



Immunomodulation in wound healing and oncogenesis

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DECLARATION OF AUTHORSHIP

I hereby confirm that this work was performed from January 2021 to July 2024 under the supervision of [REDACTED] at the Dermatology Department of the University Medical Center Mainz. I ensure that I have authored this dissertation independently without the assistance of paid third parties. All sources and aids used were specified.

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For the patients and their families

PUBLICATION LIST

The contents of this cumulative dissertation are based on the following works achieved during the doctoral studies of Emily Trzeciak (* denotes shared first authorship). From here on, these works will be referred to as **Paper 1**, **Paper 2**, and **Paper 3**:

- Paper 1:** Trzeciak*, E. R., Zimmer*, N., Kämmerer, P. W., Thiem, D., Al-Nawas, B., Tuettenberg, A., & Blatt, S. (2022). GARP Regulates the Immune Capacity of a Human Autologous Platelet Concentrate. *Biomedicines*, 10(12), 3136. <https://doi.org/10.3390/biomedicines10123136>
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- Paper 11: Schupp, J., Krebs, F. K., Zimmer, N., **Trzeciak, E.**, Schuppan, D., & Tuettenberg, A. (2019). Targeting myeloid cells in the tumor sustaining microenvironment. *Cellular immunology*, 343, 103713. <https://doi.org/10.1016/j.cellimm.2017.10.013>

ABSTRACT

Immunotherapy has emerged as a promising approach to treat a wide range of clinical conditions. Herein, immunomodulatory agents are applied to induce favorable immune responses. Depending on the condition, immune responses may be amplified (e.g., cancer) or attenuated (e.g., wound healing). Cancer and deficiencies in wound healing represent significant global healthcare burdens. Cancer is the second leading cause of death worldwide, and more than 40 million people are suffering from chronic wounds. Although the incidence of both conditions is increasing, many patients still lack effective treatment options. Therefore, the development of novel immunomodulatory approaches to treat these conditions is an utmost priority.

Before these approaches can be developed into therapies, they must first be thoroughly characterized to ensure their safety and efficacy. Therefore, this cumulative dissertation characterized two novel immunomodulatory approaches, namely the protein, glycoprotein A repetitions predominant (GARP) as a new target, and cold atmospheric plasma (CAP) as a device, in the immunological contexts of cancer and wound healing.

In the past, GARP was described as a potent mechanism of immune tolerance through its regulation of the suppressive cytokine, transforming growth factor beta (TGF- β). The present work provides some of the first insights into the role of GARP in wound healing and in cancer stem cell biology. In “GARP Regulates the Immune Capacity of a Human Autologous Platelet Concentrate”, the immunological influence of GARP in injectable platelet rich fibrin (iPRF) was investigated. It was determined that GARP mediates T cell immunity by inducing regulatory T cells, a key cell type that facilitates the wound healing process. Conversely, GARP was found to inhibit the production of proinflammatory cytokines, which can prevent effective wound healing by sustaining chronic inflammation. Application of an anti-GARP antibody reversed these effects.

The present work analyzed another aspect of GARP biology, namely its role in glioblastoma stem-like cells (GSCs). GSCs are a small subset of tumor cells, which drive tumor initiation, therapy resistance, and recurrence in glioblastoma. Prior to this study, no markers had been found that were universally expressed by all GSCs due to their innately high heterogeneity. In “Nuclear Glycoprotein A Repetitions Predominant (GARP) Is a Common Trait of Glioblastoma Stem-like Cells and Correlates with Poor Survival in Glioblastoma Patients”, GARP was evaluated and confirmed as a possible marker for GSCs. Functionally, GARP was found to be implicated in the self-renewal of GSCs. Due to the high expression of GARP on GSCs, the protein was also investigated as a prognostic biomarker for glioblastoma. Hereby, the abnormal nuclear localization of the protein was linked to reduced overall survival of patients with glioblastoma for the first time.

