UNIVERSITÀ DI SIENA 1240

Department of Biotechnology, Chemistry and Pharmacy /Department of Medical Biotechnologies

Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

XXXVI cycle

Coordinator: Prof. Ilaria Meloni

The role of myeloid CSF-1 receptor (CSF-1R) in mesothelioma cell proliferation

Scientific disciplinary sector: MED/04-General Pathology

Supervisor
Dr. Antonio Giordano
University of Siena

Co-supervisor
Dr. Emanuele Giurisato
University of Siena

PhD Candidate
Francesca Cersosimo

Firma digitale del/della candidato/a

Firmato digitalmente da CERSOSIMO FRANCESCA C=IT

Academic Year
2023/24

Università degli Studi di Siena
Dottorato in Genetica, Oncologia e Medicina Clinica
36° Ciclo

Data dell'esame finale
20/12/2023

INDEX

ACKNOWLEDGEMENTS 1

ABSTRACT 2

1. INTRODUCTION AND OVERVIEW 3

1.1. COLONY STIMULATING FACTOR 1 RECEPTOR (CSF-1R) 3
1.1.1. Structure and Function 3
1.1.2. Gene Regulation 6
1.1.3. Intracellular Signaling 6
1.1.4. Ligands: MCS-F and IL-34 8
1.1.5. Expression in Health and Disease 9
1.1.6. CSF-1R in Cancer 10
1.1.7. CSF-1R expression in Cancer Cell 10
1.1.7.1. CSF-1R in Cell Proliferation 11
1.1.7.2. CSF-1R in Cell Migration 12
1.1.7.3. CSF-1R in Drug Resistance and Stemness 13
1.1.8. Therapeutic Strategies for CSF-1R 13

1.2. CELL CYCLE 16
1.2.1. Cell cycle Regulation 17

1.3. MESOTHELIOMA 18

2. AIM 21

4. MATERIALS AND METHODS 22

3.1. Cell Culture 22

3.2. Colony Formation Assay 22

3.3. 5-ethyl-2'-deoxyuridine (Edu) Proliferation Assay 22

3.4. Cell Transfection 23

3.5. CSF-1R inhibitor 23

3.6. Antibodies 23

3.7. Flow Cytometry Staining analysis 24

3.8. mRNA Extraction, Reverse Transcription and Real Time PCR 24

3.9. Immunofluorescence 25

3.10. Immunohistochemical Analysis 25

3.11. Protein Extraction and Western Blot 25

3.12. **Synchronization Protocol** 26

3.13. **Silenced protein pocket cell lines** 26

3.14. **cDNA constructs** 27

3.15. **Statistical Analysis** 27

4. **RESULTS** 28

4.1. **Analysis of Expression Levels of CSF-1R in MM Cell Lines** 28

4.2. **Functional analysis of CSF-1R engagement in MM cell proliferation** 30

4.3. **CSF-1R expression analysis during cell cycle phase** 31

4.3.1. CSF-1R is up-regulated during G2-M phase: 31

4.3.2. CSF-1R is up-regulated during G1-S phase: 38

4.4. **Inhibition of CSF-1R activity affected cell cycle progression** 42

4.5. **CSF-1R expression in S-phase is regulated by "pocket proteins"** 44

4.6. **CSF-1R activates proliferative signaling pathway** 48

5. **DISCUSSION** 49

6. **REFERENCES** 54

7. **PUBBLICATIONS** 68

ACKNOWLEDGEMENTS

I would like to express my gratitude to everyone I've encountered during my PhD journey. I would especially like to thank my supervisor, Dr. Antonio Giordano, for giving me this opportunity, for his generosity and for encouraging me to work harder and believing in myself.

I thank all the professors who contributed to the completion of my thesis: Prof. Emanuele Giurisato, for teaching me lab techniques and helping me in the creation of this project; prof. Cristina Ulivieri for her kindness and availability as well as for her expertise in flow cytometry analysis; Dr. William Vermi's lab for their technical assistance in the creation of IHC data; prof. Federico Galvagni for helping me to realize some of RT-PCR analysis presented in this thesis; and prof. Marcella Barberino for providing us with mesothelioma cell lines. Moreover, I thank Aurora Costa for the generation of the silenced-pocket protein cell lines.

I am grateful to the Sbarro Health Research Organization (SHRO) at Temple University of Philadelphia for accompanying me on a nine-month journey. Special thank for the best dream team - Maria Carmen, Carly, Shari, Maria Chiara, Andrew, and Reyes – for their support and friendship. I appreciated all the professors who have followed my activities and assisted me in learning new technical and personal skills. I especially like to thank Elisa Ventura for inspiring me with her perseverance, patience, and inner strength.

Lastly, I would like to thank my parents, Lucia and Leonardo, my brothers, Giosue and Lorenzo, my heart, Alessio and all my past and present friends, close and far, for never making me feel alone in any place, any decision, and any time and for their support, love, and care.

ABSTRACT

Colony-stimulating factor 1 receptor (CFS-1R) is a myeloid receptor with a key role in monocyte survival and proliferation. Its overexpression is associated with aggressive tumor characterized by a tumor microenvironment enriched of M2-like tumor-associated macrophages as well as drug resistance and poor prognosis. Recently, CSF-1R was detected on cancer cell surface in different solid tumors, capturing the interest of various research group interested in investigating the role of this receptor in non-myeloid cells. Malignant Mesothelioma is an aggressive tumor of the pleura and peritoneum, characterized by minimal clinical manifestations and few therapeutic options. Because the molecular mechanisms leading to mesothelioma carcinogenesis are unique and not fully understood, the tumor is difficult to tackle both diagnostically and therapeutically. Previous research demonstrated that CSF-1R is expressed by mesothelioma cells rather than normal mesothelial cells. Additional studies showed that CSF-1R activation induced mitogenic signaling pathways and the regulation of cell cycle-related factors in both monocytes and tumor cells. The aim of this thesis is to analyze how CSF-1R supported cancer cell proliferation and the investigation of the mechanisms regulating its expression throughout cell cycle phases. The expression of CSF-1R was investigated in different mesothelioma cell lines. Cell synchronization protocols were used to assess the expression of the receptor and its activity in the various cell cycle phases. Results indicated that CSF-1R expression characterized a pool of proliferating cells. We found that CSF-1R, different from other RTKs, undergoes a fine regulation during cell cycle progression. The percentage of CSF-1R $^{+}$ cells increased in the G1 transition to S phase and in G2-M phase. Additionally, its inhibition negatively affected mitotic entry and G1-S phase transition. In terms of molecular mechanisms, using shRNA interference, we demonstrated that the Retinoblastoma protein (pRb) p105 is required for regulating CSF-1R expression in S-phase. Finally, we demonstrated that the overexpression of the receptor induced the activation of key intracellular pathway promoting cell proliferation including ERK5, ERK1/2 and AKT signaling.

Collectively these data described a unique characteristic of CSF-1R which is differentially expressed during cell cycle phases, indicating a fine tuning throughout cell cycle. These findings extend our understanding of CSF-1R role in malignancies, making it a suitable target for an anti-cancer therapy. Further studies are required to evaluate the potential impact of targeting CSF-1R and better understand the mechanisms controlling its activity in cancer.

1. INTRODUCTION AND OVERVIEW

1.1. COLONY STIMULATING FACTOR 1 RECEPTOR (CSF-1R)

1.1.1. Structure and Function

The colony Stimulating Factor 1 Receptor (CSF-1R) is a transmembrane class III tyrosine kinase receptor expressed on the surface of myeloid cells, including macrophages, monocytes, and dendritic cells, involved in pathogenic and immunological response. CSF-1R signaling drives myeloid differentiation, monocytic commitment, and macrophage survival, proliferation, and chemotaxis by modulating tyrosine phosphorylation, activation, or expression of numerous downstream proteins (Stanley & Chitu, 2014; Xiang et al., 2023). Indeed, *Csf1r* $^{-/-}$ mice reported a reduced number of macrophages in several tissues, associated with neurological and developmental defects (Dai et al., 2002) as in fact a few numbers of mice with knockout mutations of *CSF-1R* were able to survive (Rojo et al., 2017). CSF-1R is activated by binding of its specific ligands, macrophage colony stimulating factor (M-CSF) and Interleukin-34 (IL-34) (Barca et al., 2021). The receptor is encoded by the *c-fms* proto-oncogene, the human analogue of the *v-fms* oncogene of the Susan McDonough strain of feline sarcoma virus (SM-FeSV) (Walter et al., 2007). Because the discovery that *c-fms* encoded for a glycoprotein (165 kDa) with tyrosine kinase activity, capable to bind the CSF-1, *c-fms* and *csf1r* indicates today the same gene and generate the same protein (Sherr et al., 1985).

The CSF-1R is a member of the platelet-derived growth factor (PDGF) family. Structurally, it has a glycosylated extracellular region with five immunoglobulin domains, a transmembrane helix, and an intracellular consisting of a juxta membrane domain (JMD) and an intracellular region containing the tyrosine kinase domains (Stanley & Chitu, 2014). Binding of its cognate ligands occurs via the three ammino terminal Ig domains (D1, D2, D3), the others (D4 and D5) function as binding stabilizers. CSF-1R is in autoinhibitory state in the absence of ligands and upon ligand binding to CSF-1R, the JMD changes from its autoinhibitory to an active, expanded conformation (**Figure 1**) (Mun et al., 2020). Specifically, the N-terminal region of autoinhibited CSF-1R consists of a five-stranded antiparallel β-sheet (β1–β5) and a single α-helix called the αC helix. Instead, seven α-helices (αD, αE, αEF, αF–αI) and two β strands (β6 and β7) define the C-terminal portion of the receptor (**Figure 2**). The JMD (residues

542–574) includes conserved tyrosine domains, Tyr546, Tyr561 and Tyr571. Tyr561 is recognized as primary phosphorylation site, providing the binding site for Src kinase family members as well inducing CSF-1R signaling activation (Walter et al., 2007).

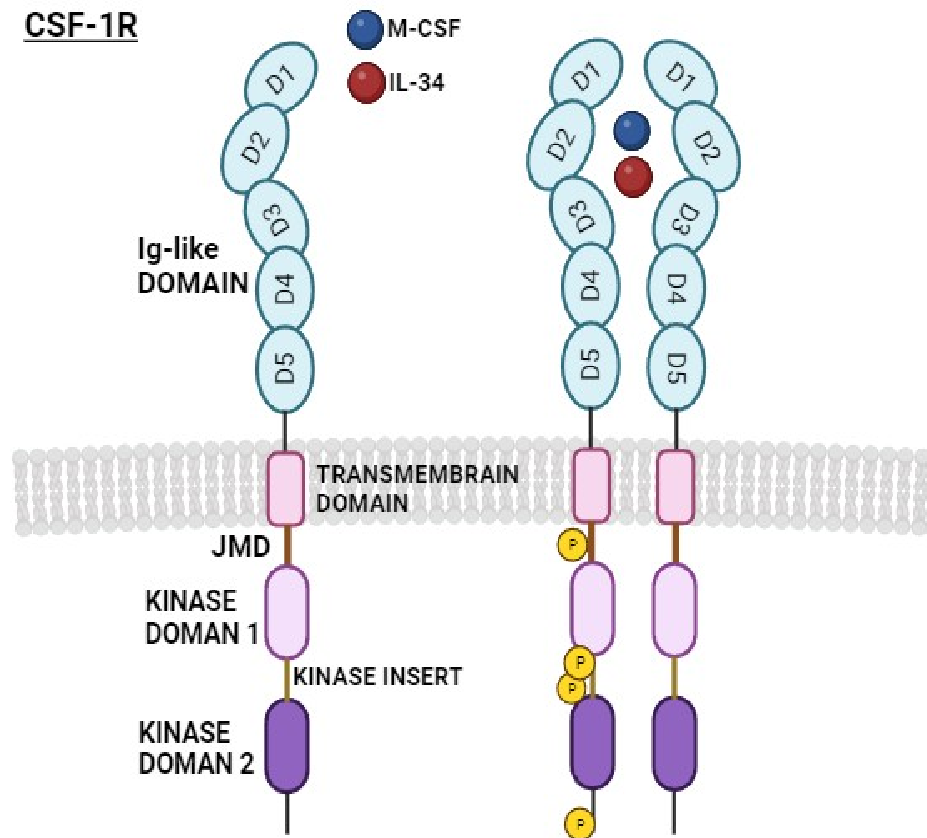


Figure 1. CSF-1R Domains Structure: five Ig-like domains, including ligand-binding D2 and D3 domains are present in the extracellular region. The transmembrane, the juxtamembrane domain (JMD), two-kinase domains, a kinase insert, and cytoplasmic domains are intracellular domains. The receptor is in autoinhibitory state in the absence of ligands. When IL-34 or M-CSF bind to CSF-1R, the JMD changes from its autoinhibitory position to an active, expanded conformation (Mun et al., 2020).

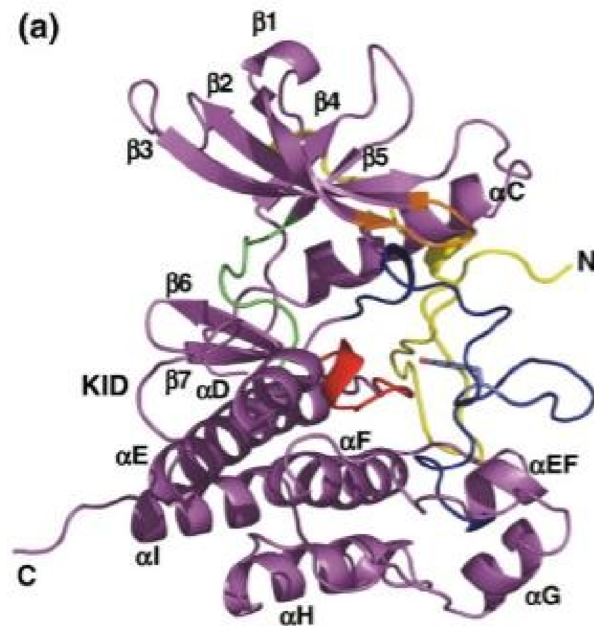


Figure 2. Crystal Structure of CSF-1R. The N and C termini are showed with the secondary structural components of the autoinhibited CSF-1R kinase domain. The JMD in yellow, the hinge region in green, the glycine loop in orange, the catalytic region in red, the activation loop in blue and the location of the KID is indicated. Image adapted from (Walter et al., 2007).

Previous study demonstrated that CSF-1R was rapidly internalized in micropinocytosis after CSF-1 stimulation and ligand binding determines cellular responses such as alterations in the actin cytoskeleton and membrane fluidity. These modifications can enhance the production of membrane ruffles and small invaginations, which are typical of micropinocytosis. Consequently, CSF-1R on the cells surface is engulfed into micropinosomes, where CSF-1R can be either sorted for recycle back to the cell surface or targeted for degradation (Lou et al., 2014). Nuclear localization of CSF-1R was also reported in human monocytes by Bencheikh et al., who observed that a portion of the receptor, followed activation, localized at chromatin level, and associated with H3K4me1 histone and EGR1 (Bencheikh et al., 2019). Nuclear localization of CSF-1R was also observed in bone marrow derived macrophages and certain tumor cell lines, including breast, ovarian and cervical cancer (Zwaenepoel et al., 2012).

1.1.2. Gene Regulation

Hematopoietic stem cells exhibit moderate levels of *CSF1R* gene expression, while Its expression increases in myeloid precursors committed to differentiate into mature macrophages (Krysinska et al., 2007). Sasmono et al. showed that murine granulocytes expressed *CSF-1R* mRNA but not the protein at the surface (Sasmono et al., 2007). Human *CSF-1R* (58 kb) is on chromosome 5q33.3 and it has 22 exons and 21 introns. The transcription of *CSF-1R* is driven by two different promoter regions: the upstream promoter, expressed in trophoblasts and mammary epithelial cells during embryonic development, and the downstream promoter, expressed and myeloid progenitors and associated to macrophage maturation (Barreda et al., 2004). The *CSF-1R* promoter region in macrophages lacks some classical elements of the transcription starting site, including the classical TATA box, which is replaced by purine-rich recognition regions for the Ets family of transcriptional factors. A conserved 300 bp enhancer located in the first intron downstream the promoter, indicated as Fms intronic regulatory elements (FIRE), is necessary for *CSF-1R* expression (Ovchinnikov et al., 2010; Rojo et al., 2017). Different transcription factors regulating monocytes development, including PU.1, RUNX1 and AP.1 bind to sites in the FIRE region. This regulatory element has antisense promoter activity, which correlated with *CSF-1R* repression in B cells (Sauter et al., 2013). Transcriptional studies revealed that FIRE sequence is conserved among birds and reptiles. The conserved regions comprise recognition sites for transcription factors AP-1 and PU.1. Previous evidence showed that *CSF-1R* expression is regulated by the synergic activity of several transcriptional factors including PU.1, Runx1, and AP-1/CREB-like elements represent the main regulators of *CSF-1R* transcription. Runx1 regulates *CSF-1R* expression directly or indirectly by regulating PU.1 expression. On the contrary, CSF-1R repression in precursors committed to other lineage is mediated by the transcriptional factors PAX5, GATA-1, MYB, FLI1, and BATF3 (Garceau et al., 2010; Rojo et al., 2017).

1.1.3. Intracellular Signaling

Binding of molecules to phosphor-tyrosine residues on CSF-1R induces the activation of mitogenic and survival pathways (Yu et al., 2012). CSF-1R signaling was initially studied in osteoclast, macrophage, and microglia (Stanley & Chitu, 2014). Ligand binding on D1-D3 domains induces receptor dimerization and autophosphorylation of intracellular tyrosine residues, providing binding sites

for src homology 2 (SH2)-containing proteins and downstream signaling (Bourette et al., 2000). Six cytoplasmic tyrosine (Tyr559, Tyr697, Tyr706, Tyr721, Tyr807 and, Tyr974) and two in the oncogenic form (Tyr544 and Tyr921) are activated and mediate intracellular signaling (Mun et al., 2020). Unphosphorylated murine Tyr559 maintains the receptor off in absence of the ligand and is the first tyrosine phosphorylated upon ligand binding. Its phosphorylation plays a crucial role in macrophage proliferation via the activation of the ERK1/2 pathway (Yu et al., 2012). Moreover, phospho-Tyr559 induces an SFK/c-Cbl pathway which promotes CSF-1R ubiquitination, which is required for signaling amplification (Xiong et al., 2011). SFK recruits the adaptor protein DAP12, which is involved in cell cycle progression by regulating nuclear localization of β-catenin, (Otero et al., 2009). ERK induction also occurs by recruitment and activation of the Grb2/Sos complex through phospho-Tyr697 binding. In addition, pTyr697 is involved in the activation of the Gab/Mona pathway, thereby promoting macrophage differentiation (Bourgin et al., 2002). On the other hand, ERK suppression is associated with CSF-1-dependent activation of the phosphatase MKP-1 (Valledor et al., 1999). Tyr921 acts as Grb2 binding site, while Tyr721 is associated with macrophage differentiation via the activation of PI3K and PLCγ (Mun et al., 2020). PI3K is also activated after Tyr706 phosphorylation (Tyr708 in human), which is required for STAT1 activation (Novak et al., 1996) while the pTyr559/Src/STAT3 pathway induced macrophage differentiation (Marks et al., 1999). A recent study demonstrated that in human macrophages, STAT3 phosphorylation at Tyr705 is ERK5-dependent (Giurisato et al., 2018). In macrophages, Tyr721 phosphorylation promoted the direct activation of Akt signaling pathway, that can be also induced by PLC signaling (Stanley & Chitu, 2014). Furthermore, Tyr807 of CSF-1R is necessary for p46/52 Shc activation, tyrosine phosphorylation, and membrane translocation of PKC-δ, which leads to increased activity of PKA-related protein kinase (Pkare), thereby contributing to monocyte differentiation (Junttila et al., 2003). CSF-1R downstream signaling contributing to actin polarization depends on PI3K and activation of small GTPases, including Cdc42, Rac, Rho, WASP and WAVE2 (Stanley & Chitu, 2014).

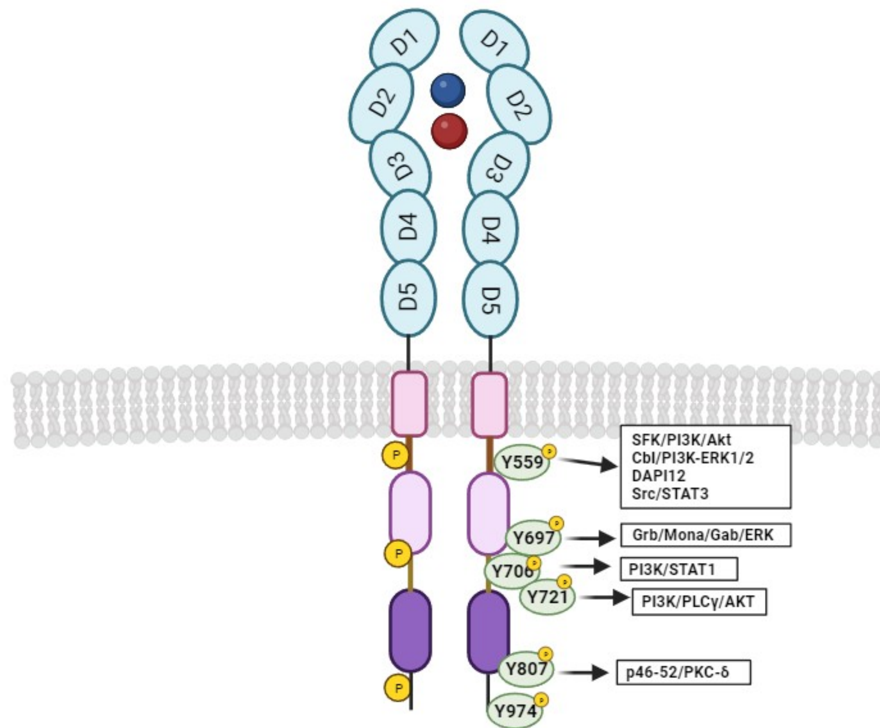


Figure 3. CSF-1R Intracellular signaling. Receptor dimerization induces its autophosphorylation and activation. Phospho-tyrosine residues represent binding sites for molecules activating mitogenic and survival pathways, including PI3K/Akt signaling, ERK1/2 and STAT molecules (Stanley & Chitu, 2014) .

1.1.4. Ligands: MCS-F and IL-34

Two different ligands activate the CSF-1R, M-CSF and IL-34 (Stanley & Chitu, 2014). M-CSF, also known as CSF-1, is the main growth factor regulating monocyte differentiation and macrophage survival (Jones & Ricardo, 2013). CSF-1 is a homodimer glycoprotein first discovered for its ability to induce maturation of progenitor cells into macrophages (Stanley et al., 1978). The human *Csf1* gene is on chromosome 1 while it is on chromosome 3 in mice. Three principal isoforms originate from *Csf1* mRNA transcription: a cell-surface glycoprotein (csCSF-1), a secreted proteoglycan (spCSF-1) and a secreted glycoprotein (sgCSF-1) (Pixley & Stanley, 2004). The human CSF-1 primary isoform is 192 amino acids long (Deng et al., 1996) and it is produced by a variety of cell types, including trophoblast, mononuclear phagocytic precursors, endothelial cells, smooth muscle, and cancer cells, in autocrine or paracrine way (Stanley & Chitu, 2014). Importantly, $Csf1^{op}/Csf1^{op}$ mice with mutations in the gene coding region displayed osteoporotic, neurological, dental, and reproductive abnormalities (Ryan et al., 2001). The second CSF-1R ligand, IL-34,

was first discovered by Lin et al. group (Haishan Lin et al., 2008). *Il34*$^{LacZ/LacZ}$ mice were used to demonstrate that IL-34 controls differentiation signals crucial for myeloid cells within the epidermis and central nervous system (Wang et al., 2012). *IL-34* mRNA, more than CSF-1, is extensively expressed in the brain, where it is involved in microglial development (Nandi et al., 2012). IL-34, but not M-CSF, is also the ligand for two other receptors, PTP-ζ and syndecan-1 (CD138). Human *IL-34* gene is on chromosome 16, encoding a 222 amino acid mature protein (Jeannin et al., 2018). Like CSF-1, the active region of IL-34 has four helical bundle folds. However, CSF-1 has a dimer with disulfide bonds interchain while IL-34 has dimers covalently bound (Ma et al., 2012; Pandit et al., 1992). IL-34 and CSF-1 bind to different positions in the D1-D3 region of the CSF-1R inducing D1-D3 dimerization. In this instance, IL-34-related D1-D3 dimerization is independent by the presence of D4-D5 Ig-like domains. On the other hand, CSF-1 is not able to form D1-D3 dimerization without D4-D5 domains interaction. Additionally, it has been demonstrated that CSF-1 and IL-34 compete for CSF-1R binding. Despite the structural differences, both ligands induced functional activation of the receptor (Ma et al., 2012; Rojo et al., 2017).

1.1.5. Expression in Health and Disease

In physiology, CSF-1R signaling plays an essential role in regulating homeostasis (Sullivan & Pixley, 2014). Previous studies demonstrated that CSF-1R signaling is required for normal development of mammary glands during puberty. Indeed, alteration of its expression are associated with breast cancer initiation (Lin et al., 2001). Various pathologies, including cancer, inflammatory conditions, and autoimmune disease, have been linked to increased CSF-1R activation (Chitu & Stanley, 2006). The activation of CSF-1R via IL-34 was detected in aggressive rheumatoid arthritis (Zhou et al., 2016). Additionally, because CSF-1R pathway is crucial for microglia functionality, abnormal CSF-1 expression in the central nervous system department enhanced the activity of defective microglia in neurological disorders including Alzheimer, Parkinson, and Huntington disease, making the receptor an intriguing therapeutic target (Han et al., 2022). It has been demonstrated that the serum of patients with chronic hepatitis C virus (HCV) is enriched in IL-34 and M-CSF, which negatively contributed to the pathogenesis of the liver fibrosis by the recruitment of hepatic macrophages secreting high content of cytokines and chemokines and inducing the production of type 1 collagen from hepatic stellate cells (Preisser et al., 2014). The

involvement of CSF-1R has been also reported in other inflammatory diseases, like pulmonary fibrosis and asthma, (Meziani et al., 2018; Moon et al., 2020).

1.1.6. CSF-1R in Cancer

In cancer, prolonged exposure to CSF-1R ligands leads to the recruitment of macrophages at tumor sites and its differentiation into pro-tumorigenic macrophages, indicated as M2-like tumor-associated macrophages (M2-TAMs) (Ries et al., 2014). Indeed, a correlation between increased circulating CSF-1 and enhanced CSF-1R+ TAMs was observed in breast cancer (Sullivan & Pixley, 2014). The development of an invasive phenotype in breast and female reproductive tract tumors was also associated with CSF-1R levels and circulating CSF-1 (Barry M. Kacinski, 1997). Gene expression profiling revealed high levels of *Csf-1* and *Csf-1r* mRNA in leiomyosarcoma patient samples. In addition, CSF-1 was secreted by stromal and tumor cells, suggesting an intricate collaboration between cells within the tumor microenvironment (TME). CSF-1R was expressed on both macrophage and tumor cell surfaces, indicating a paracrine and autocrine activation of the receptor (Espinosa et al., 2009). Blockade of the autocrine loop in ovarian cancer reversed the malignant phenotype (Toy et al., 2009). Ide et al. reported the involvement of CSF-1R in prostate cancer carcinogenesis (Ide et al., 2002). Furthermore, oncogenic mutations in c-*fms* were correlated with worse prognosis in myelodysplastic syndrome (Such et al., 2009).

1.1.7. CSF-1R expression in Cancer Cell

Abnormal expression of CSF-1R has been reported in various cancer types, playing a role in the development of an immunosuppressive TME**.** CSF-1R signaling mediates crosstalk between TME various players and the recruitment of TAMs in cancer sites (Buechler et al., 2021; Cannarile et al., 2017; Sletta et al., 2021). Genetic mutations in *CSF-1R* can induce tumorigenic characteristics and result in CFS-1-independent receptor activation (Roussel et al., 1988). In tumors, several studies reported that upregulation of the *CSF-1R* mRNA correlated with poor prognosis (Mo et al., 2021; Riaz et al., 2021). Barbetti et al. demonstrated CSF-1-dependent nuclear localization of CSF-1R in breast cancer cell lines, promoting the transcription of proliferative genes like *CCND1*, *c-JUN*, and *MYC* (Barbetti et al., 2014). Epigenetic alterations in the CSF-1R promoter led to up-

regulation in melanoma cells with BRAF mutations. Interestingly, these cancer cells did not express the transcriptional factor PU.1, a key regulator of CSF-1R expression in myeloid cells, suggesting that CSF-1R expression is regulated by a different transcriptional mechanism in tumor cells (Giricz et al., 2018).

1.1.7.1. CSF-1R in Cell Proliferation

Lee et al. (Lee et al., 1999) used breast cancer cell lines to study CSF-1R action and observed that CSF-1 stimulation produced different responses in different breast cancer cell lines. In CSF-1R-overexpressing MCF-7 cells, CSF-1 induced cell cycle arrest associated with increase in p21 levels. In contrast, in T47-D cell line CSF-1 had pro-mitogenic functions and induced low levels of p21, suggesting that the role of CSF-1R depends on cellular genetic background (Lee et al., 1999). Notably, another study in metastatic breast cancer cells showed that CSF-1R upregulation was mediated by TGF-β levels in microenvironment, as in fact CSF-1R levels decreased in primary cancer cells upon TGF-β receptor inhibition (Patsialou et al., 2009). Additional studies from the same group demonstrated that claudin-low cell lines expressed higher CSF-1R levels than luminal ones. In contrast to other reports showing that CSF-1R suppression increased *in vivo* proliferation of claudin-low cancer cells (Patsialou et al., 2015). Rovida research group demonstrated that the inhibition of the receptor reduced proliferation of claudin-low breast cancer cells (Morandi et al., 2011). The oncogenic role of CSF-1R was mediated by the interaction with the transforming growth factor-β-stimulated clone-22 (TSC-22) in cervical cancer cell lines. TSC-22 acts as onco-suppressor, and it is expressed at low levels in cancers including cervical cancer. CSF-1R was found as a target of TSC-22, that interacted with tyrosine domain (539-749 a.a.) of its intracellular region by blocking its activity (Cho et al., 2017). In melanoma cell lines, pharmacological and genetic targeting of the receptor induced lower proliferation in 3D culture and increased apoptotic rate, suggesting a pro-survival role for CSF-1R (Giricz et al., 2018). Increased cell viability, ki-67 cell positivity and colony forming ability was also observed in CSF-1R overexpressing glioma cells (Sun et al., 2019). On the contrary, in T-cell lymphoma cells, CSF-1R activation led to AKT phosphorylation in a PI3K-dependent manner (Murga-Zamalloa et al., 2020). Rattanaburee et al. identified CSF-1R as possible target of kusunokinin, a lignan molecule that has previously demonstrated anti-cancer effects. They discovered that Kusunokin down-regulated CSF-1R

expression levels by binding to the JMD of the receptor. The anti-proliferative activity of Kusunokin observed in breast cancer cells was associated to CSF-1R and AKT pathway suppression. The Kusunoki-mediated CSF-1R suppression also decreased the expression levels of G2-M markers as Cyclin B, CDK1, and c-Myc (Rattanaburee et al., 2020).

1.1.7.2. CSF-1R in Cell Migration

Cancer cell migration is a complex process involving different molecular mechanism and several evidence demonstrated that the CSF-1R is involved in promoting this process (Bouchalova & Bouchal, 2022; Huang et al., 2014). Sapi group (Sapi et al., 1998) showed that mammary epithelial cell invasiveness was associated with CSF-1R expression, and that the invasive capacity of these cells was negatively affected by a dominant negative mutant of the transcriptional component Ets2, suggesting that the involvement of Ets2 transcription factor in the CSF-1R signaling, which promoted the invasiveness of mammary epithelial cells (Sapi et al., 1998). Tumor derived from orthotopic mice injected with breast cancer cells depleted of CSF-1R, showed decreased invasive properties and reduced lung metastases compared to controls, suggesting a crucial role of CSF-1R in cancer cell motility and invasion (Patsialou et al., 2015). Partial epithelial to mesenchymal transition (EMT) was observed in inflammatory breast cancer, characterized by the expression of both Vimentin and E-cadherin. In this cancer types, treatment with BLZ945, a CSF-1R inhibitor, reduced the spindle-like phenotypes of cancer cells and reversed the partial EMT, indicating a role for CSF-1R in breast cancer invasiveness (Kai et al., 2018). The overexpression of CSF-1 in human ovarian cancer cell lines induced the acquisition of invasive and metastatic characteristics observed both *in vitro* and *in vivo*. Moreover, the metastatic activity of CSF-1 was mediated by the activation of the downstream urokinase-type plasminogen activator (uPA) pathway, which was linked to motility and invasiveness in several tumor types (Toy et al., 2009). Similarly, the administration of CSF-1 enhanced invasive potential of lung cancer in *in vivo* and *in vitro* studies (Hung et al., 2014). In human osteosarcoma cell lines, a side population of CSF-1R^{+} cells were found. The presence of the receptor was associated with the expression of mesenchymal markers and invasive capabilities, as in fact the CSF-1R-genetic silencing inhibited the EMT process and migration of tumor cells. Interestingly, the study identified *JAG1* as downstream target gene involved

in CSF-1R-related cell migration (Wen et al., 2017). Shi et al. identified the CSF-1R/STAT3/Mir-34a axis as regulator of pro-tumoral functions in colorectal cancer (CRC) (Shi et al., 2020) and CSF-1R knockdown was associated with reduced expression of the EMT marker, Vimentin in glioma cell lines (Sun et al., 2019). A functional role of CSF-1R in melanoma spread was also demonstrated by Giricz et al., showing a dose-related decrease in melanoma invasiveness after treatment with the CSF-1R inhibitor PLX-3397 (Giricz et al., 2018).

1.1.7.3. CSF-1R in Drug Resistance and Stemness

CSF-1R expression is upregulated in a 5-FU chemoresistant population of CRC cells (Shi et al., 2020). The acquisition of a resistant mechanism, as well as the expression of stem-like and EMT-related genes, was linked to downregulation of Mir-34a, implying that the CSF-1R/mir-34a pathway could be a potential target against CRC chemoresistance and invasiveness (Shi et al., 2020). Mir-34a deficiency correlated with CSF-1R up-regulation and increased the expression of the stemness marker *Lgr5,* as well as the tumor spheroid formation capabilities in intestinal adenoma cells (Liu et al., 2022). Melanoma resistant cancer cell lines showed aberrant CSF-1R expression (Giricz et al., 2018) and mesothelioma primary culture and cell lines showed that autocrine activation of CSF-1R characterized a population of malignant cells with a stem-like and chemoresistant phenotype. The study also reported that the chemoresistant phenotype of CSF-1R cells correlated with the activation of the AKT signaling pathway. Indeed, AKT blockade sensitized cells following Pemetrexed treatment (Cioce et al., 2014).

1.1.8. Therapeutic Strategies for CSF-1R

Given CSF-1R function in disease, several strategies have been developed in recent years to target the receptor. Long-term administration of GW-2580, a selective CSF-1R inhibitor, to transgenic mice model of Alzheimer's disease caused depletion of CSF-1R+ microglia and protected from synaptic damage (Olmos-Alonso et al., 2016). Numerous small compounds and monoclonal antibodies (mAbs) against CSF-1/CSF-1R axis have been developed and tested for cancer therapy purpose, both as monotherapy and in combination with immunotherapies, chemotherapeutic drugs, and target therapy. CSF-1R inhibitors have been explored in conjunction with surgery, either in the neoadjuvant or adjuvant setting, and have been shown to improve patient

outcomes (Cannarile et al., 2017; Peyraud et al., 2017). Small molecules inhibitors of CSF-1R include PLX3397, PLX7486, ARRY-382, BLZ945, and JNJ-40346527, anti-CSF1R mAbs under investigation include emactuzumab, AMG820, IMC-CS4, cabiralizumab, and lastly, anti-CSF-1 mAbs include MCS110, and PD-0360324 (Cannarile et al., 2017). The small molecule Pexidartinib or PLX3397, which inactivates the receptor's kinase domain, has been tested in pre-clinical and clinical settings of different cancer models, where the levels of CSF-1R correlated with tumor aggressiveness. Due of its anti-tumoral properties, the Food and Drug Administration (FDA) has approved PLX3397 for the treatment of Tenosynovial giant cell tumor (TGCT) in 2019 (Benner et al., 2020). However, the efficacy of Pexidartinib was preclinically evaluated in other tumors, including lung adenocarcinoma, prostate cancer, rand GIST tumor (Cuccarese et al., 2017; Kim et al., 2014; Xu et al., 2013). The combination of Paclitaxel and PLX3397 for breast tumor treatment reduced tumorigenesis and metastasis in a tumor-bearing animal model (DeNardo et al., 2011). In mice with $BRAF^{V600E}$ positive melanoma tumors, treatment with PLX3397 combined with BRAF inhibitors increased the antitumor response of the resistant cancer. The combinatory effect was given by synergic activity of the drugs which were able to negatively affect the immunosuppressive component of the TME, including M2-TAMs and to restore T-cell activity (Ngiow et al., 2016). Another small molecule for CSF-1R-inhibition, BLZ945, was also shown as effective in preclinical studies for the treatment of glioblastoma, where it improved survival rate of transgenic murine models and decreased tumor aggressiveness, as well as reduced TAMs recruitment and elicited the cytotoxic immune response in mammary and cervical tumors (Pyonteck et al., 2013; Strachan et al., 2013). In a variety of solid tumors, BLZ-945 has been tested in Phase I/II clinical studies, either as a single agent or in combination with anti-PD-1 antibodies (Peyraud et al., 2017). The Ries research group developed the first anti-CSF1R humanized mAb known as Emactuzumab or RG7155. In patients with various solid tumors including primary pleural mesothelioma, endometrial carcinoma, and colorectal cancer the research showed a significant reduction of $CSF\text{-}1R^{+}$ TAMs associated with late cancer growth and metastatic activity (Ries et al., 2014). Clinical trials for other humanized IgG mAb, Cabiralizumab, AMG-820 and IMC-CS4, which bind CSF-1R and prevent its activation are ongoing (Peyraud et al., 2017). Current clinical trials with anti-CSF-1R targeted therapy are reported in **Table 1**.

Table 1. Data are adapted from Cannarile, M. A., Weisser, M., Jacob, W., Jegg, A.-M., Ries, C. H., & Ruttinger, D. (2017). Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *Journal for ImmunoTherapy of Cancer*, *5*(1), 53.

