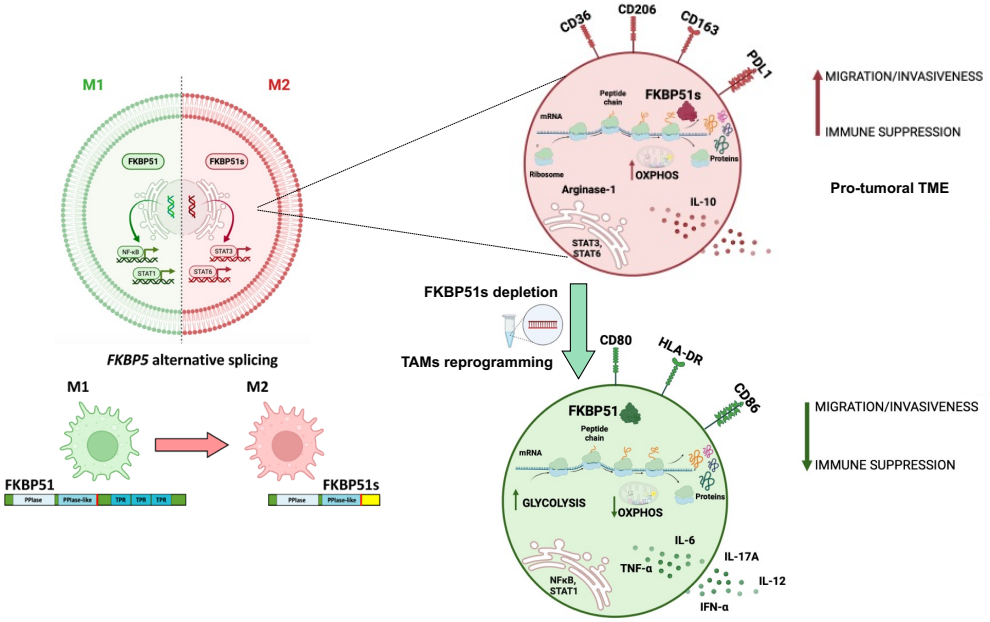
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXV CYCLE

Laura Marrone

INSIGHTS INTO THE MECHANISMS OF ALTERNATIVE MACROPHAGE POLARIZATION TO CIRCUMVENT CANCER IMMUNOTHERAPY RESISTANCE



2019-2023

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Tutor
Prof. Simona Romano

Candidate
Laura Marrone

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LIST OF ABBREVIATIONS

2-DG= 2-deoxy-glucose

APC=Allophycocyanin

AR =adenosine receptor

ARG1= Arginase 1

ATP5A = ATP synthase

CCL =C-C Motif Chemokine Ligand

CCR= C-C chemokine receptor type

CD = cluster of differentiation

CFSE = carboxyfluorescein succinimidyl ester

COX-2= cyclooxygenase-2

CSCs =Cancer Stem Cells

CSF-1=colony stimulating factor 1

CSF-1R= Colony stimulating factor 1 receptor

CTLA= cytotoxic T lymphocyte associated protein

CTLs=cytotoxic T lymphocytes

CXCL= C-X-C Motif Chemokine Ligand

CXCR= C-X-C Motif Chemokine Receptor

Cyp=cyclophilins

DCs =dendritic cells

DMEM= Dulbecco's Modified Eagle Medium

DTT=dithiothreitol

ECAR= extracellular acidification rate

ECM = extracellular matrix

EDTA=ethylene- diaminetetraacetic acid

EGF=epidermal growth factor

ELISAs=enzyme-linked immunosorbent assays

EMT =epithelial-mesenchymal transition

ER= endoplasmic reticulum

ERRα =estrogen-related receptor-α

ETC = Electron Transport Chain

FAM= 5-carboxyfluorescein

FAO=fatty acid oxidation

FBS=fetal bovine serum

FCCP= Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone

FcRs =Fc receptor

FITC= Fluorescein-5-isothiocyanate

FKBP= FK506 binding proteins

GM-CSF = granulocyte-macrophage colony-stimulating factor siRNAs =Small interfering RNAs

GPCR =G-protein-coupled receptor

HIF1α = Hypoxia Inducible Factor 1 Subunit Alpha

HSP= Heat Shock Protein

IDO =Indoleamine 2,3-dioxygenase

IFN = interferon

IGF = insulin-like growth factor

IHC =immunohistochemistry

IL-1ra = IL-1 receptor antagonist

IL-R = IL-1 decoy receptor

IL= interleukin

IMM= inner mitochondrial membrane

IRF=Interferon regulatory factor

iTregs=induced regulatory T cells

LPS = lipopolysaccharide

M-CSF= macrophage colony-stimulating factor

mAbs= Monoclonal antibodies

MARCO= Macrophage receptor with collagenous structure

MDMs =monocyte-derived macrophages

MerTK =Mer receptor tyrosine kinase

MHC =major histocompatibility complex

miRNA= microRNAs

MMPs =matrix metalloproteases

MSR1= Macrophage scavenger receptor 1

NDUFB8 NADH = ubiquinone oxidoreductase subunit B8

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells

NK=natural killer

NO= nitric oxide

NR =Nonresponsive

NRF-1=nuclear respiratory factor 1

NS RNA= non-silencing oligoribonucleotide

nTregs =recruit natural Tregs,

OCR= oxygen consumption rate

ODC =ornithine decarboxylase

OMM= mitochondrial outer membrane

OXPHOS=oxidative phosphorylation

PBMCs=peripheral mononuclear blood cells

PBS=phosphate-buffered saline

PD-1= Programmed cell death protein 1

PD-L1= Programmed cell death ligand

PE=phycoerythrin

PER=proton efflux rate

PerCP= Peridinin chlorophyll protein

PFK2= Phosphofructokinase-2

PFKFB= 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase

PI3K= PhosphatidylInositol 3-Kinase

PK= pyruvate kinase isoenzyme

PLA= Proximity ligation assay

PMA=phorbol 12-myristate 13-acetate

PMSF=phenylmethanesul- fonylfluoride

PPAR = peroxisome proliferator-activated receptor

PPIase = peptidyl-prolyl-isomerase

PTP= permeability transition pore

qRT-PCR = Quantitative real-time polymerase chain reaction

R = Responsive

REDD1= Regulated in development and DNA damage responses 1

ROS = reactive oxygen species

RPMI= Roswell Park Memorial Institute medium

SDHB= succinate dehydrogenase complex iron sulfur subunit B

SHH= Sonic hedgehog protein

siRNA = small interfering oligoribonucleotide

SOCS= suppressor of cytokine signaling

SPARC= secreted protein acidic and rich in cysteine

SRC = respiratory reserve capacity

SSOs=Splice-switching oligonucleotides

STAT = signal transducer and activator of transcription

TAMs =Tumor-associated-macrophages

TCA =The Krebs o tricarboxylic acid

TF= trigger factor

TGF = transforming growth factor

Th= T helper

TILs = tumor-infiltrating lymphocytes

TLR =Toll-like receptors

TME = Tumor microenvironment

TNF = tumor necrosis factor

TNFSF = TNF superfamily member

TOM= translocase of outer membrane complex

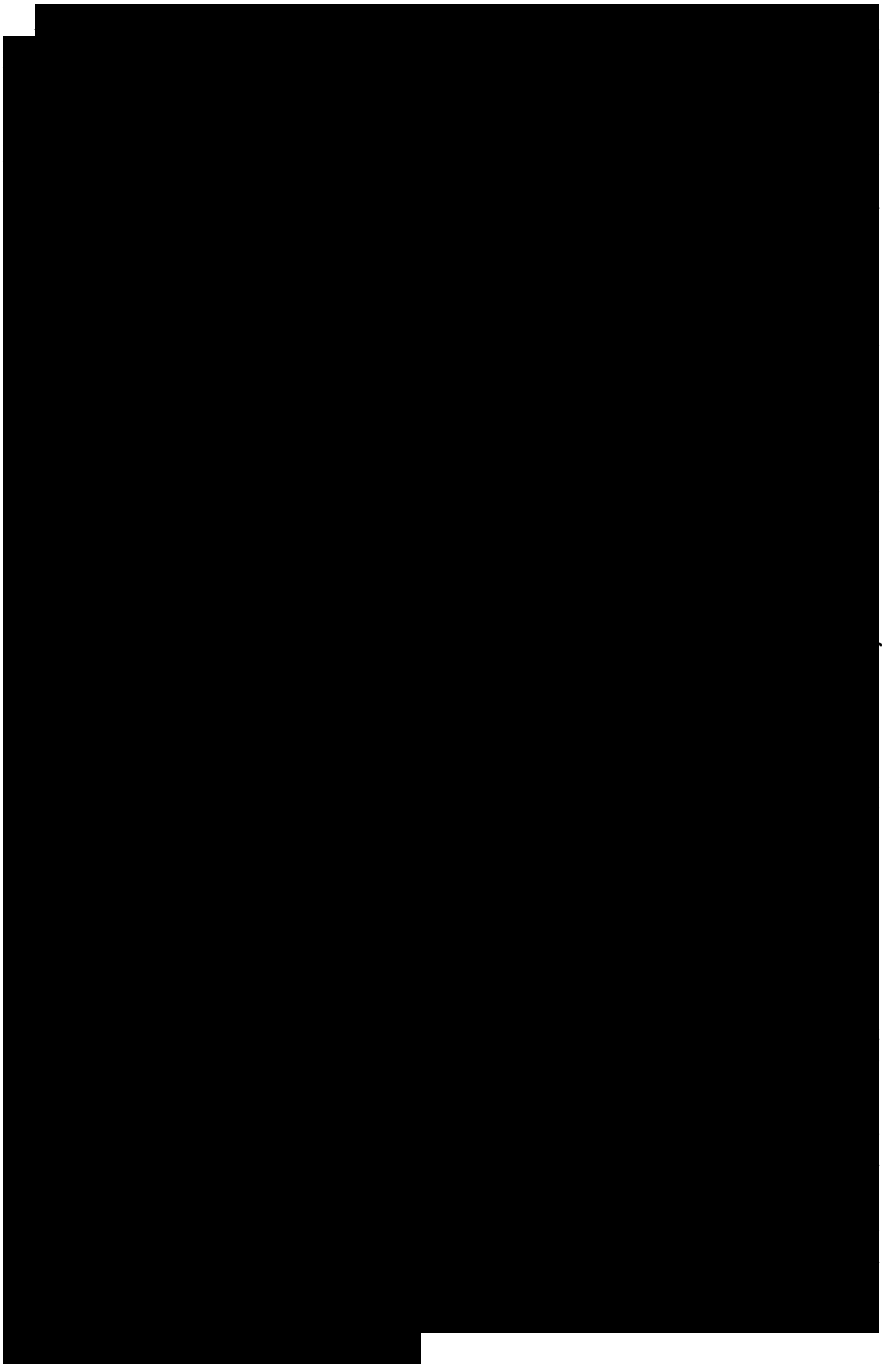
TPR = tetratricopeptide

TRMs =tissue-resident macrophages

UQCRC2 = ubiquinol-cytochrome c reductase core protein 2

VEGF =vascular endothelial growth factor

ABSTRACT



1. BACKGROUND

1.1. FKBP5 isoforms

FKBP51 is a member of the FK506 binding proteins (FKBP), these proteins, together with cyclophilins (Cyp), belong to the family of immunophilins (Dornan et al. 2003). FKBP51 was firstly cloned in lymphocytes (Baughman et al. 1995) and abundantly expressed by immune cells (Baughman et al. 1997). This protein exerts an N-terminus domain (FK1) known to be the binding domain to immunosuppressant agents, such as FK506 and rapamycin. This domain is endowed with a peptidyl-prolyl-isomerase (PPIase) enzymatic function, able to catalyze the isomerization of the prolines of FKBP51 substrates, thus guiding their proper folding and greatly improving their stability and function (Fischer et al. 2003). In humans, at least 15 FKBPs have been identified and named to reflect their molecular weights (Somarelli et al. 2008). Family members of this ubiquitous enzyme class are found in abundance in virtually all organisms and subcellular compartments. Their amino acid sequences are highly conserved phylogenetically (Fischer et al. 2003). FKBP51 also exerts tetratricopeptide (TPR) domains at the C-terminus, involved in protein-protein interactions and by which the immunophilin participates in several pathways, such as protein folding, improvement of kinase performance, receptor signaling, protein trafficking, and transcription (Dornan et al. 2003, Somarelli et al. 2008). TPR domains are responsible for protein-protein interactions with heat shock (chaperone) proteins HSP90 and HSP70 as well as with other proteins, including steroid receptors (Somarelli et al. 2008, Romano et al. 2011a). The role of this immunophilin in supporting tumor proliferation and aggression has been widely documented in many human cancers (Romano et al. 2011b). In 2014 the research group to which I belong identified, for the first time, the splicing isoform of the *FKBP5* gene, namely FKBP51s. This splicing variant is generated by alternative splicing of *FKBP5* pre-mRNA; particularly, it arises from the recognition of an intronic splice site that drives to an exon skipping and a

frameshift with the generation of a premature stop codon. As such, the splice isoform loses the C-terminus domain and generates a shorter protein, namely FKBP51s, with a new C-terminal polypeptide of 44 amino acids, in comparison with canonical FKBP51. FKBP51s retains the PPIase activity but loses the TPR domain, deputed to the interaction with multiple protein complexes through HSP90 (Romano et al. 2015) (Fig. 1).