The immunomodulatory effects of CAP were examined in the context of cancer. CAP is a partially ionized gas, which is also compromised of neutral atoms, excited electrons, and electromagnetic radiation. In contrast to thermal plasma, CAP can be applied within

physiological temperatures. CAP has been reported to selectively target cancer cells by increasing exogenous reactive oxygen and nitrogen species (RONS). Prior to this work, little was known regarding how CAP influences immune cells, key determiners of immunotherapy response in the melanoma microenvironment. In “Oxidative Stress Differentially Influences the Survival and Metabolism of Cells in the Melanoma Microenvironment”, the effects of CAP on T cells, macrophages, and tumor cells were investigated. All cell types exhibited signs of oxidative stress following CAP treatment, but T cells were the most sensitive. These effects could be partially reversed with the application of antioxidants. Surprisingly, CAP was found to influence the polarization of macrophages to an anti-inflammatory “M0/M2” phenotype.

Collectively, this work sheds light on the immunomodulatory properties and functional relevance of GARP and CAP in distinct immunological settings. In the future, these immunomodulatory mechanisms might be used as possible therapeutic approaches to elicit favorable immune responses in the treatment of cancer and the optimization of wound healing.

KURZZUSAMMENFASSUNG

Die Immuntherapie hat sich als vielversprechender Ansatz zur Behandlung eines breiten Spektrums klinischer Erkrankungen erwiesen. Dabei werden immunmodulierende Wirkstoffe eingesetzt, um gezielt Immunreaktionen hervorzurufen. Je nach Erkrankung und erwünschter Wirkung kann die Immunantwort verstärkt (z.B. Krebs) oder abgeschwächt (z.B. Wundheilung) werden. Krebs und Wundheilungsstörungen stellen weltweit eine erhebliche Belastung für die Gesundheitsversorgung dar. Krebs ist weltweit die zweithäufigste Todesursache und mehr als 40 Millionen Menschen leiden an chronischen Wunden. Obwohl die Häufigkeit beider Erkrankungen zunimmt, gibt es für viele Patienten immer noch keine wirksamen Behandlungsmöglichkeiten. Die Entwicklung neuartiger immunmodulatorischer Therapien zur Behandlung dieser Erkrankungen hat daher höchste Priorität.

Bevor diese Wirkstoffe zu Therapien weiterentwickelt werden können, müssen sie zunächst gründlich charakterisiert werden, um ihre Sicherheit und Wirksamkeit nachzuweisen. In dieser kumulativen Dissertation wurden daher zwei neuartige immunmodulatorische Ansätze, das Protein „*Glycoprotein A repetitions predominant*“ (GARP) als potenzielle Zielstruktur und kaltes atmosphärisches Plasma (CAP) als Behandlungsmethode, in den immunologischen Zusammenhängen von Krebs und Wundheilung charakterisiert.

GARP wurde bereits in der Vergangenheit als wirksamer Mechanismus der Immuntoleranz durch seine Regulierung des suppressiven Zytokins „*transforming growth factor beta*“ (TGF- β) beschrieben. Diese Arbeit liefert erste Einblicke in die Rolle von GARP sowohl bei der Wundheilung als auch in der Biologie von Krebsstammzellen. In „*GARP Regulates the Immune Capacity of a Human Autologous Platelet Concentrate*“ wurde der immunologische Einfluss von GARP in injizierbarem plättchenreichem Fibrin (iPRF) untersucht. Es wurde festgestellt, dass GARP T-Zell-Immunität vermittelt, indem es regulatorische T-Zellen induziert, einen wichtigen Zelltyp, der die Wundheilung fördert. Umgekehrt konnte gezeigt werden, dass GARP die Produktion von proinflammatorischen Zytokinen hemmt, die eine wirksame Wundheilung durch Aufrechterhaltung einer chronischen Entzündung verhindern können. Bemerkenswert ist, dass diese Effekte durch einen Anti-GARP-Antikörper verhindert werden können.

