

**SHORT THESIS 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

DEBRECEN, 2024

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

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The PhD Defense takes place at Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

at 13:00, 19th of September 2024

1. 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. Tumor stroma cells are known to significantly influence tumor behavior and response to treatments. 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. Tumor-associated MΦs (TAMs) often display characteristics similar to M2 cells, thereby promoting tumor growth and invasiveness. Within the breast tumor microenvironment, TAMs have emerged as the predominant immune cell components, exerting pivotal influence over BC tumorigenesis and progression. Therefore, their involvement in BC development has recently established TAMs as novel and promising targets for BC treatment.

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. Specifically, PARP14 is gaining attention as a potential target for chronic inflammation and has been implicated in tumor promotion, suggesting that its inhibition might be a promising strategy for cancer treatment. However, its precise roles in tumor immune evasion and involvement in TAM polarization still lacks comprehensive understanding and remains poorly understood.

Monoclonal antibody immunotherapy is a key part of BC treatment, with antibody-dependent cellular cytotoxicity (ADCC) being one of its mechanisms. 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. For instance, Trastuzumab (Herceptin), approved by the FDA in 1998, stands out as an effective treatment approach for HER2+ BC. 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. Furthermore, their activity is finely modulated by the stimulation of both activating and inhibitory receptors, such as Killer-cell immunoglobulin-like (KIR) receptors. Nonetheless, despite their significant impact in treating HER2+ BC, only about one-third of patients experience benefits from anti-HER2 Abs, with resistance arising from various factors. The evolution of immunotherapies for HER2+ BC underscores the importance of comprehending interactions among various components of the immune system. Additionally, enhancing the effectiveness of these therapies requires novel chemicals that boost cancer cell sensitivity or immune cell potency.

Multicellular tumor spheroids (MCTS) serve as prevalent three-dimensional (3D) models. 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. For investigating any disease, it is indispensable to establish pertinent and accessible models. 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. Taken together, these observations underscore the significance of tumor spheroid models as an efficacious strategy for enhancing therapeutic responses in a clinical context.

2. AIMS OF STUDY

2.1. Part I

Various aspects of MΦ biology have been shown to be regulated by the multifunctional PARP enzymes. For instance, human studies in BC have shown a direct link between poor prognosis and TAMs, and PARP14 inhibition has emerged as a promising approach to impede TAM phenotype progression. 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 M2 polarization and TAM reprogramming in murine primary bone marrow-derived macrophages (BMDMs) and primary human MΦs
- observing M2 polarization 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

2.2. Part II

Establishing models for cancer immunology research has the potential to improve our comprehension of the efficiency of drug screening and therapeutic antibodies. 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

3. MATERIALS AND METHODS

3.1. 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.

3.2. 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 and their PARP14^{+/+} littermates were used. 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).

3.3. 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 μ M of the wide-spectrum PARPi PJ34 (Sigma-Aldrich, 344458-15-7) or with 50 μ M of the specific PARP14 inhibitor MCD113 (McDaniel College, Westminster, MD, USA), in full DMEM for 1 h. 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. On day 7, cells were washed with PBS and collected for further experiments.

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

4T1 murine tumor cells were kept in culture under standard cell culture conditions and maintained in full DMEM-high glucose culture media. 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 full DMEM at a 1:1 ratio 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.

3.5. Isolation of human peripheral blood mononuclear cells (PBMCs) and macrophage (M Φ) 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 Φ 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 M2 polarization, 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.

3.6. M Φ 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 full RPMI 1640 media. To induce their differentiation into M0 M Φ s, 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 M Φ s were used for the induction of spheroids.

3.7. Generation of 3D in vitro models: JIMT-1 derived M Φ spheroids, THP-1 spheroids and JIMT-1-THP-1 co-culture spheroids

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 M Φ spheroids, 2000 cells/spheroid were seeded in full

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 M Φ s. The 1:1 ratio was optimized for better mimic the *in vivo* environment and infiltration of immune cells in the tumor. 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. 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 μ l/well) for additional 24 h to induce further polarization of M Φ s towards the M1 phenotype.

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

To investigate the role of PARylation in M Φ polarization, on day 4 after polarization with IFN- γ spheroids and co-culture spheroids were incubated with the PARPi PJ34 (25 μ M) and the PARP14i MCD113 (55 μ M) for 24 h (50 μ l/well in JIMT-1 growth media). 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.

