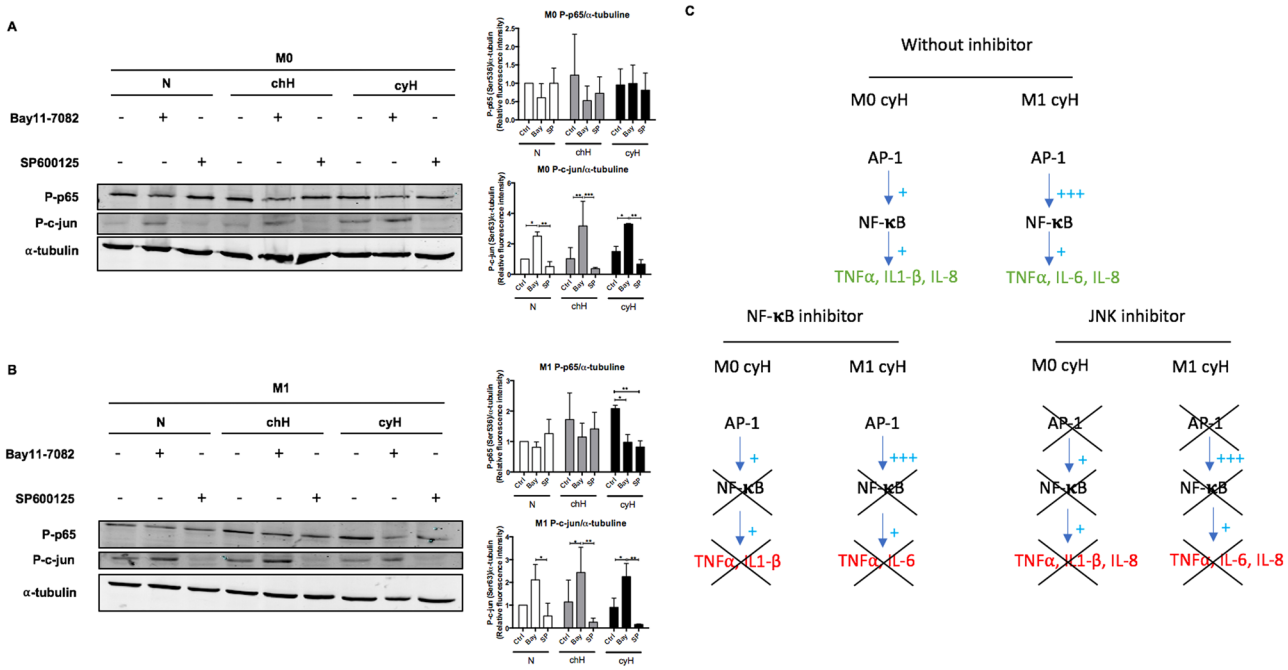

Figure 8. Effects of NF-κB and c-jun inhibition on the protein abundance of the phosphorylated form of p65 and c-jun. THP-1 M0 and M1 macrophages were incubated with Bay11-7082 (10 μM) or SP600125 (30 μM) for 1 h or 2 h, respectively. Then, these THP-1 M0 (**A**) and M1 (**B**) macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 1 h 30 min (M1) or 4 h 30 min (M0). Abundance of the phosphorylated form of p65 (Ser536) and c-jun (Ser63) were detected by western blotting (n = 3). Fluorescence intensity of each immunoblotted protein was quantified and normalized for α-tubulin. Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. The results are summarized in (**C**).

In THP-1 macrophages, cyH increased c-jun activation in M0 and M1 macrophages. In addition, it highly increased NF-κB activation and to a lesser extent STAT1 activation in M1 macrophages. No significant changes were observed in human M2 macrophages. Interestingly, we showed that p65 and JNK inhibition in M0 and M1 macrophages were both able to inhibit the pro-inflammatory phenotype induced by cyH (Fig. 7). Interestingly, JNK inhibition decreased slightly the phosphorylation of p65 in M1 macrophages exposed to N or chH and to a higher extent in M1 macrophages exposed to cyH. JNK inhibition did not changed p65 phosphorylation level in M0 macrophages (Fig. 8). Furthermore, p65 inhibitor decreased p65 phosphorylation and increased c-jun phosphorylation, which means that c-jun is unable to induce by itself and independently of NF-κB, the cyH-induced pro-inflammatory phenotype in M0 and M1 macrophages. Taken together, these results support a model wherein cyH-induced or amplified a pro-inflammatory phenotype in M0 and M1 macrophages via a c-jun/p65 signaling pathway (Summarized in Fig. 8C).

In our *in vitro* experimental model, cyH was performed by 4 cycles of 1-hour hypoxia followed by 30 minute reoxygenation. This protocol was based on *in vivo* measurements of pO_2 fluctuations in the tumor vasculature occurring at the frequency of 0.5 to 3 cycles per hour[9,37]. Furthermore, the O_2 saturation in tumor is comprised between 1 to 2% O_2 in a majority of solid tumors[38]. It was showed *in vitro* that 1-hour hypoxia causes a rapid accumulation of HIF-1α, whereas 30-minute reoxygenation is sufficient to abrogate this accumulation[39]. Moreover, a progressive accumulation of HIF-1α along cycles was observed in endothelial cells[40,41]. This *in vitro* protocol was used to demonstrate that cyH increased endothelial cell migration, tubulogenesis and endothelial cell resistance towards proapoptotic stresses, and increased tumor cell radioresistance[39,42,43]. More recently, we demonstrated that this timing of cyH amplified the TNFα-induced pro-inflammatory state of endothelial cells since an increase in both pro-inflammatory cytokine secretion and endothelial monocyte adhesion was observed[10].

In order to study the effects of obstructive sleep apnea (OSA), Murphy *et al.* showed that hypoxia/reoxygenation cycles can induce a pro-inflammatory phenotype to THP-1 M0 and M1 macrophages. The protocol of hypoxia/reoxygenation used was not relevant to cancer research. Indeed, extremely rapid changes in O_2 saturation only 8 h a day for 3 consecutive days (40 s 16% O_2, 40 s 3% O_2) were performed. Schaefer *et al.* showed that hypoxia/reoxygenation cycles (6 cycles of 40 min 1% O_2 20 min 21% O_2) induces a pro-inflammatory phenotype in THP-1 M0 macrophages characterized by an increased expression of pro-inflammatory cytokine such as TNFα, IL-6 and IL-1β. In order to see the effects of OSA on the development of atherosclerosis, Zhou *et al.* showed that hypoxia/reoxygenation cycles (6 cycles of 35 min 0.1% or 5% O_2, followed by 25 min N) induced a pro-inflammatory phenotype in unpolarized M0 THP-1 macrophages. The pO_2 saturation used in these several studies during cyH was either too low or too high for cancer research, since the O_2 saturation in tumor is comprised between 1 to 2% O_2 in a majority of solid tumors[38]. In these conditions, they showed that the advanced

glycation end-products (AGE) receptor (RAGE) was implicated in the cyH pro-inflammatory effects. Some ligands of RAGE, namely AGE and HMGB1, were also observed to induce pro-inflammatory phenotype in M0 macrophages and in human bronchial epithelial cells, respectively[44,45]. Hence, it would be interesting to study the effects of cyH in conditions relevant to cancer research on the expression and secretion of such RAGE ligands by macrophages and if there exists a crosstalk between c-jun/p65 and RAGE.

Some limitations in the study can be highlighted. The first one is the pO_2 used in the study. Indeed, in human healthy tissue, the physiological normoxia is comprised mostly between 4% O_2 (muscle) and 9.5% O_2 (kidney, outer cortex)[46,47]. In this study, normoxia and the cyH reoxygenation were performed by exposing cells to atmospheric air (21% O_2). Nonetheless, the hypoxia value that we used was physiologically relevant since O_2 saturation in tumor is comprised between 1 and 2% O_2 in a majority of solid tumors[38,47]. Secondly, we showed that cyH induced a pro-inflammatory phenotype in M0 and M1 macrophages in both BMDM and THP-1 macrophages. If there are some similarities between these two types of macrophages, we also observed some differences notably in fold induction and cytokine expression and secretion. Furthermore, the pro-inflammatory response was dependent in NF-κB and c-jun activation in THP-1 macrophages whereas cyH induced mostly STAT1 activation. The discrepancy between murine and human macrophages was well characterized in[48]. Indeed, Spiller *et al.* compared human macrophages (either derived from peripheral blood or from induced pluripotent stem cells either from THP-1 monocytes) to BMDM. Up to 800 genes were used to characterize the expression profile of each model. It was shown that human macrophages were more closely related to each other than to mouse macrophages. This could explain the differences of cyH effects between human and murine macrophages observed in our study. However, Spiller *et al.* also observed some discrepancies between human peripheral blood (PB)-derived macrophages and THP-1 macrophages[48]. Thereby, it would be interesting to confirm the effects of cyH on human PB-derived macrophages. Nonetheless, THP-1 macrophages model is a very good and reliable model which is commonly used in the literature[49]. THP-1 macrophages are more stable and show less heterogeneity in comparison to PB-derived macrophages. The difference between THP-1 macrophages and human monocyte-derived macrophages is much smaller than that of between THP-1 monocytes and human monocytes[7]. Furthermore, Shiratori *et al.* compared THP-1 macrophages and human macrophages in terms of phagocytic capacity and M1 and M2 polarization. They showed that THP-1 macrophages was an appropriate model to study the M1 polarization but less for M2 polarization. In addition, no difference in phagocytic capacity between the two models was observed. Finally, the response of THP-1 macrophages to LPS is very similar to PB-derived macrophages[50].

In conclusion, in this study, we are the first to investigate the effects of cyH and chH comparatively in human and murine macrophages and in M0, M1 and M2 macrophages. We investigated for the first time the effects of cyH on the activation of transcription factors involved in M1 polarization and on the secretory phenotype of human and murine macrophages. We demonstrated that cyH, on its own, induces or amplifies a pro-inflammatory phenotype in M0 and M1 macrophages, but not in M2 macrophages. These phenotypes were characterized by an increase in the secretion of pro-inflammatory cytokines and driven by a c-jun/p65 signaling pathway. These effects were specific to cyH, since they were not observed in cells exposed to chH. Thereby, the present work highlights the role of cyH in the amplification of inflammation, in agreement with our previous results emphasizing for the first time the link between cyH and tumor inflammation[10]. Indeed, here we demonstrated that cyH induces a pro-inflammatory phenotype in macrophages characterized by an increase in TNFα secretion, while we previously revealed that cyH amplifies the TNFα-induced inflammatory response of endothelial cells characterized by an increase in pro-inflammatory cytokine secretion and in monocyte adhesion[10]. This means that an amplification loop of the effects of cyH on tumor inflammation exists, as TNFα secreted in higher amount by cycling hypoxic macrophages could target endothelial cells and as cyH amplifies the endothelial inflammatory response to TNFα. This enhanced local inflammation could provoke an increase in blood vessel permeabilization and thus could favor cancer cell intravasation and dissemination. This could be an explanation by which cyH increases tumor metastasis[51–54].

Altogether, these results evidenced a global mechanism initiated specifically by cyH, that could account for the amplification of tumor-promoting inflammation. CyH induces a common mechanism in different cell types of the tumor microenvironment, leading to the establishment of an inflammatory microenvironment. Understanding the molecular mechanism involved in the cyH-induced effects could allow to highlight original therapeutic targets with the advantage to inhibit a global mechanism supporting tumor progression without affecting healthy tissue.

Received: 7 August 2019; Accepted: 29 December 2019;
Published online: 21 January 2020

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Acknowledgements

The authors thank the technological platforms SIAM and Morph-Im (University of Namur). Victor Delprat is a research fellow of Televie. Céline Tellier is a research fellow of FNRS (National Funds for Scientific Research, Brussels).

Author contributions

V.D. (1st co-author): wrote the main manuscript and performed Figures 7 and 8 and supplementary figures 1 and 2. C.T. (2nd co-author): participated in writing the manuscript and performed the Figures 1–6 and Supplementary Figures 3–5. C.D. (3rd author): took the pictures of Supplementary Figure 1A. M.R. (4th author): supervised the work on the murine B.M.D.M. O.F. (4th author): designed the project and supervised the writing of the manuscript. C.M. (corresponding author): designed the project and supervised the entire work.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-57677-5.

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Supplementary data :

Cycling hypoxia promotes a pro-inflammatory phenotype in macrophages via JNK/p65 signaling pathway

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Supp Table S1

qPCR primers for human genes			
RPS9	F : CTGGATGAGGGCAAGATGAAG R : GTCTGCAGGCGTCTCTCTAAGAA	HLA-DRα	F: CATAAGTGGAGTCCCTGTGCTA R: TCAGGATTCAGATAGAACTCGGC
TNFα	F : CTGCACTTTGGAGTGATCGG R : TCAGCTTGAGGGTTTGCTAC	CD80	F : ACGCCCTGTATAACAGTGTCC R : GAGGAAGTTCCCAGAAGAGGTC
CXCL10	F : AAGTGGCATTCAAGGAGTACC R : ATGCAGGTACAGCGTACAGT	IFIT1	F: CCTCCTTGGGTTCGTCTACA R: TTCTCAAAGTCAGCAGCCAGT
IL-1β	F : GCCCTAAACAGATGAAGTGCTC R : GAGATTCGTAGCTGGATGCC	Fibronectin	F : TGTGGTTGCCTTGCACGAT R : GCTTGTGGGTGTGACCTGAGT
IL-6	F : CCTGAACCTTCCAAAGATGGC R : CACCAGGCAAGTCTCCTCATT	CD206	F : GCTAAACCTACTCATGAATTACTTACAACAA R : GAAGACGGTTTAGAAGGGTCCAT
IL-8	F : TCTGTGTGAAGGTGCAGTTTT R : GGGGTGGAAAGGTTTGGAGTA	CCL22	F : TGTGGTTGCCTTGCACGAT R : GCTTGTGGGTGTGACCTGAGT
PTGS2	F : ATTAGCCTGAATGTGCCATAAGACT R : ACCCACAGTGCTTGACACAGAAT		
qPCR primers for mouse genes			
RPS9	F: GCTGTTGACGCTAGACGAGA R: AGCATTGCCTTCAAACAGACG	iNOS	F: CAATGGCAACATCAGGTCGG R: CGTACCGGATGAGCTGTGAA
TNFα	F: GAACTTCGGGGTGATCGGT R: CTCCTCCACTTGGTGGTTTG	Arg-2	F: GAGACCACAGCCTGGCAATAG R: ATGTCCGCATGAGCATCAAC
CXCL10	F: GAAATCATCCCTGCGAGCCTA R: ATCGTGGCAATGATCTCAACA	MARCO	F: ACTCCAGAGGGAGAGCACTT R: TTGTCCAGCCAGATGTTCCC
IL-1β	F: TGCCACCTTTTGACAGTGATG R: ATGTGCTGCTGCGAGATTTG	CD80	F: ACAGTCGTCGTCATCGTTGT R: CCCGAAGGTAAGGCTGTTGT
IL-6	F: CTCTGCAAGAGACTTCCATCC R: TGAAGTCTCCTCTCCGGACT	IFIT1	F: ACAGCTACCACCTTTACAGCAA R: TGAAGCAGATTCTCCATGACCT
MIP-2	F: CGCCCAGACAGAAGTCATAG R: TCCTCCTTTCCAGGTCAGTTA	Arg-1	F: GTACATTGGCTTGCGAGACG R: TTTCTTCCTTCCCAGCAGGT
KC	F: GCAGACCATGGCTGGGATT R: CCTGAGGGCAACACCTTCAA	MRC-1	F: GGATTGCCCTGAACAGCAAC R: ACTTAAGCTTCGGCTCGTCA
PTGS2	F: AGCAGATGACTGCCCAACTC R: GGGTCAGGGATGAACTCTCTC		

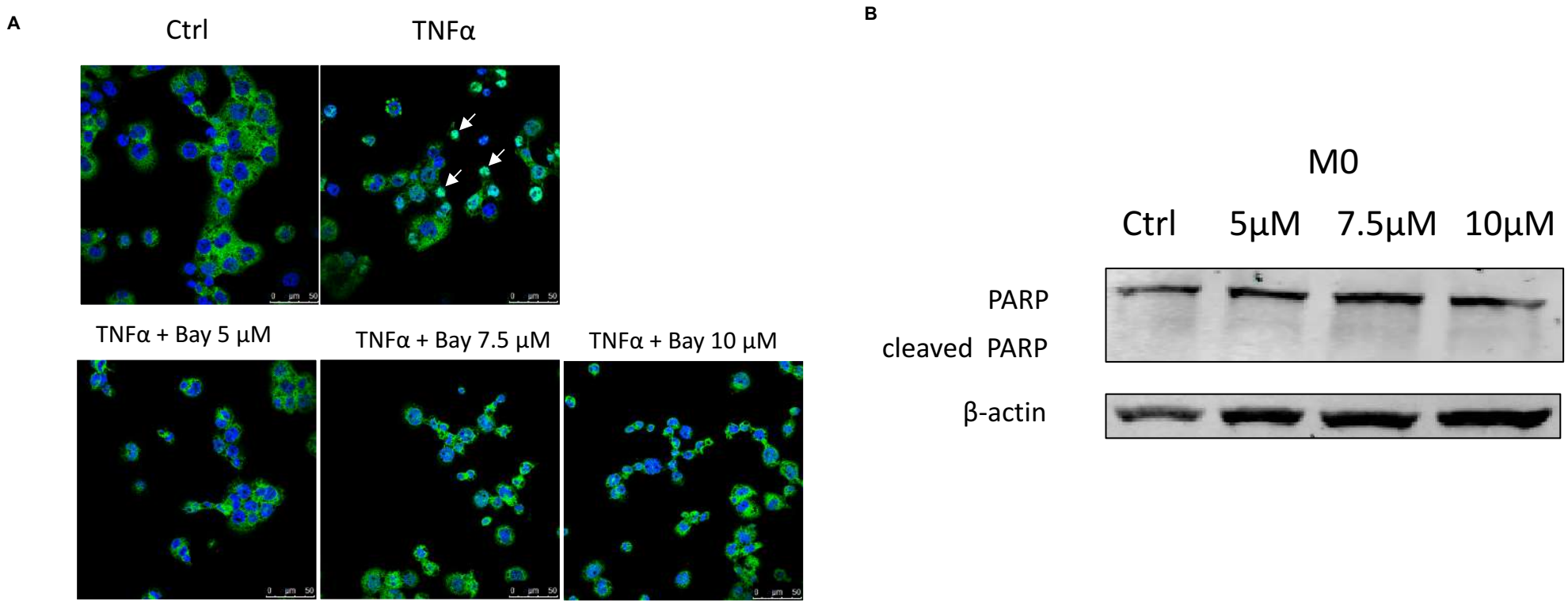
Supplementary Table S1. List of qPCR primers (5'-3')

Supp Table S2

Primary antibodies		
Reference	Incubation time	Dilution
Rabbit mAb anti-phospho-STAT1 (Tyr701) (H+M reactivity), Cell signaling, 58D6, #9167	O/N 4°C	1/1 000
Rabbit mAb anti-phospho-p65 (Ser536) (H+M reactivity), Cell signaling, 93H1, #3033	O/N 4°C	1/1 000
Rabbit polyclonal Ab anti-phospho-c-jun (Ser63) (H+M reactivity), Cell signaling, #9261	O/N 4°C	1/1 000
Mouse mAb anti-IRF5 (EPR17067) (H+M reactivity), Abcam, #ab181553	1 h RT	1/2 000
Mouse mAb anti-PARP (H reactivity), BD Pharmingen, #551024	O/N 4°C	1/2 000
Mouse mAb anti-α-tubulin (H+M reactivity), sigma, #T5168	45 min RT	1/20 000
Mouse mAb anti-β-actin (AC-15), Sigma, #A5441	30 min RT	1/10 000
Secondary antibodies		
Reference	Incubation time	Dilution
Goat anti-mouse IgG IRDye conjugated, LI-COR, Biosciences, #926-69070 (680), #926-32210 (800)	1 h RT	1/10 000
Goat anti-rabbit IgG IRDye conjugated, LI-COR, Biosciences, #926-68071 (680), #926-32211 (800)	1 h RT	1/10 000

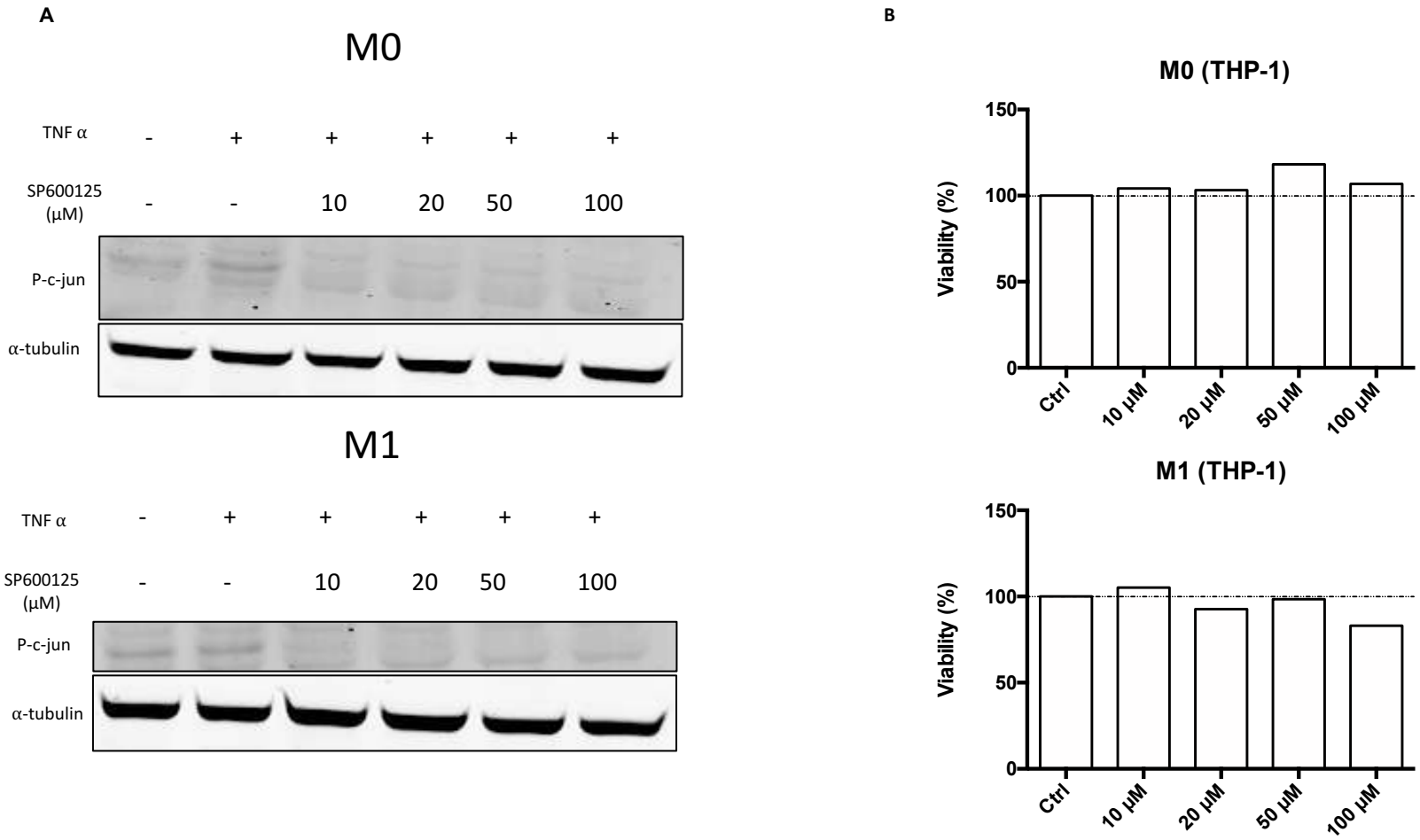
Supplementary Table S2. References of primary and secondary antibodies used for Western blot analyses.