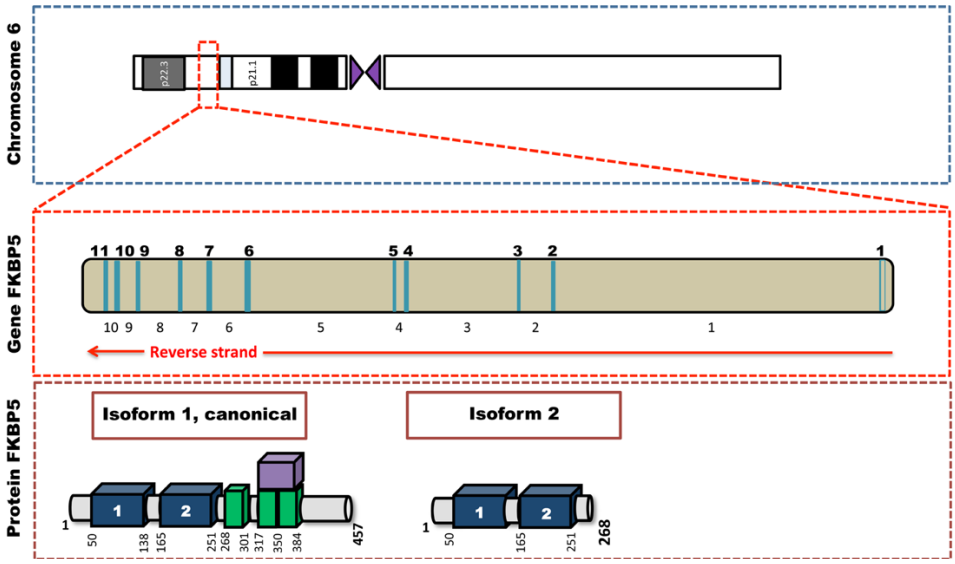


Figure 1. Schematic representation of the canonical protein FKBP51 and its splice isoform FKBP51s: *(Top) Locus on the chromosome. (Middle) The gene with exonic and intronic regions; ENSG00000096060. (Bottom) Encoded protein(s): isoform 1, NP_004108.1, NP_001139247, NP_001139248; isoform 2, NP_001139249.1. FKB51 contains a tandem FKBD separated by a short linker sequence. The N-terminal FKBD is responsible for the PPIase- and ligand-binding activities. The 2nd FKBD is inactive in those activities but seems to retain an interaction ability. This domain contains an ATP/GTP-binding sequence (D'Arrigo et al. 2016).*

1.2. FKBP51s is a PD-L1 regulator

The research group demonstrated that the splicing isoform is opportunistically exploited by melanoma to suppress undesired immunity, through the interaction of the Programmed cell death ligand (PD-L1) with its receptor PD1 (Romano et al. 2015). Particularly, they found that FKBP51s expression in the tumor-infiltrating lymphocytes (TILs) of melanoma patients was influenced by the expression of PD-L1 by the tumor (Romano et al. 2015). Furthermore, cocultures of peripheral mononuclear blood cells (PBMCs) with melanoma cells bidirectionally stimulated the expression of FKBP51s because of the immune cell/tumor interaction through PD1/PD-L1, respectively. FKBP51s, indeed, increases the expression of PD-L1 by acting as a foldase in the post-translational modifications of PD-L1 itself, which occur during the maturation of the protein and drive its expression on the plasma membrane, thus playing a relevant role in the immune suppression induced by the tumor (Romano et al. 2015; D'Arrigo et al. 2017). As shown in Fig. 2A, FKBP51s, but not FKBP51, was found in the endoplasmic reticulum (ER); moreover, treatment of the immunoprecipitated PD-L1 protein with PNGase F resulted in a decrease in the band at 68 kDa and the appearance of an additional band at about 37 kDa (Fig. 2B), demonstrating that FKBP51s has a role in catalyzing the folding of PD-L1, an essential step in glycosylation. Finally, as shown in Fig. 2C, pull-down of FKBP51s or PD-L1 confirmed that the two proteins interact with each other in the ER (D'Arrigo et al. 2017).

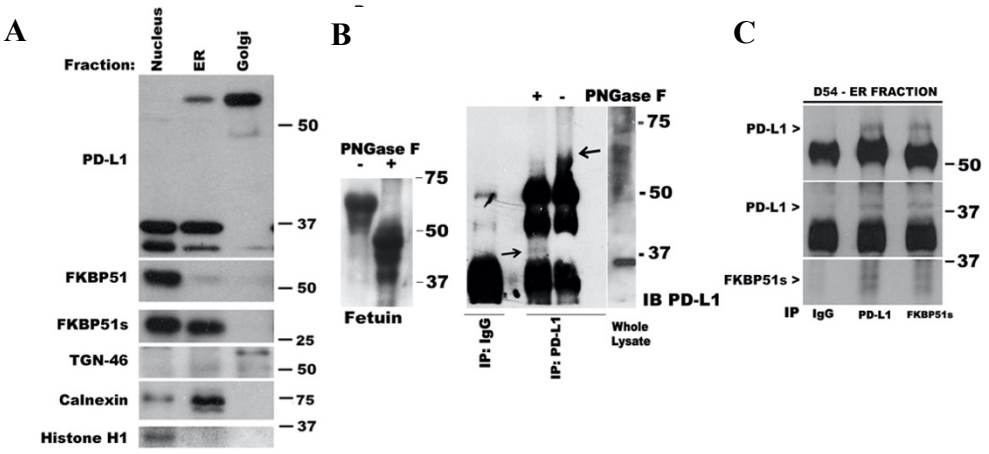


Figure 2: FKBP51s is associated with PD-L1 in ER. *(A)Immunoblot of D54 lysates obtained from sub-cellular compartments. PD-L1, FKBP51 and FKBP51s levels are shown along with relative organelle markers. (B) Whole D54 lysates immunoprecipitated with anti-PD-L1 and subjected, or not, to PNGase F treatment; the arrows indicate the higher (- PNGase F) and lower (+ PNGase F) PD-L1 band. Fetuin, on the left of the panel, was used as positive control of PNGase digestion. (C) Co-IP of PD-L1 and FKBP51s in ER fraction. ER lysate was immunoprecipitated with anti-PD-L1 and anti-FKBP51s and recognized for each protein by immunoblot (D'Arrigo et al 2017).*

1.3. FKBP51s marks PBMCs of melanoma patients

Previous data obtained by the research group led to the identification of FKBP51s as a signature generated by crosstalk between tumor and immune cells. Expression levels of the FKBP51 spliced variant was measured in 70 control subjects and 115 patients with metastatic (25) and primary (90) melanoma. The results showed that FKBP51s is present in PBMCs of melanoma patients, particularly those with metastatic melanoma (Fig. 3) (Romano et al. 2015).

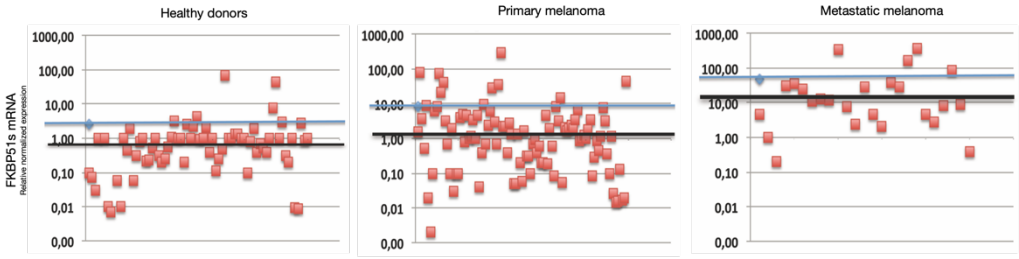


Figure 3. FKBP51s expression levels are increased in PBMCs of patients with melanoma. *FKBP51s transcript was measured in 70 control subjects and 115 patients with metastatic (25) and primary (90) melanoma, by qPCR. The values are expressed in arbitrary units (relative normalized expression) (Romano et al. 2015).*

Immunoreactivity for FKBP51s has also been found in TILs and melanomas. The expression of PDL1 and FKBP51s was investigated in a series of 76 melanoma specimens (12 from primary and 64 from metastatic patients) by immunohistochemistry (IHC). Most interestingly, in PDL-1-expressing melanomas, immunoreactivity for FKBP51s was observed in TILs (Fig. 4A). Furthermore, FKBP51s-positive TILs were closed to PDL-1-expressing macrophages, which nevertheless infiltrate PDL-1-negative tumors (Fig. 4B). These results support the idea that immunosuppressive macrophages also mediate immune evasion of tumors (Romano et al. 2015).

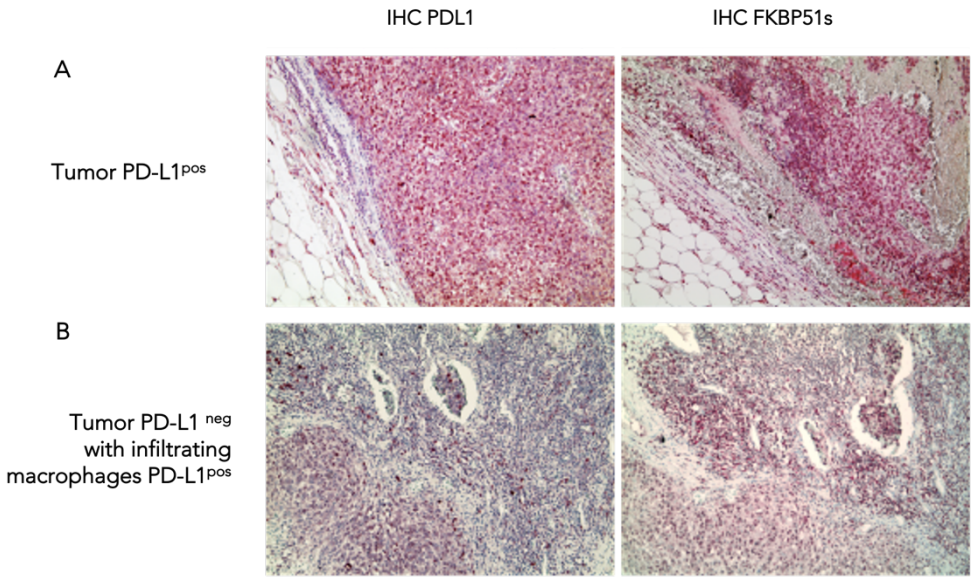


Figure 4. FKBP51s expression in TILs is increased in PDL-1-expressing melanoma*. Representative IHC of melanoma specimens. Sections from the same specimen were stained with PDL-1 and FKBP51s. (A) Left, a case of invasive primary cutaneous melanoma (vertical growth phase), showing extensive immunoreactivity for PDL1 of the tumour population. Original magnification: 9250. Right, the same case stained with FKBP51s, showing positive neoplastic melanocytes and an intense signal in the great majority of inflammatory cells. Original magnification: 9250. (B) Left, a metastatic melanoma negative for PDL-1; numerous PDL-1-positive macrophages infiltrate the tumour. Right, the same case stained with FKBP51s. No/low expression in tumour cells, numerous macrophages immunoreactive, TILs stained by FKBP51s <20%. Original magnification: 9250. (Romano et al. 2015).*

1.4. Monocytes of melanoma patients express increased levels of FKBP51s

FKBP51s isoform can be considered an immune signature, virtually associated with the tumor-induced immune tolerance and, therefore, capable of monitoring the immunotherapy response of melanoma patients. In details, by immunophenotyping 3 different cohorts of patients, 1st undergoing anti-cytotoxic T lymphocyte associated protein (CTLA)-4 (ipilimumab) immunotherapy treatment (Romano et al. 2017), 2nd receiving anti-PD1 treatment (nivolumab or pembrolizumab) (Troiani et al. 2020), and a 3rd of glioblastoma patients (Giordano et al. 2021), refractory to immunotherapy, the research group observed a cluster of differentiation (CD)14+ subset of monocytes co-expressing PD-L1 and FKBP51s. In 2017, Romano et al. measured FKBP51s expression in peripheral blood CD14 monocytes from a cohort of 118 patients and 77 age- and sex-matched healthy controls. Blood samples were collected before patients underwent ipilimumab treatment. The number of PD-L1+ monocytes was increased in the Nonresponsive (NR) patients compared to Responsive (R) patients and the controls. Subsequently, in 2020, by studying a different cohort of melanoma patients (22 patients receiving nivolumab or pembrolizumab), the research group confirmed previous findings that FKBP51s+ PD-L1+ monocytes are associated with response to checkpoint-targeted therapy. This subset significantly increased after 4 weeks from the beginning of the treatment, but only in those patients NR to immunotherapy (Fig. 5A). The observation that the treatment produced an increase in the expression levels of genes such as Arginase 1 (ARG1) and Macrophage scavenger receptor 1 (MSR1) in NR patients, while a decrease in these same transcripts was observed in R patients (Fig. 5B), supported the hypothesis of an alternative polarization of macrophages, responsible for tumor tolerance and resistance to immunotherapy (Troiani et al. 2020).

