Understanding the immunomodulatory role of PARP proteins in the response against tumors

Lucía Moreno Lama

DOCTORAL THESIS UPF/ 2019

Thesis director: Dr. José Yélamos López

Poly (ADP-ribose) polymerases group
Cancer Research Program
Hospital del Mar Medical Research Institute (IMIM)
Barcelona Biomedical Research Park (PRBB)

‘It may be that when we no longer know what to do, we have come to our real work and when we no longer know which way to go, we have begun our real journey’

Wendell Berry

A Claudia y Andrés
y, por supuesto, a Papá y Mamá

AGRADECIMIENTOS

La realización de este trabajo no habría sido posible sin la confianza que el Dr. José Yélamos ha depositado en mí durante estos años. Gracias por brindarme la oportunidad de aprender y disfrutar de la investigación biomédica. Del mismo modo agradecer a la Fundación Científica de la Asociación Española contra el Cáncer por proporcionar, en tiempos tan difíciles, la financiación necesaria para llevar a cabo este proyecto. Y mil gracias a mis compañeros de grupo y de despacho, ahora sé que en equipo todo se consigue y que hasta el día más caótico se saca adelante.

Gracias de corazón a los que me habéis recordado cada día que no estoy sola en esto de hacer ciencia ni en esto de hacer vida. He crecido estos tres años encajando pedacitos de vosotros que ya siempre estarán conmigo. Espero habéroslo expresado de muchas otras formas antes de escribirlo aquí.

Gracias Pepe. Por la tormenta y por la calma. Has sido ese inesperado desequilibrio que me ha permitido seguir adelante.

Tras todos estos pasos que torpemente he ido dando, sigue sin ser fácil encontrarme lejos de casa. Por eso agradezco a mi familia acompañarme siempre aun en la distancia. Todo lo bonito de esta vida es gracias a vosotros y, si se me permite, os dedico todos mis esfuerzos y logros, incluida esta tesis.

A mis padres, a quienes descubro en todo lo que expreso; y a mis hermanos, que son reflejo de todo lo que construyo.

This work has been entirely funded by:

Fundación científica de la Asociación Española contra el Cáncer (AECC).

ABSTRACT

Poly(ADP-ribose)-polymerases (PARP)-1 and PARP-2 play an essential role in the DNA damage response. Based on this effect of PARPs in the malignant cell itself, PARP inhibitors have emerged as new therapeutic approaches both in clinical trials and as already approved drugs. However, the complex interaction of multiple other cell types, particularly T cells, within the tumor microenvironment is determinant to either favor or limit tumorigenesis. PARP's implication in cancer immunity is still poorly understood.

Bypassing the embryonic lethality of dually PARP-1/PARP-2-deficient mice, in the present work we investigate the understudied role of these PARPs in the modulation of T cell responses against AT-3-induced breast tumors, using a PARP-1-deficient mouse with a Cd4-promoter-driven deletion of PARP-2 in T cells. We report that dual PARP-1/PARP-2-deficiency in T cells promotes tumor growth while single deficiency of each protein limits tumor progression. Analysis of tumor-infiltrating cells in dually PARP-1/PARP-2-deficiency host-mice revealed a global change in immunological profile and impaired recruitment and activation of T cells. Conversely, single PARP-1 and PARP-2-deficiency tends to produce an environment with an active and partially upregulated immune response.

Our findings pinpoint opposite effects of single and dual PARP-1 and PARP-2-deficiency in modulating the anti-tumor response with a significant impact on tumor progression; thus highlighting the importance of developing more selective PARP-centered therapies.

RESUMEN

Las enzimas poly (ADP-ribosa) polimerasas (PARP)-1 y PARP-2 juegan un papel esencial en la respuesta a daño del ADN. En base a dichos efectos de las PARP en la propia célula maligna, los inhibidores de PARP han surgido como nuevas herramientas terapéuticas actualmente en ensayos clínicos y como fármacos ya aprobados. Sin embargo, la compleja interacción de múltiples tipos celulares en el microambiente tumoral, particularmente las células T, resulta determinante a la hora de favorecer o limitar la tumorogénesis. La implicación de las PARPs en la inmunidad del cáncer continúa sin ser completamente entendida.

Superando la letalidad embrionaria de los ratones doble deficientes en PARP-1 y PARP-2, en el presente trabajo investigamos el papel de estas PARPs en la modulación de las respuestas ejercidas por las células T contra tumores de mama inducidos por la línea AT-3; utilizando para ello ratones deficientes en PARP-1 con una supresión de PARP-2 controlada bajo el promotor de CD4. Reportamos que la doble supresión de PARP-1 y PARP-2 promueve el crecimiento tumoral mientras que la supresión individual de cada proteína limita la progresión del tumor. El análisis de las células infiltrantes de tumor en ratones con deficiencia doble de PARP-1 y PARP-2 reveló un cambio global en el perfil inmunológico y alteraciones en el reclutamiento y la activación de las células T. Por el contrario, la deficiencia única de PARP-1 o PARP-2 tiende a generar un microambiente con una respuesta inmune activa y parcialmente elevada.

Nuestros hallazgos apuntan a que la deficiencia doble o única de PARP-1 y PARP-2 genera efectos contrarios en la regulación de la respuesta antitumoral con un impacto significativo en la progresión del tumor y recalcan la importancia de desarrollar terapias centradas en PARP que sean de carácter más selectivo.

PREFACE

The work submitted in this thesis has been conducted in the Poly(ADP-ribose) polymerases group in the Biomedical Research Park of Barcelona (PRBB). This group, led by Dr. José Yélamos López, is part of the Cancer Research Program at the Hospital del Mar Medical Research Institute at PRBB in Barcelona, Spain.

The main goal of the group is to contribute to the understanding of the diverse effects of poly (ADP-ribosyl)ation by PARP enzymes in the regulation of immune responses both innate and adaptive.

Particularly, the core of this PhD thesis is the study of the redundant and specific immunomodulatory roles of PARP-1 and PARP-2 in the immune response to tumors through the observation of tumor growth and the characterization of immune infiltrates in these tumors, when hosted in mice models of PARP-1 and/or PARP-2 deficiencies.

INDEX

ABBREVIATIONS

ADP: Adenosine Diphosphate

AHR: Airway Hyper-Responsiveness

AIF: Apoptosis Induction Factor

AkT/PkB: Protein kinase B

AMD: Automodification Domain

APC: Antigen Presenting Cells

APE1: AP Endonuclease 1

APLD: Aprataxin-Like Dactor

APOBEC: Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-like

ARG1: Arginase 1

ARH3: ADP-Ribosylhydrolase-3

ARTDs: ADP-Ribosyl Transferases

ATM: Atasia Elangiectasia Mutated

ATP: Adenosine Triphospate

Bcl10: B-cell lymphoma/leukemia 10

BER: Base Excision Repair

BL1/2: Basal like 1/2

BRCA1/2: *BR*east *CA*ncer susceptibility genes 1 and 2

BRCT: *BR*east *CA*ncer susceptibility Protein C

CAFs: Cancer Associated Fibroblasts

CCL: C-C Motif Chemokine Ligand

CCND1: Cyclin D1

CD: Cluster of Differentiation

CDK: Cyclin-Dependent Kinase

CHEK 2: Cell Cycle Checkpoint Kinase

CK: Cytokeratin

CTL: Cytotoxic T Lymphocyte

CTLA-4: Cytotoxic T-Lymphocyte associated Antigen 4

CXCL: Chemokine (C-X-C motif) Ligand

DBD: DNA Binding Domain

DCs: Dendritic Cells

DMEM: Dulbecco's Modified Eagle Medium

dMMR: Mismatch Repair Deficiency

DSBs: Double-Strand Breaks

EAE: Autoimmune Encephalomyelitis

EC: Endothelial Cell

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial to Mesenchymal transition

ER: Estrogen Receptor

ERK: Extracellular signal Regulated Kinase

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FDR: False Discovery Rate

FEN1: Flap Structure-Specific Endonuclease 1

FFPE: Paraffin Embedded

FGF2/bFGF: Basic Fibroblast Growth Factor

FGFR4: Fibroblast Growth Factor Receptor 4

FOXA1: Forkhead Box Protein A1

FOXP3: Forkhead Box P3

FSC: Forward Scatter

GATA3: GATA Binding Protein 3

GE: Gene Expression

GSEA: Gene Set Enrichment Analysis

GM-CSF: Granulocyte/Macrophage Colony Stimulating Factor

GZM: Granzyme

HD: Helical Domain

HDAC: Histone Deacetylase

HDL: High Density Lipoprotein

HER2neu/ERBB2: Human Epidermal Growth Factor Receptor-2

HGF: Hepatocyte Growth Factor

HLA: Human Leukocyte Antigen

HMGB1: High-Mobility Group Box 1

HR: Homologous Recombination

HSCs: Hematopoietic Stem Cells

HTLV1: Human T-cell leukemia virus 1

ICAM 1: Intercellular Adhesion Molecule 1

ICOS: Inducible Costimulator

IFN-β: Interferon-beta

IFN-γ: Interferon-gamma

IGF: Insulin Growth Factor

IHC: Immunohistochemistry

IL: Interleukin

IM: Immunomodulatory

iNOS: Inducible Nitric Oxide Synthase

IR: Infrared Radiation

IRF: Interferon Regulatory Factor

JAK3: Janus Kinase 3

KLF8: Kruppel Like Factor 8

LAG3: Lymphocyte-activation gene 3

LAR: Luminal Androgen Receptor

LDL: Low Density Lipoprotein

MAP3KI: Mitogen-Activated Protein Kinase Kinase Kinase 1

MAPK: Mitogen-Activated Protein Kinase

MDM2: Murine Double Minute 2

MDSCs: Myeloid Derived Suppressor Cells

MHCI/II: Major Histocompatibility Complex I/II

MIF: Macrophage Migration Inhibition Factor

MMTV: Mouse Mammary Tumor Virus

MRE11: Meiotic Recombination 11

MRN: RAD50-MRE11-NBS1 complex

MSI-H: Microsatellite Instability-High

MSL: Mesenchymal Stem-like

mTOR: Mammalian Target of Rapamycin

MYBL2: MYB Proto-Oncogene Like 2

NAD: β-Nicotinamide Adenine Dinucleotide

NBS1: Nijmegen Breakage Syndrome 1/ Nibrin

NER: Nucleotide Excision Repair

NES: Normalized Enrichment Score

NFAT: Nuclear factor of activated T-cells

NF-κB: Nuclear Factor Kappa B

NHEJ: Non-Homologous End Joining

NK: Natural Killer

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NoSL: Nucleolar Localization Signal

NSL: Nuclear Localization Signal

Oct-1: Octamer-Binding Transcription factor 1

PAR: Poly(ADP-ribose) Polymers

PARG: Poly(ADP-ribose) Glycohydrolase

PARP: Poly(ADP-ribose) Polymerase

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Number Antigen

PCR: Polymerase Chain Reaction

PD1/PDL-1: Programmed Cell Death Protein/Ligand-1

pDCs: Plasmacytoid Dendritic Cells

PDGF: Platelet-derived growth factor

PGC-1α: Proangiogenic Transcriptional Regulator 1α

PGE2: Prostaglandin E2

PIK3CA: Phosphatidylinositol 3-Kinase Catalytic subunit Alpha

PKs: Protein Kinases

PPAR: Proliferator-Activated Receptor

PR: Progesterone Receptor

PRD: PARP Regulatory Domain

Prf1: Perforin 1

PTEN: Phospate and Tensin homolog

PTM: Post-Translational Modification

PyMT: Murine Polyomavirus Middle T Antigen

RBC: Red Blood Cell

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute

SCID: Severe Combined Immunodeficiency

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SIRT1: Sirtuin 1

SLAM: Signaling Lymphocyte Activation Molecule

SSBs: Single-Strand Breaks

SSC: Side Scatter

STAT: Signal Transducer and Activator of Transcription

TAMs: Tumor Associated Macrophages

TAN: Tumor Associated Neutrophils

TBS: Tris-Buffered Saline

TCR: T Cell Receptor

TE: Tris-EDTA

TEF-1: Translation Elongation Factor 1

TGFβ: Transforming Growth Factor beta

Th1/2/17: T helper 1/2/17

TIF1: Transcriptional Intermediary Factor 1

TILs: Tumor Infiltrating Lymphocytes

Tim3: T-Cell immunoglobulin mucin 3

TME: Tumor microenvironment

TNBC: Triple Negative Breast Cancer

TNF-α: Tumor Necrosis Factor Alpha

Topo I: Topoisomerase I

TP53: Tumor Protein 53

Tregs: T regulatory cells

UVR: Ultraviolet Radiation

VCAM1: Vascular Cell Adhesion Molecule 1

VEGF: Vascular Epidermal Growth Factor

WGR: Tryptophan-Glycine-Arginine Rich

XBP1: X-box Dinding Protein 1

XPA: Xeroderma Pigmentosum Complementation Group A

XRCC1: X-ray Repair Cross Complementing I

ZF: Zinc finger

INTRODUCTION

1. Breast cancer

1.1 Epidemiology and etiology of breast cancer

Breast cancer remains a matter of global health urgency. Carcinoma of the breast comprises a group of biologically and molecularly heterogeneous diseases originated from the breast. It is the most common type of cancer in females, and the leading cause of morbidity and mortality of oncologic nature, affecting women worldwide today. According to statistics from Globocan, in 2018 breast cancer accounted for over 6 hundred thousand deaths, and the number of new cases surpassed the 2 million, representing 11,6% of all new cases of cancer in both sexes of all ages[1]. Survival rates have improved but they still vary worldwide being very dependent on access to early diagnosis and medical care. The five-year survival rate of early stage breast cancers is 80-90% in high resource countries; however, for the cases of more advanced stage it only reaches 24%, reflecting a critical need to improve treatment of metastatic disease[2].

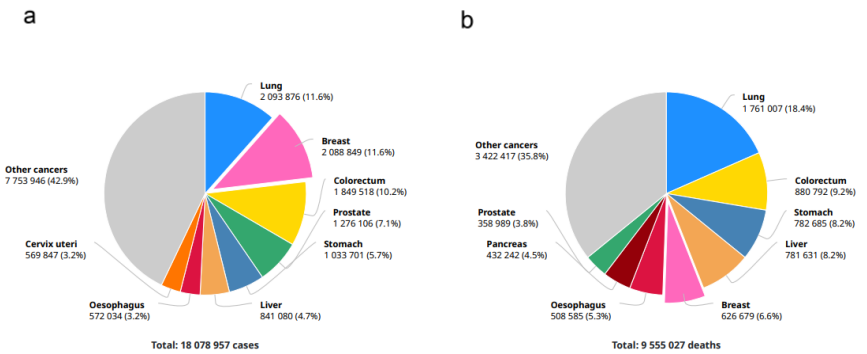


Figure 1.- Epidemiology of breast cancer. Statistics adapted from Globocan[1] breast cancer fact sheet showing (a) number of new cases and (b) number of deaths, in 2018 in both sexes and all ages.

There is a monumental body of evidence supporting the hypothesis that cancer arises from the progressive evolution of normal cells into a neoplastic state as a result of the slow accumulation of mutations[3]. Malignant transformation can be explained by the successive acquisition of the six proposed hallmarks of cancer that allow tumor growth and metastatic dissemination. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, promoting angiogenesis, and inducing invasion and metastasis. More recently two additional hallmarks have been associated with cancer pathogenesis. One involves the capability to reprogram cellular metabolism in order to sustain and promote neoplastic proliferation and the second one relies on the ability of cancer cells to evade immunological recognition and destruction[3,4].

1.2 Risk factors for breast cancer

1.2.1 Age

The main risk factor for breast cancer is age. Although it is more common after menopause, the risk of breast cancer doubles each decade until menopause after which the increase slows significantly[5]. Women who have their first pregnancy after the age of 35 and/or early menarche (before 12 years old), increase lifetime exposure to estrogen and progesterone and thus have a higher risk of developing breast cancer[2,6].

1.2.2 Geographical variation

Age standardized incidence rates to date, show the highest incidence of breast cancer in women in Australia and New Zealand followed by Western and Northern Europe and North America, whereas the lowest incidence countries are in Eastern and Middle Africa and lastly South-Central Asia[1]. Besides access to medical care, low parity and lack of lactation, which are common in high resource nations, are other

significant risk factors that are most likely related to geographical variation[7].

1.2.3 Radiation

Exposure to diagnostic and/or therapeutic ionizing radiation, even at low doses, doubles the chances of developing primary or secondary breast cancer. This is particularly significant during rapid breast formation (puberty) and in carriers of mutations in *BR*east *CA*ncer susceptibility genes (BRCA1/2)[8]. Malignant transformation of somatic cells is preceded by the affectation of cellular physiology which disturbs correct cell replication. Both hereditary and environmental factors can alter cellular pathways leading to self-sufficient growth factors signaling while evading growth suppression ques, resisting cell death, sustained proliferative signaling and uncontrolled proliferation, induction of angiogenesis, tissue invasion and metastasis[4].

1.2.4 Hormonal therapy

Epidemiological data associates hormone replacement therapy as well as the use of combined oral contraceptives (both consisting of estrogen and progesterone preparations) with an increase in relative risk for certain cancers in women, being particularly noted with early initiation of contraceptive intake (<20 years)[9].

1.2.5 Genetics and previous pathologies

Breast cancer can be sporadic or hereditary. In sporadic breast carcinoma the causes are generally of hormonal nature and result from the accumulation of acquired mutations in somatic genes[9–11]. Early stages of sporadic breast cancer most likely involve activation of oncogenes such as c-MYC, Cyclin D1 (CCND1) and Human epidermal growth factor receptor-2 (HER2neu/ERBB2)[12–14] coupled with inactivation of tumor

suppressor genes before additional mutations lead to malignant transformation of cells, tumor progression and eventually metastasis[15,16].

On the other hand, genetic predisposition accounts for up to 10% of all breast cancers in western countries. Genetic susceptibility for breast cancer is based on the transmission of a germline mutation in one allele of a high penetrance susceptibility gene which is typically inherited as an autosomal dominant with limited penetrance[6,10]. The majority of hereditary breast cancers are due to mutations in BRCA1 and BRCA2 genes which codify proteins with essential roles in Homologous Recombination (HR) repair pathway[5,6]. Mutated BRCA genes are responsible for 2 to 3% of all breast cancers and about 30 to 40% of all familial breast cancers and women with germ-line heterozygous mutations in these two genes have an expected 60 to 85% lifetime risk of developing breast (and/or ovarian) cancer[5,11]. BRCA1 and BRCA2, located on the long arms of chromosomes 17 and 13 respectively, are quite large genes and mutations can occur at any position; making first-time detections difficult. Nonetheless, some mutations can happen in defined populations with high frequency[6]. Additional susceptibility genes, likely to increase the risk of breast cancer to a moderate degree, are almost certainly still to be identified.

Besides BRCA1 and 2, germ line mutations in other genes (such as CHEK 2, TP53 or PTEN) have been associated to breast cancer susceptibility. Mutations in the cell cycle checkpoint kinase gene (CHEK 2) account for about 5% of familial cancer cases[17]. Also, although rare, Li-Fraumeni and Syndrome and Cowden's disease which are caused by inherited mutations in tumor protein TP53 and phosphate and tensin homolog PTEN genes respectively, are associated with high risk for breast cancer accounting for up to 1% of familial cases[17–19].

Furthermore, women who have had benign breast pathologies have increased probability of carcinogenesis. Women with atypical epithelial hyperplasia have a four to five times or about 30% greater risk and women with palpable cysts, complex fibroadenomas, duct papillomas, scleroadenosis, or moderate or florid epithelial hyperplasia have a 1.5 to 3 times higher risk[6,20].

1.3 Molecular subtypes

Breast cancer comprises a group of heterogeneous diseases with quite variable clinical behavior[21]. Hence, the categorization of tumors into somewhat objective subgroups is of importance for clinical trials as well as clinical management aiming for standardization of treatment and patient care[22]. Common sub-classification by immunohistochemistry is based on their ability to respond to hormonal therapy (surface expression of estrogen or progesterone receptors, ER/PR) or by histological and architectural tumor characterization including ductal, lobular and further subtypes (tubular, medullary, mucinous, micropapillary, metaplastic or secretory, among others)[23–25]. However, a more detailed means of dividing breast cancer has been established using complementary DNA microarray gene expression analysis followed by hierarchical clustering of differentially expressed genes[26]. The five main subtypes defined by gene expression profile are:

1.3.1 Luminal A breast cancer

Luminal A is the most common subtype and it is found in the clinic representing 30 to 40% of all invasive breast cancer including all races and ages. These tumors have a good prognosis are normally slow growing, HER2 negative and enriched for estrogen and progesterone receptors. With a low expression of proliferating genes, they are usually unresponsive to chemotherapy so treatment typically involves hormone

therapy alone. Luminal tumors show expression profiles that resemble those of the luminal epithelial component of the breast. Gene expression profiling includes mutations in PIK3CA, MAP3KI, GATA3 and FOXA1, and high expression of ESR1 and XBP1[21,27,28].

1.3.2 Luminal B breast cancer

Luminal B subtype accounts for 20 to 30% of all invasive cancers and has a worse prognosis. Tumors belonging to this subtype express estrogen receptors but might be PR negative and/or HER2 positive with high expression of proliferation related genes thus being better candidates for chemotherapy. They are characterized by enhanced genomic instability, TP53 and PIK3CA mutations, amplification of CD1 and MDM2 and loss of ATM[21,26–28].

1.3.3 HER2-enriched breast cancer

HER2/ERBB2 oncogene codes for a tyrosine kinase receptor that activates signal transduction pathways leading to an aggressive phenotype and poor survival in breast cancer. HER2 enriched breast cancers, however, have a good response to anti-HER therapies combined with chemotherapy. They are driven by either overexpression of human growth factor receptor-2 and HER2 amplicon or pathway-associated genes. At the same time, these tumors are estrogen and progesterone receptor-negative. The frequency among all invasive breast cancers is of 12 to 20%. They are associated with high genomic stability and gene expression profiling shows as aforementioned HER2 amplification, but also TP53, PIK3CA and APOBEC mutations, cyclin D1 amplification, and high expression of fibroblast and epidermal growth factor receptors (FGFR4 and EGFR). ERBB2 positive tumors tend to be high grade and show the worst metastasis free survival, together with the basal-like subtype[21,27–29].

1.3.4 Basal-like breast cancer

This subtype is often referred to as triple-negative breast cancer (TNBC) because they are typically negative for ER, PR and HER2, although not all TN tumors are basal tumors. They are characterized by a high index of genomic instability and upregulation of genes expressed by basal/myoepithelial cells of the mammary tissue, including cytokeratin CK5 and CK6, P-cadherin, EGFR and c-kit[21,27,30]. At the same time, TNBCs represent a diverse group of cancers that have been subcategorized into six subtypes (two basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) subtype) with unique gene expression GE profiles distinguishing different molecular drivers, clinical outcomes and response to neoadjuvant chemotherapy[31,32]. Despite being quite sensitive to chemotherapy, triple negative breast cancers are prone to relapse and metastasize[30]. Due to the lack of targeted therapies for basal-like tumors and their association with BRCA pathway dysfunction, efforts have been made towards therapies that could exploit synthetic lethality, which is based on the DNA repair status of the cell.

1.3.5 Normal Breast-like breast cancer

Normal breast-like cancers, which account for around 8% of all breast cancers, express genes of adipose and non-epithelial cells and resemble normal breast tissue. Similarly to luminal tumors, this subtype is positive for hormone receptor expression and HER2 negative. Their prognosis, although good, is slightly worse than that of Luminal A cancer[33].

Remarkably, the molecular subtypes exhibit significant differences in the prediction of overall survival, as well as disease-free survival with the basal-like/triple-negative ($ER^-/PR^-/ErbB2^-$) subtype having the lowest survival[26].

Intrinsic subtype	IHC status	Grade	Outcome	Prevalence[Δ]
Luminal A*	[ER+|PR+] HER2-KI67-	1|2	Good	23.7% [p1]
Luminal B*	[ER+|PR+] HER2-KI67+	2|3	Intermediate	38.8% [p1]
	[ER+|PR+] HER2+KI67+		|Poor	14% [p1]
HER2 over-expression*	[ER-PR-] HER2+	2|3	Poor	11.2% [p1]
Basal*	[ER-PR-] HER2-, basal marker+	3	Poor	12.3% [p1]
Normal-like	[ER+|PR+] HER2-KI67-	1|2|3	Intermediate	7.8% [p2]

Figure 2.- Breast tumors molecular subtypes. Adapted from[33]. Summary of molecular classification of breast tumor subtypes including IHC status, grade, prognosis and prevalence.

1.4 Systemic treatments

Following or in combination of surgical approaches for the elimination of breast cancer, such as mastectomy (removal of the entire breast) or lumpectomy (removal of the tumor and a small piece of healthy tissue) patients are generally treated with systemic therapies aiming to reduce the risk of cancer recurrence or metastasis. Systematic therapies usually conceived as adjuvant treatment involve drugs that spread via the bloodstream throughout the body to treat cancer cells regardless of their location and include:

1.4.1 Chemotherapy

Chemotherapeutic drugs are cytotoxic agents which disturb cell proliferation that are used to eliminate or stop fast replicating cells. Because a high proliferative rate is an intrinsic characteristic of other cell types (e.g., multipotent progenitor cells), besides cancer cells, chemotherapy causes side effects like alopecia and myelosuppression[34,35]. Chemotherapy can be given to the patient as a neoadjuvant, before surgery to shrink the tumor and facilitate the surgical removal, or as adjuvant therapy given shortly after the primary surgery to eliminate any remaining cells that could lead to relapse or spread[36].

1.4.2 Hormone therapy

Current hormone therapies aim to inhibit the proliferative effects of estrogen in ER-positive breast cancers. Estrogen-dependent growth can be blocked by anti-estrogen agents, which compete for the binding to ER, Tamoxifen being the most successful antiestrogen drug. Other endocrine therapies like aromatase inhibitors are based on reducing the levels of estrogen by blocking the transformation of androgen into estrogen, normally catalyzed by the enzyme aromatase[37].

1.4.3 Targeted therapies (including HER2 targeted therapy)

A current focus of research for breast cancer therapy is drugs that target specific molecules involved in cancer development, growth, and spread. For instance, HER2neu/ERBB2 is an ideal target for $HER2^+$ breast tumors. HER2 signaling participates in different cellular pathways (PI3K/Akt/mTOR and MAPK pathways) important for tumorigenesis such as cell proliferation, survival, motility or apoptosis resistance, among others[38]. HER2 is highly overexpressed in many human cancers compared to normal tissue, which might reduce the toxicity of treatment. Furthermore, HER2 overexpression is found in both primary and metastatic cancer cells and its level shows strong correlation with cancer pathogenesis and prognosis[39]. The first approved HER2-targeted drug was humanized monoclonal antibody Trastuzumab, followed by small-molecule EGFR/HER2 tyrosine kinase inhibitors (Lapatinib), or antibodies against vascular epidermal growth factor (VEGF) (Bevacizumab). Novel HER2-targeting drugs include inhibitors of PI3K/Akt/mTOR and ERK1/2 pathways and even poly(ADP-ribose) polymerase (PARP) inhibitors[38–40].

1.4.4 Immunotherapies

Immunotherapy is a biologic approach of treatment with the purpose of aiding the immune system response against a tumor. In this category, passive immunotherapy using trastuzumab yielded initial success treating HER-2 overexpressing tumors. As previously mentioned, trastuzumab is a HER2 targeted drug that promotes antibody-dependent cellular toxicity and the degradation of HER2 receptors. Although still being extensively researched in clinical trials, as of 2019, there are two novel immunotherapies approved to treat breast cancer, one of which is only approved for tumors positive for Programmed Cell Death protein 1 Ligand (PDL-1) expression. PD-1 is a receptor protein expressed on T cells that exerts immunosuppressive effects when engaged with its ligand PDL-1. This therapy combines atezolizumab with protein-bound Nab-paclitaxel being beneficial for locally advanced triple-negative breast cancer (that cannot be intervened by surgery) as well as for metastatic triple-negative breast cancer. The other approved immunotherapy, pembrolizumab is accepted to treat metastatic cancer or cancer that cannot be surgically removed and has a molecular alteration called microsatellite instability-high (MSI-H) or DNA mismatch repair deficiency (dMMR)[41–43].

2. The tumor microenvironment

Cancer is truly one of the most complex and dynamic diseases affecting human global health. In accordance, tumors are not only comprised of cancerous cells but are in fact multifaceted entities that include cells of different natures. Moreover, a systemic view of the tumor recognizes also non-cellular elements and soluble components, all of which participate in the active communication between cells, propagating diverse signals and thus modulating cancer progression and response to treatment. Besides a heterogeneous population of cancer cells, the tumor mass contains a

variety of resident and infiltrating host cells such as stromal cells and cells of the immune system; which are integrated within an extracellular matrix (ECM) and under constant stimulation of subcellular elements and secreted factors[44]. This collective network is known as the tumor microenvironment (TME).

Effective elimination of tumors by the immune system relies on the completion of a series of stepwise events, described in the Cancer-Immunity Cycle, with the ultimate role of $CD8^+$ cytotoxic T lymphocytes (CTLs) in the direct killing of cancer cells. In short, as tumor cells die released neoantigens are captured by the antigen-presenting cells (APCs) such as dendritic cells, which process them into peptides that bind to the major histocompatibility complex (MHC)-I and MHC-II molecules. Bound peptides are presented to T cells. While $CD8^+$ T cells recognize MHCI-peptide complexes, $CD4^+$ T cell receptors can recognize the peptide-MHC-II molecules. For this step to result in an anticancer T cell response, it must be accompanied by signals that specify immunity, so that induction of tumor tolerance is avoided. Effector T cells are then primed and activated to perform antigen-specific antitumor responses hence migrating to the tumor site and infiltrating the tumor. In this stage the final immune response heavily depends on the critical balance between T effector cells versus T regulatory cells. Once there, activated T cells can specifically bind to cancer cells and exert direct killing of the cancer cells. A series of activating steps precede cancer cell death and then, the dying cancer cell releases additional cancer-specific neoantigens sustaining the cycle and amplifying the anticancer immune response[45,46].

The cancer immunity cycle is illustrated below.

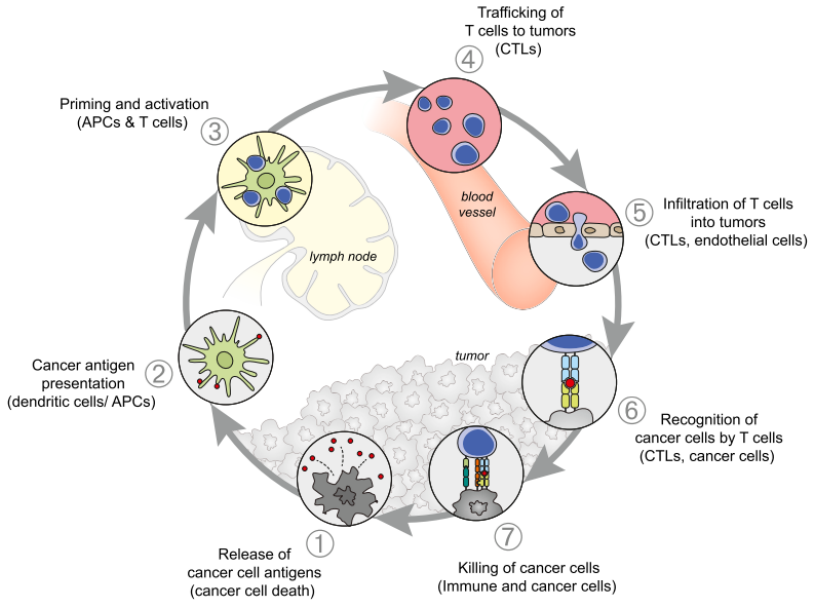


Figure 3.- The cancer-immunity cycle. Adapted from[45]. Cancer cell express neoantigens as a result of accumulated mutations during oncogenesis. Released neoantigens can be captured and processed by APC (1). In the context of immunogenic signals, APCs present them to T cells priming them (2). This is followed by the activation of effector T cell responses against the cancer-specific antigens (3). Effector T cells then have to migrate and infiltrate the tumor aiming to recognize and kill cancer cells (4,5,6 and 7).

The tumor microenvironment is characteristically inflammatory and immunosuppressive. To a different extent in different tumors, the TME contains cells of the innate immune response (including neutrophils, myeloid derived suppressor cells (MDSCs), macrophages, mast cells, dendritic cells and natural killer cells) as well as adaptive immune cells such as T and B lymphocytes. As a result of the different forms of inflammation, and the associated production of inflammatory mediators such as Interleukin (IL)-6 and prostaglandins, immunosuppressive cells are recruited to the TME where their activity paradoxically sustains the

inflammatory environment. Many cell types are thought to contribute to immune evasion including cancer-associated fibroblasts (CAFs), mesenchymal stem cells, vascular endothelial cells, lymphatic endothelial cells and immune cells such as tumor associated macrophages (TAMs), MDSCs and regulatory T cells (Tregs)[47,48]. However, it is believed that pro-tumorigenic inflammation and immune surveillance can coexist in some tumors. Stress and danger signals might prompt antigen presentation of "non-self" or modified self-antigens expressed in cancer cells, which then become targets for tumor-specific killing by activated T and NK cells[49,50]. When blood supply becomes insufficient, tumors undergo oxygen and nutrient deprivation, which induces the chronic release of necrosis-mediating pro-inflammatory factors such as IL-1 and high-mobility group box 1 (HMGB1) that in turn promote neoangiogenesis[51].

One of the major components of tumor stroma are the resident activated fibroblasts, termed cancer associated fibroblast or CAFs. Despite initial considerations, CAFs have been strongly linked to tumor progression and several pro-tumor actions within the tumor microenvironment have been described for them[52]. Driven by crosstalk with cancer cells resident fibroblasts undergo various morphological and biological transitions into CAFs which turn to play important roles in maintaining an optimal niche for cancer cell proliferation and survival[53]. Tumor cells induce fibroblast activation via secreted growth factors, cytokines and adhesion molecules (TGF-β, EGF, PDGF, FGF2, CXCL12, ICAM 1, and VCAM1). As a result, CAFs become targets and inducers of tumorigenic activation signals. CAFs promote tumor growth by secreting classical growth factors (EGF or HGF) and other membrane molecules (integrin α11 and syndecan-1) but also novel CAF-secreted proteins (secreted frizzled related protein 1, and IGF like family member (IGF) 1 and 2)[52–54]. CAFs also stimulate angiogenesis by increasing their own secretion of vascular

endothelial growth factor (VEGF) in a PDGF-mediated manner[55]. Finally, there is also evidence that suggests CAFs also recruit pro-metastatic chemokines and might be of relevance in resistance to cancer therapy[52].

The crosstalk among immune cells infiltrating tumors largely shapes the TME. Human and murine studies have identified leukocytes from the myeloid lineage and lymphoid lineages but the composition of innate and adaptive immunity cells varies between tumor types.

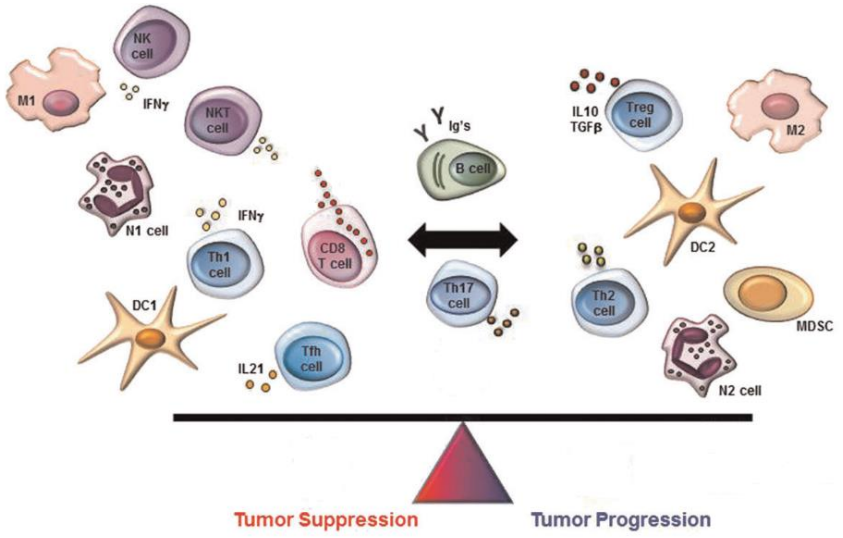


Figure 4.- Immune cellular component of TME. Adapted from[56]. Tumor-infiltrating immune cells include myeloid lineage leukocytes, tumor-associated macrophages with either protumorigenic or antitumorigenic properties, dendritic cells and myeloid-derived suppressor cells and lymphocytes such as helper T-cell subsets, cytotoxic T cells, regulatory T cells, B cells. The cross-talk between these cells modulates TME driving either anti- or protumor immune-mediated response.