Class	Compound	Combination Drug	Clinical Phase	Tumor	Clinical Trials
Small Molecules	Pexidartinib (PLX3397, PLX108-01)	Pembrolizumab (anti-PD1 mAb)	I/II	Solid tumors, malignant melanoma GIST, NSCLC, ovarian carcinoma, TNBC, SCCHN, UBC, pancreatic cancer, gastric carcinoma, leiomyosarcoma, cholangiocarcinoma, CRC (MSS)	NCT02452424
		Durvalumab (anti-PDL1 mAb)	I	Pancreatic Carcinoma, CRC	NCT02777710
	BLZ945	PDR001 (anti PD-1 mAb)	I/II	Solid tumors	NCT02829723
	Array-382	Pembrolizumab (anti-PD1 mAb)	I	Solid tumors, melanoma, NSCLC	NCT02880371
Monoclonal Antibody	Emactuzumab (RG7155)	Atezolizumab (anti-PDL1 mAb)	I	Solid tumors, TNBC, gastric cancer, soft tissue sarcoma, UBC, ovarian cancer, NSCLC, melanoma	NCT02323191
		RG7876 (CD40 agonist mAb)		TNBC, gastric cancer, mesothelioma, CRC, melanoma, pancreatic cancer	NCT02760797
	AMG820	Pembrolizumab (anti-PD1 mAb)	I	Solid Tumors	NCT02713529
	Cabiralizumab (FPA008)	Nivolumab (anti-PD1 mAb)	I	Solid tumors, NSCLC, SCCHN, pancreatic cancer, ovarian cancer, RCC, GBM	NCT02526017
	IMC-CS4 (LY3022855)	Durvalumab (anti-PDL1 mAb) or Tremelimumab (anti-CTLA4 mAb)	1	Solid tumors	NCT02718911

1.2. CELL CYCLE

The process known as *Cell cycle* includes a sequence of events which culminate in DNA replication (DNA synthesis or S-phase) and the segregation of newly duplicated chromosomes (Mitosis or M-phase) into two genetically identical daughter cells (Malumbres & Barbacid, 2009). Interphase has gaps between the M and the S-phase, including Gap1 (G1). In this phase, cell increases in size, begins to transcribe cell cycle control genes, and commit to either proliferate or to become dormant (G0). Subsequently, cells enter the S-phase, where DNA synthesis occurs. This phase is followed by another gap, Gap2 or G2, in which cells check for DNA duplication errors and commit to mitosis (Rieder, 2011). In mitosis, a series of dynamic processes lead to nuclear division. The first step of M phase is the condensation of chromosomes: duplicated DNA strands package and condense into more compact chromosomes. Eventually, the nuclear membrane breaks down, and the replicated chromosomes, each made up of two sister chromatids, attach to the mitotic spindles' microtubules. During metaphase, the chromosomes align at the equator of the mitotic spindle. Chromosomes then travel to opposite poles of the spindle during anaphase, where sister chromatids suddenly separate and recover their complete nuclei. Cell division ends with cytoplasmic division, also known as cytokinesis, when cells divide into two daughter cells (Malumbres & Barbacid, 2009) (**Figure 4**).

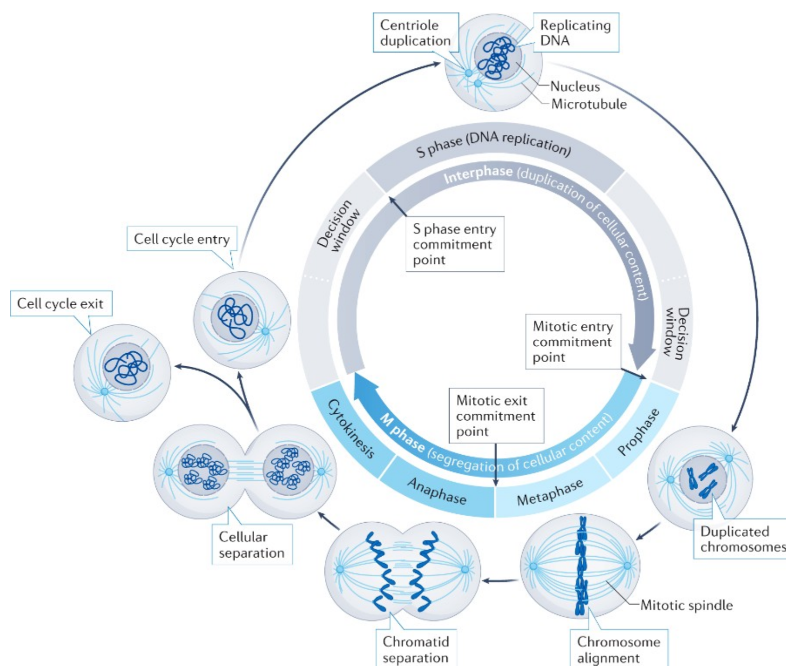


Figure 4. Schematic representation of the cell cycle phases. The process by which a cell replicates to produce two identical daughter cells is known as cell cycle. It is composed by two main phases, the interphase and mitotic or M-phase. Interphase includes the intermediate checkpoint phase and DNA replication phase, or S-phase. DNA dissociation and cellular segregation take place during the various sub-phases of the M-phase. New cells have the option to stop or restart cell division (Matthews et al., 2022).

1.2.1. Cell cycle Regulation

The cell cycle is controlled by cyclin-dependent kinases (CDKs) and cyclins. CDKs are conserved protein kinases that phosphorylate serine/threonine domain of targeted proteins (Malumbres & Barbacid, 2009). Cyclins activate the kinase activity by binding to CDKs. Cyclin D (cyclin D1, D2, or D3) interacts with CDK4 or CDK6, forming a Cyclin D–CDK4/6 complex, which phosphorylates and inactivate the RB proteins (Narasimha et al., 2014) as in fact hypo-phosphorylated RB proteins bind to the E2F transcription factors preventing cell cycle progression. At the G1- to S-phase transition, E-type cyclins (E1 and E2) bind to and activate CDK2, which promotes RB phosphorylation and detachment from E2F. Subsequently, E2F promotes the expression of genes necessary for DNA replication and cell cycle progression. Finally, numerous cellular proteins required for mitotic progression are phosphorylated by the cyclin B/CDK1 complex. CDK activity is controlled by two main classes of CDK inhibitors: the INK4 family, including p16, p15, p18, and p19, and the CIP/KIP family consisting of p21, p27 and p57 (Besson et al., 2008) (**Figure 5**). The tumor suppressor protein p53 induces transcription of p21, the first CDK inhibitor identified in mammalian cells, providing a mechanism to delay proliferation after DNA damage and allowing time for assessment and repair (Wade Harper et al., 1995). CDK inactivation prevents it from phosphorylating and inactivating downstream targets, which stop the cell cycle (Charrier-Savournin et al., 2004).

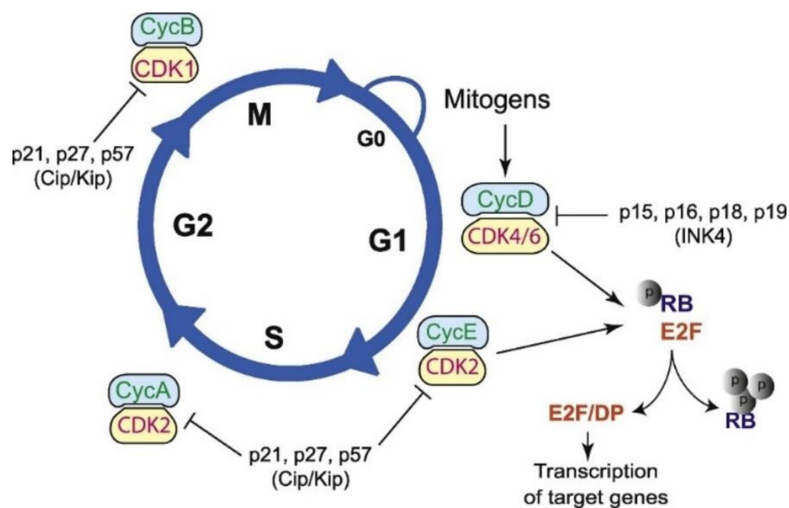


Figure 5. Schematic representation of cell cycle progression. Cell cycle progression is controlled by cell cycle regulators. During G1 phase, Cyclin D (Cyc D) and CDK4/6 complexes aggregate, phosphorylate, and inactivate the Rb proteins. E2F transcription factors are released by hyperphosphorylated Rb, promoting the transcription of target genes required for S phase advancement. The complex CDK2/Cyclin A controls the S-G2 phase transition, while Cyclin B/CDK1 complex modulates mitosis. CKIs protein families (INK4 and Cip/Kip) negatively modulate the activation and functionality of the various Cyc/CDKs complexes (Leal-Esteban & Fajas, 2020).

1.3. MESOTHELIOMA

Mesothelioma is a tumor originating from the mesothelial layer of the pleura, but also in peritoneum and tunica vaginalis. The most widespread type of mesothelioma is malignant pleural mesothelioma (MPM), accounting for the 80% of all the cases. The exposure to asbestos fibers, including occupational and environment exposure, is primarily responsible for the insurgence of MPM. Indeed, mesothelioma is considered an occupational disorder (Noonan, 2017). Mesothelioma is characterized by a long latency period, in fact, it appears clinically during the elderly. The late diagnosis is associated with a short overall survival of mesothelioma patients of approximately 6-12 months (Mott, 2012; Kusamura et al., 2023). Recent work of Carbone group (Carbone et al., 2022) reported an increase in mesothelioma cases among young patients with inherited *BAP1* germline mutations. Patients with *BAP-1*-related mesothelioma show early clinical manifestations that allow an early diagnosis and effective therapeutic intervention, as in fact, the tumor appears less aggressive with a high survival rate. In light on this, germline *BAP-1* mutations are considered positive prognostic factors for mesothelioma (Carbone et al., 2022).

The distinctive inflammatory microenvironment induced by asbestos fibers deposition is largely responsible for the pathogenesis of the malignancy. Pleural macrophages are unable to remove fibers and their accumulation leads to ROS release, abnormal signaling pathway activation and genetic changes favoring malignant transformation of mesothelial cells (Cersosimo et al., 2021). However, other non-asbestos related causes are associated with MPM development such as the exposure to radiation, other occupational minerals, pulmonary inflammation, and genetic mutations (Attanoos et al., 2018). MPM is characterized by a low mutational burden. The most frequent is the deletion of *CDKN2A* on chromosome 9, that induces alterations in the mechanisms regulating cell cycle. Other important mutations in mesothelioma are the loss of *BAP1* in chromosome 3, associated to genome instability, and mutations in *NF2* gene, and in *TP53*. Recently, it has been suggested that *BAP-1* loss and *CDKN2A* deletion are helpful indicators to increase the diagnostic sensitivity for MPM(Bueno et al., 2016; Cheng et al., 2020; Husain et al., 2018).

The prognosis of mesothelioma is highly associated with its histological presentation. Epithelioid, biphasic and sarcomatoid represent the primary histological subgroups (Franklin et al., 2016). The epithelioid subtype is characterized by the presence of epithelioid cells with a heterogenic morphology, including trabecular, tubulopapillary, micropapillary, transitional and small cellular architecture. It is associated with better prognosis because patients might benefit from surgical approaches. The sarcomatoid subtype is considered as an infiltrative proliferation of spindle cells, or cells with mesenchymal appearance. It frequently associated with poor prognosis and metastasis.

Finally, the biphasic type is considered half epithelioid and half sarcomatoid, but the proportion of one subtype vs the other is what determines the prognosis and responsiveness to surgery (Brcic & Kern, 2020) (**Figure 6**).

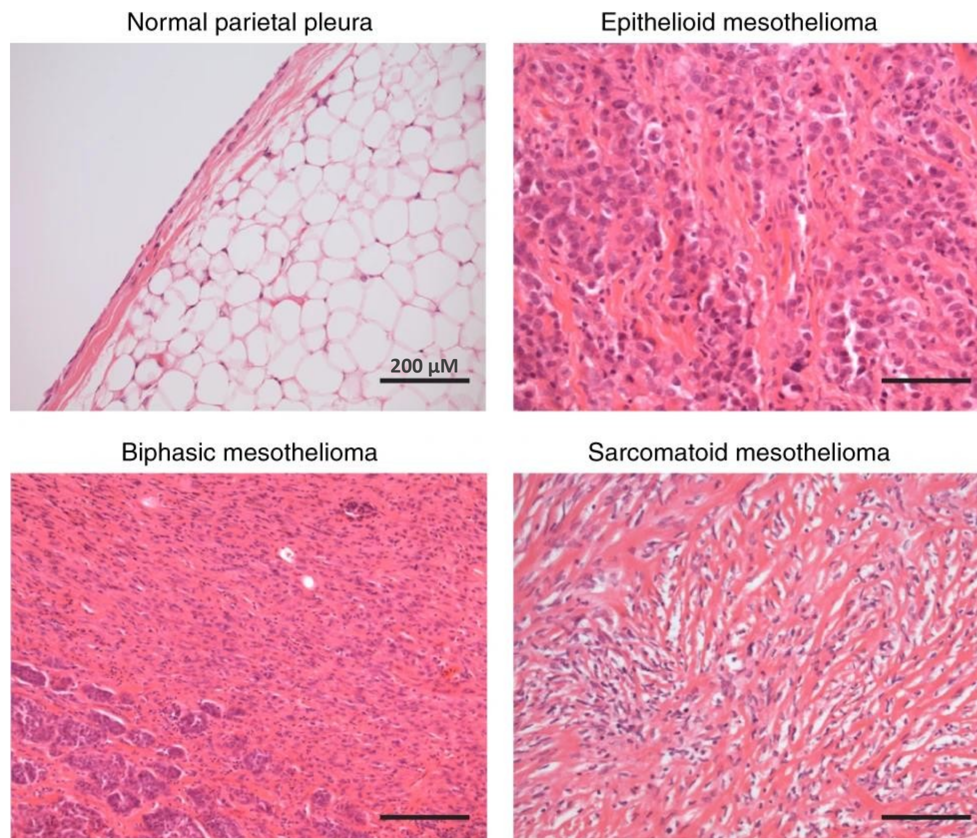


Figure 6. **H&E of Mesothelioma histotypes**. Normal tissue, epithelioid, sarcomatoid and biphasic mesothelioma histotypes were stained by hematoxylin and eosin. Images show the presence of flat and cuboidal cells in epithelioid tissue and spindle-like cells with abundant stroma in the sarcomatoid ones. The image was adopted from Obacz et al., 2021.

There are only a few treatment options for mesothelioma, which include surgery, chemotherapy, and radiotherapy. Surgery is indicated for early-stage patients or not aggressive tumor; otherwise, unresectable tumor required chemotherapy. The first-line treatment is a combination of cisplatin and pemetrexed. Bevacizumab (anti-VEGF) might be included to chemotherapy regimens for patients without cardiovascular disorders (Kindler et al., 2018). In case of symptomatic disease, radiotherapy has been used as an adjuvant or treatment (Hanna et al., 2021). The addition of immunotherapeutic such as immune checkpoint inhibitors, to systemic chemotherapy has demonstrated effective results and clinical trials are ongoing. Nivolumab (anti-PD-1) and ipilimumab (anti CTLA-4) have been just approved as first-line treatment for adult patients with unresectable MPM in October

2020 (Meirson et al., 2022; Nakajima et al., 2022). Additionally, a chimeric antigen receptor (CAR) targeting mesothelin, the cell-surface antigen highly expressed in mesothelioma was also generated and nice anti-tumoral response was reported, as single agent or in combination with anti-PD-1 (Adusumilli et al., 2019).

It has been reported that asbestos can affect several signaling pathways involved in proliferation, differentiation, and migration (Matsuzaki et al., 2012). Overexpression of receptor tyrosine kinases (RTKs) in mesothelioma was associated with the activation of mitogenic and pro-survival pathways including PI3K/Akt and MAPKs signaling (Malakoti et al., 2022). EGFR is frequently overexpressed in MPM cells**.** However, it is still debated the prognostic value of EGFR overexpression, which is not associated with EGFR mutation of amplification (Chia et al., 2019). Similarly, MPM and not mesothelial cells showed significant levels of MET receptor without evidence of genetic mutations (Thirkettle et al., 2000). Additionally, both VEGF and VEGFRs are expressed by MPM cells, and support tumor angiogenesis and MPM growth (A. Y. Lee et al., 2007). In MPM, several alterations in the RTK's downstream signaling pathway were previously investigated. The discovery of abnormal activation of signaling cascade, including those of ERK1/2, AKT, Hippo and Wnt pathways provided new potential candidates for future target therapeutic strategies (Jean et al., 2012). According to Yang et al. (Yang et al., 2023), the average level of hyperactivated MAPK/RAS signaling was the highest in MPM, compared with other tumor types. However, treatment with MEK inhibitor (MEKi) resulted ineffective in stopping tumor cell growth due to the activation of DNA protective mechanisms, such as the DNA repair protein PARP. In fact, the combined effect of MEKi and PARP inhibitors showed better efficacy, causing cancer cell death by releasing cytotoxic reactive oxygen species (ROS) (Yang et al., 2023). Alteration of the Hippo/YAP pathway have been previously described in mesothelioma. Interestingly, *in vivo* studies demonstrated that genetic inhibition of *YAP-1* induced great cancer regression and prevented the progression of the tumor (Calvet et al., 2022). Recent study demonstrated that the growth factor Progranulin is involved in the malignancy of MM. Progranulin signaling has been found to promote cell motility and tumor progression by modulating FAK activity in mesothelioma (Ventura et al., 2022) . Additionally, a recent work showed that in MM cells, Progranulin activity is mediated by EGFR and the co-receptor for the Wnt pathway, RYK (Ventura et al., 2023).

2. AIM

Malignant mesothelioma (MM) is an aggressive tumor diagnosed in advanced stage and characterized by poor prognosis due to inefficient therapeutic strategies. Recent clinical trials with immunotherapy are encouraging, but benefits are only for a small group of patients. A better understanding of cellular and molecular mechanisms regulating MM are required in order to design novel therapeutic approaches. CSF-1R expression was primarily observed in myeloid lineage cells where it is essential for survival and growth. However, several evidence demonstrated that the CSF-1R is also expressed in cancer cells of various tumor types including MM. In MM, CSF-1R expression characterized a subset of tumor cells with chemoresistant capabilities and stem-like phenotype and its inhibition negatively affected tumor growth. Additionally, both in macrophages and glioma cells CSF-1R expression correlated with the transcription of cell cycle related factors. In preliminary experiment we observed that CSF-1R was preferentially expressed by cells in the mitotic phase. However, little is known about the mechanisms regulating its expression and activity during cell cycle in cancer cells. Our hypothesis is that CSF-1R undergoes different regulation during cell cycle and it acts supporting proliferation of tumor cells. We hypothesize feedback in which CSF-1R is required for cell cycle progression and that cell-cycle related factors regulated its expression.

On the bases of this hypothesis the aim of my thesis was:

1. The characterization of CSF-1R expression in mesothelioma cell lines: CSF-1R activity in cancer cell proliferation and whether its expression is differently regulated during cell cycle phases.
2. The evaluation of the effect of CSF-1R pharmacological inhibition on cancer cell cycle progression.
3. The molecular mechanisms regulating CSF-1R expression during cell cycle.

From this study, we expected to observe different expression levels of CSF-1R among cells, based on their proliferation status, likely, to identify cell-cycle related molecules regulating CSF-1R expression in proliferating cancer cells. Our study wants to provide a new understanding of CSF-1R regulation during the cell cycle in cancer cells and to determine CSF-1R signaling cascade molecules as potential therapeutic targets.

4. MATERIALS AND METHODS

3.1. Cell Culture

MPP-89, NCI-H2052 and MSTO-211H cells are human malignant pleural mesothelioma (MPM) cell lines obtained from ATCC. All the cell lines were cultured in RPMI-1640 modified medium (GIBCO) added with 10% Fetal Bovine Serum (FBS), 1% Penicillin-streptomycin and grown at 37°C/5% CO2. Hela Cells are human cervical carcinoma cell lines obtained from ATCC, cultured in High Glucose DMEM supplemented with 10% FBS and 1% Penicillin-streptomycin at 37°C/5% CO2. SKBR3 cells are human breast cancer cell lines obtained from ATCC. Cells were cultured in DMEM medium supplemented with 10% FBS, 1% Penicillin-streptomycin and, maintained in a humidified atmosphere at 37°C/5% CO2. All the cells described are adherent ones. Cells were passed every two days, at 80% of confluence. Routine passage consisted of removing the medium, washing the cells in PBS, detachment of the cells with Trypsin-EDTA Solution 10X, centrifuging the cells at 1100 rpm for five minutes, and discarding of the supernatant. The cell pellet was resuspended in fresh medium, and the cells were counted according to the experiment setting.

3.2. Colony Formation Assay

Colony formation assay is an *in vitro* methodology used to assess the growth and survival of cells treated with cytotoxic agents, proteins, or drugs. Cells (4 x 10^3 cells/well) were cultured in a 6-well plate for 24 hours in 10% FBS medium. Cells were then cultured in medium at 0,1% FBS and stimulated with the cytokines, recombinant human M-CSF and IL-34 (Peprotech), every 48 h for seven days at a concentration of 20 ng/ml. After 7 days, cells were fixed with 4% paraformaldehyde for 15 minutes, cleaned in PBS and stained with crystal violet (0,1% in 10% EtOH) in agitation for 15 minutes. Cell count was done by ImageJ.

3.3. 5-ethyl-2'-deoxyuridine (Edu) Proliferation Assay

To assess cells in S phase, MPM cell lines were plated and, treated with either cytokines, inhibitors, or synchronization drugs. Then, cells were subjected to Click-iT® Assay by Invitrogen. Cells were pulsed with 5-ethyl-2'-deoxyuridine (Edu) labeling solution (10 µM) for 30 min at 37 °C. Cells were washed twice and fixed in 4% paraformaldehyde for 15 min at room temperature. After washing, cells were permeabilized by adding 0,03% Triton X-100 in PBS for 15 minutes. Then, cells were washed in PBS and Edu-positive cells were labeled with fluorescent azide probes before immunofluorescence labeling. DNA was stained with DAPI.

3.4. Cell Transfection

The TransIT®-2020 Transfection Reagent (Mirus) was used for cell transfection experiments. Cells were first plated (0,8-3,0 x 10^5) in complete growth medium. Cells should be 80% of confluence at the time of transfection. TransIT®-2020 reagent was warmed at room temperature and gently vortexed. 250 µL of OptiMEM® Reduced-Serum medium was placed in a sterile tube, 2,5µl of DNA (1µg/µl) and 7,5µL of TransIT®-2020 reagent were added to the tube. The mixture was incubated at room temperature for 15-30 minutes and finally added dropwise to different areas of the well. After 5-7h, cell medium was replaced with fresh medium. After 24-72h of incubation cells were harvested and prepared for the assay.

3.5. CSF-1R inhibitor

GW-2580 (Kind gift by Dr. Elisabetta Rovida, University of Florence) is a selective CSF-1R inhibitor, preventing CSF-1R autophosphorylation. The inhibitor was diluted in DMSO and used on cancer cells at the concentration of 10 µM. It was used at different time according to the experimental design.

3.6. Antibodies

Antibody	**Target**	**Company**	**Cat. Number**	**Application**
PE-anti-human CD115 Ab	CSF-1R	BioLegend	347303	FACS
CSF-1R/M-CSF-R (E4T8Z) Rabbit mAb	CSF-1R	Cell Signaling Technology	28917	WB
CSF-1R/M-CSF-R (D3O9X) XP® Rabbit mAb	CSF-1R	Cell Signaling Technology	67455	IF; IHC
Phospho-CSF-1R/M-CSF-R (Tyr923) Ab	pCSF-1R	Cell Signaling Technology	3406	WB
Anti-Histone(H3) phosphor-S10	pH3	Abcam	Ab5176	FACS/IF
Anti-FLAG M2®	FLAG	Sigma-Aldrich	F1804	WB/ IF
P44/42 MAPK Ab	ERK1/2	Cell Signaling Technology	9102	WB
Phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb	pERK1/2	Cell Signaling Technology	4370	WB
Erk5 Ab	ERK5	Cell Signaling Technology	3372	WB
Phospho-Erk5 (Thr218/Tyr220) Ab	pERK5	Cell Signaling Technology	3371	WB
Akt (pan) (C67E7) Rabbit mAb	AKT	Cell Signaling Technology	4691	WB

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb	pAKT	Cell Signaling Technology	4060	WB
Anti-β Actin mAb (C4)	β-Actin	Santa Cruz Biotechnology	Sc-47778	WB
Anti-GAPDH mAb (C9)	GAPDH	Santa Cruz Biotechnology	Sc-365062	WB
Anti-EGFR Ab-5(Hu) mAb	EGFR	Thermo Fisher Scientific	MS-316-P1	FACS
Rb D20 rabbit mAb	p105	Cell Signaling Technology	9313	WB
P107 Polyclonal Ab	p107	Proteintech	13354-1-AP	WB
RBL2 rabbit mAb D9T7M	p130	Cell Signaling Technology	13610	WB

3.7. Flow Cytometry Staining analysis

For staining of cell membrane protein cells were incubated with anti-human FcR blocking solution (Human TruStain FcX™ - BioLegend) for 15 minutes at 4 °C. Cells were then incubated with primary antibody for 30 minutes at 4°C and with secondary antibody for 15 minutes at 4°C. Where indicated, cells were incubated with anti-CD115 mAb biotin conjugated (Invitrogen, 13-1159-82), for 30 minutes at 4°C. After washing, cells were incubated with Streptavidin-FITC conjugated (Vector, A-2011) for 15 minutes at 4°C. Cells were washed, centrifuge and flow-cytometry analysis were done using a Guava EasyCyte equipment and data were analyzed by FlowJo software.

3.8. mRNA Extraction, Reverse Transcription and Real Time PCR

Cell lines were plated at a concentration of $5x10^5$ cells in complete culture medium. After 24h mRNA extraction was performed. Cells were synchronized in the desired cell cycle phase according to the synchronization protocol described below. mRNA extraction was performed by using Quick-RNA miniprep Zymo kit-lab (Zymo Research) according to the manufacturer's instructions. RNA concentrations were determined using Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). To evaluate CSF-1R mRNA, RNA samples were retro-transcribed to cDNA, 1µg RNA was retro-transcribed using High-capacity cDNA reverse transcriptase Kit with the appropriate Reverse Transcription Buffers (Applied Biosystems™). After RT-PCR, the sample were kept at -20 °C until their use. To detect mRNA levels of CSF-1R, SYBR-green real-time PCR was performed, by using the delta-CT method (Livak & Schmittgen, 2001) with β-actin as a housekeeping gene. Primers used are:

- CSF-1R fwd CCCGGATGAGTTCCTCTTCAC
- CSF-1R rev TCGATGATCTTCCAGCGGAC
- β-actin fwd CAGGGCGTGATGGTGGGC

- β-actin rev CTCGGTCAGCAGCACGG

3.9. Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized for 15 min with 0.03 % Triton X-100 in PBS. After saturation with 1% BSA for 1 hour, cells were incubated with specific antibodies over-night at 4°C or for 1h at room temperature. After 3 washes with PBS, cells were incubated with secondary antibodies for 30 min at room temperature. After washing, cell nuclei were stained with DAPI (dilution 1:2000) for 15 min.

For CSF-1R surface staining, live cells were incubated with primary antibody at 4 °C for 45min. Cells were washed in PBS and further incubated with biotinylated anti-rabbit IgG (1:600) for 15 min. After washing, cells were incubated with Streptavidin FITC (1:1000) (SA-FICT) for 15' min. Cells were then fixed in 4% paraformaldehyde for 15 min e stained with DAPI (1:2000) for 15 min at room temperature. Fluorescent images were viewed by using a Nikon eclipse E600. Image analysis was performed by using ImageJ software.

3.10. Immunohistochemical Analysis

Human breast cancer SKBR3 cells were plated on glass coverslip and synchronized with RO3306. Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized for 15 min with 0.03% Triton X-100 in PBS. Permeabilized cells were incubated with anti-CSF-1R (1:500) primary antibody 1h at room temperature. After washing, cells were incubated with secondary antibodies, HRP-conjugate anti-rabbit IgG (1:1000), for 30 min. The reaction was revealed by using Novolink Polymer (Leica Microsystems) followed by DAB. After washing, cells were stained with hematoxylin.

3.11. Protein Extraction and Western Blot

Cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific) supplemented with halt protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific) for 30 minutes on ice. The total protein concentration was estimated using a BCA assay (Thermo Fisher Scientific) and 50μg of protein samples were run on polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were then stained with 0.5% Ponceau in 5% acetic acid for 3 minutes to check the loading and then washed with a solution containing PBS 1X and Tween 0.01%. Nitrocellulose membranes were incubated with blocking solution (PBS 1X, Tween 0.01% and Milk 5% or BSA 5% according to the primary antibody). Membranes were then incubated with primary antibodies summarized in Table 1. Secondary antibodies used are anti-rabbit HRP-linked (7074) (Cell Signaling Technology) and the antibodies and m-IgGK BP HRP antibody from Santa Cruz Biotechnology (sc-516102). Signals were

detected through ECL (Thermo Fisher Scientific)and images acquired with LI-COR (Model 2800) Odyssey Fc Imager.

3.12. Synchronization Protocol

Cells were initially plated on petri dishes and grown in the appropriate mediums supplemented with 10% serum (FBS) for 24 hours.

To synchronize cells in late-G2/M phase, cells were treated with the microtubule depolymerizing agent Nocodazole (Zieve et al., 1980). Nocodazole was supplemented at a concentration of 100 ng/ml. To synchronize cells in G2/prophase, cultures were treated with nocodazole for 16 hours at 37 °C. Mitotic cells were harvested by mechanical shake-off, transferred to a tube and centrifuged at 1200×g for 5 minutes to remove the culture medium. Cell pellets were then used for FACS analysis.

Hydroxyurea (HYU) was used to synchronize cells in G1/S. HYU was used at a final concentration of 4 mM directly diluted in fresh medium for 24h and at 37 °C. At the end of the treatment, the obtained G1 phase-enriched culture was washed with PBS and incubated in fresh, 10% FBS, HYU-free medium to further progress through the S phase of the cell cycle. Samples were collected at various time points to obtain an appropriate distribution of cell cycle progression: after 3h wash-out cells are in early S-phase and after 5h in late S-phase (Apraiz et al., 2017).

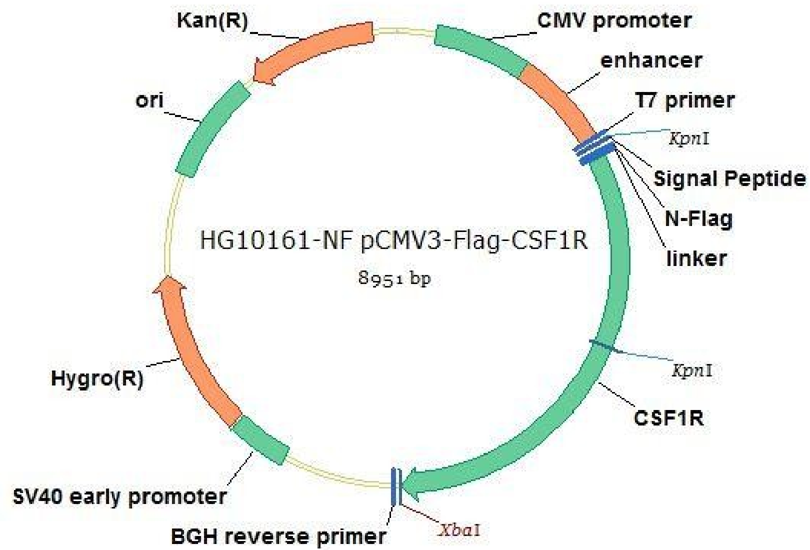
RO3306 (RO) is a CDK1 inhibitor used to synchronize cells in G2. Cells were treated with RO at a final concentration of 6μM for 18 hours at 37 °C. G2-synchronized cells were then harvested and analyzed. Then, RO was removed to allow cells to enter mitosis. After 45 min in fresh medium without RO, cells were harvested and analyzed (Tanenbaum et al., n.d.).

3.13. Silenced protein pocket cell lines

To generate RB1/p105, RBL1/p107 and RBL2/p130 depleted cells, HEK-293FT cells were transfected with PAX2 packaging plasmid, PMD2G envelope plasmid, and pLKO.1. The pLKO.1 vectors used: expressing a shRNA targeting the human RB1/p105, RBL1/p107 and RBL2/p130 mRNAs and Scrambled shRNA (pLKO.1 shSCR, gift from S. Stewart, Addgene plasmid #17920). Following transfection, supernatants were collected, filtered, and used for transducing MPM cells. Three days' post infection, cells were selected with 2μg/ml puromycin (Sigma-Aldrich).

3.14. cDNA constructs

Exogenous CSF-1R expression was performed by the transient transfection of the human MCSFR /CSF1R ORF mammalian expression plasmid expressing N-Flag tag (Sino Biological inc.) illustrated below.



3.15. Statistical Analysis

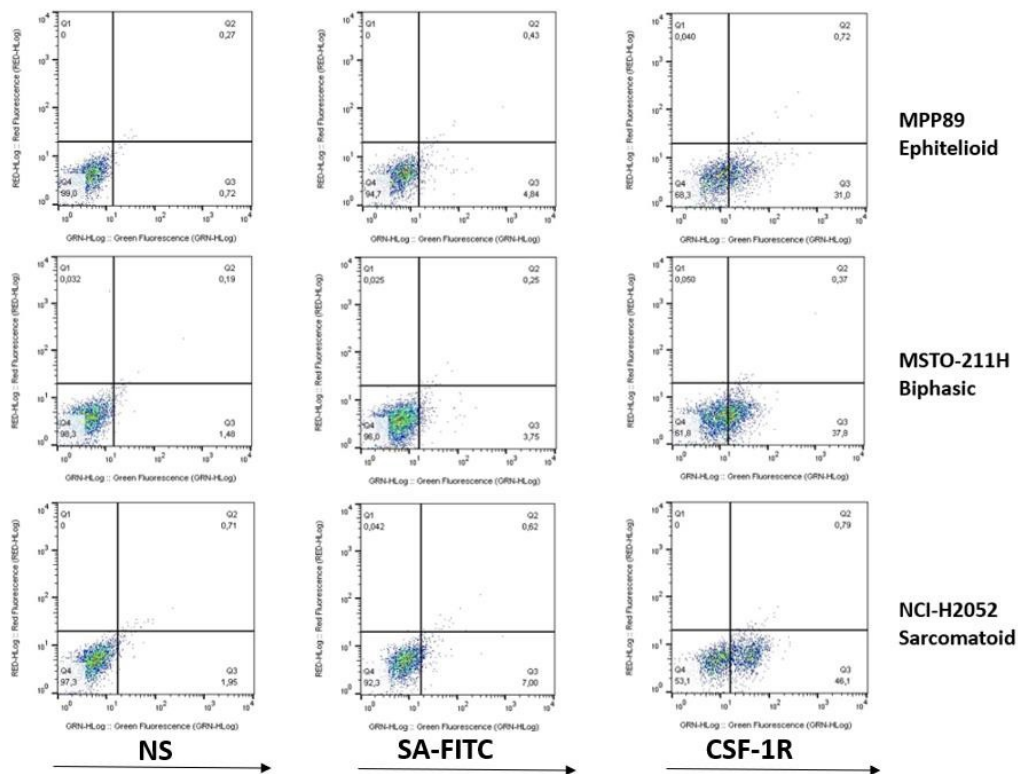
Statistical analysis was made using arithmetic average and standard deviation. Comparison of values between different experiments was performed by paired, two tailed Student's t test, using the software GraphPad Prism. Statistically significance was settled at *p<0.05, **< 0.01, ***p<0,001.

4. RESULTS

4.1. Analysis of Expression Levels of CSF-1R in MM Cell Lines

Since previous studies have shown increased expression of CSF-1R in primary MM cells compared to normal mesothelial cells (Cioce et al., 2014), we used three MPM cell lines (MPP-89, MSTO-211H, NCI-H2052) representing all main histological subtype of mesothelioma, and FACS analysis to evaluate the expression of CSF-1R at cell surface. All cell lines exhibited a sub-population positive for membrane-bound CSF-1R, with the sarcomatoid subtype - the most aggressive subtype - exhibiting the highest level of expression (**Fig. 7A**). We then used Immunofluorescence analysis on MPP-89. DAPI shape suggested that MPP-89 enhanced the expression of the receptor in cells with a mitotic-like morphology, suggesting that CSF-1R could be regulated during the cell cycle (**Fig. 7B**).

A.