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

Total RNA was isolated from murine BMDMs, human PBMC-M Φ s 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 μ g 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 μ l in triplicates, using 2X qPCR BIOSygreen Mix Lo-ROX (PCR Biosystems, PB20.11-50). 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.

3.10. 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).

3.11. 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.

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

For all HCA experiments on MΦ polarization, co-culture spheroids were generated 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. 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. 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: 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.

3.13. 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 (2×10^4 /ml cells in JIMT-1 media seeded into 96-well cell culture plates in 100 μ l/well). On day 3, spheroids were transferred in triplicates to glass bottom Cell Carrier-96 ultra microplates (PerkinElmer, 6055300). Spheroids were pre-treated for 1 h with test compound Sunitinib 40 μ M (Sigma-Aldrich, PZ0012) in fresh JIMT-1 full media (10 μ l/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 μ M CTB (Invitrogen, 1856665) for 1 h in NK medium. 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 μ g/ml of the anti-HER2 antibody Trastuzumab (Herzuma, NDC-63459-303-43) by pipetting 55 μ l/well of CTB-stained NK cells and 55 μ l/well of Trastuzumab in JIMT-1 growth medium. The total volume of treatment added for the ADCC was 110 μ l/well and the final Sunitinib concentration was 20 μ M. The plate was incubated for 24 h.

Cells were stained with Annexin V-Alexa Fluor647 conjugate (Invitrogen-ThermoFisher Scientific, A23204) for 1 h for apoptosis detection (50 μ l/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. Z-stacks were selected as described in section 5.11.1. (first plane: 0 μ m, last plane: 90 μ m), 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.

3.14. Statistical analysis

Data are presented as mean $\pm$ SEM of at least 3 independent experiments. 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.

4. RESULTS

4.1. Part I.

4.1.1. 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). 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. To confirm whether IL-4 treatments triggered M2 polarization, RT-qPCR was used to quantify the mRNA levels of a panel of M2 markers, 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).

Our main goal was to assess the role of PARylation and of specific PARP enzymes in the polarization of MΦs. PARPi. As it was described by comparing the chemical space of these inhibitors that PJ34 but not Olaparib inhibits PARP14, an enzyme which has been implicated in IL-4 response, we hypothesized that PARP14 plays a key role in mediating M2 polarization. Thus, the specific PARP14 inhibitor MCD113 (kind gift from Dana Ferraris, McDaniel College) was tested in our model. MCD113 also led to the suppression of M2 marker gene expression. To reinforce these findings and better assess the role of PARP14 in MΦ polarization, we performed the experiments with M2 polarization using BMDM-derived MΦs also isolated from PARP14KO mice. 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, thereby validating the essential role of PARP14 in M2 polarization.

4.1.2. 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) 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), 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. In the same way, lack of PARP14 in murine PARP14^{-/-}BMDMs led to the suppression of the 4T1CM-induced TAM phenotype.

4.1.3. M2 polarization in human PBMC-MΦs is regulated via PARP14

To characterize the role of PARP14 in M2 polarization of human MΦs, human peripheral blood monocyte-derived macrophages (PBMC-MΦs) 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. The cells were then collected for RT-qPCR experiments, and an expression profile analysis of human M2 markers (Arg1, Fizz1, MRC1, 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. In addition, treatments with either PJ34 or MCD113 caused a decrease in Arg1, MRC1, Fibronectin, CCL17 and CCL18 mRNA levels. 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, but in flow cytometry they appeared as a well-defined population. Confirming our results obtained from RT-qPCR, PJ34 pre-treatments led to a significant down-regulation of CD206 cell surface expression, while MCD113 treatments caused a modest but not significant reduction. Furthermore, the relative frequency of CD206⁺ cells showed a significant decrease after treatments with MCD113 inhibitor compound. These data suggest the similar role of PARP14 in both the murine and human models of M2 differentiation.

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

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 and JIMT-1 human breast cancer cells expressing EGFP. Images taken with confocal microscope on day 4 revealed that, after addition

of IFN- γ to induce M Φ polarization, spheroids did not show visible changes in size, while THP-1 derived M Φ s started to infiltrate them.