2.1 Tumor infiltrating myeloid cells

2.1.1 Tumor associated macrophages

Under physiological conditions, macrophages are tissue-resident cells with phagocytic roles essentially participating in the innate immune response[57]. Macrophages are, in fact, key modulators and effector cells in the immune response[58]. Monocytes and/or macrophages typically undergo phenotypic polarization in response to environmental signals, where M1-like (classical) and M2-like differentiation of macrophages represent the two extremes of a functional spectrum[59]. Classically activated or M1-like macrophages are stimulated by cytokines of the type 1 T helper cell (Th1) response such as interferon-γ (IFN-γ) or tumor necrosis factor (TNF), in turn producing pro-inflammatory cytokines (e.g., TNF, interleukin (IL)-12, IL-2 or IL-23, and reactive nitrogen and oxygen species. Through secretion of these immunostimulatory factors M1 macrophages exhibit antitumor activity[57,60]. On the contrary, the M2 phenotype of alternatively activated macrophages is induced by Th2 response cytokines (e.g., interleukin (IL)-4, IL-10, and IL-13)[61] and antagonizes prototypic inflammatory responses[58]. Besides participating in Th2 responses (allergy and immune response to parasites), M2-like macrophages are characteristically pro-tumorigenic and play essential roles in immune regulation as well as fibrous stroma deposition and tissue remodeling[58,60].

Tumor-associated macrophages (TAMs) closely resemble the M2-like phenotype and are the most frequently found immune infiltrating cells. Preclinical and clinical evidence associates abundance of TAMs in the TME to poor prognosis[47,61]. TAMs accumulate in the tumor after differentiating from either resident macrophages or recruited circulating monocytes[57]. To explain their role in tumor progression, a somewhat symbiotic relationship has been described for cancer cells and TAMs in which malignant cells attract and sustain survival of TAMs and, in turn,

TAMs produce and secrete important mitogens as well as various growth factors that contribute to tumor progression[62]. Tumor associated macrophages downregulate MHC class II and IL-12 expression and exhibit higher expression of the anti-inflammatory cytokine IL-10, scavenger receptor A, and arginase. All in all, TAMs stimulate proliferation, invasion, and metastasis of tumor cells, secrete angiogenesis-promoting enzymes and inhibit infiltration and antitumor function of T cells[60–62]. Inhibition of cytotoxic T cell lymphocytes (CTLs) activity is greatly mediated by TAMs through diverse mechanisms. Programmed cell death-1 (PD-1) is a surface marker of dysfunction expressed on T cells and IL-10 production by TAMs stimulates the expression of PD1 ligand (PD-L1) on monocytes, resulting in inhibition of CTL responses upon binding of said molecules[62]. In mice, L-arginine metabolism by inducible nitric oxide synthase (iNOS) or arginase I in TAMs produces nitric oxide (NO) that might act suppressing CTLs and promote angiogenesis (as iNOS expression by TAMs correlates with increased blood flow). However, the role of produced NO is controversial and it might also contribute to a tumoricidal activity of macrophages and/or monocytes[60,62]. Immunosuppression by Tregs in the tumor is also mediated partially by TAMs. TAMs secrete Treg-chemotactic factors such as CCL17 and CCL22 and TAM-derived prostaglandin E2 (PGE2) induces myeloid derived suppressor cells (MDSCs)[63] and, together with IL-10 and indoleamine 2,3-diosygenase, PGE2 is also involved in Treg activation[62]. Additionally, TAMs have been shown to induce treatment resistance in breast cancer xenografts in mice[64,65].

2.1.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature, pathologically activated, myeloid cells with a highly variable phenotype. Currently, two main MDSC populations have been

characterized: monocytic MDSCs (M-MDSC) and polymorphonuclear (also called granulocytic) MDSCs (PMN-MDSC/G-MDSC)[66]. MDSCs are defined as a population of inhibitory immune cells that accumulate upon activation by pro-inflammatory mediators in a variety of mouse and human cancers and that constitute tolerogenic, tumor-favoring microenvironments[66–68]. Tumor-infiltrating MDSCs are more suppressive than blood or splenic MDSCs on a per cell basis[68]. MDSC are suggested to promote tumor growth by suppressing antitumor immune responses at T-effector cell level (T cell anergy and/or inhibition) as well as by enhancement of tumor neovascularization and metastasis. Murine and human MDSCs suppress CD8+ T cells mainly via the production of ROS and expression of enzymes ARG1 (in the case of G-MDSCs) and NOS2 (in the case of M-MDSCs)[69–71]. In breast cancer the production of ROS by some immune cells[72] stabilizes HIF-1α, which promotes increased production of VEGF and MIF (Macrophage migration inhibition factor), facilitating cancer progression and chemotherapy resistance. MDSCs deplete the TME of non-essential aminoacid L-arginine (L-arg) by internalizing L-arg through the cationic amino-acid transporter 2B and secretion of ARG1. Depletion of by ARG1 reduces T cell expression of CD3ζ, which is required for signal transduction through the antigen-specific T cell receptor (TCR)[73,74]. Unable to upregulate cyclin D3 and cyclin-dependent kinase 4 (cdk4), L-Arg depleted T cells are arrested in cell cycle stage G0–G1[75]. NOS also metabolizes L-arginine contributing to T cell suppression also by producing nitric oxide (NO) which affect several signaling pathways (inhibiting JAK3, STAT5, ERK, and AKT) preventing IL-2 signaling, and subsequently impairing the development of effector and memory T cells[76]. MDSCs also induce the expansion of Tregs and the polarization of macrophages to a TAM-like phenotype[63,77]. In addition, MDSC can differentiate into TAMs and or neutrophils[68,71]. Finally, a direct correlation of the metastasizing capacity of cancer cells

with MDSC recruitment has been observed in vivo in murine breast cancer cells with high IL-6 expression[78].

2.1.3 Tumor associated neutrophils

Neutrophils are the most abundant white blood cells in the circulation system and are significantly represented in the tumor microenvironment. Tumor-associated neutrophils (TAN), similarly to TAMs, have been categorized as N1, anti-tumorigenic, and as N2, pro-tumorigenic, with their induction dependent on the presence of IFN-β or TGFβ, respectively[79,80]. N1 exert their anti-tumor activities through the expression of immuno-activating cytokines and chemokines, reduction of the levels of arginase, and their potential to both inhibit growth of and kill tumor cells[81,82]. N1 neutrophils promote $CD8^+$ recruitment and activation by producing attracting chemokines (such as CCL3, CXCL9, and CXCL10) and pro-inflammatory cytokines (like IL-12, TNF-α, GM-CSF, and VEGF)[83]. In addition TANs of N1 phenotype may coordinate adaptive immune responses through interactions with dendritic cells[84]. N2 neutrophils inactivate T cell effector functions in the same way that has been proposed for MDSCs and TAMs, via production of arginase contributing to arginine depletion from the TME[74,80]. N2 TANs release IL-6 which promotes tumor progression by facilitating angiogenesis and metastasis through the induction of VEGF expression[85]. Additionally, TAN release ROS that can lead to damage in DNA bases and mutations, which contribute to cancer initiation, cell proliferation, and sustain cancer-favored inflammation, immune suppression, and Epithelial to Mesenchymal transition (EMT) in multiple cancer types, including breast cancer[72,85,86].

2.1.4 Dendritic cells

Dendritic cells (DCs) are a group of heterogeneous innate cells and the most potent antigen presenting cells (APCs) playing essential roles in the priming of T cell responses. They are the first cells to reach the tumor and recognize tumor antigens, therefore, playing a pivotal role in the initiation and regulation of both innate and adaptive immunity. DCs display major histocompatibility complex molecules and costimulatory receptors upon pathogen or tumor recognition to capture, process, and present antigens to naïve T cells. The outcome is the production of cytokines that polarize and promote CD8 cytotoxic T lymphocytes (CTLs) differentiation and activation. However, DC subsets that infiltrate the tumor microenvironment can either support the anti-tumor immune response or promote tumorigenesis. Accordingly, tumors are able to modulate DC development, tumor infiltration and function[87]. DCs are generated in bone marrow from macrophage/DC progenitors that give rise to common DC progenitors (CDP), which then differentiate into two major DC subsets: classical/conventional DCs (cDCs) and plasmacytoid DCs (pDCs)[88]. Among them, conventional DCs comprise two main subsets, the $CD8\alpha^+$ and/or $CD103^+$ DC subset and the $CD11b^+$ DC subset which is more heterogeneous. $CD103^+$ DCs are frequently associated with higher cross-presentation capacity of antigens to $CD8^+$ T cells[89], which results in greater tumor-specific CTL expansion and can additionally, support $CD4^+$ T cells polarization towards Th1 responses. On that note, $CD11b^+$ DCs are IRF4 dependent and predominantly present antigens on MHC class II to $CD4^+$ T cells requiring specific activation to induce cross-presentation. T cells effector activity also depends on DC-derived cytokines such as IL-12 and type I interferons and DCs secrete chemokines that recruit circulating T cells into the TME.

Nevertheless, some cancer cell variants can exploit DC functions to promote immune T cell tolerance in the niche instead of immunity. For example, in the absence of costimulatory signals, antigen cross-presentation results in T cell anergy[90]. DCs can enhance biding of immune checkpoint receptor CTLA-4 to T cells therefore lessening costimulatory signaling and T cell activation. CTLA-4, which negatively regulates T cell responses, binds to CD80 on T cells and CD86 on DCs with higher affinity than CD28 and DCs[90]. Another means of immunosuppression by DC in tumors is the expression of inhibitory molecules such as PDL1, PDL2, Tim3 or LAG3. PDL1 and 2 inhibit proliferation and cytokine production of activated T cells expressing PD1. CD103+ DCs from tumor-draining LNs have recently been shown to have increased expression of PD-L1[88].

2.2 Tumor infiltrating lymphoid cells

2.2.1 T lymphocytes

Tumor infiltrating lymphocytes (TILs), including cytotoxic $CD8^+$ T cells, different subsets of helper $CD4^+$ T cells (Th1, Th2 and Th17), γδTCR T cells, and NKT cells, are dominant elements of the tumor microenvironment and play a central part in the antitumor immune responses. Antitumor effects are mainly mediated by the induction of a $CD8^+$ T cell response against tumor-specific antigens[91]. In this regard, three classes of antigens with high tumor specificity may be identified by T cells: antigens produced from mutated cells, cancer-germline genes and viral genes[92]. Functionally $CD8^+$ effector or CTLs lack antibody-dependent cytotoxicity and predominantly inhibit tumor proliferation upon binding to the Fas ligand (FasL), either through direct cytolytic action on tumor cells or by releasing interferon (IFN)-γ, TNF-α and granulocyte colony-stimulating factor (GM-CSF)[91,93-95]. Hence, among the effector mechanisms of T cell cytotoxicity (some shared with Natural

Killer cells) are the expression and release of death ligands (e.g., FasL and TRAIL) and granule exocytosis with subsequent secretion of perforin (Prf1) and granzymes. Prf1 is a pore forming protein that aids with entry of granzymes into the cytoplasm where they influence cell death and survival by cleaving critical intracellular substrates[96].

In accordance, a large body of evidence supports that high infiltration of $CD8^+$ T cells in the tumor is related to a good clinical prognosis in many cancers including melanoma, colorectal and breast cancer[97–99]. However, the balance between co-stimulatory and co-inhibitory signals within the TME heavily modulates functional states in $CD8^+$ and other T cell subsets[92]. For instance, the ratio of PD1/CD8 ration influences survival rates in patients with colorectal cancer being significantly worse in those with high PD1/CD8 ratio. As it has been mentioned before, tumor cells can modulate the functionality of many immune cells (e.g., TAMs, DC or T regulatory cells) to escape immunosurveillance. Tumor cells are able to induce production of immune suppression factors such as IL-10 and transforming growth factor beta (TGF-β) as well as to promote T cell inhibition by cell-mediated contact via CTLA-4 ultimately inhibiting recognition and clearance of tumor cells by CTLs[88,100,101]. Furthermore, tumors have developed other mechanisms to evade immune system recognition, such as downregulation, mutation, or loss of HLA class I molecules[100].

$CD4^+$ T helper 1 (Th1) cells support and reinforce $CD8^+$ effector T cell responses primarily by the production of the cytokines IL-2 and IFNγ and induce activation of macrophages and maturation of dendritic cells[47]. High numbers of these cells in the TME also correlate with a good prognosis[92,102]. In contrast, $CD4^+$ Th2 cells which secrete cytokines supportive of B cell responses (e.g., IL-4, IL-5 and IL-13) or Th17 cells producing IL-17A, IL-17F, IL-21 and IL-22 (that favor antimicrobial

tissue inflammation), are suggested to stimulate tumor growth[102]. Nevertheless, Th2 and Th17 populations have also been associated with a favorable outcome in breast cancer and esophageal cancers, respectively[47].

γδTCR lymphocytes show properties characteristic of innate rather than adaptive immune cells and their implication in promoting or inhibiting tumor development remains controversial. γδ T cells exhibit potent cytotoxic antitumor activity mediated by the production of proinflammatory cytokines, direct cytolytic activity, and regulation of the biological functions of other cell types. Still antitumor functions of γδ T cells might be impaired by the immunosuppressive context within the TME and, due to their plasticity; they could polarize into different functional subsets and display pro-tumoral effects instead. In this regard, γδ T cells can produce TGF-β and IL-10 immunosuppressive cytokines and, similarly to Th17 cells, they can also produce IL-17 which directly promotes proliferation and dissemination of tumor cells in breast cancer[103,104].

2.2.2 T regulatory cells

T regulatory cells (Tregs) represent a heterogeneous subset of $CD4^+$ T cells conventionally co-expressing CD25 and FoxP3 with immunosuppressive functions that have a pivotal role in modulating immune responses, dampening inflammation, maintaining homeostasis self-tolerance, and preventing autoimmunity. Tregs are also crucial players in tumor immunity and their enrichment within tumors is often associated with poor prognosis[101,105,106]. Tumor infiltrating Tregs are recognized to act typically in a pro-tumorigenic manner primarily by suppressing anti-tumor responses. Nonetheless, by downregulating tumor-promoting inflammation, Tregs may also exert an anti-tumorigenic function under certain circumstances[107]. Tregs inhibit the activities of $CD4^+$ and $CD8^+$ effector T cells, natural killer cells, NKT cells, and

antigen-presenting cells, and are considered to be the major impediment for effective antitumor T cell responses. Tregs exert their immunosuppressive functions through a variety of mechanisms which include the expression of inhibitory receptors (e.g., CTLA-4 and PD-1) secretion of immunosuppressive cytokines (IL-10, IL-35, and TGF-b) and metabolites (Adenosine), disruption of cell metabolism via IL-2 deprivation and suppression of effector functions through cell-cell direct contact, as well as direct killing of conventional T cells via cytolytic factors (Granzymes A/B and Perforin)[101,108–111].

2.2.3 NK and NKT cells

Innate cytotoxic lymphocytes, natural killer (NK) cells and natural killer T (NKT) cells, also infiltrate tumors. They are considered potent antitumor effectors cells, involved in hematological malignancies and solid tumor immunosurveillance. NK cells have intrinsic cell killing ability and they appear to be very efficient in attacking tumor cells which might be due to the relatively high expression of some NK cell-activating surface molecules on tumor cells compared to normal cells[112]. NKT cells are a subset of true T cells that work at the interface of innate and adaptive immunity and share characteristics of both T cells and NK cells. NKT cells have the potential to rapidly stimulate tumor-specific T cells and effector NK cells that can eliminate tumor cells[113]. Besides direct cytotoxicity against tumors, NK cells participate in antibody-dependent cytotoxicity directed to autologous tumor cells which can be potentiated with therapeutic antibodies[114]. Some studies however report NK cells in the TME can show an anergic phenotype induced by cancer-derived TGF-β thus being unable to exert their tumor-killing function. For a variety of cancers, tumor infiltrating NK cells are associated with improved patient prognosis and survival and, in some cases like breast cancer, an important role for NK cells in avoiding acquired resistance to systemic therapy has

been suggested[115–118]. Nonetheless tumors can escape immunosurveillance by NK cells and NKT cells for example by interfering with the expression and function of several activating receptors for NK cells[114]. In addition, natural killer cells within the TME are subject to the same suppressive factors that drive downregulation of T cells receptors[119].

2.2.4 B lymphocytes

B lymphocytes originate from hematopoietic stem cells (HSCs) in the bone marrow. Owing to their ability to produce antibodies against foreign antigens, B cells are an integral part of the humoral immune responses. In brief, tolerant immature B cells migrate from bone marrow to spleen where they differentiate into transitional immature B cells before becoming immunocompetent naïve mature B cells[120]. Tumor infiltrating B cells often co-localize with T cells, sometimes in organized lymphoid structures. Although in autoimmunity and organ transplantation B cells enhance T cell responses, increasing evidence suggests that tumor infiltrating B cells can, in fact, inhibit T cell responses in certain cancers[120]. In this regard, the activation state of B cells in the TME might be a key factor modulating their function as T cell responses appear to be inhibited by resting B cells but facilitated by activated B cells[121]. Several mechanisms of immunosuppression have been described for resting B cells including impairment of $CD8^+$ CTL priming by $CD4^+$ T cells, induction of $CD8^+$ T cell anergy via TGFβ production, downregulation of DC production of IL-12 as well as the production of cytokines that modulate Th1/Th2 differentiation[122–124]. Nevertheless, in some cases like invasive breast carcinoma, the presence of B cells correlates with good clinical prognosis[125]. B cells can therefore exert multiple anti-tumor effects including, as previously mentioned, enhancement of cytotoxic T-cell activity, by serving as local APC (if the population of DCs is depleted or dysfunctional), and producing stimulatory cytokines and chemokines.

Moreover, B cells have shown to have direct tumoricidal effect by secretion of granzyme B, or indirectly through antibody-dependent mechanisms[120,123,124]. An illustrated summary of immunosurveillance vs tumor-promoting inflammation is shown below.

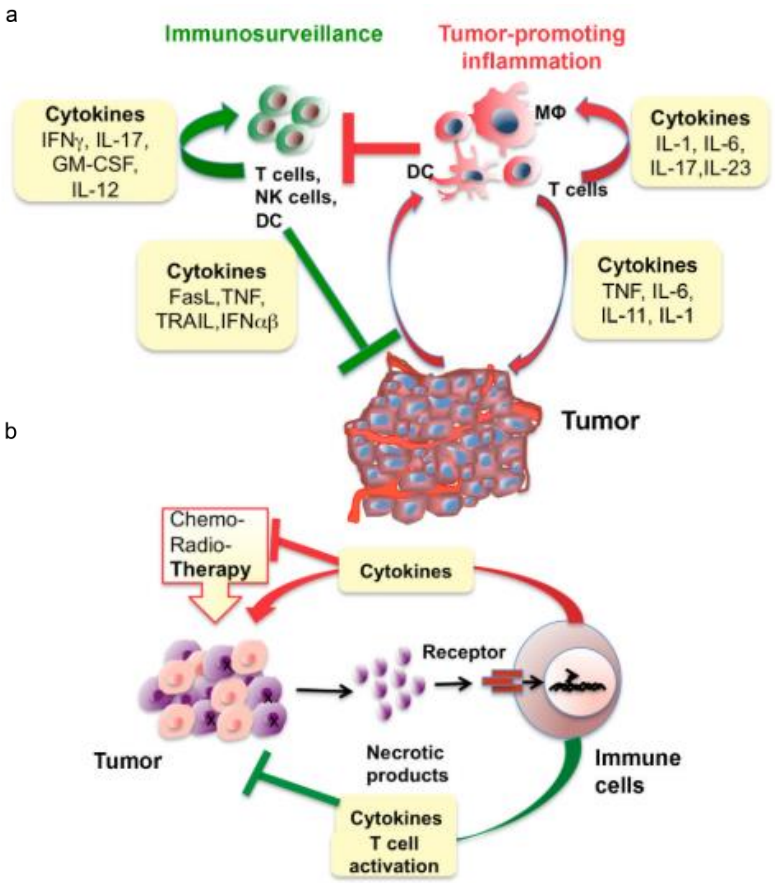


Figure 5.- Immunosurveillance, tumor-promoting and therapy-induced inflammation. Adapted from[61] (a) Tumor-derived cytokines act on transformed and immune cells to shift the balance from immunosurveillance to tumor-promoting inflammation. (b) Therapy-induced inflammation may on the one hand stimulate anti-tumor responses through antigen presentation and on the other, promote activation of pro-survival genes in residual cancer cells, rendering them resistant.

3. ADP-Ribosylation

Tight control of cell physiology is achieved by the rapid transduction of cellular signaling pathways in response to specific cues. Targeted control of protein localization and activity, RNA and protein levels, and ultimately, changes in gene expression are necessary for effective regulation. Remarkably, post-translational modification (PTM) of proteins is one of the key mechanisms in the regulation of protein catalytic activity and protein-target interactions. In human disease, cell signaling networks or their associated enzymes and proteins are frequently deregulated.

Adenosine diphosphate ribosylation or ADP-ribosylation is an evolutionarily conserved posttranslational modification found in all living cells (with the exception of yeast)[126]. It refers to the reversible transference of ADP ribose units from β-nicotinamide adenine dinucleotide (NAD^+)[127]. Single or multiple ADP-ribose moieties from NAD^+ can be covalently attached to their targets, including proteins, nucleotides and other small molecules. Intracellularly, ADP-ribosylation is controlled by diphtheria toxin-like ADP-ribosyl transferases (ARTDs) or Poly(ADP-ribose) Polymerases (PARPs) which catalyze both the initial mono-ADP-ribosylation and the subsequent elongation and branching[128,129]. Besides altering the function of modified proteins, ADP-ribosylation provides a scaffold for the recruitment of other proteins.

It was over fifty years ago that ADP-ribosylation was first identified. In parallel to the discovery of enzymes that can generate ADP-ribose polymers from NAD^+ in mammalian cell extracts[130,131], studies on the toxicity of diphtheria toxin showed that this enzyme requires NAD^+ inhibit mammalian protein synthesis[132]. Coetaneous, in 1967, the genesis of Poly(ADP-ribose) (PAR) polymers in a DNA-dependent NAD^+

consuming reaction was discovered. PARPs hence catalyze poly(ADP-ribosyl)ation and mono(ADP-ribosyl)ation (PARylation and MARylation, respectively)[133]. ADP-ribose is transferred into amino acid side chains with a nucleophilic oxygen, nitrogen, or sulfur, resulting in N-, O-, or S-glycosidic linkage to the ribose[134]. The covalent addition of the first ADP unit can occur at aspartate, glutamate, or lysine amino acid residues; then, the PAR polymer is generated via ribose-ribose bonds where the ribose group of one ADP-ribose unit is connected to the adenosine of the adjacent ADP-ribose unit. At times, branching of the polymer occurs by linkage of non-adenosine ribose groups from neighboring ADP-ribose units[135,136].

While cytoplasmic ARTs generally catalyze mono(ADP-ribosyl)ation, PARylation activity was first associated with chromatin-bound PARP-1 known to be strongly activated upon DNA damage. Thus, initial research efforts around PARPs were done in the context of the DNA damage response. Modulation of protein activity by ADP-ribose binding was suggested when consensus ADP-ribose-binding motifs were identified overlapping functional and/or binding domains of target proteins[137].

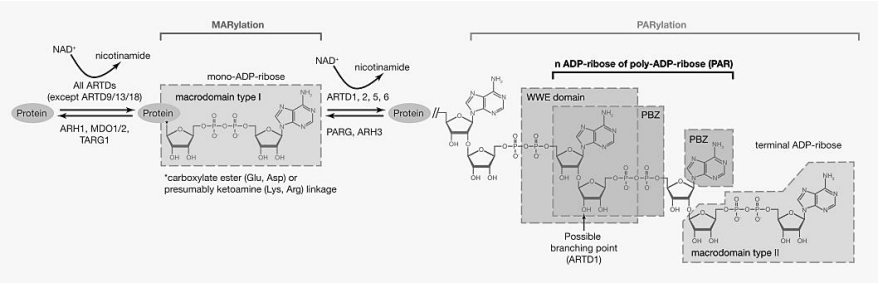


Figure 6.- ADP-Ribosylation. Adapted from[138]. Transient enzymatic reaction of mono(ADP-ribosyl)ation by mono ART and poly(ADP-ribosyl)ation by PARPs of target proteins. The reaction can be reversed by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3 (ARH3).

4. A Family of Poly(ADP-ribose) Polymerases

Poly(ADP-ribose) polymerases are a family of enzymes found in eukaryotes and prokaryotes that generate ADP-ribose modifications onto acceptor proteins[139]. After the identification of PARP-1, additional proteins with homology on their catalytic domain were identified. To date, a family of at least 17 distinct enzymes containing PARP-signature motifs, have been described in humans[128,140,141]. The family can be classified into subclasses based on their domain architectures, namely: DNA-dependent PARPs, which require DNA binding for activation; tankyrases, with protein-binding Ankyrin repairs; CCHH zinc finger PARPs, which bind viral RNA; and macro PARPs, with ADPr-binding macro domains[142]. As pointed before all ARTs share homology on their catalytic domain with conserved NAD^+ binding motifs and showing similar secondary structure. Within the NAD^+ binding motifs a His residue and a Tyr residue are crucial for positioning the A-ribose moiety and the N- ribose moiety of NAD^+ in the correct orientation. Remarkably, a conserved Glu residue in these motifs, is responsible for the ADP-ribose transference[143]. Thus, the substitution of the Glu residue can affect ART activity from PARylation to mono-(ADP-ribosyl)ation. Only PARP-9 and PARP-13 are described as enzymatically inactive while most of the family members (e.g., PARP-3, PARP-4 or PARP-16) catalyze the addition of a single ADP-ribose unit to amino acid residues of acceptor proteins. Lastly, a few members like PARP-1, PARP-2 or PARP-5 exhibit poly(ADP-ribosyl)ation activity enabling the synthesis PAR homo-polymers varying in size and branching[138].

Subfamily	Name	Aliases	Size (aa)	Subcellular Localization	Triad Motif	Key Domains	Activity
DNA dependent	ARTD1 ARTD2 ARTD3	PARP1 PARP2 PARP3	1014 583 533	Nucleus Nucleus and cytoplasm Nucleus and cytoplasm	H-Y-E H-Y-E H-Y-E	zinc fingers, BRCT, WGR WGR, DBD WGR	P P M
Tankyrase	ARTD5 ARTD6	PARP5a, TANK1 PARP5b, TANK2	1327 1166	cytoplasm cytoplasm	H-Y-E H-Y-E	Ankyrin repeat Ankyrin repeat	P P
CCCH Zn Finger	ARTD14 ARTD12 ARTD13	PARP7, tiPARP PARP12 PARP13, ZC3HAV1	657 701 902	Nucleus and cytoplasm cytoplasm cytoplasm	H-Y-I H-Y-I Y-Y-V	zinc fingers, WWE zinc fingers, WWE zinc fingers, WWE	M M I
Macro	ARTD9 ARTD8 ARTD7	PARP9, BAL1 PARP14, BAL2 PARP15, BAL3	854 1801 678	Nucleus and cytoplasm Nucleus and cytoplasm ND	Q-Y-T H-Y-L H-Y-L	macro domain macro domain, WWE macro domain	I M M
unclassified	ARTD4 ARTD17 ARTD16 ARTD10 ARTD11 ARTD15	PARP4 PARP6 PARP8 PARP10 PARP11 PARP16	1724 630 854 1025 331 322	Nucleus and cytoplasm cytoplasm cytoplasm Nucleus and cytoplasm Nucleus and cytoplasm cytoplasm	H-Y-E H-Y-Y H-Y-I H-Y-I H-Y-I H-Y-I	BRCT RRM, UIM WWE	M M M M M M
tRNA phosphotransferase	ARTD18	TRPT1, TpT1	253	cytoplasm	H-H-V		
ecto-ARTs	ARTC1 ARTC2P ARTC3 ARTC4 ARTC5	ART1 ART2P ART3 ART4 ART5	327 389 314 291	ER, plasma membrane plasma membrane, ER plasma membrane extracellular	R-S-E K-L-V G-S-E R-S-E		M M M M

Figure 7.- Family members of PARPs. Table adapted from[127] includes subfamily categorization, alternative nomenclature, amino acids motifs target for modification, and the main enzymatic activity for each member where I is inactive, M is MARylation and P is PARylation.

5. DNA-dependent PARPs

PARP-1 and PARP-2 are the two most relevant PARPs whose catalytic activity is initiated upon DNA damage[144–146]. Both PARP-1 and PARP-2, have poly(ADP-ribosyl) transferase activities and are capable of synthesizing branched PAR polymers[135,147]. Their activity has quite relevant implications in the DNA damage response, as they are largely responsible for signaling DNA breaks, as well as modification and recruitment of DNA repair proteins, chromatin relaxation, and regulation of transcription factors.

PARP-1 (113 kDa) is a highly abundant chromatin-associated protein that accounts for the majority of ADP-ribosyl transferase function in cells. It is

encoded by a gene in the position 1q41-42 of the human genome and 1H5 in the murine genome. PARP-1 is relevant for the maintenance of genomic integrity, chromatin remodeling and gene transcription. PARP-1 contains three functional domains: the N-terminal DNA-binding domain (DBD), a central automodification domain (AMD) and a C-terminal catalytic domain (CD). Subdomains include three zinc fingers motifs (ZF1-3) in the DBD, a BRCT domain in the AMD, a WGR domain, and a catalytic domain (containing two sub-domains: helical domain-HD and ADP-ribose transferase domain-ART) both in the CD domain[145,147,148].

The three zinc finger motifs of PARP-1 associate to DNA single or double-strand breaks. Because the contact is primarily with the ribose-phosphate back-bone of DNA ends, PARP-1 can be activated by DNA breaks independently of the sequence of DNA fragments[149,150]. Furthermore, PARP-1 can recognize diverse oligonucleotide structures. It is believed that ZF1 and ZF2 associate to recognize single-strand breaks (SSBs), whereas ZF1 and ZF3 together are capable of interacting with double-strand breaks (DSBs). Enzymatic activity of PARP-1 can be modulated by PARP-1 itself. Autoinhibition occurs when the catalytic domain associates with a PARP regulatory domain (PRD) present in the protein. Compared to the full-length version of the protein, when PARP-1 is bound to DNA the co-activity of the PRD and catalytic domains is reduced, allowing PARP-1 activation[151]. During DNA damage, when the two zinc finger domains bind a single or double strand break an allosteric signal is transmitted through the PRD domain and inhibition of the catalytic domain is relieved[149]. Removal of the PRD domain results in DNA-independent poly-(ADPribosyl) transferase activity[152]. DNA-dependent activity of PARP-1 results in PARylation of PAPR-1 itself and its target proteins.

PARP -2's gene (62 kDa) is encoded at positions 14q11.2 and 14C1 in human and murine genomes respectively. It was first described after evidencing residual PARP activity in embryonic PARP-1$^{-/-}$ fibroblasts[147]. The second member of the family has the highest resemblance to PARP-1, with around 69% homology on their catalytic domain, suggesting to both share functional properties. PARP-2 is involved in up to 5% of the total DNA-dependent poly(ADP-ribose) synthesis, reflecting lower abundance and/or lower catalytic activity[127].

Like PARP-1, the activity of PARP-2 is incremented in the presence of DNA and RNA in vitro[142,153] and thus is proposed to bind to nucleic acid as well, which is supported by the presence of basic residues in the N-terminal DBD. PARP-2 crystal structure differs from that of PARP-1 in the structure around the acceptor site with a very short DNA-binding domain (DBD) at the N-terminus which contributes only partially in enzyme activation[154]. PARP-2 lacks the zinc finger and BRCT domains that are present in PARP-1. It is however composed of an N-terminal region which contains nuclear and nucleoar localization signal (NSL and NoLS)[145,148], a central domain that harbors both the auto modification domain and a site of interaction with diverse targets[145,155,156] and a catalytic domain at the C-terminal. These structural distinctions may reflect the different substrate affinities that both proteins exhibit. PARP-1 shows high affinity for the linker histone H1, whereas PARP-2 seems to modify a core histone, preferentially[157]. PARP-2 also shows effective ADP-ribosylation of 5'P DNA substrates with a short double-stranded part, reflecting PARP-2's ability to PARylate DNA in a 5'-phosphorylated nick- or gap-independent manner[158]. Nevertheless, PARP-1 and PARP-2 can heterodimerize and interact with common nuclear proteins, such as X-ray repair cross complementing I (XRCC1), DNA polymerase (DNA pol)β and DNA ligaseIII, involved in DNA repair[127].

The third member described as a DNA-dependent PARP is PARP-3 (60 kDa). PARP-3 is able to modify itself, other proteins such as histone H1 and fragmented DNA in response to genotoxic stress. With the later, it may also form a specific primed structure for further use by the repair proteins[159,160]. In contrast to the other two DNA-dependent PARPs, PARP-3 mediates mono-ADP(ribosyl)ation of fragmented DNA. It effectively catalyzes MARylation of terminal phosphate residues of disrupted DSB and SSB of DNA molecules of different length. It is structurally similar to PARP-2 and shares substrate specificity with its ADP-ribosylation activity being is also independent on DNA sequence or on the nature of the phosphorylated 5-terminal nucleotide at the acceptor site of DNA. MARylated DSB termini can be subject to total degradation by PARG while MAR adducts may be left attached to aminoacid residudes[158]. Interestingly PARP-3 can interact with and activate PARP-1 in the absence of DNA. Among the key functions of PARP-3, its role in genomic integrity, mitotic division, as well as the recruitment of aprataxin-like factor (APLF) to DNA DSBs, stand out[161,162].

6. PAR Polymers: synthesis and degradation

Synthesis of PAR polymers can be summarized in three steps. First, PARPs catalyze the transfer of a single ADPr unit from a NAD^+ molecule to an acceptor amino acid residue to the target protein. Then, poly(ADP-ribose) polymers can be synthesis through the sequential transfer of ADP-ribose moieties to the initial ADPr unit linked by an α(1’’-2') ribosyl-ribose bond to the adenine ribose of the preceding ADPr molecule generating poly(ADP-ribose) linear chains. Lastly, some PARPs are able catalyze branching of the PAR polymer by transfering of ADP-ribose moieties from NAD^+ to the linear chain via1’’-2’’ glycoside branch[127,134,164].

PAR polymers have a short life and they are quickly removed from acceptor proteins mainly by two distinct enzymes. In its majority, catabolism of PAR is mediated PARG, an enzyme with both exo- and endoglycosidase activities that hydrolyze the glycosidic linkages between the ADP-ribose units of PAR generating free ADP-ribose[165,166]. Despite PARP-1 being present at 5 to 20-fold over PARG in some cell types, diverse regulatory mechanisms ensure tight control of PAR levels in the nucleus[165,167]. In this sense, PARG's enzymatic activity is increased with increased PAR length[168] and it is also affected by the nature of the acceptor protein or the cell cycle's phase. On the other hand, the basal activity of PARP-1 is quite low and it is only stimulated upon binding to targets[169,170]. Another PAR-degradation pathway is mediated by ARH3, a structurally unrelated enzyme able to split the ADP-ribose glycosidic linkages by hydrolysis[164]. PARG and ARH3 are proposed to act in tandem, regulating nuclear and cytoplasmic PAR degradation upon hydrogen peroxide (H_2O_2) exposure[171]. Unbound PAR, the product of catabolic activity of PARG, can be translocated to the cytoplasm where it may trigger *pharthanatos*, a cell death pathway mediated by apoptosis induction factor (AIF). ARH3's protective role entails lowering PAR levels in the nucleus and cytoplasm thus preventing translocation of the polymers[171,172].

7. PARP-1 and PARP-2: Physiological and pathological implications

PARP-1 and PARP-2 belong to the superfamily of proteins that catalyze the transference of ADP-ribose to acceptor proteins in a PTM manner known as PARylation. Mice that are deficient for PARP-1 or PARP-2 exhibit important alterations in biological processes such as the maintenance of chromatin structure and DNA repair pathways, suggesting some redundancy in the biological functions of these proteins[163]. In fact, the double deficiency of PARP-1 and PARP-2 in mice is lethal at embryonic stage[146].

7.1 PARP-1 and PARP-2 in genomic stability, DNA damage and repair

Genome integrity is under constant threat from DNA damaging agents of both endogenous and exogenous origins[173]. Metabolic products (e.g., reactive oxygen species, eroded telomeres or immune mediators) and environmental genotoxic elements (e.g., radiation and drugs) continuously cause DNA lesions that must be effectively recognized and repaired. As DNA-dependent PARPs, PARP-1 and PARP-2 are able to sense DNA breaks and have a direct implication in cellular response to DNA damage by regulating the activity of DNA repair proteins and facilitating their recruitment to the DNA strand lesion[137,147,174–176]. Accordingly evidence supports that the depletion of nuclear NAD^+ upon DNA damage is a consequence of the cofactor serving as a substrate for poly(ADP-ribosyl)ation[177]. The complete reversibility of DNA ADP-ribosylation suggests that modification by PARP of DNA strand break termini serves rather as transient marks as compared to that of proteins[158].

Furthering evidence shows that PARP-1 knockout mice are viable and fertile, but are susceptible to genomic instability as well as dysregulation of gene expression[132,174,178]. Likewise, PARP-2$^{-/-}$ mice are sensitive to ionizing radiation and treatment with alkylating agents also proved to increase genomic instability and chromosome missegregation in PARP-2$^{-/-}$ embryonic fibroblasts[146]. In vivo studies thus show that depletion of PARP-1 or PARP-2 results in hypersensitivity to alkylating agents, oxidative stress and high doses of ionizing radiation[145,146,174] with PARP-2 but not PARP-1 deficiency also producing 'hyperradiosensitivity' to low-dose IR (<2 Gy)[179]. Accordingly, PARP-1$^{-/-}$ and PARP-2$^{-/-}$ cells exhibit increased spontaneous genomic instability[180,181].