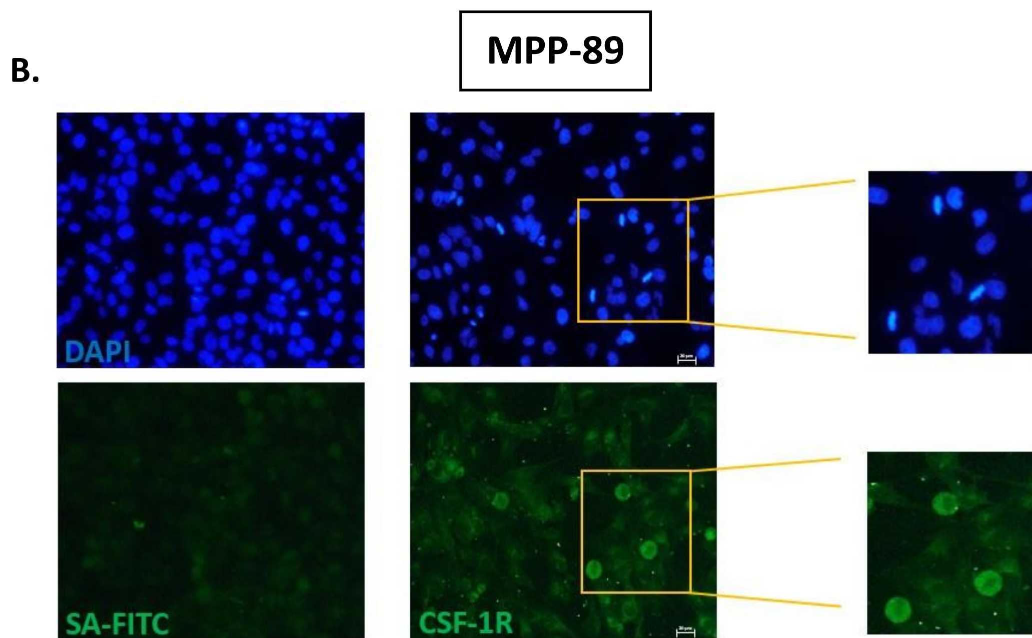
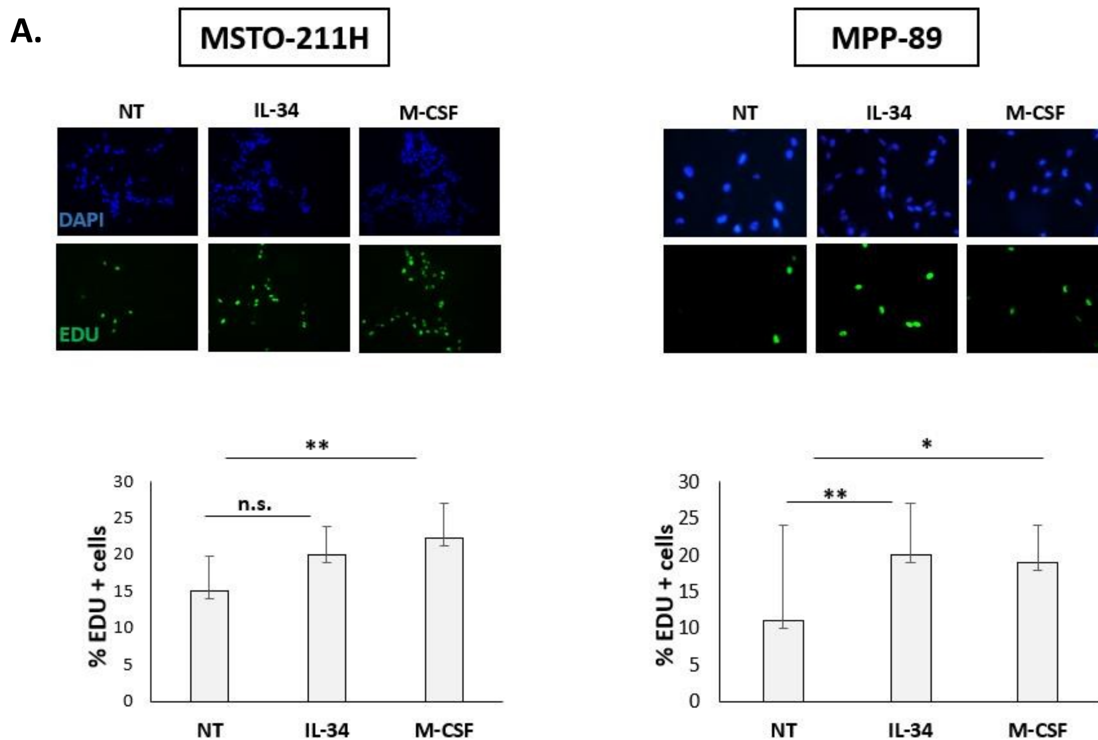


Figure 7. Characterization of CSF-1R expression in MM. (A) Surface CSF-1R was detected by Flow cytometry in MPP-89, MSTO-211H and NCI-H2052 cell lines with anti-CSF-1R-SA-FITC. (B) Representative Immunofluorescence images after staining against CSF-1R (green) in MPP-89 cells. DNA was visualized by DAPI staining (blue). NS= No staining. SA-FITC= Streptavidin-FITC. Scale bar=20μm.

4.2. Functional analysis of CSF-1R engagement in MM cell proliferation

To evaluate the functional role of the receptor, we assessed proliferative index of mesothelioma cells after stimulation with the CSF-1R ligands, IL-34 and M-CSF, using 5-ethynyl-2'-deoxyuridine (Edu) cell proliferation assay. MSTO-211H and MPP-89 cells were stimulated with the cytokines for 48 hours in serum-free media and then incubated with Edu kit according to the manufacturer's instructions and the number of Edu $^{+}$ cells (green) were analyzed by immunofluorescence. The results indicated that, stimulation of CSF-1R with its cognate ligands, enhanced the percentage of mesothelioma cells in S-phase as compared to non-stimulated cells (**Fig. 8A**). Next, we tested colony forming capabilities of MPM cells. Cells were stimulated with IL-34 and M-CSF every 48 hours. After 7 days of treatment in 0,1% of FBS-RPMI we measured the clonogenic ability of these cells. As shown (**Fig. 8B**), the activation of the CSF-1R pathway enhanced cell growth and colony formation as compared to unstimulated control cells, indicating that CSF-1R has a role in promoting cell proliferation of MM cells.



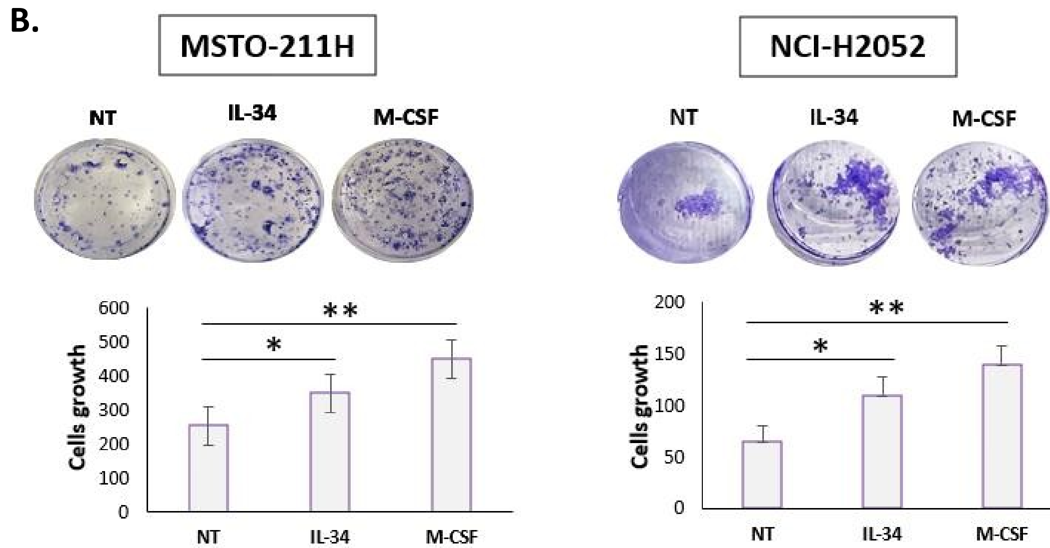


Figure 8. CSF-1R-induced cell growth and proliferation in MM cells. (A) Edu $^{+}$-incorporating MM cells were analyzed after 48h stimulation with Interleukin-34 (IL-34) and Monocyte Colony Stimulating Factor (M-CSF). The graph indicates the percentage of EDU $^{+}$ cells. The image is representative of two independent experiments. n.s.: not significant; **: p<0,01; *: p<0,05. (B) Colony formation assay of MSTO-211H and NCI-H2052 with or without IL-34 and M-CSF stimulation every 48h for 7 days in 0,1% FBS growing medium. Colonies were quantified by using ImageJ software. Graphical quantification of cell growth rate is indicated. +/- SD. **: p<0,01; *: p<0,05.

4.3. CSF-1R expression analysis during cell cycle phase

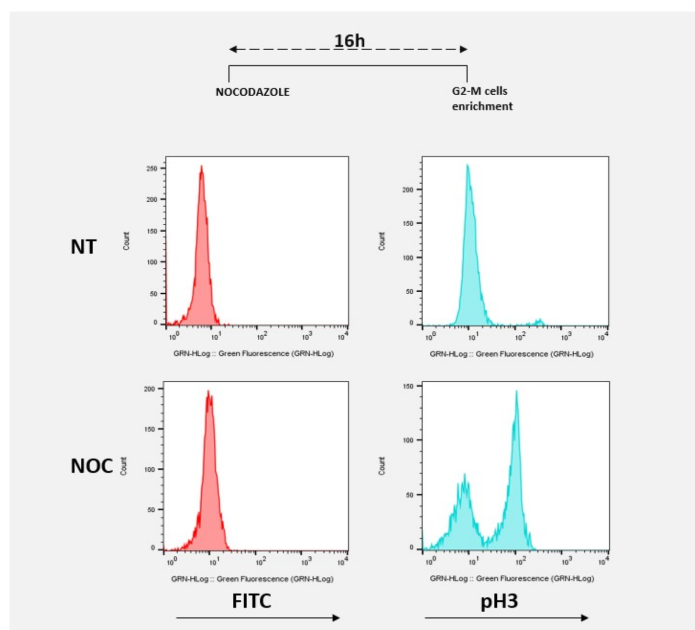
4.3.1. CSF-1R is up-regulated during G2-M phase:

Because our previous results indicated that CSF-1R can modulate cancer cell proliferation (**Fig. 8A**) and CSF-1R $^{+}$ cells with high expression were observed in mitotic phase (**Fig. 7B**), we investigated whether CSF-1R expression is regulated during cell proliferation. MPP-89 cells at 60% of confluence were synchronized in G2-M phase by incubating cells with the microtubule interfering agent, Nocodazole (100 ng/mL), for 16 hours (Zieve et al., 1980). Cell synchronization was confirmed by phospho-Histone-3 (pH3) staining, which is a well-known marker of cell mitosis (**Fig. 9A**). After treatment, we analyzed synchronized cells by flow cytometry staining in order to detect CSF-1R surface levels. The analysis revealed that nocodazole increased the percentage of CSF-1R $^{+}$ cells compared with non-treated (NT) condition (**Fig. 9B**). The expression levels of EGFR were assessed under the same conditions to rule out the possibility of non-specific nocodazole effect on other receptors. As shown in Fig 9C, we found that that EGFR levels did not change after Nocodazole treatment, suggesting that CSF-1R expression is positively regulated during G2-M phase (**Fig. 9C**). To better confirm the expression of CSF-1R in mitotic cells, Nocodazole-synchronized cells were stained for both pH3 and CSF-1R and analyzed by flow cytometry. As illustrated in Fig.

10A, most of pH3+ cells co-expressed CSF-1R, confirming that the receptor is positively regulated in mitotic cells (**Fig. 10A**).

To confirm this result, on CSF-1R modulation, we extended these experiments to other tumor cell types. The plots in Fig. 11A indicated the percentage of CSF-1R+ and EGFR+ cells in Hela cell lines, after Nocodazole treatment (**Fig. 11A**). We observed a shift in favor of CSF-1R+ cells. As control, we evaluated EGFR expression in comparison with CSF-1R levels in the G2-M population. We showed an increase in the number of CSF-1R+ cells after G2-M synchronization, whereas EGFR expression levels were almost unchanged in both treated and untreated cells (**Fig. 11A-B**). Moreover, we performed immunofluorescence staining in not-synchronized Hela cells. The analysis revealed a different localization of CSF-1R based on the cell cycle phase of the cells. Indeed, CSF-1R showed a surface, perinuclear and nuclear localization (**Fig. 11C**). Additional data in support to the hypothesis that CSF-1R is positively regulated during mitosis were indicated in Fig. 12. We synchronized SKBR3 cell lines in G2 phase by using the CDK1 inhibitor, RO3306 (RO), and by RO wash-out for 45 min we move cells to the M phase. Cells in the different cell cycle phases were identified based on the chromatin morphology stained with the DNA probe DAPI. At RO 45 min wash-out it was evident the metaphase plate representative of mitotic cells (**Fig. 12A**). We performed immunohistochemical analysis with anti-CSF-1R on SKBR3 synchronized respectively in G2 (RO, T0) and M phase (RO, 45min). As shown, the most CSF-1R+ cells were those with mitotic shape (spherical and less adherent), as in fact, the highest percentage of CSF-1R+ cells are in the condition of cells synchronized in M phase (RO 45min) (**Fig.12B**). Collectively, these data indicate that CSF-1R is positively regulated during G2-M cell cycle phase.

A.



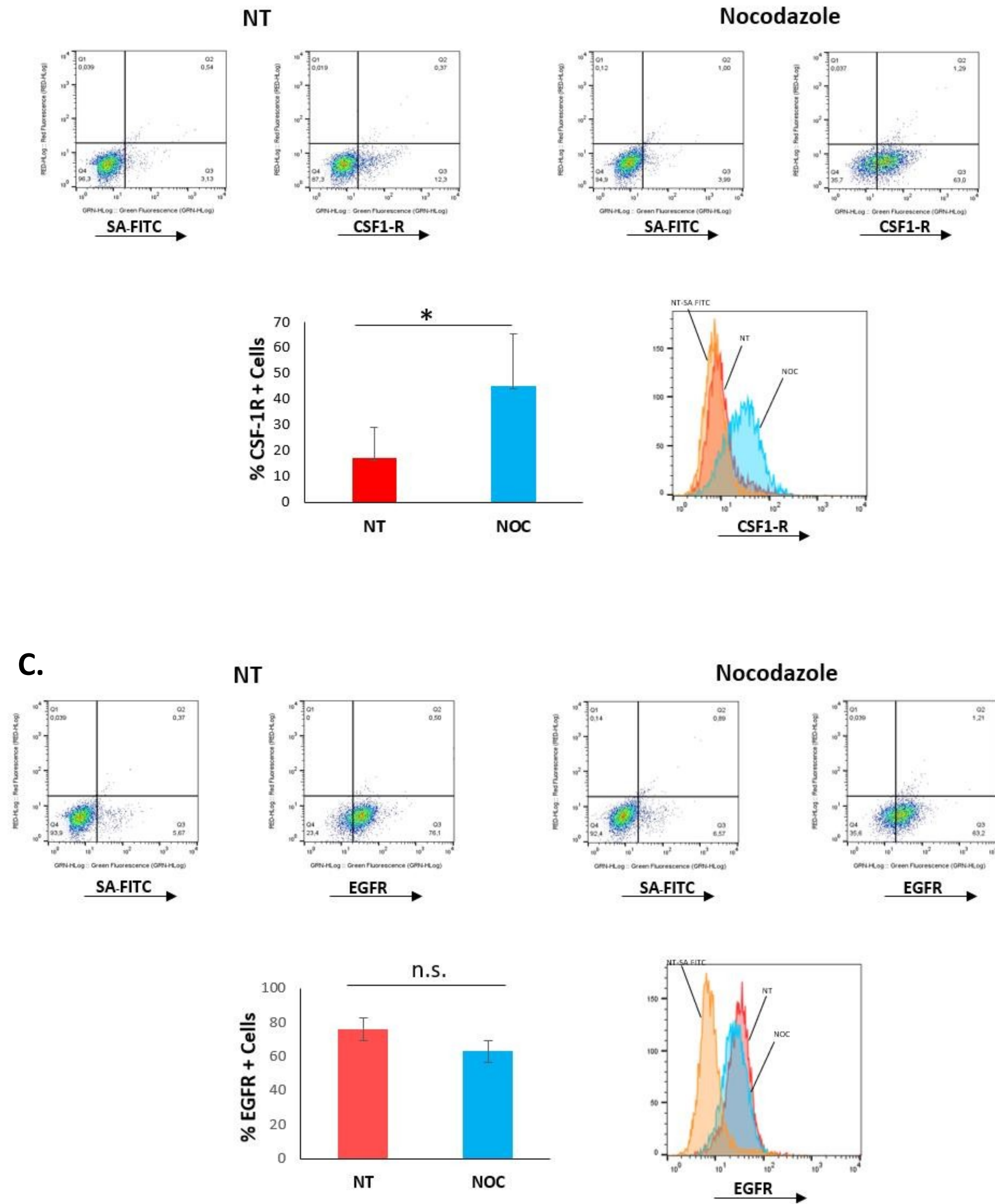


Figure 9. CSF-1R up-regulation during G2/M phase. (A) Time-course of Nocodazole treatment. Histograms are representative of phospho-H3 (pH3) intensity staining after Nocodazole treatment. (B) Representative flow cytometry plots of Streptavidin Fitc (SA-FITC) and CSF-1R in Non-treated (NT) and Nocodazole (NOC) treated MPP-89 cells. Graphs show the percentage of cells expressing CSF-1R in both conditions. The histogram is representative of the CSF-1R expression intensity in the flow cytometry analysis. (C) Representative flow cytometry plots of Streptavidin Fitc (SA-FITC) and EGFR in NT and NOC-treated MPP-89 cells. Graphs show the percentage of cells expressing EGFR in both conditions. The histogram is representative of the flow cytometry analysis. +/- SD, n.s.: not significant; *: p<0,05.

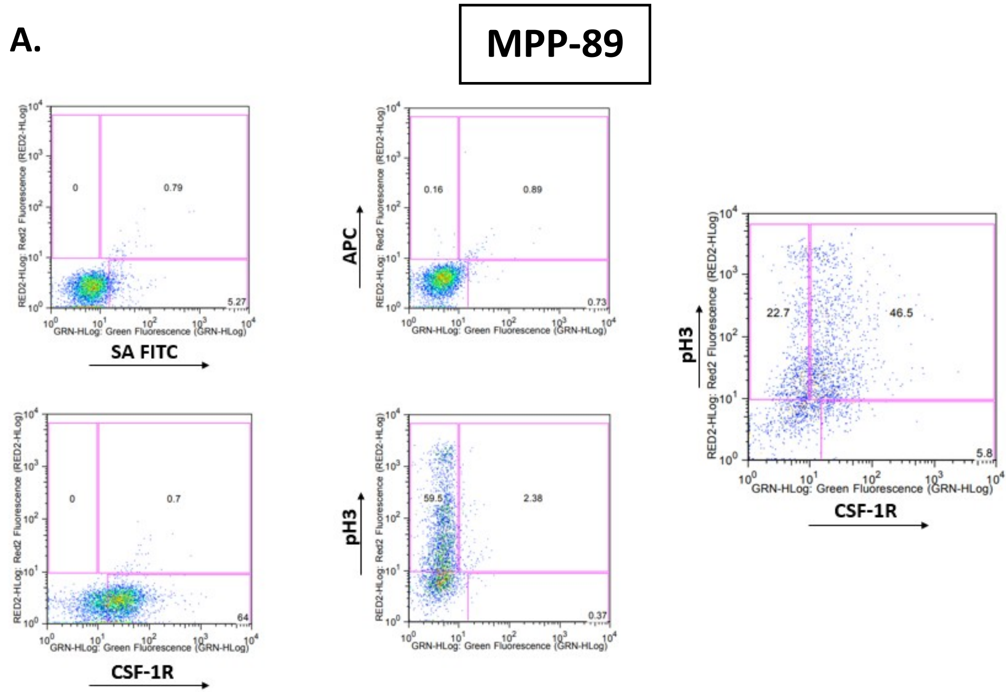
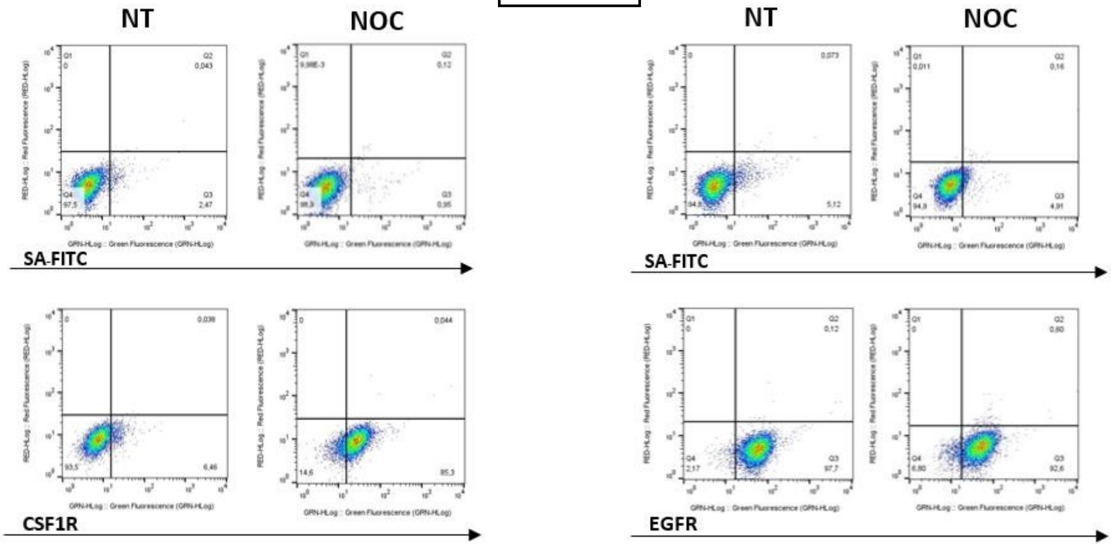
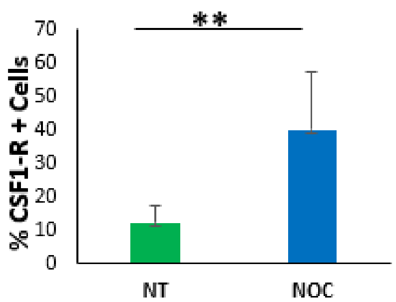
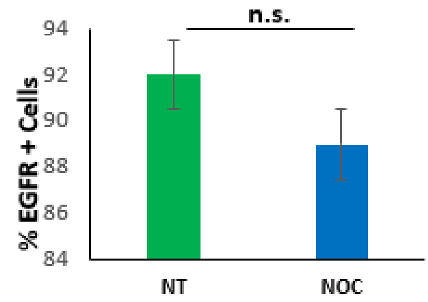


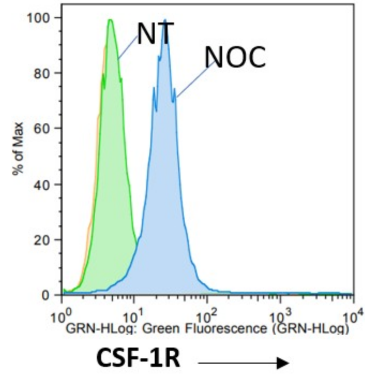
Figure 10. CSF-1R is expressed by pH3⁺ cells. (A) Representative flow cytometry plots of Streptavidin Fitc (SA-FITC), CSF-1R, Isotype-APC, phospho-histone-3 (pH3) and combined staining of CSF-1R/pH3 in Nocodazole-treated MPP-89 cells.

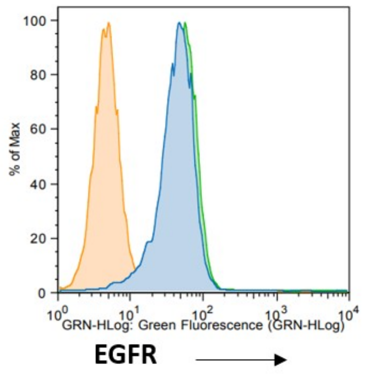






B.





	CSF-1R - MFI
NT	7,5
NOC	30

	EGFR - MFI
NT	59
NOC	49

C.

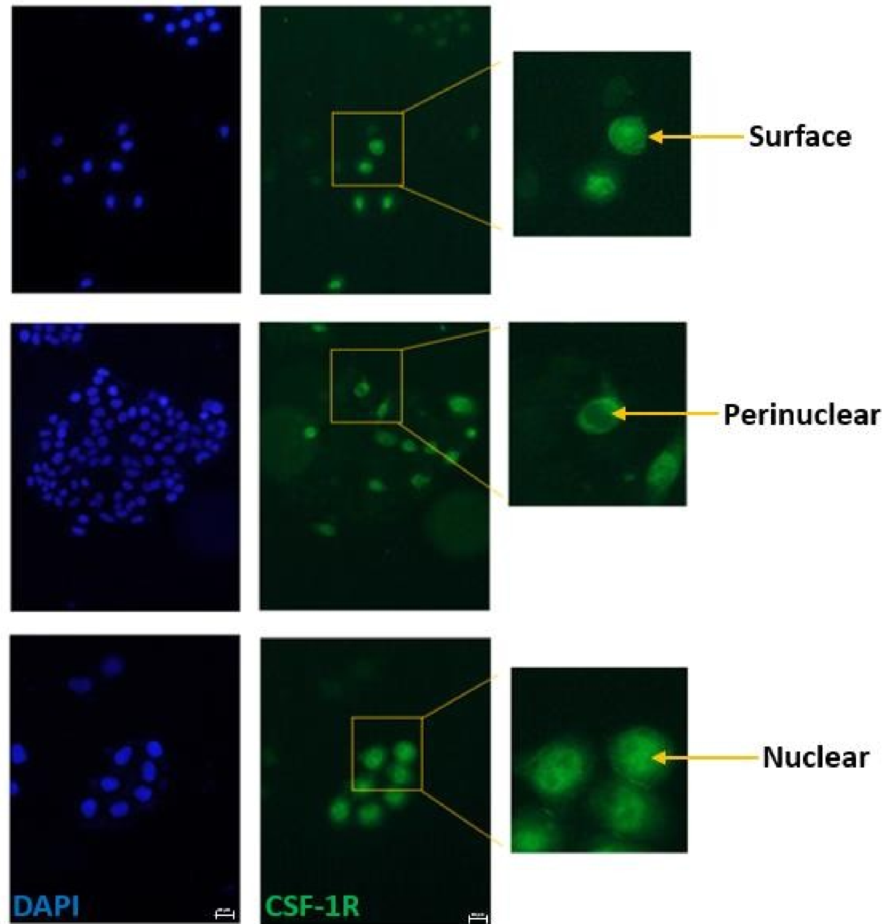


Figure 11. CSF-1R expression in mitotic tumor cells. (A) Representative plots of flow cytometry analysis of Hela cell lines with anti-CSF-1R and anti-EGFR in non-treated (NT) cells and Nocodazole-treated (NOC) cells. Graphical representation of A is indicated. (B) The histograms represent the mean fluorescent intensity of the CSF-1R (left) and EGFR (right) in Hela cells. CSF-1R and EGFR levels were measured in Nocodazole (NOC) treated cells (blue) and untreated (NT) cells (green), isotype (orange). (C) Representative Immunofluorescence images after staining against CSF-1R (green) in HELA cell lines. DNA was visualized by DAPI staining (blue). SA FITC= Streptavidin FITC. As mean +/-SD, n.s.: not significant, **: p<0,01.

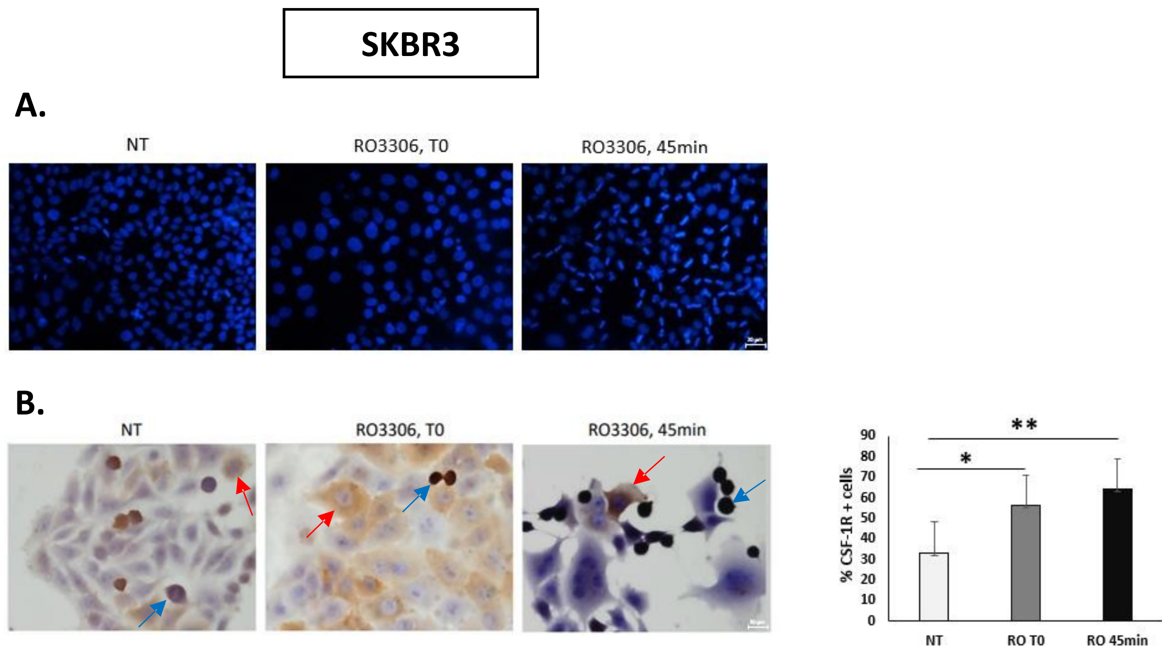
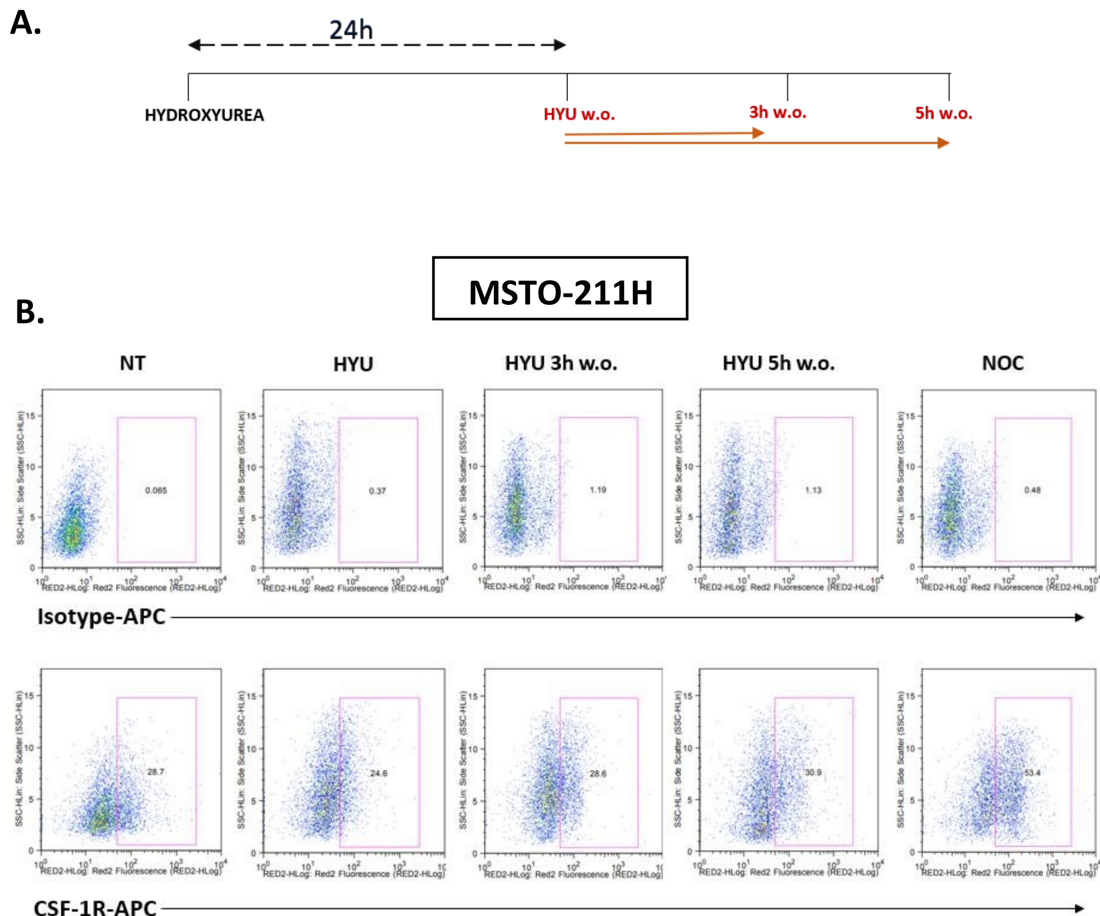
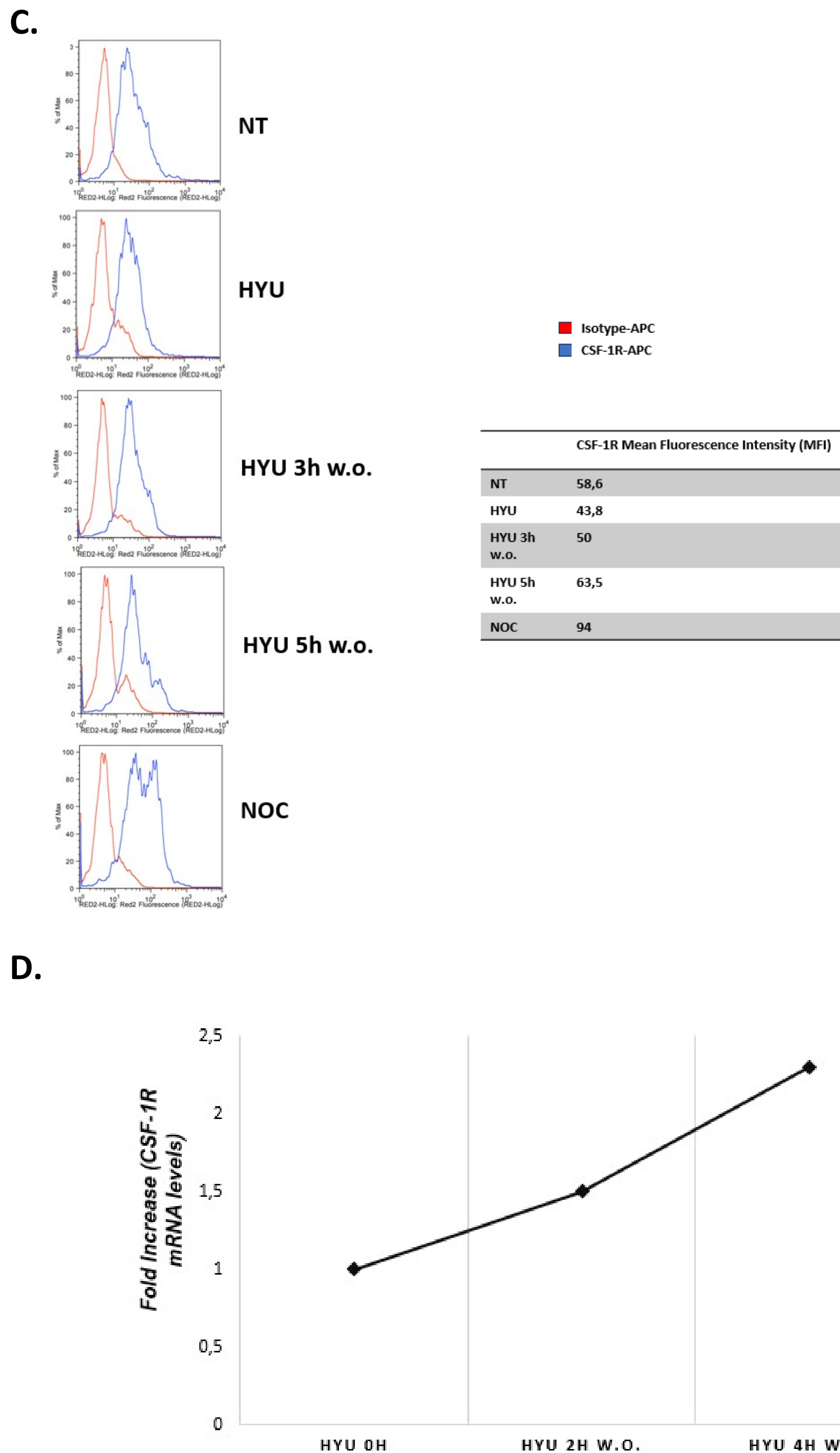


Figure 12. CSF-1R⁺ cells increased during G2-M phase transition. (A) DNA content of SKBR3 was analyzed in G2-synchronized cells (RO3306, T0) and M-synchronized cells (RO3306, 45min). DNA was stained with DAPI (blue). The mitotic cells were identified by microscopy based on chromosome condensation. Scale bar 20µm. (B) Representative images of immunohistochemical analysis of SKBR3 cells with anti-CSF-1R (brown). Staining was performed on non-treated cells (NT), G2-synchronized (RO3306, T0) and M-synchronized (RO3306, 45min) cells. Sections were counterstained with hematoxylin. Red arrow: CSF-1R in G2 phase, blue arrow: CSF-1R in M phase. Scale bar 50µm. The quantification of $CSF\text{-}1R^+$ cells is indicated in the graphs. As mean +/- SD, *: $p<0,05$, **: $p<0,01$.

4.3.2. CSF-1R is up-regulated during G1-S phase:

We observed in previous analysis that there is a population of pH3$^-$/CSF-1R$^+$ indicated that CSF-1R expression is not restricted to the G2-M phase but receptor expression could be also regulated in other cell cycle phases. In order to test this hypotheses, MSTO-211H cells were synchronized in G1-S transition cell cycle phase by the treatment with Hydroxyurea (HYU) at 4 mM and block release at different time as indicated in the timetable in **Fig. 13A**. Cells were also synchronized in G2-M phase with Nocodazole (NOC) for 16h. We then analyzed the percentage of CSF-1R$^+$ cells by flow cytometry and the CSF-1R expression intensity by calculating the Mean Fluorescence Intensity (MFI) (**Fig. 13B-C**). CSF-1R mRNA levels were measured by RT-PCR in MSTO-211H synchronized with HYU and pushed through G1-S and S phase by releasing the block (**Fig. 13D**). Results indicated that CSF-1R expression was upregulated with cell cycle progression. The experiment was replicated on Hela cells synchronized in G1, G1-S and S phase via the administration of HYU for 24 hours and HYU wash out at 3 and 5 hours (**Fig.14 A-B**). In parallel, at the end of indicated treatment, Hela cells were incubated with thymidine-analogue Edu, fixed, and stained with Edu-Click, pH3 and DAPI to quantify the number of cells progressed from G1 to S and G2/M phase (**Fig.14 C**). Altogether, data indicated that CSF-1R expression is positively regulated during the proliferative phase, reaching the peak of expression during the S phase.

