

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

The role of poly(ADP-ribose) polymerase 14 in shaping the phenotype
of tumor-associated macrophages in breast cancer

by

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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CONTENTS

1. ABBREVIATIONS	4
2. INTRODUCTION.....	7
3. THEORETICAL BACKGROUND.....	9
3.1. Breast Cancer	9
3.1.1. Epidemiology and Biology of Breast Cancer.....	9
3.1.2. Current Treatments for Breast Cancer: Strengths and Weaknesses.....	10
3.1.3. Anti-HER2 Therapy and Trastuzumab	12
3.2. The Tumor Stroma: Impact on Drug Resistance.....	14
3.3. Stromal Cells in the Breast Cancer Tumor Microenvironment	18
3.4. Macrophages (MΦs) and Tumor-Associated MΦs (TAMs).....	19
3.4.1. The Origin of MΦs.....	19
3.4.2. MΦ Polarization and TAMs.....	20
3.4.3. TAMs as a Promising Therapeutic Target for Cancer Treatment.....	23
3.4.4. The Role of TAMs in Breast Cancer.....	24
3.5. The Poly(ADP-ribose) Polymerase (PARP) Superfamily and Poly(ADP-ribosyl)ation (PARylation).....	26
3.6. The Potential Role of PARylation and PARP14 in MΦ Biology.....	30
3.7. Multicellular Tumor Spheroids (MCTS).....	32
3.8. Applications of MCTS: Impact in Cancer Research and Drug-screening.....	34
4. AIMS OF STUDY	36
4.1. Part I.....	36
4.2. Part II.....	36
5. MATERIALS AND METHODS	37
5.1. Chemicals and Reagents	37
5.2. Cell culture and maintenance	38
5.3. Isolation and differentiation of murine bone marrow-derived macrophages (BMDMs).....	39
5.4. Treatments with PARP inhibitors to study BMDMs polarization.....	39
5.5. TAM phenotype induction by 4T1 conditioned media (CM)	40
5.6. Isolation of human peripheral blood mononuclear cells (PBMCs) and macrophage (MΦ) polarization.....	40
5.7. MΦ differentiation from THP-1 monocyte cells.....	41
5.8. Generation of 3D in vitro models: JIMT-1 derived MΦ spheroids, THP-1 spheroids and JIMT-1-THP-1 co-culture spheroids	41
5.9. Treatments of spheroids and co-culture spheroids with PARP inhibitors.....	42
5.10. Total RNA isolation, reverse transcription and quantitative real-time PCR (RT-qPCR)	42
5.11. Flow cytometry	44

5.12. Measurement of the cytokine concentrations	44
5.13. High-Content Analysis (HCA) on live cells: cell death in spheroids	45
5.13.1. Effect of PARP inhibitors on JIMT-1-THP-1 MΦ spheroid co-cultures.....	45
5.13.2. Effect of test compounds on ADCC in a JIMT-1+NK92.CD16 spheroid model	46
5.14. Statistical analysis	47
6. RESULTS.....	48
6.1. Part I.....	48
6.1.1. PARP14 mediates IL-4-induced M2 polarization in murine BMDMs	48
6.1.2. PARP14 mediates TAM polarization in murine BMDMs.....	50
6.1.3. M2 polarization in human PBMC-MΦs is regulated via PARP14.....	51
6.1.4. THP-1 derived MΦs spontaneously undergo polarization toward an M2-like phenotype when co-cultured with JIMT-1 breast cancer cells in spheroids.....	55
6.1.5. PARP inhibitors suppress M2 marker expression in JIMT-1-THP-1 derived MΦ spheroid co-cultures.....	57
6.1.6. Production of soluble mediators by THP-1 derived MΦs is modulated by PARP enzymes.....	59
6.2. Part II.	61
6.2.1. High-Content Analysis (HCA) for studying spheroid co-cultures.....	61
6.2.2. HCA on a 3D model of ADCC for the identification of effector compound.....	63
7. DISCUSSION	67
8. SUMMARY	77
9. REFERENCES	78
10. PUBLICATION LIST PREPARED BY THE KENÉZY LIFE SCIENCES LIBRARY	88
11. KEYWORDS.....	90
12. ACKNOWLEDGEMENTS.....	91
13. APPENDIX.....	93

1. ABBREVIATIONS

3D - three-dimensional

7-AAD - 7-aminoactinomycin-D

ADCC - antibody-dependent cellular cytotoxicity

ADP - adenosine diphosphate

ADPRT- ADP-ribosyl transferase

Arg1 - Arginase 1

ARH3 - (ADP-ribosyl) hydrolase 3

BC - breast cancer

BMDM - bone marrow-derived macrophage

BRCA1 - breast cancer gene 1

CAR - chimeric antigen receptor

CCL - chemokine (c-c motif) ligand

CD - cluster of differentiation

CHIL1 - chitinase-3-like protein 1

CM - conditioned media

CTB - Cell Tracker Blue

CXCL - chemokine ligand

DAPI - 4',6-diamidino-2-phenylindole

DC - dendritic cell

DMEM - Dulbecco's Modified Eagle Medium

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

ECM - extracellular matrix

EMT - epithelial-mesenchymal transition

EGFP - enhanced green fluorescent protein

ER - estrogen receptor

Fab - fragment antigen binding

FBS - fetal bovine serum

FcγR - F c gamma receptor

FCS - fetal calf serum

FDA - Food and Drug Administration

Fizz1 - Resistin-like molecule alpha (found in inflammatory zone protein)

HCA - High-Content Analysis

HER2 - human epidermal growth factor receptor 2

HR - hormone receptor

IFN- γ - interferon- γ

IL - interleukin

KO - knockout

KIR - killer-cell immunoglobulin-like receptor

LCN2 - lipocalin-2

LPS - lipopolysaccharide

MARylation - mono(ADP-ribosyl)ation

M-CSF - macrophage colony-stimulating factor

MIF - macrophage migration inhibitory factor

MMP-9 - matrix metalloproteinase-9

MRC1 - mannose receptor C-type 1

M Φ - macrophage

NAD - nicotinamide adenine dinucleotide

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

NK - Natural Killer

NKT - Natural Killer T

NLG - N-linked glycosylation

NO - nitric oxide

OPN - osteopontin

PD-L1 - programmed death-ligand 1

PAI-1 - plasminogen activator inhibitor-1

PAMPs - pathogen-associated molecular patterns

PAR - poly(ADP-ribose)

PARG - poly(ADP-ribosyl) glycohydrolase

PARP - poly(ADP-ribose) polymerase
PARPi - poly(ADP-ribose) polymerase inhibitor
PARylation - poly(ADP-ribosyl)ation
PBMC - peripheral blood mononuclear cells
PBS - phosphate-buffered saline
PMA - phorbol 12-myristate 13-acetate
PJ34 - (2-(dimethylamino)-N-(6-oxo-5H-phenanthridin-2-yl)acetamide hydrochloride
PR - progesterone receptor
RCF - relative centrifugal force
RPMI - Roswell Park Memorial Institute
RNA - ribonucleic acid
ROS - reactive oxygen species
RT-qPCR - reverse transcription-quantitative polymerase chain reaction
RTK - receptor tyrosine kinase
SEM - standard error of mean
SSC - side-scattered light
STING - stimulator of interferon genes
TAM - tumor-associated macrophages
TAN - tumor-associated neutrophils
TGF- β 1 - transforming growth factor-beta 1
Th2 - T-helper 2
TLR - Toll-like receptor
TME - tumor microenvironment
TNBC - triple-negative breast cancer
TNF - tumor necrosis factor
Tregs - regulatory T cells
VEGF - vascular endothelial growth factor
WHO - World Health Organization

2. INTRODUCTION

Breast cancer (BC) stands as the most prevalent malignant tumor among females. Tailored treatments may involve monoclonal antibody therapy and hormone therapy, potentially improving prognosis [1]. Tumor stroma cells are known to significantly influence tumor behavior and response to treatments [2]. For instance, macrophages (MΦs) are highly plastic innate immune cells commonly abundant in tumors that can transition between pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes in response to environmental cues [3]. Tumor-associated MΦs (TAMs) often display characteristics similar to M2 cells, thereby promoting tumor growth and invasiveness [4]. Within the breast tumor microenvironment, TAMs have emerged as the predominant immune cell components, exerting pivotal influence over BC tumorigenesis and progression [5]. Therefore, their involvement in BC development has recently established TAMs as novel and promising targets for BC treatment [6]. ADP-ribosylation is a posttranslational modification mediated by a family of poly(ADP-ribose) polymerase (PARP) enzymes, known to regulate various cellular functions e.g. DNA repair, gene transcription and cell death in different cell types, including MΦs [7]. Specifically, PARP14 is gaining attention as a potential target for chronic inflammation and has been implicated in tumor promotion [8]. However, its precise roles in tumor immune evasion and involvement in TAM polarization still lacks comprehensive understanding and remains poorly understood. In the first study, we investigated the role of PARP enzymes in this process. We found that the PARP inhibitor (PARPi) PJ34 and the specific PARP14 inhibitor MCD113 reduced the expression of M2 marker genes in primary murine, in THP-1 monocytic human and in primary human monocyte-derived MΦs polarized with IL-4. Mice lacking PARP14 showed reduced ability to differentiate into M2 cells. In mouse and human models of TAMs, both PARPis and PARP14 knockout (KO) weakened TAM polarization, leading to increased cancer cell apoptosis. Moreover, lipocalin-2 (LCN2), macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) were identified by protein profiling arrays as potential (ADP-ribosyl)ation-dependent mediators of TAM differentiation. Our results suggest that inhibiting PARP14 could be a promising strategy for cancer treatment by reprogramming TAM behavior to enhance anti-cancer immune responses.

Monoclonal antibody immunotherapy is a key part of BC treatment, with antibody-dependent cellular cytotoxicity (ADCC) being one of its mechanisms [9]. In this scenario, the antibody binds to cell surface antigens on the cancer cells and engages Fc receptors on immune cells, e.g. natural killer (NK) cells, activating cytotoxic immune responses, to eliminate the tumor. Enhancing the effectiveness of these therapies requires novel chemicals that boost cancer cell

sensitivity or immune cell potency [9]. Based on our recent findings [10], in the second project we developed a cancer ADCC spheroid model and outline a protocol to find ADCC-modulating drugs. Three-dimensional (3D) models e.g. cancer spheroids are superior to 2D cultures in predicting *in vivo* responses of tumors to anticancer therapies [11]. By utilizing 3D models, we established co-cultures of human epidermal growth factor receptor 2-positive (HER2+) BC cells and NK cells, inducing ADCC with Trastuzumab. Using a sensitive probe to measure apoptotic cell death and image analysis with an automated microscope, we distinguished live and dead killer and target cells. With our method, compatible with high-throughput screening, we demonstrated that Sunitinib, a Food and Drug Administration (FDA)-approved cancer drug, significantly reduces ADCC. This approach offers a platform for screening ADCC-modulating compounds in cancer cell spheroids, and, importantly, it can be applied to several assays of interest.

3. THEORETICAL BACKGROUND

3.1. Breast Cancer

3.1.1. Epidemiology and Biology of Breast Cancer

Breast cancer (BC) stands as the predominant form of malignant tumor among women on a global scale, with a minority of cases (1-2%) affecting men. This type of cancer typically originates within the milk ducts or the glandular tissue responsible for milk production within the breast. As per the World Health Organization (WHO), the global incidence of new cases surpasses 2 million annually, with nearly 700,000 women succumbing to the disease yearly [12].

The clinical categorization of this heterogeneous disease relies on the identification of cytoplasmic hormone receptors (HR), such as estrogen (ER) and progesterone (PR) receptors. Additionally, the presence of overexpressed or amplified human epidermal growth factor receptor 2 (HER2) protein/oncogene further contributes to the classification [13]. Four subtypes of BC are typically identified based on the immunohistochemical expression of these receptors: luminal A (HR+/HER2-), luminal B (HR+/HER2+), HER2-positive (HR-/HER2+), and triple-negative BC (TNBC, HR-/HER2-). Luminal A subtype constitutes approximately 60% of BC cases and is linked to a favorable prognosis. On the other hand, Luminal B subtype, representing 30% of the cases, is characterized by elevated Ki67 levels (>14%), indicative of high proliferation, and is associated with a less favorable prognosis [13, 14]. Constituting 10-15% of BC cases, the HER2-positive subgroup is characterized by elevated HER2 expression alongside the absence of ER and PR. These tumors generally exhibit a poorer prognosis compared to luminal tumors and necessitate specialized treatments targeting the HER2/neu protein [15]. Finally, TNBC accounts for 15–20% of BC cases. This subtype is noted for its heightened aggressiveness and poorer prognosis compared to other molecular subtypes, often manifesting in younger women [16]. The characteristics of BC across different molecular subtypes are presented in **Figure 1**.

Molecular subtype	Luminal A	Luminal B	HER2	TNBC
ER/PR	+			−
HER2	−	+		−
Frequency ^a	50–60%	30%	10%	10–20%
Grade ^b	Low		High	
Prognosis ^c	Good		Poor	
5–y survival rate ^d	94.3%	90.5%	84.0%	76.9%
Treatment	Endocrine therapy			
		Anti-HER2 therapy		
	Chemotherapy			

Figure 1. Characteristics of molecular subtypes in breast cancer. ER (estrogen receptor), PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), and TNBC (triple-negative breast cancer). ^a The frequency data is sourced from Al-thoubaity et al. [17] and Hergueta-Redondo et al.[18]. ^b Grade information is extracted from Engstrom et al.[19]. ^c Prognosis details are based on Hennigs et al. [20]. ^d The 5-year survival rate is obtained from the most recent SEER survival statistics. Source: [12].

The 5-year relative BC-specific survival rate is encouraging, standing at 90.3% across all subtypes and stages. However, the 5-year relative cancer-specific survival rate for metastatic BC remains dishearteningly low at 29%, irrespective of subtype, and can plummet to 12% for metastatic TNBC [9].

This underscores the inadequacy of current treatment strategies for metastatic BC patients in ensuring satisfactory survival rates. Therefore, there is a critical need to explore novel solutions for the treatment of metastatic BC.

3.1.2. Current Treatments for Breast Cancer: Strengths and Weaknesses

The selection of treatment is tailored to the grade, stage, and molecular subtype of BC, aiming to provide the most personalized, safe, and effective therapy possible. Conventional treatment modalities encompass the surgical excision of the tumor, cytotoxic chemotherapy and radiotherapy. Additionally, tailored treatment strategies are selected based on the tumor's cancer marker profile, particularly emphasizing hormone receptor (HR) expression (e.g. ER and PR receptors) and the overexpression of the oncogene HER2. This approach may incorporate

monoclonal antibody therapy and hormone therapy, offering potential enhancements to the disease prognosis [15]. **Figure 2** illustrates the algorithm for breast cancer treatment.

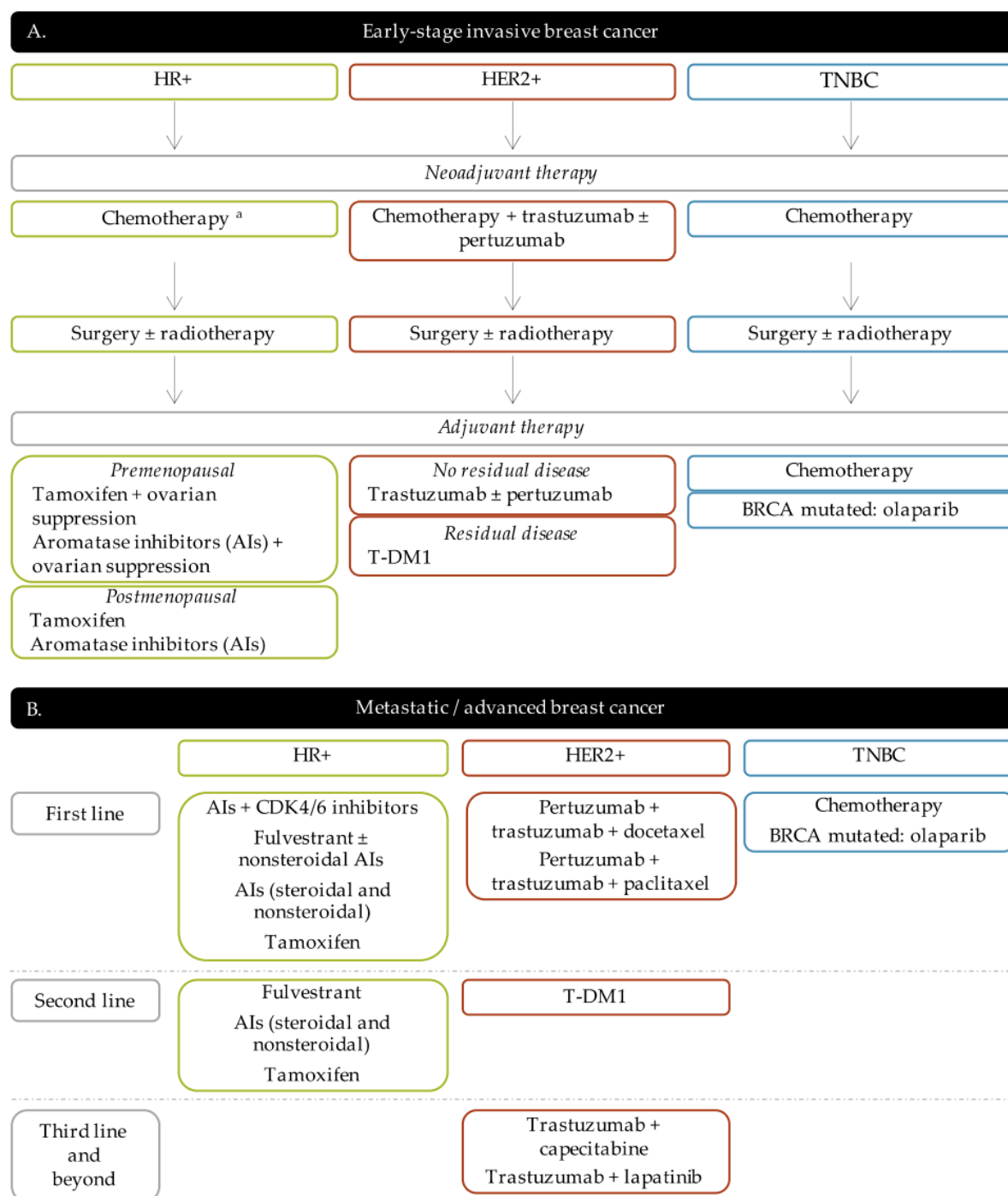


Figure 2. Flowchart for breast cancer treatment. Early-stage BC (A). Metastatic or advanced BC (B). ^a Neoadjuvant chemotherapy for hormone receptor-positive BC patients is not universally prescribed. It is primarily recommended for luminal B BC patients and/or older BC patients. HR+: hormone receptor positive; HER2+: human epidermal growth factor receptor 2 positive; TNBC: triple-negative BC; AIs: aromatase inhibitors; T-DM1: trastuzumab-emtansine. Source: [12].

BC progression entails a multi-step process, emphasizing the critical importance of early detection for effective disease management [21]. Despite the advent of personalized treatment modalities for BC, such as endocrine and anti-HER2 antibody therapies, the complexity of this disease persists as a formidable challenge for oncologists. Similarly to conventional treatments like surgery, chemotherapy and radiotherapy, these personalized therapies may induce significant adverse effects, and patients may develop resistance to them over time. Consequently, determining the optimal treatment strategy remains a long-term challenge in combating BC [22]. Therefore, enhancing our comprehension of the dynamic relationship between the tumor and its microenvironment is crucial, offering promising avenues for the advancement of tailored treatments that consider the unique characteristics of various BC subtypes [23].

3.1.3. Anti-HER2 Therapy and Trastuzumab

HER2 overexpression correlates with inferior survival outcomes in comparison to BC that is HR-positive and HER2-negative. Therefore, therapies directed at HER2 are crucial for the management of HER2+ BC [24]. A burgeoning array of immunotherapeutic approaches, including antibody drug conjugates, adoptive T-cell therapies, vaccines, and innovative HER2-directed monoclonal antibodies (mAbs), is under investigation across diverse patient populations with HER2-expressing tumors [9].

For instance, Trastuzumab (Herceptin), approved by the FDA in 1998, stands out as an effective treatment approach for HER2+ BC. One aspect of its mechanism involves facilitating fragment crystallizable gamma receptor (Fc γ R)-dependent activities. Fc γ Rs are characterized by their affinity for the Fc fragment and the ensuing immune response they elicit. Activation of Fc γ RIIIa (CD16A) on natural killer (NK) cells is pivotal for orchestrating antibody-dependent cellular cytotoxicity (ADCC), while triggering Fc γ RIIa (CD32A) and Fc γ RIIIa on macrophages prompts antibody-dependent cellular phagocytosis (ADCP) [9, 25] (**Figure 3**).

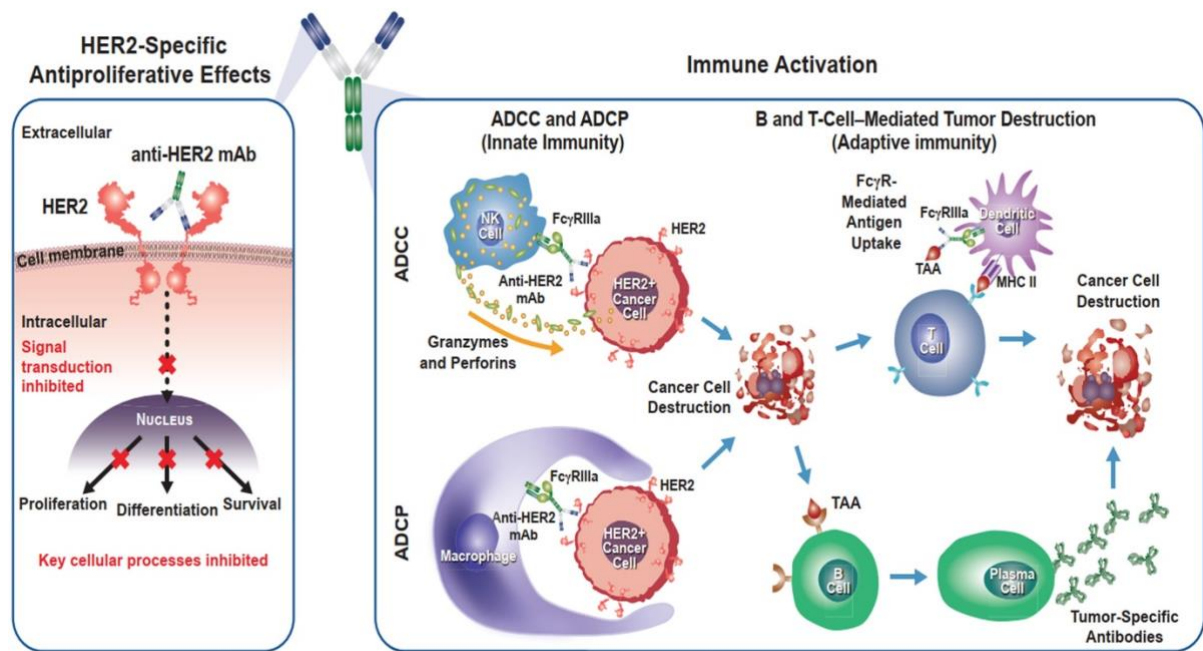


Figure 3. Mechanism of action of anti-HER2 monoclonal antibodies involves antiproliferative effects and immune activation. The clinical effectiveness of monoclonal antibodies (mAbs) stems from both non-immune and immune-mediated pathways. Non-immune mechanisms arise from the direct binding of the Ab's fragment antigen-binding (Fab) domains to HER2 receptors on tumor cells, disrupting HER2 signaling and leading to anti-proliferative effects. On the other hand, immune-related mechanisms involve the engagement of the Ab's fragment crystallizable (Fc) domains with Fc receptors (FcRs) on immune cells attached to tumor cells. FcRs facilitate communication between innate and adaptive immune responses, and their polymorphic variants exhibit various activation properties. ADCC: antibody-dependent cellular cytotoxicity; ADCP: antibody-dependent cellular phagocytosis; FcγR: fragment crystallizable gamma receptors; MHC II: major histocompatibility complex class II; TAA: tumor-associated antigens. Red X in the left panel indicates inhibition. Modified from: [9].

Interestingly, research conducted on animal models has demonstrated that mice deficient in FcγRI (CD64) and FcγRIII (CD16) receptors failed to mount protective immune responses against tumor-specific antigens. This finding suggests that ADCC is likely a primary mechanism of action for the mAb Trastuzumab [25].

As NK cells rely on tumor cell-bound Abs for the elimination of cancer cells via ADCC, the expression of Fc receptors plays a crucial role in the effectiveness of Trastuzumab treatment [26]. Furthermore, their activity is finely modulated by the stimulation of both activating and inhibitory receptors, such as Killer-cell immunoglobulin-like (KIR) receptors [27].

The evolution of immunotherapies for HER2-positive BC underscores the importance of comprehending interactions among various components of the immune system. Additionally, extensive exploration of combination therapies, spanning traditional, immune, or cell-based treatments, has been undertaken to pinpoint synergistic combinations [28].

Nonetheless, despite their significant impact in treating HER2+ BC, only about one-third of patients with HER2+ tumors experience benefits from anti-HER2 Abs [29]. Resistance may arise from various factors. One hypothesis suggests that structural changes in HER2 hinder Ab binding. These changes may be due to alternative splicing leading to a truncated isoform known as p95HER2, which remains constitutively active [30]. Additionally, overexpression of alternative tyrosine kinases like IGF-1R and c-Met can bypass HER2 signaling pathways, thereby promoting resistance to anti-HER2 Abs [31]. Another potential mechanism involves intracellular alterations in HER2 downstream signaling pathways. HER2 activates PI3K/Akt signaling, which is inhibited by PTEN. Loss of PTEN function or constitutive activation of PI3K due to mutations can lead to poorer therapeutic outcomes with Trastuzumab [32]. Moreover, HER2 N-linked glycosylation (NLG) has been shown to significantly contribute to the development of resistance as well [33]. NLG is an essential process for the maturation of transmembrane receptor tyrosine kinase (RTK) glycoproteins. Several studies examined the impact of NLG on the binding of Herceptin to the HER2 protein in BC, highlighting the potential of N-glycosylation inhibitors as promising combination for BC and thereby underscoring their importance in improving the clinical efficacy of Trastuzumab [34, 35]. Based on these observations, a valuable future objective in treating HER2+ BC is to advance combination therapies that more effectively eliminate HER2+ cancer cells by enhancing immune-mediated mechanisms.