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, Fibronectin, CCL17, CCL18). IFN- γ was added to the spheroids to initiate polarization of M Φ s toward M1. The gene expression profiles of JIMT-1-THP-1 spheroid co-cultures and JIMT-1 cancer cell spheroids were compared. Results intriguingly showed that M Φ only spheroids (not kept in co-culture) displayed significantly higher levels of M1 markers when compared to M Φ -cancer cell co-culture spheroids. Moreover, in the case of JIMT-1 spheroids the mRNA levels of M1 markers were found to be always low, thereby suggesting that the M Φ markers were not expressed by the cancer cells but specifically by M Φ s. M2 marker levels were also determined in co-culture spheroids. We observed that, when compared to “pure” M Φ spheroids, the co-cultures with JIMT-1 cells induced M2 marker expression in THP-1-derived M Φ s.

These findings confirm our hypothesis that M2-like polarization of M Φ s is triggered by signals of the TME, and corroborate our results obtained from 4T1CM transfers in the murine M Φ model.

4.1.5. PARP inhibitors suppress M2 marker expression in JIMT-1-THP-1 derived M Φ spheroid co-cultures

To better understand the involvement of ADP-ribosylation in our 3D M Φ polarization system, spheroids were grown in JIMT-1-THP-1-derived M Φ co-cultures or in THP-1 derived M Φ s 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 M Φ s. In this panel, Arg1 and Fizz1 were also included, along with the MRC1, fibronectin, CCL17 and CCL18. Our obtained data showed that spheroid co-cultures significantly elevated the M2 markers expression compared to M Φ only spheroid cultures, in the presence of IFN- γ . In addition to that, co-cultures showed a significant mRNA level decrease of all the M2 markers, when treated with either the PARPi PJ34 or the PARP14i MCD113.

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.

4.1.6. *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. 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. 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. Furthermore, treatments of spheroids with the two ADP-ribosylation inhibitors caused a decrease in the mRNA levels of the mentioned cytokines.

4.2. *Part II.*

4.2.1. *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. Moreover, a diminished compactness was detected after treatment with PJ34.

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.

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

Spheroids were generated using JIMT-1-EGFP cells, and were allowed to clump for 3 days. Sunitinib (20 μ M) was then added to the 3D cultures as a test compound, since it was previously demonstrated that it can influence ADCC. Induction of ADCC was initiated by adding NK cells (20:1) to the spheroids, along with the anti-HER2 antibody Trastuzumab (10 μ g/mL) for 1 day in the absence or presence of Sunitinib. 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. 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). 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.

5. DISCUSSION

The TME significantly influences both tumor progression and tumor responsiveness to therapeutic interventions. 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. 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. 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 M2 polarization induced by IL-4. Consistent with existing literature, our results verified the role of PARP enzymes in this phenomenon. It is noteworthy that MCD113 was specifically designed as a PARP14i, whereas PJ34 primarily inhibits PARP1 and PARP2, although exhibiting a weaker inhibitory effect on PARP14. 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 M2 polarization 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. 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 M2 polarization. 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 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 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. 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, 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. 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. Additionally, cytoplasmic MIF has been suggested to hold prognostic values in patients with squamous cell

carcinoma. 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. Moreover, it serves as a significant MΦ-induced mediator of cancer progression. Studies have demonstrated the involvement of PAI-1 in M2 polarization in various tumor-related contexts. 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.

Previous reports have indicated that targeting PARP14 can induce a shift in MΦs from an M2 to an M1 phenotype. 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.

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 PARPis in BRCA mutant BC. TAMs have been documented to play a role in conferring resistance to PARPi compounds, and strategies involving the reprogramming of TAMs, such as through STING agonism, have proven effective in restoring PARPi sensitivity in breast cancer. This suggests that PARPis 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 BC cells induce the polarization of MΦs towards a TAM phenotype. Our findings underline the effectiveness of PARPis 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. PARylation is deemed essential for the development of the TAM phenotype, with PARP14 being identified as a key player in this process. 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. As one of the main immune component of the TME, TAMs wield significant influence over the initiation and progression of cancer. 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. 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 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. 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. In line with the literature, we also detected an increment in the spheroids' size with signs of disintegration in the outer region upon treatment with PARPi compounds. Overall, considering the varied responses of drugs across different cell lines and their potential cell line-specific effects, 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. 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. 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.