Supp Fig.1



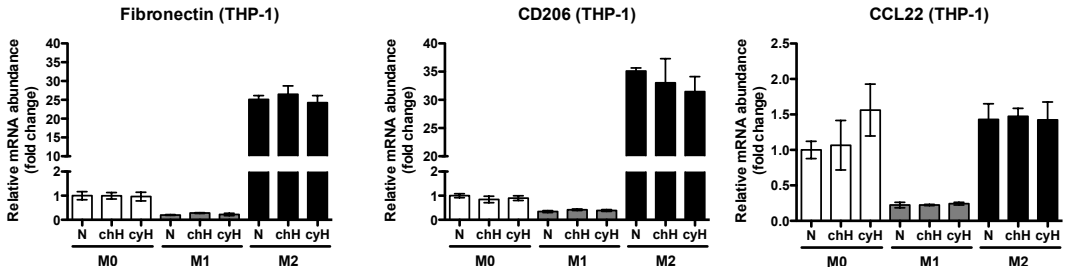
Supplementary Figure 1. Bay11-7082 efficiency and toxicity. (A) THP-1 M0 macrophages were incubated with Bay11-7082 at 5, 7.5 and 10 µM during 1h. Then, cells were treated 30 min with 20 ng/ml of TNFα. Immunofluoresnce staining of p65 was then analyzed to measure the effects of Bay11-7082 on p65 translocation into the nucleus (white arrows ; n=1). (B) THP-1 M0 macrophages were incubated with Bay11-7082 at 5, 7.5 or 10 µM during 7h. Then, the total abundance of PARP and cleaved PARP was analyzed by western blotting (n=1).

Supp Fig.2



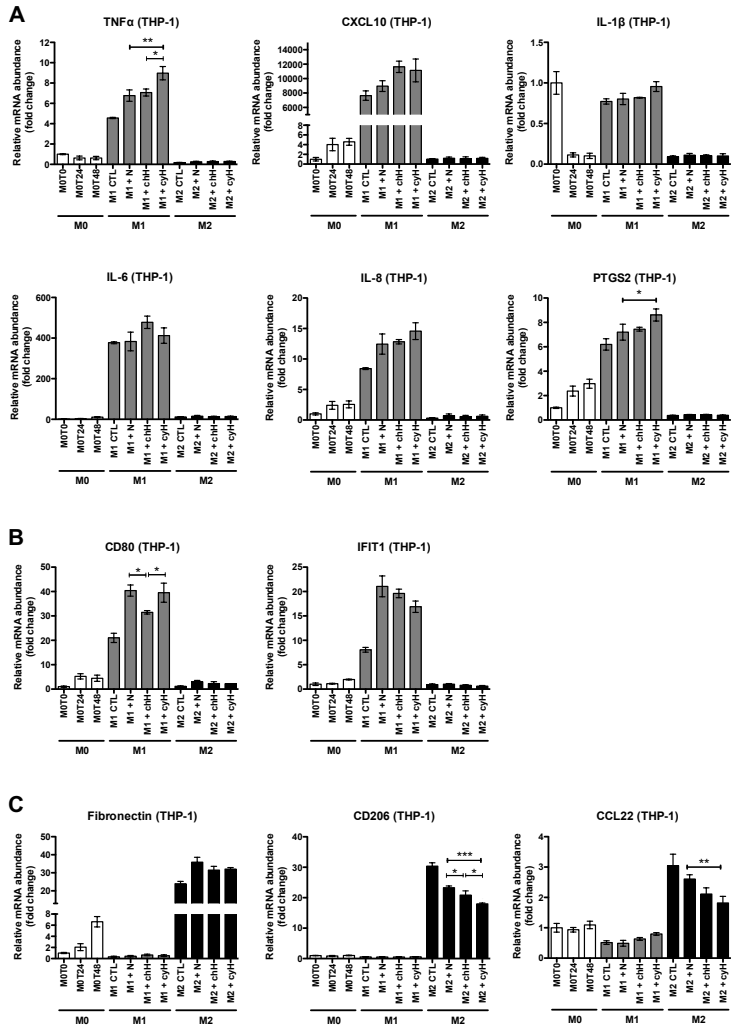
Supplementary Figure 2. SP600125 efficiency and toxicity. (A) THP-1 M0 and M1 macrophages were incubated with SP600125 at 10, 20, 50 or 100 μM during 2h. Then, cells were treated 45 min with 20 ng/ml of TNFα. The abundance of P-c-jun was then analyzed by western blotting to measure the efficiency of SP600125 to inhibit the phosphorylation of c-jun (n=1). (B) THP-1 M0 and M1 macrophages were incubated with SP600125 at 10, 20, 50 or 100 μM during 8h. Then, the viability of cells was analyzed by MTT assay (n=1).

Supp Fig.3



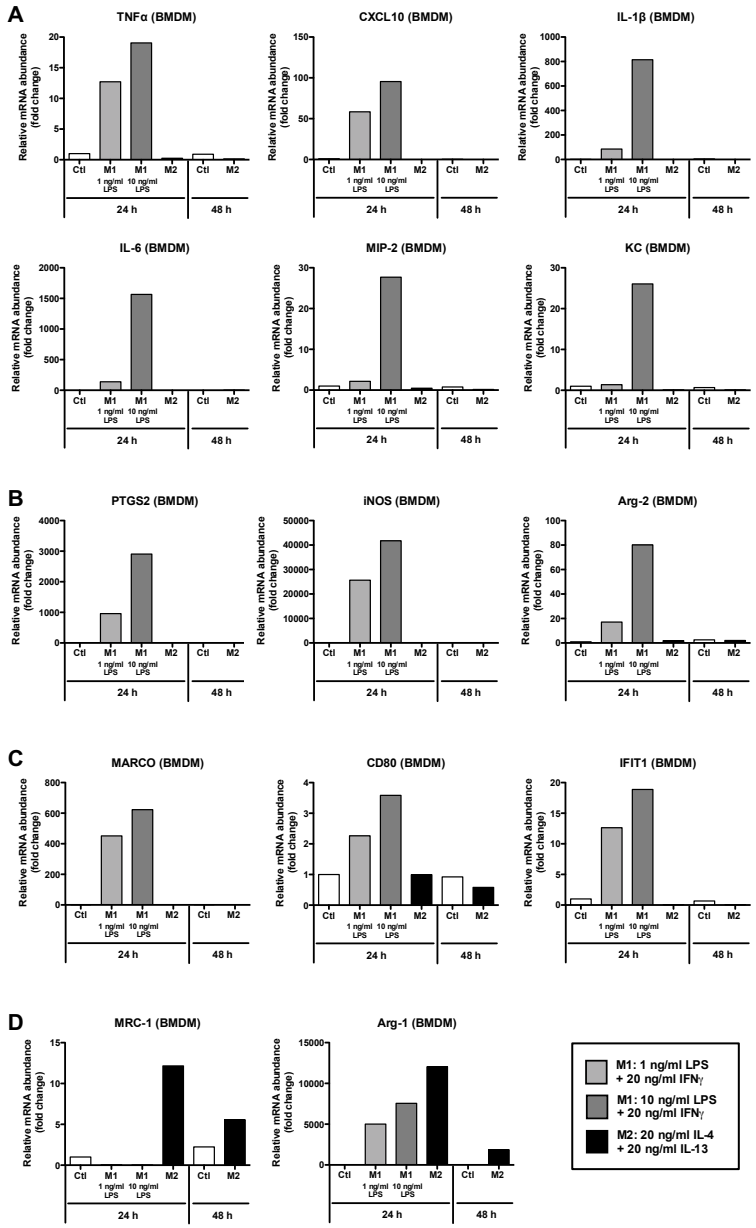
Supplementary Figure 3. Effects of cycling hypoxia on the mRNA expression of M2 markers in human M0, M1 and M2 macrophages. THP-1 M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. mRNA expression of M2 markers was evaluated directly after the incubation by RT-qPCR (n=3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test.

Supp Fig.4



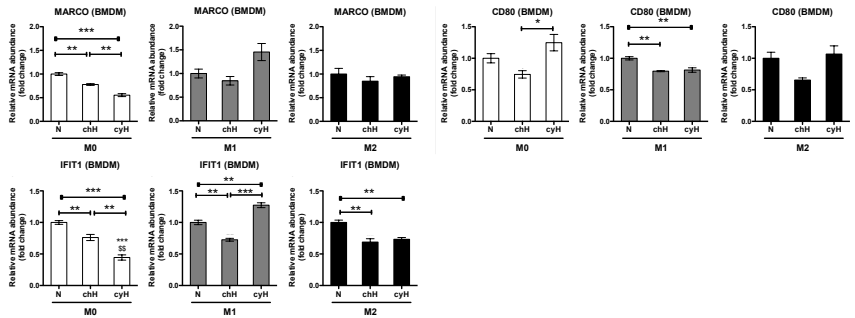
Supplementary figure 4. Modulation of the mRNA expression of M1 and M2 markers by a simultaneous exposure of human macrophages to cycling hypoxia and polarization molecules. THP-1 M0 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h simultaneously with the beginning of M1 or M2 polarization. After the 6 h of co-incubation, cell medium was replaced and M1 or M2 polarization stimulation was continued (+ 18 h for M1 and + 42 h for M2). mRNA expression of M1 markers (A, B) and M2 markers (C) was evaluated after the complete polarization (24 h for M1 and 48 h for M2) by RT-qPCR (n=3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Supp Fig.5



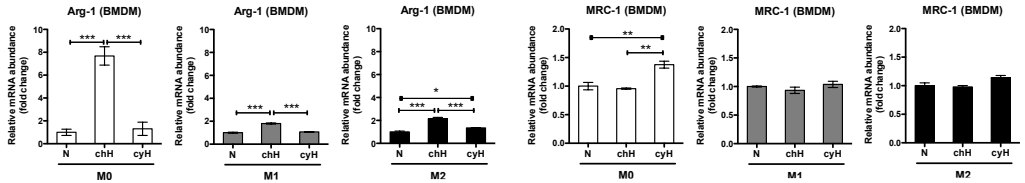
Supplementary Figure 5. Validation of M1 and M2 polarization of murine bone marrow-derived macrophages (BMDM). For M1 polarization, BMDM were incubated with IFNγ (20 ng/ml) and LPS (either 1 or 10 ng/ml) for 24 h. For M2 polarization, BMDM were incubated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) either for 24 h or for 48 h. Control BMDM (Ctl) were kept in the cell culture medium without polarization molecules for 24h or for 48 h. mRNA expression of M1 markers (A, B, C) and M2 markers (D) was evaluated by RT-qPCR (n=1).

Supp Fig.6



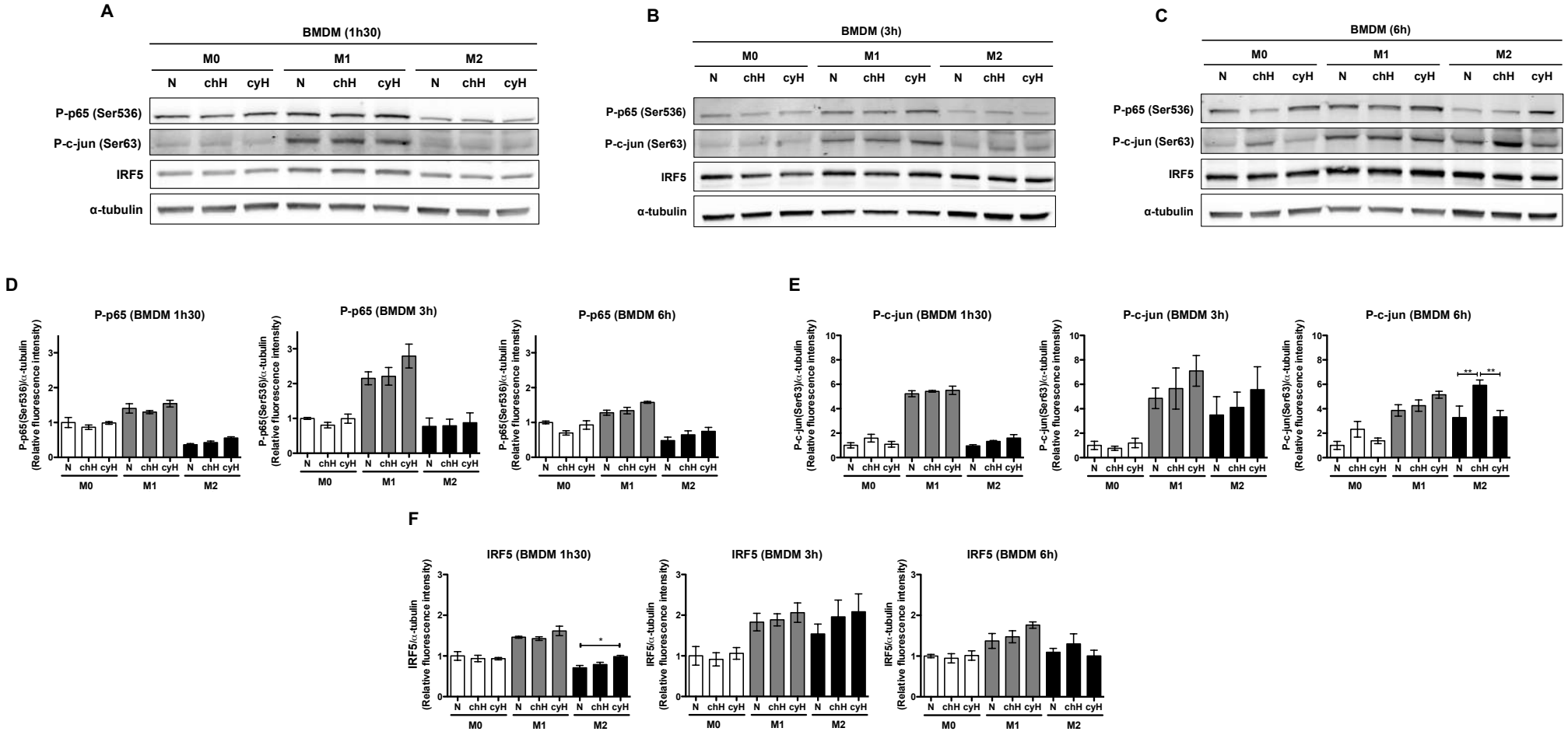
Supplementary Figure 6. Effects of cycling hypoxia on the mRNA expression of intracellular host defense markers in murine M0, M1 and M2 macrophages. M0, M1 and M2 macrophages (BMDM) were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. mRNA expression of intracellular host defense markers was evaluated directly after the incubation by RT-qPCR (n=3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Supp Fig.7



Supplementray Figure 7. Effects of cycling and chronic hypoxia on the mRNA expression of M2 markers in murine M0, M1 and M2 macrophages. M0, M1 and M2 macrophages (BMDM) were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 6 h. mRNA expression of M2 markers was evaluated directly after the incubation by RT-qPCR (n=3, mean ± 1 SEM). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Supp Fig.8



Supplementary Figure 8. Effects of cycling hypoxia on the abundance of the phosphorylated form of p65 and c-jun as well as on the abundance of IRF5 in murine M0, M1 and M2 macrophages. M0, M1 and M2 macrophages (BMDM) were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) for 1h30 (A), for 3 h (B) or for 6 h (C), and then total protein extraction was performed. Abundance of the phosphorylated form of p65 (Ser536) and c-jun (Ser63) as well as the total abundance of IRF5 was detected by western blotting (n=3). α-tubulin was used as loading control. Fluorescence intensity of each immunoblotted protein was quantified and normalized for α-tubulin (D, E, F, G). Statistical analysis was performed by two-way ANOVA and Holm-Sidak test as post hoc test. *, $, #, $P < 0.05$; **, $$, ##, $P < 0.01$

6. Part II

6.1 Effects of cycling hypoxia-exposed macrophages on endothelial cell phenotype

6.1.1 Context

Endothelium is composed of a monolayer of ECs in direct contact with the blood. This is a physical barrier between blood and tissues. ECs regulates several features of the blood vessel such as blood vessel permeability, blood coagulation, vascular tone, angiogenesis, regulate leukocyte infiltration in tumors, leukocyte extravasation during inflammation and cancer cell intravasation and extravasation during cancer cell dissemination (metastasis) (Michiels, 2003; Wettschureck et al., 2019).

In tumors, there are reciprocal interactions between monocytes/macrophages and ECs (Delprat and Michiels, 2021). More particularly TAMs enhance tumor angiogenesis and modify ECs phenotype towards a pro-metastatic one. On the other hand, ECs regulates monocytes/macrophage infiltration in tumors and shift macrophage towards an immunosuppressive M2 phenotype. Interestingly, the infiltration of inflammatory monocyte into secondary tumor site strongly enhances cancer cell seeding (Qian et al., 2011).

ECs phenotype and permeability are strongly involved in tumor metastasis and leukocyte infiltration/extravasation. Several steps are observed during leukocyte extravasation, such as leukocyte rolling, firm adhesion on ECs, followed by extravasation (Vestweber, 2015; Wettschureck et al., 2019). These steps depend on adhesion molecules expression by ECs. ICAM1 and VCAM1 are involved in leukocyte adhesion, whereas E-selectin regulates leukocyte rolling. Interestingly, the expression of these adhesion molecules in ECs also regulates cancer cell adhesion, a step involved in their extravasation, and thereby in their dissemination.

Previously, we have shown that cyH promotes a pro-inflammatory phenotype in M0 and M1 human and murine macrophages (Delprat et al., 2020). This was dependent to JNK/p65 signaling pathway in human THP-1-derived macrophages. Since tumor inflammation is involved in metastasis and that ECs inflammation promotes experimental metastasis, we wanted to investigate the effect of cyH-exposed M0, M1 and M2 macrophages to ECs phenotype. More particularly, we wanted to investigate the effect of macrophages on ECs inflammatory proteins expression and secretion, on ECs adhesion molecule expression and protein abundance. Furthermore, we aim to test if macrophages could modulate the ability of ECs to bind monocytes and cancer cells *in vitro*. The objectives are represented in Fig. 29.

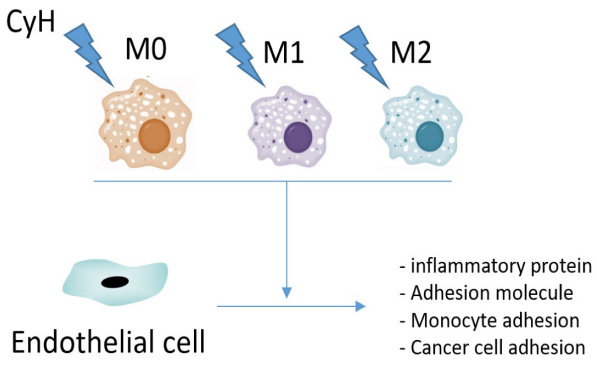


Fig. 29: Schematic representation of the objectives of the present work.

6.1.2 Experimental model

In order to test the impact of macrophages on ECs, THP-1-derived M0, M1 and M2 macrophages were incubated with CO_2 independent medium in normoxia, chH and cyH during 6h, followed by 16h of reoxygenation. Macrophage media were harvested, their pH were adjusted to pH of CO_2 independent medium, and the media were stored either at 4°C or stored at -80°C after rapid-freezing in liquid nitrogen. Thereafter, EA.hy926 cells and HUVECs were incubated with the macrophage media during 24h or 48h, and appropriate experiments were performed (Fig. 30).