The DNA perturbations observed in genetic knockouts reflect the central role that PARP-1 and PARP-2 have on the cellular response to DNA damage. Both proteins share some key functions in DNA damage response including chromatin de-condensation, recruitment and/or modification of PAR-binding factors and transcriptional regulation. Some of their target proteins (e.g. XRCC1, DNA pol-β, the RAD50-MRE11-NBS1 (MRN) complex, Topo I) are involved in DNA repair. PARylation by PARPs provides a scaffold for the recruitment of DNA repair machinery. PARP-1 and PARP-2 therefore contribute to a different extent to Single-Strand Break or Base Excision repair (SSB/BER)[156,182], and Double-Strand Break (DBS) repair[183–186]. A summary of PARP implication in the DNA damage response is illustrated next:

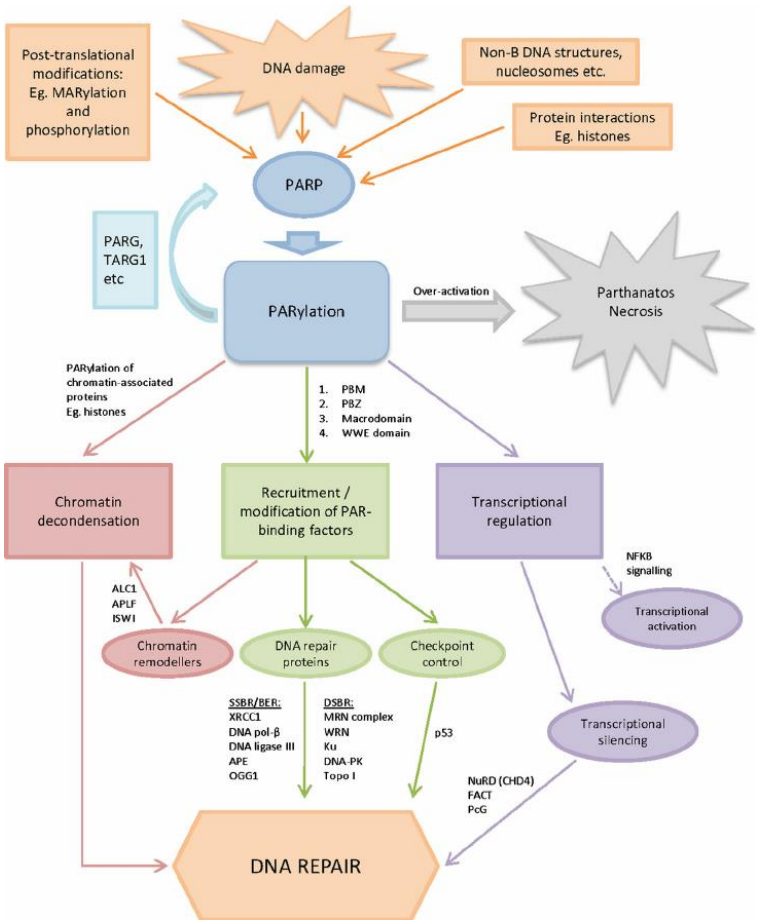


Figure 8.- PARylation in the DNA damage response. Adapted from[187]. Apart from other stimuli, PARP is activated upon binding to damaged DNA. The roles of PARylation in the DNA damage response can be divided into: chromatin decondensation, recruitment and/or modification of PAR-binding factors and transcriptional regulation. Excessive PARylation leads to cell death by parthanatos or necrosis pathways.

7.1.1 PARylation in Base excision repair BER

Sometimes, DNA lesions are presented as small alterations of bases, such as deamination, methylation or hydroxylation consequence of cellular metabolism; or as oxidation and alkylation of nucleotides, caused by endogenous or environmentally-induced ROS[188,189]. Base excision repair

(BER) is used by the cell to resolve single strand breaks and correct damaged DNA bases. The reaction is initiated by DNA glycosylases which, by hydrolysis of the N-glycosyl bond, generate AP sites that are further transformed into a single nucleotide gap primarily by AP endonuclease APE1[182,190]. The single strand break site becomes a substrate for DNA polβ which processes and fills the gap that is then subsequently ligated and sealed by the XRCC-1-ligaseIIIα complex[190].

DNA bound PARPs suffer auto poly(ADP-ribosyl)ation which results in their release from DNA thereby granting access of BER machinery to the damage DNA[168,180]. Although PARP-1 and PARP-2 are simultaneously required for the formation of the BER complex, the recruitment of XCCR1 is only dependent on PARP-1[191,192]. Likewise, PARP-2 also has unique partners, such as the telomeric protein TRF-2 and it is not regulated by autoPARylation[146,156,193]. PARP-1 and PARP-2 have different incorporation kinetics to the BER complex. In accordance to its higher affinity for SSB, PARP-1 shows a transient but rapid accumulation whereas the addition of PARP-2 is delayed and persists associated with later BER intermediates such as gaps or flaps structures[156,194]. Interaction with the BER complex inhibits the DNA synthetic and endonuclease activities of FEN1 and DNA Ligase III, respectively. This inhibition can be relieved by PARP-1 but not PARP-2 PARylation but PARP-2 acts to abrogate the restorative polymerisation of PARP-1[195].

7.1.2 PARylation in Nucleotide excision repair NER

Nucleotide excision repair (NER) pathway is required to prevent DNA mutations and chromosome aberrations caused by UV radiation, mutagenic chemicals, or chemotherapeutic drugs[196]. NER pathway detects DNA helix distorting photolesions, product of ultraviolet radiation (UVR) exposure. Increasing evidence designs a role of PARP-1 in UV-induced DNA damage response and a role in lesion recognition steps in NER. For

instance, significant accumulation of UVR-induced photoproducts is observed after genetic silencing or pharmacological inhibition of PARP-1[197]. On the other hand PARP activation following UVR exposure promotes the association between PARP-1 and XPA, a central protein in NER which has a repair-promoting function when bound to DNA[198] and regulates XPA its association with chromatin[197].

7.1.3 PARylation in Double strand break repair DSBR

Double strand breaks are arguably the most significant type of DNA lesions. Not only can they result in cell death if left unresolved but, misrepaired DSB can lead to chromosome loss, chromosomal rearrangements, apoptosis, or carcinogenesis[199]. Causes of DSBs include environmental factors (ROS, IR or X-Ray and specific antineoplastic drugs) and endogenous determinants mainly during the cell cycle and DNA replication[200,201]. Spontaneous DSB may be generated indirectly from two closely located single-strand breaks, or during the repair of other lesions[186]. DSB can be repaired by Non-homologous end-joining (NHEJ) and homologous recombination (HR) requiring different degrees of chromatin remodeling and thus being determined by chromatin state and phase of the cell cycle[201-203]. Growing evidence implicates poly(APD-ribosyl)ation in these repair pathways as cells deficient in proteins involved in HR and NHEJ are exceptionally sensitive to PARP inhibition[204-206].

Besides supporting DNA replication and telomere maintenance, HR serves as a template-dependent DNA repair mechanism at the S or G2 phase of the cell cycle where sister chromatid or a homologous chromosome are available[207,208]. HR is a multistep process entailing contribution of numerous proteins, beginning with immediate phosphorylation of H2AX followed by rapid migration of BRCA1 protein.

Initial recruitment of MRN complex for HR repair requires BRCA1 as a scaffold protein. The MRN complex has 3'-5' exonuclease activity that enables the initial processing of the 3' ends at either side of DSBs[209,210]. Later steps involve BRCA2 for the recruitment stabilization of RAD51 which then mediates the alignment of the damaged strand and its complementary sequence and further processing of the lesion[211,212]. The 3' is finally extended by DNA polymerases and replication is resolved releasing each strand to their original chromosome[201]. meiotic recombination 11 (MRE11) has a putative PAR binding domain and rapid accumulation of MRE11 and NBS1 at sites of DNA damage requires PARP-1[213]. PARP-1 and PARP-2 are relevant in early detection of stalled or collapsed forks and rapid recruitment of MRE11 and so they may be crucial for the choice of DNA repair pathway, shifting the decision towards HR[214-216].

NHEJ is the main repair pathways used in mammalian cells throughout the cell cycle, preferably acting at G1 phase when the DNA template required for HR is absent[203]. NHEJ relies essentially on the binding of DNA dependent protein kinase subunit (DNA-PKs) to Ku70/Ku80 heterodimer and XRCC4-DNA ligase complex IV. Numerous reports account for a functional interaction between PARPs and NHEJ related proteins like Ku70/Ku80-independent simulation of DNA-PKcs activity upon PARylation[217]. In addition, in vivo studies report embryonic lethality in mice with a PARP-1/Ku80 double deficiency and mice deficient for ATM and PARP-1 or PARP-2[218,219].

7.2 PARP-1 and PARP-2 in chromatin structure and transcription

Chromatin is a highly dynamic macromolecular entity which is subject to continuous changes in structure and organization. Remodeling of local

chromatin domains allows DNA replication, transcription, repair and recombination and depends on the core domains of histones that mediate compaction of DNA[220]. These core domains are subject to various forms of PTMs including acetylation, methylation, ubiquitylation, phosphorylation and ADP-ribosylation[221–223]. In this regard, increasing evidence designs an important role of PARPs in regulation of gene expression by at least two, non-redundant, mechanisms: through the modulation of chromatin structure[144] or functioning as part of enhancer/promoter binding complexes in conjunction with other DNA binding factors and coactivators (e.g., direct interaction with transcription factors and/or transcription factor binding sites)[224]. PARPs catalyze direct PARylation of several nuclear proteins such as histones and other chromatin structure modulators, but also DNA polymerases, topoisomerases I and II, Ca_2^+ and Mg_2^+ endonucleases and proliferating cell number antigen (PCNA), hence modulating epigenetic marks and overall chromatin structure[126,225,226].

Over 20 years ago chromatin decondensation upon PARP-1 activation was described[163]. Mechanistically, a bulk negative charge is added onto chromatin by direct PARylation of DNA-associated histones which generates a strong electrostatic repulsion and ultimately their release from the DNA prompting chromatin relxation[173,227]. This is for example the case for heterochromatin-promoting histone H1, whose PARylation mediated removal from chromatin promotes chromatin decondensation[228,229]. Likewise, PARP activity is required for chromatin loosening in stress induced genes in drosophila where PARP-1 presence is described at transcriptionally repressed chromatin regions[230]. Histones H1 and H2B show the most poly(ADP-ribosyl)ation in vivo and, while PARP-1 preferentially targets linker histone H1, H2A/H2B are the favored targets of PARP-2[149,231,232]. Of late, covalent modifications on

specific lysine residues of all four histones have been ascribed to PARP-1 but not PARP-2[233]. Chromatin modulation by PARPs is also affected by modification and recruitment of non-histone chromosomal proteins such as High Mobility Group Proteins (HMGP) and heterochromatin proteins HP1a and HP1b[234].

Related to their role in chromatin dynamics contribution of PARP and poly(ADP-ribosyl)ation to transcription regulation of specific genes have been demonstrated. For instance PARP-1 can directly alter the activity of enhancers and promoters (e.g., NF-κB, MYBL2, Oct-1, nuclear receptors, and the HTLV Tax-1 protein) acting like a "classical" transcriptional regulator or coregulator[235–237]. Likewise PARP-2 also interacts with transcription factors such as nuclear Estrogen Receptor (ER)α and Peroxisome Proliferator-Activated Receptor (PPAR) and with transcriptional Intermediary Factor 1 (TIF)β[238,239]. Furthermore, trans-activating factors such as NFAT, Klf8 and Tef-1 are associated with, and activated by PAR polymers probably functioning as scaffolds to retain these factors at target genes[240–242]. Although PARylation normally leads to protein activation, it can also mediate functional suppression of chromatin remodelers such as nucleosome-remodeling ATPase Iswi which promotes the association between H1 and DNA[243]. Other non-enzymatic mechanism of transcriptional repression by PARP-1 has been described at a subset of promoters where, under unstimulated conditions, PARP-1 presents together with nucleolin, nucleophosmin, and Hsp70 forming a corepressor complex that is released upon signal activation of PARP-1[243]. Recent evidence involves PARP-1 also in bridging chromatin to RNA and recruiting splicing factors highlighting its involvement in co-transcriptional splicing. It may also modulate alternative splicing through the regulation of RNA Polymerase II elongation[224].

7.3 PARP-1 and PARP-2 in cell differentiation processes

Increasing evidence reveals unique functions of poly(ADP-ribose) polymerases PARP-1 and PARP-2 in cell differentiation. For example Dnmt1, a recognized target of PArylation and DNA methylation, is known to be required for the proper expression of specific, lineage and function-defining genes in T cells and other somatic mammalians cell. Precisely, PARP-1 is able to directly influence DNA methylation patterns controlling transcription and activity of Dnmt1 which functions to preserve the methylation state of differentially methylated regions (DMRs) of imprinted genes[244–246]. These data seems to reflect a role of poly(ADP-ribosyl)ation also as a regulatory mechanism of epigenetic-driven events. As previously mentioned, PARP-1 and PARP-2 are able to physically and functionally interact, to a different extent, with endodermal-differentiation factors TIF1β and HP1[238]. Lately, PARP-2 but not of PARP-1, has also been associated with a number of differentiation processes, including adipogenesis, spermatogenesis and T lymphocyte development[145,247]. On the other hand, PARP-1 has been implicated in both B and T cell maturation where it acts as anti-recombinant factor, and even in terminal osteogenic differentiation as suggested by the presence of PAR polymers in the extracellular matrix of dead osteoblasts[248,249].

7.4 PARP-1 and PARP-2 in replicative stress and cell cycle regulation

Stringent regulation of replication of DNA is required to ensure genome integrity. In mammalian cells the velocity of replication forks during S phase, is adjusted to ensure DNA replication in pace with the cell cycle. Fork stalling induced by replicative stress prompts genome instability and carcinogenesis[250]. Besides PARPs role in stalled fork detection and resolution via MRE-11-induced HR[209,210], recent studies describe a

coordinated regulatory network where p53, and PARP-1, mediate modulation of fork progression by PARylation and the PCNA interactor P21Cip1 (P21) which act as fork speed suppressor[251]. P21 is an important regulator of the cell cycle acting at G1/S and G2/M phases and also presents functional interaction with PARP-2 as suggested by the embryonic lethality phenotype observed in double null mice[252]. PARP-2 also represses several other cell cycle related genes such as RB, E2F1 and oncogene c-MYC, through its interaction with different histone modifier enzymes (e.g., HDAC5, HDAC7 and histone methyltransferase G9a[253]. Furthermore, PARP-2 deficiency in erythroblasts triggers replicative stress, as indicated by the presence of micronuclei, the accumulation of γ-H2AX (phospho-histone H2AX) in S-phase cells and constitutive CHK1 and replication protein A phosphorylation[252].

7.5 PARP-1 and PARP-2 in cell death

Contrasting their previously described relevance in cell survival PARPs have a role in promoting cell death when DNA lesions are severe rather than mild. PAR's most remarkable contribution is perhaps to parthanatos (or PAR-mediated cell death) via an apoptosis-inducing factor (AIF)-mediated mechanism. Upon extensive DNA damage or likewise toxic stimuli (e.g., ROS or oxidative stress); and following excessive PARP activation, a vast number of PAR polymers are produced prompting a parthanic toxic cascade[254]. In brief, nuclear-to-mitochondrial translocation of PAR triggers the translocation of AIF to the nucleus and mediates a caspase-independent cell death by chromatin condensation and fragmentation[255,256]. In support of this model fibroblasts isolated from PARP-1 knockout mice showed suppression of cell death and persistence of AIF in mitochondria[257].

During caspase-dependent apoptosis, PARP-1 and PARP-2 (although delayed in time) are inactivated upon cleavage by caspase 3 and caspase

7, which releases its DNA-binding domain from its catalytic domain[146]. As a consequence of PARP inactivation further depletion of cellular NAD^+ is stopped likely avoiding the toxic effects associated with high levels of ATP (e.g., necrotic cell death, damage to neighboring cells and inflammation). In the same way, inactivation of PARP-1 and PARP-2 reduces the ability of the cell to repair DNA damage and therefore increases the rate of apoptotic cell death[229]. In addition PARP relevant apoptosis related genes such as Bcl10, c-Rel, and tumor necrosis factor-related apoptosis-inducing ligand receptor-1 and - 2 are differentially expressed after induction of apoptosis in a PARP-1 but not PARP-2 - dependent manner[258].

Some roles for PARPs in necroptosis have also been suggested. A novel pathway of programmed necrosis involving Receptor-Interacting- Proteins (RIPs) implicates PARP-1 through direct interaction with RIPs (containing PARP binding motif) and/or poly(ADP-ribosyl)ation of necroptosis effectors[259].

7.6 PARP-1 and PARP-2 in metabolic regulation and disease

Metabolic pathways are subject to strict regulating on various levels, including co-enzyme availability, allosteric regulation via various metabolic intermediates, and post-translational modification of metabolic enzymes. Cofactor NAD^+ is a vital player in cell metabolism through its electron transfer function in redox reactions. In many cellular response pathways, PARP-1 and PARP-2 functions are mediated by the synthesis of PAR polymers using NAD^+ as a donor of ADP-ribose units. Thus, PARP activity has a strong impact intracellular NAD^+ homeostasis and the broader metabolic profile of the cell[224,260]. Persistent activation of PARP forces the cell to consume ATP for NAD^+ recovery creating a feedback loop that can compromise cell survival[261].

Related to its influence on NAD^+ metabolism, ADP-ribosylation has also been implicated, directly or indirectly, in other processes such as glycolysis and oxidative phosphorylation or lipid metabolism[163]. PARP- 1 and PARP-2 interact with a large number of nuclear receptors and transcription factors regulating mitochondrial and lipid oxidation genes, such as PPARγ, FOXO1, and ER with both repression an activation impact[261]. Additionally, PARP deletion or pharmacological inhibition correlates with a protective phenotype in aging and high-fat induced obesity models, with PARP-1 and PARP-2 knock out mice exhibiting higher catabolism rates respectively in brown adipose tissue and liver[261]. Additionally, inhibition of PARP-1 normalizes, through an unknown mechanism, pathological Low Density/High Density Lipoprotein (LDL/HDL) ratios and PARP-$2^{-/-}$ mice display impaired cholesterol transport as evident by their reduced levels of HDL in serum[261,262]

Several studies appoint that PARP-1 deletion enhances mitochondria biogenesis in skeletal muscle by improving insulin sensitivity. On its part, PARP-2 knock outs do not respond properly to glucose intake and show smaller islets and diminished insulin content, suggesting a pancreatic role of PARP-2 in β cell proliferation. These observations certainly implicate PARPs in metabolic syndrome and Type I-Type II diabetes, which are pathologies characterized by impaired glucose/lipid metabolism and insulin sensibility[261].

Finally, NAD^+-dependent type III deacetylase SIRT1 plays a major role in global metabolic homeostasis functioning as a metabolic sensor to modulate the use of different energetic substrates and their correspondent transcription programs. PARP-1 compromises SIRT1 activity due to the avid consumption of NAD^+, as suggested by the increased SIRT1 activation and consequent mitochondrial metabolism enhancement observed in PARP-$1^{-/-}$ mice[263]. Similarly, PARP-2 acts as a direct negative

regulator of SIRT1 promoter and thus, loss of PARP-2 function produces upregulation of SIRT1 promoting energy expenditure, and increasing mitochondrial content in mice[264].

7.7 PARP-1 and PARP-2 in angiogenesis and inflammation

The angiogenic response, a process of new blood vessel formation, is required in a plethora of tightly regulated physiological processes such as tissue damage healing or endometrial hyperplasia, but serves as a pathological contributor in tumor expansion and psoriasis if uncontrolled. The activity of PARP-1 is implicated in various angiogenesis-related properties of endothelial cells (EC). PARP-1 pharmacological inhibition reduces angiogenesis by blocking EC proliferation, capillary morphogenesis and vascular endothelial growth factor (VEGF) activation in vitro and in vivo[265–267]. EC adhesion and tube-formation are also impaired when PARP-1 or PARP-2 are inhibited[232]. Additionally, knock-down of PARP-1 correlates with a significant reduction of VEGF mRNA a protein expression in human-derived ovarian cells[268].

Besides its role in the modulation of energetic metabolism, aforementioned SIRT1 exerts a proangiogenic effect through several mechanisms that include repression of the antiangiogenic transcription factor FOXO1 and activation of the proangiogenic transcriptional regulator PGC-1α[269]. In addition, SIRT1 is also involved in the Notch signaling pathway, an important regulator of blood vessel modeling and growth[232,270].

Inflammation is the initial defensive process employed by mammalian cells against tissue damage or infections. This complex response involves cells of leukocyte origin (namely macrophages, neutrophils, and lymphocytes) which release substances that mediate the inflammatory process towards restoration of the affected tissue. On the other hand,

tumorigenic properties have been correlated with a pro-inflammatory tumor microenvironment[4,271]. PARPs play major roles in promoting inflammatory responses primarily sustaining the expression of cytokines, chemokines and other mediators such as tumor necrosis factor-a (TNF-a), interleukin-1 (IL-1), IL-6, interferon-c (IFN-c), CCL3 and inducible nitric-oxide synthase (iNOS); and through stimulation of pro-inflammatory signal transduction pathways. PARP-1 and PARP-2 seem to have overlapping regulatory roles for some genes such as iNOS, Il-1β and TNFa whose levels are lowered in the absence of either protein[272]. PARPs also regulate different EC surface adhesion molecules (I-CAM, V-CAM, L-CAM) and selectins which mediate migration of inflammatory cells to the site of inflammation. Consistently, PARP gene knock-down or pharmacological inhibition halts inflammation response through impairment of cell migration to inflammatory sites. Additionally, PARP-induced necrotic cell death releases intracellular components such as HMGB1 protein that contribute to promote inflammation in surrounding tissues.

Regarding the specific role of PARP-1 in inflammation, most studies point to its functional interaction with NF-κB, an important transcriptional regulator of inflammation, and to NAD^+ depletion as a consequence of PARP-1 over-activation. Additionally, PARP-1 is involved in gene expression and activation of neutrophils, macrophages, dendritic cells, microglia and other cell types[273–275]. Specific roles of PARP-2 in the inflammatory process are still elusive, while it may also act as cofactor of pro-inflammatory cytokines and regulatory factors, namely NF-κB - dependent expression of adhesion factors; its implication has been stronger in the regulation of non-classical regulators such as SIRT1.

A wide spectrum of inflammatory diseases are PARP mediated and they affect multiple systems in the organism including neuroinflammation and

the central nervous system, gastrointestinal tract (e.g., colitis), cardiovascular system (e.g., atherosclerosis), respiratory system, and tissues such as skin, kidneys, bones or muscle[163,276]. Furthermore, the two enzymes have been associated with autoimmune pathologies like autoimmune encephalomyelitis (EAE) or immune nephritis[277].

7.8 Immunomodulatory roles of PARP-1 and PARP-2

Immunological roles of PARP-1 and PARP-2 are subject of growing interest in recent years. As advanced earlier, PARP-1 and PARP-2 are involved in the development and maturation of immune cells and erythrocytes and undoubtedly relevant in the modulation of both innate and adaptive immune responses. For instance, PARPs influence on inflammation also contributes to the upregulation of danger signals, creating the conditions to initiate and sustain the innate immune response[275]. Moreover, PARP-1 has been implicated in terminal differentiation of monocytes, dendritic cells and natural killer cells[275,278,279]; whereas PARP-2, has shown to shorten the lifespan of erythrocytes and cause chronic anemia through impairment of erythroid progenitors differentiation[252].

PARylation also regulates the development and function of T cells, a major player in the adaptive immune response. For instance a role for PARP-2 but not PARP-1, has been reported in thymocyte development without affecting peripheral T cell homeostasis[147]. PARP-2$^{-/-}$ DP thymocytes harbor defects in TCRα expression that have been correlated to DP thymocyte death soon after the initiation of TCRα rearrangement[280]. However, mice with a dual deficiency for PARP-1 and PARP-2 show affectation of T cell maturation in the thymus and periphery, due to a DNA damage accumulation and concomitant cell death rather than in proliferation reflecting in the different T cell compartments. In addition, T cell specific double mutant mice exhibit faulty T-dependent antibody

response and the generation of spontaneous T cell lymphomas[280]. Some non-redundant roles have been ascribed to the two enzymes. PARP-1 modulates the activity of certain transcription factors that are important in T cell development and function (e.g., NFAT and FoxP3) and is described to promote Th1 responses[281–283]. In contrast PARP-2 was found to be relevant in Th1 and Th17 cell infiltration of central nervous system in a model of EAE[277].

In accordance with these observations, poly(ADP-ribosyl)ation is frequently associated with immune-mediated pathologies including autoimmune diseases, infection and allergies. The first evidence of PARP-1 involvement in an immune-mediated diseases was observed when genetic silencing of PARP-1 gene in a mouse model of rheumatoid arthritis ameliorated the severity of the disease, showing reduced bone and cartilage damage correlating with lower expression of monocyte chemoattractant[284]. Similarly, inhibition of poly(ADP-ribosylation) prevents T cell driven immunopathology and reduces inflammation in H. Pylori infected gastric epithelial cells preventing and reverting pre-neoplastic lesions[285]. PARP-1 modulates STAT-6 dependent gene transcription of IL-5 influencing the pathogenesis of allergen-induced inflammation and airway hyper-responsiveness (AHR)[286]. Nevertheless, further studies are required to understand the specific function of PARP-1 and PARP-2 in immunomodulation and its role in tumor destruction and immune evasion.

8. PARP inhibitors in cancer

8.1 Therapeutic rationale behind PARP inhibitors

Acting as molecular sensors of DNA damage, PARPs contribute to the maintenance of genome integrity and cell survival with key roles regarding the spatial and temporal organization of DNA lesions. Thus, great interest soon arouse in generating PARP inhibitory molecules for the treatment of pathologies related with genomic integrity but also stress response and inflammation where over activation of PARP-1 is one of the known origins. The rationale behind targeting PARP for pharmacological inhibition exploits the impairment of the DNA repair cascade initiated by PARP. In this sense, the antitumoral effects of PARP inhibition are a consequence of two different but complementary approaches, synthetic lethality and chemosensitization[287].

Mechanistically, after detecting DNA single strand breaks, PARPs bind to DNA and catalyze a series of PARylation events concluding autoPARylation and subsequent release of the enzyme from DNA. This allows recruitment of DNA repair factors and their access to the lesion in order to solve them by BER[168,180]. PARP inhibitors prevent the release of PARP from DNA by hindering autoPARylation. The result is the persistence of SSBs and stalled forks during replication followed by degeneration of the nicks into DSBs. Halting PARP-mediated repair fires backup DNA repair mechanisms in the cell, such as homologous recombination[287–289]. While either loss of function alone is viable, co-occurrence of HR deficiencies and PARP's pharmacological inhibition is fatal for the cell. Synthetic lethality thus occurs when, unrepaired DNA accumulate in DSB form and these cells undergo chromatid instability leading to cell cycle arrest and eventually to cell death[288]. When combined with cytotoxic therapy (DNA damaging agents or IR), PARP inhibition

enhances the antitumoral effect sensitizing and reducing resistance to chemotherapy.

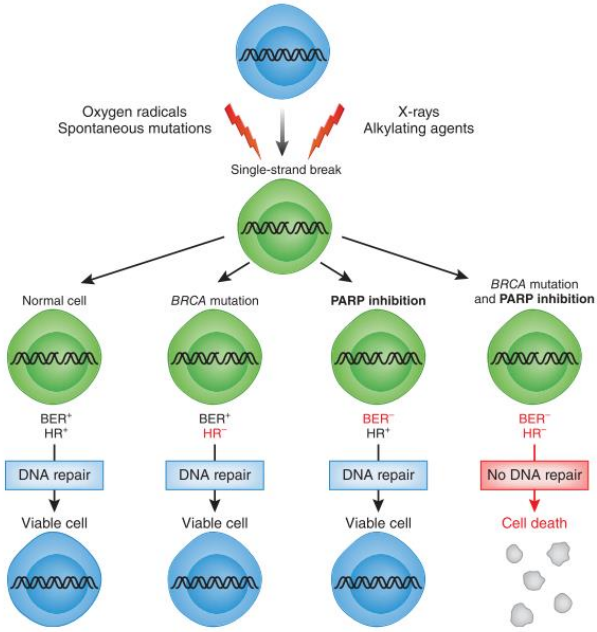


Figure 9.- PARP pharmacological inhibition as cancer therapy. Adapted from[287]. PARP inhibitors cause death by synthetic lethality of cancer cells in which DNA repair by homologous recombination is s impaired (e.g., BRCA mutated cancer cells).

As mentioned previously BRCA1 and BRCA2 proteins play quite relevant roles in cell cycle control, transcription regulation and DSB repair by HR. For that reason these proteins are classical lethal partners of PARP inhibition[290].

Hence, the impression that certain tumors defective in homologous recombination mechanisms, may rely on PARP-mediated DNA repair for survival, has motivated the development of PARP inhibitors in cancer therapy to potentiate, the cytotoxicity of anticancer drugs and radiotherapy[291,292]. Over the last decade growing evidence shows that BRCA1/2-deficient mammary tumors, and derived cell lines, are more

sensitive to PARP inhibition than proficient cell lines[293,294]. Nonetheless, selective sensitivity of BRCA1/2- deficient cells was discovered to be dependent on the specificity and potency of the PARP inhibitor[290]. The pipeline of PARP inhibitor development has had to overcome several obstacles and clinical setbacks. For instance, certain tumors with frame-shift BRCA1/2 mutations are able to develop resistance to PARP inhibition and chemotherapy through secondary mutations that restore the expression and HR function of these proteins[295] and some of the most promising candidates failed to pass phase 3 of clinical trials for TNBC[287].

8.2 PARP inhibitors

Most PARP inhibitors prevent auto PARylation and poly(ADP-ribosyl)ation of target proteins by mimicking the NAD^+ moiety and competing for the catalytic domain of the enzyme[296]. Cytotoxicity of the PARP inhibitors mainly depends on the trapping potential and formation of PARP-DNA complexes. All PARP inhibitors target indistinctly PARP-1 and PARP-2 in vitro at nanomolar concentrations but exhibit different PARP-trapping efficacy on the DNA SSB sites[289]. The new generation of PARP inhibitors derive primarily from the structure of NAD^+ analogue, 3-aminobenzamide (3-AB), and include different types of molecules such as benzimidazoles (ABT-888 or veliparib), dihydroisoquinolinones (INO1001), pthalazinones (AZD-2281 or olaparib), tryciclic indoles (AG-014699) as well as other not disclosed structures (BSI-201 or iniparib). Most recent clinical trials in ovarian cancer include four already approved compounds: olaparib, rucaparib, talazoparib and niraparib[289,297].

HYPOTHESIS AND OBJECTIVES

Tumors are complex entities harboring an intricate network of tumor cells, stromal cells and tumor-infiltrating immune cells of both innate and adaptive lineages. Generally these immune cells are not able to mount a proper anti-tumor response which has generated an imperative need to understand how these diverse cells communicate with each other in order to modulate the immune response. Despite the considerable enthusiasm about the prospect of anti-cancer compounds that act through targeting PARP-proteins, no attention has been taken regarding the role of these proteins in the modulation of the immune response to tumors. However, both PARP-1 and PARP-2 play specific roles in the immune system cells.

The aim of the present thesis is therefore to explore the immunomodulatory functions of PARP-1 and PARP-2 and their relevance on the immune response to breast cancer. To achieve our general objective we propose the following goals:

1. To explore the impact of PARP-1 and/or PARP-2 deficiencies mice harboring syngeneic tumors.
2. To elucidate the mechanisms by which PARP-1 and PARP-2 specifically modulate the immune responses to cancer cells.
3. To study the differential expression of tumor-infiltrating T cells harboring different PARP-1 and PARP-2 deficiencies.

MATERIALS AND METHODS

1. Mouse model

1.1 Mice

Total Parp-1-deficient mice (Parp-$1^{-/-}$), Parp-$2^{flox/flox}$ and transgenic mice for cre-recombinase driven by Cd4 promoter (Cd4-cre) have been previously describe[146,245,280]. Cd4-cre; Parp-$2^{flox/-}$; Parp-$1^{+/-}$ heterozygous mice were backcrossed to generate all the possible cohorts, with our interest in Cd4-creParp-$1^{+/+}$Parp-$2^{+/+}$, Cd4-creParp-$1^{-/-}$Parp-$2^{+/+}$, Cd4-cre Parp-$1^{+/+}$Parp-$2^{f/f}$, and Cd4-cre Parp-$1^{-/-}$Parp-$2^{f/f}$ mice[280].

C57BL/6J and SCID Beige mice were obtained from Charles River Laboratories. All mice were reared under specific pathogen-free conditions at the Animal House Facility of Barcelona Biomedical Research Park (PRBB). The PRBB Institutional Animal Care and Use Committee approved the studies and all experiments were performed in accordance with relevant guidelines and regulations.

1.2 Mouse genotyping

1.2.1 Tail/ear biopsy

During mice weaning a tail and/or ear biopsy was obtained by technicians from the Animal House facility and put to our disposal in order to characterize the genotype of each animal. All littermates in each cage were properly identified by small ear perforations and assigned an identification number linked to the Animal Facility platform. Tail/ear tissue was kept at -20ºC in an eppendorf tube with the corresponding ID number until genomic DNA extraction.

1.2.2 Genomic DNA extraction

DNA extraction from tail/ear tissue was performed using an isopropanol precipitation protocol as follows:

1. Add to each sample 400µl of lysis buffer (100 mM Tris-HCl pH=8.5, 5mM EDTA, 200mM NaCl, 1% SDS)
2. Add to each sample 10µl of Proteinase K (stock 20 mg/ml) (Roche, Basel, Switzerland)
3. Vortex and incubate at 55ºC overnight
4. Spin 8min at 17000xg
5. Recollect 350µl of supernatant
6. Add 350µl of Isopropanol
7. Mix by inversion until precipitated DNA is apparent
8. Spin 5' at 17000xg and discard supernatant
9. Resuspend pellet in 350µl of Ethanol 70%
10. Spin 5' at 17000xg, discard supernatant and let pellet dry at RT
11. Add 500µl of TE and leave at RT o/n or until use

1.2.3 End-point PCR

PCR for genotypic characterization was performed in any of two thermo cyclers (MyCycler Thermalcycler, BioRad®, Hercules, CA and MJ MiniTM Personal Thermal Cycler; BioRad®, Hercules, CA). All reagents that were used, except primers, were from Roche® and were stored at -20ºC. Primers at 10µM from Sigma-Aldrich® (St.Louis, MO) were stored at -20ºC.

Table 1.- Primer sequences for PCR genotyping.

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')	Primer 3 (5'-3')	WT	KO
Parp-1	ggccagatgcgcctgtccaagaag	ggcgaggatctcgtcgtgaccatg	cttgatggccgggagctgcttcttc	200 bp	700 bp

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	WT	Transgene / KO
CD4-cre	tcgatgcaacgagtgatgaggttcg	acagcattgctgtcacttggtcgtg	No band	300 bp
Parp-2 flox/flox	cccaaaccagagtcccatcc	ctcgagtgtttcactgtgagggag	497 bp	657 bp

Table 2.- Sample mix composition and specific PCR conditions.

Gene	Mix composition/sample	PCR conditions
Parp-1	10,2µl H2O 2µl Buffer 10x (without Mg) 1,2µl Mg1Cl 50mM 0,2µl dNTPs 25mM 1µl primer 1, 10µM 1µl primer 2, 10µM 2µl primer 3, 10µM 0,3µl Taq Polymerase 2µl tail-extracted DNA	94ºC-3 min 35 cycles: 94ºC-30sec; 66ºC-30sec; 72ºC 1 min 72ºC 5 min Keep at 4ºC
Cd4-cre	11,05µl H2O 2µl Buffer 10x (without Mg) 0,6µl Mg1Cl 50mM 0,15µl dNTPs 25mM 2µl primer forward, 10µM 2µl primer reverse, 10µM 0,2µl Taq Polymerase 2µl tail-extracted DNA	94ºC-3 min 35 cycles: 94ºC-30sec; 62ºC-30sec; 72ºC 1 min 72ºC 5 min Keep at 4ºC
Parp-2$_{flox/flox}$	12,88µl H2O 2µl Buffer 10x (without Mg) 1,2µl Mg1Cl 50mM 0,16µl dNTPs 25mM 0,8µl primer forward, 10µM 0,8µl primer reverse, 10µM 0,16µl Taq Polymerase 2µl tail-extracted DNA	94ºC-5 min 35 cycles: 94ºC-30sec; 56ºC-30sec; 72ºC 30 sec 72ºC 5 min Keep at 4ºC

2. Tumor cell line

2.1 AT-3 breast cancer tumor cell line

Syngeneic AT-3[298] tumor cell line, derived from a primary mammary gland carcinoma of MMTV-PyMT/B6 transgenic mice was cultured at 37ºC in a cell incubator at 5% CO2 using DMEM (Invitrogen Life Technologies) culture media supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin/100 g/ml streptomycin solution, 50 μM β-mercaptoethanol, and 10% heat inactivated FBS.

