Universidade de Aveiro
2020

CAROLINA FERREIRA NETO

MODULATION OF MICRORNAS EXPRESSION FOR TAM PHENOTYPE REPROGRAMMING AS A CANCER IMMUNOTHERAPY APPROACH

MODULAÇÃO DA EXPRESSÃO DE MICRORNA'S PARA REPROGRAMAR O FENÓTIPO DOS MACRÓFAGOS COMO IMUNOTERAPIA ANTI-CANCRO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Rui Medeiros, coordenador do grupo de Oncologia Molecular e Patologia Viral do IPO do Porto e sob a coorientação da Doutora Maria Adelaide Almeida, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à minha avó.

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palavras-chave oncologia, polarização, modulação, cancro, pós-transcrição, ativação

resumo

Os macrófagos desempenham um papel importante na progressão tumoral e metástase devido à plasticidade demonstrada durante a sua ativação, sendo especialmente percetível *in vivo*. Dependendo dos sinais presentes no microambiente tumoral (TME), os macrófagos podem ter um fenótipo pró-inflamatório, conhecidos como macrófagos M1, ou um fenótipo anti-inflamatório, conhecidos como macrófagos M2. Os macrófagos M1 estão associados à inflamação comum nos estágios iniciais da tumorigénese. Posteriormente, os sinais do TME podem polarizar macrófagos em direção a um fenótipo semelhante ao dos macrófagos M2, denominados como macrófagos associados ao tumor (TAMs), uma resposta pró-tumor, que leva à progressão tumoral, angiogénese e metástase. A presença de TAMs no TME está normalmente associada a um prognóstico negativo para pacientes oncológicos.

O estado de ativação e, consequentemente, as funções dos macrófagos são estritamente reguladas por microRNAs (miRNAs). Os miRNAs regulam a expressão génica após a transcrição e modulam muitos aspetos das respostas imunes, como a diferenciação, proliferação, função das células, e também, libertação de citocinas. Os miRNAs podem atuar como oncogenes ou como supressores de tumor, afetando a polarização dos macrófagos. Existem miRNAs envolvidos tanto na promoção quanto na inibição de fenótipos dos macrófagos, além de existirem miRNAs envolvidos na diferenciação celular. Alterações na expressão de miRNAs podem conduzir a oncogénese, progressão tumoral e metástase, contribuindo para a geração de TAMs.

Os TAMs em conjunto com miRNAs são ótimos candidatos terapêuticos. A regulação terapêutica dos níveis de miRNA pode estar ligada ao tratamento do cancro e à regulação da resposta imune. A reativação de genes pró-inflamatórios nos TAMs pode ajudar a promover a rejeição do tumor.

Este projeto tem como objetivo compilar uma revisão da literatura sobre a modulação de miRNAs de encontro à imunoterapia contra o cancro, mas também avaliar o efeito de compostos na expressão de miRNAs selecionados, com foco na reprogramação do fenótipo dos TAMs.

keywords

oncology, polarization, modulation, cancer, posttranscriptional, activation

abstract

Macrophages play an important role in tumour progression and metastasis due to the plasticity expressed during activation, especially noticeable *in vivo*. Depending on the signals present in the tumour microenvironment (TME), macrophages can have a pro-inflammatory phenotype, known as M1 macrophages, or an anti-inflammatory phenotype, known as M2 macrophages. M1 macrophages are associated with the cancer-related inflammation common in the early stages of tumorigenesis. Later, signals from the TME can polarize macrophages towards an M2-like phenotype, referred as tumour-associated macrophages (TAMs), a pro-tumour response, which leads to tumour progression, angiogenesis, and metastasis. The presence of TAMs in the TME is normally linked to a poor prognosis for cancer patients.

The activation state and, consequently, the functions of macrophages are tightly regulated by microRNAs (miRNAs). miRNAs regulate gene expression after transcription and modulate many aspects of the immune responses such as differentiation, proliferation, cells' function, but also, cytokine responses. miRNAs can act either as oncogenes or as tumour suppressors, by affecting the polarization of macrophages. There are miRNAs involved both in the promotion or inhibition of macrophage activated phenotypes, besides miRNAs involved in differentiation. Alterations in miRNA expression can drive oncogenesis, tumour progression and metastasis, by contributing to the generation of TAMs.

TAMs in conjunction with miRNAs are great therapeutic candidates. Therapeutically regulating miRNA levels may be linked to cancer treatment and to the regulation of the immune response. Reactivating pro-inflammatory genes in TAMs might help to promote tumour rejection.

This project aims to compile a review of the literature concerning miRNA's modulation towards cancer immunotherapy, but also to assess the effect of anticancer drugs in the expression of selected miRNAs, focusing on reprogramming TAMs phenotype.

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List of Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cellular Phagocytosis
Arg	Arginase
ARE	AU-Rich Elements
ASOs	Antisense Oligos
BMDM	Bone-Marrow Derived Macrophages
CCL	Chemokine C-C Motif Ligand
CLIP	Cross-Linked Immunoprecipitation
COX	Cyclooxygenase-2
CSF	Colony Stimulating Factor
CSFR	Colony Stimulating Factor Receptor
CXCL	Chemokine (C-X-C Motif) Ligand
DAMPs	Damage Associated Molecular Patterns
dsDNA	Double Strand DNA
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Erg	Early Growth Response transcriptional factor
ERK	Extracellular-Signal-Regulated Kinase
FACS	Fluorescence-Associated Cell Sorting
FGF	Fibroblast Growth Factor
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
HIF	Hypoxia Inducible Factor
IFN	Interferon

IGF	Insulin-like Growth Factor
IκB	Inhibitor of NF-κB
IL	Interleukin
IL-R	Interleukin-Receptor
iNOS	Inducible Nitric Oxide Synthase
IRAK	IL-1R–Associated Kinase
IRF	Interferon Regulatory Factor
JAK	Janus-Activated Kinase
JNK	c-Jun N-terminal Kinase
LPS	Lipopolysaccharide
MAPKs	Mitogen-Activated Protein Kinases
M-CSF	Macrophage-Colony Stimulating Factor
MDMs	Monocyte Derived Monocytes
MENK	Methionine Enkephalin
MHC	Major Histocompatibility Complex
miRNA	microRNA
MKPs	MAPKs Phosphatases
MMPs	Matrix Metalloproteinases
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer
NOS2	Nitric Oxide Synthetase
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase chain reaction
PDGF	Platelet-Derived Growth Factor

PI3K	Phosphoinositide 3-Kinase
PMs	Peritoneal Macrophages
PPAR	Peroxisome Proliferator-Activated Receptor
pri-miRNA	Primary miRNA
RISC	RNA-Induced Silencing Complexes
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAGE	Serial Analysis of Gene Expression
SOCS	Suppressor of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
TAM	Tumour Associated Macrophage
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
TRAF	TNF Receptor-Associated Factor
TRIF	Toll/IL-1R Domain-Containing Adaptor-Inducing IFN-β
UTR	Untranslated Region
VEGF	Vascular Epithelial Growth Factor

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1. Literature review

1.1. GENERAL KNOWLEDGE OF MACROPHAGES

Macrophages (from the Greek μακρός = makrós = large, φαγεῖν = phagein = to eat) were originally identified by Ilya Metchnikoff in 1882 in infected *Daphnnia magna*. In 1908, Metchnikoff received the Nobel Prize of Physiology and Medicine for their discovery and for describing their phagocytic nature, proposing insights that led to the concept of innate immunity, including the participation of macrophages in the maintenance of tissue integrity and homoeostasis [64,73]. These large cells derive from monocytes and are recruited to tissues by different stimuli. Macrophages are one of the essential components of innate immunity, since the early stages of fetal development, inhibiting or promoting immune cell proliferation and tissue repair [69]. Macrophages work as phagocytes and antigen-presenting cells, promoting inflammation and its resolution, as well as wound healing, displaying great functional diversity.

Besides acting on the fetal and postnatal development, remodelling of several tissues such as brain, bone and mammary gland tissues, macrophages are extremely important after the organism development. In the adult mammal organism, these large cells detect damage, regulate metabolism and angiogenesis through several mechanisms like compounds synthesis [9,55]. Macrophages originate from embryo progenitors or from the bone marrow. Macrophages from embryo progenitors seed growing tissues, having key roles in the removal of dead cells and tissue remodelling, required for the development of rapidly growing and differentiating tissues, during the fetal development. In the adult life, the bone marrow supplies monocytes to seed tissues and inflammatory lesions. Both monocyte-derived macrophages and embryonic-derived macrophages can be activated when given the appropriate cues [70].

Bone marrow or monocyte-derived macrophages originate from a mobile population of monocytes circulating in the peripheral blood able to transmigrate into tissues during inflammation [38,39]. When given the appropriate stimuli, monocytes are recruited from the circulation and differentiate into macrophages [21]. The development of phagocytes like macrophages from monocyte progenitor cells is directed by colony stimulating factors (CSF's), including macrophage colony stimulating factor (M-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF). M-CSF regulates macrophages without altering their activation state, while GM-CSF is involved in the activation of monocytes and macrophages [73].

Macrophages derived from embryonic haematopoiesis colonize different tissues during the fetal development [75]. Major tissue resident macrophages developed during fetal development, including liver, lung alveolar, peritoneal and splenic macrophages, are established prior to birth and their maintenance is independent of the blood replenishment in the adult life [73].Tissue macrophages seeded by progenitor cells from the fetal yolk sac can locally self-renew and can also be replenished by bone marrow-derived monocytes. The location in the

tissues of resident macrophages allows a fast response to infections. Tissue macrophages are essential for the health of specialized parenchymal and stromal cells by maintaining tissue integrity through the repair or replacement of cells and matrices lost through senescence or after injury, by producing growth factors and interleukins (ILs) [63,64].

The innate immune system evolved to protect the host from invading pathogens, allergens, and xenobiotics [73]. Innate immunity plays an important role in host defence to invading microbial pathogens and also in the repair of tissue damage. In this process, the involvement of multiple immune cells is critical. Macrophages are an important component of the innate immune response, with a key role in clearance of foreign microorganisms, and in tissue healing [76]. To overcome the daily dermal insults and injuries there is a complex mechanism for repair [5]. In response to pathogen invasion, tissue injury, physical stress or exposure to irritants, macrophages are activated in order to respond to the insult [9, 69]. Macrophages play an important role in wound repair by regulating many processes, such as removal of dead cells, cellular debris and pathogens, extracellular matrix (ECM) deposition, re-vascularisation and re-epithelialisation [5,9].

In order to perform this wide range of functions, macrophages present phenotype plasticity throughout the different stages of wound healing. As a classification based on the role played in wounds, macrophage phenotypes are divided into two major categories: the M1 phenotype (also known as classically activated macrophages) and the M2 phenotype (also known as alternatively activated macrophages). The term classical activation for M1 macrophages was used by Nathan in 1983 referring to the first activated macrophage population to be formally defined [64,71]. The M1/M2 nomenclature is derived from the cytokines that are associated with these macrophage phenotypes, as these cytokines are linked with T helper 1- and T helper 2-type immune responses, respectively [21]. Since T cells do not recognize pathogens directly, macrophages instruct T cells to what type of response to make [64]. T cells depend on macrophages to present antigens in combination with major histocompatibility class (MHC) I or II [66]. M1 macrophages direct T cells to produce Th1-like cytokines that stimulate specific cytolytic T cells and further activate more M1 macrophages. In contrast, M2 macrophages stimulate T cells to produce Th2-like cytokines that cause B cell proliferation and antibody production, also further amplifying M2 responses. So, macrophages actually use T cells as the intermediator to further amplify M1 or M2 responses [64]. The ability to direct T cells in these different ways allows macrophages to outplay pathogens by stimulating different adaptive responses that are effective and pathogen specific [66].

While M1 macrophages are crucial for the resistance to pathogens and their elimination, M2 macrophages are important for maintaining tissue integrity and reestablishment of homeostasis. In the early stages of wound healing, M1 macrophages are needed to create an inflammatory reaction and phagocytose

the invading bacteria [9]. After ingestion, macrophages destroy pathogens by several antimicrobial mechanisms, including lysosomal degradation, antimicrobial peptides, cytokine-mediated mechanisms and production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), recruit inflammatory immune cells to create a local inflammatory response and present antigens to T cells for initiation of acquired immunity [15]. Although M1 macrophages are initially beneficial by eliminating invading organisms, their response is associated with collateral tissue damage due to the toxic activity of ROS and RNS [9,35]. So, macrophages are re-educated toward an M2 phenotype and begin tissue regeneration. Macrophages switch into the anti-inflammatory phenotype facilitating tissue repair and wound closure. M2 macrophages ingest the subsequent remainings, help restore lost cells and intercellular matrices through the production of growth factors. M2 macrophages act to switch off inflammation, but also to regulate re-vascularisation, fibroblast regeneration and myofibroblast differentiation, as well as collagen production [3, 5,10].

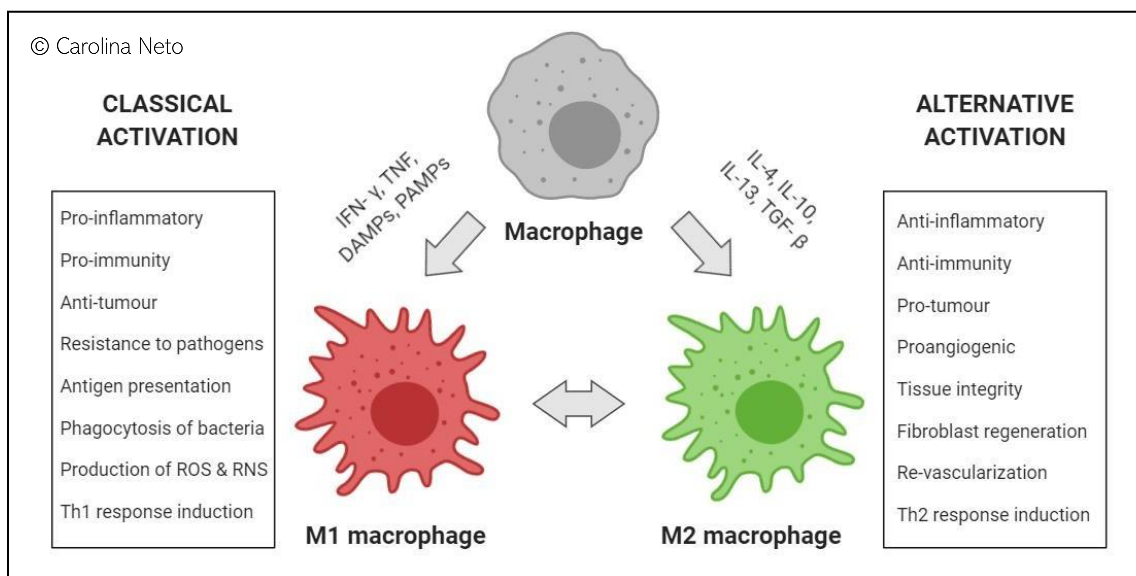

Figure 1 – Dualistic model of macrophage polarization: classical activation resulting in the M1 phenotype and alternative activation resulting in the M2 phenotype, including mediators and key functions *(original illustration adapted from [10, 21,69])*

So, the M1 phenotype correspond to pro-inflammatory and pro-immunity macrophages, while the M2 phenotype correspond to anti-inflammatory, immune suppressive and proangiogenic macrophages. Macrophages have a unique metabolic machinery referred to as plasticity that allows a rapid switch from phenotypes [63]. For instance, M2 macrophages can be activated and adopt an M1 phenotype, and vice-versa. This process, also called re-education or repolarization, is crucial *in vivo* where the cells need to be plastic and adapt to an ever-changing environment. Macrophage polarization is driven by signals in the

tissue microenvironment, which include cytokines, growth factors and pathogen-associated molecular patterns (PAMPs). These signals are thought to dictate a transcriptional response that shapes the phenotype and function of the macrophages based on the physiological or pathophysiological context [21].

The repair response of the body to injury is highly complex, consisting of three stages: inflammation phase, proliferation phase and remodelling phase, involving complex interactions between immune cells and ECM components, as well as other mediators that help orchestrate this mechanism [5]. The phenomenon of the repair response is regulated by macrophages, due to their dual pro- and anti-inflammatory action [73].

In the beginning of tissue injury, blood loss is stopped thanks to the formation of a blood clot containing thrombocytes, erythrocytes, leukocytes, and fibrin fibers. Immune cells are attracted by soluble factors released from thrombocytes and from the injured tissue. Within hours neutrophils are the first responders to reach the injured tissue and eliminate potential pathogens. Neutrophil numbers peak 1–2 days after injury, emitting many mediators that attract other immune cells to the site, before apoptosing. Following this stimulation, monocytes migrate to the wound, differentiating into macrophages or tissue resident macrophages are activated in order to fulfil their roles [5]. During the inflammation phase, most of macrophages have an M1 phenotype, in order to recognize and eradicate invading agents [76]. Inflammation is an important component of the immune response, that limits responses to cellular or tissue damage [74]. M1 macrophages secrete pro-inflammatory mediators, such as cytokines, chemokines, and others like ROS, RNS and nitric oxide (NO) [5]. NO is not only cytotoxic, but it generates many downstream toxic metabolites that constitute the M1 killing machinery. Rapid killing is important since pathogens proliferate fast unless killed by immune responses [10]. The production of NO, a gas that freely diffuses in all directions and across membranes, kills whatever is nearby thus causing collateral damage to normal tissue [64,66]. Apoptosed neutrophils and remaining pathogens are removed from the site by phagocytosis. During the proliferation phase, macrophages stimulate fibroblasts, keratinocytes, and endothelial cells to differentiate, proliferate and migrate, leading to the deposition of new ECM, re-epithelialisation, and revascularisation of the damaged tissue. As the wound matures, 5 days post injury, a small percentage of macrophages have an M1 phenotype and the wound is primarily populated by M2 macrophages. M2 macrophages dominate the wound, along with high levels of anti-inflammatory cytokines and growth factors. In the remodelling phase, M2 macrophages release enzymes that alter the composition of the ECM [5]. M2 macrophages are responsible for induction of angiogenesis and decreased apoptosis that induces the ECM remodelling but also the process of regeneration. M2 macrophages support cell proliferation to alleviate the hypoxia caused by the inflammatory tissue insult. The growth factors released from these macrophages stimulate the differentiation of fibroblast into myofibroblasts, facilitating the contraction and closure of the wound along with the synthesis of ECM components. M2 macrophages also promote the proliferation of many neighbour

parenchymal and stromal cells, attracting stem cells and progenitor cells to help the wound repair process. The impairment of macrophages at any stage of the repair response may lead to poor wound healing [73].

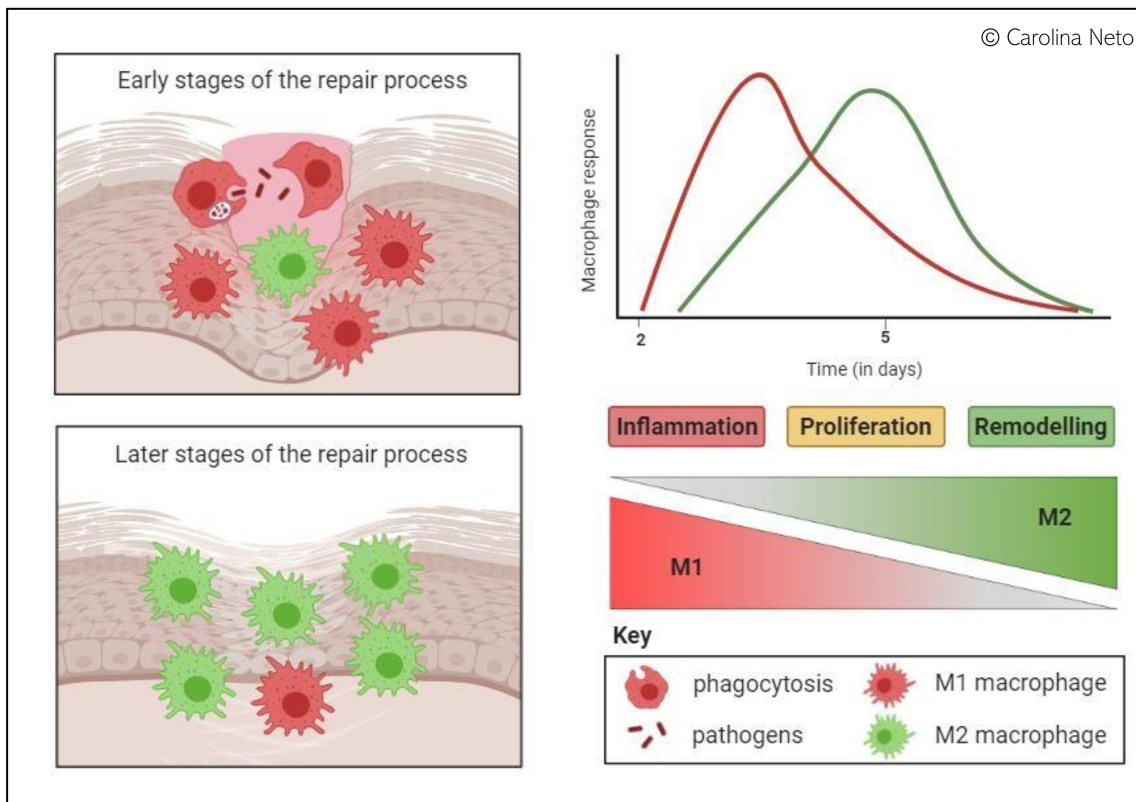

Figure 2 - Ratio of M1 and M2 macrophages throughout the stages of repair response to injuries, referred as inflammation, proliferation, and remodelling phases *(original illustration adapted from [5, 10])*

Macrophages regulate wound closure and scar formation, performing different functions at different stages of the repair process [5]. Macrophage activation occurs to maintain the homeostasis or during different pathological conditions. Macrophage polarization is regulated by epigenetic, external stimuli and tissue environment. So, the activation of macrophages is not a simple process due to the integration of multiple signals [73]. Studies conducted by Lucas et al, in 2010, show that the loss of macrophages during the later stages of the repair process has no impact on tissue maturation like scar formation, but the removal of macrophages in the proliferation phase results in haemorrhaging wounds. M2 macrophages are found to be required for vascular stability through their production of vascular epithelial growth factor-A (VEGF-A) and transforming growth factor-β (TGF-β) [5]. In 2020, Bhat related that the overactivation of M2 macrophages during the wound repair process leads to the development of fibrosis, causing ultimate organ failure and death [73]. Timed macrophage modulation considering the different phases of the repair process might be a good target for immune responses manipulation in therapeutical approaches [5].

1.1.1. M1 CLASSICAL ACTIVATED MACROPHAGES

The M1 phenotype of macrophages is linked to resistance against intracellular pathogens, including infection with *Listeria monocytogenes, Salmonella typhi, Salmonella typhimurium,* as well as the early phases of infection with *Mycobacterium tuberculosis, Mycobacterium ulcerans,* and *Mycobacterium avium [65].*

The classical activation of macrophages occurs following an injury or infection by agents such as microbial products or pro-inflammatory cytokines including bacterial lipopolysaccharides (LPS), interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α) [69].

LPS is a glycolipid component of the outer membrane in gram-negative bacteria [61]. Proteins encoded by LPS-induced genes, such as cytokines and chemokines, are intended to initiate microbial clearance [48].

IFN-γ, a cytokine secreted by activated CD4 T/CD8 T cells and Natural Killer (NK) cells, is required for classical activation, inducing high levels of pro-inflammatory cytokines and intensifying the ability of macrophages to kill pathogens [13,15]. The priming effect of IFN-γ induce the secretion of higher level of pro-inflammatory cytokines and lower level of anti-inflammatory cytokines, enhancing microbicidal and tumoricidal activity of macrophages [13]. The lack of IFN-γ signalling predisposes individuals to infections by pathogens [14].

TNF-α plays a crucial role in the control of mycobacterial growth in macrophages through the activation of macrophage-mediated mycobactericidal mechanisms and in protective immunity, especially in pathogenic bacteria. The regulation of expression and secretion of TNF is a key factor in host cell immune responses [20].

Pathogen recognition is linked to M1 macrophages, being able to identify PAMPs unique to the microbial world [7]. PAMPs are detected by receptors expressed in immune cells. Pro-inflammatory responses to microbial infections are mediated by binding of PAMPs to evolutionary conserved toll-like receptors (TLRs), which are expressed at high levels on macrophages [22,48]. TLRs bind microbial factors at the cell surface or through endosomes, thus activating cytoplasmic signal transduction pathways that initiate the pro-inflammatory response [28].

Binding of TLRs to their respective ligands initiates a wide range of responses including phagocytosis and production of cytokines, that shape and enhance the inflammatory and adaptive immune responses. After pathogen recognition, TLRs 1, 2, 5, 6, 7, 8 and 9 signal through adaptor proteins of the myeloid differentiation factor 88 (MyD88) family to activate several downstream signal transduction pathways, leading to the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways [16,22,52]. NF-κB is a nuclear transcription factor that regulates the expression of many genes critical for inflammatory responses [46]. MAPKs are a group of highly

conserved serine/threonine protein kinases [59]. Members of the MAPK family, including the extracellular-signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38, play crucial roles in multiple aspects of inflammatory responses. The JNK signalling pathway can be activated by stimuli, such as cytokines, fatty acids, and ROS, being related to the gene expression of pro-inflammatory cytokines. The activation of downstream pathways results in the production of inflammatory cytokines and antimicrobial mediators, such as TNF and NO [16,22]. TLR3 and TLR4 use Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) to control the downstream signalling. TRIF is believed to activate the type I interferon pathway via either C- or N-terminal phosphorylation of interferon regulatory factor 3 (IRF3) [99].

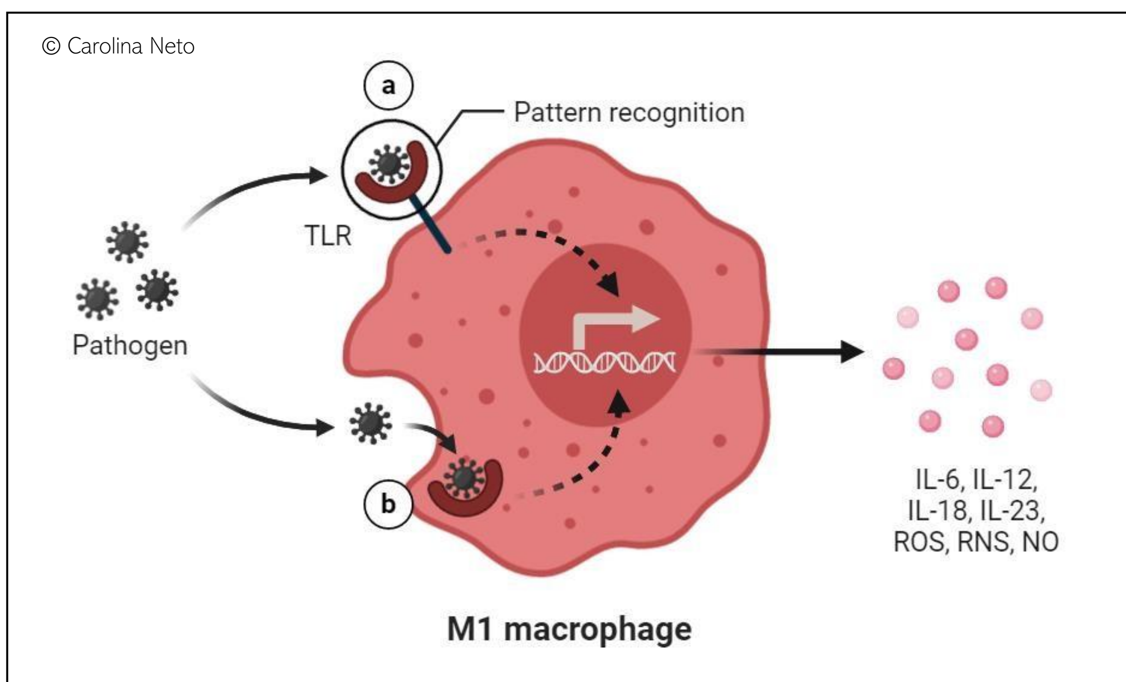

Figure 3 – Pathogen recognition by M1 macrophages through TLR biding to PAMPS at the cell surface (a) and/or through endosomes (b) resulting in the release of inflammatory cytokines and interferons *(original illustration adapted from [100])*

TLRs are expressed on the plasma membrane (TLR1, TLR5, TLR6, and TLR10), in intracellular endosomes (TLR3, TLR7, TLR8, TLR9, and TLR11), or in both compartments (TLR2 and TLR4). TLRs bind to a number of diverse molecular structures, including lipids (TLR4 binds to LPS, while TLR2 binds to lipoproteins), proteins (TLR5 binds to flagellin, while TLR2 and TLR4 both bind to HMGB1), and nucleic acids (TLR3 recognize dsRNA, TLR7 and TLR 8 recognize ssRNA, and TLR9 identify unmethylated CpG motifs in bacterial, viral, and fungal DNA) [100].

Engagement of TLRs with DAMPs activates immune cells to produce pro-inflammatory mediators, such as cytokines and ROS, which are essential to the eradication of microbial infection [24,43,61].

Engagement of TLR4 results in the recruitment of mitochondria to macrophage phagosomes and augments mitochondrial ROS production [35]. Stimulation of TLR4 by LPS triggers the activation of multiple signalling pathways, most importantly the MAPK and NF-κB pathways [46]. The GM-CSF signalling pathway, including janus-activated kinase (JAK), signal transducer and activator of transcription (STAT), MAPK, and phosphoinositide 3-kinase (PI3K) molecules, ultimately acts to trigger NF-κB activation [7]. GM-CSF-induced human macrophages have an M1-like phenotype, with increased expression of pro-inflammatory cytokines and increased ability to activate Th1 cell immune responses [21].