In der vorgelegten Arbeit wurde ein weiterer Aspekt der GARP-Biologie untersucht, nämlich die Rolle des Proteins in Glioblastom-Stammzellen (GSCs). GSCs sind eine kleine Untergruppe von Tumorzellen, die für die Tumorentstehung, Therapieresistenz und das Wiederauftreten von Glioblastomen verantwortlich sind. Bis zu dieser Studie gab es keine Marker, die universell von allen GSCs exprimiert werden, da diese von Natur aus sehr heterogen sind. In der Studie „*Nuclear Glycoprotein A Repetitions Predominant (GARP) Is a Common Trait of Glioblastoma Stem-like Cells and Correlates with Poor Survival in Glioblastoma Patients*“ wurde GARP untersucht und als möglicher Marker für GSCs bestätigt. Funktionell konnte gezeigt werden, dass GARP an der Selbsterneuerung von GSCs beteiligt ist. Aufgrund der hohen Expression von GARP auf GSCs wurde das Protein auch als prognostischer Biomarker für das Glioblastom untersucht. Erstmals konnte eine abnorme nukleare Lokalisation dieses Proteins mit einer

verkürzten Gesamtüberlebenszeit von Glioblastom-Patienten in Verbindung gebracht werden.

Im Zusammenhang mit Krebs wurden die immunmodulatorischen Wirkungen von CAP untersucht. CAP ist ein teilweise ionisiertes Gas, das zudem neutrale Atome, angeregte Elektronen und elektromagnetische Strahlung enthält. Im Gegensatz zu thermischem Plasma kann es bei physiologischen Temperaturen angewendet werden. Es wurde berichtet, dass CAP selektiv auf Krebszellen wirkt, indem es die exogenen reaktiven Sauerstoff- und Stickstoffspezies (RONS) erhöht. Vor dieser Arbeit war wenig darüber bekannt, wie CAP Immunzellen beeinflusst, die für das Ansprechen auf eine Immuntherapie in der Mikroumgebung des Melanoms entscheidend sind. In *„Oxidative Stress Differentially Influences the Survival and Metabolism of Cells in the Melanoma Microenvironment“* wurden die Auswirkungen von CAP auf T-Zellen, Makrophagen und Tumorzellen untersucht. Alle Zelltypen wiesen nach der CAP-Behandlung Anzeichen von oxidativem Stress auf, wobei die T-Zellen am stärksten betroffen waren. Diese Auswirkungen konnten durch die Anwendung von Antioxidantien teilweise rückgängig gemacht werden. Überraschenderweise wurde festgestellt, dass CAP die Polarisierung von Makrophagen in Richtung eines immunsuppressiven „M0/M2“-Phänotyps fördert.

Insgesamt erklärt diese Arbeit die immunmodulatorischen Eigenschaften und die funktionelle Bedeutung von GARP und CAP in verschiedenen immunologischen Umgebungen. In Zukunft könnten diese immunmodulatorischen Mechanismen als mögliche therapeutische Ansätze genutzt werden, um günstige Immunreaktionen in der Krebstherapie und zur Optimierung der Wundheilung zu induzieren.

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LIST OF ABBREVIATIONS

Meaning

Glycoprotein A repetitions predominant
Cold atmospheric plasma
Transforming growth factor beta
Injectable platelet rich fibrin
Glioblastoma stem-like cells
Reactive oxygen and nitrogen species
Deutscher Akademischer Austauschdienst
Collaborative Research Center 1066
Mainz Research School of Translational Biomedicine
Gutenberg Academy Fellows Program
International Studies and Language College
Regulatory T cells
Human Integrated Protein-Protein Interaction
rEference database
Physical Protein-Protein Interactions With
Reality Scores database
Dendritic cell
Pattern recognition receptors
Pathogen-associated molecular patterns
T lymphocyte
B lymphocyte
T cell receptor
B cell receptor
Antigen presenting cell
Major histocompatibility complex
CD40 ligand
Interleukin 2
Effector T cells
Cytotoxic T lymphocytes
T follicular helper cells
Helper T cell
Interferon-gamma
Interleukin 12
T-box expressed in T cells
Classically activated macrophages
Transforming growth factor beta
Interleukin 6
Forkhead box P3
Interleukin 10

