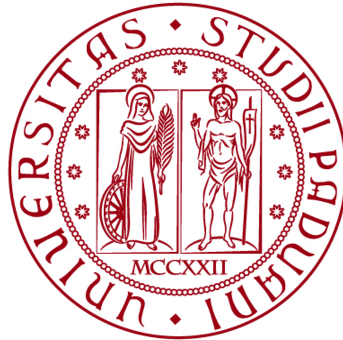
UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Biologia Sanitaria



TESI DI LAUREA

Investigating the role of the mitochondrial chaperone TRAP1 in protumoral polarization of macrophages

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ANNO ACCADEMICO 2023/2024

ABSTRACT

Macrophages are immune cells present in all tissues that can be found also in different tumors with diverse functions. Tumor microenvironment can educate them toward proneoplastic functions, characterized by proangiogenic and proinvasive features that are driven by specific macrophage metabolic alterations. In cancer cells, such as Malignant Peripheral Nerve Sheath Tumor cells (MPNSTs), the mitochondrial chaperone TRAP1 can contribute to metabolic regulation through succinate mediated stabilization of HIF1α that induces protumoral gene transcription. However, the role played by TRAP1 in macrophage metabolic regulation is still unknown.

This project aims to investigate whether TRAP1 has a role in macrophage polarization, thus enhancing their protumoral functions. The use of mouse bone-marrow derived macrophages has revealed that they undergo a proneoplastic polarization when exposed to MPNST cell media, as assessed by measuring phenotypic and functional features. Importantly, the onset of these changes is reduced when TRAP1 expression is knocked out in macrophages. Succinate administration rescues the protumoral functions of these cells, suggesting the presence of a mechanistic link between TRAP1 and HIF1α that is crucial for the proneoplastic functions of macrophages. These findings indicate that the chaperone TRAP1 is a crucial regulator of macrophage metabolism, paving the way for novel therapeutic approaches aiming at hitting macrophages in the MPNST microenvironment.

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List of abbreviations

AGM	Aorta-gonad-mesonephros
AKT	Serine/Threonine kinase
ARG1	Arginase 1
BMDM	Bone marrow derived macrophages
CD206 or MMR	Macrophage mannose receptor
CM	Conditioned media
cNF	Cutaneous neurofibroma
CSF1R	Colony stimulating factor 1 receptor
CypD	Cyclophilin D
DMS	Dimethyl succinate
ECM	Extracellular matrix
EMP	Erythromyeloid progenitors
EMT	Epithelial to mesenchymal transition
ENO1	Enolase 1
ERK1/2	Extracellular regulated kinase 1/2
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
Grb2	Growth factor receptor-bound protein 2
GS	Glutamine synthetase
HIF1α	Hypoxia inducible factor 1 alpha
HK2	Hexokinase 2
HRE	Hypoxia responsive elements
HSC	Hematopoietic stem cells
HSP (75/90)	Heat shock protein (75/90)
IDO	Indoleamine 2,3 dioxygenase
IFNγ	Interferon gamma
IL-	Interleukin-
iNOS	Inducible nitric oxide synthase
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
MAPK or MEK	Mitogen-activated protein kinase
M-CSF or CSF1	Macrophage colony stimulating factor
MCT	Monocarboxylate transporter
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MPNST	Malignant Peripheral Nerve Sheath Tumor
mTOR	Mammalian target of rapamycin
NF1	Neurofibromatosis type 1
Nf1	Neurofibromin
NK	Natural killer
NO	Nitric oxide

OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PDGF	Platelet-derived growth factor
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol biphosphate
PIP3	Phosphatidylinositol trisphosphate
PKM2	Pyruvate Kinase M2
pNF	Plexiform neurofibroma
PTEN	Phosphatase and tensin homolog
PTP	Permeability transition pore
RAF	Serine/Threonine kinase
RAS	Rat sarcoma virus guanosine triphosphatase
RAS-GAP	RAS GTPase activating protein
ROS	Reactive oxygen species
RPLP0	Ribosomal protein lateral stalk subunit P0
RTK	Receptor tyrosine kinase
RTM	Resident tissue macrophages
SC	Schwann cells
SDH	Succinate dehydrogenase
SOS	Son of Sevenless, guanine nucleotide exchange factor
SUCNR1	Succinate receptor 1
TAM	Tumor associated macrophages
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alfa
TP53	Tumor protein 53
TRAP1	Tumor necrosis factor receptor associated protein 1
Th1/2	Helper T lymphocytes 1/2
Treg	Regulatory T lymphocytes
UDP-GlcNAc	N-acetylglucosamine
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau

1 INTRODUCTION

1.1 Macrophages

Macrophages are cells of the innate immune system with diverse functions, from the primary immune response to tissue homeostasis. They are highly plastic cells exerting a role during infections, thanks to their pathogen killing and antigen presentation capacities, but also in the subsequent restoration of healthy environment through wound healing and tissue remodeling processes.[1] Macrophages were first identified at the end of the 19th century by Elie Metchnikoff as phagocytic cells involved in development and inflammatory responses.[1,2] Similar phagocytic cells have been found in many phylogenetic groups, including invertebrates and mammals[3]. In the latter, macrophages can be distinguished into **resident tissue macrophages** (RTMs) and **monocyte-derived macrophages**, with different developmental origins. At the level of the embryo, all of them derive either from the yolk sac or from the aorta-gonad-mesonephros (AGM) region, both of them originating from the mesoderm and giving rise to a lineage of progenitors.[4] From the yolk sac, the erythromyeloid progenitors (EMPs) emerge in two distinct waves: the first one produces the primitive macrophages, which remain only to form the definitive microglia, while during the second one EMPs migrate to the fetal liver, where they expand and mature into fetal liver monocytes.[4,5] These are able to colonize fetal tissues where they become RTMs, a population of long-lived and self-renewing cells[5] (examples are Kupffer and Langerhans cells)[3,4]. The other lineage emerges from the AGM within the embryo, to form the hematopoietic stem cells (HSCs).[4] These cells migrate and proliferate in the fetal liver, and, after proper maturation, move to the bone marrow, where they permanently reside. Here, HSCs can self-renew but they also participate in the hematopoietic process as they are the progenitors of different blood cell lineages, such as erythrocytes, lymphoid cells and myeloid cells[3]. Bone marrow derived monocytes are circulating, short-lived cells that are continuously replenished from HSCs.[4] For example, during infections new monocytes are produced and reach a target tissue, where they eventually differentiate into macrophages to exert their functions. Despite their origins, a major common lineage regulator has been identified, the cytokine macrophage colony stimulating factor (M-CSF, or CSF1), as well as its receptor CSF1R.[2]

As previously mentioned, macrophages intervene in infections or injuries, as they are recruited from the circulation to the damaged tissue, where they initially assume a proinflammatory phenotype. This is fundamental to initiate the immune response by activation of their killing mechanisms (for example phagocytosis) and the secretion of proinflammatory mediators to attract other cells such as T lymphocytes. Resolution of the inflammatory process requires a reparative step that is orchestrated by macrophages. To this aim, they shift their phenotype toward an anti-inflammatory one, thus promoting wound healing, tissue repair and homeostasis.[2] This double nature of macrophages is commonly referred to as M1/M2 polarization.

1.1.1 M1/M2 polarization

Polarization can be defined as the "morphological, functional and phenotypic differentiation of macrophages under the action of different microenvironmental signaling *in vitro* and *in vivo*".[6] In tissues, macrophages are normally in a resting state defined naïve or M0 state. Upon stimuli from the environment, they undergo a metabolic reprogramming and they can assume either the M1 state, which is proinflammatory, or the M2 state, anti-inflammatory.[6] (see ***Fig. 1.1***)

1.1.1.1 <u>M1 polarization</u>

The **classically activated** (M1) macrophages are proinflammatory and antitumoral and this state can be activated upon stimulation with interferon-gamma (IFN-γ), bacterial moieties such as lipopolysaccharide (LPS), toll-like receptor (TLR) activation[6] and also TNFα (tumor necrosis factor).[1] M1 macrophages have a role in host recognition and killing, tumor resistance and promotion of inflammatory response. They produce agents such as ROS (reactive oxygen species) and NO (nitric oxide) to kill pathogens, but also proinflammatory cytokines to engage a more robust immune response.[6] Released cytokines comprehend TNFα, interleukin 1 (IL-1) and IL-6 to facilitate inflammation[6], IL-12 to promote Th1 differentiation and consequent antigen phagocytosis, and IL-23 to recall Th17 for inflammatory response[1]. They also express complement factors and Major Histocompatibility Complex (MHC) I, thus facilitating complement-mediated phagocytosis[1], and MHC II to perform antigen presentation to T lymphocytes[7]. Secretion of chemokines (CXCL9, CXCL5, CXCL10) is fundamental to recruit other immune cells such as T lymphocytes and Natural Killer (NK) cells that will aid in pathogen killing.[1] This polarization state can be recognized through the expression of a typical marker, inducible nitric oxide synthase (iNOS), which converts arginine into citrulline and NO, the latter being used to enhance macrophage killing capacities.[6]

1.1.1.2 <u>M2 polarization</u>

Conversely, the **alternatively activated** (M2) macrophages are anti-inflammatory and protumoral: this state can be induced by several cytokines, such as IL-4, IL-10 and IL-13[6], and by environmental cues such as hypoxia[8] and lactate[6]. The most important physiological functions of M2 macrophages are immunomodulation, immunosuppression and tissue remodeling[6]; however, in the tumor microenvironment they favor angiogenesis and neoplastic growth.[8] Markers of M2 polarization are the enzyme arginase 1 (ARG1) and the scavenger receptor CD206.[6] ARG1 converts arginine into ornithine, promoting collagen and extracellular matrix (ECM) production that are functional to the wound healing process.[1] M2 macrophages also synthesize anti-inflammatory cytokines such as IL-10, which favors immunomodulation and tumor progression, and promotes Th2 secretion of IL-4 and IL-13; transforming growth factor β (TGFβ), which deepens immunosuppression by inhibiting NK cell activity and enhances tumor cell invasiveness; chemokines such as CCL24, CCL17, CCL22 that recruit Th2 cells, further promoting the anti-inflammatory response.[1]

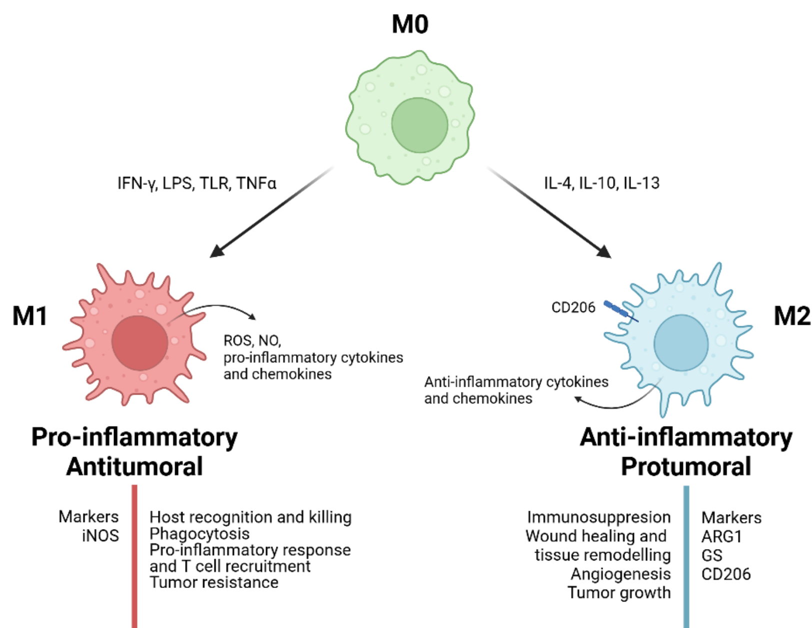

Figure 1.1 - Overview of macrophage polarization. *In this scheme, M1/M2 polarization is summarized, depicting the principal drivers, markers and functions of the two states. - Created with BioRender.com*

1.1.2 TAMs: tumor associated macrophages

As the name suggests, **tumor associated macrophages** (TAMs) are present within various neoplasms and they represent the most abundant immune cells in the tumor microenvironment (TME).[6,9] TME consists of ECM, blood and lymphatic vessels, various cells among which endothelial cells, fibroblasts and immune cells such as macrophages and lymphocytes.[1,9] Typically, TAMs are bone marrow derived macrophages[9] (BMDM) that assume an M2-like phenotype, as they support tumor growth and suppress the immune response.[10] However, it has been shown that during the early phases of neoplastic formation, macrophages adopt an M1-like phenotype, mediating a proinflammatory and antitumor response, while in later stages, the TME is able to induce an M2-like polarization in TAMs.[8,11] This progression from proinflammatory to protumoral phenotype is possibly driven by the accumulation of cancer cell byproducts, such as lactic acid, whose formation depends on the so-called Warburg effect (enhanced glycolysis independently of oxygen supply[12]), and by hypoxia, typically found in tumors because of the abnormal vasculature and poor perfusion of these masses.[8,13] TAM polarization not only changes over time, but analysis of many tumors has revealed that different TAMs distribute in distinct areas of the TME. In hypoxic regions, TAM recruitment is enhanced, predominantly with an M2-like phenotype[11], whereas M1-like TAMs

generally distribute in less hypoxic areas[14]. Indeed, proangiogenic TAMs accumulate in perivascular regions, where they can interact with endothelial cells to enhance vessels formation[11,15]. Protumoral TAMs are also prevalently found at the invasive margins of tumors, helping in the epithelial to mesenchymal transition (EMT) and metastatic processes[15]. These differential temporal and spatial distribution of TAMs in the TME underline the high diversity and complexity of these immune cells and the numerous functions that TAMs can exert in this context.

Albeit being useful, the classic dichotomic categorization of M1 and M2 macrophages only represents the extremes of a continuum of polarization states[10,11], where M1 macrophages assume proinflammatory functions while M2 are considered anti-inflammatory and capable of sustaining tumor growth[1]. Indeed, in an *in vivo* situation TAMs are exposed to and integrate a plethora of stimuli that drive their polarization process[13], resulting in different TAM populations[9] that occupy all the polarization spectrum, from M1-like to M2-like TAMs, including also TAMs expressing both M1 and M2 typical markers[11]. However, TME signals most commonly induce an M2-like polarization of TAMs[8], thus skewing these cells to tumor sustaining functions.

All polarization states are tightly linked to metabolic changes in macrophages, that in turn sustain and drive the polarization process[13]. Cues in the TME are able to induce the activation of specific metabolic programs causing a consistent metabolic rewiring in TAMs[13], that in turn determines specific TAM functions, such as the proangiogenic and proinvasive functions exerted by proneoplastic TAMs[1]. The specific TAM features that are determined by this metabolic rewiring are fundamental to shape the TME itself[13]. Therefore, there exists a strong interplay between environmental cues, metabolic programs and TAM functions to maintain their protumoral activity and allow tumor progression.

1.1.2.1 TAM metabolism

One of the main drivers of macrophage polarization is tumor hypoxia: low oxygen can be sensed by macrophages, promoting the stabilization of HIF1α (hypoxia inducible factor 1 alpha), a transcription factor that elicits the induction of various metabolic enzymes. Also lactic acid[16] and succinate that can derive either from the TME or from the macrophage metabolism can be responsible for HIF1α stabilization, fostering several TAM metabolic adaptations spanning from enhanced glucose utilization to lipid accumulation (see ***Fig. 1.2***).

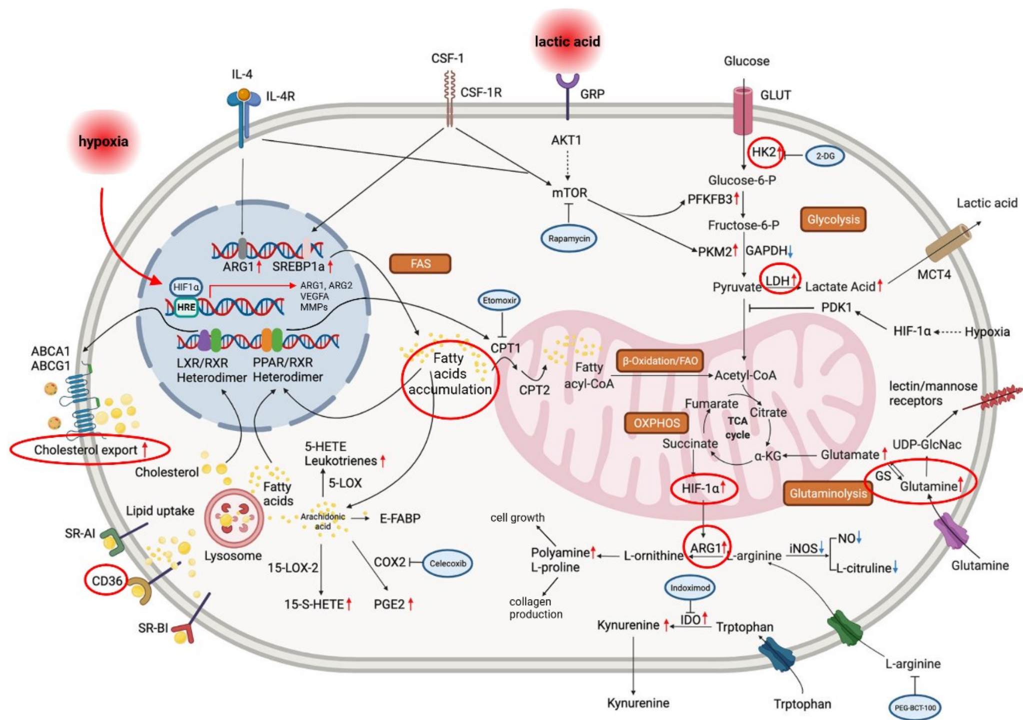

Figure 1.2 - TAM metabolism. *Lactic acid and hypoxia (red clouds) induce a protumoral polarization of TAMs. Hypoxia stabilizes HIF1α, which promotes the transcription of proneoplastic genes. In red circles, main upregulated pathways are evidenced: glycolysis (with hexokinase 2, HK2, upregulation) leads to pyruvate production, which is converted into lactic acid (lactate dehydrogenase, LDH, upregulation). Glutamine synthetase (GS) and glutamine are increased, together with Arginase 1 (ARG1), a typical M2 enzyme that ultimately promotes collagen production and cell growth. Fatty acid accumulation and cholesterol export are also increased. – Modified from (13).*

Glucose metabolism

To fulfil their energetic demand, macrophages adopt different strategies based on their functional state. M1 macrophages generally rely on aerobic glycolysis for rapid energy extraction, which seems to be useful for fighting infections, as these M1 cells are part of the innate immune response and immediately intervene after an infection. Indeed, they require large amount of rapidly available energy to exploit their high bactericidal and phagocytic potential[17], the latter through the formation of an autophagosome containing the pathogen, later sent to the lysosome for degradation[7]. On the other hand, M2 macrophages prioritize the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS) for a more efficient ATP production.[13] In TAMs, glycolytic enzymes like hexokinase 2 (HK2), enolase1 (ENO1), pyruvate kinase M2 (PKM2) are upregulated[10] and pyruvate is redirected to be converted into lactate.[8] TAMs metabolism is thus shifted toward aerobic glycolysis, with an increased glucose uptake, but it still retains an intact OXPHOS activity (M2-like), as proved by the normal oxygen consumption rate (OCR)

measured in TAMs.[13] In hypoxic conditions, TCA cycle and OXPHOS can be fueled by lactate present in the TME[18], that can be imported through monocarboxylate transporters (MCT)[10], and converted into pyruvate. This in turn is oxidized to acetylCoA which ultimately enters the TCA cycle[18]. This mixed behavior in glucose utilization is typical of hypoxic TAMs, and it highlights the intermingled M1/M2 nature of these cells.