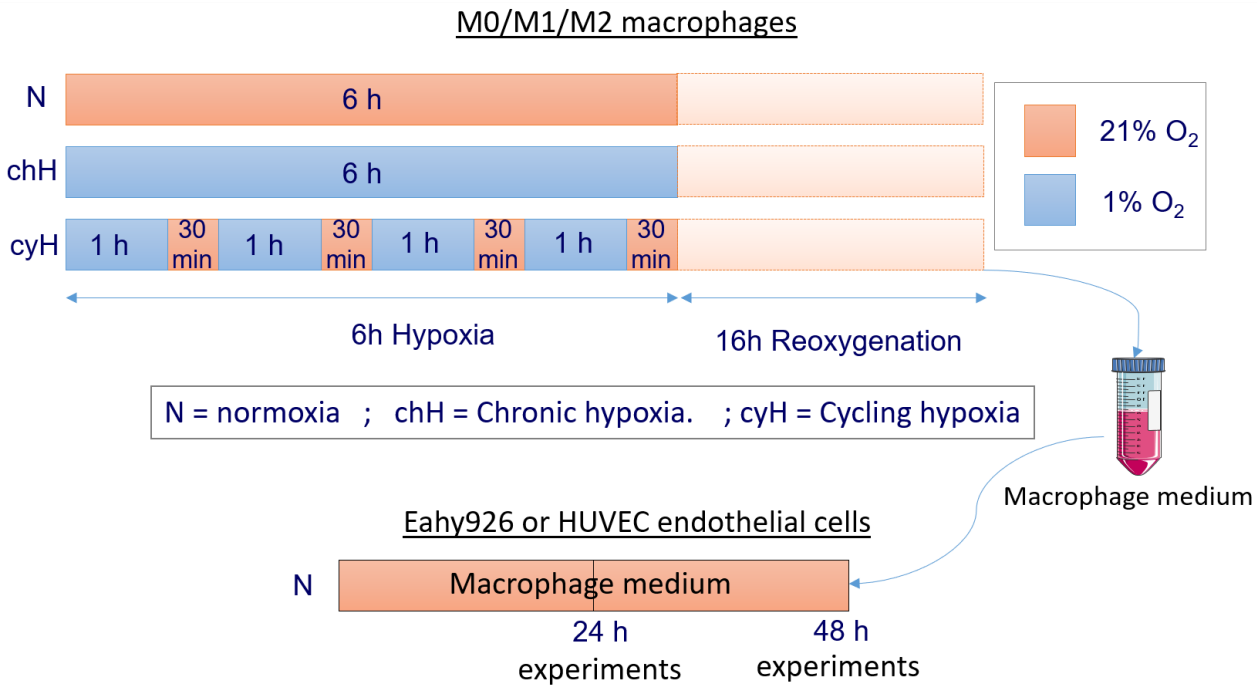


Fig. 30 : Schematic representation of the experimental procedure of the present work

6.1.3 pH adjustement

The pH of macrophage media was significantly lower in M1 and M2 chH media, compared to the respective N and cyH media (Fig. 31). Furthermore, pH of M2 chH medium was lower than the pH of M1 chH medium. M1 macrophages secrete more lactate than M2 macrophages, hence M2 chH medium is lower than M1 chH medium likely independently of lactate release (Caputa et al., 2019).

Tumor acidosis has strong impact in the TME, promotes tumor metastasis and induces immunosuppression (Bohn et al., 2018; Corbet and Feron, 2017; Ibrahim-Hashim and Estrella, 2019). Furthermore, acidosis and hypoxia are both responsible for the promotion of the polarization of macrophage into M2 phenotype. Hence, the effect of chH on the reduction of the pH of M1 and M2 macrophage is very interesting since it could exist a reciprocal loop between hypoxia and acidosis in the induction of M2 polarization. Nonetheless, the effect of chH on the pH of macrophage is, to our knowledge, not observed *in vivo*. More particularly, it is unknown if macrophages alone can be responsible for tumor acidosis. Several studies showed that pH, and more particularly acidic pH alters ECs phenotype (Dong et al., 2013; Thews and Riemann, 2019). In our experiments, M2 chH slightly induced eNOS expression by

ECs (vs N or cyH). Adjustement of pH prevented this effect (data not shown). For these reasons and in order to avoid to study the impact of pH modulation by macrophages (which could be an *in vitro* artefact), the pH of macrophage media was adjusted prior to incubation with ECs.

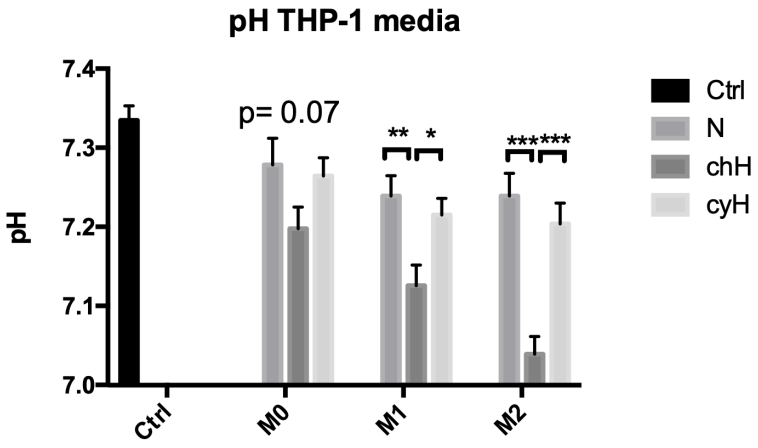


Fig. 31: pH of THP-1-derived macrophage conditioned media.

6.1.4 EA.hy926 cell line and HUVECs

EA.hy926 are immortalized EC cell line. The fusion of HUVEC with lung cancer cell A549 allowed the generation of EA.hy926 in 1983 (Bouis et al., 2001). These cells are responsive to TNFα since TNFα treatment increases EA.hy926 ICAM1, VCAM1 and E-selectin expression and monocyte adhesion. This cell line is often used for leukocyte adhesion assay. Nonetheless, their expression of E-selectin and VCAM1 are very low (Bouis et al., 2001). Hence, the difference of adhesion ability into TNFα-treated vs basal EA.hy926 cells is strongly lower than in primary endothelial cells such as HUVECs.

HUVECs are ECs isolated from human umbilical vein. These cells were firstly isolated in 1963 by Maruyama et al. (Maruyama et al., 1963). This is a very good model to study endothelial cell biology. These primary cells are now routinely isolated and commercialized. HUVEC used in this work were purchased from LONZA (CC-2519) and used between passages 2 and 5.

6.1.5 EA.hy926 cell and HUVEC incubation with macrophage medium

In order to perform adhesion test, it is needed that ECs form a confluent monolayer.

EA.hy926 cells were incubated with 100% of macrophage medium, during 24h or 48h. In this conditions, EA.hy926 cells were able to grow until a confluent monolayer (data not shown).

In these conditions, HUVEC were unable to form confluent monolayer, even after only 22h incubation (Fig. 32). Hence, we incubated cells with 75% macrophage medium + 25% EGM-2 (HUVEC culture medium). At this concentration, HUVEC formed a confluent monolayer, even after 48h incubation (Fig. 32).

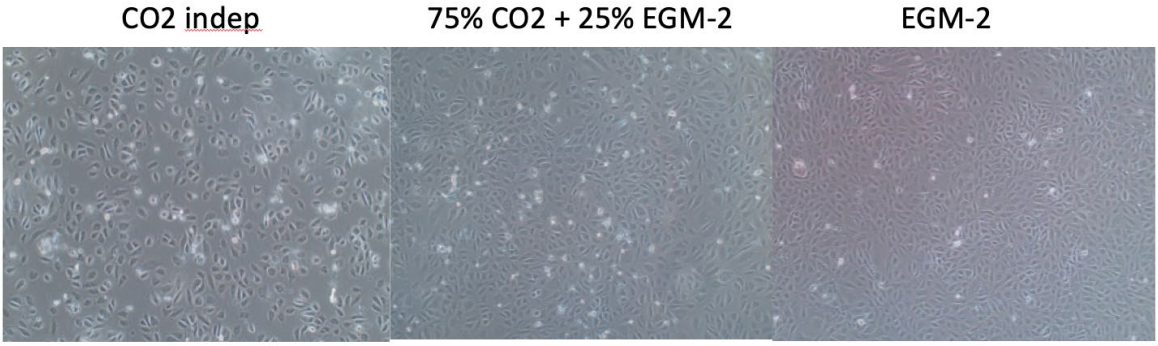


Fig. 32: Pictures of HUVECs after 48h incubation with CO_2 independent medium, 75% CO_2 independent medium + 25%EGM-2, and EGM-2.

Furthermore, the impact of fibronectin coating on HUVEC responsiveness to TNFα was assessed. The idea was to choose the concentration of coating which shows the largest difference between non-activated HUVECs (no TNFα) and activated HUVECs (TNFα) in adhesion molecule and pro-inflammatory markers expression, in order to likely maximize the effect of macrophage media on HUVECs. Coating of the wells with 1 μg/cm^2 fibronectin enhanced the effect of TNFα on HUVEC E-selectin expression (Fig. 33A). This effect was similar when other markers were assessed. Hence, we coated the wells with 1 μg/cm^2 fibronectin before HUVEC seeding.

The impact of the addition of serum to the level of EGM-2 medium (2%) to the expression of adhesion molecule expression and pro-inflammatory gene expression was assessed. The addition of serum decreased the basal expression (without TNFα stimulation) of adhesion molecule and pro-inflammatory proteins expression (Fig. 33B). This effect was similar when other markers were assessed.

Overall, we chose to coat the well with 1 μg/cm^2 fibronectin, to add serum in order to reach 2% in macrophage media, and to incubate HUVECs with 75% macrophage media + 25% EGM-2.

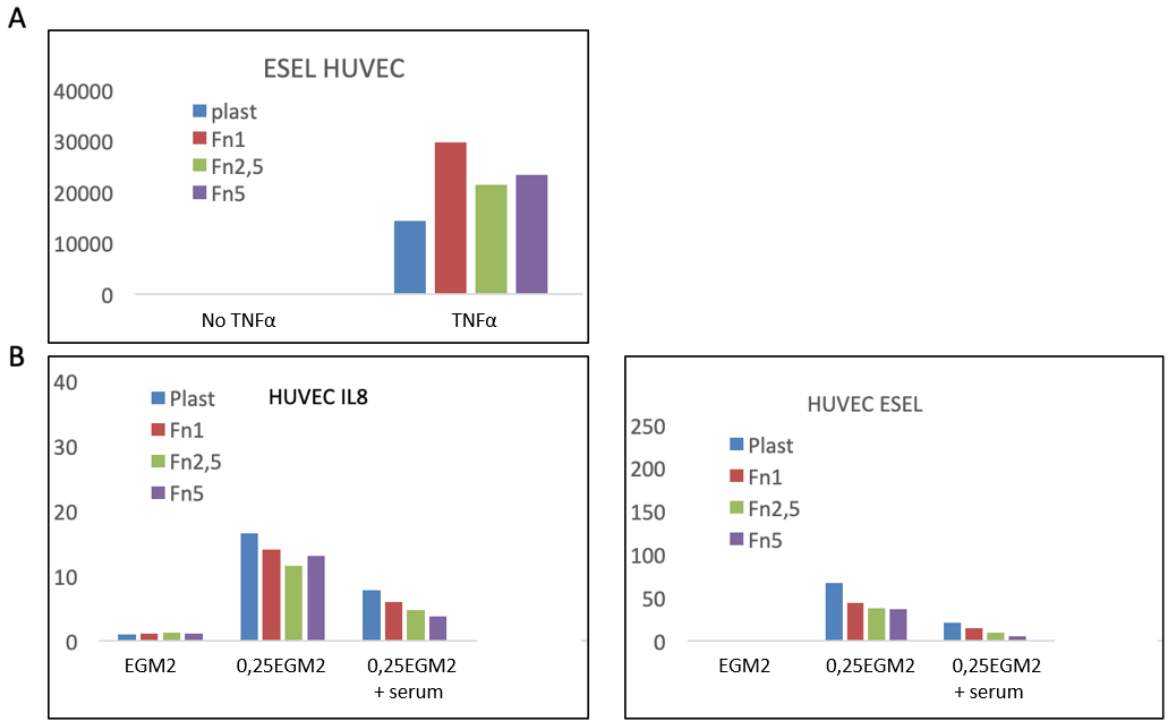


Fig. 33 : Effect of fibronectin coating on HUVEC responsiveness to TNFα and of the addition of serum on basal expression of adhesion molecule and pro-inflammatory protein expression. A) the addition of fibronectin enhanced the responsiveness of HUVECs to TNα. This was more pronounced with 1 μg/cm^2 fibronectin. B) The addition of serum in 75% CO_2 independent medium + 25% EGM-2 decreased the basal expression of IL8 and E-selectin (and other markers).

6.1.6 Discussion about the model used

In this work, M0, M1 and M2 macrophages were incubated 6h in N, chH and cyH, followed by 16h reoxygenation. Then, macrophage media were harvested and pH adjustement was performed. Then, EA.hy926 cells and HUVEC were incubated 24h or 48h with macrophage media. Some limitations of the present model should be discussed. The fact that macrophages are reoxygenated in N (and not in chH or cyH) has already been discussed in section 5.1.3.

First, it is well described that hypoxia strongly modulates macrophage metabolism and hence modulates nutrient consumption by macrophages as well as their metabolism. The global composition of CO_2-independent medium is unknown, but this is known that it contains gluccose and glutamine since we directly put these nutrients in the medium. Glucose uptake and glycolysis are enhanced by hypoxia in macrophages (Roiniotis et al., 2009), and glutamine uptake is enhanced in several cell types by hypoxia (Yoo et al., 2020). Interestingly, an increase in glucose uptake by TAMs has been shown to induce vascular modification (notably vascular normalization) both *in vitro* and *in vivo* (Wenes et al., 2016). On the other hand, little is known about the effect of cyH on cellular metabolism (Bader et al., 2020). To our knowledge, only one study showed that cyH increases the expression of glucose metabolism genes in breast cancer cells (Jarrar et al., 2020). Furthermore, the difference between chH and cyH in cell metabolism has, to our knowledge, not yet been investigated. In conclusion, the effects of

macrophage media on ECs are likely due to secreted molecule by macrophages, but some effects could be due to the diminution of some nutrients in the media of chH and cyH-exposed macrophages.

Secondly, cancer cells are absent in our model. Cancer cells and TME have strong impact on the macrophage and EC phenotype. Cancer cells promote macrophage M2 polarization and immunosuppressive phenotype, and TME is responsible for the induction of EC anergy. EC anergy corresponds to the unresponsiveness of EC to pro-inflammatory cytokines such as TNFα. Hence, first, it is very likely that, since cancer cells modulate macrophage phenotype, the composition of macrophage media (and hence their effects on ECswould be strongly different in the presence of cancer cells. Secondly, tumor ECs are strongly different from healthy tissue blood vessel ECs, and it is possible that tumor ECs would differently respond than the ECs used in the present work. It is therefore possible that the impact of macrophage media observed in our model, notably in terms of EC activation, could be different in tumors. In order to confirm the effect of macrophage on ECs observed in this work *in vivo*, it would be interesting to study if it exists correlation between cyH markers and EC adhesion molecule, and between macrophage markers and EC adhesion molecule expression in human cancers.

6.2 Submitted work : “The impact of macrophages on endothelial cells is potentiated by cycling hypoxia : enhanced tumor inflammation and metastasis

The impact of macrophages on endothelial cells is potentiated by cycling hypoxia : enhanced tumor inflammation and metastasis

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Research article : Tumor immunology and Microenvironment.

Abstract

In cancers, the interaction between tumor-associated macrophages and endothelial cells (ECs) regulates tumor inflammation and metastasis. Due to temporary occlusions in tumor blood vessels leading to altered blood flow, these cells are both affected by cycling hypoxia (cyH), also called intermittent hypoxia, a feature of the tumor microenvironment. cyH is also known to favor tumor inflammation and metastasis. Nonetheless, the potential impact of cyH on the dialog between macrophages and ECs is still unknown. In this work, the effects of M0, M1 and M2 macrophages exposed to normoxia, chronic hypoxia (chH) and cyH on endothelial adhesion molecule expression, pro-inflammatory gene expression and on EC adhesiveness for monocytes and cancer cells was investigated. M1 macrophages were the most potent and M2 macrophages the least potent inducers of adhesion molecule expression and pro-inflammatory gene expression in ECs. CyH increased the ability of M0 and M1 macrophages to induce EC inflammation and expression of the EC endothelial adhesion molecule ICAM1, respectively. M0, M1 and M2 macrophages were all able to promote EC adhesive properties toward cancer cells. Furthermore, the ability of macrophages (mostly M1) to shift EC phenotype towards one allowing cancer cell and monocyte adhesion onto ECs was potentiated by cyH. These effects were specific to cyH since they were not observed with chH. Altogether, these results show that cyH amplifies the effects of macrophages on ECs which may promote tumor inflammation and metastasis.

Keywords: cycling hypoxia, macrophages, endothelial cells, cancer cells, cancer.

What's new ?

Cycling hypoxia (cyH), neo-angiogenesis and tumor-associated macrophages are key features of the tumor microenvironment. In this study, we demonstrate that cyH potentiates the induction by M0 and M1 macrophages of endothelial inflammatory phenotype and adhesiveness for monocytes and cancer

cells. This process triggers a positive feedback loop sustaining tumor inflammation. This work opens the door for innovative therapeutic strategies in order to treat tumor inflammation and metastasis.

Abbreviations

chH, chronic hypoxia; cyH, cycling hypoxia; ECs, endothelial cells; ESM1, endothelial-cell specific molecule 1; HUVEC, human umbilical vein endothelial cells; ICAM1, intercellular adhesion molecule 1; MMP9, matrix metalloproteinase 9; N, normoxia; N-cadherin, neuronal cadherin; TAMs, tumor-associated macrophages; TME, tumor microenvironment ; VCAM1, vascular cell adhesion molecule 1.

1. Introduction

The tumor microenvironment (TME) is composed of cancer cells and stromal cells (*e.g* fibroblasts, endothelial and immune cells) which are exposed to several physicochemical stresses. These stromal cells are strongly involved in the modulation of tumor growth, metastasis and tumor inflammation[1-3]. One of the most important physicochemical feature which modifies the TME is the reduction of oxygenation, a phenomenon called hypoxia. In tumors, two types of hypoxia occur: cycling hypoxia (cyH) and chronic hypoxia (chH)[4]. ChH is the result of uncontrolled proliferation of cancer cells which leads to some cells being too far from blood vessels to be oxygenated. CyH, also called acute, intermittent or cyclic hypoxia, is due to intermittent erythrocyte flow into the tumor blood vessels which leads to periods of hypoxia followed by periods of reoxygenation. Another cause of cyH in cancer is sleep apnea in which periods of hypoxia/reoxygenation are shorter and more numerous. Previously in the lab, we showed that cyH induces endothelial cell activation via NF-κB pathway activation, hence promoting tumor inflammation[5]. Accordingly, we showed that cyH favors inflammatory phenotype in M0 and M1 macrophages, which could be one explanation of cyH-induced tumor inflammation[6]. Furthermore, it is well known that cyH promotes metastasis[4, 7]. Several reports showed that the exposure of mice carrying spontaneous or induced tumors to cyH increased the metastatic spread of cancer cells[7]. Both metastases and tumor inflammation are hallmarks of cancer[8, 9]. Metastasis is involved in most of the cancer-related deaths and hence it is very important to understand this process. Inflammation is a cause of genetical instability, but it also enhances angiogenesis, cancer cell proliferation and survival as well as metastasis[9, 10]. Hence, metastasis and inflammation are linked to poor disease outcome.

Stromal cells are involved in the promotion of metastasis and inflammation. Among these cells, tumor-associated macrophages (TAMs) and endothelial cells (ECs) are strongly involved in these processes[11-15]. In tumors, macrophages originate mostly from blood monocytes which infiltrate the tumors and then differentiate in macrophages. Macrophages are usually classified according to the M1 and M2 polarization axis. M1 macrophages are pro-inflammatory and induced by IFNγ and/or LPS stimulation, whereas M2 macrophages are anti-inflammatory and induced notably by IL-4 and/or IL-13 stimulation[16]. The endothelium is composed of an EC monolayer which lines the inner side of blood vessels in direct contact with the circulation. ECs play essential roles in the exchanges of gas and metabolites between blood and tissues, in the regulation of vascular dilation, in thrombosis, in angiogenesis, and in the immune response[13, 17]. Blood vessels are essential to the growth of most solid tumors since they sustain a constant supply of nutriments and oxygen to the tumors[18]. Furthermore, ECs are strongly involved in tumor metastasis. Indeed, the metastasis process corresponds to the spread of cancer cells from a primary tumor site into a secondary site. This process is composed of several steps and ECs participate in some of them such as cancer cell migration towards blood vessels, intravasation and extravasation[14, 19, 20]. In tumors, ECs and macrophages strongly interact with each

other in a bi-directional dialog which influences the tumor progression[15]. Indeed, TAMs favor angiogenesis, lymphangiogenesis and shift EC phenotype towards one promoting cancer cell intravasation and extravasation[15, 21, 22]. On the other hand, ECs are critically involved in the regulation of monocyte/macrophage infiltration in the tumor and infiltrated monocytes/macrophages enhance cancer cell extravasation and seeding as well as regulates tumor inflammation[12, 13, 15]. At the molecular level, the transmigration of immune cells through the endothelium is well described and depends on a complex set of interactions between leukocyte and EC membrane receptors[23, 24]. In case of endothelial activation, ECs strongly express leukocyte adhesion receptors at their luminal face, such as E-selectin, intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1). These receptors favor the rolling, arrest, firm adhesion, and extravasation of immune cells from the circulation toward the tissues[23]. On the other hand, cancer cell binding onto endothelium is less well described. ICAM1, E-selectin, neuronal cadherin (N-cadherin) and integrins are among the best characterized molecules of the ECs involved in cancer cell adhesion onto endothelium[14, 20, 25-27].