Abbreviation or symbol

GARP
CAP
TGF- β
iPRF
GSCs
RONS
DAAD
SFB1066
TransMed
GAFF
ISSK
Tregs
HIPPIE database

HitPredict database

DC
PRRs
PAMPs
T cell
B cell
TCR
BCR
APC
MHC
CD40L
IL-2
Teff
CTL
Tfh
Th
IFN- γ
IL-12
Tbet
M1 macrophages
TGF- β
IL-6
Foxp3
IL-10

IL-2 receptor α chain	CD25
IL-7 receptor α chain	CD127
Immune dysregulation Polyendocrinopathy, Enteropathy, X-linked	IPEX
Natural killer cell	NK cell
Cytotoxic T-lymphocyte-associated Protein 4	CTLA-4
Lymphocyte activation gene	LAG3
Cyclic adenosine monophosphate	cAMP
Alternatively activated macrophages	M2 macrophages
Leucine rich repeats	LRRs
Cysteine	Cys
Latent TGF- β	LTGF- β
Soluble GARP	sGARP
Nuclear GARP	GARP ^{NU}
Latency associated peptide	LAP
Experimental autoimmune encephalomyelitis	EAE
TGF- β type II receptor	TGF- β RII
Graft-versus-host disease	GvHD
Tumor necrosis factor alpha	TNF- α
Vascular endothelial growth factor	VEGF
Extracellular matrix	ECM
Tissue inhibitor of metalloproteinases	TIMPs
Matrix metalloproteinases	MMPs
Tumor microenvironment	TME
Ultra-violet	UV
Programmed death ligand 1	PD-L1
Myeloid-derived suppressor cells	MDSC
Epithelial-mesenchymal transition	EMT
World Health Organization	WHO
Computed tomography	CT
Magnetic resonance imaging	MRI
Isocitrate dehydrogenase	IDH
Not otherwise specified	NOS
Not elsewhere classified	NEC
2-hydroxyglutarate	2-HG
O6-methylguanine-DNA methyltransferase	MGMT
Temozolomide	TMZ
Epidermal growth factor receptor	EGFR
Mitogen-activated protein kinase	MAPK
Phosphatidylinositol 3	PI3K
Blood brain barrier	BBB
Tumor mutational burden	TMB

Glioma associated macrophages	GAMs
Gross total resection	GTR
Subtotal resection	STR
Bevacizumab	Avastin®
Vascular endothelial growth factor A	VEGF-A
Vascular endothelial growth factor receptor 1	VEGFR1
Vascular endothelial growth factor receptor 2	VEGFR2
Erlotinib	Tarceva®
Gefitinib	Iressa®
Depatuxizumab-Mafodotin	Depatux-M
Cetuximab	Erbitux®
Immune checkpoint inhibitors	ICIs
Programmed death-1	PD-1
Programmed death ligand-2	PD-L2
Nivolumab	Opdivo®
Pembrolizumab	Keytruda®
Prominin-1	CD133
Reactive oxygen species	ROS
Asymmetry, Border, Color, Diameter, and Elevation	ABCDE
Tumor, Node, Metastasis	TMN
Tumor infiltrating lymphocytes	TILs
Vemurafenib	Zelboraf®
Dabrafenib	Tafinlar®
Extracellular signal-regulated kinases	ERK
Trametinib	Mekinist®
Ipilimumab	Yervoy®
Antibody-dependent cellular cytotoxicity	ADCC
Immune related adverse events	irAE
Atezolizumab	Tecentriq®
Cobimetinib	Cotellic®
Cyclin-dependent kinase inhibitor 1	p21
Hematopoietic stem cells	HSCs
Signal transducer and activator of transcription 3	STAT3
Decapping mRNA 2	DCP2
Protein phosphatase 1 regulatory subunit 42	PPP1R42
Nuclear localization sequence	NLS
Arginine	R
Lysine	K
Immunogenic cell death	ICD
Glial fibrillary acidic protein	GFAP
Platelet-derived growth factor receptor alpha	PDGFR- α
Aldehyde dehydrogenase 1 family member A3	ALDH1A3