3.2. *The Tumor Stroma: Impact on Drug Resistance*

The primary defense mechanism of the immune system resides in the innate immune response, which includes several cells such as macrophages, dendritic cells, granulocytes, mast cells, NK cells, and natural killer T (NKT) cells. Conversely, the adaptive immune system features key players like CD8+ T lymphocytes, B-lymphocytes, and CD4+ T cells, which regulate both arms of adaptive immunity and are integral components of the tumor-associated immune network [36]. There is a growing acknowledgment of the significant impact exerted by tumor stroma cells on the behavior of tumors, including tumor cell proliferation and their responsiveness to therapies [2]. The tumor microenvironment (TME) encompasses the local biological surroundings where tumor cells reside, with cancer cell presence ranging from 5% to 100% [37]. It is characterized as an acidic and hypoxic milieu infiltrated by cancer cells alongside accessory cells, including stromal cells, endothelial cells, immune cells, mesenchymal cells, adipocytes, and pericytes, in addition to extracellular matrix (ECM)

components and cytokines [38, 39]. Within the tumor stroma, this diverse array of immune cells from both the innate and adaptive immune systems plays crucial roles, contributing to both beneficial and detrimental effects on the overall disease outcome [40]. Functioning as a protective shield for tumor cells, the TME offers mechanical support and secretes various cytokines to evade treatment [41] (**Figure 4**).

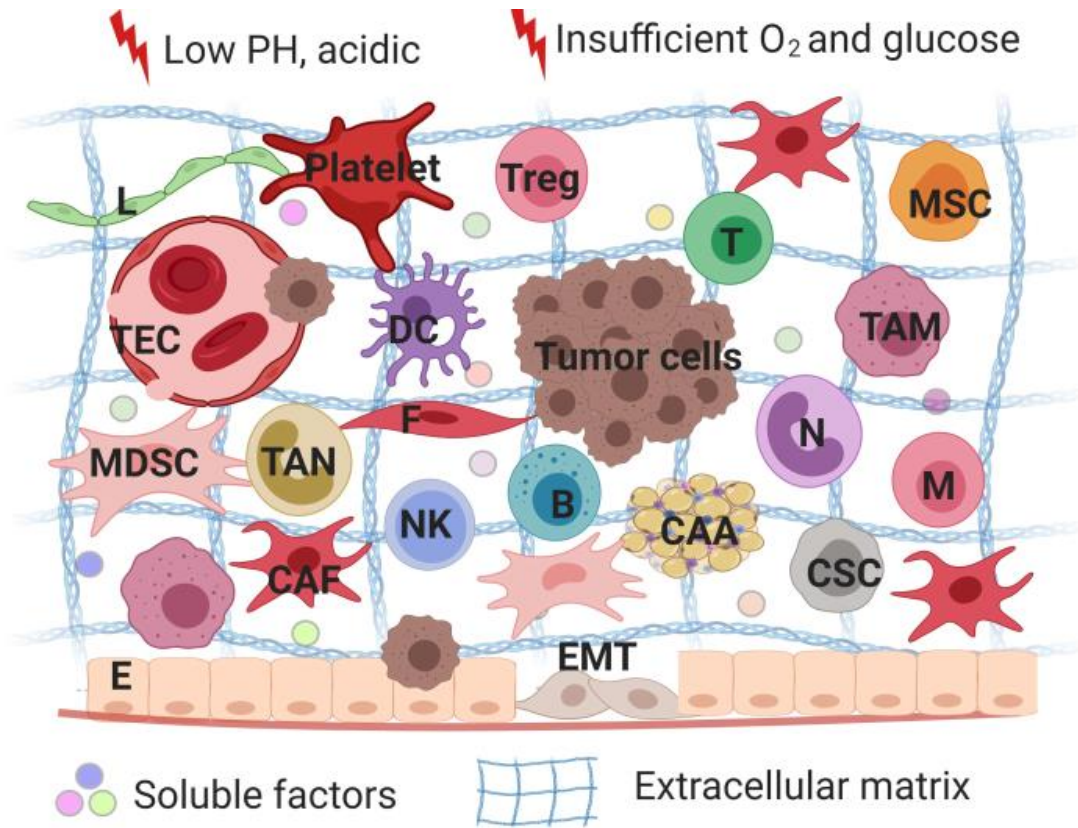


Figure 4. Schematic illustration of the tumor microenvironment. The tumor microenvironment (TME) denotes the local biological milieu hosting tumor cells, composed of tumor cells, stromal cells, and immune cells. The TME is intricately involved in initiating and perpetuating non-cell autonomous resistance through various means, encompassing hypoxia, acidic pH, alterations in immune cell populations, vascular irregularities, and a diverse array of secreted molecules originating from stromal cells, including secretomes, exosomes, and soluble factors. Additionally, the TME acts as a protective barrier for tumor cells, offering mechanical reinforcement and secreting diverse cytokines as evasion tactics against treatment. Treg: regulatory T lymphocytes; MSC: mesenchymal stem cells; T: T lymphocytes; B: B lymphocytes; DC: dendritic cells; N: neutrophils; M: macrophages; NK: natural killer cells; E: epithelial cells; F: fibroblasts; CAA: cancer-associated adipocytes; L: lymphatic endothelial cells; TEC: tumor endothelial cells; CSC: cancer stem cells; TAM: tumor-associated macrophages; CAF: cancer-associated fibroblasts; MDSC: myeloid-derived suppressor cells; EMT: epithelial-mesenchymal transition. Source: [42].

This complex network of diverse heterogeneous cells and their secretory cytokines enables tumor cells to proliferate rapidly, maintain stemness, become insensitive to medication,

and evade immune surveillance. Non-inherent adaptive resistance, also known as non-cell-autonomous resistance, is dependent on therapeutic interventions [43].

Resistance of cancer cells to various therapies remains a significant challenge in contemporary oncology. Initially, scientific research predominantly focused on the intrinsic mechanisms within tumor cells themselves as the primary drivers of acquired drug resistance. However, recognition of the pivotal role of the TME in modulating immune evasion, drug resistance, tumor progression, and metastasis, prompted further research to explore cancer-stroma interactions [42].

The majority of cancers exhibit heterogeneity, wherein different subpopulations of cancer cells coexist within a single tumor. Theoretically, the greater the diversity of subpopulations within a tumor, the greater the likelihood that one of them will develop drug resistance (**Figure 5**), resulting in the emergence of a pool of surviving cells known as minimal residual disease. Tumor cell heterogeneity thus grants the tumor resilience, enabling it to endure drug treatment, even if only a small fraction of cells manage to survive [44].

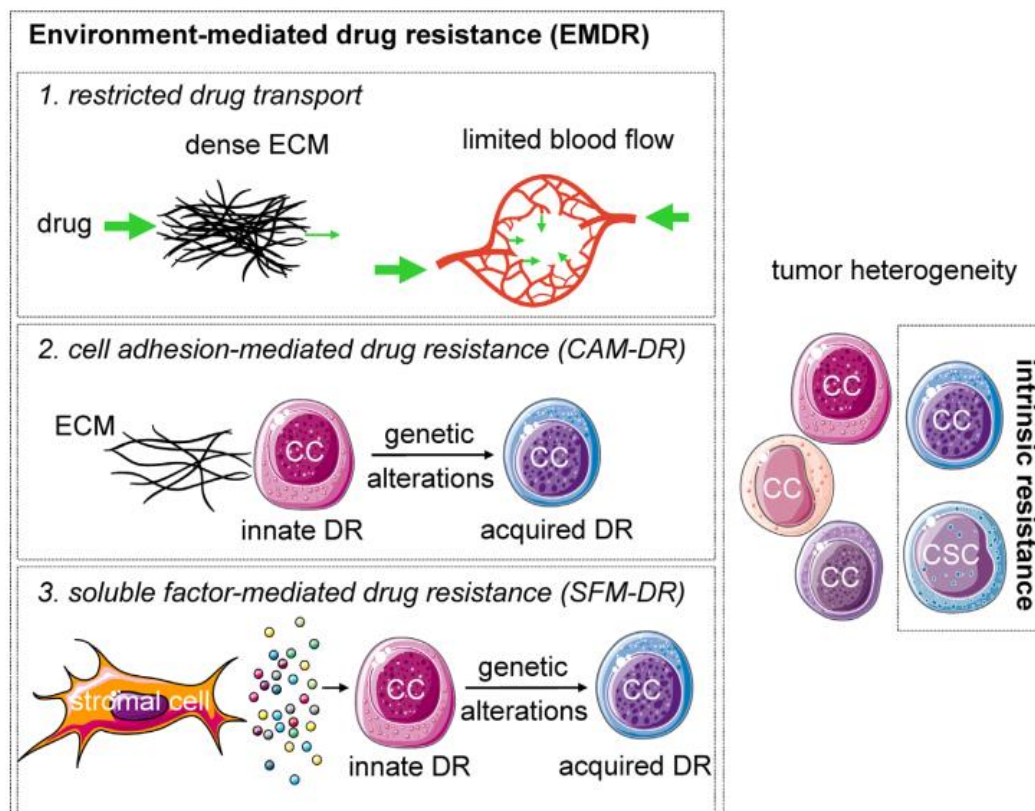


Figure 5. Schematic overview of environment-mediated drug resistance. Left Panel: varieties of environment-mediated drug resistance. A dense extracellular matrix (ECM) or limited blood flow might impede the drug's access to certain areas of the tumor (1). Interactions between cancer cells (CC) and ECM proteins could activate pro-survival signaling pathways, offering immediate protection against

drugs (innate drug resistance). This interaction might also enable cancer cells to survive long enough to develop resistance through genetic alterations (2). Stromal cells might release pro-survival factors, potentially leading to acquired drug resistance in cancer cells over time (3). Right Panel: intrinsic drug resistance. Tumors comprise diverse subpopulations of cancer cells, contributing to tumor heterogeneity. Certain subpopulations of cancer cells, such as cancer stem cells (CSCs), may inherently possess drug resistance. Source: [44].

While traditional tumor treatment strategies primarily targeted cancer cells directly by chemotherapy, radiotherapy, and targeted therapy, the significance of immune cells in anti-tumor therapy has gradually gained recognition with the discovery of immune checkpoints [45]. Emerging strategies targeting immune cells for tumor treatment have demonstrated significant clinical efficacy and are endorsed by corresponding guidelines. These include T cell-based immune checkpoint inhibitors such as programmed cell death 1/programmed death-ligand 1 (PD-1/PD-L1) antibodies, cytotoxic T lymphocyte-associated protein-4 (CTLA-4) antibodies, and chimeric antigen receptor T (CAR-T) cell therapy [46]. They work by either disrupting tumor-induced immunosuppressive signals or by arming effector immune cells e.g. cytotoxic T cells, with tools for recognizing tumor antigens, like CARs, thereby enhancing their ability to eliminate tumor cells [46, 47]. Additionally, approaches targeting immune cells encompass dendritic cell (DC) vaccines, reduction of bone marrow-derived suppressor cells (MDSCs), regulatory T cells (Tregs), regulatory B cells (Bregs), and reprogramming of M2 tumor-associated macrophages (TAMs) and N2 tumor-associated neutrophils (TANs) into tumor-killing cells [48].

Moreover, stromal cells present within the TME communicate with other components through cytokines and mediators either in a paracrine manner or via cell-cell interactions, thereby promoting tumor invasion, metastasis, angiogenesis, drug resistance, and recurrence [49, 50]. Consequently, the TME emerges as an appealing target for enhancing tumor sensitivity to traditional therapies and as an alternative approach for managing resistant diseases.

Understanding the physiological roles of each component of TME is essential for comprehending their impact on tumor behavior and may provide insights into novel approaches for treating tumors based on stromal cells and overcoming resistance. Targeting stromal cells within the TME could serve as a valuable complement to immunotherapy and targeted therapy. Additionally, there's a pressing need for novel strategies aimed at targeting and reshaping the microenvironment to disrupt pro-tumor communication and restore immune surveillance [40, 42].

3.3. *Stromal Cells in the Breast Cancer Tumor Microenvironment*

The immune milieu of BC is notably abundant in diverse immune cell populations [51]. Although, as described above, the notable residence of immune cells within the tumor does not always translate into an effective anti-tumor immune response. Quite the contrary, many tumors emit suppressive signals that can coerce immune cells into adopting a tolerant or even a tumor growth supporting phenotype [52]. The significance of tumor-stroma interactions in BC development, invasion and metastasis and the TME's role in resistance mechanisms is increasingly being elucidated [53]. Both direct and indirect interactions between tumor and stromal cells have been demonstrated to influence these processes [42]. Furthermore, studies have shown that the presence of a high stromal content in the primary tumor is considered a prognostic parameter, with patients exhibiting primary tumors containing a high proportion of tumor stroma experiencing significantly worse outcomes [44]. Recent literature advancements have also highlighted the crucial role of tumor-stroma interactions in BC chemotherapy resistance, posing a significant challenge in BC treatment [54].

Stromal cells, such as CAFs and TAMs, emerge as major players in contributing to chemotherapy resistance through the secretion of various factors, including cytokines and chemokines, and thereby represent a promising emerging area in BC research [55]. Various stroma-specific therapies have been explored, targeting both stromal cells and tumor-stroma interactions. However, stroma-specific therapy remains underdeveloped, with only a single clinical trial currently evaluating this therapeutic approach [56]. Further research is warranted to refine strategies and develop agents tailored to the diverse tumor heterogeneity and BC subtypes, ultimately paving the way for new stroma-specific targeted therapies. For instance, the remarkable clinical efficacy demonstrated by CAR-T cells and immune checkpoint inhibitors has overturned previous beliefs by showcasing that potent antitumor immune responses can indeed be successfully unleashed in BC [57].

Efforts to understand and counteract stromal cells-mediated resistance hold potential for improving treatment outcomes in BC. As the stroma co-evolves alongside tumor cells, it is probable that each individual tumor possesses an unique stromal composition [53]. To realize the objective of truly personalized therapy, it is essential to understand both the molecular characteristics of tumor cells and those of the tumor-associated stromal cells.

3.4. Macrophages (MΦs) and Tumor-Associated MΦs (TAMs)

3.4.1. The Origin of MΦs

Macrophages (MΦs) are highly plastic innate immune cells which fulfill numerous significant functions, including defending the host and regulating inflammatory reactions, essential for preserving tissue equilibrium and safeguarding against invading pathogens [58] [59]. MΦs differentiate from monocytes derived from bone marrow progenitors, migrating from the bloodstream into tissues during both homeostasis and inflammation [60]. These progenitors proliferate or differentiate *in situ* and generate offspring throughout their lifespan, contributing to populations such as alveolar MΦs, brain MΦs, and Kupffer cells [61, 62]. Upon recruitment and activation by various signals in the TME, MΦs undergo substantial alterations that profoundly impact tumor progression and metastasis. The cellular lineage of MΦs is illustrated in **Figure 6**.

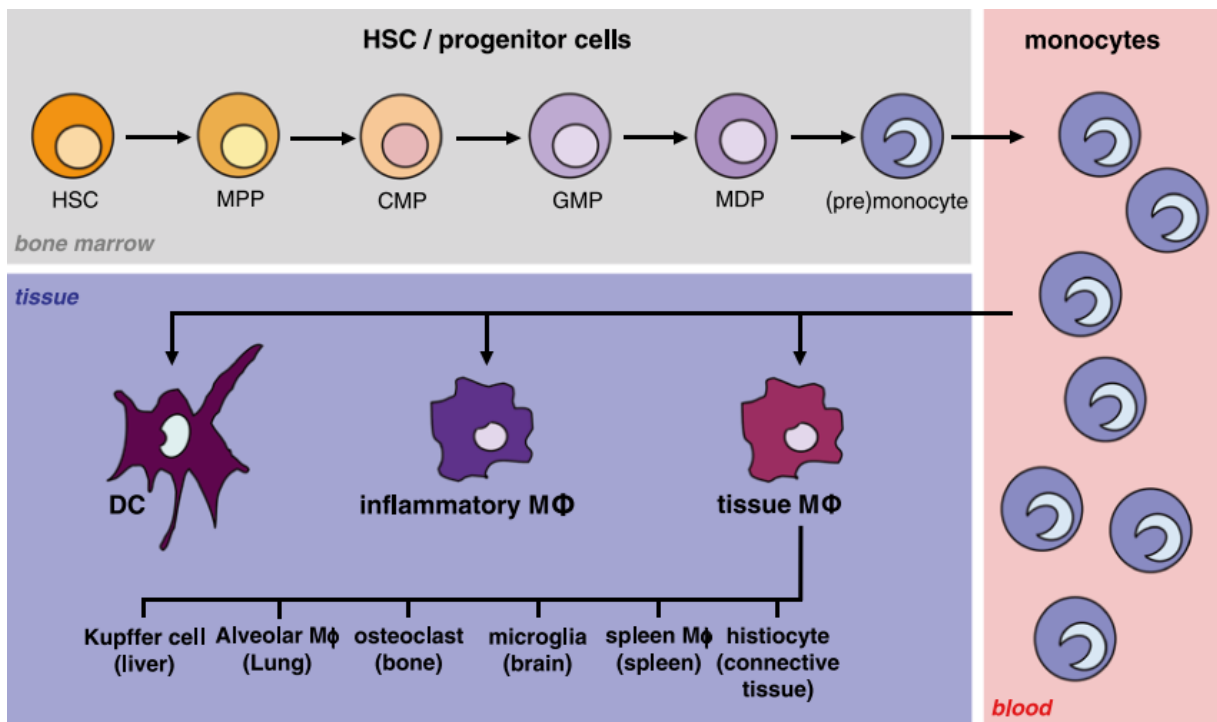


Figure 6. Cellular origin and development of MΦs. The scheme illustrates various myeloid and premonocytic stages of monocytic maturation. It commences with self-renewing hematopoietic stem cells in the bone marrow, progressing through several myeloid progenitors to ultimately form blood monocytes. Subsequent developmental stages lead to the generation of MΦs and dendritic cells in various tissues. Monocytes migrate to tissues and differentiate *in situ*, contributing to populations such as alveolar MΦs, brain MΦs and Kupffer cells. MΦs respond to various signals within the TME, where they become recruited and activated. HSC: hematopoietic stem cell; MPP: multipotent progenitor; CMP: common myeloid progenitor; GMP: granulocyte/monocyte progenitor; MDP: monocyte/macrophage and dendritic cell progenitor. Source: [59].

This migration is prompted by local growth factors, pro-inflammatory cytokines, and microbial products. Additionally, MΦ populations exhibit distinct temporal and spatial distributions within the TME [63]. Tissue-resident MΦs migrate to nearby tumor cells early in the initial stages, promoting epithelial-mesenchymal transition (EMT) and facilitating invasion. Moreover, they enhance the proliferation of regulatory T cells, fostering immune evasion by tumor cells [64]. For this reason, they represent a potentially novel target for tumor therapy.

3.4.2. MΦ Polarization and TAMs

MΦs are frequently found in abundance within tumors, often constituting the predominant immune cell population. Nonetheless, their presence in the tumor stroma is typically associated with poor prognosis [65, 66]. MΦs, being highly adaptable immune cells, are capable of assuming various phenotypes in response to environmental cues. They can display two distinct phenotypes, ranging from the classically activated, pro-inflammatory (M1) phenotype to the alternatively activated, anti-inflammatory (M2) phenotype, which plays a role in resolving inflammation [3]. Both M1 and M2 MΦs possess considerable plasticity and can transition into each other in response to signals from the TME or therapeutic interventions [67]. M1 MΦs play crucial roles in host defense mechanisms against microbial challenges such as bacteria, viruses, and protozoa, as well as in antitumor immunity. Activation of M1 MΦs typically occurs through stimuli like bacterial lipopolysaccharides (LPS), interferon-gamma (IFN- γ), and Toll-like receptor (TLR) activation, leading to the secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-12, IL-23, tumor necrosis factor (TNF), and chemokines (CXCL9, CXCL10, CXCL11) [68, 69]. Additionally, M1 MΦs exhibit antitumorigenic functions by efficiently recognizing and eliminating cancer cells through phagocytosis and cytotoxicity mediated by the release of tumor-killing molecules like reactive oxygen and nitrogen species (ROS and RNS, respectively) [70, 71]. In contrast, the alternatively activated M2 phenotype is associated with promoting tumor growth. M2 MΦs are induced by transforming growth factor- β (TGF- β), by interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-13 (IL-13), typically produced by T-helper 2 (Th2) cells [72]. M2 MΦs are characterized by the expression of markers such as CCL18, CD163, mannose receptor 1 (MRC1), and transforming growth factor-beta 1 (TGF- β 1) [73, 74]. Infiltration of M2-like MΦs is commonly associated with poor prognosis in various human malignancies [64, 75]. Within the TME, polarized M2 MΦs exert suppressive effects on antitumor immunity, cell migration,

and invasion by secreting cytokines such as TGF- β 1, vascular endothelial growth factor (VEGF), promoting angiogenesis, and facilitating tumor metastasis [76].

The distinction between M1 and M2 M Φ s and their differential chemokine secretion within the TME is shown in **Figure 7**.

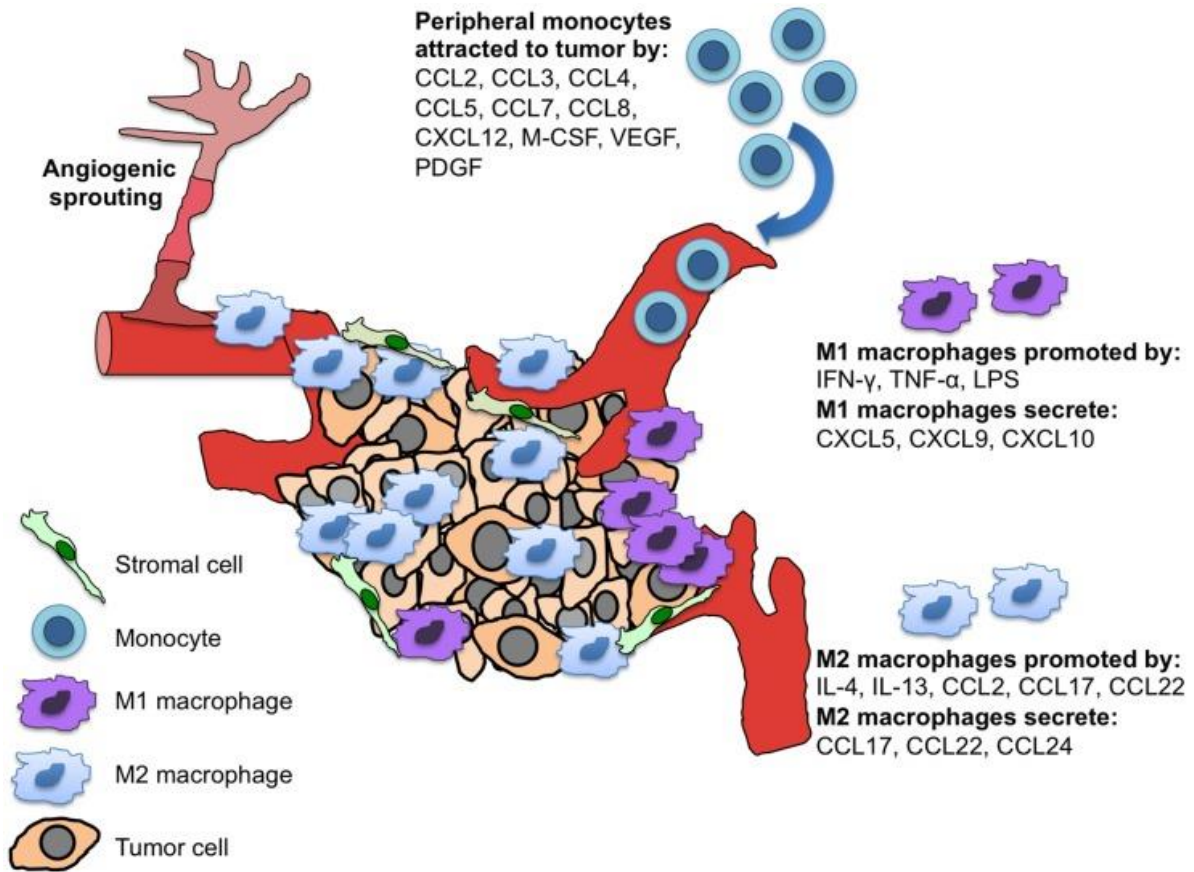


Figure 7. Schematic illustration of M1 and M2 M Φ s and their respective patterns of chemokine secretion within the TME. Tumor cells and stromal cells release chemokines and cytokines that attract monocytes to the tumor site. Upon arrival, these peripheral monocytes undergo differential polarization into tissue M Φ s (M1 or M2), each exhibiting unique chemokine secretion profiles and functions within the tumor milieu. Source: [77].

The prevalence of M2 M Φ s within solid tumors tends to surpass that of M1 M Φ s. Nevertheless, the balance between these phenotypes is dictated by the nature and concentration of various signals present in the TME [78].

Within the tumor, TAMs often exhibit a distinctive phenotype akin to M2 cells [79]. TAMs exhibit multifaceted roles in tumor progression, encompassing cancer initiation, promotion, immune regulation, metastasis, and angiogenesis [68], as depicted in **Figure 8**. For instance,

TAM-derived cytokines IL-6 and IL-10 have been identified as triggers for tumor-elicited inflammation, subsequently fueling tumor growth [80] (**Figure 8**). Additionally, research indicates that heightened levels of TAM-derived IL-6 contribute to an exacerbated inflammatory response, thereby facilitating the onset and advancement of hepatocellular carcinoma through STAT3 signaling [81].