The effect of cyH on the effects of macrophages on ECs is still unknown. Since cyH, macrophages and ECs are involved in tumor metastasis and inflammation, it is very important to investigate the effect of cyH on the interaction between macrophages and ECs. In this work, the impact of M0, M1 and M2 macrophages exposed to cyH on EA.hy926 cell (EC lineage) and on primary human umbilical vein endothelial cell (HUVEC) phenotype was studied. Results showed that the adhesion of monocytes onto ECs incubated with M1 cyH medium was strongly enhanced compared to ECs incubated with M1 N and M1 chH media. Furthermore, macrophages exposed to cyH (i) increased pro-inflammatory cytokine and protein expression in EA.hy926 cells (ii) increased the mRNA expression and the protein abundance of ICAM1 in ECs compared to macrophages exposed to N or chH. The adhesion of breast cancer cells onto ECs was enhanced by M0, M1 and M2 macrophages and the effect of M1 macrophages was potentiated by cyH. Altogether, these results show that cyH initiates an amplification positive feedback loop between TAMs and ECs to maintain tumor inflammation and metastasis.

2. Materials and methods

2.1 Cell culture

THP-1 monocytes (ATCC ; TIB-202 ; RRID:CVCL_0006) were cultured in RPMI 1640 medium (21875, Gibco), supplemented with 10% heat-inactivated fetal bovine serum (HIS), 10 mM HEPES, 1 mM pyruvate, 2,5 g/L glucose and 50 μM β-mercaptoethanol. EA.hy926 cells (RRID:CVCL_3901) and primary human umbilical vein endothelial cells (HUVEC) were from ATCC (CRL-2922) and from LONZA (CC-2519), respectively. EA.hy926 endothelial cells were cultivated in DHGL-1 medium (DMEM high glucose, low $NaHCO_3$, without sodium pyruvate) supplemented with 10% fetal bovine serum. HUVEC were grown in EGM-2 medium (LONZA), until passage 7. MDA-MB-231 breast cancer cells (ATCC ; HTB-26 ; RRID:CVCL_0062) were cultivated in DMEM high glucose (Gibco), 10% HIS, 0.5 mM glutamine.

All cell lines have been authenticated using short tandem repeat profiling within the last three years.. All experiments were performed with mycoplasma-free cells.

2.2 Macrophage-derived conditioned-media

The protocol used to differentiate and polarize human THP-1 monocytes into M0, M1 and M2 macrophages was setup in[16] and used as in[6].

M0, M1 and M2 macrophages were then exposed to normoxia, chronic hypoxia and cycling hypoxia during 6h, and were then left for 16h in normoxic air (21% O_2). Chronic hypoxia incubation

corresponded to 6h of 1% O_2, whereas cycling hypoxia corresponded to 4 cycles of 1h 1% O_2 and 30 min 21% O_2. The incubation of cells in hypoxia was performed as in[6]. During this 22h (6h + 16h) incubation period, macrophages were incubated in CO_2 independent medium (18015, Gibco) supplemented with 4 mM glutamine (G8540, Sigma) and 3.75 g/L glucose (6877, Roth). After incubation, conditioned-media were harvested and the pH of each medium was adjusted to that of CO_2 independent medium pH ± 0.02. Conditioned-media were either stored at 4°C for a maximum period of 3 days and used for experiments, or were frozen in liquid nitrogen (snap-freeze) and stored at -70 °C and were used afterwards for experiments. The media which were stored at 4°C no more than 3 days or which were snap-frozen and stored at -70°C showed the same biological effects (data not shown).

2.3 Incubation of endothelial cells with macrophage conditioned-media

EA.hy926 cells were incubated with 100% of macrophage conditioned-media, during the corresponding incubation time. Before seeding HUVEC, plates were coated with 1 µg/mL fibronectin (1030-FN, R&D systems) during 45 min at 37°C. Fibronectin was removed and plates were rinsed with PBS. Then, HUVEC were seeded, and incubated 24h at 37°C and 5% CO_2 prior to incubation with 23.5% EGM-2, 1.5% HIS and 75% macrophage conditioned-media, during the corresponding incubation time.

2.4 Immunofluorescence labelling

EA.hy926 cells and HUVEC were seeded in 24-well plates at 7 500 and 25 000 cells/well, respectively. Endothelial cells (ECs) were incubated 24h at 37°C and 5% CO_2 prior to incubation 48h in 1 mL of control medium or macrophage conditioned-media as indicated in section 2.3. As a positive control, cells were incubated 16h with 1 ng/mL TNFα (210-TA, R&D systems). After incubation, ECs were fixed 15 min in 400 µL of 4% paraformaldehyde in PBS. Cells were washed 3 times in PBS and were blocked in PBS containing 2% of bovine serum albumin (BSA) during 30 min. Then, cells were incubated O/N at 4 °C with anti-ICAM1 primary antibody (diluted 1 : 60 in PBS BSA 2% ; #BBA3 ; R&D systems). Cells were washed 30 min in PBS BSA 2% 30 min, and were incubated with secondary antibody (Alexa Fluor 488-conjugated anti-rabbit IgG antibody; diluted 1 : 1000 in PBS BSA 2% ; #A1103; Molecular Probes). Cells were then incubated with DAPI (diluted 1 : 2500 in PBS ; 10236276001, Sigma) in order to stain the nucleus. The coverslips were mounted in Mowiol (Sigma) and pictures taken with a confocal microscope (SP5, Leica). Pictures were analyzed using image J software.

2.5 MTT assay

EA.hy926 cells and HUVEC were seeded in 24-well plates at 50 000 and 25 000 cells/well, respectively. ECs were then incubated 24h at 37°C and 5% CO_2 prior to incubation 48h in 500 µL of control medium or macrophage conditioned-media as indicated in section 2.3. Then, 500 µL of 2.5 mg/mL of MTT solution (M2128, Sigma) in PBS were added and cells were incubated 2h at 37°C and 5% CO_2. Media were removed and cells were lysed in 1 mL DMSO (A994, Roth) and under slight agitation, during 1h. Afterwards, absorbance was read at 570 nm.

2.6 ELISA

EA.hy926 cells and HUVEC were seeded in 24-well plates at 50 000 and 25 000 cells/well, respectively. ECs were then incubated 24h at 37°C and 5% CO_2 prior to incubation in 1 mL of control medium or macrophage conditioned-media as indicated in section 2.3. In parallel, 1 mL of each corresponding conditioned-medium was incubated at 37°C during the same period (24h). Supernatants were harvested and centrifuged at 200g during 5 min at 4°C. Supernatants were stored at -70°C and used afterwards for cytokine quantification. In parallel, ECs were lysed with 200 µL of NaOH 0.5 N and frozen at - 20°C. The concentration of IL-6 and IL-8 in supernatants was analyzed using human quantikine

ELISA kits (R&D systems) which were used according to manufacturer indications. The concentration of ESM1 protein in supernatant was analyzed using human ESM1 ELISA kit (ab213776, abcam) and used according to manufacturer indications. In order to quantify only the protein secreted by ECs, the proteins contained in the macrophage supernatant were subtracted from each corresponding EC supernatant. Cytokine concentrations (pg/mL) were normalized by total protein concentration (µg) contained in ECs and macrophages determined by the Folin Method after 200 µL (ECs) or 1 mL (macrophages) NaOH 0.5 N lysis.

2.7 Western Blot

EA.hy926 cells were seeded in 25cm^2 flasks at 5000 cells/cm^2. EA.hy926 cells were then incubated 24h at 37°C and 5% CO_2 prior to incubation in 5 mL of control media or macrophage conditioned-media. Then, cells were lysed and western blots for ICAM1 were performed as in[5]. Antibodies used are listed in supplementary table S1.

2.8 RT-qPCR

EA.hy926 cells and HUVEC were respectively seeded in 6-well plates and 12-well plates at 250 000 and 30 000 cells/well, respectively. ECs were then incubated 24h at 37°C and 5% CO_2 prior to incubation 24h in 2 mL or 1 mL of control medium or macrophage conditioned-media as indicated in section 2.3. Total RNA extraction was performed using ReliaPrep RNA Tissue Miniprep Systems (Z6111, Promega) according to manufacturer's instruction. Total RNA extract was quantified with nanophotometer (N60, IMPLEN) and 2 µg of RNA was reverse transcribed using GoScript Reverse Transcription Mix, Oligo(dT) (#A2791, Promega). qPCR was performed as in[6]. RPL13A and β-2-microglobulin genes were used for normalization in EA.hy926 cells and HUVEC samples, respectively.

2.9 THP-1 monocyte adhesion onto endothelial cells

EA.hy926 cells or HUVEC were grown to confluence in 96-well plates and 24-well plates, respectively. ECs were incubated with 1 mL or 200 µL of control medium or macrophage conditioned-media as indicated in section 2.3, respectively. THP-1 monocytes were rinsed in RPMI without phenol red (11835, Gibco) and were labelled with 5 µM calcein-AM at a concentration of 5.10^6 cells/mL, during 30 min at 37°C and 5% CO_2. EA.hy926 cells and HUVEC were rinsed with RPMI and with EGM-2, respectively. TNFα at 1 ng/mL during 6h was used as positive control. THP-1 monocytes were then seeded and incubated 1h with EA.hy926 cells or HUVEC, at a density of 50 000 cells/well for EA.hy926 cells or of 100 000 cells/well for HUVEC. Directly after THP-1 seeding, plates were read with fluorimeter (485 nm excitation; 520 nm emission) to ensure equal seeding between each well (INPUT). After 1h, cells were washed with RPMI and then read with fluorimeter (485 nm excitation; 520 nm emission). Each well was normalized according to INPUT and data were presented as % of adherent THP-1 monocytes.

2.10 MDA-MB-231 breast cancer cell adhesion onto HUVEC

HUVEC were grown to confluence in 24-well plates. HUVEC were incubated with 1 mL of control medium or macrophage conditioned-media as indicated in section 2.3. MDA-MB-231 cells were rinsed in RPMI without phenol red and were labelled with 5 µM calcein-AM (V13181, ThermoFisher) at a concentration of 5.10^6 cells/mL, during 30 min at 37°C. HUVEC were rinsed with EGM-2 and MDA-MB-231 cells were then seeded and incubated 1h on HUVEC, at a density of 40 000 cells/well. Directly after MDA-MB-231 cell seeding, plates were read with fluorimeter (485 nm excitation; 520 nm emission) to ensure equal seeding between each well (INPUT). After 1h, cells were washed with RPMI and then read with fluorimeter (485 nm excitation; 520 nm emission). Each well was normalized according to INPUT and data were presented as % of adherent MDA-MB-231 cells.

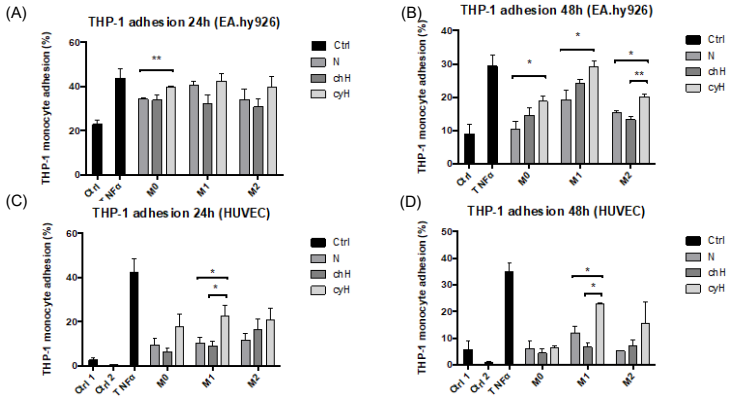

Fig. 1. Adhesion of THP-1 monocytes on EA.hy926 cells or HUVEC incubated with macrophage-conditoned media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Confluent monolayer of EA.hy926 cells **(A and B)** and HUVECs **(C and D)** were incubated 24h **(A and C)** or 48h **(B and D)** with macrophage conditioned-media. Thereafter, the adhesion of THP-1 monocytes **(A, B, C and D)** on endothelial cells was assessed by adhesion assay (n = 3, mean ± 1 SEM). Ctrl 1 and Ctrl 2 correspond to endothelial cells incubated with CO_2 independent medium or EGM-2 medium, respectively. Incubation of endothelial cells with 1ng/mL TNFα during 6h was used as positive control. Statistical analysis was performed using Student's t test. *p < 0.05 ; **p < 0.01.

2.11 Statistical analysis

Data are reported as mean ± 1 SEM. Statistical analyses were performed using SigmaPlot Software. Corresponding statistical tests are outlined in figure legends.

3. Results

In this work, we investigated the impact of THP-1-derived M0, M1 and M2 macrophages exposed to cyH on EA.hy926 cells and HUVEC endothelial cells, respectively. The experiments were performed initially on the EA.hy926 cell line and confirmed on primary HUVEC. To this purpose, THP-1 monocytes were differentiated and polarized in M0, M1 and M2 macrophages as in[6]. In order to produce macrophage conditioned-media, M0, M1 and M2 macrophages were exposed to N, chH and cyH during 6h and were left for 16h in N. Macrophage conditioned-media were harvested and ECs were incubated in these media during the indicated times. Effects of macrophages exposed to cyH on ECs were systematically compared to the effects of macrophages exposed to chH.

3.1 Cycling hypoxia amplifies the effect of macrophages on EC adhesiveness for monocytes

First, we tested if macrophage media were toxic to endothelial cells by assessing cell viability using a MTT assay. No effects of conditioned media on cell viability were observed when incubating EA.hy926 cells or HUVEC with macrophage media for 48h (Supplementary Fig. 1).

Previously, we showed that cyH amplified the pro-inflammatory phenotype of M0 and M1 macrophages[6]. Therefore, we tested the functional impact of the incubation of EA.hy926 cells and HUVEC with cyH-exposed macrophage media on undifferentiated THP-1 monocyte adhesion onto the endothelium (Fig. 1 and Supplementary Fig. 2). To this purpose, EA.hy926 cells and HUVEC were incubated 24h or 48h with the media of M0, M1 and M2 macrophages exposed to N, chH and cyH. Afterwards, the adhesion of THP-1 monocytes onto ECs monolayer was assessed. The adhesion of THP-1 monocytes onto EA.hy926 cells was slightly and significantly increased when EA.hy926 cells were incubated 24h with M0 cyH medium compared to M0 N medium (Fig. 1A). After 48h incubation, THP-1 monocyte adhesion onto EA.hy926 was increased when treated with M0 cyH, M1 cyH and M2 cyH media compared to the respective normoxic media (Fig. 1B). The adhesion of THP-1 monocytes onto HUVEC was significantly increased in HUVEC incubated 24h and 48h with M1 cyH medium compared to HUVEC incubated 24h and 48h with M1 N and M1 chH media (Fig. 1C, 1D and Supplementary Fig. 2). Altogether, these results showed that cyH amplifies the effect of macrophages on EC adhesiveness for monocytes.

3.2 Cycling hypoxia amplifies the effect of M0 macrophages on EC inflammatory protein expression and secretion

We then compared the effects of cyH-exposed macrophage media to N and chH-exposed macrophage media on EA.hy926 cells and HUVEC mRNA expression and secretion of pro-inflammatory cytokines (IL-6 and IL-8) and proteins (ESM1). To this purpose, EA.hy926 cells and HUVEC were incubated 24h with macrophage media and the mRNA expression and secretion of the pro-inflammatory cytokines in ECs were assessed by qPCR and ELISA, respectively. The mRNA expression of IL-6, IL-8 and ESM1 was significantly higher in EA.hy926 cells incubated with M0 cyH medium than in EA.hy926 cells incubated with M0 N and M0 chH media (Fig. 2A). These results were consistent with protein profiles since the secretion of IL-6 and IL-8 was significantly higher in EA.hy926 cells incubated with M0 cyH medium than in EA.hy926 cells incubated with M0 N and M0 chH media (Fig. 3A). Furthermore, ESM1 protein secretion tends to increase in EA.hy926 cells incubated with M0 cyH medium compared to M0 N and M0 chH media, although these differences did not reach statistical significance (Fig. 3A). EA.hy926 cells incubated with M2 cyH medium displayed a significantly higher IL-6 expression compared to EA.hy926

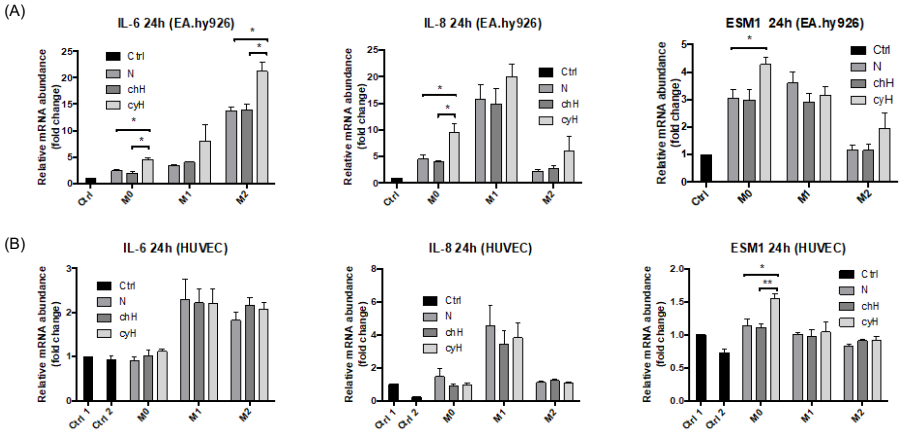

Fig. 2. Endothelial pro-inflammatory mRNA expression in EA.hy926 cells and HUVECs incubated 24h with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells **(A)** and HUVEC **(B)** were incubated for 24h with macrophage conditioned-media and their mRNA expression for IL-6, IL-8 and ESM1 was assessed by RT-qPCR (n=4, mean ± 1 SEM). Ctrl 1 and Ctrl 2 correspond to endothelial cells incubated with CO_2 independent medium or EGM-2 medium, respectively. Statistical analysis was performed using Student's t test. *p < 0.05 ; **p < 0.01.

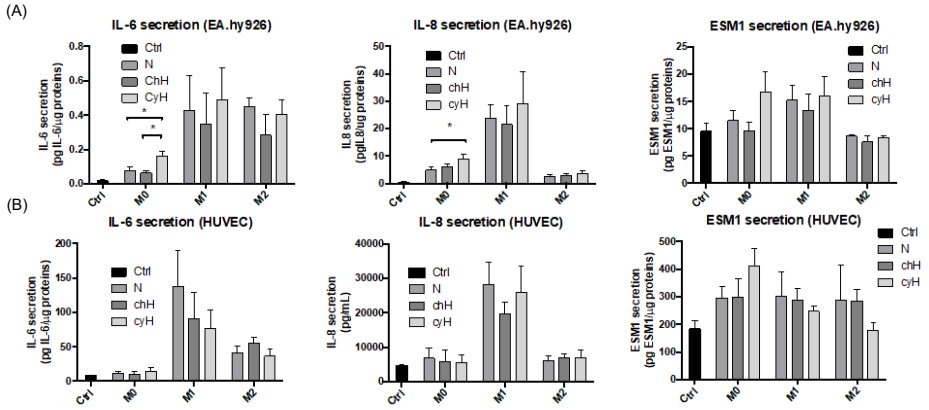

Fig. 3. Endothelial pro-inflammatory protein secretion in EA.hy926 cells and HUVEC incubated 24h with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells **(A)** and HUVEC **(B)** were incubated for 24h with macrophage conditioned-media and the secretion of IL-6, IL-8 and ESM1 in the endothelial cells was assessed by ELISA (n=3, mean ± 1 SEM). Ctrl corresponds to endothelial cells incubated with CO_2 independent medium. Statistical analysis was performed using Student's t test. *p < 0.05

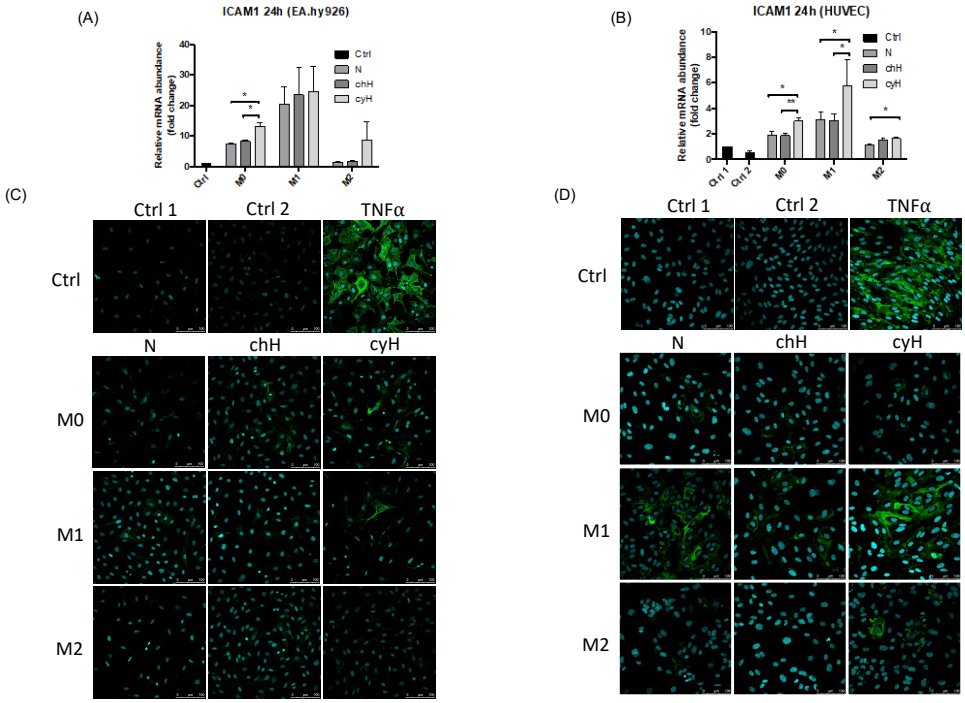

Fig. 4. ICAM1 expression and protein abundance in EA.hy926 cells and HUVEC incubated with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells and HUVEC were incubated for 24h **(A and B)** or 48h **(C and D)** with macrophage conditioned-media. mRNA expression of ICAM1 in EA.hy926 cells **(A)** and HUVEC **(B)** was assessed by RT-qPCR (n=4, mean ± 1 SEM). ICAM1 protein abundance in EA.hy926 cells **(C)** and HUVEC **(D)** was analyzed by immunofluorescence (n = 2). Ctrl 1 corresponds to endothelial cells incubated with CO_2 independent medium. Ctrl 2 corresponds to endothelial cells incubated with DHGL-1 **(C)** or EGM-2 **(B and D)** medium. Incubation of endothelial cells with 1ng/mL TNFα during 16h was used as positive control. Statistical analysis was performed using Student's t test. *p < 0.05 ; **p < 0.01.