2.2 EG7-OVA thymoma cell line

The syngeneic E.G7-OVA cell line was derived from a murine thymoma line, EL-4, by transfection with a neomycin-selectable vector expressing full-length chicken ovalbumin[299]. EG7-OVA cells were cultured at 37 ºC in a cell incubator at 5% CO2 using RPMI-1640 (Invitrogen Life Technologies) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM Lglutamine, 15 mM HEPES, 100 U/ml penicillin/100 g/ml streptomycin solution, 50 μM β-mercaptoethanol, and 10% heat-inactivated FBS.

3. Western Blot

Cells were PBS-washed, counted and lysed in 50μl per 10^6 cells of homemade SDS-lysing buffer lysing buffer (Tris-HCl 67mM pH6.8 + 2% SDS) made in house, and adjusted with in house Laemmli Buffer 4X (Tris-HCl 62,5mM pH6,8 + 5% β-mercaptoethanol + 2% SDS + 40% glicerol + 0,05% bromophenol blue). Proteins were resolved by SDS-PAGE, and analyzed by standard western blotting techniques as described below:

1. Mix samples with Laemmli Buffer 4X (dilution 1:1) and heat them for 5 min at 95°C.
2. Depending on molecular weight of targeted protein, load onto a 10% or 12% SDS-PAGE gel and run samples until protein of interest reaches the center of the gel.
3. Transfer onto a PVDF membrane at 360 mA for 1h or 90 minutes.
4. Block membrane with 5% milk in TBS (TrisHCl 50mM pH7,4 + 150mM NaCl) for 1h at R.T.
5. Incubate with primary antibody o/n at 4°C.
6. Wash 3 times for 5 min with TBST in agitation.
7. Incubate with secondary antibody for 1h at R.T.
8. Wash 3 times for 5 min with TBST in agitation.
9. Enhace chemiluminescence treatment of membranes with ECL reactives (GE Healthcare Europe GmbH, Barcelona, Spain) and/or ECL prime for 5 min
10. Expose to a medical X-ray film (Agfa-Gevaert N.V., Mortsel, Belgium).

The following antibodies were used for the western blot:

Table 3.- Antibodies for Western Blot

Antigen	Origin	Molecular weight	Company	Clone	Working dilution	Incubation
β-actin	mouse	42kDa	Sigma-Aldrich	AC-15	1/5000 (in TBST + 5% milk)	Overnight
Parp-1	mouse	110kDa	in-house	A6.4.12	1/20 (in TBST + 5% milk)	Overnight
Parp-2	rabbit	65kDa	in-house	Polyclonal	1/1000 (in TBST + 5% milk)	1h

Primary antibody binding was detected using peroxidase-coupled rabbit anti-mouse (GE Healthcare) or goat anti-rabbit Ig antibodies (Dako) according to the manufacturer's protocol.

4. Clonogenic assay

In vitro colony formation assay or clonogenic assay tests proliferative potential of cells by evaluating the ability of a single cell to divide into a colony attached to the plastic surface of a culture plate. Single cell suspension was plated in 6 well plates in triplicate and cultured at 37ºC using the previously described culture media. After 24 hours of attachment, cells were untreated or treated with 1 μM, or 0,5 μM (AZD-2281, Shelleck Chemicals). Drug and solvent containing medium was replace with fresh medium and olaparib treatment daily for a duration of 10 days. At the end of the study, cells were washed with PBS and stained with Cristal Violet solution (0,06% Crystal Violet-Sigma, 10% ethanol, 10% acetic acid). Stained cells were scanned and colonies were manually counted.

5. Syngeneic tumor implantation

5.1 Orthotopic implantation

Cell culture medium of AT-3 was changed and cells were re-plated or split into cell culture dishes for expansion to ensure an adequate growth rate and a sufficient number of the cells the day of injection. To do so, medium was first aspirated and cells washed twice with 10 ml of sterile PBS. Cells were carefully treated with 1 ml of sterile 0,25% Trypsin-EDTA solution (Sigma-Aldrich®) and incubated for 1 minute at 37ºC for complete detachment of cells. When the cells are detached and floating, trypsin is neutralized by adding 9 mL of medium containing 10% FBS.

The day of the surgical implantation, cells have ideally reached a confluence of 70% and they are carefully trypsinized and prepared for injection in a 2:1 PBS:Matrigel mixture (Corning® DDBIOLAB) (1000µl PBS +500µl Matrigel), as follows:

Anesthesia flow is set by opening the oxygen tank valve to a 0.8 liters/minute flow and the isoflurane valve to 3 liters/minute flow. The animal is placed inside the anesthesia chamber and the stopcock for isoflurane and oxygen mix is open. Once completely anesthetized the animal is laid on its back on the surgical table. The animal's head is placed inside an anesthetic mask so that it is in contact with the respiratory airways and anesthesia is maintained throughout the procedure. The lower lefts region of the abdomen are shaved and iodine gel is applied to ensure asepsis on the incision point. With sterile tweezers and surgical scissors small incision of about 4 mm transversal to the groin is performed. After the small cut, skin is dissected until adipose panicle (Fat-pad) is visible. With the help of surgical tweezers the Fat-pad is exposed to inject, with a 1ml sterile syringe, 50µl of matrigel cell suspension containing 0.5 $x10^5$ cells. The incision is retracted and sealed with a surgical stapler. The animal is placed into a clean cage over a heating blanket to facilitate recovery from anesthesia. Staples are removed 7 days after the surgery.

5.2 Subcutaneous implantation

Cell culture medium of EG7-OVA was changed and cells were re-plated or split into cell culture dishes for expansion to ensure an adequate growth rate and a sufficient number of the cells the day of injection. Cultured cells were washed and prepared in a PBS 1X suspension for implantation. $1x10^6$ cells were subcutaneously injected into the right flank of mice.

6. Tumor growth monitoring

Tumor growth was measured twice a week by caliper measurement and tumor volume was calculated using the formula $(w2 \times l)/2$, where w is width, and l is length.

7. In vivo treatment

Olaparib (AZD-2281, Shelleck Chemicals) was resuspended in DMSO and stored in 50mg/kg aliquots at -80ºC for in vivo studies. C57BL/6J and SCID mice with AT-3 implanted tumors were treated daily via intraperitoneal injection of 100µl of 50mg/kg Olaparib or 100µl of vehicle (PBS 1X 10% 2-Hydroxypropyl β-cyclodextrins) starting 48h prior to tumor implantation. Tumor growth was followed up for 22days

8. Cell extraction protocols

8.1 Splenic cell extraction

For all cell preparations, mice were previously sacrificed by CO2 asphyxia. Total splenocytes were retrieved from the Spleen, located at the left superior quadrant and inside the abdominal cavity. Intact spleen was harvested during necropsy and kept on 5ml of PBS + 5% Fetal Bovine Serum (FBS) + 2mM EDTA in 50ml sterile (BD Falcon) tubes. Tissue disaggregation was achieved by dispersing the organ through a 100µm Nylon strainer into a new 50ml Falcon tubes using a 2ml syringe plunger (B. Braun Melsungen AG, Germany). Splenocytes cell suspension was kept in 10 ml PBS +5% FBS + 2mM EDTA to avoid cell aggregates.

8.2 Tumor dissection

Tumors were dissected during necropsy of the euthanized mice, weighted and divided into two halves. One was maintained in PBS for enzymatic digestion and phenotypic characterization by flow cytometry staining and the other was maintained in formol for histology analysis.

8.3 Intratumoral immune cell isolation

To obtain infiltrating leukocytes, solid tumors were processed by mechanic and enzymatic digestion. Tumor tissue was submerged in 2 ml of PBS and cut in small pieces with a razor to aid cell disaggregation and removal of connective tissue. 5 ml of in-house made dissociation buffer (PBS 1X + 5% FBS; EDTA 0.5M; collagenase IV (gibco, life technologies) and DNAse I (New England BioLabs Inc) was added to dissected tumor tissue and incubated for enzymatic digesting for 40 minutes at 37ºC with gentle shaking. Afterwards, cell and tissue suspension was passed through cell strainer into 50 ml falcon tubes with the help of a syringe plunger and filled up to 15 ml with PBS. Collected suspension was centrifuged at low velocity to remove tumoral detritus (10xg for 10 min or 30xg for 5 min). Supernatant was collected and centrifuged for 5 minutes at 300xg rpm. Supernatant was discarded and PBS was added to the cell pellet and accordingly aliquoted before proceeding with flow cytometry staining.

9. Viable cell count

For specific experiments, number of viable cells was counted manually during culture and all cell preparations. Splenocytes were treated for red blood cell (RBC) lysis by mixing the cells with TURK solution (1% acetic

acid +1/2500 Blue Giemsa in H_2O) at 1:2 dilution for erythrocyte lysis to contrast live cells. PBS was added then to obtain a final 1:10 dilution before its count. Trypan Blue staining was used to discriminate non-viable cells, blue stained, from membrane-intact viable cells that remain unstained. Cells were counted using a Bürke chamber (Brand Scientific GMBH, Wertheim, Germany).

10. Flow cytometry

10.1 Cell surface staining

A volume corresponding to $1x10^6$ cells suspensions was pipetted into an Eppendorf tube, and then processed as indicated:

1. Wash with 1ml PBS. Spin down tubes at 300xg for 5 minutes.
2. Discard supernatant and resuspend pellet in staining buffer (PBS + 10% FBS) containing the appropriate antibodies in a 1/100 dilution (1µl antibody per 10^6 cells in 100µl of staining buffer). If necessary cells are pretreated with purified anti-CD16 (BD Biosciences) to block cell surface FcRs.
3. Incubate for 30 minutes at 4ºC in the dark.
4. After incubation, wash cell suspensions with 1 ml PBS 1X, centrifuge for 5 min at 300xg and discard supernatant.
5. Resuspend pellet in 150µl of ACK RBC lysis buffer and incubate for 3 minutes at RT.
6. After the RBC lysis, wash with 1mL of PBS 1X to recover osmolarity and centrifuge for 5 min at 300xg.
7. Finally, prepare cell suspension for FACS acquisition by resuspension of the pellet in 300µL of PBS 1X + 2µg/ml DAPI and keep at 4ºC protected from light until acquisition.

10.2 Intracellular cell staining

For intracellular staining, cells were first stained for cell surface markers (if necessary), fixed and made permeable by using BD Cytofix/Cytoperm (BD Bioscience), and finally stained for specific intracellular antigens as described below.

1. If required, stain for cell surface markers
2. Fix and permeabilize cells.
    a. Resuspend cells in 100µl of BD Cytofix/Cytoperm Buffer.
    b. Incubate for 15-30 min at 4ºC protected from the light
    c. Wash with 1 ml of Perm Wash Buffer 1X and centrifuge at 300xg for 5 min.
    d. Resuspend in 300µl of PBS 1X + 5%FBS and leave samples overnight at 4ºC or continue with step 3.
3. Incubate with antibodies against intracellular antigens for 20 minutes at R.T, protected from light.
4. Wash twice with 1ml of Wash Buffer 1X and centrifuge a 300xg for 5 min.
5. Wash with 1ml of PBS 1X and centrifuge at 300xg for 5 min.
6. Resuspend cells in 300ml of PBS.

10.3 FACS acquisition and analysis

All samples were acquired using FACS Fortessa (BD Bioscience) and analyzed using FACS DIVA (BD Bioscience) software. Cell doublets were excluded from all analyses using FSC-H/FSC-W and SSC-H/SSC-W.

10.4 Cell sorting

Cells were appropriately stained for CD3, CD4 and CD8 cell surface markers and sorted using cells sorters FACs ARIAIISORP and BD influx (BD Bioscience).

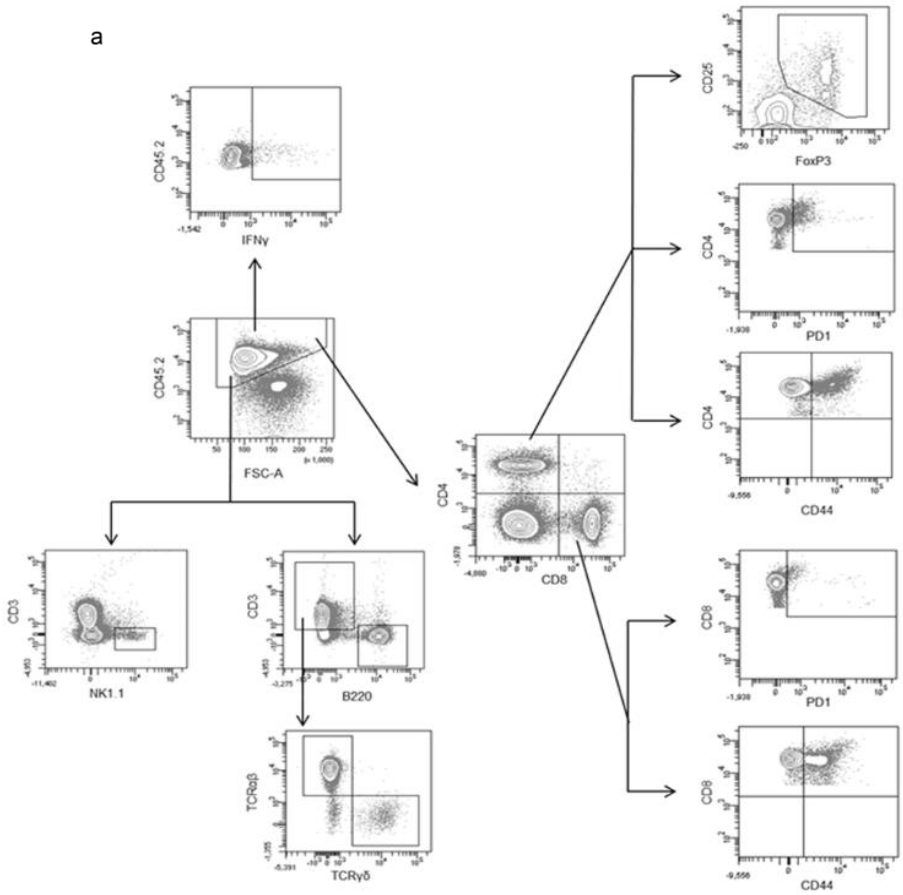
Table 4.- List of antibodies used for flow cytometry analysis and cell sorting.

Antigen	Fluorochrome	Clone	Isotype	Dilution	Company
CD3	APC-Cy7	17A2	Rat (SD) IgG2b, K	1/100	BD Biosciences Pharmingen™
CD4	PE	GK1.5	Rat (LEW) IgG2b, κ	1/100	BD Biosciences Pharmingen™
CD4	PE-Cy7	GK1.5	Rat IgG2b, κ	1/100	BioLegend ®
CD8	PE	H35-17.2	Rat IgG2b, κ	1/100	BD Biosciences Pharmingen™
CD8	APC-Cy7	53-6.7	Rat IgG2a, κ	1/100	BioLegend ®
CD25	APC	PC61.5	Rat IgG1	1/100	Invitrogen
CD11.b	FITC-c	M1/70	Rat IgG2b, κ	1/100	BD Biosciences Pharmingen™
CD11c	PerCP	N418	Armenian Hamster IgG2, k	1/100	BioLegend ®
CD16/CD32	purified	2.4G2	Rat IgG_{2b}, κ	1/00	BD Biosciences Pharmingen™
CD44	PerCP	IM7	Rat IgG2b, κ	1/100	BD Biosciences Pharmingen™
CD45.2	FITC-c	104	Mouse SJL IgG2a, κ	1/100	BioLegend ®
CD45.2	BV605	104	Mouse SJL IgG2a, κ	1/00	BioLegend ®
CD45R (B220)	FITC-c	RA3-6B2	Rat IgG2a, κ	1/00	BD Biosciences Pharmingen™
CD62L	BV605	MEL-14	Rat IgG2a, κ	1/100	BD Biosciences Pharmingen™
CD80	PE-CF594	16.10A1	Armenian Hamster IgG2, k	1/00	BD Biosciences Pharmingen™
Ly6-C	APC	HK1.4	Rat IgG2c, κ	1/100	eBioscience
Ly6-G	PE-Cy7	1A8	Rat IgG2a, κ	1/100	BioLegend ®

CD279 (PD-1)	APC	J43	Armenian Hamster IgG2, k	1/00	BD Biosciences Pharmingen™
CD278 (ICOS)	PE	7E.17G9	Rat IgG2b, κ	1/00	BioLegend ®
CD103	APC-R700	M290	Rat IgG2a, κ	1/00	BD Biosciences Pharmingen™
F4/80	PE	BM8	Rat IgG2a, κ	1/00	BioLegend ®
β chain TCR	PE-Cy7	H57-597	Armenian Hamster IgG2	1/00	BioLegend ®
γδTCR	PE	UC7-13D5	Armenian Hamster IgG2	1/00	BioLegend ®
IA/IE MHC II	APC	M5/114.15.2	Rat IgG2b, κ	1/00	BioLegend ®
IA/IE MHC II	APC-Cy7	M5/114.15.2	Rat IgG2b, κ	1/00	BioLegend ®
FoxP3	PE	FJK16S	Rat IgG_{2a}, κ	5/100	Invitrogen
NK1.1	APC-Cy7	PK136	Mouse C3H x BALB/c IgG2a, κ	1/00	BD Biosciences Pharmingen™

Table 5.- Phenotypic characterization of immune cell populations.

	Population name	Cell surface phenotype
Lymphoid lineage	Single positive CD4	$CD45^+CD4^+$
	Single positive CD8	$CD45^+CD8^+$
	Naïve CD4	$CD4^+$ $CD62L^+$ $CD44^{low}$
	Naïve CD8	$CD8^+$ $CD62L^+$ $CD44^{low}$
	Memory CD4	$CD4^+$ $CD62L^-$ $CD44^{high}$
	Memory CD8	$CD8^+$ $CD62L^-$ $CD44^{high}$
	B cells	$CD45^+B220^+$
	T regulatory cell	$CD4^+$ $CD25^{high}$ $FoxP3^+$
	γδ T cells	$CD3^+$ $\alpha\beta TCR^-$ $\gamma\delta TCR^+$
	NK cells	$CD45^+CD3^-NK1.1^+$
Myeloid lineage	M-MDSC	$CD11b^+$ $Ly6G^{low}$ $Ly6C^{high}$
	G-MDSC	$CD11b^+$ $Ly6G^+$ $Ly6C^{low/-}$
	Macrophage subset 1	$CD45^+F4/80^+CD11b^+Ly6C^{low}MHCII^{high}$
	Macrophage subset 2	$CD45^+F4/80^+CD11b^+Ly6C^{low}MHCII^{low}$
	Macrophage subset 3	$CD45^+F4/80^+CD11b^+Ly6C^{high}MHCII^{high}$
	Macrophage subset 4	$CD45^+F4/80^+CD11b^+Ly6C^{high}MHCII^{low}$
	Dendritic cell subset 1	$CD45^+MHCII^{high}CD11b^+CD103^-$
	Dendritic cell subset 2	$CD45^+MHCII^{high}CD11b^-CD103^+$


a
CD45.2
IFNγ
CD45.2
FSC-A
CD3
NK1.1
CD3
B220
TCRαβ
TCRγδ
CD4
CD8
CD25
FoxP3
CD4
PD1
CD4
CD44
CD8
PD1
CD8
CD44

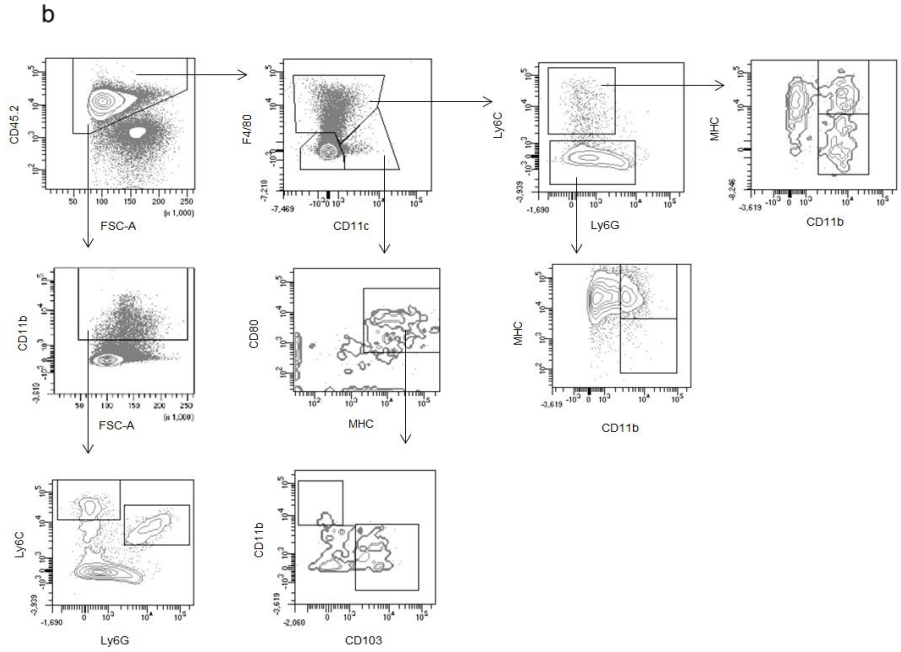


Figure 10.- Immunophenotyping by flow cytometry. Gating strategies for flow cytometry analysis of (a) lymphoid lineage and (b) myeloid lineage cell subsets.

11. Immunohistochemistry

Tumor samples were formalin-fixed for 24 hours, processed and paraffin embedded (FFPE). Three micrometers- thick sections from FFPE tissues were obtained, placed in positively charged glass slides and dried. IHC was performed using a commercial staining system kit (Dako Envision, Dako). After deparaffination and rehydration, a heat-induced demasking antigen procedure was carried out by using a commercial solution (Dako Demasking Antigen Solution High Ph., Dako), followed by an endogenous peroxidase-blocking procedure.

Sections were then incubated overnight at 4ºC with the following primary antibodies: polyclonal rabbit anti-CD3 (A0452, Dako), monoclonal rabbit-

anti-CD4 (ab18368 Abcam), monoclonal rabbit anti-CD8 (ab203035, Abcam).

The second day, sections were incubated with the secondary anti-rabbit labelled polymer (Dako) for 30 minutes at 10 37ºC, and finally revealed with 3-3´Diaminobencidine (DAB). Positive reaction was identified as a dark-brown pericellular precipitate. Representative pictures of anti-CD3, anti-CD4 and anti-CD8 staining of fixed AT-3-induced tumor tissue.

12. RNA extraction

Total RNA was isolated from sorted CD4+ and CD8+-tumor-infiltrating T cells by using the Rneasy Total RNA Isolation kit (Qiagen), with on-column DNase I (Qiagen) digestion, in accordance to the manufacturer's instructions as follows:

1. For $5x10^6$ cells add 10µl β-ME per 1 ml Buffer RLT. Add, 350µl mix in each sample.
2. Homogenize the lysate by vortexing for 1 min.
3. Add 350µl of 70% ethanol to the homogenized lysate, and mix cell by pipetting.
4. Transfer up to 700µl of the sample, including any precipitate that may have formed, to a RNeasy spin column placed in a 2ml collection tube. Close the lid gently, and centrifuge for 15s at 17000xg.
5. Add 350µl buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 17000xg to wash the spin column membrane. Discard the flow-through.
6. Add 10µl DNase I stock to 70µl Buffer RDD. Mix gently by inverting the tube, and do a quick spin down to collect the residual liquid from the sides of the tube.

7. Add the DNase I incubation mix (75µl) directly to the RNeasy spin column membrane, and place on the benchtop (20-30ºC) for 15 min.
8. Add 350µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 17000xg. Discard the flow-through.
9. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 17000xg. Discard the flow-through.
10. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 17000xg to wash the spin column membrane.
11. Place the RNeasy spin column in a new 2mk collection tube and discard the old collection tube. Close the lid gently and centrifuge at full speed for 1 min.
12. Place the RNeasy spin column in a new 1.5ml collection tube. Add 30-50µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 15s at 17000xg to elute the RNA.

13. Microarray

Microarray gene analysis was carried out by the MARGenomics service at IMIM. Samples were processed according to the following protocols: GeneChip Pico Reagent kit (P/N 703308 Ver.4, 2016) and GeneChip™ Expression Wash, Stain and Scan User Guide (P/N 702731, 2017) (ThermoFisher Scientific). Microarray used was the Clariom S Mouse (ThermoFisher Scientific).

14. RT-qPCR

Quantity and integrity of the RNA was assessed by nanoelectrophoresis using the Pico lab-on-a-chip assay for total eukaryotic RNA using Bioanalyzer 2100 (Agilent Technologies). For mRNA expression analysis total RNA (5 ng) from each sample was used for cDNA synthesis using the GeneChip Pico Reagent Kit (Thermo Fisher Scientific). For cDNA reverse transcription an initial step of desnaturalization for 10 min at 65ºC was done with the MJ MiniTM Personal Thermal Cycler (BioRad®, Hercules, CA) RT reaction was performed using the MyCycler Thermalcycler (BioRad®, Hercules) under the following conditions:

1. RT reaction 50ºC 1h
2. Inactivation step 85ºC 5 min
3. End point 4ºC forever

RT-qPCR was carried out using 15 ng cDNA per sample using platinum SYBRR Green Master mix (Applied Biosystems). Assays were run in triplicate on the ABI 7900HT system. Samples were normalized according to β-actin expression levels.
Data analysis was done with SDS 2.4 and DataAssist 3.0 softwares from Applied Biosystems.

Table 6.- List of primers used for RT-qPCR:

Gene	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)
Gzma	GGGGCTCACTCAATCAATAAGG	CATCCTGCTACTCGGCATCT
Gzmb	TCTCGACCCTACATGGCCTTA	TCCTGTTCTTTGATGTTGTGGG
Gzmc	CAGAGGAGATAATCGGAGGCA	ACGAATTTGTCTCGAACCAGG

Gzmbk	TGGCTGGCGTTTATATGTCTTC	TCTGGGAAACCAAGAGTAGCA
Fasl	CAGCCCATGAATTACCCATGT	ATTTGTGTTGTGGTCCTTCTTCT
Cd244	ACAGGCGGAAAAGTGTGCAA	GGCCTTCAGGTTAGGGGTC
Prf1	CTGCCACTCGGTCAGAATG	CGGAGGGTAGTCACATCCAT
Cd80	TCAGTTGATGCAGGATACACCA	AAAGACGAATCAGCAGCACAA
Cd83	CGCAGCTCTCCTATGCAGTG	GTGTTTTGGATCGTCAGGGAAT
Cd86	TCAATGGGACTGCATATCTGCC	GCCAAAATACTACCAGCTCACT
Pdcd1	CAGCTTGTCCAACTGGTCG	GCTCAAACCATTACAGAAGGCG
Ctla4	CATGGTGTCGCCAGCTTTC	GGTAATCTAGGAAGCCCACTGTA
Icos	ATGAAGCCGTACTTCTGCCG	CGCATTTTTAACTGCTGGACAG
Lgals1	AACCTGGGGAATGTCTCAAAGT	GGTGATGCACACCTCTGTGA
Ccr2	ATCCACGGCATACTATCAACATC	TCGTAGTCATACGGTGTGGTG
Ifng	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
Il12rb1	GCTGCTGCGTTGAGAAGACA	CACAGGACGTGAGAAACATTGT
Il23r	AACAACAGCTCGGATTTGGTAT	ATGACCAGGACATTCAGCAGT
Il10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
Cxcr3	GGTTAGTGAACGTCAAGTGCT	CCCCATAATCGTAGGGAGAGGT
Ccl1	TGCCGTGTGGATACAGGATG	GTTGAGGCGCAGCTTTCTCTA
Cxcl13	GGCCACGGTATTCTGGAAGC	ACCGACAACAGTTGAAATCACTC
Cxcl10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Cxcl16	CCTTGTCTCTTGCGTTCTTCC	TCCAAAGTACCCTGCGGTATC
Tbx21	AGCAAGGACGGCGAAT	GTGGACATATAAGCGGTT

	GTT	CCC
Ccr6	TGGGCCATGCTCCCTAGAA	GGTGAGGACAAAGAGTATGTCTG
Cxcr4	GACTGGCATAGTCGGCAATG	AGAAGGGGAGTGTGATGACAAA
Ccl4	TTCCTGCTGTTTCTCTTACACCT	CTGTCTGCCTCTTTTGGTCAG
Ccl5	TTTGCCTACCTCTCCCTCG	CGACTGCAAGATTGGAGCACT
Ccr5	ATGGATTTTCAAGGGTCAGTTCC	CTGAGCCGCAATTTGTTTCAC
Nlrp3	ATTACCCGCCCGAGAAAGG	CATGAGTGTGGCTAGATCCAAG
Xcl1	TAGCTGTGTGAACTTACAAACCC	ACAGTCTTGATCGCTGCTTTC

15. Statistical analysis

Results are presented as mean values ± SEM. The log-rank test was used to determine the statistical of animal survival. An unpaired two-tailed Mann-Whitney was used to analyze all the experiments. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Host-mice with deficiencies of PARP-1 and/or PARP-2 display differential changes in the growth of tumors harboring both PARP-1 and PARP-2 proteins

Tumor growth complexity can be comprehended only as the evolution of a cancerous tumor together with its environment. Tumor parenchyma, comprised of cancerous cells, is in constant interaction with a dynamic multicellular ecosystem conformed by non-malignant cells and connective tissue known as the tumor microenvironment. The cross-talk between tumor cells and infiltrating immune cells is a critical factor either promoting or hindering tumor growth. The adaptive response is responsible for direct destruction of the cancerous cells being T lymphocytes the major players in this response.

In here, we sought to study the role of PARP-1 and/or PARP-2 in the modulation of the T cell immune response against tumors, independently of their activity on the tumor cells. To do so, we have orthotopically implanted the syngeneic breast cancer tumor cell line AT-3, proficient for both proteins (Figure 11a), in host-mice harboring different cell deficiencies of PARP-1 and/or PARP-2 and monitored tumor growth by measuring tumor volume for 25 days. Host-mice comprise control (Cd4-creParp-$1^{+/+}$Parp-$2^{+/+}$), single PARP-1-deficient (Cd4-creParp-$1^{-/-}$Parp-$2^{+/+}$), single PARP-2-deficiency only in T cells (hereafter T cell-specific PARP-2-deficient) (Cd4-creParp-$1^{+/+}$Parp-$2^{f/f}$), and dually PARP-1 and PARP-2-deficient (Cd4-creParp-$1^{-/-}$Parp-$2^{f/f}$) mice.

Interestingly, we observed a significant reduction in AT-3-induced tumor growth in both single PARP-1-deficient and single PARP-2-deficient host-mice compared to control (Figure 12a). These data suggest that single deficiency of either PARP-1 or PARP-2 in T cells enhances the

immune response against tumors. On the contrary, tumor growth was remarkably faster in dually PARP-1 and PARP-2 deficient mice compared to control mice (Figure 12a). Weight of tumor mass at the last day of monitoring was in accordance with tumor growth rate (Figure 12b). Similar results were obtained when we carried out the same experiment with a subcutaneous tumor induced by the syngeneic thymoma EG7-OVA cell line (Figure 12c and 12d), which is also proficient for both PARP-1 and PARP-2 proteins (Figure 11b).

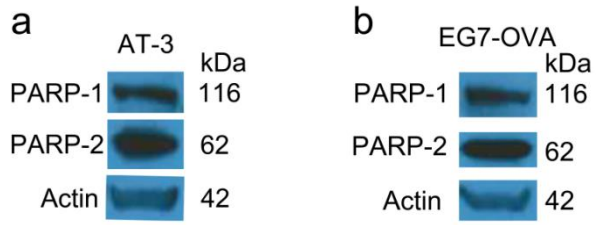


Figure 11.- Tumor cell lines AT-3 and EG7-OVA are proficient for PARP-1 and PARP-2 expression. (a) Western-blot of PARP-1 and PARP-2 protein levels in the AT-3 breast tumor cell line[298]. (b) Western-blot of PARP-1 and PARP-2 protein levels in the EG7-OVA breast tumor cell line[299].

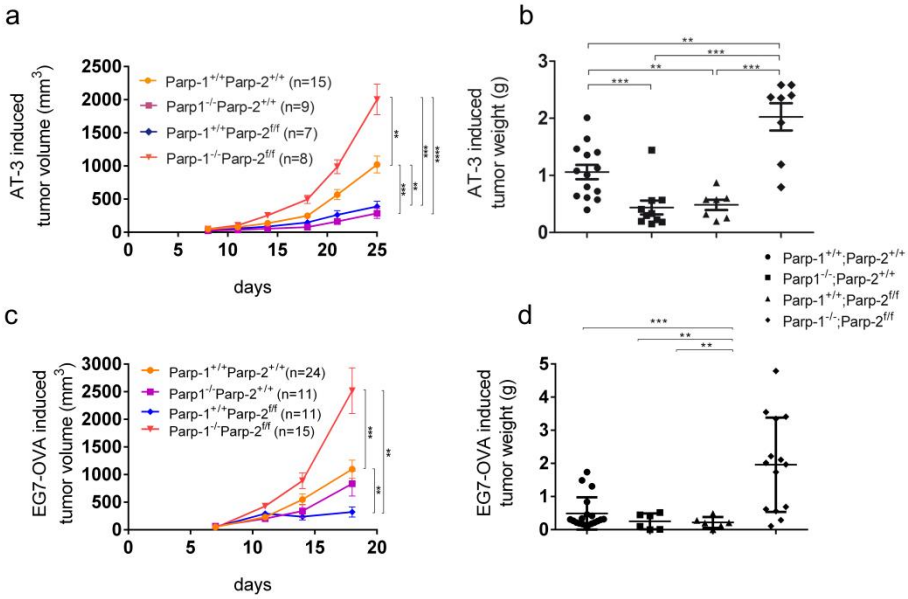


Figure 12.- Host-mice with deficiencies of PARP-1 and/or PARP-2 display differential changes in the growth of tumors harboring both PARP-1 and PARP-2 proteins. (a) Tumor growth monitoring and (b) tumor weight at day 25, after orthotopical implantation of $5x10^5$ AT-3 breast tumor cells into the fat pad of a single mammary gland of female mice of the indicated genotypes; *Cd4-creParp-1$^{+/+}$Parp-2$^{+/+}$; Cd4-creParp-1$^{-/-}$Parp-2$^{+/+}$; Cd4-creParp-1$^{+/+}$Parp-2$^{f/f}$ and Cd4-creParp-1$^{-/-}$Parp-2$^{f/f}$*, all of them in C57BL/6J background. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Then, we questioned whether the simultaneous pharmacological inhibition of PARP-1 and PARP-2 using olaparib, results in the same effects on tumor growth as the simultaneous genetic silencing of both proteins. The AT-3 cell line is sensitive to olaparib in vitro as observed in the proliferation assay where concentrations of 0,5 μM and 1 μM significantly impacted AT-3 colony formation showing reduced number of colonies in comparison with untreated AT-3 cells (Figure 13a). Furthermore, when the tumor cell line was implanted in immunodeficient mice (SCID mice), a significant decrease in tumor growth was observed in mice treated with

olaparib compared to the group treated with vehicle (Figures 13b). However, the anti-tumor effect conferred by olaparib disappears when the tumor cell line is implanted in immunocompetent wild-type mice (C57 mice) (Figure 13c). Tumor weights at endpoint of the experiment correlate with the observations in tumor growth (Figure 13d).

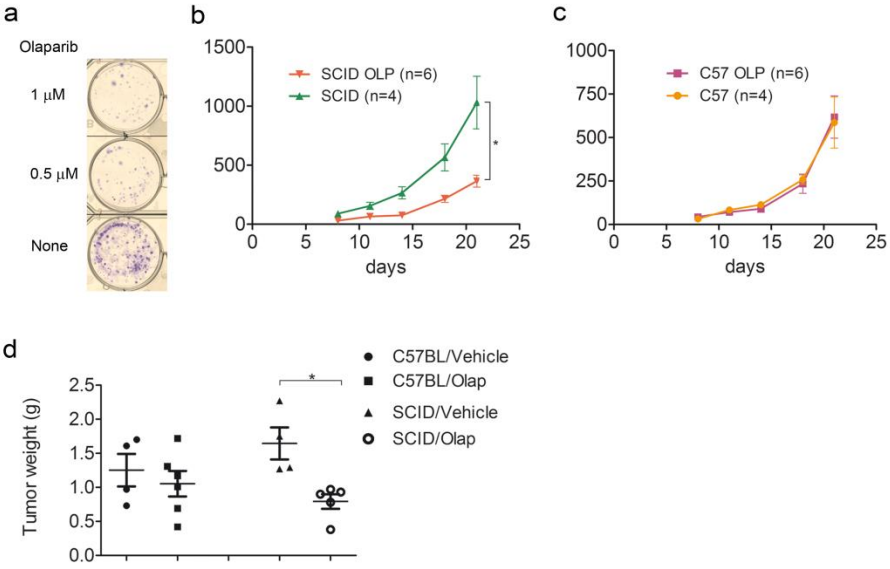


Figure 13.- Effect of olaparib on the AT-3 tumor cells in vitro and AT-3-induced tumor growth in vivo. (a) In vitro proliferation assessed by clonogenic assay, of AT-3 cells treated with 0,5μM olaparib, AT-3 cells treated with 1μM olaparib and untreated AT-3 cells, after 10 days of culture. (b) Effect of olaparib on the AT-3-induced tumor growth in vivo in SCID mice. (c) Effect of olaparib on the AT-3-induced tumor growth in vivo in C57BL/6J mice. (d) AT-3 tumor weight at 22 days after implantation in immunocompetent and immunodeficient mice treated with olaparib or vehicle. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Interestingly, the EG7-OVA cell line implanted in SCID mice is resistant to olaparib (Figure 14a). Remarkably, in immunocompetent mice implanted with EG7-OVA cells, olaparib treatment resulted in significantly accelerated tumor growth compared to treatment with vehicle

(Figure 14b). This observation suggests a possible negative pro-cancer effect of olaparib on the immune system.