Amino acid metabolism

Amino acids are the building blocks of proteins, but they also serve as precursors for metabolite production. An example is constituted by arginine, which can be found in the TME where it is useful for stimulating T cells and NK cells cytotoxicity[6], but it can also be imported in macrophages (thus competing with T cells[13]) for production of molecules such as NO and citrulline or to take part in the urea cycle. Arginine catabolism is used to distinguish between the M1 and M2 macrophages: M1 upregulates iNOS which converts arginine in NO and citrulline, while M2 express ARG1 that mediates the production of urea and ornithine.[10] NO and citrulline may have antitumoral functions:[13] NO is important for antimicrobial activity and to mediate the production of reactive nitrogen species, while citrulline is an intermediate for the restoration of arginine levels, to further fuel NO production. On the other hand, M2-derived ornithine is used as a substrate for production of polyamines, small molecules involved in various processes such as DNA replication, cell growth and immune reactions. Ornithine is also important for the synthesis of proline, essential for collagen production, thus assuming a role in ECM remodelling.[1] High levels of ARG1 have been found in many TAMs, consistent with their M2-like polarization and their functions in tumor progression.[1]

Glutamine metabolism is another key determinant of macrophage polarization: high levels of glutamine synthetase (GS), the enzyme catalyzing the condensation of glutamate and ammonia to form glutamine, sustain M2 polarization, whereas the absence of this enzyme skews macrophages toward an M1 state[19]. High glutamine is thus essential for protumoral polarization and increases in both GS and glutamine are observed in TAMs. Glutamine can support ATP production through the anaplerotic conversion into α-ketoglutarate[7], that fuels the TCA cycle, but its predominant role in TAMs is for the synthesis of N-acetylglucosamine (UDP-GlcNAc), a molecule required for glycosylation in endoplasmic reticulum and branching reactions in Golgi.[13] The glycosylation process involves also scavenger receptors[13] such as CD206, also known as MMR (macrophage mannose receptor), which is a c-type lectins receptor and considered another M2 marker.[2]

Another interesting amino acid used in immunoregulation is tryptophan, which can be catabolized by indoleamine 2,3 dioxygenase (IDO) in the kynurenine pathway, leading to kynurenine production with a consequent immunosuppressive action. In TAMs, high levels of IDO and kynurenine have been reported, coherently with the immune downregulation function of these cells.[10]

Lipid metabolism

Fatty acid metabolism is important in lipid homeostasis and dichotomic subdivision identifies fatty acid synthesis (FAS) as an M1 feature, while fatty acid oxidation (FAO) as belonging to M2 macrophages. The hallmark for TAMs is lipid accumulation[13], which can be achieved in different ways. On the one hand, there is the increase in fatty acid uptake from the environment by scavenger receptors (CD36) and the enhancement of FAS due to the high levels of acetylCoA. On the other hand, this accumulation is also functional to the use of lipids as a carbon source in FAO and ATP production, in an M2 fashion[10], thus resulting in a complex equilibrium in lipid utilization for TAMs. However, cholesterol accumulation can suppress protumoral functions, and its efflux is strongly favored in TAMs.[10,13]

1.1.2.2 Succinate in TAMs

Succinate is an intermediate of the TCA cycle that is normally oxidized into fumarate by succinate dehydrogenase (SDH). This enzyme is also known as the complex II of the mitochondrial respiratory chain and it couples fumarate production to ubiquinone reduction into ubiquinol, allowing electron transport to proceed. Succinate is an oncometabolite that inhibits α-ketoglutarate dependent dioxygenases[20], eliciting a variety of transcriptional and epigenetic effects that enhance tumor growth, cancer cell migration and invasion, but it has also a role in TAM metabolism[21]. Succinate is mainly described as a proinflammatory metabolite, as its accumulation has been demonstrated to prompt proinflammatory genetic programs in LPS-activated macrophages through HIF1α stabilization[22]. Indeed, an increase in succinate oxidation by SDH drives proinflammatory polarization of macrophages, whereas an SDH inhibition increases succinate levels and shifts macrophages toward an anti-inflammatory state.[22] Another line of evidence has shown that TAMs can be polarized toward an M2-state by extracellular succinate released by cancer cells. This metabolite binds to the receptor SUCNR1 on the macrophage membrane and drives the activation of the PI3K/AKT pathway that ultimately leads to HIF1α activation and consequent transcription of M2 markers (such as ARG1).[21] SUCNR1 has been found to be upregulated in M2 macrophages and its activation can polarize these macrophages toward this anti-inflammatory phenotype.[23] Based on the different context, succinate can thus favor the proinflammatory M1 polarization of macrophages or it can assume an anti-inflammatory role when SDH is inhibited (M2 polarization). Furthermore, extracellular succinate present in the TME can skew macrophage polarization toward the M2 state through SUCNR1 activation.

1.1.2.3 TAM functions

As previously observed, distinct metabolic changes establish roles and functions of TAMs, in general facilitating tumor growth and metastasis formation (see ***Fig. 1.3***). HIF1α is a major driver of the immunosuppressive and proangiogenic TAM phenotype.[13]

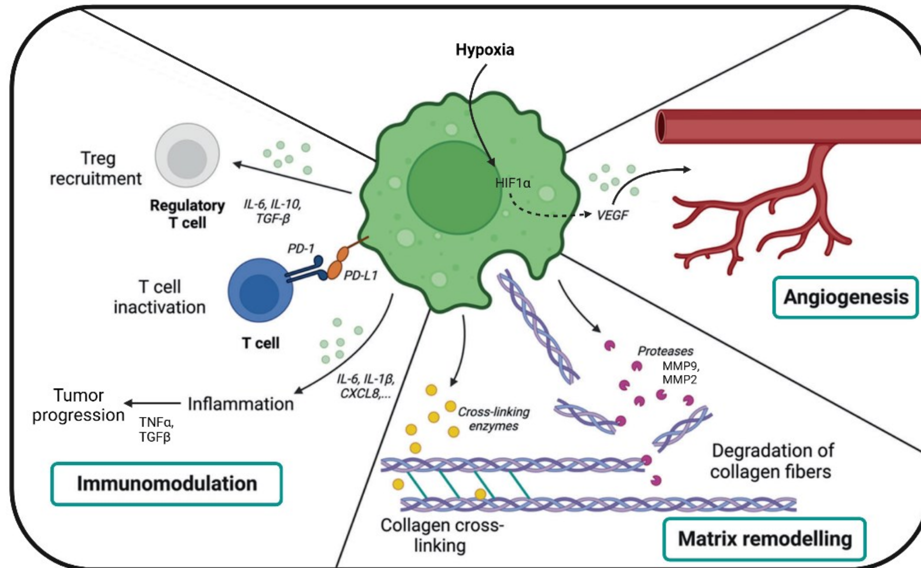

Figure 1.3 - TAM functions. *Some of TAM functions are depicted in the Figure: immunomodulation through inflammation, T_{reg} recruitment and T cell inactivation to sustain tumor growth; matrix remodeling through degradation of collagen (matrix metalloproteinases release, MMPs); angiogenesis through VEGF secretion. – Modified from (9)*

Immunomodulation

TAMs are able to operate an immunomodulation in the TME. They can promote inflammation through cytokine release, promoting an inflammatory state that could eventually lead to increased genetic instability in cancer cells.[8] At the same time, tumor-related inflammation can be useful for tumor growth itself, as in the case of the TAM-secreted TNFα, a proinflammatory cytokine that promotes cancer cell proliferation and survival through NF-kB pathway. Also TGFβ can exert a protumoral activity by enhancing invasion[1,7] and by promoting recruitment of regulatory T cell (T_{reg}), which in turn deactivate cytotoxic T cells.[9] Furthermore, TAMs can directly inhibit cytotoxic T cells and NK cells activity through the PD-L1/PD-1 immune checkpoint inhibitors[7,9] and through ARG1-mediated TCR (T cell receptor) unresponsiveness[1], due to a drop on arginine levels that impairs TCR assembly and signaling. The creation of a permissive environment is fundamental to foster tumor growth and metastasis formation, also through the realization of other processes such as angiogenesis and ECM remodeling.

Angiogenesis

The formation of new vasculature, known as angiogenesis, is an important feature to guarantee oxygen and nutrient supply to cancer cells, and TAMs are key players in this process.[8] Hypoxia drives HIF1α stabilization in TAMs, in turn activating the expression of proangiogenic genes such as those encoding for key proangiogenic

factors (VEGF-A, PDGF, TGFβ). Hence, TAMs contribute to angiogenesis initiation and to the maintenance of this process, thus sustaining tumor growth[1].

ECM remodeling
In tumors, ECM represents a structural scaffold that provides tracks for angiogenesis as well as for tumor cell invasion, the starting point for tumor intravasation and metastasis formation. ECM can be remodeled under hypoxic conditions by the activity of a variety of proteases.[8] TAMs express different types of such enzymes, such as **matrix metalloproteinases** (MMPs, with MMP2 and MMP9 being among the most important) and cathepsins, to degrade the ECM, in particular collagen fibers, which then undergo turnover and degradation in lysosomes.[9] MMP contribution is also important during angiogenesis, as these proteinases are able to degrade ECM and "create space" for new vessel formation.[7] The ECM remodeling is the initial step of metastasis: together with EMT, another process stimulated by TAMs in which cancer cells assume invasive and migratory properties, cancer cells can intravasate in the circulation and disseminate to distal organs, where permissive metastatic niches can develop.[9]

Due to their contribution in tumor progression, TAMs are now examined as possible immunotherapeutic target for future antineoplastic treatments. Analysis of the metabolism can be useful to deeply understand TAM features and functions. Furthermore, this kind of investigation can reveal one or more metabolic regulators that can act as key factors in TAM protumoral polarization, thus opening the way for novel TAM targeting systems. In this perspective, one possible novel target could be the mitochondrial chaperone TRAP1.

1.2 TRAP1

TRAP1 is the mitochondrial paralogue of the HSP90 chaperone family. It is also called heat shock protein 75 (HSP75) and it is encoded by the *TRAP1* gene located on chromosome 16p13.[24] Chaperones are proteins responsible for the correct folding of other proteins, termed clients. Both TRAP1 and HSP90 are defined foldases, for their ATP-dependent role in folding nascent or unfolded proteins, thus contributing to cellular proteostasis.[25] Despite TRAP1 high homology with HSP90, they do not share the same functions: HSP90 is a cytosolic protein fundamental to guarantee homeostasis, whereas TRAP1, although normally exerting its foldase role, is not essential and its ablation does not impair cell viability.[25] In contrast, TRAP1 is involved in regulating mitochondrial metabolic changes and adaptations under stress conditions, during which an increase in its expression and activity has been observed. Moreover, this upregulation is also present in many cancer cell types and it seems to sustain neoplastic progression.[24]

1.2.1 Structure and physiological function

In its active form, TRAP1 is a homodimer composed by two protomers, each characterized by three distinct domains: an N-terminal domain (NTD) with ATP-binding and hydrolytic activity and a mitochondrial import sequence (cleaved after reaching the mitochondrial matrix)[25]; a middle domain (MD) forming the client binding site and collaborating in the ATP-pocket formation; a C-terminal domain (CTD) encompassing the dimerization interface.[24] (***Fig. 1.4-A***) The catalytic cycle starts from the open "apo" state, in which two ATP molecules can bind to the protomers causing a shift to the closed state characterized by an asymmetric conformation. The hydrolysis of the first ATP promotes client remodeling while the hydrolysis of the second one causes its dissociation, followed by TRAP1 return to the open state.[24] (***Fig. 1.4-B***)

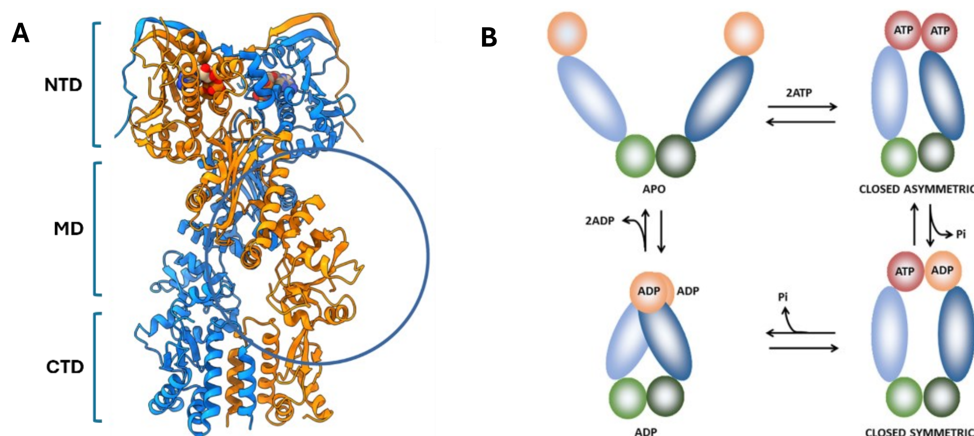

Figure 1.4 - TRAP1 structure and function. A) *Representation of TRAP1 structure, with the two protomers (orange and blue) and the different domains (N-terminal, NTD; middle, MD; C-terminal, CTD). The circle indicates the catalytic site.*

***B)** Graphical representation of TRAP1 catalytic cycle to perform client remodeling. Image (A) modified from (24), (B) from (26).*

1.2.2 TRAP1 in tumors

In neoplastic conditions, TRAP1 is active and plays an important role in regulating mitochondrial metabolism, interacting with a variety of different proteins (see ***Fig. 1.5***).

First, it works as a negative regulator of mitochondrial respiration, since it favors a shift toward aerobic glycolysis (Warburg effect), decreasing OXPHOS and enhancing glucose uptake. Indeed, TRAP1 can downregulate complex IV (cytochrome *c* oxidase) and complex II (succinate dehydrogenase, SDH)[24,25] of the electron transport chain. SDH normally oxidizes succinate into fumarate, a reaction in which electrons are transferred to ubiquinone *via* FAD, thus enabling the electron transport chain to proceed.[24] This enzyme is at the crossroads of two distinct metabolic pathways: it participates both to OXPHOS and TCA cycle, thus inhibition of SDH impairs both processes.

Second, TRAP1 plays an antioxidant and prosurvival role in tumor conditions. Downregulating OXPHOS leads to a decrease in ROS formation, and this can contribute to create favorable conditions for cancer cell survival. Moreover, the prosurvival action is exerted through the inhibition of Cyclophilin D (CypD), an isomerase that contributes to the PTP (permeability transition pore) opening when a cell is primed for cell death. Upon TRAP1 interaction with the PTP, CypD cannot perform its death-inducing action, and cancer cells can continue to proliferate.[24]

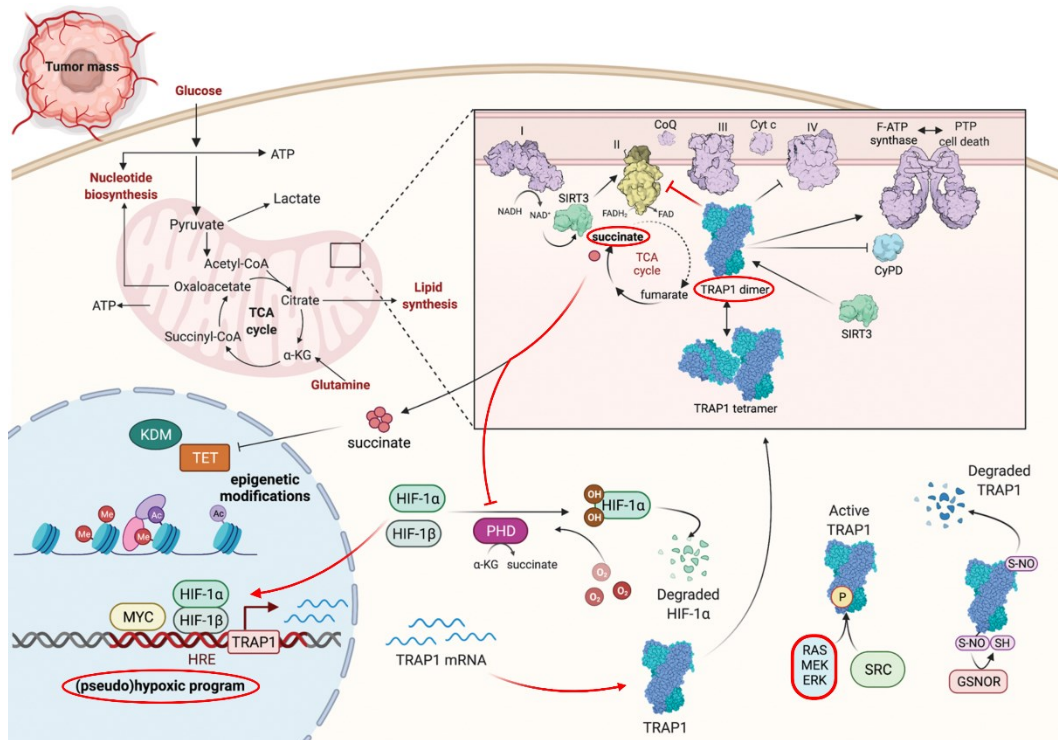

Figure 1.5 - TRAP1 in cancer. *In neoplastic conditions, TRAP1 exerts various roles: the most important pathway for the formation of a pseudo-hypoxic environment is highlighted in red. Active TRAP1 inhibits SDH (succinate dehydrogenase) provoking succinate accumulation, which in turn stabilizes HIF1α. In the nucleus, HIF1α triggers transcription of hypoxic genes, including TRAP1 itself (HRE region in the promoter). On the bottom right part, activation of TRAP1 is mediated by RAS-MEK-ERK pathway activation. – Modified from (24).*

1.2.2.1 TRAP1 and the pseudo-hypoxic phenotype

Succinate accumulation following SDH inhibition by TRAP1 is involved in the induction of a pseudo-hypoxic phenotype. Indeed, succinate can flow to the cytoplasm where it inhibits prolyl hydroxylase (PHD), an enzyme of the α-ketoglutarate-dependent hydroxylase family that is responsible for the proteasomal-dependent degradation of HIF1α.[24]
Under normoxia, the constitutively expressed HIF1α is first hydroxylated by the O_2-sensitive PHD, then it is targeted and ubiquitinated by the VCB complex that comprises the von Hippel-Lindau (VHL) and the Elongin C and B proteins, and eventually it is recognized and degraded by the proteasome.
Under hypoxic conditions, HIF1α is stabilized because PHD is unable to work in the absence of oxygen; so, HIF1α can enter the nucleus and promote the transcription of different genes involved in the response to hypoxia.
Even in the presence of oxygen, succinate is able to block PHD (acting as a competitive inhibitor of α-ketoglutarate), preventing HIF1α degradation and hence inducing a hypoxia-responsive transcriptional program even under normoxic conditions, thus determining a pseudo-hypoxic phenotype. In the nucleus, HIF1α can bind DNA in the HRE (hypoxia responsive element) regions, present at the level of the promoter.[20] Interestingly, TRAP1 promoter possesses HREs and its transcription can be induced by HIF1α itself. A feedforward loop exists between TRAP1 that stabilizes HIF1α, and HIF1α that triggers TRAP1 expression. The pseudo-hypoxic condition is typical of neoplastic environments, where induction of the HIF1α-dependent transcriptional program stimulates cancer cell invasiveness and angiogenesis.[24]

1.2.2.2 Modulation of TRAP1 activity

The TRAP1 chaperone activity can be consistently modulated by post-translational modifications. One example is protein phosphorylation that is performed by ERK1/2, kinases that are part of the RAS signaling cascade. TRAP1 phosphorylation increases its activity, further contributing to the metabolic shift explained so far.[24] RAS pathway is often dysregulated in different tumors, and its hyperactivation can contribute to the subsequent enhancement of TRAP1 functions. A strong example of this can be found in a tumor known as MPNST (Malignant Peripheral Nerve Sheath Tumor).