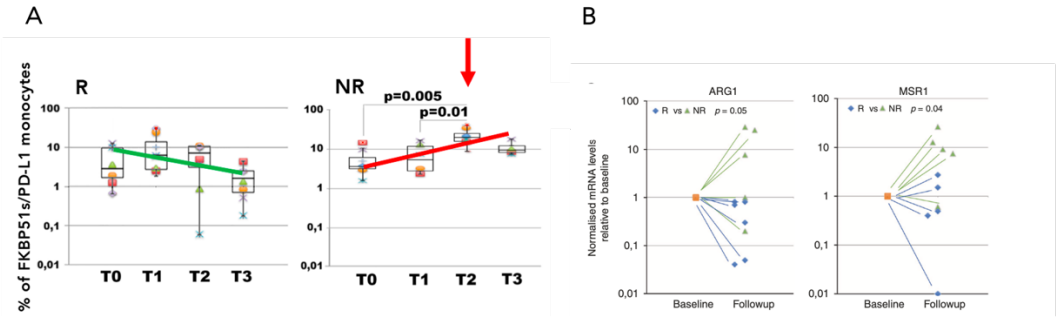


Figure 5. Increased counts of FKBP51s$^+$ PD-L1$^+$ monocytes in non-responder patients to anti-PD1*. (A) Graphic representation of FKBP51s$^+$ PD-L1$^+$ monocyte counts at baseline and during treatments from 8 R and 8 NR patients. (B) Levels of ARG1 and MSR1 in 6 R and 5 NR patients were assessed by qPCR at baseline and at T2/T3 (follow-up) (Troiani et al. 2020).*

In a third cohort of glioblastoma patients, a tumor NR to immunotherapy, notwithstanding high expression of PD-L1, they confirmed the existence of this monocyte subset even more numerous in this case, but they observed a further phenotype more stringent related to the tumor and co-expressing FKBP51s along with CD163, the most represented M2 marker of glioblastoma peripheral monocytes. Particularly, FKBP51s and CD163 marked circulating monocytes associated with the presence of the tumor upon surgical resection: a dramatic decrease of such a subset was observed in those patients with radical surgery, but not in those with a partial surgical removal of the tumor (Fig. 6) (Giordano et al. 2021).

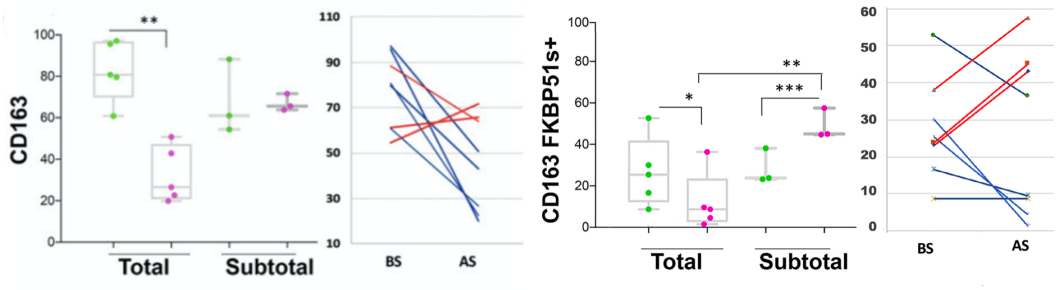


Figure 6. ***CD163+FKBP51s+ monocytes are sensitive to tumor removal.*** *Representative Boxplots of CD163/FKBP51s monocytes. In patients with incomplete resection, no significant changes in whole CD163 monocyte count were registered after surgery, but the fraction co-expressing FKBP51s+ resulted in significant increase (Giordano et al. 2021).*

1.5. Macrophage heterogeneity, plasticity, and nomenclature

Macrophages are a heterogeneous and complex population of immune cells with roles in host defense against pathogens, maintenance of tissue homeostasis, and tissue architecture (Italiani et al. 2014). Macrophages are highly plastic cells and in response to microenvironment signals such as chemokines and cytokines they differentiate/polarize into distinct phenotypes with specific functionalities. Multiple macrophage populations are known to occur within the same microenvironment, and each phenotype has a distinctive combination of receptor expression, chemokine secretion, and cytokines (DeNardo et al. 2019). The current classification of macrophages is based on their function and response to polarizing agents (Fig. 7).

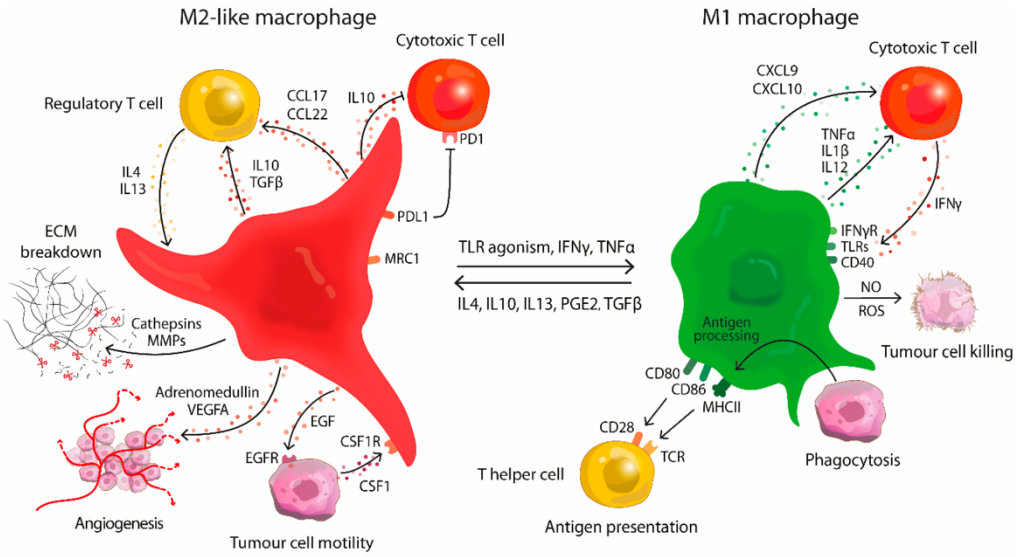


Figure 7. Schematic representation of M1 and M2-like macrophages functions in tumor development. *During the early stages of tumorigenesis, activated macrophages (M1) present antigens and support cytotoxic T lymphocytes (CTLs) by producing proinflammatory cytokines. They eliminate tumor cells with nitrogen radicals and oxygen or by phagocytosis. These anti-tumor macrophages can be captured by the tumor and shifted to the M2-like state by secretion of immunosuppressive cytokines. The formed M2-like macrophages suppress the function of CTLs and redirect them to immunosuppressive T-cell subgroups. M2-polarized TAMs support tumor growth at all stages of the disease, including proliferation, angiogenesis, and metastasis. (van Dalen et al. 2018)*

Macrophages can be schematically divided into non-polarized macrophages, called M0 (naïve), classically activated macrophages M1, with pro-inflammatory activity and induced by T helper (Th) 1 cells, and macrophages derived from alternative activation M2, with anti-inflammatory activity and mainly induced by stimulation of Th2 cells. This classification also includes M2a, M2b, M2c, and M2d subtypes (Martinez et al. 2008). Mantovani et al. (2004) defined classically activated macrophages or M1 those resulting from stimulation with interferon (IFN) γ combined with lipopolysaccharide (LPS), or tumor necrosis factor (TNF). Macrophages alternatively activated macrophages *in vitro* by interleukin (IL) 4 were, instead, renamed M2a. Two other M2-like macrophage phenotypes were induced by activation of the Fc receptor (FcRs) by

immune complexes (M2b) or glucocorticoids and IL-10 (M2c) (Mantovani et al. 2004).

1.5.1. M1 macrophages

M1 macrophages exert tumor suppressors and pro-inflammatory functions; they produce pro-inflammatory cytokines, mediate resistance to pathogens, and exhibit strong microbicidal properties, but also contribute to tissue damage. M1 macrophages, also known as inflammatory macrophages, are activated mainly by IFN-γ secreted by Th1 cells, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells; they are also activated upon long exposure to microorganisms or microbial products such as LPS, a component of the outer membrane of Gram-negative bacteria and granulocyte-macrophage colony-stimulating factor (GM-CSF) that stimulates the production of pro- inflammatory cytokines (Fleetwood et al. 2007). These cells show enhanced antigen presentation and phagocytosis capacity and secrete high levels of proinflammatory factors and cytokines, such as TNF-α, IL-1β, IL-12 and IL-18, nitric oxide (NO), thus participating in the type I immune response (Rhee et al. 2020). These factors, in turn, exert positive feedback on unpolarized macrophages by attracting them into the M1 state. Key transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription (STAT) 1, STAT5, Interferon regulatory factor (IRF) 3, and IRF5 have been shown to regulate the expression of M1 genes. It seems that NF-κB and STAT1 are the two major pathways involved in M1 macrophage polarization and result in microbicidal and tumoricidal functions (Yao et al. 2019). Phenotypically, M1 macrophages express high levels of major histocompatibility complex (MHC) II, CD68, as well as costimulatory molecules CD80 and CD86 (Raggi et al. 2017).

1.5.2. M2 macrophages.

M2 macrophages possess tissue repair and tumor growth promotion activities. M2 macrophages, also known as anti-inflammatory macrophages, are mainly activated by IL-4, IL-13, colony stimulating factor 1 (CSF-1), IL-10, transforming growth factor (TGF) β and helminth infections through activation of STAT6, peroxisome proliferator-activated receptor (PPAR) γ, suppressor of cytokine signaling (SOCS) 2. Key transcription factors, such as STAT6, IRF4 and PPARγ have been shown to regulate the expression of M2 genes (Murray et al. 2017). M2 macrophages produce many anti-inflammatory factors, including IL-10, TGF-β and ARG1, participating in the type II immune response, which plays a central role in the response to parasites, tissue remodeling, angiogenesis, and allergic diseases. ARG1 is an enzyme of the urea cycle; its action catalyzes the hydrolysis of arginine to ornithine. Ornithine is the substrate for ornithine decarboxylase (ODC). This pathway regulates a multitude of cellular processes like DNA replication, protein translation, cell growth, and differentiation (Murray et al. 2014). Up-regulation of cytokines and chemokines, such as IL-10, TGF-β, C-C Motif Chemokine Ligand (CCL) 1, CCL17, CCL18, CCL22, and CCL24 also attract unpolarized macrophages to polarize into the M2 state (Gordon et al. 2010; Mulder et al. 2014). Phenotypically, M2 macrophages are characterized by the expression of specific membrane glycoproteins. CD206 is a transmembrane glycoprotein that binds and internalizes collagen glycoproteins and ligands. Macrophages expressing CD206 have unfavorable profibrotic effects, as they promote fibroblast growth through secretion of TGF-β and CCL18 (Bellón et al. 2011). CD163 is a scavenger receptor for haptoglobin. It is an M2 marker protein, mainly due to its upregulated expression in response to IL-4, IL-10, and glucocorticoids (Murray et al. 2014). Macrophages co-expressing CD206 and CD163 are large producers of IL-10, IL-1 receptor antagonist (IL-1ra) and CCL18. They also have high uptake of apoptotic cells (Zizzo et al. 2012). The CD36 scavenger receptor is a membrane glycoprotein that acts as a receptor for a wide range of ligands, including fibronectin, collagen,

and ligands of a lipid nature such as fatty acids. Cellular responses to these ligands are involved in angiogenesis, inflammatory response, and fatty acid metabolism (Liang et al. 2018).