The NF-κB pathway can be activated by various stimuli, such as TNF-α and LPS [74]. Binding of ligands to TLRs activates the transcriptional factors NF-κB and IRF3/7. NF-κB activation upon TLR2 stimulation is mediated by MyD88, while activation of IRF3 in response to TLR3 engagement is dependent on TRIF. TLR4 intracellular domain recruits both MyD88 and TRIF, leading to activation of both NF-κB and IRF3 after LPS stimulation. NF-κB activation results in the expression of numerous pro-inflammatory mediators, including the cytokines TNF- and IL-6. The activation of IRF3 leads to enhanced expression of type I IFN, which activates the STAT family of transcriptional factors and results in STAT-dependent amplification of IFN-associated responses [60]. TLR2 has been reported to signal the presence of bacterial lipoproteins and lipoteichoic acids. A polymorphism in TLR2 in humans has been associated with increased susceptibility to infection with Staphylococcus aureus. These findings demonstrate the role of TLR2 in responses to bacterial lipoproteins. TLR3 has shown to be more responsive to Gram-positive bacteria than to Gram-negative bacteria. Treatment with *E. coli* shows significant changes in the expression of several TLRs and associated molecules. TLR1, TLR6, and TLR9 are down-regulated, whereas TLR3, TLR7 and MyD88 are up-regulated [78].

TLR4 mediates phagocytosis and translocation of Gram-negative bacteria. The MyD88, an essential component of the TLR signalling, is required for TLR-mediated phagocytosis [3]. Stimulation of TLR4 by LPS triggers the recruitment of adaptor protein MyD88, which in turn transmits a series of signalling cascades that lead to the activation of MAPKs. Once activated, MAPKs phosphorylate downstream protein kinases and transcription factors, leading to the production of proinflammatory cytokines, such as TNF-a, IL-1, IL-6, and IL-8. Deactivation of MAPKs is regulated mainly by a family of MAPK phosphatases (MKPs), vital negative regulators in the innate immune system [59]. Polymorphisms in TLR4 are associated LPS hyperresponsiveness showing the importance of intact PAMP recognition systems in human health [78].

TLR5 is a signalling mediator of bacterial flagellin and may play a role in resistance to *Salmonella* infection. Co-transfection of different TLRs may lead to

either enhancement or inhibition of the recognition of specific PAMPs, suggesting that cellular responses to PAMPs are dependent on the total repertoire of TLRs displayed on a cell, necessary cofactors, and the levels of each protein present. Some TLRs act as coreceptors like TLR1 and TLR6 with other TLRs like TLR2 and can promote or inhibit cellular responsiveness to activating ligands [78].

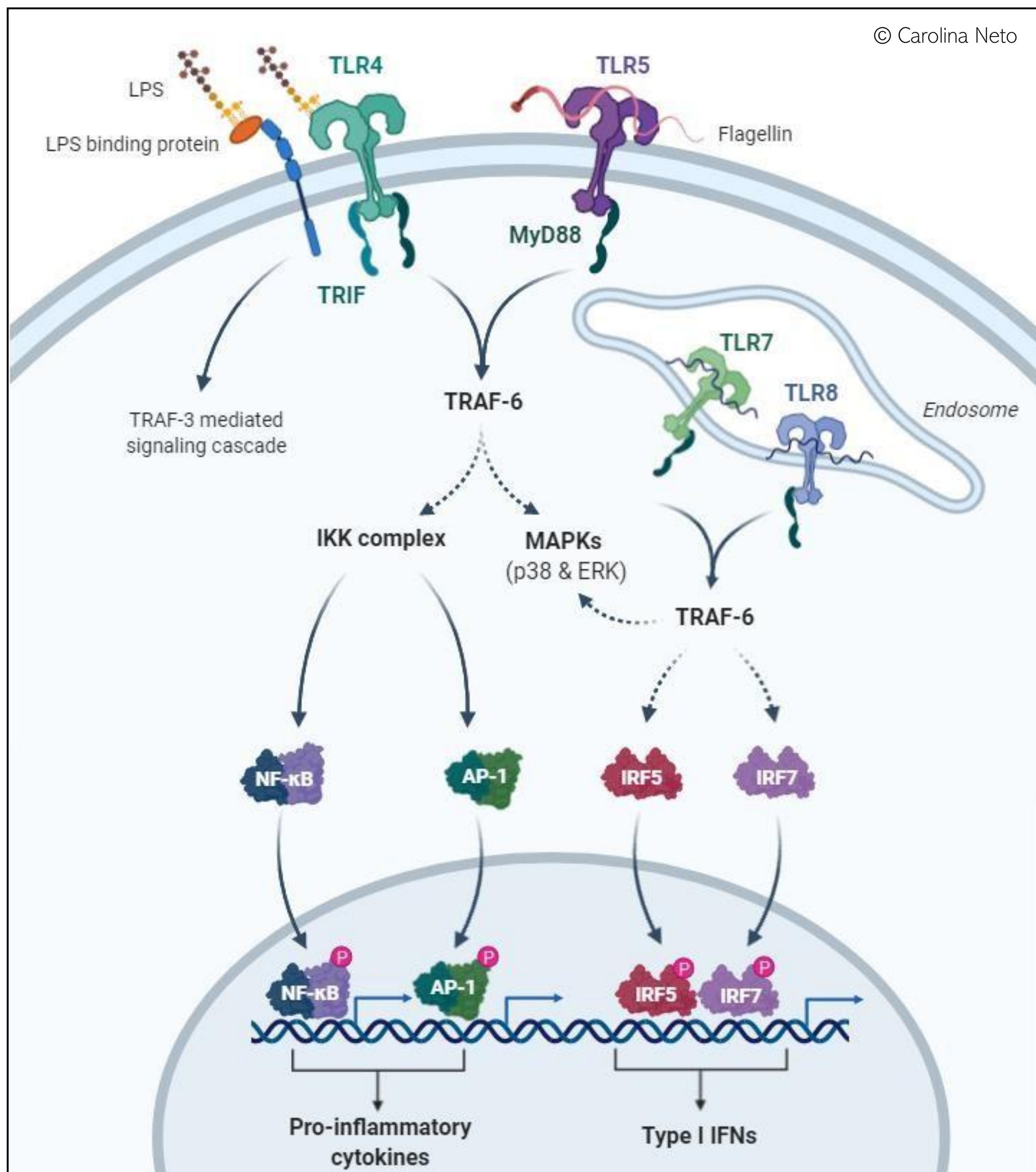

Figure 4 – Signalling cascade of TLR5, TLR5, TLR7 and TLR8, representing the multidirectional pathways of the initiation of the inflammatory response of M1 macrophages *(original illustration adapted from [7, 21, 59, 60, 78])*

TLR 7 activates both NF-κB and IRF7 signalling in macrophages, which in turn leads to IFN-α production. TLR9 activation leads to the activation of the IL-

1R–associated kinase (IRAK)/TNF receptor-associated factor (TRAF) pathway which in turns activated NF-κB signalling [87].

Both components of the TLR1/TLR2 heterodimer contribute to lipopeptide binding at the convex dimerization interface [100]. A key intermediate in TLR1/2/4 signalling is TRAF6, leading to ROS production [99].

Each one of these receptors is specific for certain PAMPs, and therefore different pathogens will be sensed differently. These differences allow for the fine-tuning of the immune response to the type of invading pathogen [85].

M1 macrophages are characterized by the production of pro-inflammatory cytokines and high levels ROS and NO [69]. The M1 phenotype of macrophages is characterized by increased microbicidal activity including the expression of nitric oxide synthase (NOS2), high antigen-presenting activity associated with elevated MHC class II expression and increased production of IL-12 [21]. The action of NOS2 and the production of NO is a powerful and necessary antibacterial defence mechanism against invading organisms. The cytoplasmic expression of NOS2 mediates NO production. NOS2 is induced by IFN-γ or by nonspecific bacterial components like LPS, mediating NO production [15]. NO is generated by inducible nitric oxide synthase (iNOS)-mediated metabolism of amino acid L-arginine. Lipoproteins from the infectious organisms stimulate TLR-dependent transcription of iNOS and subsequent production of NO in macrophages, to kill intracellular invading organisms directly [16]. The production of NOS2 is promoted by IFN-γ-mediated JAK–STAT signalling [21]. Similar to IFN-γ, IFN-β is produced in response to viral and bacterial infections and plays an important role in macrophage activation. A subset of TLR-responsive genes requires IFN-β autocrine/paracrine signalling for their induction [52]. Stimulation of the IFN-γ receptor triggers JAK-mediated tyrosine phosphorylation and subsequent dimerization of STAT1, which binds as a homodimer to *cis* elements known as γ-activated sequences in the promoters of the genes encoding NOS2 and IL-12, among others. A deficiency in STAT1 shows severe defects in immunity to intracellular bacterial and viral pathogens, which is dependent on the IFN response. LPS-activated macrophages show increased expression of MHC class II molecules, NOS2 and IL-12, through the autocrine production of IFN-β, which requires TRIF-dependent signalling from TLR4 to IRF3, inducing the STAT1–STAT2 heterodimer. STAT1 activity is crucial for M1 macrophage polarization in the presence of IFN-γ, which can be derived from innate lymphocytes or TH1 cells [21]. M1 macrophages secrete high levels of inflammatory cytokines, like interleukins IL-12 and IL-23. IL-12 induces the activation and clonal expansion of Th17 cells, which secrete high amounts of IL-17, and thus contribute to inflammation [8]. IRF5 is required for optimal expression of IL-12 and pro-inflammatory cytokines in mice, and thus M1 polarization of macrophages [21].

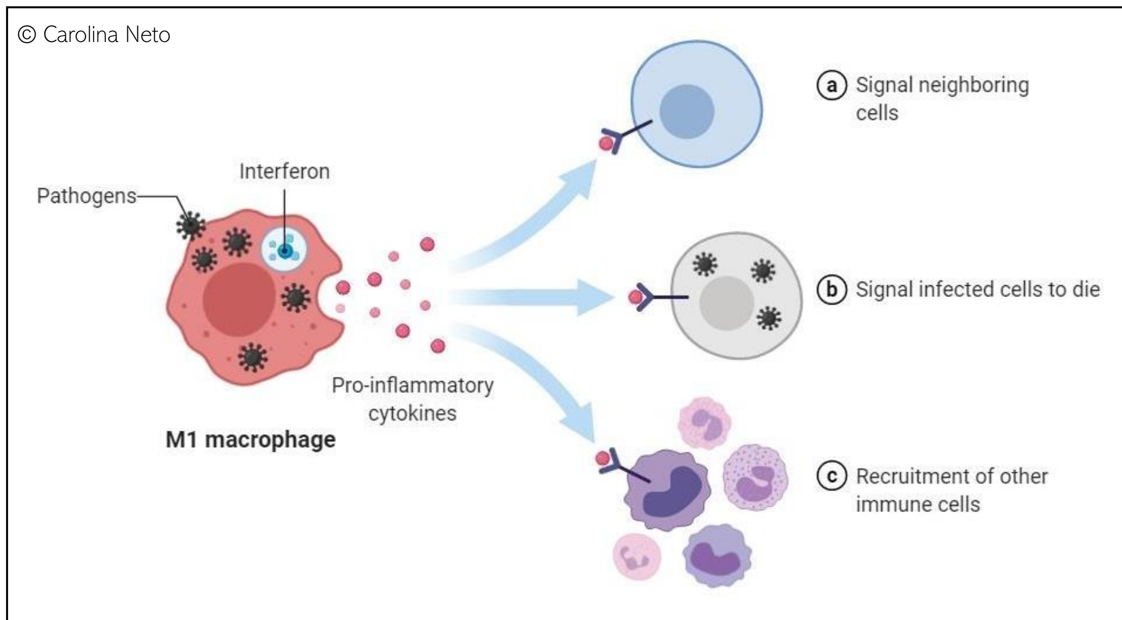

Figure 5 – Interferons induction of high levels of pro-inflammatory cytokines, enhancing microbicidal, pro-immunity and anti-tumour activity of M1 macrophages *(original illustration)*

M1 macrophages secrete pro-inflammatory mediators, such as cytokines (IL-1β, IL-6, IL-12, IL-18, IL-23, TNF), chemokines, like chemokine C-X-C motif ligand 1 (CXCL1), CXCL10, CXCL 11, CXCL 16, chemokine C-C motif ligand 2-5 (CCL2-5) and CCL8-11 and others (ROS, RNS and NO). Pro-inflammatory mediators such IFN-γ, TNF and DAMPs activate M1 macrophages [5]. The M1 phenotype activates the NF-κB and STAT1 signalling pathways, leading to the production of ROS, NO and proinflammatory cytokines, that enhance antimicrobial and anti-tumour immunity and facilitate the initial stages of wound healing. These mediators promote peripheral insulin resistance, necessary for an effective defence against pathogens, since M1 macrophages use glycolysis to fuel their functions in host defence [5,9]. M1 macrophages exhibit increased glycolysis, increased release of lactate, decreased oxygen consumption and glutaminolysis [73]. In response to M1 polarization cues, macrophages display a metabolic shift towards the anaerobic glycolytic pathway, due to the need of a fast microbicidal response and the need to deal with the hypoxic tissue environment. So, an anaerobic process like glycolysis is best-suited to meet the high energy demands [91]. LPS stimulation enhances the levels of succinate, followed by up-regulation of glycolytic genes. Inhibition of glycolysis in macrophages decreases IL-1β production [97].

IL-6 is a key component of the LPS-induced proinflammatory cytokine response during pathogen invasion and helps to induce the innate immune response [27]. IL-6 is secreted by macrophages and T cells to stimulate immune response during infection or trauma. IL-6 is one of the most important inflammatory factors in fever and the acute phase response. The fine tuning of IL-6 expression is crucial since dysregulation of IL-6 has been attributed to many

diseases such as atherosclerosis, diabetes, multiple myeloma, depression, Alzheimer′s Disease, prostate cancer, and rheumatoid arthritis, among others [26].

IRAK has also been implicated in the signal transduction of TLR/IL1R family. IRAK-1 and IRAK-4, along with their two inactive counterparts IRAK-2 and IRAK-M, play important roles in mediating NF-κB and MAPK signalling. After TLR4 activation, TLR4, MyD88, IRAK-4, and IRAK-1 form a complex, that later phosphorylates, subsequently activating TRAF6 and the NF-κB and JNK/p38 pathways. The negative regulators of TLR/IL-1R–mediated signalling include IRAK-2 and IRAK-M. IRAK-M inhibits IRAK-1 dissociation from the receptor complex and blocks LPS-induced IRAK-1–TRAF6 interaction and NF-κB activation. TLRs, along with the MyD88, IRAKs, and p38, facilitate the up-regulation of scavenger receptors, leading to an increase in phagocytosis of bacteria [35].

Macrophages become apoptotic after phagocytosis of extracellular bacteria. The phagocytosis and subsequent apoptosis of macrophages correlate with the decreased capacity for antigen processing and presentation. Macrophage apoptosis diminishes the pro-inflammatory immune response [35].

1.1.2. M2 ALTERNATIVE ACTIVATED MACROPHAGES

Since overly exuberant inflammation can cause collateral tissue damage that leads to organ injury, an anti-inflammatory response is needed [43]. The immune system is equipped with a wide range of negative regulatory mechanisms that restrain excessive inflammatory responses in the activation process of macrophages. The production of anti-inflammatory cytokines regulates the excessive production of pro-inflammatory cytokines [48]. For example, massive stimulation of innate immunity, due to severe Gram-negative bacterial infections, leads to excess production of cytokines and other molecules, causing the development of a fatal syndrome, known as the septic shock [49]. Under physiological conditions, macrophages can become less susceptible to extracellular bacterial stimuli, for example, in re-exposure to LPS; this desensitized state protecting against septic shock is referred to as endotoxin tolerance and results from a loss of MyD88 recruitment to TLR4 and thus NF-κB activity [83].

M2 macrophages are activated by IL-4, IL-13, IL-1 receptor ligands, IL-6 and IL-10, secreting anti-inflammatory cytokines such as IL-10 and chemokines CCL18 and CCL22 [69].

Epithelium-derived alarmins and type 2 cytokines, IL-4 and IL-13, result in the alternatively activated M2 macrophages. This activation culminates in ECM remodelling, wound-healing, angiogenesis, and fibrosis, by expressing TGF-β1, platelet-derived growth factor (PDGF), VEGF, and matrix metalloproteinases (MMPs) that regulate myofibroblast activation and the deposition of ECM

components (like collagen) [9]. PDGF is crucial for fibroblast differentiation and TGF-β is related to epithelial development, while VEGF is need to vasculogenesis and endothelial cell differentiation [92].

Cytokines IL-4 and IL-13 play important roles in the polarization of macrophages towards an M2 phenotype [103]. IL-13 is a key cytokine that plays a central role in some chronic inflammatory diseases such as asthma and ulcerative colitis [55]. Both cytokines bind to IL-4 receptor (IL-4R) and signal through membrane receptor dimers. IL-13 signals exclusively through a heterodimer composed of IL-4Rα and IL-13Rα1, while IL-4 signals through an IL4Rα/IL13Rα1 heterodimer or through an IL-4Rα/γc heterodimer. Engagement of these receptors leads to phosphorylation and activation of JAK proteins, believed to be bound to these cytokine receptors in unstimulated cells. The active phospho-JAK proteins phosphorylate the IL-4R chain, providing docking sites for STAT6. Once bound to the receptor, STAT6 is also phosphorylated by JAKs, which causes its activation, dimerization, and translocation to the nucleus, where it exerts its transcriptional roles. STAT6 is the main mediator in the IL-13/IL-4 signalling pathway, becoming phosphorylated and active upon stimulation with these cytokines [55,103]. STAT6 is required to drive M2 macrophage activation during Th2 cell-mediated immune responses in the presence of IL-4 and/or IL-13. IL-4R signals through a JAK–STAT6 pathway, and many of the genes associated with M2 macrophages are regulated by STAT6, including Arginase 1 (Arg1). IL-4 triggers the activation of other signalling proteins, such as PI3K, which is also crucial for cell responses to IL-4 [21].

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-activated transcription factor belonging to the nuclear receptor superfamily. It plays a pivotal role in the control of lipid metabolism in macrophages and maintenance of energetic homeostasis [86]. PPAR-γ has long been known to inhibit pro-inflammatory gene expression through several mechanisms, including the transrepression of NF-κB. Deficiency in PPAR-γ results in macrophages that are resistant to M2 polarization. PPAR-γ expression can be induced by IL-4 and IL-13 [21]. The nuclear receptors PPAR-γ and PPAR-δ control distinct subsets of genes associated with M2 macrophage activation and oxidative metabolism. Interestingly, STAT6 coordinates and synergizes with PPAR-γ for macrophage activation [65]. For macrophage phenotype reprogramming towards M2 polarization, the activation of PPAR-γ is considered to be critical [86].

TGF-β is a pleiotropic cytokine with important actions in wound healing and tissue remodelling, being pro-fibrotic, pro-angiogenic, and anti-proliferative. TGF-β can also act as an immunosuppressive and anti-inflammatory cytokine. Increase in TGF-β has been associated with the promotion of fibrosis and ECM deposition, being also known to modulate angiogenesis and to be an immunomodulatory cytokine [41]. TGF-β is critical for epithelial-mesenchymal transition and epithelial motility [92]. TGF-β dysregulation leads to pathological deposition of ECM, aberrant remodelling, cell proliferation, and uncontrolled angiogenesis [41].

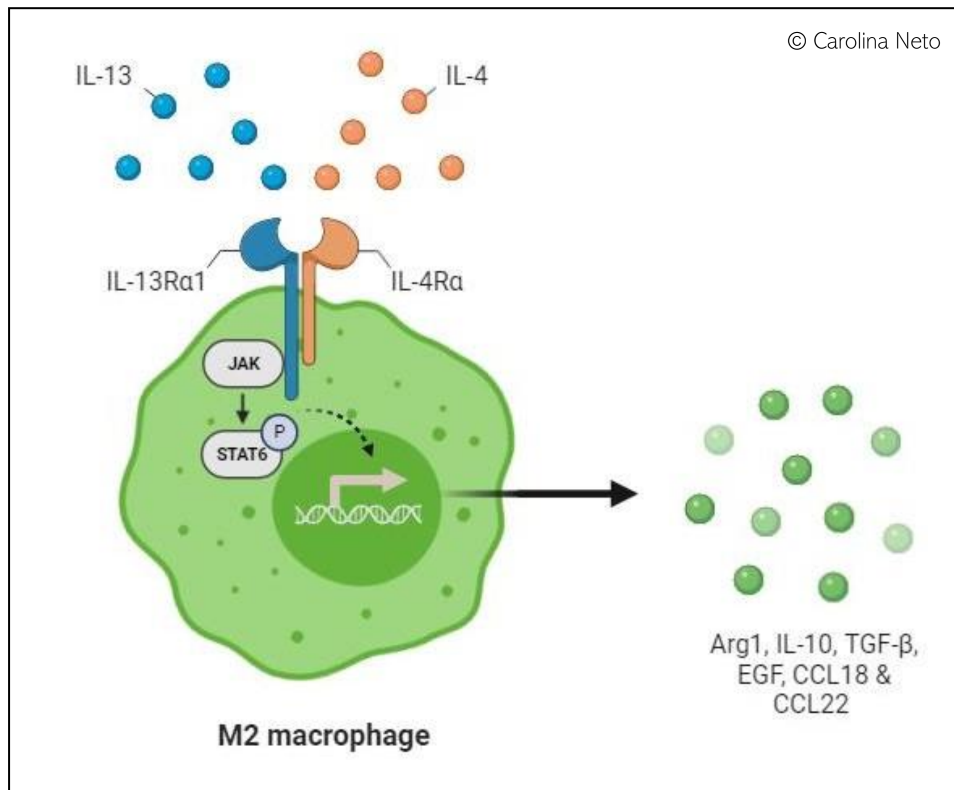

Figure 6 – Activation of the M2 phenotype on macrophages by interleukins IL-4 and IL-13, including signalling pathways and secreted molecules *(original illustration adapted from [21, 55])*

Profibrogenic or M2 macrophages are a critical source of TGF-β, which is a key driver of fibrosis. IL-4 and IL-13 contribute to extensive tissue fibrosis and activate fibroblasts by secreting profibrogenic cytokines. IL-4/IL-13-induced macrophages activate fibroblasts by secreting profibrogenic cytokines. CCL18, a chemokine induced by IL-4/IL-13 induces collagen production by fibroblasts. Although IL-4/IL-13 activates the STAT6 pathway for M2 polarization, these cytokines induce negative feedback to inhibit STAT6 phosphorylation. PPAR-γ which is up-regulated by IL-4/IL-13 and interacts with STAT6 at target gene promoters, is also necessary for M2 activation. PPAR-γ, a primary coordinator of STAT6, acts along with STAT6 to fully induce several hallmark M2 genes. PPAR-γ is down-regulated in M1 macrophages but up-regulated in M2 macrophages [36].

In parasite infections, macrophages generally undergo a dynamic switch towards M2 polarization. IRF4, induced by IL-4, specifically regulates M2 macrophage polarization in response to parasites or fungal cell wall components, and inhibit IRF5-mediated M1 polarization [65].

M2 macrophages express a variety of immunoregulatory proteins, like Arg1 and IL10 that regulate the intensity and duration of the immune responses [9]. Alternatively, activated macrophages are anti-inflammatory and aid in the

process of angiogenesis and tissue repair. Since M2 macrophages are not capable of efficient antigen presentation, they produce large quantities of IL-10 and other anti-inflammatory cytokines. Expression of IL-10 by M2 macrophages promotes a Th2 response, and Th2 cells, in turn, up-regulate the production of IL-3 and IL-4. IL-4 is an important cytokine in the healing process by contributing to the production of the ECM [8].

IL-10 is one of the most important anti-inflammatory mediators that prevents excessive inflammation and autoimmune pathologies [13,43]. This interleukin is crucial for dampening the inflammatory response after pathogen invasion and acts to protect the host from excessive inflammation [50]. IL-10 is one of the most effective suppressors of inflammatory cytokine production induced by TLR, displaying a powerful inhibitory action on innate immune cells, by direct inhibition of cytokine transcription, by destabilizing the coding RNA or by blocking the translation [45,51]. IL-10 mediates an anti-inflammatory effect through the down-regulation of pro-inflammatory genes induced downstream of TLR2 signalling like those encoding IL-1, IL-12, TNF, and IL-6, accomplished through the JAK1-STAT3 pathway. This interleukin mediates lots of immunoregulatory events, such as negative regulation of maturation and activation of macrophages. IL-10 is important to counteract the above pro-inflammatory effects, and functions to moderate TNF-α and IL-1α production, preventing autocrine-induced cell death or endotoxin shock [83]. Dysregulation of IL-10 leads to various immunological diseases, such as ulcerative colitis, Crohn disease, asthma, and also cancer [44,50].

Arg1 is highly expressed by M2 macrophages, catalysing polyamines production, which is necessary for collagen synthesis, cell proliferation, fibrosis and other tissue remodelling functions [91].

Even though IL-33 induces the production of a range of pro-inflammatory cytokines and chemokines and promotes macrophage activation during the development of the repair responses. IL-33 promotes the polarization of alternatively activated macrophages [30].

The inhibitor of transcription factor NF-κB (IκB) α is synthesized by NF-κB to block excessive transcription factor activity after LPS stimulation. The production of anti-inflammatory cytokines is also induced by LPS signalling, including IL-10, which has paracrine effects on neighbour cells to negatively regulate the action of NF-κB [53].

While M1 macrophages obtain energy through aerobic glycolysis, M2 macrophages rely on free fatty acid and oxidative metabolism [64]. M2 macrophages are dependent on fatty acid oxidation and mitochondrial oxidative phosphorylation. So, succinate, a signalling metabolite, regulates the process of activation as well as the process of inflammation by the release of IL-4 [73]. Since M2 macrophages only require a sustained supply of energy, unlike M1 macrophages, it is fulfilled by the oxidative glucose metabolism (fatty acid

oxidation), in order to perform functions like tissue remodelling, tissue repair and wound healing [91].

The transitions between phenotypes are influenced by multiple factors and pathways. An optimum balance between M1 and M2 macrophages is very important for immune regulation as any imbalance can cause dysregulation and disease like cancer. The plasticity of macrophage transition might be attributed to the complexity of the signalling pathways associated with the two phenotypes (for example, the multiple molecules common to both phenotypes) [69].

1.1.3. MACROPHAGES HETEROGENEITY AND PLASTICITY

Heterogeneity and plasticity are hallmarks of activated macrophages. Macrophage polarization refers to a dynamic process whereby macrophages can modify their phenotypes in a plastic manner in response to different stimuli [64]. The most remarkable characteristics of macrophages are their plasticity and heterogeneity, depending on the specific task carried out. This is reflected by their ability to reverse their phenotype and reprogram their gene expression profiles, presenting in between phenotypic profiles and a constituting a heterogeneous population [55].

Macrophages are the most functionally diverse type of leukocyte, not only because of the diverse range of fulfilled roles but also due to the ability to dynamically switch its function in response to changing stimuli. Macrophage plasticity is especially noticeable in the M1 or M2 activation of macrophages, in which the alterations in stimuli results in rapid changes at the level of gene transcription, miRNA regulation, and protein expression [7]. The M1 to M2 switch of macrophage phenotype during the progression of the inflammatory response enables the dual role of macrophages in orchestrating the onset of inflammation and subsequently promoting healing and repair [21]. The M1-M2 switch observed during the transition from acute to chronic infection may provide protection against overwhelming uncontrolled inflammation [65]. The dual functions of macrophages are unique and necessary for the survival of multicellular animals [66]. Thus, polarization is not fixed: macrophages are sufficiently plastic to integrate multiple signals, from microbes, damaged tissues, and the normal tissue environment [70]. The functional properties of macrophages vary depending on their anatomical location (tissue specific) and physiological context [37]. Macrophages are highly plastic cells with polarized populations that differ in terms of effector functions, cell surface receptor expression, and cytokine production. The hight plasticity of macrophages allows them to modify and reprogram their effector functions in response to immune stimuli [75].

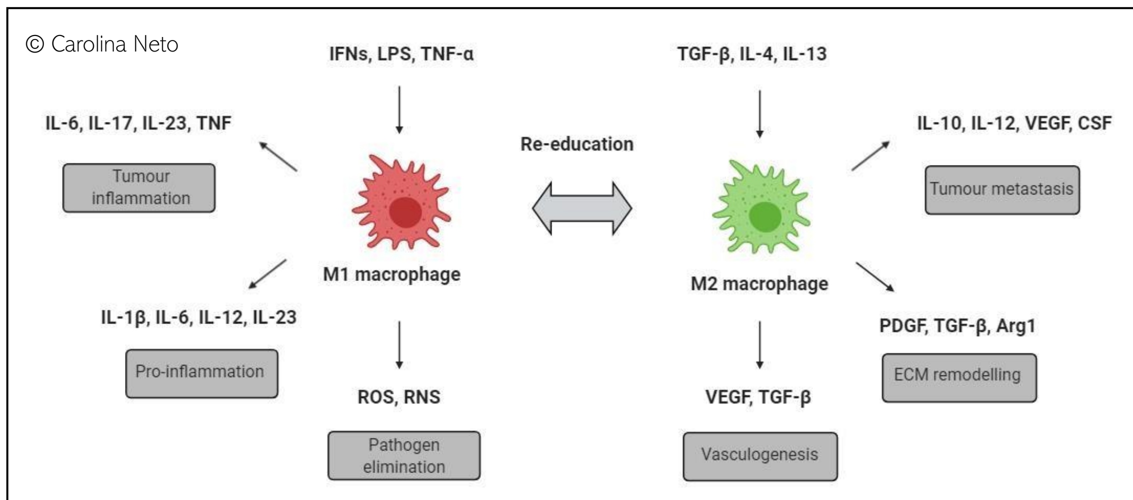

Figure 7 – Macrophage plasticity conferring them the ability to integrate multiple signals, display several functions and different cytokine production *(original illustration adapted from [5, 10, 21])*

However, the initial M1/M2 model proposed only represents two polar ends of a continuum exhibiting pro-inflammatory and anti-inflammatory activities, respectively. These two polar ends do not fully capture the wide functional plasticity and high heterogeneity of macrophage populations, neither take account all the activation states [75]. Different combinations of external stimuli lead to distinct polarized activation patterns, resulting in a spectrum of possible macrophage activation phenotypes (M1, M2a, M2b, M2c and M2d macrophages) [81]. Macrophage heterogeneity results in the acquisition of an array of phenotypes and functional properties in response to different microenvironmental cues, and manifests as a spectrum of different functional states, oversimplified by the dual model of M1 and M2 macrophages [76]. In terms of both phenotype and function, macrophages have remarkable heterogeneity, which reflects the specialization of tissue-resident macrophages in different microenvironments such as liver, brain, and bone. The macrophage lineage includes a remarkable diversity of cells with different functions and functional states that are determined by a complex interplay between microenvironmental signals received from neighbouring cells and intrinsic differentiation pathways [21]. Macrophages are heterogenous in their function as well as in their phenotypes, thanks to a wide range of receptors and an efficient metabolic machinery [73]. Macrophages present a wide range of phenotypic profiles and defence mechanisms depending on the tissue context and the stimuli presented (pathogens, cytokines, apoptotic cells, among others).