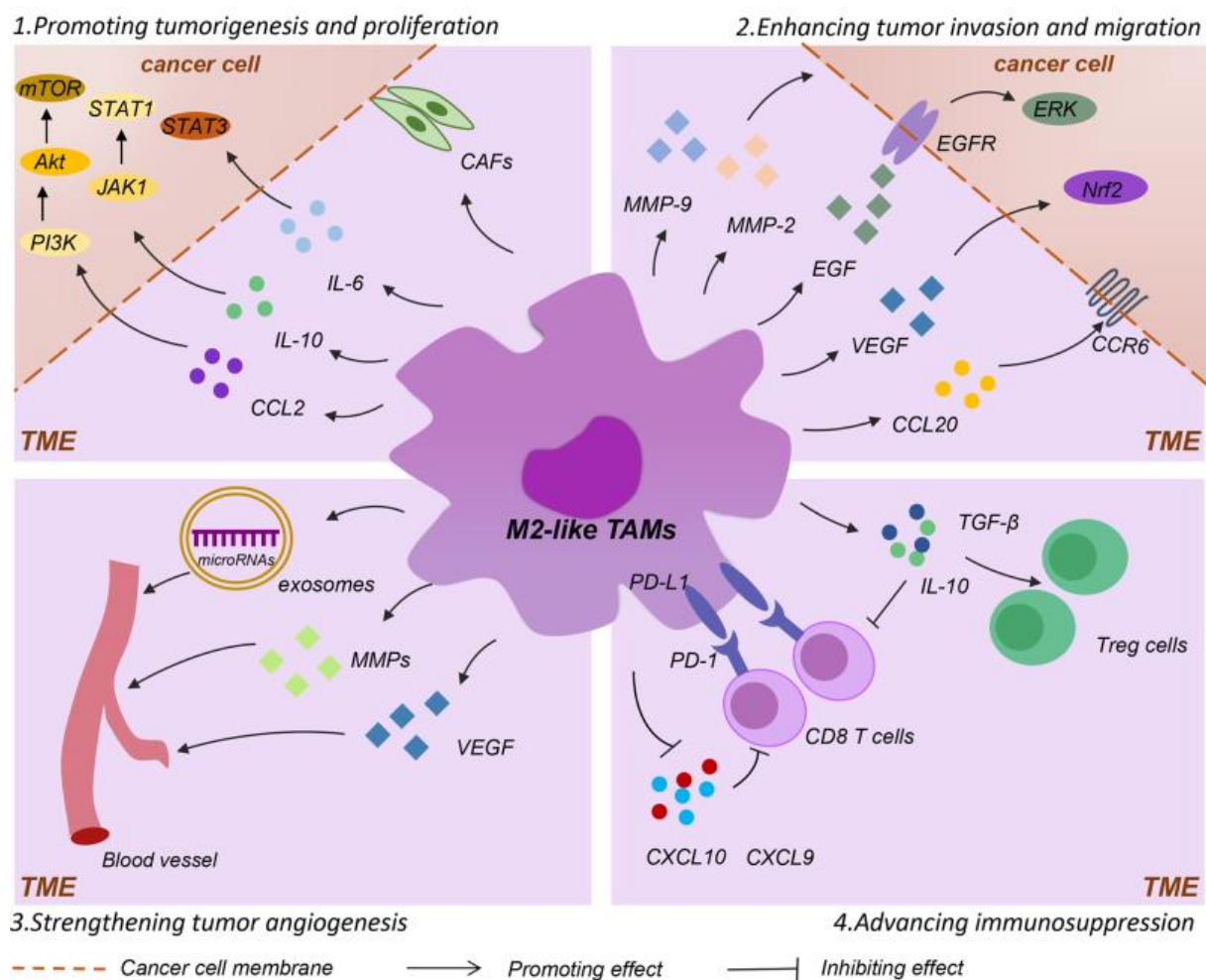


Figure 8. Protumoral functions and immunosuppressive activity of M2-like TAMs. TAMs play a crucial role in enhancing cancer progression through four main mechanisms. **1.** Promotion of cell proliferation and tumorigenesis: M2-like TAMs secrete growth factors such as CCL2, IL-6, and IL-10, which activate proliferation pathways in cancer cells. Additionally, they induce ECM degradation in cancer-associated fibroblasts (CAFs). **2.** Induction of EMT and enhancement of tumor invasion and migration: M2-like TAMs secrete EGF/VEGF to activate EMT-related pathways, and produce MMPs and CCL20, which facilitate tumor invasion and migration. **3.** Stimulation of tumor angiogenesis: M2-like TAMs contribute to tumor angiogenesis by secreting exosomes, MMPs, and VEGF. **4.** Facilitation of immunosuppression: M2-like TAMs downregulate CXCL9/CXCL10 and secrete IL-10 and TGF- β , inhibiting the recruitment of CD8 T cells. Furthermore, IL-10 and TGF- β promote the proliferation of regulatory T cells (Tregs), contributing to tumor immune evasion. TAMs: tumor-associated macrophages; TME: tumor microenvironment; CAFs: tumor-associated fibroblasts; CCL2: chemokine

(C-C motif) ligand 2; MMPs: matrix metalloproteinases; VEGF: vascular endothelial growth factor; PD-1: programmed cell death 1(PD-1); PD-L1: PD ligand 1; Tregs: regulatory T cells. Source: [81].

3.4.3. TAMs as a Promising Therapeutic Target for Cancer Treatment

Discoveries of molecular details of TAM behavior laid a solid groundwork for targeting these cells and/or their precursors to enhance patient outcomes. Presently, therapeutic approaches fall into four main categories: (1) inhibition of monocyte/M Φ recruitment (strategies aim to impede the influx of monocytes and M Φ s into the tumor microenvironment), (2) restoration of M Φ phagocytic activity (efforts focus on enhancing M Φ ability to engulf and eliminate tumor cells), (3) reprogramming M2-like M Φ s into M1 anti-tumoral phenotype (therapeutic interventions seek to promote tumor suppression), (4) monocyte/TAM depletion (reducing the population of monocytes and TAMs within the TME, thereby mitigating their pro-tumoral effects) [82].

By targeting these cells and their precursors through these diverse therapeutic strategies, there is potential to significantly improve patient outcomes in cancer treatment [3, 80]. Therapeutic strategies targeting TAMs are summarized in **Table 1** below.

Table 1. Summary of the promising therapeutic strategies targeting TAMs. Study references are the following. Inhibition of monocytes/macrophage recruitment: [83-86]. Depletion of monocytes/macrophages: [87-91]. Restoring macrophage intrinsic functions: [84, 92-95]. Modified from: [80].

Strategy	Examples	Benefits	Side Effects	
Inhibition of monocyte/ macrophage recruitment	M-CSF/CSF1R blockade	Small molecules: PLX3397, PLX7486, JNJ-40346527, ARRY-382, BLZ945, CS2164 Monoclonal antibodies: RG7155, IMC-CS4, R05509554, RG7155, FPA008, AMG820, LY3022855, PD-0360324	Eliminate circulating monocytes/TAMs and restore T cell infiltration	Systemic symptoms including Anemia; neutropenia; hepatotoxicity; hypertension
	CCL2/CCR2 blockade	CCL2 inhibitor (Carlumab) CCR2 inhibitor (PF-04136309)	Eliminate circulating monocytes/TAMs	No effect on resident TAMs; relapse; pulmonary toxicity
Depletion of monocytes/ macrophages	Inorganic compounds: Bisphosphonates and its derivatives		Useful in killing monocytes, macrophages, and tumor cells in bone metastases	Short plasma half-life, rapid kidney clearance
	Chemical agents: Trabectedin, lurbinctedin		Cytotoxic to tumor cells, monocytes, TAMs, and MDSCs	Anaphylaxis, neutropenic sepsis, rhabdomyolysis, hepatotoxicity, cardiomyopathy, capillary leak syndrome, and tissue necrosis
	M2 targeting peptides: NW, M2pep, UNO, Melittin, RP182, LyP-1		Highly specific; drug delivery and imaging; less immunogenic; cheap and easy to synthesize; high penetration rate	Unstable bio-stability

Restoring macrophage intrinsic functions	Enhance phagocytosis	CD47 targeting peptides: Anti-CD47 antibodies: Hu5F9-G4, CC-90002, SRF231, IBI188, ZL1201, TTI-621, ALX148 pep-20, pep20-D12, TAX2 Anti-SIRP α antibodies: Velcro-CD47, KWAR23, ADU-1805	Enhance macrophage functions (phagocytosis, antigen presentation, ADCC); induce tumor cell apoptosis; repolarize TAMs towards M1	Anemia; nausea; headache; isolated neutropenia
	Enhance antigen presentation	Anti-CD40 antibodies: CP-870,893, ChiLob7/4, RO7009789	Prime T cell activation; promote anti-tumoral factors secretion; alter M2 cytokine profile	Cytokine release syndrome; liver toxicity

3.4.4. The Role of TAMs in Breast Cancer

In addition to immune cells, the breast TME comprises various cell types, including mesenchymal cells, fibroblasts, endothelial cells, and adipocytes. The combination of mesenchymal cells and fibroblasts constitutes the connective tissue [96]. These cells, in collaboration with tumor cells, secrete proteases and pro-tumor factors that influence the recruitment and metabolism of tumor cells [97]. Within the breast TME, M2-like TAMs have emerged as the predominant immune cell components, exerting pivotal influence over BC progression. Specifically, they have been shown to contribute to the alteration of the TME by secreting or modulating cytokine production, thereby promoting increased blood flow, angiogenesis, and other inflammatory responses [98]. Moreover, TAMs have been reported to play a supportive role in tumor growth by instigating cancer stemness, modulating energy metabolism, and fostering immune suppression in BC [4] (**Figure 9**).

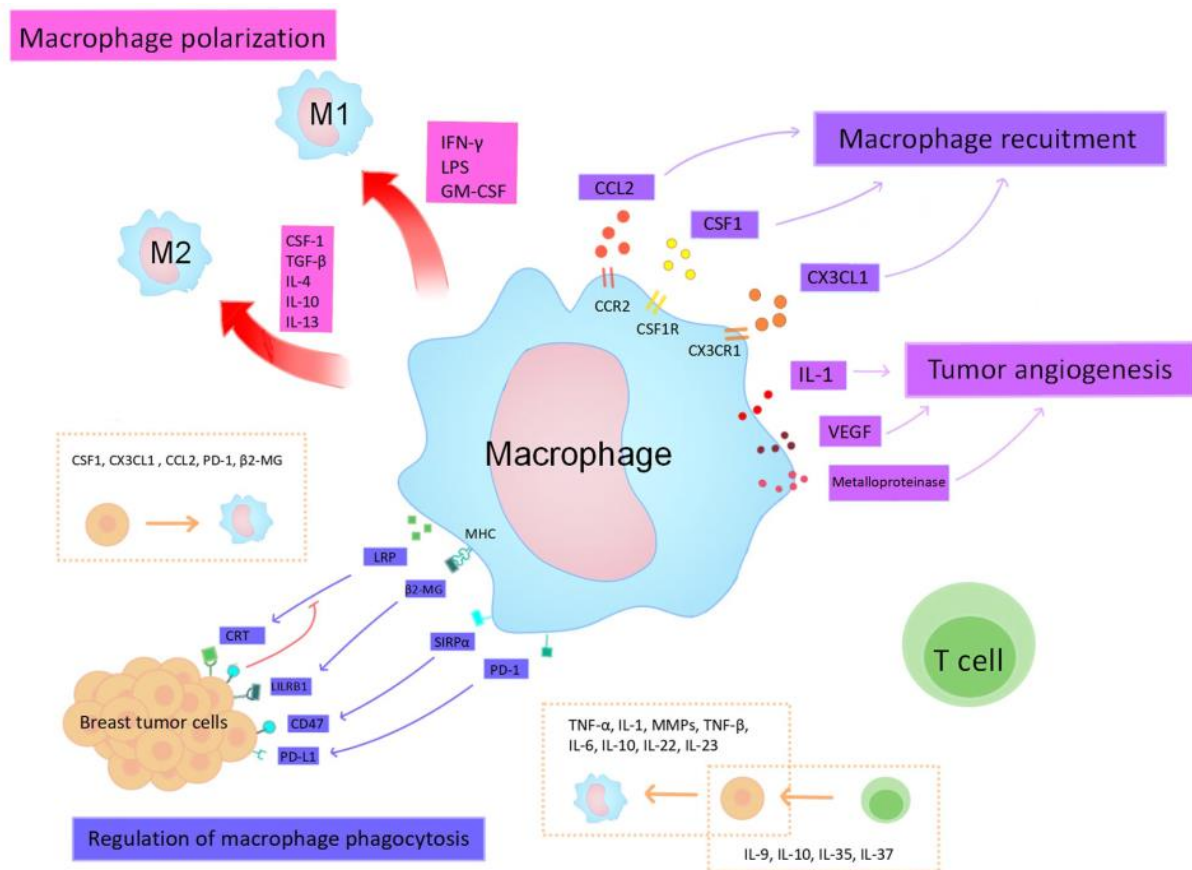


Figure 9. The breast TME modulates TAMs and facilitates their interaction with breast tumor cells. TAM polarization is intricately controlled, with M1 polarization regulated by factors such as IFN- γ , LPS, and GM-CSF, while M2 polarization is influenced by CSF-1, TGF- β , IL-4, IL-10, and IL-13; TAMs are recruited to the tumor site through signaling molecules including CSF1, CX3CL1, and CCL2; the microenvironment promotes blood vessel growth via factors like VEGF, IL-1, and metalloproteinases, thus facilitating tumor angiogenesis; TAM phagocytic activity is modulated by factors such as CRT/LRP, LILRB1/ β 2-MG, CD47/SIRP α , and PD-1/PD-L1 interactions. GM-CSF: granulocyte-macrophage colony-stimulating factor; CSF-1: colony stimulating factor 1; CRT/LRP: calreticulin/low density lipoprotein receptor-related protein; LILRB1/ β 2-MG: leukocyte immunoglobulin like receptor B1/ β 2 microglobulin; CD47/SIRP α : cluster of differentiation 47/signal regulatory protein α ; PD-1/PD-L1: programmed cell death protein/programmed cell death-ligand 1. Yellow dashed box highlights TAM-cancer cell interactions in BC, and the impact of T cells on BC cells within this dynamic microenvironment. Source: [96].

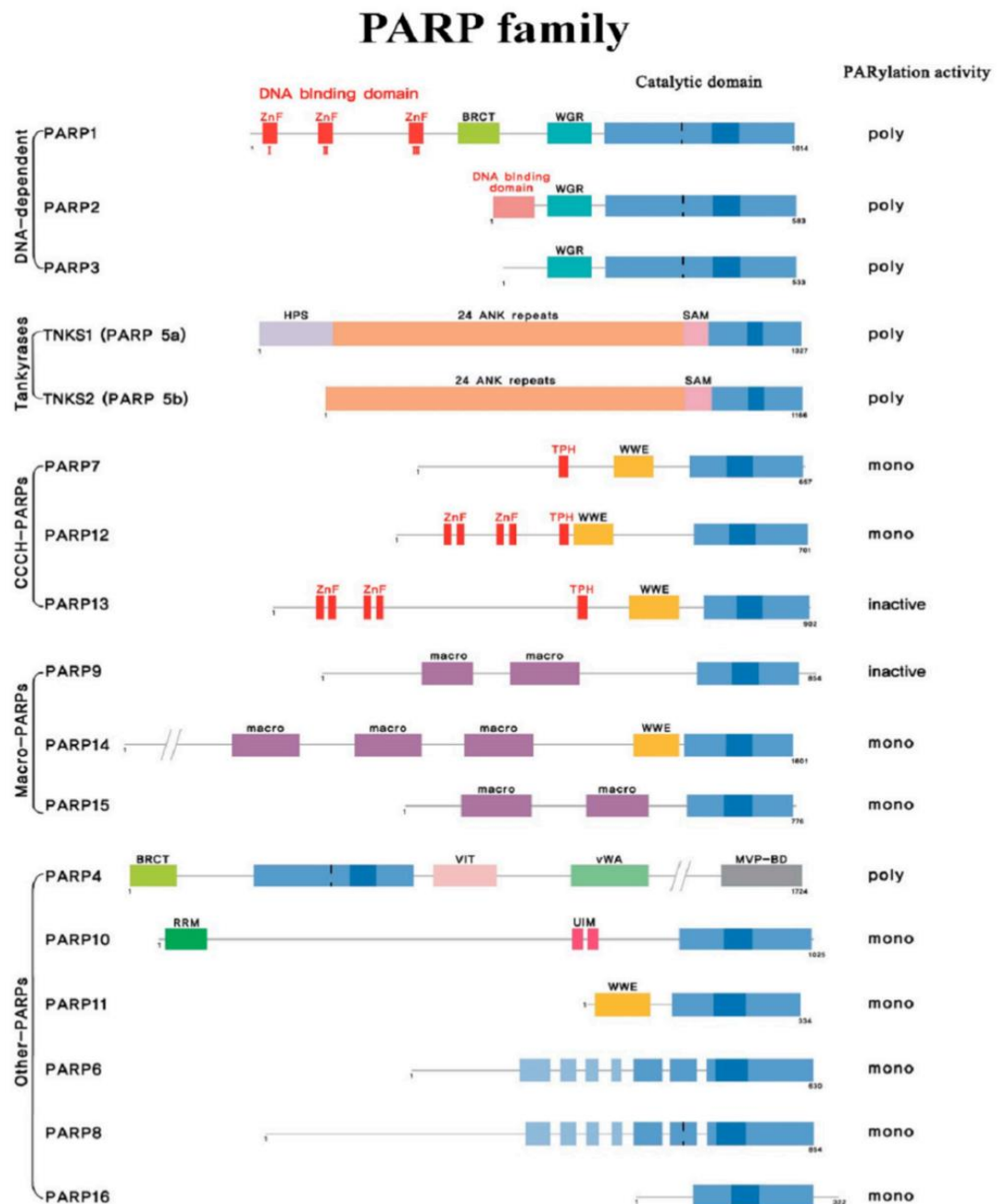
This evidence provides a foundation for comprehensive investigation into leveraging immune cells to influence breast tumors and steer anti-tumor trends. As TAMs exhibit notable phenotypic plasticity, they have been recently established as novel and promising targets for BC treatment [6]. In specific, their transformation into M1 M Φ s has demonstrated potential for promoting breast tumor regression in several studies [5]. For instance, Liu Y. *et al.* demonstrated that the administration of multifunctional nanoparticles to a 4T1 breast tumor model or M2 M Φ s resulted in the accumulation of ROS, mitochondrial damage and apoptosis

induction. These findings were further validated in an animal model of BC, showing the successful targeting of tumors and TAMs, by the nanoparticles [99]. Studies on an *in vivo* 4T1.2 cancer model also showed that ultrasmall nanocarriers led to increased accumulation of therapeutic agents in tumor tissues and notably enhanced anti-tumor efficacy. Importantly, this anti-tumor activity was attributed to a synergistic targeting of both tumor cells and TAMs [100]. Moreover, another study demonstrated that STING agonists reprogram pro-tumor M2-like MΦs into an anti-tumor M1-like state in a BRCA1-deficient breast tumor mouse model [101]. Such studies carry significant implications for both the prevention and treatment of BC. However, further investigation is required to explore the selective reprogramming of M2 MΦs [82].

3.5. The Poly(ADP-ribose) Polymerase (PARP) Superfamily and Poly(ADP-ribosyl)ation (PARylation)

Poly(ADP-ribosyl)ation (PARylation) is a post-translational modification catalyzed by the ADP-ribosyl transferases (ADPRT), alternatively known as poly(ADP-ribose) polymerase (PARP) enzyme family [102]. PARPs catalyze the attachment of mono(ADP-ribose) (MAR) or poly(ADP-ribose) (PAR) to target proteins, utilizing nicotinamide adenine dinucleotide (NAD⁺) as a donor. PARylation represents a reversible process, wherein covalently linked linear or branched chains of PAR can be hydrolyzed by PAR glycohydrolase (PARG) or PAR hydrolase (ARH3) to release PAR polymers or oligomers [7, 103]. Humans express 17 PARP enzymes that play pivotal roles in numerous cellular processes, including DNA repair, transcriptional regulation, chromatin remodeling, cell death, and energy metabolism [104]. PARP enzymes possess a conserved catalytic domain containing the PARP signature motif, a highly conserved sequence critical for forming the active site [105] (**Figure 10**).

A



B

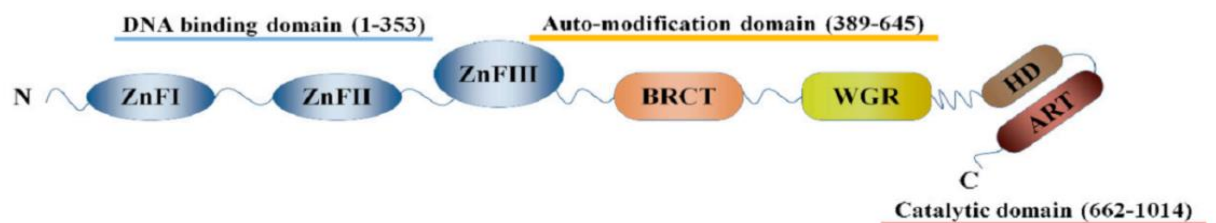


Figure 10. The structure of PARP superfamily. The PARP family comprises 17 members. Based on domain structure and function, they are categorized into five subgroups: DNA damage-dependent PARPs (PARP1, PARP2, and PARP3), tankyrases (tankyrase1/PARP5 and tankyrase2/PARP5b),

CCCH-type PARPs (PARP7, PARP12, and PARP13), macro-PARPs (B-aggressive lymphoma 1 (BAL1)/PARP9, BAL2/PARP14, and BAL3/PARP15), and other PARPs (PARP4, PARP6, PARP8, PARP10, PARP11, and PARP16). The catalytic domain at the C-terminus contains additional zinc fingers, BRCA C-terminus-like (BRCT) motifs, ankyrin repeats, macro domains and WWE domains. It is conserved in all members (A). PARP1 comprises seven major domains, including three zinc-finger domains in the DNA binding domain, the BRCT domain in the auto-modification domain, and the centrally located pADPr accepting WGR domain (W). The C-terminus contains two catalytic domains: ART and a helical domain (HD) (B). Source: [105].

Initially, the involvement of PARPs was investigated primarily in the context of DNA damage response. In the case of single-strand DNA breaks, PARP enzymes bind to the damaged DNA strand, facilitating the binding of NAD⁺ to their active sites [106]. ADP-ribose units from NAD⁺ are then transferred to acceptor proteins through PARylation, which subsequently recruit single-strand DNA repair effectors to repair the DNA lesions [107]. The initial attachment of the first ADPr moiety to proteins occurs covalently to D, E, S and K residues [108]. Subsequent ADPr units bind to the former unit via a O-glycosidic bond, potentially forming branched structures [102] (**Figure 11**). Additionally, various proteins can bind PAR non-covalently through diverse PAR-binding motifs, each with varying dissociation constants (K_d) that influence cellular responses [109] (**Figure 11**).

The variability in PAR structures and PAR-binding motifs suggests a role in transmitting signals between cellular pathways during responses to stressors, thereby ultimately influencing cell fate [102]. Due to technical constraints in chemical measurement and synthesis, the precise conformation and nature of PAR remain poorly defined. On the other hand, the enzymes responsible for PAR chain initiation, formation, extension, and branching are well studied [108]. Importantly, recognition of PAR by different proteins depends on PAR structure and target protein binding motifs, which may also be influenced by varying K_d values ranging from high to low affinities. Thus, PAR-binding by different target proteins/partners likely serves as significant signaling to downstream effectors [109].

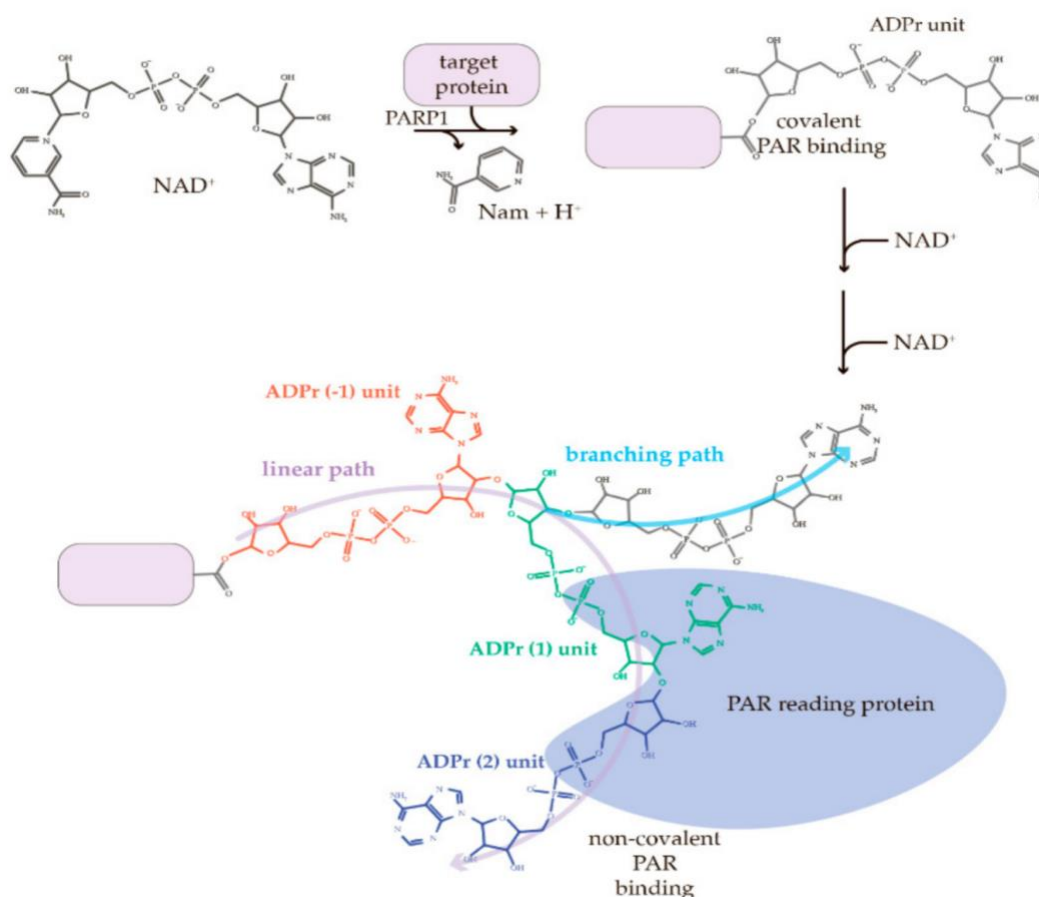


Figure 11. PARylation reaction cycle. The glycosidic bond between nicotinamide and ribose of NAD^+ is cleaved by PARP1. This facilitates the covalent attachment of ADP-ribose (ADPr) onto target proteins. Subsequent NAD^+ molecules are utilized for further chain elongation via an O-glycosidic bond. Proteins containing specific and distinct PAR-binding motifs can recognize and bind PAR chains non-covalently. The scheme illustrates the process of reading via the PBZ domain. Source: [109].

ADP-ribosylation has shown its capacity to regulate a broad spectrum of cellular functions, encompassing DNA repair, gene transcription, telomere maintenance, genomic stability, RNA degradation, mitosis, protein translation and cell death [103]. For this reason, an area of rapidly expanding research currently focuses on the roles of various PARPs and their substrates in diverse diseases. Moreover, several studies demonstrated the potential of targeting these DNA damage repair mechanisms with PARP inhibitors (PARPis) as an emerging as an innovative approach for use in relevant pathological conditions e.g. in cancer therapy [105, 110]. In this context, PARP1, the most studied member of the PARP family, stands out as a significant target in numerous cancer types, including breast, ovarian and prostate cancer [111-113]. Inhibition of PARP leads to the accumulation of single-strand breaks, which eventually convert to double-strand breaks during DNA replication. These double-strand breaks are lethal to cells deficient in homologous recombination repair, a common defect in many BRCA1/2-mutated tumors [105, 114]. Furthermore, currently four PARPis, Olaparib, rucaparib, niraparib and talazoparib,

are approved for the treatments of these types of cancers [115]. Numerous studies have elucidated the differential specificity of various PARPis towards distinct PARP isoforms, thereby influencing their efficacy and safety profiles. For instance, Olaparib demonstrates greater potency towards PARP1 and PARP2, while Rucaparib exhibits affinity towards PARP1, PARP2, and PARP3. Beyond merely inhibiting PARylation, these inhibitors also trap PARP enzymes on DNA, further blocking repair and enhancing cytotoxicity in homologous recombination-deficient cells. This dual mechanism-PARP inhibition and trapping-differentiates the various PARPis and contributes to their therapeutic efficacy [116, 117]. However, the specificity of PARPis for cancer cells with defective homologous recombination pathways means they have relatively low toxicity in normal cells, which maintain functional homologous recombination repair mechanisms. This specificity underscores their advantage over traditional chemotherapy, which often affects both cancerous and normal cells indiscriminately [114, 118]. The effectiveness of PARPis in cancer treatment also involves their potential synergistic effects when combined with other therapeutic modalities. For example, combining these inhibitors with cytotoxic chemotherapeutics or radiation therapy can potentiate DNA damage in cancer cells, leading to enhanced cell death [116]. These combination therapies are being actively explored in clinical trials to maximize the therapeutic benefits while minimizing resistance.