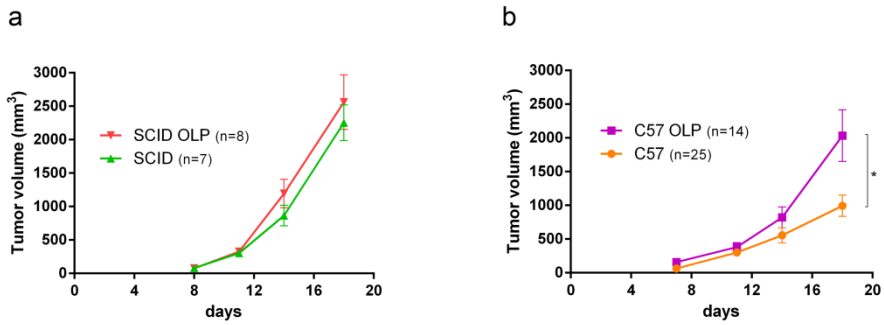


Figure 14.- Effect of olaparib on the EG7-OVA-induced tumor growth in vivo. (a) Effect of olaparib on the AT-3-induced tumor growth in vivo in SCID mice. (b) Effect of olaparib on the AT-3-induced tumor growth in vivo in C57BL/6J mice. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

T cell response to AT-3-induced breast tumors is modulated by PARP-1 and PARP-2 proteins

In light of these results, we found interest in studying the different populations of immune cells that are potentially implicated in the host rejection of tumors, and checking for discrepancies between genotypes with special attention to anomalies in the PARP-$1^{-/-}$; PARP-$2^{f/f}$ T cells. So, to explore in what way PARP-1 and/or PARP-2 deficiencies in host-mice affect the immune response against AT-3-induced tumors, we took an initial look at the immune landscape at secondary lymphoid organ level. Hence we analyzed, by flow cytometry, splenic lymphocytes at 25-days after tumor cell line implantation. We observed a striking reduction in the number of T lymphocytes, but not B lymphocytes (Figure 15a), in host mice with T cell-specific dual deficiency in PARP-1 and PARP-2. The T cell reservoir in spleen of double mutant mice only reached around 9% in

average of all CD45.2$^+$ cells compared to the 30-42% showed in control and single mutant mice (Figure 15a). T cell reduction affected both CD4$^+$ and CD8$^+$ T cell subsets of double knock out mice (Figure 15b) in an equal manner. Percentage of CD4$^+$ and CD8$^+$ cell populations in control and single PARP-1 and T cell-specific PARP-2 mice averaged 18-20% and 11-13%, respectively, whereas double deficient subsets exhibited 8.4% of CD4$^+$ and 3.1% of CD8$^+$ T cells in average.

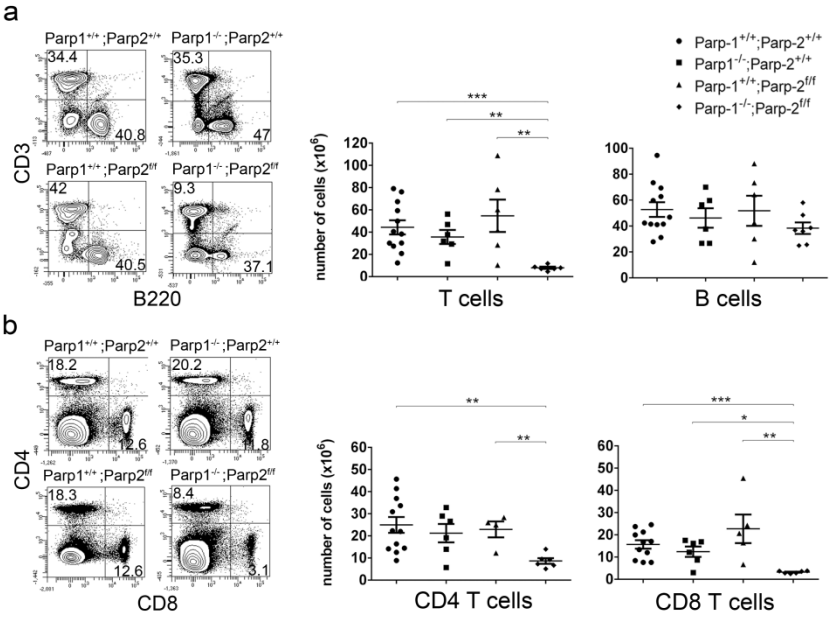


Figure 15.- Analysis of splenic B and T lymphocytes from mice hosting AT-3-induced tumors. Representative dot-plots and absolute number of: (a) T (CD3$^+$) and B (B220$^+$) cells and (b) CD4 and CD8 T cells. Percentage of cells in the individual subpopulations indicated in each quadrant. Dots represent individual mice and horizontal lines represent median values of number of cells. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Further characterization of $CD4^+$ and $CD8^+$ naïve and memory compartment revealed that the splenic T lymphopenia was occurring in the naïve and memory compartments of both lineages alike (Figure 16a and 16b).

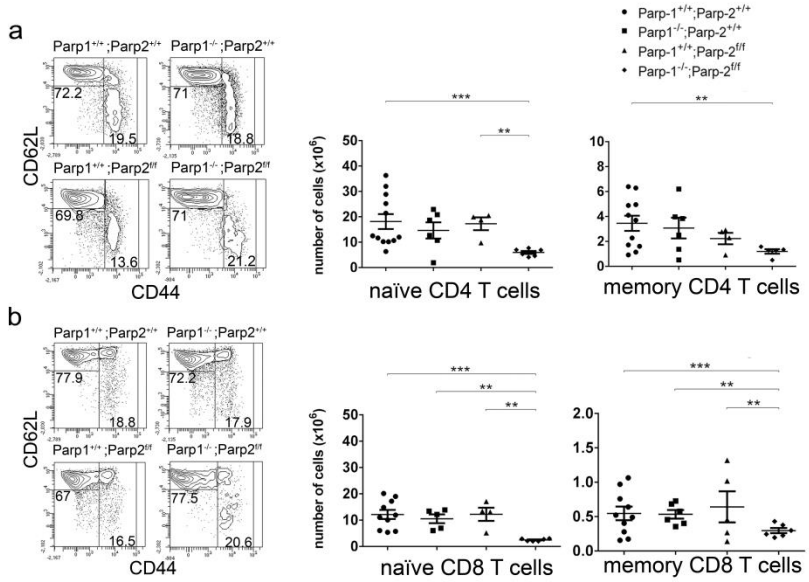


Figure 16.- Analysis of naïve and memory compartments of splenic $CD4^+$ and $CD8^+$ T lymphocytes from mice hosting AT-3-induced tumors. Representative dot-plots and absolute number of: (a) $CD4^+$ naïve and memory cells and (b) $CD8^+$ naïve and memory cells. Percentage of cells in the individual subpopulations indicated in each quadrant. Dots represent individual mice and horizontal lines represent median values of number of cells. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

No differences were observed between the four genotypes in the percentage or total number of T regulatory cells in spleen (Figure 17a). Interestingly, splenic T lymphocytes expressing γδTCR were considerably increased in mice with the single, T-cell specific PARP-2 deletion. The expansion of γδ T cells was however abolished by PARP-1 deficiency (Figure 17b). Of, note the number of splenic NK cells was significantly

reduced in host mice deficient for both PARP-1 and PARP-2 compared with the controls.

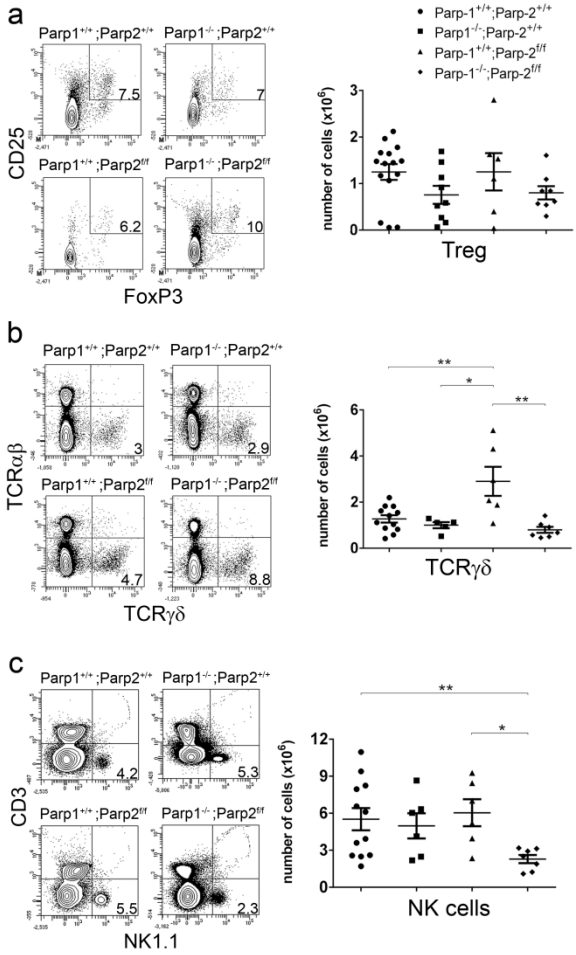


Figure 17.- Analysis of splenic subpopulations of T lymphocytes and Natural Killer cells from mice hosting AT-3-induced tumors. Representative dot-plots and absolute number of: (a) T regulatory cells ($CD4^+$,$CD25^+$,$FoxP3^+$), (b) γδ T cells and (c) natural killer cells ($CD3^-$,$NK1.1^+$). Percentage of cells in the individual subpopulations indicated in each quadrant. Dots represent individual mice and horizontal lines represent median values of number of cells. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Furthermore, we wondered whether the impact of the double deficiency was similar in the TME compared to that of secondary lymphoid organs, particularly in spleen. First, we determined infiltration of T lymphocytes in AT-3-induced tumors. Immunohistochemistry was performed at 25-days post-implantation. Infiltrating $CD3^+$ T cell numbers were significantly decreased in tumors hosted in dually PARP-1 and PARP-2-deficient mice compared with single PARP-1-deficient, single PARP-2-deficient or control host mice. This reduction was due to diminution of both $CD4^+$ and $CD8^+$ T cells. By the contrary, a slight increase in T cell infiltration was observed in tumors hosted by single mutant mice compared to control (Figures 18a and 18b).

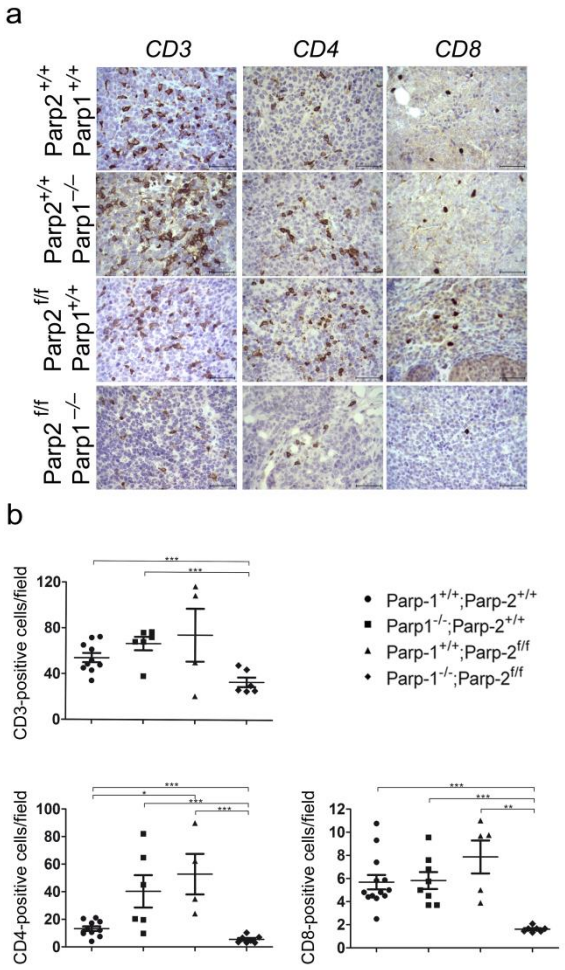


Figure 18.- T cell response to AT-3-induced breast tumors is modulated by PARP-1 and PARP-2 proteins. Immunohistochemical analyis. (a) Immunohistochemical staining of AT-3-induced breast tumors for T cell subsets. (b) Quantification of infiltrating cells determined by microscopy analysis. Dots represent individual mice and horizontal lines represent median values of number of cells. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

We performed immunophenotyping by flow cytometry to further characterize leukocyte infiltration in AT-3 induced tumors. To do so we used antibodies specific for lymphoid cell markers. Gating strategies to define each population are shown in section 10 of materials and methods.

In agreement with our immunohistochemistry observations, we report a significant reduction in the percentage of infiltrating T cells (Figure 19a) in tumors hosted in double PARP-1 and PARP-2 deficient mice compared to the control.

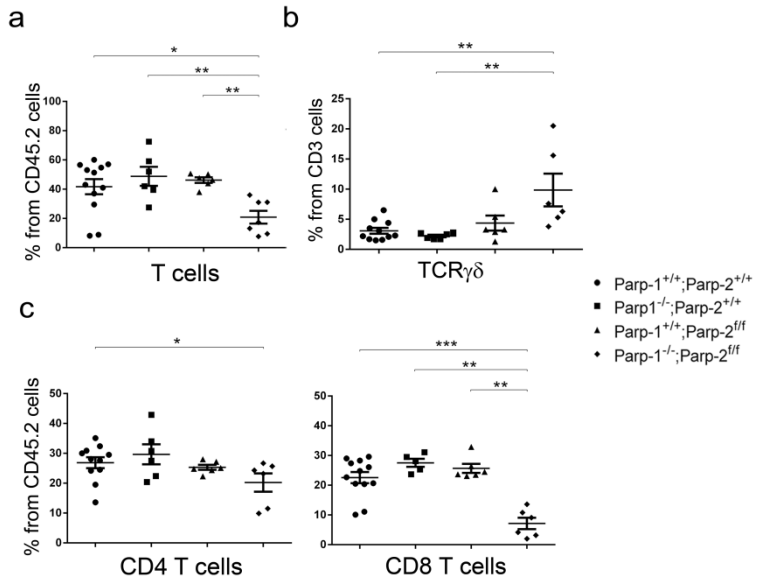


Figure 19.- T cell response to AT-3-induced breast tumors is modulated by PARP-1 and PARP-2 proteins. Flow cytometry analysis of tumor-infiltrating T cell subsets. Percentage of (a) T ($CD3^+$) lymphocytes from tumor infiltrating $CD45.2^+$ cells; (b) γδ T cells ($TCR\gamma\delta^+$) from tumor infiltrating $CD3^+$, and (c) $CD4^+$ and $CD8^+$ T lymphocyte subsets, from tumor infiltrating $CD45.2^+$ cells. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

The reduction was more significant for the $CD8^+$ T cell subset whereas we only detected a minor decrease of infiltrating $CD4^+$ cells in tumors from dual deficient mice (Figure 19c). Interestingly, additional T cell markers revealed a significant increase in the percentage of γδ T cells in tumors hosted in dually PARP-1 and PARP-2 deficient mice, compared to control

host-mice. PARP-2 single deficiency mice exhibited increased γδ T cells (Figure 19b) although to a lesser degree than in spleen.

Further identification of infiltrating $CD4^+$ and $CD8^+$ T cells, revealed that tumors hosted in mice with a single deficiency in either PARP-1 or PARP-2 showed an increased percentage of $CD8^+$ T cells (Figure 19b) which accounted for the memory but not naïve compartment (Figure 20a). The aforementioned reduction of $CD8^+$ T cells infiltrating tumors hosted in dually deficient mice affected only the memory compartment. In fact, a higher percentage of naïve $CD8^+$ T cells was detected in these tumors. Because the reduction of tumor infiltrating $CD8^+$ T cells in dual PARP-1 and PARP-2 deficient mice is disturbing the effector T cells we decided to also analyze PD-1 expression on tumor infiltrating T cells of the different genotypes. A reduction in the percentage of memory $CD4^+$ and memory $CD8^+$ T cells expressing PD-1 was detected in tumors hosted in dually PARP-1 and PARP-2 deficient mice, compared to control host-mice. Conversely, single PARP-1 deficient or single PARP-2 deficient host-mice display a slightly increased percentage of infiltrating PD-1-expressing memory T cells, mainly affecting the $CD8^+$ lineages, compared to control mice (Figure 20b).

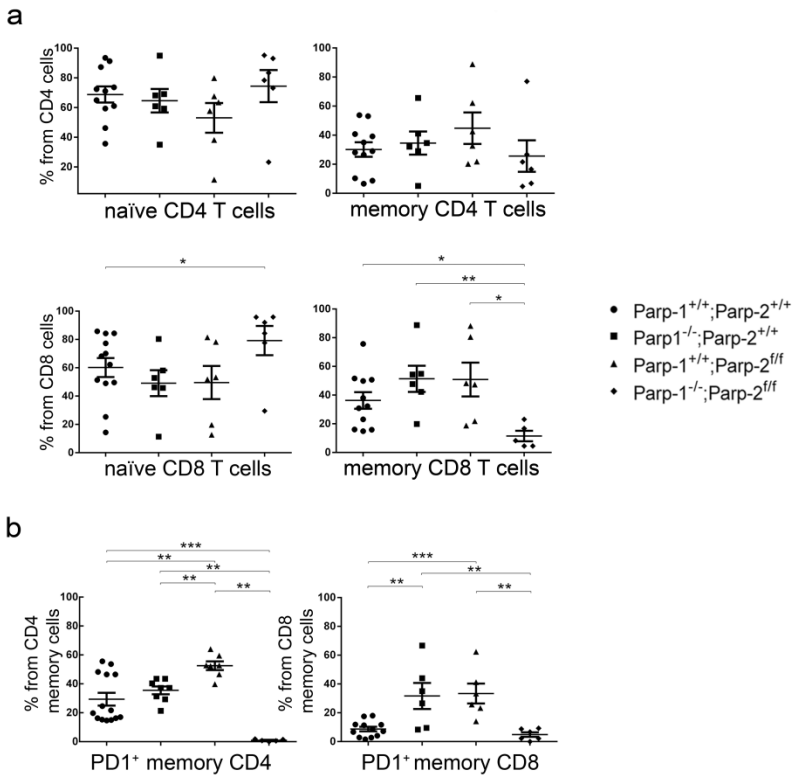


Figure 20.- T cell response to AT-3-induced breast tumors is modulated by PARP-1 and PARP-2 proteins. Flow cytometry analysis of tumor-infiltrating T cell subsets. Percentage of (a) naïve ($CD44^{low}$, $CD62L^{+}$) and memory ($CD44^{high}$, $CD62L^{-}$) of $CD4^{+}$ and $CD8^{+}$ T lymphocyte subsets. (b) PD1 expression in memory $CD4^{+}$ and $CD8^{+}$ T lymphocytes. Gating strategies to define each population are show on section 10 of Material and Methods. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Of note, the percentage of Treg cells, expressing CD4, CD25 and FoxP3, was slightly reduced in tumors hosted in dually PARP-1 and PARP-2 deficient mice (Figure 21a) and the percentage of tumor-infiltrating cells producing IFNγ was increased in single PARP-1 and PARP-2 deficient host-mice but decreased in dually PARP-1 and PARP-2 deficient host-

mice compared to control host-mice (Figure 21b), although did not reach statistical significance.

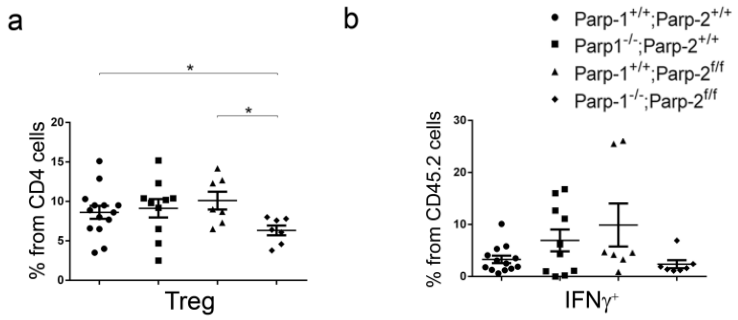


Figure 21.- T cell response to AT-3-induced breast tumors is modulated by PARP-1 and PARP-2 proteins. Flow cytometry analysis of tumor-infiltrating lymphoid lineage. Percentage of (a) T regulatory cells ($CD4^+$,$CD25^+$,$FoxP3^+$) from tumor infiltrating $CD4^+$ lymphocytes and (b) of tumor infiltrating $CD45.2^+$ lymphocytes producing IFNγ. Dots represent individual mice and horizontal lines represent median values. Values represent represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Although our mouse models are targeting the T cell compartment, modification in this compartment and its interactions could also modify other cellular components of the immune system in the tumor microenvironment. Accordingly, we have also used surface markers to study B cells, NK cells and myeloid cell subsets in tumors hosted by mice of the different genotypes. Similar percentages of B cells were observed in tumors hosted in dual or single PARP-1 and PARP-2 deficient mice or controls next to a slight decrease in dually deficient mice (Figure 22a). The percentage of infiltrating NK cells was similar in dually PARP-1 and PARP-2 deficient hosted mice and control mice, while a slight increase was observed in single PARP-1 deficient or single PARP-2 deficient host-mice (Figure 22b) which could be contributing to the better antitumor response that these groups exhibit.

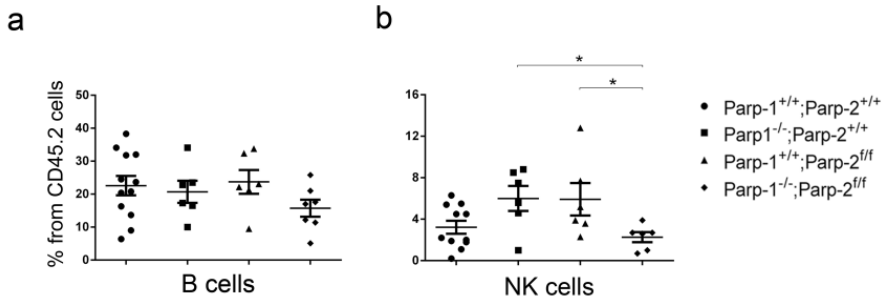


Figure 22.- Flow cytometry analysis of tumor-infiltrating lymphoid lineage cell subsets. Percentage of (a) B lymphocytes ($B220^{+}$) and (b) Natural killer cells ($CD3^{-}$; $NK1.1^{+}$) from tumor infiltrating CD45.2+. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Dually PARP-1 and PARP-2-deficiency in host-mice impact on the myeloid-derived cell subsets

Interestingly, the percentage of tumor infiltrating macrophages was significantly increased in tumors hosted in dually PARP-1 and PARP-2 deficient mice compared to single deficient or control mice (Figure 23). Augmented macrophage subpopulation comprised mainly the MHCII^{low} macrophages, which have been previously correlated with tumor progression[300].

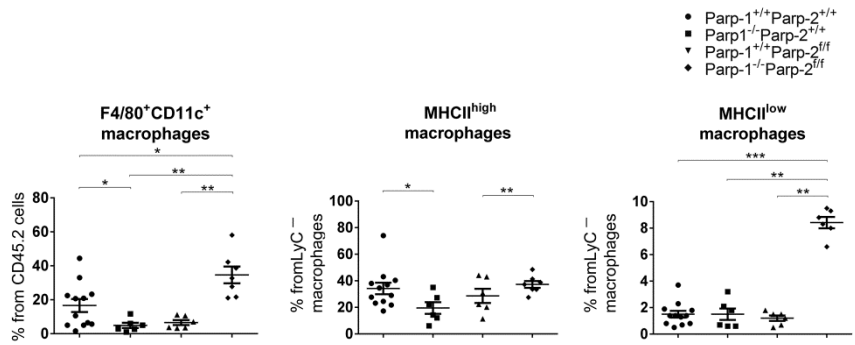


Figure 23.- Double PARP-1 and PARP-2-deficiency in host-mice impact on the tumor-infiltrating macrophages. Flow cytometry analysis of tumor-infiltrating myeloid lineage cell subsets. Percentage of total macrophages (F4/80$^+$;CD11c$^+$) from tumor infiltrating CD45.2$^+$; MHCII^{high} and MHCII^{low} macrophage subsets. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

In addition, tumors hosted in dually PARP-1 and PARP-2 deficient mice display higher percentage of CD11b$^+$ dendritic cells than single and control hosted mice while dendritic cells expressing CD103, generally associated to induction of antitumor T cell responses, was slightly decreased (Figure 24). Altogether, our data suggest that single deficiency of PARP-1 or PARP-2 promotes an antitumor environment while double deficiency of these proteins would result in the opposite effect.

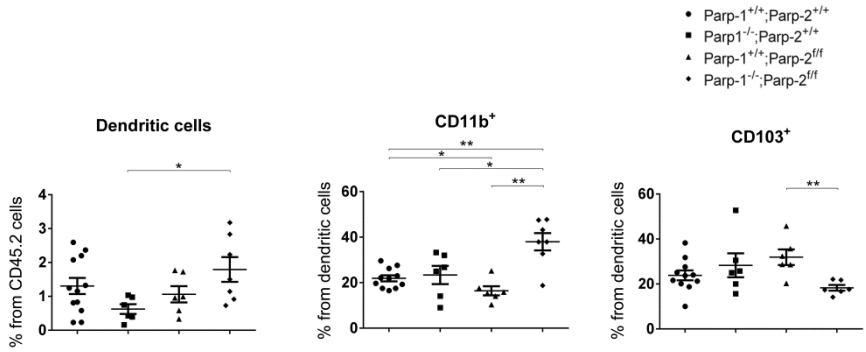


Figure 24.- Double PARP-1 and PARP-2-deficiency in host-mice impact on the dendritic cell subsets. Flow cytometry analysis of tumor-infiltrating myeloid lineage cell subsets. Percentage of total dendritic cell and DC subsets. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Interestingly, the percentage of $CD11b^+$ monocytes was significantly increased in tumors hosted in dual T cell-specific PARP-1 and PARP-2 deficient mice in contrast to the single deficient PARP-2 mice whose tumors display a reduced $CD11b^+$ cell population. Surprisingly, when analyzing the two myeloid-derived suppressor cell compartment we observed that both M-MDSCs and G-MDSCs subsets where actually depleted in the double PARP-1 and PARP-2 knock out suggesting that immature monocytes are probably not differentiating into MDSCs in this mouse model.

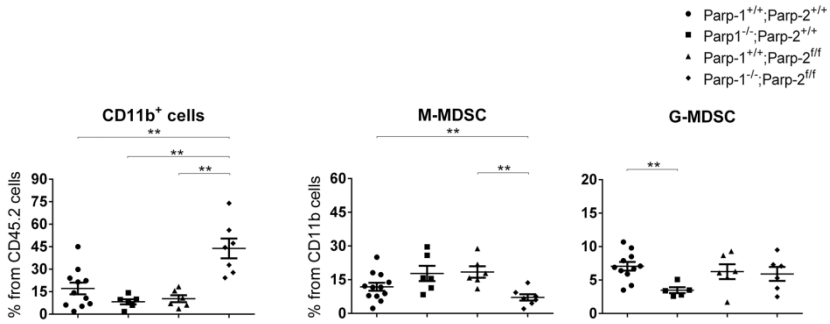


Figure 25.- Double PARP-1 and PARP-2-deficiency in host-mice impact on the Myeloid-derived suppressor cells. Flow cytometry analysis of tumor-infiltrating myeloid lineage cell subsets. Percentage of total immature monocytes and MDSCs subsets. Dots represent individual mice and horizontal lines represent median values. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

Dual PARP-1 and PARP-2 deficiency impacts on the expression of genes involved in cell migration and activation in tumor-infiltrating T cells.

To gain further insights into the faulty T cell response against AT-3-induced tumors in dually PARP-1 and PARP-2-deficient host-mice, we performed a microarray analysis of purified $CD4^+$ and $CD8^+$ tumor-infiltrating T cells, from the four genotypes, to evaluate differential expression profiles. Figures from Gene Set Enrichment analysis (GSEA), including False Discovery Rate (FDR) and Normalized Enrichment Score (NES), can be found in supplementary data.

Based on the GSEA analysis from the microarray data we followed up with validating by RT-qPCR a set of differentially expressed genes involved in crucial pathways including migration, activation and cytotoxicity of T cells. A significant decrease in the expression of genes involved in chemotaxis specially CCR2, CCR5, CCR6, CCL1, CCL4, CCL5, CXCL13, XCL1 and TBX21 was observed in dually PARP-1 and PARP-2 deficient host-mice compared to control (Figure 26). Similarly, expression of T cell activation genes such as CD80, CD83, CD86, PD1, CTLA4, IL23R were diminished in double knock out T cells (Figure 27). Remarkably, mRNA expression of granzymes and CD244, which are related to T cell-mediated cytotoxicity[96,301], were significantly down-regulated in infiltrating T cells from dually PARP-1 and PARP-2 deficient host-mice compared to control and single deficient host-mice (Figure 28). Other cellular attack mechanisms of cytotoxic T cells are mediated by the expression and release of death ligands such as FasL[96]. Of note, FasL expression was also significantly down-regulated in infiltrating T cells from dually PARP-1 and PARP-2 deficient host-mice compared to control and single deficient host-mice (Figure 28).

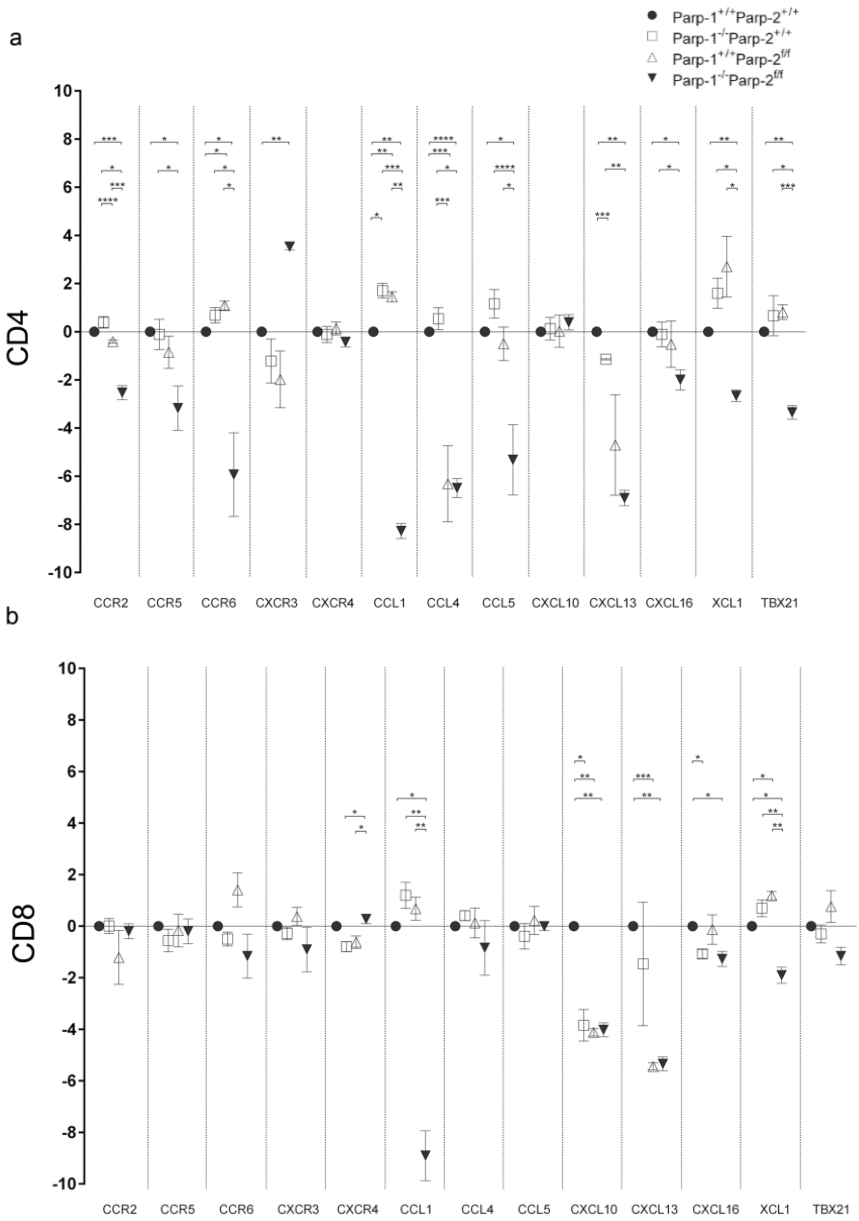


Figure 26.- Dual PARP-1 and PARP-2 deficiency impacts on the expression of genes involved in cell migration of tumor-infiltrating T cells. Quantitative RT-qPCR analysis of cell migration, genes mRNA expression in sorted (a) $CD4^+$ and (b) $CD8^+$ T cells infiltrating the AT-3-induced breast tumors hosted in the indicated genotypes. Samples were normalized according to β-actin expression levels. Results are expressed as log2-fold expression compared with levels measured in T cells from hosted control mice. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

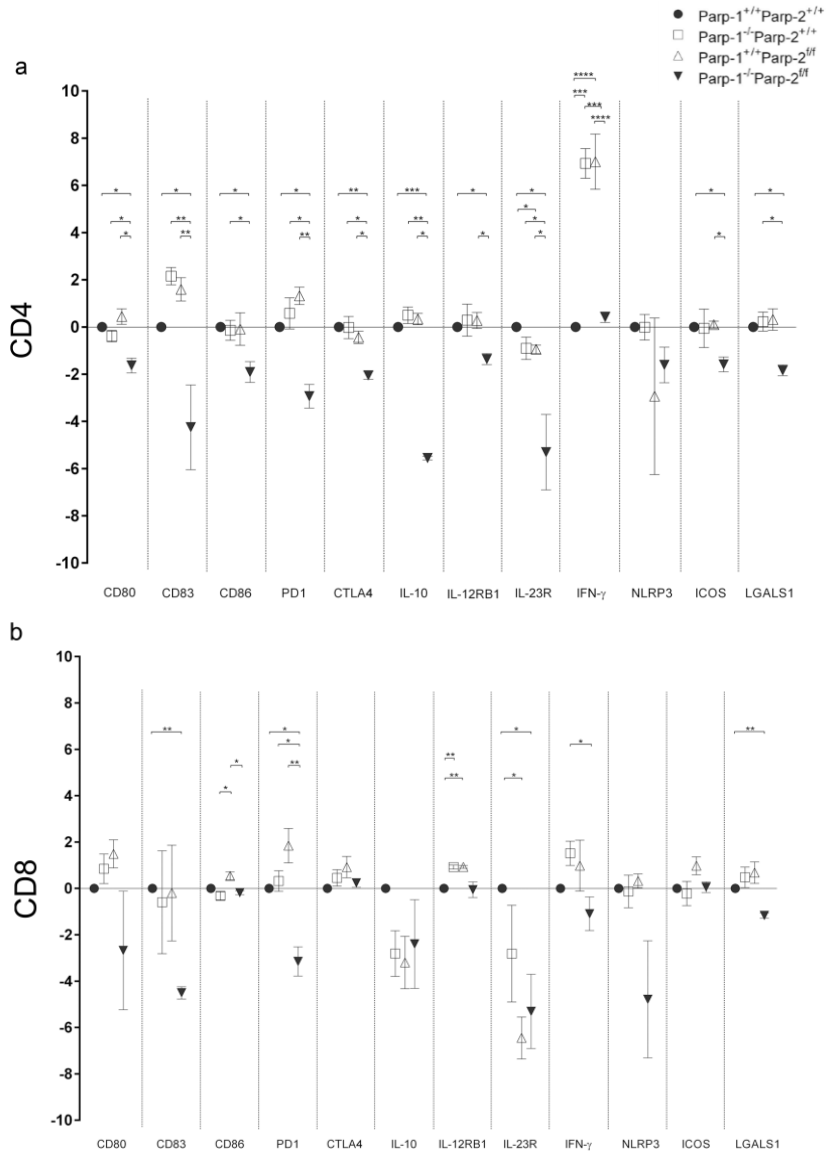


Figure 27.- Dual PARP-1 and PARP-2 deficiency impacts on the expression of genes involved in T cell activation of tumor-infiltrating T cells. Quantitative RT-qPCR analysis of cell activation, genes mRNA expression in sorted (a) $CD4^+$ and (b) $CD8^+$ T cells infiltrating the AT-3-induced breast tumors hosted in the indicated genotypes. Samples were normalized according to β-actin expression levels. Results are expressed as log2-fold expression compared with levels measured in T cells from hosted control mice. Values represent the mean ± SEM. Statistically significant differences*P < 0.05; **P < 0.01; ***P < 0.001.