Macrophages switch between forms in response to stimuli, a process that involves a complicated and sophisticated mechanism and gene expression regulation. The STAT family, the nuclear receptor PPAR-γ, the IRFs, and the NF-κB family all participate in the regulation of polarization, involving many signalling pathways including JAK/STAT, JNK, PI3K, and Notch, among others [33]. The

STAT pathway has been shown to play roles in the signalling cascades triggered by LPS, IFN and other cytokines [28]. M1 polarization cues such as IFN-γ or LPS induce IRF3, IRF5, IRF7, STAT1 and P50-P65 NF-κβ signalling leading to a proinflammatory response. In contrast, M2 stimuli as IL4, IL10 and IL13 activate IRF4, STAT3, STAT6 and P50-P50 NF-κβ signalling [87]. Molecular pathways regulating the plasticity of macrophages have been an area of intensive research, however there are still unknown or unclear pathways since the phenotypic diversity of macrophages is controlled by a tight regulatory machinery [37].

A network of signalling molecules, transcription factors, epigenetic mechanisms, and posttranscriptional regulators underlies the different phenotypes of macrophages. The balance between activation of STAT1 and STAT3/STAT6 finely regulates macrophage polarization and activity [75]. A predominance of NF-κB and STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and inflammatory functions. In contrast, a predominance of STAT3 and STAT6 activation results in M2 macrophage polarization, associated with immune suppression and tumour progression [65]. IRF/STAT signalling pathways are activated by IFNs and TLR signalling to guide macrophage polarization toward the M1 phenotype via STAT1 or by IL-4 and IL-13 instructing macrophages toward the M2 phenotype via STAT6 [36,65]. The M1 macrophage phenotype is controlled by STAT1 and IRF5, whereas STAT6, IRF4 and PPAR-γ regulate M2 macrophage polarization [21]. M1 macrophages up-regulate IRF5, which is essential for induction of cytokines IL-12, IL-23 and TNF. The IL-4 receptors activate STAT6, which in turn activates transcription of genes typical of M2 polarization. IL-10 activates STAT3-mediated expression of genes associated with the M2 phenotype. STAT-mediated activation of macrophages is regulated by members of the suppressor of cytokine signalling (SOCS) family. IL-4 and IFN-γ upregulate SOCS1 and SOCS3, which in turn inhibit the action of STAT1 and STAT3, respectively [65]. IL-4 induces the M2-polarizing IRF-4 pathway to inhibit IRF5-mediated M1 polarization. IL-10 promotes M2 polarization through the induction of NF-κB and STAT3 activities [75]. STAT3 is activated by cytokines including IL-1β, IL-6, IL-11, IL-10 and IFNs, mediating both anti-inflammatory and proinflammatory responses. For instance, activation of STAT3 promotes IL-6 production, and IL-6 itself can lead to the phosphorylation of STAT3 [28].

In vivo, macrophage phenotypes are not activated by individual cytokines. Instead, the infection itself induces the M1 mode and the absence of pathogens keeps macrophages in M2 mode [10]. *In vitro*, after a few hours following stimulation, M1 macrophages become unable to reactivate pro-inflammatory genes following a secondary stimulation. However, they retain the ability to induce the expression of many other genes, including interleukins. This altered state of responsiveness to secondary stimulation is commonly referred to as endotoxin tolerance and results in a sustained switch from a M1 pro-inflammatory phenotype to an M2 anti-inflammatory phenotype. Endotoxin tolerance is a gene-specific response and a progressive attenuation of pro-inflammatory gene

expression that aims to decrease the risks of excessive inflammation while keeping antimicrobial systems fully active [21].

The phagocytosis of apoptotic cells is one of the major roles of tissue macrophages, specifically M2 macrophages. On the other hand, efferocytosis by macrophages can reinforce signalling pathways that reprogram macrophages toward an anti-inflammatory M1 phenotype. After engulfment of apoptotic cells, macrophages down-regulate M1 signalling pathways such as TLR and NF-κB and up-regulate gene markers of M2 polarization including PPAR-γ [64].

Macrophages undergo dynamic changes during different phases of wound healing. Moreover, macrophages with different phenotypes can coexist. Macrophages adopt different activation states in response to stimuli from the local environment (like microbial products or damaged cells) [9,65]. As stated earlier, there are many intermediate stages that cannot be accurately described in the simplistic M1/M2 model for macrophages' phenotypes. So, the M2 phenotype for macrophages has been subdivided into four types: M2a, M2b, M2c and M2d based on their function and key markers [5,8].

M2a macrophages are induced by interleukins IL-4 or IL-13 and are usually referred as profibrotic. They mainly induce a Th2 response and tissue repair [8]. The M2a phenotype secretes cytokines like IL-1ra, sIL-1R and IL-10, chemokines like CCL17, CCL18, CCL22, CCL24, and other compounds including epidermal growth factor (EGF), TGF-β, insulin-like growth factor (IGF) 1 e Arg1, having key roles in tissue remodelling, inhibition of inflammation, promotion of type II inflammation allergy and parasite killer [5,81,88]. EGF is crucial for cell proliferation, differentiation, and survival, while IGF is important for cell proliferation and inhibition of cell death [92,98]. M2a macrophages express high levels of surface molecules and receptors, such as CD14+, CD16++, CD68, CD206, CD163, CD23, CD209, Fizz1, Arg1, IL-4R, CXCR1, CXCR2, and Dectin-1 [98].

M2b macrophages are activated by immune complexes (such as the combination of TLR and apoptotic cells or of LPS and specific receptors) and by IL1-R ligands. These macrophages are involved in antigen presentation leading to Th2 activation and immune regulation [8, 75,81]. The M2b phenotype plays a role in immunoregulation by secreting interleukins IL-1β, IL-6, IL-10 and TNF, and chemokines CCL1 CCL20, CXCL1 and CXCL3 and cyclooxygenase-2 (COX-2) [5,88]. M2b macrophages express high levels of the surface molecules CD14+, CD16++, CD68, CD80 and CD86 [98].

M2c macrophages are activated by glucocorticoid hormones, IL-10 or TGF-β, and are involved in immune suppression, tissue repair and matrix remodelling [8,98]. The M2c phenotype acts on immunoregulation, matrix deposition, tissue remodelling by secreting interleukins and chemokines like CCL16, CCL18, CXCL13, CCR2, IL–10 and TGF-β, but also EGF, TGFβ and Arg1 [5,88]. Even though these macrophages are known as 'deactivated macrophages', they still retain some functions such as phagocytosis [81]. M2c

macrophages express high levels of surface receptors and molecules, including CD14+, CD16++, CD50, CD68, CD150, CD163 and CD206 [98].

M2d macrophages are activated by adenosine and IL-6 and exhibit functions that help tumour progression by allowing new blood vessel growth, which feeds the malignant mass of cells, promoting their growth [8,98]. The M2d phenotype promotes angiogenesis by producing interleukins IL-10, IL-12, TGF-β and TNF, chemokines CXCL-10, CXCL-16, CCL-5 and CCL-18, and also VEGF, TGF-β and iNOS [5,64,88]. M2d macrophages also express high levels of CD14+, CD16++, CD68 and CD163 [98].

The IL-4/IL-13-induced phenotype is defined as the M2a subset of the M2 phenotype. The M2b subset of the M2 phenotype for macrophages is also called IL-10/TGF-b-induced phenotype [39].

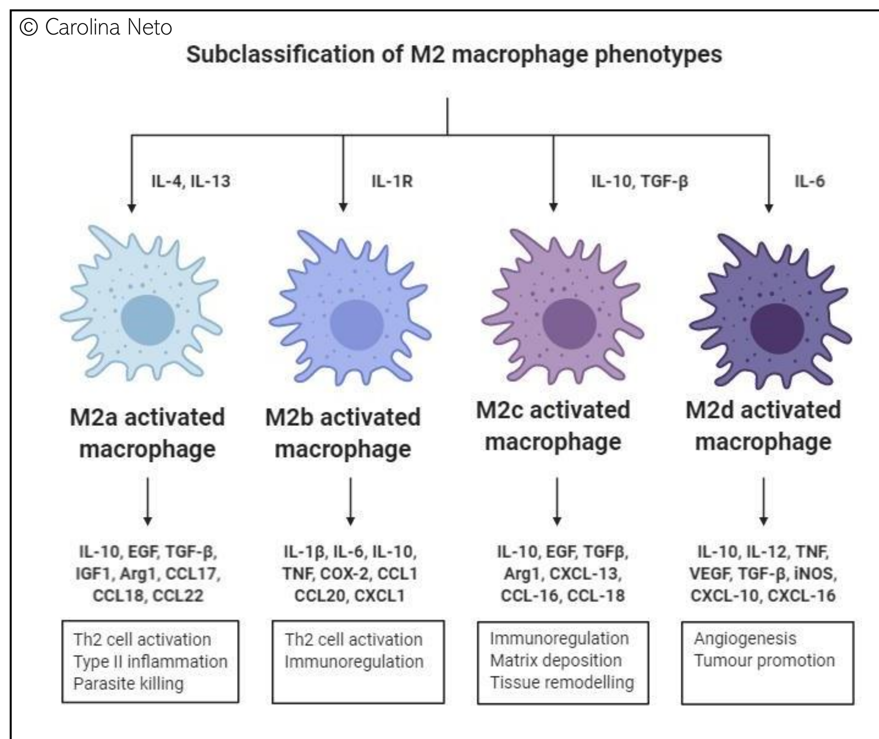

Figure 8 – Subclassification of M2 macrophage phenotypes in M2a, M2b, M2c and M2d macrophages, including key inducer and secreted molecules (*original illustration adapted from [8, 39, 88,98,110])*

STAT-1, STAT-4 and SOCS-3 regulate M1 polarization, while STAT-3 mediates the M2a phenotype. SOCS3+ regulates M2b activation, whereas M2c activation is controlled by STAT-6 and SOCS3+ [5].

Heterogeneity and plasticity are hallmarks that permit the essential immune roles of macrophages in maintaining the homeostasis to be fulfilled. The full spectrum of macrophage activation and functions *in vivo* is less straightforward since macrophages can encounter both M1 and M2 polarizing

signals [75]. A variety of stimuli can make macrophages resistant to activation or suppress the function of already activated macrophages. For example, IL-10 may suppress the pro-inflammatory activation of macrophages by limiting the production of pro-inflammatory cytokines. Macrophage deactivation is a phenomenon that can occur before or after polarization. An environment shifting from one that is rich in pro-inflammatory signals to one that is rich in anti-inflammatory signals will be accompanied by an M1 phenotype deactivation [7].

Table I below summarizes the key activating molecules for each phenotype described (M1, M2a, M2b, M2c and M2d), as well as secreted molecules (chemokines, cytokines, growth factors and others), signalling pathways and marker expression.

	Induced by	Secreting			Signalling pathways	Marker expression
		Chemokines	Cytokines	Others (e.g. growth factors)		
M1	IFN-γ, TNF, DAMPs, PAMPs, GM-CSF	CXCL1, CXCL10, CXCL 11, CXCL 16, CCL2-5, CCL8-11	IL-1β, IL-6, IL-10, IL-12, IL-18, IL-23, TNF	ROS, RNS, NO, iNOS	STAT-1, STAT-4, SOCS-3	CD14++, CD16−, CD68, CD86, CD80, MHC II high
M2a	IL-4,IL-13	CCL17, CCL18, CCL22, CCL24	IL-1ra, sIL-1R, IL-10	EGF, TGF-β, IGF1, Arg1	STAT-3	CD14+, CD16++, CD68, CD163, MHC-II low
M2b	IL-1R	CCL1, CCL20, CXCL1, CXCL3	IL-1β, IL-6, IL-10, TNF	COX-2	SOCS3+	CD14+, CD16++, CD68, CD80, CD86, MHC-II+
M2c	IL-10, TGF-β	CXCL13, CCL16, CCL18, CCR2	IL-10	EGF, TGF-β, Arg1	STAT-6, SOCS3+	CD14+, CD16++, CD68, CD150, CD163, CD206
M2d	IL-6	CXCL10, CXCL16, CCL5	IL-6, IL-10, IL-12, TNF	VEGF, TGF-β, iNOS		CD14+, CD16++, CD68, CD163

Table I – Signalling pathways, marker expression, inducer and secreted molecules (chemokines, cytokines, growth factors and others) for each macrophage phenotype *(adapted from [5,8,69,110])*

Macrophage polarization is more accurately viewed as a continuum with overlapping cell surface expressions, cytokine secretions, and transcriptional regulators, than a fixed or permanent process [75].

1.2. RELATIONSHIP BETWEEN TUMOURS AND MACROPHAGES

Despite macrophages playing key roles in maintaining homeostasis, their dysregulation and dysfunction have been implicated in several disease pathologies. Macrophage plasticity has been linked to many pathological conditions being an attractive target for drug development and therapeutic interventions [73]. There are several chronic pathological findings associated with increased tissue levels of M2 macrophages, including cancer. High macrophage infiltration and high densities of cells expressing macrophage-associated markers are linked to worse prognoses in various tumours, including breast cancer, lung cancer, and lymphomas [67,68]. Tumour progression is accompanied by an

altered myelopoiesis causing the accumulation of immunosuppressive cells, such as M2-like macrophages [54].

Tumours are abundantly populated by macrophages, formerly part of the anti-tumour response, but later promoting tumour initiation, progression, and metastasis. Even though M1 macrophages can eliminate cancer cells, M2 macrophages are believed to promote cancer growth [10]. M1-macrophages release cytokines that inhibit the proliferation of malignant cells. In contrast, M2-macrophages release cytokines that promote tumour growth and dissemination [69]. M1 macrophages play an important role in the recognition and destruction of cancer cells. After recognition, malignant cells can be destroyed through several mechanisms, including contact-dependent phagocytosis and cytotoxicity (release of cytokines) [8]. In response to persistent infections or chronic irritation, M1 macrophages produce inflammatory cytokines, like IFN-γ, TNF-α and IL-6, sustaining the inflammation, common in tumour initiation and promotion. A tumour-promoting microenvironment plays an important role in tumour development and hampers the onset of an immune response against tumour cells. The tumour microenvironment (TME) consists of ECM, an expanding vasculature, stromal cells, and various types of immune cells [87]. A plethora of extrinsic and intrinsic factors including communication between tumour cells and immune cells, fibroblasts, epithelial cells, vascular and lymphatic endothelial cells, cytokines and chemokines are part of the TME. Altogether the cells part of the TME can modify the neoplastic properties of the tumour [92]. Once tumours become established, signals from the TME affect macrophages polarization. The TME itself promotes the activation of immune suppressive cells, that favour angiogenesis and tumour growth while suppressing anti-tumour immunity. The TME polarizes macrophages from a pro-immunity phenotype (M1 macrophages) to adopt an immunosuppressive phenotype (M2-like macrophages), creating an anti-inflammatory response, which can lead to tumour progression, angiogenesis, and metastasis [1,7,9]. These pro-tumour macrophages are called tumour associated macrophages (TAMs). TAMs can actually account for up to 30% of the tumour mass and up to 85% of the total macrophage population in the tumour and some authors describe a symbiotic relationship between TAMs and cancer cells [87,95].

Cancer cells display, among others, unregulated cell growth, tumour-promoting inflammation, evasion of the immune system and dysregulation of metabolic pathways [8]. These events are in fact linked to macrophage polarization. Even though macrophages in the TME should have an anti-tumour effect, either by direct killing of tumour cells or by presenting tumour-related antigens to the immune system to induce tumour removal, this does not occur [33]. Starting from the early steps of tumorigenesis, cancel cells modify the surrounding environment to evade host immune response. Moreover, the recruitment to tumour mass and secondary lymphoid organs of myeloid cells with immunosuppressive activity is a prominent feature of tumour progression [54]. TAMs actually function as an accomplice in the promotion of tumour progression,

especially after being programmed and polarized into a proangiogenic and immune-suppressive M2 phenotype by the TME [33].

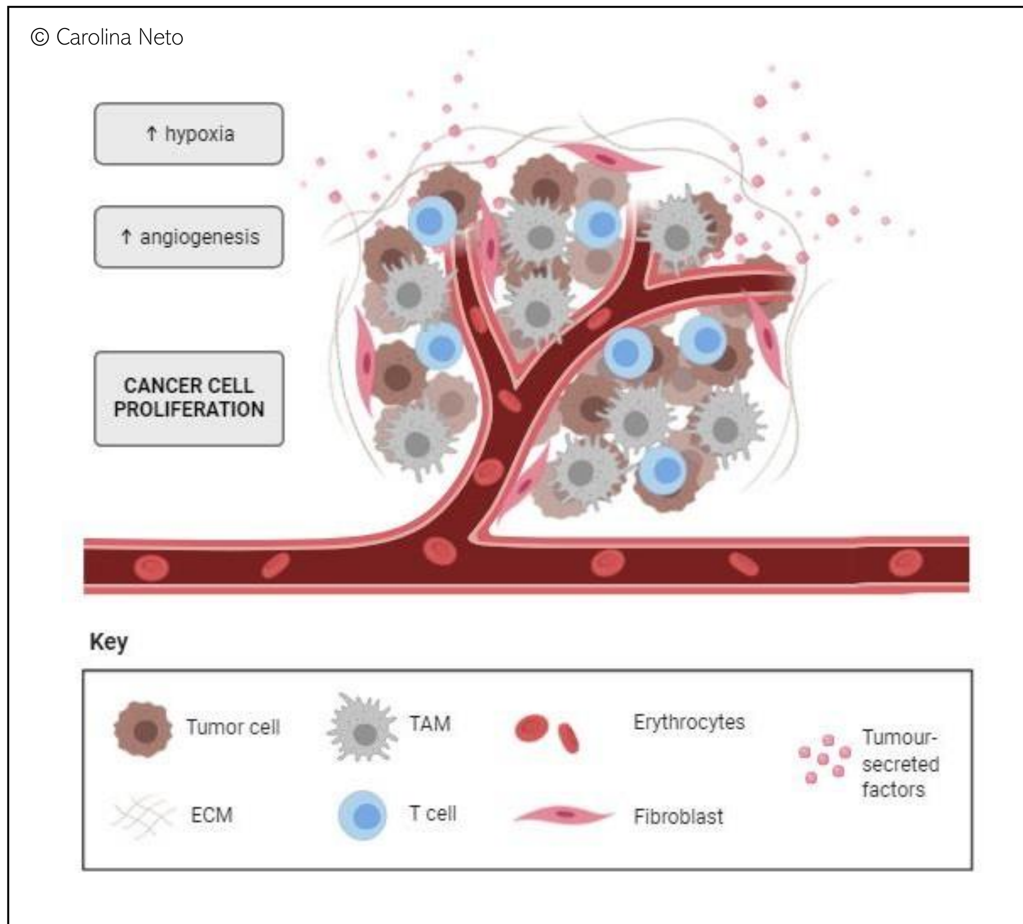

Figure 9 – The activation of TAMs in the TME thus promoting hypoxia and angiogenesis, contributing to cancer cell proliferation and poor clinical prognosis *(original illustration)*

The prognosis of cancer patients is dependent on the ratio of M1 and M2 macrophages in the TME [86]. For instance, in pulmonary carcinoma 70% of macrophages in the TME presented an M2-like phenotype [96]. The mechanisms that underlie the transformation of M1 macrophages into M2 macrophages and vice-versa influence the progression of many chronic diseases like cancer [9]. During the late stages of tumour progression, TAMs acquire a prevalent M2-like phenotype characterized by low IL-12 expression and high IL-10 expression [54]. Although TAMs have some characteristics of the M2 phenotype for macrophages, TAMs have a transcriptional profile that is quite distinct from the referred as M2 phenotype [21]. In contrast to M1 macrophages, TAMs have poor anti-tumoral activity, promoting tissue remodelling and angiogenesis, favouring metastatic dissemination. In the TME, where increased IL-6 levels are found, monocytes consume M-CSF, and this event is linked to the skewing towards M2-like macrophages [54]. Tumour cell products, including ECM components, IL-10,

CSF-1, and chemokines (CCL2, CCL18, CCL17, and CXCL4), set macrophages in an M2-like cancer-promoting mode [65]. CSF-1 is considered as the primary factor that attracts macrophages to the tumour periphery, and CSF-1 production by cancer cells predicts lower survival rates and indicates a poor prognosis for patients [8]. Furthermore, other chemokines such as IL-4, IL-13, CCL7, CCL8, CCL9, and CXCL12 are also highly expressed in tumours and involved in TAM recruitment and polarization [101].

TAMs are originated from tissue resident macrophages or monocytes recruited to the tumour site by chemotactic factors, like CCL2, or other like VEGF and M-CSF. The higher expression of these mediators is correlated to cancer growth. TAMs establish a symbiotic relationship with the cancer cells [95]. TAMs are able to promote tumorigenesis owing to their pro-angiogenic and immune-suppressive functions in the TME, thus promoting tumour initiation, progression and metastasis [40]. In established tumours, TAMs stimulate tumour migration, invasion, intravasation and angiogenesis, events required for tumour cells to become metastatic, facilitating the escape through the circulatory or lymphatic system. TAMs support the growth, proliferation and migration of endothelial cells and blood vessel formation by the release of VEGF-A, as well as FGF from the TME [73]. FGF is important for endothelial cell proliferation, fibroblast proliferation, and epithelial cells proliferation, migration, and differentiation [92].

M1 macrophages have the potential to contribute to the earliest stages of neoplasia, because the free radicals produced can lead to the DNA damage that causes mutations and predispose host cells to transformation [88]. The start of tumorigenesis is often accompanied by a non-resolving inflammation, influenced by macrophages, that provide a mutagenic environment by producing nitrogen or oxygen radicals and secreting a variety of growth factors and interleukins like IL-23, IL-17 and IL-6. Following the development of an intraepithelial lesion, the macrophage phenotype is shifted towards a M2-like phenotype by tumour-derived cytokines as IL-4, IL-10 and IL-13 [87,101]. Recent studies indicate that the induction of a M2-like phenotype of macrophage population in tumours may occur through the MyD88-dependent activation of NF-κB [88]. The tumour-educated M2-like macrophages (or TAMs) down-regulate tumoricidal activity via IL-10 secretion. They support immunosuppression leading to immune evasion and proliferation of the tumour. Tumour expansion is followed by neovascularisation, the key step in the benign-to-malignant transition [87]. TAM accumulate in hypoxic regions of the tumour, which triggers the pro-angiogenic functions of these cells [94]. In response to the increased hypoxia, TAMs secrete angiogenic factors such as VEGF, and produce proangiogenic cytokines as TNF-α and IL1β. Moreover, TAMs facilitate angiogenesis by releasing growth factors from the ECM and by cleaving ECM components facilitating recruitment of endothelial cells. The last stage in tumorigenesis is dissemination from the primary site. TAM-derived proteases support intravasation by degrading the epithelial basement membrane and cleave cell adhesion proteins. Following the invasion of blood or lymphatic vasculature, a specialised TAM subset primes the metastatic site by deposition of fibronectin and release of VEGFs and

angiopoietins. Furthermore, this pre-metastatic niche provides a platform of interleukins (IL-1, IL-6 and TNF-α), growth factors and adhesion molecules, enabling circulating tumour cells to engraft the secondary site [87].

Hypoxia, which results from tumour cells with a status of vigorous metabolism and rapid growth, is a common feature occurring in the majority of solid tumours. Hypoxia promotes malignancy by various mechanisms, such as inducing immune escape, promoting glycolysis, antagonizing apoptosis, and promoting TAM differentiation. The gradients of chemokines induced by hypoxia, such as CCL2, CCL5, CSF-1, VEGF, among others, are responsible for the migration of TAMs into the hypoxic areas of tumours. Hypoxia further traps the seeding macrophages by down-regulating their chemokine receptors. Hypoxia modulates the TAM phenotype toward a pro-tumoral profile by various factor [101].

IFN-γ, IFN-β, and TNF-α are intended to initiate microbial clearance. However, excess or inappropriate production of cytokines may cause tissue damage and result in pathological conditions such as cancer [24,52]. IFN-γ is an important mediator of tumour immune surveillance [21].

In the TME, Notch signalling plays an important role in the polarization of M1 macrophages. Macrophages that are deficient in Notch signalling express an M2 phenotype regardless of other extrinsic inducers [8].

Persistent infection with intracellular pathogens like *Chlamydia* sp., *Mycobacteria* sp., *H. pylori* and others can exploit macrophage polarization for cancer development [104]. The immune system protects the host from bacterial and viral induced tumours by eliminating or supressing infections. Timed elimination of pathogens and resolution of inflammation can prevent the formation of an environment conductive to tumorigenesis [92]. Even though phagocytosis is a key function of macrophages, there is a difference between the phagocytosis of bacteria which is typically pro-inflammatory and the uptake of apoptotic cells which is typically anti-inflammatory [7]. TLRs, a fundamental signalling pathway that triggers inflammation, have been demonstrated to be generally expressed in tumour cells. Tumour cells expressing TLRs inhibit tumour inflammation, that promotes the initiation, promotion, and progression of tumorigenesis [29]. TLR engagement leads to NF-κB activation and production of inflammatory mediators associated with M1 macrophages. However, NF-κB activation also activates a genetic program essential for resolution of inflammation and for M2 polarization of TAMs [65]. TAMs are thought to be able to maintain their M2 phenotype by receiving polarization signals from cancer cells such as IL-1R, which are mediated through the NF-κB signalling cascades. In fact, inhibition of NF-κB in TAMs promotes classical activation resulting in pro-immunity and anti-tumoral activity. Also, by inhibiting NF-κB transcription factors in cancer cells, tumour growth and angiogenesis are reduced [8]. The activation of NF-κB and the activation of the members of the STAT families are essential for a balanced immune response under inflammatory conditions. Their continuous activation or overexpression has been found in many different cancer malignancies [62].

In response to LPS there is a production of different pro-inflammatory cytokines, including TNF-α (the main cytokine produced by macrophages in response to LPS) and IL-1, involved in pathogen clearance. LPS stimulation of macrophages leads to the activation of the NF-κB pathway, responsible for the transcription of several pro-inflammatory genes. However, during pathological situations, the same mediators and the activated pathways can also play a role in conditions such as cancer [48,49,94]. The response of macrophages to LPS signalling through NF-κB results in the activation of the M1 phenotype involved in anti-tumoral functions [9]. TAMs display NF-κB activation in response to LPS and TNF-α, producing low rates of NO [94]. TAMs are activated by IL-10 and IL-4 or IL-13 that signal to STAT3 and STAT6, respectively [9]. STAT3 activation promotes neoplastic cell proliferation and survival, and several tumour cell lines exhibit IL-6- or STAT3-dependent chemoresistance *in vitro* [67]. Colony stimulating factor 1 receptor (CSF1R) also signals to a wide range of transcriptional factors, including Myc. Myc signalling has been shown to be important for pro-tumoral phenotypes. CSF1R expression is regulated by transcription factors, and genetic ablation of this factors in macrophages in tumours recapitulates the loss of CSF1 in tumours, resulting in inhibition of angiogenesis and tumour growth decreasing [9].

Oncogene Myc is an interesting orchestrator of macrophage function in tumours, since it mediates the tumorigenic mechanisms of cancer cells and macrophages. Expression of the Myc oncogene accounts of approximately 40% of the transcriptional fingerprint of human M2•macrophage activation, and Myc is overexpressed in human TAMs. In cancer cells, the oncogenic protein MYC has been shown to induce expression of the anti-phagocytosis signal CD47. Expression of Myc seems to enable the suppression of innate immunity by cancer cells, through macrophage-mediated phagocytosis, via CD47 expression, as well as activation of effective adaptive anti-tumour immunity [6].

Tumour angiogenesis is known to promote dissemination of cancer cells from the primary tumour into the circulation by increasing the density of leaky vessels and enhancing tumour cell invasion [95]. TAMs display a key role in tumour angiogenesis by regulating the increase in vascular density, switching to the malignant state. TAMs secrete angiogenic molecules such as VEGF, TNF-a, IL-1b, IL-8, PDGF, EGF and FGF [9]. It has been shown that VEGF inhibitors can decrease macrophage recruitment, and this effect may contribute to their anti-angiogenic activity [65]. Although macrophages are not necessarily a dominant source of VEGF, deletion of VEGF in macrophages revealed their role in driving abnormal vascular phenotypes in tumours [67]. EGF enhances invasion ability and motility by increasing matrix degradation [95]. TAMs also promote angiogenesis by secreting chemokines CXCL1, CXCL8, CXCL12, CXCL13, CCL2 and CCL5 [92]. The promotion of angiogenesis is one of the key functional properties of M2 macrophages. The growth of new blood vessels not only fuels tumour growth but also provides additional routes by which tumour cells may migrate and metastasize. As tumour growth progresses, TAMs produce great quantities of M-CSF and TGF-β, but also great quantities of the potent

deactivating cytokine, IL-10 [7]. TGF-β is a cytokine that exerts important effects on processes such as fibrosis, angiogenesis, and immunosuppression. However, TGF-β also facilitates metastasis by altering the homeostasis of TGF-β signalling in advanced malignancies [62].

From local invasion, intravasation into vessels, and extravasation at peripheral sites, macrophages have been implicated as regulators of all stages of the metastatic process, often through positive feedback pathways involving CCL2 and/or CSF-1 [92]. The metastasis process of tumour cells is a multistep sequence mainly including invasion in the primary sites, intravasation into the vasculature, survival in the circulation, extravasation out of the vasculature, and adaption and growth in the metastatic sites (colonization) [101].