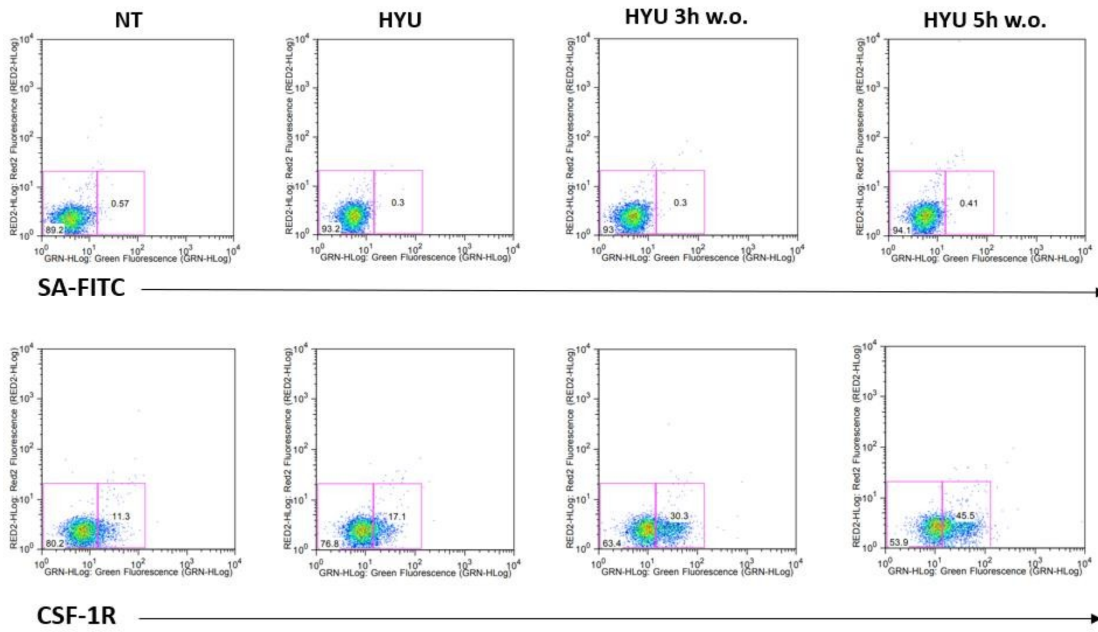




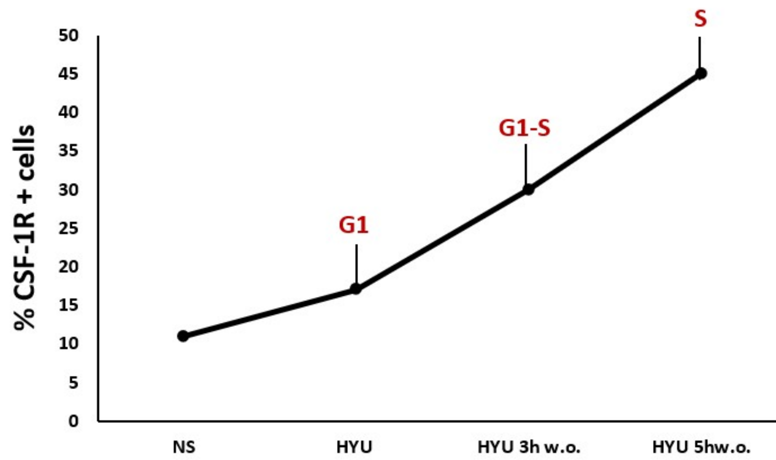
	CSF-1R Mean Fluorescence Intensity (MFI)
NT	58,6
HYU	43,8
HYU 3h w.o.	50
HYU 5h w.o.	63,5
NOC	94

Figure 13. CSF-1R up-regulation in G1-S phase transition in MSTO-211H. (A) Timetable of the experimental design. MSTO-211H were synchronized with HYU for 24h and HYU wash out (w.o.) at different time points to synchronize cells at different phase of the cell cycle. (B) Representative flow-cytometry analysis of CSF-1R (CSF-1R-APC) expression in Non-treated (NT), G1 (HYU), G1-S (HYU 3h w.o.), S (HYU 5h w.o.), and G2-M (NOC) synchronized cells. (C) Histograms of CSF-1R expression as in B. The levels of CSF-1R expression are indicated as mean fluorescence intensity (MFI). (D) mRNA *CSF-1R* levels in synchronized MSTO-211H at G1, G1/S phase and S phase. The fold increase of CSF-1R mRNA was normalized to HYU 0h.

A.



B.



C.

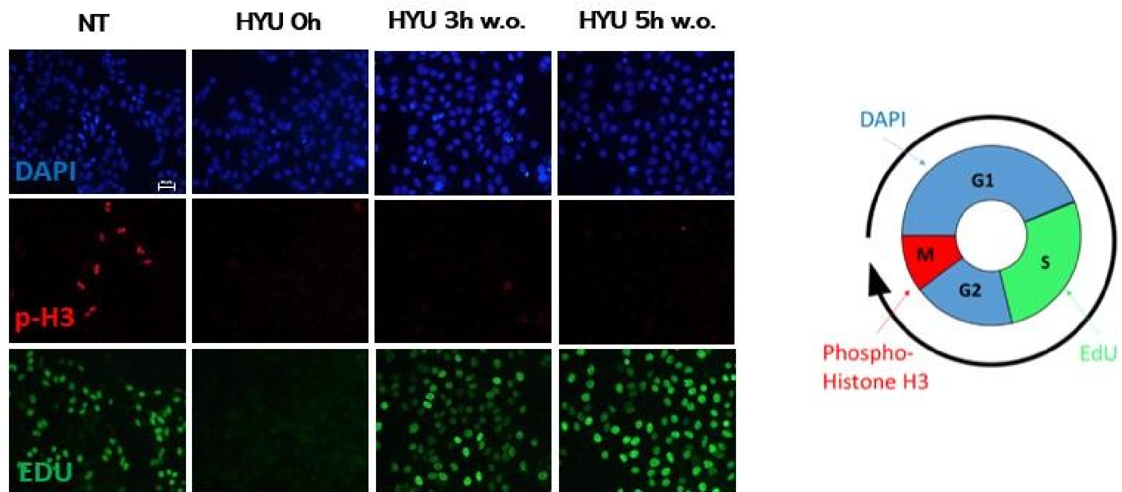


Figure 14. CSF-1R up-regulation in G1-S phase transition in cancer cell. (A) Representative flow-cytometry analysis of CSF-1R expression in Non-treated (NT) Hela cells and synchronized cells with HYU during the different phases of the cell cycle: G1 (HYU), G1-S (HYU 3h w.o.), S (HYU 5h w.o.) phase. (B) The graphical representation of the plots is indicated. (C) Analysis of transition G1/S in HYU-synchronized HELA cells. Asynchronized (NT) cells or HYU-synchronized cells were examined by Edu (green) and pH3 (red) staining. Nuclear staining was imaged by DAPI (blue).

4.4. Inhibition of CSF-1R activity affected cell cycle progression

What is the impact of CSF-1R activity in G2/M cell? To answer this point, we analyzed the activity of the receptor during G2 phase transition in an asynchronous culture of MPP-89 cell lines. Cells were treated for 2 hours with a specific inhibitor of CSF-1R kinase activity, GW-2580, and we performed immunofluorescence staining with anti-phosphoHistone3 (pH3) as indicator of cells in G2/M phase. As shown in **Fig. 15A-B,** 50% of the mitotic populations was reduced after two hours of GW-2580 treatment.

To determine the role of CSF1R activity during G1/S transition, MSTO-211H were synchronized in G1 phase with HYU (HYU) for 24h, and then HYU was removed for 2h (HYU 2h w.o.) and 5h (HYU 5h w.o.) without or with GW-2580 administered 1h before the end of HYU treatment and during the time of wash out (**Fig. 16A**). After the treatment, cells were incubated with Edu, fixed, stained with Edu Click and DAPI. As shown (**Fig. 16B-C)**, immunofluorescence revealed that CSF-1R inhibitor decreased the number of cells entering the S phase (Edu+ cells) compared to control cells. Indeed, the percentage of Edu+ cells was significant impaired after HYU 5h w.o, indicating that the CSF1R kinase activity is required for G1-S phase transition.

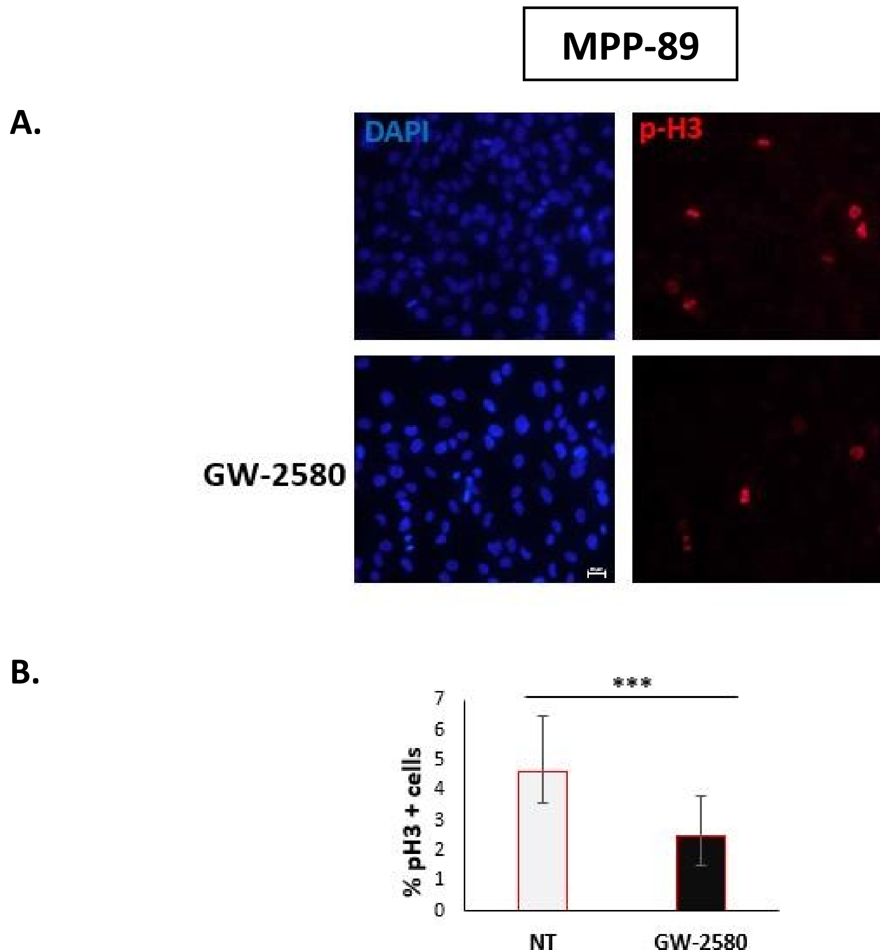


Figure 15. CSF-1R inhibition negatively affected mitosis. (A) Immunofluorescence of an asynchronous culture of MPP-89 cell lines treated with GW-2580 (10μM) for 2h. Cells were stained anti-phospho-Histone 3 (pH3) in red and nuclei are marked with DAPI in blue. Number of pH3 $^{+}$ cells were compared between Non-treated (NT) and GW-2580-treated cells. Scale bar 20μm. (B) The percentage of pH3 $^{+}$ cells as indicated in A were reported in the graph as mean +/- SD. ***: p<0,001. This graph is representative of two independent experiments.

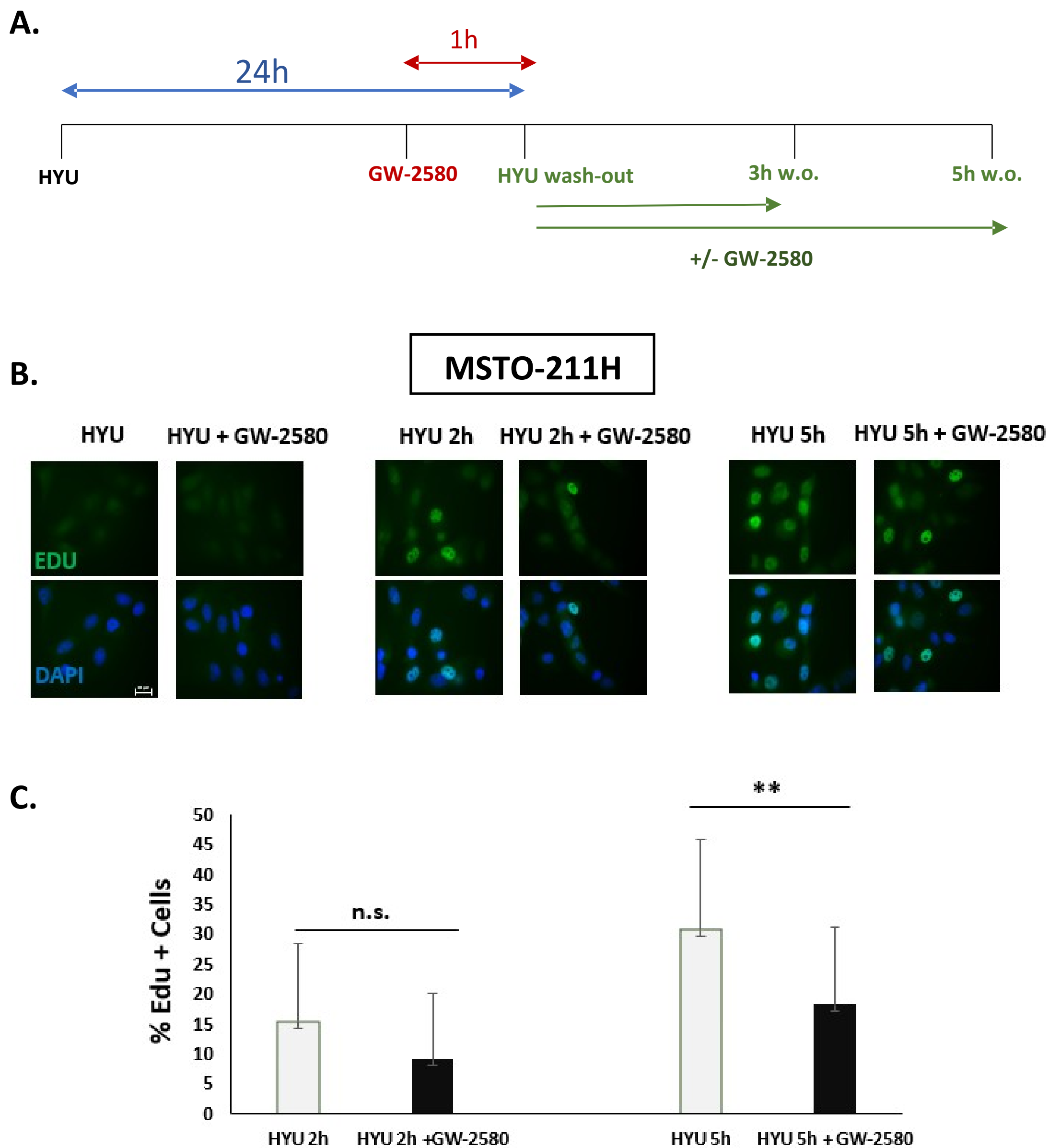


Figure 16. CSF-1R inhibition negatively affected G1-S transition. (A) Time course of the experimental design. (B) Analysis of G1-S phase progression in synchronized MSTO-211H treated with or w/o GW-2580 for the time indicated in A. Cells entering the S-phase are represented by Edu incorporating cells (green). DNA is stained with DAPI (blue). Scale bar 20μm. (C) Graphical representation of the percentage of Edu $^{+}$ cells showed in B, as mean +/- SD. n.s.: not significant; **: p<0,01. This graph is representative of two independent experiments.

4.5. CSF-1R expression in S-phase is regulated by "pocket proteins"

The RB family of "pocket proteins" is a key cell cycle regulator, and includes the retinoblastoma protein RB1 (p105), as well the pRB-related proteins RBL1 (p107) and RBL2 (p130). The functions of these proteins, which control E2F-related genes, is critical for regulating G1-S cell cycle phase transition (Cobrinik, 2005). In a prior study, Iavarone et al. found that the expression of CSF-1R in fetal livers was impaired in Rb-deficient embryos fetal livers (Iavarone et al., 2004). On the base of this results, we hypothesized that other RB family members could be involved in the regulation of CSF1R during cell proliferation in tumor cells. In order to test the correlation between Rb proteins and CSF-1R expression, we depleted MSTO-211H cells of pocket proteins using shRNA approaches targeting p105/RB1, p107/RBL1 and p130/RBL2, using MSTO-211H transduced with non-target scrambled shRNA (shSC) as controls. The efficacy of shRNA-mediated pocket protein suppression was confirmed by immunoblot analysis (**Fig. 17A**). We then evaluated the impact of Rb proteins depletion on cell growth by clonogenic assay. p105/RB1 and p107/RBL1 proteins genetic depletion reduced the colony formation ability of tumor cell lines, demonstrating the essential role of Rb expression on cancer cell growth activity. p130/RBL2 deficiency did not affected colony formation ability of MSTO-211H (**Fig. 17B**). To study the activity of Rb proteins in CSF-1R regulation during the S-phase of the cell cycle, Rb proteins depleted-cells were synchronized in the S-phase by treatment with HYU for 24h and HYU wash out for 5h. At the end of the treatment, we analyzed cell surface expression of CSF-1R by Flow cytometry (**Fig. 18A-B**). Data indicated that the number of CSF-1R^{+} cells decreased in all cells in which the pocket proteins were genetically depleted compared to parental and control cells MSTO-211H. Given that MSTO shp105 showed the lowest expression levels of CSF-1R, we focused on the activity of p105 on CSF-1R expression by analyzing CSF-1R mRNA levels in MSTO shSC and MSTO shp105. Results indicated that *CSF-1R* expression in non-synchronized cells and cells in S phase was negatively affected in MSTO shp105 cells compared with control cells (**Fig 19A-B**). The data obtained indicated a role for p105/RB1 in the regulation of *CSF-1R* mRNA transcription during the S phase.

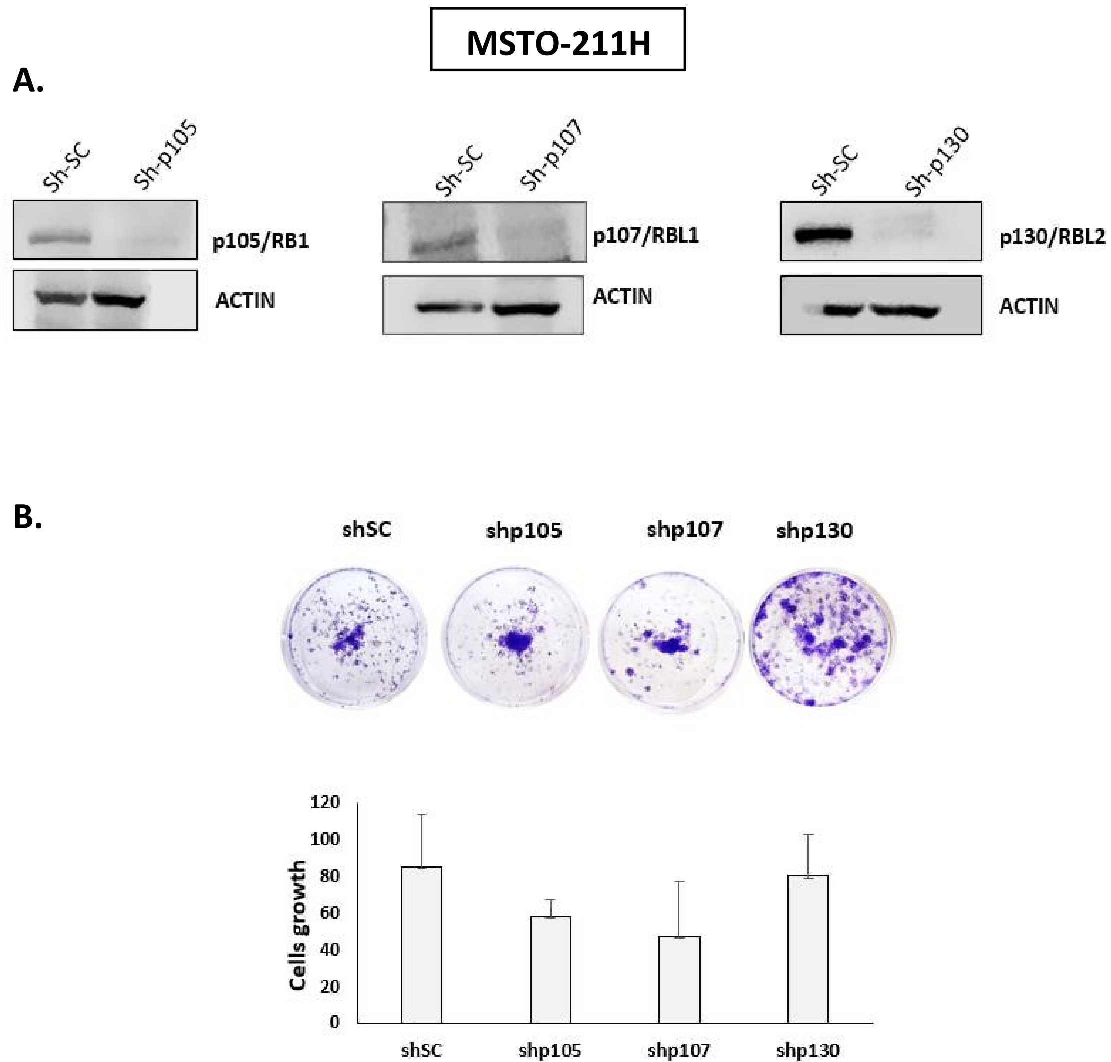
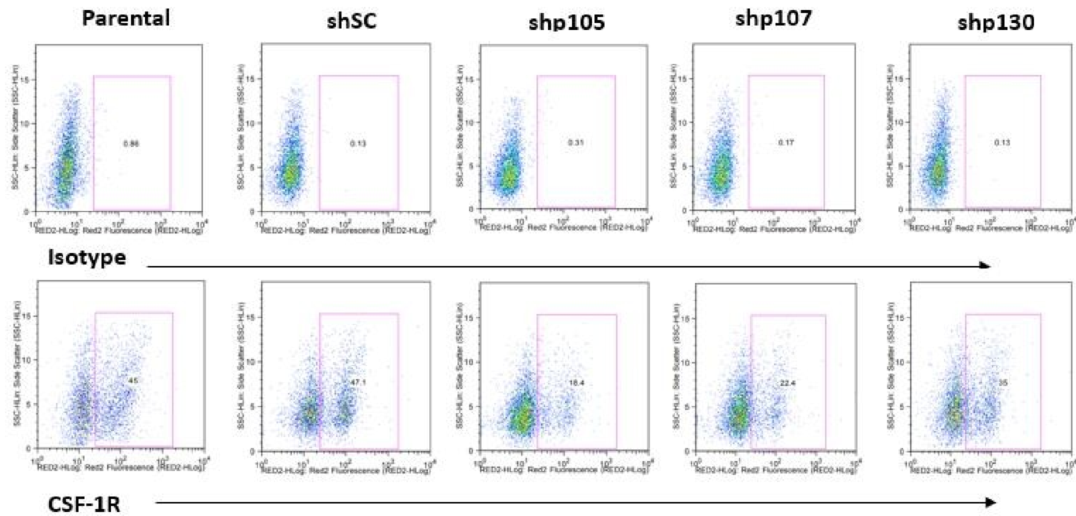


Figure 17. Genetic suppression of pocket proteins in MSTO-211H cells. (A) Western Blot analysis of MSTO-211H cell lines indicates that p105/RB1 p107/RBL1 and p130/RBL2 protein expression levels were suppressed in cells treated specific small hairpin RNA compared to control shRNA (shSC) treated cells. (B) Representative image of colony formation assay of shSC, shp105, shp107, shp130-MSTO-211H cells after 7 days cultured in 10% FBS medium. Colonies were quantified by using ImageJ software. Images are representative of three independent experiments. Graphical quantification of cell growth rate is indicated, as mean +/- SD.

A.



B.

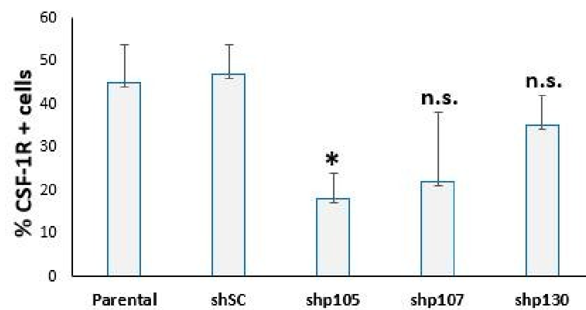


Figure 18. Rb proteins regulated CSF-1R expression in S phase. (A) Flow cytometry analysis of CSF-1R expression levels (CSF-1R APC) in parental, shSC, shp105, shp107 and shp130 MSTO-211H cells synchronized in S phase by treatment with HYU for 24h and HYU wash out for 5h. (B) Graphical representation of the percentage of CSF-1R $^{+}$ cells indicated in the plots. Experiments were replaced twice. +/- SD, n.s.: not significant; *: p<0,05.

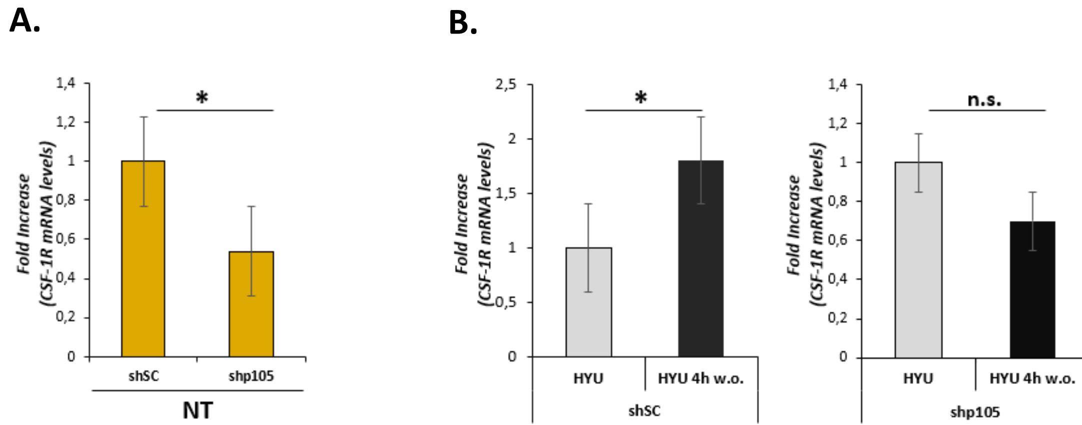


Figure 19. p105 depletion impaired CSF-1R mRNA expression level in G1-S transition phase. (A) Graphical representation of RT-PCR in non-treated (NT) shSC and shp105 MSTO-211H (B) RT-PCR analysis of CSF-1R mRNA levels on MSTO-shSC and MSTO-shp105 cells synchronized in G1 (HYU) and S (HYU 4h w.o.) cell cycle phases. The fold increase of CSF-1R mRNA was normalized to shSC (in A) and to HYU (in B). Error bars = mean +/- SD, *: p<0.05., n.s.: non-significant.

4.6. CSF-1R activates proliferative signaling pathway

As shown above, we detected CSF-1R expression in MM cells. However, the endogenous CSF-1R is expressed at low levels, thus, in order to investigate CSF-1R downstream signaling, we transiently transfected MSTO-211H with a FLAG-tagged human CSF-1R-expressing plasmid. The efficiency of the trasnfection was tested by Immunofluorescence staining and western blot analysis with anti-FLAG M2 and anti-CSF-1R antibodies 24h after transfection (**Fig. 20A**). As shown in Fig. 20B, overexpressed CSF-1R was constitutively activated in MSTO-211H cells as compared to transfected control cells, and CSF-1R phosphorylation was abolished by incubating the cells with the specific CSF-1R inhibitor GW-2580. Accordingly, downstream ERK5 and AKT pathways were activated in CSF-1R-overexpressing MSTO-211H cells over control cells, and ERK5 and AKT activity was sensitive to CSF-1R inhibition (**Fig. 20B**). Additionally, in our conditions, CSF-1R overexpression had also effect on the activation of the ERK1/2 pathway. These results indicate some pathway-specificity of signaling downstream of upregulated CSF-1R in MM cells.

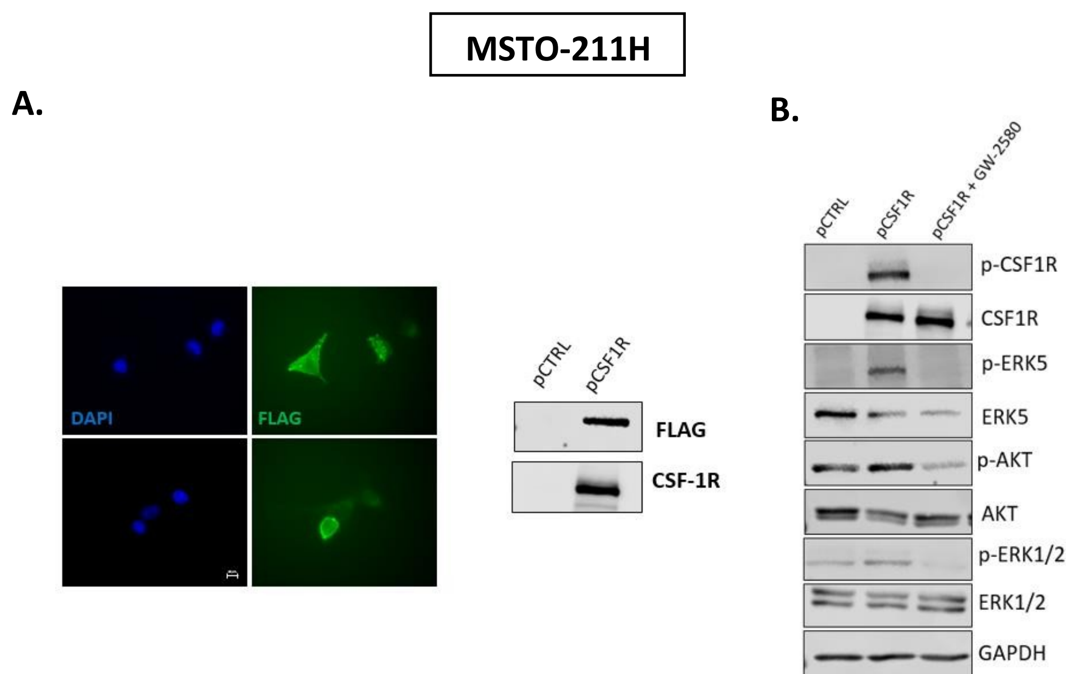


Figure 20. Western blot analysis of CSF-1R-overexpressing cells. (A) Immunofluorescence and immunoblot detection of CSF-1R using anti-FLAG in MSTO-211H cells transfected with a FLAG-tagged human CSF-1R-expressing plasmid (pCSF-1R) and empty vector (pCTRL). (B) Protein lysates from MSTO-211H cells cultured in 10% FBS medium and transfected with pCDNA3-CSF-1R or pCDNA3 were analyzed by immunoblot. The main downstream signaling pathways involved in cell proliferation were analyzed by immunoblots with specific antibodies.

5. DISCUSSION

In this study, we demonstrated that *i)* a sub-population of CSF-1R$^+$ cells are detectable in MM cell lines, *ii)* the activation of CSF-1R increased MM cell proliferation and their clonogenic ability, *iii)* CSF-1R expression is regulated during the cell cycle as in fact it is up-regulated in G1-S transition and in G2-M phase, *IV)* pharmacological inhibition of CSF-1R negatively affected the number of mitotic cells and the transition to G1-S phase, *V)* CSF-1R regulation during the S phase is mediated by Retinoblastoma proteins, VI) CSF-1R overexpression activated important signaling pathway promoting cell proliferation, such as ERK5, ERK1/2 and AKT.

Previous research demonstrated the expression of CSF-1R and its ligand CSF-1 in different tumor tissues and cancer cell lines. In certain tumors, such as breast, pancreatic, bone cancer and sarcoma CSF-1R expression levels correlate with malignancy and have a prognostic value (Abiatari et al., 2018; Espinosa et al., 2009; Richardsen et al., 2015; Ries et al., 2014). *In vitro* studies demonstrated that CSF-1R inhibition reduced cancer cell proliferation, invasiveness and EMT processes in breast cancer (Morandi et al., 2011; Kai et al., 2018). Moreover, genetic alteration of *CSF-1R* were found in tumor cell lines, including in melanoma and breast cancer, associated with the acquisition of stem-like and chemoresistance mechanisms (Barbetti et al., 2014; Giricz et al., 2018; Liu et al., 2022; Roussel et al., 1988). CSF-1R$^+$ cells were isolated and characterized in MM, as in fact, a CSF-1R$^+$ self-renewing cell pool with strong tumorigenic traits like drug resistance and clonogenicity was identified. Moreover, autocrine activation of CSF-1R was observed in MM cell lines. Given that the previous study showed increased *CSF-1R* mRNA in mesothelioma compared to mesothelial tissue (Cioce et al., 2014), we focused our study on MM cell lines representing all major mesothelioma histological subtypes, epithelioid, sarcomatoid and biphasic (Franklin et al., 2016).

Our data by FACS and Immunofluorescence analysis demonstrated the presence of a CSF-1R$^+$ subset of cells within mesothelioma cell lines. However, we noticed that its expression on cell membrane varied based on the confluence status of the cell culture. Fully confluent cells expressed less receptor than the non-confluent cells, indicating that CSF-1R expression is growth-dependent and decreases upon cell growth reduction. By immunofluorescence, we observed that CSF-1R is highly expressed by mitotic-shaped cells with a perinuclear localization around the mitotic plate. Previous study demonstrated that CSF-1R selectively localized in the nucleus of breast cancer cell lines in connection with chromatin sub-regions. Nuclear CSF-1R interacted with the promoter of genes involved in proliferation including *CCND1*, *c-JUN* and *c-MYC* (Barbetti et al., 2014). Moreover, nuclear CSF-1R was discovered in

a variety of cancer cell types, in response to CSF-1 stimulation (Zwaenepoel et al., 2012). Notably, nuclear CSF-1R associated with chromatin was also observed in monocytes, similarly to other RTKs, which were detected in nuclei linked with transcriptional factors (Bencheikh et al., 2019). The significance of nuclear CSF-1R is still not fully elucidated and further studies are required.

Our results suggest a role of ligand-activated CSF-1R in MM cells. However, mesothelioma cells react differently when stimulated by different specific CSF-1R ligands. It is not known whether the proliferative role of CSF-1R is preferentially induced by one specific cognate ligand, given that they bind on different sites of the receptor and generate different biological responses in myeloid cells (Chihara et al., 2010). It is important to note that, in addition to CSF-1R, IL-34 can also bind to two other cell membrane receptors, that are PTP-ζ which is expressed by neural cells, and CD138, found in epithelial and cancer cells. Whereas CSF-1R is the exclusive receptor for CSF-1 but not for IL-34 (Lelios et al., 2020; Saqi et al., 2005).

We carefully examined in which cell cycle phase CSF-1R might function by evaluating its expression levels in the different phases. The use of Nocodazole allowed the synchronization of cells in the G2-M window and this step increased the percentage of CSF-1R positive cells as well as enhanced membrane receptor expression levels. Changes of CSF-1R levels were specific as in fact EGFR levels did not change in the G2-M window, indicating that the mitotic population did not change EGFR levels compared to the asynchronous population. Furthermore, inhibition of CSF-1R activity using the kinase inhibitor GW-2580 reduced the number of G2-M cells, expressed in $pH3^+$ cells.

The results were in line with a previous observation demonstrating that CSF-1R inhibition decreased the expression of G2-M markers like Cyclin B, CDK1 and c-Myc in breast cancer cells (Morandi et al., 2011). Additionally, a recent work demonstrated that human colon crypts in large intestinal cells express high levels of CSF-1R (Huynh et al., 2013). Interestingly, *Csf-1r* $^{-/-}$ colonic crypts were characterized by reduced expression of *Cyclin B* and *c-myc,* as well as by decreased $pH3^+$ nuclei, supporting the hypothesis that the CSF-1R affects cell proliferation (Huynh et al., 2013). We found that pH3 has variable expression levels among mitotic cells. Based on pH3 expression levels we can distinguish three populations of mitotic cells: intermediate (G2), high (M), and low (telophase). Given that the pH3-intermediate population co-expressed CSF-1R at the high levels, we hypothesized that these cells are the fraction entering the G2/M phase and not in the one in early or late mitosis, suggesting a role for CSF-1R in the progression to the G2-M phase.

To determine whether CSF-1R shares common mechanism with various cancers, we expanded our investigation and included cells from other tumor models. We choose cervical and breast cancer cells since previous research have shown that they express and activate CSF-1R (Barbetti et al., 2014; Cho et al., 2017; Morandi et al., 2011). IHC analysis on RO3306-synchronized SKBR3 cells clearly demonstrated the increase of CSF-1R expression in G2 phase and in mitotic cells. Further research is needed to better understand the function of CSF-1R in the mitotic window and the involvement of cytokines in controlling G2-M window.

In glioma cells, genetic suppression of the CSF-1R reduced the number of cells in the S-phase cell and delayed G1-S phase transition by activating p27 and suppressing Cyclin E/CDK2 activity and Rb phosphorylation (Sun et al., 2019). In MM cells, our results demonstrated that CSF-1R expression was crucial for the G1-S phase transition, as supported by the fact that CSF-1R inhibition prevented cells from entering the S-phase. Russel et al. (Russel MF, 1997) proposed a model according to which CSF-1R activation by CSF-1 stimulation led to signaling pathways that converged to the expression of *myc* and cyclin-D, which are necessary for Rb proteins modulation and subsequent G1 progression or DNA synthesis (Russel MF, 1997). Iavarone et al. (Iavarone et al., 2004) also explored the correlation between Rb proteins and CSF-1R, and they observed that phosphorylated Rb can bind Id2, which release and activate the transcriptional factor PU.1., a crucial regulator of *CSF-1R* transcription. Indeed, Rb-deficient embryos showed almost undetectable levels of CSF-1R in fetal livers (Iavarone et al., 2004). On the base of these evidence, we tested the hypothesis that Rb proteins could be involved in the regulation of CSF-1R expression in cancer cells. In our results, the genetic silencing of Rb pocket proteins in MM cells altered the expression of the CSF-1R at protein levels during the S phase. The alteration was more evident in RB1/p105 depleted cells, probably because p105 plays a key role in the G1-S phase transition (Helmbold et al., 2012). Moreover, we observed that silenced-p105/RB1 MM cells decreased CSF-1R mRNA levels during G1-S transition phase. Collectively, these data described for the first time a mechanism by which CSF-1R expression increased during the cell cycle and is regulated by Rb/p105 which is involved in *CSF-1R* transcription. Although it has been reported that overexpression of Rb/p107 in fibroblasts inhibits Cdk2 activation and delays S phase entry (Rodier et al., 2005), our results suggested that Rb/p107 suppression did not significantly affect CSF-1R expression in mesothelioma cells. In addition, we observed that the suppression of Rb/p130 did not impair the CSF-1R expression in MM cell, supporting the idea that Rb/p130 is not associated with G1-S phase transition (Helmbold et al., 2009). Further investigation will be required to understand how exactly Rb proteins regulate CSF-1R expression. One avenue to explore is a possible crosstalk between Rb-Id2-PU.1.