The spheroids were formed using a human tumor cell line engineered to stably express EGFP. 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. 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. 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.

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. 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. This approach accounts for the inherent differences between 2D and 3D environments, ensuring a more comprehensive assessment of compound efficacy.

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 MΦs or NK92.CD16 cells to further enhance our understanding of the molecular mechanisms underlying the action of PARPi compounds and Trastuzumab.

This might present an opportunity to predict the therapeutic potential and efficacy of PARP inhibition and Trastuzumab-dependent ADCC *in vivo*. 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.

6. 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. 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 IL4. Mice lacking PARP14 showed reduced ability to differentiate into M2 cells. In mouse and human models of TAMs, both PARPis and PARP14 KO weakened TAM polarization, leading to increased cancer cell apoptosis. Moreover, lipocalin-2, macrophage migration inhibitory factor and plasminogen activator inhibitor-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, 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. 3D models e.g. cancer spheroids are superior to 2D cultures in predicting *in vivo* responses of tumors to anticancer therapies. 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. 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.

7. PUBLICATION LIST PREPARED BY THE KENÉZY LIFE SCIENCES LIBRARY



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Registry number: DEENK/198/2024.PL
Subject: PhD Publication List

Candidate: Isotta Sturniolo
Doctoral School: Doctoral School of Molecular Medicine

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.
Int. J. Mol. Sci. 25 (7), 1-21, 2024.
DOI: <http://dx.doi.org/10.3390/ijms25073601>
IF: 5.6 (2022)
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J. Vis. Exp. [Epub ahead of print], 2024.
DOI: <http://dx.doi.org/10.3791/65922>
IF: 1.2 (2022)

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.
DOI: <http://dx.doi.org/10.3389/fonc.2019.00542>
IF: 4.848

Total IF of journals (all publications): 24,087

Total IF of journals (publications related to the dissertation): 6,8

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 April, 2024



8. ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof. Dr. László Virág, who always supported and encouraged me throughout my doctoral journey. I am thankful for his invaluable guidance and help in improving and managing myself in a scientific manner. I am incredibly grateful for all the opportunities he gave me to attend several International Conferences to develop my scientific knowledge and participate in great scientific discussions. His expertise, patience, and unwavering commitment to excellence have been instrumental in shaping my research and personal growth.

I would like to thank all my great colleagues, Dr. Zsolt Regdon, Dr. Csaba Hegedűs, Dr. Máté Demény, Dr. Zsuzsanna Polgár, Dr. Katalin Kovács, Dr. Alexandra Kiss and Dr. Endre Kókai, and my dear friends at the Department of Medical Chemistry, University of Debrecen, Ilka Keller, Ádám Ungvári, Dr. Eliza Guti, Marvi Ghani and Márton Fonódi, whose support and resources have facilitated my academic endeavors and provided a stimulating intellectual environment. I am deeply appreciative of the laboratory assistance, staff and faculty members, Éva Neiszné Kovács, Andrea Gregus, Andrea Tankáné Farkas, Dávid Varga and Zsolt Márton. I extend my heartfelt thanks to my enthusiastic students, especially Artjola Puja (Molecular Biology MSc), Csongor Váróczy and Ákos Máté Bede (General Medicine students), for contributing our common works and for their encouragement, which have made the research journey both productive and enjoyable.

Special thanks are due to my collaborators and co-authors, in particular Prof. Dr. Attila Bácsi and Dr. Anett Mázló from the Department of Immunology, University of Debrecen, and to my precious friend Giorgia Intili from the University of Palermo (Department of Biomedicine, Neuroscience and advance Diagnostics), for their incredible help and contributions to various aspects of this research project. Their competence has greatly enhanced the quality and scope of my work.

This acknowledgment would not be complete without giving thanks to all my friends from home, my foreigner friends and loved ones, who were always interested in my scientific progress and were always there for me during this journey.

I must acknowledge with deep thanks my family. My supporting and brave parents, always present during these years through thick and thin. I am so grateful for my whole family's encouragement, understanding and unwavering love. Their support has been my source of strength and motivation.