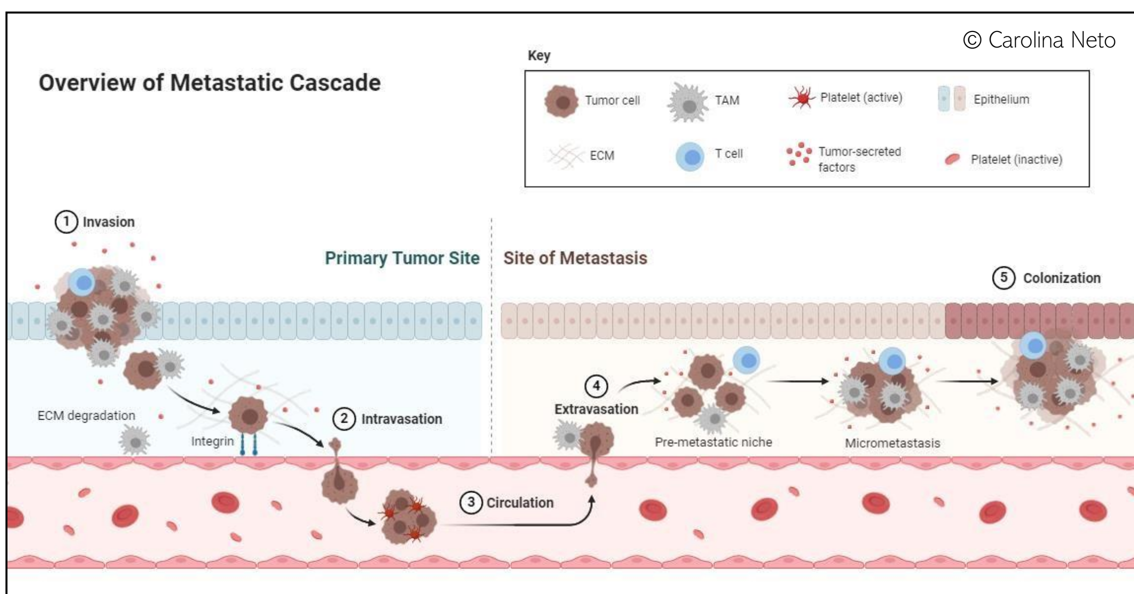

Figure 10 – TAMs as helpers of all the stages of the metastatic process including invasion, intravasation, circulation entry, extravasation, and colonization *(original illustration adaptaded from [6, 101])*

Metastasis begins with tumour cells obtaining the ability of escaping from the confines of the basement membrane into the surrounding stroma, known as invasion [101]. Neoplastic cell invasion of ectopic tissue can be promoted through the directed release of cytokines and chemokines such as EGF and CCL18 or through protease-dependent ECM remodelling that may directly affect neoplastic migration. EGF expression is driven by signalling through the CSF-1R via neoplastic cell production of CSF-1 as well as T cell-derived IL-4 [67,92]. TAMs are capable of secreting a number of proteolytic enzymes, including cathepsins, matrix metalloproteinases (MMPs) and serine proteases, which are important components mediating ECM degradation and cell-ECM interactions [101]. By producing proteolytic enzymes that digest the ECM, TAMs pave the way to tumour-cell dissemination from the primary tumour site, thus contributing to metastasis [6].

Tumour cells squeezing through small pores in the vascular endothelium, known as intravasation, to gain access to the host vasculature is another critical step in metastasis. On one hand, macrophages break down the ECM around the endothelium by a number of proteolytic enzymes such as cathepsins, matrix metalloproteinases, and serine proteases. On the other hand, TAMs hijack tumour cells into the circulation by a positive feedback loop consisting of tumour cell-produced CSF-1 and TAM-produced EGF [101].

Once penetrated into the vasculature, the tumour cells have to be fit for survival and exit the circulation. Clots packed around the tumour cells alleviate stress from NK cells, for example, and help the tumour thrive in the circulation [101].

Once the tumour cells settle in the capillaries of the targeted organs, they try to attach and extrude through the vessel walls with the assistant of macrophages. This phenomenon is known as extravasation [101].

Moreover, macrophages produce factors that promote the accumulation of tumour cells at distant sites, and TAMs in metastatic sites can provide a supportive niche for metastatic cells [6]. Cancer-related inflammation conditionate the premetastatic niche, to favour a secondary localization of cancer [65]. TAMs play important roles in forming premetastatic niches in the organs to which tumour cells eventually metastasize. For instance, TAM-secreted TNF-α, VEGF, and TGF-β, are believed to be transported through the bloodstream to distant organs where they induce tissue-resident macrophages to recruit tumour cells to the secondary sites and promote the formation of metastatic foci [102].

TAM-dependent immunosuppression is an essential program that accompanies tumour cells through the metastatic process [102]. Moreover, the tumour stromal cells produce growth factors and chemokines such as CSF1, CCL2, CCL3, CCL5, that recruit macrophages to the TME. Macrophages activate the angiogenic switch, by producing high levels of IL-10, TGF-β, Arg1 and low levels of IL-12, TNF-α, and IL-6. The levels of expression of these cytokines suggest that macrophages modulate immune evasion [8]. This way, tumours can escape the immune surveillance and prevent the detection and elimination by the immune system [92]. In solid tumours, macrophages are attracted by hypoxic environments and respond by producing HIF-1α and HIF-2α, which regulate the transcription of genes associated with angiogenesis. During the angiogenic switch, macrophages can also secrete VEGF (stimulated by the NF-κB pathway), which will promote blood vessel maturation and vascular permeability [8]. Chemokines CCL18 and CCL22 are secreted by TAMs and display immunosuppressive functions. The tumour not only can evade the immune system, but also select the most suitable immune cells with reduced tumorigenesis to use in its advantage, growth promotion and survival. Tumours can gain an impressive arsenal of weapons to hamper the induction of anti-tumour immune activity [92]. TAM have poor antigen presenting capacity and can actually supress T cell activation and proliferation, by secreting IL-10 and TGF-β.

The low production of IL-12 results in inability to trigger Th1 immune responses [94]. TAMs can also inhibit T cell activity by the depletion of L-arginine, required for T cell functions, in the TME by secreting Arg1 [106].

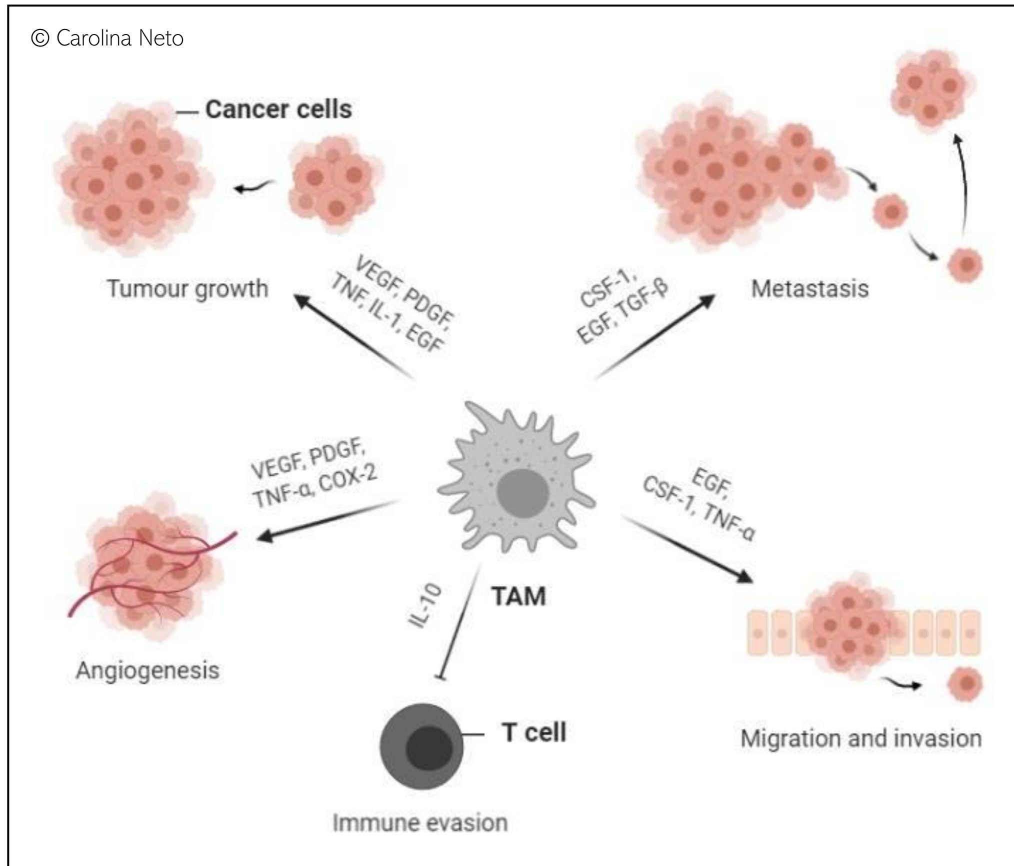

Figure 11 – TAMs promotion of cancer cell growth, angiogenesis, metastasis, migration, and invasion, as well as suppression of the immune responses, including key mediators involved *(original illustration adapted from [8,92,101, 102])*

Oxidative stress can be a consequence of the increased generation of ROS and RNS produced in order to eliminate invading pathogens by the immune responses. In the context of oxidative stress, the secretion of a large amount of ROS and RNS recruits more inflammatory immune cells. Once the combination between inflammation and oxidative stress becomes chronic, excessive cellular ROS and RNS is produced. This exacerbating loop can result in the oxidation of intracellular proteins, lipids, and nucleic acids, leading to aberrant genetic changes and epigenetic alterations such as the dysregulation of oncogenes and tumour-suppressor gene expression [62].

TAMS support tumour growth by the secretion of cytokines like IL-10, TNF-β, IL-10 and TNF-β, chemokines like CCL2 and CXCL12, and growth factors such as VEGF, PDGF and EGF [92].

M2 activity, for example Arg, is strongly up-regulated in growing tumours [64]. TAMs promotion of cancer growth is associated with the way macrophages metabolize arginine. Arginine is a limiting metabolite at sites of inflammation, since both M1 and M2 macrophages avidly consume this amino acid. M2 macrophages metabolize arginine to ornithine and urea through the Arg pathway. Macrophage production of ornithine is required for many repair processes, including cell proliferation and collagen biosynthesis [10]. In M1 macrophages, NO is generated by the mediated metabolism of amino acid L-arginine mediated by iNOS. Microbial products, pro-inflammatory cytokines and interferons are inducers of iNOS [16]. In M1 macrophages the arginine metabolism produces toxic NO and citrulline. Although iNOS expression is low in human blood monocyte-derived macrophages, iNOS is expressed at greater levels in inflamed tissue macrophages and infiltrating monocyte-derived macrophages [10]. L-arginine metabolism exhibits discrete shifts important to cytokine expression in macrophages and is exemplary of distinct metabolic pathways altering TAM-tumour cell interactions. M1 macrophages favour iNOS. The iNOS pathway produces cytotoxic NO, and cells consequently exhibit anti-tumour behaviour. M2 macrophages have been shown to favour the Arg pathway, and produce urea and l-ornithine, which contribute to tumour cell growth [8].

Below there is table II compiling key mediators secreted by TAMs that ultimately promote cancer proliferation, growth and survival. The contribution of TAMs to angiogenesis, tumour growth, metastasis, immune suppression, and migration was separated according to cytokines, chemokines, and growth factors production. An overlap in molecules can be clearly distinguished while analysing the table. IL-4, IL-10 and TNF-α are the more prevalent cytokines secreted by macrophages that are involved in cancer promotion. CCL2 is the most prevalent chemokine and TGF-β, EGF and VEGF are the more prevalent growth factors secreted by TAMs that contribute to cancer cell proliferation.

Contribution of TAMs to cancer cell proliferation	Key mediators secreted		
	Cytokines	Chemokines	Growth factors
Angiogenesis	IL-1, IL-4, IL-6, ,IL-10, IL-12, IL-13, TNF-α	CXCL1, CXCL8, CXCL12, CXCL13, CCL2, CCL5	VEGF, PDGF, FGF, HIF, TGF-β
Tumour growth	IL-10, TNF-β	CCL2, CXCL12	VEGF, PDGF, EGF
Metastasis	IL-4, TNF-α	CCL2, CCL18	CSF-1, VEGF, EGF
Migration	IL-1, IL-4, TNF-α	CCL2, CCL18	EGF, CSF-1, TGF-β
Immune evasion	IL-4, IL-6, IL-10, IL-12, TNF-β	CCL2, CCL3, CCL5, CCL17, CCL22	TGF-β

Table II – Contribution of TAMs to the proliferation of cancer including cells cancer cell growth, angiogenesis, metastasis, migration and invasion, and immune evasion *(adapted from [5,8,21,69,92])*

1.3. CURRENT METHODS FOR IMMUNOTHERAPY TARGETING TAMs TOWARDS CANCER TREATMENT

The concept of cancer therapy revolves around the elimination of cancer cells or the inhibition of its growth [8]. Surgical removal of the primary tumour is not successful in cases diagnosed in later stages of the disease due to the highly immunosuppressive TME. Traditional therapies like chemotherapy and radiation also exhibit limited efficacy, due to the variable responses, toxicity, and lack of treatment durability [93]. On the other hand, immunotherapy relays on the induction, reinforcement, or suppression of immune responses. Cancer immunotherapy involves the stimulation of the immune system to recognize, reject and destroy cancer cells [8]. Immunotherapy allows the tailoring of individualized therapies towards precision, however drug resistance and the tumour anti-immunity signals present challenges for developing multitargeted immunotherapies that are cost-effective and safe [93].

Tumour cells are able to promote its own growth and spread, by suppressing the adaptive immunity played by T-cells, enhancing angiogenesis, cell invasion and intravasation into blood vessels, events promoted by TAM's. Since cancer cells associate themselves with the non-cancer cells of the stroma for its own survival and spread, the TME can be targeted for therapy [1,105]. Immunosuppression is a key factor that tumour cells take advantage off to exploit the immune system in their favour. Readjusting host immune response is believed to offer tremendous therapeutic advantages. Modulating the cytokine network between TAMs and cancer cells could limit tumour progression [104]. Targeted TAM therapy in conjunction with other anticancer agents may improve traditional cancer therapy effects [68].

The understanding of the molecular mechanisms implicated in TAMs promotion of tumour growth allows for the development of original anti-tumour therapeutic strategies, and some of them are currently under evaluation in clinical trials [105]. Based on compelling preclinical data from numerous laboratories indicating that macrophage presence and activity are malleable *in vivo*, clinical studies are now ongoing in several solid tumours where macrophages are being targeted [67]. Macrophages are great therapeutic targets since their function can be augmented or inhibited to influence on disease outcome [9]. Therapeutic strategies targeting macrophages as tumour-promoting factors, aimed at macrophage activation and re-education are undergoing clinical assessment; such strategies have the potential to complement cytoreductive, antiangiogenic, and immune-checkpoint-inhibitor treatments [3].

Macrophages can be sourced for *ex vivo* modification for therapeutic purposes in two manners: from cell lines or primary cells. Even though cell lines have longer survival and better proliferation ability, they are poor candidates for clinical translation because of the differences comparing to primary cells, potential for virus shedding, and tumorigenicity. Primary cells can be collected in

two ways. One of the methods is to collect monocytes from blood or bone marrow and differentiate these cells in a M-CSF medium. The other method includes taking resident macrophages from body cavities (peritoneal or alveolar) and incubate these cells with macrophage polarizing agents to induce the desired phenotype. Macrophages isolated from bone marrow, blood and body cavities only produce very low numbers of cells, also usually failing to proliferate and being difficult to genetically modify. Furthermore, novel strategies to generate M2-like macrophages in a large scale using embryonic stem cells have also shown success [64].

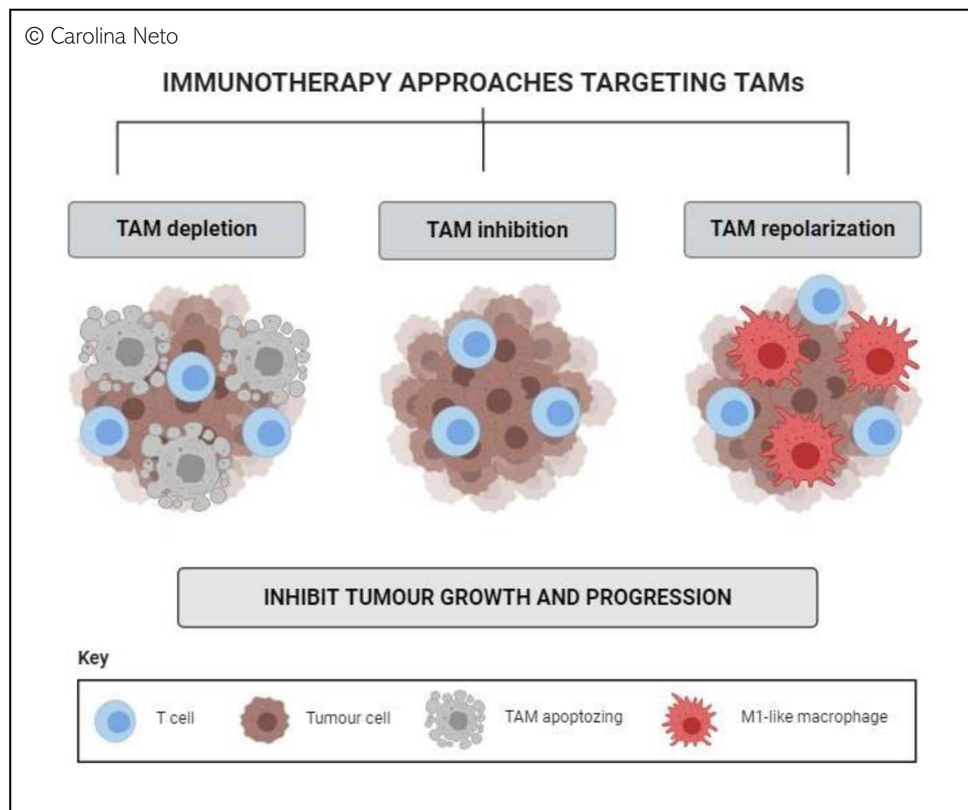

Figure 12 – Immunotherapy approaches including TAM depletion, TAM recruitment inhibition and TAM repolarization, as inhibitors of tumour growth and progression *(original illustration)*

The way neoplastic cells respond to therapy is not solely dependent on their complexity of the genomic aberrations but is also regulated by numerous dynamic properties of the microenvironment. Most current cancer therapies focus on killing malignant cells which are often genetically unstable and can become resistant to chemotherapy [68]. TAMs can limit the anti-tumour activity of conventional chemotherapy and radiotherapy by orchestrating a tumour-promoting repair response to tissue damage, and by providing a protective niche for cancer stem cells [6]. TAMs limit the anti-tumour activity of both chemotherapy and radiotherapy by creating a tumour-promoting repair response to tissue damage and by protecting cancer cells [3]. Macrophages mediate chemotherapy

resistance by providing survival factors and/or activating anti-apoptotic programs in malignant cells. Although soluble factors secreted by macrophages have usually been implicated, it is also possible that ECM deposition and/or remodelling or direct cell-cell interactions are involved too [67]. TAMs are therefore potential targets for adjuvant anticancer therapies since they can contribute to chemotherapy resistance in tumours [68]. Blocking the function of TAMs in TME is a potential therapeutic strategy to inhibit tumorigenesis [40]. Also, re-programming or re-polarizing TAMs to express a pro-immunity M1 phenotype may be another therapeutic approach [33]. Targeting TAMs potentiate traditional therapy, selectively destroying this subpopulation of macrophages without targeting resident macrophages that are essential for homeostasis and critical to host defence. However, preferential removal of TAMs remains challenging.

TAMs represent an attractive target to recalibrate immune responses in the TME. Initial TAM-targeted strategies, such as macrophage depletion or disruption of TAM recruitment, have shown beneficial effects in preclinical models and clinical trials. Alternatively, reprogramming TAMs towards a pro-inflammatory and tumoricidal phenotype has also become an attractive strategy in immunotherapy [87]. The response of tumours to irradiation and anticancer drugs can be improved by blocking macrophages entry in tumours or by suppressing their polarization from an M1 to an M2 phenotype [1]. Studies conducted by Ruffell and Coussens (2015) show that preclinical mouse models of tumour development (mammary, pancreas, glioblastoma, etc.) in which macrophages have either been depleted (but not completely) or reprogrammed exhibit decreased metastasis. Therapies targeting macrophage presence and/or polarization can ameliorate metastasis in cancer patients [67]. Cancer immunotherapy using macrophages can be achieved by TAM repolarization, inhibition, or depletion [97].

Therapeutic targeting the signalling pathways that regulate the switch between macrophage phenotypes can control immunosuppression in cancer. Therapeutic approaches that stimulate pro-inflammatory gene expression may enhance cancer patient survival [11]. Reactivating pro-inflammatory genes in TAMs might help to promote tumour rejection [21]. Since the tumour mass contains a great number of M2-like macrophages or TAMs, they can be used as targets for cancer treatment. Considering that the TME strongly polarizes macrophages towards an M2-like phenotype, that aids cancer cell proliferation, repolarizing macrophages towards an M1 phenotype in the presence of cancer can help the immune system reject tumours [1]. A lot of effort has been put in on the identification of molecules associated with the activation of M1 and M2 macrophage phenotypes [106]. TAMs can be repolarized into an M1-like phenotype *in vitro*, increasing the expression of TNF-α and iNOS and decreasing IL-10 and Arg1 expression, in order to inhibit the proliferation and invasion of cancer cells and tumour growth [87]. Many natural and synthetic pharmacological agents that modulate the polarization pathway of macrophages have been identified [106]. Strategies to reprogram TAMs phenotype for anti-tumour therapy include the targeting of PI3Kγ. Activation of PI3Kγ signalling in TAMs inhibits

adaptive immune responses and promotes immune cells invasion into tumours. In mouse models, pharmacological inhibition of PI3Kγ using a PI3Kγ-selective inhibitor, results in macrophage reprogramming, which reduced pro-tumour macrophages while increasing anti-tumour macrophages and T cell responses [105]. TAM repolarization shows an interesting interplay with the tumour vasculature and decreased expression of angiogenic growth factors leading to vessel normalisation. Vessel normalisation has shown to sensitize the tumour to chemotherapeutic regimes and improved NK cell infiltration [87].

Reducing the number of macrophages in the TME can likewise help destroy cancer cells or at least impair tumour growth [1]. The reduction of the number of TAMs in the TME can be achieved by either TAM depletion or blockage of TAM recruitment.

One of the strategies for reducing TAMs numbers in the TME is to block their recruitment or infiltration of monocytes/macrophages into tumours [106]. The accumulation of macrophages in tumours is due to the continuous recruitment of monocytes from the circulation by tumour-derived factors, including CSF-1, CCL2, and VEGF. Since CCL2 targets though CCR2, blockade of CCR2 can suppress the accumulation of TAMs in tumours. CCR2 inhibitors and anti-CCL2 antibodies have shown efficacy in reducing tumour growth and metastasis in several pre-clinical murine models specially when administered simultaneously [105]. The administration of certain drugs or stromal factors can stimulate TAMs to produce specific cytokines and chemokines leading to the recruitment of various tumour-infiltrating lymphocytes. For instance, in skin cancer, targeting the CSFR in TAMs led to tumour growth suppression [93]. CSF1 controls proliferation, differentiation, recruitment, survival, and function of mononuclear phagocytes, such as monocytes and macrophages. Blockade of CSF1R decreases the accumulation of TAMs in the TME but can also induce the restoration of glycolysis to favour the maintenance of the M1-like phenotype [106]. The most straightforward method of inducing the M1 phenotype activation in TAMs is through stimulation with TLR ligands, that activates pro-inflammatory gene regulators such as STAT1. For instance, TLR4 is able to reprogram TAM phenotype with an increased IL12, iNOS and MHC II expression and a decreased IL10, Arg1, VEGF and matrix metalloproteinases expression. TLR3 reprogrammed macrophages express IL-6, IL-12, TNF-α and iNOS and show an enhancement in antigen uptake. TLR7 activation is another attractive target tor TAM repolarization since it activates both NF-κB and IRF7 signalling in macrophages, that stimulates IFN-α production [87].

Another strategy for reducing TAMs numbers in the TME is direct depletion. One approach for depleting TAMs is to trigger their apoptosis, which could effectively inhibit tumour growth and restore local immune surveillance in the TME. Several compounds have been shown to induce apoptosis of macrophages including zoledronate, clodronate, and trabectedin. Bisphosphonates are used to taken up by phagocytosing cells and have cytotoxic effects on immune cells. Bisphosphonates exert several anti-tumour effects,

including inhibition of tumour cell proliferation and induction of tumour cell apoptosis, inhibition of tumour cell adhesion and invasion, inhibition of angiogenesis, and enhancement of immune surveillance [106].

There are several methodologies used in cancer immunotherapy. One of the approaches to cancer immunotherapy includes the use of cytokines or chemokines (like IFN-α and IFN-β) to recruit activated macrophages and other immune cells to the tumour site and allow the tumour cells to be recognized and destroyed. This technique inhibits tumour progression, induces the apoptosis of cancer cells and depletes TAMs from the TME [8]. For example, in skin cancer, since CCL17 and CCL22 are up-regulated in TAMs as an immunosuppressive mechanism, repolarization of TAMs by immunomodulatory reagents like IFN-β are useful for down-regulating these chemokines and supressing tumour growth [93]. Vaccines are also used as immunotherapeutic agents, being able to activate the immune system to attack the tumour, presenting antigens in combination with adjuvants. Even though a combination of vaccines is normally used in immunotherapy towards cancer treatment, the immunosuppressive TME drives the process towards immune escape [93]. Some trials are currently investigating the use of specific mRNA tumour vaccines encoding neo-antigens, in several cancer models [105]. For example, since CCL2 is required for the recruitment of TAMs, treatment with anti-CCL2 neutralizing antibodies reduces the number of macrophages in the tumour site, ultimately reducing cancer cell proliferation [95]. Antibodies are able to shut down a specific M2 signalling pathway, decreasing TAM tumour infiltration [87]. Adoptive cell transfer therapies, that employ either naturally occurring or genetically engineered immune cells, can act on tumour regression [93]. For instance, single adoptive transfer of macrophages from systemically irradiated mice resulted in enhanced cellular immunity [104]. Immune checkpoints are represented by a family of proteins on the surface of T cells, which interact with specific ligands on macrophages or cancer cells and inhibit their activation. Anti-checkpoint antibodies have been used in tumour immunotherapy. However, it has been showed to be less efficient in some types of cancer, including pancreatic, colorectal, and ovarian cancer [105]. The use of inorganic molecules in cancer immunotherapy also shows positive results. Methionine enkephalin (MENK) has antitumor properties *in vivo* and *in vitro*, by polarizing macrophages from the M2 to the M1 phenotype, down-regulating Arg1 and IL-10 and up-regulating TNF-α and NO. Bisphosphonates are also inorganic molecules employed in cancer immunotherapy using macrophages. These molecules can induce apoptosis in TAMs in order to reduce metastasis, reduce the number of infiltrating TAMs in the tumour site and impair angiogenesis. For example, since TAMs also produce CCL18, responsible for tissue remodelling in the TME and related to STAT1 signalling, depletion of TAMs through bisphosphonates could be a useful strategy for melanoma immunotherapy [93].

Drug delivery is achieved by the used of hydrogels, cellular vehicles, nanocarriers and microparticles. For example, liposomes are nanoparticles used to deliver drugs and deplete TAMs from the TME [8]. These nanosized vesicles are used to encapsulate drugs, reducing the systemic toxicity profiles thanks to

improved pharmacokinetics and biodistribution, showing great advantage in delivering adjuvants with toxic side effects, like IL-12 and IFN-α [93]. The use of these vesicles results in depletion of IL-1β-producing cancer cells and inhibition of the production of angiogenic factors such as VEGF-A and IL-8 [8]. Trials in tumour-bearing mice pre-treated with clodronate liposomes are successful in macrophage depletion but presents high toxicity [105]. For instance, liposomes containing clodronate have been developed and successfully applied in several cancer models, leading to the regression of tumour growth, angiogenesis, and metastasis. Macrophages have also received considerable attention as a drug delivery carrier due to their tropism to hypoxia and their ability to migrate and infiltrate into tumours [106].