CSF-1R downstream signaling pathway is still very poorly understood in non-myeloid cells. We expressed CSF-1R in MM cells and CSF-1R upregulation induced constitutive receptor activation. The constitutive activation of the receptor may be explained by the autocrine activation of the CSF-1R described in other tumor types. Data in melanoma cells have suggested the presence of a feedback regulatory mechanisms connecting CSF-1R activation, the activation of nuclear transcriptional factor such as RUNX1 that in turn can elicits the transcription of *CSF-1R* and *IL-34* (Giricz et al., 2018). The autocrine activation of the receptor was observed in other tumors including breast cancer, mesothelioma, and ovarian cancer, according to the studies, tumor cells were able to secrete CSF-1R ligands which in turn activated the receptor on cell surface resulting in pro-tumoral activity (Cioce et al., 2014; Patsialou et al., 2009; Toy et al., 2009). According to these data, our results suggested that MM cells were able to produce CSF-1R ligands that induced the basal phosphorylation of the receptor that was inhibited in presence of CSF-1R inhibitor. Additionally, CSF-1R upregulation elicited the phosphorylation of downstream signaling pathways, including ERK5, AKT and to a lesser extent ERK1/2. It has been observed that myeloid cells respond to CSF-1 stimulation by activating the mitogen-activated protein kinase ERK5, which is involved in macrophage proliferation (Giurisato et al., 2018; Rovida et al., 2008). Additionally, key role for ERK5 in cell proliferation was described in different cancer types, including mesothelioma (Shukla et al., 2013). The activation of AKT pathways was previously seen in mesothelioma correlated with resistance to pemetrexed (Cioce et al., 2014). Although it is generally established that AKT signaling affects cancer cell survival, more research is needed to determine how CSF-1R might work to affect AKT activity and viability of MM cells. Moreover, CSF-1R inhibition also induced ERK1/2 dephosphorylation, suggesting that the receptor is also involved in the regulation of ERK1/2 signaling. Similarly, in glioma and breast cancer, malignant behaviors were associated with the engagement of ERK1/2 followed CSF-1-mediated CSF-1R activation (Morandi et al., 2011; Sun et al., 2019). More studies are necessary to understand the signaling cascade activated by CSF-1R and mediating its proliferative activity in MM.

Overall, our findings showed that proliferative MM cells express CSF-1R, which play a role in regulating cell cycle progression. Different from other membrane receptors, CSF-1R expression is finely regulated throughout the G2-M and S phase of the cell cycle. The study offers unique information regarding the molecular mechanism controlling cell cycle in tumor cells. Based on these results, CSF-1R become an important mitotic and proliferative marker and possible target for therapeutic intervention, with the aim to limit the aberrant growth of MM cancer cells. Additional studies are required to investigate the autocrine activation of CSF-1R correlated with cell proliferation in MM and whether one of CSF-1R

ligands preferentially modulate cell cycle phase progression. These results can also be extended to other tumor types. Further studies are ongoing to fully define the involvement of CSF-1R in cancer-stem cells (CSCs) behavior, given the characteristic proliferative potential of these cells.

6. REFERENCES

Abiatari, I., Midelashvili, T., Motsikulashvili, M., Tchavtchavadze, A., Tawfeeq, S., & Amiranashvili, I. (2018). Overexpressed Progenitor Gene Csf1r In Pancreatic Cancer Tissues And Nerve Invasive Pancreatic Cancer Cells. *Georgian Medical News*, *285*, 96–100. http://europepmc.org/abstract/MED/30702078

Adusumilli, P. S., Zauderer, M. G., Rusch, V. W., O'Cearbhaill, R. E., Zhu, A., Ngai, D. A., McGee, E., Chintala, N. K., Messinger, J. C., Vincent, A., Halton, E. F., Diamonte, C., Pineda, J., Modi, S., Solomon, S. B., Jones, D. R., Brentjens, R. J., Rivière, I., & Sadelain, M. (2019). Abstract CT036: A phase I clinical trial of malignant pleural disease treated with regionally delivered autologous mesothelin-targeted CAR T cells: Safety and efficacy. *Cancer Research*, *79*(13_Supplement), CT036–CT036. https://doi.org/10.1158/1538-7445.AM2019-CT036

Apraiz, A., Mitxelena, J., & Zubiaga, A. (2017). Studying cell cycle-regulated gene expression by two complementary cell synchronization protocols. *Journal of Visualized Experiments*, *2017*(124). https://doi.org/10.3791/55745

Attanoos, R. L., Churg, A., Galateau-Salle, F., Gibbs, A. R., & Roggli, V. L. (2018). Malignant Mesothelioma and Its Non-Asbestos Causes. *Archives of Pathology & Laboratory Medicine*, *142*(6), 753–760. https://doi.org/10.5858/arpa.2017-0365-RA

Barbetti, V., Morandi, A., Tusa, I., Digiacomo, G., Riverso, M., Marzi, I., Cipolleschi, M. G., Bessi, S., Giannini, A., Di Leo, A., Dello Sbarba, P., & Rovida, E. (2014). Chromatin-associated CSF-1R binds to the promoter of proliferation-related genes in breast cancer cells. *Oncogene*, *33*(34), 4359–4364. https://doi.org/10.1038/onc.2013.542

Barca, C., Foray, C., Hermann, S., Herrlinger, U., Remory, I., Laoui, D., Schäfers, M., Grauer, O. M., Zinnhardt, B., & Jacobs, A. H. (2021). The Colony Stimulating Factor-1 Receptor (CSF-1R)-Mediated Regulation of Microglia/Macrophages as a Target for Neurological Disorders (Glioma, Stroke). In *Frontiers in Immunology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2021.787307

Barreda, D. R., Hanington, P. C., & Belosevic, M. (2004). Regulation of myeloid development and function by colony stimulating factors. In *Developmental and Comparative Immunology* (Vol. 28, Issue 5, pp. 509–554). Elsevier Ltd. https://doi.org/10.1016/j.dci.2003.09.010

Kacinski M. Barry. (1997). CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. *MOLECULAR REPRODUCTION AND DEVELOPMENT, 46*, 71–74.

Bencheikh, L., Diop, M. K., Rivière, J., Imanci, A., Pierron, G., Souquere, S., Naimo, A., Morabito, M., Dussiot, M., De Leeuw, F., Lobry, C., Solary, E., & Droin, N. (2019). Dynamic gene regulation by nuclear colony-stimulating factor 1 receptor in human monocytes and macrophages. *Nature Communications*, *10*(1), 1935. https://doi.org/10.1038/s41467-019-09970-9

Benner, B., Good, L., Quiroga, D., Schultz, T. E., Kassem, M., Carson, W. E., Cherian, M. A., Sardesai, S., & Wesolowski, R. (2020). Pexidartinib, a novel small molecule csf-1r

inhibitor in use for tenosynovial giant cell tumor: A systematic review of pre-clinical and clinical development. In *Drug Design, Development and Therapy* (Vol. 14, pp. 1693–1704). Dove Medical Press Ltd. https://doi.org/10.2147/DDDT.S253232

Besson, A., Dowdy, S. F., & Roberts, J. M. (2008). CDK Inhibitors: Cell Cycle Regulators and Beyond. *Developmental Cell*, *14*(2), 159–169. https://doi.org/https://doi.org/10.1016/j.devcel.2008.01.013

Bouchalova, P., & Bouchal, P. (2022). Current methods for studying metastatic potential of tumor cells. *Cancer Cell International*, *22*(1), 394. https://doi.org/10.1186/s12935-022-02801-w

Bourette, R. P., Rohrschneiderb, L. R., Rohrschneider, L. R., & Bourette, R. P. (n.d.). Early Events in M-CSF Receptor Signaling. In *Growth Factors* (Vol. 17).

Bourgin, C., Bourette, R. P., Arnaud, S., Liu, Y., Rohrschneider, L. R., & Mouchiroud, G. (2002). Induced Expression and Association of the Mona/Gads Adapter and Gab3 Scaffolding Protein during Monocyte/Macrophage Differentiation. *Molecular and Cellular Biology*, *22*(11), 3744–3756. https://doi.org/10.1128/mcb.22.11.3744-3756.2002

Brcic, L., & Kern, I. (2020). Clinical significance of histologic subtyping of malignant pleural mesothelioma. *Translational Lung Cancer Research*, *9*(3), 924–933. https://doi.org/10.21037/tlcr.2020.03.38

Buechler, M. B., Fu, W., & Turley, S. J. (2021). Fibroblast-macrophage reciprocal interactions in health, fibrosis, and cancer. In *Immunity* (Vol. 54, Issue 5, pp. 903–915). Cell Press. https://doi.org/10.1016/j.immuni.2021.04.021

Bueno, R., Stawiski, E. W., Goldstein, L. D., Durinck, S., De Rienzo, A., Modrusan, Z., Gnad, F., Nguyen, T. T., Jaiswal, B. S., & Chirieac, L. R. (2016). Comprehensive genomic analysis of malignant pleural mesothelioma identifies recurrent mutations, gene fusions and splicing alterations. *Nature Genetics*, *48*(4), 407–416.

Calvet, L., Dos-Santos, O., Spanakis, E., Jean-Baptiste, V., Le Bail, J.-C., Buzy, A., Paul, P., Henry, C., Valence, S., Dib, C., Pollard, J., Sidhu, S., Moll, J., Debussche, L., & Valtingojer, I. (2022). YAP1 is essential for malignant mesothelioma tumor maintenance. *BMC Cancer*, *22*(1), 639. https://doi.org/10.1186/s12885-022-09686-y

Cannarile, M. A., Weisser, M., Jacob, W., Jegg, A.-M., Ries, C. H., & Rttinger, D. (2017). Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *Journal for ImmunoTherapy of Cancer*, *5*(1), 53. https://doi.org/10.1186/s40425-017-0257-y

Carbone, M., Pass, H. I., Ak, G., Alexander, H. R., Baas, P., Baumann, F., Blakely, A. M., Bueno, R., Bzura, A., Cardillo, G., Churpek, J. E., Dianzani, I., De Rienzo, A., Emi, M., Emri, S., Felley-Bosco, E., Fennell, D. A., Flores, R. M., Grosso, F., … Hassan, R. (2022). Medical and Surgical Care of Patients with Mesothelioma and Their Relatives Carrying Germline BAP1 Mutations. In *Journal of Thoracic Oncology* (Vol. 17, Issue 7, pp. 873–889). Elsevier Inc. https://doi.org/10.1016/j.jtho.2022.03.014

Cersosimo, F., Barbarino, M., Lonardi, S., Vermi, W., Giordano, A., Bellan, C., & Giurisato, E. (2021). Mesothelioma Malignancy and the Microenvironment: Molecular Mechanisms. *Cancers*, *13*(22). https://doi.org/10.3390/cancers13225664

Charrier-Savournin, F. B., Château, M. T., Gire, V., Sedivy, J., Piette, J., & Dulić, V. (2004). p21-mediated nuclear retention of cyclin B1-Cdk1 in response to genotoxic stress. *Molecular Biology of the Cell*, *15*(9), 3965–3976. https://doi.org/10.1091/mbc.E03-12-0871

Cheng, Y. Y., Yuen, M. L., Rath, E. M., Johnson, B., Zhuang, L., Yu, T., Aleksova, V., Linton, A., Kao, S., & Clarke, C. J. (2020). CDKN2A and MTAP are useful biomarkers detectable by droplet digital PCR in malignant pleural mesothelioma: A potential alternative method in diagnosis compared to fluorescence in situ hybridisation. *Frontiers in Oncology*, *10*, 579327.

Chia, P. L., Scott, A. M., & John, T. (2019). Epidermal growth factor receptor (EGFR)-targeted therapies in mesothelioma. In *Expert Opinion on Drug Delivery* (Vol. 16, Issue 4, pp. 441–451). Taylor and Francis Ltd. https://doi.org/10.1080/17425247.2019.1598374

Chihara, T., Suzu, S., Hassan, R., Chutiwitoonchai, N., Hiyoshi, M., Motoyoshi, K., Kimura, F., & Okada, S. (2010). IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell Death & Differentiation*, *17*(12), 1917–1927. https://doi.org/10.1038/cdd.2010.60

Chitu, V., & Stanley, E. R. (2006). Colony-stimulating factor-1 in immunity and inflammation. *Current Opinion in Immunology*, *18*(1), 39–48. https://doi.org/10.1016/J.COI.2005.11.006

Cho, M.-J., Lee, J.-Y., Shin, M.-G., Kim, H.-J., Choi, Y.-J., Bae Rho, S., Kim, B.-R., Jang, I. S., & Lee, S.-H. (2017). TSC-22 inhibits CSF-1R function and induces apoptosis in cervical cancer. *Oncotarget*. 2017;8(58):97990-98003. Published 2017 Aug 16. doi:10.18632/oncotarget.20296

Cioce, M., Canino, C., Goparaju, C., Yang, H., Carbone, M., & Pass, H. I. (2014). Autocrine CSF-1R signaling drives mesothelioma chemoresistance via AKT activation. *Cell Death and Disease*, *5*(4). https://doi.org/10.1038/cddis.2014.136

Cobrinik, D. (2005). Pocket proteins and cell cycle control. *Oncogene*, *24*(17), 2796–2809. https://doi.org/10.1038/sj.onc.1208619

Cuccarese, M. F., Dubach, J. M., Pfirschke, C., Engblom, C., Garris, C., Miller, M. A., Pittet, M. J., & Weissleder, R. (2017). Heterogeneity of macrophage infiltration and therapeutic response in lung carcinoma revealed by 3D organ imaging. *Nature Communications*, *8*. https://doi.org/10.1038/ncomms14293

Dai, X.-M., Ryan, G. R., Hapel, A. J., Dominguez, M. G., Russell, R. G., Kapp, S., Sylvestre, V., & Stanley, E. R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood*. 2002;99(1):111-120. doi:10.1182/blood.v99.1.111

DeNardo, D. G., Brennan, D. J., Rexhepaj, E., Ruffell, B., Shiao, S. L., Madden, S. F., Gallagher, W. M., Wadhwani, N., Keil, S. D., Junaid, S. A., Rugo, H. S., Hwang, E. S., Jirström, K., West, B. L., & Coussens, L. M. (2011). Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discovery*, *1*(1), 54–67. https://doi.org/10.1158/2159-8274.CD-10-0028

Deng, P., Wang, Y. L., Pattengale, P. K., & Rettenmier, C. W. (1996). The Role of Individual Cysteine Residues in the Processing, Structure, and Function of Human Macrophage Colony-Stimulating Factor. *Biochemical and Biophysical Research Communications*, *228*(2), 557–566. https://doi.org/10.1006/BBRC.1996.1698

Espinosa, I., Beck, A. H., Lee, C. H., Zhu, S., Montgomery, K. D., Marinelli, R. J., Ganjoo, K. N., Nielsen, T. O., Gilks, C. B., West, R. B., & Van De Rijn, M. (2009). Coordinate expression of colony-stimulating factor-1 and colony-stimulating factor-1-related proteins is associated with poor prognosis in gynecological and nongynecological leiomyosarcoma. *American Journal of Pathology*, *174*(6), 2347–2356. https://doi.org/10.2353/ajpath.2009.081037

Franklin, P., Alfonso, H., Reid, A., Olsen, N., Shilkin, K. B., Brims, F., Klerk, N. de, & Musk, A. W. (2016). Asbestos exposure and histological subtype of malignant mesothelioma. *Occupational and Environmental Medicine*, *73*(11), 749. https://doi.org/10.1136/oemed-2016-103721

Garceau, V., Smith, J., Paton, I. R., Davey, M., Fares, M. A., Sester, D. P., Burt, D. W., & Hume, D. A. (2010). Pivotal Advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. *Journal of Leukocyte Biology*, *87*(5), 753–764. https://doi.org/10.1189/jlb.0909624

Giricz, O., Mo, Y., Dahlman, K. B., Cotto-Rios, X. M., Vardabasso, C., Nguyen, H., Matusow, B., Bartenstein, M., Polishchuk, V., Johnson, D. B., Bhagat, T. D., Shellooe, R., Burton, E., Tsai, J., Zhang, C., Habets, G., Greally, J. M., Yu, Y., Kenny, P. A., … Verma, A. K. (2018). The RUNX1/IL-34/CSF-1R axis is an autocrinally regulated modulator of resistance to BRAF-V600E inhibition in melanoma. *JCI Insight*, *3*(14). https://doi.org/10.1172/jci.insight.120422

Giurisato, E., Xu, Q., Lonardi, S., Telfer, B., Russo, I., Pearson, A., Finegan, K. G., Wang, W., Wang, J., Gray, N. S., Vermi, W., Xia, Z., & Tournier, C. (2018). Myeloid ERK5 deficiency suppresses tumor growth by blocking protumor macrophage polarization via STAT3 inhibition. *Proceedings of the National Academy of Sciences*, *115*(12), E2801–E2810. https://doi.org/10.1073/pnas.1707929115

Lin, H., Lee, E., Hestir, K., Leo, C., Huang, M., Bosch, E., Halenbeck, R., Wu, G., Zhou, A., Behrens, D., Hollenbaugh, D., Linnemann, T., Qin, M., Wong, J., Chu, K., Doberstein, S. K., & Williams, L. T. (2008). Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science (New York, N.Y.)*, *320*(5877), 807–811. https://doi.org/10.1126/science.1154370

Han, J., Chitu, V., Stanley, E. R., Wszolek, Z. K., Karrenbauer, V. D., & Harris, R. A. (2022). Inhibition of colony stimulating factor-1 receptor (CSF-1R) as a potential therapeutic strategy for neurodegenerative diseases: opportunities and challenges. *Cellular and Molecular Life Sciences*, *79*(4), 219. https://doi.org/10.1007/s00018-022-04225-1

Helmbold, H., Komm, N., Deppert, W., & Bohn, W. (2009). Rb2/p130 is the dominating pocket protein in the p53-p21 DNA damage response pathway leading to senescence. *Oncogene*, *28*(39), 3456–3467. https://doi.org/10.1038/onc.2009.222

Helmbold, H., Galderisi, U., & Bohn, W. (2012). The switch from pRb/p105 to Rb2/p130 in DNA damage and cellular senescence. *Journal of cellular physiology*, *227*(2), 508–513. https://doi.org/10.1002/jcp.22786

Hanna, G. G., John, T., & Ball, D. L. (2021). Controversies in the role of radiotherapy in pleural mesothelioma. *Translational Lung Cancer Research*, *10*(4), 2079–2087. https://doi.org/10.21037/tlcr-20-583

Huang, L., Xu, X., & Hao, Y. (2014). The possible mechanisms of tumor progression via CSF-1/CSF-1R pathway activation. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie*, *55*(2 Suppl), 501–506.

Hung, J. Y., Horn, D., Woodruff, K., Prihoda, T., Lesaux, C., Peters, J., Tio, F., & Abboud-Werner, S. L. (2014). Colony-stimulating factor 1 potentiates lung cancer bone metastasis. *Laboratory Investigation*, *94*(4), 371–381. https://doi.org/10.1038/LABINVEST.2014.1

Husain, A. N., Colby, T. V, Ordonez, N. G., Allen, T. C., Attanoos, R. L., Beasley, M. B., Butnor, K. J., Chirieac, L. R., Churg, A. M., & Dacic, S. (2018). Guidelines for pathologic diagnosis of malignant mesothelioma 2017 update of the consensus statement from the International Mesothelioma Interest Group. *Archives of Pathology & Laboratory Medicine*, *142*(1), 89–108.

Huynh, D., Akçora, D., Malaterre, J., Chan, C. K., Dai, X.-M., Bertoncello, I., Stanley, E. R., & Ramsay, R. G. (2013). CSF-1 Receptor-Dependent Colon Development, Homeostasis and Inflammatory Stress Response. *PLOS ONE*, *8*(2), e56951-. https://doi.org/10.1371/journal.pone.0056951

Iavarone, A., King, E. R., Dai, X.-M., Leone, G., Stanley, E. R., & Lasorella, A. (2004). Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. *Nature*, *432*(7020), 1040–1045. https://doi.org/10.1038/nature03068

Ide, H., Seligson, D. B., Memarzadeh, S., Xin, L., Horvath, S., Dubey, P., Flick, M. B., Kacinski, B. M., Palotie, A., & Witte, O. N. (2002). Expression of colony-stimulating factor 1 receptor during prostate development and prostate cancer progression. *Proceedings of the National Academy of Sciences*, *99*(22), 14404–14409. https://doi.org/10.1073/pnas.222537099

Jean, D., Daubriac, J., Le Pimpec-Barthes, F., Galateau-Salle, F., & Jaurand, M.-C. (2012). Molecular Changes in Mesothelioma with an Impact on Prognosis and Treatment. *Archives of Pathology & Laboratory Medicine*, *136*(3), 277–293. https://doi.org/10.5858/arpa.2011-0215-RA

Jeannin, P., Paolini, L., Adam, C., & Delneste, Y. (2018). The roles of CSFs on the functional polarization of tumor-associated macrophages. In *FEBS Journal* (Vol. 285, Issue 4, pp. 680–699). Blackwell Publishing Ltd. https://doi.org/10.1111/febs.14343

Jones, C. V., & Ricardo, S. D. (2013). Macrophages and CSF-1: Implications for development and beyond. In *Organogenesis* (Vol. 9, Issue 4, pp. 249–260). https://doi.org/10.4161/org.25676

Junttila, I., Bourette, R. P., Rohrschneider, L. R., & Silvennoinen, O. (2003). M-CSF induced differentiation of myeloid precursor cells involves activation of PKC-δ and expression

of Pkare. *Journal of Leukocyte Biology*, *73*(2), 281–288. https://doi.org/10.1189/jlb.0702359

Kai, K., Iwamoto, T., Zhang, D., Shen, L., Takahashi, Y., Rao, A., Thompson, A., Sen, S., & Ueno, N. T. (2018). CSF-1/CSF-1R axis is associated with epithelial/mesenchymal hybrid phenotype in epithelial-like inflammatory breast cancer. *Scientific Reports*, *8*(1), 9427. https://doi.org/10.1038/s41598-018-27409-x

Kim, T. S., Cavnar, M. J., Cohen, N. A., Sorenson, E. C., Greer, J. B., Seifert, A. M., Crawley, M. H., Green, B. L., Popow, R., Pillarsetty, N., Veach, D. R., Ku, A. T., Rossi, F., Besmer, P., Antonescu, C. R., Zeng, S., & DeMatteo, R. P. (2014). Increased KIT Inhibition Enhances Therapeutic Efficacy in Gastrointestinal Stromal Tumor. *Clinical Cancer Research*, *20*(9), 2350–2362. https://doi.org/10.1158/1078-0432.CCR-13-3033

Kindler, H. L., Ismaila, N., Armato 3rd, S. G., Bueno, R., Hesdorffer, M., Jahan, T., Jones, C. M., Miettinen, M., Pass, H., Rimner, A., Rusch, V., Sterman, D., Thomas, A., & Hassan, R. (2018). Treatment of Malignant Pleural Mesothelioma: American Society of Clinical Oncology Clinical Practice Guideline. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, *36*(13), 1343–1373. https://doi.org/10.1200/JCO.2017.76.6394

Krysinska, H., Hoogenkamp, M., Ingram, R., Wilson, N., Tagoh, H., Laslo, P., Singh, H., & Bonifer, C. (2007). A Two-Step, PU.1-Dependent Mechanism for Developmentally Regulated Chromatin Remodeling and Transcription of the c-fms Gene. *Molecular and Cellular Biology*, *27*(3), 878–887. https://doi.org/10.1128/MCB.01915-06

Leal-Esteban, L. C., & Fajas, L. (2020). Cell cycle regulators in cancer cell metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1866*(5), 165715. https://doi.org/https://doi.org/10.1016/j.bbadis.2020.165715

Lee, A. W., Nambirajan, S., & Moffat, J. G. (1999). CSF-1 activates MAPK-dependent and p53-independent pathways to induce growth arrest of hormone-dependent human breast cancer cells. *Oncogene*, *18*(52), 7477–7494. https://doi.org/10.1038/sj.onc.1203123

Lee, A. Y., Raz, D. J., He, B., & Jablons, D. M. (2007). Update on the molecular biology of malignant mesothelioma. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *109*(8), 1454–1461.

Lelios, I., Cansever, D., Utz, S. G., Mildenberger, W., Stifter, S. A., & Greter, M. (2020). Emerging roles of IL-34 in health and disease. *Journal of Experimental Medicine*, *217*(3), e20190290. https://doi.org/10.1084/jem.20190290

Lin, E. Y., Nguyen, A. V, Russell, R. G., & Pollard, J. W. (2001). Colony-stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. In *J. Exp. Med* (Vol. 193, Issue 6). http://www.jem.org/cgi/content/full/193/6/727

Liu, F., Bouznad, N., Kaller, M., Shi, X., Ko nig, J., Jaeckel, S., & Hermeking, H. (2022). Csf1r mediates enhancement of intestinal tumorigenesis caused by inactivation of Mir34a. *Int J Biol Sci*, *18*, 5415–5437. https://doi.org/10.7150/ijbs.75503

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/https://doi.org/10.1006/meth.2001.1262

Lou, J., Low-Nam, S. T., Kerkvliet, J. G., & Hoppe, A. D. (2014). Delivery of CSF-1R to the lumen of macropinosomes promotes its destruction in macrophages. *Journal of Cell Science*, *127*(24), 5228–5239. https://doi.org/10.1242/jcs.154393

Ma, X., Lin, W. Y., Chen, Y., Stawicki, S., Mukhyala, K., Wu, Y., Martin, F., Bazan, J. F., & Starovasnik, M. A. (2012). Structural Basis for the Dual Recognition of Helical Cytokines IL-34 and CSF-1 by CSF-1R. *Structure*, *20*(4), 676–687. https://doi.org/10.1016/J.STR.2012.02.010

Malakoti, F., Targhazeh, N., Abadifard, E., Zarezadeh, R., Samemaleki, S., Asemi, Z., Younesi, S., Mohammadnejad, R., Hadi Hossini, S., Karimian, A., Alemi, F., & Yousefi, B. (2022). DNA repair and damage pathways in mesothelioma development and therapy. *Cancer Cell International*, *22*(1), 176. https://doi.org/10.1186/s12935-022-02597-9

Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer*, *9*(3), 153–166. https://doi.org/10.1038/nrc2602

Marks, D. C., Csar, X. F., Wilson, N. J., Novak, U., Ward, A. C., Kanagasundarum, V., Hoffmann, B. W., & Hamilton, J. A. (1999). Expression of a Y559F mutant CSF-1 receptor in M1 myeloid cells: a role for Src kinases in CSF-1 receptor-mediated differentiation. *Molecular cell biology research communications: MCBRC*, *1*(2), 144–152. https://doi.org/10.1006/mcbr.1999.0123

Matsuzaki, H., Maeda, M., Lee, S., Nishimura, Y., Kumagai-Takei, N., Hayashi, H., Yamamoto, S., Hatayama, T., Kojima, Y., Tabata, R., Kishimoto, T., Hiratsuka, J., & Otsuki, T. (2012). Asbestos-Induced Cellular and Molecular Alteration of Immunocompetent Cells and Their Relationship with Chronic Inflammation and Carcinogenesis. *Journal of Biomedicine and Biotechnology*, *2012*, 492608. https://doi.org/10.1155/2012/492608

Matthews, H. K., Bertoli, C., & de Bruin, R. A. M. (2022). Cell cycle control in cancer. *Nature Reviews Molecular Cell Biology*, *23*(1), 74–88. https://doi.org/10.1038/s41580-021-00404-3

Meirson, T., Nardone, V., Pentimalli, F., Markel, G., Bomze, D., D'Apolito, M., Correale, P., Giordano, A., Pirtoli, L., Porta, C., Gray, S. G., & Mutti, L. (2022). Analysis of new treatments proposed for malignant pleural mesothelioma raises concerns about the conduction of clinical trials in oncology. *Journal of Translational Medicine*, *20*(1), 593. https://doi.org/10.1186/s12967-022-03744-6

Meziani, L., Mondini, M., Petit, B., Boissonnas, A., De Montpreville, V. T., Mercier, O., Vozenin, M. C., & Deutsch, E. (2018). CSF1R inhibition prevents radiation pulmonary fibrosis by depletion of interstitial macrophages. *European Respiratory Journal*, *51*(3). https://doi.org/10.1183/13993003.02120-2017

Mo, H., Hao, Y., Lv, Y., Chen, Z., Shen, J., Zhou, S., & Yin, M. (2021). Overexpression of macrophage-colony stimulating factor-1 receptor as a prognostic factor for survival in cancer: A systematic review and meta-analysis. *Medicine*, *100*(12). https://journals.lww.com/md-journal/fulltext/2021/03260/overexpression_of_macrophage_colony_stimulating.85.aspx

Moon, H. G., Kim, S. jae, Lee, M. K., Kang, H., Choi, H. S., Harijith, A., Ren, J., Natarajan, V., Christman, J. W., Ackerman, S. J., & Park, G. Y. (2020). Colony-stimulating factor 1 and its receptor are new potential therapeutic targets for allergic asthma. *Allergy: European Journal of Allergy and Clinical Immunology*, *75*(2), 357–369. https://doi.org/10.1111/all.14011

Morandi, A., Barbetti, V., Riverso, M., Dello Sbarba, P., & Rovida, E. (2011). The Colony-Stimulating Factor-1 (CSF-1) receptor sustains ERK1/2 activation and proliferation in breast cancer cell lines. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0027450

Mott, F. E. (2012). Mesothelioma: A Review. *The Ochsner Journal*, *12*, 70–79.

Mun, S. H., Park, P. S. U., & Park-Min, K. H. (2020). The M-CSF receptor in osteoclasts and beyond. In *Experimental and Molecular Medicine* (Vol. 52, Issue 8, pp. 1239–1254). Springer Nature. https://doi.org/10.1038/s12276-020-0484-z

Murga-Zamalloa, C., Rolland, D. C. M., Polk, A., Wolfe, A., Dewar, H., Chowdhury, P., Onder, O., Dewar, R., Brown, N. A., Bailey, N. G., Inamdar, K., Lim, M. S., Elenitoba-Johnson, K. S. J., & Wilcox, R. A. (2020). Colony-Stimulating Factor 1 Receptor (CSF1R) Activates AKT/mTOR Signaling and Promotes T-Cell Lymphoma Viability. *Clinical Cancer Research*, *26*(3), 690–703. https://doi.org/10.1158/1078-0432.CCR-19-1486

Nakajima, E. C., Vellanki, P. J., Larkins, E., Chatterjee, S., Mishra-Kalyani, P. S., Bi, Y., Qosa, H., Liu, J., Zhao, H., Biable, M., Hotaki, L. T., Shen, Y.-L., Pazdur, R., Beaver, J. A., Singh, H., & Donoghue, M. (2022). FDA Approval Summary: Nivolumab in Combination with Ipilimumab for the Treatment of Unresectable Malignant Pleural Mesothelioma. *Clinical Cancer Research*, *28*(3), 446–451. https://doi.org/10.1158/1078-0432.CCR-21-1466

Nandi, S., Gokhan, S., Dai, X. M., Wei, S., Enikolopov, G., Lin, H., Mehler, M. F., & Richard Stanley, E. (2012). The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation. *Developmental Biology*, *367*(2), 100–113. https://doi.org/10.1016/J.YDBIO.2012.03.026

Narasimha, A. M., Kaulich, M., Shapiro, G. S., Choi, Y. J., Sicinski, P., & Dowdy, S. F. (2014). Cyclin D activates the Rb tumor suppressor by mono-phosphorylation. *ELife*, *3*, e02872. https://doi.org/10.7554/eLife.02872

Ngiow, S. F., Meeth, K. M., Stannard, K., Barkauskas, D. S., Bollag, G., Bosenberg, M., & Smyth, M. J. (2016). Co-inhibition of colony stimulating factor-1 receptor and BRAF oncogene in mouse models of BRAFV600E melanoma. *OncoImmunology*, *5*(3), e1089381. https://doi.org/10.1080/2162402X.2015.1089381

Noonan, C. W. (2017). Environmental asbestos exposure and risk of mesothelioma. *Annals of Translational Medicine*, *5*(11), 234. https://doi.org/10.21037/atm.2017.03.74

Novak, U., Nice, E., Hamilton, J. A., & Paradiso, L. (1996). Requirement for Y706 of the murine (or Y708 of the human) CSF-1 receptor for STAT1 activation in response to CSF-1. *Oncogene*, *13*(12), 2607—2613. http://europepmc.org/abstract/MED/9000134

Obacz, J., Yung, H., Shamseddin, M., Linnane, E., Liu, X., Azad, A. A., Rassl, D. M., Fairen-Jimenez, D., Rintoul, R. C., Nikolic , M. Z., & Marciniak, S. J. (2021). Biological basis for novel mesothelioma therapies. *British Journal of Cancer*, *125*(8), 1039–1055. https://doi.org/10.1038/s41416-021-01462-2

Olmos-Alonso, A., Schetters, S. T. T., Sri, S., Askew, K., Mancuso, R., Vargas-Caballero, M., Holscher, C., Perry, V. H., & Gomez-Nicola, D. (2016). Pharmacological targeting of CSF1R inhibits microglial proliferation and prevents the progression of Alzheimer's-like pathology. *Brain*, *139*(3), 891–907. https://doi.org/10.1093/brain/awv379

Otero, K., Turnbull, I. R., Poliani, P. L., Vermi, W., Cerutti, E., Aoshi, T., Tassi, I., Takai, T., Stanley, S. L., Miller, M., Shaw, A. S., & Colonna, M. (2009). Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and β-catenin. *Nature Immunology*, *10*(7), 734–743. https://doi.org/10.1038/ni.1744

Ovchinnikov, D. A., DeBats, C. E. E., Sester, D. P., Sweet, M. J., & Hume, D. A. (2010). A conserved distal segment of the mouse CSF-1 receptor promoter is required for maximal expression of a reporter gene in macrophages and osteoclasts of transgenic mice. *Journal of Leukocyte Biology*, *87*(5), 815–822. https://doi.org/10.1189/jlb.0809557

Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Koths, K., & Kim, S.-H. (1992). Three-Dimensional Structure of Dimeric Human Recombinant Macrophage Colony-Stimulating Factor. *Science*, *258*(5086), 1358–1362. http://www.jstor.org/stable/2880431

Patsialou, A., Wang, Y., Pignatelli, J., Chen, X., Entenberg, D., Oktay, M., & Condeelis, J. S. (2015). Autocrine CSF1R signaling mediates switching between invasion and proliferation downstream of TGFβ in claudin-low breast tumor cells. *Oncogene*, *34*(21), 2721–2731. https://doi.org/10.1038/onc.2014.226

Patsialou, A., Wyckoff, J., Wang, Y., Goswami, S., Stanley, E. R., & Condeelis, J. S. (2009). Invasion of Human Breast Cancer Cells In vivo Requires Both Paracrine and Autocrine Loops Involving the Colony-Stimulating Factor-1 Receptor. *Cancer Research*, *69*(24), 9498–9506. https://doi.org/10.1158/0008-5472.CAN-09-1868

Peyraud, F., Cousin, S., & Italiano, A. (2017). CSF-1R Inhibitor Development: Current Clinical Status. *Current Oncology Reports*, *19*(11), 70. https://doi.org/10.1007/s11912-017-0634-1

Pixley, F. J., & Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends in Cell Biology*, *14*(11), 628–638. https://doi.org/https://doi.org/10.1016/j.tcb.2004.09.016

Preisser, L., Miot, C., Elene Le Guillou-Guillemette, H., Beaumont, E., Foucher, E. D., Garo, E., Blanchard, S., Fr, I., Crou, A., Fouchard, I., Lunel-Fabiani, F., Er^ Ome Boursier, J., Roingeard, P., Cale, P., Delneste, Y., & Jeannin, P. (2014). IL-34 and Macrophage Colony-Stimulating Factor Are Overexpressed in Hepatitis C Virus Fibrosis and Induce Profibrotic Macrophages That Promote Collagen Synthesis by Hepatic Stellate Cells. *HEPATOLOGY*, *60*, 1879–1890. https://doi.org/10.1002/hep.27328/suppinfo

Pyonteck, S. M., Akkari, L., Schuhmacher, A. J., Bowman, R. L., Sevenich, L., Quail, D. F., Olson, O. C., Quick, M. L., Huse, J. T., Teijeiro, V., Setty, M., Leslie, C. S., Oei, Y., Pedraza, A., Zhang, J., Brennan, C. W., Sutton, J. C., Holland, E. C., Daniel, D., & Joyce, J. A. (2013). CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nature Medicine*, *19*(10), 1264–1272. https://doi.org/10.1038/nm.3337

Rattanaburee, T., Tipmanee, V., Tedasen, A., Thongpanchang, T., & Graidist, P. (2020). Inhibition of CSF1R and AKT by (±)-kusunokinin hinders breast cancer cell proliferation. *Biomedicine and Pharmacotherapy*, *129*. https://doi.org/10.1016/j.biopha.2020.110361

Riaz, N., Burugu, S., Cheng, A. S., Leung, S. C. Y., Gao, D., & Nielsen, T. O. (2021). Prognostic significance of csf-1r expression in early invasive breast cancer. *Cancers*, *13*(22). https://doi.org/10.3390/cancers13225769

Richardsen, E., Uglehus, R. D., Johnsen, S. H., & Busund, L.-T. (2015). Macrophage-Colony Stimulating Factor (CSF1) Predicts Breast Cancer Progression and Mortality. *Anticancer Research*, *35*(2), 865. http://ar.iiarjournals.org/content/35/2/865.abstract

Rieder, C. L. (2011). Mitosis in vertebrates: the G2/M and M/A transitions and their associated checkpoints. *Chromosome Research*, *19*(3), 291–306. https://doi.org/10.1007/s10577-010-9178-z

Ries, C. H., Cannarile, M. A., Hoves, S., Benz, J., Wartha, K., Runza, V., Rey-Giraud, F., Pradel, L. P., Feuerhake, F., Klaman, I., Jones, T., Jucknischke, U., Scheiblich, S., Kaluza, K., Gorr, I. H., Walz, A., Abiraj, K., Cassier, P. A., Sica, A., … Rüttinger, D. (2014). Targeting Tumor-Associated Macrophages with Anti-CSF-1R Antibody Reveals a Strategy for Cancer Therapy. *Cancer Cell*, *25*(6), 846–859. https://doi.org/10.1016/J.CCR.2014.05.016

Rodier, G., Makris, C., Coulombe, P., Scime, A., Nakayama, K., Nakayama, K. I., & Meloche, S. (2005). p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein Skp2. *Journal of Cell Biology*, *168*(1), 55–66. https://doi.org/10.1083/jcb.200404146

Rojo, R., Pridans, C., Langlais, D., & Hume, D. A. (2017). Transcriptional mechanisms that control expression of the macrophage colony-stimulating factor receptor locus. In *Clinical Science* (Vol. 131, Issue 16, pp. 2161–2182). Portland Press Ltd. https://doi.org/10.1042/CS20170238

Roussel, M. F., Downing, J. R., Rettenmier, C. W., & Sherr, C. J. (1988). A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. *Cell*, *55*(6), 979–988. https://doi.org/https://doi.org/10.1016/0092-8674(88)90243-7

Rovida, E., Spinelli, E., Sdelci, S., Barbetti, V., Morandi, A., Giuntoli, S., & Dello Sbarba, P. (2008). ERK5/BMK1 Is Indispensable for Optimal Colony-Stimulating Factor 1 (CSF-1)-Induced Proliferation in Macrophages in a Src-Dependent Fashion1. *The Journal of Immunology*, *180*(6), 4166–4172. https://doi.org/10.4049/jimmunol.180.6.4166

Russel MF. (1997). Regulation of cell cycle entry and G1 progression by CSF-1. *Molecular Reproduction and Development*, *46*(1), 11–18.