Mean fluorescence intensity	MFI
Antibody	Ab
Wildtype	WT
GARP overexpression	GARP ⁺
Empty vector	EV
Newly diagnosed glioblastoma	ndGB
Recurrent glioblastoma	recGB
Glioblastoma	GB
Lower left	LL
Lower right	LR
Upper right	UR
Upper left	UL
Relative fluorescence units	RFU
Propidium iodide	PI
Oxygen consumption rate	OCR
Extracellular acidification rate	ECAR
Transmembrane	TM
University Medical Center Mainz	UMC-Mainz
Johannes Gutenberg University Mainz	JGU
Arbeitsgemeinschaft Dermatologische Forschung	ADF
Arbeitsgemeinschaft Dermatologische Onkologie	ADO

1. INTRODUCTION

Modulation of immune responses has emerged as a promising way to treat a wide range of clinical conditions¹⁻⁷. This cumulative dissertation characterized two novel immunomodulatory approaches, namely the suppressive protein, glycoprotein A repetitions predominant (GARP) and cold atmospheric plasma (CAP). This work provides valuable insights into the functional role of GARP in two distinct immunological settings: wound healing (**Paper 1**) and cancer (**Paper 2**)^{8,9}. Furthermore, the influence of CAP on immune cells found in the melanoma microenvironment was investigated (**Paper 3**)¹⁰.

1.1 THE IMMUNE SYSTEM

The immune system is an intricate biological network, consisting of physical and chemical barriers, organs, cells, and proteins¹¹. The principal function of the immune system is to protect the cells of the body, either from foreign material, like viruses and bacteria, or from abnormal cells, such as cancer, all the while maintaining immune homeostasis. The immune system can be subdivided into two categories, namely innate and adaptive immunity.

1.1.1 INNATE AND ADAPTIVE IMMUNITY

Innate immunity is made-up of the skin and mucosal membranes, phagocytic cells, anti-microbial proteins, and the complement system^{11,12}. Acting as the first line of defense, the skin and mucosal membranes form an external barrier, preventing the entry of foreign material into the body. Epithelial cells, that form these barriers, secrete antimicrobial peptides and enzymes, which can disrupt the membranes and cell walls of invading microbes, leading to pore formation¹¹. If these protective barriers become compromised due to injury, plasma proteins in the blood that form the complement system can bind to and coat the surface of the intruding microbe in a process called opsonization^{11,12}. This not only can neutralize the microbe but also increase the recognition efficiency by phagocytic cells, like macrophages, dendritic cells (DCs), and neutrophils.

Phagocytic cells can also detect microbes with pattern recognition receptors (PRRs), which bind to evolutionary conserved structures on microbes, called pathogen-associated molecular patterns (PAMPs)^{11,12}. Upon recognition, phagocytic cells engulf the invading cells and destroy them¹¹. Besides opsonization, proteins from the complement system can also trigger inflammation and lyse microbes independently of phagocytic cells. Destruction of microbes by the innate immune system occurs rapidly within the scope of hours^{11,12}. If innate immunity alone fails to clear an infection, an adaptive immune response will take place.

One-way innate immunity differs from adaptive immunity is that it is unspecific, meaning that upon repeated exposure to the same foreign material, neither recognition of nor immune response against it is improved. The cellular and humoral components of adaptive immunity consist of T and B lymphocytes (T cells, B cells) as well as antibodies, produced by B cells¹¹. In contrast to innate immunity, adaptive immune responses are specific, meaning that immune responses are orchestrated against a particular foreign antigen¹¹⁻¹³. In more detail, lymphocytes can specifically bind to foreign antigens with T or B cell receptors (TCR, BCR) and

thereby initiate an adaptive immune response^{11,13}. Upon repeated exposure to the same foreign antigen, both the speed and strength of the adaptive immune response improves with time.

1.1.2 T CELL ACTIVATION

Unlike innate immunity, adaptive immune responses can take days to occur. This is due to the time needed for T and B cells, expressing TCRs and BCRs specific to the foreign antigen, to be activated, undergo proliferation, and subsequent differentiation into specialized effector or memory cells^{11,13}. This process is collectively referred to as priming¹¹.