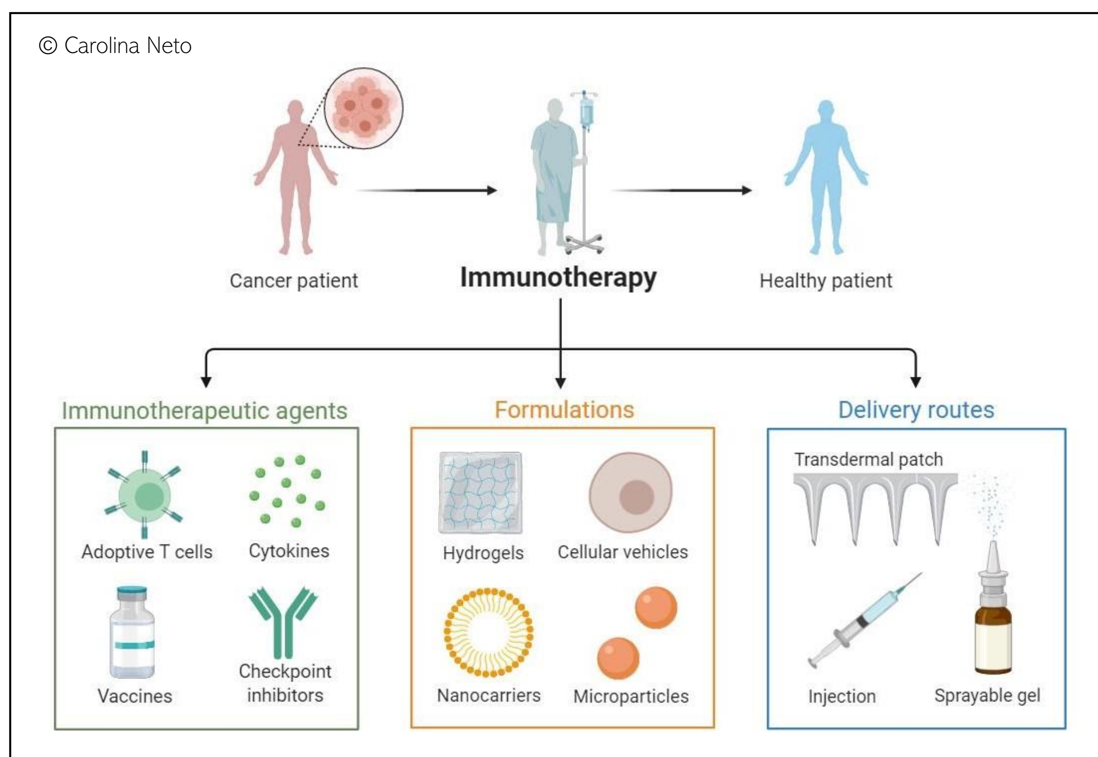

Figure 13 – Immunotherapy methodologies for cancer patients, including immunotherapeutic agents, formulations, and delivery routes *(original illustration adapted from [67, 105])*

TAMs contribute to the anti-tumour activity of some chemotherapeutic agents, such as doxorubicin (under certain conditions), and of monoclonal antibody therapies via antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Of note, macrophage depletion has a key role in the anti-tumour activity of the anticancer agent trabectedin [3,106]. Trabectedin is able to block the production of some pro-metastatic cytokines (like CCL2, CXCL8, IL-6, and VEGF) in macrophages and induces their rapid apoptosis as well. Trabectedin is a cytotoxic agent that directly kills cancer cells by interfering with several transcription factors, DNA-binding

proteins, and DNA repair pathways [101]. In addition to targeting tumour cells, trabectedin can partially deplete circulating monocytes and TAMs through apoptosis [105].

Many natural plant products possess anti-oxidative and anti-inflammatory properties that are associated with their protective effects against tumorigenesis [62]. For example, neferine from green seed lotus embryos exerts an antitumor effect on angiogenesis by regulating the polarization of TAMs in ovarian cancer. Deoxyschizandrin, a phytochemical, from berries can inhibit the activity of M2 macrophages, and onionin A not only increases cytotoxicity against ovarian cancer cells but also suppresses the activation of M2 macrophages. TAM repolarization can also be mediated by the natural compound baicalin [98].

Zoledronic acid is an agent that suppresses M2 polarization and enhances M1 activity in macrophages thus inhibiting TAMs supportive roles in tumours is feasible. Zoledronic acid is able to reverse the polarization of TAMs from M2-like to M1-like by attenuating IL-10, VEGF, and MMP-9 production and recovering iNOS expression [101]. Since the deletion of VEGF in macrophages results in abnormal vascular phenotypes in tumours, VEGF antagonists can induce vascular normalization and increased uptake of chemotherapeutics due to reduced vessel leakiness and interstitial fluid pressure [67]. The pharmacological blocking of angiogenesis, for example, with anti-VEGF approaches, can cause blood vessel damage, which induces hypoxia and stimulates the secretion of immune cell chemoattractants. Anti-VEGF approaches shows higher therapeutic efficacy and higher inhibition of vessel formation, upon depletion of TAMs with zoledronic acid, in several tumour models [105].

Macrophage recruitment to the tumour site is a feature of poor cancer prognosis. As a matter of fact, several inhibitors and antibodies targeting the TAM recruiting factors are being evaluated in early clinical trials across various types of cancers. It was reported that both small molecular inhibitors and antibodies targeting either CCL2 or CSF-1 signalling inhibited the mobilization of monocytes and macrophages accumulation in the TME. CSF-1 is another potent determinant factor of M2 macrophage in tumours polarization. CSF-1 overexpression is observed at the invasive edge of various tumours and correlates with a significant increase in metastasis. CSF-1 depletion has shown to greatly reduce macrophage density, delay tumour progression, and severely inhibit metastasis [101].

TNF-α may be one of the critical factors mediating chemoprotection, either directly through NF-κB activation, or indirectly through induced IL-6 expression and subsequent STAT3 activation. Macrophages can be a critical source of TNF-α *in vivo* and a great candidate for therapies and drug development [67]. IL-17 has also been found to direct anti-tumour responses to tumour cell lines following treatment with chemotherapy [67]. Other cytokines such as TNF-α and IL-6 have also been linked to the recruitment of macrophages to the TME [8]. With the critical role for CCL2 and CSF-1 in recruiting macrophages to neoplastic tissue, there is growing interest in therapeutics targeting these ligands and/or their

respective receptors to ablate the pro-tumorigenic properties of macrophages [67].

Immunotherapy approaches using macrophages	Cytokines and chemokines		Inorganic molecules			Vesicles
	IFN-α/IFN-β	CCR2	MENK	Biphosphonates	Polymers	Endossomes
Main results	Inhibition of cancer cell apoptosis & monocyte-macrophage differentiation	Blockage of the recruitment of immunce cells to the tumour site	Activation of the M1 macrophage, up-regulating NO & TNF-α	Depletion of TAMs & impairment of angiogenesis	Induction of Th1 response & up-regulation of IL12	Inhibition of angiogenic factors

Table III – Types of cancer immunotherapy approaches using macrophages, including main results obtained *(adapted from [8,69])*

The key driver of metastasis is TGF-β, which can be produced by TAMs; TGF-β triggers cancer cell motility and immunosuppression [105]. Some tumours have reduced responsiveness to the anti-proliferative effects of TGF-β as a consequence of modulation of TGF-β signalling pathways [41].

The overactivation of the epidermal growth factor receptor (EGFR) signalling pathway by either overexpression or mutation is frequently involved in tumour initiation, growth, and metastasis. EGFR signalling not only promotes proliferation and invasion of tumour cells, but also adjusts the TME by regulating macrophage recruitment and M2-like polarization. Disrupted EGFR signalling by results in less TAMs and better prognosis in colon cancer models of mice [101].

IFN-y correlates with increased response to immunotherapy and chemotherapy approaches [97]. Other inflammatory signals such as LPS or TNF-α can easily polarize macrophages towards an M1 phenotype *in vitro*. Activated M2 can be produced *in vitro* through the addition of IL-4 or IL-13 [5]. Alternative activation can also be induced by stimulating macrophages with prostaglandins. The functional effects of prostaglandins are, however, closely connected to other stimuli, such as corticosteroids and adenosine [7]. However, the use of substances such an LPS and TNF-α *in vivo* exacerbate a whole-body inflammatory response involving cells of both the innate and adaptive immune systems. These substances can cause fever and inflammation in several tissues including the mucosal surfaces and the lungs. These inflammatory signals are highly cytotoxic and can be detrimental to cancer patients and compromise their health. Since immunotherapy requires the activation of the immune system, it is difficult to find a cytokine, chemokine, compound, or biomaterial that will not produce some side effects. However, considering that macrophages belong to the innate immune system and exhibit pro- and anti-inflammatory properties, these cells are ideal immunotherapy candidates [8].

Although a goal of immunotherapy using macrophages is to reduce the presence of TAMs, it is important not to eradicate pro-inflammatory macrophages

and resident macrophages that are essential for homeostasis [68]. Those remaining macrophages must be reprogrammed toward an anti-tumour phenotypic state to support T cell responses and, together with cytotoxic therapy, limit ongoing tumour growth [67].

Evidence demonstrate that macrophages play an important role in supporting tumorigenesis and that novel approaches can target their recruitment and functions in cancer, as adjuvants of immunotherapy [96].

1.4. THE ROLE OF miRNAs ON MACROPHAGE ACTIVATION

Although inflammation provides protection against pathogens and promotes healing, it may cause more damage than the inciting event if its magnitude and duration are not controlled by regulators, such as microRNAs (miRNAs) [48]. Precise control of innate immune cells and their production of cytokines is therefore critical [85]. miRNAs are endogenous RNA molecules usually involved in post-transcriptional gene silencing by inducing mRNA degradation or translational repression by binding to a target mRNA [74]. Negative regulation of the immune response controls the homeostasis of the immune system and prevent the development of diseases [45].

Produced from genes within the genomic loci of cells, microRNAs (miRNAs) are short (approximately 22 nucleotides long) non-coding RNA molecules capable of regulating gene expression at the transcriptional and post-transcriptional level.

miRNA is processed from a long primary transcript known as primary miRNA (pri-miRNA), transcribed by RNA polymerase II in the nucleus, leading to an hairpin precursor step with approximately 70 nucleotides known as pre-miRNA, by the microprocessor complex, which includes the Drosha enzyme and the double-stranded RNA-binding protein DiGeorge syndrome critical region 8 gene, prior to their transport into the cytoplasm. In the cytoplasm, the pre-miRNA is cleaved by the cytoplasmic ribonuclease Dicer to generate a double stranded RNA duplex, resulting in the mature forms of miRNA. One of the strands corresponds to the major stable form, known as mature miRNA and is then loaded onto the ribonucleoprotein complex dubbed RNA-induced silencing complex (RISC), in order to perform the recognition and translational repression or degradation of target mRNAs. The other strand, the antisense miRNA* is less stable and degraded. The sequences of the two forms are partially complementary and have the potential to regulate different sets of target genes, so both forms can be functionally active [22,47,77].

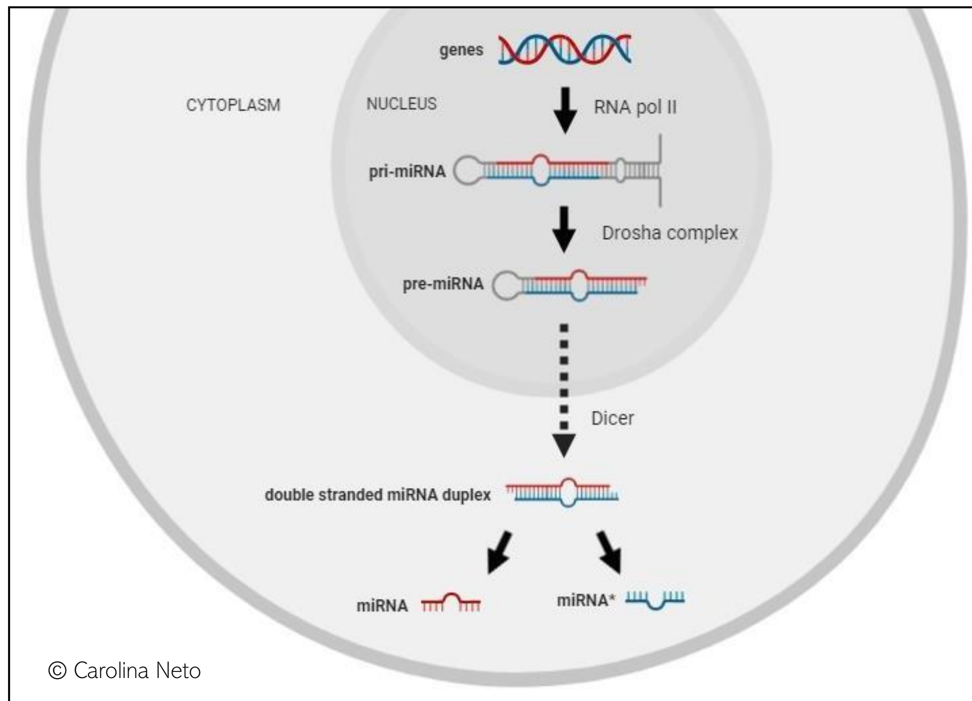

Figure 14 – Pathway and mechanisms of miRNA processing or biogenesis, including phases as mediators involved *(original illustration adapted from [22, 47, 77])*

miRNA act as endogenous short hairpin RNA that down-regulates target genes by binding through nucleotide complementarity to sequences in the 3'-untranslated region (UTR) extremity to decrease/reduce both mRNA and protein expression [12,15,17]. By targeting 3′UTR of mRNA, microRNAs can inhibit translation or promote partial or full degradation of mRNA transcripts [16,47]. miRNAs control gene expression by regulating mRNA translation and/or stability in the cytoplasm [48]. miRNAs participate in signal transduction circuits by maintaining the expression of signalling proteins [36]. The post-transcriptional regulatory functions of miRNAs are key to allow cells to rapidly respond to different cellular cues, thus representing an important component of cellular networks [76].

With more than 1100 described human miRNAs, these molecules are vital regulators of human biological processes such as development, differentiation, inflammation, fibrosis, apoptosis, neoplasia, regulation of insulin secretion, among many others. It is estimated that 30% of protein-coding genes are regulated by miRNAs, being also involved in the eukaryotic response to pathogens. microRNAs regulate innate and adaptive immunity, having a crucial role in development of inflammation in macrophages [12,15,17,47].

There is much discussion about the origin and function of miRNAs, like if these molecules are products of cell excretion or if they undergo programmed secretion. However, some studies suggest that they have important functions, including the ability to modulate immune cells [74]. miRNAs play important roles

in innate immunity by regulating the expression of cytokines and molecules involved in pathogen recognition [30]. The efficacy of macrophage responses to environmental cues relies on the coordinated expression of key factors, which expression is regulated at both transcriptional and post-transcriptional level [77]. The expression of microRNAs is subjected to regulation by transcriptional factors in response to cellular stimuli [60]. This regulation includes a variety of effects depending on different cellular and tissue contexts [41]. For instance, stimulation of macrophages with TLR ligands (like LPS) significantly alters the expression profile of miRNAs, which, in turn, regulates macrophage activation. miRNA expression profiling of different macrophage phenotypes after activation suggests important regulatory roles for a wide-range of miRNAs [30].

miRNAs have the ability to transfer themselves to other cells or organs by exosomes [74]. Exosome-derived miRNAs released by immune cells operate as extracellular soluble mediators with regulatory effects on adjacent and remote cells or tissues through endocrine or paracrine signalling.

miRNAs also have the ability to modulate the magnitude of the innate immune response, participating as integral components of the feedback loop regulatory mechanism. They are able to tune macrophage differentiation and polarization [76]. During the inflammatory process, genes encoding proteins can be regulated at the transcriptional level by miRNAs. The miRNA transcriptional process can be induced by inflammation, and these molecules rapidly become active as they do not require to be translated or translocated back into the nucleus to repress their targets. Some proteins, for example, cytokines induced during the inflammatory response can regulate the processing of miRNAs. miRNAs can also regulate several mechanisms that are involved in the initiation and resolution of inflammation, such as macrophage activation [74]. miRNAs regulate not only gene expression via mRNA degradation but also transcription factors in macrophage polarization [98].

Inflammation involves a coordinated, sequential, and self-limiting sequence of events controlled by positive and negative regulatory mechanisms. Macrophages are extremely plastic cells, and both epigenetic changes and noncoding RNAs can contribute to their polarization [54]. Macrophages present transcriptional complexity, being capable of phenotypic switch according to the homeostatic demands [9]. It is important to understand how miRNAs can promote or inhibit the phenomenon of plasticity and the mechanisms underlying the transition of macrophages between states of opposing functionality [7]. Multiple miRNAs regulate macrophage polarization since the over-expression of a single miRNA is not enough to induce phenotypic changes [37]. Macrophage polarization is probably regulated by several miRNAs via a combination of direct or indirect regulation. The indirect regulation includes miRNA targeting of associated surface receptors, intracellular signalling molecules, and components of the protein secretion machinery [7].

miRNAs undergo the same regulatory mechanisms of any other protein-coding gene, being in normal conditions transactivated or silenced by specific

transcription factors, affected by chromosomal deletions or amplifications, and/or point mutations. Moreover, their expression level is also affected by extensive epigenetic regulatory mechanisms, such as promoter methylation and histone modifications [76]. Polarization of macrophages is regulated by specific epigenetic mechanisms of histone modifications. Histone methyltransferases and demethylases are upregulated in M1 macrophages, while M2 macrophages are characterized by DNA methyltransferases and deacetylases. MAPKs are a central signalling pathway for macrophage polarization, through histone modification. However, miRNAs regulate macrophage polarization mainly through SOCS1 [75].

There are several miRNAs involved in macrophage recruitment and activation, that have been identified by several studies [7,76]. For instance, the activation of pro-inflammatory cells via TLRs triggers the expression of several miRNAs, which target different components of the TLR signalling itself, fine-tuning the overall response. Thus, given the importance of miRNAs in the modulation of TLR signalling, anti-inflammatory stimuli can either increase or suppress miRNA expression, according to the role played in sustaining or repressing TLR-mediated cell activation [51].

In particular, mi155 has been extensively studied in the context of macrophage polarization and inflammation. Its expression levels largely increase when macrophages are polarized towards the M1 phenotype, whereas in M2 macrophages mi155 levels are strongly decreased. Interestingly, mi155 inhibition results in impaired M1 polarization and its overexpression induces a re-polarization towards a M1-like phenotype. Although the mechanism by which mi155 directs macrophage M1 polarization has not been completely elucidated, evidence indicates that mi155 expression is induced by TLR agonists and pro-inflammatory cytokines through an NF-κB-mediated mechanism [76].

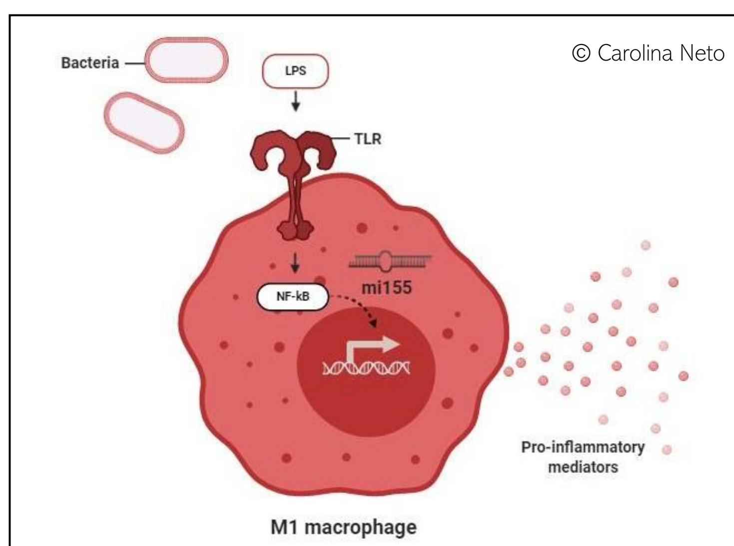

Figure 15 – The role of mi155 in the induction of the pro-inflammatory response in macrophages after LPS stimulation, including signalling pathways *(original illustration adapted from [47])*

When inducing the differentiation of human monocytes into M1 macrophages, GM–CSF provokes a decrease in the expression of mi223, mi15a, and mi16. The down-regulation of these miRNAs may contribute to the activation of this specific phenotype [23].

M1 polarization is also supported by mi127 with consequent increased expression of pro-inflammatory cytokines. mi127 leads to increased phosphorylation of JNK, and its knockdown resulted in reduced expression of M1 signature genes and promoted the transcription of M2-related genes. Other miRNAs associated with M1 polarization include mi181 and mi451 [76].

The first miRNA associated with M2 polarization was mi146a since its expression levels rise after M2 macrophage activation. Enforced expression of mi146a in macrophages results in reduced levels of M1-marker genes (iNOS, CD86, TNF, IL-12 and IL-6), and increased production of M2-phenotype markers (Arg1, CCL17, CCL22 and CD206). Mechanistically, it was demonstrated that mi146a modulates macrophage polarization at least in part by targeting Notch1 and PPAR-γ. Other miRNAs highly expressed in M2 macrophages are mi146a, mi125a, mi145-5p, mi511-3p, mi223, and let-7c, all of which have been shown to promote M2 polarization. Also relevant for macrophage polarization is the mi23a/27a/24-2 cluster, which is down-regulated by M1-type stimuli and up-regulated by M2-type stimuli [76].

miRNAs regulated by LPS target genes that contribute to the inflammatory phenotype [80]. Several miRNAs are induced during infection, by LPS and participate in the proinflammatory response of M1 macrophages [16,36]. The inflammatory response is valuable for dealing with pathogens and, if unregulated, can lead to serious disease. Thus, understanding the regulators of the inflammatory response is very important [22]. The stability of the mRNAs of inflammatory mediators, encoded in the 3'UTR, partially directs the sequential initiation, propagation and resolution of inflammation. The AU-rich elements (ARE) located in the 3'UTR of these inflammatory mediators are regulated by miRNAs [44]. In macrophages activated by LPS, transcripts encoding cytokines, such as TNF-α, IL-1, IL-6, COX-2 and CXCL1, have ARE in their 3'UTR [44]. LPS induces a robust transcriptional induction of a large number of genes that is accompanied by the increase of the half-life of a more restricted number of transcripts [48].

Conflicting reports highlight the tissue-specific nature of TGF-β regulation of miRNA's [42]. The different outcomes are likely to be due to changes in the transcriptome depending on the cell type and associated differences in the pool of miRNAs targets being expressed and subjected to repression [47].

The production of TNF-α for instance by macrophages is regulated by several miRNAs. This regulation includes miRNA targeting of associated surface receptors, intracellular signalling molecules, and components of protein secretion machinery. Many of these mechanisms of suppression involve the targeting of

multiple components of a signalling pathway by a single miRNA [7]. TNF contains an ARE in its mRNA 3′ UTR that is frequently targeted by RNA binding proteins for degradation [20]. The induction of TNF-α by LPS is mediated at both transcriptional and posttranscriptional levels. Posttranscriptional control of TNF-α expression is achieved by regulating mRNA stability, polyadenylation, and translational initiation. This posttranscriptional regulation depends on ARE located in the 3'-UTR of TNF-α mRNAs. In resting macrophages, the 3'-UTR of TNF-α mRNA is implicated in the repression of their own translation. However, after activation the LPS signal targets the 3'-UTR of TNF-α and relieve this self-inhibitory effect., leading to TNF-α production and secretion. The inhibition of the MAPKs pathways blocks the induction of TNF- by LPS. While ERK1/2 control the nucleocytoplasmic transport of TNF-α mRNAs thus allowing the translation to occur, the other pathways control the stability or enhance the translation of TNF-α mRNAs [48,49].

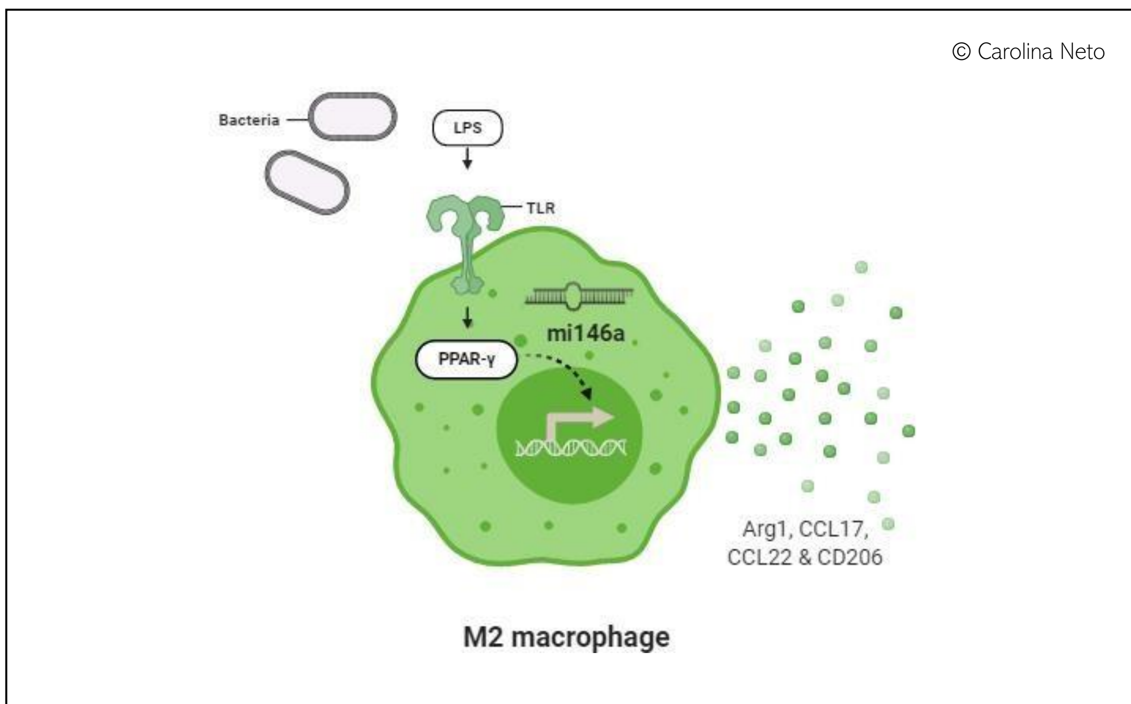

Figure 16 – The role of mi146a in the induction of the anti-inflammatory response in macrophages after LPS stimulation, including signalling pathways *(original illustration adapted from [76])*

mi155 and mi125b are clear important regulators of macrophage polarization and potential therapeutic targets. mi155 is up-regulated by NF-κB, enhancing TNF-α translation and supresses anti-inflammatory pathways like SOCS1 signalling. mi125b supresses IRF4 that negatively regulates IRF5 signalling [87].

In M1 macrophages, the expression of iNOS is regulated at the transcriptional level and NO production is regulated by several factors at both

transcriptional and post-transcriptional levels. Microbial products, pro-inflammatory cytokines and interferons are inducers of iNOS. These factors trigger NF-κB, MAPK or STAT1 pathways, which initiate the transcription of the iNOS gene. Nevertheless, whether microRNAs regulate iNOS expression and NO production during innate immune response remains unknown [16].

The role of miRNAs as regulators of gene expression, especially those miRNAs relevant to human diseases, has opened up the possibility of modulating miRNA expression for therapeutic purposes. A key requirement to assess the potential use of miRNA antagonists in the clinic is the ability to synthesize stable and specific miRNA antagonists on a scale suitable for in vivo studies [47]. Given that most miRNAs lead to RNA degradation, it is expected that over-expression of a specific miRNA will lead to down-regulation of specific mRNA targets [37]. The knowledge about miRNA-mediated regulation of macrophage plasticity and polarization can be used to modulate functional phenotypes in order to create therapies against the pathogenesis of numerous diseases, including cancer [7].

1.4.1. RELATIONSHIP BETWEEN miRNAs AND TUMOURS

Dysregulated miRNA levels are associated with several types of malignancies including colon, breast, and lung cancers [52]. Modulation and dysregulation of miRNAs has been witnessed during bacterial infections in order to evade the immune system response and to promote pathogen survival [14,15]. Tumours are able to modulate immune responses in order to favour their growth through several mechanisms. One of such mechanisms is the release of microparticles that lower tumour inflammation via transferring miRNAs to nearby macrophages [29]. So, microRNAs are not restricted to their parent cells; they can be transferred to other cells for functional expression through multiple transferring pathways. For example, during apoptosis, cells shed vesicles (known as microparticles) of components of the plasma membrane encapsulating cytoplasmic elements into the extracellular space. Microparticles can contain enzymes, proteins, RNAs and DNAs, and are capable of transferring messenger molecules including miRNAs from one cell to another, functioning as vectors for delivering molecular messages among cells. Tumour cell-derived microparticles transfer inflammation-regulating miRNAs to macrophages, thus tuning immune responses. Tumour-infiltrating immune cells, especially macrophages are central players in the development of tumour inflammation and capable of taking up microparticles [29].

miRNA expression is dysregulated in cancer malignancies. The underling mechanisms include chromosomal abnormalities, transcriptional control changes, epigenetic changes, and defects in the miRNA biogenesis machinery [107]. Dysregulated processing of a miRNA presents great importance in cancer initiation and progression. miRNAs dysregulation is associated with the development of cancer malignancy, by causing dysfunction in immune responses [48]. Given the sensitivity displayed by miRNAs to a wide range of stimuli, it

seems likely that altered miRNA expression contributes to the generation of TAMs [7]. The link between aberrant miRNA expression and carcinogenesis may be attributed to the fact that more than 50% of the genes that encode these miRNAs are in cancer-associated regions with chromosomal instability. Based on the properties of the target genes, miRNAs can function as either oncogenic miRNAs or tumour-suppressive miRNAs. Most oncogenic miRNAs down-regulate the expression of tumour suppressors and are overexpressed in cancer cells [62].

The first miRNAs described to be involved in human cancer were mi15a and mi16-1. Both are down-regulated in the majority of leukaemia cases. mi15 and mi16-1 acts as tumour suppressors to induce apoptosis [107].

miRNAs can act as either oncogenes or tumour suppressors. miRNAs considered oncogenes usually target mRNAs encoding oncoproteins, while miRNAs exhibiting tumour suppressor properties are able to promote cancer target mRNAs encoding tumour suppressor proteins [90].