Ryan, G. R., Dai, X. M., Dominguez, M. G., Tong, W., Chuan, F., Chisholm, O., Russell, R. G., Pollard, J. W., & Stanley, E. R. (2001). Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood*, *98*(1), 74–84. https://doi.org/10.1182/blood.v98.1.74

Sapi, E., Flick, M. B., Rodov, S. M., & Kacinski, B. M. (1998). Ets-2 transdominant mutant abolishes anchorage-independent growth and macrophage colony-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. *Cancer Research*, *58(5)*, 1027–1033.

Saqi, A., Yun, S. S., Yu, G. H., Alexis, D., Taub, R. N., Powell, C. A., & Borczuk, A. C. (2005). Utility of CD138 (syndecan-1) in distinguishing carcinomas from mesotheliomas. *Diagnostic Cytopathology*, *33*(2), 65–70. https://doi.org/https://doi.org/10.1002/dc.20297

Sasmono, R. T., Ehrnsperger, A., Cronau, S. L., Ravasi, T., Kandane, R., Hickey, M. J., Cook, A. D., Himes, S. R., Hamilton, J. A., & Hume, D. A. (2007). Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. *Journal of Leukocyte Biology*, *82*(1), 111–123. https://doi.org/10.1189/jlb.1206713

Sauter, K. A., Bouhlel, M. A., O'Neal, J., Sester, D. P., Tagoh, H., Ingram, R. M., Pridans, C., Bonifer, C., & Hume, D. A. (2013). The Function of the Conserved Regulatory Element within the Second Intron of the Mammalian Csf1r Locus. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0054935

Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T., & Stanley, E. R. (1985). The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF 1. *Cell*, *41*(3), 665–676. https://doi.org/https://doi.org/10.1016/S0092-8674(85)80047-7

Shi, X., Kaller, M., Rokavec, M., Kirchner, T., Horst, D., & Hermeking, H. (2020). Characterization of a p53/miR-34a/CSF1R/STAT3 Feedback Loop in Colorectal Cancer. *CMGH*, *10*(2), 391–418. https://doi.org/10.1016/j.jcmgh.2020.04.002

Shukla, A., Miller, J. M., Cason, C., Sayan, M., MacPherson, M. B., Beuschel, S. L., Hillegass, J., Vacek, P. M., Pass, H. I., & Mossman, B. T. (2013). Extracellular Signal–Regulated Kinase 5: A Potential Therapeutic Target for Malignant Mesotheliomas. *Clinical Cancer Research*, *19*(8), 2071–2083. https://doi.org/10.1158/1078-0432.CCR-12-3202

Sletta, K. Y., Castells, O., & Gjertsen, B. T. (2021). Colony Stimulating Factor 1 Receptor in Acute Myeloid Leukemia. In *Frontiers in Oncology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fonc.2021.654817

Stanley, E. R., Chen, D.-M., & Lin, H.-S. (1978). Induction of macrophage production and proliferation by a purified colony stimulating factor. *Nature*, *274*(5667), 168–170. https://doi.org/10.1038/274168a0

Stanley, E. R., & Chitu, V. (2014). CSF-1 receptor signaling in myeloid cells. In *Cold Spring Harbor Perspectives in Biology* (Vol. 6, Issue 6). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/cshperspect.a021857

Strachan, D. C., Ruffell, B., Oei, Y., Bissell, M. J., Coussens, L. M., Pryer, N., & Daniel, D. (2013). CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8+ T cells. *OncoImmunology*, *2*(12), e26968. https://doi.org/10.4161/onci.26968

Such, E., Cervera, J., Valencia, A., Garcia-Casado, Z., Senent, M. L., Sanz, M. A., & Sanz, G. F. (2009). Absence of mutations in the tyrosine kinase and juxtamembrane domains of C-FMS gene in chronic myelomonocytic leukemia (CMML). *Leukemia Research*, *33*(9), e162–e163. https://doi.org/10.1016/J.LEUKRES.2009.03.018

Sullivan, A. R., & Pixley, F. J. (2014). CSF-1R signaling in health and disease: A focus on the mammary gland. *Journal of Mammary Gland Biology and Neoplasia*, *19*(2), 149–159. https://doi.org/10.1007/s10911-014-9320-1

Sun, L., Liang, H., Yu, W., & Jin, X. (2019). Increased invasive phenotype of CSF-1R expression in glioma cells via the ERK1/2 signaling pathway. *Cancer Gene Therapy*, *26*(5), 136–144. https://doi.org/10.1038/s41417-018-0053-y

Tanenbaum, M. E., Stern-Ginossar, N., Weissman, J. S., & Vale, R. D. (2015). Regulation of mRNA translation during mitosis. *eLife*, *4*, e07957. https://doi.org/10.7554/eLife.07957

Thirkettle, I., Harvey, P., Hasleton, P. S., Ball, R. Y., & Warn, R. M. (2000). Immunoreactivity for cadherins, HGF/SF, met, and erbB-2 in pleural malignant mesotheliomas. *Histopathology*, *36*(6), 522–528.

Toy, E. P., Azodi, M., Folk, N. L., Zito, C. M., Zeiss, C. J., & Chambers, S. K. (2009). enhanced ovarian cancer tumorigenesis and macrophage colony-stimulating factor. *Neoplasia*, *11*(2), 136–144. https://doi.org/10.1593/neo.81150

Valledor, A. F., Xaus, J., Marque s, L., & Celada, A. (1999). Macrophage Colony-Stimulating Factor Induces the Expression of Mitogen-Activated Protein Kinase Phosphatase-1 Through a Protein Kinase C-Dependent Pathway1. *The Journal of Immunology*, *163*(5), 2452–2462. https://doi.org/10.4049/jimmunol.163.5.2452

Ventura, E., Belfiore, A., Iozzo, R. V, Giordano, A., & Morrione, A. (2023). Progranulin and EGFR modulate receptor-like tyrosine kinase sorting and stability in mesothelioma cells. *American Journal of Physiology-Cell Physiology*, *325*(2), C391–C405. https://doi.org/10.1152/ajpcell.00248.2023

Ventura, E., Xie, C., Buraschi, S., Belfiore, A., Iozzo, R. V, Giordano, A., & Morrione, A. (2022). Complexity of progranulin mechanisms of action in mesothelioma. *Journal of Experimental & Clinical Cancer Research: CR*, *41*(1), 333. https://doi.org/10.1186/s13046-022-02546-4

Wade Harper, J., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L.-H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., Pat Fox, M., Wei, N., & McLean, M. (1995). Inhibition of Cyclin-dependent Kinases by p21. In *Molecular Biology of the Cell* (Vol. 6).

Walter, M., Lucet, I. S., Patel, O., Broughton, S. E., Bamert, R., Williams, N. K., Fantino, E., Wilks, A. F., & Rossjohn, J. (2007). The 2.7 A Crystal Structure of the Autoinhibited Human c-

Fms Kinase Domain. *Journal of Molecular Biology*, *367*(3), 839–847. https://doi.org/10.1016/j.jmb.2007.01.036

Wang, Y., Szretter, K. J., Vermi, W., Gilfillan, S., Rossini, C., Cella, M., Barrow, A. D., Diamond, M. S., & Colonna, M. (2012). IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nature Immunology*, *13*(8), 753–760. https://doi.org/10.1038/ni.2360

Wen, Z.-Q., Li, X.-G., Zhang, Y.-J., Ling, Z.-H., & Lin, X.-J. (2017). Osteosarcoma cell-intrinsic colony stimulating factor-1 receptor functions to promote tumor cell metastasis through JAG1 signaling. In *Am J Cancer Res* (Vol. 7, Issue 4). www.ajcr.us/

Xiang, C., Li, H., & Tang, W. (2023). Targeting CSF-1R represents an effective strategy in modulating inflammatory diseases. In *Pharmacological Research* (Vol. 187). Academic Press. https://doi.org/10.1016/j.phrs.2022.106566

Xiong, Y., Song, D., Cai, Y., Yu, W., Yeung, Y. G., & Stanley, E. R. (2011). A CSF-1 receptor phosphotyrosine 559 signaling pathway regulates receptor ubiquitination and tyrosine phosphorylation. *Journal of Biological Chemistry*, *286*(2), 952–960. https://doi.org/10.1074/jbc.M110.166702

Xu, J., Escamilla, J., Mok, S., David, J., Priceman, S., West, B., Bollag, G., McBride, W., & Wu, L. (2013). CSF1R Signaling Blockade Stanches Tumor-Infiltrating Myeloid Cells and Improves the Efficacy of Radiotherapy in Prostate Cancer. *Cancer Research*, *73*(9), 2782–2794. https://doi.org/10.1158/0008-5472.CAN-12-3981

Yang, H., Gao, Y., Xu, D., Xu, K., Liang, S.-Q., Yang, Z., Scherz, A., Hall, S. R. R., Forster, S., Berezowska, S., Yao, F., Ochsenbein, A. F., Marti, T. M., Kocher, G. J., Schmid, R. A., Dorn, P., & Peng, R.-W. (2023). MEK1 drives oncogenic signaling and interacts with PARP1 for genomic and metabolic homeostasis in malignant pleural mesothelioma. *Cell Death Discovery*, *9*(1), 55. https://doi.org/10.1038/s41420-023-01307-2

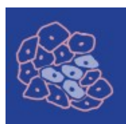
Yu, W., Chen, J., Xiong, Y., Pixley, F. J., Yeung, Y. G., & Stanley, E. R. (2012). Macrophage proliferation is regulated through CSF-1 receptor tyrosines 544, 559, and 807. *Journal of Biological Chemistry*, *287*(17), 13694–13704. https://doi.org/10.1074/jbc.M112.355610

Zhou, R. P., Wu, X. S., Xie, Y. Y., Dai, B. B., Hu, W., Ge, J. F., & Chen, F. H. (2016). Functions of interleukin-34 and its emerging association with rheumatoid arthritis. In *Immunology* (Vol. 149, Issue 4, pp. 362–373). Blackwell Publishing Ltd. https://doi.org/10.1111/imm.12660

Zieve, G. W., Turnbull, D., Mullins, J. M., & McIntosh, J. R. (1980). Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Nocodazole accumulated mitotic cells. *Experimental Cell Research*, *126 2*, 397–405. https://api.semanticscholar.org/CorpusID:32476870

Zwaenepoel, O., Tzenaki, N., Vergetaki, A., Makrigiannakis, A., Vanhaesebroeck, B., & Papakonstanti, E. A. (2012). Functional CSF-1 receptors are located at the nuclear envelope and activated via the p110δ isoform of PI 3-kinase. *The FASEB Journal*, *26*(2), 691–706. https://doi.org/10.1096/fj.11-189753

7. PUBBLICATIONS



cancers

Review

Mesothelioma Malignancy and the Microenvironment: Molecular Mechanisms

Francesca Cersosimo [1], Marcella Barberino [2,3], Silvia Lonardi [4], William Vermi [4,5], Antonio Giordano [2,3], Cristiana Bellan [2] and Emanuele Giurisato[1,6,*]

[1]Department of Biotechnology Chemistry and Pharmacy, University of Siena, 53100 Siena, Italy; francesca.cersosi@student.unisi.it

[2]Department of Medical Biotechnologies, University of Siena, 53100 Siena, Italy; marcella.barbarino@unisi.it (M.B.); antonio.giordano@unisi.it (A.G.); cristiana.bellan@unisi.it (C.B.)

[3]Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA 19122, USA

[4]Department of Molecular and Translational Medicine, University of Brescia, 25100 Brescia, Italy; silvia.lona@gmail.com (S.L.); william.vermi@unibs.it (W.V.)

[5]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63130, USA

[6]Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine, and Health,

The University of Manchester, Manchester M13 9PL, UK

*Correspondence: giurisato2@unisi.it; Tel.: +39-057-723-2125

Simple Summary: In the tumor microenvironment, interaction among tumor cells, immune cells, stromal cells, and the extracellular matrix is vital to support pro-tumor mechanisms such as drug resistance and metastases. Malignant pleural mesothelioma has a unique and complex tumor microenvironment. Several reports underlined the key role of immune and stromal cells in tumorigenesis and progression of mesothelioma. These non-cancer cells, via a reciprocal informational exchange with tumor cells, established a chronic inflammatory microenvironment that support the malignancy and the chemoresistant phenotype of the tumor. The knowledge of the cellular and molecular mechanisms underlying tumor microenvironment interconnection was recently considered a crucial point for the design of more effective therapeutic strategies. In this review, we summarize the molecular mechanisms by which stroma and immune cells support the malignancy of mesothelioma and their potential therapeutic targeting.

Abstract: Several studies have reported that cellular and soluble components of the tumor microen vironment (TME) play a key role in cancer-initiation and progression. Considering the relevance and the complexity of TME in cancer biology, recent research has focused on the investigation of the TME content, in terms of players and informational exchange. Understanding the crosstalkbetween tumor and non-tumor cells is crucial to design more beneficial anti-cancer therapeutic strategies . Malignant pleural mesothelioma (MPM) is a complex and heterogenous tumor mainly caused by asbestos exposure with few treatment options and low life expectancy after standard therapy. MPM leukocyte infiltration is rich in macrophages. Given the failure of macrophages to eliminate asbestos fibers, these immune cells accumulate in pleural cavity leading to the establishment of a unique inflammatory environment and to the malignant transformation of

mesothelial cells. In this inflammatory landscape, stromal and immune cells play a driven role to support tumor development and progression via a bidirectional communication with tumor cells. Characterization of the MPM microenvironment (MPM-ME) may be useful to understand the complexity of mesothelioma biology, such as to identify new molecular druggable targets, with the aim to improve the outcome of the disease. In this review, we summarize the known evidence about the MPM-ME network, including its prognostic and therapeutic relevance.

Keywords: mesothelioma; tumor microenvironment; inflammation; macrophages; cancer stem cells

1. Introduction

Malignant pleural mesothelioma is the main cancer affecting the pleural membranes covering the lungs. It is considered a disease of the elderly and generally manifests in an advanced stage after decades from environmental carcinogen exposure [1]. In addition, its intrinsic heterogeneity, lack of effective targeted therapies and still insufficient knowledge of MPM biology, compromise the quality of life and the prognosis of MPM patients. For sixteen years the only approved therapy has been the combination of platinum and antifolates; recently, nivolumab, a PD-1 blocking antibody, in combination with ipilimumab, a CTLA-4 inhibitor, has also been approved as first-line therapy for unresectable MPM [2]. MPM pathogenesis has unique features because it is mainly related to the exposure of external carcinogens that are mostly represented by asbestos fibers [3,4]. Despite the efforts to limit asbestos use, it is actually banned in 30% of countries. For this reason and for the increasing concerns about the carcinogenicity of new fibrous materials similar to asbestos, the incidence of mesothelioma is not expected to decrease in the coming years [5]. In addition, cases of mesothelioma have also been reported in chronic inflammatory conditions, in absence of fibers exposure, such as chronic pleural diseases, chronic empyema or therapeutic pneumothorax [6]. MPM is characterized by a low tumor mutation burden (TMB) [7], uncommon genetic aberrations, and recurrent somatic mutations in tumor suppressor genes, in both asbestos and non-asbestos induced tumors [8]. The first and most common mutation described in mesothelioma is the deletion of the Cyclin D dependent Kinase inhibitor 2A (CDKN2A) gene on chromosome 9 [9], accounting for approximately 70% of MPM cases [10]. The deletion of this gene affected the cell cycle regulating function of pRB and p53. For its proximity to CDKN2A, the methylthioadenosine phosphorylase (MTAP) gene is frequently co-deleted in different cancer types, including malignant mesothelioma [11,12]. Other common mutations in mesothelioma are in chromosome 3, involving the loss of the BAP1 gene, in chromosome 22 enclosing the neurofibromin 2 (NF2) gene, and in TP53 [7,13–15]. BRCA1-associated protein–1 (BAP1) has many biological activities, including genome stability, DNA damage repair, modulation of the cellular metabolism, regulation of transcription and cell death, among others. BAP-1 loss, together with MTAP/CDKN2A deletion, has been recently proposed as useful markers to improve the diagnostic sensitivity for MPM [16] (Figure 1). Mutation in the NF2 gene, encoding the cell growth-regulating protein Merlin, has been described in about 50% of MPM [17], and has been linked to mesothelioma progression. Alteration in NF2 function has been recently related to the tumor immune microenvironment and proposed as biomarker for MPM patient's stratification for immune-checkpoint blockade (ICB) therapies [18]. It has been reported that in BNC mice, where the specific disruption of the Bap1, Nf2, Cdkn2ab tumor suppressor loci in the mesothelial lining of the thoracic cavity leads to a highly aggressive MM, an infiltration of leukocytes was found [19]. In particular, a significant number of macrophages, T cells, including regulatory T cells (Tregs), B cells and NK cells was observed. This recapitulates the histological features

and gene profile observed in human patients carrying combined BAP1, NF2 and CDKN2A alterations [20], indicating that the combined deletion of these tumor-suppressor genes creates a mesothelioma-specific microenvironment. The enrichment of NF-kB signaling pathway in BNC tumors likely contributes to the recruitment of immune cells to these tumors. In support of the link between TME and different genetic background, Yang H and collaborators provided evidence that $CD8^+$ T cells were mainly enriched in the MPM harboring Large Tumor Suppressor Kinase [1] (LATS1/2 mutation compared with NF2-mutant cancer. In addition, MPM tumors harboring LATS1/2 mutation is associated with high PD-L1 expression and rather than NF2-mutant MPM, display enriched Tregs signature and plasma B cell signature [18], suggesting that different tumor-infiltrating immune cell patterns exist between dysregulation of NF2 and Hippo-YAP signaling in MPM. More recently, the relationship between p14/ARF encoded by CDKN2A and tumor microenvironment was evaluated. Pezzuto et al. found that p14/ARF-negative tumors are characterized by a high percentage of $CD163^+$ cells and low PD-L1 and CD4 expression, correlated with an immune microenvironment less sensitive to immune checkpoint inhibitors [21]. Collectively, these data support the idea that genomic intratumor heterogeneity shapes the MPM tumor microenvironment and modulates host immune surveillance or immune escape in MPM [22].

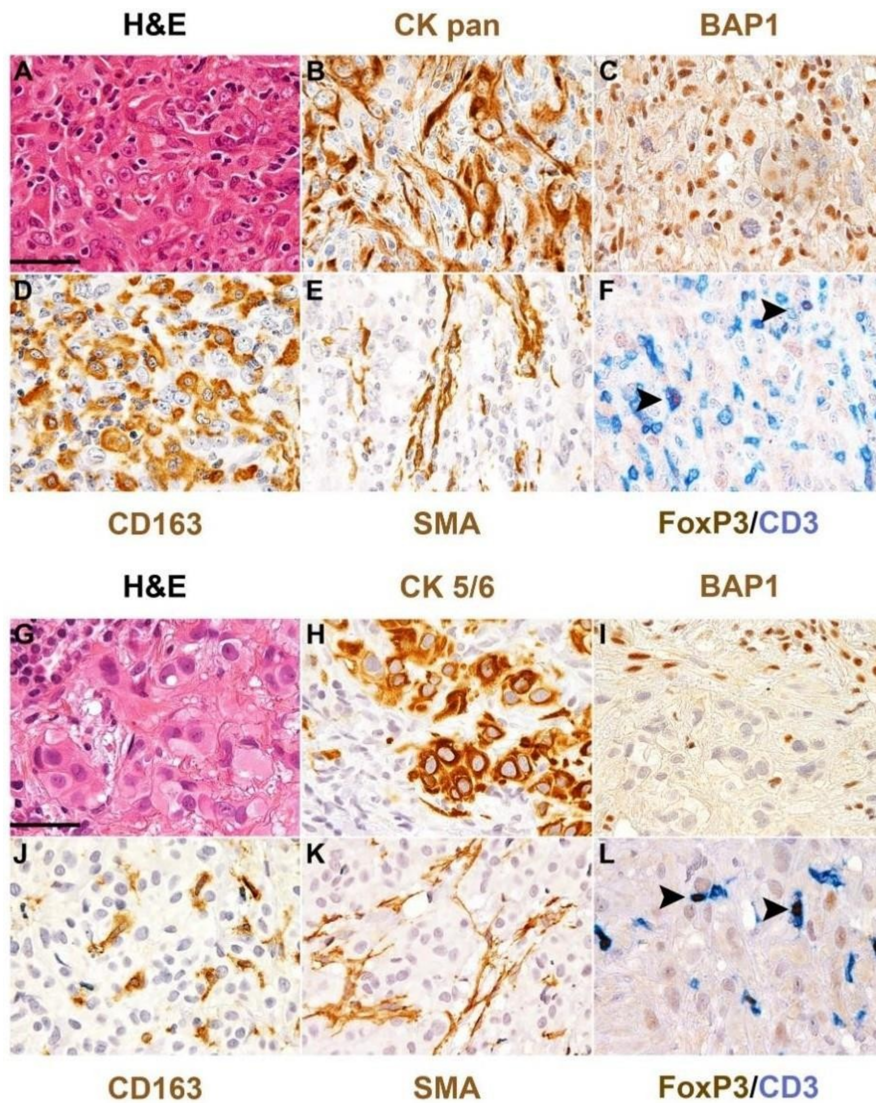


Figure 1. BAP1 loss and microenvironment in human mesothelioma. Hematoxylin and eosin stainand a set of immunostainings performed on formalin-fixed paraffin-embedded sections of two cases of human epithelioid mesothelioma of the pleura showing loss of BAP1. The upper case (**A**–**F**) results massively infiltrated by CD163$^+$ macrophages, SMA$^+$ fibroblasts and CD3$^+$ T-cells including the FOXP3$^+$ regulatory population, whereas the bottom case (**G**–**L**) displays a scant macrophage infiltration. Primary antibodies included broad-spectrum cytokeratin (CK, **B**) or cytokeratin 5/6 (**H**) as mesothelioma markers; anti-BAP1 (**C**,**I**); CD163 (**D**,**J**), for tumor associated macrophages (TAMs); smooth muscle actin (SMA, **E**,**K**) for cancer-associated fibroblasts (CAFs); CD3 (**F**,**L**) for T-cells and FoxP3 for regulatory T-cells. Arrows (**F**,**L**) highlight representative regulatory T-cells. Original magnification 400×, scale bar 50 μM.

According to the low TMB, microsatellite instability and deficiency of DNA mismatch repair system proteins (dMMR) (MutS Homolog 2 (MSH2), MutS Homolog 6 (MSH6), MutL Homolog 1 (MLH1)) due to gene mutation or epigenetic silencing, have been found in a small subset of MPM [23–25]. In this scenario of a low mutational burden, epigenetic regulation is considered to significantly contribute to the malignant mesothelial transformation. Particularly for cancers linked to environmental insults such as mesothelioma, the study of epigenetics has been demonstrated to be a useful indicator of disease risk, with diagnostic and prognostic value [26–29]. However, the deregulation of genes and protein expression described above contribute to, rather than determine, the MPM onset. Different from other cancers, driver mutations have not been found in mesothelioma and the pathogenesis of this cancer is undoubtedly related principally to the inflammatory microenvironment created by asbestos deposition in the pleura that stimulates the immune response. A crucial role in this process is recognized to the pleural macrophages recruited at the inflammatory site, which, failing the attempts to eliminate the fibers, are subject to frustrated phagocytosis, a process leading to activation of Nicotinamide adenine dinucleotide phosphate (NADPH), the generation of reactive oxygen species (ROS) and release of proinflammatory molecules (IL-1β, IL-8, IL-6 and TNF-α) [30]. Accumulation of asbestos fibers leads to aberrant activation of intracellular pathways and transcriptional processes responsible for the malignant transformation of mesothelial cells and the development of a unique inflammatory microenvironment [31]. Indeed, tumor-associated macrophages (TAMs), T regulatory cells (T_{reg}), such as cancer-associated fibroblasts (CAFs), are the most abundant population of MPM infiltration, that, in response to pro-tumoral signals, acquire malignant and immunosuppressive properties, influencing the progression of the tumor [32] (Figure 1). Tumor cells have developed different mechanisms to escape immune surveillance, such as the activation of inhibitory pathway (PD-1/PD-L1) leading to T-cell exhaustion and suppression of cytotoxicity. Overexpression of PD-L1 on immune cells and PD-1 of tumoral-origin was also described in MPM [33]. Moreover, resistance to anti-cancer drugs is another feature of MPM cells, that is supported by the TME crosstalk [34]. Given the importance of the interactions between the surrounding and neoplastic cells, several research groups have investigated the content of TME in terms of cellular component and exchanged soluble factors with the aim to improve the immunotherapy. However, its variability contributes to the complexity and the difficulty to evaluate it in terms of diagnosis, prognosis and therapeutic approach [32]. According to Linton et al., inflammation in mesothelioma could be considered a “friend” or a “foe”, because chronic inflammation could be established as a favorable environment for cell survival on one hand and could induce the suppression of anti-cancer response on the other hand [35]. For this, the inflammatory landscape of mesothelioma TME is required to be deeply investigated in order to restore the immune response and develop strategies with better therapeutic benefits. In this review, we summarize the known evidence on the MPM TME, focusing on the inflammatory population, the interplay between immunity, stroma and tumor cells, such as mechanisms regulating immune-evasion and drug resistance. Additionally, several studies reported the prognostic value of immune cells in the mesothelioma microenvironment. High incidence of $CD8^{+}$ T cells was associated with a better prognosis and favorable outcome; on the contrary, the presence of M2-like macrophages that represent the most abundant immune population in MPM-TME, was correlated with a worse prognosis and no improvement in the overall survival [36,37]. Thus, here we described the potential prognostic role of these cells and highlight the importance

to under- stand the MPM-TME players as potential candidates for promising mesothelioma therapy.

2. Mesothelioma Stem Cells (MSCs) and Chemoresistance

A sub-population of cancer cells, indicated as cancer stem cells (CSCs), was assumed to be involved in the tumorigenesis and metastatic progression. These cells display the capability of self-renewal via asymmetrical division, maintaining the stem niche in tumor sites. CSCs theory is based on a hierarchical organization of cells in the tumor, where only a sub-set of cells with high tumor-forming properties can give rise to a heterogenouscancerous cell population associated with rapid recurrence [38,39]. Different studies identified the presence of CSCs within malignant mesothelioma (MM) tissue as responsible for tumor heterogeneity [40], chemoresistance and relapse after therapy [41]. MM, as with other tumor types, is strongly characterized by inter and intra-tumor heterogeneity and the persistence of resistant CSCs in tumor tissue represent the main cause of treatment failure in cancer therapy [42,43]. Kay K et al. demonstrated that cisplatin-based treatment upon MM cell lines favored the selection of a side population with increased replicative potential and high expression levels of stemness-related genes, including OCT4, NOTCH1 and BMI1 [44]. The presence of SOX2/OCT4 positive cells in chemotherapy-resistant MM has been later confirmed by Blum et al. who additionally demonstrated the tumorigenic properties of these cells by in vivo studies [45]. Various tumors have shown the presence of a chemoresistant sub-population of cells expressing high levels of ABC-transporters protein and increased aldehyde dehydrogenase (ALDH1) activity [46]. Similarly, overexpression of ABCGE protein was found in MM cells expressing stem-like characteristics, such as a therapy resistant phenotype [44] and an elevated ALDH1 activity characterized by cells inMM with CSCs properties [47]. Up-regulation of ABCB-5 drug transporter was observed in MM CSCs as necessary for the acquisition and maintenance of a stemness and chemoresistant phenotype. Additionally, intrinsic mechanisms, such as increased expression of theautocrine loops Wnt/GSK3β/β-catenin/c-myc in MM CSCs support the over-activation of ABCB-5 as well as drug resistance of these cells [48]. Conino et al. have demonstrated that Pemetrexed induced rapid senescence in MM cells associated with the production of cytokines and pro-inflammatory molecules as well as the involvement of STAT3 signaling, that, in turn, activate epithelial-to-mesenchymal transition (EMT) programs, via the release of invasion-promoting factors (MMP-2) and the emergence of chemoresistant, clonogenic and $ALDH^{+}$ cells [49]. The deficiency of the tumor suppressor gene, NEF2, in mesothelioma mouse models was correlated with an increase of CSCs in the tumor tissue [50]. Recent studies demonstrated a link between the presence of NF2-negative cell populations and high sensitivity to FAK-signaling inhibitors in MM cells [51]. The tumorigenic role of FAK and its contribution to self-renewal and aggressiveness of CSCs in tumors has been widely reported [52]. CSCs elimination by using FAK inhibitors treatment in MM cell lines and NOD/SCID mice injected with MM stem-like cells has been observed. Since CSCs constitute the main targets for drug resistance, a combined approach with FAK inhibitors and chemotherapeutic agents could represent a new potential cancer treatment strategy to eradicate CSCs and overcome the mechanism of chemoresistance [45,51]. Nowadays, less is known about molecular mechanisms underlying CSCs chemoresistance in MM. Therefore, a better characterization of MM CSCs biology is required to fight drug resistance and improve MM treatment.

3. Mesothelioma Stem Cells Malignancy and TME

Different studies have highlighted the metastatic ability of CSCs as key players of tumor progression [53], also in MM [54–56]. In this regard, biochemical analysis has shown that spheroid-derived mesothelioma stem-cells (MSCs), expressing increased levels of the cancer cell survival-related protein transaglutaminase (TG2), had more invasive and migratory capabilities compared with monolayer-derived mesothelioma cells. TG2 has been reported to have important roles in CSC-phenotype acquisition and invasiveness of tumor cells. Inactivation of TG2 has been shown to decrease the expression of epithelial-to-mesenchymal transition markers (Fibronectin, MMP-9, Slug and Snail), the Matrigel invasive abilities and increased the levels of the pro-apoptotic factors as caspase-9 and PARP activity [55]. Moreover, a recent study demonstrated that inhibition of YAP1/TAZ/TEAD signaling pathway in MSCs from peritoneal and pleural-derived mesothelioma cells reduced migration and invasiveness of these cells, such as increased pro-apoptotic markers expression and negatively affected the spheroid forming ability of these MM stem-like cells [56]. A heterogenous population of cells, expressing stromal, immune e stem-cell markers, was identified by the analysis of the tumor spheroid derived from orthotopic MM murine model [54]. Several reports have documented the importance of TME interactions to sustain tumor growth and metastasis [57]. Indeed, a study demonstrated that stem cells number in tumor spheroid raised in response to chemotactic signals, specifically increased expression of the SDF1/CXCR4 axis, which induced the recruitment of these cells at the tumor sites [54]. These data highlight the potential impact of investigating TME signaling in order to find targetable molecules involved in cancer cell dissemination to secondary sites.

4. Mesothelioma Microenvironment Crosstalk: Molecular Mechanisms

4.1. Mesothelioma and Stroma

Tumor growth depends not only on cancer cells activity, but also by the interactions between neoplastic cells, stroma, extracellular soluble factors, and inflammatory cells that collaborate to support the tumor progression. Tumor cells are able to change the surrounding microenvironment by the release of soluble factors that induce the malignant transformation of resident stromal cells. The communication with the stroma is necessary to create a permissive environment allowing cancer cells to escape immune defensive mechanisms and to metastasize [58,59]. Thus, understanding this complex network that orchestrates the TME is required for the discovery of promising therapeutic target therapy. Different studies have investigated the content of MPM tumoral stroma, as a source of prognostic markers and potential therapeutic targets. In mesothelioma, as with other tumor types, the stromal component plays an important role to support tumor growth and invasion [60] (Figure 2). Lievense et al. identified high expression of pro-inflammatory soluble cytokines in pleural effusion of MPM patients as well as in MPM cell line super- natant. These cytokines, including IL-6, TGF-β, VEGF and IL-12, are known for taking part in the malignancy of the tumor, including the invasive, angiogenetic, and immuno- suppressive mechanisms [61]. Proteins of the extracellular matrix (ECM) resulted in being up-regulated, especially in the most aggressive histological subtypes of MM. Integrins, collagen, fibronectin and metalloproteinases have been highly produced by mesotheliomacell lines to make ECM permissive for chemotaxis and invasion [36]. A previous study identified the involvement of TGF-β pathway in the expression of connective-tissue growth factors (CTGFs) in MM tumor cells and surrounding stromal cells [62]. CTGF expression has been found in several cancer types

to be correlated with malignant features, such as angiogenesis, invasion, and metastasis [63]. In MPM, the CTGF expression was found to participate in the modulation of the ECM via secretion of matrix-associated proteins in favor of tumor progression [62]. Cancer-associated fibroblasts (CAFs) are one of the main components recruited in the tumoral stroma. These cells interact with tumor cells, and recruit immune and vascular cells at the tumor site through the release of soluble factors, such as cytokines and chemokines [59] (Figure 2). CAFs are able to remodel ECM by the production of several ECM-related proteins (e.g., integrins) that mediate the crosstalk with tumor cells and promote local invasion and metastatic spread [64]. These spindle-like cells are also found in mesothelioma tissue, associated with pro-tumor functions, such as stromal remodeling and tumor invasion, and are correlated with poor prognosis [65]. Ohara et al. proposed a mechanism by which fibroblasts participate to pleural fibrosis in the early mesothelioma phases in response to ROS production by frustrated macrophages. Activated fibroblasts exerted a pro-tumor role by the expression of CTGF and other cy- tokines [65]. Li Q. et al. suggested positive feedback between MPM cells and CAFs: tumor cells producing growth factors, such as FGF-2 and PDGF-AA, promote the growth and the activity of CAFs that in response secrete cytokine HGFs enhancing the migratory and invasive abilities of tumor cells [66]. Furthermore, MPM is indicated as a tumor with a high tendency to angiogenesis [67] and it is well known that CAFs are pro-angiogenic factors in tumors [68]. Serum and pleural effusion from mesothelioma patients have shown high levels of angiogenic cytokines as VEGF and FGF-2 that are linked with the development of new blood vessels in tumors [67,69,70]. Additionally, a lower infiltration of immune cells has been correlated with high expression of stromal-related and connective-related genes in mesothelioma tissues [33]. MM contains a heterogenous population of immune cells, changing among patients and histological subtypes [32,60]. Because of the prognostic and therapeutical role of immune cells, the investigation of this area has recently attracted the interest of immune oncology research. Ujii et al., with the aim to characterize MPM TME, identified immune markers with prognostic value in tumor and tumoral stroma of epithelioid MPM tissue. Specifically, they found that markers of tumor-infiltrating lymphocytes (CD8, CD20) correlated with a better prognosis. Instead, high density of M2-like TAMs ($CD163^+$) and cytokine receptors ($IL\text{-}7R^+$) expression by tumor cells resulted with decreased patient survival [71]. Several findings have demonstrated that TME of MPM is enriched with a high number of immunosuppressive cells, among these M2-like TAMs and regulatory T cells represent the most abundant immune cells in the MM microenvironment [32,36]. Pleural effusions of MPM patients have shown a strong infiltration of activated cytotoxic and helper T cells; however, most of them are associated with T cell exhaustion markers, such as $PD\text{-}1^+$, TIM-3, LAG-3, that negatively regulate lymphocyte activity [72]. Additionally, evidence shows that PD-L1 signaling in tumors promoted T helper-1 (Th-1) cells reprogramming in T-regulatory cells [73]. The high levels of PD-L1 in mesothelioma tissue have made it an eligible tumor for immune checkpoint inhibitor-based therapy [33] (Figure 2). Recently, Klampatsa et al. identified, by flow cytometry analysis on MPM patient samples, a proportion of $CD8^+$ tumor-infiltrated lymphocytes (TIL) and tissue-resident memory (Trm) cells with hypofunction that was related not to the expression of inhibitor molecules but to the higher degree of Tregs in TME and to the expression of the Eomes transcriptional factor, known as regulator of $CD8^+$ cell functions [74]. A link between high density of $CD8^+$ immune cells and improved MPM patient outcomes has also been demonstrated [71,75,76], suggesting a potential therapeutic strategy. Natural killer (NK) cells were also present

in MPM samples; however, these cells showed an immunosuppressive profile and lower cytotoxic functions [77]. Given the heterogeneity of immune cell content in MM TME and the pro-tumor functions of the stromal content, an understanding of these components is required for better understanding MM pathogenesis and for the development of an efficacious anti-cancer targeted therapy.