1.5.3. The Subsets of M2 Macrophages and Their Characteristics

As mentioned above, M2 macrophages are subgrouped into M2a, M2b, M2c, and M2d (Fig. 8).

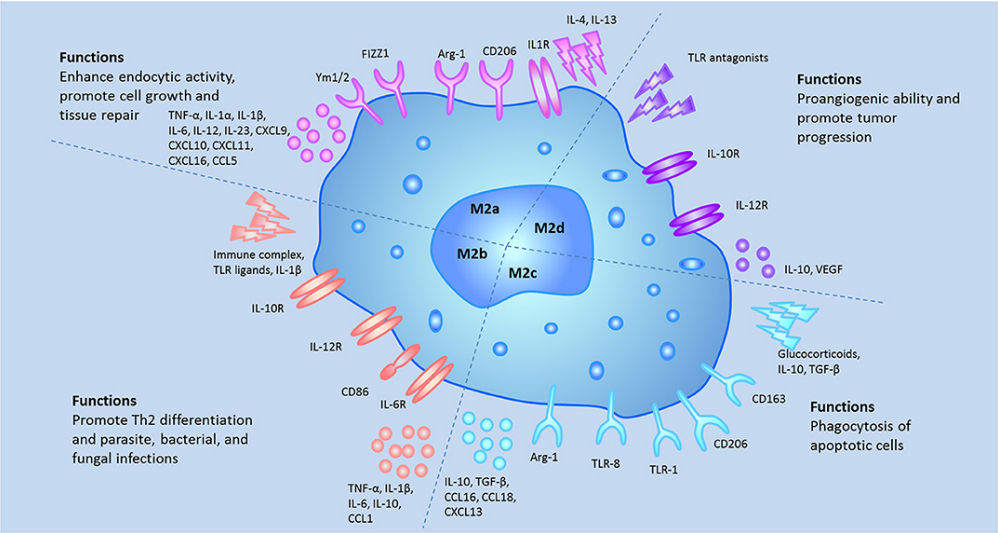


Figure 8. Schematic representation of M2 macrophages subsets and their characteristics. *The different stimuli, surface markers, secreted cytokines, and biological functions of the M2 macrophage subsets (M2a, M2b, M2c, M2d) were summarized (Yao et al. 2019).*

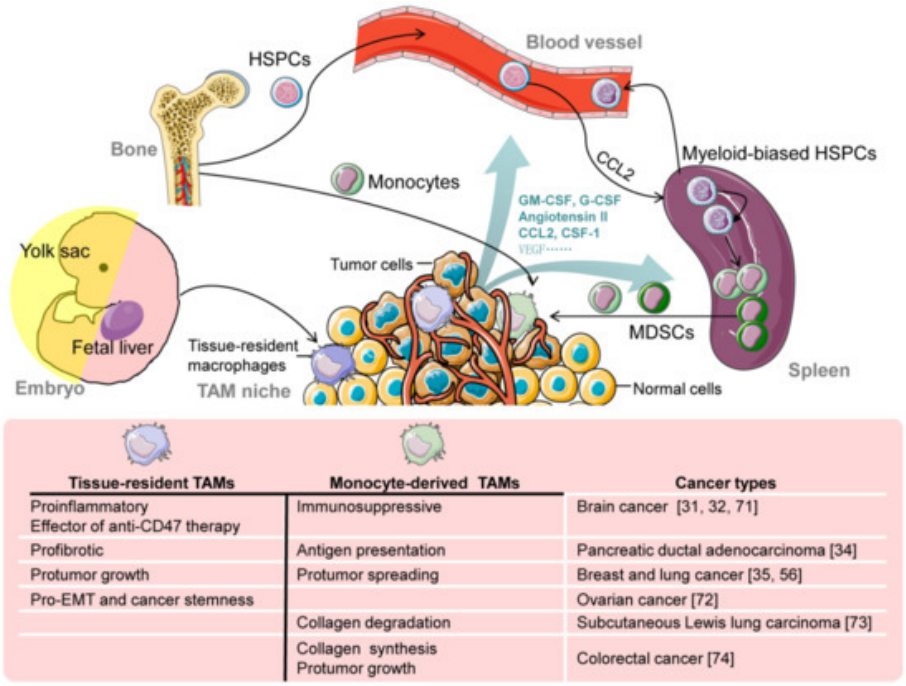
M2a macrophages, also called wound-healing macrophages, enhance endocytic activity, promote cell growth, and tissue repair. They are induced by IL-4 and IL-13 and express high levels of CD206, IL-1 decoy receptor (IL-R) and CCL17 and secrete pro-fibrotic factors such as TGF-β, insulin-like growth factor (IGF) and fibronectin to contribute to tissue repair (Mantovani et al. 2004). M2b macrophages, also known as regulatory macrophages, regulate the extent and depth of immune responses and inflammatory reactions. They can be induced by

combined exposure to Toll-like receptors (TLR) agonists or IL-1R agonists and express high levels of CCL1 and TNF superfamily member (TNFSF) 14 (Mantovani et al. 2004). In addition to proinflammatory cytokines (IL-1β, IL-6 and TNF-α), M2b cells also express and secrete substantial amounts of the anti-inflammatory cytokine IL-10 and low levels of IL-12, which is the functional inverse of M1 cells (Yue et al. 2017). M2c macrophages, also known as inactivated macrophages, play crucial roles in the process of phagocytosis of apoptotic cells. They are induced by glucocorticoids, IL-10 and TGF-β through activation of STAT3 and show strong anti-inflammatory activity by releasing large amounts of IL-10 and pro-fibrotic activity by secreting high levels of TGF-β (Mantovani et al. 2004). In addition, M2c macrophages exhibit high expression of Mer receptor tyrosine kinase (MerTK), resulting in efficient phagocytosis of apoptotic cells (Zizzo et al. 2012). M2d macrophages promote angiogenesis and tumor progression; they are induced by costimulation with TLR ligands and adenosine receptor (AR) 2 agonists or by IL-6 (Shapouri-Moghaddam et al. 2018). These cells are mainly characterized by high production of IL-10, TGF-β and vascular endothelial growth factor (VEGF) and low production of IL-12, TNF-α and IL-1β (Martinez et al. 2008).

1.6. Origins of Tumor-associated-macrophages (TAMs)

Macrophages have two main origins: tissue-resident macrophages (TRMs) and monocyte-derived macrophages (MDMs). TRMs develop from embryonic precursors (yolk sac or fetal liver progenitors), whereas MDMs develop from bone marrow hematopoietic cell progenitors (Blériot et al. 2020) and play different roles in both health and disease. TRMs are widely distributed throughout the body and, depending on their location, are called osteoclasts in bone, alveolar macrophages in the lungs, microglial cells in the central nervous system, and Kupffer cells in the liver. In general, they play tissue-specific

homeostatic roles, as well as basic functions such as removing dying cells. MDMs are recruited to tissues during inflammation, secrete pro-inflammatory cytokines, help clear infection, regulate the immune response, and serve as a reservoir for macrophage reconstitution and are recruited in pathology (De Nardo et al. 2019). Under physiological conditions, TRMs and MDMs have distinct tissue distribution: (i) both subpopulations are found in the liver, pancreas, lung, heart, kidney, and spleen; (ii) only TRMs are found in the brain; and (iii) MDMs predominate in the gut and dermis (Zhu et al. 2017). Therefore, in pathological situations, macrophages are a heterogeneous population. Compared with tissue homeostasis, cancer is characterized by increased recruitment of monocytes and/or expansion of TRMs, with both populations involved in tumorigenesis (De Nardo et al. 2019) (Fig. 9).



Tissue-resident TAMs	Monocyte-derived TAMs	Cancer types
Proinflammatory Effector of anti-CD47 therapy	Immunosuppressive	Brain cancer [31, 32, 71]
Profibrotic	Antigen presentation	Pancreatic ductal adenocarcinoma [34]
Protumor growth	Protumor spreading	Breast and lung cancer [35, 56]
Pro-EMT and cancer stemness		Ovarian cancer [72]
	Collagen degradation	Subcutaneous Lewis lung carcinoma [73]
	Collagen synthesis Protumor growth	Colorectal cancer [74]

Figure 9. Schematic representation of the two main origins of TAMs: TRMs and MDMs. *TRMs develop from embryonic precursors (yolk sac or fetal liver progenitors), whereas MDMs develop from bone marrow hematopoietic cell progenitors. Tissue-resident TAMs and monocyte-derived TAMs have different functions in tumor progression (Zhang et al. 2021).*

Several pathways are involved in monocyte recruitment to the tumor, including cytokines, chemokines, and growth factors that recruit monocytes. Inhibition of the recruitment of monocyte-derived macrophages is a promising strategy to reduce the TAM population and enhance the antitumor response. The M-CSF/CSF-1R, CCL2/CCR2, CCL5/CCR5 and CX3CL1/CX3CR1 pathways are major targets showing potential in cancer therapy (Peyraud et al. 2017). In general, TAMs are thought to closely resemble M2 macrophages with Th2 immune response and immunosuppressive features. However, current studies have shown that the TAM population is in a state of constant transition between the two forms of M1 and M2 types (Pan et al. 2020) (Fig. 10).

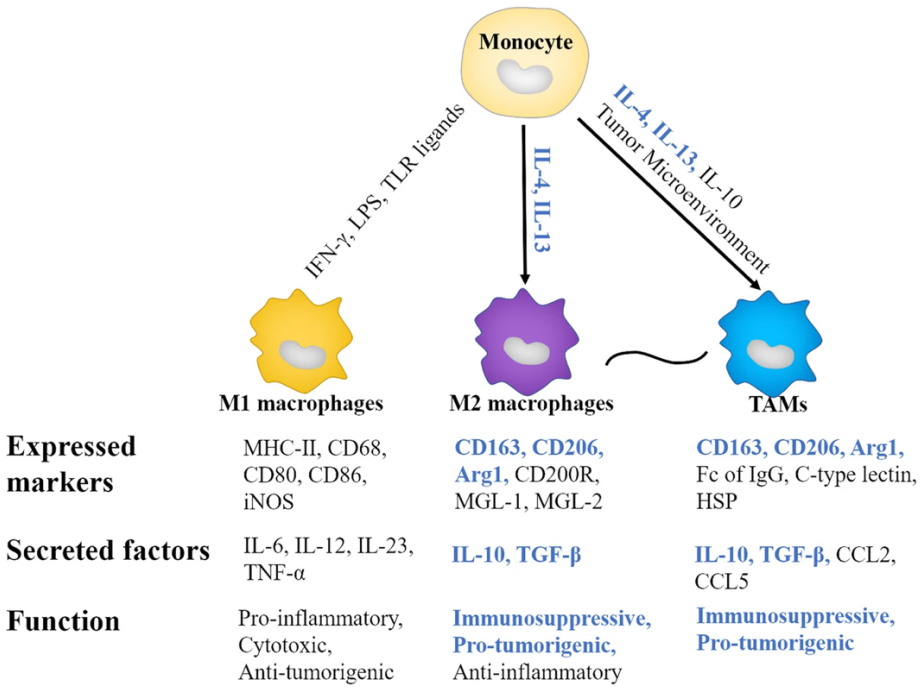


Figure 10. Schematic representation of TAMs characteristics. *M1 macrophages exert pro-inflammatory, cytotoxic and tumoricidal roles. On the contrary, M2 macrophages and TAMs exert immunosuppressive and pro-tumorigenic roles. In general, TAMs are thought to more closely resemble M2 macrophages, however they show some different characteristics (Chen el al. 2019).*

The proportion of each form is determined by the type and concentration of different signals in the tumor microenvironment. It is known that the population of TAMs within the Tumor microenvironment (TME) is phenotypically heterogeneous (De Nardo et al. 2019), and the total number of TAMs accumulated within a tumor is not considered in estimating clinical prognosis. However, the M1/M2 ratio is considered an important prognostic marker (Dan et al. 2020). A low M1/M2 TAM ratio is associated with tumor progression and poor prognosis, whereas a high M1/M2 ratio tends to correlate with positive outcomes in ovarian cancer, gastric cancer, colorectal cancer, osteosarcoma, lung cancer, and oral squamous cell carcinoma. Numerous studies have shown that M2 TAMs play an important role in promoting tumor growth, angiogenesis,

extracellular matrix modification, inhibition of antitumor immunity, metastasis, immunotherapy resistance, and recurrence (Hourani et al. 2021).