1.3 MPNST

MPNST is an aggressive and invasive cancer that represents around 10% of all soft tissue sarcomas. Morphologically, it appears as a growing soft mass associated with peripheral nerve roots and bundles and often invading nerves themselves or surrounding structures. Histological analysis highlights the presence of alternating hypocellular and hypercellular regions within MPNST, but also areas of necrosis, high mitotic activity and hyperchromatic nuclei of spindle cells.[27] Due to this particular cell shape, it is also classified as a spindle cell sarcoma with a neural crest origin. The strong proximity to nerve networks often hinders the possibility of surgical resection, thus chemo- and radiotherapy become the only available options, but standardized successful treatments are currently lacking.[27] Around 50% of all MPNST cases arises from malignant transformation of plexiform neurofibromas (pNF) in patients affected by the tumor-predisposing syndrome Neurofibromatosis type 1 (NF1), while the other half occurs sporadically or sometimes in association with radiotherapy.[27]

1.3.1 NF1-related MPNST

NF1 is an autosomal dominant disease caused by a germline inactivating mutation in the *NF1* gene, encoding the tumor suppressor protein **neurofibromin**.[28] This is a RAS-GAP, meaning a GTPase activating protein: when RAS is bound to GTP (active form), neurofibromin can promote RAS GTPase activity, thus leading to RAS-GDP formation (inactive) and to the switching off of the RAS signaling pathway. Mutated neurofibromin is unable to work as a RAS negative regulator, thus resulting in hyperactivation of the pathway and in promotion of cell survival and proliferation.[27,28] This explains why inactivating mutations in the second copy of the *NF1* gene (loss of heterozygosity, LOH) induces the onset of different tumor types in NF1 patients. Neurofibroma is a characteristic NF1-related tumor that develops from LOH in **Schwann cells**. It is a benign nerve sheath tumor associated with spinal, peripheral or cranial nerves and it can be either cutaneous (cNF), affecting single nerves in the skin, or plexiform (pNF), involving larger nerve plexuses.[28] About 8 to 13% of pNFs can evolve to MPNST through the accumulation of further mutations in other genes, such as PTEN, a PI3K and mTOR pathway inhibitor, and TP53, encoding a transcription factor that regulates cell cycle arrest and apoptosis.[27] As a consequence, dysregulation of the aforementioned pathways can drive MPNST development, even though there are many other mutated cascades that can be responsible for the formation of this sarcoma (see ***Fig. 1.6***).

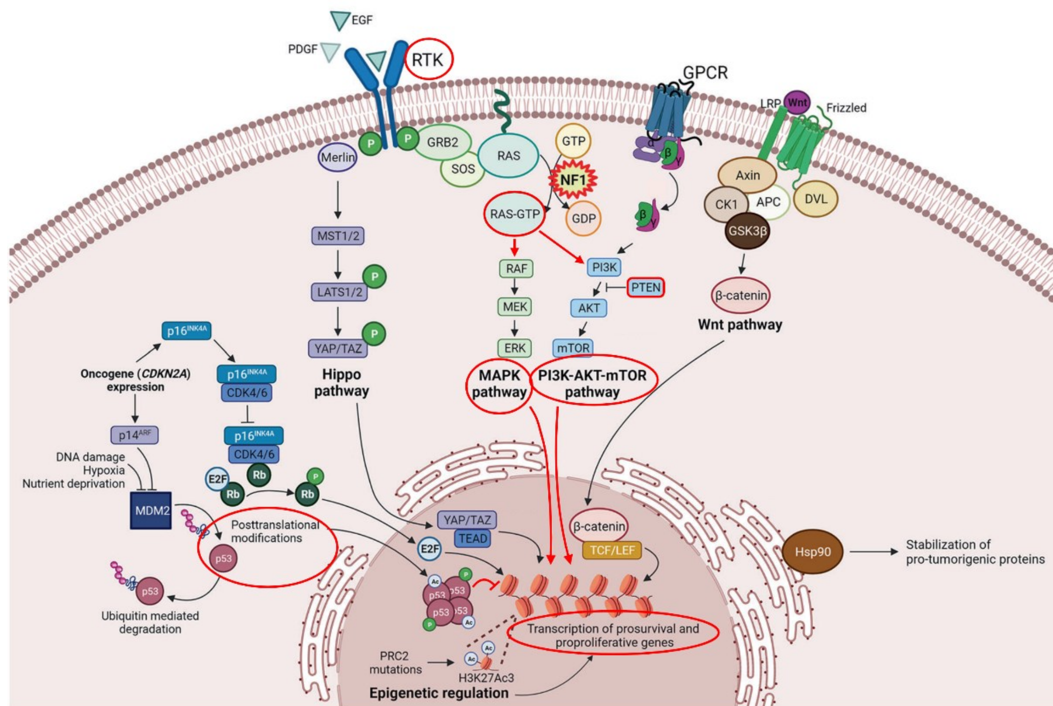

Figure 1.6 - Dysregulated pathways in NF1-related MPNST. *Upon ligand binding, RTK (receptor tyrosine kinase) triggers RAS activation (RAS-GTP) and mutated neurofibromin (NF1) cannot mediate its switching off. MAPK and PI3K pathways are upregulated (red circles) and lead to prosurvival and proproliferative gene transcription. Mutate PTEN (red circle) is no longer able to turn off PI3K signaling cascade. P53 (left part, red circle) normally blocks cell cycle, but mutations hinder its activity, with prosurvival effects. – Modified from (27)*

1.3.1.1 RAS pathway

Deregulation of this pathway is the key driver of NF1-related pNF development, which underpins further evolution into MPNST. RAS activates downstream to growth factor binding to receptor tyrosine kinases (RTKs), which elicits receptor dimerization and transphosphorylation, adaptor protein (Grb2) and guanine nucleotide exchange factor (SOS) recruitment activation that lead to the activating binding of GTP to RAS. Due to neurofibromin loss, RAS remains constitutively bound to GTP in its active conformation. As a consequence, effector proteins, such as the kinases RAF, MEK and ERK1/2 maintain the whole signaling cascade in its active mode, prompting the transcription of genes involved in cell survival, proliferation and growth.[27]

1.3.1.2 PI3K pathway

Another RAS effector is phosphatidylinositol 3-kinase (PI3K), which phosphorylates the substrate PIP2 mediating its conversion in PIP3, an important second messenger that triggers the Ser/Thr kinase activity of AKT. This initiates

different prosurvival signaling cascades, but it can also induce mTOR (mammalian target of rapamycin) activation that enhances cell growth. In this set-up, the phosphatase PTEN acts as a negative regulator of the cascade because it can convert PIP3 into PIP2, thus blocking the following steps.[27] As previously described, mutations in PTEN in MPNSTs can remove the inhibition exerted by this phosphatase, hence inducing a constitutive activation of the PI3K/mTOR pathway with even stronger cell survival and growth effects.

1.3.1.3 TP53

This gene encodes the transcription factor p53 that undergoes ubiquitin-mediated proteasomal degradation in physiological conditions, but responds to several stresses, mainly those inducing DNA damage, by blocking cell division or by inducing senescence or cell death. Mutated p53 is no longer able to induce cell cycle blockage or cell death, thus cell proliferation can continue under conditions of potential genomic instability. Through these mechanisms, p53 inactivating mutations contribute to pNF progression towards MPNST in NF1 patients.[28]

1.3.2 Sporadic MPNST

Little is known about sporadic MPNST formation: in general, it has a later onset, a smaller size and a better prognosis than the NF1-associated counterpart, but cellular origin and driver mutations are still under investigation. Among the various reported mutations, $BRAF^{V600E}$ is likely to be a driver mutation due to its presence being restricted only to this tumor type compared to neurofibromas.[27] Indeed, this kinase is part of the RAS pathway, which is known to induce cell proliferation and survival, and BRAF mutations have been found in different cancer types.

1.4 MPNST, TRAP1 and TAMs

1.4.1 MPNST and TRAP1

It has been demonstrated that TRAP1 is present and active in *Nf1*$^{-/-}$ mouse fibroblasts[29] and subsequently in NF1-related MPNST cells[30]. Hyperactivation of the RAS pathway in MPNSTs triggers ERK1/2 phosphorylation activity, which has been found to be directed also toward TRAP1.[29] Phosphorylation of this chaperone enhances its activity, in turn causing the inhibition of SDH and subsequent succinate accumulation[24], but it also stabilizes the mitochondrial fraction of ERK1/2.[29] The formation of a complex between ERK1/2 – TRAP1 – SDH has been identified as a proof of their interaction in *Nf1*-deficient cells[29], ultimately causing the creation of a pseudo-hypoxic environment through succinate-dependent HIF1α stabilization. Additionally, TRAP1 ablation in this model abolishes cancer growth[29], reinforcing its role in neoplastic induction.

1.4.2 MPNST and TAMs

Just like pNFs, MPNSTs comprise a mixture of different cells, from Schwann cells (SCs), to fibroblasts, to immune cells such as macrophages,[28] that become tumor associated macrophages. It has been demonstrated that TAMs are recruited by both neurofibromas and MPNSTs.[31] Also, TAMs density seems to correlate with MPNST progression and malignancy, in accordance with TAM protumoral roles. Indeed, macrophage depletion could reduce neurofibroma growth.[31] These observations highlight a possible contribution of TAMs to the progression of this particular tumor, making them suitable for further investigations as possible therapeutical targets.

1.4.3 TAMs and TRAP1

As underlined up to this point, TRAP1 is a regulator of cell metabolism especially in cancer conditions, as demonstrated in the case of MPNST. TRAP1-mediated succinate accumulation has been proven to drive cancer growth by leading to HIF1α stabilization. Succinate-HIF1α axis is also critical for macrophage polarization. Still, no information about TRAP1 contribution in macrophage differentiation and function have ever been gathered. In this context, it is extremely interesting to investigate whether TRAP1 has a role in TAM protumoral polarization.

1.5 Preliminary data and working hypothesis

From unpublished data of our laboratory, we now know that TRAP1 presence influences TAM proneoplastic phenotype and functions in a model of macrophages conditioned with MPNST cell medium (*for detailed information, see Materials and Methods section*). In this experimental set-up, it has been shown that TRAP1 KO macrophages are not able to express typical M2 markers (***Fig. 1.7*** for M0-derived macrophages, ***Fig. 1.8*** for M2-derived macrophages) and that their proangiogenic and promigratory functions are significantly impaired if compared to TRAP1 WT macrophages. Therefore, TRAP1 seems to have a regulatory role in TAM functions, but the underlying mechanisms are still unclear.

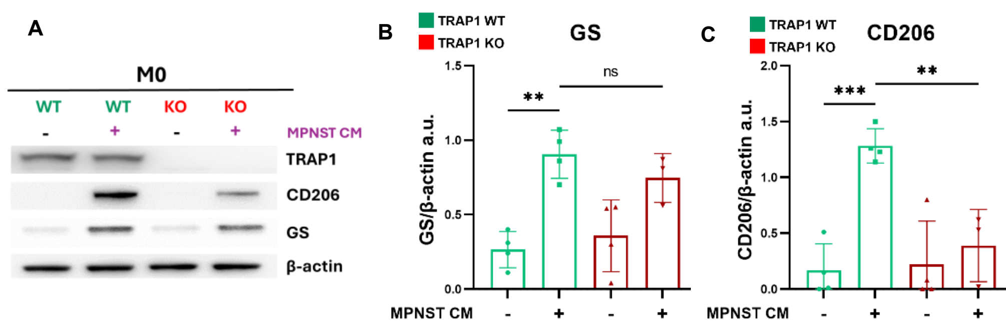

Figure 1.7 - M2 marker expression in M0-derived macrophages. A) *Representative image of a Western Blot showing the expression of typical M2 markers (GS, CD206) in M0-derived macrophages, either TRAP1 WT (green) or TRAP1 KO (red), exposed for 48 h to MPNST CM (+) or to control media (-). Upregulation of these markers was visible in MPNST CM exposed macrophages, confirming their M2 nature. Significantly lower expression of CD206 in TRAP1 KO MPNST-exposed macrophages was detectable, coherently with the hindered M2 polarization due to the ablation of the chaperone. β-actin was used as a loading control, TRAP1 was used as a WT/KO control.* ***B)*** *Quantification of GS protein levels and* ***C)*** *of CD206 protein levels both normalized on β-actin. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+). Data were analyzed through One-way Anova and displayed as mean ± SD. (*) p < 0,05, (**) p < 0,01, (***) p < 0,001.*

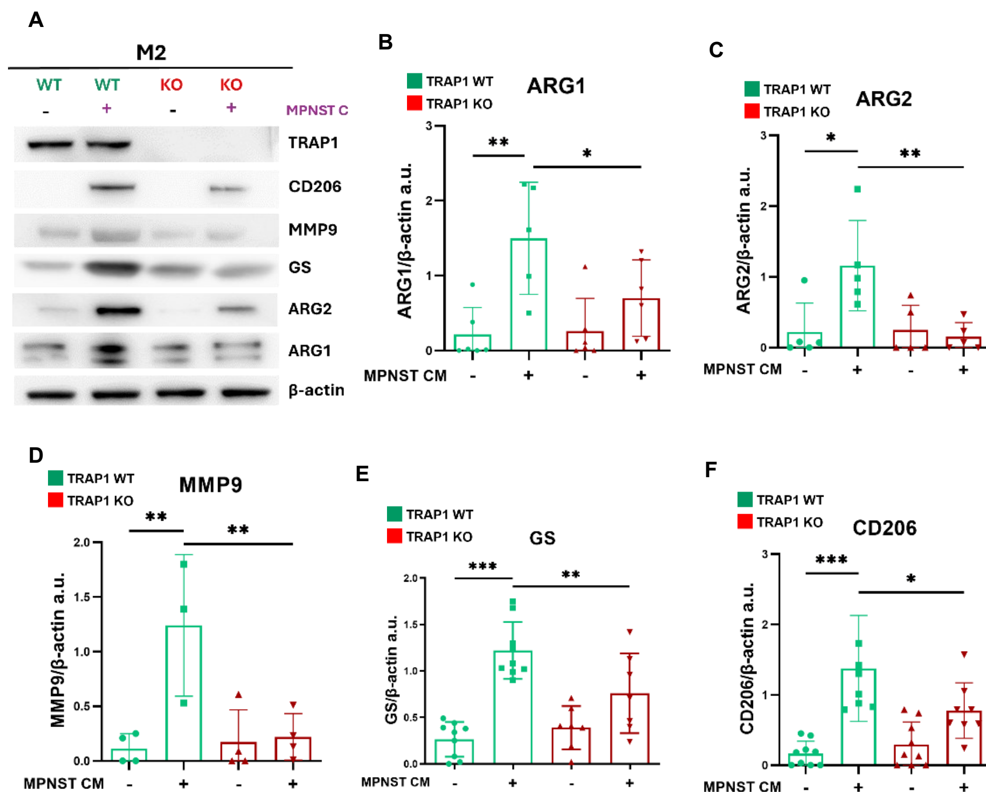

Figure 1.8 - M2 marker expression in M2-derived macrophages. A) *Representative image of a Western Blot showing the expression of typical M2 markers (ARG1, ARG2, GS, MMP9, CD206) in M2-derived macrophages, either TRAP1 WT (green) or TRAP1 KO (red), exposed for 48 h to MPNST CM (+) or to control media (-). Upregulation of these markers was visible in MPNST CM exposed TRAP1 WT macrophages, compared to their KO counterpart, confirm the crucial role of TRAP1 in macrophage polarization. β-actin was used as a loading control, TRAP1 was used as a WT/KO control.* ***B)*** *Quantification of ARG1,* ***C)*** *ARG2,* ***D)*** *MMP9,* ***E)*** *GS and* ***F)*** *CD206 protein levels, normalized on β-actin. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+). Data were analyzed through One-way Anova and displayed as mean ± SD. (*) $p < 0,05$, (**) $p < 0,01$, (***) $p < 0,001$.*

Given the fact that TRAP1 normally works by inhibiting SDH and favoring HIF1α stabilization[24] and that HIF1α-dependent genetic programs are important drivers of several TAM functions, it could be possible that this chaperone operates through succinate accumulation to promote protumoral functions in macrophages. Indeed, succinate not only prompts HIF1α stabilization but, upon secretion in the TME, it is also implicated in TAM M2 polarization by binding to macrophage succinate receptor (SUCNR1).[21]

Therefore, the aim of this work is to understand whether it does exists a TRAP1 – succinate – HIF1α axis in TAMs, that mediates their protumoral role. The hypothesis is that TRAP1 works through succinate accumulation, which in turn can lead to pseudo-hypoxic environment formation, and ultimately to macrophage polarization (***Fig. 1.9***).

To pursue this aim, I focused on the comparison of the phenotypical and functional traits of macrophages with or without TRAP1 induced by external succinate administration.