Adaptive immune responses begin with the recognition and phagocytosis of foreign material by antigen presenting cells (APCs), frequently DCs¹¹. Once internalized, APCs degrade the material, breaking down proteins into smaller units known as peptides. These peptides undergo additional processing to be loaded onto major histocompatibility (MHC) receptors, which present the antigen on the surface of APCs^{11,14}. Recognition of foreign material by DCs not only results in antigen presentation but also triggers DC maturation and migration to neighboring lymph nodes. Once in the lymph nodes, DCs encounter circulating naïve T cells.

If the TCR of the naïve T cell can bind to the presented antigen on the DC, the first stage of T cell activation begins (**Fig. 1**)^{11,13}. Mature DCs express costimulatory molecules, like CD40 as well as the B7 molecules CD80/86, which bind to CD40 ligand (CD40L) and CD28, expressed on the surface of T cells; this forms the second step of T cell activation (**Fig. 1**)^{11,13,14}. If this step fails to occur, the T cell will become functionally inactivated or anergic^{11,13}. This serves as a mechanism to protect against the generation of mature autoreactive T cells, which recognize self-antigens and thus could enact destructive immune responses against the healthy tissues of the body.

Figure 1: Activation & differentiation of T cells

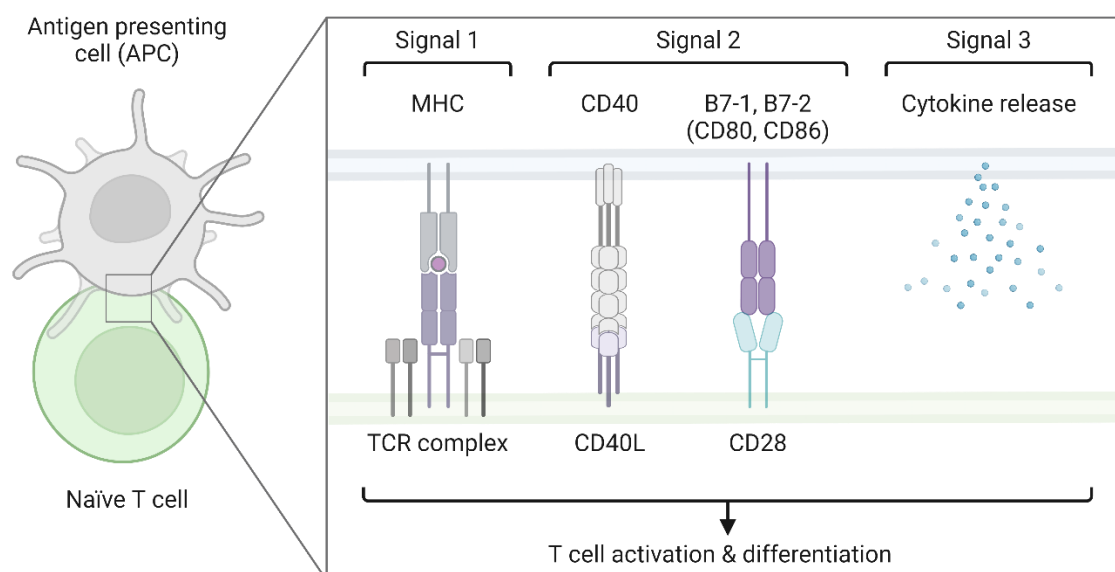


Fig. 1: Schematic representation of T cell activation and differentiation. All signals must be present for successful activation to occur¹¹. T cell differentiation is dependent on the

cytokines present during priming. Adapted from “T Cell Co-stimulation”, by BioRender.com (2024)¹⁵.