1.7. TAMS in tumor initiation

Tumors acquire mutations in oncogenes or suppressor genes that allow them to progress to malignancy. For years cancer research focused on these mutations, and cancer therapies also aimed to target oncogenes. Nowadays it is known that stromal cells in the microenvironment evolve along with the tumor and provide essential support for their malignant phenotype (Joyce and Pollard 2009). The presence of inflammation is a shared feature of many cancers: more than 20 percent of neoplasms are induced or aggravated by infection, chronic inflammation, or autoimmunity in the same tissue or organ (Grivennikov et al. 2010). Activated macrophages are central to this type of immune response and work in concert with other immune cells (Balkwill et al. 2005) (Fig. 11).

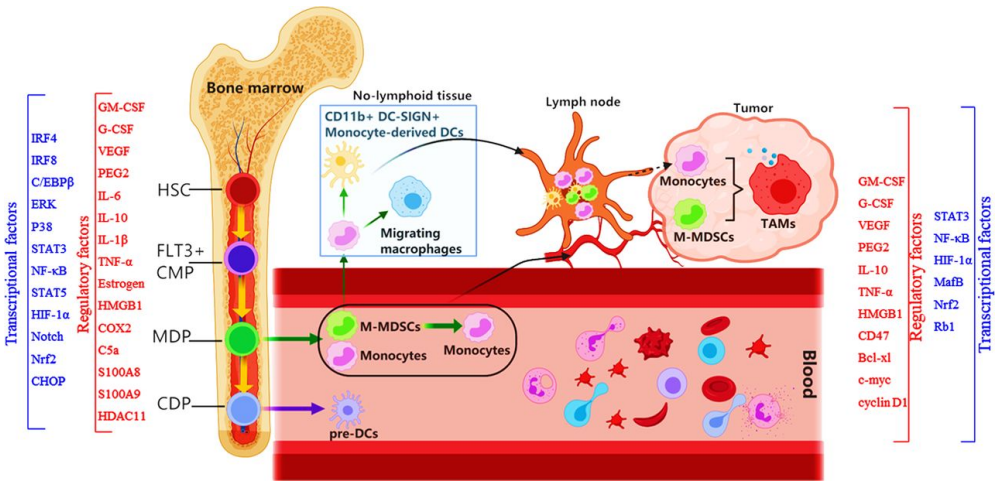


Figure 11. Overview of macrophages involvement in myeloid cell differentiation in cancer. *Macrophages development, accumulation, suppressive activity, and survival are controlled by a complex network of transcription factors, cytokines, and non-cytokine immune regulatory factors. Under different conditions such as the tumor microenvironment, a variety of factors promote cancer risk, facilitate cancer onset and progression, and polarize TAMs (Li et a.l 2021).*

It has been hypothesized that these immune cells produce a mutagenic environment by generating reactive nitrogen and oxygen species that cause mutations in the adjacent epithelial cells (Pang et al. 2007). In addition, there is evidence that the inflammatory microenvironment also promotes genetic instability within the developing tumor epithelial cells (Colotta et al. 2009). In either case, the mutations are fixed after replication of the epithelial cells, a process that is stimulated by growth factors synthesized by the infiltrating or resident immune cells that include macrophages. Macrophages are a major infiltrating immune cell in chronic inflammation, secreting inflammatory factors and cytokines and influencing angiogenesis and tumor metastasis, such as IL-6, TNF-α and IFN-γ (Grivennikov et al. 2010). TNFα action through NF-κB is a causal agent in this promotion through mechanisms that act directly on epithelial cells and on the inflammatory cells in the surrounding stroma, particularly the macrophages (Balkwill et al. 2009). Together these data strongly support causal roles for inflammation in cancer initiation and promotion. Although not

definitive, given that macrophages have not been uniquely targeted in any system, the data suggest that macrophages are key cells in cancer induced by inflammation.

1.8. TAMs functions in the Primary Tumor

The macrophage phenotype associated with cancer initiation and promotion is comparable to the pro-inflammatory (or activated) one (Gordon et al. 2003). However, once tumors are established, macrophages are educated to become pro-tumor (Qian and Pollard. 2010). At various stages of tumor mass development, immune system cells with Th1 type inflammatory action, initially recruited to fight it, are gradually and progressively modified to create a Th2 type immune environment. Macrophage plasticity is a characteristic that is opportunistically exploited by the tumor. Macrophages are, in fact, drawn into the tumor tissue as M1 macrophages and progressively "reprogrammed" into M2, with the result that they are deprived of their antitumor functions and diverted to contribute to the growth and spread of malignant cells (Fig. 12) (Yunna et al. 2020).

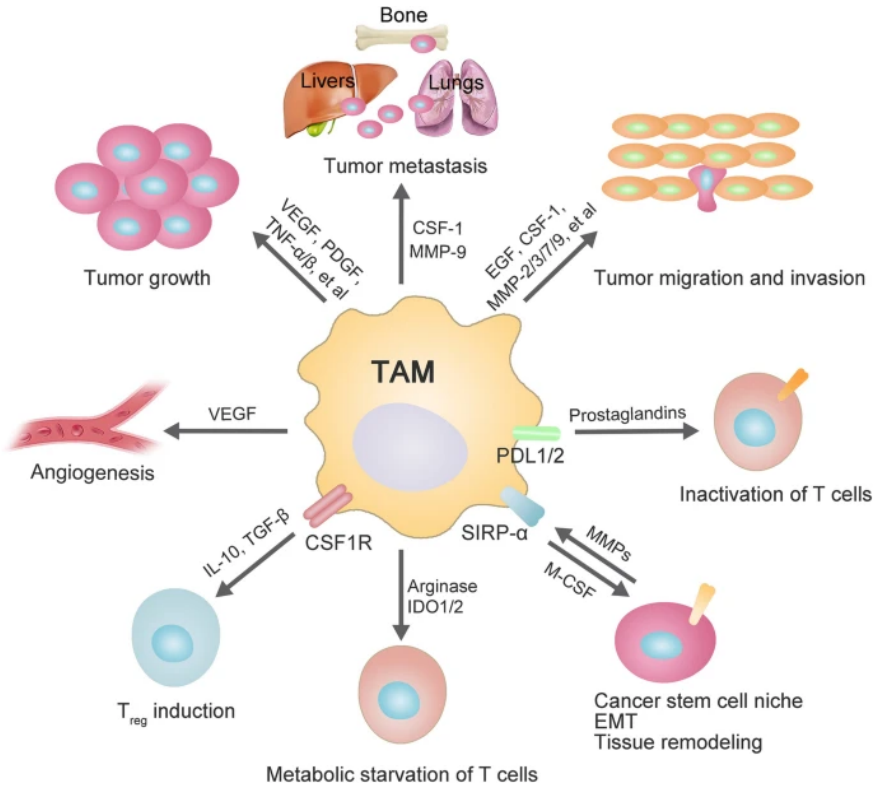


Figure 12. Schematic representation of main roles of TAMs in tumorigenesis*. TAMs promote tumor growth, angiogenesis, Treg cells induction, metabolic starvation of T cells, cancer stem cells induction, T cells inactivation, epithelial-mesenchymal transition (EMT), invasion, migration and metastasis (Chen el al. 2019).*

Macrophage plasticity can also be used as an opportunity to cure cancer by repolarizing TAMs to become anticancer. Several options are currently used to select the M1 phenotype from the TAMs or to reprogram the TAMs from the M2 to M1 phenotype: TLR agonists, Monoclonal antibodies (mAbs) targeting inhibitory proteins of the M1 phenotype, and other compounds. TLR agonists represent a promising anticancer therapy (De Meyer et al. 2012). An anti-scavenger receptor CD204 antibody conjugated to the gustin toxin reduced the number of vascular leukocytes and inhibited tumor progression in a murine ovarian cancer model (Bak et al. 2007). Macrophage receptor with collagenous

structure (MARCO) is a pattern recognition scavenger receptor and is expressed by immunosuppressive TAMs. Targeting this receptor is a promising new way to treat breast cancer, colon cancer and melanoma by reprogramming immunosuppressive TAMs to a pro-inflammatory phenotype and increasing the tumor immune response (Georgoudaki et al. 2016).

1.9. TAMs in promoting tumor invasion, migration, and intravasation

Tumor metastasis is an important feature of poor prognosis after tumor therapy. The main reason for tumor cell migration and metastasis is the degradation and damage of the basement membrane of endothelial cells in the tumor tissue. It has been reported that activated TAMs exert a direct effect on promoting metastasis through the direct production of soluble factors. They are the key that opens the gate for tumor cells to escape. Mechanistically, tumor cells synthesize CSF-1, which stimulates macrophages to move and produce epidermal growth factor (EGF), which in turn activates tumor cell migration (Wyckoff et al. 2004). Macrophages and tumor cells move in tandem, and inhibition of EGF or CSF-1 signaling pathways results in inhibition of migration and chemotaxis of both cell types (Wyckoff et al. 2007) (Fig.13).

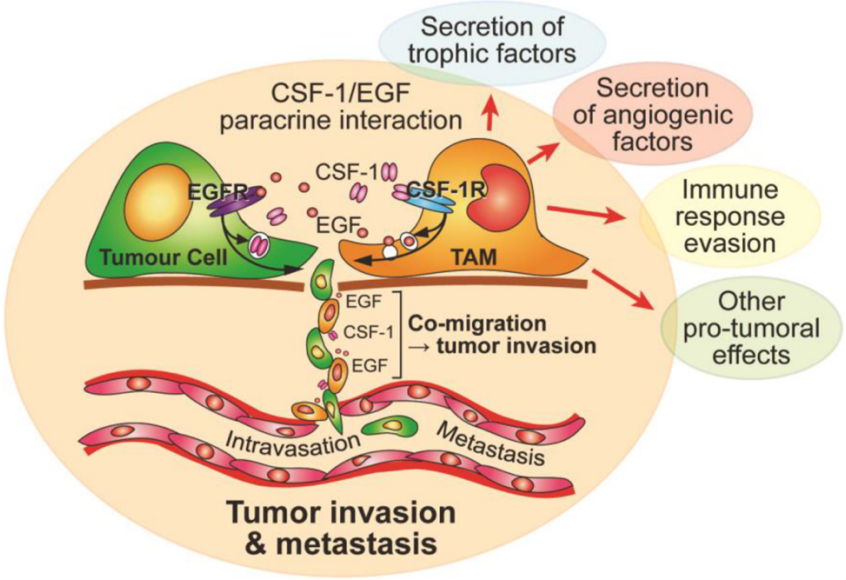


Figure 13. Overview of contribution by TAM to tumor invasion and metastasis*. TAMs EGF and tumour cells secrete CSF-1 to induce a paracrine loop-driven co-migration and invasion of both cells type towards blood vessels (Dwyer et al. 2017).*

The interaction between tumor cells and TAMs plays a key role in the epithelial-mesenchymal transition (EMT) process. TAMs induce EMT in tumor cells by secreting a series of cytokines and growth factors, such as TGF-β, TNF-α, IL-6, and IL-8, thereby promoting tumor invasion and metastasis. TAMs not only contribute to early EMT of tumor cells, but also help to prepare a distant site ready to support metastatic growth (Li et al. 2022). The extracellular matrix (ECM) plays an important role in modifying tumor cell invasiveness. Macrophages synthesize secreted protein acidic and rich in cysteine (SPARC)/osteonectin, which is important for collagen IV deposition, increased tumor cell invasion, and adhesion to other ECM components (such as fibronectin). SPARC/osteonectin has been shown to be required for spontaneous metastasis formation from the primary tumor (Sangaletti et al. 2008). Fibrillar collagen 1 also promotes the invasion process, as tumor cells and macrophages

move ~10 times faster on these structures than through the stroma itself. This has the unfortunate consequence of recruiting cells to blood vessels, as these collagen fibrils also anchor these structures (Condeelis and Segall 2003). Macrophages on the vessels then give approach signals that cause tumor cells to migrate along the collagen fibrils toward the vessels, where the tumor cells escape into the vasculature aided by the macrophages. Tumor cell migration also requires proteolytic destruction of the matrix to allow tumor cells to escape from the borders of the basement membrane. Subsequently, proteolysis is required for tumor cell migration through the dense stroma. Macrophages are potent producers of many proteases, including cathepsins, matrix metalloproteases (MMPs) and serine proteases (Egeblad and Werb 2002). M2- like TAMs induce proteolytic clearance of interstitial collagen through upregulated MMP expression, such as MMP-2, MMP-9, and MMP-12, accompanied by increased endocytosis and lysosomal degradation of collagen (Madsen et al. 2017). In addition to ECM degradation, in a colorectal cancer model, TAMs contribute to ECM deposition. Interestingly, this study found that here TAMs are the major cell type to upregulate synthesis and assembly of collagens, specifically collagen types I, VI and XIV, and induce deposition, cross- linking and linearization of these collagen fibers near invasive tumor cells (Afik et al. 2016). These data add to growing evidence that immune cells contribute to ECM deposition, as macrophages also deposit the glycoprotein osteonectin, which promotes stromal invasion in a mouse model of breast cancer (Sangaletti et al. 2008).