3.6. The Potential Role of PARylation and PARP14 in MΦ biology

Evidence suggests that ADP-ribosylation plays a role in inflammation [119-121]. Particularly noteworthy is the role of PARP1 in influencing inflammatory gene expression, primarily by acting as a co-activator of NFκB [122]. PARP1 has also been implicated in the mechanisms underlying the responses of MΦs or MΦ-like cell lines to pathogen-associated molecular patterns (PAMPs), such as LPS [123-127]. Moreover, PARP1 was reported to exert proinflammatory effects on other MΦ-related cell types, such as Kupffer cells in the fatty liver and microglia in the injured brain [128, 129]. In some instances, PARP1-mediated induction of proinflammatory mediators does not rely on its enzymatic activity, suggesting that the mechanisms by which PARP1 impacts inflammation may vary depending on the context or its targets [124, 126]. While PARP1 has been implicated in various proinflammatory roles, the specific contributions of other members of the PARP family in MΦ activation remain incompletely understood.

Of note, it has recently been demonstrated that both PARP9 and PARP14 are involved in the activation in human MΦs [8]. Specifically, they have been documented to exert cross-regulatory effects on the polarization of M0 towards the M1 and M2 phenotype in the setting of coronary atherosclerosis [8].

Proteomic analysis conducted on human and mouse MΦ-like cell lines treated with IFN γ or IL-4, representing proinflammatory M1 versus non/anti-inflammatory M2 cells led to the identification of molecules that exhibited increased expression with IFN γ treatment and decreased expression with IL-4 treatment [130]. Remarkably, among the proteins meeting these stringent criteria, PARP14 emerged as the sole candidate, and PARP9 was found to exhibit similar responses. Further investigation revealed that PARP14 suppresses proinflammatory IFN γ –STAT1 signaling and activates the anti-inflammatory IL-4–STAT6 pathway in primary human MΦs [131] (**Figure 12**).

PARP9 also appeared to interfere with the suppressive action of PARP14 on the IFN γ –STAT1 axis, thereby promoting proinflammatory MΦ activation [130] (**Figure 12**).

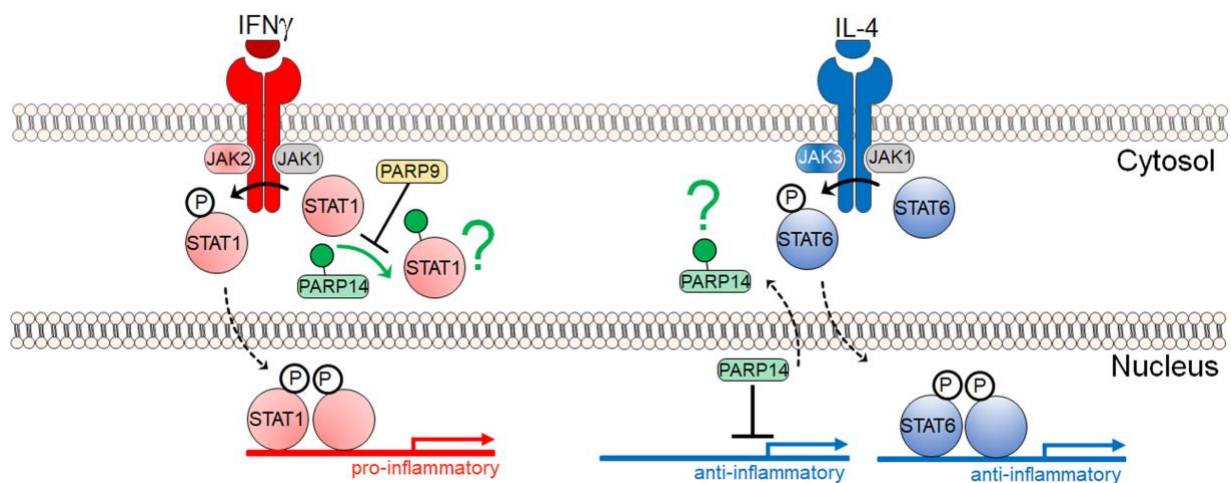


Figure 12. Schematic representation of the functions of PARP14 and PARP9 in MΦ activation. Both *in vivo* and *in vitro* investigations related to IFN γ signaling in MΦs suggest that PARP14 moderates proinflammatory phosphorylated STAT1 via ADP-ribosylation. On the other hand, PARP9 may serve to inhibit PARP14 enzymatic activity [8]. *In vitro* studies concerning IL-4 signaling in B-cell biology contexts propose that under non-stimulating conditions, PARP14 functions as a transcriptional repressor of STAT6 target genes. Upon activation by IL-4, PARP14 dissociates from a DNA-protein complex, facilitating STAT6 binding and subsequent transcriptional activation of anti-inflammatory markers. Conversely, PARP9 may act as an inhibitor of PARP14. In response to IFN γ signaling, PARP14 is activated, promoting the STAT1 pathway and thus fostering pro-inflammatory MΦ activation [132]. The green question mark symbolizes uncertainty regarding the fate of ADP-ribosylated substrates. Although the mechanisms involving IFN γ and IL-4 appear distinct, they may represent partial and complementary aspects of a complex biological process. Source: [130].

For instance, Iqbal *et al.* demonstrated in MΦs that PARP14 reduces mRNA stability, resulting in decreased expression levels of tissue factor, a surface glycoprotein with a significant thrombogenic role in MΦ-rich atherosclerotic lesions [133]. Specifically, PARP14 can function as a transcriptional repressor of STAT6 target genes, but upon activation by IL-4, it undergoes auto-ADP-ribosylation, leading to dissociation from DNA-protein complexes and thereby facilitating STAT6 binding [131, 132].

While PARP14 has been relatively overlooked compared to other PARP enzymes, it is now emerging as an appealing potential target for addressing chronic inflammation. Recent studies have highlighted PARP14 involvement in promoting tumors [134, 135].

Recently, novel anticancer immunotherapy strategies have emerged, targeting TAMs [136]. Strategies aimed at combating TAMs include inhibition of the M2 phenotype, induction of M1 MΦ activation, or reprogramming M2-to-M1 [137, 138]. Both M1 and M2 differentiation pathways can be targeted with PARPis, but the specific mechanism and the PARP isoforms responsible for defining the M1 phenotype require further exploration. Clinical trials have demonstrated that PARP inhibition can attenuate polarization of M0 MΦs towards M1 and restrain the release of cytokines that may negatively impact cancer growth or survival [139, 140].

Yet, despite its recognition as an oncoprotein, there remains a scarcity of information regarding the precise roles of PARP14 in tumor immune evasion. Moreover, there remains a paucity of understanding regarding the involvement of ADP-ribosylation in the polarization of TAMs. Therefore, it is essential to conduct further characterization of each compound to elucidate their precise effects. Such insights could pave the way for identifying novel therapeutic targets for infections or immune-related disorders.

3.7. Multicellular Tumor Spheroids (MCTS)

Multicellular tumor spheroids (MCTS) serve as prevalent three-dimensional (3D) models, arising from the natural tendency of adherent cells to aggregate [141]. They constitute a valuable resource for delving into the mechanistic intricacies of cancer cell biology [11]. Compared to traditional 2D cell culture methods, these 3D *in vitro* models offer superior capabilities to mimic key characteristics of *in vivo* tumors, including hypoxia, structural organization, cell-cell and cell-matrix interactions, thereby providing more physiologically relevant insights [142] (**Figure 13**). Spheroids with a diameter larger than 400–500 μm typically exhibit a structured composition, consisting of an outer layer of proliferating cells, an

intermediate layer of quiescent cells, and an internal necrotic core (**Figure 13**) [11, 143]. Consequently, the protein and gene expression profiles of cancer cells within spheroids closely resemble those observed in tumor cells *in vivo* [144].

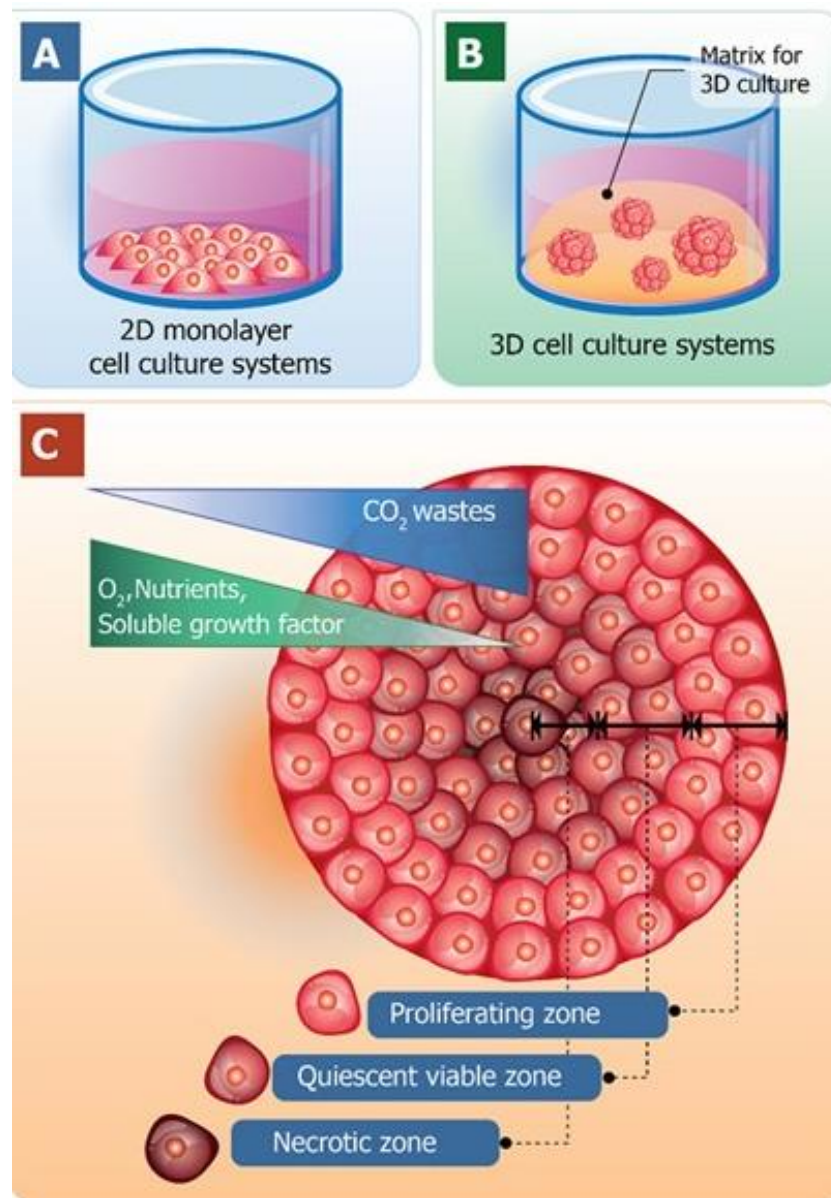


Figure 13. Schematic representation of traditional two-dimensional monolayer cell culture and three-dimensional cell culture. Two-dimensional monolayer cell cultures (A). Three-dimensional cell culture systems (B). In three-dimensional spheroids, various zones of cells are organized with different characteristics of oxygenation, nutrition, and CO₂ removal. The spheroid structure comprises distinct regions, including the necrotic zone (innermost), the quiescent viable cell zone (middle), and the proliferating zone (outermost), progressing from inside to outside (C). Modified from: [144].

In conventional 2D models, cells grow on a flat surface, typically within a Petri dish or culture flask, which offers mechanical support. In these 2D monolayers, cells have access to a uniform distribution of nutrients, signal molecules, and growth factors present in the medium, resulting

in homogeneous growth and proliferation [145]. However, the natural architecture of the TME *in vivo* is more complex, leading to variability in nutrient availability and inducing heterogeneous cell population growth. Moreover, most 2D methods lack control over cell shape, which can impact gene expression patterns, cellular topology, and biochemistry [146]. Additionally, these models often induce an apical-basal polarity, which may alter native cell functions such as proliferation, migration, and intercellular signaling [142, 147]. Indeed, cells cultured in 3D environments exhibit gene expression and transcription patterns that closely resemble *in vivo* changes in proliferation rates, morphology, and drug resistance. These characteristics contribute to the creation of models that are better representatives of tumor tissues [148, 149].

MCTS can be maintained in culture for 2–4 weeks, allowing for long-term experiments. Furthermore, to better mimic the TME and its cell-cell interactions, hybrid cell spheroids, also referred to as mixed-cell spheroids, can be easily generated by co-culturing cancer cells with immune cells, fibroblasts, endothelial cells and other relevant cell types present in the TME [144, 150]. MCTS can be generated using various protocols, although the core principle, which relies on anchorage-independent methodology, remains consistent. By creating conditions where cell-cell adhesive forces outweigh cell-substrate adhesive forces, cancer cells aggregate to form spheroids instead of adhering to a substrate [151]. The most commonly used methods for producing MCTS include hanging drop and liquid overlay techniques. However, alternative methods such as agitation-induced systems, microencapsulation, and magnetic levitation have also been proposed [152, 153].

3.8. Applications of MCTS: Impact in Cancer Research and Drug-screening

For investigating any disease, it is indispensable to establish pertinent and accessible models. Designing models for cancer immunology research poses particular challenges due to the intricate nature of the immune system, which encompasses numerous cell types, each with multiple subtypes and a diverse range of activation states [154]. This complexity is further compounded by the interactions between cancer cells and various immune cell types, as well as other components of the TME, all of which collectively shape the disease's progression and outcome [146]. Therefore, it is crucial to establish reliable and precise platforms that can predictively enhance the drug-testing process, while considering the intricate pathophysiology of the TME. Conventional 2D *in vitro* cell culture techniques are inadequate in capturing these intricate cellular interactions, lacking translational relevance and predictive capacity for drug

response at the systemic level, such as within tissues [155, 156]. Additionally, mouse models, while commonly used, present significant limitations owing to inherent disparities between human and murine immune systems [146].

By replicating the biological behavior of tumor cells, particularly the mechanisms underlying therapeutic evasion and drug resistance, MCTS provide a more representative platform and are extensively employed in 3D cancer research. Their utilization holds promise for advancing the development of efficacious drugs targeting various cancer types [151, 157, 158]. Therefore, 3D culture systems offer a promising avenue to address existing model deficiencies, offering an alternative approach and enhancing our understanding of cancer immunology [159]. Spheroid models could be of utmost importance in evaluating immunotherapies, particularly the efficacy of therapeutic antibodies and drug screening [160]. Additionally, the potential of MCTS consisting of cells in varied proliferative and metabolic states has been extensively showcased in studying interactions between cancer cells and stromal cells (such as MΦs, fibroblasts, lymphocytes), as well as in the development of novel anticancer approaches [161].

To mitigate biases and enhance data accuracy, various tumor spheroid models have been developed, emphasizing the importance of meticulously selecting the most suitable spheroid colonies, particularly in drug screening studies [162]. Additionally, several recent studies emphasized the benefits of utilizing 3D cultures in preclinical oncology drug development, revealing that drug screening conducted in 3D spheroid models can substantially expedite the process while yielding highly reproducible results [163]. Moreover, 3D growth assays offer the capability to address several critical aspects of preclinical research, including cell motility, genetic and phenotypic analysis, and pharmacodynamic testing under physiological-like conditions (such as hypoxia, nutrient availability and ECM composition) [162].

Notably, various 3D models of HER2-positive BC have been employed in drug discovery. As an example, Balalaeva *et al.* utilized SKBR-3 spheroids with HER2 overexpression to evaluate the cytotoxic effects of the HER2-targeted immunotoxin 4D5scFv-PE40 [164]. In a separate investigation, researchers developed a 3D matrigel-based culture system for HER2-positive BC, aiming to assess cell proliferation in response to Trastuzumab and endocrine agents [165]. Taken together, these studies underscore the significance of tumor spheroid models comprising HER2-overexpressing cancer cells as an efficacious strategy for enhancing therapeutic responses in a clinical context [166].

4. AIMS OF STUDY

4.1. Part I

Various aspects of MΦ biology have been shown to be regulated by the multifunctional PARP enzymes [167]. For instance, human studies in BC have shown a direct link between poor prognosis and TAMs [168], and PARP14 inhibition has emerged as a promising approach to impede TAM phenotype progression [132, 134]. However, little is known regarding the suppressive signaling in TAMs. These observations prompted us to set up *in vitro* models in which to investigate the role of ADP-ribosylation in the polarization into the TAM phenotype. In specific, our study aimed at:

- examining polarization towards M2 MΦs and TAM reprogramming in murine primary bone marrow-derived macrophages (BMDMs) and primary human MΦs
- observing MΦ polarization towards M2 in 3D models of spheroid co-cultures of human THP-1 derived MΦs and JIMT-1 BC cells
- assessing whether certain cytokines could act as key mediators of TAM differentiation
- understanding the role of PARP14 in TAM differentiation

4.2. Part II

Establishing models for cancer immunology research has the potential to improve our comprehension of the efficiency of drug screening and therapeutic antibodies [146]. This improvement aims to boost immune cell infiltration and anti-tumoral effects against spheroid targets, thereby identifying adjuvant chemicals to increase the sensitivity of cancer cells. With these goals in mind, we aimed at:

- establishing 3D spheroid co-cultures for studying cancer cells-immune cells interactions using high-content screening compatible techniques
- setting up a spheroid ADCC model for high-throughput screening applications for ADCC-modulating compounds
- testing the effects of bioactive molecules on the spheroid cultures

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents

Dulbecco's Modified Eagle's Medium-High glucose (DMEM, D6429) and recombinant mouse IL-4 (I1020) were purchased from Sigma-Aldrich (Budapest, Hungary). Fetal bovine serum (FBS, FB-1090/500), and penicillin-streptomycin (LM-A4118/100) were from Biosera (Cholet, France). PARP14^{-/-} mice [169] were a kind gift of Mark Boothby (Vanderbilt University, Nashville, TN, USA). Recombinant mouse M-CSF (416-ML-010/CF) was purchased from R&D Systems (Minneapolis, Minnesota, USA). PJ34 (2-(dimethylamino)-N-(6-oxo-5H-phenanthridin-2-yl)acetamide hydrochloride, 344458-15-7) was obtained from Sigma-Aldrich (Budapest, Hungary), and MCD113 was a kind gift from Herwig Schöler and Dana Ferraris (McDaniel College, Westminster, MD, USA) [170]. L-glutamine (BEBP17-605E) and PBS (BE17-517Q) were from Lonza (Basel, Switzerland). Trypan blue (T8154) was from Sigma (Budapest, Hungary), and Trypsin-EDTA (LM-T1706) was from Biosera (Cholet, France). Ficoll-Paque Plus (17-1440-03) was obtained from Amersham Biosciences (Amersham, UK). Anti-CD14-conjugated microbeads (130-050201) were from MiltenyiBiotec (BergischGladbach, Germany), and RPMI (R5886-500ML) was from Sigma-Aldrich (Budapest, Hungary). FCS (10270-106) and antibiotic/antimycotic solution (15140-122) were from Gibco (Waltham, Massachusetts, USA). Human M-CSF (300-25) and IL-4 (200-04) were purchased from PeproTech (Waltham, Massachusetts, USA). Phorbol 12-myristate 13-acetate (PMA, P1585), DMEM/F-12 (D8437) and human IFN- γ (13255) were purchased from Sigma-Aldrich (Budapest, Hungary). RPMI 1640 (L0501-500) was from Biowest (Nuaille, France), and insulin Humulin R (HI0219) was obtained from Eli Lilly Canada Inc. (Toronto, Ontario). TRI Reagent (TR118) was from Molecular Research Center (Cincinnati, OH, USA), High Capacity cDNA Reverse Transcription Kit (4668814) was from Applied Biosystems (Foster City, CA, USA), and 2X qPCRBIOSygreen Mix Lo-ROX (PB20.11-50) was from PCR Biosystems (London, UK). Oligonucleotides were purchased from IDT (Coralville, Iowa, USA) or Sigma-Aldrich (St. Louis, Missouri, USA). Anti-CD206-allophycocyanin (APC 550889) antibody conjugate was from BioLegend (San Diego, CA, USA). 7-aminoactinomycin-D (7-AAD, A1310) and pluronic-F127 (P2443) were obtained from Sigma-Aldrich (Budapest, Hungary). Cell Tracker Blue (CTB, 1856665) and Annexin V-Alexa Fluor 647 conjugate (A23204) were purchased from Invitrogen-ThermoFisher Scientific (Waltham, MA, USA). Agarose (A9539) and Dimethyl sulfoxide (DMSO, D8418) were from Sigma (Budapest, Hungary). Glass bottom Cell Carrier-96 ultra microplates (6055300) were from

PerkinElmer (Waltham, MA, USA). The Proteome Profiler Human XL Cytokine Array Kit (ARY022B) was from R&D Systems (Minneapolis, Minnesota, USA), and the IRDye 800CW Streptavidin (926-32230) was from LI-COR (Lincoln, NE, USA).

α -MEM (M8042) and Sunitinib malate (PZ0012) were obtained from Sigma-Aldrich (Budapest, Hungary), glutamine (35, 050-061) and MEM Non-Essential Amino Acids (MEM-NEAA, 11,140-050) were from Gibco (Waltham, Massachusetts, USA). Human IL2 (PHC0026) was from Proleukin, Novartis Hungária Kft. (Budapest, Hungary), Na-pyruvate (BE13-115E) was from Lonza (Basel, Switzerland), and Trastuzumab (humanized anti-HER2 monoclonal antibody, NDC-63459-303-43) was from Herzuma, EGIS Pharmaceuticals (Budapest, Hungary). Trastuzumab-F(ab')₂ was a kind gift of Prof. György Vereb and Árpád Szöör (Department of Biophysics and Cell Biology, University of Debrecen, Debrecen, Hungary).

5.2. Cell culture and maintenance

Mouse mammary 4T1 tumor cells were cultured in Dulbecco's Modified Eagle's Medium-High glucose (Sigma-Aldrich, D6429), supplemented with 10% FBS (Biosera, FB-1090/500), 5% L-glutamine (Lonza, BEBP17-605E), and 5% penicillin-streptomycin (Biosera, LM-A4118/100). The human monocytic cell line THP-1 was cultured in RPMI 1640 (Biowest, L0501-500), supplemented with 10% FBS (Biosera, FB-1090/500) and 5% penicillin/streptomycin (Biosera, LM-A4118/100).

JIMT-1 breast cancer cells were cultured in DMEM/F-12 (Sigma, D8437), supplemented with 20% FBS (Biosera, FB-1090/500), 0.3 U/ml insulin (100 NE/ml, Humulin R, HI0219) and 1% penicillin-streptomycin (Biosera, LM-A4118/100). An Enhanced Green Fluorescent Protein (EGFP) expressing subline (JIMT-1-EGFP) was generated as previously described [10] and was used for High-Content Analysis (HCA) experiments.

CD16.176V.NK-92 cells (hereinafter referred to as NK cells) were obtained from Dr. Kerry S. Campbell (Fox Chase Center, Philadelphia, PA, on behalf of Brink Biologics, Inc. San Diego, CA), and were licensed by Nantkwest, Inc. (Culver City, CA, USA). NK cells were cultured in α -MEM (Sigma, M8042), supplemented with 20% FBS (Biosera, FB-1090/500), 1% glutamine (Gibco, 35, 050-061), 1% MEM-NEAA (Gibco, 11,140-050), 1% Na-pyruvate (Lonza, BE13-115E), 100 IU/mL of IL-2 (Proleukin, PHC0026) and 1% penicillin-streptomycin (Biosera, LM-A4118/100).

All cell lines were cultured under standard conditions in a humidified atmosphere, with 5% CO₂ at 37 °C. Cells were routinely checked for the absence of mycoplasma contamination.

5.3. Isolation and differentiation of murine bone marrow-derived macrophages (BMDMs)

For bone marrow cells isolation, 10-12-week-old male C57/BL6 mice or PARP14^{-/-} mice [169] and their PARP14^{+/+} littermates were used, as previously reported [171]. Murine bone marrow macrophages collection was approved by the Regional Research Ethics Committee (15/2016/DEMÁB), in accordance with the Declaration of Helsinki. Cells were seeded into 6-well tissue culture plates (10⁶ cells/ml) in DMEM-high glucose (DMEM, Sigma-Aldrich, D6429), supplemented with 10% heat inactivated fetal bovine serum (FBS, Biosera, FB-1090/500), 5% L-glutamine (Lonza, BEBP17-605E) and 5% penicillin-streptomycin (Biosera, LM-A4118/100), and they were incubated at 37 °C and 5% CO₂. On day 3 and day 5 of culture, 25 ng/ml of recombinant mouse M-CSF (R&D Systems, 416-ML-010/CF) were added to induce differentiation. On the next day, cells were fully differentiated and used as bone marrow-derived macrophages (BMDMs).

5.4. Treatments with PARP inhibitors to study BMDMs polarization

In order to determine the effect of PARP inhibitor (PARPi) compounds on the polarization of macrophages and to specifically assess the role of PARP14 in this process, BMDM cells were pre-treated with either 20 µM of the wide-spectrum PARPi PJ34 (Sigma-Aldrich, 344458-15-7) or with 50 µM of the specific PARP14 inhibitor MCD113 (McDaniel College, Westminster, MD, USA), in complete DMEM for 1 h. PJ34 is here indicated as “wide-spectrum” inhibitor compound since it has been shown to inhibit primarily PARP1 and PARP2, but to exert a weak inhibitory effect on PARP14 as well [105, 110]. PBS (Lonza, BE17-517Q) was used to wash the cells twice, and polarization towards M2 macrophages was then induced with incubation of the cells with 5 ng/ml recombinant mouse IL-4 (Sigma-Aldrich, 11020) for 1 day. BMDMs were also polarized towards TAMs with 4T1 conditioned media (4T1CM) for 1 day (see details below). On day 7, cells were washed with PBS and collected for further experiments.

5.5. TAM phenotype induction by 4T1 conditioned media (4T1CM)

4T1 murine tumor cells were kept in culture under standard cell culture conditions and maintained in complete DMEM-high glucose culture media as specified in 5.2. Once reached 80% confluency approximately, they were seeded into 6-well plates and incubated for 1 day at 37°C (10^6 /ml in cell culture media). 4T1 cell culture supernatant was then carefully collected and centrifuged at room temperature (150 RCF for 3 min) to remove cell debris. Subsequently, the supernatant was added to BMDM cells to induce polarization into TAM phenotype. Specifically, 4T1CM was diluted in complete DMEM (see composition above) at a 1:1 ratio [172, 173] and added to BMDMs. After an incubation time of 24 h, cells were washed with PBS (Lonza, BE17-517Q) and then collected for further experiments. (Note: to assess the levels of IL-4 protein in 4T1CM, the bead assay BD CBA Mouse IL-4 Flex Set (BD Biosciences, 558298) was used for quantitation, and no IL-4 was detected, data not shown).