LPS enhances the level of mi15a/16, leading to decreased phagocytosis and bactericidal functions, as well as decreased generation of mitochondrial ROS. Inhibition of mi15a/16 up-regulates the expression of TLR4, facilitating the release of pro-inflammatory cytokines (IL1b, IL-6, and IL-21) and chemokines from macrophages. For instance, mi15a and mi16 primarily act as tumour suppressors. Expression of these miRNAs inhibits cell proliferation and promotes apoptosis of cancer cells, via targeting multiple oncogenes [35]. In brain cancer, since mi124 is down-regulated; so, treatment with mi124 may have a beneficial effect by restricting proliferation and by deactivating macrophages [38]. The mi23a/27a/24-2 cluster was shown to be significantly decreased in TAMs of breast cancer patients, and macrophages overexpressing the mi23a/27a/24-2 cluster inhibited tumour growth *in vivo*. This might be related to the TME signalling or tumorigenic factors including the activation balance between NF-κB and STAT6 pathway [33]. mi31 expression inversely correlates with the metastatic ability of breast cancer cells, so it may be linked to M1 phenotype activation. mi31 blocks several steps of metastasis, including local invasion, extravasation, and metastatic colonization [90]. In prostate cancer, mi221 and mi222 are able to reduce tumour growth. These miRNAs decrease the expression of epithelial-specific genes and increase the expression of mesenchymal-specific genes [90]. The mi101–coding genes are located in genomic loci with a high frequency of allelic losses in several types of cancer. The aberrant expression profile of mi101 also is shown in the majority of cancer cell lines and cancer tissues examined, suggesting that it may act as a cancer-related miRNA. Genes identified as targets of mi101 have been found to be involved in tumorigenesis and implicate mi101 as a potent tumour suppressor in cancer therapy [59].

mi155 can act in some situations as an oncogenic miRNA, as it is up-regulated and seems to be a key factor in the carcinogenic process in neoplastic disease [41]. mi155 has been linked with Myc overexpression in cancer cells [108]. BMMs with mi3473b overexpression are less efficient in inducing tumour

cell apoptosis, suggesting that mi3473b suppresses the tumoricidal capacity of M1 macrophages [13]. mi10b is associated with the metastatic ability of cancer cells and its inhibition, both *in vivo* and *in vitro,* decreases the formation of cancer metastasis [98]. Lymphomas from mice expressing both Myc and the mi17/19b-1 cluster are characterized by increased cell proliferation and decreased apoptosis levels. Myc regulates the expression of the cluster [108]. mi21 has been described by several authors as a key regulator of the link between macrophages and cancer cells including processes like metastasis and angiogenesis [53, 107]. mi130a is another miRNA that acts as an oncogene and is up-regulated in M1 macrophages, down-regulating M2 macrophage activation. Down-regulation of mi130a predicts a poor prognosis in cancer patients by leading to cancer cell proliferation and metastasis [86]. mi192, mi194, mi15a, mi21, mi122, mi221, mi222 and mi215 are all known to protect cancer cells from apoptosis, thus resisting cell death include [107].

However, there are some miRNAs that can display both function as oncogenes and tumour suppressors. mi720 is abnormally expressed in cancer and plays an important role in tumour progression depending on cancer type. For example, in oesophageal squamous cell carcinoma cells, mi720 inhibits cell proliferation. The elevated expression of mi720 has been reported in human colorectal cancer, while decreased expression was reported in breast cancer [40].

miRNAs released by cancer cells have been shown to bind to TLR7 and TLR8, inducing a pro-metastatic inflammatory response [76]. TLR activation affects the expression of many miRNAs (including mi132, mi9, mi147 and mi346). As many of those miRNAs regulated by TLR signalling are also dysregulated in cancer, it is possible that miRNAs form a key link between inflammation and cancer and that the induction of specific miRNAs by TLRs may be a key step in tumour progression [53]. Among TLR family members, TLR4 is typically highly expressed in tumour cells [29].

Some miRNAs functionally integrate multiple critical cell proliferation pathways, and the dysregulation of these miRNAs is responsible for sustaining proliferative signalling in cancer cells. For example, mi486, significantly downregulated in non-small-cell lung cancer, was found to affect cell proliferation and migration through IGF and PI3K signalling. Other miRNAs involved in cancer cell proliferation include mi221, mi222, mi486, mi545 and mi663 [107].

Evasion of apoptosis is another significant hallmark of tumour progression, which is believed to be regulated by miRNAs. For instance, mi192, mi194 and mi215 are known to protect mRNA from degradation and prevent cancer cell apoptosis. Other miRNAs involved in resisting cell death include mi15a, mi21, mi122, mi221 and mi222 [107].

Growing evidences show that miRNAs have an important role in invasion and metastasis. TGF-β-regulated miRNAs were found to engage in TGF-β signalling to induce invasion and facilitate metastasis in advanced malignancies.

mi155 is one of the miRNAs involved in this regulation process, overexpressed by cancer cells. The knockdown of mi155 suppresses TGF-β-induced invasion and tight junction dissolution, as well as cell migration. Other important miRNAs involved in regulating metastasis include mi9, mi200 and mi212. [107].

Several miRNAs were also linked to angiogenesis, by targeting HIF or VEGF signalling pathway. mi125b, mi210 and mi424 are induced during hypoxia that promote angiogenesis by targeting those pathways [107].

There are also several miRNAs linked to TAMs activity. Down-regulation of let-7 in macrophages suppresses the inflammatory response and leads to tumour progression. Stimulation of the expression of let-7 in macrophages can promote the production of pro-inflammatory cytokines, including TNF and IL-1β, by induction of the NF-κB pathway. Overexpression of mi21 in macrophages can promote angiogenesis and tumour growth, by activating the NF-κB pathway and suppressing IL-10 expression. The inhibition of miR33 expression is responsible for M2 polarization through the targeting of AMPK. mi146a has long been regarded as an anti-inflammatory miRNA highly expressed in M2 macrophages. Downregulation of this miRNA can effectively reduce the expression of several cytokines related to the M1 phenotype, such as IL-6, IL-12, TNF-α, and iNOS, and increase the levels of M2 markers, including Arg1, CD206, CCL17, and CCL22 [109].

Genome-wide profiling demonstrates that miRNA expression signatures are associated with tumour type, stage, and clinical outcomes, so miRNAs could be potential candidates for diagnostic biomarkers, prognostic biomarkers, therapeutic targets or tools. There is growing interest in the role of miRNAs in cancer chemoprevention, and several naturally occurring chemopreventive agents have been found to be modulators of miRNA expression both *in vitro* and *in vivo* [62].

1.4.2. CURRENT METHODS FOR miRNA's ROLE TESTING IN MACROPHAGE ACTIVATION

miRNAs play important roles in the cell proliferation, apoptosis, and differentiation that accompany pro- and anti-inflammatory responses [79]. Autoimmune diseases are a great candidate for miRNA-based therapies, including pathologies mediated by immune cells like macrophages. Since macrophages are key mediators of inflammation and immunopathology, modulating macrophage polarization and plasticity via miRNA manipulation is a promising therapeutic strategy. Since macrophages are highly plastic cells, manipulation of miRNA expression in macrophages has the potential to deliver clinical benefit across opposite ends of the inflammatory, immunological, and pathological spectrums [7].

To study the interaction of the factors and regulatory molecules, such as miRNAs, that promote the pro-tumour phenotype in macrophages requires a

sophisticated genomic analysis to help differentiate the regulation of the multiple subsets associated [9]. However, adjusting the polarization of macrophages through microRNAs may be more effective for disease preventing than for modulating the effect of a single cytokine [36]. Also, miRNA-based diagnoses are more accurate than mRNA-based approaches due to their regulatory role. The ability to profile the miRNA expression of tumours with accuracy and reproducibility in a clinical setting is a valuable medical tool [108].

The adaptation of high-throughput technologies has facilitated the study of the expression of multiple miRNAs in a given sample, making it possible to profile substantial sample numbers with relative ease. Among the most important methods for miRNA profiling is the use of oligonucleotide miRNA microarray analysis, which allows the researcher to simultaneously determine the expression levels of hundreds of miRNAs in a given sample. Other important approaches are also available, such as quantitative polymerase chain reaction (PCR) for precursor miRNAs or mature miRNAs, genome-wide approaches with serial analysis of gene expression (SAGE), and bead-based flow cytometric techniques [108].

Alterations in miRNA expression are associated with a variety of pathologies, and the guided alterations of specific miRNAs have been suggested as novel approaches for the development of innovative therapeutic protocols. miRNA therapeutics is a novel field in which miRNA activity is the major target of intervention. The inhibition of miRNA activity can be achieved by the use of small miRNA inhibitors, oligomers, including RNA, DNA and DNA analogues (miRNA antisense therapy). On the contrary, an increase in miRNA activity (miRNA replacement therapy) can be achieved by the use of modified miRNA mimetics, such as plasmid or lentiviral vectors carrying miRNA sequences [90].

There are many techniques and technologies used for miRNA research today. For example, high throughput sequencing of RNA immunoprecipitated with Argonaut proteins can identify miRNAs and targets from the Ago silencing complex. Three different methods based on cross-linked immunoprecipitation (CLIP) followed by next generation sequencing techniques, have helped make great strides toward global miRNA targets identification with higher confidence. The findings can be further validated by luciferase reporter assay that is commonly adopted for the purpose of predicted miRNA–mRNA target interactions [7].

Besides using miRNA in therapy, its antisense sequence can also be used. Antisense oligos (ASOs) are used as synthetic miRNAs to target mRNA of therapeutic value, while anti-miRNAs (miRNA inhibitors) can bind mature miRNA and block their post-transcriptional activity. One of the obstacles for RNA-based therapeutics is the susceptibility to endogenous RNase activity. Various approaches have been developed to address the issue of miRNA stability for *in vivo* studies. Enzyme-resistant biochemical modifications of synthetic RNA molecules enhance stability of miRNA targeting molecules from the degradation by serum or intracellular RNases. For instance, non-binding oxygen in ASOs

were replaced with sulfur to generate phosphorotiorate nucleotides. Furthermore, introduction of 2′-O-methyl groups rendered improve nuclease resistant and increase binding affinity to target miRNA, thus enhancing sequence specificity. In yet another approach, 2′-oxygen and 4′-carbon on ribonucleotide backbone were chemically locked [7].

Anti-miRNAs have emerged as useful tools for inhibiting the function of miRNAs. Key issues for successful clinical application are stability, safety, and successful delivery to the appropriate tissue and cell type. Thus, the activity of miRNA modulators has been enhanced via chemical modification, conjugation, and encapsulation, aiming at protecting therapeutic molecules from biological degradation and clearance and also at improving their delivery to target cells. Chemically modified anti-miRNAs have demonstrated effectiveness *in vivo*. Chosen chemicals should show high affinity and sequence specificity for complementary RNA and DNA, high chemical and metabolic stability, possess antisense biological activities *in vivo* without toxicity, no alterations in the expression of genes, and no other unwanted off-target side effects [47].

Many of the commonly used *in vitro* techniques for delivering miRNA mimics or inhibitors into cells present obstacles for clinical use, including *in vivo* instability, transfection reagent toxicity, lack of cell or tissue specific targeting, susceptibility to endogenous RNase activity, among others [7].

A big challenge in using miRNA-based therapeutics is the delivery. Several approaches such as liposomes, dendrimers, cholesterol conjugation, polyethyleneimine, and pH-based peptide are promising vehicles to achieve efficient miRNA delivery. Nonetheless, due to the target tissue heterogeneity, location and cytotoxicity, the delivery may be hampered. Therefore, employing more than one delivery systems or testing of various vehicles in order to monitor tissue-specific efficiency may prove beneficial. The use of nanoparticles as delivery vectors also appears promising. Nanoparticles can be formed from various molecules, and when combined with certain modifications, may target specific cell type. Exosome-based strategy to deliver miRNAs is embraced as novel, non-immunogenic, broader, or cell-specific miRNA delivery methods. These membrane enclosed miRNA containing endosome-derived nanovesicles are ubiquitously secreted by cells for communication [7].

let7b is a synthetic miRNA mimic that engages TLR7 and redirects TAMs to a pro-inflammatory phenotype, increasing the expression of IL-12 and iNOS. The use of let7b in immunotherapeutic approaches has been revealed effective in inhibition of tumour growth. TLR7 and TLR8 engagement has also shown potent re-education of TAMs phenotype. An important consideration of TLR agonism is off-target inflammation, which severely limits the therapeutic window, that can be circumvented by selective delivery of the TLR agonist [87]. TAMs treated with let-7b inhibitors displayed characteristics of a M1 activated macrophage, with a significantly higher expression of pro-inflammatory cytokines (such as IL-10, IL-12, and IL-23), and down-regulated pro-tumoral cytokines such as TNF-α [101].

Overexpression of m125a in macrophages by transfection notably enhances their phagocytic activity and represses tumour growth. Overexpression of mi125b in macrophages can make macrophages more responsive to IFN-γ. mi125b represses the expression of IRF4, and in turn, activates macrophages and endows them with the ability to kill tumour cells more effectively. Recently, using nanoparticles containing mi125b, transfected TAMs showed a remarkable increase in the M1 to M2 ratio, proved by an increase in the iNOS/Arg1 ratio [109].

Currently there are no FDA-approved miRNA therapies available; however, commercial development of miRNA therapies is underway and are in preclinical stages for the treatment of several types of cancer, cardiovascular disease, and fibrosis [42].

A pressing challenge to the use of miRNAs as therapeutic tools concerns finding methods to deliver synthetic miRNAs to the desired tissues in a targeted and effective manner. Given that these molecules cannot discriminate between healthy and malignant cells, side effects of treatment remain a concern. Upon reaching a deeper understanding of the mechanism of miRNA biogenesis and action, and the development of new delivery technologies, these small RNAs might well fulfil their promise as valuable therapeutics [108].

2. Goals

Macrophages are an essential player of the immune system and have been widely studied and discussed. Many aspects of the macrophage biology have been described, but the field is evolving at such pace that is very challenging to keep up with all the information available [73]. The current review is therefore intended to provide a summary of the knowledge and new developments of macrophage biology and its links to cancer. This review discusses the characteristics and functions of polarized macrophages, their role in cancer, their activation pathways, and metabolic functions, and also their potential use in cancer immunotherapy approaches. Topics include the origin of macrophages, the contribution of macrophages to homeostasis, macrophage polarization and plasticity, relationship between miRNAs and macrophages, methodologies for miRNA role testing, relationship between macrophages and cancer cells, methodologies for immunotherapy practises, and a characterization of the transcriptional and post-transcriptional networks that regulate macrophage activation and function in the TME.

Macrophages are undoubtedly attractive therapeutic targets, with their heterogeneity and plasticity providing both opportunities and challenges. Many new discoveries, some of which have been discussed in this article, have led to paradigm shifts but have added complexity to the field. This review summarizes key experimental findings about macrophage polarization and attempts to define some of the major questions in this field.

The coexisting macrophages with different phenotypes, the impact of dynamic changes during disease evolution, and the molecular networks orchestrating this plethora of phenotypes needed to be carefully studied. The macrophage plasticity required a deep analysis in different pathological conditions. The identification of mechanisms and molecules associated with macrophage plasticity and polarized activation provides a basis for macrophage-centred diagnostic and therapeutic strategies [65]. Achieving the full therapeutic potential of macrophages requires a better understanding of their fundamental biology. Improving our understanding of cytokines synthesis, secretion, and mode of action leads to the development of drugs better suited to diseases, as well as to the design of new therapeutic approaches [49].

Since macrophages are induced to adopt a spectrum of widely divergent phenotypes in response to diverse external stimuli, the present study was based upon the hypothesis that miRNAs are regulators and coordinators of the changes in the expression of a number of genes that occur after macrophage exposure to different cues. It was hypothesized that miRNAs are involved, not only in macrophage responses to inflammatory conditions, but also in the modifications of gene expression required to generate a spectrum of macrophage phenotypes. This study has examined the global expression patterns of miRNAs in macrophage polarization and contributed to the growing understanding of the role

of miRNAs in macrophage exposure to different activating conditions. Thus, miRNA profiling reveals novel molecules and signatures associated with activation of immune responses which may be a candidate target in pathophysiology [77].

Identification of disease-specific miRNAs and their targets is critical for understanding their role in pathogenesis of certain diseases and may help define novel therapeutic targets [34]. This project provides an overview of current knowledge regarding the role of miRNA in shaping macrophage polarization and plasticity through targeting of various pathways and genes. The data compiled highlights an immunoregulatory role for miRNA's on macrophage function, having physiological and pathophysiological significance. Considering the evidence demonstrating their contribution to disease and their roles in cellular mechanisms like differentiation, metabolism, and immunity, miRNAs exhibit a potential link between inflammatory diseases and cancer [50]. Since the immune response dysregulation is linked to pathologies and to miRNAs, an investigation on the regulation of macrophage pro- or anti- immune responses by miRNAs was led. The way miRNAs target regulatory mechanisms in innate immunity is not completely understood [43]. The fact that miRNAs are implicated in a wide array of cellular processes prompted investigators to look for miRNAs whose levels would change in innate immune response and investigate their potential functions. This study was aimed to identify miRNAs that might be possibly involved in the innate immune response and assess their potential use in laboratory experiments [49]. An investigation was led on the contribution of miRNAs to macrophage activation. In this review, we highlight the essential molecular mechanisms underlying the different phases of the innate immunity responses that are targeted by miRNAs to inhibit or promote M1 or M2 macrophage polarization. We further review the impact of miRNA-dependent.

Both miRNAs and microbially induced inflammation are associated with cancer [52]. Stratification of patients according to their immune status in the course of the disease will help identify high-risk patient populations that need close follow-up and aggressive treatment [96]. Profiling differential miRNA patterns in a variety of cancer types may provide potential cancer-specific miRNA signatures for diagnostic and therapeutic applications [62]. The identification of the various cellular and molecular pathways that participate in inflammation in different human cancers is required for the understanding of cancer-related inflammation to meaningful therapeutic advances [65].

In this study, the immune regulation of TAMs and the relative implication for tumour biology was investigated. The TME itself is a bidirectional, dynamic, and intricate network of interactions between the immune cells and cancer cells, in which immune cells contribute to an immunosuppressive environment and immune evasion, ultimately contributing to tumour progression. Therefore, a full understanding of both tumour biology and the molecular mechanism underlying tumour development and malignant progression requires attention [92]. This review focus on the macrophage and cancer cell interactions in the TME,

highlighting functions in cancer progression and discovering a network connections and communication. The purpose of this review was to analyse the viability of implementing TAM's targeting, in order to improve the response of tumours to cancer therapies.

Five populations of activated macrophages have been identified with unique characteristics, in which macrophages share characteristics between populations, such as cytokines involved. Also, 45 miRNAs were found to be involved in macrophage polarization in different phenotypes, through an intricate network. The identification of biochemical markers that are specific for each of these macrophage populations may allow us to deactivate specific populations of macrophages in the TME, or to diagnose disease progression or resolution by the presence or absence of these populations. The identification of the mechanisms that promote TAM activation and deactivation may disclose new valuable therapeutic targets against tumours.

3. Methods and thesis outline

In order to find suitable articles for this review, an online research was conducted. The used explorers were Academic Google, Research Gate, PNAS, Spandido Publications, Europe PMC and PubMed (US National Library of Medicine National Institutes of Health). The searched keywords or expressions include the following: macrophages, microRNAs, macrophage polarization, macrophage manipulation, macrophage modulation, macrophages' phenotypes, macrophage activation, modulation of macrophage phenotypes, microRNA's modulation, miRNA's manipulation, immunotherapy, tumour microenvironment, tumour associated macrophages, m1 macrophages, m2 macrophages, classical activation of macrophages, alternative activation of macrophages, among others. When possible, filters such as ordering by relevance or date were used. The date of publication of the articles was taken in consideration, conferring more focus in articles published after 2015, given that the information is more up to date.

The information gathered to accomplish the literature review was organised in: general knowledge on macrophages, relationship between macrophages and tumours, current methods for immunotherapy targeting TAMs towards cancer treatment, role of miRNAs in macrophage activation, and current methods on miRNAs role testing in macrophages.

Tables I, II and III were accomplished thanks to the information gathered. Table I compiles the information about the molecule network involved in the macrophage phenotypes, including inducer molecules, secreted chemokines, cytokines and other such as growth factors, signalling pathways and markers expression. Table II gathers the information related to the contribution of TAMs to cancer cell proliferation (angiogenesis, tumour growth, metastasis, migration, and immune evasion), including key mediators secreted, like cytokines, chemokines, and growth factors. Table III summarizes the main results of the several immunotherapy approaches using macrophages, including cytokines, chemokines, inorganic molecules and vesicles like endosomes.

Data collected from trials conducted about miRNAs role in macrophage polarization was compiled in the tables shown in the results section. The information on the results tables was gathered from experimental studies conducted on miRNA(s) role testing in macrophage activation. Data including article reference, used model(s) (cell lines and/or animals), miRNA(s) in study, and main results obtained were compiled on table IV. Table IV was divided in four parts, being the different effects of miRNA(s) in macrophage activation. Table V includes the miRNAs involved in

The illustrations are originals conceived using Biorender, summing up the information and data collected from the literature review. The illustrations serve as complementary graphic summaries of the theorical information gathered, that ease the reading and help with the consolidation of topics. The illustrations were made according to scientific accuracy, optimal usability and industry-leading design standards for science communication.

4. Results

4.1. DATA COMPILATION

Since miRNAs show such complex pathways and ways of action, it is hard to understand the exact function of each miRNA that contributes to macrophage activation. Most studies focus on one or some miRNAs, becoming difficult to see the big picture and to design suitable drugs.

Below there is a compilation of the information gathered from the articles of trials regarding the role of several miRNAs in shaping macrophage polarization and plasticity through targeting of various pathways and genes. Table IV provides insights of the mechanism underlying the influence of miRNAs in the polarization of macrophages and the consequent activated phenotypes. Table IV was divided in four parts according to the miRNAs effect on macrophage polarization: miRNAs involved in the promotion of the activation of an M1-like phenotype in macrophages (a); miRNAs involved in the promotion of the activation an M2-like phenotype in macrophages (b); miRNAs involved in the inhibition of the activation of an M1-like phenotype in macrophages (c); miRNAs involved in the inhibition of the activation of an M2-like phenotype in macrophages (d).

Ref	Model(s)	microRNA(s)	Main results
[18]	MDMs	**mi144**	Down-regulation after *M. tuberculosis* infection, promotes the secretion of pro-inflammatory factors TNF-α, IL-1β and IL-6 , by targeting ERK signalling pathway and by inducing ERK1/2 phosphorylation
[20]	THP-1, Human MDM's	**mi155**	*M. smegmatis* induce high mi55 expression & high TNF production; *M. tuberculosis* induce low mi155 expression & low TNF production
[34]	THP-1	**mi615-3p**	Overexpression enhances the phagocytic capacity of splenic macrophages, by enhancing the PPAR-γ mRNA and protein levels
[33]	RAW 264.7, PMs, BMDMs	**mi23a/27a/24-2 cluster**	Down-regulated by M1 stimulation (LPS through the negative regulation of NF-κB activation) & up-regulated by M2 stimulation (IL-4 through STAT6 positive regulation)
[49]	RAW 264.7, HEK- 293T, C57BL/6	**mi155**	Up-regulated by LPS, has a role in the regulation of TNF-α production and in M1 macrophage phenotype activation
[52]	BMDMs, C57BL/6	**mi155**	Oncogenic miRNA up-regulated by IFN-β and TLRs, involving the TNF-α, JNK and MAPK signalling, acts as a component of primary macrophage response to different types of inflammatory mediators
[59]	RAW264.7	**mi101**	Induced by TLR4 stimulation, inhibits MPK-1, a phosphatase deactivator of MAPKs induced by LPS, in order to prolong the activation of the MAPKs and promote the production of TNF
[81]	MDMs	**mi125a, mi26a-2*, mi155, mi29b**	Up-regulation of mi29b and mi125a enhances NF-κB signaling; mi155 (induced by LPS), mi26a-2* and mi29b (induced by IFN-γ) regulate M1 macrophage activation
[84]	MCF7, Jurkat, RAW 264.7	**mi155, mi125b**	LPS stimulation results in the up-regulation of mi155 and down-regulation of mi125b levels, enhancing TNF-α translation
[85]	BMDMs, C57BL/6	**mi455, mi125a, mi146, mi155**	Both *Candida albicans* and LPS up-regulate mi455, mi125a, mi146 and mi155 through the NF-Kb signalling pathway; IL-10 induces mi146, but inhibits mi155
[86]	THP-1	**mi130a**	Up-regulated in M1 macrophages, promoting the release of pro-inflammatory cytokines and supressing PPAR-γ expression, also down-regulating M2 macrophage activation
[27]	RAW264.7, BALB/c	**mi142-3p**	Down-regulated by histone deacetylation leading to functional impairment of macrophages and age-related inflammatory diseases, by the increased production of IL-6

Table IV (a) – miRNAs involved in the promotion of the activation of a M1-like phenotype including references, model(s) used and main results obtained

The miRNAs involved in the promotion of the M1-like phenotype are: mi26a-2*, mi29b, mi101, mi125a, mi 125b, mi130a, mi144, mi155, mi455, mi615-3p, mi1246m and the cluster mi23a/27a/24-2.

LPS stimulation results in the up-regulation of mi155 and down-regulation of mi125b levels, activating pathways that ultimately enhance TNF-α translation.

LPS/TNF-α-dependent regulation of mi155 and mi125b may be implicated in the response to endotoxin shock, thus offering new targets for drug design [84].

The abundance of mi125a3p is significantly increased in MDMs exposed to M1 polarizing conditions. mi222* expression is reduced under M1-polarizing conditions. mi155*, mi26a-2* and mi29b-1* are also induced in M1 conditions, suggesting these may be LPS-responsive. Experimental augmentation of mi29b or mi155 results in increases in the M1 marker, CXCL9, as well as the M1/M2b markers IL-6 and TNF-α. IFN-γ alone accounts for the increase in mi29b expression, whereas LPS accounts for increases in mi125a-5p, mi146a, and mi155 expression. IFN-γ antagonizes the LPS-induced increase in mi125a-5p and mi146a expression. Transfection with mi155 and mi29b mimics induces macrophage expression of IL-6, TNF, and CXCL9, characteristically expressed during M1 polarization. The mi125a-5p mimic induced the expression of CXCL9, an M1 marker [81].

mi101 attenuates the translation of MKP-1 (a phosphatase that deactivates MAPKs) induced by LPS, prolonging the activation of p38 and JNK. The decrease of MKP-1 by mi101 promotes the biosynthesis of pro-inflammatory cytokines like TNF. Treatment of cells with dexamethasone, an anti-inflammatory agent, inhibits mi101 expression and enhances the expression of MKP-1 in LPS-stimulated macrophages [59].

mi130a is up-regulated in M1 macrophages, promoting the release of pro-inflammatory cytokines and supressing PPAR-γ expression, also down-regulating the M2-like phenotype activation [86].

mi144 was found to be down-regulated in (monocyte-derived macrophages) MDMs infected with *Mycobacterium tuberculosis* and directly binds to the 3'-UTR of MAP3K8, acting as a negative regulator. Moreover, inhibition of mi144 or over-expression of MAP3K8 can activate the ERK signalling pathway by inducing ERK1/2 phosphorylation. TNF-α, IL-1β and IL-6 secretion were also demonstrated to be significantly augmented. The results suggested that mi144 is expressed at a low level in infected MDMs and is closely connected with ERK signalling that regulates inflammatory factor secretion [18].

Some studies show that mi155 enhances TNF production by increasing TNF mRNA half-life. Macrophages incubated with *Mycobacterium tuberculosis* induce low mi155 expression as well as low TNF production, since it leads to reduced translation and rapid degradation of TNF mRNA. In contrast, incubation with *Mycobacterium smegmatis* induces high mi155 expression and high TNF production thanks to TLR2 recognition [20].

Results by other authors shows that mi155 is also related to TNF production. LPS stimulation results in up-regulation of mi155. The overexpression of this miRNA induced by LPS results in enhanced translation and production of TNF-α, by enhancing the stability of its transcript. Since TNF-α transcripts are expressed but not translated in cells unstimulated by LPS, the up-regulation of

mi155 levels in response to LPS may play a role in the regulation of TNF-α production. The up-regulation of mi155 in response to LPS is considered an indicator of increased sensibility to endotoxin shock and its cytotoxic effects [49]. The cytokine IFN-β and TLRs also both up-regulate mi155, involving TNF-α signalling. mi155 expression, regulated by MAPK and JNK signalling, is a component of the primary macrophage response to different types of inflammatory mediators. Up-regulation of mi155 due to exposure to several inflammatory mediators emphasizes that the avoidance of oncogenic transformation justifies the need to rapidly resolve inflammatory responses [52].

Both *Candida albicans* and LPS up-regulate mi455, mi125a, mi146 and mi155 through the NF-κB signalling pathway. mi146 and mi155 target proteins that are involved in inflammatory signalling, including IRAK1. IL-10, an anti-inflammatory cytokine, induces mi146 but inhibits mi155 [85].

Trials done on THP-1 cells, show that the overexpression of mi615-3p enhances the phagocytic capacity of splenic macrophages, by enhancing PPAR-γ mRNA and protein levels [34].

The mi23a/27a/24-2 cluster is down-regulated by M1 stimulation. IL-4 promotes the cluster transcription through STAT6-mediated positive regulation and LPS inhibits the cluster transcription through the negative regulation of NF-κB activation. NF-κB binds the promoter of the mi23a/27a/24-2 cluster thus repressing its expression and STAT6 binds to the cluster promoter thus promoting its expression [33].

Ref	Model(s)	microRNA(s)	Main results
[36]	MDMs, BALB/C	**mi142-5p, mi130a-3p**	Increased mi142-5p and reduced mi130a-3p expressions result in M2 macrophage phenotype activation and profibrogenesis
[37]	BMDMs, C57BL/6	**mi511**	Up-regulated in M2 macrophages following IL-4- and IL-13-stimulation and down-regulated in M1 macrophages, being involved in M2 macrophage activation
[38]	BMDMs, C57BL/6	**mi124**	Down-regulated in microglia results in deactivation of macrophages; its overexpression inhibits TNF-α and iNOS and promotes TGF-β1, reducing inflammation
[39]	RAW 264.7, BMDMs	**mi124**	Up-regulated by IL-4 & IL-13, resulting in up-regulation of M2 markers & down-regulation of M1 markers (CD86, iNOS, TNF), contributing to the M2 phenotype activation
[56]	HEK-293, RAW 264.7	**mi98**	Down-regulated after LPS exposure promotes IL-10 production & decreases COX-2 expression, is involved in fine tuning of the critical level of IL-10 expression in endotoxin tolerance
[53]	RAW264.7, THP-1, BMDMs	**mi21**	After its induction, LPS decreases PDCD4, a tumour supressor, blocking NF-κB activity and promoting IL-10 production; it's a negative regulator of TLR4 signalling through the targeting of PDCD4
[82]	MDMs	**mi511**	Up-regulated in macrophages; it functions as a positive regulator of TLR4, by regulating IL-2 and the JAK/STAT signalling pathways
[89]	BMDMs, C57BL/6	**mi125a-5p**	Up-regulated n response to TLR2/4 stimulation requires MyD88 signalling, promoting M2 activation and repressing the production of M1 markers

Table IV (b) – miRNAs involved in the promotion of the activation of a M2-like phenotype in macrophages, including references, model(s) used and main results obtained

The miRNAs involved in the promotion of the activation of a M2-like phenotype in macrophages are: mi21, mi98, mi124, mi125a-5p, mi130a-3p, mi142-5p, and mi511.