1.10. TAMs promote the cancer stemness

Cancer Stem Cells (CSCs) are cellular elements in the tumor tissue with stem-like properties which have been demonstrated to play a key role in disease progression and tumor recurrence. They represent a distinctive cell subset within the tumoral mass and are characterized by unlimited self-renewal properties,

tumor initiation ability and chemo-resistance (Kreso and Dick 2014). In tumors, also the CSCs reside in a cancer niche that defends them from stress signals, such as apoptosis- inducing chemotherapeutic agents and from attacks by the immune system (Plaks et al. 2015). Key players in the cancer niche are TAMs, which indeed secrete a variety of soluble factors and physically interact with CSCs to protect them from environmental damage (Liguori et al. 2021). As mentioned above, TAMs are active producers of matrix-degrading enzymes and also of ECM macromolecules, thus contributing to the incessant remodeling of the tumor stroma (Liguori et al. 2011). Matrix components are crucial for preserving the niche architecture as well as for the communication between CSCs and the surrounding cells. Notably, the physical interaction between macrophages and CSCs appears crucial to support stemness features, as demonstrated in studies specifically addressing the importance of juxtacrine signaling mechanisms. Cell–cell contact activates several pathways that are important for CSC, such as: SHH, NOTCH, STAT3 (Yang et al. 2013), PI3K/AKT, WNT/b-catenin, NANOG (Morgan et al. 2018) and NF-kB (Galoczova et al. 2018).

1.11. TAMs in immunosuppression

The coordinated interaction between the innate immune system, represented by macrophages and dendritic cells (DCs), and the adaptive immune system, consisting largely of T lymphocytes, is essential to prevent the development and progression of neoplastic cells (Koebel et al. 2007). While the immune surveillance process functions normally in noncancer hosts, a key problem in cancer immunology is to combat immunosuppressive factors within the ECM, which tame normal antitumor responses. Substantial evidence has supported that TAMs change the composition of immune cells within the TME, reducing

antitumor immune cells and simultaneously increasing the presence of immunosuppressive cell types to accelerate tumorigenesis (Fig. 14).

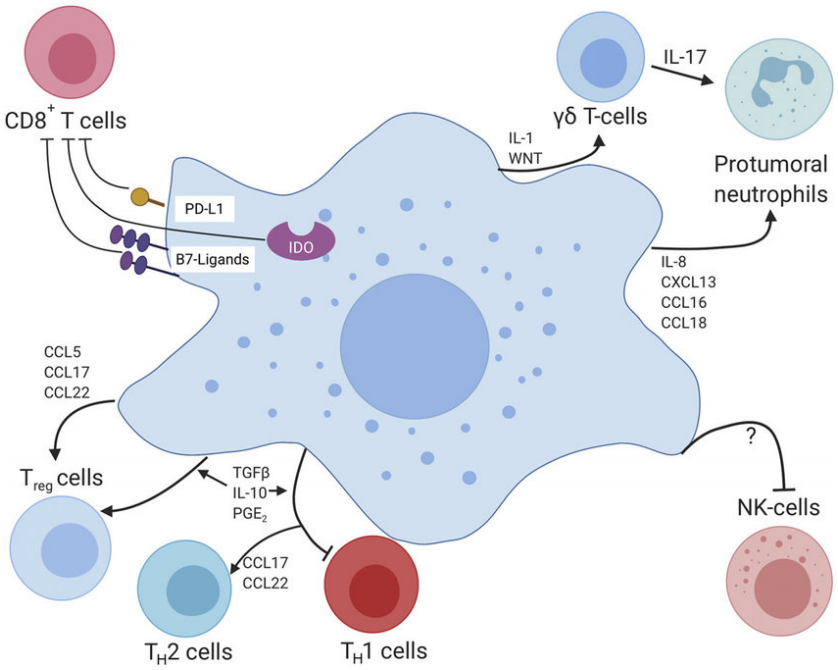


Figure 14. Schematic representation of the immunosuppressive role of TAMs. *Immunosuppressive TAMs express immune checkpoint ligands, which directly inhibit the functions of CTLs. TAMs also promote immunosuppression by recruiting Th2 cells and T reg cells through the production of chemokines (Lopez-Yrigoyen et al. 2021).*

The immunosuppressive effects of TAMs consist of direct interactions with CTLs in an antigen-specific and antigen-non-specific manner or indirect overpressure of effector T cells through Treg expansion (Petty and Yang 2017). Cathepsin K, cyclooxygenase (COX) 2, ARG1 and MMPs secreted by TAMs can directly inhibit the effector function of CD8+ and CD4+ T cells. In addition, these TAM-derived chemokines, cytokines, and enzymes can also stimulate the generation of induced regulatory T cells (iTregs) and recruit natural Tregs

(nTregs), which perform an immunosuppressive function by directly inhibiting effector T cells or secreting immunosuppressive factors. In turn, Treg cells indirectly promote an M2-like phenotype of TAMs and support their survival by suppressing CD8+ T cells in the TME (Liu.C et al. 2019). In addition, some studies suggest that TAMs also induce NK cell dysfunction; in fact, TAM-derived IL-10 inhibits the local production of IL-12, a cytokine essential for NK cell cytotoxicity (Sica et al. 2000). Finally, TAMs further promote dysfunction of TILs by expressing inhibitory receptor ligands of PD- 1 and CTLA- 4 (Kuang et al. 2009). Taken together, this evidence suggests that TAMs are an important force in disrupting antitumor responses by effector cells in the TME and remain a significant obstacle to effective immunotherapy.

1.12. Macrophage metabolism

Macrophage polarization also involves metabolic reprogramming, their metabolism in fact clearly reflects their functions. An M1 macrophage is part of the first line of defense of the innate immune system, which takes place over hours or days, compared with an M2 macrophage, which plays a greater role in the resolution phase and thus has more long-term functions. We now know that the metabolism of M1 macrophages is characterized by high expression of iNOS and NO production, glycolysis, and low oxidative phosphorylation (OXPHOS) while the metabolism of M2 macrophages is characterized by high levels of ARG1, fatty acid oxidation (FAO), and OXPHOS (Fig.15).

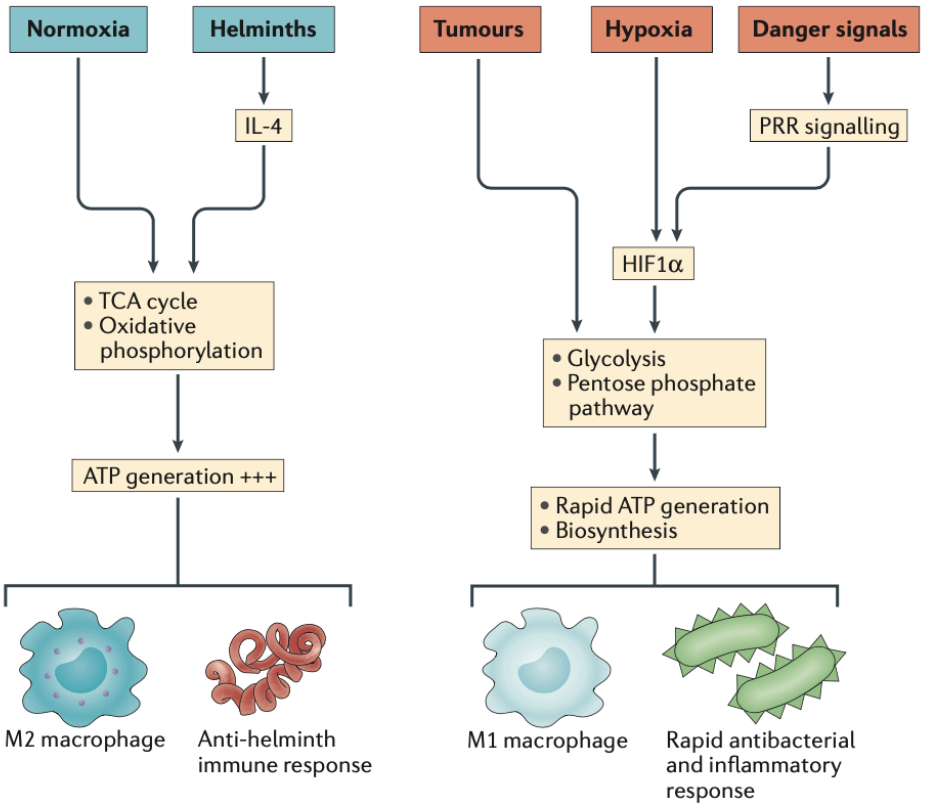


Figure 15. Schematic representation of immunometabolic pathways in macrophages. *Pro-inflammatory macrophages are more glycolytic, reflecting their need to rapidly meet the energy requirements of acute inflammation in the form of ATP. By contrast, anti-inflammatory macrophages utilize fatty acid OXPHOS to slowly but efficiently generate ATP to support the resolution of inflammation (O'Neill et al 2016)*

In M1 macrophages, upon activation, aerobic glycolysis is induced, resulting in increased glucose uptake and conversion of pyruvate to lactate. At the same time, respiratory chain activities are attenuated, allowing the production of reactive oxygen species (ROS). In addition, the pentose phosphate pathway is also induced following classical action. This pathway is critical for the generation of NADPH for NADPH oxidase, which is important for ROS production, but also for nitric oxide synthesis (Aktan et al. 2004). Thus, these metabolic events can provide the cell with energy and fast reducing equivalents, which are necessary for bactericidal activity. A key mechanism for increased glycolysis in the

presence of activated macrophages is the induction of pyruvate kinase isoenzyme (PK) M2. This form of PKM (which is an enzyme that generates pyruvate and ATP from phosphoenolpyruvate and ADP during glycolysis) is regulated to slow down glycolytic flux and allow the detour of glycolytic intermediates to biosynthetic pathways. PKM2 also has a separate function outside of its role in glycolysis. It translocates to the nucleus, where it interacts with Hypoxia Inducible Factor 1 Subunit Alpha (HIF1α) and promotes the expression of HIF1α-dependent genes, including those encoding the glycolytic enzymes and inflammatory factors, such as IL-1β. Also interesting is the observation that a small molecule that forces PKM2 into a tetrameric state (in which it is unable to enter the nucleus and is more active in glycolysis than dimeric PKM2) reprograms macrophages to become more M2-like in their gene expression profiles (Palsson-McDermott et al. 2015). This indicates that inhibition of HIF1α (as will occur in this situation, since PKM2 is no longer nuclear) will change the macrophage phenotype from pro-inflammatory M1 to a pro-reparative (or alternatively activated) M2 phenotype (O'Neill et al. 2016). In M2 macrophages, NO production is low, allowing OXPHOS to be maintained. High ARG1 activity is associated with the metabolism of arginine to proline, a component of collagen (Gordon et al. 2003). Collagen production can stimulate matrix synthesis, which is necessary for tissue repair and granuloma formation, both of which are important for the resolution of inflammation. Unlike M1 macrophages, M2 macrophages do not show increased glycolytic activity (Galvan-Pena and O'Neill 2014). Following alternative activation, the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) 1 gene is expressed instead of PFKFB3, resulting in higher levels of the hepatic isoform of Phosphofructokinase-2 (PFK2) and lower levels of fructose-2,6-bisphosphate. The lower glycolytic levels are compensated by increased OXPHOS. Following macrophage activation with IL4, an oxidative metabolic program is induced, ranging from fatty acid uptake and oxidation to OXPHOS and mitochondrial respiration. The mechanism underlying this increase is

somewhat better understood than that of glycolysis in M1 macrophages. Following treatment with IL4, the transcription factor STAT6, which is responsible for mediating the transcriptional responses of this cytokine, is activated. Active STAT6 can induce PPARγ-coactivator-1β (PGC-1β). PGC-1β can induce mitochondrial respiration and mitochondrial biogenesis. Moreover, together with the transcription factors, nuclear respiratory factor 1 (NRF-1) and estrogen-related receptor-α (ERRα), it drives the production of key mitochondrial components, such as cytochrome c and ATP synthase (St-Pierre et al. 2003). It is therefore not surprising that PGC-1β is considered the key player responsible for metabolic change in M2 macrophages. The Krebs o tricarboxylic acid (TCA) cycle and OXPHOS have been extensively studied in immune cells. In M2 macrophages, there is an intact TCA cycle that is coupled to OXPHOS. This allows the generation of UDP-GlcNAc intermediates, which are required for glycosylation of M2-associated receptors, such as the mannose receptor (Jha et al. 2015). However, in M1 macrophages the situation is quite different. In these cells, the TCA cycle has been shown to be interrupted at two points: after citrate (due to decreased expression of isocitrate lyase) and after succinate (Tannahill et al. 2013). It has been shown that citrate that accumulates in M1 macrophages is exported from mitochondria via the citrate transporter. It is then used to produce fatty acids, which in turn are used for membrane biogenesis. The succinate that accumulates in M1 macrophages because of TCA cycle disruption has a direct impact on cytokine production by macrophages (Tannahill et al. 2013). One mechanism involved is the inhibition of prolyl hydroxylases by succinate, which leads to stabilization of HIF1α and sustained production of IL-1β. This pathway functions in both normoxia and hypoxia and thus represents a mechanism for HIF1α activation under aerobic conditions (O'Neill et al 2016).