5.6. Isolation of human peripheral blood mononuclear cells (PBMCs) and macrophage ($M\Phi$) polarization

In accordance with the written approval of the Regional and Institutional Research Ethical Committee of the University of Debrecen (Faculty of Medicine, Debrecen, Hungary) and the Director of the National Blood Transfusion Service, heparinized leukocyte-enriched buffy coats were acquired from the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) from healthy blood donors (protocol number: OVSzK 3572-2/2015/5200). Prior to blood donation, informed and written consent was obtained from the blood donors. Ficoll-Paque Plus (Amersham Biosciences, 17-1440-03) gradient centrifugation was used to separate peripheral blood mononuclear cells (PBMCs) from buffy coats. Purification of monocytes from PBMCs was conducted by positive selection with anti-CD14-conjugated immunomagnetic microbeads (MiltenyiBiotec, 130-050201), according to the manufacturer's instructions. As measured by flow cytometry, almost 96-99% of the cells appeared as CD14⁺ monocytes following separation on a VarioMACS magnet. Isolated monocytes were plated at a density of 1.5×10^6 cells/ml into 24-well tissue culture plates and cultured for a total time of 5 days in RPMI (Sigma-Aldrich, R5886-500ML) supplemented with 10% FCS (Gibco, 10270-106), 1% antibiotic/antimycotic solution (Gibco, 15140-122), in the presence of 50 ng/ml human M-CSF (PeproTech, 300-25) for $M\Phi$ polarization. On day 2 of differentiation, fresh medium was added and complemented with 50 ng/ml M-CSF.

On day 5, cells were pre-treated for 1 h with the PARPi PJ34 (20 μ M) or the PARP14i MCD113 (50 μ M) and were washed twice with PBS (Lonza, BE17-517Q). To induce polarization towards M2, cells were then further cultured in the presence of human 100 ng/ml IL-4 (PeproTech, 200-04) in RPMI supplemented with 10% FCS and 1% antibiotic/antimycotic solution for additional 24 h. On day 6, they were washed with PBS and collected for further experiments.

5.7. *MΦ differentiation from THP-1 monocyte cells*

THP-1 cells were collected and seeded at 1.5×10^6 /ml density into a 6 cm Petri dish in complete RPMI 1640 media. To induce their differentiation into M0 MΦs, THP-1 cells were incubated for 48 h with 100 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P1585). With the use of a cell scraper, cells were carefully detached from the Petri dish and centrifuged at room temperature for 3 min at 150 RCF. Afterwards, M0 MΦs were used for the induction of spheroids (as detailed below).

5.8. *Generation of 3D in vitro models: JIMT-1 derived MΦ spheroids, THP-1 spheroids and JIMT-1-THP-1 co-culture spheroids*

Spheroids were grown as formerly reported [174], with modifications as follows. In order to form a cell-repellent, U-shaped bottom, a solution of 0.5% agarose (Sigma, A9539) in PBS was added into 96-well cell culture plates for pre-coating the wells (30 μ l/well). For formation of JIMT-1 and THP-1 derived MΦ spheroids, 2000 cells/spheroid were seeded in complete DMEM/F-12 (2×10^4 cells/ml in 100 μ l) into the 96-well plates' pre-coated wells. Cells were then allowed to clump for 3 days, and size and shape of spheroids were regularly checked with an inverted microscope.

For the generation of spheroid co-cultures, JIMT-1 cells were mixed at a 1:1 ratio with THP-1 derived M0 MΦs (see 5.7.). The 1:1 ratio was optimized for better mimic the *in vivo* environment and infiltration of immune cells in the tumor [175]. Each of the two cell populations was seeded into the pre-coated 96-well plates in 50 μ l/well at 2×10^4 cells/ml, and they were allowed to clump together as previously described. Specifically, 2000 cells/cell type were seeded for each spheroid (4000 cells in total) in an overall volume of 100 μ l/well in JIMT-1 growth media. On day 3, recombinant human IFN- γ (Sigma, 13255) was added at a

concentration of 20 ng/ml to the spheroids (50 µl/well) for additional 24 h to induce further polarization of MΦs towards the M1 phenotype.

5.9. Treatments of spheroids and co-culture spheroids with PARP inhibitors

To investigate the role of PARylation in MΦ polarization, on day 4 after polarization with IFN-γ spheroids and co-culture spheroids were incubated with the PARPi PJ34 (25 µM) and the PARP14i MCD113 (55 µM) for 24 h (50 µl/well in JIMT-1 growth media). It is well known that cell-to-cell contacts in spheroids induce cell resistance to toxic stimuli [176]. For this reason, concentrations of PARPi compounds used in these experiments were slightly increased, and the incubation time in their presence was extended, as a 24 h incubation was used instead of the previous 1 h pre-incubation. On the following day, spheroids were centrifuged at 150 RCF at room temperature for 3 min after being transferred to 15 ml Falcon tubes, and the cell pellet was collected for further experiments.

5.10. Total RNA isolation, reverse transcription and quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from murine BMDMs, human PBMC-MΦs and spheroid cultures with the TRIzol Reagent (Molecular Research Center, TR118) according to the manufacturer's instructions. The quality and quantity of RNA samples were detected by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 1 µg of total RNA was used for reverse transcription (RT) and cDNA synthesis was accomplished by PCR (Applied Biosystems, Foster City, CA, USA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4668814), based on the manufacturer's guidelines. Diluted cDNA samples were used for quantitative real-time PCR analysis. PCR was performed on a Roche Lightcycler 480II (Basel, Switzerland) in a final volume of 10 µl in triplicates, using 2X qPCR BIOSygreen Mix Lo-ROX (PCR Biosystems, PB20.11-50). The mouse and human primers used in the present study are summarized in **Table 2** and **Table 3**, respectively. Oligonucleotides were purchased from IDT (Coralville, Iowa, USA) or Sigma-Aldrich (St. Louis, Missouri, USA). Relative quantification of gene expression was calculated by the Delta Delta Ct approach. The data output was determined as fold changes in expression and assessed using geometrical means of a combination of housekeeping genes as endogenous control for mRNA expression (mouse HPRT, B2M, GAPDH, Cyclophilin A, 36B4 and human GAPDH, Cyclophilin A, 36B4). All fold changes are expressed normalized to the untreated control.

Table 2. List of mouse primer pairs used for RT-qPCR gene expression analysis

Gene name	Forward primer	Reverse primer
mArg1	CTTGCGAGACGTAGACCCTG	GGCCTTTTCTTCCTTCCCAG
mFizz-1	GCCAATCCAGCTAACTATCCCT	CAACGAGTAAGCACAGGCAG
mMRC1 (CD206)	GCAAATGGAGCCGTCTGTGC	CTCGTGGATCTCCGTGACAC
mHPRT	AGTGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT
mB2M	CTGCTACGTAACACAGTTCCACC C	CATGATGCTTGATCACATGTCTC G
mGAPDH	ACAGTCCATGCCATCACTGCC	GCCTGCTTCACCACCTTCTTG
mCyclophilin A	TCCGACTGTGGACAGCTCTA	ATTGCGAGCAGATGGGGTAG
m36B4	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT

Table 3. List of human primer pairs used for RT-qPCR gene expression analysis

Gene name	Forward primer	Reverse primer
hCD-14	GGAAGACTTATCGACCATGGA	GCTGAGGTTTCGGAGAAGTTG
hIL-12	CCAAGAAGCTTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC
hTNFa	TGGGTCTATTCCGTTGTGTC	GAGGGCTGATTAGAGAGAGGTC
hARG1	TCATCTGGGTGGATGCTCACAC	GAGAATCCTGGCACATCGGGAA
hRETNLB (Fizz-1)	GCAAGAAGCTCTCGTGTGCTAG	AACATCCCACGAACCACAGCCA
hMRC1 (CD206)	CGAGGAAGAGGTTTCGTTCCACC	GCAATCCCGGTTCTCATGGC
hFibronectin	CCACCCCCATAAGGCATAGG	GTAGGGGTCAAAGCACGAGTCAT
hCCL17	ACTTCAAGGGAGCCATTCCC	CCCTGCACAGTTACAAAA
hCCL18	CTCTGCTGCCTCGTCTATACCT	CTTGTTAGGAGGATGACACCT
hLCN2 (Lipocalin2)	GTGAGCACCAACTACAACCAGC	GTTCCGAAGTCAGCTCCTTGGT
hMIF	AGAACCGCTCCTACAGCAAGCT	GGAGTTGTTCCAGCCCACATTG

hPAI-1 (Serpin E1)	CTCATCAGCCACTGGAAAGGCA	GACTCGTGAAGTCAGCCTGAAAC
hGAPDH	AGCCTCAAGATCAGCAATG	ATGGACTGTGGTCATGAGTCCTT
hCyclophilin A	GTCTCCTTTGAGCTGTTTGCAGAC	CTTGCCACCAGTGCCATTATG
h36B4	CCATTGAAATCCTGAGTGATGTG	GTCGAACACCTGCTGGATGAC

5.11. Flow cytometry

Phenotyping of human monocyte-derived MΦs in the presence of the PARPi PJ34 or with MCD113 PARP14i was performed by flow cytometry using anti-CD206-allophycocyanin antibody conjugate (BioLegend, APC 550889). Cells were labeled with antibodies on ice for 30 min according to the manufacturer's instructions. 7-aminoactinomycin-D (7-AAD, Sigma-Aldrich, A1310) staining was used to assess cell viability. Immediately before flow cytometric analysis, samples were stained with 10 µg/ml 7-AAD for 10 min. Fluorescence intensities and the frequency of CD206+ cells were measured with a Novocyte2000R Flow Cytometer (Agilent (Acea) Biosciences Inc., USA), and data were analyzed with the FlowJo v X.0.7 software (Tree Star).

5.12. Measurement of the cytokine concentrations

The concentration of secreted chitinase-3-like-protein 1 (CHI3L1), interleukin-8 (IL-8), lipocalin 2 (LCN2), C-C motif chemokine ligand 2 (CCL2), macrophage migration inhibitory factor (MIF), matrix metalloproteinase-9 (MMP-9), osteopontin (OPN) and plasminogen activator inhibitor-1 (PAI-1) cytokines were measured using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, ARY022B), according to the manufacturer's protocol. Spheroids grown from THP-1 derived MΦs and JIMT-1-MΦ co-cultures were kept in culture for a total time of 4 days (1 day in the presence of 20 ng/ml IFN-γ), collected and centrifuged at 150 RCF for 3 min at room temperature. 500 µl of culture supernatants were added to each membrane array and levels of the selected soluble proteins mentioned above were detected. IRDye 800CW Streptavidin (LI-COR, 926-32230) was added to the membranes based on the manufacturer's instructions, and LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) was used for imaging the arrays.

5.13. High-Content Analysis (HCA) on live cells: cell death in spheroids

Our next goal was to establish co-culture spheroid models in which the cellular interactions between cancer cells and immune cells could be studied with HCA. In specific, we aimed at setting up 3D systems in which we could distinguish between the two cell populations, to assess 1) how MΦ phenotype was changing and if MΦs were infiltrating the spheroids and 2) the effects of certain compounds on Trastuzumab-induced ADCC.

5.13.1. Effect of PARP inhibitors on JIMT-1-THP-1 MΦ spheroid co-cultures

For all HCA experiments on macrophage polarization, co-culture spheroids were generated as described in section 5.8. with JIMT-1-Enhanced Green Fluorescent Protein (EGFP) cells and THP-1 derived MΦs. Staining of M0 THP-1 derived MΦs (differentiated as indicated above) with Cell Tracker Blue (CTB, Invitrogen, 1856665) before seeding (10 μM in THP-1 growth medium for 1 h), allowed the distinction between the two cell populations, making visualization and HCA measurements more precise, easier and less time consuming. After incubation time, M0 MΦs were washed in RPMI serum-free media (Biowest, L0501-500) twice in order to eliminate the excess of the dye (150 RCF for 3 min at room temperature). Stained cells were subsequently mixed at a 1:1 ratio with JIMT-1-EGFP cells in JIMT-1 media and seeded into a 96-well plates pre-coated with 0.5% agarose in PBS. In total, 2×10^4 cells/ml were used for each cell population, in a volume of 100 μl (see details in 5.8.).

After 3 days, spheroids were transferred with the use of a 1 ml pipette to glass bottom Cell Carrier-96 ultra microplates (PerkinElmer, 6055300) in triplicates. Before transferring the spheroids, wells were coated with 0.5% pluronic-F127 (Sigma, P2443) in DMSO (Sigma, D8418) for 45 min at room temperature (50 μl/well). Wells were then washed with 100 μl DMEM/F-12-serum free media twice. This step was crucial to prevent cell attachment to the bottom of the wells, as spheroids were kept in culture for additional 48 h. Human IFN-γ (Sigma, 13255) was added in 50 μl/well to the spheroids (20 ng/ml) to induce MΦ polarization towards M1. On the next day, cells were incubated for additional 24 h with 25 μM PJ34 or 55 μM MCD113 (50 μl/well in JIMT-1 growth media) to study the effects of the PARPi compounds on tumor apoptosis and morphological changes of spheroids.

Next, Annexin V-Alexa Fluor647 conjugate (Invitrogen-ThermoFisher Scientific, A23204) was used for staining the cells (1:100 in JIMT-1 growth media, 50 μl/well) to detect apoptotic cell death. After 1 h staining, the plate was imaged with Opera Phenix High-Content Analysis

equipment (Perkin Elmer, Waltham, MA, USA) and data were analyzed with the Harmony software (Perkin Elmer). Images of 3 spheroids/condition were taken in confocal mode with a 10x air objective and analyzed for Annexin V-647 intensity of tumor cells in each well, and for area of the spheroids. 10 planes of Z-stacks were selected, as cells in the spheroids tend to be on different focal planes of the microscope (lower plane: 0 μm ; higher plane: 90 μm). Following channels were selected in order to detect fluorescence intensity: EGFP (ex: 488 nm, em: 500-550 nm), DAPI (ex: 405 nm, em: 435-480 nm) and Alexa647 (ex: 640 nm, em: 650-760 nm). Using the “Find Texture Regions” option, spheroids were identified by the EGFP fluorescence of JIMT-1 cells and were filtered out by size ($> 25,000$). Annexin-positive (red) cells in green (JIMT-1-EGFP cells) were identified as apoptotic tumor cells with the “Select population” option and were measured for the Annexin V fluorescence intensity. Annexin intensities were determined and expressed as mean intensity.

5.13.2. Effect of test compounds on ADCC in a JIMT-1+NK92.CD16 spheroid model

For HCA experiments on detection of ADCC effector compounds, spheroids were generated using JIMT-1-EGFP cells as described in section 5.7. ($2 \times 10^4/\text{ml}$ cells in JIMT-1 media seeded into 96-well cell culture plates in 100 $\mu\text{l}/\text{well}$). On day 3, spheroids were transferred in triplicates to glass bottom Cell Carrier-96 ultra microplates (PerkinElmer, 6055300). Before transferring, plates were coated with 0.5% Pluronic-F127 (Sigma, P2443) in DMSO (Sigma, D8418) for 45 min as described above to prevent cell attachment to the bottom of the wells. Spheroids were pre-treated for 1 h with test compound Sunitinib 40 μM (Sigma-Aldrich, PZ0012) in fresh JIMT-1 complete media (10 $\mu\text{l}/\text{well}$). ADCC was then induced by adding the NK effector cells to the JIMT-1-EGFP target cells at an effector-to-target (E:T) ratio of 20:1 (40,000 NK cells/well). To distinguish between the two cell populations, NK cells were stained with 10 μM CTB (Invitrogen, 1856665) for 1 h in NK medium (α -MEM, see composition above). Cells were washed twice with α -MEM serum-free media (Sigma-Aldrich, M8042) to eliminate the excess of the dye (150 RCF for 3 min at room temperature). Stained NK cells were then added to the spheroids along with 10 $\mu\text{g}/\text{ml}$ of the anti-HER2 antibody Trastuzumab (Herzuma, NDC-63459-303-43) by pipetting 55 $\mu\text{l}/\text{well}$ of CTB-stained NK cells and 55 $\mu\text{l}/\text{well}$ of Trastuzumab in JIMT-1 growth medium. The total volume of treatment added for the ADCC was 110 $\mu\text{l}/\text{well}$ and the final Sunitinib concentration was 20 μM . The plate was incubated for 24 h.

As NK92 cells are known to exert non-specific cytotoxic functions, for control (CTL) JIMT-1 cells were co-incubated with both NK cells alone and with a F(ab')₂ Ab. Specifically, F(ab')₂-Trastuzumab (TR-F(ab')₂) was used as a negative CTL in this study. TR-F(ab')₂ fragment was prepared as reported by Tóth *et al.* [177] and added in the same volume as Trastuzumab along with NK cells as described above. Concentration was adjusted to 6.6 µg/ml corresponding to an equimolar concentration with Trastuzumab.

Cells were stained with Annexin V-Alexa Fluor647 conjugate (Invitrogen-ThermoFisher Scientific, A23204) for 1 h for apoptosis detection (50 µl/well, 1:100 in JIMT-1 media). Images were taken with Opera Phenix High-Content Analysis equipment (Perkin Elmer, Waltham, MA, USA) and data were analyzed with the Harmony software (Perkin Elmer). Images of 3 spheroids/condition were taken (10x air objective with 0.3 numerical aperture in confocal mode) and analyzed for the intensity of Annexin V-647 in the surrounding area of the spheroids, since target cells killed by ADCC appear in an apoptotic “ring” of the spheroids. This yielded a more pronounced ADCC effect and lower experimental errors rather than measuring either the whole spheroid area or the entire well area. Z-stacks were selected as described in section 5.13.1. (first plane: 0 µm, last plane: 90 µm), and fluorescence intensity was detected at the following channels: EGFP (ex: 488 nm, em: 500-550 nm) and Alexa647 (ex: 640 nm, em: 650-760 nm). To visualize the NK cells within the spheroids, DAPI channel was selected (ex: 405 nm, em: 435-480 nm). Spheroids were identified by the EGFP fluorescence of JIMT-1 cells by using the “Find Texture Regions” option, and filtered out by size (> 25,000). Red cells (Annexin-positive cells) were selected by the “Select Population” option. Annexin intensities were expressed as mean intensity and measured in this spheroid “ring” with the “Select Region” option to determine apoptotic cell death.

5.14. Statistical analysis

Data are presented as mean ± SEM of at least 3 independent experiments (detailed in figure legends). Shapiro-Wilk test was used to analyze normality. If the data distribution was normal, one-way ANOVA complemented by Tukey's, Dunnett's or Bonferroni's post-hoc test was used. Kruskal-Wallis test followed by Dunn's post-hoc test was performed when distribution of data was not normal. Statistical analysis was performed using GraphPad Prism 9.5.1 (GraphPad Software Inc., San Diego, CA, USA). P values <0.05 were considered significant, as stated in figure legends.

RESULTS

6.1. Part I.

6.1.1. PARP14 mediates IL-4-induced M2 polarization in murine BMDMs

To investigate M2 polarization, we first established a murine *in vitro* model of 2D bone marrow derived macrophages (BMDMs). As described in section 5.2. of “Materials and Methods”, M-CSF was added to the cells for 6 days to induce differentiation. Once differentiated, BMDMs were polarized with IL-4 towards the M2 phenotype [178]. To confirm whether IL-4 treatments triggered M2 polarization, RT-qPCR was used to quantify the mRNA levels of a panel of M2 markers [179], and to characterize the functional state of macrophages. Analyses revealed an increase in all M2 marker genes Arginase1 (Arg1), Fizz1 and Mannose Receptor 1 (MRC1/CD206) (**Figure 14**).

Our main goal was to assess the role of PARylation and of specific PARP enzymes in the polarization of MΦs. Our preliminary experiments with the clinically used specific PARP1/2 inhibitor Olaparib revealed no involvement of PARP1 in MΦ polarization towards M2 (data not shown), in line with a recent study by Wang L. *et al.* [180]. Instead, a significant decrease of all M2 markers was detected at relatively high concentration of PJ34, another wide-spectrum PARPi (**Figure 14A**). As it was described by comparing the chemical space of these inhibitors that PJ34 but not Olaparib inhibits PARP14 [181], an enzyme which has been implicated in IL-4 response [8, 130], we hypothesized that PARP14 plays a key role in mediating polarization of MΦs to M2. Thus, the specific PARP14 inhibitor MCD113 (kind gift from Dana Ferraris, McDaniel College) [170] was tested in our model. MCD113 also led to the suppression of M2 marker gene expression (**Figure 14B**). To reinforce these findings and better assess the role of PARP14 in MΦ polarization, we performed the experiments with polarization towards M2 using BMDM-derived MΦs also isolated from PARP14 KO mice [169]. Interestingly, our RT-qPCR data confirmed that PARP14^{-/-} MΦs stimulated with IL-4 did not express the M2 markers and did not polarize toward the M2 phenotype (**Figure 14C**), thereby validating the essential role of PARP14 in MΦ polarization towards M2.

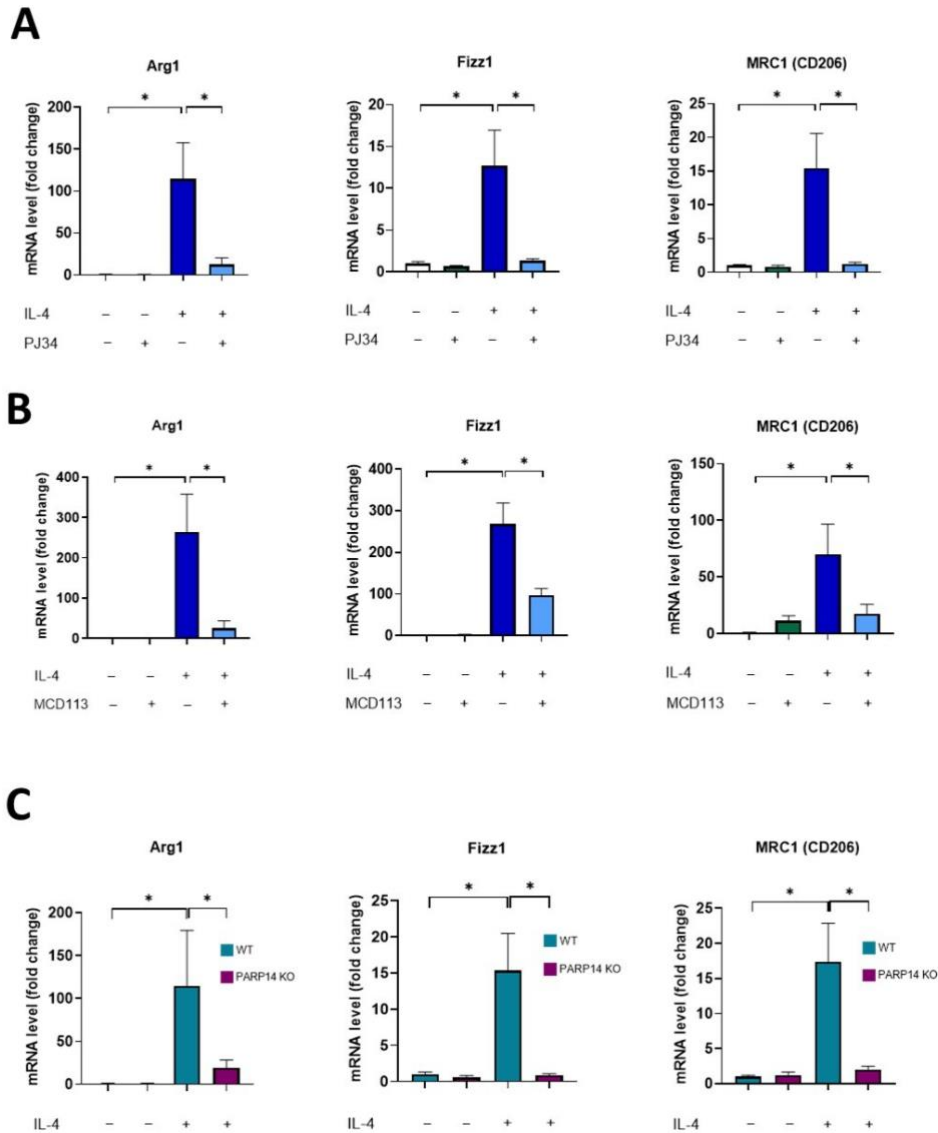


Figure 14. PJ34 and MCD113 PARPi decrease expression of M2 markers and PARP14 mediates M2 polarization in IL-4-polarized murine BMDMs. Bone marrow-derived macrophages (BMDMs) were isolated from C57/BL6 mice and were differentiated for 6 days in the presence of 25 ng/ml M-CSF. After pre-treatment for 1 h with PARPi compounds PJ34, 20 μ M (**A**) or MCD113, 50 μ M (**B**), BMDMs were polarized towards M2 M Φ s with IL-4 for 24 h (5 ng/ml). On day 7, RNA extraction was performed and mRNA expression levels of M2 markers Arg1, Fizz1 and MRC1 (CD206) were studied by RT-qPCR. BMDMs were also differentiated from PARP14 KO mice and their wild type counterparts (6 days with 25 ng/ml M-CSF as stated above) and polarized towards M2 phenotype with IL-4 for 24 h (5 ng/ml). On day 7 RT-qPCR was conducted (**C**). Light blue and purple colors in panel (**C**) show WT and PARP14 KO groups, respectively. Graphs show mean of 4 independent experiments ($\pm$ SEM). Statistical evaluation was performed with Kruskal-Wallis test followed by Dunn's post-hoc test (panel A and Arg1 in panel B) or with one-way ANOVA and Tukey's post-hoc test (Fizz1 and MRC1/CD206 in panel B and panel C) (* $p < 0.05$).

6.1.2. PARP14 mediates TAM polarization in murine BMDMs

For further studying PARP14 role in the polarization of TAMs, we established an *in vitro* model of BMDMs in which transfer of cancer cell supernatant (conditioned media; CM) were performed. Murine BMDMs were isolated from C57/BL6 mice and differentiated for a total time of 6 days in the presence of M-CSF, as specified above in “Materials and Methods”. Then, MΦs were cultured with CM derived from 4T1 mouse mammary carcinoma cells (diluted at a 1:1 ratio with full DMEM) [172, 173] for 24 h. Data obtained from RT-qPCR showed that 4T1CM transfers resulted in a significant increase in the expression levels of M2 marker mRNA (Arg1, Fizz1 and MRC1/CD206) (**Figure 15**), indicating an M2-like polarization in BMDMs. To determine the role of PARylation in TAM polarization, the PARPi PJ34 and the PARP14i MCD113 were tested in this model. Both inhibitors significantly reduced the expression of the M2 markers, indicating the contribution of PARP14-dependent ADP-ribosylation in the induction of the TAM phenotype (**Figure 15A, B**). In the same way, lack of PARP14 in murine PARP14^{-/-} BMDMs led to the suppression of the 4T1CM-induced TAM phenotype (**Figure 15C**). Taken together, our results reveal that M2-like polarization of murine BMDMs is triggered by 4T1CM, and demonstrate that PARP14 plays a fundamental role in TAM polarization.