The tumour suppressor PDCD4 is a pro-inflammatory protein that promotes activation of the transcription factor NF-κB and supresses IL-10 translation. Induction of mi21 lowers PDCD4 expression, blocking NF-κB activity

and promoting IL-10 production in response to LPS. Thus, mi21 regulates PDCD4 expression after LPS stimulation. LPS modulates the expression of PDCD4 through the induction of mi21, negatively regulating NF-κB activity while promoting IL-10 production [53].

Results from trials shows thats mi98 targets the 3'UTR of IL-10 transcripts. Overexpression of mi98 inhibits TLR4-triggered IL-10 production and promotes COX-2 expression. mi98 is down-regulated after LPS exposure promoting IL-10 production. mi98 is involved in fine tuning the critical level of IL-10 expression in endotoxin tolerance [56].

mi124 is reported to be involved in the promotion of the activation of M2 macrophages. Exposure to IL-4 and IL-13 results in the up-regulation of mi124 in macrophages. Overexpression of mi124 causes up-regulation of M2 markers like CD206, suggesting that this microRNA contributes to the M2 phenotype development and maintenance [39]. mi124 expression inhibits TNF-α and iNOS but promotes TGF-β1, reducing inflammation. mi124 can promote the polarization of M2 macrophages [38].

Up-regulation of mi125a-5p after TLR2/TLR4 induction requires the adaptor MyD88, promoting M2 activation. The data suggests that mi125a-5p has an important role in suppressing the classical activation of macrophages while promoting alternative activation [89].

IL-4 and IL-13 both up-regulate mi142-5p via STAT6 and down-regulate mi130a-3p by histone deacetylation, sustaining the profibrogenic effect of macrophages and controlling M2 polarization. Overexpression of mi142-5p prolongs STAT6 phosphorylation and inhibition of mi130a relieves the inhibition of PPAR-γ. Increased mi142-5p and reduced mi130a-3p expression result in M2 polarization of macrophages and profibrogenesis. Aberrant mi142-5p and mi130a-3p expression might be involved in the development of pulmonary and liver fibrosis [36].

mi511 is increased in M2 macrophages following IL-4 and IL-13 stimulation and decreased in M1 macrophages, both *in vitro* and *in vivo* suggesting that it is one of the regulators of the M2 macrophage activation. The results establish mi511 as a M2-associated miRNA, suppressed under M1-polarizing conditions. However, over-expression of mi511 alone following M2 or M1-activation signals is not sufficient to affect the phenotype of macrophages [37].

mi511 targets TLR4 and CD80 and is overexpressed after LPS stimulation. mi511 seems to function as a positive regulator of TLR4, being a novel potent modulator of human immune response. Inhibition of mi511 results in reduced specific intercellular adhesion molecules non-integrins levels. These data together indicate that mi511, depending on the target mRNA 3'-UTR and cellular environment, can either up-regulate or down-regulate the target gene expression [82].

Ref	Model(s)	microRNA(s)	Main results
[13]	HEK293T, EL4, BMDMs	mi3473b	Down-regulated after IFN-γ stimulation, promoting pro-inflammatory cytokine production; its overexpression promotes IL-10 production
[14]	MDMs, HEK293	mi132, mi26a	Up-regulated after *Mycobacterium tuberculosis* infection, negatively regulates the IFN-γ signalling cascade and impair macrophage response to this cytokine
[15]	RAW264.7, HEK293T, THP-1	mi155	Up-regulated after *Mycobacterium marinum* infection, decreasing the production of NO and promoting bacteria survival as a mechanism for evasion to immune responses
[16]	RAW264.7, BMDMs	mi146a	Up-regulated after *Mycobacteirum bovis* infection, represses iNOS expression and NO generation, by suppressing NF-κB and MAPKs pathways since it targets TRAF6, thus facilitating bacterial surviving
[17]	C57BL/6, RAW264.7	mi223	Supresses pro-inflammatory activation of Kupffer cells (kidney macrophages), by inhibiting IL-1β production and decreasing Arg1 levels, in acute liver failure
[20]	THP-1, Human MDMs	mi125b	*Mycobacterium megmatis* induces low mi125b expression & high TNF production; *M. tuberculosis* induce high mi125b expression & low TNF production
[22]	THP-1,U937,HL-60, WEHI-3, BJAB, Mono-Mac-6	mi146a, mi146b	Induced by microbial components and pro-inflammatory mediators, base-pairs with sequences in the 3'UTRs of the TRAK6 and IRAK1 genes
[25]	RAW264.7	mi146a	Up-regulated after LPS stimulation, negatively regulating TLR4 activation, decreases both mRNA and protein expression of IL-6, thus having anti-inflammatory properties
[26]	RAW264.7 & HEK-293	mi181b	After LPS stimulation, targets the 3'UTR of IL-6, decreasing IL-6 at protein levels to avoid excessive immune response in a NF-κB-dependent manner
[28]	RAW264.7, BALB/c	mi223	Down-regulated after LPS stimulation, promoting the expression of IL-6 and IL-1β by an increase in STAT3 protein expression
[29]	RAW264.7, BALB/c	let7b	Transferred to macrophages by microparticles released by tumour cells due to TLR4 signalling, resulting in down-regulation of IL-6, attenuating tumour inflammation
[30]	BMDMs, BALB/c	mi487b	Down-regulated by LPS, while IL-33 production increases, resulting in increased expression of iNOS, as well as other pro-inflammatory molecules (IL-1β, IL-6, and TNF-α)
[31]	BMDMs, C57BL/6	mi24, mi30b, mi142-3p	Overexpression negatively impacts the ability to phagocytose bacteria, by regulating antigen presentation, resulting in low IFN-γ and TNF-α secretion
[32]	BALB/C	mi16	Up-regulated after LPS stimulation, significantly reduces TNF-α and IL-12p40 expression, thus suppressing inflammation
[42]	C57BL/6	mi29b	Up-regulated by TGF-β, causes dysfunction of alveolar macrophages due to a decrease in DNA methyltransferases and hypomethylation of COX-2 promoter, after bone marrow transplantation
[43]	BMDMs, C57BL/6	mi27a	Down-regulated after LPS stimulation (TLR2 & TLR4 engagement), increasing IL-10 and decreasing IL-1β, IL-6, IL-12, and TNF-α production, in order to restrain excessive inflammatory responses
[44]	HEK293T, RAW264.7, 3LL, C57BL/6	mi466l	Up-regulates mRNA and protein expression of IL-10, by binding to the IL-10 3'UTR, competing with tristetraprolin to prevent mRNA degradation and avoid excessive inflammation
[45]	THP-1, HEK-293T	mi146b	After LPS stimulation is induced by IL-10, leads ro reduction of pro-inflammatory cytokines and chemokines (IL-6, TNF-α, IL-8, CCL3, CCL2, CCL7 & CXCL10), by targeting the TLR4 signalling pathway
[46]	RAW264.7, THP-1	mi155	Expression inhibited by glucocorticoids through the suppression of NF-κB activation, decreasing the release of pro-inflammatory molecules TNF-α, IL-6, and NO
[48]	BMDMs, RAW 264.7	mi155	Induced by LPS, regulates indirectly the expression of cytokines and chemokines, both pro-inflammatory meadiators (IL1B, IL12, CCL5, CXCL10, CXCL11) and anti-inflammatory mediators like IL10
[49]	RAW 264.7, HEK- 293T, C57BL/6	mi125b	Down-regulated by LPS having a role in the post-transcriptional repression of TNF-α mRNAs in the absence of bacterial infection
[50]	BMDMs, C57BL/6, RAW264.7	mi155	IL-10 inhibits the expression of this miRNA in response to LPS stimulation and TLR4 engagement, in a STAT3-dependent manner at the transcriptional level (pri- and pre-miRNA)
[53]	RAW264.7, THP-1, BMDMs	mi21	After its induction, LPS decreases PDCD4, a tumour supressor, blocking NF-κB activity and promoting IL-10 production; it's a negative regulator of TLR4 signalling through the targeting of PDCD4
[57]	HEK-293, RAW 264.7, C57BL/6J	mi210	Up-regulated by LPS decreases IL-6 and TNF-a secretion and expression level of mRNA for iNOS, by targeting NF-κB and inhibiting its activation
[58]	RAW264.7, C57BL/6	mi34a	Down-regulated after LPS stimulation, decreases the expression of IL-6 and TNF-α and supress the activation of NF-κB
[60]	HEK-293, RAW 264.7, THP-1, C57BL/6	mi147	Up-regulated after LPS stimulation and TLR2/3/4 engagement, requiring NF-κB and IRF3 activation, decreasing pro-inflammatory cytokines release (TNF-α and IL-6)
[61]	RAW264.7	mi27a*, mi532-5p, mi146a, mi155	Down-regulation of miR-27a* & miR-532-5p and up-regulation of miR-146a & miR-155 after LPS stimulation, by targeting IL-10 and NF-κB
[79]	MDMs, THP-1	mi125-3p	Highly up-regulated compared to other miRNAs; inhibits the secretion of pro-inflammatory cytokines IL-6, IL-2, TGF-b, and TNF-a, by decreasing cell lipid uptake
[80]	BMDMs, RAW264.7, C57BL/6	mi181c, mi155, let7e	Akt1, activated by LPS, up-regulates let-7e (that represses TLR4 signalling) and mi181c, but down-regulates mi155 (that represses SOCS1 signalling), regulating endotoxin tolerance
[83]	RAW 264.7, HeLa, MEF-1	let7b	LPS and TLR4 induce its repression, that promotes the expression of IL-6 and IL-10; down-regulated in macrophages, in response to *Salmonella* and *E. coli* infection
[89]	BMDMs, C57BL/6	mi125a-5p	Up-regulated in response to TLR2/4 stimulation through MyD88, promoting M2 activation and repressing the production of M1 markers
[38]	BMDMs, C57BL/6	mi124	Down-regulated in microglia results in deactivation of M1 macrophages, by inhibiting TNF-α and iNOS and promoting TGF-β1, reducing inflammation
[39]	RAW 264.7, BMDMs	mi124	Overexpression induced by exposure to IL-4 and IL-13 causes down-regulation of M1 markers including CD86, iNOS, TNF
[24]	BMDMs	mi146a	Up-regulated after LPS stimulation, inhibits the activation of the TLR4-dependent signalling pathway and decreases the expression of both mRNA and protein expression IL-6

Table IV (c) – miRNAs involved in the inhibition of the activation of a M1-like phenotype in macrophages including references, model(s) used and main results obtained

The miRNAs involved in the inhibition of the activation of a M1-like phenotype in macrophages are: let7b, let7e, mi16, mi21, mi24, mi26a, mi27a, mi29b, mi30b, mi34a, mi124, mi125-3p, mi125a-5p, mi125b, mi132, mi142-3p, mi146b, mi147, mi210, mi155, mi181b, mi181c, mi223, mi466l, mi487b, mi532-5p and mi3473b.

mi21, as described earlier, is a negative regulator of TLR4 signalling through the targeting of PDCD4. This microRNA is up-regulated in many cancers, including lymphoma and leukaemia, since PDCD4 is targeted for degradation during tumour promotion [53].

mi27a is down-regulated in macrophages following LPS stimulation and TLR2 and TLR4 engagement, increasing IL-10 expression in order to prevent excessive inflammatory responses. Up-regulation of mi27a causes the expression of pro-inflammatory cytokines in macrophages activated by TLR2 and TLR 4, by diminishing IL-10 expression. Overexpression of mi27a enhances inflammatory response of macrophages after TLR4 stimulation, increasing the LPS-induced expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α. So, mi27a downregulation serves as an important negative regulating mechanism by which macrophages restrain an excessive inflammatory response [43].

Down-regulated after LPS stimulation, mi34a decreases the expression of IL-6 and TNF-α and supresses the activation of NF-κB. mi34a is known as a tumour suppressor by regulating inflammation [58].

Overexpression of mi24, mi30b, and mi142-3p negatively impacts phagocytosis of bacteria, by regulating antigen presentation, resulting in low IFN-γ and TNF-α secretion [31]. Upregulation of mi16 level in colonic macrophages significantly reduces TNF-α and IL-12p40 expression, which suppresses inflammation after LPS stimulation [32].

Increased TGF-β signalling, overproduced by alveolar epithelial cells after bone marrow transplantation, induces high levels of mi29b. TGF-β down-regulates the expression of DNA methyltransferases in alveolar macrophages, via mi29b-induced hypomethylation of the COX-2 promoter, resulting in enhanced COX-2 expression. This causes defective phagocytosis (for example, unable to phagocytize *P. aeruginosa*) and bacterial-killing response of macrophages, resulting in susceptibility to bacterial infections mortality for bone marrow transplantation patients. Inhibition of mi29b restores methylation, bacterial killing, and phagocytic function. Hypomethylation and increased COX-2 expression in response to TGF-β have also been demonstrated to happen in cancer [42].

Both mi132 and mi26a are involved in the inhibition of the activation of M1 macrophages during bacterial infection. Induced by *Mycobacterium tuberculosis*, mi132 and mi26a are negative regulators of the IFN-γ signalling cascade, impairing transcriptional, translational, and functional responses of macrophages

to IFN-γ. Induction of these miRNAs diminishes transcription of IFN-γ–induced genes and macrophage responsiveness to this key cytokine, contributing for bacterial survival [14].

Several microbial infections (such as Dengue virus infection) can up-regulate mi146a expression. Mycobacteria-induced mi146a represses iNOS expression and NO production in macrophages, which facilitates mycobacterial survival. mi146a suppresses NF-κB and MAPKs pathways by targeting TRAF6, thus inhibiting iNOS expression. Results from an experiment done by Li et al. show that the level of mi146a is higher after *Mycobacterium bovis* infection than the level in uninfected controls, suggesting that infection induces mi146a expression in murine macrophages [16].

mi125b binds to the 3′UTR region of TNF mRNA and destabilizes the transcript. Results show that macrophages incubated with *M. tuberculosis* induce high mi125b expression as well as low TNF production, since it leads to reduced translation and rapid degradation of TNF mRNA. In contrast, incubation with *M. smegmatis* induces low mi125b expression and high TNF production thanks to TLR2 recognition. These results are explained mi125b targets the 3′ UTR region of TNF mRNA transcript destabilizing it and inducing degradation [20].

Other studies show that LPS stimulation results in down-regulation of mi125b levels. mi125b acts as a post-transcriptional repressor of TNF-α mRNAs, hence the need for its down-regulation for proper TNF-α production. The high levels of mi125b in the absence of LPS is needed in macrophages to help ensure that the LPS pathway remains switched off in the absence of microbial infection. mi125b may use different mechanisms to down-regulate post-transcriptionally the expression of TNF-α, like repression of translation by targeting its 3'-UTR, de-adenylation of TNF-α transcripts, or both [49].

mi124 is reported to be involved in the inhibition of the activation of M1 macrophages. Exposure to IL-4 and IL-13 results in the up-regulation of mi124 in macrophages. Overexpression of mi124 causes down-regulation of M1 markers (CD86, iNOS, TNF) [39]. Down-regulated mi124 in microglia results in deactivation of M1 macrophages. mi124 expression inhibits TNF-α and iNOS but promotes TGF-β1, reducing inflammation [38].

Trials conducted on bone-marrow derived macrophages (BMDMs) and C57BL/6 models show that the up-regulation of mi125a-5p after TLR2/TLR4 induction requires the adaptor MyD88, repressing the production of M1 markers. mi125a-5p regulates phagocytic and bactericidal activities of macrophages [89]. Other studies also show that mi125a-3p is up-regulated in macrophages and decrease lipid uptake. Trials with a mi125a-3p inhibitor increased the secretion of pro-inflammatory cytokines IL-6, IL-2, TGF-β, and TNF-α [79].

mi142-3p expression is reduced in macrophages from aged mice, which are less responsive to modulation by TLR stimulation. Furthermore, IL-6 mRNA is a target of mi142-3p, suggesting that mi142-3p is a potential target for the

development of treatments for age-related inflammatory diseases. Inhibition of mi142-3p by histone deacetylation is involved in the increased production of IL-6 in response to LPS observed in aged mice. Down-regulated expression of mi142-3p contributes to the age-associated dysfunction of macrophages [27].

Expression of mi146a and mi146b respond to a variety of microbial components and pro-inflammatory cytokines, by base-pairing with the 3'UTR region of the TRAF6 and IRAK1 genes. Induced by LPS, these two microRNAs control TLR and cytokine signalling through negative regulation of IRAK1 and TRAF6 protein levels. So, IRAK1 and TRAF6 genes are targets for post-transcriptional repression by mi146a and mi146b. NF-κB plays a critical role in induction of transcription of mi146a by LPS, TNF, and IL-1β [22].

Other studies involving mi146 have similar results. The expression of mi146a is upregulated in macrophage in response to LPS stimulation in a dose- and time-dependent manner. In addition, mi146a mimics decreased, while mi146a inhibitor increased, the expression of IL-6, but did not affect TNF-α expression. Bioinformatics analyses predicted Notch1 as a potential target of mi146a. Moreover, mi146a overexpression in LPS-treated macrophages did significantly decrease Notch1 mRNA and protein levels. These results suggested that mi146a may function as a novel feedback negative regulator to LPS-induced production of inflammatory cytokines, at least in part, via inhibiting the expression of Notch1 [25].

mi146a decreases the expression of both mRNA and protein expression IL-6 in LPS-stimulated macrophages, acting as a negative regulator of production of pro-inflammatory cytokines. This miRNA is up-regulated after LPS stimulation, mi146a negatively regulates TLR4 and thereby inhibits the activation of the TLR4-dependent signalling pathway. mi146a has anti-inflammatory properties by attenuating the expression of inflammatory cytokines [24].

Also, higher expression of mi146b monocytes lead to a reduction in the LPS-dependent production of several pro-inflammatory cytokines and chemokines, including IL-6, TNF-α, IL-8, CCL3, CCL2, CCL7, and CXCL10. By direct targeting multiple elements involved in the TLR4 signalling pathway, this miRNA is able to prevent excessive inflammatory activity through enrichment in the RISC [45].

LPS induces the expression of mi155, and the maturation from its precursors (pri-mi155 transcription). Inhibition of mi155 or its maturation (resulting in accumulation of pri-mi155) causes LPS to induce the overexpression of inflammation mediators. mi155 regulates indirectly the extent of the response to LPS, specifically the expression of cytokines and chemokines. mi155 controls the expression of pro-inflammatory mediators (IL1B, IL12, CCL5, CXCL10, CXCL11) and molecules with an anti-inflammatory function like IL10 [48].

mi155 was the first oncogenic miRNA to be discovered. IL-10 inhibits mi155 induction by LPS and TLR4 engagement in a STAT3-dependent manner

5 at the transcriptional level. IL-10 can inhibit the generation of both pre-mi155 and pre-mi155. Overexpression of mi155 leads to B cell malignancies and myeloid leukaemia, by decreasing the expression of its target, an inositol phosphatase. TLR signalling can promote the pro-inflammatory response through the activation of PI3K, resulting in activation of MAPK and NF-κB. In this setting, IL-10 switches the pro-inflammatory response off [50].

mi155 expression is significantly increased in macrophages after *Mycobacterium marinum* infection, decreasing the production of NO and promoting bacteria survival. The up-regulation of miR155 after infection impairs bacterial clearance, by down-regulating NO production. These findings identify an evasion strategy from the immune responses in which bacteria regulate the host miRNA to inhibit pathogen killing [15]. Glucocorticoids, used as anti-inflammatory therapy, inhibit the expression of both pri-mi155 and pre-mi155 in LPS-induced macrophages through suppression of NF-κB activation. mi155 reverses the anti-inflammatory effect of glucocorticoids, by enhancing the production of TNF-α, IL-6, and NO. Since mi155 is overexpressed in TLR4-activated macrophages and becomes a contributor to inflammation, downregulation of mi155 can enhance the anti-inflammatory effect of glucocorticoids therapy [46].

Up-regulation of mi181b after LPS exposure contributes to down-regulation of IL-6, which might be an important regulatory mechanism for controlling pro-inflammatory immune response and induction of endotoxin tolerance [26].

mi210 inhibits LPS-induced production of pro-inflammatory cytokines, by targeting NF-κB in order to decrease its activation. mi210 decreases IL-6 and TNF-a secretion and expression level of mRNA for iNOS. After TLR4 activation by LPS, this miRNA targets molecules downstream to inhibit NF-κB activation and subsequent production of pro-inflammatory cytokines [57].

mi223 is an important regulator of macrophage activation during different inflammatory responses. In acute liver failure, the activation of Kupffer cells (liver macrophages) shows high pro-inflammatory activity in the initial phase since the expression of mi223 decreases. When mi223 expression finally starts to increase, it induces the anti-inflammatory response by favouring the M2 phenotype and decreasing IL-1β expression. Once again, mi223 levels drop and the pro-inflammatory response is established, as Arg1 levels decrease, IL-1β expression level increase and the M1 phenotype is promoted. Declined expression of mi223 contribute to pro-inflammatory activation of Kupffer cells, by enhancing the secretion of IL-1β. The drastic declining of mi223 at the later stage of acute liver failure might be one of the most important reasons that contributes to the long lasting pro-inflammatory response and ultimately leading to hepatocytes injury [17].

Down-regulation of mi223 promotes TLR-triggered production of pro-inflammatory cytokines IL-6 and IL-1β in macrophages by targeting the activation

of STAT3. In turn, IL-6 down-regulates mi223, thus promoting the augmented activation of STAT3 and facilitating the production of IL-6 and IL-1β, which forms a positive regulatory loop for pro-inflammatory cytokine production. This miRNA is then associated with the regulation of inflammatory responses in macrophages during microbial infection [28].

mi466l upregulates both mRNA and protein expression of IL-10 in TLR-activated macrophages. Binding of the 3'UTR of IL-10 prevents IL-10 mRNA degradation mediated by tristetraprolin, resulting in extended half-life of IL-10 mRNA and elevated IL-10 expression. The overexpression of mi466l leads to the formation of more RISC, that occupies the ARE of IL-10 mRNA, reducing the chance of tristetraprolin competitive binding and preventing mRNA degradation [44].

mi487b is a negative regulator of M1 macrophage activation by suppressing the levels of mRNA and protein for IL-33 during the differentiation of BMDMs. Exposure to LPS decreases mi487b expression, increases IL-33 transcript levels, and induces the production of pro-inflammatory mediators (iNOS, IL-1β, IL-6, and TNF-α) [30].

mi3473b, which is significantly down-regulated after IFN-γ stimulation, attenuates the classical activation of macrophages and the cytotoxicity of TLR-activated macrophages. mi3473b promotes IL-10 production and inhibits innate pro-inflammatory responses. The inhibition of this microRNA can promote pro-inflammatory cytokine production [13].

let7b repression is induced by LPS and TLR4 after infection. The down-regulation of let7b promotes the expression of IL-6 and IL-10 and is down-regulated in macrophages, in response to *Salmonella* and *E. coli* infections [83].

TLR4 signalling expressed by tumour cells triggers the release of microparticles, which transfers let-7b to macrophages and results in the down-regulation of the pro-inflammatory cytokine IL-6, attenuating tumour inflammation and progression [29].

mi27a* & mi532-5p are down-regulated and mi146a & mi155 are up-regulated in macrophages after LPS stimulation. The mi27a is known to target the innate immune response, including the inflammatory response, and the apoptosis process, involving IL-10 [61].

In vitro and *in vivo* studies conducted by Androulidaki et al. (2009) show that Akt1, a protein kinase activated by LPS, up-regulates let-7e (that represses TLR4 signalling) and mi181c but downregulates mi155 (that represses SOCS1 signalling) and mi125b. TLR4 and SOCS1 are key regulators of macrophage sensitivity to LPS, and their expression affects the magnitude of the response and the amount of pro-inflammatory mediators secretion. Akt1 is responsible for the inhibitory feedback loop, that controls macrophage hyperresponsiveness to LPS [80].

Ref	Model(s)	microRNA(s)	Main results
[12]	MLE-12, C2C12, EL4, H820, A549 & H441	mi133, mi133b	Induced during hyperoxia, supresses M-CSF expression that is induced by IL-10 and TNF, augmenting the susceptibility to tissue injury
[40]	THP-1, MDA-MB-23	mi720	Overexpression inhibits M2 polarization, by decreasing the production of IL-10 and CCL17, thus supressing cell migration and phagocytic activity
[33]	RAW 264.7, PMs, BMDMs	mi23a/27a/24-2 cluster	Down-regulated by M1 stimulation (LPS through the negative regulation of NF-κB activation) and up-regulated by M2 stimulation (IL-4 through STAT6 positive regulation)
[41]	THP1–155, HeLa	mi155	Induced by LPS and IFN-β, its overexpression decreases TGF-β signaling pathways & activation, with important effects on fibrosis, angiogenesis, and immunity
[23]	K562	mi142-3p	Down-regulated during human macrophage differentiation, by binding to the 3'UTR of Egr2 which acts as transcription repressor of the miRNA itself
[54]	HEK293T, C57BL/6, BALB/c	´mi142-3p	Overexpression represses M2 macrophages during cancer-promoted myelopoiesis, through the alteration of IL-6 expression and signalling, ultimately controlling tumour growth
[55]	THP1, HeLa	mi155	Up-regulation leads to a decrease in IL-13R protein levels and diminished STAT6 phosphorylation reducing the expression of anti-inflammatory cytokines IL-4 & IL-13
[86]	THP-1	mi130a	Up-regulated in M1 macrophages, promoting the release of pro-inflammatory cytokines and supressing PPAR-γ expression, also down-regulating M2 macrophage activation

Table IV (d) – miRNAs involved in the inhibition of the activation of a M2-like phenotype in macrophages including references, model used(s) and main results obtained

The miRNAs involved in the inhibition of the activation of a M2-like phenotype in macrophages are: mi130a, mi133, mi133b, mi142-3p, mi155, mi720, and the cluster mi23a/27a/24-2.

mi130a is up-regulated in M1 macrophages, also down-regulating M2 macrophage activation. Down-regulation of mi130a predicts a poor prognosis in cancer patients since it leads to cancer cell proliferation and metastasis [86].

M-CSF presents anti-apoptotic and anti-injury actions, involving mi133 and mi133b. M-CSF mRNA is expressed by stimulation with IL-1β or TNF, but inhibited during hyperoxia, augmenting the susceptibility to tissue injury [12].

Exposure of human monocytes to CSF1 (important for macrophage differentiation) induces a decrease in the expression of mi142-3p. mi142-3p is down-regulated during human macrophage differentiation, by binding to the 3′UTR of Early Growth Response (Erg) 2 transcription factor mRNA and leading to low expression of Egr2 protein. Egr2, which acts as a transcription repressor of mi142-3p gene, is highly expressed upon CSF1, inhibiting mi142-3p expression, thus promoting early macrophage differentiation [23].

Other studies also demonstrate that mi142-3p down-regulation promotes macrophage differentiation and determines the acquisition of their immunosuppressive function in tumours. mi142-3p is critical for preventing macrophage differentiation during tumour-induced myelopoiesis. mi142-3p overexpression favours anti-tumour immune response controlling tumour growth. mi142-3p controls the generation of M2 macrophages during cancer-promoted myelopoiesis, affecting the regulation of the immunological activity of TAMs. mi142-3p modulates macrophage differentiation through the alteration of IL-6 expression and signalling [54].

Results show that overexpression of mi155 altered the cellular responses to TGF-β by changing the expression of genes that are involved in inflammation, fibrosis, and angiogenesis. Induced by LPS and IFN-β, mi155 regulates the ability

of myeloid cells to respond to TGF-β, with important effects on fibrosis, angiogenesis, and immunity. An increase in mi155 can significantly reduce the levels of TGF-β in macrophages [41].

Other authors report that mi155 is involved in the regulation of the M1/M2 balance in macrophages by modulating IL-13 effects. mi155 up-regulation blocks the translation of the IL-13Rα1, leading to diminished activation of STAT6. mi155 affects the IL-4- and IL-13-dependent phosphorylation of STAT6. mi155 levels modulate the response of macrophages to IL-13, leading to a change in their genetic profile. mi155 favours the M1 phenotype for macrophages reducing the expression of anti-inflammatory cytokines [55].

Overexpression of mi720 inhibits M2 polarization, by inhibiting the production of M2 associated cytokine, IL-10 and chemokine, CCL17. This miRNA is down-regulated in TAMs since it supresses cell migration and phagocytic activity [40].

The mi23a/27a/24-2 cluster is up-regulated by M2 stimulation. Overexpression of mi23a/27a/24-2 promotes the expression of pro-inflammatory cytokines, negatively regulating M2 polarization. mi23a and mi27a inhibit M2 polarization by targeting JAK1/STAT6 and IRF4/PPAR-γ, respectively, thus repressing M2-associated transcription factors. The mi23a/27a/24-2 cluster is decreased in TAMs, and overexpression of the cluster in macrophages suppresses tumour growth *in vivo*. This cluster might be functionally important for the regulation of macrophage polarization and balancing the M1/M2 macrophage ratio in the TME [33].