1.13. TAMs metabolism

The homeostasis and evolution of the TME are governed by an intimate exchange within and between all cellular compartments, including malignant, endothelial, stromal, and immune cells. This complex interaction often involves extracellular metabolites, which not only provide a source of energy but also act as communication signals between different cellular compartments. TAMs react with metabolic changes in response to stimuli in the TME and actively engage in metabolic crosstalk with tumor (and other) cells, which often promotes cancer progression (Vitale et al. 2019) (Fig. 16).

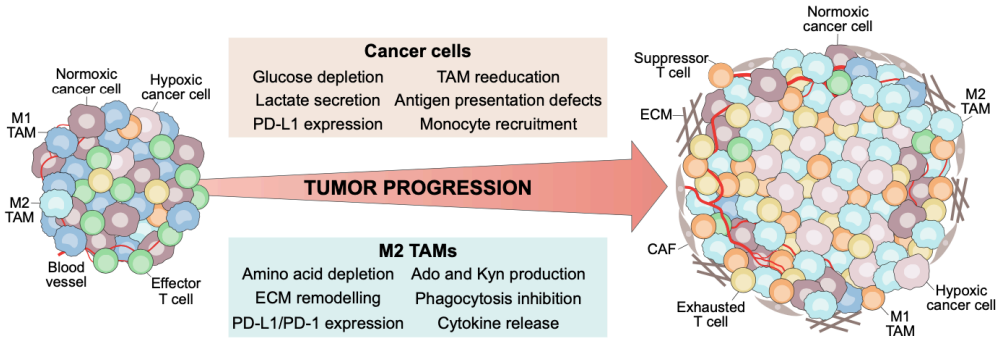


Figure 16. Schematic representation of TAMs and cancer cells crosstalk. *Early neoplastic lesions exhibit limited degree of hypoxia, abundant infiltration by effector T cells, and a TAM compartment largely polarized toward an immunostimulatory M1-like state. As disease progresses, cancer cells avidly deplete the TME of glucose as they produce increased amounts of lactate and secrete cytokines that favor the recruitment of blood-borne monocytes and their polarization toward an immunosuppressive M2-like state (Vitale et al 2019).*

A major metabolic pathway in macrophages that has been shown to influence tumor growth is amino acid metabolism, and as mentioned above, protumoral TAMs highly express ARG1 (Murray et al. 2014). Tumor-associated myeloid cells express cationic amino acid transporters 1 and 2B at higher levels than non-

tumor-associated myeloid cells, leading to increased uptake of arginine and its depletion in the TME (Geiger et al 2016). This phenomenon leads to three results. First, ARG1 converts arginine to ornithine and urea, inhibiting tumor NO synthesis. Second, arginine is metabolized into ornithine and polyamines, which promote tumor growth. Myeloid-specific deletion of ODC, a rate-limiting factor in the polyamine biosynthesis pathway, also leads to increased production of M1-associated cytokines, including TNFα, IL-1β, IFNγ, and NOS2, resulting in reduced tumor burden and improved survival in carcinogenesis. Third, depletion of arginine from TME suppresses the antitumor activity of T cells (Singh et al. 2018). Tryptophan metabolism is also involved in the modulation of antitumor immune responses. The enzyme Indoleamine 2,3-dioxygenase (IDO) performs the first step of the kynurenine pathway, which converts tryptophan to N-formyl kynurenine. In the context of tumors, this has been linked to T-cell suppression through depletion of tryptophan from TME and promotion of regulatory T-cell responses (Campesato et al. 2020). However, tumors overexpressing IDO also recruit more TAMs into the TME, where they express CD206 and high levels of TGFβ, but low levels of NOS2, CD86, and IL-12, characteristic of protumorigenic macrophages (Campesato et al. 2020). Tumor cells and TAMs also engage in metabolic crosstalk (Fig. 14). Cancer cells are typically highly glycolytic due to exclusive expression of the M2 isoform of PKM2 (Christofk et al. 2008), which can lead to competition for nutrients with other glycolytic cells in the TME (Chang et al. 2015). The product of cancer cell glycolysis is lactate, which promotes VEGF and ARG1 production in TAMs to support vascularization and tumor proliferation. Lactate metabolism is particularly relevant not only because of the metabolic symbiosis between hypoxic (lactate-generating) and normoxic (lactate-importing) tumor cells (Allen et al. 2016), but also because of the ability of hypoxic tumor cells to re-educate TAMs toward a poorly glycolytic M2 profile, with increased FAO, decreased antigen-presenting capacity (Liu.N et al. 2019) and, at least in glioblastoma, increased expression of immunosuppressive molecules (Kren et

al. 2010). The M2 bias of melanoma-associated TAMs appears to be promoted by a mechanism involving a G-protein-coupled receptor (GPCR) that senses TME acidification induced by increased glycolysis of cancer cells (Bohn et al. 2018). Based on these observations, the development of treatments that target key metabolic enzymes could have important clinical benefits (Fig. 17).

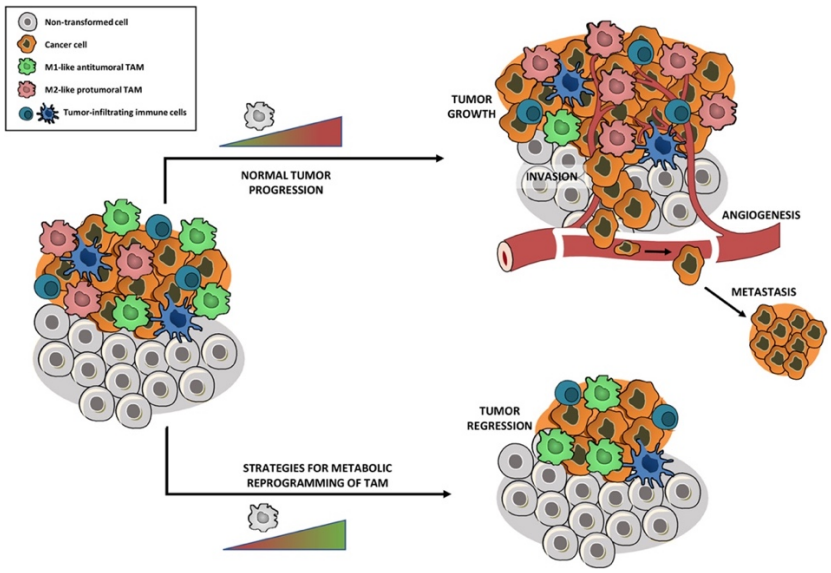


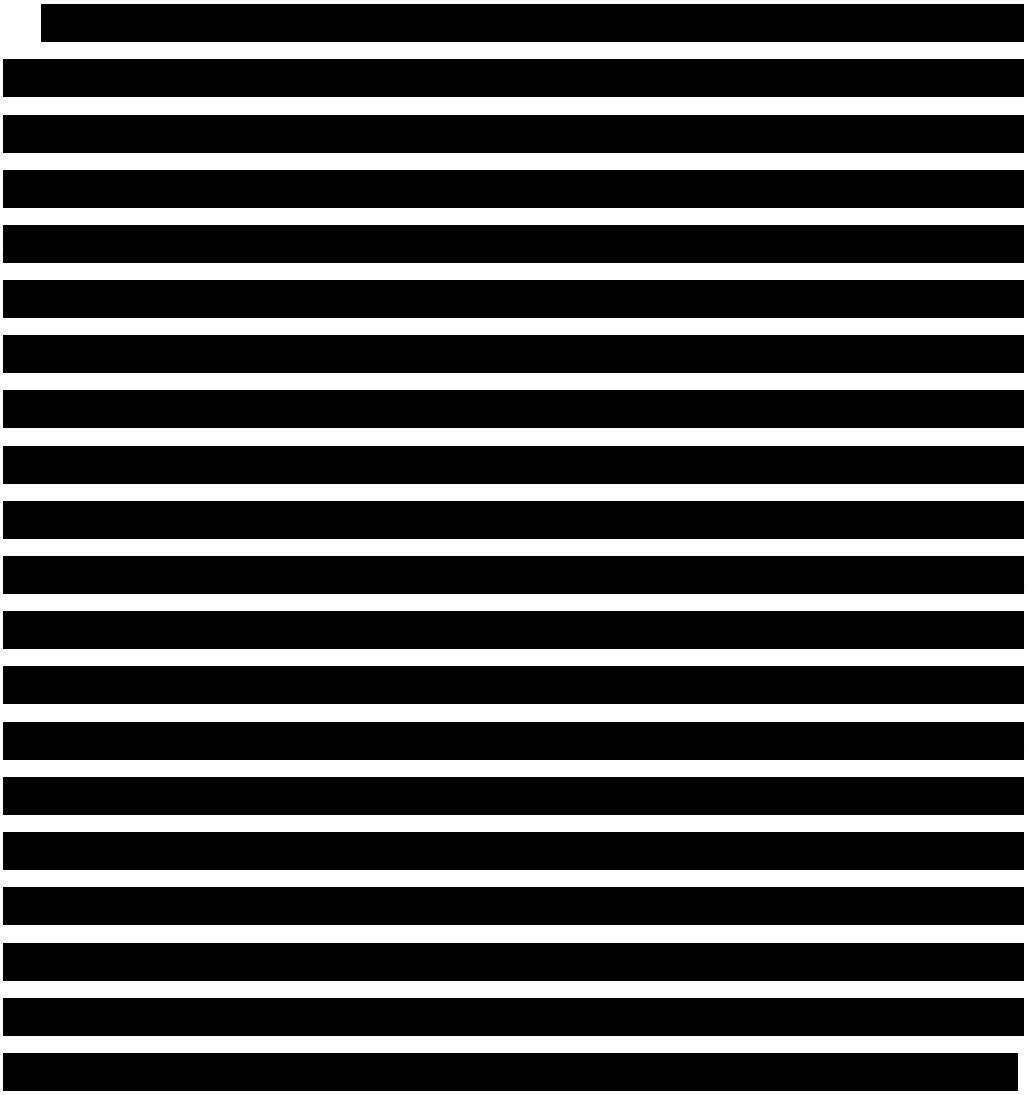
Figure 17. Schematic representation of metabolic reprogramming of TAMs toward an antitumoral phenotype*. Strategies that metabolically reprogram protumoral M2 TAM into an antitumoral M1 phenotype, without depleting the full TAM population, could reduce tumor growth and metastasis and allow re-establishment of conventional cancer therapies (Geeraerts et al. 2017).*

A prototypical inhibitor of mTOR, rapamycin, repolarizes protumoral macrophages towards an antitumoral phenotype by suppressing mitochondrial ROS and NLRP3 inflammasomes, suggesting that targeting upper stream factor of glucose would be beneficial for antitumoral responses in TAMs (Aslam et al. 2017). As expected, the glucose-lowering drug, metformin, reduces M2

polarization in TAMs in murine pancreatic tumors and osteosarcoma tumor models and reduces IL-1β production (Uehara et al. 2019).

A study by the group of Mazzone revealed that TAM metabolism directly affects tumor vasculature and metastasis, making the link between TAM metabolism and its protumoral functionality. Regulated in development and DNA damage responses 1 (REDD1), an inhibitor of mTOR, is highly expressed by TAM in the hypoxic regions of the tumor, which have been described previously as more M2-like macrophages with high angiogenic potential. Genetic deletion of REDD1 in hypoxic TAM induced mTOR activity, which in turn increased glucose uptake and directed hypoxic macrophage metabolism toward glycolysis. Enhanced glycolysis upon REDD1 deletion caused competition for glucose between hypoxic TAM and tumor endothelial cells (Wenes et al. 2016). Together, these observations suggest that altering the metabolic programs of TAMs could be a useful strategy for repolarizing macrophages to promote antitumor effector functions.