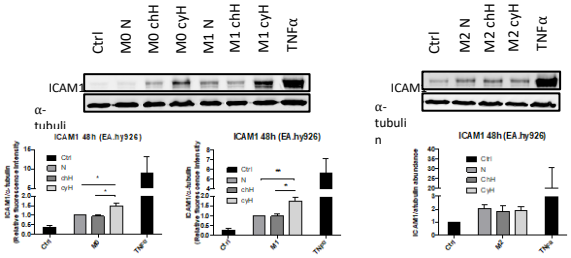

Fig. 5. ICAM1 protein abundance in EA.hy926 cells incubated with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells were incubated for 48h with macrophage conditioned-media. ICAM1 protein abundance in EA.hy926 cells was analyzed by western blot (n = 3, mean ± 1 SEM) Ctrl corresponds to endothelial cells incubated with CO_2 independent medium. Incubation of endothelial cells with 1ng/mL TNFα during 16h was used as positive control. Statistical analysis was performed using Student's t test. *p < 0.05 ; **p< 0.01.

cells incubated with M2 N and M2 chH media. In HUVEC, only ESM1 mRNA expression was significantly increased by M0 cyH medium compared to the M0 N and M0 chH media (Fig. 2B). Consistently, ESM1 protein secretion tends to increase in HUVEC incubated with M0 cyH medium compared to HUVEC incubated with M0 N and M0 chH media but this effect did not reach statistical significance (Fig. 3B). In conclusion, M0 exposed to cyH induced a pro-inflammatory phenotype in EA.hy926 cells and HUVEC, and this pro-inflammatory phenotype was stronger in EA.hy926 cells than in HUVEC.

3.3 Cycling hypoxia amplifies the effect of macrophages on the induction of endothelial ICAM1 expression

Since the adhesion of THP-1 monocytes on ECs was increased by macrophages cyH media compared to macrophage N and chH media, we studied the impact of macrophage on expression and protein abundance of ECs adhesion molecules ICAM1, VCAM1 and E-selectin. To this purpose, EA.hy926 cells and HUVEC were incubated with M0, M1 and M2 macrophages media exposed to N, chH and cyH. mRNA expression of endothelial adhesion molecules was assessed after 6h (Supplementary Fig. 2B) and 24h (Fig. 4 and Supplementary Fig. 3A) incubation, whereas protein abundance of ICAM1 was assessed after 24h (Supplementary Fig. 5) and 48h (Figs. 4 and 5 and Supplementary Fig. 4). The mRNA expression levels of adhesion molecules were higher in HUVEC and EA.hy926 cells incubated with M1 media compared to HUVEC or EA.hy926 incubated with M2 media (Fig. 4 and Supplementary Fig. 3A and 3B), except for VCAM1 expression in HUVEC incubated 24h with media which was not different in HUVEC incubated with M1 macrophage media compared to HUVEC incubated with M2 macrophage media (Supplementary Fig. 3A). This is consistent with a study in which endothelial adhesion molecules expression in ECs was more strongly induced by M1 macrophages than M2 macrophages[28]. Interestingly, ICAM1 mRNA expression was significantly higher in EA.hy926 cells incubated 24h with M0 cyH medium, compared to EA.hy926 cells incubated 24h with M0 N or M0 chH media (Fig. 4A). Furthermore, ICAM1 mRNA expression in HUVEC was significantly increased by M0 cyH, M1 cyH and M2 cyH media compared to the respective normoxic macrophages media (Fig. 1B). In HUVEC, the expression of VCAM1 or E-selectin was unaltered by cyH-exposed macrophage media compared to the related N- or chH-exposed macrophage media. On the other hand, E-selectin expression was significantly higher in HUVEC incubated 6h with M2 cyH medium compared to HUVEC incubated 6h with M2 N or M2 chH media (Supplementary Fig. 3B).

Since ICAM1 mRNA expression in ECs was increased by cyH-exposed macrophage, we studied if ICAM1 protein abundance was correspondingly increased. In EA.hy926 cells incubated 48h with macrophage media, ICAM1 protein abundance was significantly increased by M0 cyH and M1 cyH media compared the respective N-exposed macrophage media, whereas no differences were observed when ECs were incubated with M2 macrophage media (Figs. 4C and 5 and Supplementary Fig. 4A). ICAM1 protein abundance was noticeably higher in EA.hy926 cells incubated 24h with M1 media compared to EA.hy926 cells incubated with M2 media (Supplementary Fig. 5). No differences in ICAM1 protein abundance were observed in EA.hy926 cells incubated 24h with cyH-exposed macrophages media compared to N- or chH-exposed macrophages media (Supplementary Fig. 5). In HUVEC, ICAM1 protein abundance was slightly increased by M1 cyH medium and strongly increased by M2 cyH medium compared to the respective N and chH media (Fig. 4D and Supplementary Fig. 4B).

3.4 Macrophages increase the adhesiveness of endothelial cells for MDA-MB-231 breast cancer cells and this effect is potentiated by cyH

We showed that the impact of macrophages on EC adhesiveness for THP-1 monocytes was increased by cyH (Fig. 1). Furthermore, we showed that endothelial ICAM1 expression and protein abundance were enhanced by cyH-exposed macrophage compared to N- or chH-exposed macrophages (Fig. 1 and

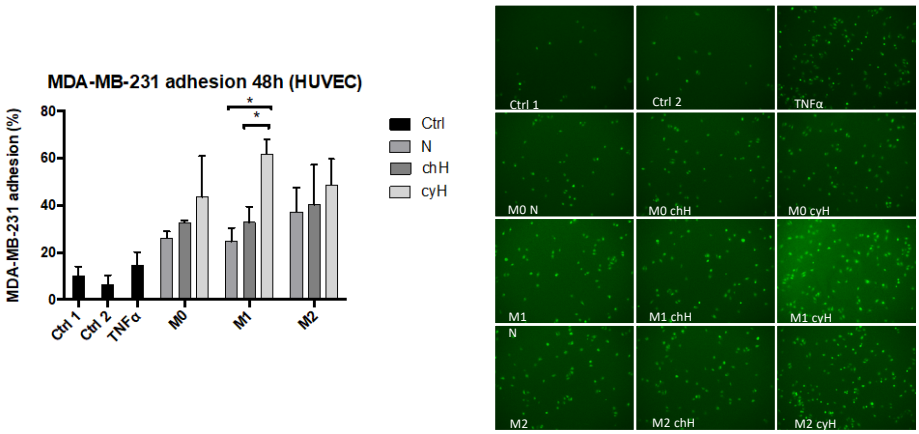

Fig. 6. Adhesion of MDA-MB-231 cancer cells on HUVEC incubated with macrophage-conditoned media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Confluent monolayers of HUVEC were incubated 48h with macrophage conditioned-media. Thereafter, the adhesion of MDA-MB-231 cancer cells on HUVEC was assessed by adhesion assay (n = 3, mean ± 1 SEM). Ctrl 1 and Ctrl 2 correspond to endothelial cells incubated with CO_2 independent medium or EGM-2 medium, respectively. Incubation of endothelial cells with 1ng/mL TNFα during 6h was used as positive control. Left : quantification of MDA-MB-231 adhesion of HUVEC. Right : Representative picture of adherent calcein-labelled MDA-MB-231 breast cancer cells on HUVEC incubated 48h with macrophage conditioned-media. Statistical analysis was performed using Student's t test. *$p < 0.05$.

Supplementary Fig. 4 and 5). Some studies showed that macrophages increased the extravasation of cancer cells towards EC monolayer[29-31]. Nonetheless, the effect of macrophages on EC adhesiveness for cancer cells is still unknown. Hence, we tested the impact of the incubation of HUVEC with M0, M1 and M2 macrophages exposed to N, chH and cyH on breast cancer cell adhesion onto the endothelium (Fig. 6). Adhesion of MDA-MB-231 breast cancer cells onto HUVEC was higher in HUVEC incubated 48h with M0, M1 and M2 macrophages compared to HUVEC incubated with control medium (Fig. 6). Furthermore, the adhesion of MDA-MB-231 cancer cells onto HUVEC was significantly higher in HUVEC incubated 48h with M1 cyH medium compared to HUVEC incubated 48h with M1 N and M1 chH media (Fig. 6). Altogether, these results showed that the incubation of ECs with macrophages media increases their adhesiveness for breast cancer cells and that the effect of M1 macrophages on ECs adhesiveness for breast cancer cells is potentiated by cyH.

4. Discussion

Tumor inflammation and metastasis are two hallmarks of cancers[9, 32] associated with bad prognosis and CyH, TAMs and ECs are critically involved in these processes[4, 5, 11-14]. For example, EC adhesiveness for monocytes and cancer cells is needed for cancer metastasis. Previously, we showed that cyH induced tumor inflammation in a murine tumor model, and induced macrophage and ECs pro-inflammatory phenotype[5, 6]. In this work, the impact of N-, chH- and cyH-exposed macrophages on EC pro-inflammatory phenotype, EC adhesion molecules expression and EC ability to bind monocytes and cancer cells was investigated. We showed that cyH-exposed macrophages increased ICAM1 expression in ECs, and that M0 cyH-exposed macrophages increased the expression and secretion of pro-inflammatory cytokines in ECs. ECs incubated with M1 cyH medium were more prone to bind monocytes and cancer cells than those incubated with M1 N and M1 chH media. These two processes are involved in the regulation of tumor inflammation and cancer metastasis[12-14, 29, 33].

M0 macrophages exposed to cyH induced more strongly the expression and secretion of pro-inflammatory cytokines (IL-6 and IL-8) and protein (ESM1) by ECs, compared to M0 macrophages exposed to N and chH (Figs. 2 and 3). Interestingly, IL-6 is strongly expressed by ECs in human and murine glioblastoma tumors[34, 35]. Furthermore ECs-derived IL-6 is a cytokine that favors macrophage M2 polarization, at least in murine glioblastoma[34]. M2 macrophage polarization is associated with bad prognosis in most cancer types[36]. Furthermore, ESM1 is an EC biomarker that is strongly expressed in tumor ECs in several murine tumor models[37]. ESM1 expression in cancer is correlated with bad prognosis in human gastrointestinal and hepatocellular carcinomas[38]. Furthermore, a study showed that ESM1 induces ICAM1 expression in ECs, and that could explain the stronger induction of ICAM1 expression in EA.hy926 cells by cyH-exposed M0 macrophages than N- or chH-exposed M0 macrophages[39].

ICAM1 expression and protein abundance in ECs and VCAM1 and E-Selectin expression in ECs were mostly induced by M1 macrophages and were least induced by M2 macrophages (Fig. 4, Supplementary Figs. 3, 4 and 5). This is consistent with a previous study showing that M1 macrophages were more potent than M2 macrophages to induce adhesion molecule expression in ECs[28]. CyH enhanced the ability of each macrophage type to induce ICAM1 mRNA expression in HUVEC (Fig. 4B). Furthermore, cyH enhanced the ability of M1 macrophages to increase ICAM1 protein abundance in both EC types (Figs. 4C, 4D, 5 and Supplementary Fig. 4), whereas M0 and M2 macrophages exposed to cyH were more potent (than those exposed to N or chH) inducers of ICAM1 protein expression in EA.hy926 cells or in HUVEC, respectively (Fig. 4C and 4D). Altogether, these results showed that cyH enhanced the ability of macrophages to induce endothelial ICAM1 protein abundance. Endothelial ICAM1 expression is involved in the metastatic cascade. Indeed, EC ICAM1 is involved in cancer cell

adhesion and extravasation of several cancer cell types, which are two needed steps for the metastatic spread of cancer cells[40]. Furthermore, ICAM1 expression is involved in melanoma cell infiltration into liver upon their injection into the tail vein of mice[41].

In this study, macrophages modulated EC phenotype toward one allowing monocyte and cancer cell binding to EC (Figs. 1, 6 and Supplementary Fig. 2). This is consistent with a previous study showing that incubation of ECs with the conditioned-media of inflammatory macrophages promoted monocyte adhesion onto endothelium[42]. Interestingly, we showed in this work that the three macrophages types - namely M0, M1 and M2 - were able to increase breast cancer cell binding onto endothelium (Fig. 6). Furthermore, cyH enhanced the ability of macrophages (mostly M1 macrophages) to induce monocyte and cancer cell binding onto endothelium (Figs. 1, 6 and Supplementary Fig. 2). These two features are the first steps of their infiltration into tumors and are strongly involved in tumor metastasis[14, 29, 33]. Indeed, the blockade of monocyte or macrophage infiltration into secondary tumor sites strongly inhibits metastatic formation[29, 33]. Furthermore, cancer cell binding onto endothelium is a critical step of the metastatic cascade[14]. The impact of macrophages on the ability of EC to allow for the adhesion of cancer cells was poorly known. It was previously shown that pro-inflammatory macrophages promoted cancer cell binding onto lymphatic ECs[43], likely via IL-1β secretion, whereas macrophages treated with carcinoembryonic antigen (CEA) were able to promote colon carcinoma HT-29 cancer cell binding onto ECs[44], but no data were generated without pre-treatment of macrophages with CEA. Here, we showed for the first time that M0, M1 and M2 macrophages, on their own, were able to modulate EC phenotype towards one allowing breast cancer cell binding onto ECs, and that the effect of M1 macrophages was potentiated by cyH. It would be interesting to investigate the molecular mechanism involved in this process, such as molecules involved in EC-cancer cell interaction and it is important to note that the increase in ICAM1 expression in ECs observed in this study could be involved in this process. Other EC proteins involved in cancer cell binding are notably N-cadherin, E-selectin and integrins[14, 27]. Monocytes and macrophages affect cancer cell extravasation notably via VEGF and MMP9-mediated EC permeability[29, 30, 45]. It would then be worth investigating the impact of cyH-exposed M0, M1 and M2 macrophages on cancer cell extravasation and EC permeability.

In this work, we show that the incubation of macrophages with cyH increased their ability to induce pro-inflammatory cytokine expression and secretion in ECs, ICAM1 expression in ECs, and to shift EC phenotype towards one allowing the adhesion of monocytes and cancer cells. This effect is specific to cyH since it was not observed in ECs incubated with macrophages exposed to chH or N. Strikingly, cyH, on its own, induces similar effects to ECs than macrophages exposed to cyH[5, 39], except for cancer cell adhesion which was not investigated in ECs exposed to cyH to our knowledge. Short-term exposure of ECs to cyH (6h) had an impact on EC alone with pro-inflammatory cytokine stimulation[5] since the effects were only observed with EC incubated with TNFα, whereas the effects of long-term exposure of ECs to cyH (> 48h) were independent of pre-existing inflammation[39]. Since some common effects of cyH-exposed macrophages and cyH on ECs are shared, it would be interesting to investigate if some levels of synergy exist between the two kinds of stimulation. Furthermore, this is physiologically relevant, since in tumors, some macrophages – called perivascular macrophages - are localized near to ECs[46] and that these two cell types should hence be submitted together to cyH in the TME.

In conclusion, we compared the impact of M0, M1 and M2 macrophages on ECs. Furthermore, we studied the effects of the pre-exposure of macrophages with N, chH and cyH on their impact on ECs. We show that M0, M1 and M2 macrophages, on their own, shift EC phenotype towards one allowing cancer cell binding onto ECs and that this effect was enhanced in M1 macrophages exposed to cyH (Fig. 6). Altogether, these results further enhance the previous results obtained in the lab that evidence that cyH induced tumor inflammation, promoted EC inflammation and promoted a pro-inflammatory

phenotype in human and murine M0 and M1 macrophages[5, 6]. These effects are specific to cyH since they are not observed with chH. It would be interesting to investigate by which secreted molecules macrophages induces their effects on ECs and to investigate by which intracellular mechanisms macrophages induce these effects in ECs. This may lead to the discovery of new therapeutic strategies for the treatment of tumor inflammation and metastasis.

Acknowledgements

The authors are thankful to the technological platform Morph-Im (University of Namur). We thank Catherine Demazy for the pictures taken under confocal microscopy. Victor Delprat is a research fellow of Televie. The authors thank Dr. Anthony Treizebre for MDA-MB-231 cells gift. This work was supported by TRANSUNIV-Interreg, ERASMUS and FNRS mobility grants.

Conflict of interest

The authors declare no conflicts of interest.

Author's contribution

V.D performed the experiments and wrote the main manuscript. O.F designed the project and critically revised the manuscript. F.S supervised the whole part performed on HUVEC and critically revised the manuscript. C.M. (corresponding author) designed the project, critically revised the manuscript and supervised the entire work.

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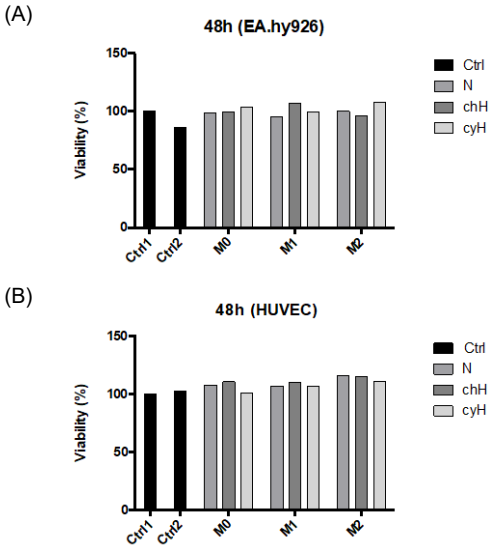
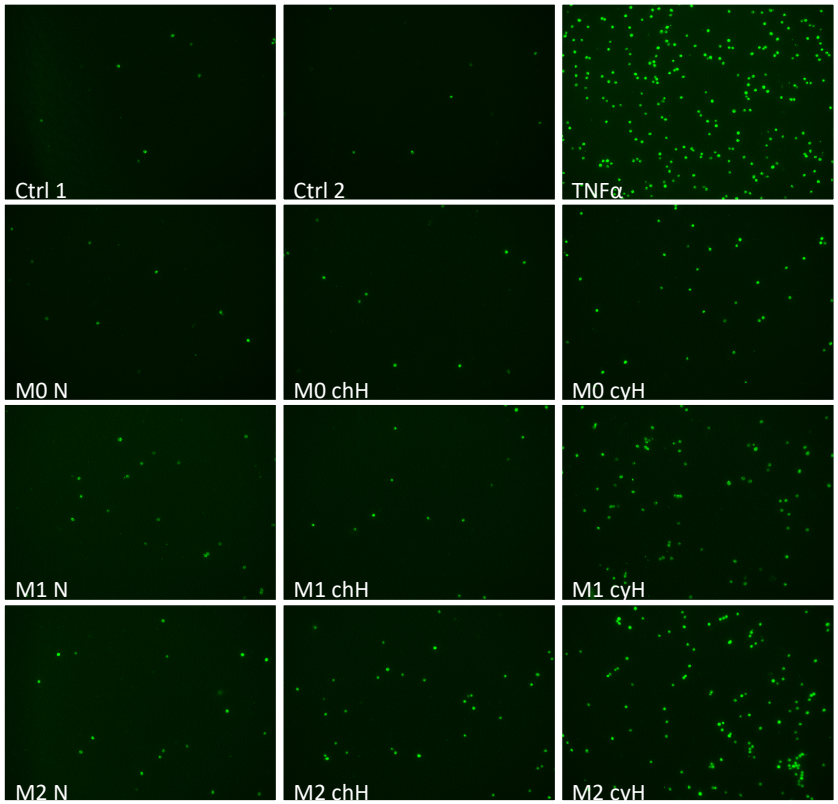
Supplementary data of the second work : " The impact of macrophages on endothelial cells is potentiated by cycling hypoxia : enhanced inflammation and tumor metastasis

Primary antibodies		
Reference	Incubation time	Dilution
Rabbit polyclonal Ab anti-ICAM1 (H reactivity), CST, #4915	O/N 4°C	1/1 000
Mouse mAb anti-α-tubulin (H+M reactivity), Sigma, #T5168	30 min RT	1/10 000
Secondary antibody		
Reference	Incubation time	Dilution
Goat anti-mouse IgG IRDye conjugated, LI-COR, Biosciences, #926-69070 (680)	1h RT	1/10 000
Goat anti-rabbit IgG IRDye conjugated, LI-COR, Biosciences, #926-68071 (680)	1h RT	1/10 000