Effect on macrophages	microRNAs involved
Inhibition of M1 polarization	let7b, let7e, mi16, mi21, mi24, mi26a, mi27a, mi29b, mi30b, mi34a, mi124, mi125-3p, mi125a-5p, mi125b, mi132, mi142-3p, mi126a, mi146a, mi146b, mi147, mi155, mi181b, mi181c, mi210, mi223, mi466l, mi487b, mi532-5p & mi3473b
Inhibition of M2 polarization	mi130a, mi133, mi133b, mi142-3p, mi155, mi720 & the mi23a/27a/24-2 cluster
Promotion of M1 polarization	mi26a-2*, mi29b, mi101, mi125a, mi 125b, mi130a, mi144, mi155, mi455, mi615-3p, mi1246m & the mi23a/27a/24-2 cluster
Promotion of M2 polarization	mi21, mi98, mi124, mi125a-5p, mi130a-3p, mi142-5p & mi511
TAM activation and promotion of tumour progression	mi21, mi29b, mi34a, mi130a, mi142-3p, mi155, mi720 & the mi23a/27a/24-2 cluster

Table V – miRNAs effects on the activation or promotion of the activation of macrophages phenotypes M1 and M2

Graff et al. (2012) are the authors of the only article found that concerns the role of miRNAs in the activation of the five macrophage phenotypes (M1, M2a, M2b, M2c and M2d). While mi193b expression increases in MDMs exposed to M2a polarizing conditions, mi27a*, mi29b-1*, mi132*, and mi222 increase in MDMs exposed to M2b polarizing conditions. mi222* expression is increased in both M2a and M2b polarized MDMs. mi155* is induced by M2b conditions, which are induced by LPS. mi26a-2* and mi29b-1* were also induced in M2b conditions. Transfection with mi155 and mi29b mimics induces macrophage expression of IL-6 and TNF, characteristically expressed during M2b polarization [81].

Given the multiple interactions between miRNA and 3′ UTRs, and the existence of multi-miRNA regulation of a single target, the identification of multiple interactions that together explain the polarization of macrophages was expected.

4.2. DATA ANALYSIS

The data compiled highlights the complexity of the molecules networks that are involved in the process of the activation of the immune response and its link to diseases. The results in the Table II show that microRNAs do not have a clear function on the manipulation of macrophage polarization. miRNAs send signals that promote or repress macrophage activation and require a thorough study.

To date, the study of miRNAs in macrophages has been largely focused on the expression and function of miRNAs associated with M1 macrophage activation and their expression associated with M2 macrophage polarization received considerably less attention [37]. This review has identified 45 miRNAs that are involved in macrophage polarization, after revising the cited articles (53 references). Of the 45 miRNAs identified, 29 miRNAs were identified as involved in the inhibition of M1 polarization, 7 miRNAs were identified as involved in the inhibition of M2 polarization, 14 miRNAs were found to be involved in the promotion of the M1 phenotype of macrophages and 6 miRNAs were found to reported in the inhibition of M2 phenotype activation.

The tables show that there are clearly more miRNAs involved in M1 macrophage activation and less miRNAs involved in M2 polarization. There is a lot more information available about the miRNAs network involved in M1 macrophage activation, maybe due to the public-health interest interplay, since these macrophages are linked to several common inflammatory diseases.

The results identify mi124, mi146a, mi155, and mi142-3p, as the miRNAs, that mostly influence macrophage activation, with the highest number of appears in the used references.

In particular, mi155 has been extensively studied in the context of macrophage polarization and inflammation. Its expression levels largely increase due to M1 activation of macrophages. whereas in M2-polarized macrophages mi155 levels are strongly decreased. Interestingly, mi155 has a role in inhibiting

or promoting M1 macrophage polarization since its overexpression induces a re-education of macrophages. mi155 suppresses the activation of NF-κB, decreasing the release of pro-inflammatory molecules like TNF-α, IL-6, and NO [46]. mi155 is down-regulated by Akt1 and IL-10 and up-regulated by LPS, IFNs and TLRs, in macrophages, enhancing TNF-α translation and decreasing TGF-β signalling pathways & activation, with important effects on fibrosis, angiogenesis, and immunity [49,50,52,80]. Its up-regulation leads to a decrease in IL-13 receptor protein levels and diminished STAT6 phosphorylation reducing the expression of anti-inflammatory cytokines IL-4 and IL-13 [55]. mi155 is up-regulated after *M. marinum* infection, decreasing the production of NO and promoting bacteria survival as a mechanism for evasion to immune responses [15]. This particular miRNA shows a complex interaction with macrophages, being involved in the promotion and inhibition of M1 polarization but also in the inhibition of M2 macrophages.

mi124 stands out as the miRNA more influencing on M2 polarization of macrophages. mi124 functions both as an inhibitor of M1 activation and as a promoter of M2 polarization. mi124 is capable of inhibiting M1 markers such as CD86, TNF-α and iNOS and promoting M2 markers CD206 and TGF-β1, reducing inflammation. Treatment with mi124 may have a beneficial effect by restricting cell proliferation [38,39].

The miRNA more associated with the inhibition of M1 polarization was mi146a. Enforced expression of mi146a in peritoneal macrophages (PMs) resulted in reduced levels of M1-marker genes (for example, iNOs, CD86, TNF, IL-12 and IL-6), and increased production of M2-phenotype markers (like Arg1, CCL17, CCL22 and CD206). In contrast, mi146a knockdown promotes M1 macrophage polarization and inhibits M2 macrophage polarization [76]. mi146a is up-regulated after LPS stimulation and inhibits the activation of the TLR4-dependent signalling pathway, decreasing the expression of both mRNA and protein expression IL-6 [24]. This miRNA represses iNOS expression and NO generation, by suppressing NF-κB and MAPKs pathways since it targets TRAF6, thus facilitating bacterial surviving [16].

mi142-3p is linked to inhibition and promotion of M1 polarization and inhibition of M2 polarization of macrophages. It is down-regulated by histone deacetylation leading to functional impairment of macrophages and age-related inflammatory diseases, by the increased production of IL-6 [27]. mi142-3p negatively impacts phagocytosis of bacteria, by regulating antigen presentation and inducing low IFN-γ and TNF-α secretion [31]. Its overexpression represses M2 macrophages during cancer-promoted myelopoiesis, through the alteration of IL-6 expression and signalling, ultimately controlling tumour growth and progression [54].

The phenomenon of different miRNAs converging at the level of phenotype regulation while diverging at the mechanistic level suggests that a multi-miRNA approach to miRNA-based therapies may be advantageous to prevent loss of efficacy due to redundancy and/or to achieve a phenotypic profile better tailored to the treatment of specific disease parameters. This approach would need to be

based on a thorough understanding of how each miRNA functions alone and together at the level of coordinated network regulation [7].

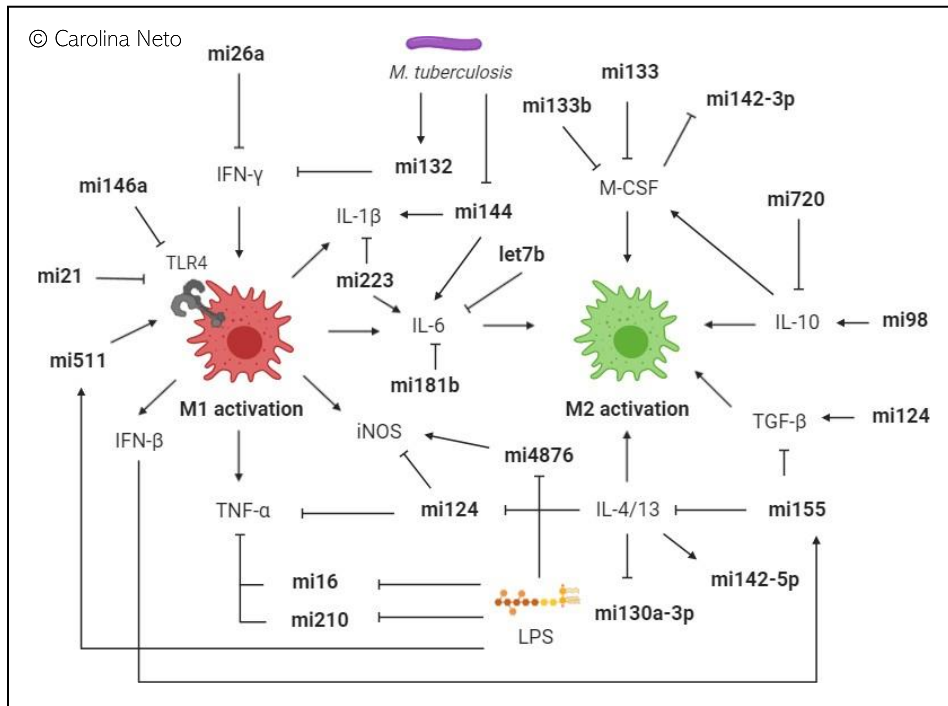

Figure 17 – Selected combining evidence of the complex miRNAs involvement in macrophage polarization towards the M1- and the M2-like phenotype including key mediators *(original illustration)*

Collectively, these results identify unique and complex miRNA profiles in the activated phenotype of macrophages. There is a clear link between miRNAs and macrophage activation. Genome-wide studies profiling transcriptional and epigenetic modifications reveal profound dynamic changes at gene loci associated with macrophage polarization, resulting in the coordinated action of distinct signalling pathways and transcription factors. The identification of specific subsets of miRNAs differentially expressed under distinct polarizing conditions ultimately leads to knowledge of the impact of miRNA deregulation in macrophage polarization [76].

5. Discussion

Since this field of study is so complex and lacks information, there are a few misconceptions in the subject. Given that macrophage polarization is controlled by the interlocking pathways of extrinsic factors, intrinsic factors, and the tissue environment, the molecular pathways involved in macrophage polarization are complex and still poorly defined [70].

The different origin of macrophages itself creates some doubts on functional distinction. It is not fully understood how much this populations differ from each other and how they contribute to the different stages of the inflammatory response. The future of monocyte-derived macrophages is also a wondering question. Although it is known that they undergo apoptosis, there is some debating around their contribution to the immune memory or even to the tissue-resident macrophage population [75]. The relationship between the origin of macrophages and their activation state and subsequent functions is also unclear.

Even though, most articles report days of duration of the macrophage response, the reality is different. In some cases, recruitment, repair, and resolution are rapid taking only minutes or a few days for instance in minor cuts or even toxin-induced muscle damage. By contrast, the immune response to schistosome eggs lodged in the liver and its accompanying Th2 response, which drives M2 polarization of macrophages, takes months to years to eliminate the eggs and restore normal liver architecture. Nonstop inflammation such as cancer occurs over months to years to decades, causing macrophages to adopt whatever activation signals they receive across such wide time ranges. Also, the recruitment of other immune cells causes dynamic changes in polarization that are difficult to assess from a single endpoint experiment taken after months of inflammatory disease [70].

The first dualistic model of macrophage activation presented by Mills in 2000 is obviously oversimplistic, since the variables that affect polarization are themselves heterogenous, as well as time and tissue dependent. Macrophage polarization is far too multidimensional to be boxed in the M1/M2 model. Macrophage functions are not limited to make "stop" or "go" signals, as the 'kill or repair" notion mentioned [70]. The macrophage lineage includes a variety of cells with different functions and functional states that are specified by the complex interplay between microenvironmental signals and a complex polarization mechanism that determines the macrophage phenotype [72]. Exposure of macrophages in culture to inflammatory stimuli induces, with different kinetics, both proinflammatory and anti-inflammatory mediators. Some genes are activated early and transiently, while others are induced at later time points, up to 24h post-stimulation [48].

For instance, multiple extrinsic factors contribute to M1 polarization, such as IFN-γ and its receptor combined with TLR and IL1R signalling, as well as TNF. The precise contribution of each factor to M1 polarization is unclear and probably varies given the inflammatory context.

The links between IL-10 and macrophage polarization are also poorly defined. IL-10 is produced in many settings from different immune cell types and it is an essential and irreplaceable anti-inflammatory factor. IL-10 is required to suppress all forms of inflammation and it is mediated by numerous pathways. In macrophage activation, IL-10 production seems to be graded depending on the polarization status: M1 macrophages make IL-10, but M2 macrophages make more IL-10. IL-10 has many additional functions in macrophage biology, often overlooked: IL-10 increases the amount of IL-4R chain on the cell surface, making macrophages more sensitive to IL-4 and IL-13, and thus more sensitive to being polarized into M2 macrophages.

Although technology has played a vital role in understanding macrophage polarization, numerous questions remain. The specific roles of hallmark factors elicited in M1 and M2 macrophages in well-characterized models of pathophysiology are not defined, as well as the specific M1 and M2 factors and their roles in humans as opposed to rodents. How macrophage polarization is modulated in specific cellular contexts remains unanswered. The roles of polarized macrophages in different tissues and the different functions between resident and recruited macrophages are both unclear. It is also needed to further investigate the relationships between metabolism at the cellular and tissue levels and polarized macrophages [70].

Therapeutic approaches not originally designed as macrophage oriented or specific have been found to affect macrophage activation and polarization. These off-target examples provide insights and lessons for the development of more specifically directed approaches [65].

Recently, there have been several studies in which investigators have successfully shown that the inflammatory pathway can be diverted from a pro-inflammatory to an anti-inflammatory path by utilizing the pharmacological application of various natural analogues [69]. However, the knowledge acquired until now about immunotherapy is mainly derived from *in vitro* experiments using limited ligands, which does not fully capture the functional diversity of macrophages *in vivo* [64]. Macrophages not only depend on the type of stimulating agent, but also on its concentration and time of exposure, which are difficult to replicate [75]. Most researches done on macrophage polarization use simple *in vitro* techniques. So, macrophages derived from *in vitro* cultures in survival cytokines (CSF-1 and GM-CSF, that are essential for the maintenance of normal macrophage numbers) are stimulated with M1 or M2 polarizing agents. M1 polarization typically involves IFN-γ with a TLR agonist, like LPS. M2 polarization involves stimulation with IL-4 or IL-13. This approach is designed to mimic the exposure of macrophages to polarized CD4+ T cells producing their distinctive cytokine combinations (for example, IFN-γ from TH1, or IL-4 and IL-13 from TH2). *In vitro* polarization via extrinsic signals has the advantages of simplicity, short time windows, and cost effectiveness ratio. The disadvantages are the lack of realism. The macrophage target population is not necessarily representative of the macrophages found in the *in vivo* environment, and few if

any *in vitro* settings mirror the tidy constraints of *in vivo* polarization. In addition, there is significant interlaboratory variation in the experimental setup and an inconsistency in the used markers. GM-CSF and CSF-1 also block apoptosis by enhancing the expression of proteins that block the mitochondrial intrinsic death pathway. Thus, in conditions of inflammation, such as the tumour microenvironment, incoming macrophages are bathed in survival cytokines, increasing both their longevity and their ability to be polarized. Even though M2 macrophages are associated with poor outcomes in cancer and several products of M2 macrophages, such as Arg1, are known immunosuppressive factors, it has not been tested in a systematic way [70].

Given the important roles of TAMs in orchestrating tumour progression, targeting TAMs offers a novel approach to improving anti-tumour therapy. Although depletion of TAMs can be an attractive approach and delay tumour progression, whether only immunosuppressive myeloid cells are targeted remain unclear. Likely, pro-immunity cells will also be depleted, causing severe adverse effects, such as bacterial infections. Hence, complete deletion approaches for macrophages are not feasible in the context of cancer [106].

Even though macrophages are essential immune effectors, some pathogens can use these cells to survive and thrive [75]. Some pathogens can exploit changes in macrophage physiology and use macrophages for intracellular growth [88]. Metchnikoff himself described how some pathogens can survive apoptosis and grow within the cytoplasm of macrophages, being able to spread [71]. *Mycobacterium tuberculosis* is also able to infect macrophages, replicate and persist inside the cell, causing chronic infections [75]. Many bacterial pathogens actively invade host cells or become intracellular after ingestion by macrophages. During infection, intracellular pathogens extensively manipulate the signalling and gene expression cascades of the host for survival and replication. Some DNA viruses encode miRNAs and utilize the host machinery to regulate either host or viral mRNAs for their own benefit [83]. So, it is normal to find tumour cells being able to modulate macrophage regulation by miRNAs in order to benefit its own proliferation.

miRNA expression is affected by a cross-talk between signalling pathways that are simultaneously activated during the polarization of different macrophage phenotypes. Thus, variations in miRNA expression can result from cross-talk between signalling cascades initiated by different external stimuli. Given the complex variability of macrophage activation, the involvement of miRNAs gives the cell a logical means of translating a great number of external stimuli into a spectrum of responses. Adding to this complexity, multiple miRNAs can contribute to the expression of one transcript and one miRNA can simultaneously modify the expression of many genes. When it comes to modulation of miRNA expression towards immunotherapy approaches, the subsequent effect on the expression of target and off-target genes needs to be taken in consideration [81].

Since mi155 and mi125b are key regulators of M1 macrophage polarization, they are great targets for immunotherapy approaches that focus in TAM phenotype reprogramming.

TAMs phenotype has been linked to a M2-like phenotype, but they can, in fact, exhibit phenotypes anywhere in between tumoricidal M1 type and pro-tumoral M2 type [101]. Some studies link the spatial distribution of macrophages in the TME with their polarization. The majority of macrophages located in tumour islets present a M1-like phenotype, while macrophages located in the tumour stroma have a M2-like phenotype. So, TAMs phenotype may be linked to the secreted molecules by the tumour itself since macrophage phenotype in the TME depends on tumour distance [96]. For instance, IL-10 promotes the differentiation of monocytes in M2c macrophages and blocks the differentiation in dendritic cells. This interleukin is very tightly correlated with pro-tumoral and anti-immunity functions of TAMs. Since TAMs produce large quantities of IL-10 but low quantities of TNF-α, TAMs phenotype may be related to the M2c macrophage phenotype [94,98]. However, TAMs phenotype may also be linked to the M2b macrophage phenotype, since TAMs produce high levels of IL10 and low levels of IL-12. The M2d phenotypes has also been linked to TAMs due to their expression of high levels of VEGF and IL-10 [98]. The role of immune cells like macrophages in cancer progression depends on spatial distribution, density, and interaction with other cells in the TME [96].

Blocking macrophage recruitment into tumours, inhibiting pro-tumour polarization, or directly promoting M1 macrophage activation have all been used successfully in preclinical models to enhance the response to cytotoxic therapy. The question remains which of these approaches will be the most effective when combined with cytotoxic, targeted, or immune checkpoint blockade therapy. It is critical to understand whether depletion, or instead repolarization, is the best therapeutic approach to accompany combination therapy, for which tumour types, and at which stage of tumour progression (primary or metastatic disease). It is also important to evaluate the durability of the resultant anti-tumour immune responses formed [67].

Molecular and cell-biological details involved in promoting metastasis might be more complicated than expected. Various major points of regulation networks remain elusive [101]. The fact that malignant cells likely already reside in secondary metastatic niches long before the clinical presentation of malignant primary disease shows that this therapeutic approach needs to be evaluated carefully. The development of macrophage-directed therapeutics aiming to minimize or eradicate metastasis first requires the identification of the clear pathways that drive neoplastic cell survival, proliferation, angiogenesis, and immune suppression. Nevertheless, it remains uncertain whether macrophages are important in mediating therapeutic resistance at metastatic sites or even the degree to which they are involved in mediating metastatic outgrowth. Since most patients succumb to metastatic disease, this is an urgent area of research that has been largely unexplored, in part because of experimental obstacles [67].

The complex network of chemokines present at the tumour site can play a role in the induction of adaptive immunity, recruiting TAMs and monocytes. Some chemokines may enhance specific host immunity against tumours, but other chemokines can contribute to immune evasion [94]. Some ligands can bind to multiple receptors and vice-versa, but the binding affinities of ligands to receptors are largely different. The expression of ligands and receptors is spatially and temporally regulated, which means each ligand-receptor pair selectively regulates the positioning of immune cells for host defence and immunity. For example, CCL2 production that recruits TAMs to the tumour site differs between tumour types. Solid tumours use chemokines and their receptors to accomplish successful metastasis. Blocking chemokine signalling should be carefully considered as a strategy to prevent malignant tumour development by disrupting the accumulation of TAMs on the tumour site and preventing the formation of lethal metastatic tumours. However, chemokine signalling can also switch in response to environmental changes caused by chemotherapy and hormonal treatments, making it a less reliable method for immunotherapy approaches [95].

The mechanisms of resistance to therapies are mediated not only by genetic mutations and therapy-resistant tumour clones but also by the TME, which allows tumour cells to escape from the toxicity of chemotherapy, survive, and become resistant [102]. The TME influences the response to therapy and outcome in cancer. The metabolic changes in the TME alter the phenotype and function of immune cells, contributing to the failure of the current immunotherapy approaches. A major goal of immunotherapy is to overcome the immunosuppressive effect of the tumour itself [97]. For example, immunotherapy methods like vaccines in patients with established and advanced tumours do not have the desired effect, as it is difficult to achieve a continued activation of the immune system, due to the very immunosuppressive TME [93]. The use of metabolism-targeting drugs could offer new opportunities to improve cancer immunotherapies [97].

TAMs can limit the efficacy of chemotherapy either directly by adhesion-dependent mechanisms that involve direct contact between macrophages and tumour cells (juxtracrine mechanisms) or adhesion-independent mechanisms through the secretion of soluble products (paracrine mechanism); or indirectly by modulating the immune system [102]. TAMs in tumours treated with chemotherapeutic agents are frequently responsible for chemoresistance. Cancer cells treated with chemotherapy are more susceptible to the cytotoxic effect of macrophages. On the other hand, TAMs hamper the efficacy of chemotherapeutic drugs by increased recruitment of immuno-suppressive cells, suppression of adaptive anti-tumour immune responses and activation of anti-apoptotic programs in cancer cells [105]. The reactive oxygen and nitrogen intermediates (ROS and RNS) generated by TAMs contribute to cancer-cell genetic instability and tissue damage that also limits the effectiveness of chemotherapy and targeted therapies [6].

Chemotherapy-induced metastasis is also an emerging concept in the treatment of cancer. The molecular mechanisms behind the pro-metastatic phenotypes induced by chemotherapy are triggered as a stress response to the cytotoxic effects of chemotherapy. The increase of TAMs following chemotherapy is mostly the result an increased expression of chemotactic agents known to recruit macrophages (including CSF1, CXCL12, and CCL2), which are often up-regulated in tumour cells in response to cytotoxic chemotherapy [102].

Even though immunotherapy is emerging as a promising approach on cancer treatment, long-term clinical benefits are not achieved in most patients. The overlapping nature of many metabolic pathways calls for caution in target selection and drug design. Better understanding of metabolic regulation provides new avenues to improve cancer immunotherapy. Novel platforms that can deliver metabolic reprogramming agents in a cell type-specific manner will be necessary to achieve selective targeting [97].

A major challenge of TAM repolarisation are also off-target inflammatory responses. To prevent systemic inflammation targeting strategies can be adopted to selectively reach TAMs or the TME. For instance, antibodies directed against upregulated antigens could be linked to nanoparticle-encapsulated molecules or directly conjugated to small molecule TLR ligands. Lastly, conjugating multiple TLR ligands together could be an attractive strategy to potentiate TAM reprogramming [87].

TAMs do not exert functions in isolation; the TME is a complex system consisting of a plethora of cells other than TAMs, such as fibroblasts, epitheliums, neutrophils, mesenchymal stem cells, myeloid cell-derived suppressor cells, and mast cells. All of the cells involved are tightly linked and interacted with each other constantly. Preclinical experiments targeting TAMs without considering the intricacy and versatility in their interactions are prone to fail in future effective therapeutic approaches in the clinic [101]. Because there are complex intercellular interactions involving TAMs in the TME, targeting TAMs may trigger multifaceted stromal reactions in the TME that are difficult to predict and may vary from patient to patient. [106] Also, many of the studies use murine models that may not represent the reality of human cells. For example, markers commonly used to identify murine-activated macrophages, such as Arg1 and iNOS, do not exhibit similar up-regulation of homologs in humans [68].

Since TAMs adapt their polarization in order to overcome obstacles that tumour cells meet while in the process of the metastatic dissemination, future therapies must consider the limiting effect of TAMs on chemotherapy success.

In clinical practice, pathology reports do not routinely describe TAM features in tumour samples, making it difficult to identify potential TAM-target beneficiaries and creating a gap in knowledge between the clinic and tumour immunology research. Hence, figuring out TAM-related features, such as density, phenotypes, and cytokine profiles on pathology reports, or even assessing circulating M2 macrophage numbers as well as systemic CSF1 and CCL2 levels

might provide a tool for better predicting cancer progression. Further studies are needed to evaluate their therapeutic effectiveness both as a single agent or as part of a combination therapy [101].

In addition to an overview of the immune context of the tumour, the contribution to immune responses of other host specific factors (such as genetics, nutrition and individual microbiota) must be first determined as they interplay with the immunotherapeutic agents [93,97]. Tumours with different histological types and gradings, different genetic background, as well as diverse local inflammatory profiles, might have heterogenous responses to the same treatment [101].

The past decades have witnessed significant advance in the understanding of tumour immunology and the development of immunotherapeutic drugs. Sadly, there is still need for advances in technology, such as an unmet requirement for bioinformatics platforms and deep-learning algorithms that can assist scientists with analysing massive datasets [93].

TAM represents a novel and attractive target that may alter the landscape of future cancer therapy, although many critical obstacles are still lying ahead, and more studies need to be done.

6. Conclusions and future perspectives

Macrophages possess a broad array of cell surface receptors, intracellular mediators, and essential secretory molecules for recognition and destruction of invading pathogens and tissue repair. Macrophages participate in the inflammatory process by adapting their functional phenotype according to microenvironmental cues. Plasticity confers them the ability to coordinate host defence mechanisms to eliminate pathogens and re-establish homeostasis [76]. Macrophages are abundant in diverse tissues and organs where they function as immune effectors, immune regulators and/or tissue remodelling coordinators. External stimuli can cause macrophages to undergo a dramatic and coordinated change in expression of multiple gene products, changing the functional state of the cell [81].

It is necessary to understand macrophage diversity and clearly define their phenotypes according to anatomical location and function, and according to the regulation of the set-points that define types of macrophages. Macrophage biology in humans is poorly developed because of the technical limitations of obtaining fresh material for fluorescence-associated cell sorting (FACS) and the over-reliance of functional and genomic studies on cell lines such as the myelomonocytic leukemic cell line THP1 or the *in vitro* differentiation of circulating monocytes by CSF1 [9].

Progress has been made in defining the molecular networks underlying polarized activation of macrophages. miRNAs have emerged as regulators of phagocyte activation and function, but their role in macrophage polarization needs to be better defined [65]. Although miRNA biology is not fully understood, the knowledge gathered so far, and the progress made in the development of strategies to block or enhance miRNA activity, have propelled the therapeutic promise of these small RNAs. Taken together, these data highlight miRNAs as potential therapeutic targets for improving immune responses in oncological patients [42].

Correlating the up-regulation and down-regulation of miRNAs with the apoptosis process, inflammatory response, cytokine production is important for further understanding of the immune response. However, few of these targets and regulatory pathways have been experimentally validated, the functional confirmation of them will be the further focus [61].

Significant progress has been already made in identifying the role of some miRNAs in regulating macrophage polarization and plasticity. miRNAs, as important mediators of the immune regulatory machinery, have answered the question of how macrophages are able to respond to stimuli in such a dynamic fashion. miRNAs represent an important mechanism for altering macrophage function without requiring changes in gene transcription. Identifying the complete repertoire of direct miRNA targets in different cell types and diseased tissues will prove extremely valuable in employing miRNA therapeutics with higher confidence. Furthermore, the multi-functional role of macrophages in initiating

and resolving inflammation makes it a very attractive therapeutic target for many types of disease, such as cancer. Continued progress in the identification of miRNAs, along with descriptions of their complex regulatory properties, both in the context of disease and macrophage function, brings scientists closer to the dawn of a new class of therapeutic agents [7].

One of the hallmarks of malignancy is the repolarization of TAMs from a pro-immunity M1 phenotype to an immunosuppressive M2 phenotype. The two distinct subsets, which coexist in tumours, adapt to the changing TME, and can be re-educated by immunoregulatory cues. This event is interesting for therapies development, with the aim of skewing TAMs to an M1-like phenotype. Nonetheless, only a few molecules have been identified to orchestrate this process thus far [86]. Since M1 macrophage tumour infiltration is normally related to a good prognosis, the total depletion of macrophages in the TME may not be such a good idea [87].

TAMs are able to promote immunosuppression, angiogenesis, invasion, intravasation, migration, metastasis, and colonization [102]. It is obvious that macrophages play a significant role in cancer progression, and immunotherapies involving macrophages should be considered in the treatment of this disease. The polarization of macrophages towards an M1 response with minimal side effects may prove to be a powerful therapy against solid tumours [8].

Although macrophages have been discovered a century ago and revolutionized the immunology research, much more is still remaining to explore in macrophage biology and its role in diseases like cancer, that may influence immunotherapeutic approaches [73]. Besides TAMs, other subpopulations of macrophages have been identified like CD169+ macrophages and TCR+ macrophages. The list of macrophages subpopulations is still growing and there is a lot of investigation to be done [75].

Identification of pathways shared by cancer cells and immune cells like macrophages will allow the selection of appropriate metabolism-targeting drugs as potential immune modulators [97].

Since recent advances in immunotherapy are encouraging, it is critical to continue to explore strategies that will expand treatment options and optimize clinical outcomes and patient survival rates. The latest findings reinvigorate the pursuit for cutting-edge approaches that take advantage of the potential of macrophages as stimulators of targeted anti-tumour immune responses. Further studies on patient samples are required for the clinical application of immunotherapeutic approaches.

Further work is needed to identify substances and protocols that can adeptly re-educate the immune system to attack cancer cells, prevent angiogenesis and metastasis, and to protect the host from developing a damaging inflammatory response [8].

There is also the necessity of broadening communication between, and collaboration among, research groups that focus on TAM repolarization immunotherapy approaches.

The sum of pre-clinical animal and correlative human studies suggest that targeting TAMs could significantly improve the efficacy of immunotherapeutic approaches. However, in spite of the clinical interest and a few suggestive early trial outcomes, the optimum therapeutic approach has yet to be identified. This may be partially due to a lack of data on key clinical parameters that may determine the success or failure of therapeutic approaches. Other questions include what type of approach (depletion or reprogramming) should be employed, and whether the best therapeutic modalities might be cancer type dependent. Finally, it will be critical to determine how exactly to employ these therapeutic approaches, including data driven considerations of dosing strategies and sequencing to minimize potential toxicities and maximize immune stimulatory properties. The challenge will be to empirically design trials rather than base them on historical doses, clinical practicality, or financial considerations. In spite of these challenges, there remains significant potential to harness macrophage biology to improve outcomes for cancer patients.

7. References

The illustrations in this review are originals and were created thanks to Biorender, online illustrator from Canada.

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