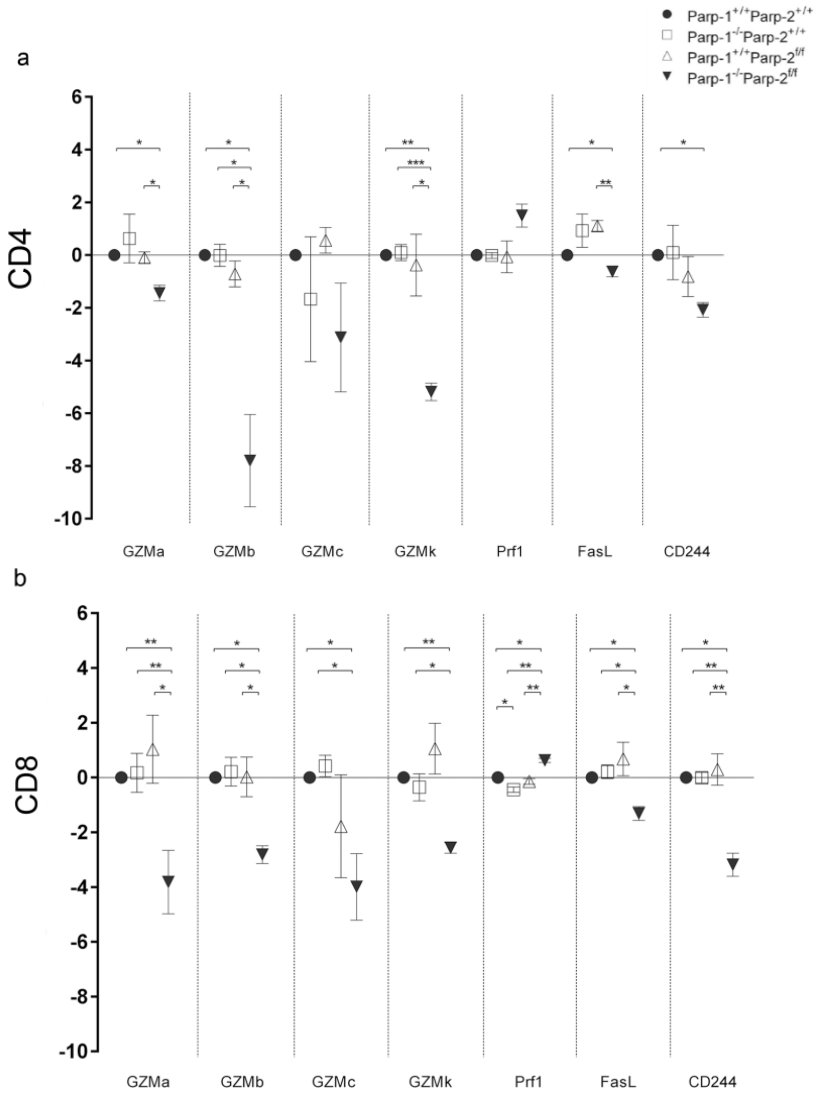


Figure 28.- Dual PARP-1 and PARP-2 deficiency impacts on the expression of genes involved in cytotoxicity of tumor-infiltrating T cells. Quantitative RT-qPCR analysis of cytotoxicity genes mRNA expression in sorted (a) $CD4^+$ and (b) $CD8^+$ T cells infiltrating the AT-3-induced breast tumors hosted in the indicated genotypes. Samples were normalized according to β-actin expression levels. Results are expressed as log2-fold expression compared with levels measured in T cells from hosted control mice. Values represent the mean ± SEM. Statistically significant differences*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

DISCUSSION

The crucial role of PARP proteins in the DNA damage response is, at present, still being explored for therapeutic purposes against cancer[224,288,297]. Through physical association and/or PARylation of their partner proteins, PARP-1 and PARP-2 are able to induce chromatin decondensation and initiate DNA repair processes[163]. Cells with an inadequate DNA stability system are thus more sensitive to cytotoxicity of DNA-methylating agents and ionizing radiation. Hence, it is of no surprise that in recent years we have witnessed an upswing and considerable enthusiasm surrounding anti-tumor therapies that target PARP proteins, with non-specific PARP inhibitors currently in clinical trials or already approved for clinical use[206]. Nevertheless, the potential detracting effects of such inhibiting strategies on the immune system have been alarmingly ignored so far. This reaches a stronger relevance under the notion that tumors are composed of an intimate mixture of cancer cells and non-cancer cells such as infiltrating immune cells comprising the tumor microenvironment. Accordingly, in the present work we have studied the immunomodulatory implications of PARP-1 and PARP-2 in the response to tumors and their influence on the different immune cell populations.

Beyond DNA damage repair, a wide array of biological functions has been attributed to PARP-1 and PARP-2, including gene transcription regulation, cell death and energy homeostasis among others[139,163]. In addition, certain roles in innate and adaptive immune responses have been described for PARP-1 and PARP-2[129,275]. Notably, whereas mice with PARP-1 or PARP-2 single deficiencies exhibit normal peripheral T cell homeostasis[247], PARP-1; PARP-2 doubly-deficient mice display T lymphomas and faulty T cell driven immune responses against viruses[280]. Dampened immune responses observed in this mouse model seem to be in agreement with the results presented on this thesis regarding tumor growth progression.

Indeed, tumor growth using a syngeneic tumor model of breast cancer, induced by the cell line AT-3, is significantly faster when mice double deficient for PARP-1 and PARP-2 are used as host, compared with control and single deficient hosts. These data shows consistency and was reproducible when tumors were induced with EG7-OVA, a syngeneic thymoma cell line, further indicating that an inefficient immune response is likely to be behind the higher tumor growth rate observed in doubly-deficient mice.

Moreover, when we explored whether the simultaneous pharmacological inhibition of PARP-1 and PARP-2 by olaparib had the same effects on tumor growth as the double genetic inactivation, we observed that, when olaparib-sensitive tumor cell line was implanted in a context of immune deficiency mice (SCID), a significant decrease in tumor burden was observed as a result of unspecific PARP inhibition within the tumor. The antitumor effect was however lost when the tumor cell line was implanted in the context of an intact immune system. And furthermore, in immunocompetent mice (C57BL/6J), PARP pharmacological inhibition in fact accelerated growth of a olaparib-resistant cell line. Altogether, these data suggest that the anti-tumor effect of olaparib on a sensitive tumor cell line is blunted by an intact immune system. It is possible that the positive anti-cancer effect of olaparib on the cancer competes with the potentially negative pro-cancer effect of olaparib on the immune system and as such when the cancer is resistant to olaparib such as with EG7-OVA cells, there is a net tumorigenic effect from the immune effects of olaparib.

As previously mentioned, progression of tumors is subjective to their interaction with the cellular component of the tumor microenvironment, especially infiltrating immune cells. As main players in adaptive immune response, T cells, particularly $CD8^+$ effector T cells, have the ability to directly target tumor cells for their elimination. Consensus exists in that

sufficient infiltration and activation of T cells are two key factors for improved prognosis[56]. Thus, recruitment, hyper-proliferation and proper function (cytotoxicity and/or cytokine secretion) of effector memory T cells is of vital importance to mount a proper immune response.

In our analysis, a significant depletion of $CD8^+$ T cells was consistently observed in mice in which T cells where affected by genetic ablation of PARP-1 and PARP-2 both in the tumor and in secondary lymphoid organ spleen. Previously published data[280] suggests a compensatory role of PARP-1 and PARP-2 in T cell homeostasis, as evident by the T cell lymphopenia described in mice bearing a T cell specific deficiency of PARP-2 within a PARP-1 deficient background, being particularly severe in the memory T cell compartment. Further analyses report an increased cell death rate and no defect in the passage to S-phase of peripheral T cells from PARP-1/PARP-2 doubly deficient mice[280]. In accordance, impairment in the generation or survival of the memory T compartment could explain the imbalance ratio of naïve/memory doubly-deficient T cells. In view of that, the alterations in the percentage of infiltrating $CD8^+$ lymphocytes that result from PARP-1 and PARP-2 silencing may explain, at least partially, the differences in the progression of AT-3-induced tumors observed in our models. Indeed, the reduction of infiltrating $CD8^+$ T cells in tumor hosted by dually PARP-1 and PARP-2-deficient mice is affecting the effector cells while the percentage of infiltrating naïve $CD8^+$ T cells is similar to that in tumors hosted in control mice.

Failure of cancer immunosurveillance is also largely attributed to the inadequate activation of tumor-specific $CD8^+$ cytotoxic T lymphocytes. With the aim of characterizing activation state of T cells we analyzed expression of PD-1 on $CD4^+$ and $CD8^+$. PD-1 is an immune-checkpoint molecule expressed in chronically stimulated $CD4^+$ and $CD8^+$ T cells and its expression has been associated with T cell activation but also with T

cell dysfunction[302]. Our observations associate single deficiency of either PARP-1 or PARP-2 to a higher percentage of PD-1 expressing $CD8^+$ memory T cells whereas tumors of mice with T cell specific dual deficiency exhibit reduced percentages of $CD4^+$ and $CD8^+$ memory T cells expressing PD-1. PD-1 binding to its ligands PD-L1 and PD-L2 impairs TCR signaling and co-stimulation[303] leading to T cell dysfunction and tumor escape and anti-PD-1 antibodies have been used successfully in cancer therapy as immune checkpoint inhibitors[304]. However, PD-1 is also marker of T cell activation and different studies have associated its expression on $CD8^+$ cells with a favorable clinical outcome[305]. The presence of tumor-infiltrating T cells expressing PD-1 together with production of IFNγ has been associated with an active tumor microenviroment[302]. In this regard, we also report an increased percentage of IFNγ-producing cells infiltrating tumors in single PARP-1 and PARP-2 deficient host-mice and the opposed observation in dually PARP-1 and PARP-2 deficient host-mice where the percentage of infiltrating IFNγ-producing cells is reduced. All in all, the diminished tumor growth we report in our single knock outs, which have larger percentage of PD-1 expressing $CD8^+$ memory T cells, might be explained, at least partially, by the presence of more activated T cells.

Unsurprisingly, when we analyzed expression profiles of TILs from mice with different PARP deficiencies we observed, in dual deficient mice, a remarkable decrease in the expression of genes involved in T cell activation including above discussed, PD-1. We detected a reduced expression of CD80 and CD86, which are the cognate ligands for CD28 and CTLA-4. They are induced in T cells after activation and their expression on $CD4^+$ T cells have been shown to play an essential role in proliferation and survival[306]. Likewise, interaction of CTLA-4 (also decreased in the expression analysis) with CD80 and CD86 is important in

maintenance of peripheral tolerance by induction of anergy[307]. Additionally, we report downregulation of IL-23 receptor which has been suggested to specifically modulate regulatory T cell induction[308]; as well as memory T cell proliferation and function including secretion of important cytokines such as IL-17 and IFN-γ[309]. This might also be related with our data reporting decreased IFN-γ-producing leukocytes within the tumor of PARP-1 and PARP-2 dual deficient mice.

The reduced T cell recruitment to tumor in our models of double deficiency of PARP-1 and PARP-2 is also supported by the data from our gene expression analysis where we report a reduced expression of chemotaxis-related genes that are relevant for T lymphocyte migration to the tumor sites. These mice exhibit downregulation of CCL4 and CCL5 (secreted by CD8 memory T cells) which are known to actively recruit T lymphocytes[310,311]; as well as CCL1 that, in turn, attracts T regulatory cells[312]. These chemokine expression patterns are in accordance with our observations in the percentage of infiltrating memory and regulatory T cells. Likewise, expression of chemokine receptor CCR5, known to play a crucial role in early recruitment of memory $CD8^+$ T cells in viral infections[313] was also reduced in double mutant T cells. Of special interest is the downregulation of CCR2 which has been associated with intrinsic regulation of inflammatory T cell cytokine expression and regulation of effector/ regulatory T cell ratio[314]. In addition we observed downregulation of XCL1, specialized in antigen cross-presentation.

Effector functions of CTLs include granulocyte exocytosis and the death ligand/death receptor system[96]. We report a downregulation of the genes encoding perforin1 and granzymes a, b c and k in mice with double deficiency in PARP-1 and PARP-2 suggesting impaired cytotoxicity as one of the reasons behind the accelerated tumor growth observed in this mice when they host syngeneic tumors. Release of death ligands is another mechanism of

effector cytotoxicity[96]. Of note, we detected that FasL expression was also significantly lessened in infiltrating T cells from dually PARP-1 and PARP-2 deficient host-mice compared to controls, pointing to a compromised FasL-mediated immunosurveillance as another contributor to tumor progression in these mice models. Interestingly, we also observed a reduction in mRNA expression of CD244 which is a signaling lymphocyte activation molecule (SLAM) found on many immune cell types including a subset of T cells and NKs and whose high expression has been associated with T cell/NK inhibition or exhaustion[315]. In sum, these data could be a reflection of an impaired differentiation of tumor infiltrating T cells into effector cytotoxic T cells and a dysfunction of these CTLs.

Nevertheless, $CD8^+$ T cell cytotoxic antitumor activity is heavily modulated by the TME by either, non-cellular components, the influence of tumor cells or through the activity of other immune cell populations. As the main antigen presenting cells, tumor resident DCs play a pivotal role in priming T cells and inducing effective T cells responses against tumor-specific antigens. We report that tumors hosted in dually PARP-1 and PARP-2 deficient mice exhibit higher percentage of $CD11b^+$ dendritic cells than single and control hosted mice while the percentage of dendritic cells expressing CD103 is decreased. Functions of intratumoral $CD103^+$ DCs include antigen cross presentation, and production of IL-12, and have been therefore associated to tumor rejection[89,144,316]. On the other hand, $CD11b^+$ dendritic cells have been associated primarily with the induction of Th2 cell responses[316]. These data further supports an effect of the double deficiency of PARP-1 and PARP-2 in shifting the balance of immune populations creating a rather tolerogenic TME, which might facilitate tumor progression[61].

Tumor associated macrophages are considered the main tumor-promoting, immunosuppressive cells (involved in tumor development, metastasis

formation and resistance to therapy) in the TME after polarization to a M2-like phenotype[317]. Whereas M1 macrophages are pro-inflammatory and contribute to Th1 anti-tumor response, M2 TAMs (which downregulate expression of MHCII) support tumor survival inhibiting T cell responses, by exerting a negatively influence on cytotoxic T cells and recruiting T regulatory cells. Accordingly, tumor progression generally correlates with the level of TAM infiltration[300]. Percentage of $MHCII^{low}$ macrophages was significantly increase in tumors from PARP-1 and PARP-2 double deficient mice compared to single deficient and control mice. Interestingly, macrophage infiltration was considerably reduced in tumors hosted in PARP-1 and PARP-2 single knock out mice which could be indicative of a less immunosuppressive TME. These data suggest that T cell responses are dampened in dual deficient mice partially due to the negative immune regulation that TAMs exert on cytotoxic T cells.

A number of studies have also implicated immature monocytes in dampening T cell responses in tumors[318]. These cells are precursors of macrophages, granulocytes, DCs and myeloid suppressor cells at early stages of differentiation. In our analysis we report an increased percentage of $CD11b^+$ cells in tumors growing in mice with double deficiency in PARP-1 and PARP-2 compared to controls with no correlation with either MDSC cell subset suggesting that circulating immature monocytes might be directly differentiating into TAMs or $CD11b^+$ DCs in these mice.

In summary, our study demonstrates that individual and dual PARP-1 and PARP-2 deficiency in T cells mediate contradictory effects on the response to PARP-1 and PARP-2-proficient tumor growth which, in turn, impact on tumor progression. Thus, while the single deficiency of any of these proteins exerts an antitumor effect, the double deficiency favors tumor growth, independently of the tumor cells. We found differential effects of single and double PARP deficiency on immunological profiles

of tumor infiltration. Dually PARP-1 and PARP-2 deficient mice display reduced infiltration of $CD4^+$ and $CD8^+$ T cells associated to the memory cell compartment with a concomitant reduction in PD-1 and IFNγ expressing memory T cells and an increase in γδ T cells compared to controls with a largely opposite pattern in single deficient mice.

Single and double deficient mice also differ in terms of NK and myeloid cell subset profiles with a notable increase in $MHCII^{low}$ macrophages and $CD11b^+$ dendritic cells and a reduction in $CD103^+$ dendritic cells in a dually deficient mice, changes predicted to be associated with increased tumor progression[61,89]. In addition, we observed a reduction in expression of chemotaxis, T cell activation and specific T cell-mediated cytotoxicity genes in dually deficient mice compared to the other genotypes. It can thus be seen that single and double deficiency of PARP-1 and PARP-2 exert differential effects on the intra-tumor immunological landscape which are likely to impact the antitumor response and contribute to our observe differences in tumor progression between genotypes. Given the well-known promiscuity of existing PARP inhibitors used as chemical tool compounds or as clinically trialed or approved therapeutics[290,292], a better understanding of the impact of single and multi-isoform inhibition will help to inform the design of novel PARP inhibitors with fewer off-target effects and an optimized anti-tumor immune response.

CONCLUSIONS

The work collected in this thesis emphasizes the urgency of understanding the immunomodulatory effect of PARP proteins in the cross-talk between immune cells in the tumor microenvironment and their effector functions, particularly in response to cancer. From our results we can conclude that:

1. Simultaneous deficiency of PARP-1 and PARP-2 in host T cells promotes growth of PARP-proficient syngeneic tumors.
2. Single deficiency of PARP-1 or PARP-2 limits tumor progression of PARP-proficient syngeneic tumors.
3. An intact immune system can dampen the anti-tumor effect of PARP-inhibitor Olaparib on a sensitive tumor cell line.
4. Single and double deficiency of PARP-1 and PARP-2 entail differences in global immunological profiles of secondary lymphoid organs.
5. Mice with T cell specific double-deficiency in PARP-1 and PARP-2 exhibit a significant depletion of splenic and tumor infiltrating $CD8^+$ T lymphocytes with a stronger effect in $CD8^+$ memory T cell compartment implicated in direct killing of tumor cells.
6. T cells with double deficiency in PARP-1 and PARP-2 show reduced PD-1 expression on tumor infiltrating T cells.
7. Tumors from mice harboring a PARP-1 and PARP-2 dual deficiency exhibit a reduced infiltration of Natural Killer cells.
8. Tumors from mice harboring a PARP-1 and PARP-2 dual deficiency exhibit a higher infiltration of M2-like tumor associated macrophages, $CD11b^+$ dendritic cells and immature monocytes, all of which have immunosuppressive characteristics.
9. T cell-specific ablation of PARP-1 and PARP-2 downregulates expression of genes involved in chemotaxis, activation and cytotoxicity in T cells infiltrating PARP proficient tumors.

SUPPLEMENTARY DATA

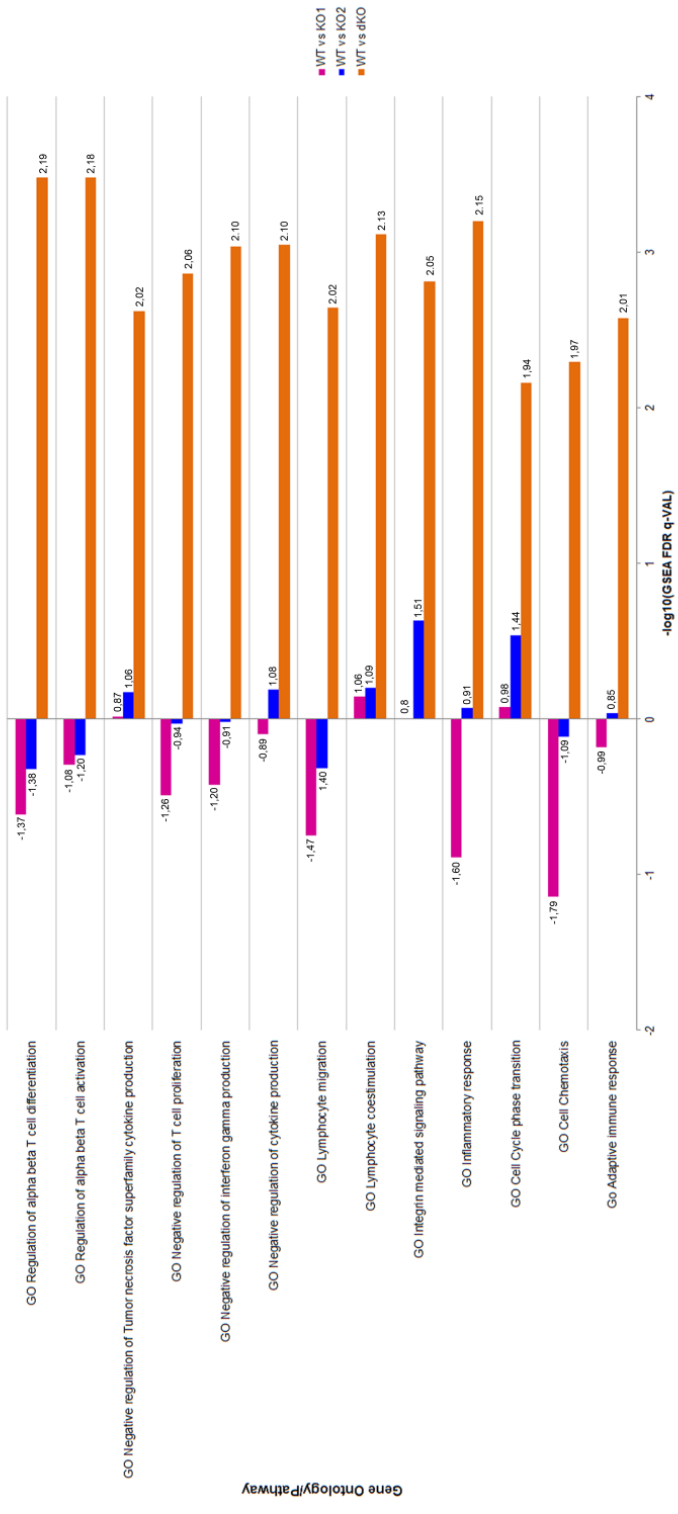

WT vs KO1
WT vs KO2
WT vs dKO
-log10(GSEA FDR q-VAL)
Gene Ontology/Pathway
GO Regulation of alpha beta T cell differentiation
GO Regulation of alpha beta T cell activation
GO Negative regulation of Tumor necrosis factor superfamily cytokine production
GO Negative regulation of T cell proliferation
GO Negative regulation of interferon gamma production
GO Negative regulation of cytokine production
GO Lymphocyte migration
GO Lymphocyte coestimulation
GO Integrin mediated signaling pathway
GO Inflammatory response
GO Cell Cycle phase transition
GO Cell Chemotaxis
Go Adaptive immune response
-1,37
-1,38
2,19
-1,08
-1,20
2,18
0,87
1,06
2,02
-1,26
-0,94
2,06
-1,20
-0,91
2,10
-0,89
1,08
2,10
-1,47
1,40
2,02
1,06
1,09
2,13
0,8
1,51
2,05
-1,60
0,91
2,15
0,98
1,44
1,94
-1,79
-1,09
1,97
-0,99
0,85
2,01

Figure S1.- GO enrichment analysis from GSEA in CD4$^+$ T cells. Selected Gene Ontology terms enriched comparing WT vs KO1, WT vs KO2 and WT vs dKO CD4$^+$ T cells. Bars represent $-\log_{10}$(FDR q-value) and sign and number tags represent NES score. Several gene sets related to T cell activation, and coestimulation of T cells are down-represented in infiltrating CD4$^+$ T cells from mice with dual deficiency of PARP-1 and PARP-2.

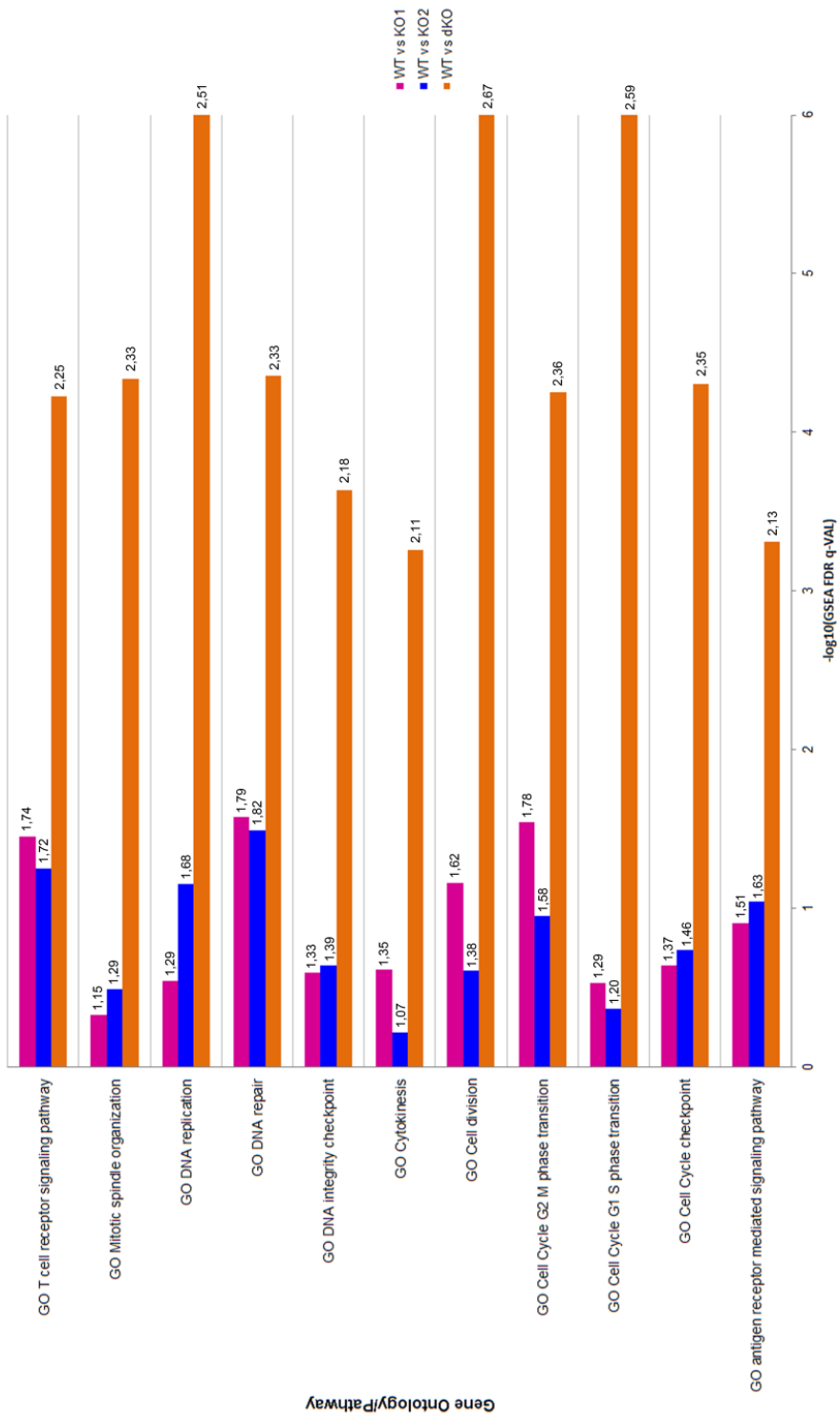
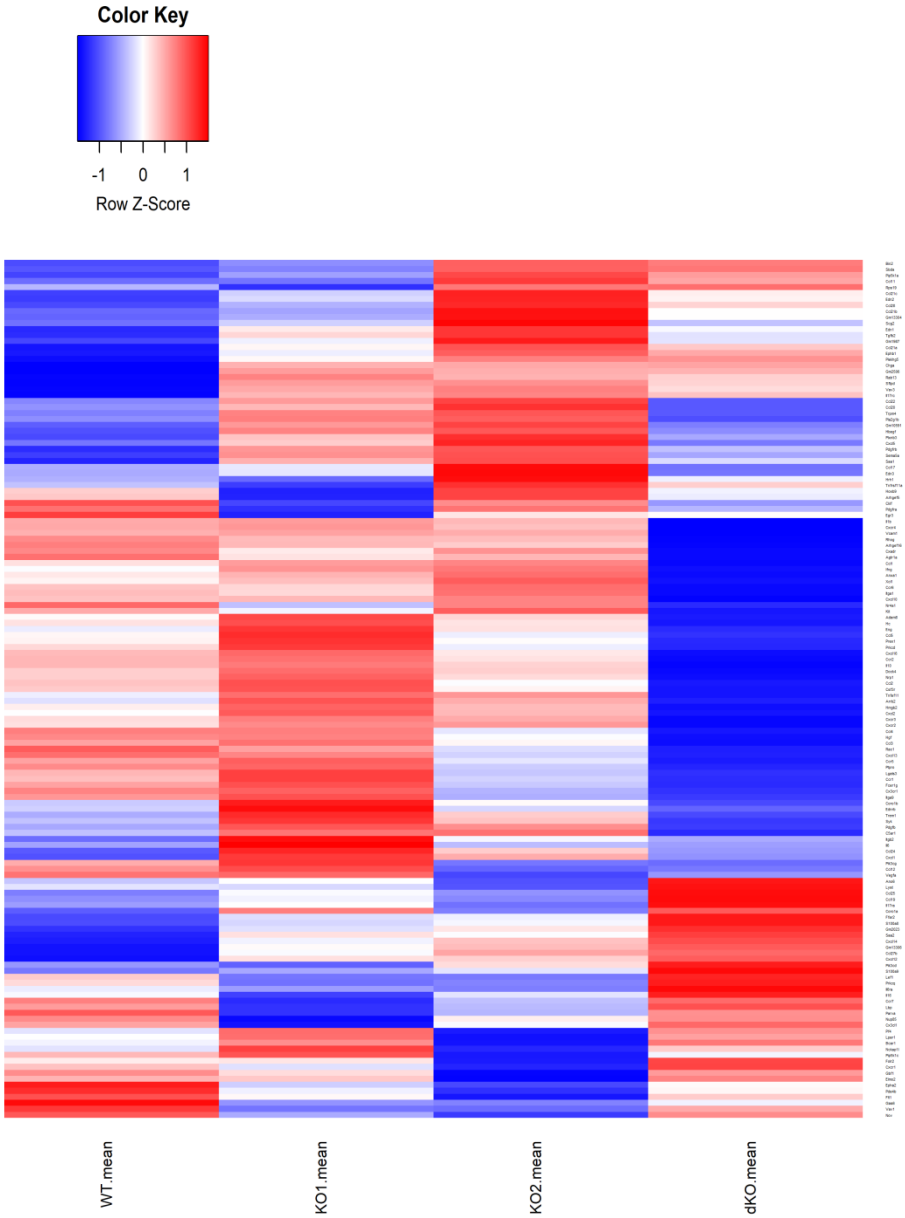

WT vs KO1
WT vs KO2
WT vs dKO
GO T cell receptor signaling pathway
GO Mitotic spindle organization
GO DNA replication
GO DNA repair
GO DNA integrity checkpoint
GO Cytokinesis
GO Cell division
GO Cell Cycle G2 M phase transition
GO Cell Cycle G1 S phase transition
GO Cell Cycle checkpoint
GO antigen receptor mediated signaling pathway
1,74
1,72
2,25
1,15
1,29
2,33
1,29
1,68
2,51
1,79
1,82
2,33
1,33
1,39
2,18
1,35
1,07
2,11
1,62
1,38
2,67
1,78
1,58
2,36
1,29
1,20
2,59
1,37
1,46
2,35
1,51
1,63
2,13
0
1
2
3
4
5
6
-log10(GSEA FDR q-VAL)
Gene Ontology/Pathway

Figure S2.- GO enrichment analysis from GSEA in CD8$^+$ T cells. Selected Gene Ontology terms enriched comparing WT vs KO1, WT vs KO2 and WT vs dKO CD8$^+$ T cells. Bars represent $-\log_{10}$(FDR q-value) and sign and number tags represent NES score. Several gene sets related to T cell receptor signaling, DNA repair and cell cycle checkpoints are down-represented in infiltrating CD8$^+$ T cells from mice with dual deficiency of PARP-1 and PARP-2.

a



b

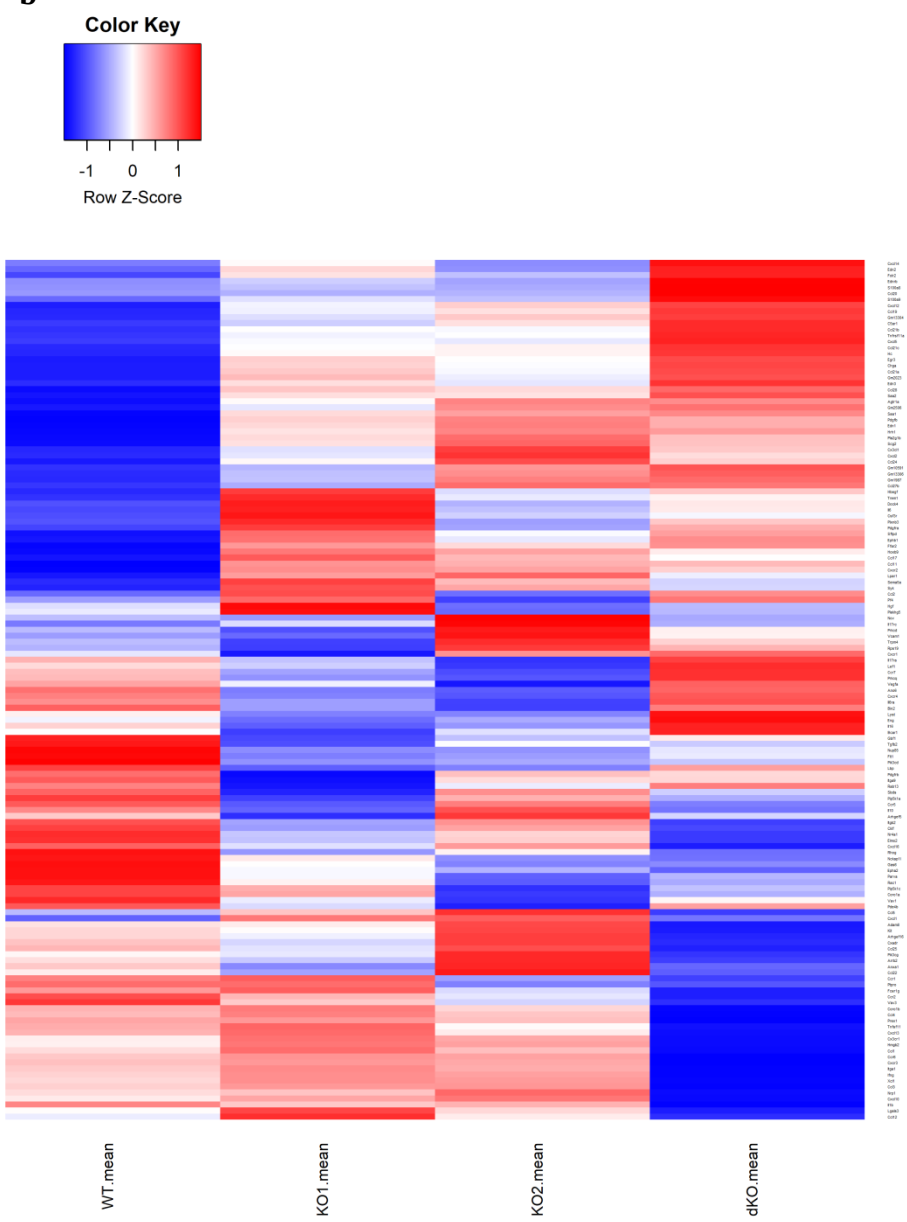
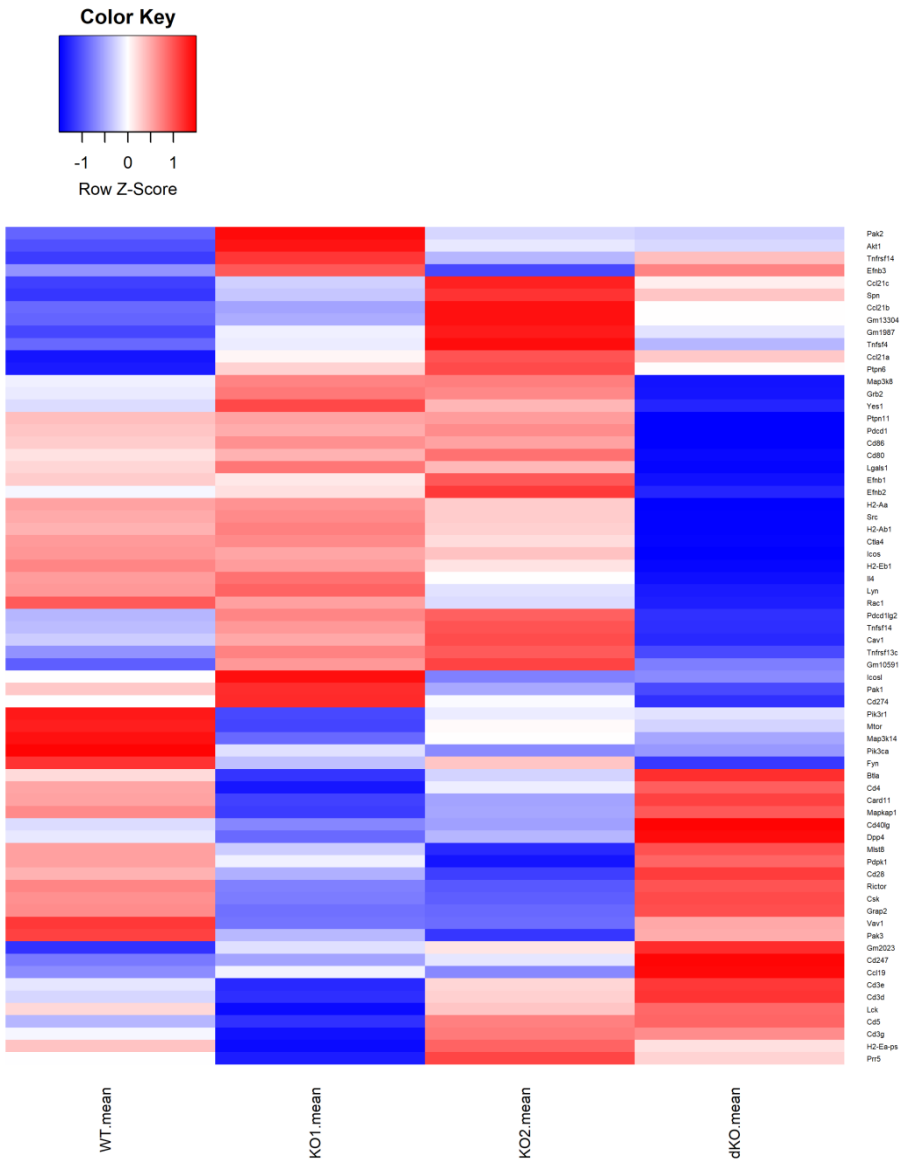