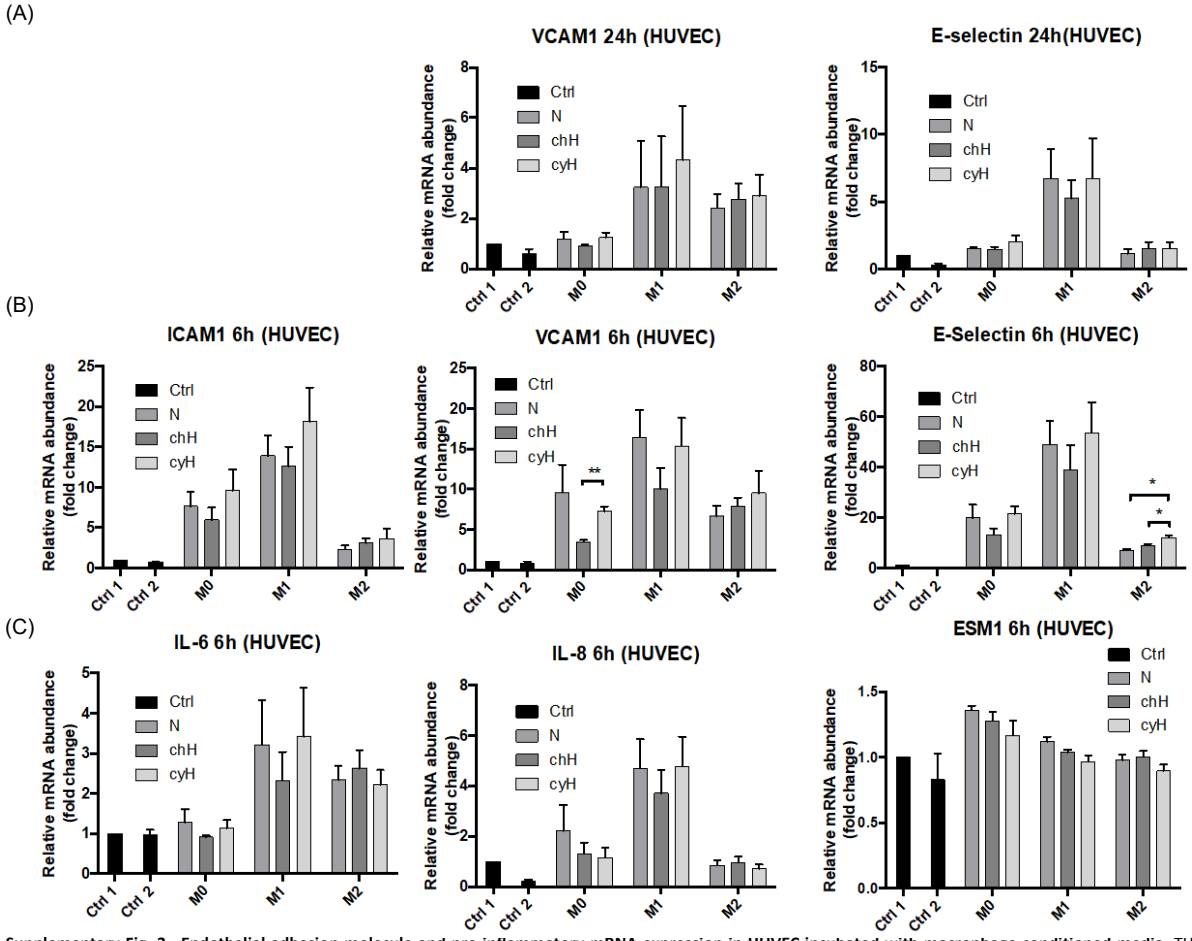
Supplementary Table S1. References of primary and secondary antibodies used for Western blot analyses



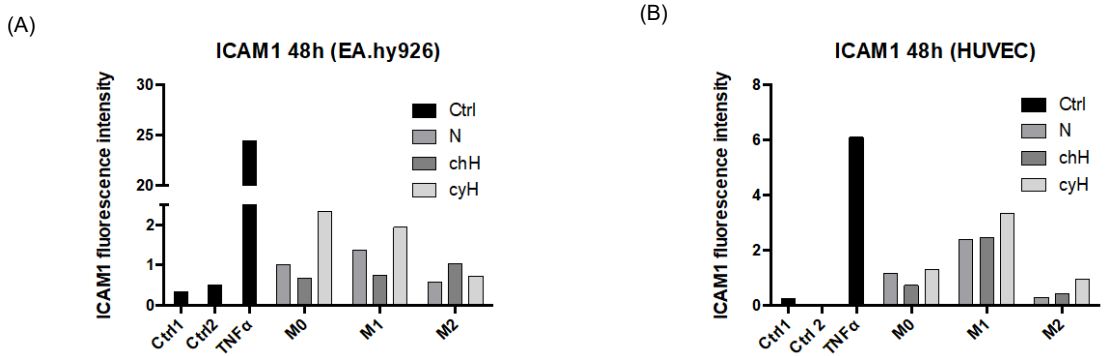
Supplementary Fig. 1. Inocuity of macrophage conditioned-media on EA.hy926 cells and HUVEC. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells **(A)** and HUVEC **(B)** were incubated for 24h with macrophage conditioned-media and the viability of endothelial cells was assessed by MTT assay (n=1). Ctrl 1 corresponds to endothelial cells incubated with CO_2 independent medium. Ctrl 2 corresponds to endothelial cells incubated with DHGL-1 **(A)** or EGM-2 **(B)** medium.



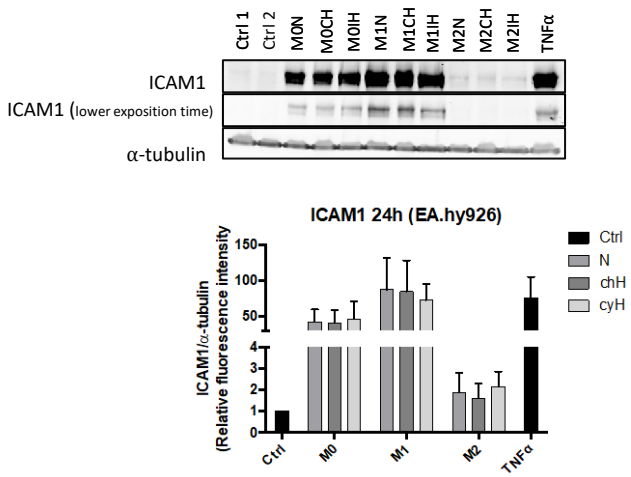
Supplementary Fig. 2. Pictures of adherent calcein-labeled THP-1 monocytes on HUVEC incubated 24h with macrophage conditioned-media (Related to Fig. 1C)



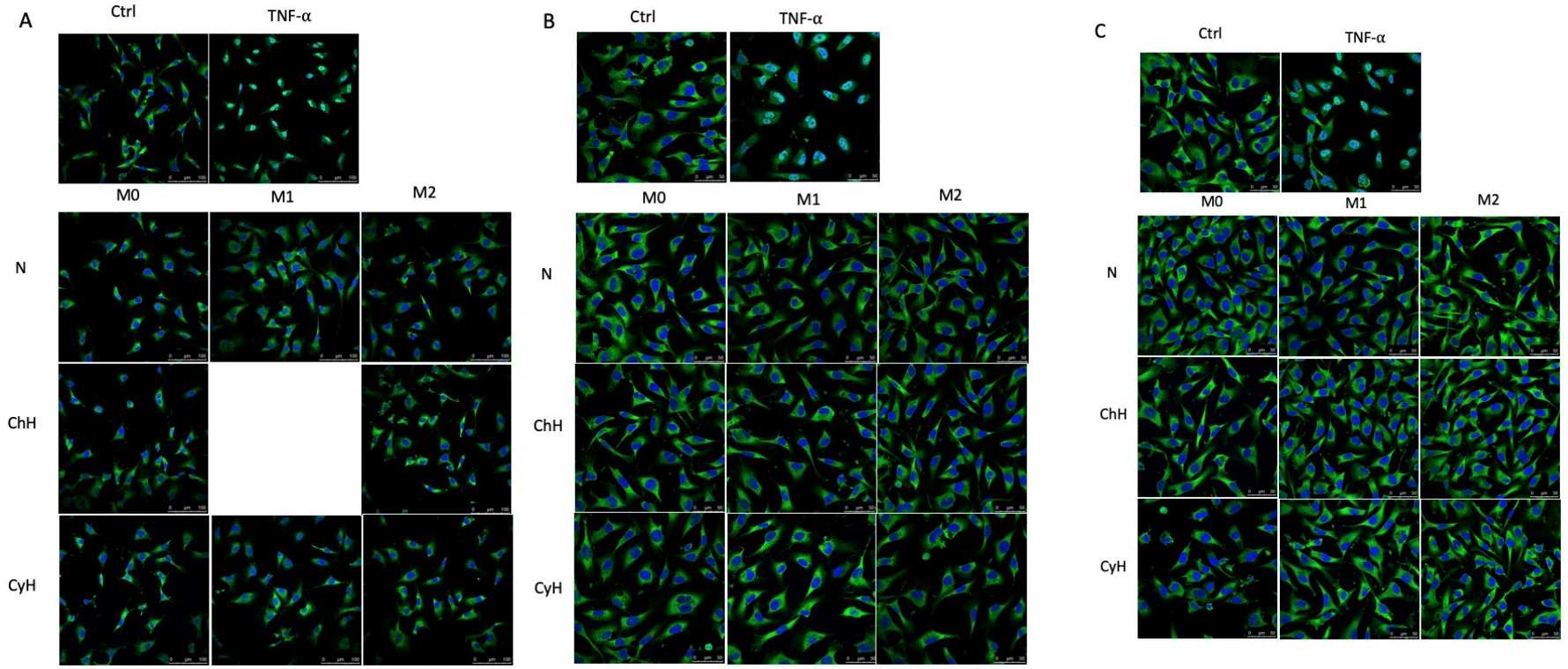
Supplementary Fig. 3. Endothelial adhesion molecule and pro-inflammatory mRNA expression in HUVEC incubated with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, HUVEC were incubated for 24h **(A)** or 6h **(B and C)** with macrophage conditioned-media. mRNA expression of endothelial adhesion molecules **(A and B)** and pro-inflammatory cytokines and gene expression **(C)** was assessed by RT-qPCR (n=4, mean ± 1 SEM). Ctrl 1 and Ctrl 2 correspond to endothelial cells incubated with CO_2 independent medium or EGM-2 medium, respectively. Statistical analysis was performed using Student's t test. *p < 0.05



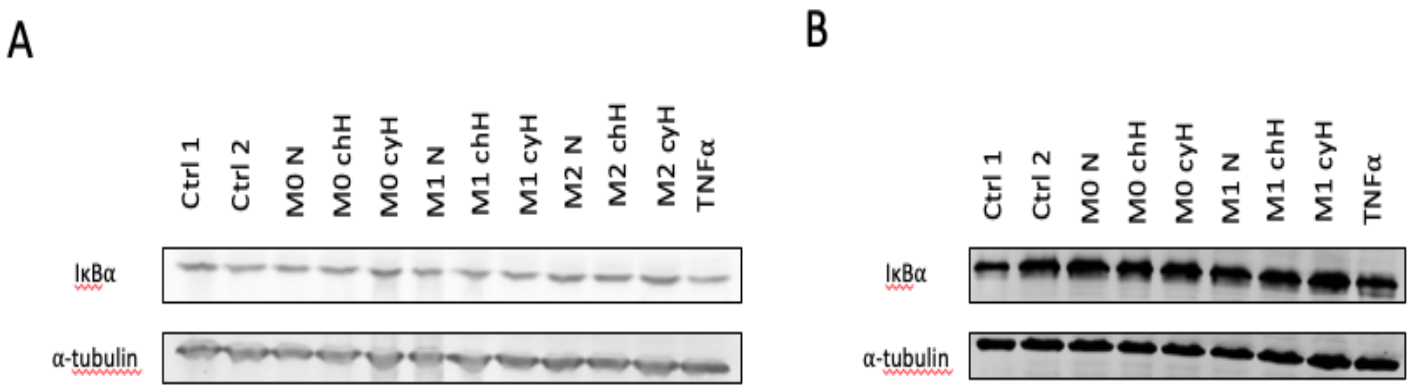
Supplementary Fig. 4. Quantification of ICAM1 immunofluorescence labeling intensity shown in figure 1. EA.hy926 (A) or HUVEC (B) were incubated 48h with macrophage conditioned-media, and ICAM1 immunofluorescence labeling was performed. These results are the mean of two independent experiments. n=2



Supplementary Fig. 5. ICAM1 protein abundance in EA.hy926 incubated 24h with macrophage conditioned-media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells were incubated for 24h with macrophage conditioned-media. ICAM1 protein abundance in EA.hy926 cells was analyzed by western blot (n = 3, mean ± 1 SEM). Ctrl corresponds to endothelial cells incubated with CO_2 independent medium. Incubation of endothelial cells with 1ng/mL TNFα during 16h was used as positive control. Statistical analysis was performed using Student's t test.



Supplementary Fig. 6 : p65 nucleus translocation is not observed in EA.hy926 cells incubated 30 min, 3h or 6h with macrophage media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells were incubated for 30 min (**A)**, 3h **(B)** or 6h **(C)** with macrophage conditioned-media. p65 nucleus translocation in EA.hy926 cells was analyzed by immunofluorescence labeling (n = 1). Ctrl corresponds to EA.hy926 cells incubated with CO_2 independent medium. Incubation of EA.hy926 cells with 1 ng/mL TNFα during 45 min was used as positive control of p65 nucleus translocation.



Supplementary Fig. 7 : IκBα is not degraded in EA.hy926 cells incubated 24h or 48h with macrophage media. THP-1-derived M0, M1 and M2 macrophages were exposed to normoxia (N), chronic hypoxia (chH) or cycling hypoxia (cyH) during 6h and were then left for 16h in normoxic air in order to produce macrophage conditioned-media. Thereafter, EA.hy926 cells were incubated for 24h (**A)** or 48h **(B)** with macrophage conditioned-media. IκBα protein abundance in EA.hy926 cells was analyzed by western blot (n = 1). Ctrl 1 corresponds to EA.hy926 cells incubated with CO_2 independent medium. Ctrl 2 corresponds to EA.hy926 cells incubated with DHGL1 medium. Incubation of EA.hy926 cells with 1 ng/mL TNFα during 16h was used as positive control for IκBα degradation.

C. Discussion, perspectives and general conclusion

7. Part I results

7.1 Cycling hypoxia and macrophage phenotype

CyH strongly promotes tumor inflammation in tumor-bearing mice *in vivo* (Li et al., 2018; Tellier et al., 2015). Tumor inflammation is a hallmark of cancer which is associated with a bad prognosis and which increases tumor metastasis, favors cancer development and progressively modifies TME into an immunosuppressive one (Colotta et al., 2009; Greten and Grivennikov, 2019). Macrophages are the main cells which infiltrate solid tumors, and strongly regulate tumor inflammation (Gentles et al., 2015; Mantovani et al., 2008). Macrophages are classified among the oversimplified M1 and M2 polarization axis, in which M1 macrophages are pro-inflammatory and M2 macrophages are anti-inflammatory. *In vitro*, M0 macrophages correspond to unpolarized macrophages. In the first part of the "results" section, the effects of cyH on human THP-1-derived and murine bone marrow-derived M0, M1 and M2 macrophages were investigated and compared to the effect of normoxia or chH.

In both models of macrophages, cyH induced a pro-inflammatory phenotype in M0 macrophages and amplified the pro-inflammatory phenotype of M1 macrophages, whereas the phenotype of M2 macrophages was not or slightly altered by cyH. CyH strongly promoted the expression of TNFα in M0 and M1 in both human THP-1 and murine macrophages. Accordingly, the secretion of TNFα was increased by cyH in M0 and M1 human macrophages and in murine M1 macrophages. Additionally, in human macrophages, cyH increased the expression of IL-1β in M0 macrophages, IL-6 in M1 macrophages and IL-8 in M0, M1 and M2 macrophages. The secretion of IL-8 was also enhanced by cyH in these macrophages. In murine BMDM, cyH increased the expression of CXCL10 and iNOS in M1 macrophages, increased the expression of COX2 and CXCL2 in M0 and M1 macrophages, and increased the expression of keratinocyte chemoattractant (KC) in the three types of macrophages.

In the literature, the impact of cyH has also been investigated in human THP-1 derived macrophages. Nonetheless, it was either investigated only in M0 or in M0 and M1 macrophages, but the impact of cyH on M2 macrophage had never been investigated before our study. Additionally, the comparison between cyH and chH is not performed in each study. Furthermore, we were the first to investigate the effect of cyH on murine BMDM.

The results obtained in our study are consistent with those described in the literature. It was shown that cyH induced or amplified the pro-inflammatory phenotype in THP-1-derived M0 and M1 macrophages, characterized by an increase in TNFα, IL-8 and IL-6 expression (Murphy et al., 2017). Furthermore, other studies showed that cyH increased the expression of iNOS, IL-6, TNFα and IL-1β in THP-1 derived M0 macrophages (Schaefer et al., 2017; Zhou et al., 2018). These effects – except for iNOS which was not studied - were dependent to the activation of advanced glycation end-products (AGE) receptor (RAGE) activation since RAGE shRNA prevented these effects (Zhou et al., 2018). More recently, the impact of cyH on primary M0 and M1 BMDM has been investigated (Fitzpatrick et al., 2020). CyH increased the expression of IL-6 and the expression and secretion of IL-1β in M0 and M1 macrophages. The

expression of iNOS and the secretion of IL-6 was also increased by cyH in M0 BMDM macrophages (Fitzpatrick et al., 2020). Hence, this is consistent with the conclusion of our study arguing that cyH increases or amplifies the pro-inflammatory phenotype in M0 and M1 murine BMDM, respectively (Delprat et al., 2020). Nonetheless, we did not observe an induction of IL-6 nor IL-1β by cyH in murine BMDM. This could be explained by the difference of the duration of cyH incubation, which was 6h in our model and 8 hours a day during 2 days in their study (Fitzpatrick et al., 2020).

Since cyH increased the expression of M1 polarization markers in M0 and M1 macrophages, we therefore tested the effect of cyH on the activation of signalling pathway involved in M1 polarization, namely NF-κB, STAT1, IRF5 and AP-1 (Lawrence and Natoli, 2011). We found that cyH increased the protein abundance of the phosphorylated form of c-jun (P-cjun ; AP-1) in human M0 and M1 macrophages and of the phosphorylated form of p65 (P-p65 ; NF-κB) and STAT1 (P-STAT1) in human M1 macrophages. In murine macrophages, only the abundance of the phosphorylated form of STAT1 was increased by cyH. In both human and murine macrophages, P-STAT1 was detected only in M1 macrophages. CyH did not alter IRF5 protein abundance in both human and murine models. Overall, these results are consistent with the literature since several studies showed that cyH induces NF-κB activation (including one in M0 macrophages) and one study showed that cyH induces AP-1 activation in PC12 cancer cells (Bader et al., 2020; Yuan et al., 2004; Zhou et al., 2018).

Since cyH increased the activation of several signalling pathways, we wondered if the activation of these signalling pathways by cyH was involved in the effects of cyH in macrophages. We firstly tried to inhibit the expression of p65, STAT1 or AP-1 by siRNA. Since these transcription factors are involved in M1 polarization, THP-1-derived macrophages had to be transfected after the polarization. However, THP-1-derived macrophages are very tough to transfect. INTERFERin (Polyplus) is a very efficient THP-1 monocyte transfection reagent but did not allow for the transfection of THP-1-derived macrophages in our hands. We then tried to transfect THP-1 derived macrophages using several transfection reagents -including Dharmafect- but that did not work due to toxicity and because the transfection reagents strongly increased the expression of pro-inflammatory cytokines in THP-1-derived macrophages (data not shown).

Hence, we chose to inhibit STAT1, NF-κB or AP-1 with chemical inhibitors. Only one molecule is described to inhibit STAT1 without impacting the activation of the other STATs, namely fludarabine (Selleckchem). Nonetheless, fludarabine is a purine analog which inhibits DNA synthesis. Hence, fludarabine is also used in clinic as a chemotherapeutic drug for the treatment of leukemia and lymphoma (Ricci et al., 2009). In THP-1 derived macrophages, fludarabine did not decrease the phosphorylation of STAT1, and even increased it, and hence cannot be used in our model (data not shown). On the other hand, the inhibition of NF-κB abolished the impact of cyH on the expression of TNFα, IL-6 in human M0 and M1 macrophages, respectively. Furthermore, the inhibition of JNKs abolished the overexpression of TNFα, IL-1β IL-8 and IL-6 induced by cyH. JNK inhibition also inhibited the phosphorylation of c-jun and hence likely inhibited the activation of AP-1. Overall, these results strongly suggest

that cyH exerts its effects via NF-κB and JNKs signalling, and it is possible that AP-1 is also involved in this.

Since NF-κB and JNK inhibition both impair the pro-inflammatory phenotype induced by cyH in human M0 and M1 macrophages, we wondered if these two signalling pathways were linked together. We showed that the inhibition of JNKs decreased the activation of c-jun, and that the inhibition of NF-κB decreased the activation of p65. Interestingly, the inhibition of JNKs in human M1 macrophages also strongly diminished the activation of p65, specifically under cyH conditions. Overall, these results suggest that JNKs/p65 signaling pathway is specifically activated under cyH in M1 macrophages, and that this signalling is responsible for at least some of the effects of cyH in M1 macrophages.

It is important to note that chemical inhibitors have also a lot of side effects, and likely much more than siRNA. Bay11-7082, the chemical inhibitor of NF-κB used in this work, impairs the phosphorylation of IκBα, and hence its proteasomal degradation. Interestingly, Bay11-7082 impairs the DNA-binding and transcriptional activities of NF-κB but does not impair the one of AP-1 (Mori et al., 2002). Bay11-7082 also targets the ubiquitin system and directly inhibits the NLRP3 inflammasome (Juliana et al., 2010; Strickson et al., 2013). NLRP3 inflammasome is a component of the innate immune system, which increases the expression and secretion of IL-1β (Kelley et al., 2019). On the other hand, SP600125, the inhibitor of JNKs used in this work, also targets the serine/threonine kinases, Aurora A and tropomyosin receptor kinase A (TrkA). In conclusion, since chemical inhibitors have a lot of side effects, it would be interesting to confirm the results by using a second type of NF-κB and JNK inhibitors.

Overall, our study shows that cyH increases or amplifies the pro-inflammatory phenotype in M0 macrophages and M1 human and murine macrophages, respectively. These results are specific to cyH since they are not observed under chH. Furthermore, JNK/p65 signaling pathway is involved in these effects. The results are summarized in Fig. 34.