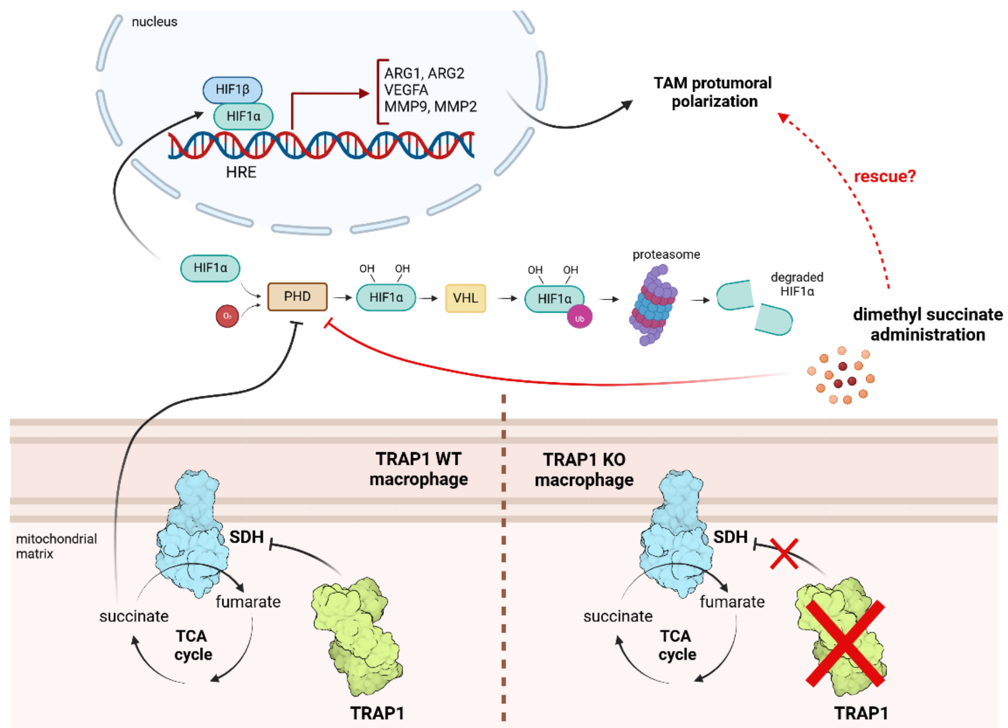

Figure 1.9 - Working hypothesis. *Graphical representation of the project's aim: evaluation of the potential phenotypical and functional rescue of TRAP1 KO upon dimethyl succinate administration, to determine whether TRAP1-mediated protumoral polarization of TAMs could be driven by succinate – HIF1α pathway. – Created with BioRender.com*

2 MATERIALS AND METHODS

2.1 Cell lines

In this project, different immortalized cell lines have been cultured both to prepare cell conditioned media and to perform functional assays.

2.1.1 sMPNST

This immortalized cell line derives from a murine Malignant Peripheral Nerve Sheath Tumor and it is both Nf1- and p53-deficient. These cells represent a good model for NF1-related MPNST, as they carry the LOH in the neurofibromin gene and another driving mutation (TP53 deletion) typical of the malignant phenotype. These cancer cells are characterized by an elongated shape and a rapid proliferation, which is not inhibited by cell-cell contact (a common feature of neoplastic cells).
This cell line was chosen as a tumor model to mimic the *in vivo* environment in which the tumor itself (MPNST) can interact and polarize TAMs. sMPNSTs were thus cultured with a double purpose: to produce a conditioned medium for macrophage polarization and to be used in functional assays.
Cells were cultured in complete DMEM + 10% heat inactivated fetal bovine serum (hi-FBS) + 1% Penicillin and Streptomycin (PenStrep; *for detailed information, see Section 2.3*). They were grown in adhesion and they were kept in an incubator at 37°C and in a humidified atmosphere with 5% CO_2.

2.1.2 L929

This is a murine fibroblast cell line, derived from the connective tissue of a male CH3/An mouse, more precisely from a clone of normal subcutaneous areolar and adipose tissue. They grow in adhesion and assume a regular polygonal shape with short projections. These cells have been selected for their ability to secrete M-CSF under particular culturing conditions[32], producing a necessary medium for macrophage differentiation.
Cells were grown in complete DMEM + 10% hi-FBS + 1% PenStrep and they were maintained in an incubator at 37°C and in a humidified atmosphere with 5% CO_2.

2.1.3 Cell line maintenance

If not otherwise specified, cells were maintained in a T75 standard adhesion flask at an 80% confluence and split regularly. To do this, cell growth medium was first removed, and cells were washed with 3 mL of phosphate buffer saline (*Dulbecco's phosphate buffered saline, DPBS 1X, Sigma-Aldrich – Catalog #D8537*) and then detached with 1 mL of 0,25% Trypsin-EDTA (*Gibco™ – Catalog #25200056*).

Trypsin was then inactivated with 4 mL of complete growth medium and cells were resuspended. Cells were seeded in a T75 flask with 10 mL of complete DMEM + 10% hi-FBS + 1% PenStrep and placed again in the incubator (37°C, humidified, 5% CO_2) to grow.

2.2 Animals

Primary cell lines of macrophages were isolated from femur/tibia of female mice belonging to the C57BL/6 strain, either TRAP1 wild-type (WT) or TRAP1 knock-out (KO), at about 3 months of age.

2.3 Cell culture media preparation and usage

2.3.1 Common cell culture media and their components

All cell culture media were prepared starting from a commercial medium which was supplemented with heat inactivated FBS and PenStrep (*Gibco™ PenStrep, 10.000 U/mL Penicillin, 10.000 µg/mL Streptomycin – Catalog #15140122*). After complementation, media underwent a vacuum filtration step through a 0,22 µm pore membrane (250 mL or 500 mL *Stericup® Quick Release - Catalog #S2GPU05RE*) to ensure complete sterilization.

2.3.1.1 Heat inactivated FBS (hi-FBS)

Fetal bovine serum is commonly used to provide nutrients and growth factors for cell proliferation, but it contains also the complement factors that can activate the immune response in cells like macrophages and lymphocytes. Therefore, the inactivation step must be performed to avoid complement-mediated cell activation. To do so, FBS (*Gibco™ One Shot FBS – Catalog #A5670402*) was thawed in a water bath at room temperature, then heated at 56°C for 45 min. After this step, FBS was quickly frozen at -20°C and stored there until usage.

2.3.1.2 Complete DMEM medium

Complete DMEM (*Dulbecco's Modified Eagle's Medium, Gibco™ - Catalog #11995073*) contains 4,5 g/L D-glucose, 4,0 mM L-glutamine, 1,0 mM Sodium Pyruvate, supplemented with 10% hi-FSB and 1% PenStrep. It was used for sMPNST and L929 culture.

2.3.1.3 Complete RPMI medium

This medium consists of RPMI 1640 (*Roswell Park Memorial Institute, Gibco™ - Catalog #21875034*) supplemented with vitamins and glutathione, to which HEPES (*HEPES Solution, Sigma-Aldrich – Catalog #H3537*) was added as a buffer solution to a final concentration of 0.025 M, and then the pH was adjusted to 7,4. Hi-FBS and PenStrep addition followed as previously described. This medium was used in

the initial steps of BMDM extraction and culture, since it is suitable for monocytes growth and to prepare differentiation media.

2.3.2 *Conditioned media*

All the following media derive from the supernatant of cells kept in different culture conditions, to allow the secretion of various factors.

2.3.2.1 <u>L929 conditioned medium and differentiation medium</u>

L929 conditioned medium

To produce the L929 medium, cells were seeded in T175 standard adhesion flasks with 20 mL of DMEM + 10% hi-FBS + 1% PenStrep and incubated at 37°C in a humidified atmosphere with 5% CO_2 until they reached about 100% confluence. At this point, old medium was removed and 20 mL of fresh DMEM + 10% hi-FBS + 1% PenStrep were added and cells were left to grow for 10 days. During this period, L929 cells produce and release various substances in the supernatant, among which the M-CSF, the molecule responsible for monocyte-to-macrophage differentiation. When ready, supernatant was collected in 50 mL tubes and centrifuged at 750 *g* for 10 min to pull down residual cells or debris. This conditioned medium was then filtered, and 10 mL aliquots were prepared for long term storage at -80°C.

Differentiation medium

The differentiation medium was prepared with complete RPMI 1640 + 10% hi-FBS + 1% PenStrep and 20% L929 conditioned medium. This was used for monocyte culture and differentiation into macrophages.

2.3.2.2 <u>MPNST conditioned medium</u>

Starting from a MPNST cell culture at about 80% confluence, cells were seeded at 800.000 cells/plate in standard adhesion 10 cm cell-culture dishes with 10 mL complete DMEM + 10% hi-FBS + 1% PenStrep. These were incubated for 4 days at 37°C in a humidified atmosphere with 5% CO_2. The supernatant was then collected in 50 mL tubes and centrifuged at 400 *g* for 5 min to pellet cells or debris, and finally filtered. This medium was freshly prepared right before its usage and kept at 4°C for the shortest time possible; the soluble factors responsible for the conditioning are still unknown and prolonged storage was avoided as it could potentially lead to their degradation.

2.4 Bone marrow derived macrophages extraction and culture

Macrophage extraction and culture is the process through which monocytes are isolated from bone marrow of 3-months old mice and placed in culture with differentiation medium to obtain a primary cell culture of macrophages. These can be subjected to further treatment, such as the exposure to cancer cell conditioned media.

2.4.1 BMDM extraction

Prior to the extraction, instruments such as tweezers and scissors underwent sterilization with UV light for 20 min, in a Becker with ultrapure water and 70% ethanol.

Initially, mice were sacrificed through head dislocation and quickly washed with 70% ethanol. Their legs were cut apart and placed in a cell-culture dish filled with PBS. Under the biological hood, legs were quickly rinsed in 70% ethanol and moved to an empty dish that acts as a clean surface for dissection. The limb was held firmly with the tweezers, while skin, flesh and muscles were removed, after carefully eliminating all hairs. At the level of the ankle, tendons were cut to mobilize the foot and facilitate its removal and finally femur and tibia were separated. It is fundamental to clean thoroughly the bones to avoid fibroblasts contamination, therefore bones were left in PBS for a few minutes to facilitate the removal of eventual muscle residuals. To retrieve the bone marrow, both ends of femur and tibia were snipped to create a hole. A 10 mL syringe with 21G needle was prepared and filled up with complete RPMI + 10% hi-FBS + 1% PenStrep. By inserting the needle in the bone end, the bone marrow was gently flushed out with 5 mL of media and collected in a 50 mL tube. The cell suspension was filtered with a 40 µm pore strainer (*SARSTEDT – Catalog #83.3945.040*) and centrifuged at 400 *g* for 5 min to pellet all cells. After removing the supernatant, cells were resuspended in 10 mL Red Blood Cells Lysing Buffer (*Sigma-Aldrich – Catalog #R7757*) and let at room temperature for 10 min. This passage is essential to remove erythrocytes that can interfere with the following steps. An equivalent volume of media was then added to stop the reaction, and another centrifugation step was performed (400 *g*, 5 min). Supernatant was discarded, cells were resuspended in 10 mL of differentiation medium, and then monocyte number was determined by using either the cell counter or the Burker chamber. Finally, cells were seeded in low adhesion 10 cm cell-culture dishes (*SARSTEDT – Catalog #83.3902.500*) at 800.000 cells/plate in 10 mL differentiation medium, and placed in the incubator at 37°C, humidified atmosphere, 5% CO_2.

To obtain fully differentiated macrophages, culture must be carried on for 7 days. During this period, one medium refresh was performed at day 4 by adding 5 mL of

differentiation medium to each plate without removing any of the old one. In the first days, monocytes/macrophages appear as round and fluctuating cells, while at the end of the culture period they will have considerably replicated, and they will be attached to the plate and slightly elongated.

2.4.2 *Macrophage polarization*

At day 7, macrophages were fully differentiated in an M0 state and ready to undergo eventual treatments, such as polarization to an M2 state, which was normally performed in half of the plates. To do so, cell medium was changed with 10 mL of polarization medium composed of RPMI 1640 + 10% hi-FBS + 1% PenStrep additioned with recombinant mouse IL-4 (*ImmunoTools – Catalog #12340045*), which was previously dissolved in 0,1% BSA (Bovine Serum Albumine) and used at the final concentration of 10 ng/mL. Macrophages were incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2.

2.4.3 *Conditioned or control medium exposure*

Macrophages can be either conditioned with cancer cell medium, to simulate the TME, or cultured with control medium, to obtain a non-conditioned counterpart.

2.4.3.1 Conditioned medium exposure

To obtain TAM-like macrophages, exposure to MPNST cell conditioned medium (CM) is necessary in order to mimic the TME to which TAMs could be exposed in an *in vivo* situation. Macrophages were exposed to this medium for a total of 48 hours. After removing the old medium, 10 mL of freshly collected CM were added to the plate for the first 24 hours, then they were substituted with other 10 mL of fresh CM for the remaining time. The medium was kept at 4°C between the two exposures and heated in a 37°C water bath 30 min before usage. Given that this CM was derived from another cell culture, it could be depleted of nutrients; for this reason, its refreshment every 24 hours is essential to guarantee enough nourishment to macrophages.

2.4.3.2 Control medium exposure

In parallel to CM administration, some of the plates were exposed to 10 mL of control media, consisting of complete DMEM + 10% hi-FBS + 1% PenStrep supplemented with 20% L929, for the same 48 hours without any refresh. L929 addition is fundamental to maintain a differentiation signal in the control macrophages.

2.4.4 *Dimethyl succinate treatment*

Dimethyl succinate (DMS) is the permeable form of succinate, a TCA cycle metabolite. Due to its capacity to cross the membranes, this compound was administered directly to the plate by pipetting it in the cell culture medium. The treatment was performed using 1 mM dimethyl succinate for 48 hours. This compound was prepared from a 7.8 M stock (*Catalog #W23,960-7-K*) diluted in complete DMEM + 10% hi-FBS + 1% PenStrep to obtain a 100 mM working dilution. This treatment was added once in the control medium and twice in the CM, following the refreshment step.

2.4.5 *Experimental conditions*

At the end of this 10-days process, we obtained various experimental conditions based on the starting mouse strain (WT or TRAP1 KO) and on the different media to which macrophages were exposed. To sum up, ***Table 2.1*** lists the different conditions at the end of the process:

Table 2.1 - Experimental conditions

TRAP1 WT	TRAP1 KO
M0 WT + DMEM	M0 KO + DMEM
M0 WT + CM	M0 KO + CM
M0 WT + DMEM + DMS	M0 KO + DMEM + DMS
M0 WT + CM + DMS	M0 KO + CM + DMS
M2 WT + DMEM	M2 KO + DMEM
M2 WT + CM	M2 KO + CM
M2 WT + DMEM + DMS	M2 KO + DMEM + DMS
M2 WT + CM + DMS	M2 KO + CM + DMS

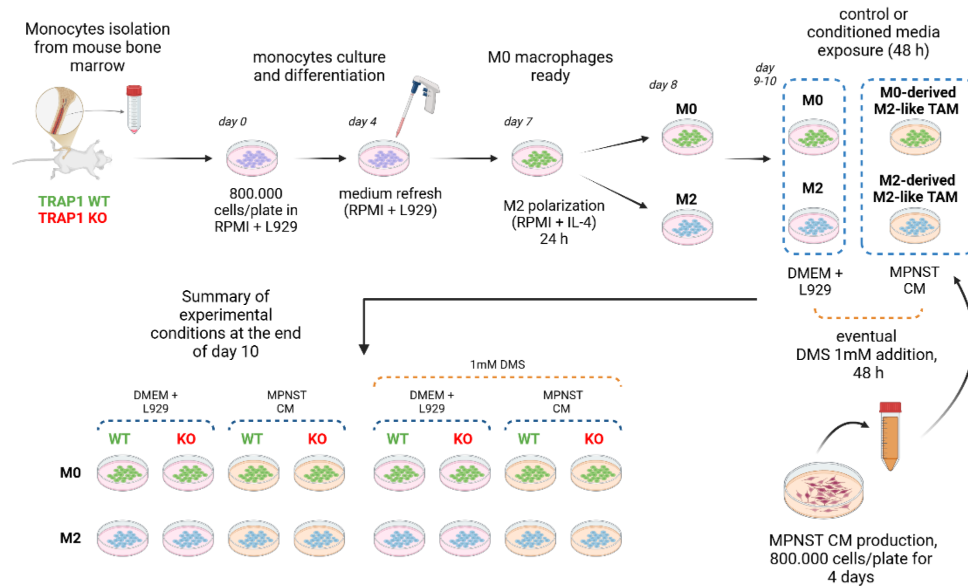

***Figure 2.1 - Visual summary of BMDM extraction and culture** – Created with BioRender.com*

2.5 *Western Blot*

This semiquantitative technique is carried out in denaturing conditions, allowing the separation of proteins through an electrical field based on their molecular weight.

2.5.1 *Sample collection and preparation*

At the end of the culture period, macrophages were ready to be collected for protein lysate preparation. Cell media were recovered from the plate and stored apart (*see Section 2.7.1 for details*). Working on ice, a quick wash with PBS was performed and 70 µL of lysis buffer were added to the plate and all the material was scraped down and collected in a 1.5 mL tube.

A stock of lysis buffer was prepared in advance and stored at -20°C by mixing RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7,4, 5 mM EDTA, 10% glycerol, 1% Triton X-100) with 1:100 phosphatase and protease inhibitors (*Sigma-Aldrich – Catalog #P5726-5ML, #P0044-5ML, #P8340-5ML*).

2.5.1.1 Sample clarification

Samples must undergo sonication and clarification to allow a complete cell rupture. During sonication, high frequency sound waves were applied to completely shear cells and solubilize the intracellular content. Tubes were inserted in the sonicator, filled with ice and water, and cycles of 30 seconds waves and 30 seconds rest were

applied for a total of 6 min. Samples were then centrifuged at 17900 *g*, at 4°C, for 30 min to pull down all the undesired material. The supernatant was then retrieved and placed in a clean tube on ice.

2.5.1.2 Sample quantification and preparation

The quantification step allows the determination of protein concentration in the lysates, and it was performed using the BCA method (bicinchoninic acid). This technique relies on the ability of peptide bonds to reduce Cu^{2+} (present in the solution reagents) to Cu^{+}, suddenly forming a purple complex with two BCA molecules, which can be detected through absorbance measurements at 562 nm. Following the kit instructions (*Pierce™ BCA Protein Assay Kit – Catalog #23227*), a standard curve was generated using BSA (0-2 mM concentration range) and sample concentrations were determined.

Western Blot samples were prepared using 40 µg of proteins in a total volume of 15 µL: the correct amount of sample was mixed with ultrapure water and LB (loading buffer) 5X. The latter is composed of 4% SDS (sodium dodecyl sulphate) to denature proteins and negatively charge them; 10% β-mercaptoethanol to break disulfide bonds; 20% glycerol, to make the preparation heavier and easier to load; 0,004% bromophenol blue, a colorant, to track the migration front; 0,125 M Tris-HCl at pH 6,8. SDS and β-mercaptoethanol are fundamental to obtain linear proteins that will migrate according to their molecular weight. Before loading, samples were heated at 50°C under shaking (400 rpm).