4.2. Macrophages in Mesothelioma

Macrophages are a heterogenous population of immune cells acting by phagocytosis and destruction of foreign antigens [78]. Failed phagocytosis of asbestos fibers by macrophages represents one of the possible mechanisms promoting neoplastic transformation of mesothelial cells [79]. Phagocytic macrophages, unable to eliminate fibers, release oxidative molecules and pro-inflammatory cytokines that promote a pro-inflammatory environment and activate signaling pathways in tumor cells that help them to survive despite the asbestos-related damage [80]. A critical pro-inflammatory mediator in the mesothelial transformation process has been identified in high-mobility group protein box 1 (HMGB1), a cytokine that, upon asbestos exposure, is released by mesothelial cells recruit- ing and activating macrophages. Moreover, HMGB1 impairs macrophage phagocytosis and induces the secretion of tumor necrosis factor-α (TNF-α), protecting the mesothelial cells from death signals and sustaining the chronic inflammatory response [81–83]. The binding of HMGB1 to specific receptors on macrophages activates the NLRP3 inflammasome and induces the secretion of IL-1β, IL-18, IL-1α, and HMGB1 itself, establishing a chronic inflammatory loop [84]. By this mechanism, the new mesothelioma cells are able to proliferate, giving rise to a progeny of neoplastic cells [85]. Additionally, MM tissue has been shown to express high levels of the "don't eat me" signal CD47 that help tumor cells to escape from the immune surveillance systems, including macrophage phagocytosis [86]. Macrophages are plastic cells that, in response to environmental signals, may acquire different phenotypes [87]. The classical or pro-inflammatory (M1) and the alternative or anti- inflammatory (M2) profiles represent the main phenotypes of polarized macrophages [88]. Tumor-associated macrophages (TAMs) are generally infiltrating immune cells that preferentially polarize to M2-like phenotypes [89]. These M2-like TAMs favor a pro-tumor microenvironment via the production of several growth factors and enzymes promoting angiogenesis, immunosuppression and metastases [90]. In mesothelioma microenvironments, TAMs represent the most abundant immune population, and the high prevalence of these cells has been associated with poor prognosis in mesothelioma patients [36] (Figure 2). The expansion of $CD206^+$-M2-like macrophages during mesothelioma progression was observed in tumor tissue of an orthotopic model developed to mimic MPM-ME [91]. MPM cells, by producing high levels of the monocyte chemoattractant protein CCL2, induced the recruitment of monocytes at tumor sites [92]. In various cancers, the activation of the CCL2/CCR2 axis, that mediates the crosstalk between TAMs and tumor cells, was associated with metastases and cancer progression. Moreover, targeting CCL2 in lung cancer has been shown to reduce the macrophages recruitment and the M2-polarization rate, such as with active cytotoxic T cells [93]. A previous study described MM cells as able to induce the M2-like polarization, the release of pro-inflammatory cytokines (TNF-α; IL-10) and the acquisition of an immunosuppressive profile [94]. Indeed, high expression of immunomodulatory cytokines. including TGF-β, IL-10 and M-CSF, have been found in pleural effusions of MPM patients [36]. Therefore, an increased number of infiltratingmacrophages ($CD68^+$) with a M2-like phenotype ($CD163^+$; $CD206^+$; IL-4Rα^+) was found in MPM and peritoneal

mesothelioma [92,95]. Concurrently, TAMs communicate with MPM cells via the IL-1β/IL-1R signaling and the activation of the IL-1R pathway in tumor cells was shown to correlate with the acquisition of a CSC-like phenotype [96]. In addition, the presence of M2-like TAMs in MPM-ME was linked with an increased proliferation rate of tumor cells, such as decreased efficacy of chemotherapeutic drugs [92]. Growing evidence has shown that the release of molecules, such as IL-6, IL-10 and IL-34 by TAMs, contributed to the acquisition of a chemo/radioresistant phenotypes in tumor cells [97].Previous studies have reported the presence of CSF-1R ligands, M-CSF and IL-34, in pleural effusion of MPM patients associated with short survival. However, only the presence of M-CSF was associated with M2-like markers expression, suggesting a different role for IL-34 in TME [98]. More recently, a chemoresistant phenotype of a CSF-1R^{+} population of mesothelioma cells, detected in primary cultures and MPM cell lines, resulted in being supported by the expression of both IL-34 and M-CSF ligands [99]. Accordingly, in vitro and in vivo studies have demonstrated that inhibition of CSF-1R might restore the CD8^{+} T cell anti-tumor response [98,100]. Inhibition of CSF-1R not only avoided mesothelioma progression and enhanced T cell response but was also shown to increase the sensitivity of mesothelioma to PD-L1 inhibitors [101]. Additionally, in vivo studies have demonstrated the efficacy of a recently developed monoclonal antibody anti-CSF-1R (RG7155) in the reduction of CD68^{+}/CD163^{+} TAMs in mesothelioma biopsies [102]. Thus, targeting the IL-34, M-CSF and CSF-1R may represent a potential therapeutical approach to suppress both mesothelioma cells and pro-tumor macrophages. TAMs may also exert immunomodulatory functions, by defending tumor cells from immune attack [103]. Lievense et al. reported the immunosuppressive properties of macrophages in MPM-ME, by a mechanism directly affecting CD4/CD8-T cell proliferation. In addition, they linked high levels of the prostanoid PGE_2 in the pleural effusion from MPM patients with the increased number of M2-like macrophages, suggesting a potential role of PGE_2 as promotor of a suppressive macrophage profile [61]. Miselis et al. demonstrated that targeting M2-like TAMs reduced mesothelioma growth and metastases [104]. Moreover, zoledronic acid has been reported to exhibit inhibitory functions on M2-macrophage differentiation and TAMs accumulation in mesothelioma [105]. Different strategies have been designed to reduce TAMs infiltration showing therapeutic benefits, via blocking their recruitment or direct killing, or aiming at reprogramming their anti-tumor abilities [106]. New evidence suggests that TAMs proliferate in tumors, including mesothelioma. These proliferative macrophages have been shown as a common hallmark of human solid tumors and as a potentially important prognostic marker of malignancy [107]. The mechanism that regulates TAMs self-renewal is still under investigation as a new potential therapeutic target. Little evidence exists regarding the known molecular mechanisms regulating TAMs in mesothelioma and few therapeutic strategies targeting TAMs have been developed. However, given the involvement of TAMs in drug resistance and mesothelioma progression, targeting TAMs alone or in combination with other treatments may be a promising therapeutic strategy for cancer therapy.

4.3. Adenosine Pathway and Mesothelioma Microenvironment

Extracellular amounts of adenosine (ADO) in tumor tissues are higher than in normal tissues because of accumulation of ATP [108]. Adenosine is an ATP-AMP metabolite that accumulates in the tumor and its expression is essentially mediated by CD39 and CD73 ectonucleotidase expression [108]. Besides tumor cells, CD39 and CD73 are also expressed on a broad range of cells of TME, such as T-cells, macrophages, MDSC, B cells, epithelial

cells, and also on tumor-derived exosomes [109,110]. Extracellular ATP (eATP) is released by dying and damaged cells, and functions as immunostimulatory signal. The key event activating the adenosinergic pathway (AP) is the conversion of extracellular adenosine triphosphate (eATP) to 5′-AMP by CD39 (ectonucleoside triphosphate diphosphohydrolase-1), and then the production of ADO from 5′-AMP by CD73 (ecto-5′-nucleotidase) [111,112] (Figure 2). ADO binds and activates four different receptors (G-protein-coupled receptors A1R, A2AR, A2BR, and A3R) with different affinity and cellular specificity [111]. The AP is principally mediated by the binding of ADO to the high affinity A2AR presents on the surface of macrophages, T cells, NKs, neutrophils and dendritic cells, and on epithelial, mesothelial and cancer cells [113,114]. The balance between ATP and ADO receptors level and the expression of CD39 and CD73 ectonucleases determines the activation of an inflammatory or an anti-inflammatory response. While under physiological conditions, the AP is precisely controlled in pathologic conditions, such as cancer, the signaling is deregulated precluding autoimmunity and providing protection for malignant cells. AP in cancer, sustaining immunosuppressive cell types and thus the release of cytokines and immune modulatory factors, such as VEGF, IL6, IL10, and TGFβ, and activating survival pathways, inhibits immunosurveillance and enhances tumor survival, metastasis and therapy resistance [115]. AP deregulation in cancer has been principally linked to ADO accumulation in the TME that promotes regulatory T-cells (Tregs) activity and polarization of myeloid cells to immunosuppressive and pro-angiogenic phenotypes and affects NKs and effector T cells (CD8+), thus enhancing tumor growth. Many studies have reported that increased expression of CD73 in cancer relates to different outcomes, such as progression, poor prognosis, metastasis, and weak response to chemotherapy agents [116]. Although ADO signaling has been poorly investigated in MPM, available data support a role for AP in MPM immunosuppression. Al-Taei and collaborators [117] observed CD73 expression on TAMs in pleural effusion (PE) but not peripheral blood of mesothelioma patients or healthy donors, reinforcing the hypothesis that TAMs immunosuppressive function can involve, at least in part, ADO signaling. In addition, CD73 expression can be induced by PGE_2, cAMP or adenosine on human $CD14^+$ cells, demonstrating the existence of an autocrine loop that, upon A2AR activation, leads to the upregulation of CD73 on human CD14+ monocytes. Activation of A2A receptors primarily has multiple inhibitory effects on the M1 macrophage subset, while adenosine receptors induce the M2 macrophage subset by up-regulating the expression of several markers, such as arginase 1 (Arg-1), tissue inhibitor of matrix metalloproteinase 1, and macrophage galactose-type C lectin 1 [118]. Several lines of evidence also support the idea that adenosine can increase VEGF secretion by macrophages through the activation of A2A receptors [119]. In addition, Cekic et al. [120] reported that A2A expression on myeloid cells, specifically TAMs, in- directly mediated suppression of T cells and NK cells in the tumor microenvironment. eATP hydrolytic activity to produce ADO has also been documented in exosomes isolated from MPM PE estimated to contribute for 20% of the total ATP-hydrolytic activity in MPM PE [121]. In addition, adenosine inhibits TNF-α and IL-12 release and augments IL-10 and vascular endothelial growth factor (VEGF) [122] production by LPS or bacteria-activated macrophages [123–126] and promotes alternative macrophage activation [127]. More recently, it has been reported that adenosine is also involved in TAMs proliferation [128]. Mechanistic studies have demonstrated that adenosine released from hepatoma cells could promote macrophages proliferation through the A2A receptor, and tumor-derived adenosine functions synergistically with autocrine GM-CSF released in TME,

supporting macrophages proliferation (Figure 2). Despite the lack of information on the role of AP in MPM, these preliminary results support the rationale of investigating AP in MPM as a new therapeutic opportunity to improve the response to current therapeutic regimen and the response to the emerging immunotherapies.

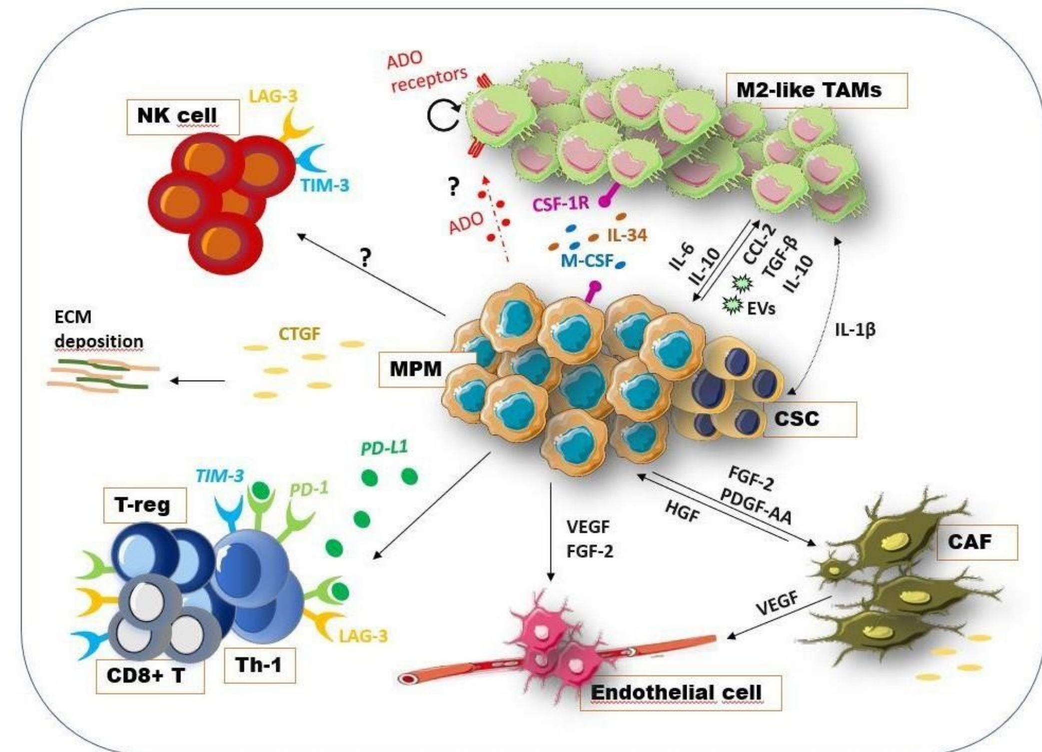


Figure 2. Cellular and soluble factors in MPM-ME. The interconnection between tumor cells and the surrounding stromal and immune component is necessary to create a permissive environment for cancer growth, immune escape and invasiveness [59]. Tumor cells, by the release of the growth factors FGF-2 and PDGF-AA, recruited fibroblast at tumor sites promoting their pro-tumoral activity as CAFs. In turn, these CAFs secreted the HGFs, produced ECM-related proteins, expressed the CTGF and other cytokines supporting tumor growth [65,66]. Additionally, MPM cells have been found to express the CTGF as modulator of ECM-related proteins and supporter of cancer invasiveness [62]. CAFs as well as tumor cells, via the release of the angiogenic VEGF, promoted the recruitment of endothelial cells and the vasculogenesis [67,69,70]. Exhausted Th and CD8$^+$ T cells are also present in TME, characterized by the expression of immune checkpoint molecules, such as PD-1, TIM3, LAG3 [72]. Moreover, PD-L1 signaling induced Th cells reprogramming in Treg cells [73]. Moreover, theNK cells showed an immunosuppressive phenotype and low cytotoxicity [77]. TAMs, which represent the most abundant immune population [36], are recruited at tumor sites by tumor produced CCL2 [92]. MPM cells enhance the malignancy of macrophages via the release of TGF-β, IL-10, exosomes (EVs) and M-CSF [36]. The presence of M-CSF and IL-34 was associated with short survival and chemoresistance [98,99]. Recently, the inhibition of CSF-1R has been shown to reduce mesothelioma progression and increase the susceptibility of MPM to immune checkpoint inhibitors [101]. The activation of the IL-1β/IL-1R signaling pathway in tumors by TAMs is correlated with the acquisition of a CSC-like phenotype [96]. As observed in other tumor types, adenosine (ADO) pathways may be involved in MM cells-TAMs interaction, inducing the release of pro-tumoral cytokines and promoting TAMs proliferation [115,127,128]. Although limited data indicate its involvement in MPM immunosuppression [117], a deep investigation is required. This figure was prepared using a template on the Servier medical art website (http://smart.servier.com/).

5. Intra-Tumor Heterogeneity within MPM Subtypes and TME

Several studies have highlighted how the immune landscape among MPM histological variants impact on the clinics and the immunotherapeutic response. Different microenvironmental stimuli drive the activation of different signaling pathways and genetic events that induce tumors to acquire distinct phenotypes and behaviors. Thus, the knowledge of the TME complexity has become important in order to understand the molecular profile of the tumor and its involvement in therapy resistance [42]. The intra-tumoral heterogeneity was investigated by Blum et al. who identified two distinct populations with epithelioid and sarcomatoid traits in different sites of MPM samples. The non-epithelioid sites resulted in being enriched in T cells, monocytes, fibroblasts, and endothelial cells as well as high expression of the immune checkpoint molecules PD-L1 and CTLA-4, determining an immunosuppressive environment [129]. Similarly, two different cellular sub-populations with distinct immunological phenotypes were discovered within MPM tumor immune microenvironment (TiME) by a CyTOF analysis on 12 tumor samples. In the study, a higher number of PD-1$^+$CTLA-4$^+$ CD8$^+$ T cells was found in a subtype and more ICOS$^+$-CTLA4$^+$ T-regs and PD-1$^+$ TAMs in the other one, suggesting a different response to immune-checkpoint inhibitors (ICIs) [130]. Additionally, increased macrophage infiltration was detected in the non-epithelioid MPM and a high ratio CD8$^+$/CD68$^+$ was correlated with a worse prognosis [34,131]. Non-epithelioid subtypes were also associated with PD-L2 positivity, and they were more likely to have a high infiltration with TIM3+ lymphocytes. Furthermore, an immune checkpoint score composition, comprehending the expression of PD-L1, PD-L2and TIM3, when divided into three groups, identified that patients with high scores were more likely to be of non-epithelioid histology and had greater TILs [132]. On the other hand, in the epithelioid compartment, enrichment of NK cells and innate immunity markers was found, such as high prevalence of CD4$^+$ and CD20$^+$ cells, together with the expression of the T-cell immune suppressor VISTA [34,129,133]. As described by Yang et al., it is crucial to understand the genomic, molecular and histological heterogeneity among MPM patients given its importance at prognostic and therapeutic level and with the attempt to move to personalized treatment options [134]. Another study documented the up-regulation of VISTA on inflammatory cells in the epithelioid subtype; however, correlated it with a better overall survival. In contrast, the augmented PD-L1 expression levels in the sarcomatoid MPM were associated with a poor patient outcome [133]. Recently, Alcala et al. classified MPM types from a “hot” to “cold” tumors, based on the immune content and the overall survival [135]. Interesting data were also reported by an analysis along MPM histotypes, highlighting that T-cell immune response was progressively lost in the more aggressive sarcomatoid histotype. However, an immunosuppressive network provided by the expression of immunosuppressive cytokines and functionally impaired T-cells was also observed in the epithelioid subtype [136]. Lately, an immune-related classification of MPM patients associated with the overall survival and drug response to chemotherapeutics and ICIs was also suggested by Alay et al. [137]. Although the role of the TME network in tumor resistance mechanisms has previously reported [138], the correlation between TME heterogeneity and therapy response in MPM requires more investigation.

6. Therapeutic Approach Targeting TME

The standard treatment for MPM is provided by surgical resection combined with radio/chemotherapy (multimodality treatment) or radio and chemotherapy alone for unresectable tumors. However, the life expectancy of patients remains low [139]. The

reduced efficacy of standard treatment is mainly related to the development of resistant mechanisms, such as the complexity and the heterogeneity of this tumor among patients [140]. Thus, a better knowledge of mesothelioma biology and chemoresistance mechanisms is required to design successful therapeutic strategies. We have previously described the known evidence about the mesothelioma microenvironment. Because most of the resistance mechanisms were mediated by the crosstalk between tumor and the surrounding microenvironmental cells via the exchange of pro-tumoral signals, cancer research has recently focused on the investigation of TME content with the aim to find new molecules to target as monotherapy or in combination with the standard treatment [36]. Different immunotherapeutic approaches for MPM have been investigated by pre-clinical and clinical studies with the aim to restore the anti-tumor immune response. Pre-clinical studies based on the use of animal models and MM cell lines have aimed to investigate mechanisms able to elicit the cytotoxicity against tumor cells and to deplete the immunosuppressive cells. These studies tested the use of antibodies targeting T regs, such as anti-CD25, or liposome encapsulated clodronate (CLIP) to reduce M2-like TAMs, and the use of dendritic cell (DC) immunotherapy or chimeric antigen receptor (CAR) T cell therapy, such as the development of chemokine/cytokine-blocking molecules [141]. The available drugs targeting TME tested in clinical trials for mesothelioma treatment are indicated in Table 1. Mesothelioma is a highly angiogenic tumor and overexpression of vascular-endothelial factor (VEGF), its receptor and other angiogenic factors, has been observed in tumor tissues. Bevacizumab is a VEGF-targeted humanized monoclonal antibody approved by FDA in different cancer types. Phase II-III clinical trials for VEGF inhibitor (Bevacizumab) used in combination with the standard chemotherapeutic drugs pemetrexed plus cisplatin showed a significant improvement in the overall survival of advanced MPM patients [142,143]. Additionally, phase II studies on the use of the VEGF, PDGF and FGF receptors inhibitor (Nintedanib) plus chemotherapeutics showed better response to therapy and improved OS, mainly for the epithelioid histologic subtypes [144]. Active immunization with DCs-conjugated with the mesothelioma antigen WT1 was tested in combination with standard chemotherapy (platinum/pemetrexed-based therapy). Phase I/II studies on DCs vaccination for MPM treatment resulted in being safe and efficacious in the stimulation of anti-cancer immune response [145]. Furthermore, DC-based immunochemotherapy was proven, with the addition of metronomic cyclophosphamide (mCTX), to deplete T reg cells and improve the anti-tumor immunomodulatory effects. The novel strategy was reported as safe and immunostimulatory, and beneficial effects in term of survival were observed in MPM patientsafter DC/mCTX-based treatment [146]. Mesothelioma cells are known to develop mechanisms for escaping immune surveillance, leading to T cell exhaustion via up-regulation of immune checkpoint molecules such as CTLA-4 [140]. In this regard, monoclonal antibodies targeting CTLA-4 have been developed, inducing the activation of cytotoxic T immunity. Among these inhibitors, tremelimumab and ipilimumab have been tested in clinical trials; however, no improvement in terms of overall survival for mesothelioma patients was observed [147]. Monoclonal antibodies targeting the PD-1/PD-L1 axis are also tested for mesothelioma treatment. PD-1 is generally expressed on the immune cells' surface and once activated in response to ligand binding, they negatively regulate T cell activation. The anti-PD-1 antibodies, pembrolizumab, nivolumab and durvalumab have shown promising results in clinical trials; however, a heterogenic response among MPMpatients was reported [147,148]. To increase the efficacy of these treatment modalities, combinatory strategies are under

clinical investigation, with both CTLA-4 and PD-1 inhibitorsas well as PD-1 inhibitors plus chemotherapy [147,149]. Other checkpoints on immune cells and up-regulated in MPM tissue as LAG-3 and TIM-3 have been recently investigated as promising targets for immunotherapy [149]. Tumor cells also increase the expression of different molecules involved in the invasive and metastatic process. Among these, TWIST1 is a key regulator of the EMT event, highly expressed by malignant mesothelioma cells. Recently, Tan et al. investigated, in pre-clinical studies, the potential of a therapeutic strategy based on the use of a DNA vaccine expressing soluble PD-1 (sPD-1) linked with TWIST1 construct, with the aim to block the immunotolerance for the self-antigen TWIST1 and destroy cancer cells. Then, because the combination of tumor vaccine and ICIs reported benefits in clinical and pre-clinical trials for cancer therapy, the hypothesis of the sPD-1-TWIST1 vaccination combined with anti-CTLA-4 was also tested, resulting in elicited long-lasting T cell immunity and mesothelioma reduction [150]. The benefits of the CAR-T cell therapy were also investigated in mesothelioma. CAR-T cells targeting the specific mesothelioma antigen surface mesothelin were evaluated, resulting in enhanced T cell activity and tumor cell destruction. However, phase I and II for CAR-T therapy are outstanding [151]. CAR-T targeting stromal factors, such as the fibroblast-activating protein (FAP) and VEGF-R2, have been developed for MPM treatment and pre-clinical data in mesothelioma mouse models showing efficacy and low toxicity [152]. Cytokine-based therapy included the intrapleural administration of interferon-α/β (IFN-α/β), resulting in partial tumor reduction and cytotoxicity, and the administration of IL-2, with therapeuticeffect on pre-clinical models but controversial results, based on the route of administration, in clinical trials (intraperitoneal vs. subcutaneous/intravenous) [139,141]. The administration of emactuzumab (mAb anti-CSF-1R) was also investigated as monotherapy or combined with paclitaxel for malignant mesothelioma treatment [153]. Studies on the use of oncolytic immunotherapy demonstrated that some MPM cases, characterized by a defective IFN- α/β response, were sensitive to the oncolytic function of attenuated strains of the measles virus [154]. Additionally, mutations in the IFN-I genes correlated with CDKN2A homozygous deletion as well as oncolytic therapy response may be associated with the genetic status of BAP1. Thus, it is important to understand the defective genomic landscape in MPM patients for an appropriate therapeutic choice [155].

Table 1. Agents targeting mesothelioma microenvironment in clinical trials.

Drug Name	Therapeutic Strategy	Molecular Target	Phase	Clinical Trials	Ref
Mesothelin-targeted CAR-T	CAR-T	Mesothelin	Phase I Phase II	NCT02414269 NCT03054298	[152]
Nivolumab	mAb	PD-1	Phase I Phase II Phase III	NCT02497508 JapicCTI163247 NCT03063450	[148]
Pembrolizumab	mAb	PD-1	Phase I Phase II Phase III	NCT02054806 NCT02399371 NCT02991482	[147]
Tremelimumab	mAb	CTLA-4	Phase II	NCT01843374	[147]
Bevacizumab	mAb	VEGF		NCT00651456	[142,143]
FAP-targeted CAR-T	CAR-T	Fibroblast-activating protein (FAP)	Phase I	NCT01722149	[152]
WT1- DCV	DC vaccination	Wilms tumor protein 1 (WT1)	Phase I/II	NCT02649829	[145]
Adenoviral-mediated IFN-α/β	Cytokine-based gene therapy	Interferon	Phase I Phase I	NCT01212367 NCT01119664	[140,141]
Nintedanib	Tyrosine kinase Inhibitor	VEGF, PDGF, FGF receptors	Phase II	NCT01907100	[144]
Emactuzumab	mAb	CSF-1 receptor	Phase I	NCT01494688	[153]

7. Conclusions

The malignant mesothelioma microenvironment is unique and the complex landscape of MPM TME is characterized by the communication mechanisms between cancer and stromal cells. Several players take part to the tumor organization and to the principal pro-tumoral functions, making tumor a complex object to analyze and understand. Different genomic defects and different immune landscape have been observed not only among patients, but also in different areas of the same tumors, highlighting the great intra-tumoral and inter-tumoral heterogeneity characterizing MPM [129]. Many interesting data correlated the malignancy of MPM TME with the infiltration of immunosuppressive TAMs [36], the high expression of immune checkpoint molecules that leads to the exhaustion of T cell activity [72], as well as the presence of CAFs promoting metastatic spread and pleural fibrosis [65,66], and the abundant content of pro-inflammatory cytokines from MPM pleural effusions [61]. Although the data reported in this review provide several information about the MPM TME players and its close connections, many questions related to their clinicopathological impact are still opened and a better knowledge of them may help cancer research for developing TME-based therapeutics able to overcome the poor overall survival of MPM patients, the chemoresistance mechanisms and the relapse of the tumor. Although the last therapy targeting TME in mesothelioma clinical trials including the immunotherapy and the combination immune/chemotherapy are promising therapeutic approaches [148–150], the efficacy of these strategies remains limited. A wide consideration of TME as the main player of cancer initiation and progression is required, in order to have in-depth knowledge of TME complexity and to design new drugs that target not only tumor cells but also the TME players supporting cancer.

Author Contributions: F.C. and E.G. conceived and designed the main content; F.C., M.B., S.L., and E.G. reviewed the literature and wrote the manuscript; methodology was carried out by S.L. and W.V.; A.G., C.B. and W.V. provided valuable critical revisions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by PSR 2021 (University of Siena), Sbarro Health Research Organization (http://www.shro.org), and SL was supported by Fondazione Beretta (Brescia).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Thomas, A.; Chen, Y.; Yu, T.; Gill, A.; Prasad, V. Distinctive clinical characteristics of malignant mesothelioma in young patients. *Oncotarget* **2015**, *6*, 16766–16773. [CrossRef]
2. FDA: U.S. Food and Drug Administration. FDA Approves Drug Combination For Treating Mesothelioma. Press release. Available online: https://www.fda.gov/news-events/press-announcements/fda-approves-drug-combination-treating-mesothelioma (accessed on 26 September 2021).
3. Yap, T.A.; Aerts, J.G.; Popat, S.; Fennell, D.A. Novel insights into mesothelioma biology and implications for therapy. *Nat. Rev. Cancer* **2017**, *17*, 475–488. [CrossRef]
4. Mangone, L.; Storchi, C.; Bisceglia, I.; Romanelli, A. Malignant Mesothelioma in the Italian Region Emilia-Romagna: Incidence and Asbestos exposure update to 2020. *Ann. Res. Oncol.* **2021**, *1*, 199–208. [CrossRef]
5. Barbarino, M.; Giordano, A. Assessment of the Carcinogenicity of Carbon Nanotubes in the Respiratory System. *Cancers* **2021**, *13*, 1318. [CrossRef]
6. Hillerdal, G.; Berg, J. Malignant Mesothelioma Secondary to Chronic Inflammation and Old Scars. Two New Cases and Review of the Literature. *Cancer* **1985**, *55*, 1968–1972. [CrossRef]
7. Bueno, R.; Stawiski, E.W.; Goldstein, L.D.; Durinck, S.; De Rienzo, A.; Modrusan, Z.; Gnad, F.; Nguyen, T.T.; Jaiswal, B.S.; Chirieac, L.R.; et al. Comprehensive Genomic Analysis of Malignant Pleural Mesothelioma Identifies Recurrent Mutations, Gene Fusions and Splicing Alterations. *Nat. Genet.* **2016**, *48*, 407–416. [CrossRef]
8. Testa, J.R.; Berns, A. Preclinical Models of Malignant Mesothelioma. *Front. Oncol.* **2020**, *10*, 101. [CrossRef] [PubMed]
9. Husain, A.N.; Colby, T.V.; Ordonez, N.G.; Allen, T.C.; Attanoos, R.L.; Beasley, M.B.; Butnor, K.J.; Chirieac, L.R.; Churg, A.M.; Dacic, S.; et al. Guidelines for Pathologic Diagnosis of Malignant Mesothelioma 2017 Update of the Consensus Statement From the International Mesothelioma Interest Group. *Arch. Pathol. Lab. Med.* **2018**, *142*, 89–108. [CrossRef] [PubMed]
10. Illei, P.B.; Rusch, V.W.; Zakowski, M.F.; Ladanyi, M. Homozygous Deletion of CDKN2A and Codeletion of the Methylthioadeno- sine Phosphorylase Gene in the Majority of Pleural Mesotheliomas. *Clin. Cancer Res.* **2003**, *9*, 2108–2113. [PubMed]
11. Bertino, J.R.; Waud, W.R.; Parker, W.B.; Lubin, M. Targeting Tumors That Lack Methylthioadenosine Phosphorylase (MTAP) Activity: Current Strategies. *Cancer Biol. Ther.* **2011**, *11*, 627–632. [CrossRef]
12. Kirovski, G.; Stevens, A.P.; Czech, B.; Dettmer, K.; Weiss, T.S.; Wild, P.; Hartmann, A.; Bosserhoff, A.K.; Oefner, P.J.; Hellerbrand, C. Down-Regulation of Methylthioadenosine Phosphorylase (MTAP) Induces Progression of Hepatocellular Carcinoma via Accumulation of 5′-Deoxy-5′-Methylthioadenosine (MTA). *Am. J. Pathol.* **2011**, *178*, 1145–1152. [CrossRef] [PubMed]
13. Carbone, M.; Adusumilli, P.S.; Alexander, H.R.J.; Baas, P.; Bardelli, F.; Bononi, A.; Bueno, R.; Felley-Bosco, E.; Galateau-Salle, F.; Jablons, D.; et al. Mesothelioma: Scientific Clues for Prevention, Diagnosis, and Therapy. *CA Cancer J. Clin.* **2019**, *69*, 402–429. [CrossRef] [PubMed]
14. Lu, Y.Y.; Jhanwar, S.C.; Cheng, J.Q.; Testa, J.R. Deletion Mapping of the Short Arm of Chromosome 3 in Human Malignant Mesothelioma. *Genes. Chromosomes Cancer* **1994**, *9*, 76–80. [CrossRef]

[PubMed]

15. Hmeljak, J.; Sanchez-Vega, F.; Hoadley, K.A.; Shih, J.; Stewart, C.; Heiman, D.; Tarpey, P.; Danilova, L.; Drill, E.; Gibb, E.A.; et al. Integrative Molecular Characterization of Malignant Pleural Mesothelioma. *Cancer Discov.* **2018**, *8*, 1548–1565. [CrossRef]
16. Cheng, Y.Y.; Yuen, M.L.; Rath, E.M.; Johnson, B.; Zhuang, L.; Yu, T.K.; Aleksova, V.; Linton, A.; Kao, S.; Clarke, C.J.; et al. CDKN2A and MTAP Are Useful Biomarkers Detectable by Droplet Digital PCR in Malignant Pleural Mesothelioma: A Potential Alternative Method in Diagnosis Compared to Fluorescence *In Situ* Hybridisation. *Front. Oncol.* **2020**, *10*, 579327. [CrossRef]
17. Thurneysen, C.; Opitz, I.; Kurtz, S.; Weder, W.; Stahel, R.A.; Felley-Bosco, E. Functional Inactivation of NF2/Merlin in Human Mesothelioma. *Lung Cancer* **2009**, *64*, 140–147. [CrossRef]
18. Yang, H.; Hall, S.; Sun, B.; Zhao, L.; Gao, Y.; Schmid, R.A.; Tan, S.T.; Peng, R.W.; Yao, F. NF2 and Canonical Hippo-YAP Pathway Define Distinct Tumor Subsets Characterized by Different Immune Deficiency and Treatment Implications in Human Pleural Mesothelioma. *Cancers* **2021**, *13*, 1561. [CrossRef] [PubMed]
19. Badhai, J.; Pandey, G.K.; Song, J.Y.; Krijgsman, O.; Bhaskaran, R.; Chandrasekaran, G.; Kwon, M.C.; Bombardelli, L.; Monkhorst, K.; Grasso, C.; et al. Combined deletion of Bap1, Nf2, and Cdkn2ab causes rapid onset of malignant mesothelioma in mice. *J. Exp. Med.* **2020**, *217*, e20191257. [CrossRef]
20. Newman, A.M.; Liu, C.L.; Green, M.R.; Gentles, A.J.; Feng, W.; Xu, Y.; Hoang, C.D.; Diehn, M.; Alizadeh, A.A. Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods* **2015**, *12*, 453–457. [CrossRef]
21. Pezzuto, F.; Lunardi, F.; Vedovelli, L.; Fortarezza, F.; Urso, L.; Grosso, F.; Ceresoli, G.L.; Kern, I.; Vlacic, G.; Faccioli, E.; et al. P14/ARF-Positive Malignant Pleural Mesothelioma: A Phenotype with Distinct Immune Microenvironment. *Front. Oncol.* **2021**,*11*, 653497. [CrossRef]
22. Zhang, M.; Luo, J.L.; Sun, Q.; Harber, J.; Dawson, A.G.; Nakas, A.; Busacca, S.; Sharkey, A.J.; Waller, D.; Sheaff, M.T.; et al. Clonal architecture in mesothelioma is prognostic and shapes the tumour microenvironment. *Nat. Commun.* **2021**, *12*, 1751. [CrossRef] [PubMed]
23. Le, D.T.; Durham, J.N.; Smith, K.N.; Wang, H.; Bartlett, B.R.; Aulakh, L.K.; Lu, S.; Kemberling, H.; Wilt, C.; Luber, B.S.; et al. Mismatch Repair Deficiency Predicts Response of Solid Tumors to PD-1 Blockade. *Science* **2017**, *357*, 409–413. [CrossRef]
24. Le, D.T.; Uram, J.N.; Wang, H.; Bartlett, B.R.; Kemberling, H.; Eyring, A.D.; Skora, A.D.; Luber, B.S.; Azad, N.S.; Laheru, D.; et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N. Engl. J. Med.* **2015**, *372*, 2509–2520. [CrossRef] [PubMed]
25. Cedres, S.; Ponce-Aix, S.; Iranzo, P.; Callejo, A.; Pardo, N.; Navarro, A.; Martinez-Marti, A.; Gomez-Abecia, S.; Zucchiatti, A.C.;
Sansano, I.; et al. Analysis of Mismatch Repair (MMR) Proteins Expression in a Series of Malignant Pleural Mesothelioma (MPM) Patients. *Clin. Transl. Oncol.* **2020**, *22*, 1390–1398. [CrossRef] [PubMed]
26. Sinn, K.; Mosleh, B.; Hoda, M.A. Malignant Pleural Mesothelioma: Recent Developments. *Curr. Opin. Oncol.* **2020**, *33*, 80–86.
[CrossRef]
27. Rozitis, E.; Johnson, B.; Cheng, Y.Y.; Lee, K. The Use of Immunohistochemistry, Fluorescence in Situ Hybridization, and Emerging Epigenetic Markers in the Diagnosis of Malignant Pleural Mesothelioma (MPM): A Review. *Front. Oncol.* **2020**, *10*, 1742. [CrossRef]
28. Ferrari, L.; Carugno, M.; Mensi, C.; Pesatori, A.C. Circulating Epigenetic Biomarkers in Malignant Pleural Mesothelioma: State of the Art and Critical Evaluation. *Front. Oncol.* **2020**, *10*, 445. [CrossRef]
29. McLoughlin, K.C.; Kaufman, A.S.; Schrump, D.S. Targeting the Epigenome in Malignant Pleural Mesothelioma. *Transl. Lung Cancer Res.* **2017**, *6*, 350–365. [CrossRef]
30. Benedetti, S.; Nuvoli, B.; Catalani, S.; Galati, R. Reactive oxygen species a double-edged sword for mesothelioma. *Oncotarget* **2015**,
6, 16848–16865. [CrossRef] [PubMed]
31. Mossman, B.T.; Shukla, A.; Heintz, N.H.; Verschraegen, C.F.; Thomas, A.; Hassan, R. New insights into understanding the mechanisms, pathogenesis, and management of malignant mesotheliomas. *Am. J. Pathol.* **2013**, *182*, 1065–1077. [CrossRef]
32. Minnema-Luiting, J.; Vroman, H.; Aerts, J.; Cornelissen, R. Heterogeneity in Immune Cell Content in Malignant Pleural Mesothelioma. *Int. J. Mol. Sci.* **2018**, *19*, 1041. [CrossRef]