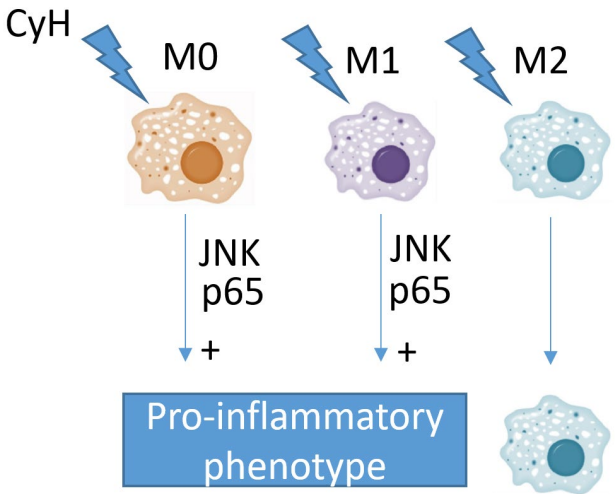


Fig. 34 : Schematic representation of the results of the first part of the thesis.

7.1.1 Are ROS responsible for the phenotype induced by cycling hypoxia in macrophages?

Several studies showed that cyH increases the production of ROS *in vitro* and *in vivo*, to a higher extent than chH (Bader et al., 2020). Nonetheless, although the effect of cyH in the overproduction of ROS by cancer cells and ECs is well described (Chen et al., 2015; Hsieh et al., 2012a; Hsieh et al., 2010; Hsieh et al., 2011; Hsieh et al., 2012b; Li et al., 2018; Malec et al., 2010; Rouschop et al., 2013; Rouschop et al., 2009; Toffoli et al., 2009b), the effect of cyH on the production of ROS by macrophages is not yet well known. Indeed, only one study showed that rat liver tissue-resident macrophages (called Kupffer cells) pre-incubated with lipids and exposed to one cycle of hypoxia/reoxygenation produce more ROS than control ones (Wang et al., 2013b). It would hence be interesting to investigate the effect cyH on ROS production in other macrophage models.

In general, the production of ROS in M1 macrophages is higher than in M2 macrophages, and M1 macrophages have glycolytic metabolism, whereas M2 macrophages have oxidative metabolism (Galvan-Pena and O'Neill, 2014). Interestingly, ROS generation promotes M1 polarization, whereas the impact of ROS in M2 macrophages is less well clear (Tan et al., 2016). For example, the induction of ROS production by macrophages by adenosine triphosphate (ATP) strongly induces IL-1β secretion in a ROS dependent manner (Cruz et al., 2007). Stimulation of TLR4 by LPS induces ROS production in macrophages, and the induction of IL-1β by LPS is impaired with antioxidant treatment in macrophages (Mills et al., 2016). Accordingly, the induction of the expression or secretion of M1 markers iNOS CD80, IL-6, IL-1β and TNFα by LPS and IFNγ in macrophages is impaired by an inhibitor of the ETC complex III (an inducer of ROS) (Cameron et al., 2019). Mitochondrial ROS production in macrophages strongly enhances their bactericidal activity, an ability associated with M1 macrophages (West et al., 2011). Hence, ROS are mostly produced by M1 macrophages and ROS are involved in the production and secretion of pro-inflammatory cytokines in macrophages.

Interestingly, ROS are involved in the activation of several signaling pathway, including NF-κB and JNKs (Zhang et al., 2016b). The treatment of cells with H_2O_2 induces NF-κB p65 phosphorylation, nucleus translocation, DNA binding and transcriptional activity (Schoonbroodt et al., 2000; Takada et al., 2003). The activation of NF-κB by H_2O_2 is slower than the one induced by pro-inflammatory cytokines such as TNFα and IL-1β. It takes approximately 1h for H_2O_2 to induces maximal NF-κB activation whereas only 15-30 min for pro-inflammatory cytokines. The induction of NF-κB activation by H_2O_2 takes place via the tyrosine 42 phosphorylation of IκBα, and IκBα is not degraded upon H_2O_2 treatment (Schoonbroodt et al., 2000; Takada et al., 2003). The activation of NF-κB by H_2O_2 is independent of IKK, and involves casein kinase II and Syk, since their inhibition impairs or inhibits NF-κB activation (Schoonbroodt et al., 2000; Takada et al., 2003). On the other hand, JNKs are activated by both pro-inflammatory cytokines and oxidative stress. It is described that oxidative stress induces JNK activation by activating upstream of JNK activator– such as ASK1 – (Matsukawa et al., 2004). Once activated JNKs activate several transcription factors such as ETS like-1 (Elk1), c-jun and ATF2 (Zhang et al., 2016b). Accordingly, some studies showed that oxidative stress also induces c-jun and c-fos activation (Goitre et al., 2014; Janssen et al., 1997). Overall, these data show that ROS are involved in the activation of NF-κB, JNKs and c-jun.

In conclusion, cyH is a well-known inducer of ROS, notably in cancer cells and ECs, although it is not well known if cyH induces ROS production in macrophages. Nonetheless, oxidative stress promotes M1 polarization and pro-inflammatory cytokine production in macrophages, and triggers notably NF-κB, JNK and c-jun signaling activation. In our study, we showed that cyH induces pro-inflammatory phenotype in macrophages, via JNK/p65 signaling. It would hence be very interesting to see if cyH induced these effects by a ROS-dependent mechanism. In this purpose, we could study the impact of cyH in the production of ROS in our human and murine macrophage models. If cyH induces ROS production, it would hence be interesting to use antioxidant (such as Tempol), to see if it decreases the cyH-induced effects in macrophages, such as the increase in pro-inflammatory cytokine expression and the activation of signaling pathways.

Interestingly, *in vivo*, cyH promotes cancer development. For example, patients with severe OSA have higher risk to develop cancers and long-term exposure of mice to severe cyH increases the spontaneous formation of tumors, although the underlying mechanisms are still not known (Cao et al., 2015a; Gallego-Martin et al., 2017). Furthermore, cyH promotes the development of CAC in mice, and the effects of cyH are reduced by antioxidant (Yoon et al., 2019). On the other hand, although postulated since a while, it was only recently shown that the production of ROS in myeloid cells -including macrophages- spontaneously promotes the formation of tumors in mice (Canli et al., 2017). Hence, if cyH promotes the formation of ROS in macrophages, it would be very interesting to study if cyH promotes cancer development by myeloid cell (or macrophages)-dependent ROS production. To this purpose, the impact of macrophage/myeloid cell depletion or antioxidant in the effect of long-term exposure of mice to cyH (model of Gallego-Martin et al., 2017) in the promotion of cancer development could be investigated.

In conclusion, this would be very interesting to study the impact of cyH on ROS production by macrophages for several reasons. First, it would more precisely explain the effect of cyH in the induction of a pro-inflammatory phenotype in macrophages. Secondly, it could be clinically relevant, since that could explain why cyH promotes cancer development, and hence may lead to the development of innovative therapeutic strategies.

7.1.2 Cycling hypoxia, IL-6 and M2 macrophage polarization in the tumor microenvironment

Although several studies show that cyH promotes M1 polarization, several studies also show that cyH increases the proportion of M2 macrophages among total macrophages in mice lung cancers, and hence increases the M2/M1 ratio (Almendros et al., 2014; Campillo et al., 2017; Delprat et al., 2020; Torres et al., 2020; Zhang et al., 2014a). It is hence interesting to know if cyH could directly trigger M2 polarization. Interestingly, our study – and other studies – show that the exposure of macrophage to cyH does not directly induce M2 polarization (Delprat et al., 2020). Furthermore, in the supplementary Fig. S4 of our study, we co-incubated M2 macrophages with IL-4 and IL-13 (M2 polarizing cytokines). CyH decreased the M2 polarization induced by IL-4 and IL-13, characterized by a decrease in the expression of the M2 markers CD206 and CCL22. Hence, cyH does not increase the IL-4- or IL-13-induced M2 polarization. Furthermore, it is not known if cyH impacts the level of IL-4 and/or IL-13 in tumor or *in vitro*. Very recently, the pro-inflammatory cytokine IL-6 has been shown to trigger M2 polarization

and to modify macrophage phenotype to a more responsive one to IL-4-induced M2 polarization (Fernando et al., 2014; Fu et al., 2017). The effect of cyH on M2 polarization in mice tumor could involve IL-6 for several reasons which are described below.

First, cyH increases the production of IL-6 both *in vitro* and in mice tumor. *In vitro*, cyH increases the secretion of IL-6 by ECs (Feng et al., 2007; Tellier et al., 2015). The expression of IL-6 by breast cancer cells and lung cancer cells is also increased by cyH (Chen et al., 2018; Liu et al., 2010). *In vivo*, the expression of IL-6 is higher in LLc tumor-bearing mice exposed to cyH compared to those exposed to normoxia (Tellier et al., 2015). The expression of IL-6 is higher in experimental melanoma lung metastasis in which mice were exposed to cyH compared to those exposed to chH or N (Li et al., 2018).

Secondly, IL-6 triggers, on its own, M2 polarization and also enhances IL-4-induced M2 polarization. The incubation of peripheral blood mononuclear cells (PBMC)-derived macrophages with IL-6 increases their expression and secretion of the M2 markers IL-10 and TGF-β and decreases their secretion of the M1 marker IL-12 (Fu et al., 2017). IL-6 also increases their expression of the M2 marker CD206. Furthermore, IL-6 increases human PBMC-derived STAT3 activation, and STAT3 activation is responsible for the M2 polarization induced by IL-6. The induction of IL-10 expression and secretion by IL-10 in murine macrophages also depend on Jak since it is prevented by JAK inhibitors (Kothari et al., 2014). On the other hand, IL-6 renders macrophage more responsive to IL-4-induced M2 polarization (Fernando et al., 2014; Mauer et al., 2014). IL-6 increases the expression of IL-4 receptor α chain (IL4-RA) in macrophages, likely via STAT3 binding to IL4-RA promoter. Furthermore, the incubation of macrophages with IL-4 + IL-6 strongly increases IL-4-induced M2 polarization, in an IL-6 receptor α chain (IL6-RA) dependent mechanism. Overall, these results show that IL-6, on its own, triggers M2 polarization via STAT3 (and to a lesser extent JAK) activation. Furthermore, IL-6 and IL-4 synergistically trigger M2 polarization.

Finally, cyH increases the M2 polarization induced by IL-6 (Zhang et al., 2014a). Indeed, IL-6 increases the proportion of murine Raw macrophages expressing the M2 markers CD209 and CD206, and this effect of IL-6 is potentiated by cyH. Furthermore, cyH increases the induction of Arg-1 and Ym1 by IL-6 in macrophages. Nonetheless, the underlying mechanism remains unknown, and it would hence be very interesting to investigate this. To this purpose, the impact of cyH + IL-6 on the activation of signaling pathway activation involved in M2 polarization could be studied. Such signaling pathways are notably STAT3, STAT6, IRF4, c-myc, peroxisome proliferator activated receptor γ (PPARγ), cAMP response element binding protein (CREB), CCAAT-enhancer-binding proteins β (C/EBPβ) and HIF2α (Lawrence and Natoli, 2011; Liu et al., 2014; Sica and Mantovani, 2012). Furthermore, it would be interesting to see if cyH potentiates the IL-6-induced increase in macrophage IL-4 responsiveness towards M2 polarization. To do this, we could study if cyH increases the ability of IL-6 to enhance the expression of IL4-RA, and to see if cyH enhances the synergistic effect of IL-6 + IL-4 in the induction of M2 polarization.

In vivo, the impact of IL-6 inhibition - by IL-6 antibody or IL6-RA KO for example - in the promotion of M2 macrophage population by cyH in murine lung tumor (and also other tumors) should be investigated. It would also be interesting to see if that could have an impact in other

tumor features such as tumor angiogenesis, metastasis and tumor growth. If the underlying mechanism by which cyH potentiates the M2 polarization induced by IL-6 is discovered, it would be interesting to investigate whether the inhibition of this factor impairs the induction of M2 macrophage population in murine tumors, and whether that also decreases other tumor features.

In preclinical models, two monoclonal antibodies targeting IL-6 (siltuximab) and IL-6 receptor (tocilizumab) show strong positive effects in many cancer types (Johnson et al., 2018). In clinic, several phase II clinical trials show that siltuximab has very limited effects (Johnson et al., 2018). Tocilizumab has been approved by FDA for the treatment of chimeric antigen receptor T cell-induced cytokine syndrome in acute B lymphoblastic leukemia and is currently in clinical trials for metastatic $HER2^+$ breast cancer, ovarian cancer, pancreatic cancer and B cell chronic lymphocytic leukaemia (Johnson et al., 2018; Kotch et al., 2019). Since siltuximab shows very limited effects in clinic, it may be interesting to use this therapy in combination with other therapies.

In conclusion, cyH induces M2 macrophage population in lung tumor by an unknown mechanism. This mechanism could involve IL-6 because (i) cyH increases IL-6 expression in murine tumor, (ii) IL-6 triggers M2 polarization and (iii) cyH potentiates the induction of M2 polarization by IL-6. Hence, the mechanism by which cyH potentiates the IL-6-induced M2 polarization should be investigated and could lead to innovative therapies.

8. Part II results

8.1 Effects of macrophages exposed to cycling hypoxia on endothelial cell phenotype: possible involvement in tumor metastasis

Tumor inflammation and metastasis are two hallmarks of cancer which are associated with poor prognosis (Hanahan and Weinberg, 2011). Metastasis is related to up to 90% of cancer-related deaths (Seyfried and Huysentruyt, 2013). Tumor inflammation is regulated by TAMs, and cyH increases inflammation both *in vitro* and in tumor-bearing mice (Li et al., 2018; Mantovani et al., 2008; Tellier et al., 2015). Furthermore, cyH, TAMs and ECs strongly regulate tumor metastasis. CyH has been shown to promote tumor metastasis to a higher extent than chH. TAMs are involved in most cancer metastasis steps (Delprat and Michiels, 2021) and ECs strongly regulate tumor cell migration towards blood vessels, intravasation and extravasation of cancer cells (Delprat and Michiels, 2021; Reymond et al., 2013; Wettschureck et al., 2019). The extravasation of cancer cells requires first the adhesion of cancer cells on ECs. TAMs and ECs strongly reciprocally interact each other (Delprat and Michiels, 2021). Indeed, ECs regulate monocytes/TAM infiltration and induce an immunosuppressive M2 phenotype in TAMs. Interestingly, monocyte infiltration in secondary tumor site strongly enhances tumor metastasis (Qian et al., 2011). On the other hand, TAMs induce angiogenesis, lymphangiogenesis, and modify EC phenotype towards a pro-metastatic one. Nonetheless, the effect of macrophages on ECs in the modulation of the adhesion of cancer cell on ECs is quite unknown. Furthermore, the effect of cyH in the regulation of the dialog between macrophages and ECs remains unknown. Hence, the aims of the second part of the thesis were to study the effects of macrophages on EC inflammatory phenotype, on EC adhesion molecule expression

and on EC ability to bind monocytes and cancer cells. Furthermore, we wanted to study the effect of cyH on this dialog. To this purpose, human THP-1-derived M0, M1 and M2 macrophages were exposed to N, chH and cyH. Then, EA.hy926 cells and HUVECs were incubated with macrophage media, and the expression of adhesion molecule, the expression of pro-inflammatory proteins and the adhesion of monocytes and cancer cells onto these ECs were studied.

The expression of IL-6, IL-8 and ESM1 in EA.hy926 cells was increased by cyH-exposed M0 media compared to the respective N and chH media. The expression of ESM1 in HUVEC was also increased by cyH-exposed M0 macrophages, but IL-6 and IL-8 expression remained unaltered. Accordingly, the secretion of IL-6 and IL-8 in EA.hy926 was increased by M0 cyH media (vs N or chH media), whereas a trend to the increases of the secretion of ESM1 in both ECs types was observed. Nonetheless, although IL-6 secretion was increased by M0 cyH medium, M1 media were the most potent to induce IL-6 secretion from ECs. Interestingly, it was recently shown that IL-6 induces M2 polarization and amplifies the M2 polarization induced by IL-4 (Fu et al., 2017; Mauer et al., 2014). Furthermore, in murine GBM, IL-6 derived mostly from ECs, since a specific inducible deletion of IL-6 in ECs strongly diminishes the level of IL-6 in murine GBM (Wang et al., 2018). Accordingly, EC-derived IL-6 induces TAM M2 polarization in murine GBM. Overall, these data show that IL-6 secretion by EA.hy926 cells is increased by M0 cyH medium although IL-6 secretion is more strongly induced by M1 macrophages. Hence, it would be interesting to see if ECs exposed to M0 or M1 cyH media could promote M2 polarization to a higher extent than ECs exposed to M0 or M1 N or chH media.

ICAM1, VCAM1 and E-selectin expression in ECs was mostly induced by M1 media. Furthermore, ICAM1 protein abundance was mostly induced by M1 media. In EA.hy926 cells, the expression of ICAM1 was increased by M0 cyH media compared to the respective N and chH media. Furthermore, a trend to the increase of ICAM1 by M2 cyH medium was observed. ICAM1 protein abundance was strongly increased by M0 cyH and M1 cyH media compared to the respective N and chH media. In HUVECs, the expression of ICAM1 was increased by M0, M1 and M2 cyH media vs the respective corresponding N and chH media. The protein abundance of ICAM1 was strongly increased by M2 cyH medium (vs the respective N and chH media) and was slightly increased by M1 cyH medium (vs the respective N and chH media), although this was not significant since this was performed only twice. ICAM1 expression in ECs promotes the adhesion of bladder, breast and fibrosarcoma cancer cells onto ECs (Benedicto et al., 2019; Laurent et al., 2014; Park et al., 2009). ICAM1 expression in cancer cells or ECs are linked to tumor metastasis (Benedicto et al., 2017). Furthermore, ICAM1 expression in ECs also strongly regulates leukocyte-including monocyte- adhesion (Gerhardt and Ley, 2015; Vestweber, 2015).

The effect of macrophage media on EC adhesiveness for monocyte was then assessed. To this purpose, ECs were incubated 24h and 48h with macrophage media and monocyte adhesion assays were performed. At 48h, monocyte adhesion was higher on EA.hy926 cells incubated with M0, M1 and M2 cyH media, compared to the respective N and chH media. At 24h, only M0 cyH slightly increased the adhesion of monocytes. In HUVECs, M1 cyH medium increased

the adhesiveness of ECs for monocytes compared to M1 N and M1 chH media. This effect is very interesting since the adhesion of monocytes onto ECs is involved in their extravasation, and that monocyte infiltration in secondary tumor site strongly enhances tumor metastasis (Qian et al., 2011; Vestweber, 2015). On the other hand, each macrophage medium increased the adhesion of cancer cells onto HUVECs. The effect of M1 macrophages to increase cancer cell adhesion was potentiated by cyH. This is the first time that such results are observed. Indeed, it was only shown that PBMC-derived M1 macrophages promote breast cancer cell adhesion onto lymphatic ECs, likely via IL-1β secretion, and that CEA-treated THP-1-derived M0 macrophages increased HUVEC adhesiveness for HT-29 colorectal cancer cells (Aarons et al., 2007; Storr et al., 2017). Overall, these results show that macrophages increase the adhesiveness of ECs for monocytes and cancer cells and that these effects are potentiated by cyH.

Monocyte adhesion and extravasation are nonetheless not always related. Indeed, an increase in monocyte adhesion can occur without increasing monocyte extravasation. For example, the adhesion of monocytes is increased by the overexpression of microRNA 126-5p in HUVECs, whereas this treatment decreases the transmigration of monocytes through HUVEC monolayer (Poissonnier et al., 2014). Such an observation is likely to occur also for cancer cells. Hence, it would be very interesting to study the impact of macrophage media on monocyte and cancer cell extravasation. The ways to performs such experiments are reviewed in (Cheng and Cheng, 2021). Such experiments can be performed using Boyden chamber/transwell or in microfluidic platform. Boyden chamber/transwell device are easier to use and should hence be tried first.

Overall, these results show that macrophages enhance the pro-inflammatory phenotype of ECs, enhance EC adhesion molecules expression, and enhance EC adhesiveness for monocytes and cancer cells. Furthermore, their effects are potentiated by cyH. Since these features are involved in cancer metastasis, this could have an impact on cancer metastasis. These results are summarized in the Fig. 35.

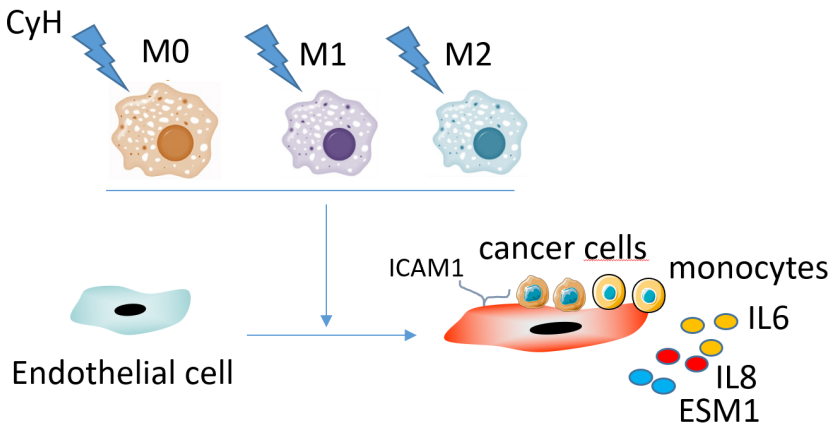


Fig. 35 : Schematic representation of the results of the second part of the thesis.

8.1.1. How to discover the underlying mechanisms of the observed results

Although the results found in the second part of the thesis are encouraging, it would be interesting to investigate on the underlying mechanisms. Several research perspectives should be envisaged.

First, each macrophage medium increased EC adhesiveness for breast cancer cells. It would be interesting to know by which ECs molecule this adhesiveness is mediated. To this purpose, the effect of macrophage media on EC expression and protein abundance of known proteins involved in cancer cell adhesion should be investigated. Such proteins are E-selectin, ICAM1, VCAM1, JAM-B, JAM-C, integrin α5, CD146, L1CAM, N-cadherin, galectin-3 and CD44H (Glinsky et al., 2001; Price et al., 1996; Wettschureck et al., 2019; Yu et al., 2007). If a protein expression and/or abundance is enhanced by macrophage media, it would then be interesting to perform cancer cell adhesion assay with a blocking antibody specific to this protein. The same work could be performed about the effect of macrophage media on monocyte adhesion.