2. AIM OF THE STUDY



3. MATERIALS AND METHODS

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4. RESULTS

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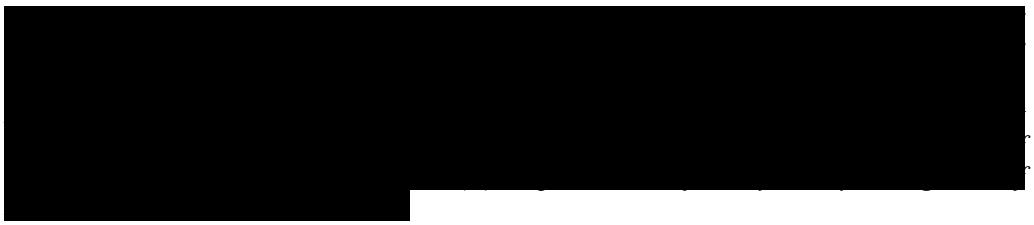

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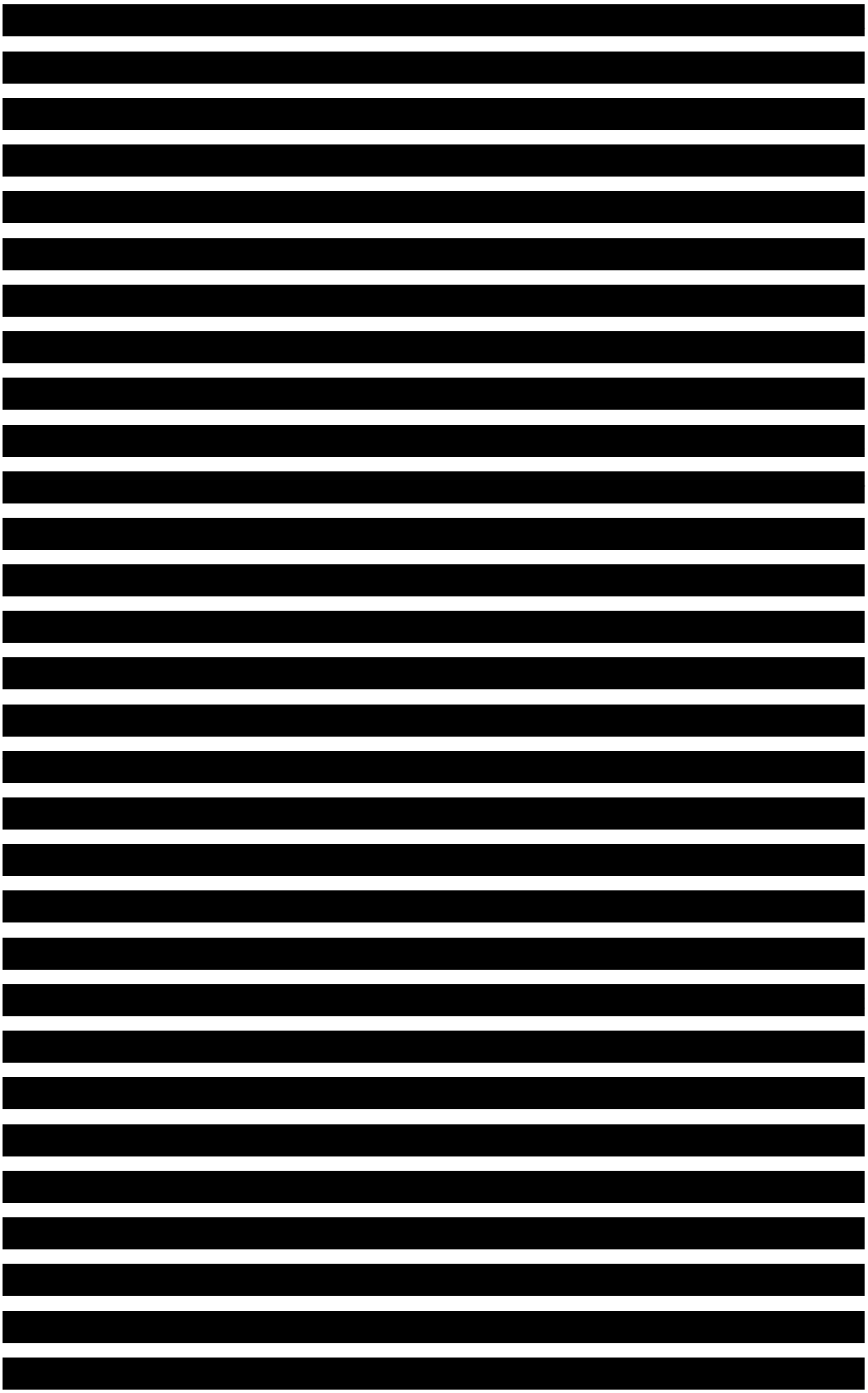

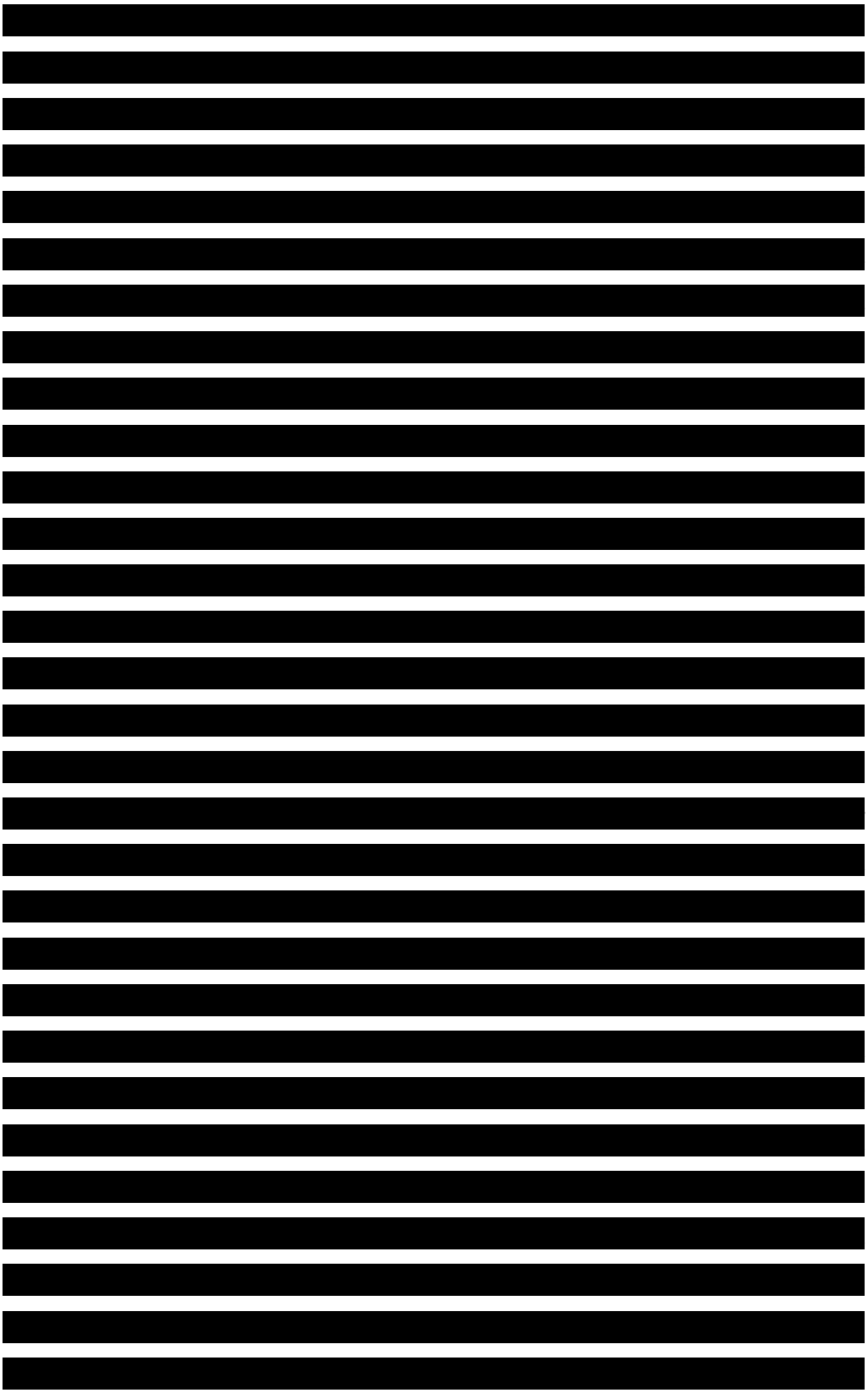

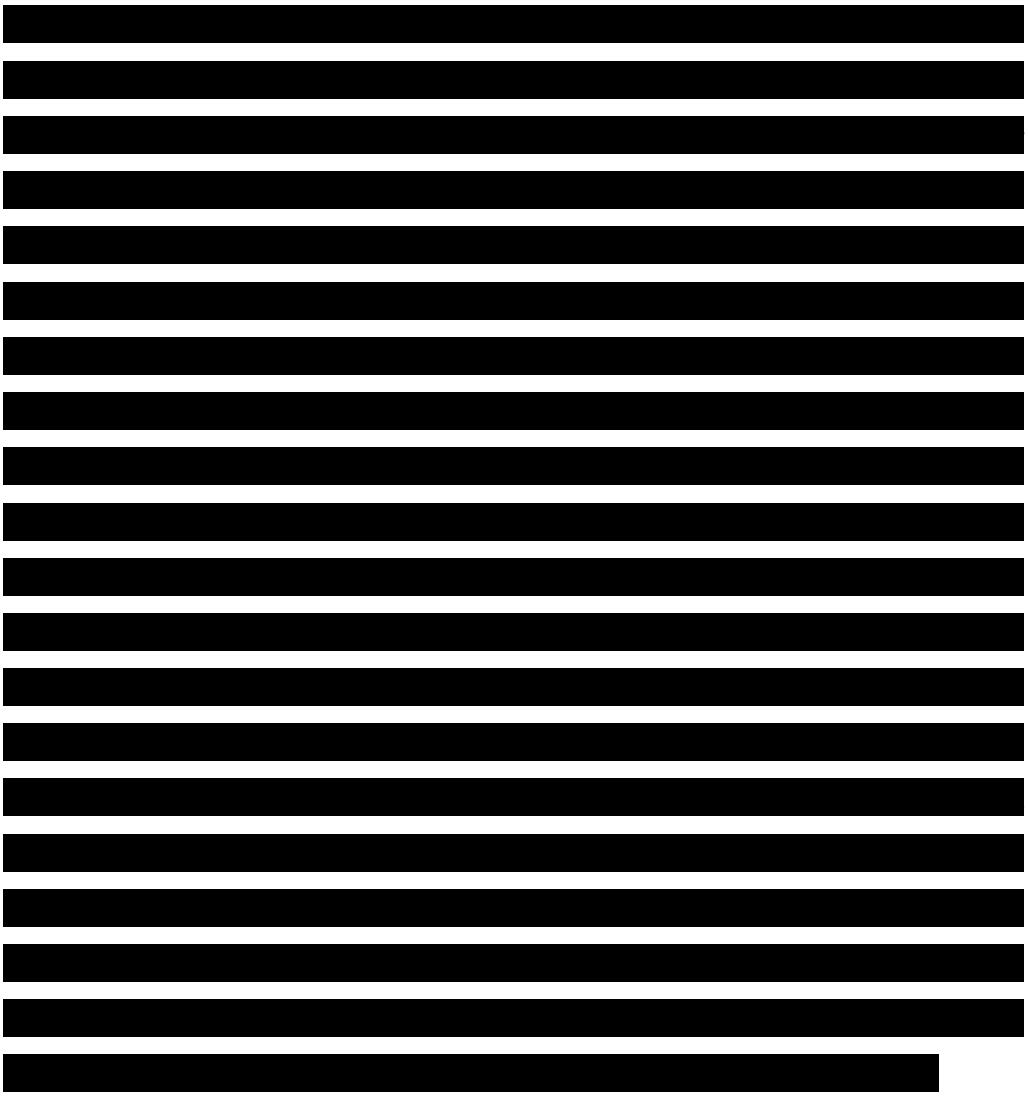

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8. LIST OF PUBLICATIONS

1. Tufano, M *****., **Marrone, L ***., D'Ambrosio, C. *et al.* FKBP51 plays an essential role in Akt ubiquitination that requires Hsp90 and PHLPP. *Cell Death Dis* **14**, 116 (2023). https://doi.org/10.1038/s41419-023-05629-y ***Co-first author**
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3. Yin M, **Marrone L**, Peace CG, O'Neill LAJ. NLRP3, the inflammasome and COVID-19 infection. QJM. 2023 Jan 20: hcad011. doi: 10.1093/qjmed/hcad011. Epub ahead of print. PMID: 36661317.
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