Upon successful costimulatory binding, activated T cells undergo rapid proliferation, in a process known as clonal expansion¹¹. Clonal expansion is dependent on the cytokine, interleukin 2 (IL-2), which is secreted by DCs and other activated T cells (**Fig. 1**)^{11,13}. Clonal expansion occurs over a period of several days and results in the formation of differentiated effector T cells (Teff)¹¹. Once differentiated, Teff leave the lymph nodes and reenter circulation, where they can bind to target cells using their TCR without need of further costimulation, and thereby enact adaptive immune responses. Therefore, T cell activation represents a key first step in adaptive immune responses.

1.1.3 CD4⁺ AND CD8⁺ T CELLS

Teff can be broadly classified into two categories based on the surface expression of the MHC co-receptors, CD4 and CD8, as well as the class of MHC receptors that their respective TCRs bind to^{11,13}. Differentiated CD8⁺ T cells, also known as cytotoxic T lymphocytes (CTL), mainly function to directly kill cells that present foreign antigens in MHC class I receptors. In more detail, CTLs play a vital role in defending the body from intracellular pathogens, including viruses and bacteria, as well as from cancer cells^{11,16}. Of note, all healthy nucleated cells in the human body express MHC class I receptors, allowing CTLs to scan them for signs of intracellular infection^{11,17}. Once CTLs identify a target cell via TCR binding, CTLs release cytotoxic granules, containing perforin, granzymes, and granulysins^{11,16}. Perforin forms pores in the membrane of the target cell, enabling the entry of the apoptosis, or programmed cell death, inducing factors, granzyme and granulysin. Collectively, this results in the rapid death of the target cell.

CD4⁺ T cells differ from CD8⁺ T cells in several important ways^{11,13}. CD4⁺ T cells bind antigens presented in MHC class II receptors and can differentiate into various T cell subsets depending on the cytokines present in their environment during priming (**Fig. 1**). There are five main subsets of CD4⁺ T cells including helper Th1, Th2, Th17, and T follicular (Tfh) cells as well as regulatory T cells (Tregs)¹¹. Some subsets of CD4⁺ T cells are referred to as “helper” T cells (Th cells) as they can enhance the function and/or the recruitment of other immune cells, like aiding in the clearance of pathogens or cancer cells from the body. For example, Tfh cells aid in the activation of B cells and thus enable them to differentiate into mature antibody producing plasma cells and memory B cells. This differs from CD8⁺ T cells, which exert their function directly on target cells. CD4⁺ T cell subsets are distinguished from each other based off the cytokines they produce as well as the surface markers and transcription factors they express upon differentiation^{11,13}.

1.1.4 T CELL SUBSETS

This work primarily focused on the examination of CD4⁺ Th1 cells and Tregs, which will be discussed now in further detail.

1.1.5 TH1

Th1 cells result from the abundance of the cytokines, interferon-gamma (IFN- γ), and interleukin 12 (IL-12), during the T cell activation process^{11,13}. This induces the expression of the transcription factor, T-box expressed in T cells (Tbet), the master transcription factor in Th1 differentiation^{11,13,18}. Expression of Tbet leads to the production of IFN- γ , the principal cytokine produced by Th1 cells, which can act on both cells from the innate and adaptive immune system.

The main role of Th1 cells in the body is to aid in the clearance of intracellular infections, especially in the case of macrophages^{11,18}. Upon recognition of a presented microbial antigen on the surface of an infected macrophage, Th1 cells release proinflammatory IFN- γ ¹¹. This results in the classical activation of macrophages, also known as proinflammatory M1 macrophages. These M1 macrophages display increased antigen presentation ability and production of proinflammatory cytokines, like IL-12, as well as reactive oxygen and nitrogen species (RONS) that aid in the killing of intracellular pathogens^{11,19}. Altogether, IFN- γ produced by Th1 cells enhances the abilities of infected macrophages to both engage with other immune cells and to clear intracellular infections.