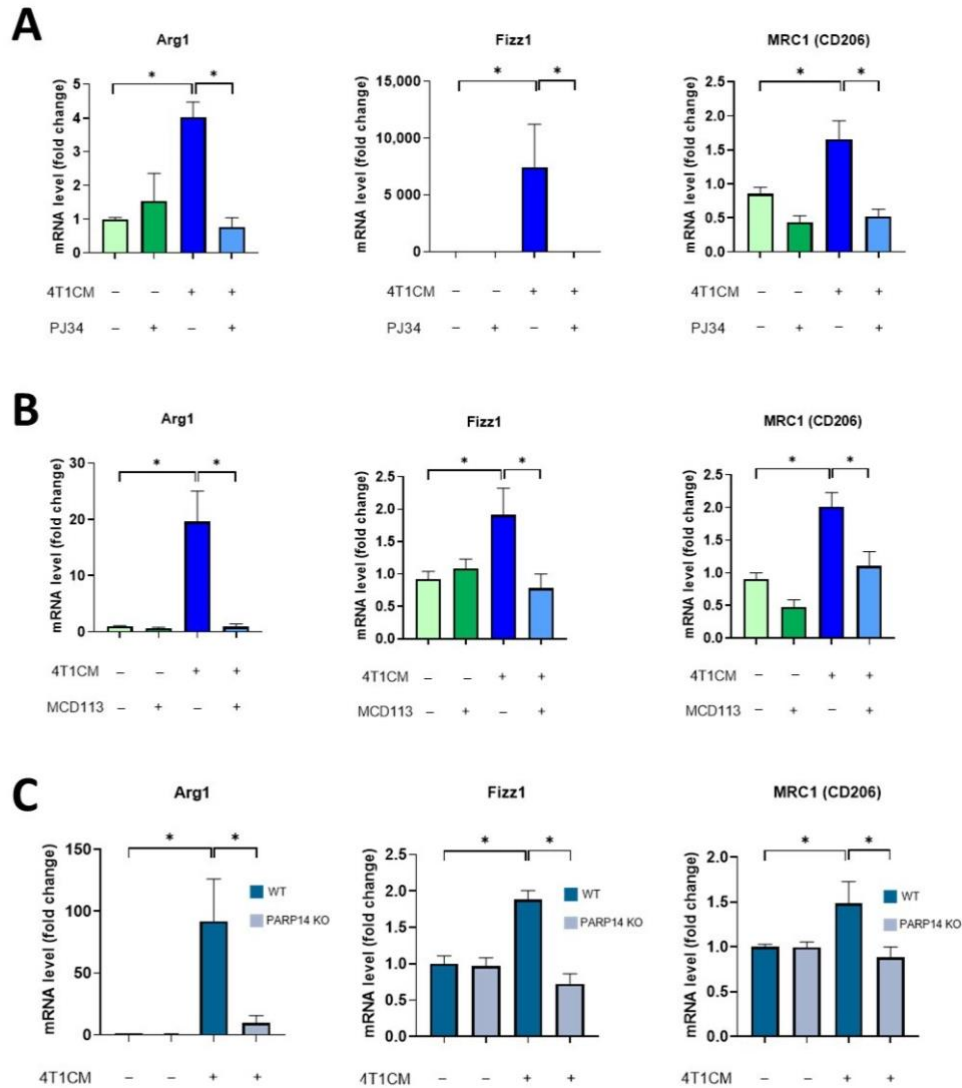


Figure 15. 4T1 mouse breast cancer cells induce reprogramming of murine BMDMs toward an M2-like TAM phenotype in a PARP14-dependent manner. BMDMs were isolated and differentiated from C57/BL6 mice for 6 days with M-CSF (25 ng/ml). They were pre-treated for 1 h with either 20 μ M PJ34 (A) or 50 μ M MCD113 (B). Then, BMDMs were kept in culture for 24 h with 4T1 conditioned media (4T1CM; 1:1 dilution in full DMEM) for polarization into TAMs. On day 7, RT-qPCR was performed to quantify the mRNA levels of M2 markers. BMDMs were differentiated from PARP14 KO mice and their wild type counterparts as well, for 6 days with 25 ng/ml M-CSF, followed by treatments with 4T1CM for 24 h. RNA extraction and RT-qPCR were conducted on day 7 (C). Dark blue and grey colors in panel (C) show WT and PARP14 KO groups, respectively. The graphs show mean of 4 independent experiments ($\pm$ SEM). Data were analyzed using Kruskal-Wallis and Dunn's post-hoc test (* $p < 0.05$).

6.1.3. M2 polarization in human PBMC-M Φ s is regulated via PARP14

To characterize the role of PARP14 in the polarization of human M Φ s towards M2, human peripheral blood monocyte-derived macrophages (PBMC-M Φ s) were differentiated in the

presence of M-CSF and used for this study. Pre-treatments were performed with incubation of PBMC-MΦs for 1 h with PJ34 or MCD113. Cells were then polarized to the M2 phenotype with a 24 h stimulation with IL-4. It was confirmed, on day 6, that treatments did not affect cell viability but, as indicated by formation of cell clusters, the microscopic phenotype of PBMC-MΦs was found to be changed (**Figure 16**). The cells were then collected for RT-qPCR experiments, and an expression profile analysis of human M2 markers (Arg1, Fizz1, MRC1/CD206, Fibronectin, CCL17 and CCL18) was performed.

Our data showed that, compared to the non-polarized control cells, IL-4 stimulated PBMC-MΦs expressed a significantly higher level of all the M2 markers (**Figure 17A**). In addition, treatments with either PJ34 or MCD113 caused a decrease in Arg1, MRC1 (CD206), Fibronectin, CCL17 and CCL18 mRNA levels. In the case of Fizz1, both compounds decreased the levels of mRNA, although this change was statistically significant only for PJ34, but not for MCD113 ($p = 0.06$) (**Figure 17A**). Since the expression of MRC1 (CD206) was significantly altered by both inhibitors, the presence of this M2 marker on the surface of PBMC-MΦs and the frequency of CD206⁺ cells were examined. 1 h treatment of MΦs in the presence of MCD113 slightly affected the granulation and size of the cells (**Figure 16B**), but in flow cytometry they appeared as a well-defined population. The expression of CD206 was increased in PBMC-MΦs upon their polarization with IL-4, as it was expected. Confirming our results obtained from RT-qPCR, PJ34 pre-treatments led to a significant down-regulation of MRC1 (CD206) cell surface expression, while MCD113 treatments caused a modest but not significant reduction (**Figure 17B**). Furthermore, the relative frequency of CD206⁺ cells showed a significant decrease after treatments with MCD113 inhibitor compound (**Figure 17B**). These data suggest the similar role of PARP14 in both the murine and human models of M2 differentiation.

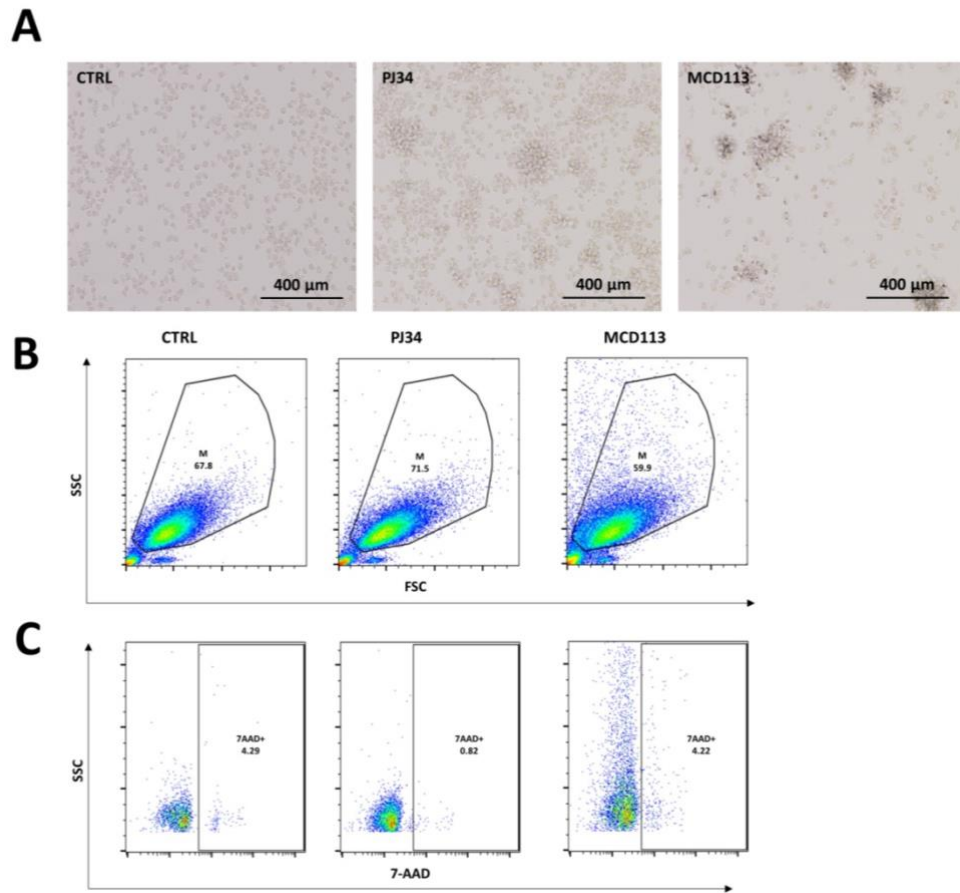


Figure 16. Treatments with PARPis PJ34 and MCD113 cause cell clumping but are non-toxic to MΦs. MΦs were differentiated from human monocytes for 5 days in the presence of M-CSF (50 ng/ml). Cells were pre-treated for 1 h with PJ34 (20 μM) or with MCD113 (50 μM). After, MΦs were washed twice with PBS and further cultured with IL-4 (100 ng/ml) for additional 24 h. The morphology of monocyte-derived cells was investigated on day 6 using an OLYMPUS IX-81 microscope (A). Images were taken at 10x magnification. The granulation and size of the cells (B) as well as their viability (7-AAD staining) (C) were analyzed by flow cytometry.

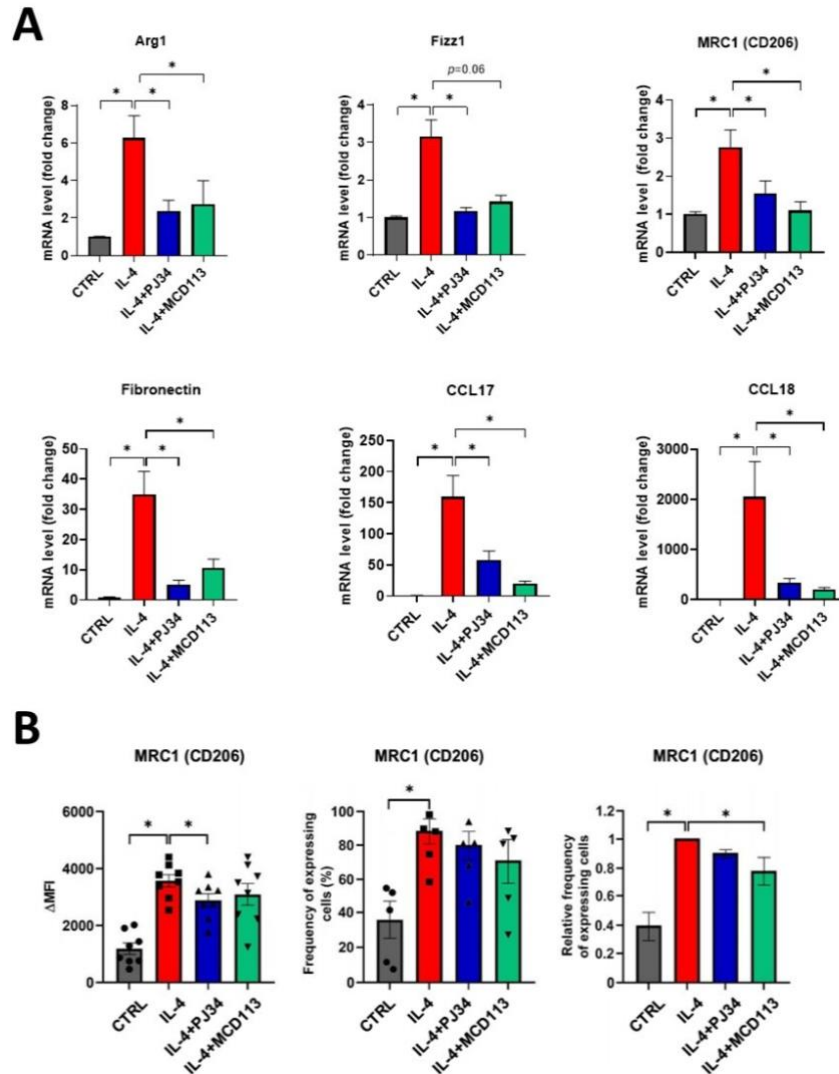


Figure 17. The PARPi PJ34 and the PARP14i MCD113 cause a reduced expression of M2 markers in human MΦs. MΦs were differentiated from human monocytes for 5 days in the presence of 50 ng/ml M-CSF. On day 5, cells were pre-treated for 1 h with either PJ34 20 μ M or with MCD113 50 μ M and were stimulated for 24 h with IL-4 100 ng/ml to induce polarization toward the M2 phenotype. MΦs were collected on day 6 for RNA extraction and RT-qPCR was performed to measure M2 markers mRNA levels. Histograms show mean of 4 independent experiments ($\pm$ SEM). Statistical evaluation was performed with Kruskal-Wallis test and Dunn's post-hoc test (* p < 0.05, ns: not significant) (A). On day 6, cells were collected and labelled with APC-conjugated anti-human mouse CD206 monoclonal antibody. Flow cytometry was used to measure fluorescence intensities and the ratio of MRC1 (CD206)-expressing cells. 8 independent experiments ($\pm$ SEM) were used to calculate the mean value of MFIs (Median Fluorescence Intensity) of CD206 cell surface molecule. 5 independent experiments ($\pm$ SEM) were used to calculate the mean value of frequency of CD206+ cells. The relative frequency of CD206-expressing cells was assessed as follows. For each donor, the frequency of CD206+ cells stimulated with IL-4 was considered to be 1, and the frequency of untreated cells and cells treated with both IL-4 and PARPis was compared to this. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test (left panel) or by Kruskal-Wallis test and Dunn's post-hoc test (middle and right panels) (* p < 0.05) (B).

6.1.4. THP-1 derived MΦs spontaneously undergo polarization toward an M2-like phenotype when co-cultured with JIMT-1 breast cancer cells in spheroids

Next, we aimed at setting up cellular models that could better mimic *in vivo* clinical settings or tumor features. To determine the role of ADP-ribosylation in TAM differentiation and the effects of tumor-derived signals, we established an *in vitro* model of 3D tumor spheroids in which the interactions between MΦs and cancer cells could be studied in their complexities. Co-culture spheroids were grown using human THP-1-derived MΦs [182, 183] and JIMT-1 human BC cells expressing EGFP. The co-cultures were established as previously reported [10]. MΦs were differentiated from THP-1 cells and were stained with the fluorescent dye Cell Tracker Blue-CTB for 1 h before seeding as described in section 5.13.1. Images taken with confocal microscope on day 4 revealed that, after addition of IFN-γ to induce MΦ polarization, spheroids did not show visible changes in size, while THP-1 derived MΦs started to infiltrate them (**Figure 18A**).

Gene expression analysis from RT-qPCR was used to check the mRNA levels in the spheroid co-cultures of a panel of both M1 markers (CD14, IL12, TNF) and M2 markers (MRC1/CD206, Fibronectin, CCL17, CCL18), keeping in mind that human and murine markers of MΦ polarization are considerably different [3]. IFN-γ was added to the spheroids to initiate polarization of MΦs toward M1. The gene expression profiles of JIMT-1-THP-1 spheroid co-cultures and JIMT-1 cancer cell spheroids were compared, since it was not possible to exclude that cancer cells could induce the expression of some MΦ markers. Results intriguingly showed that MΦ only spheroids (not kept in co-culture) displayed significantly higher levels of M1 markers when compared to MΦ-cancer cell co-culture spheroids (**Figure 18B**). Moreover, in the case of JIMT-1 spheroids the mRNA levels of M1 markers were found to be always low, thereby suggesting that the MΦ markers were not expressed by the cancer cells but specifically by MΦs. As it is known that TAMs more likely display an M2-like phenotype in the tumor microenvironment (TME) [168, 175], M2 marker levels were also determined in co-culture spheroids. We observed that, when compared to “pure” MΦ spheroids, the co-cultures with JIMT-1 cells induced M2 marker expression in THP-1-derived MΦs (**Figure 18C**).

These findings confirm our hypothesis that M2-like polarization of MΦs is triggered by signals of the TME, and corroborate our results obtained from 4T1CM transfers in the murine MΦ model (see above). In general, M1 and M2 marker expression in our 3D system validated the polarization of THP-1 derived MΦs in co-culture spheroids.

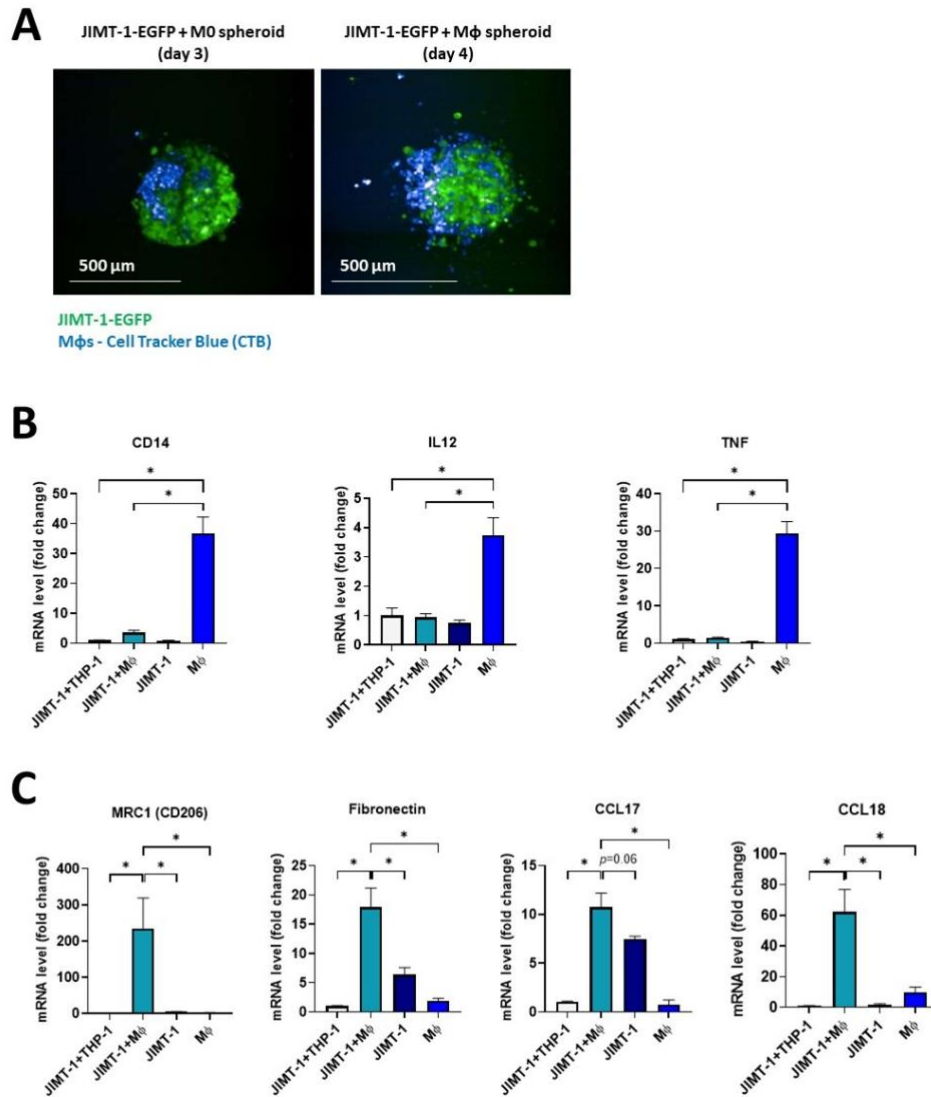


Figure 18. *MΦs differentiated from THP-1 cells polarize toward the M2 phenotype when incorporated into JIMT-1 breast cancer cell spheroids.* Spheroids were formed with JIMT-1-EGFP cancer cells and THP-1-derived MΦs (differentiated with PMA 100 ng/ml for 48 h). CTB-stained MΦs (blue) and JIMT-1-EGFP cells (green) were mixed at a 1:1 ratio in JIMT-1 growth media before seeding. Images were taken after induction of spheroids on day 3 (left, JIMT-1+M0) and after treatments with IFN- γ 20 ng/ml on day 4 (right, JIMT-1+MΦ) (A). Expression levels of M1 (B) and M2 markers (C) were assessed by RT-qPCR. Co-culture spheroids were kept in cultures for 4 days in total, with the presence of IFN- γ for 1 day. Columns represent the relative mRNA expression levels of the polarization markers measured in spheroid co-cultures JIMT-1+THP-1 and JIMT-1+MΦ, and in spheroids JIMT-1 and MΦs, respectively. The graphs show mean of 4 independent experiments ($\pm$ SEM). Statistical evaluation was performed with Kruskal-Wallis test followed by Dunn's post-hoc test or with one-way ANOVA and Tukey's post-hoc test (CD14 in panel B and Fibronectin in panel C) (* $p < 0.05$).

6.1.5. PARP inhibitors suppress M2 marker expression in JIMT-1-THP-1 derived MΦ spheroid co-cultures

To better understand the involvement of ADP-ribosylation in our 3D MΦ polarization system, spheroids were grown in JIMT-1-THP-1-derived MΦ co-cultures or in THP-1 derived MΦs for 3 days. Spheroids were treated and incubated for 24 h with the PARPi compounds PJ34 and MCD113. A panel of M2 markers was utilized for a gene expression characterization via RT-qPCR of the functional state of MΦs [179]. In this panel, Arg1 and Fizz1 were also included, along with the MRC1 (CD206), Fibronectin, CCL17 and CCL18. Supporting the results displayed in **Figure 18C**, our obtained data showed that spheroid co-cultures significantly elevated the M2 markers expression compared to MΦ only spheroid cultures, in the presence of IFN-γ (**Figure 19**). In addition to that, co-cultures showed a significant mRNA level decrease of all the M2 markers, when treated with either the PARPi PJ34 (**Figure 19A**) or the PARP14i MCD113 (**Figure 19B**). Different was the case of CCL18, in which there was no significant reduction in the levels of M2 markers, even though this change was visible (**Figure 19A, B**). Once again, our results highlight PARP enzymes (especially PARP14) as potential key mediators in M2 polarization, and are in line with our data obtained in mice models.

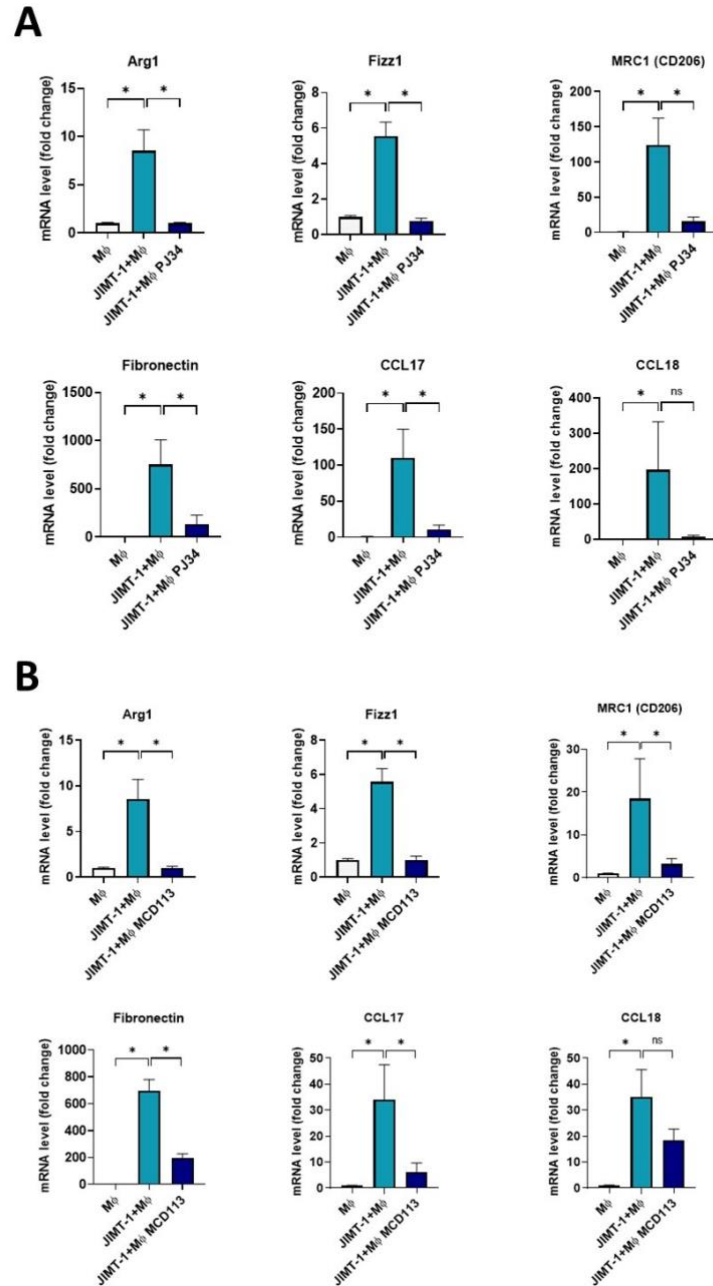


Figure 19. PARPis cause a decrease in M2 markers in JIMT-1- MΦ spheroid co-cultures. JIMT-1 cells and THP-1-derived MΦs were used to grow spheroid co-cultures, and THP-1-derived MΦs were used to grow pure spheroid cultures. On day 3, spheroids were kept in culture for an additional 24 h with 20 ng/ml IFN- γ . They were then treated for 1 day with either PJ34 25 μ M (**A**) or with MCD11355 μ M (**B**). Columns indicate the relative expression of M2 marker mRNA levels in spheroids and co-culture spheroids measured with RT-qPCR. Histograms show mean of 4 independent experiments ($\pm$ SEM). Statistical evaluation was performed with one-way ANOVA and Tukey's post-hoc test (MRC1/CD206 and CCL17 in panel A, Fibronectin in panel A and B), or with with Kruskal-Wallis test and Dunn's post-hoc test (* $p < 0.05$, ns: not significant).