Secondly, once the protein involved in the increased ECs adhesiveness for cancer cells, it would be interesting to search for the underlying signalling pathway which increases its expression. It is likely that this is independent of canonical NF-κB signaling since neither p65 translocation, nor IκBα degradation was observed, at least in EA.hy926 cells. It is interesting to note that identifying the proteins involved in cancer cell adhesion would strongly facilitate the discovery of the underlying signaling pathway.

Finally, it would be interesting to know which molecule(s) secreted by macrophage induce(s) the observed effects. In order to see if this molecule is a protein or a microRNA, it would be envisaged to treat media with proteinase K or RNAse, respectively. If this molecule is a protein, it would be interesting to perform 2D-DIGE followed by mass spectrometry or to perform mass spectrometry alone in order to identify this protein. Furthermore, in order to have more information about the size of the molecule, a centrifugation of the media with different size cut-off (such as 3-Kda and 30 kDa) could be performed in order to separate molecules of more 3 kDa (or 3 kDa) than those of less than 3 kDa (or 30 kDa).

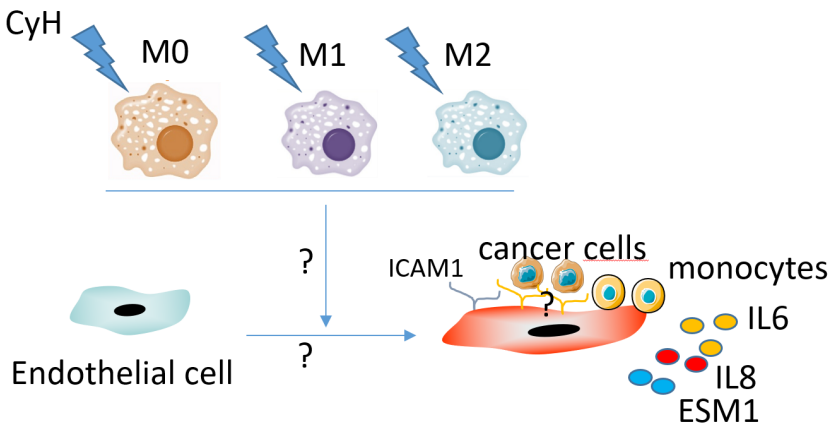


Fig. 36 : Schematic representation of the perspectives of the second part of the thesis.

8.1.2 Effect of macrophages on EC permeability

Vascular permeability strongly enhances cancer metastasis (Garcia-Roman and Zentella-Dehesa, 2013). Metastases are strongly increased by TAMs notably via an enhancement of vascular permeability (Delprat and Michiels, 2021). Furthermore, cyH is a known inducer of ROS, and ROS are involved in a loss of EC integrity (Bader et al., 2020; Boueiz and Hassoun, 2009). Hence, it would be interesting to study the impact of macrophages exposed to cyH on EC permeability. EC permeability is regulated by EC-EC junction and notably by VE-cadherin (Hordijk et al., 1999). In our model, HUVECs are incubated with 75% CO_2 independent medium 25% EGM-2. Despite this is suitable for the experiments performed in the second part of the thesis, we observed that in these conditions, HUVEC VE-cadherin protein abundance is reduced compared to HUVECs incubated in 100% EGM-2 (data not shown). In our model, macrophages are incubated in CO_2 independent medium since hypoxia incubation in the homemade incubator used in the work has to be performed outside of the CO_2 incubator. In order to study the impact of macrophage cyH media on EC permeability it would be interesting to incubate macrophages in hypoxia in 100% EGM-2 medium + 10-25 mM HEPES (HEPES is a well described CO_2 independent cell culture medium buffer), and then to see the impact of the macrophage media on HUVECs.

8.1.3 Effect of cyH on EC-induced macrophage M2 polarization

In the second part of the thesis, the effect of macrophages exposed to cyH on EC phenotype was studied. In tumors, ECs induce M2 polarization, notably via the secretion of HSP90α and IL-6 (Delprat and Michiels, 2021; Fan et al., 2019; Wang et al., 2018). IL-6 promotes IL-4-induced M2 polarization and also promotes M2 polarization on its own (Fu et al., 2017; Mauer et al., 2014). ECs are major source of IL-6, at least in murine GBM (Wang et al., 2018). CyH increases IL-6 secretion and expression in ECs *in vitro* and increases IL-6 expression in tumor bearing mice (Feng et al., 2007; Li et al., 2018; Tellier et al., 2015). Since cyH increases IL-6 secretion in ECs, it would be interesting to test the effect of the exposure of ECs to cyH in their ability to induce macrophage M2 polarization, in the presence or absence of IL-4. Since cyH increases the ability of IL-6 to induce M2 polarization, it would be interesting to expose macrophages to cyH when incubated with ECs.

9. General conclusion

In the present thesis, the effects of cyH on THP-1-derived macrophage and BMDM phenotype were studied. We showed that cyH enhances the pro-inflammatory phenotype of M0 and M1 macrophages, and that this was dependent on JNK/p65 signaling pathway for human THP-1-derived macrophages. These effects were specific to cyH since they were not observed with chH. Then, the effects of macrophage exposed or not to cyH on EC phenotype were invertigated. Macrophages increased EC adhesiveness for monocytes and cancer cells, and this was potentiated by cyH. Interestingly, cyH promotes both tumor inflammation and metastasis in tumor-bearing mice. The results obtained *in vitro* suggest that cyH could, at least partially, promote these features by altering macrophage phenotype and macrophage/EC interaction. This is very encouraging and several works could be performed in order to find innovative and specific therapeutic strategies aimed at targeting this new positive feedback loop.

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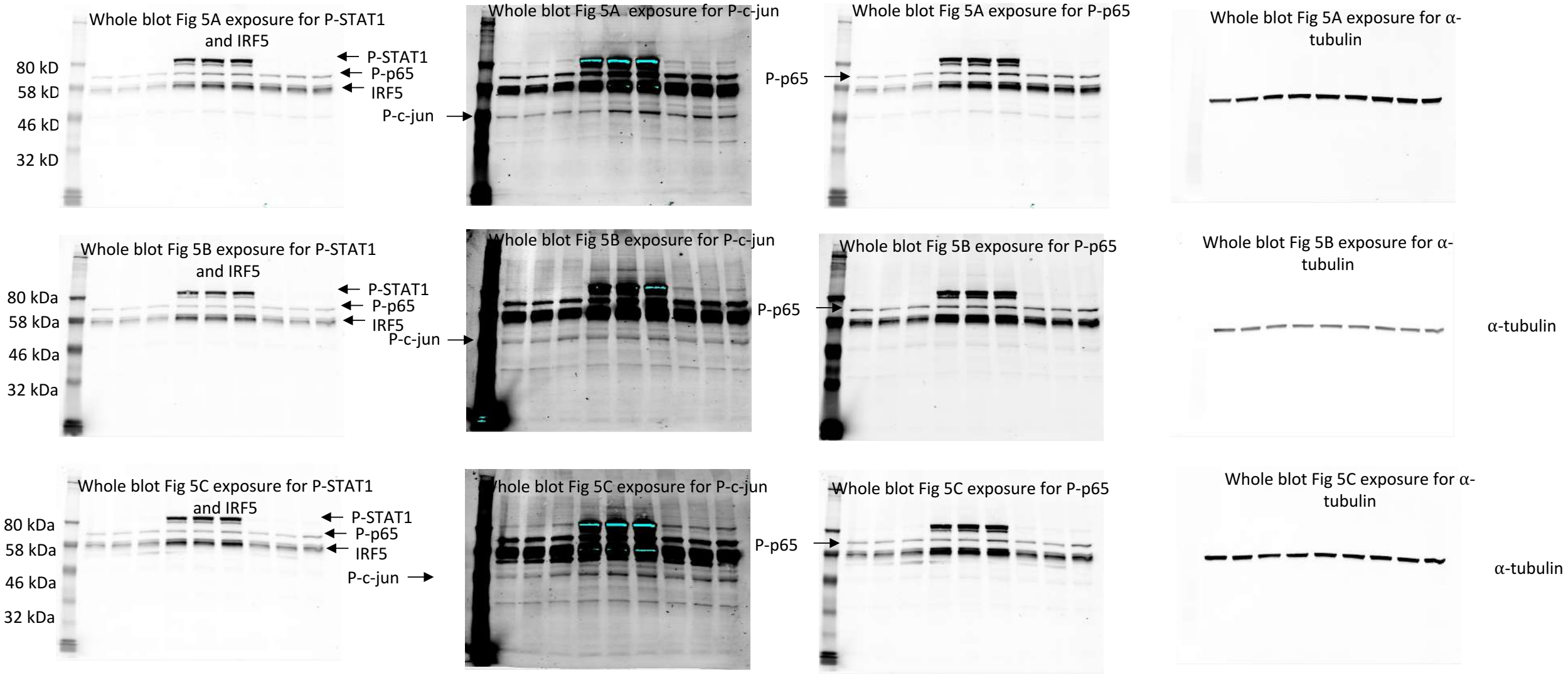
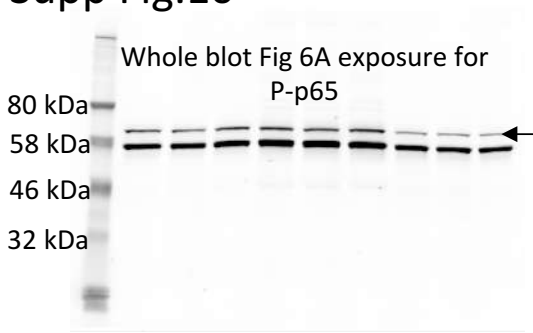
E. Whole blots of the article “Cycling hypoxia promotes a pro-inflammatory phenotype in macrophages via JNK/p65 signaling pathway”

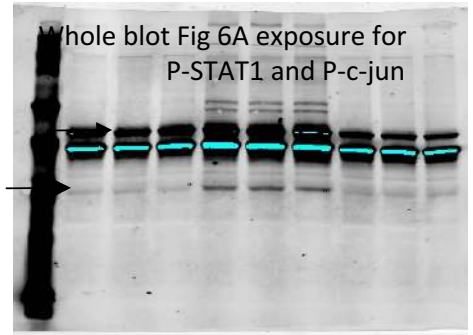
Supp Fig.9



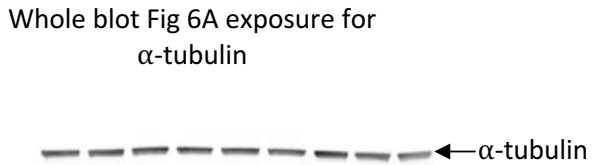
Supplementary Figure 9. Figure 5 whole blots, with the different exposure times used for each protein. The same membrane was incubated with antibodies against P-STAT1, IRF5, P-p65,P-c-jun and α-tubulin. We used red fluorescence for P-STAT1 , IRF5, P-p65, P-c-jun. We used green fluorescence for α-tubulin.

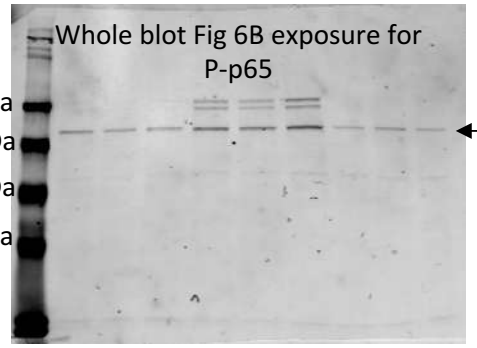
Supp Fig.10

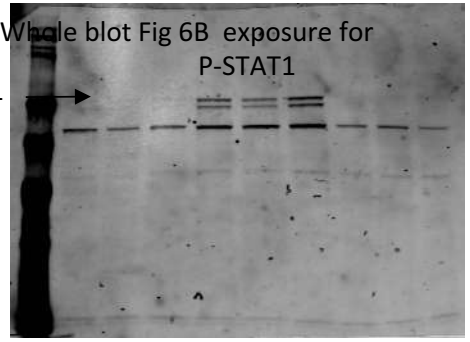

Whole blot Fig 6A exposure for
P-p65
80 kDa
58 kDa
46 kDa
32 kDa
P-STAT1
P-p65

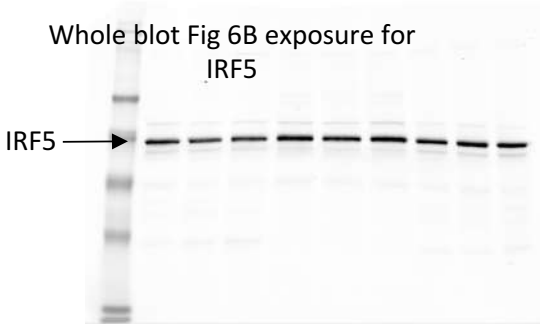

Whole blot Fig 6A exposure for
P-STAT1 and P-c-jun
P-c-jun

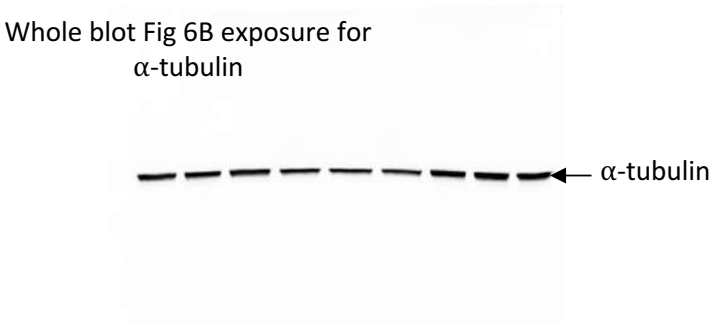

Whole blot Fig 6A exposure for
IRF5
IRF5

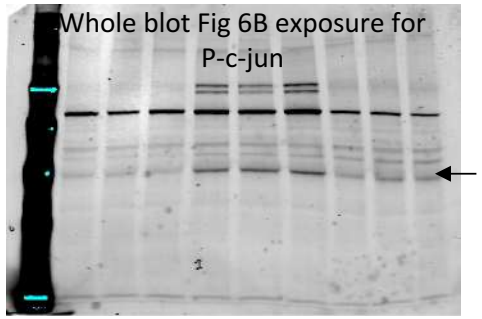

Whole blot Fig 6A exposure for
α-tubulin
α-tubulin


Whole blot Fig 6B exposure for
P-p65
80 kDa
58 kDa
46 kDa
32 kDa
P-STAT1
P-p65

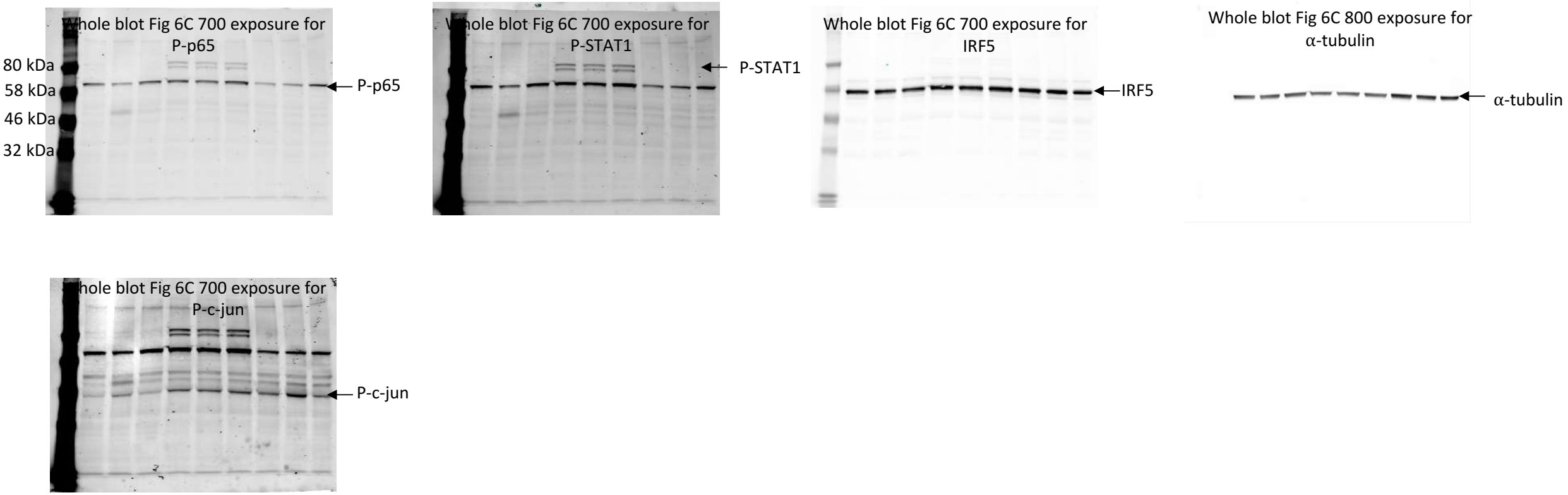

Whole blot Fig 6B exposure for
P-STAT1


Whole blot Fig 6B exposure for
IRF5
IRF5


Whole blot Fig 6B exposure for
α-tubulin
α-tubulin

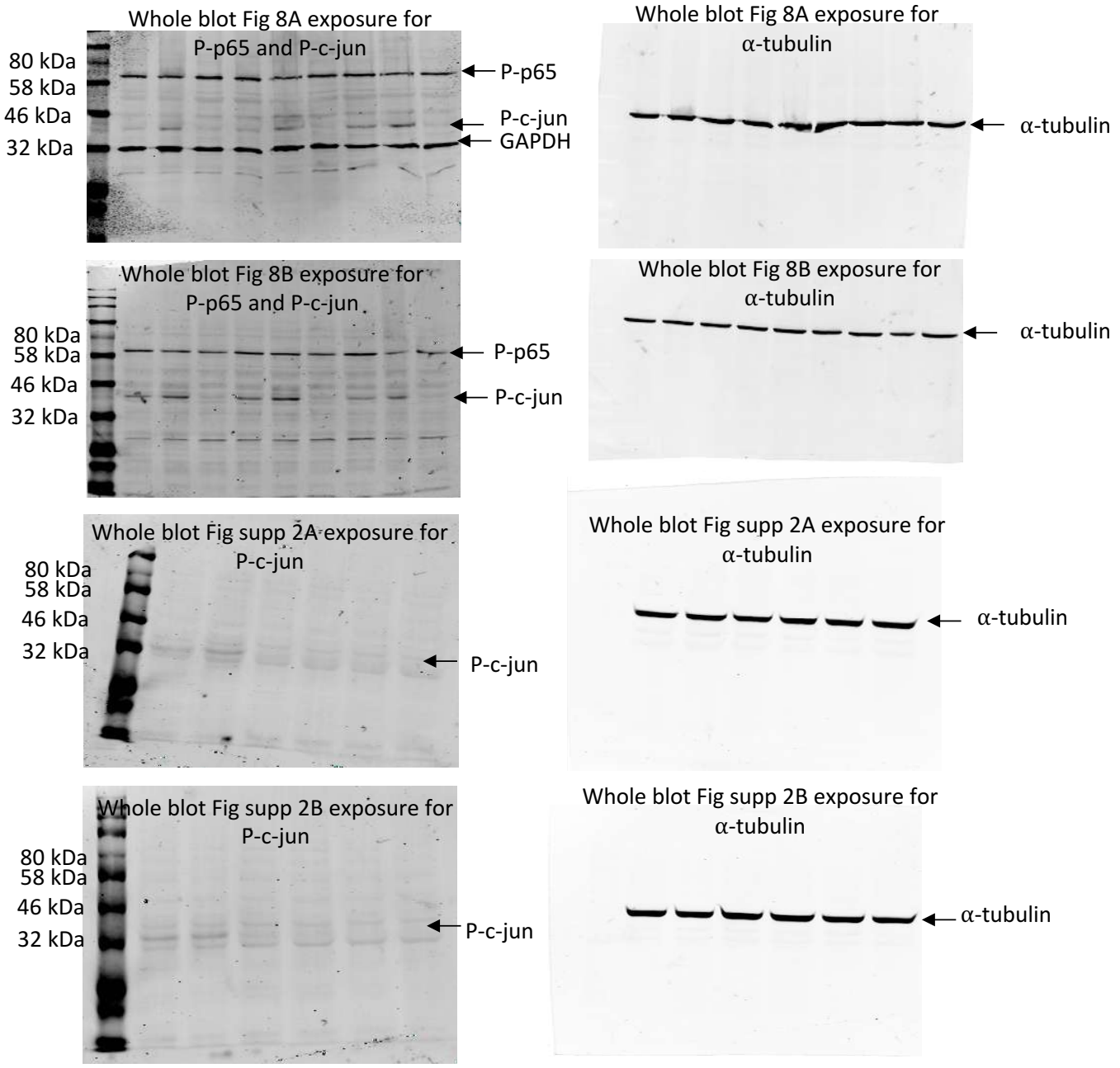

Whole blot Fig 6B exposure for
P-c-jun
P-c-jun

Supp Fig.10 (continued)



Supplementary Figure 10. Figure 6 whole blots, with the different exposure times used for each protein. The same membrane was incubated with antibodies against P-STAT1, IRF5, P-p65,P-c-jun and α-tubulin. We used red fluorescence for P-STAT1 , IRF5, P-p65, P-c-jun. We used green fluorescence for α-tubulin.

Supp Fig.11



Supplementary Figure 11. Figure 8 and supplementary Fig 2B whole blots, with the different exposure times used for each protein. In figure 8, the same membrane was incubated with antibodies against P-p65, P-c-jun and α-tubulin. We used red fluorescence for P-p65 and P-c-jun and green fluorescence for α-tubulin. In Supplementary Fig 2B, the same membrane was incubated with antibodies against P-c-jun and α-tubulin. We used red fluorescence for P-c-jun and green fluorescence for α-tubulin.