2.5.2 Western Blot

2.5.2.1 Gel electrophoresis

This is the true separation step, in which negatively charged proteins migrate throughout the gel following the electrical field and according to their molecular weight.

Polyacrylamide gradient gel 4-12% (*NuPAGE™ Bis-Tris Mini Protein Gels Invitrogen, Catalog #NP0322BOX*) was assembled in the electrophoresis chamber and covered with MOPS running buffer 1X, prepared from 20X MOPS-SDS (*NuPAGE™ MOPS SDS Running buffer 20X - Catalog #NP0001*) diluted in deionized water. After loading the samples and a molecular weight marker (*Precision Plus Protein™ Dual Color Standards, BioRad – Catalog #1610394*), current intensity was set at 30 mA and the electrophoresis was left on for 2 hours, until the front of migration had reached the end of the gel.

2.5.2.2 Protein transfer

In this passage, proteins are transferred to a nitrocellulose membrane with the wet transfer technique to allow for their immobilization and subsequent visualization.

The transfer setup was assembled by placing the nitrocellulose membrane (*Amershan™ Potran® 0,2 µm Nitrocellulose Blotting Membrane – Catalog #GE10600001*) in contact with the gel and covering them from both sides with

paper and soaked sponges; the membrane must face the positive electrode for correct migration. The transfer buffer 1X (*NuPAGE Transfer Buffer 20X – Catalog #NP0006-1* and 20% Methanol in deionized water) was added to cover the system and the voltage was fixed at 30 V for 90 minutes.

2.5.2.3 Saturation and incubation with antibodies

Once the transfer was concluded, the membrane was colored with Ponceau Red solution (*Ponceau S Solution, Sigma-Aldrich – Catalog #P7170-1L*), a protein staining solution, to assess transfer quality. The membrane was then saturated with 5% BSA (*Bovine Serum Albumine, Sigma-Aldrich – Catalog #A7030*) in TBS-tween 0,1% for 1 hour, to fill all the unbound areas of the membrane and avoid non-specific antibody binding. Membrane was then incubated with primary antibodies overnight at 4°C under mild shaking conditions (see ***Table 2.2*** for primary antibodies' list). All the primary antibodies were diluted in 5% BSA in TBS-tween 0,1%.

The following day, the membrane was washed 3 times with TBS-tween 0,1%, 10 min for each washing, then it was incubated with the secondary antibody (see ***Table 2.3***) for 1 hour at room temperature. All the secondary antibodies were diluted (1:10000) in 5% non-fat dried milk (*Nonfat dried milk powder, PanReac AppliChem – Catalog #A0830)* in TBS-tween 0,1%. The membrane was washed again as previously described and the visualization was performed by adding the HRP (Horseradish peroxidase) substrate (*Immobilion Forte Western HRP Substrate, Millipore – Catalog #WBULS0100*). This substrate reacts with the HRP bound to the secondary antibody and it produces a chemiluminescent signal that was detected through the instrument UVITEC. Final images were quantified through ImageJ Software by calculating the peak area for each band, followed by the normalization of each signal against the loading control marker (β-actin in this case).

Table 2.2 - Western Blot primary antibodies. *List of primary antibodies used in Western Blot to evidence the presence of the corresponding proteins.*

Primary antibodies	
Antibody	**Description and technical specifications**
β-actin	It is a cytoskeletal protein, ubiquitously expressed in eukaryotic cells, used as housekeeping
	Santa Cruz Biotechnology, host: mouse, dilution 1:4000 – Catalog #sc-47778
Arginase 1 (ARG1)	It converts arginine in urea and ornithine, used as an M2 marker
	Cell Signaling technology, host: rabbit, dilution 1:2000 – Catalog #93668S

Arginase 2 (ARG2)	Mitochondrial enzyme that converts arginine in urea and ornithine, important in the anti-inflammatory phenotype of macrophages
	Abcam, host: rabbit, dilution 1:1000 – Catalog #ab264066
Glutamine Synthetase (GS)	It converts glutamate in glutamine, and it plays a crucial role in M2-polarization; used as an M2 marker
	Genetex, host: rabbit, dilution 1:10000 – Catalog #GTX109121
TRAP1	It is a mitochondrial chaperone involved in tumorigenesis, used as a WT/KO control
	BD Transduction Laboratories, host: mouse, dilution 1:500 – Catalog #612344
Matrix metalloproteinase 9 (MMP9)	It degrades ECM to promote collagen turnover and cancer cell migration; used as a protumoral macrophage marker
	ThermoFisher Scientific, host: rabbit, dilution 1:500 – Catalog #PA5-27191
HIF1α	Hypoxia associated transcription factor, it is possibly involved in protumoral polarization of macrophages; used to detect its presence in different conditions
	Novus Biologicals, host: rabbit, dilution 1:500 – Catalog #NB100-449
CD206	It is a mannose receptor; used as an M2-polarization marker
	ThermoFisher Scientific, host: rabbit, dilution 1:1000 – Catalog #PA5-114370

Table 2.3 - Western Blot secondary antibodies. *List of secondary antibodies used to detect and amplify the signal of primary antibodies.*

	Secondary antibody
Antibody	**Specification**
Anti-mouse	It detects primary antibodies produced in mouse by Fc fragment recognition; conjugated with HRP
	Goat anti-mouse IgG (H+L)-HRP Conjugate, BIORAD – Catalog #170-6516

Anti-rabbit	It detects primary antibodies produced in rabbit by Fc fragment recognition; conjugated with HRP
	Goat anti-rabbit IgG (H+L)-HRP Conjugate, BIORAD – Catalog #170-6515

2.6 Real-time PCR

Also known as qPCR (quantitative polymerase chain reaction), this technique allows the detection of the transcription products and their quantification over time, since it monitors the amplicon formation as PCR progresses.

2.6.1 RNA extraction

To perform the real-time PCR, RNA must be extracted from macrophages at the end of the culture period. Cell media was saved for other assays (*see Section 2.7.1*), and cells were quickly washed with PBS. According to the kit instructions (*E.Z.N.A. Total RNA Kit I, Omega – Catalog #R6834*), while working on ice, 350 μL of RNA lysis buffer (supplemented with β-mercaptoethanol) were added directly to the plate and the material was scraped and collected in a 1.5 mL tube. Extraction was then performed following the instruction through the mini-column set-up, a system in which the matrix inside the columns can reversibly bind to RNA and hold it until elution. The eluted RNA was then quantified through the instrument NanoDrop.

2.6.2 Reverse transcription

In this step, RNA was converted into cDNA through the reverse transcription reaction to perform the following PCR. Using a kit (*SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR, Invitrogen – Catalog #11752*), the reaction mix was added to 1 μg of RNA samples, which were incubated in a thermocycler (25°C for 10 min, 50°C for 30 min, 85°C for 5 min) to permit single-strand cDNA formation. A subsequent step with RNAse H was carried out to remove the RNA template. Samples were diluted in RNase-free water to reach a concentration of 20 ng/μL and stored at -20°C.

2.6.3 qPCR

To perform the quantitative PCR, a reaction mix, primers and cDNA are required. The qPCR master mix (*GoTaq qPCR Master Mix, Promega – Catalog #A6002)* comprises the enzyme (Hot Start Polymerase), dNTPs, $MgCl_2$, reaction buffer and a DNA-binding fluorescent dye (to detect DNA production). Primers were diluted

in nuclease-free water at a 100 µM concentration and a 10 µM working dilution of forward and reverse primers was prepared (see ***Table 2.4*** for complete primer list). The PCR plate (96-well) was then loaded so that each well contained a total volume of 20 µL, of which 10 µL of qPCR Master Mix (2X), 0,3 µL of 10 µM primer mix, 1 µL of cDNA and the remaining volume of nuclease-free water. The plate was then sealed with sealing tape (*SARSTEDT – Catalog #95.1994*) and spinned at 200 *g* for 1 min to collect all the reagents at the bottom of the wells. To start the reaction, the plate was incubated in a thermocycler with these parameters: an initial 2 min step at 95°C to activate the enzyme, followed by 40 PCR cycles, composed of a denaturation step (95°C for 15 sec) and an annealing and extension step (60°C for 1 min). At the end, data were retrieved and normalized through the ΔΔCt method: ΔCt between target gene and housekeeping gene was calculated for each sample, followed by normalization against the control sample ($\Delta Ct_{sample} - \Delta Ct_{control}$) and calculation of the fold change.

Table 2.4 - qPCR primers. *List of primers with their sequences used for qPCR; all of them are designed for mouse DNA.*

Primers	
Target transcript	**Information and sequence (5’ → 3’)**
m-VEGFA	Vascular endothelial growth factor A; upregulated in protumoral conditions, it mediates angiogenesis
	For: CCACGACAGAAGGAGAGCAGAAGTCC Rev: CGTTACAGCAGCCTGCACAGCG
m-ARG1	Arginase 1, M2 marker, it converts arginine in ornithine and urea
	For: CCACAGTCTGGCAGTTGGAAG Rev: GGTTGTCAGGGGAGTGTTGATG
m-ARG2	Arginase 2, mitochondrial isoform of ARG1
	For: CTTGCGTCCTGACGAGATCC Rev: CCACTCCTAGCTTCTTCTGTCC
m-MMP9	Matrix metalloproteinase 9, it degrades collagen
	For: GCTCCTGGCTCTCCTGGCTT Rev: GTCCCACCTGAGGCCTTTGA
m-MMP2	Matrix metalloproteinase 2, it degrades collagen
	For: CAGAGACCTCAGGGTGACAC Rev: GAAGAAGTTGTAGTTGGCCA
m-RPLP0	Ribosomal protein lateral stalk subunit P0, used as housekeeping gene
	For: TCACTGTGCCAGCTCAGAAC Rev: CTCCCACCTTGTCTCCAGTC

2.7 Migration assay

Also known as Boyden chamber assay, this technique aims at evaluating the migratory capacity of cells in the presence of different stimuli to determine whether different conditions can improve cell motility. To induce migration, a gradient must be created between the upper and lower part of the well, so that the chemoattractant is placed on the bottom and cells can move through the Transwell membrane.

2.7.1 Macrophage media recovery

Macrophage conditioned media were used to assess if they could tune the migratory phenotype in sMPNST cells. Macrophage media were recovered from the plates at the end of the 10-days culture period, centrifuged at 400 *g* for 8 min to pellet cells and debris and the supernatant was moved to a new 15 mL tube. These media were stored at -80°C until usage.

2.7.2 Boyden chamber assay

The setup for this assay comprises a Transwell basket with a polyethylene terephthalate membrane with 8 µm pores (*24-well Insert 8,0 µm PET clear, CellQart – Catalog #9328012*) and a 24-well plate in which Transwells are inserted. sMPNST were detached and seeded at 20.000 cells/well in 200 µL of complete DMEM + 10% hi-FBS + 1% PenStrep above the Boyden chamber membrane. In the lower part of the well, 800 µL of the selected medium were added to create the potential gradient. Due to the high variability of this test, three technical replicates were prepared for each condition (see ***Table 2.1*** *for the list of experimental conditions*). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 18 hours. After that time, Boyden membranes were retrieved and emptied from medium, then the excess was dried with a cotton swab. They were stained with a kit (*RAL Diff-Quick™ - Catalog #10736131*) composed of 3 different solutions to obtain a variation of the Romanowsky staining: the first one is a fixative solution, the second and third solutions are for cell staining, composed of eosin, methylene blue and Azure A. After drying, images of the membranes were captured for subsequent quantification using ImageJ Software, that measured the area covered by cell staining. Mean areas were calculated for each sample and normalized against the control, obtaining a fold change measure.

As a control, complete DMEM + 1% PenStrep but without hi-FBS was used, in this way the gradient cannot form, and basal migratory ability of cells could be evaluated.

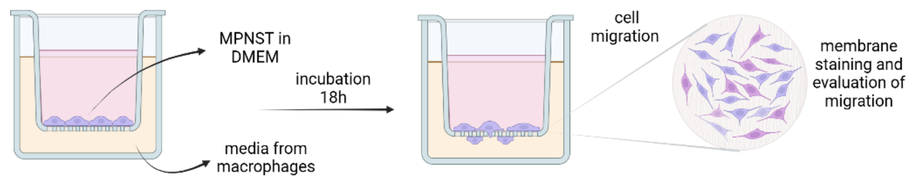

Figure 2.2 - Boyden chamber assay. *Graphical representation of the migration assay in which the capability of cells to cross the Transwell membrane is measured. In my experiments, the chemoattractants in the bottom chamber were the macrophage media. – Created with BioRender.com*

2.8 Statistical analysis

Analysis was conducted through the software GraphPad Prism. If not otherwise specified, all experiments were performed at least three times. Analysis of difference between groups was performed through One-Way Anova test with p<0,05 (*), p<0,01 (**), p<0,001 (***).

3 RESULTS

Considering the preliminary data of the laboratory proving the contribution of TRAP1 in TAM-like macrophage polarization (*see Section 1.5*), the focus of my work was to investigate the possible molecular mechanism that withstands this process. More specifically, my purpose was to investigate the possible contribution of the TRAP1 – succinate – HIF1α axis in macrophage protumoral polarization.

To pursue this aim, different assays were performed in TAM-like macrophages to evaluate the possible phenotypical and functional rescue of the protumoral state in TRAP1 KO macrophages upon treatment with DMS, a permeable form of succinate.

3.1 *Effect of DMS administration on HIF1α expression in TAM-like macrophages*

Since macrophage polarization can be driven by HIF1α and its stabilization could depend on TRAP1-mediated succinate accumulation, the expression of this transcription factor in TAM-like macrophages has been studied with respect to exposure to MPNST conditioned medium (CM) and to DMS.

Analysis of M0-derived TAM-like macrophages showed that the presence of HIF1α was restricted only to TRAP1-expressing macrophages following exposure to MPNST CM. Upon DMS addition, HIF1α was detectable also in TRAP1 KO M0-derived TAM-like cells exposed to the CM, to levels comparable with M0-derived WT TAM-like cells. HIF1α was never detectable if macrophages were not exposed to the MPNST CM, independently of DMS (***Fig. 3.1***).

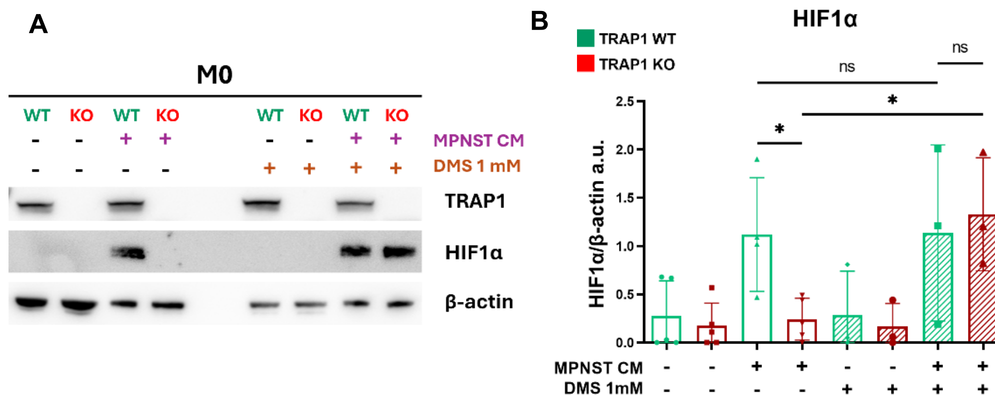

Figure 3.1 *-* ***HIF1α rescue in M0-derived TAM-like macrophages by DMS treatment. A)*** *Representative image of Western Blot results, showing HIF1α expression levels in M0 samples after 48 h of culture with MPNST CM (+) or control media (-), with or without 1mM DMS (+/-). β-actin was used as the loading*

control, TRAP1 was used as the WT/KO control. ***B)*** *Quantification of HIF1α protein levels in the various M0 samples, normalized on β-actin. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Data were analyzed through One-way Anova and represented as mean ± SD. (*) p < 0,05, (**) p < 0,01, (***) p < 0,001*

Similarly, Western Blot analyses on M2-derived TAM-like macrophages revealed that HIF1α was detectable only in M2-derived TRAP1 WT macrophages exposed to MPNST CM, and in KO M2-derived TAM-like cells in the presence of both MPNST CM and DMS. Instead, HIF1α was never visible if macrophages were not exposed to the MPNST CM, independently of DMS (***Fig. 3.2***).

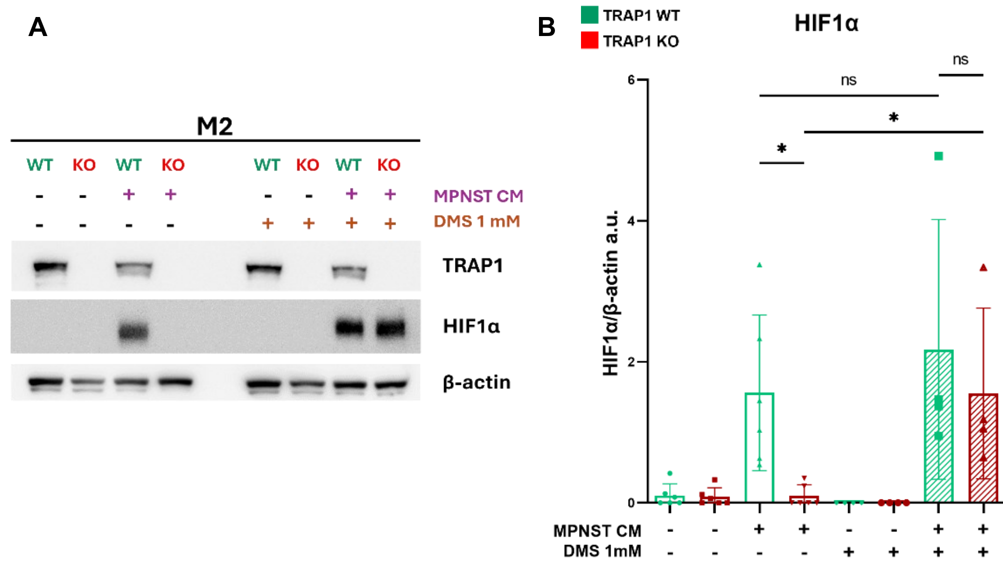

Figure 3.2 - HIF1α rescue in M2-derived TAM-like macrophages by DMS treatment. A) *Representative image of Western Blot results, showing HIF1α expression levels in M2 samples after 48 h of culture with MPNST CM (+) or control media (-), with or without 1mM DMS (+/-). β-actin was used as the loading control, TRAP1 was used as the WT/KO control.* ***B)*** *Quantification of HIF1α protein levels in the various M2 samples normalized on β-actin. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Data were analyzed through One-way Anova and represented as mean ± SD. (*) p < 0,05, (**) p < 0,01, (***) p < 0,001.*

Taken together, these observations point out that HIF1α stabilization in macrophages is induced upon exposure to MPNST CM, but only in the presence of TRAP1, or in its absence if cells are provided with succinate. These results indicate that TRAP1-mediated succinate accumulation stabilizes HIF1α when M0/M2 macrophages receive soluble signals from MPNST cells.