6.1.6. Production of soluble mediators by THP-1 derived MΦs is modulated by PARP enzymes

To understand the underlying mechanisms of JIMT-1 cells in inducing TAM differentiation, the presence and levels of soluble mediators in cell culture supernatants of spheroid cultures were monitored by a human protein array. Collection of the supernatants was performed on day 4 from THP-1-derived MΦs or from THP-1-MΦ-JIMT-1 co-culture spheroids, and the secretion of cytokines was assessed with human Proteome Profiling arrays (**Figure 20A**). Specifically, the mediators were measured with the Human XL Cytokine Array Kit. LCN2, MIF and PAI-1 concentration in the co-cultures were distinctly elevated, in comparison to the spheroids of THP-1 derived MΦs only. There was also a noticeable but slight increase in the levels of CCL2 (**Figure 20B**).

To estimate whether ADP-ribosylation regulates the secretion of these cytokines, the spheroids were then treated with the PARPi PJ34 and the PARP14i MCD113, and RT-qPCR analyses were performed. At the mRNA level, LCN2, MIF and PAI-1 expression in the THP-1-MΦ only spheroids was significantly lower than in the spheroid co-cultures, confirming the data obtained from cytokine arrays (**Figure 20C**). Furthermore, treatments of spheroids with the two ADP-ribosylation inhibitors caused a decrease in the mRNA levels of the mentioned cytokines (**Figure 20C**).

These results suggest the implication of LCN2, MIF and PAI-1 in the polarization of TAMs, and indicate that PARP14 may have a role in regulating their gene expression [184, 185].

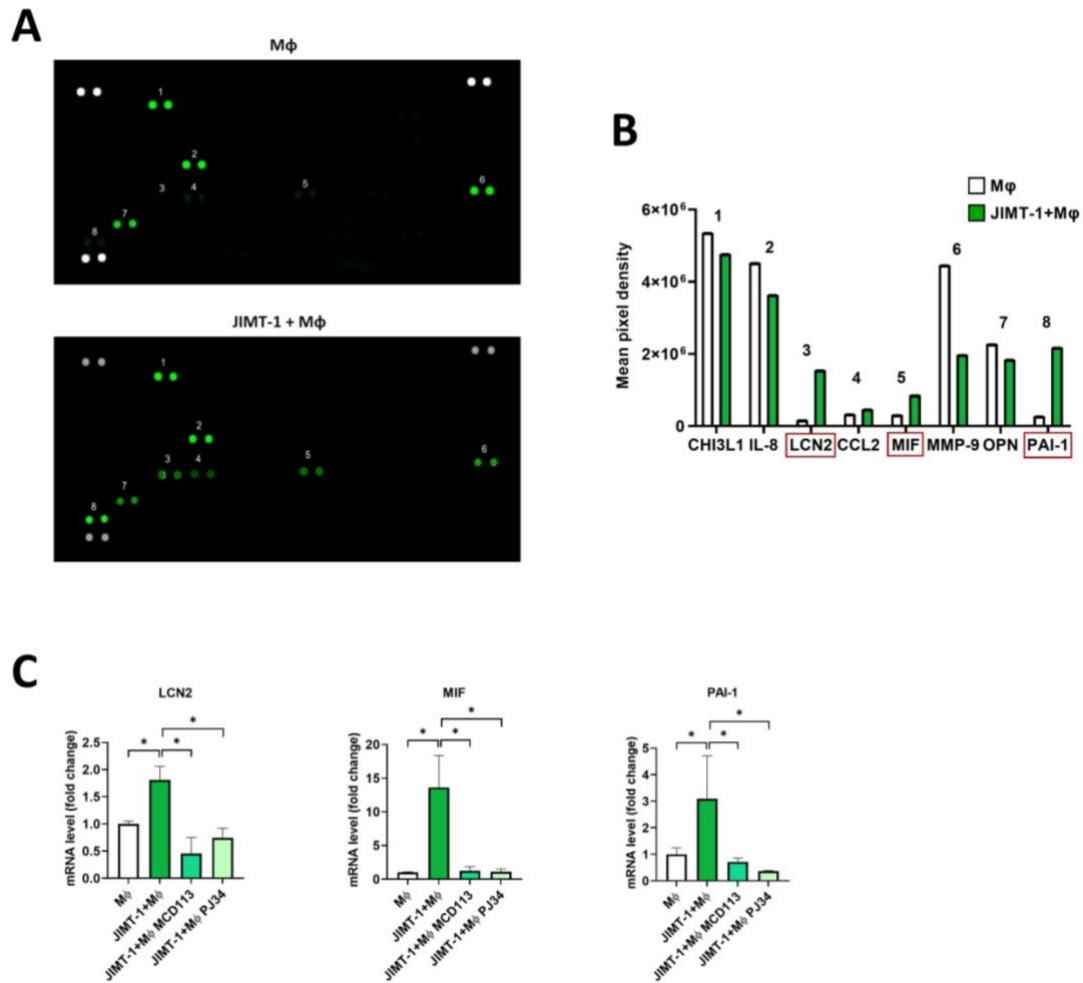


Figure 20. Proteome Profiling Array suggests that LCN2, MIF and PAI-1 cytokines are involved in TAM polarization. THP-1-derived MΦs and JIMT-1 cells were used to generate spheroids or spheroid co-cultures. On day 3 they were kept in culture for 24 h with 20 ng/ml IFN- γ , and on day 4 cell supernatants were collected (500 μ l) and added to each membrane array (A). Each membrane contained 105 different capture antibodies, printed in duplicate. Detection of analytes was performed by proteome profiler as described in the methods section (B). RNA extraction from spheroids was conducted under the same conditions, and the same set of cytokines were quantified at the mRNA level by RT-qPCR. Addition of the PARPi PJ34 (25 μ M) and the PARP14i MCD113 (55 μ M) led to a decrease in the cytokine expression in a significant way (C). The numbers in panels (A) and (B) depict the different cytokines detected. The red rectangles in panel (B) indicate the three cytokines used for RT-qPCR analyses, as shown in panel (C). Graphs represent mean of 3 independent experiments ($\pm$ SEM). Statistical evaluation was performed with one-way ANOVA and Tukey's post-hoc test (* p < 0.05).

6.2. Part II.

6.2.1. High-Content Analysis (HCA) for studying spheroid co-cultures

Next, we aimed at investigating PJ34 and MCD113 effects on the 3D co-culture spheroids in terms of tumor cell death and phenotypical modifications. JIMT-1 cells Expressing Green fluorescent protein (JIMT-1-EGFP) were co-cultured with THP-1-derived MΦs (pre-stained with CTB dye) in order to grow spheroids. Following induction of MΦ polarization with IFN- γ treatments for 1 day, PJ34 or MCD113 were added to the spheroids on day 4 for an additional 24 h. Annexin V-Alexa Fluor 647 was then used to stain the spheroids for 1 h before imaging in order to measure apoptotic cell death. HCA performed on 3D cell cultures with confocal microscope showed that treatments with both PARPi compounds caused cell death in JIMT-1 cells as suggested by the higher fluorescence intensity of Annexin V. Annexin V-positive cells (red) appeared in the outer area of treated spheroids (**Figure 21**). Moreover, a diminished compactness was detected after treatment with PJ34, along with evidence of disintegration in the peripheral area of the spheroids (**Figure 21A**), whilst no visible difference was observed in morphology and size after IFN- γ addition (**Figure 21A, B**). Additionally, spheroid size was found to be significantly increased after co-incubation with both PJ34 and MCD113 in comparison to untreated spheroids (**Figure 22**).

Together, our data indicate that reprogramming of TAMs regulated by ADP-ribosylation might have functional consequences, e.g. inhibition of cancer cell killing mediated by MΦs.

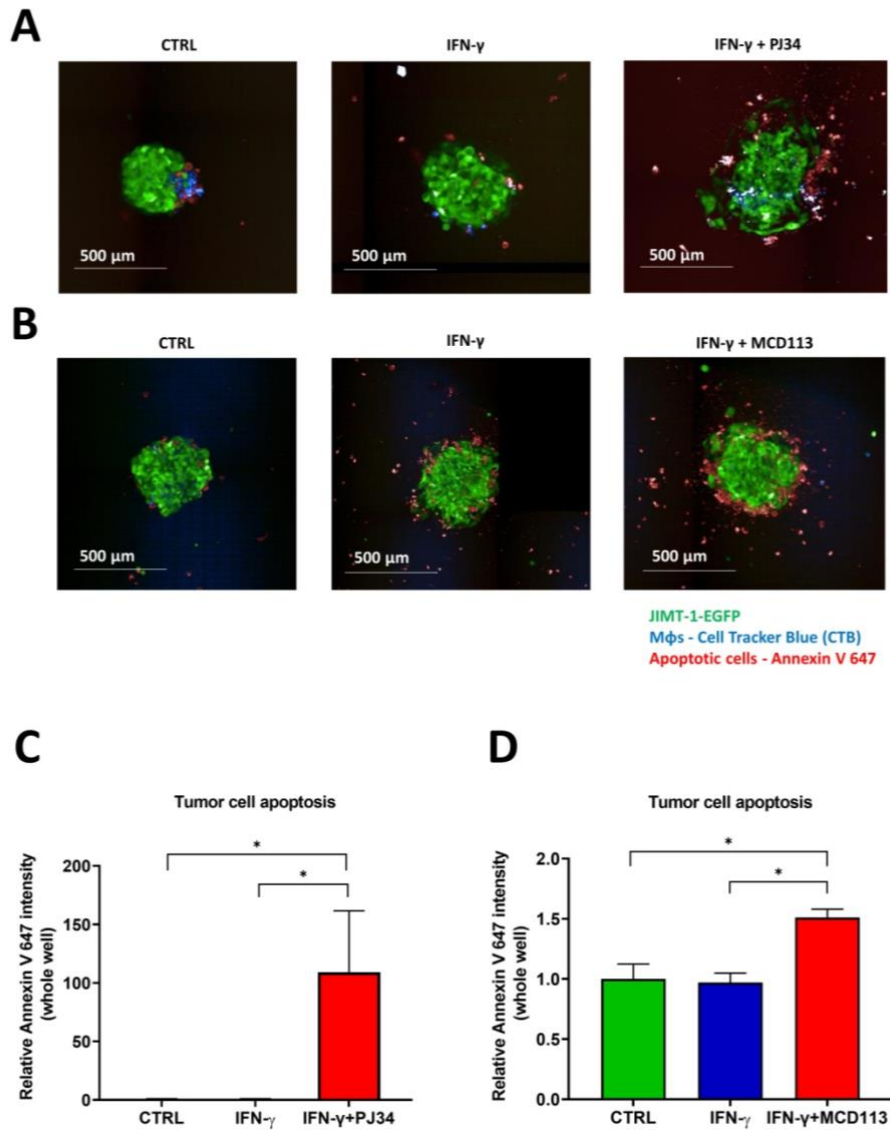


Figure 21. Tumor apoptosis is triggered by PARP inhibitors in co-culture spheroids. JIMT-1-EGFP (green) cells and THP-1-derived M ϕ s (CTB-stained, blue) were used to generate spheroid co-cultures (mixed at 1:1 ratio). On day 4 after addition of 20 ng/ml IFN- γ for 24 h, spheroids were kept in culture for an additional 24 h with PJ34 25 μ M or MCD11355 μ M. Annexin V 647 was used to detect tumor cell death. Images were taken on day 5 after treatments with the PARPis PJ34 (**A**) and MCD113 (**B**), respectively. Images of 3 spheroids/treatment were analyzed in tumor cells/each well for Annexin V-Alexa 647 fluorescence intensity (**C**) and (**D**). Means $\pm$ SEM of 3 independent experiments are depicted in the columns. The statistics were calculated with one-way ANOVA followed by Dunnett's post-hoc test (* $p < 0.05$).

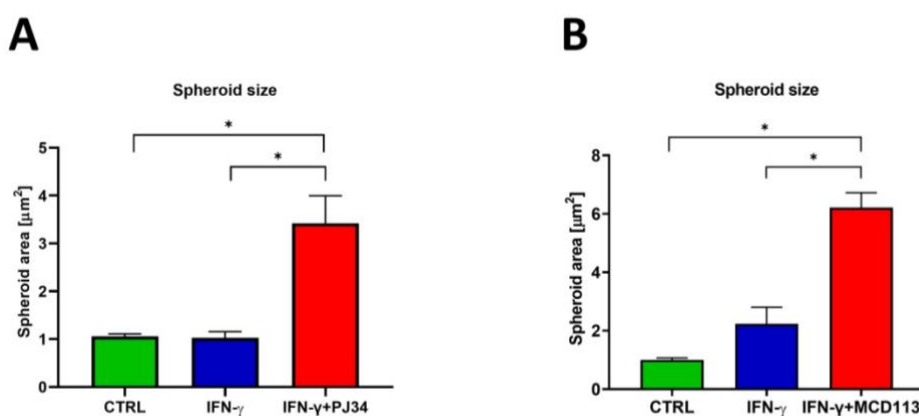


Figure 22. Co-culture spheroids show phenotypic alteration after treatments with PARP inhibitors. JIMT-1-EGFP cells and THP-1 derived M Φ s (CTB-stained) were used to generate co-culture spheroids (mixed at 1:1 ratio). On day 3, spheroids were kept in cultures for 24 h in the presence of 20 ng/ml IFN- γ , and on day 4, they were co-incubated with PJ34 (25 μM) or with MCD113 (55 μM) for additional 24 h. Images of 3 spheroids/treatment were taken and analyzed for area following PJ34 (**A**) and MCD113 (**B**) treatments, respectively. Means $\pm$ SEM of 3 independent experiments are shown in the columns. The statistics were calculated with one-way ANOVA followed by Dunnett's post-hoc test (panel A) and with Kruskal-Wallis and Dunn's post-hoc test (panel B) (* $p < 0.05$).

6.2.2. HCA on a 3D model of ADCC for the identification of effector compounds

Spheroids were generated using JIMT-1-EGFP cells as described in section 5.8., and were allowed to clump for 3 days. Sunitinib (20 μM) was then added to the 3D cultures as a test compound, since it was previously demonstrated that it can influence ADCC [10]. Induction of ADCC was initiated by adding NK cells (20:1) to the spheroids, along with the anti-HER2 antibody Trastuzumab (10 $\mu\text{g/ml}$) for 1 day in the absence or presence of Sunitinib. For the optimization of assay conditions (E:T ratio and the concentration of antibody), we performed preliminary experiments (**Figure 23**), relying on our previous publication [10]. We optimized the protocol with an E:T ratio of 20:1 for the 3D model, from a 2:1 ratio for the 2D assay [10], since it is possible that drugs mostly influence outer layers of spheroids and are unable to penetrate in their core. Changes in the concentrations of Trastuzumab, however, did not change the action of ADCC (**Figure 23**).

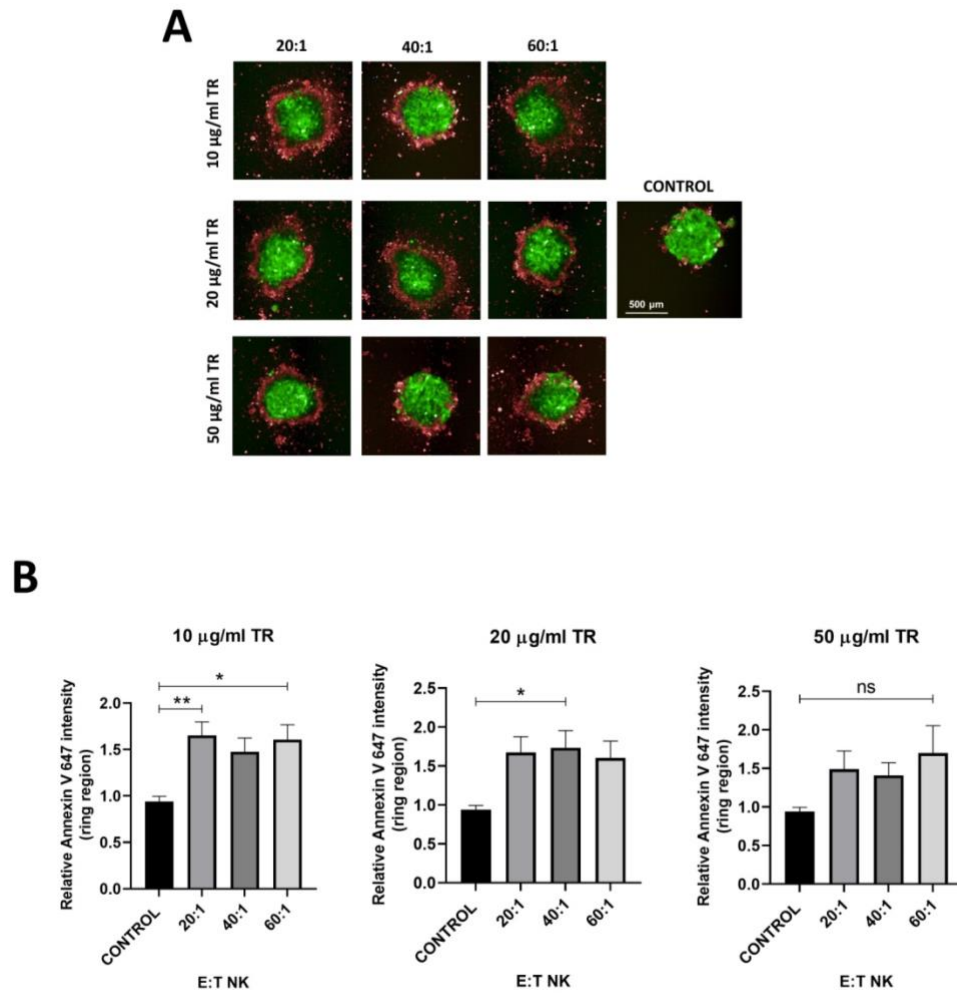


Figure 23. Optimization of the E:T ratio and Trastuzumab concentration in the 3D ADCC spheroid model. Spheroids were generated with JIMT-1-EGFP cells ($1 \times 10^6/\text{ml}$). The spheroids were transferred to a HCS plate on day 3, ADCC was induced by adding NK cells at different E:T ratios (20:1, 40:1, 60:1), along with Trastuzumab (TR) 10, 20 and 50 $\mu\text{g}/\text{ml}$. JIMT-1 growth media was added to the control (JIMT-1-EGFP). After 1 day, Annexin V-647 (red) was used to stain the spheroids for 1 h in order to measure apoptotic cell death (A). Histograms show means of 3 independent experiments ($\pm\text{SEM}$). The columns represent Annexin V-647 intensity around JIMT-1 spheroids (B). Images of 3 spheroids/condition were taken analyzed for the fluorescence intensity of Annexin V-647 in the ring region. Data were analyzed with one-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, ns: not significant).

As additional controls (CTRLs), JIMT-1 spheroids and JIMT-1 cells co-incubated with NK cells (20:1) were utilized. After incubation with drugs and immune cells, Annexin V-647 was used for staining the spheroids for 1 h in order to assess cell apoptosis. Results obtained from imaging with confocal microscope indicated that in the presence of Trastuzumab, NK cells triggered the death of JIMT-1 cells, as suggested by Annexin V staining. As shown in **Figure**

24A, apoptotic cells were found in the peripheral area of the spheroids (apoptotic “ring”), while NK cells alone did not affect tumor cell viability (no apoptotic changes were detected).

To investigate the biological effect of Trastuzumab and to explore its action in ADCC, TR-F(ab')₂ lacking functional FcR binding capability was used as a negative CTRL of Trastuzumab in this study (6.6 µg/ml) [177]. As TR-F(ab')₂ was ineffective (**Figure 24B**), the effect is likely to be mediated via ADCC. In addition, Annexin V staining in the spheroids was reduced by pre-treatment with Sunitinib, unveiling the inhibition of apoptosis in JIMT-1 cancer spheroids co-incubated with Trastuzumab and NK cells and validating the resistance of Sunitinib-induced ADCC.

In summary, the present High-Content Screening assay could be ideal for the discovery of ADCC effector compounds.

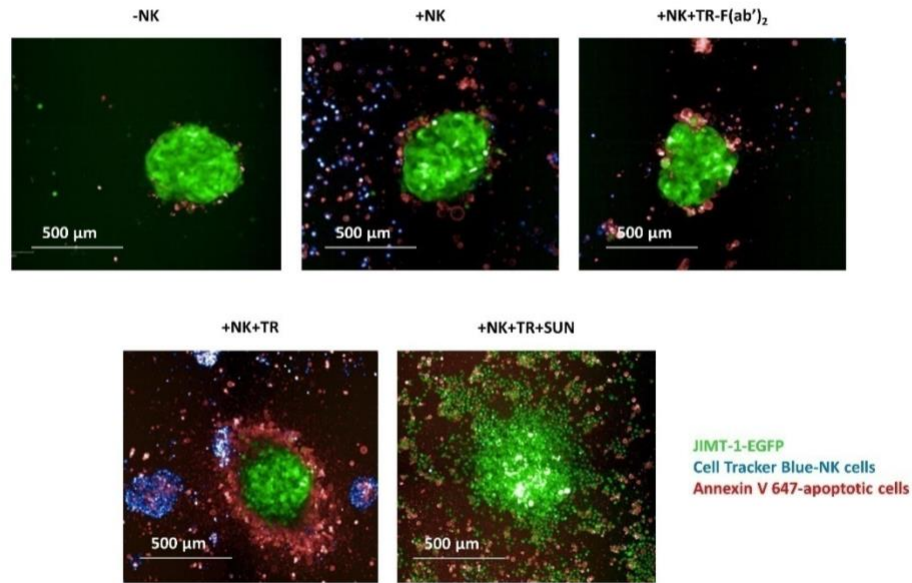
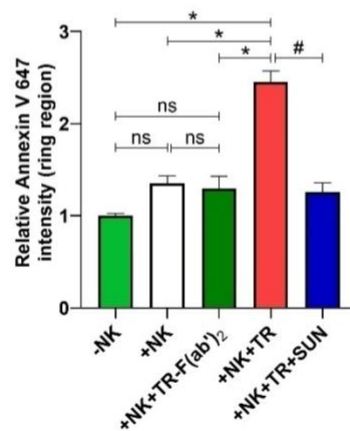
A**B**

Figure 24. Discovering ADCC effector compounds with HCA in a 3D spheroid model. JIMT-1-EGFP cells were used to grow tumor spheroids. On day 3, spheroids were transferred to a pre-coated HCS plate (Pluronic F-127 in DMSO) to prevent cell attachment. ADCC was induced on day 4 by addition of 10 $\mu\text{g/ml}$ Trastuzumab (TR) (or equimolar 6.6 $\mu\text{g/ml}$ TR-F(ab')₂ as negative CTRL) and NK92.CD16 cells (pre-stained with Cell Tracker Blue, 20:1), in the absence or the presence of 20 μM Sunitinib (SUN). JIMT-1 spheroids and JIMT-1 + NK (20:1) spheroids were used as additional CTRLs. Annexin V-647 was added after 24 h to stain the spheroids (1 h) in order to stain apoptotic cells red (A). Images of 3 spheroids/condition were taken and analyzed for Annexin V-647 intensity in the outer area of the spheroids (ring region) (B). Histogram shows 4 independent experiments (means $\pm$ SEM). Data were analyzed using Kruskal-Wallis test followed by Dunn's post-hoc test (* $p < 0.05$, # $p < 0.05$, ns: not significant).

7. **DISCUSSION**

The TME significantly influences both tumor progression and tumor responsiveness to therapeutic interventions [66]. MΦs stand out as the most prevalent cell type in numerous cancers, and their presence in the tumor microenvironment is frequently associated with a negative prognosis. Studies conducted on human BC have also revealed a connection between TAMs and adverse prognosis for tumors [168]. There is a relatively limited understanding concerning the mechanisms by which cancer cells reprogram TAMs into a tumor promoting M2-like phenotype. Additionally, the processes underlying the suppression of anti-tumor inflammatory signaling pathways in TAMs remain inadequately understood. Members of the PARP enzyme family have recently been identified as novel contributors to the regulation of MΦ polarization [167]. However, their specific impact on shaping the phenotype of TAMs remains unclear. For this reason, our objective was to explore the influence of ADP-ribosylation on MΦ polarization towards the TAM phenotype.

Initially, we examined the impact of ADP-ribosylation inhibitors PJ34 and MCD113 on the MΦ polarization towards M2 induced by IL-4. Consistent with existing literature [186, 187], our results verified the role of PARP enzymes in this phenomenon. It is noteworthy that MCD113 was specifically designed as a PARP14i [170], whereas PJ34 primarily inhibits PARP1 and PARP2, although exhibiting a weaker inhibitory effect on PARP14 [181]. Consequently, our pharmacological experiments indicated the potential involvement of PARP14 in the process of M2 polarization. Hence, we proceeded to demonstrate that MΦs lacking PARP14 (PARP14^{-/-}) exhibited diminished IL-4-induced expression of all tested M2 markers. This observation suggests that the mediation of IL-4-induced polarization toward the M2 phenotype is attributed to PARP14. Similar results were observed in human MΦs. In fact, in our human model, six M2 markers were used, all of which exhibited a comparable pattern of alterations-specifically, induction by IL-4 and suppression by both PARylation inhibitors.

The mentioned findings aligned with prior reports [8, 58]. However, the role of PARylation in TAMs remains less comprehensively elucidated. Consequently, we proceeded to establish a tumor cell-induced polarization of TAMs using 4T1 mouse breast carcinoma cells and murine BMDMs. The transfer of conditioned media from the tumor cells effectively led to MΦ reprogramming toward a TAM phenotype reminiscent of polarization to M2. Given that both PARPis and the PARP14 KO phenotype markedly diminished the expression levels of M2 markers, we deduced that PARP14 plays a pivotal role in the development of the TAM phenotype. Analogous results were detected in 3D human spheroids generated through the co-culture of JIMT-1 breast carcinoma cells and THP-1 derived MΦs. Within these co-culture

spheroids, the upregulation of M2 markers was evident, accompanied by a reduction in the expression of M1 markers, implying a shift from an M1 to an M2 phenotype. In JIMT-1-THP-1 co-culture spheroids, both PARPi effectively impeded the induction of M2 marker genes. Additionally, we also showed (**Figure 21**) that PARP inhibition resulted in the functional reprogramming of MΦs, as evidenced by an elevated level of apoptosis in JIMT-1 cells co-cultured with THP-1-derived MΦs.

Previous studies have proposed various tumor-derived metabolites, including lactate, as well as cytokines and chemokines [188-191] to be potential inducers of TAM polarization. In our study we employed a membrane-based human proteome profiler assay to assess alterations in the expression levels of over a hundred human cytokines, chemokines, growth factors, and other soluble proteins in MΦ-tumor cell co-cultures, comparing them to pure MΦ cultures. Three proteins were discerned with elevated expression levels in the co-cultures of JIMT-1 cells and MΦs, and subsequent reduction in the expression following pre-treatments with PARPi compounds (**Figure 20**). The MΦ proteins revealing PARylation-dependent upregulation in the co-cultures included lipocalin-2 (LCN2), macrophage migration inhibitory factor (MIF), and plasminogen activator inhibitor-1 (PAI-1).