Figure S3.- Heatmaps of cell chemotaxis related genes in infiltrating T cells. (a) $CD4^+$ T cells and (b) $CD8^+$ T cells infiltrating tumors. From left to right genotypes are *Cd4-creParp-1*$^{+/+}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{-/-}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{+/+}$*Parp-2*$^{f/f}$ *and Cd4-creParp-1*$^{-/-}$*Parp-2*$^{f/f}$. Heatmaps with the genes belonging to each GO biological pathway (rows) are grouped by hierarchical clustering method average with correlation distances. Columns represent the mean intensity for each condition. Red represents highly-expressed genes and blue represents low gene expression.

a


Color Key
-1
0
1
Row Z-Score
Pak2
Akt1
Tnfrsf14
Efnb3
Ccl21c
Spn
Ccl21b
Gm13304
Gm1987
Tnfsf4
Ccl21a
Ptpn6
Map3k8
Grb2
Yes1
Ptpn11
Pdcd1
Cd86
Cd80
Lgals1
Efnb1
Efnb2
H2-Aa
Src
H2-Ab1
Ctla4
Icos
H2-Eb1
Il4
Lyn
Rac1
Pdcd1lg2
Tnfsf14
Cav1
Tnfrsf13c
Gm10591
Icosl
Pak1
Cd274
Pik3r1
Mtor
Map3k14
Pik3ca
Fyn
Btla
Cd4
Card11
Mapkap1
Cd40lg
Dpp4
Mst6
Pdpk1
Cd28
Rictor
Csk
Grap2
Vav1
Pak3
Gm2023
Cd247
Ccl19
Cd3e
Cd3d
Lck
Cd5
Cd3g
H2-Ea-ps
Prr5
WT.mean
KO1.mean
KO2.mean
dKO.mean

b

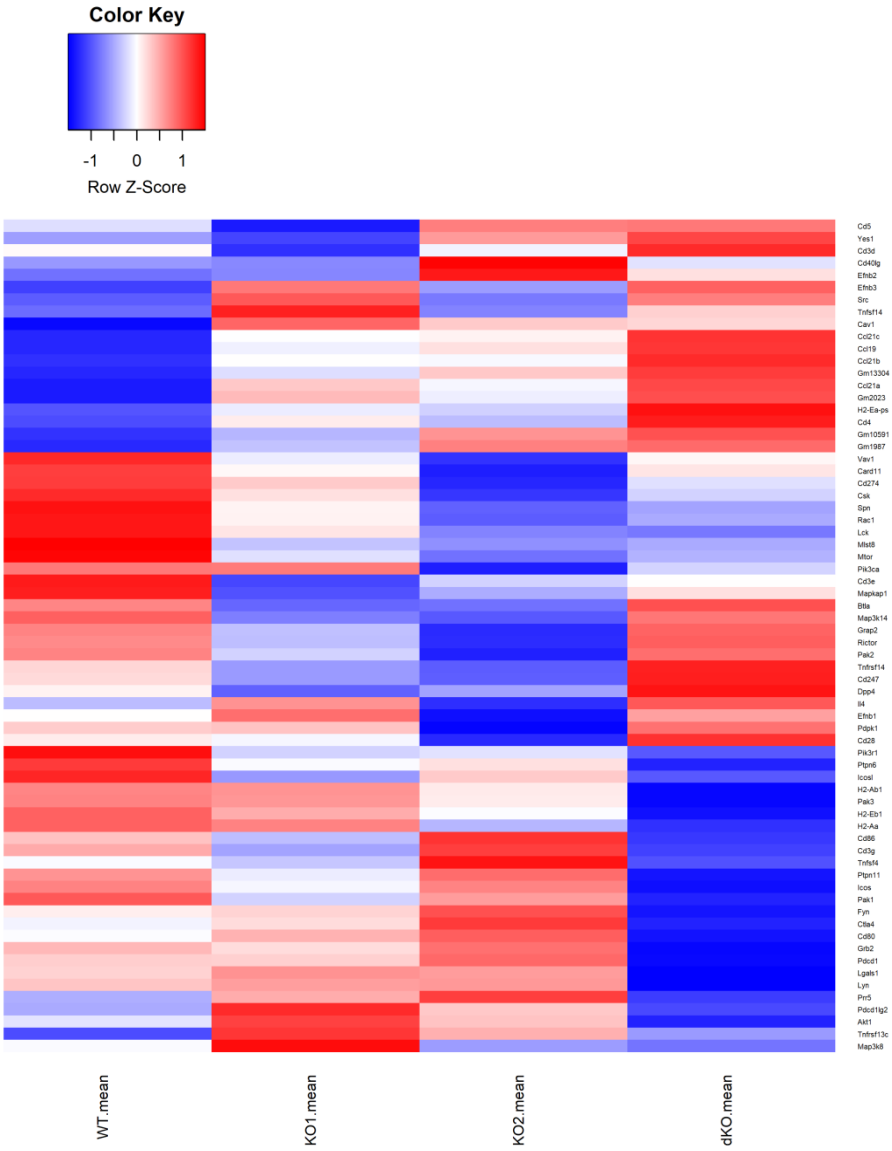
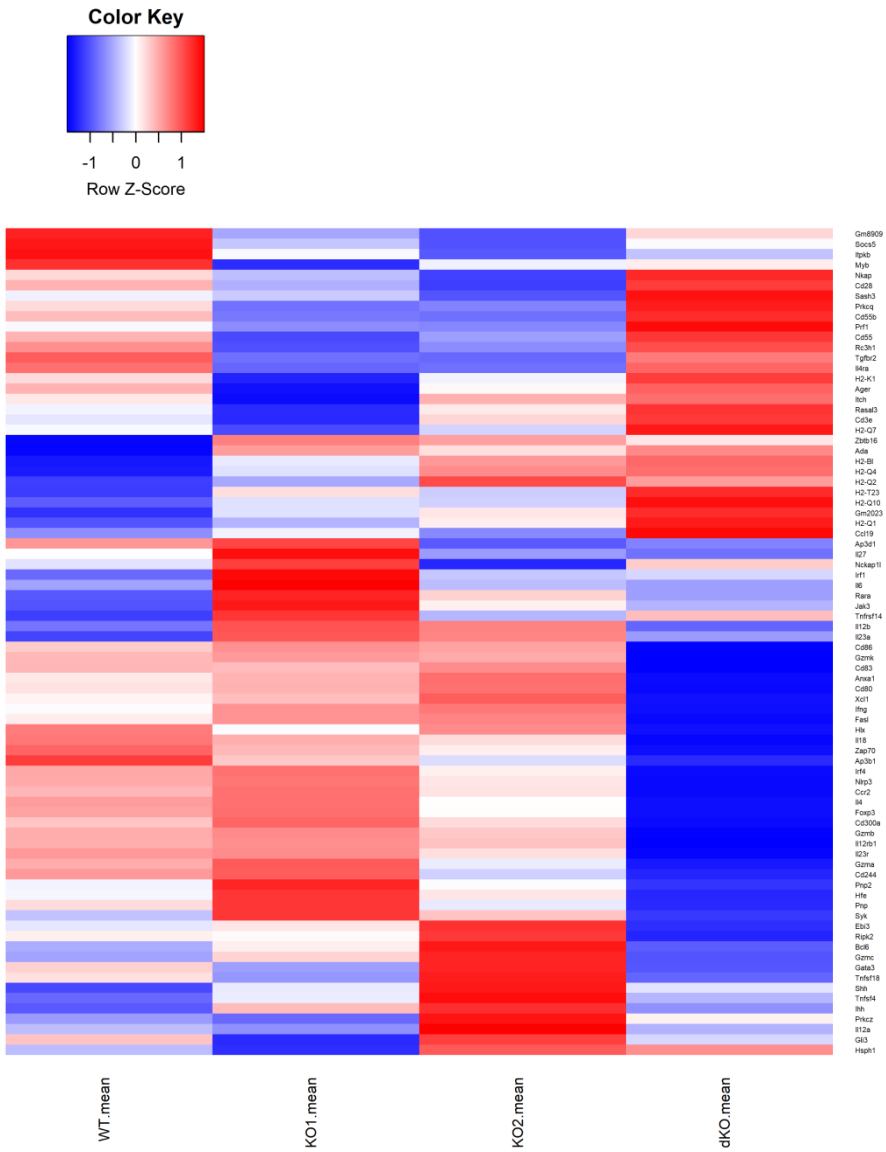

Color Key
-1
0
1
Row Z-Score
Cd5
Yes1
Cd3d
Cd40g
Efnb2
Efnb3
Src
Tnfsf14
Cav1
Ccl21c
Ccl19
Ccl21b
Gm13304
Ccl21a
Gm2023
H2-Ea-ps
Cd4
Gm10591
Gm1987
Vav1
Card11
Cd274
Csk
Spn
Rac1
Lck
Mst8
Mtor
Pik3ca
Cd3e
Mapkap1
Btla
Map3k14
Grap2
Rictor
Pak2
Tnfrsf14
Cd247
Dpp4
Il4
Efnb1
Pdpk1
Cd28
Pik3r1
Ptpn6
Icosl
H2-Ab1
Pak3
H2-Eb1
H2-Aa
Cd86
Cd3g
Tnfs14
Ptpn11
Icos
Pak1
Fyn
Ctla4
Cd80
Grb2
Pdcd1
Lgals1
Lyn
Prr5
Pdcd1lg2
Akt1
Tnfrsf13c
Map3k8
WT.mean
KO1.mean
KO2.mean
dKO.mean

Figure S4.- Heatmaps of coestimulation related genes in infiltrating T cells. (a) $CD4^+$ T cells and (b) $CD8^+$ T cells infiltrating tumors. From left to right genotypes are *Cd4-creParp-1*$^{+/+}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{-/-}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{+/+}$*Parp-2*$^{f/f}$ *and Cd4-creParp-1*$^{-/-}$*Parp-2*$^{f/f}$. Heatmaps with the genes belonging to each GO biological pathway (rows) are grouped by hierarchical clustering method average with correlation distances. Columns represent the mean intensity for each condition. Red represents highly-expressed genes and blue represents low gene expression.

a


Color Key
-1
0
1
Row Z-Score
Gm8909
Socs5
Itpkb
Myb
Nkap
Cd28
Sash3
Prkcq
Cd55b
Prf1
Cd55
Rc3h1
Tgfbr2
Il4ra
H2-K1
Ager
Itch
Rasal3
Cd3e
H2-Q7
Zbtb16
Ada
H2-Bl
H2-Q4
H2-Q2
H2-T23
H2-Q10
Gm2023
H2-Q1
Ccl19
Ap3d1
Il27
Nckap1l
Irf1
Il6
Rara
Jak3
Tnfrsf14
Il12b
Il23a
Cd86
Gzmk
Cd83
Anxa1
Cd80
Xcl1
Ifng
Fasl
Hlx
Il18
Zap70
Ap3b1
Irf4
Nlrp3
Ccr2
Il4
Foxp3
Cd300a
Gzmb
Il12rb1
Il23r
Gzma
Cd244
Pnp2
Hfe
Pnp
Syk
Ebi3
Ripk2
Bcl6
Gzmc
Gata3
Tnfsf18
Shh
Tnfsf4
Ihh
Prkcz
Il12a
Gli3
Hsph1
WT.mean
KO1.mean
KO2.mean
dKO.mean

b

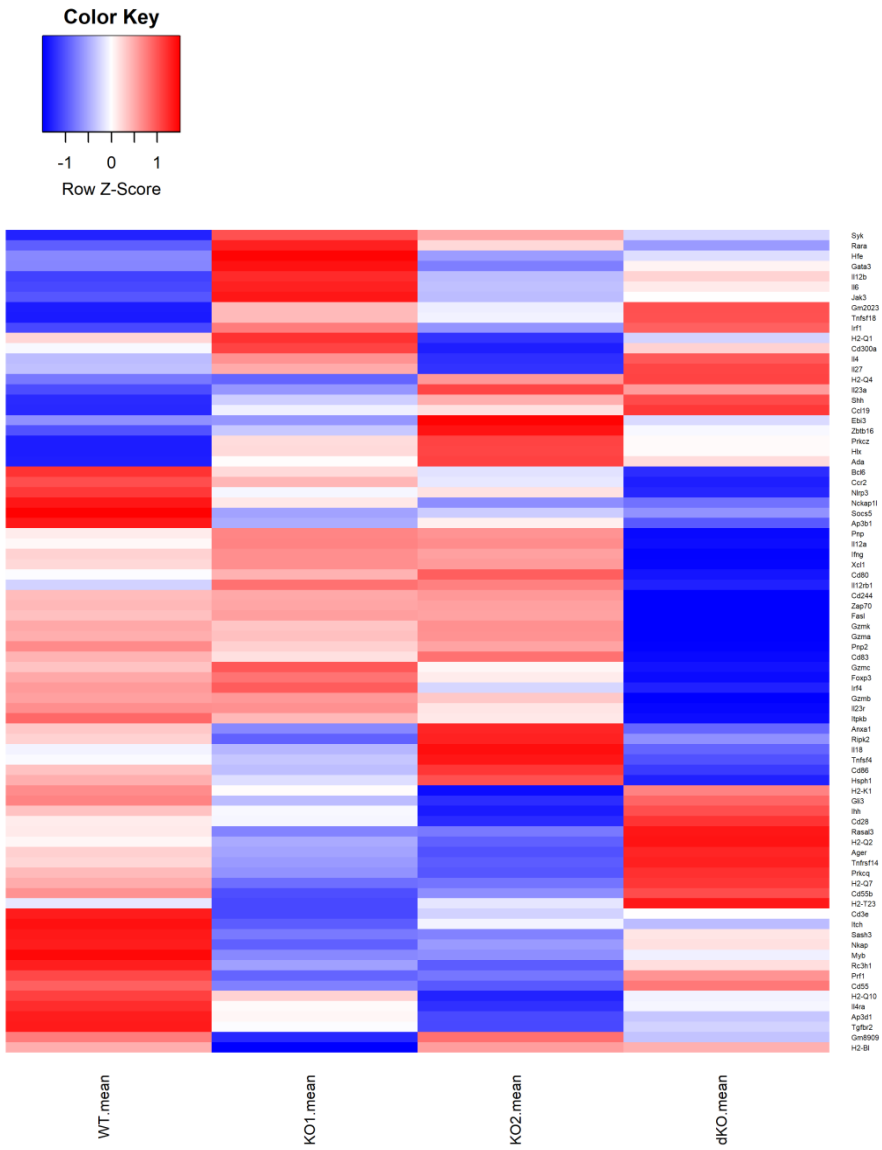

Color Key
-1
0
1
Row Z-Score
Syk
Rara
Hfe
Gata3
Il12b
Il6
Jak3
Gm2023
Tnfsf18
Irf1
H2-Q1
Cd300a
Il4
Il27
H2-Q4
Il23a
Shh
Ccl19
Ebi3
Zbtb16
Prkcz
Hlx
Ada
Bcl6
Ccr2
Nlrp3
Nckap1l
Socs5
Ap3b1
Prnp
Il12a
Ifng
Xcl1
Cd80
Il12rb1
Cd244
Zap70
Fasl
Gzmk
Gzma
Prnp2
Cd63
Gzmc
Foxp3
Irf4
Gzmb
Il23r
Itpkb
Anxa1
Ripk2
Il18
Tnfsf4
Cd86
Hsph1
H2-K1
Gli3
Ihh
Cd28
Rasal3
H2-Q2
Ager
Tnfrsf14
Prkcq
H2-Q7
Cd59b
H2-T23
Cd3e
Itch
Sash3
Nkap
Myb
Rc3h1
Prf1
Cd55
H2-Q10
Il4ra
Ap3d1
Tgfbr2
Gm8909
H2-Bl
WT.mean
KO1.mean
KO2.mean
dKO.mean

Figure S5.- Heatmaps of αβ T cell activation related genes in infiltrating T cells. (a) $CD4^+$ T cells and (b) $CD8^+$ T cells infiltrating tumors. From left to right genotypes are *Cd4-creParp-1*$^{+/+}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{-/-}$*Parp-2*$^{+/+}$*; Cd4-creParp-1*$^{+/+}$*Parp-2*$^{f/f}$ *and Cd4-creParp-1*$^{-/-}$*Parp-2*$^{f/f}$. Heatmaps with the genes belonging to each GO biological pathway (rows) are grouped by hierarchical clustering method average with correlation distances. Columns represent the mean intensity for each condition. Red represents highly-expressed genes and blue represents low gene expression.

BIBLIOGRAPHY

1. World Health Organization. Breast fact sheet. **876**, 2 (2019).

2. World Cancer Research Fund. Diet, nutrition, physical activity and breast cancer. *Contin. Updat. Proj. Expert Rep. 2018* 50 (2018). doi:10.1007/s12082-007-0105-4

3. Schedin, P. & Elias, A. Multistep tumorigenesis and the mircroenvironment. *Breast Cancer Res.* **6**, 93–101 (2004).

4. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).

5. Ford, D. *et al.* Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. *Am. J. Hum. Genet.* **62**, 676–689 (1998).

6. Dixon, J. M. ABC of Breast Disease. **321**, 24–29 (2006).

7. Anstey, E. H. *et al.* Breastfeeding and Breast Cancer Risk Reduction: Implications for Black Mothers. *Am. J. Prev. Med.* **53**, S40–S46 (2017).

8. Drooger, J. C. *et al.* Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for BRCA1 and BRCA2 mutation carriers: A critical review of the literature. *Cancer Treat. Rev.* **41**, 187–196 (2015).

9. International Agency for Research on Cancer. Combined Estrogen-Progestogen Contraceptives. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. **2005**, 100A:283-317 (2012).

10. Kenemans, P., Verstraeten, R. A. & Verheijen, R. H. M. Oncogenic pathways in hereditary and sporadic breast cancer. *Maturitas* **49**, 34–43 (2004).

11. Hilakivi-clarke, L. Estrogens , BRCA1 , and Breast Cancer. 4993–5001 (2000).

12. Mitri, Z., Constantine, T. & O'Regan, R. The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemother. Res. Pract.* **2012**, 1–7 (2012).

13. Velasco-Velázquez, M. A. *et al.* Examining the role of cyclin D1 in breast cancer. *Futur. Oncol.* **7**, 753–765 (2011).

14. Nass, S. J. & Dickson, R. B. Defining a role for c-Myc in breast tumorigenesis. *Breast Cancer Research and Treatment* **44**, 1–22 (1997).

15. Beckmann, M. W., Niederacher, D., Schnüren, H. G., Gusterson, B. A. & Bender, H. G. Multistep carcinogenesis of breast cancer and tumour heterogeneity. *J. Mol. Med.* **75**, 429–439 (1997).

16. Dunning, A. M. *et al.* A systematic review of genetic polymorphisms and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **8**, 843–854 (1999).

17. Lu, H. M. *et al.* Association of Breast and Ovarian Cancers with Predisposition Genes Identified by Large-Scale Sequencing. *JAMA Oncol.* **5**, 51–57 (2019).

18. Tan, M.-H. *et al.* Lifetime Cancer Risks in Individuals with Germline PTEN Mutations. *Clin. Cancer Res.* **18**, 400–407 (2012).

19. Masciari, S. *et al.* Breast cancer phenotype in women with TP53 germline mutations: A Li-Fraumeni syndrome consortium effort. *Breast Cancer Res. Treat.* **133**, 1125–1130 (2012).

20. Kader, T., Hill, P., Rakha, E. A., Campbell, I. G. & Gorringe, K. L. Atypical ductal hyperplasia: Update on diagnosis, management, and molecular landscape. *Breast Cancer Res.* **20**, 1–11 (2018).

21. Provenzano, E., Ulaner, G. A. & Chin, S. F. Molecular Classification of Breast Cancer. *PET Clin.* **13**, 325–338 (2018).

22. Russnes, H. G., Lingjærde, O. C., Børresen-Dale, A. L. & Caldas, C. Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters. *Am. J. Pathol.* **187**, 2152–2162 (2017).

23. Weigelt, B., Geyer, F. C. & Reis-Filho, J. S. Histological types of breast cancer: How special are they? *Mol. Oncol.* **4**, 192–208 (2010).

24. Hollern, D. P., Swiatnicki, M. R. & Andrechek, E. R. *Histological subtypes of mouse mammary tumors reveal conserved relationships to human cancers*. *PLoS Genetics* **14**, (2018).

25. Malhotra, G. K., Zhao, X., Band, H. & Band, V. Histological, molecular and functional subtypes of breast cancers. *Cancer Biol. Ther.* **10**, 955–960 (2010).

26. Sørlie, T. *et al.* Gene Expression Patterns of Breast Carcinomas Distinguish Tumor Subclasses with Clinical Implications. (2016).

27. Fragomeni, S. M., Sciallis, A. & Jeruss, J. S. Molecular Subtypes and Local-Regional Control of Breast Cancer. *Surg. Oncol. Clin. N. Am.* **27**, 95–120 (2018).

28. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumors. *Nature* **490**, 61–70 (2012).

29. Prat, A. *et al.* Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. *J. Natl. Cancer Inst.* **106**, 1–8 (2014).

30. Podo, F. *et al.* Triple-negative breast cancer: Present challenges and new perspectives. *Mol. Oncol.* **4**, 209–229 (2010).

31. Lehmann, B. D. *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. **121**, (2011).

32. Lehmann, B. D. *et al.* Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. *PLoS One* **11**, e0157368 (2016).

33. Dai, X. *et al.* Breast cancer intrinsic subtype classification, clinical use and future trends. *Am. J. Cancer Res.* **5**, 2929–2943 (2015).

34. Hassan, M. S. ., Ansari, J., Spooner, D. & Hussain, S. . Chemotherapy for breast cancer (Review). *Oncol. Rep.* **24**, 1121–1131 (2010).

35. Nurgali, K., Jagoe, R. T. & Abalo, R. Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae? *Front. Pharmacol.* **9**, 1–3 (2018).

36. Richards, M. A. Systemic treatment of breast cancer. *J. R. Soc. Health* **111**, 141–145 (1991).

37. Hussain, S. A., Williams, S., Stevens, A. & Rea, D. W. Endocrine therapy for early breast cancer. *Expert Rev. Anticancer Ther.* **4**, 877–888 (2004).

38. Thill, M., Kraft, C. & Friedrich, M. Targeted Therapy in HER2-Positive Breast Cancer. *Oncol. Res. Treat.* **39**, 295–302 (2016).

39. Dent, S., Oyan, B., Honig, A., Mano, M. & Howell, S. HER2-targeted therapy in breast cancer: A systematic review of neoadjuvant trials. *Cancer Treat. Rev.* **39**, 622–631 (2013).

40. Meric-Bernstam, F. *et al.* Advances in HER2-targeted therapy: Novel agents and opportunities beyond breast and gastric cancer. *Clin. Cancer Res.* **25**, 2033–2041 (2019).

41. Marker, S., Perner, A. & Møller, M. H. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast. *N. Engl. J. Med.* **380**, 985 (2019).

42. Adams, S. *et al.* Current Landscape of Immunotherapy in Breast Cancer: A Review. *JAMA Oncol.* 1–10 (2019). doi:10.1001/jamaoncol.2018.7147

43. Vonderheide, R. H., Domchek, S. M. & Clark, A. S. Immunotherapy for breast cancer: What are we missing? *Clin. Cancer Res.* **23**, 2640–2646 (2017).

44. Grizzi, F. & Chiriva-Internati, M. Cancer: Looking for simplicity and finding complexity. *Cancer Cell Int.* **6**, 1–7 (2006).

45. Chen, D. S. & Mellman, I. Oncology meets immunology: The cancer-immunity cycle. *Immunity* **39**, 1–10 (2013).

46. Pio, R., Ajona, D., Ortiz-Espinosa, S., Mantovani, A. & Lambris, J. D. Complementing the cancer-immunity cycle. *Front. Immunol.* **10**, 1–12 (2019).

47. Balkwill, F. R., Capasso, M. & Hagemann, T. The tumor microenvironment at a glance. *J. Cell Sci.* **125**, 5591–5596 (2012).

48. Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. *Nat. Rev. Cancer* **18**, 485–499 (2018).

49. Dunn, G. P., Old, L. J. & Schreiber, R. D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21**, 137–148 (2004).

50. Bui, J. D. & Schreiber, R. D. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Curr. Opin. Immunol.* **19**, 203–208 (2007).

51. Vakkila, J. & Lotze, M. T. Inflammation and necrosis promote tumour growth. *Nat. Rev. Immunol.* **4**, 641–648 (2004).

52. Tao, L., Huang, G., Song, H., Chen, Y. & Chen, L. Cancer associated fibroblasts: An essential role in the tumor microenvironment (review). *Oncol. Lett.* **14**, 2611–2620 (2017).

53. Cirri, P. & Chiarugi, P. Cancer-associated-fibroblasts and tumour cells: A diabolic liaison driving cancer progression. *Cancer Metastasis Rev.* **31**, 195–208 (2012).

54. Östman, A. & Augsten, M. Cancer-associated fibroblasts and tumor growth - bystanders turning into key players. *Curr. Opin. Genet. Dev.* **19**, 67–73 (2009).

55. Gueths Gomes, F., Nedel, F., Menna Alves, A., Nör, J. E. & Tarquinio Chaves, S. B. Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. *Life Sci.* **92**, 101–107 (2013).

56. Salgado, R. *et al.* The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: Recommendations by an International TILS Working Group 2014. *Ann. Oncol.* **26**, 259–271 (2015).

57. Qiu, S. Q. *et al.* Tumor-associated macrophages in breast cancer: Innocent bystander or important player? *Cancer Treat. Rev.* **70**, 178–189 (2018).

58. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage

activation: Time for reassessment. *F1000Prime Rep.* **6**, 1–13 (2014).

59. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958–969 (2008).

60. Yang, L. & Zhang, Y. Tumor-associated macrophages: from basic research to clinical application. *J. Hematol. Oncol.* **10**, 58 (2017).

61. Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883–899 (2010).

62. Bingle, L., Brown, N. J. & Lewis, C. E. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. *J. Pathol.* **196**, 254–265 (2002).

63. Sinha, P., Clements, V. K., Fulton, A. M. & Ostrand-Rosenberg, S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res.* **67**, 4507–4513 (2007).

64. Shiao, S. L. *et al.* T H 2-polarized CD4 + T Cells and macrophages limit efficacy of radiotherapy. *Cancer Immunol. Res.* **3**, 518–525 (2015).

65. Xu, M. *et al.* Intratumoral Delivery of IL-21 Overcomes Anti-Her2/Neu Resistance through Shifting Tumor-Associated Macrophages from M2 to M1 Phenotype. *J. Immunol.* **194**, 4997–5006 (2015).

66. Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. *Bone* **23**, 1–7 (2011).

67. Sica, A. & Bronte, V. Altered macrophage differentiation and immune dysfunction in tumor development Find the latest version : Review series Altered macrophage differentiation and immune dysfunction in tumor development. *J. Clin. Invest.* **117**, 1155–1166 (2007).

68. Parker, K. H., Beury, D. W. & Ostrand-rosenberg, S. Myeloid-Derived Suppressor Cells: Critical Cells Driving Immune Suppression in the Tumor Microenvironment. *Adv Cancer Res* **128**, 95–139 (2015).

69. Bronte, V. *et al.* IL-4-Induced Arginase 1 Suppresses Alloreactive T Cells in Tumor-Bearing Mice. *J. Immunol.* **170**, 270–278 (2003).

70. Raber, P., Ochoa, A. C. & Rodríguez, P. C. Metabolism of L-Arginine by Myeloid-Derived Suppressor Cells in Cancer: Mechanisms of T cell suppression and Therapeutic Perspectives. *Bone* **23**, 1–7 (2011).

71. Monu, N. R. & Frey, A. B. Myeloid-Derived Suppressor Cells and anti-tumor T cells: a complex relationship. *Immunol. Invest.* **41**, 595–613 (2013).

72. Parekh, A. *et al.* Multi-nucleated cells use ROS to induce breast cancer chemo-resistance in vitro and in vivo. *Oncogene* **37**, 4546–4561 (2018).

73. Zea, A. H. *et al.* L-Arginine modulates CD3ζ expression and T cell function in activated human T lymphocytes. *Cell. Immunol.* **232**, 21–31 (2004).

74. Rodriguez, P. C. *et al.* Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* **64**, 5839–5849 (2004).

75. Rodriguez, P. C., Quiceno, D. G. & Ochoa, A. C. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* **109**, 1568–1573 (2007).

76. Mazzoni, A. *et al.* Myeloid Suppressor Lines Inhibit T Cell Responses by an NO-Dependent Mechanism. *J. Immunol.* **168**, 689–695 (2002).

77. Huang, B. *et al.* Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* **66**, 1123–1131 (2006).

78. Oh, K. *et al.* A mutual activation loop between breast cancer cells and myeloid-derived suppressor cells facilitates spontaneous metastasis through IL-6 trans-signaling in a murine model. *Breast Cancer Res.* **15**, R79 (2013).

79. Pylaeva, E., Lang, S. & Jablonska, J. The essential role of type I interferons in differentiation and activation of tumor-associated neutrophils. *Front. Immunol.* **7**, 1–13 (2016).

80. Fridlender, Z. G. *et al.* Polarization of TAN phenotype by TGFb: N1 versus N2 TAN. **16**, 183–194 (2010).

81. Mócsai, A. Diverse novel functions of neutrophils in immunity, infammation, and beyond. *J. Exp. Med.* **210**, 1289–1299 (2013).

82. Sagiv, J. Y. *et al.* Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep.* **10**, 562–573 (2015).

83. Scapini, P. *et al.* The neutrophil as a cellular source of chemokines. *Immunol. Rev.* **177**, 195–203 (2000).

84. Van Gisbergen, K. P. J. M., Sanchez-Hernandez, M., Geijtenbeek, T. B. H. & Van Kooyk, Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J. Exp. Med.* **201**, 1281–1292 (2005).

85. Wu, L., Saxena, S., Awaji, M. & Singh, R. K. Tumor-associated neutrophils in cancer: Going pro. *Cancers (Basel).* **11**, (2019).

86. Uribe-Querol, E. & Rosales, C. Neutrophils in cancer: Two sides of the

same coin. *J. Immunol. Res.* **2015**, (2015).

87. Wylie, B., Macri, C., Mintern, J. D. & Waithman, J. Dendritic cells and cancer: From biology to therapeutic intervention. *Cancers (Basel).* **11**, 1–21 (2019).

88. Fu, C. & Jiang, A. Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. *Front. Immunol.* **9**, 3059 (2018).

89. Ma, Q. *et al.* Dissecting the Tumor Myeloid Compartment Reveals Rare Activating Antigen Presenting Cells, Critical for T cell Immunity. *Cancer Cell* **26**, 638–652 (2014).

90. Wculek, S. K. *et al.* Dendritic cells in cancer immunology and immunotherapy. *Nat. Rev. Immunol.* (2019). doi:10.1038/s41577-019-0210-z

91. Gisterek, I., Frydecka, I., Światoniowski, G., Fidler, S. & Kornafel, J. Tumour-infiltrating CD4 and CD8 T lymphocytes in breast cancer. *Reports Pract. Oncol. Radiother.* **13**, 206–209 (2008).

92. Giraldo, N. A. *et al.* The immune contexture of primary and metastatic human tumours. *Curr. Opin. Immunol.* **27**, 8–15 (2014).

93. Gingras, I., Azim, H. A., Ignatiadis, M. & Sotiriou, C. Immunology and breast cancer: Toward a new way of understanding breast cancer and developing novel therapeutic strategies. *Clin. Adv. Hematol. Oncol.* **13**, 372–382 (2015).

94. Coombs, R. R. A. & Ashby, J. Lymphocytes Infiltrating Human Breast Cancers. 166–176 (1981).

95. Caruana, I., Simula, L., Locatelli, F. & Campello, S. T lymphocytes against solid malignancies: winning ways to defeat tumours. *Cell Stress* **2**, 200–212 (2018).

96. Martínez-Lostao, L., Anel, A. & Pardo, J. How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clin. Cancer Res.* **21**, 5047–5056 (2015).

97. Ziai, J. *et al.* CD8+ T cell infiltration in breast and colon cancer: A histologic and statistical analysis. *PLoS One* **13**, 1–18 (2018).

98. Fu, Q. *et al.* Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis. *Oncoimmunology* **8**, 1–14 (2019).

99. Matkowski, R. *et al.* The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. *Anticancer Res.* **29**, 2445–2451 (2009).

100. Diefenbach, A. & Raulet, D. H. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunol. Rev.* **188**, 9–21 (2002).

101. Chao, J. L. & Savage, P. A. Unlocking the Complexities of Tumor-Associated Regulatory T Cells. *J. Immunol.* **200**, 415–421 (2018).

102. Fridman, W. H., Pagès, F., Saut̀s-Fridman, C. & Galon, J. The immune contexture in human tumours: Impact on clinical outcome. *Nat. Rev. Cancer* **12**, 298–306 (2012).

103. Murugaiyan, G. & Saha, B. Protumor vs Antitumor Functions of IL-17. *J. Immunol.* **183**, 4169–4175 (2009).

104. Presti, E. Lo *et al.* γδ T cells and tumor microenvironment: From immunosurveillance to tumor evasion. *Front. Immunol.* **9**, 1–10 (2018).

105. Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **10**, 942–949 (2004).

106. Leong, P. P. *et al.* Phenotyping of lymphocytes expressing regulatory and effector markers in infiltrating ductal carcinoma of the breast. *Immunol. Lett.* **102**, 229–236 (2006).

107. Gallimore, A. M. & Simon, A. K. Positive and negative influences of regulatory T cells on tumour immunity. *Oncogene* **27**, 5886–5893 (2008).

108. Nagarsheth, N., Kryczek, I., Wei, S., Frankel, T. & Zou, W. Regulatory T Cells in Tumor Immunity. *Encycl. Immunobiol.* **4**, 451–459 (2016).

109. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* **6**, 295–307 (2006).

110. Tanaka, A. & Sakaguchi, S. Regulatory T cells in cancer immunotherapy. *Cell Res.* **27**, 109–118 (2017).

111. Chaudhary, B. & Elkord, E. Regulatory T cells in the tumor microenvironment and cancer progression: Role and therapeutic targeting. *Vaccines* **4**, 1–25 (2016).

112. Höglund, P. & Ljunggren, H. G. Natural killer cells in cancer. *Nat. Kill. Cells* **16**, 55–64 (2010).

113. Krijgsman, D., Hokland, M. & Kuppen, P. J. K. The role of natural killer T cells in cancer-A phenotypical and functional approach. *Front. Immunol.* **9**, (2018).

114. Pahl, J. H. W. *et al.* Antibody-dependent cell lysis by NK cells is preserved after sarcoma-induced inhibition of NK cell cytotoxicity. *Cancer Immunol. Immunother.* **62**, 1235–1247 (2013).

115. Mariel, G. C. *et al.* Expression of NK cell surface receptors in breast cancer tissue as predictors of resistance to antineoplastic treatment. *Technol. Cancer Res. Treat.* **17**, 1–11 (2018).