3.2 Effect of DMS administration on the expression of M2 markers in TAM-like macrophages

As already mentioned, macrophages exposed to MPNST CM assume an M2-like phenotype, expressing the typical markers of this state (ARG1, GS, CD206). Indeed, this protumoral polarization seems to be tied to TRAP1 presence, as TRAP1 KO TAM-like cells show significantly reduced expression of M2 markers (*see Section 1.5, **Fig. 1.7**, **Fig. 1.8***).

To further clarify whether TRAP1 could act through succinate – HIF1α axis to induce this proneoplastic phenotype, I evaluated the effect of DMS treatment on the expression of M2 markers in macrophages exposed to MPNST CM. In particular, I focused on HIF1α-target genes, such as ARG1, ARG2 and MMP9, in order to detect whether their expression is regulated by succinate levels.

In the case of the M0-derived TAM-like macrophages, expression of ARG1, ARG2 and MMP9 was not detectable in any of the genotypes or experimental conditions (*not shown*). Despite the absence of these proteins, a significant expression of other typical M2 markers such as GS and CD206 was still observable in the cells exposed to MPNST CM, independently of TRAP1 expression (***Fig. 3.3-A***). Considering that neither GS nor CD206 are HIF1α targets, DMS addition was not expected to cause any change in their expression levels. Coherently, Western Blot analysis showed no significant upregulation of these markers in samples exposed to exogenous succinate (***Fig. 3.3-B, C***). Altogether, these results suggest that HIF1α induction in M0 TAM-like cells by MPNST CM is not sufficient to elicit the transcription of some of its target genes, and that the appearance of the M2 markers GS and CD206 occurs independently of the TRAP1 – succinate – HIF1α signaling axis.

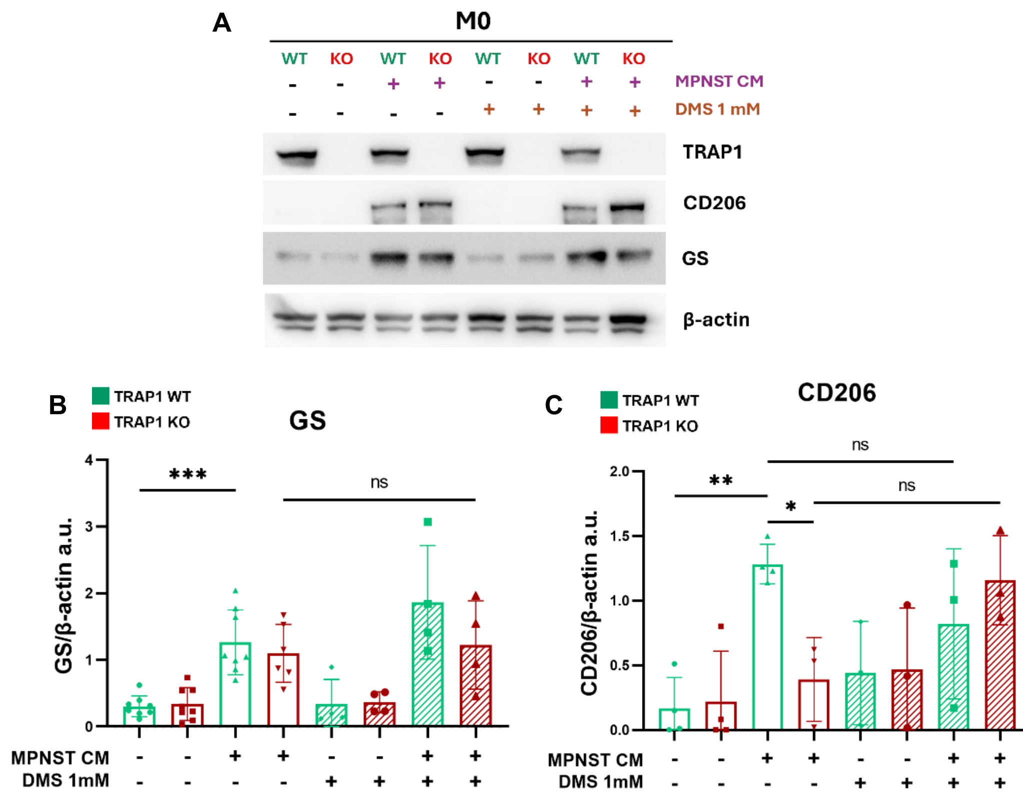

Figure 3.3 - M2 markers in M0-derived macrophages. A) *Representative image of a Western Blot showing GS and CD206 expression levels in macrophages exposed for 48 h to MPNST CM (+) or to control media (-), with or without DMS (+/-). Upregulation of these markers was visible in MPNST CM exposed macrophages. β-actin was used as a loading control, TRAP1 was used as a WT/KO control.* ***B)*** *Quantification of GS protein levels and* ***C)*** *of CD206 protein levels both normalized on β-actin, showing no relevant differences in TRAP1 KO TAM-like cells without (-) or with (+) DMS. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Data were analyzed through One-way Anova and displayed as mean ± SD. (*) $p < 0,05$, (**) $p < 0,01$, (***) $p < 0,001$.*

Similarly to what observed in M0-derived macrophages, in M2-derived TAM-like cells the M2 markers GS and CD206 were markedly induced by exposure to MPNST CM independently of both TRAP1 and DMS presence, even if they were already detectable in untreated cells (***Fig. 3.4-A, E, F***)
However, at variance from M0-derived cells, expression of the HIF1α targets ARG1, ARG2 and MMP9 was markedly induced by MPNST CM only in TRAP1-expressing M2-derived TAM-like macrophages. Importantly, administration of DMS was able to increase the expression of ARG1 and MMP9 also in M2-derived TAM-like cells that do not express TRAP1 when exposed to the CM, whereas ARG2 was insensitive to DMS (***Fig. 3.4-A, B, C, D***).

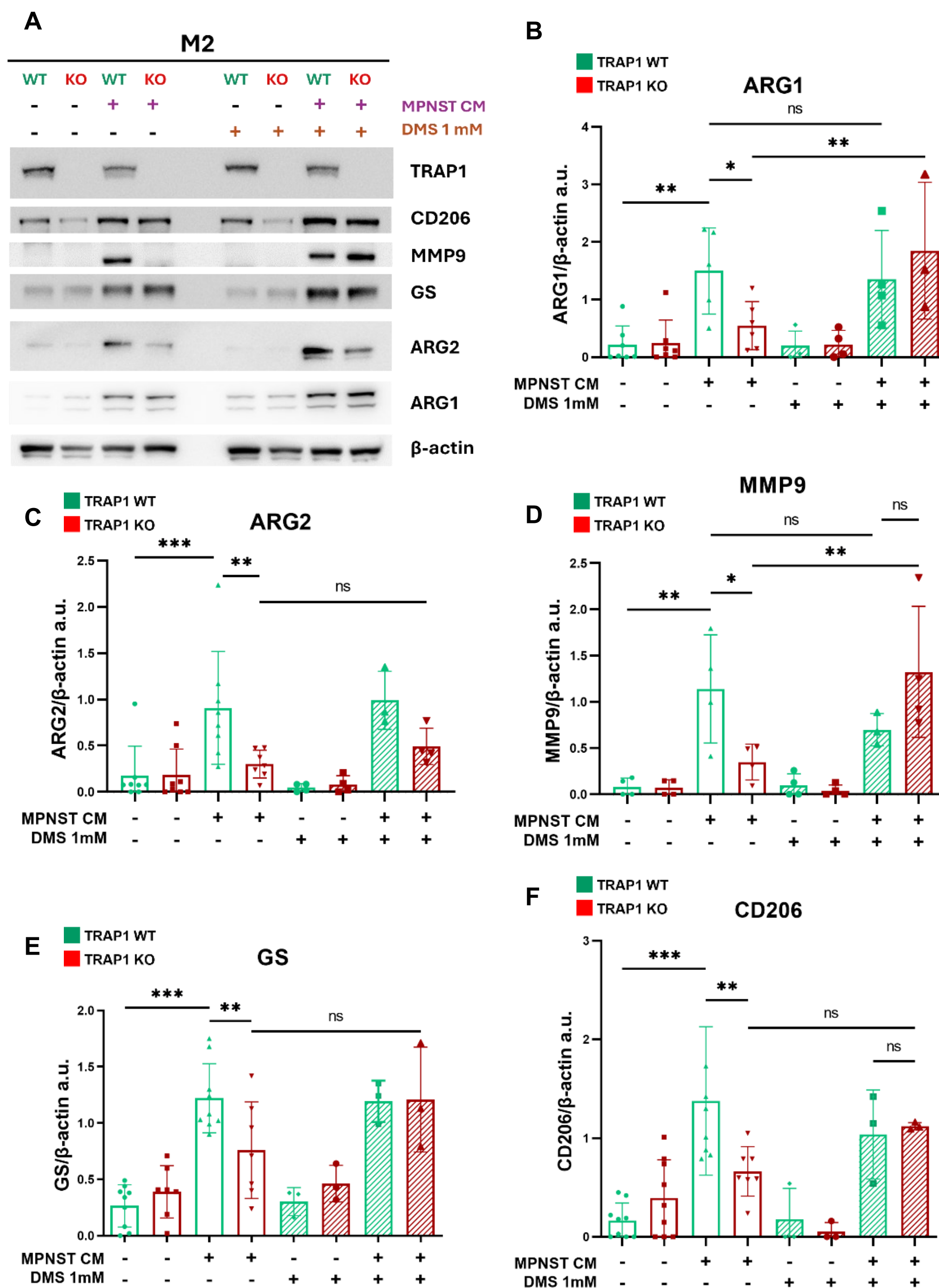

***Figure 3.4 - M2 markers in M2-derived macrophages**. **A)** Representative image of a Western Blot showing the expression levels of ARG1, ARG2, GS, MMP9 and CD206 in M2-derived macrophages cultured for 48 h with MPNST CM (+) or control medium (-), with or without DMS (+/-). β-actin was used as a loading control, TRAP1 was used as the WT/KO control. **B)** Quantification of ARG1, **C)** ARG2, **D)** MMP9, **E)** GS and **F)** CD206 protein levels normalized on β-actin, showing increased expression in M2-derived TRAP1 WT TAM-like cells for all markers, but phenotypical rescue in TRAP1 KO TAM-like cells only for ARG1 and MMP9. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to*

control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Data were analyzed through One-way Anova and displayed as mean ± SD. (*) $p < 0,05$, (**) $p < 0,01$, (***) $p < 0,001$.

From this analysis of the M2-derived TAM-like macrophages, expression of HIF1α-dependent protumoral markers only in TRAP1 WT macrophages cultured with MPNST CM confirmed the role of the chaperone in this polarization process. Furthermore, the increased expression of ARG1 and MMP9 in M2-derived TRAP1 KO TAM-like cells upon DMS administration indicates that activation of a TRAP1-succinate-HIF1α axis elicits the expression of specific M2-like markers.

3.3 Effect of DMS administration on the expression of mRNA for M2 markers in TAM-like macrophages

The previous analysis of HIF1α-dependent M2 markers has shown their upregulation in TRAP1 KO TAM-like macrophages when exposed to DMS, evidencing the succinate-dependent induction of the protumoral phenotype. However, as not all proteins were detectable through Western Blot technique (as in the case of M0 samples), it became necessary to consider also the mRNA expression levels of HIF1α target genes, either to better elucidate the phenotype of M0-derived macrophages and to possibly validate the Western Blot results.

To pursue this aim, RT-qPCR was performed in M0-derived and M2-derived macrophages evaluating the mRNA levels of genes involved in protumoral features: ARG1, ARG2, MMP9, MMP2, another matrix metalloproteinase, and VEGF-A, a released factor responsible for the angiogenesis process. Expression level of RPLP0 was used as a loading control, as it is a housekeeping gene for macrophages.

For the M0 samples, mRNA levels of VEGF-A, ARG1 and MMP9 showed a significant upregulation in the M0-derived WT TAM-like cells following exposure to the MPNST CM compared to the control (macrophages exposed to DMEM) and to the TRAP1 KO counterpart. Upon DMS treatment, a relevant increase in the expression of all the three mRNAs was detected in M0-derived TRAP1 KO TAM-like cells (***Fig. 3.5***).
In the case of ARG2 and MMP2, transcript expression was not measurable as it was outside the range of detection. Indeed, threshold cycles (Ct) for the various samples were too high to be considered, as the fluorescent signal was observable generally after 35 cycles out of a total of 40 (see ***Tab. 3.1*** for example values).

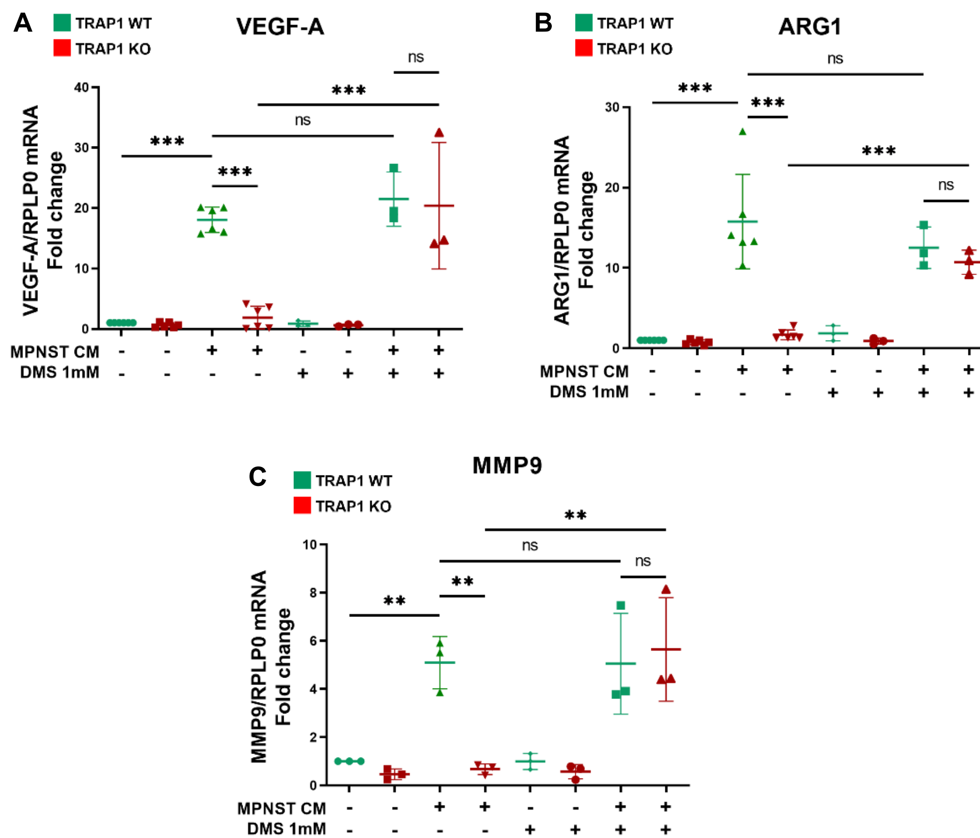

Figure 3.5 - M2-like mRNAs expression in M0-derived macrophages. *Quantification of expression levels of* ***A)*** *VEGF-A,* ***B)*** *ARG1 and* ***C)*** *MMP9 all normalized on RPLP0 expression levels. Rescue of mRNAs expression is evident in TRAP1 KO TAM-like cells exposed to DMS. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Raw data were normalized through the ΔΔCt method, followed by calculation of the Fold Change. Data were analyzed through One-way Anova and represented as FC mean ± SD. (*) p < 0,05, (**) p < 0,01, (***) p < 0,001.*

Table 3.1 - qPCR Ct values in M0 samples*. Representative mean Ct values of qPCR for ARG2 and MMP2 in M0-samples, outside the detection range (Ct > 35).*

Sample	Target	
	ARG2	MMP2
	Mean Ct	Mean Ct
M0 WT DMEM	35,57	36,40
M0 KO DMEM	35,91	36,01
M0 WT CM	36,69	35,78
M0 KO CM	35,46	35,88
M0 WT DMEM + DMS	36,51	35,34
M0 KO DMEM + DMS	36,41	36,06
M0 WT CM + DMS	36,86	35,89
M0 KO CM + DMS	37,29	35,49

For the M2 samples, mRNA levels of VEGF-A, ARG1, MMP9 and also ARG2 were highly increased in the TRAP1 WT TAM-like cells, compared to the DMEM-exposed sample and to the TRAP1 KO TAM-like macrophages. When exposed to both MPNST CM and DMS, TRAP1 KO macrophages showed a significant induction in the expression of all the above-mentioned mRNAs (***Fig. 3.6***).
On the contrary, MMP2 transcript was not detectable, as the fluorescent signal was generally measured after 35 out of 40 cycles, thus it was considered background signal (*see* ***Tab. 3.2*** *for example values*).