LCN2 is a multifunctional protein primarily recognized for its role as a siderophore, facilitating the binding and transportation of iron. In addition, it serves as a carrier for several hydrophobic metabolites, such as prostaglandins, steroids, and certain hormones. Given the dysregulated iron homeostasis and the elevated demand for iron observed in cancer, which is considered an official hallmark of the disease [192-194], it is reasonable to propose that the upregulation of LCN2 induced by JIMT-1 cells in MΦs may play a role in the polarization of TAMs. In fact, several pieces of evidence in the literature support the notion that elevated levels of LCN2 are related to poor prognosis and therapy resistance in BC [195-197]. Furthermore, LCN2 is recognized for its association with MMP-9, impeding the degradation of this metalloproteinase, which plays a crucial role in the degradation of ECM and thereby enhances cancer metastasis [198]. However, it appears unlikely that this latter scenario is significant in our model, as the upregulation of LCN2 was accompanied by the downregulation of MMP-9 (**Figure 20B**). The mechanisms through which PARPi compounds suppress the expression of LCN2 necessitate further investigation. Indeed, it is plausible that both inhibitors exerted their effects through the inhibition of PARP14. However, considering the involvement of the NFκB pathway in the regulation of the expression of LCN2 and the co-activator role of PARP1 in NFκB signaling, the effect of PJ34 could also be attributed to the inhibition of NFκB signaling.

MIF also surfaced as another possible mediator of the PARylation-dependent TAM polarization. MIF was also induced in MΦs by JIMT-1 cells, in a similar way to LCN2, and both PARPi compounds attenuated this induction. MIF1 has previously been associated with the polarization of MΦs toward an M2 phenotype, as evidenced in studies involving MΦs from glioblastoma, cisplatin-resistant lung cancer, and oral squamous cell carcinomas [199-201]. Additionally, cytoplasmic MIF has been suggested to hold prognostic values in patients with squamous cell carcinoma [202]. Given these findings, it is conceivable that the inhibition of MIF expression by the PARPis may have a hand in the reversal of the TAM phenotype in BC.

The serine protease inhibitor PAI-1 is implicated in recruiting MΦs to tumor sites [203]. Moreover, it serves as a significant MΦ-induced mediator of cancer progression [204]. Studies have demonstrated the involvement of PAI-1 in MΦ polarization towards M2 in various tumor-related contexts [204-206]. Hence, the PARylation-dependent induction of PAI-1 is expected to contribute to TAM polarization, thus presenting a potential therapeutic target in the treatment of BC.

Considering the outlined observations, we believe that further investigation into the role of PARylation in regulating the expression of LCN2, MIF and PAI-1 is warranted, as it could potentially contribute to the anticancer action of PARylation across several cancer types characterized by high levels of infiltrating MΦs.

An important aspect to consider is the contribution of various PARP family members to the development of TAM phenotype. Our present findings emphasize the significance of PARP14 not only in IL-4-induced polarization to M2, but also in cancer cell-induced TAM polarization (**Figure 25**).

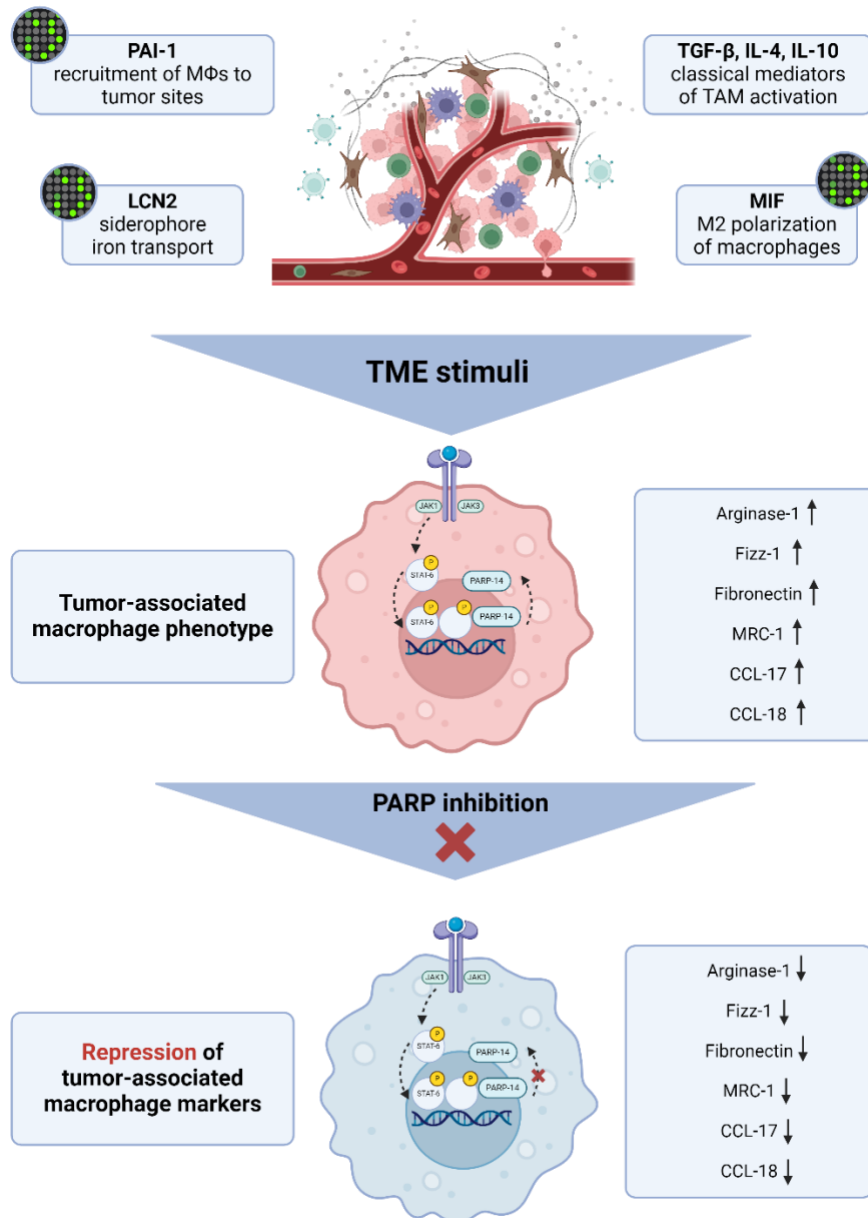


Figure 25. Mechanism of PARP14 activation and function in TAM polarization. In response to stimulation by classical TAM inducers (e.g. TGF- β , IL-4 and IL-10) or TME-derived stimuli (e.g. PAI-1, LCN2, MIF and tumor-conditioned media), PARP14 is believed to become enzymatically active, thereby activating target genes that foster tumor progression and inhibition of inflammation. In vitro studies suggest that this mechanism is likely to be mediated via the dissociation of PARP14 from the promoter(s) and the binding of phosphorylated STAT6 [132]. On the opposite, inhibition of PARP14 does not activate STAT6 pathway, thus promoting repression of TAMs markers and pro-inflammatory macrophage activation (the figure was created using the Biorender software; Biorender.com).

Previous reports have indicated that targeting PARP14 can induce a shift in M Φ s from an M2 to an M1 phenotype [8, 207]. Additionally, the potential involvement of PARP1 in the polarization of TAMs cannot be precluded. This scenario finds support in studies indicating that Olaparib, a clinically used specific PARP1/2 inhibitor, effectively inhibited tumor growth.

Notably, this effect was nullified when MΦs were depleted [208]. Nevertheless, findings from Wang *et al.* indicated that Olaparib-induced MΦ reprogramming occurs even in the absence of PARP1 or PARP2 [180]. Consequently, it appears reasonable to hypothesize that both PARP14 and PARP1/2 may contribute to TAM polarization, depending on the particular model and context.

An intriguing implication of our findings is that inhibiting the development of the TAM phenotype, for instance through the inhibition of PARP14, could potentially enhance the efficacy of treatments with PARPi in BRCA mutant breast cancer. TAMs have been documented to play a role in conferring resistance to PARPi compounds [58], and strategies involving the reprogramming of TAMs, such as through STING agonism, have proven effective in restoring PARPi sensitivity in breast cancer [101]. This suggests that PARPi targeting both DNA damage-responsive PARPs (PARP1 and PARP2) and PARP14 may have an enhanced anticancer effect in BC.

Taken together, our data support a scenario wherein signals derived from breast cancer cells induce the polarization of MΦs towards a TAM phenotype. Our findings underline the effectiveness of PARPi in downregulating protumor genes, emphasizing the significance of a deeper understanding of the mechanisms underlying the transition of TAMs from an M2 to an M1 phenotype as a potentially efficacious treatment strategy for cancers [209]. PARylation is deemed essential for the development of the TAM phenotype, with PARP14 being identified as a key player in this process [8]. Our results indicate that PARP14 targeting in BC holds therapeutic potential by reprogramming tumor-promoting MΦs towards an anti-cancer phenotype. Further investigation is needed to determine whether PARP14 plays similar roles in other types of cancer and whether inhibition of PARP14 significantly reverses the TAM phenotype, thereby unleashing anti-tumor effector mechanisms of the adaptive immune system. Recently, 3D tumor culture systems e.g. spheroids and tumor organoids, have emerged as cost-effective and readily implementable models which closely mimic key aspects of tumor features, surpassing traditional monolayer cultures in representation [210]. The spatially coordinated interaction between cancer cells and different tumor-associated immune cells represents, for instance, one distinguishing feature. This characteristic renders 3D co-culture models superior tools over 2D assays for drug screening, in particular for the identification of modulators targeting immune cells to treat malignancies [211]. As one of the main immune component of the TME, TAMs wield significant influence over the initiation and progression of cancer [212]. Moreover, the substantial morbidity and mortality associated with BC despite substantial advancements in treatment underscore the ongoing need for exploration into the underlying

molecular mechanisms, as well as the establishment of robust screening platforms aimed at recognizing novel molecules suitable for therapeutic intervention [213, 214]. To this end, we aimed at developing advanced imaging and analysis methods capable of high-throughput and high-content screening, specifically tailored for examining phenotypic alterations within human cancer spheroids and for enabling real-time tracking of compound treatments. Therefore, we created a 3D co-culture model comprising JIMT-1 human breast carcinoma cells and THP-1-derived MΦs to replicate the intricate interactions between immune cells and cancer cells.

The use of JIMT-1-EGFP cells [10] and CTB-staining for MΦs allowed us to monitor the size of spheroids and to observe whether MΦs were infiltrating them. We then further investigated the effects of PARPis on phenotypic changes and on tumor apoptosis. Our data demonstrated that inhibition of PARP resulted in increased apoptosis levels in JIMT-1 cells co-cultured with THP-1-derived MΦs (**Figure 21**). This is in line with previous studies which have shown that PARPis (including PJ34) impede proliferation of tumor cells and induce apoptosis in solid tumors, including BC, in both murine and human models [215-217]. In line with the literature [218, 219], we also detected an increment in the spheroids' size with signs of disintegration in the outer region upon treatment with PARPi compounds. Imaging was optimized by employing a maximum projection algorithm, which consolidates cellular data from numerous slices across a 3D object into a single image. This approach facilitated a streamlined comparison of diverse spheroid phenotypes efficiently. Overall, considering the varied responses of drugs across different cell lines and their potential cell line-specific effects [11], the co-culture spheroid models we developed show potential in providing valuable insights for researchers to better understand breast cancer pathophysiology and assess novel therapies more effectively.

Immunotherapy has emerged as a promising approach to treating several forms of cancer. Use of immune cells, such as NK cells, along with small molecule drugs and antibodies through ADCC has been investigated as a potential combination therapy for some difficult to treat solid tumors [26, 220]. Nevertheless, there remains a need to develop tools that support co-culture of target cancer cells and effector immune cells in a contextually relevant 3D environment to provide a rapid means to screen for and optimize ADCC-drug combinations.

In light of this evidence, we went on and extended our method to encompass a novel 3D model with the aim to investigate NK-cell-mediated Trastuzumab-dependent ADCC against JIMT-1 breast carcinoma cells. Additionally, we outlined an automated imaging and analysis workflow for assessing the killing of cancer cells mediated by NK cells. The methodology was executed using a high-content imager and an image analysis software, enabling the surveillance of NK cell-mediated cancer cell apoptosis, which is relevant to disease progression. Previously, the

multitargeted tyrosine kinase inhibitor Sunitinib was identified, in 2D cultures of JIMT-1 cells, as a compound that induces resistance to Trastuzumab-dependent ADCC. Specifically, Sunitinib was found to disrupt the cytotoxic function of NK cells, to trigger autophagy, to reduce HER2 expression, and to promote the adhesion of JIMT-1 cells [10]. To confirm our analysis workflow, Sunitinib was tested as a model compound. We demonstrated that it impedes the action of NK cells in eliminating cancer cell spheroids, replicating the effect observed in monolayer cultures.

The approach we used in our study is straightforward, and it consists of culturing spheroids in 96-well culture plates pre-coated with agarose. These spheroids are then transferred in a single-step onto a glass-bottom microplate specifically designed for HCS and HCA purposes. Test compounds are then introduced together with separately stained NK cells, which are activated by Trastuzumab administration to induce cytotoxicity. Following this, a one-time staining process with vital dyes to detect cell death is performed, followed by microscopic analysis. Importantly, the wells were not significantly altered once the co-culturing of the two cell types begins and the drug treatment is initiated, ensuring the integrity of the spheroids. The assay can be completed within a timeframe of just 4 days. The coating of the bottoms of 96-well tissue culture plates with 1% low-melting point agarose serves as a cost-effective substitute for utilizing ultra-low attachment plates. This step effectively prevents the adherence of JIMT-1 cells to the surface of the plates, and facilitates the natural formation of tumor cells spheroids through spontaneous organization. Due to the limitation posed by the agarose cushion and the U-shaped plastic surface, capturing images directly is not feasible. Consequently, the spheroids must be carefully transferred into the imaging plate for image capture. An essential aspect of the process prior to the spheroid transfer was the addition of the bio-inert surfactant, Pluronic-F127, to the wells of the HCS plate. This crucial step prevented spheroids from dispersing and spreading out on the bottom of the wells, thereby facilitating both imaging and analysis.

As described above, the spheroids were formed using a human tumor cell line engineered to stably express EGFP [10]. This genetically encoded marker provided continued fluorescence throughout the protocol, eliminating the need for cell stains. Unsupervised texture analysis of EGFP fluorescence serves as the foundation for identifying the compact core of the spheroids, encompassing the entire structure without inducing cell death. However, upon the addition of NK cells and the induction of ADCC, the pronounced disintegration of the contours occasionally presented a challenge for the image analysis algorithm in delineating the spheroid's outline. As the presence of Annexin V-positive cells primarily located in the peripheral area of the spheroids, this issue was overcome by the adjustment of cell death

evaluation. The latter was in fact assessed within a peripheral annular region created by extending the well-defined spheroid core using the software's built-in image segmentation module. By visually curating hundreds of spheroid images, the expansion factor determining the outer boundary of this region was attentively selected to encompass the penumbra of dying, shredded, and dispersed cells consistently across all cases. Assessing the total fluorescence of the apoptotic ring area in the maximal projections of Z-stacks enabled us to reduce experimental errors. We carefully selected fluorescent labels for marking the BC cells, using EGFP, and for detecting apoptotic cell death, using Alexa 647, thereby eliminating the necessity for channel separation. We chose an end-point readout relying on fluorescent labeling for exposed phosphatidylserine, an apoptotic marker. This method was preferred over monitoring the decline in EGFP fluorescence intensity in order to assess the viability of spheroids over time, although this could represent an alternative. The Annexin staining method facilitated a more precise identification of cell death. Theoretically, enhancing the existing one-dimensional staining approach could involve supplementing it with additional and more specific cell death markers capable of discerning the modality of cell death (such as fluorescent caspase substrates), and identifying the type of cells that have perished.

With minimal modifications, this method can be adapted to investigate other types of effector immune cells such as macrophages (as we showed), T cells and chimeric antigen receptor (CAR)-expressing immune cells, as well as activator molecules like interleukins and cytokines. With a focus on efficiency, robustness and simplicity, we have intentionally not fully explored the potential of HCA in this assay. The imaging and analysis protocols can easily accommodate the incorporation of additional fluorescent labels, offering flexibility for further experimentation and enhancement. The versatility of this assay extends to the investigation of more complex spheroids by introducing various cell types and employing multiplexed fluorescent probes. These enable to differentiate the distinct cell types, while specific fluorescent markers can be utilized to correlate them with ongoing cellular events [146, 154]. The protocol outlined is adaptable to any HC imager, and the resulting images can be analyzed using any image analysis software that includes a texture analysis module. This flexibility allows for seamless integration with different imaging platforms and analysis tools, enhancing the accessibility and applicability of the method.

As the significance of this protocol lies in spheroid analysis, several parameters must be taken into account. It is well-established that 3D cell cultures exhibit a diminished response to compounds compared to those grown in monolayers [164, 166]. This disparity emphasizes the importance of carefully considering and adjusting experimental conditions when working with

spheroid cultures. In reality, solely relying on 2D cell cultures does not provide an accurate prediction of the effective concentration range of a test agent with the entire organism. In developing our 3D models, we examined higher compound concentrations (of PARPi in the JIMT-1-EGFP-THP-1-derived M Φ co-cultures) and E:T ratios (NK:JIMT-1-EGFP co-cultures) than those typically reported in 2D cultures. This approach accounts for the inherent differences between 2D and 3D environments, ensuring a more comprehensive assessment of compound efficacy. As part of the optimization process, we refined the protocol by enhancing the E:T ratio. Specifically, we increased it from 2:1, as employed in our 2D ADCC assay [10], to 20:1 for the cancer spheroids. One possible explanation for this is that the drugs may have been unable to effectively penetrate the core of the spheroids, primarily impacting the cells in the outer layer. Conversely, despite increasing Trastuzumab concentration, there was no significant augmentation observed in the ADCC response (**Figure 23**). This choice could potentially vary depending on the specific cell lines and targets involved. Therefore, it would be valuable to evaluate this protocol using other cell lines, such as a non-HER2 breast cancer cell line. It is important to consider here that the level of HER2 expression plays a crucial role in determining the ADCC response [165].

In conclusion, our novel method presents a rapid and reliable platform for analyzing the impact of compounds within a 3D immune-cancer model, offering the potential for high-throughput drug screening applications. This highlights the importance of utilizing HER2+ BC spheroids in conjunction with M Φ s or NK92.CD16 cells to further enhance our understanding of the molecular mechanisms underlying the action of PARPi compounds and Trastuzumab (**Figure 26**).

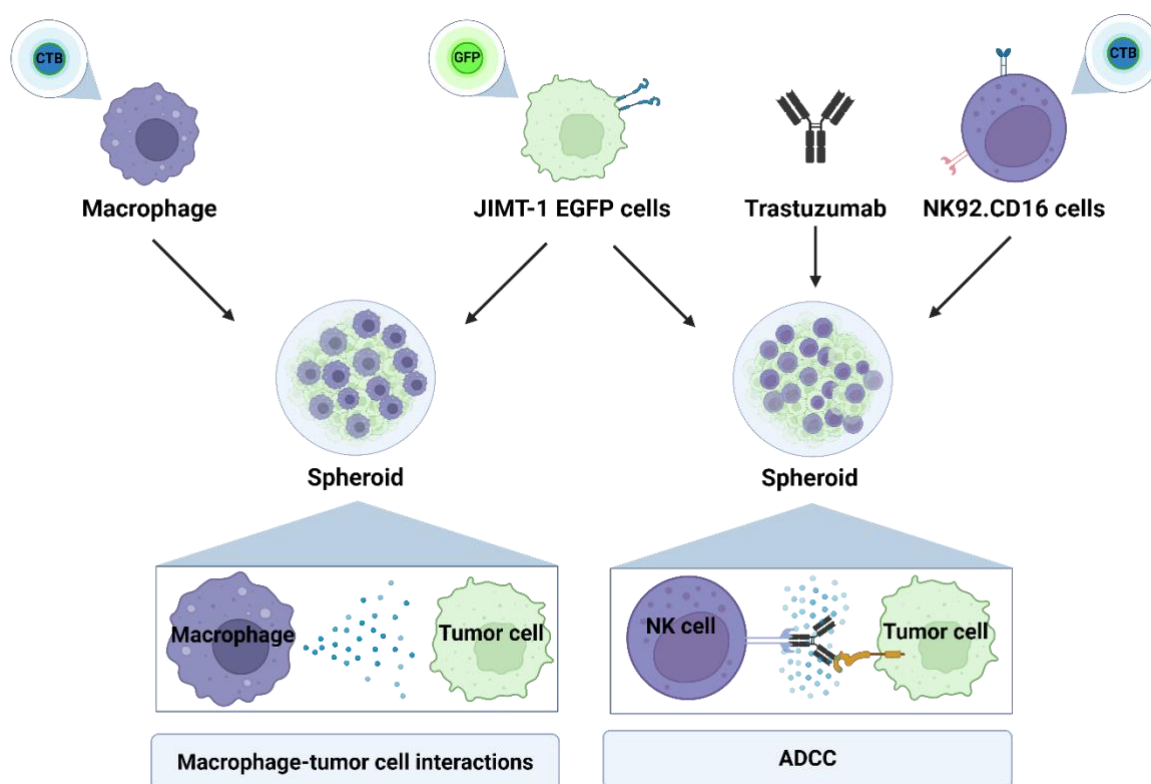


Figure 26. Setting up a tumor 3D spheroid model to investigate the interactions between immune cells and cancer cells. Our experimental setup utilizes a 3D spheroid system, which offers a more faithful representation of the *in vivo* microenvironment compared to traditional 2D models. JIMT-1-EGFP breast cancer cells were seeded onto a concave cell-repellent surface to facilitate the formation of spherical cellular clusters, known as spheroids. To study the interactions of tumor cells with MΦs, JIMT-1-EGFP cells (green) and THP-1-differentiated MΦs (blue) were mixed at a 1:1 ratio. Spheroids were treated with PARPi compounds for 24 h. On the other hand, to detect ADCC effector compounds, NK92.CD16 natural killer cells (blue) were introduced to the JIMT-1-EGFP cells (green) to induce ADCC at a ratio of 20:1 along with the anti-HER2 monoclonal antibody Trastuzumab. Cells were stained with Annexin V-647 to detect apoptosis. This experimental model has demonstrated effectiveness and ease of use in our study (the figure was created using the Biorender software; Biorender.com).

This might present an opportunity to predict the therapeutic potential and efficacy of PARP inhibition and Trastuzumab-dependent ADCC *in vivo* [221]. The pursuit of novel anti-tumor immune modulators stands to gain significant benefits from this assay. Moreover, the method holds promise for expansion into personalized chemosensitivity testing using patient-derived tumor cell cultures. This application could facilitate the approach to cancer treatment by offering tailored therapeutic strategies based on individual patient responses. Our findings indicate that the conventional 2D co-cultures typically employed in immune cell killing and ADCC tests can be successfully adapted into 3D spheroid assays, likely increasing their translational relevance in biomedical research.

8. SUMMARY

Breast cancer (BC) is the most prevalent malignancy in women, often treated with personalized approaches considering tumor marker profiles e.g. hormone receptor (HR) expression and HER-2 overexpression. Hormone therapy and monoclonal antibody therapy are common personalized treatments that may help in prognosis improvement. Tumor stroma cells, including macrophages (MΦs) and Natural Killer (NK) cells, significantly influence tumor behavior and responses to treatment. Tumor-associated MΦs (TAMs), resembling M2 cells, promote tumor growth within the breast tumor microenvironment, establishing TAMs as promising BC treatment targets.

ADP-ribosylation, mediated by PARP enzymes, regulates various cellular functions, including tumor promotion. PARP14, implicated in chronic inflammation, has not been characterized in detail regarding its roles in TAM polarization and in tumor immune evasion. Our study investigated the role of PARP enzymes in TAM polarization. We found that inhibiting PARP14 with the wide-spectrum PARPi PJ34 and the specific PARP14 inhibitor MCD113 could reduce M2like polarization of TAMs, suggesting PARP14 inhibition as a potential cancer treatment strategy for BC.

Monoclonal antibody immunotherapy, crucial in BC treatment, operates via antibody-dependent cellular cytotoxicity (ADCC), where antibodies activate immune cells like NK cells to kill tumor cells. It is essential to identify compounds that can enhance ADCC efficacy and address potential drug interactions in cancer patients. In our second project, we developed a cancer ADCC spheroid model suitable for drugs screening. Using HER2+ BC cell and NK cell co-cultures in 3D spheroids, we demonstrated the utility of this platform for high-throughput screening, verifying the multitargeted receptor tyrosine kinase inhibitor Sunitinib as a significant Trastuzumab-dependent ADCC inhibitor. This approach offers a versatile platform for screening compounds in cancer cell spheroids to identify ADCC activator substances.

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List of publications related to the dissertation

1. **Sturniolo, I.**, Váróczy, C., Regdon, Z., Türk-Mázló, A., Muzsai, S., Bácsi, A., Intili, G., Hegedűs, C., Boothby, M. R., Holechek, J., Ferraris, D., Schöler, H., Virág, L.: PARP14 contributes to the development of the tumor-associated macrophage phenotype.
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IF: 5.6 (2022)
2. **Sturniolo, I.**, Váróczy, C., Bede, Á. M., Hegedűs, C., Demény, M. Á., Virág, L.: Quantifying antibody-dependent cellular cytotoxicity in a tumor spheroid model: application for drug discovery.
J. Vis. Exp. [Epub ahead of print], 2024.
DOI: <http://dx.doi.org/10.3791/65922>
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List of other publications

3. Gutí, E., Regdon, Z., **Sturniolo, I.**, Kiss, A., Kovács, K., Demény, M. Á., Szőör, Á., Vereb, G., Szöllősi, J., Hegedűs, C., Polgár, Z., Virág, L.: The multitargeted receptor tyrosine kinase inhibitor sunitinib induces resistance of HER2 positive breast cancer cells to trastuzumab-mediated ADCC.
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Front Oncol. 9, 1-14, 2019.
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11. KEYWORDS

Macrophage, ADP-ribosylation, breast cancer, Poly(ADP-ribose)polymerase, PARP14, PARP inhibitors, MIF, PAI-1, LCN2, spheroids, tumor-associated macrophage, macrophage polarization, natural killer cells, ADCC, immunotherapy.

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APPENDIX

The thesis is based on the following publications:

Isotta Sturniolo, Csongor Váróczy, Zsolt Regdon, Anett Mázló, Szabolcs Muzsai, Attila Bácsi, Giorgia Intili, Csaba Hegedüs, Mark R. Boothby, Jacob Holechek, Dana Ferraris, Herwig Schuler and László Virág. *PARP14 Contributes to the Development of the Tumor-Associated Macrophage Phenotype*. International Journal of Molecular Sciences, 2024, DOI: 10.3390/ijms25073601

Isotta Sturniolo, Csongor Váróczy, Ákos Máté Bede, Csaba Hegedüs, Máté Á. Demény and László Virág. *Quantifying Antibody-Dependent Cellular Cytotoxicity in a Tumor Spheroid Model: Application for Drug Discovery*. JoVE, 2024, DOI: 10.3791/65922