116. Takanami, I., Takeuchi, K. & Giga, M. The prognostic value of natural killer cell infiltration in resected pulmonary adenocarcinoma. *J. Thorac. Cardiovasc. Surg.* **121**, 1058–1063 (2001).

117. Rusakiewicz, S. *et al.* Immune in filtrates are prognostic factors in localized gastrointestinal stromal tumors. *Cancer Res.* **73**, 3499–3510 (2013).

118. Klanova, M. *et al.* Prognostic Impact of Natural Killer Cell Count in Follicular Lymphoma and Diffuse Large B-cell Lymphoma Patients Treated with Immunochemotherapy. *Clin. Cancer Res.* **25**, 4634–4643 (2019).

119. Nicholson, S. E., Keating, N. & Belz, G. T. Natural killer cells and anti-tumor immunity. *Mol. Immunol.* **110**, 40–47 (2019).

120. Nelson, B. H. CD20 + B Cells: The Other Tumor-Infiltrating Lymphocytes . *J. Immunol.* **185**, 4977–4982 (2010).

121. Wang, S. si *et al.* Tumor-infiltrating B cells: their role and application in anti-tumor immunity in lung cancer. *Cell. Mol. Immunol.* **16**, 6–18 (2019).

122. Watt, V., Ronchese, F. & Ritchie, D. Resting B cells suppress tumor immunity via an MHC class-II dependent mechanism. *J. Immunother.* **30**, 323–332 (2007).

123. Tsou, P., Katayama, H., Ostrin, E. J. & Hanash, S. M. The emerging role of b cells in tumor immunity. *Cancer Res.* **76**, 5591–5601 (2016).

124. Sarvaria, A., Madrigal, J. A. & Saudemont, A. B cell regulation in cancer and anti-tumor immunity. *Cell. Mol. Immunol.* **14**, 662–674 (2017).

125. Mahmoud, S. M. A. *et al.* The prognostic significance of B lymphocytes in invasive carcinoma of the breast. *Breast Cancer Res. Treat.* **132**, 545–553 (2012).

126. Abplanalp, J. & Hottiger, M. O. Cell fate regulation by chromatin ADP-ribosylation. *Semin. Cell Dev. Biol.* **63**, 114–122 (2017).

127. Liu, C. & Yu, X. ADP-Ribosyltransferases and Poly ADP-Ribosylation. *Curr. Protein Pept. Sci.* **16**, 491–501 (2015).

128. Hottiger, M. O., Hassa, P. O., Lüscher, B., Schüler, H. & Koch-Nolte, F. Toward a unified nomenclature for mammalian ADP-ribosyltransferases. *Trends Biochem. Sci.* **35**, 208–219 (2010).

129. Gibson, B. A. & Kraus, W. L. New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nat. Rev. Mol. Cell Biol.* **13**, 411–424 (2012).

130. Chambon, P., Weill, J. D., Doly, J., Strosser, M. T. & Mandel, P. On the formation of a novel adenylic compound by enzymatic extracts of liver nuclei. *Biochem. Biophys. Res. Commun.* **25**, 638–643 (1966).

131. Nishizuca, Y., Ueda, K., Nakazawa, K. & Hayaishi, O. Studies on the Polymer of Adenosine. **242**, 3164–3171 (1967).

132. Honjo, T., Nishixuka, Y. & Kato, I. Diphteria Toxin-dependent Adenosine Diphosphate Ribosylation of Aminoacyl Transferase II and Inhibition of Protein Synthesis. (1963).

133. Kraus, W. L. PARPs and ADP-Ribosylation: 50 Years . . . and Counting. *Mol. Cell* **58**, 902–910 (2015).

134. Lüscher, B. *et al.* ADP-Ribosylation, a Multifaceted Posttranslational Modification Involved in the Control of Cell Physiology in Health and Disease. *Chem. Rev.* **118**, 1092–1136 (2018).

135. Jacobson, E. L. & Jacobson, M. K. Poly(ADP-ribose). 607–610 (1982).

136. Leung, A. K. L. Poly(ADP-ribose): An organizer of cellular architecture. *J. Cell Biol.* **205**, 613–619 (2014).

137. Pleschke, J. M., Kleczkowska, H. E., Strohm, M. & Althaus, F. R. Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J. Biol. Chem.* **275**, 40974–40980 (2000).

138. Hottiger, M. O. SnapShot: ADP-Ribosylation Signaling. *Mol. Cell* **58**, 1134.e1-1134.e1 (2015).

139. Bunney, P. E., Zink, A. N., Holm, A. A., Billington, C. J., & Kotz, C. M. A systematic analysis of the PARP protein family identifies new functions critical for cell physiology. *Physiol. Behav.* **176**, 139–148 (2017).

140. Amé, J. C., Spenlehauer, C. & De Murcia, G. The PARP superfamily. *BioEssays* **26**, 882–893 (2004).

141. Feijs, K. L. H., Forst, A. H., Verheugd, P. & Lüscher, B. Macrodomain-containing proteins: Regulating new intracellular functions of mono(ADP-ribosyl)ation. *Nat. Rev. Mol. Cell Biol.* **14**, 445–453 (2013).

142. Vyas, S. *et al.* Family-wide analysis of poly (ADP-ribose) polymerase activity. *Nat Commun* 1–29 (2015). doi:10.1038/ncomms5426.Family-wide

143. Otto, H. *et al.* In silico characterization of the family of PARP-like

poly(ADP-ribosyl)transferases (pARTs). *BMC Genomics* **6**, 1–23 (2005).

144. Schreiber, V., Dantzer, F., Amé, J. C. & De Murcia, G. Poly(ADP-ribose): Novel functions for an old molecule. *Nat. Rev. Mol. Cell Biol.* **7**, 517–528 (2006).

145. Yélamos, J., Schreiber, V. & Dantzer, F. Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends Mol. Med.* **14**, 169–178 (2008).

146. Ménissier de Murcia, J. *et al.* Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. **22**, 2255–2263 (2003).

147. Amé, J. C. *et al.* PARP-2, a novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. *J. Biol. Chem.* **274**, 17860–17868 (1999).

148. Pion, E. *et al.* DNA-induced dimerization of poly(ADP-ribose) polymerase-1 triggers its activation. *Biochemistry* **44**, 14670–14681 (2005).

149. Langelier, M., Planck, J. L., Roy, S. & Pascal, J. M. Structural basis for DNA-dependent poly(ADP-ribosyl)ation by human PARP-1. **336**, 728–732 (2002).

150. Ali, H. R., Chlon, L., Pharoah, P. D. P., Markowetz, F. & Caldas, C. Patterns of Immune Infiltration in Breast Cancer and Their Clinical Implications: A Gene-Expression-Based Retrospective Study. *PLoS Med.* **13**, 1–24 (2016).

151. Yang, G., Sau, C., Lai, W., Cichon, J. & Li, W. Structural Basis for Potency and Promiscuity in Poly(ADP- ribose) Polymerase (PARP) and Tankyrase Inhibitors. **344**, 1173–1178 (2015).

152. Dawicki-mckenna, J. M. *et al.* PARP-1 activation requires local unfolding of an autoinhibitory domain. **60**, 755–768 (2016).

153. Léger, K., Bär, D., Savić, N., Santoro, R. & Hottiger, M. O. ARTD2 activity is stimulated by RNA. *Nucleic Acids Res.* **42**, 5072–5082 (2014).

154. Langelier, M. F., Riccio, A. A. & Pascal, J. M. PARP-2 and PARP-3 are selectively activated by 5' phosphorylated DNA breaks through an allosteric regulatory mechanism shared with PARP-1. *Nucleic Acids Res.* **42**, 7762–7775 (2014).

155. Yelamos, J., Farres, J., Llacuna, L., Ampurdanes, C. & Martin-Caballero, J. PARP-1 and PARP-2: New players in tumour development. *Am. J. Cancer Res.* **1**, 328–346 (2011).

156. Schreiber, V. *et al.* Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and

XRCC1. *J. Biol. Chem.* **277**, 23028–23036 (2002).

157. Oliver, A. W. *et al.* Crystal structure of the catalytic fragment of murine poly(ADP-ribose) polymerase-2. *Nucleic Acids Res.* **32**, 456–464 (2004).

158. Zarkovic, G. *et al.* Characterization of DNA ADP-ribosyltransferase activities of PARP2 and PARP3: New insights into DNA ADP-ribosylation. *Nucleic Acids Res.* **46**, 2417–2431 (2018).

159. Belousova, E. A., Ishchenko, A. A. & Lavrik, O. I. DNA is a New Target of Parp3. *Sci. Rep.* **8**, 1–12 (2018).

160. Loseva, O. *et al.* PARP-3 is a mono-ADP-ribosylase that activates PARP-1 in the absence of DNA. *J. Biol. Chem.* **285**, 8054–8060 (2010).

161. Boehler, C. *et al.* Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2783–2788 (2011).

162. Augustin, A. *et al.* PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. *J. Cell Sci.* **116**, 1551–1562 (2003).

163. Bai, P. Biology of Poly(ADP-Ribose) Polymerases: The Factotums of Cell Maintenance. *Mol. Cell* **58**, 947–958 (2015).

164. Boulikas, T. Poly(ADP-ribose) synthesis and degradation in mammalian nuclei. *Anal. Biochem.* **203**, 252–258 (1992).

165. Davidovic, L., Vodenicharov, M., Affar, E. B. & Poirier, G. G. Importance of poly(adp-ribose) glycohydrolase in the control of poly(adp-ribose) metabolism. *Exp. Cell Res.* **268**, 7–13 (2001).

166. Mortusewicz, O., Fouquerel, E., Amé, J. C., Leonhardt, H. & Schreiber, V. PARG is recruited to DNA damage sites through poly(ADP-ribose)- and PCNA-dependent mechanisms. *Nucleic Acids Res.* **39**, 5045–5056 (2011).

167. Min, W. & Wang, Z. Q. Poly (ADP-ribose) glycohydrolase (PARG) and its therapeutic potential. 1619–1626 (2009).

168. D'Amours, D., Desnoyers, S., D'Silva, I. & Poirier, G. G. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem. J.* **342**, 249–268 (1999).

169. Kun, E., Kirsten, E. & Ordahl, C. P. Coenzymatic activity of randomly broken or intact double-stranded DNAs in auto and histone H1 trans-poly(ADP-ribosylation), catalyzed by poly(ADP-ribose) polymerase (PARP I). *J. Biol. Chem.* **277**, 39066–39069 (2002).

170. Oei, S. L. & Shi, Y. Transcription factor Yin Yang 1 stimulates

poly(ADP-ribosyl)ation and DNA repair. *Biochem. Biophys. Res. Commun.* **284**, 450–454 (2001).

171. Mashimo, M., Kato, J. & Moss, J. ADP-ribosyl-acceptor hydrolase 3 regulates poly (ADP-ribose) degradation and cell death during oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 18964–18969 (2013).

172. Mashimo, M. & Moss, J. Functional Role of ADP-Ribosyl-Acceptor Hydrolase 3 in poly(ADPRibose) Polymerase-1 Response to Oxidative Stress. *Curr. Protein Pept. Sci.* **17**, 633–640 (2016).

173. Wei, H. & Yu, X. Functions of PARylation in DNA Damage Repair Pathways. *Genomics, Proteomics Bioinforma.* **14**, 131–139 (2016).

174. Ménissier De Murcia, J. *et al.* Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7303–7307 (1997).

175. Chen, Q., Kassab, M. A., Dantzer, F. & Yu, X. PARP2 mediates branched poly ADP-ribosylation in response to DNA damage. *Nat. Commun.* **9**, (2018).

176. Gupte, R., Liu, Z. & Kraus, W. L. Parps and adp-ribosylation: Recent advances linking molecular functions to biological outcomes. *Genes Dev.* **31**, 101–126 (2017).

177. Whish, W. J. D., Davies, M. I. & Shall, S. Stimulation of poly(ADP-ribose) polymerase activity by the anti-tumour antibiotic, streptozotocin. *Biochem. Biophys. Res. Commun.* **65**, 722–730 (1975).

178. Simbulan-Rosenthal, C. M. *et al.* Misregulation of gene expression in primary fibroblasts lacking poly(ADP-ribose)polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11274–11279 (2000).

179. Farreś, J. *et al.* Parp-2 is required to maintain hematopoiesis following sublethal γ-irradiation in mice. *Blood* **122**, 44–54 (2013).

180. Trucco, C., Oliver, F. J., De Murcia, G. & Ménissier-De Murcia, J. DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res.* **26**, 2644–2649 (1998).

181. Nicolás, L. *et al.* Loss of poly(ADP-ribose) polymerase-2 leads to rapid development of spontaneous T-cell lymphomas in p53-deficient mice. *Oncogene* **29**, 2877–2883 (2010).

182. Dantzer, F. *et al.* Base excision repair is impaired in mammalian cells lacking poly(ADP- ribose) polymerase-1. *Biochemistry* **39**, 7559–7569 (2000).

183. Sunada, S., Nakanishi, A. & Miki, Y. Crosstalk of DNA double-strand

break repair pathways in poly(ADP-ribose) polymerase inhibitor treatment of breast cancer susceptibility gene 1/2-mutated cancer. *Cancer Sci.* **109**, 893–899 (2018).

184. Wang, M. *et al.* PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res.* **34**, 6170–6182 (2006).

185. Audebert, M., Salles, B. & Calsou, P. Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J. Biol. Chem.* **279**, 55117–55126 (2004).

186. Pears, C. J. *et al.* The role of ADP-ribosylation in regulating DnA double-strand break repair. **11**, 48–56 (2012).

187. Tallis, M., Morra, R., Barkauskaite, E. & Ahel, I. Poly(ADP-ribosyl)ation in regulation of chromatin structure and the DNA damage response. *Chromosoma* **123**, 79–90 (2014).

188. Martin, L. J. DNA damage and repair: Relevance to mechanisms of neurodegeneration. *J. Neuropathol. Exp. Neurol.* **67**, 377–387 (2008).

189. De Bont, R. & van Larebeke, N. Endogenous DNA damage in humans: A review of quantitative data. *Mutagenesis* **19**, 169–185 (2004).

190. Wallace, S. S. Base excision repair: A critical player in many games Susan. *Bone* **23**, 1–7 (2008).

191. Masson, M. *et al.* XRCC1 Is Specifically Associated with Poly(ADP-Ribose) Polymerase and Negatively Regulates Its Activity following DNA Damage. *Mol. Cell. Biol.* **18**, 3563–3571 (1998).

192. Caldecott, K. W., Aoufouchi, S., Johnson, P. & Shall, S. XRCC1 polypeptide interacts with DNA polymerase β and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. *Nucleic Acids Res.* **24**, 4387–4394 (1996).

193. Kutuzov, M. M. *et al.* Interaction of PARP-2 with AP site containing DNA. *Biochimie* **112**, 10–19 (2015).

194. Bian, C. *et al.* NADP + is an endogenous PARP inhibitor in DNA damage response and tumor suppression. *Nat. Commun.* **10**, 1–14 (2019).

195. Kutuzov, M. M. *et al.* Interaction of PARP-2 with DNA structures mimicking DNA repair intermediates and consequences on activity of base excision repair proteins. *Biochimie* **95**, 1208–1215 (2013).

196. Marteijn, J. A., Lans, H., Vermeulen, W. & Hoeijmakers, J. H. J. Understanding nucleotide excision repair and its roles in cancer and

ageing. *Nat. Rev. Mol. Cell Biol.* **15**, 465–481 (2014).

197. King, B. S., Cooper, K. L., Liu, K. J. & Hudson, L. G. Poly(ADP-ribose) contributes to an association between Poly(ADP-ribose) polymerase-1 and xeroderma pigmentosum complementation group A in nucleotide excision repair. *J. Biol. Chem.* **287**, 39824–39833 (2012).

198. Fadda, E. Role of the XPA protein in the NER pathway: A perspective on the function of structural disorder in macromolecular assembly. *Comput. Struct. Biotechnol. J.* **14**, 78–85 (2016).

199. Hoeijmakers, J. H. J. The consequences of DNA injury Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366–374 (2001).

200. Jeggo, P. A. & Löbrich, M. DNA double-strand breaks: Their cellular and clinical impact? *Oncogene* **26**, 7717–7719 (2007).

201. Khanna, K. K. & Jackson, S. P. DNA double-strand breaks: Signaling, repair and the cancer connection. *Nat. Genet.* **27**, 247–254 (2001).

202. Kakarougkas, A. & Jeggo, P. A. DNA DSB repair pathway choice: An orchestrated handover mechanism. *Br. J. Radiol.* **87**, (2014).

203. Ray Chaudhuri, A. & Nussenzweig, A. The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nat. Rev. Mol. Cell Biol.* **18**, 610–621 (2017).

204. McCabe, N. *et al.* Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res.* **66**, 8109–8115 (2006).

205. Farmer, H. *et al.* Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**, 917–921 (2005).

206. Bryant, H. E. *et al.* Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913–917 (2005).

207. Li, X. & Heyer, W.-D. Homologous recombination in DNA reapir and DNA. *Cell Res.* **18**, 99–113 (2008).

208. Sonoda, E. *et al.* Sister Chromatid Exchanges Are Mediated by Homologous Recombination in Vertebrate Cells. *Mol. Cell. Biol.* **19**, 5166–5169 (1999).

209. Zhong, Q. *et al.* Association of BRCA1 with the hRad5O-hMrel 1 -p95 Complex and the DNA Damage Response. **285**, 747–750 (2016).

210. Paull, T. T. *et al.* A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. 886–895 (2014).

211. Davies, A. A. *et al.* Role of BRCA2 in Control of the RAD51 Recombination and DNA Repair Protein. **7**, 273–282 (2001).

212. Scates, D. K., Clark, S. K., Phillips, R. K. S. & Venitt, S. BRCA2 Is Required for Homology-Directed Repair of Chromosomal Breaks. *Br. J. Surg.* **85**, 965–969 (1998).

213. Haince, J. F. *et al.* PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. *J. Biol. Chem.* **283**, 1197–1208 (2008).

214. Bryant, H. E. *et al.* PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *EMBO J.* **28**, 2601–2615 (2009).

215. Ying, S., Hamdy, F. C. & Helleday, T. Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Res.* **72**, 2814–2821 (2012).

216. Gottipati, P. *et al.* Poly(ADP-ribose) polymerase is hyperactivated in homologous recombination-defective cells. *Cancer Res.* **70**, 5389–5398 (2010).

217. Ruscetti, T. *et al.* Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase. *J. Biol. Chem.* **273**, 14461–14467 (1998).

218. Huber, A., Bai, P., Murcia, J. M. De & Murcia, G. De. PARP-1, PARP-2 and ATM in the DNA damage response: Functional synergy in mouse development. *DNA Repair (Amst).* **3**, 1103–1108 (2004).

219. Henrie, M. S. *et al.* Lethality in PARP-1/Ku80 double mutant mice reveals physiological synergy during early embryogenesis. *DNA Repair (Amst).* **2**, 151–158 (2003).

220. Arents, G. & Moudrianakis, E. N. The histone fold: A ubiqu'itous architectural motif utilized in DNA compaction and protein dimerization (paired-element motif/archaeal histones/centromeric CENP-A/transcription/evolution). *Biochemistry* **92**, 11170–11174 (1995).

221. Cheung, P., Allis, C. D. & Sassone-Corsi, P. Signaling to chromatin through historic modifications. *Cell* **103**, 263–271 (2000).

222. Kurdistani, S. K. & Grunstein, M. Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* **4**, 276–284 (2003).

223. Rupp, R. A. W. & Becker, P. B. Gene regulation by histone H1: New links to DNA methylation. *Cell* **123**, 1178–1179 (2005).

224. Javle, M. & Curtin, N. The role of PARP in DNA repair and its therapeutic exploitation. *Curr. Sports Med. Rep.* **16**, 467–468 (2017).

225. Aldinucci, A. *et al.* A Key Role for Poly(ADP-Ribose) Polymerase-1 Activity during Human Dendritic Cell Maturation. *J. Immunol.* **179**, 305–312 (2007).

226. Prosperi, E. & Scovassi, A. I. Dynamic Interaction between PARP-1, PCNA and p21waf1/cip1. *Poly(ADP-Ribosyl)ation* 67–74 (2008). doi:10.1007/0-387-36005-0_7

227. Guy, C. T., Cardiff, R. D. & Muller, W. J. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol. Cell. Biol.* **12**, 954–961 (1992).

228. de Murcia, G. *et al.* Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation. *J. Biol. Chem.* **261**, 7011–7017 (1986).

229. Rouleau, M., Aubin, R. A. & Poirier, G. G. Pol (ADP-ribosyl)ated chromatin domains: Access granted. *J. Cell Sci.* **117**, 815–825 (2004).

230. Tulin, A. & Spradling, A. Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci. *Science (80-.).* **299**, 560–562 (2003).

231. Martin-Hernandez, K., Rodriguez-Vargas, J. M., Schreiber, V. & Dantzer, F. Expanding functions of ADP-ribosylation in the maintenance of genome integrity. *Semin. Cell Dev. Biol.* **63**, 92–101 (2017).

232. Ali, S. O., Khan, F. A., Galindo-Campos, M. A. & Yélamos, J. Understanding specific functions of PARP-2: New lessons for cancer therapy. *Am. J. Cancer Res.* **6**, 1842–1863 (2016).

233. Messner, S. *et al.* PARP1 ADP-ribosylates lysine residues of the core histone tails. *Nucleic Acids Res.* **38**, 6350–6362 (2010).

234. Bustin, M. High Mobility Group Proteins. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1799**, 1–2 (2010).

235. Anderson, M. G., Scoggin, K. E. S., Simbulan-Rosenthal, C. M. & Steadman, J. A. Identification of Poly(ADP-Ribose) Polymerase as a Transcriptional Coactivator of the Human T-Cell Leukemia Virus Type 1 Tax Protein. *J. Virol.* **74**, 2169–2177 (2000).

236. Cervellera, M. N. & Sala, A. Poly(ADP)-ribose) polymerase is a B-MYB coactivator. *J. Biol. Chem.* **275**, 10692–10696 (2000).

237. Hassa, P. O. & Hottiger, M. O. The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-κB in inflammatory disorders. *Cell. Mol. Life Sci.* **59**, 1534–1553 (2002).

238. Quénet, D. *et al.* The histone subcode: Poly(ADP-ribose) polymerase-1

(Parp-1) and Parp-2 control cell differentiation by regulating the transcriptional intermediary factor TIF1β and the heterochromatin protein HP1α. *FASEB J.* **22**, 3853–3865 (2008).

239. Szántó, M. *et al.* Poly(ADP-ribose) polymerase-2: Emerging transcriptional roles of a DNA-repair protein. *Cell. Mol. Life Sci.* **69**, 4079–4092 (2012).

240. Lu, H. *et al.* Identification of poly (ADP-ribose) polymerase-1 (PARP-1) as a novel krüppel-like factor 8-interactingand-regulating protein. *J. Biol. Chem.* **286**, 20335–20344 (2011).

241. Butler, A. J. & Ordahl, C. P. Poly(ADP-Ribose) Polymerase Binds with Transcription Enhancer Factor 1 to MCAT1 Elements To Regulate Muscle-Specific Transcription. *Mol. Cell. Biol.* **19**, 296–306 (1999).

242. Olabisi, O. A. *et al.* Regulation of Transcription Factor NFAT by ADP-Ribosylation. *Mol. Cell. Biol.* **28**, 2860–2871 (2008).

243. Sala, A. *et al.* The nucleosome-remodeling ATPase ISWI is regulated by poly-ADP-ribosylation. *PLoS Biol.* **6**, 2329–2342 (2008).

244. Ciccarone, F. *et al.* Poly(ADP-ribosyl)ation Acts in the DNA Demethylation of Mouse Primordial Germ Cells Also with DNA Damage-Independent Roles. *PLoS One* **7**, (2012).

245. Lee, P. P. *et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function and survival. **15**, 763–774 (2001).

246. Zampieri, M. *et al.* ADP-ribose polymers localized on Ctcf-Parp1-Dnmt1 complex prevent methylation of Ctcf target sites. *Biochem. J.* **441**, 645–652 (2012).

247. Yélamos, J. *et al.* PARP-2 deficiency affects the survival of CD4+CD8+ double-positive thymocytes. *EMBO J.* **25**, 4350–4360 (2006).

248. Brown, M. L., Franco, D., Burkle, A. & Chang, Y. Role of poly (ADP-ribosyl) ation in DNA-PK cs - independent V (D) J recombination. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 4532–4537 (2002).

249. Chow, W. Y. *et al.* NMR spectroscopy of native and in vitro tissues implicates polyADP ribose in biomineralization. *Science (80-.).* **344**, 742–746 (2014).

250. Halazonetis, T. D., Gorgoulis, V. G. & Bartek, J. An oncogene-induced DNA damage model for cancer development. *Science (80-.).* **319**, 1352–1355 (2008).

251. Maya-Mendoza, A. *et al.* High speed of fork progression induces DNA replication stress and genomic instability. *Nature* **559**, 279–284 (2018).

252. Farrés, J. *et al.* PARP-2 sustains erythropoiesis in mice by limiting replicative stress in erythroid progenitors. *Cell Death Differ.* **22**, 1144–1157 (2015).

253. Liang, Y. C., Hsu, C. Y., Yao, Y. L. & Yang, W. M. PARP-2 regulates cell cycle-related genes through histone deacetylation and methylation independently of poly(ADP-ribosyl)ation. *Biochem. Biophys. Res. Commun.* **431**, 58–64 (2013).

254. Fatokun, A. Parthanatos: Poly adp ribose polymerase (parp)-mediated cell death. *Apoptosis Beyond Many Ways Cells Die* 535–558 (2018). doi:10.1002/9781119432463.ch25

255. Virág, L., Robaszkiewicz, A., Rodriguez-Vargas, J. M. & Oliver, F. J. Poly(ADP-ribose) signaling in cell death. *Mol. Aspects Med.* **34**, 1153–1167 (2013).

256. Cregan, S. P., Dawson, V. L. & Slack, R. S. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23**, 2785–2796 (2004).

257. Yu, S. *et al.* Mediation of Poly (ADP-Ribose) Polymerase-1-Dependent Cell Death by Apoptosis-lnducing Factor. **297**, 259–263 (2016).

258. Cohausz, O. & Althaus, F. R. Role of PARP-1 and PARP-2 in the expression of apoptosis-regulating genes in HeLa cells. *Cell Biol. Toxicol.* **25**, 379–391 (2009).

259. Aredia, F. & Scovassi, A. I. Poly(ADP-ribose): A signaling molecule in different paradigms of cell death. *Biochem. Pharmacol.* **92**, 157–163 (2014).

260. Hopp, Grüter & Hottiger. Regulation of Glucose Metabolism by NAD+ and ADP-Ribosylation. *Cells* **8**, 890 (2019).

261. Bai, P. & Cantó, C. The role of PARP-1 and PARP-2 enzymes in metabolic regulation and disease. *Cell Metab.* **16**, 290–295 (2012).

262. Szántó, M. *et al.* Deletion of PARP-2 induces hepatic cholesterol accumulation and decrease in HDL levels. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1842**, 594–602 (2014).

263. Bai, P. *et al.* PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. **13**, 461–468 (2012).

264. Bai, P. *et al.* PARP-2 regulates SIRT1 expression and whole-body energy expenditure. *Cell Metab.* **13**, 450–460 (2011).

265. Pyriochou, A., Olah, G., Deitch, E. A., Szabó, C. & Papapetropoulos, A. Inhibition of angiogenesis by the poly(ADP-ribose) polymerase inhibitor

PJ-34. *Int. J. Mol. Med.* **22**, 113–118 (2008).

266. Tentori, L. *et al.* Poly(ADP-ribose) polymerase (PARP) inhibition or PARP-1 gene deletion reduces angiogenesis. *Eur. J. Cancer* **43**, 2124–2133 (2007).

267. Mao, H., Lockyer, P., Davin Townley-Tilson, W. H., Xie, L. & Pi, X. LRP1 Regulates Retinal Angiogenesis by Inhibiting PARP-1 Activity and Endothelial Cell Proliferation. *Arterioscler. Thromb. Vasc. Biol.* **36**, 350–360 (2016).

268. Wei, W., Li, Y., Lv, S., Zhang, C. & Tian, Y. PARP-1 may be involved in angiogenesis in epithelial ovarian cancer. *Oncol. Lett.* **12**, 4561–4567 (2016).

269. Potente, M. *et al.* SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev.* **21**, 2644–2658 (2007).

270. Guarani, V. *et al.* Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. *Nature* **473**, 234–238 (2011).

271. de Visser, K. E. & Coussens, L. M. The inflammatory tumor microenvironment and its impact on cancer development. *Contrib. Microbiol.* **13**, 118–137 (2006).

272. Bai, P. & Virág, L. Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes. *FEBS Lett.* **586**, 3771–3777 (2012).

273. Oliver, F. J. *et al.* Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly (ADP-ribose) polymerase-1 deficient mice. *EMBO J.* **18**, 4446–4454 (1999).

274. Giansanti, V., Donà, F., Tillhon, M. & Scovassi, A. I. PARP inhibitors: New tools to protect from inflammation. *Biochem. Pharmacol.* **80**, 1869–1877 (2010).

275. Rosado, M. M., Bennici, E., Novelli, F. & Pioli, C. Beyond DNA repair, the immunological role of PARP-1 and its siblings. *Immunology* **139**, 428–437 (2013).

276. Haskó, G. *et al.* Poly(ADP-ribose) polymerase is a regulator of chemokine production: Relevance for the pathogenesis of shock and inflammation. *Mol. Med.* **8**, 283–289 (2002).

277. Kamboj, A. *et al.* Poly(ADP-ribose) polymerase 2 contributes to neuroinflammation and neurological dysfunction in mouse experimental autoimmune encephalomyelitis. *J. Neuroinflammation* **10**, (2013).

278. Szabó, C. *et al.* Inhibition of poly (ADP-ribose) synthetase attenuates

neutrophil recruitment and exerts antiinflammatory effects. *J. Exp. Med.* **186**, 1041–1049 (1997).

279. Laudisi, F., Sambucci, M. & Pioli, C. Poly (ADP-Ribose) Polymerase-1 (PARP-1) as Immune Regulator. *Endocrine, Metab. Immune Disord. - Drug Targets* **11**, 326–333 (2012).

280. Navarro, J. *et al.* PARP-1/PARP-2 double deficiency in mouse T cells results in faulty immune responses and T lymphomas. *Sci. Rep.* **7**, 1–14 (2017).

281. Zhang, P. *et al.* PARP-1 controls immunosuppressive function of regulatory T cells by destabilizing Foxp3. *PLoS One* **8**, 2–9 (2013).

282. Valdor, R. *et al.* Regulation of NFAT by poly(ADP-ribose) polymerase activity in T cells. *Mol. Immunol.* **45**, 1863–1871 (2008).

283. Luo, X. *et al.* Poly(ADP-ribosyl)ation of FOXP3 Protein Mediated by PARP-1 Protein regulates the function of regulatory t cells. *J. Biol. Chem.* **290**, 28675–28682 (2015).

284. García, S. *et al.* Partial protection against collagen antibody-induced arthritis in PARP-1 deficient mice. *Arthritis Res. Ther.* **8**, 1–9 (2005).

285. Toller, I. M., Altmeyer, M., Kohler, E., Hottiger, M. O. & Müller, A. Inhibition of ADP ribosylation prevents and cures Helicobacter-induced gastric preneoplasia. *Cancer Res.* **70**, 5912–5922 (2010).

286. Datta, R. *et al.* PARP-1 deficiency blocks IL-5 expression through calpain-dependent degradation of STAT-6 in a murine asthma model. *Allergy Eur. J. Allergy Clin. Immunol.* **66**, 853–861 (2011).

287. Guha, M. PARP inhibitors stumble in breast cancer. *Nat. Biotechnol.* **29**, 373–374 (2011).

288. Morales, J. C. *et al.* Review of Poly (ADP-ribose) Polymerase (PARP) Mechanisms of Action and Rationale for Targeting in Cancer and Other Diseases. *Crit Rev Eukaryot Gene Expr* **24**, 15–28 (2014).

289. Jiang, X., Li, W., Li, X., Bai, H. & Zhang, Z. Current status and future prospects of PARP inhibitor clinical trials in ovarian cancer . *Cancer Manag. Res.* **Volume 11**, 4371–4390 (2019).

290. Ekblad, T., Camaioni, E., Schüler, H. & Macchiarulo, A. PARP inhibitors: Polypharmacology versus selective inhibition. *FEBS J.* **280**, 3563–3575 (2013).

291. Jones, P., Wilcoxen, K., Rowley, M. & Toniatti, C. Niraparib: A Poly(ADP-ribose) polymerase (PARP) inhibitor for the treatment of tumors with defective homologous recombination. *J. Med. Chem.* **58**,

3302–3314 (2015).

292. Curtin, N. J. PARP Inhibitors and Cancer Therapy. *Poly(ADP-Ribosyl)ation* 218–233 (2008). doi:10.1007/0-387-36005-0_18

293. Evers, B. *et al.* Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. *Clin. Cancer Res.* **14**, 3916–3925 (2008).

294. Rottenberg, S. *et al.* High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 17079–17084 (2008).

295. Wang, W. & Figg, W. D. Secondary BRCA1 and BRCA2 alterations and acquired chemoresistance. *Cancer Biol. Ther.* **7**, 1004–1005 (2008).

296. Southan, G. J. & Szabó, C. Poly (ADP-Ribose) Polymerase Inhibitors. (2014). doi:10.2174/0929867033368376

297. Underhill, C., Toulmonde, M. & Bonnefoi, H. A review of PARP inhibitors: From bench to bedside. *Ann. Oncol.* **22**, 268–279 (2011).

298. Stewart, T. J. & Abrams, S. I. Altered Immune Function during Long-Term Host-Tumor Interactions Can Be Modulated to Retard Autochthonous Neoplastic Growth. *J. Immunol.* **179**, 2851–2859 (2007).

299. Moore, M. W., Carbone, F. R. & Bevan, M. J. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **54**, 777–785 (1988).

300. Wang, B. *et al.* Transition of tumor-associated macrophages from MHC class IIhito MHC class IIlowmediates tumor progression in mice. *BMC Immunol.* **12**, 43 (2011).

301. Lee, K.-M. *et al.* Cutting Edge: The NK Cell Receptor 2B4 Augments Antigen-Specific T Cell Cytotoxicity Through CD48 Ligation on Neighboring T Cells. *J. Immunol.* **170**, 4881–4885 (2003).

302. Simon, S. & Labarriere, N. PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *Oncoimmunology* **7**, 1–7 (2017).

303. Hui, E. *et al.* T cell costimulatory receptor CD28 is a primary target for PD-1– mediated inhibition. **355**, 1428–1433 (2018).

304. Seidel, J. A., Otsuka, A. & Kabashima, K. Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations. *Front. Oncol.* **8**, 1–14 (2018).

305. Badoual, C. *et al.* PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-Associated head and neck cancer. *Cancer Res.* **73**, 128–138 (2013).

306. Mukherjee, S., Maiti, P. K. & Nandi, D. Role of CD80, CD86, and CTLA4 on mouse CD4+ T lymphocytes in enhancing cell-cycle progression and survival after activation with PMA and ionomycin. *J. Leukoc. Biol.* **72**, 921–931 (2002).

307. Perez, V. L. *et al.* Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* **6**, 411–417 (1997).

308. Izcue, A. *et al.* Interleukin-23 Restrains Regulatory T Cell Activity to Drive T Cell-Dependent Colitis. *Immunity* **28**, 559–570 (2008).

309. Li, Y., Wang, H., Lu, H. & Hua, S. Regulation of Memory T cells by interleukin-23. *Int. Arch. Allergy Immunol.* **169**, 157–162 (2016).

310. Castellino, F. *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* **440**, 890–895 (2006).

311. Marçais, A. *et al.* Cell-Autonomous CCL5 Transcription by Memory CD8 T Cells Is Regulated by IL-4. *J. Immunol.* **177**, 4451–4457 (2006).

312. Kuehnemuth, B. *et al.* CCL1 is a major regulatory T cell attracting factor in human breast cancer. *BMC Cancer* **18**, 1–6 (2018).

313. Kohlmeier, J. E. *et al.* CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. **29**, 101–113 (2009).

314. Bakos, E. *et al.* CCR2 Regulates the Immune Response by Modulating the Interconversion and Function of Effector and Regulatory T Cells. *J. Immunol.* **198**, 4659–4671 (2017).

315. Agresta, L., Hoebe, K. H. N. & Janssen, E. M. The emerging role of CD244 signaling in immune cells of the tumor microenvironment. *Front. Immunol.* **9**, 1–9 (2018).

316. Veglia, F. & Gabrilovich, D. I. Dendritic cells in cancer: the role revisited. *Curr. Opin. Immunol.* **45**, 43–51 (2017).

317. Quaranta, V. & Schmid, M. C. Macrophage-Mediated Subversion of Anti-Tumour Immunity. *Cells* **8**, 747 (2019).

318. Kusmartsev, S., Nagaraj, S. & Gabrilovich, D. I. Tumor-Associated CD8 + T Cell Tolerance Induced by Bone Marrow-Derived Immature Myeloid Cells . *J. Immunol.* **175**, 4583–4592 (2005).