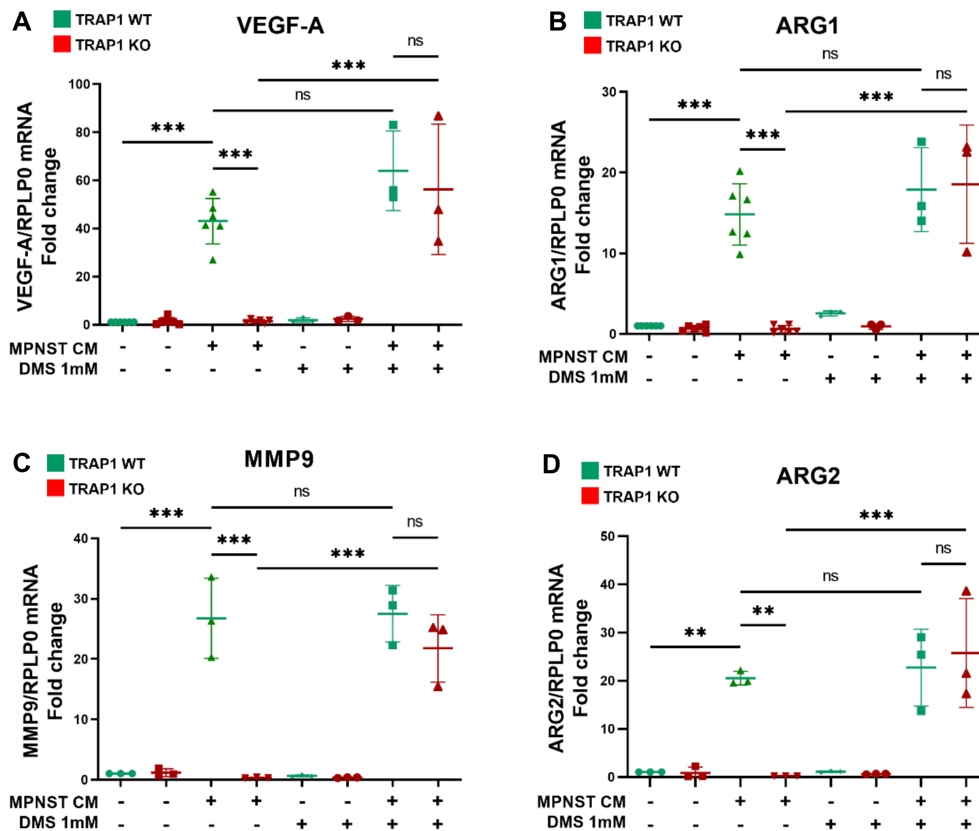

Figure 3.6 - M2-like mRNAs expression in M2-derived macrophages. *Quantification of expression levels of* ***A)*** *VEGF-A,* ***B)*** *ARG1,* ***C)*** *MMP9 and* ***D)*** *ARG2 all normalized on RPLP0 expression levels. Rescue of mRNAs expression is evident in TRAP1 KO TAM-like cells exposed to DMS. TRAP1 WT (green) and TRAP1 KO (red) samples were exposed either to control medium (-) or to MPNST CM (+), in absence or presence of DMS (-/+). Raw data were normalized through the ΔΔCt method, followed by calculation of the Fold Change. Data were analyzed through One-way Anova and represented as FC mean ± SD. (*) p < 0,05, (**) p < 0,01, (***) p < 0,001.*

Table 3.2 - qPCR Ct values in M2 samples. *Representative mean Ct values of qPCR for MMP2 in M2 samples, outside the detection range (Ct > 35).*

	Target
	MMP2
Sample	**Mean Ct**
M2 WT DMEM	36,88
M2 KO DMEM	37,74
M2 WT CM	36,91
M2 KO CM	37,12
M2 WT DMEM + DMS	35,69
M2 KO DMEM + DMS	37,65
M2 WT CM + DMS	37,20
M2 KO CM + DMS	36,65

These results highlight the role of succinate in rescuing the protumoral phenotype in the TRAP1 KO TAM-like macrophages, both M0- and M2-derived.

3.4 Effects of macrophage CM and DMS on the migration of MPNST cells

Macrophage protumoral polarization is a process that involves both phenotypical and functional characteristics; indeed, not only mRNA and protein expression can be modified but also macrophage functions can be altered. As explained earlier in the ***Introduction***, TAMs are generally able to induce migration of cancer cells and proneoplastic angiogenesis. Up to now, data from our laboratory have shown that the promigratory function exerted by TAM-like cells seems to be linked to TRAP1 presence, as the ablation of this chaperone (TRAP1 KO TAM-like macrophages) strongly impairs the promigratory capability of macrophages. To test whether the TRAP1 – succinate – HIF1α axis could be responsible for migration induction, I performed Boyden chamber migration assays. In these experiments, I assessed the different migratory ability of MPNST cells when exposed to the media derived from M0- or M2-derived TAM-like macrophages cultured either with or without DMS addition.

For all the following analyses, migration in DMEM was used as the control condition. Migration was calculated as the area covered by cells in the bottom part of the Transwell membrane, measured after proper staining with eosin, methylene blue and Azure A (commercial kit, see *Section 2.7.2*).

With the M0-derived media, migration was significantly increased when MPNST cells were cultured with the medium conditioned by M0-derived WT TAM-like

cells, compared to the control medium and to the one collected from M0-derived TRAP1 KO TAM-like cells. However, when cultured with the media coming from M0 macrophages treated with 1 mM DMS, MPNST cell migration was enhanced in presence of the medium coming from M0-derived TRAP1 KO TAM-like cells to a comparable level to the TRAP1 WT counterpart exposed to MPNST CM and DMS. (***Fig. 3.7***).

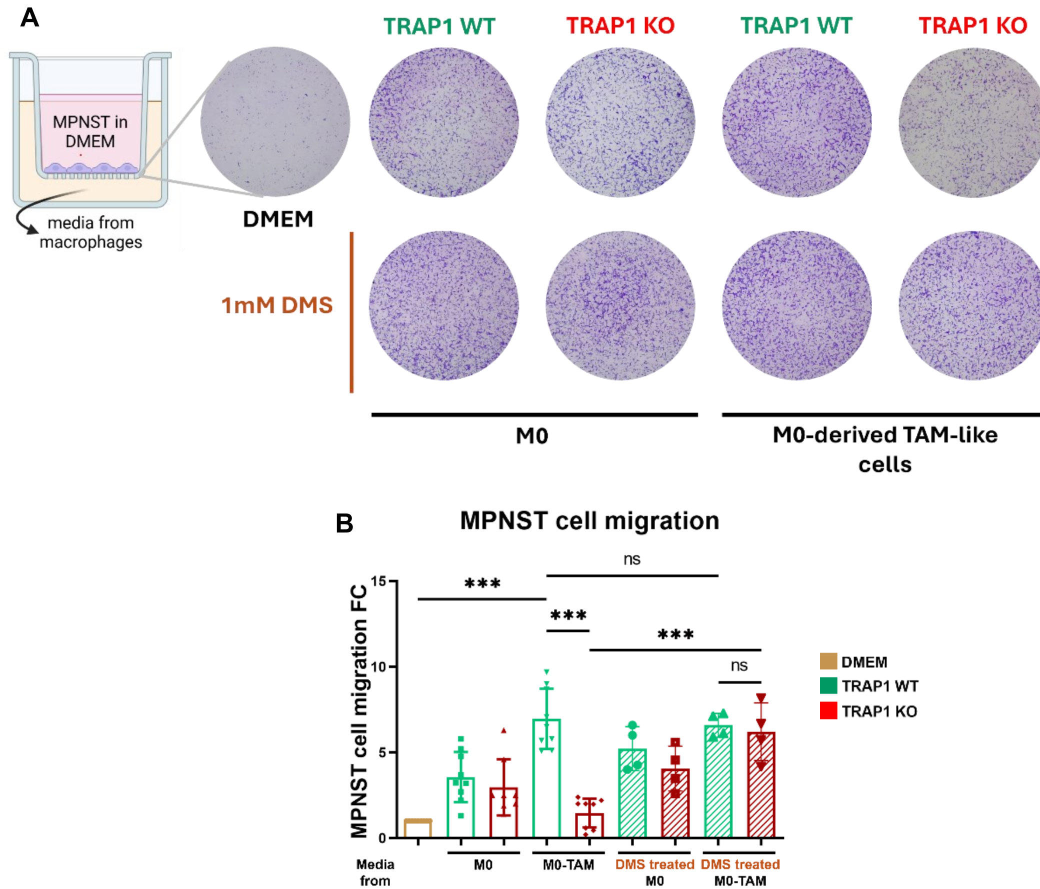

Figure 3.7 - Boyden chamber migration assay with M0-derived media. ***A)*** *Representative images of the different Transwell membranes after staining, indicating MPNST migration (purple areas are indicative of migrated cells) when exposed to the different macrophage-derived media (M0 or M0-derived TAM-like cells).* ***B)*** *Quantification of the migration calculated as the area covered by MPNST cells, normalized on DMEM control. Cells were exposed to control media (DMEM, yellow), to M0- or M0-TAM derived media either from TRAP1 WT (green) and TRAP1 KO (red) macrophages, without or with ("DMS treated", orange) 1mM DMS treatment during culture period (48 h). Data were analyzed through One-way Anova and displayed as FC mean ± SD. (*) $p < 0{,}05$, (**) $p < 0{,}01$, (***) $p < 0{,}001$.*

When performing the same assay with the M2-derived media, migration was found to be consistently enhanced in presence of media conditioned by M2-derived WT

TAM-like cell, compared to the control and the KO counterpart (M2-derived TRAP1 KO TAM-like cells). Also in this case, the media coming from M2-derived TRAP1 KO TAM-like cells treated with DMS was able to induce a significant increase in MPNST migration, to a similar level as the M2-derived WT TAM-like cells treated with DMS (***Fig. 3.8***).

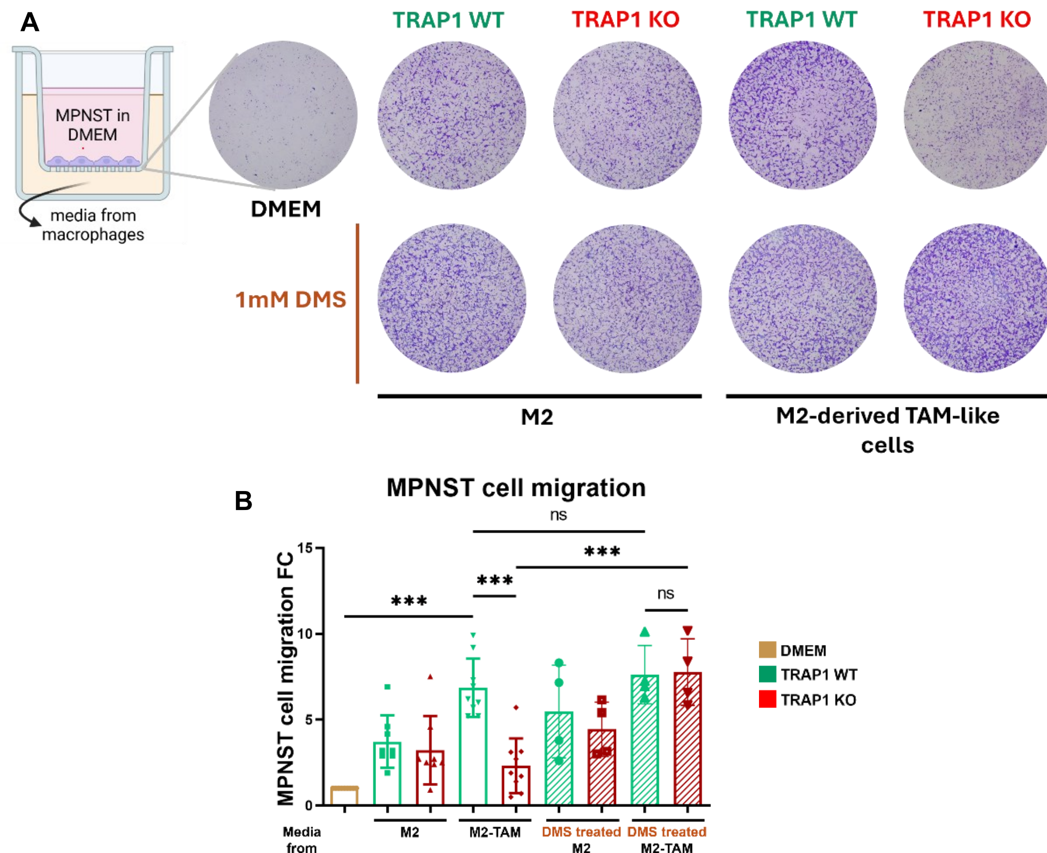

Figure 3.8 - Boyden chamber migration assay with M2-derived media. A) *Representative images of the different Transwell membranes after staining, indicating MPNST migration (purple areas are indicative of migrated cells) when exposed to the different macrophage-derived media (M2 or M2-derived TAM-like cells).* ***B)*** *Quantification of the migration calculated as the area covered by cells, normalized on DMEM control. Cells were exposed to control media (DMEM, yellow), to M2- or M2-TAM derived media either from TRAP1 WT (green) and TRAP1 KO (red) macrophages, without or with ("DMS treated", orange) 1mM DMS treatment during culture period (48 h). Data were analyzed through One-way Anova and displayed as FC mean ± SD. (*) $p < 0,05$, (**) $p < 0,01$, (***) $p < 0,001$.*

Taken together, these results first validate the role of TRAP1 in the protumoral polarization of TAM-like cells (M0 or M2 macrophages exposed to MPNST CM). Indeed, the rise in migration capability of MPNST cells was detected solely when incubated with the media collected from M0/M2-derived WT TAM-like cells and

not in the presence of the media coming from macrophages lacking the chaperone TRAP1 (M0/M2-derived TRAP1 KO TAM-like cells). When performing the assay with DMS-treated macrophage media, MPNST migratory ability was also induced in the setting employing M0/M2-derived TRAP1 KO TAM-like cells. This suggests that succinate addition during macrophage culture in the presence of MPNST CM can restore their protumoral polarization even when TRAP1 is knocked-out, thus pointing to a metabolite-mediated TRAP1 role in macrophage tumor supporting functions.

4 DISCUSSION

Tumor associated macrophages are immune cells present in the TME of different tumors that generally sustain tumor growth, metastasis and angiogenesis, mostly assuming an M2-like phenotype.[1] Their protumoral polarization is mainly driven by environmental cues and one key player in this process is tumor hypoxia, which can lead to HIF1α stabilization in TAMs. This transcription factor can in turn activate different genetic programs that promote the phenotypic and functional rewiring of macrophages toward a proneoplastic state.[8]
Given that many metabolic changes occur within TAMs when in contact with the TME, it could be of great interest to find key metabolic drivers that promote their protumoral polarization, as these could open new perspectives in targeting TAMs and TME of the various tumors.

In this work, I focused my attention on the mitochondrial chaperone TRAP1, whose presence has been found to contribute to *in vitro* TAM proneoplastic polarization (*unpublished data*). Indeed, TRAP1 plays a role in the regulation of mitochondrial metabolism in neoplastic cells, as it is implicated in the promotion of tumor growth and in the formation of a pseudo-hypoxic environment. The chaperone exerts its action in cancer cells through the inhibition of SDH and consequent succinate accumulation, ultimately leading to HIF1α stabilization.[24,29]

Considering that succinate-dependent HIF1α stabilization contributes to macrophage polarization[22] and that TRAP1 can regulate the metabolism by inhibiting SDH and stabilizing this transcription factor[24], my interest was to understand the possible presence of a signaling axis involving TRAP1, succinate and HIF1α in TAMs.

This hypothesis has been evaluated using an *in vitro* model, where BMDMs were exposed to a conditioned medium obtained from MPNST cancer cells. Single cell analysis of MPNSTs have shown that the TME of these tumors comprises a variety of cells, among which one of the major populations consists of proneoplastic TAMs[33]. As macrophages are naturally present within the TME of these sarcomas, MPNST cells have been considered a good model to mimic the tumor microenvironment in order to obtain *in vitro* TAM-like cells. Moreover, the lack of successful therapies for this aggressive cancer has raised the interest toward the study of its TME.

Both M0-derived and M2-derived macrophages have been considered in this work, in order to gain a more complete vision of the effects of MPNST CM exposure in different macrophage populations. M0-derived cells are basal macrophages normally found in resting conditions prior to any polarizing stimulus, whereas M2-derived macrophages are already primed to exert protumoral functions before exposure to the cancer cell medium. In this perspective, considering both

populations was fundamental to gain an insight of the different responses of TAM-like macrophages when exposed to CM.

Previous results collected in the laboratory (*unpublished data*) have shown that TRAP1 has a fundamental role in driving macrophage polarization, as its ablation hinders the ability of these cells to acquire an M2-like state, both at the phenotypical and at the functional level.
In this work, the analyses were directed at understanding the mechanism of TRAP1 action, proposing a possible axis between the chaperone, succinate and HIF1α. This aim was pursued by evaluating the potential rescue of the protumoral state in TRAP1 KO TAM-like cells when treated with a permeable form of succinate (DMS).

Initial phenotypical investigations through Western Blot analyses have demonstrated the rescue of HIF1α stabilization both in M0- and M2-derived TRAP1 KO macrophages when treated with DMS. Succinate administration was thus fundamental to bypass the absence of the chaperone, restoring the presence of one key polarization driver (HIF1α).

HIF1α triggers the transcription of different genes containing hypoxia responsive elements (HRE) in their promotor[20] and many M2-markers are targets of this transcription factor. Examples are ARG1, a fundamental enzyme that converts arginine into ornithine and urea and is upstream to polyamines and proline synthesis, and defines anti-inflammatory macrophages; and MMP9, an enzyme responsible for ECM degradation and matrix remodeling. Both of them are HIF1α targets and their presence has been recovered in M2-derived TRAP1 KO TAM-like cells when supplemented with succinate, thus stressing the phenotypical rescue operated by this metabolite. The observation that succinate is able to restore HIF1α and M2 marker expression in KO cells reinforces the hypothesis that the chaperone works by inducing succinate accumulation.
However, not all M2 markers have resulted in this clear upregulation, as in the case of ARG2. This result could simply be due to the lack of sufficient biological replicates, as an increase in its levels is evident in M2-derived TRAP1 KO TAM-like cells treated with DMS, though not significative.

In M0-derived cells, expression of the aforementioned HIF1α-dependent markers was not at all detectable. Still these cells can be classified as M2-like macrophages because they upregulate other M2 typical markers, such as GS and CD206. Considering that they are a different population compared to the M2-derived macrophages, possibly a different expression pattern could be at the basis of the lack of some markers. On the other hand, enzymes such as matrix metalloproteinases are normally secreted in the extracellular space[9], where they exert their function. In this sense, the release of enzymes could be another possible explanation for the failed visualization of proteins through Western Blot.

As expected, markers such as GS and CD206, that are not HIF1α targets, were not upregulated in macrophages when exposed to succinate. This strengthens the idea that succinate could be tightly linked to the stabilization of HIF1α, without exerting other broad effects.

To further clarify the phenotypical polarization of TAM-like cells, evaluation of HIF1α-dependent transcripts was performed. These results evidence a significant rescue pattern in the expression of some mRNAs upon DMS treatment. For example, in M0-derived cells it was possible to notice the expression of VEGF-A, ARG1 and MMP9 mRNAs with increased levels in the TRAP1 KO TAM-like cells upon DMS exposure. Along with these observations, also in M2-derived TRAP1 KO macrophages the expression of VEGF-A, ARG1, MMP9 and ARG2 was induced following DMS administration. These observations together with the Western Blot analyses underline the complex nature of TAM-like cells, as some M2 markers can be found either as mRNAs or as proteins, with high levels of variability. In this sense, the presence of M2-like mRNAs in M0-derived TAMs suggests that they are actually expressing HIF1α-dependent M2 markers, although some other processes could be engaged thus impeding their visualization as proteins in cell lysates.

Limiting the analysis to a phenotypic investigation would have been belittling, thus an evaluation of TAM functions was also executed. One of the main characteristics of protumoral TAMs is promoting cancer cell migration, thus this particular feature was investigated to deepen the comprehension of these immune cells. As the hypothesis takes into account TRAP1 role in both phenotypical and functional TAM polarization, also in this situation succinate administration was expected to rescue the promigratory function in TRAP1 KO macrophages. Coherently, both M0- and M2-derived TRAP1 KO TAM-like cells restored their ability to induce migration of MPNST cells when exposed to succinate. Because the assay was performed using media deriving directly from macrophage cultures, it sounds logical to ask whether migration could depend on residual succinate presence, as this metabolite is generally implicated in cancer cell growth and survival.[21] However, if this was the case, also control macrophages treated with succinate should have displayed a strong increase in this function, but that was not evidenced. Consequently, migration was promoted only by macrophage released factors that are present in the media, even though their nature is still unknown.