[PubMed]
33. Patil, N.S.; Righi, L.; Koeppen, H.; Zou, W.; Izzo, S.; Grosso, F.; Libener, R.; Loiacono, M.; Monica, V.; Buttigliero, C.; et al. Molecular and Histopathological Characterization of the Tumor Immune Microenvironment in Advanced Stage of Malignant Pleural Mesothelioma. *J. Thorac. Oncol.* **2018**, *13*, 124–133. [CrossRef] [PubMed]
34. Pasello, G.; Zago, G.; Lunardi, F.; Urso, L.; Kern, I.; Vlacic, G.; Grosso, F.; Mencoboni, M.; Ceresoli, G.L.; Schiavon, M.; et al. Malignant pleural mesothelioma immune microenvironment and checkpoint expression: Correlation with clinical-pathological features and intratumor heterogeneity over time. *Ann. Oncol.* **2018**, *29*, 1258–1265. [CrossRef]
35. Linton, A.; van Zandwijk, N.; Reid, G.; Clarke, S.; Cao, C.; Kao, S. Inflammation in malignant mesothelioma—Friend or foe? *Annal. Cardiothorac. Surg.* **2012**, *1*, 516–522. [CrossRef]
36. Chu, G.J.; van Zandwijk, N.; Rasko, J. The Immune Microenvironment in Mesothelioma: Mechanisms of Resistance to Im- munotherapy. *Front. Oncol.* **2019**, *9*, 1366. [CrossRef]
37. Cornelissen, R.; Lievense, L.A.; Maat, A.P.; Hendriks, R.W.; Hoogsteden, H.C.; Bogers, A.J.; Hegmans, J.P.; Aerts, J.G. Ratio of intratumoral macrophage phenotypes is a prognostic factor in epithelioid malignant pleural mesothelioma. *PLoS ONE* **2014**, *9*, e106742. [CrossRef]
38. Guo, W.; Lasky, J.L.; Wu, H. Cancer stem cells. *Pediatr. Res.* **2006**, *59 Pt 2*, 59R–64R. [CrossRef]
39. Kuşoğlu, A.; Biray Avcı, Ç. Cancer stem cells: A brief review of the current status. *Gene* **2019**, *681*, 80–85. [CrossRef]
40. Pasdar, E.A.; Smits, M.; Stapelberg, M.; Bajzikova, M.; Stantic, M.; Goodwin, J.; Yan, B.; Stursa, J.; Kovarova, J.; Sachaphibulkij, K.; et al. Characterisation of mesothelioma-initiating cells and their susceptibility to anti-cancer agents. *PLoS ONE* **2015**, *10*, e0119549. [CrossRef] [PubMed]
41. Cortes-Dericks, L.; Carboni, G.L.; Schmid, R.A.; Karoubi, G. Putative cancer stem cells in malignant pleural mesothelioma show resistance to cisplatin and pemetrexed. *Int. J. Oncol.* **2010**, *37*, 437–444. [CrossRef]
42. Oehl, K.; Vrugt, B.; Opitz, I.; Meerang, M. Heterogeneity in Malignant Pleural Mesothelioma. *Int. J. Mol. Sci.* **2018**, *19*, 1603. [CrossRef]
43. Colak, S.; Medema, J.P. Cancer stem cells–important players in tumor therapy resistance. *FEBS J.* **2014**, *281*, 4779–4791. [CrossRef] [PubMed]
44. Kai, K.; D'Costa, S.; Yoon, B.I.; Brody, A.R.; Sills, R.C.; Kim, Y. Characterization of side population cells in human malignant mesothelioma cell lines. *Lung Cancer* **2010**, *70*, 146–151. [CrossRef]
45. Blum, W.; Pecze, L.; Felley-Bosco, E.; Wu, L.; de Perrot, M.; Schwaller, B. Stem Cell Factor-Based Identification and Functional Properties of In Vitro-Selected Subpopulations of Malignant Mesothelioma Cells. *Stem Cell Rep.* **2017**, *8*, 1005–1017. [CrossRef]
46. Begicevic, R.R.; Falasca, M. ABC Transporters in Cancer Stem Cells: Beyond Chemoresistance. *Int. J. Mol. Sci.* **2017**, *18*, 2362. [CrossRef] [PubMed]
47. Cortes-Dericks, L.; Froment, L.; Boesch, R.; Schmid, R.A.; Karoubi, G. Cisplatin-resistant cells in malignant pleural mesothelioma cell lines show ALDH(high)CD44(+) phenotype and sphere-forming capacity. *BMC Cancer* **2014**, *14*, 304. [CrossRef] [PubMed]
48. Milosevic, V.; Kopecka, J.; Salaroglio, I.C.; Libener, R.; Napoli, F.; Izzo, S.; Orecchia, S.; Ananthanarayanan, P.; Bironzo, P.; Grosso,F.; et al. Wnt/IL-1β/IL-8 autocrine circuitries control chemoresistance in mesothelioma initiating cells by inducing ABCB5. *Int. J. Cancer* **2020**, *146*, 192–207. [CrossRef] [PubMed]
49. Canino, C.; Mori, F.; Cambria, A.; Diamantini, A.; Germoni, S.; Alessandrini, G.; Borsellino, G.; Galati, R.; Battistini, L.; Blandino, R.; et al. SASP mediates chemoresistance and tumor-initiating-activity of mesothelioma cells. *Oncogene* **2012**, *31*, 3148–3163. [CrossRef]
50. Menges, C.W.; Kadariya, Y.; Altomare, D.; Talarchek, J.; Neumann-Domer, E.; Wu, Y.; Xiao, G.H.; Shapiro, I.M.; Kolev, V.N.; Pachter, J.A.; et al. Tumor suppressor alterations cooperate to drive aggressive mesotheliomas with enriched cancer stem cells via a p53-miR-34a-c-Met axis. *Cancer Res.* **2014**, *74*, 1261–1271. [CrossRef]
51. Shapiro, I.M.; Kolev, V.N.; Vidal, C.M.; Kadariya, Y.; Ring, J.E.; Wright, Q.; Weaver, D.T.; Menges, C.; Padval, M.; McClatchey, A.I.; et al. Merlin deficiency predicts FAK inhibitor sensitivity: A synthetic lethal relationship. *Sci. Transl. Med.* **2014**, *6*, 237ra68. [CrossRef]

52. Kolev, V.N.; Tam, W.F.; Wright, Q.G.; McDermott, S.P.; Vidal, C.M.; Shapiro, I.M.; Xu, Q.; Wicha, M.S.; Pachter, J.A.; Weaver, D.T. Inhibition of FAK kinase activity preferentially targets cancer stem cells. *Oncotarget* **2017**, *8*, 51733–51747. [CrossRef]
53. Ayob, A.Z.; Ramasamy, T.S. Cancer stem cells as key drivers of tumour progression. *J. Biom. Sci.* **2018**, *25*, 20. [CrossRef]
54. Lau, B.W.; Kane, A.B. SDF1/CXCL12 is involved in recruitment of stem-like progenitor cells to orthotopic murine malignant mesothelioma spheroids. *Anticancer Res.* **2010**, *30*, 2153–2160.
55. Adhikary, G.; Grun, D.; Alexander, H.R.; Friedberg, J.S.; Xu, W.; Keillor, J.W.; Kandasamy, S.; Eckert, R.L. Transglutaminase is a mesothelioma cancer stem cell survival protein that is required for tumor formation. *Oncotarget* **2018**, *9*, 34495–34505. [CrossRef] [PubMed]
56. Kandasamy, S.; Adhikary, G.; Rorke, E.A.; Friedberg, J.S.; Mickle, M.B.; Alexander, H.R.; Eckert, R.L. The YAP1 Signaling Inhibitors, Verteporfin and CA3, Suppress the Mesothelioma Cancer Stem Cell Phenotype. *Mol. Cancer Res.* **2020**, *18*, 343–351. [CrossRef] [PubMed]
57. Hinshaw, D.C.; Shevde, L.A. The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Res.* **2019**, *79*, 4557–4566. [CrossRef]
58. Werb, Z.; Lu, P. The Role of Stroma in Tumor Development. *Cancer J.* **2015**, *21*, 250–253. [CrossRef] [PubMed]
59. Bremnes, R.M.; Dønnem, T.; Al-Saad, S.; Al-Shibli, K.; Andersen, S.; Sirera, R.; Camps, C.; Marinez, I.; Busund, L.T. The role of tumor stroma in cancer progression and prognosis: Emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J. Thorac. Oncol.* **2011**, *6*, 209–217. [CrossRef]
60. Napoli, F.; Listı , A.; Zambelli, V.; Witel, G.; Bironzo, P.; Papotti, M.; Volante, M.; Scagliotti, G.; Righi, L. Pathological Characteriza- tion of Tumor Immune Microenvironment (TIME) in Malignant Pleural Mesothelioma. *Cancers* **2021**, *13*, 2564. [CrossRef]
61. Lievense, L.A.; Cornelissen, R.; Bezemer, K.; Kaijen-Lambers, M.E.; Hegmans, J.P.; Aerts, J.G. Pleural Effusion of Patients with Malignant Mesothelioma Induces Macrophage-Mediated T Cell Suppression. *J. Thorac. Onc.* **2016**, *11*, 1755–1764. [CrossRef]
62. Fujii, M.; Toyoda, T.; Nakanishi, H.; Yatabe, Y.; Sato, A.; Matsudaira, Y.; Ito, H.; Murakami, H.; Kondo, Y.; Kondo, E.; et al. TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. *J. Exp. Med.* **2012**, *209*, 479–494. [CrossRef]
63. Chu, C.Y.; Chang, C.C.; Prakash, E.; Kuo, M.L. Connective tissue growth factor (CTGF) and cancer progression. *J. Biomed. Sci.* **2008**, *15*, 675–685. [CrossRef] [PubMed]
64. Jang, I.; Beningo, K.A. Integrins, CAFs and Mechanical Forces in the Progression of Cancer. *Cancers* **2019**, *11*, 721. [CrossRef] [PubMed]
65. Ohara, Y.; Enomoto, A.; Tsuyuki, Y.; Sato, K.; Iida, T.; Kobayashi, H.; Mizutani, Y.; Miyai, Y.; Hara, A.; Mii, S.; et al. Connective tissue growth factor produced by cancer-associated fibroblasts correlates with poor prognosis in epithelioid malignant pleural mesothelioma. *Oncol. Rep.* **2020**, *44*, 838–848. [CrossRef]
66. Li, Q.; Wang, W.; Yamada, T.; Matsumoto, K.; Sakai, K.; Bando, Y.; Uehara, H.; Nishioka, Y.; Sone, S.; Iwakiri, S.; et al. Pleural mesothelioma instigates tumor-associated fibroblasts to promote progression via a malignant cytokine network. *Am. J. Pathol.* **2011**, *179*, 1483–1493. [CrossRef]
67. Kumar-Singh, S.; Weyler, J.; Martin, M.J.; Vermeulen, P.B.; Van Marck, E. Angiogenic cytokines in mesothelioma: A study of VEGF, FGF-1 and -2, and TGF beta expression. *J. Pathol.* **1999**, *189*, 72–78. [CrossRef]
68. Wang, F.T.; Sun, W.; Zhang, J.T.; Fan, Y.Z. Cancer-associated fibroblast regulation of tumor neo-angiogenesis as a therapeutic target in cancer. *Oncol. Lett.* **2019**, *17*, 3055–3065. [CrossRef]
69. Chia, P.L.; Russell, P.; Asadi, K.; Thapa, B.; Gebski, V.; Murone, C.; Walkiewicz, M.; Eriksson, U.; Scott, A.M.; John, T. Analysis of angiogenic and stromal biomarkers in a large malignant mesothelioma cohort. *Lung Cancer* **2020**, *150*, 1–8. [CrossRef]
70. Hegmans, J.P.; Hemmes, A.; Hammad, H.; Boon, L.; Hoogsteden, H.C.; Lambrecht, B.N. Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses. *Eur. Resp. J.* **2006**, *27*, 1086–1095. [CrossRef]
71. Ujiie, H.; Kadota, K.; Nitadori, J.I.; Aerts, J.G.; Woo, K.M.; Sima, C.S.; Travis, W.D.; Jones, D.R.; Krug, L.M.; Adusumilli, P.S. The tumoral and stromal immune microenvironment in malignant pleural mesothelioma: A comprehensive analysis reveals prognostic immune markers. *Oncoimmunology* **2015**, *4*, e1009285. [CrossRef]

72. Marcq, E.; Waele, J.; Audenaerde, J.V.; Lion, E.; Santermans, E.; Hens, N.; Pauwels, P.; van Meerbeeck, J.P.; Smits, E. Abundant expression of TIM-3, LAG-3, PD-1 and PD-L1 as immunotherapy checkpoint targets in effusions of mesothelioma patients. *Oncotarget* **2017**, *8*, 89722–89735. [CrossRef] [PubMed]
73. Amarnath, S.; Mangus, C.W.; Wang, J.C.; Wei, F.; He, A.; Kapoor, V.; Foley, J.E.; Massey, P.R.; Felizardo, T.C.; Riley, J.L.; et al. The PDL1-PD1 axis converts human TH1 cells into regulatory T cells. *Sci. Transl. Med.* **2011**, *3*, 111ra120. [CrossRef]
74. Klampatsa, A.; O'Brien, S.M.; Thompson, J.C.; Rao, A.S.; Stadanlick, J.E.; Martinez, M.C.; Liousia, M.; Cantu, E.; Cengel, K.; Moon, E.K.; et al. Phenotypic and functional analysis of malignant mesothelioma tumor-infiltrating lymphocytes. *Oncoimmunology* **2019**, *8*, e1638211. [CrossRef]
75. Anraku, M.; Cunningham, K.S.; Yun, Z.; Tsao, M.S.; Zhang, L.; Keshavjee, S.; Johnston, M.R.; de Perrot, M. Impact of tumor- infiltrating T cells on survival in patients with malignant pleural mesothelioma. *J. Thorac. Cardiovasc. Surg.* **2008**, *135*, 823–829. [CrossRef] [PubMed]
76. Yamada, N.; Oizumi, S.; Kikuchi, E.; Shinagawa, N.; Konishi-Sakakibara, J.; Ishimine, A.; Aoe, K.; Gemba, K.; Kishimoto, T.; Torigoe, T.; et al. CD8+ tumor-infiltrating lymphocytes predict favorable prognosis in malignant pleural mesothelioma after resection. *Cancer Immunol. Immunother.* **2010**, *59*, 1543–1549. [CrossRef] [PubMed]
77. Hiltbrunner, S.; Mannarino, L.; Kirschner, M.B.; Opitz, I.; Rigutto, A.; Laure, A.; Lia, M.; Nozza, P.; Maconi, A.; Marchini, S.; et al. Tumor Immune Microenvironment and Genetic Alterations in Mesothelioma. *Front. Oncol.* **2021**, *11*, 660039. [CrossRef]
78. Hirayama, D.; Iida, T.; Nakase, H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis.
Int. J. Mol. Sci. **2017**, *19*, 92. [CrossRef] [PubMed]
79. Carbone, M.; Bedrossian, C.W. The pathogenesis of mesothelioma. *Semin. Diagn. Path.* **2006**, *23*, 56–60. [CrossRef] [PubMed]
80. Desage, A.L.; Karpathiou, G.; Peoc'h, M.; Froudarakis, M.E. The Immune Microenvironment of Malignant Pleural Mesothelioma: A Literature Review. *Cancers* **2021**, *13*, 3205. [CrossRef]
81. Wang, M.; Gauthier, A.; Daley, L.; Dial, K.; Wu, J.; Woo, J.; Lin, M.; Ashby, C.; Mantell, L.L. The Role of HMGB1, a Nuclear Damage-Associated Molecular Pattern Molecule, in the Pathogenesis of Lung Diseases. *Antiox. Redox Signal.* **2019**, *31*, 954–993. [CrossRef]
82. Carbone, M.; Yang, H. Mesothelioma: Recent highlights. *Ann. Transl. Med.* **2017**, *5*, 238. [CrossRef] [PubMed]
83. Hiraku, Y.; Guo, F.; Ma, N.; Yamada, T.; Wang, S.; Kawanishi, S.; Murata, M. Multi-walled carbon nanotube induces nitrative DNA damage in human lung epithelial cells via HMGB1-RAGE interaction and Toll-like receptor 9 activation. *Part. Fibre Toxicol.* **2016**, *13*, 16. [CrossRef]
84. Boyles, M.S.; Young, L.; Brown, D.M.; MacCalman, L.; Cowie, H.; Moisala, A.; Smail, F.; Smith, P.J.; Proudfoot, L.; Windle, A.H.; et al. Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. *Toxicol. In Vitro* **2015**, *29*, 1513–1528. [CrossRef] [PubMed]
85. Carbone, M.; Yang, H. Molecular pathways: Targeting mechanisms of asbestos and erionite carcinogenesis in mesothelioma. *Clin.
Cancer Res.* **2012**, *18*, 598–604. [CrossRef]
86. Schurch, C.M.; Forster, S.; Bruhl, F.; Yang, S.H.; Felley-Bosco, E.; Hewer, E. The "don't eat me" signal CD47 is a novel diagnostic biomarker and potential therapeutic target for diffuse malignant mesothelioma. *Oncoimmunology* **2017**, *7*, e1373235. [CrossRef]
87. Cersosimo, F.; Lonardi, S.; Bernardini, G.; Telfer, B.; Mandelli, G.E.; Santucci, A.; Vermi, W.; Giurisato, E. Tumor-Associated Macrophages in Osteosarcoma: From Mechanisms to Therapy. *Int. J. Mol. Sci.* **2020**, *21*, 5207. [CrossRef] [PubMed]
88. Martinez, F.O. Regulators of macrophage activation. *Eur. J. Immunol.* **2011**, *41*, 1531–1534. [CrossRef]
89. Xu, F.; Wei, Y.; Tang, Z.; Liu, B.; Dong, J. Tumor-associated macrophages in lung cancer: Friend or foe? (Review). *Mol. Med. Rep.*
2020, *22*, 4107–4115. [CrossRef]
90. Yang, Q.; Guo, N.; Zhou, Y.; Chen, J.; Wei, Q.; Han, M. The role of tumor-associated macrophages (TAMs) in tumor progression and relevant advance in targeted therapy. *Acta Pharm. Sin. B* **2020**, *10*, 2156–2170. [CrossRef] [PubMed]
91. Colin, D.J.; Cottet-Dumoulin, D.; Faivre, A.; Germain, S.; Triponez, F.; Serre-Beinier, V. Experimental

Model of Human Malignant Mesothelioma in Athymic Mice. *Int. J. Mol. Sci.* **2018**, *19*, 1881. [CrossRef]

92. Chene, A.L.; d'Almeida, S.; Blondy, T.; Tabiasco, J.; Deshayes, S.; Fonteneau, J.F.; Cellerin, L.; Delneste, Y.; Gregoire, M.; Blanquart,
C. Pleural Effusions from Patients with Mesothelioma Induce Recruitment of Monocytes and Their Differentiation into M2 Macrophages. *J. Thorac. Oncol.* **2016**, *11*, 1765–1773. [CrossRef] [PubMed]

93. Sarode, P.; Schaefer, M.B.; Grimminger, F.; Seeger, W.; Savai, R. Macrophage and Tumor Cell Cross-Talk Is Fundamental for Lung Tumor Progression: We Need to Talk. *Front. Oncol. 2020. 10*, 324. [CrossRef]
94. Izzi, V.; Chiurchiu , V.; D'Aquilio, F.; Palumbo, C.; Tresoldi, I.; Modesti, A.; Baldini, P.M. Differential effects of malignant mesothelioma cells on THP-1 monocytes and macrophages. *Int. J. Oncol.* **2009**, *34*, 543–550. [CrossRef]
95. Burt, B.M.; Rodig, S.J.; Tilleman, T.R.; Elbardissi, A.W.; Bueno, R.; Sugarbaker, D.J. Circulating and tumor-infiltrating myeloid cells predict survival in human pleural mesothelioma.*Cancer* **2011**, *117*, 5234–5244. [CrossRef]
96. Horio, D.; Minami, T.; Kitai, H.; Ishigaki, H.; Higashiguchi, Y.; Kondo, N.; Hirota, S.; Kitajima, K.; Nakajima, Y.; Koda, Y.; et al. Tumor-associated macrophage-derived inflammatory cytokine enhances malignant potential of malignant pleural mesothelioma. *Cancer Sci.* **2020**, *111*, 2895–2906. [CrossRef] [PubMed]
97. Chen, Y.; Song, Y.; Du, W.; Gong, L.; Chang, H.; Zou, Z. Tumor-associated macrophages: An accomplice in solid tumor progression. *J. Biomed. Sci.* **2019**, *26*, 78. [CrossRef]
98. Blondy, T.; d'Almeida, S.M.; Briolay, T.; Tabiasco, J.; Meiller, C.; Chene, A.L.; Cellerin, L.; Deshayes, S.; Delneste, Y.; Fonteneau, J.F.; et al. Involvement of the M-CSF/IL-34/CSF-1R pathway in malignant pleural mesothelioma. *J. Immunother. Cancer* **2020**, *8*,e000182. [CrossRef]
99. Cioce, M.; Canino, C.; Goparaju, C.; Yang, H.; Carbone, M.; Pass, H.I. Autocrine CSF-1R signaling drives mesothelioma chemoresistance via AKT activation. *Cell Death Dis.* **2014**, *5*, e1167. [CrossRef] [PubMed]
100. Dammeijer, F.; Lievense, L.A.; Kaijen-Lambers, M.E.; van Nimwegen, M.; Bezemer, K.; Hegmans, J.P.; van Hall, T.; Hendriks, R.W.; Aerts, J.G. Depletion of Tumor-Associated Macrophages with a CSF-1R Kinase Inhibitor Enhances Antitumor Immunity and Survival Induced by DC Immunotherapy. *Cancer Immunol. Res.* **2017**, *5*, 535–546. [CrossRef]
101. Magkouta, S.F.; Vaitsi, P.C.; Pappas, A.G.; Iliopoulou, M.; Kosti, C.N.; Psarra, K.; Kalomenidis, I.T. CSF1/CSF1R Axis Blockade Limits Mesothelioma and Enhances Efficiency of Anti-PDL1 Immunotherapy. *Cancers* **2021**, *13*, 2546. [CrossRef]
102. Ries, C.H.; Cannarile, M.A.; Hoves, S.; Benz, J.; Wartha, K.; Runza, V.; Rey-Giraud, F.; Pradel, L.P.; Feuerhake, F.; Klaman, I.; et al. Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy.*Cancer Cell* **2014**, *25*, 846–859. [CrossRef]
103. Noy, R.; Pollard, J.W. Tumor-associated macrophages: From mechanisms to therapy.*Immunity* **2014**, *41*, 49–61. [CrossRef] [PubMed]
104. Miselis, N.R.; Wu, Z.J.; Van Rooijen, N.; Kane, A.B. Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma. *Mol. Cancer Ther.* **2008**, *7*, 788–799. [CrossRef] [PubMed]
105. Veltman, J.D.; Lambers, M.E.; van Nimwegen, M.; Hendriks, R.W.; Hoogsteden, H.C.; Hegmans, J.P.; Aerts, J.G. Zoledronic acid impairs myeloid differentiation to tumour-associated macrophages in mesothelioma. *Br. J. Cancer* **2010**, *103*, 629–641. [CrossRef] [PubMed]
106. Andon, F.T.; Digifico, E.; Maeda, A.; Erreni, M.; Mantovani, A.; Alonso, M.J.; Allavena, P. Targeting tumor associated macrophages: The new challenge for nanomedicine. *Semin. Immunol.* **2017**, *34*, 103–113. [CrossRef] [PubMed]
107. Giurisato, E.; Lonardi, S.; Telfer, B.; Lussoso, S.; Risa-Ebrı , B.; Zhang, J.; Russo, I.; Wang, J.; Santucci, A.; Finegan, K.G.; et al. Extracellular-Regulated Protein Kinase 5-Mediated Control of p21 Expression Promotes Macrophage Proliferation Associated with Tumor Growth and Metastasis. *Cancer Res.* **2020**, *80*, 3319–3330. [CrossRef]
108. Allard, B.; Beavis, P.A.; Darcy, P.K.; Stagg, J. Immunosuppressive activities of adenosine in cancer. *Curr. Opin. Pharmacol.* **2016**, *29*,
7–16. [CrossRef]

109. Whiteside, T.L. The potential of tumor-derived exosomes for noninvasive cancer monitoring.*Expert.*

Rev. Mol. Diagn. **2015**, *15*, 1293–1310. [CrossRef]

110. Allard, B.; Longhi, M.S.; Robson, S.C.; Stagg, J. The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. *Immunol Rev.* **2017**, *276*, 121–144. [CrossRef] [PubMed]
111. Ghalamfarsa, G.; Kazemi, M.H.; Raoofi Mohseni, S.; Masjedi, A.; Hojjat-Farsangi, M.; Azizi, G.; Yousefi, M.; Jadidi-Niaragh, F. CD73 as a potential opportunity for cancer immunotherapy. *Expert. Opin. Ther. Targets* **2019**, *23*, 127–142. [CrossRef]
112. Hatfield, S.M.; Kjaergaard, J.; Lukashev, D.; Belikoff, B.; Schreiber, T.H.; Sethumadhavan, S.; Abbott, R.; Philbrook, P.; Thayer, M.; Shujia, D.; et al. Systemic oxygenation weakens the hypoxia and hypoxia inducible factor 1α-dependent and extracellular adenosine-mediated tumor protection. *J. Mol. Med.* **2014**, *92*, 1283–1292. [CrossRef]
113. Novitskiy, S.V.; Ryzhov, S.; Zaynagetdinov, R.; Goldstein, A.E.; Huang, Y.; Tikhomirov, O.Y.; Blackburn, M.R.; Biaggioni, I.; Carbone, D.P.; Feoktistov, I.; et al. Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* **2008**, *112*, 1822–1831. [CrossRef] [PubMed]
114. Deaglio, S.; Dwyer, K.M.; Gao, W.; Friedman, D.; Usheva, A.; Erat, A.; Chen, J.F.; Enjyoji, K.; Linden, J.; Oukka, M.; et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **2007**, *204*, 1257–1265. [CrossRef]
115. Reyna-Jeldes, M.; Dı az-Munoz, M.; Madariaga, J.A.; Coddou, C.; Va zquez-Cuevas, F.G. Autocrine and paracrine purinergic signaling in the most lethal types of cancer. *Purinergic Signal.* **2021**, 1–26. [CrossRef]
116. Yang, H.; Yao, F.; Davis, P.F.; Tan, S.T.; Hall, S. CD73, Tumor Plasticity and Immune Evasion in Solid Cancers. *Cancers* **2021**, *13*, 177. [CrossRef]
117. Al-Taei, S.; Salimu, J.; Spary, L.K.; Clayton, A.; Lester, J.F.; Tabi, Z. Prostaglandin E $_2$-mediated adenosinergic effects on CD14$^+$ cells: Self-amplifying immunosuppression in cancer. *Oncoimmunology* **2016**, *6*, e1268308. [CrossRef]
118. H askọ G.; Pacher, P. Regulation of macrophage function by adenosine. *Arterioscler. Thromb. Vasc. Biol.* **2012**, *32*, 865–869. [CrossRef] [PubMed]
119. Ernens, I.; Leonard, F.; Vausort, M.; Rolland-Turner, M.; Devaux, Y.; Wagner, D.R. Adenosine up-regulates vascular endothelial growth factor in human macrophages. *Biochem. Biophys. Res. Commun.* **2010**, *392*, 351–356. [CrossRef] [PubMed]
120. Cekic, C.; Day, Y.J.; Sag, D.; Linden, J. Myeloid expression of adenosine A2A receptor suppresses T and NK cell responses in the solid tumor microenvironment. *Cancer Res.* **2014**, *74*, 7250–7259. [CrossRef]
121. Clayton, A.; Al-Taei, S.; Webber, J.; Mason, M.D.; Tabi, Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J. Immunol.* **2011**, *187*, 676–683. [CrossRef]
122. Pinhal-Enfield, G.; Ramanathan, M.; Hasko, G.; Vogel, S.N.; Salzman, A.L.; Boons, G.J.; Leibovich, S.J. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. *Am. J. Pathol.* **2003**, *163*, 711–721. [CrossRef]
123. Csoka, B.; Nemeth, Z.H.; Virag, L.; Gergely, P.; Leibovich, S.J.; Pacher, P.; Sun, C.X.; Blackburn, M.R.; Vizi, E.S.; Deitch, E.A.; et al. A2A adenosine receptors and C/EBPbeta are crucially required for IL-10 production by macrophages exposed to Escherichia coli. *Blood* **2007**, *110*, 2685–2695. [CrossRef]
124. H askọ G.; Kuhel, D.G.; Chen, J.F.; Schwarzschild, M.A.; Deitch, E.A.; Mabley, J.G.; Marton, A.; Szabo, C. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J.* **2000**, *14*, 2065–2074. [CrossRef]
125. Kreckler, L.M.; Wan, T.C.; Ge, Z.D.; Auchampach, J.A. Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 172–180. [CrossRef]
126. Nemeth, Z.H.; Lutz, C.S.; Cso ka, B.; Deitch, E.A.; Leibovich, S.J.; Gause, W.C.; Tone, M.; Pacher, P.; Vizi, E.S.; Hasko, G. Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. *J. Immunol.* **2005**, *175*, 8260–8270. [CrossRef]
127. Cs oka, B.; Selmeczy, Z.; Koscso, B.; Nemeth, Z.H.; Pacher, P.; Murray, P.J.; Kepka-Lenhart, D.; Morris, S.M., Jr.; Gause, W.C.; Leibovich, S.J.; et al. Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB J.* **2012**, *26*, 376–386. [CrossRef] [PubMed]
128. Wang, J.; Wang, Y.; Chu, Y.; Li, Z.; Yu, X.; Huang, Z.; Xu, J.; Zheng, L. Tumor-derived adenosine

promotes macrophage proliferation in human hepatocellular carcinoma. *J. Hepatol.* **2021**, *74*, 627–637. [CrossRef] [PubMed]

129. Blum, Y.; Meiller, C.; Quetel, L.; Elarouci, N.; Ayadi, M.; Tashtanbaeva, D.; Armenoult, L.; Montagne, F.; Tranchant, R.; Renier, A.; et al. Dissecting heterogeneity in malignant pleural mesothelioma through histo-molecular gradients for clinical applications. *Nat. Commun.* **2019**, *10*, 1333. [CrossRef]
130. Lee, H.S.; Jang, H.J.; Choi, J.M.; Zhang, J.; de Rosen, V.L.; Wheeler, T.M.; Lee, J.S.; Tu, T.; Jindra, P.T.; Kerman, R.H.; et al. Comprehensive immunoproteogenomic analyses of malignant pleural mesothelioma. *JCI Insight* **2018**, *3*, e98575. [CrossRef]
131. Wadowski, B.; Bueno, R.; De Rienzo, A. Immune Microenvironment and Genetics in Malignant Pleural Mesothelioma. *Front. Oncol.* **2021**, *11*, 684025. [CrossRef]
132. Thapa, B.; Salcedo, A.; Lin, X.; Walkiewicz, M.; Murone, C.; Ameratunga, M.; Asadi, K.; Deb, S.; Barnett, S.A.; Knight, S.; et al. The Immune Microenvironment, Genome-wide Copy Number Aberrations, and Survival in Mesothelioma. *J. Thorac. Oncol.* **2017**, *12*, 850–859. [CrossRef]
133. Muller, S.; Victoria Lai, W.; Adusumilli, P.S.; Desmeules, P.; Frosina, D.; Jungbluth, A.; Ni, A.; Eguchi, T.; Travis, W.D.; Ladanyi, M.; et al. V-domain Ig-containing suppressor of T-cell activation (VISTA), a potentially targetable immune checkpoint molecule, is highly expressed in epithelioid malignant pleural mesothelioma. *Mod. Pathol.* **2020**, *33*, 303–311. [CrossRef] [PubMed]
134. Yang, H.; Xu, D.; Schmid, R.A.; Peng, R.W. Biomarker-guided targeted and immunotherapies in malignant pleural mesothelioma. *Ther. Adv. Med. Oncol.* **2020**, *12*, 1758835920971421. [CrossRef]
135. Alcala, N.; Mangiante, L.; Le-Stang, N.; Gustafson, C.E.; Boyault, S.; Damiola, F.; Alcala, K.; Brevet, M.; Thivolet-Bejui, F.; Blanc- Fournier, C.; et al. Redefining malignant pleural mesothelioma types as a continuum uncovers immune-vascular interactions. *EBioMedicine* **2019**, *48*, 191–202. [CrossRef]
136. Tazzari, M.; Brich, S.; Tuccitto, A.; Bozzi, F.; Beretta, V.; Spagnuolo, R.D.; Negri, T.; Stacchiotti, S.; Deraco, M.; Baratti, D.; et al. Complex Immune Contextures Characterise Malignant Peritoneal Mesothelioma: Loss of Adaptive Immunological Signature in the More Aggressive Histological Types. *J. Immunol. Res.* **2018**, 5804230. [CrossRef] [PubMed]
137. Alay, A.; Cordero, D.; Hijazo-Pechero, S.; Aliagas, E.; Lopez-Doriga, A.; Marı, R.; Palmero, R.; Llatjos, R.; Escobar, I.; Ramos, R.; et al. Integrative transcriptome analysis of malignant pleural mesothelioma reveals a clinically relevant immune-based classification. *J. Immunother. Cancer* **2021**, *9*, e001601. [CrossRef] [PubMed]
138. Junttila, M.R.; de Sauvage, F.J. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* **2013**, *501*, 346–354. [CrossRef] [PubMed]
139. Kotova, S.; Wong, R.M.; Cameron, R.B. New and emerging therapeutic options for malignant pleural mesothelioma: Review of early clinical trials. *Cancer Manag. Res.* **2015**, *7*, 51–63. [CrossRef]
140. Bronte, G.; Incorvaia, L.; Rizzo, S.; Passiglia, F.; Galvano, A.; Rizzo, F.; Rolfo, C.; Fanale, D.; Listı , A.; Natoli, C.; et al. The resistance related to targeted therapy in malignant pleural mesothelioma: Why has not the target been hit yet?*Crit. Rev. Oncol. Hematol.***2016**, *107*, 20–32. [CrossRef]
141. Bograd, A.J.; Suzuki, K.; Vertes, E.; Colovos, C.; Morales, E.A.; Sadelain, M.; Adusumilli, P.S. Immune responses and immunother- apeutic interventions in malignant pleural mesothelioma. *Cancer Immunol. Immunother.* **2011**, *60*, 1509–1527. [CrossRef]
142. Chia, P.L.; Russell, P.A.; Scott, A.M.; John, T. Targeting the vasculature: Anti-angiogenic agents for malignant mesothelioma. *Expert Rev. Anticancer Ther.* **2016**, *16*, 1235–1245. [CrossRef] [PubMed]
143. Tsao, A.S.; Lindwasser, O.W.; Adjei, A.A.; Adusumilli, P.S.; Beyers, M.L.; Blumenthal, G.M.; Bueno, R.; Burt, B.M.; Carbone, M.; Dahlberg, S.E.; et al. Current and Future Management of Malignant Mesothelioma: A Consensus Report from the National Cancer Institute Thoracic Malignancy Steering Committee, International Association for the Study of Lung Cancer, and Mesothelioma Applied Research Foundation. *J. Thorac. Oncol.* **2018**, *13*, 1655–1667. [CrossRef] [PubMed]
144. Grosso, F.; Steele, N.; Novello, S.; Nowak, A.K.; Popat, S.; Greillier, L.; John, T.; Leighl, N.B.; Reck, M.; Taylor, P.; et al. Nintedanib Plus Pemetrexed/Cisplatin in Patients With Malignant Pleural Mesothelioma: Phase II Results From the Randomized, Placebo-Controlled LUME-Meso Trial. *J. Clin. Oncol.* **2017**, *35*, 3591–3600. [CrossRef] [PubMed]

145. Berneman, Z.N.; Germonpre, P.; Huizing, T.M.; Van de Velde, A.; Nijs, G.; Stein, B.; Van Tendeloo, V.F.; Lion, E.; Smits, E.L.; Anguille, S. Dendritic cell vaccination in malignant pleural mesothelioma: A phase I/II study. *J. Clin. Oncol.* **2014**, *32* (Suppl. 15), 7583. [CrossRef]
146. Noordam, L.; Kaijen, M.; Bezemer, K.; Cornelissen, R.; Maat, L.; Hoogsteden, H.C.; Aerts, J.; Hendriks, R.W.; Hegmans, J.; Vroman, H. Low-dose cyclophosphamide depletes circulating naıve and activated regulatory T cells in malignant pleural mesothelioma patients synergistically treated with dendritic cell-based immunotherapy. *Oncoimmunology* **2018**, *7*, e1474318. [CrossRef] [PubMed]
147. Nicolini, F.; Bocchini, M.; Bronte, G.; Delmonte, A.; Guidoboni, M.; Crino , L.; Mazza, M. Malignant Pleural Mesothelioma: State-of-the-Art on Current Therapies and Promises for the Future. *Front. Oncol.* **2020**, *9*, 1519. [CrossRef]
148. Obacz, J.; Yung, H.; Shamseddin, M.; Linnane, E.; Liu, X.; Azad, A.A.; Rassl, D.M.; Fairen-Jimenez, D.; Rintoul, R.C.; Nikolić, M.Z.; et al. Biological basis for novel mesothelioma therapies. *Br. J. Cancer* **2021**, *10*, 1–17. [CrossRef]
149. Cinausero, M.; Rihawi, K.; Cortiula, F.; Follador, A.; Fasola, G.; Ardizzoni, A. Emerging therapies in malignant pleural mesothe- lioma. *Crit. Rev. Oncol. Hematol.* **2019**, *144*, 102815. [CrossRef]
150. Gray, S.G.; Mutti, L. Immunotherapy for mesothelioma: A critical review of current clinical trials and future perspectives. *Trans. Lung Cancer Res.* **2020**, *9* (Suppl. 1), S100–S119. [CrossRef]
151. Tan, Z.; Chiu, M.S.; Yan, C.W.; Wong, Y.C.; Huang, H.; Man, K.; Chen, Z. Antimesothelioma Immunotherapy by CTLA-4 Blockade Depends on Active PD1-Based TWIST1 Vaccination. *Mol. Ther. Oncolytics* **2020**, *16*, 302–317. [CrossRef]
152. Klampatsa, A.; Albelda, S.M. Current Advances in CAR T Cell Therapy for Malignant Mesothelioma. *J. Cell. Immunol.* **2020**, *2*, 192–200. [CrossRef] [PubMed]
153. Gomez-Roca, C.A.; Italiano, A.; Le Tourneau, C.; Cassier, P.A.; Toulmonde, M.; D'Angelo, S.P.; Campone, M.; Weber, K.L.; Loirat, D.; Cannarile, M.A.; et al. Phase I study of emactuzumab single agent or in combination with paclitaxel in patients with advanced/metastatic solid tumors reveals depletion of immunosuppressive M2-like macrophages. *Ann. Oncol.* **2019**, *30*, 1381–1392. [CrossRef] [PubMed]
154. Delaunay, T.; Achard, C.; Boisgerault, N.; Grard, M.; Petithomme, T.; Chatelain, C.; Dutoit, S.; Blanquart, C.; Royer, P.J.; Minvielle, S.; et al. Frequent Homozygous Deletions of Type I Interferon Genes in Pleural Mesothelioma Confer Sensitivity to Oncolytic Measles Virus. *J. Thorac. Oncol.* **2020**, *15*, 827–842. [CrossRef] [PubMed]
155. Yang, H.; Xu, D.; Gao, Y.; Schmid, R.A.; Peng, R.W. Oncolytic Viral Therapy for Malignant Pleural Mesothelioma. *J. Thorac. Oncol.* **2020**, *15*, e111–e113. [CrossRef] [PubMed