Taken together, all these results validate the initial hypothesis that TRAP1-mediated proneoplastic polarization of macrophages occurs through succinate accumulation and subsequent HIF1α stabilization. This has been confirmed by both the phenotypical and functional rescues obtained through succinate addition in M0-derived and M2-derived TRAP1 KO TAM-like cells.

Even though no direct experiments have properly addressed whether protumoral polarization was actually dependent on stabilization of HIF1α, some clues about the

role of this transcription factor can be found. In particular, the fact that HIF1α itself was stabilized in Western Blot analysis and that only the expression of HIF1α-dependent M2 markers was upregulated if compared to other proteins, allow us to speculate that the main actor of the axis could actually be HIF1α. To have definitive proof of this hypothesis, silencing of the transcription factor in TRAP1 WT macrophages should be performed, followed by exposure to tumor CM. In this way, the so far evidenced protumoral polarization should not be expected, thus confirming the key role of HIF1α in this process.

However, due to the known role of this transcription factor in the activation of many different protumoral genetic programs, I can support the model in which the TRAP1 chaperone drives macrophage protumoral polarization through succinate – HIF1α axis.

Looking forward, the identification of TRAP1 as one key driver in the polarization of these immune cells could open innovative perspectives for TME targeting. Future strategies could take into consideration the modulation of this chaperone activity to rewire macrophages toward a proinflammatory and anti-tumoral state, in order to indirectly hit cancer cells.

5 BIBLIOGRAPHY

1. Hao NB, Lü MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol*. 2012;2012. doi:10.1155/2012/948098

2. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;496(7446):445-455. doi:10.1038/nature12034

3. Cox N, Pokrovskii M, Vicario R, Geissmann F. Origins, Biology, and Diseases of Tissue Macrophages. Published online 2021:6. doi:10.1146/annurev-immunol-093019

4. Lee CZW, Ginhoux F. Biology of resident tissue macrophages. *Development (Cambridge)*. 2022;149(8). doi:10.1242/dev.200270

5. Yona S, Kim KW, Wolf Y, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*. 2013;38(1):79-91. doi:10.1016/j.immuni.2012.12.001

6. Wang S, Liu G, Li Y, Pan Y. Metabolic Reprogramming Induces Macrophage Polarization in the Tumor Microenvironment. *Front Immunol*. 2022;13. doi:10.3389/fimmu.2022.840029

7. Chen S, Saeed AFUH, Liu Q, et al. Macrophages in immunoregulation and therapeutics. *Signal Transduct Target Ther*. 2023;8(1). doi:10.1038/s41392-023-01452-1

8. He Z, Zhang S. Tumor-Associated Macrophages and Their Functional Transformation in the Hypoxic Tumor Microenvironment. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.741305

9. Bied M, Ho WW, Ginhoux F, Blériot C. Roles of macrophages in tumor development: a spatiotemporal perspective. *Cell Mol Immunol*. 2023;20(9):983-992. doi:10.1038/s41423-023-01061-6

10. Xiao L, Wang Q, Peng H. Tumor-associated macrophages: new insights on their metabolic regulation and their influence in cancer immunotherapy. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1157291

11. Basak U, Sarkar T, Mukherjee S, et al. Tumor-associated macrophages: an effective player of the tumor microenvironment. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1295257

12. Warburg O. The Metabolism of Carcinoma Cells. *J Cancer Res*. 1925;9(1):148-163. doi:10.1158/jcr.1925.148

13. Li J, DeNicola GM, Ruffell B. Metabolism in tumor-associated macrophages. In: *International Review of Cell and Molecular Biology*. Vol 367. Elsevier Inc.; 2022:65-100. doi:10.1016/bs.ircmb.2022.01.004

14. Wu K, Lin K, Li X, et al. Redefining Tumor-Associated Macrophage Subpopulations and Functions in the Tumor Microenvironment. *Front Immunol*. 2020;11:1731. doi:10.3389/fimmu.2020.01731

15. Chu X, Tian Y, Lv C. Decoding the spatiotemporal heterogeneity of tumor-associated macrophages. *Mol Cancer*. 2024;23(1). doi:10.1186/s12943-024-02064-1

16. Colegio OR, Chu NQ, Szabo AL, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 2014;513(7519):559-563. doi:10.1038/nature13490

17. Galván-Peña S, O'Neill LAJ. Metabolic reprogramming in macrophage polarization. *Front Immunol*. 2014;5(AUG). doi:10.3389/fimmu.2014.00420

18. Geeraerts X, Fernández-Garcia J, Hartmann FJ, et al. Macrophages are metabolically heterogeneous within the tumor microenvironment. *Cell Rep*. 2021;37(13). doi:10.1016/j.celrep.2021.110171

19. Palmieri EM, Menga A, Martín-Pérez R, et al. Pharmacologic or Genetic Targeting of Glutamine Synthetase Skews Macrophages toward an M1-like Phenotype and Inhibits Tumor Metastasis. *Cell Rep*. 2017;20(7):1654-1666. doi:10.1016/j.celrep.2017.07.054

20. Liao C, Zhang Q. Understanding the Oxygen-Sensing Pathway and Its Therapeutic Implications in Diseases. *American Journal of Pathology*. 2020;190(8):1584-1595. doi:10.1016/j.ajpath.2020.04.003

21. Wu JY, Huang TW, Hsieh YT, et al. Cancer-Derived Succinate Promotes Macrophage Polarization and Cancer Metastasis via Succinate Receptor. *Mol Cell*. 2020;77(2):213-227.e5. doi:10.1016/j.molcel.2019.10.023

22. Mills EL, Kelly B, Logan A, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell*. 2016;167(2):457-470.e13. doi:10.1016/j.cell.2016.08.064

23. Trauelsen M, Hiron TK, Lin D, et al. Extracellular succinate hyperpolarizes M2 macrophages through SUCNR1/GPR91-mediated Gq signaling. *Cell Rep*. 2021;35(11). doi:10.1016/j.celrep.2021.109246

24. Masgras I, Laquatra C, Cannino G, Serapian SA, Colombo G, Rasola A. The molecular chaperone TRAP1 in cancer: From the basics of biology to pharmacological targeting. *Semin Cancer Biol*. 2021;76:45-53. doi:10.1016/j.semcancer.2021.07.002

25. Joshi A, Ito T, Picard D, Neckers L. The Mitochondrial HSP90 Paralog TRAP1: Structural Dynamics, Interactome, Role in Metabolic Regulation, and Inhibitors. *Biomolecules*. 2022;12(7). doi:10.3390/biom12070880

26. Masgras I, Sanchez-Martin C, Colombo G, Rasola A. The chaperone TRAP1 as a modulator of the mitochondrial adaptations in cancer cells. *Front Oncol*. 2017;7(MAR). doi:10.3389/fonc.2017.00058

27. Somatilaka BN, Sadek A, McKay RM, Le LQ. Malignant peripheral nerve sheath tumor: models, biology, and translation. *Oncogene*. 2022;41(17):2405-2421. doi:10.1038/s41388-022-02290-1

28. Walker JA, Upadhyaya M. Emerging therapeutic targets for neurofibromatosis type 1. *Expert Opin Ther Targets*. 2018;22(5):419-437. doi:10.1080/14728222.2018.1465931

29. Masgras I, Ciscato F, Brunati AM, et al. Absence of Neurofibromin Induces an Oncogenic Metabolic Switch via Mitochondrial ERK-Mediated Phosphorylation of the Chaperone TRAP1. *Cell Rep*. 2017;18(3):659-672. doi:10.1016/j.celrep.2016.12.056

30. Masgras I, Cannino G, Ciscato F, et al. Tumor growth of neurofibromin-deficient cells is driven by decreased respiration and hampered by NAD+ and SIRT3. *Cell Death Differ*. 2022;29(10):1996-2008. doi:10.1038/s41418-022-00991-4

31. Prada CE, Jousma E, Rizvi TA, et al. Neurofibroma-associated macrophages play roles in tumor growth and response to pharmacological inhibition. *Acta Neuropathol*. 2013;125(1):159-168. doi:10.1007/s00401-012-1056-7

32. Heap RE, Marín-Rubio JL, Peltier J, et al. Proteomics characterisation of the L929 cell supernatant and its role in BMDM differentiation. *Life Sci Alliance*. 2021;4(6). doi:10.26508/LSA.202000957

33. Wu LMN, Zhang F, Rao R, et al. Single-cell multiomics identifies clinically relevant mesenchymal stem-like cells and key regulators for MPNST malignancy. *Sci Adv*. 2022;8(44). doi:10.1126/sciadv.abo5442

6 RIASSUNTO

6.1 Introduzione e scopo del progetto

I macrofagi sono cellule del sistema immunitario innato che si trovano in tutti i tessuti con vari ruoli, dalla rapida risposta alle infezioni fino al mantenimento dell'omeostasi tissutale. Queste cellule sono altamente plastiche e normalmente presenti in uno stato basale (detto M0) e, a seguito di stimolazioni dall'ambiente esterno, potranno subire un processo cosiddetto di polarizzazione, ossia una riprogrammazione metabolica, fenotipica e funzionale finalizzata allo svolgimento dei loro diversi ruoli. In modo semplicistico, i macrofagi vengono classificati come M1, proinfiammatori e antitumorali, che intervengono durante le infezioni per fagocitare il patogeno e reclutare altre cellule immunitarie; o M2, antinfiammatori e protumorali, deputati allo spegnimento dell'infiammazione, alla guarigione delle ferite e al rimodellamento tissutale. Questi ultimi sono anche in grado di sostenere un'eventuale crescita tumorale e favorire processi migratori e di neoangiogenesi (***Fig. 1.1*** *per i dettagli della polarizzazione M1/M2*).

Oltre alle funzioni appena elencate, i macrofagi hanno un ruolo importante anche nella formazione e sviluppo dei tumori, all'interno dei quali assumono il nome di **TAM** (macrofagi associati al tumore). In questo contesto, il microambiente tumorale è in grado di favorire una polarizzazione dei TAM generalmente verso un fenotipo M2 (protumorale), portando ad una riorganizzazione metabolica degli stessi che sta alla base delle loro caratteristiche e funzioni. I tratti distintivi del metabolismo dei TAM comprendono: un incremento marcato dei processi glicolitici (anche in presenza di ossigeno) pur mantenendo intatti il ciclo di Krebs e la fosforilazione ossidativa; un aumento dell'espressione di enzimi quali Arginasi 1 (ARG1), per la conversione dell'arginina in ornitina e successivamente in poliammine e prolina, e Glutammina Sintetasi (GS), fondamentale per mantenere elevati i livelli di glutammina; infine un accumulo degli acidi grassi, utilizzati in diversi processi (***Fig. 1.2*** *per i dettagli del metabolismo dei TAM*).

Si aggiunge al quadro il succinato, un metabolita normalmente ossidato dalla succinato deidrogenasi (SDH) all'interno del ciclo di Krebs, che risulta però coinvolto nel processo di polarizzazione M2 dei TAM. Il suo accumulo a seguito dell'inibizione della SDH porta alla stabilizzazione di HIF1α, fattore di trascrizione che promuovere l'espressione di marcatori M2 (ad esempio ARG1, metalloproteasi MMP9, fattore di crescita dell'endotelio vascolare VEGF-A). Inoltre, il succinato extracellulare può legarsi al suo recettore (SUCNR1) sulla membrana dei macrofagi favorendo l'attivazione della via del PI3K/AKT/HIF1α che conduce ad un fenotipo antinfiammatorio.

I TAM nel contesto neoplastico possono svolgere diverse funzioni, tra cui le più importanti riguardano l'immunomodulazione, l'angiogenesi, ossia la formazione di nuovi vasi (regolata dal VEGF-A), e il rimodellamento della matrice extracellulare,

guidato da enzimi come le metalloproteasi (MMP9, MMP2) per favorire i successivi processi di migrazione e metastatizzazione (***Fig. 1.3*** *per i dettagli delle funzioni dei TAM*).

Essendo implicati nei processi di crescita tumorale, i TAM vengono studiati come possibili bersagli terapeutici. Si rende perciò indispensabile una maggiore comprensione dei regolatori metabolici che stanno alla base della loro polarizzazione. In questo lavoro, lo chaperone mitocondriale TRAP1 è stato proposto come possibile modulatore del loro metabolismo.

TRAP1 è uno chaperone mitocondriale (famiglia HSP90) che fisiologicamente assiste al corretto ripiegamento delle proteine. Tuttavia, la sua attività è stata maggiormente correlata a condizioni patologiche quali i tumori. In questo contesto TRAP1 interagisce con diversi enzimi, tra cui la SDH, che viene inibita dallo chaperone portando ad un accumulo del succinato che stabilizza HIF1α, il quale infine induce l'attivazione di programmi genetici pseudo-ipossici (***Fig. 1.5*** *per i dettagli delle interazioni di TRAP1 nel contesto neoplastico*).

Il coinvolgimento di TRAP1 è stato descritto anche nel sarcoma associato alla Neurofibromatosi di tipo 1 chiamato **MPNST** (tumore maligno della guaina dei nervi periferici). In questo tumore, una mutazione germinale causa la perdita della neurofibromina (proteina deputata all'inattivazione di RAS) portando ad un'attivazione costitutiva della via di RAS/MEK/ERK che sfocia nell'induzione di programmi proneoplastici. ERK fosforilata è anche in grado di interagire e fosforilare TRAP1, aumentandone l'attività. Quest'ultimo agisce inibendo la SDH e portando al conseguente accumulo del succinato e alla stabilizzazione di HIF1α.

Il tumore MPNST è un sarcoma molto aggressivo per il quale attualmente mancano delle cure definitive. All'interno del suo microambiente, è stata evidenziata la presenza di TAM, che sembra essere correlata con una progressione maligna del sarcoma stesso. Da qui la scelta di utilizzare cellule di MPNST come modello per la polarizzazione *in vitro* dei macrofagi, con l'obiettivo di indagare il ruolo di TRAP1 come regolatore metabolico dei TAM, visto il contributo di questo chaperone nel contesto neoplastico.
In particolare, lo scopo di questo lavoro è di chiarire il possibile meccanismo di funzionamento di TRAP1 nella polarizzazione dei macrofagi, proponendo l'esistenza di un asse TRAP1 – succinato – HIF1α che sottostà al processo di polarizzazione (***Fig. 1.9*** *per l'ipotesi sperimentale*).

6.2 Risultati

Al fine di validare l'ipotesi, ho analizzato due diverse popolazioni di macrofagi, TRAP1 WT e TRAP1 KO, estratti come monociti dal midollo osseo di femore e tibia di topo e successivamente differenziati in macrofagi basali (M0). I macrofagi sono stati poi mantenuti come M0 o indotti a M2 e successivamente condizionati con il mezzo derivante dalle cellule tumorali MPNST per 48 ore, oppure lasciati in

terreno di controllo (***Tab. 2.1** per le condizioni sperimentali e **Fig. 2.1** per il processo completo di estrazione, differenziamento e condizionamento*).

L'esposizione al mezzo condizionato induce una polarizzazione proneoplastica nei macrofagi, che diventano così simili ai TAM (simil-TAM). Inoltre, l'assenza di TRAP1 (TRAP1 KO) riduce il livello di polarizzazione dei simil-TAM, mostrando come lo chaperone svolga un ruolo fondamentale in questo processo (***Fig. 1.7** e **Fig. 1.8** per i dati preliminari sugli effetti di TRAP1 nella polarizzazione dei macrofagi*).

Per poter elucidare il meccanismo d'azione dello chaperone, ho aggiunto ai macrofagi in coltura il dimetil succinato (DMS), una forma permeabile del succinato, per verificare un eventuale ripristino della polarizzazione protumorale nei simil-TAM TRAP1 KO.

Le prime analisi fenotipiche, condotte tramite la tecnica del Western Blot, hanno dimostrato un ripristino della stabilizzazione del fattore di trascrizione HIF1α nei macrofagi TRAP1 KO esposti al mezzo condizionato e al DMS, ad un livello comparabile con i simil-TAM TRAP1 WT, sia nella popolazione M0 che M2 (***Fig. 3.1** e **Fig. 3.2***).

Successivamente, è stata analizzata l'espressione dei geni M2 dipendenti da HIF1α, quali ARG1, ARG2 e MMP9, da cui è emerso un ripristino significativo dei livelli di ARG1 e MMP9 nei simil-TAM TRAP1 KO quando trattati con il DMS, ma solamente per la popolazione degli M2 (***Fig. 3.4***). Per la popolazione degli M0, questi marcatori non erano rilevabili in Western Blot, ma sono stati comunque evidenziati altri marcatori M2, indipendenti da HIF1α (GS e CD206), nei simil-TAM che non hanno dimostrato alterazioni a seguito dell'aggiunta del DMS (***Fig. 3.3***). Ciò suggerisce la presenza di altri meccanismi di polarizzazione indipendenti dall'asse succinato-HIF1α, al contempo rafforzando l'idea che il succinato agisca solo tramite l'asse ipotizzato.

Si è quindi proceduto con un'analisi dei trascritti tramite la tecnica della qPCR, valutando anche VEGF-A e MMP2 in aggiunta ai marcatori già citati. È stato rilevato un aumento significativo dei livelli dei trascritti di VEGF-A, ARG1 e MMP9 nei simil-TAM TRAP1 KO trattati con il DMS, sia per gli M0 che per gli M2 a livelli comparabili con i simil-TAM TRAP1 WT (***Fig. 3.5*** e ***Fig. 3.6***). Negli M2, anche ARG2 ha mostrato la stessa tipologia di incremento.

Per un'analisi più approfondita, ho valutato anche la funzione promigratoria di questi TAM esercitata sulle cellule MPNST, tramite la tecnica della migrazione in camera di Boyden. È stato osservato un notevole aumento della migrazione delle cellule MPNST in presenza del terreno derivante dai simil-TAM TRAP1 KO quando trattati con DMS, sia per gli M0 che per gli M2 (***Fig. 3.7*** e ***Fig. 3.8***).

Considerando tutti questi risultati, si può notare come l'aggiunta di succinato porti ad un ripristino del fenotipo e delle funzioni protumorali dei macrofagi esposti al mezzo tumorale anche in assenza di TRAP1. Nonostante la variabilità riscontrata, questi dati nel complesso confermano l'esistenza di un asse TRAP1 – succinato – HIF1α che guida la polarizzazione dei macrofagi nel contesto neoplastico. Tale osservazione può porre le basi per nuove terapie in grado di bersagliare i macrofagi nel microambiente tumorale, con lo scopo di colpire indirettamente il tumore.