IFN- γ produced by Th1 cells not only activates macrophages but also CTLs. The cytolytic capability of CD8⁺ T cells is dependent on exposure to IFN- γ ^{11,19}. Upon exposure, CD8⁺ T cells upregulate expression of the IL-2 receptor and granzyme. Furthermore, IFN- γ influences the proliferation of CD8⁺ T cells upon antigen exposure^{19–21}. Responding to IL-2 is critical in the activation and differentiation of naïve CD8⁺ T cells to CTLs as previously described in “1.1.2 T cell activation”^{11,13}. Additionally, as mentioned in “1.1.3 CD4⁺ and CD8⁺ T cells”, granzyme can induce apoptosis in CTL target cells^{11,16}. Of note, Th1 cells have an important function in anti-cancer immunity by producing IFN- γ and enhancing the cytolytic function and proliferation of tumor killing CTLs^{11,19–22}.

Although Th1 type 1 immune responses mediated by IFN- γ help clear intracellular infections, dysregulated production of IFN- γ has been linked to several autoimmune diseases, like rheumatoid arthritis and systemic lupus erythematosus^{11,18,22}. Therefore, it is important to tightly regulate Th1 immune responses to maintain immune homeostasis. This is where Tregs come into play.

1.1.6 REGULATORY T CELLS (TREGS)

In contrast to Th cells, Tregs mediate immune tolerance^{11,23,24}. This means that they suppress immune responses against antigens that their TCRs recognize¹¹. Therefore, Tregs are essential in preventing autoimmunity by maintaining immunological tolerance to the body's own tissues^{11,23,24}. Additionally, they regulate homeostasis of the immune system by suppressing immune responses. This includes but is not limited to regulating inflammation in wound healing as well as maintaining peripheral tolerance to commensal bacteria and consumed food^{11,25–27}.

Tregs are differentiated by the cytokines, transforming growth factor beta (TGF- β) and IL-2, in the absence of pro-inflammatory cytokines, like interleukin 6 (IL-6), during T cell activation¹¹. In the gastrointestinal tract, all-trans-retinoic acid and TGF- β are essential for the development of intestinal Tregs. However, it is important to clarify that this work only focused on immune cells isolated from peripheral blood.

Tregs express the master Treg transcription factor, forkhead box P3 (Foxp3)^{11,23,24,28}. This transcription factor regulates the expression of TGF- β and interleukin 10 (IL-10), two key anti-inflammatory cytokines produced by Tregs. In addition to the expression of Foxp3, Tregs can be identified by surface expression of CD4, CD25 (high), and CD127 (low) (CD4⁺CD25^{high}CD127^{low}/-Foxp3⁺ Tregs)²⁸. CD25, or IL-2 receptor α chain, is upregulated upon T cell activation and forms part of the high affinity IL-2 receptor with IL-2 receptor β and common γ chains¹¹. Because Tregs cannot produce their own IL-2, CD25 is constitutively expressed to obtain IL-2 from the local environment with high affinity. CD127, or IL-7 receptor α chain, is normally expressed on the surface of naïve T cells, but it is downregulated upon T cell activation. Activated Tregs can be identified by the upregulation of the transmembrane protein GARP onto the cell surface^{28–30}.

Foxp3 plays an essential role in both the differentiation and suppressive capacity of Tregs^{31,32}. Missense mutations in the FOXP3 gene have been found to lead to the development of x-linked recessive autoimmune syndrome IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) in humans^{31,33–35}. Patients with the disease lack functional Tregs and as a result, experience severe unchecked autoimmunity. Symptoms include but are not limited to severe allergic inflammation and autoimmune polyendocrinopathy (autoimmune responses against multiple endocrine organs), often resulting in premature death. In mice, a frameshift mutation of the FOXP3 gene, commonly referred to as the “*scurfy*” mutation, renders Scurfin, the murine equivalent of Foxp3, incapable of binding DNA, and thus unable to exhibit its function as a transcription factor³⁶. Mice with *scurfy* mutations have lower numbers of Tregs, resulting in the development of fatal autoimmune disease.

1.1.7 SUPPRESSIVE MECHANISMS OF TREGS

Tregs suppress a wide variety of immune cells, including both adaptive (T cells and B cells) and innate (DCs, natural killer (NK) cells, macrophages, and monocytes) immune cells^{23,28,37,38}. The suppressive functions of Tregs are mediated through multiple mechanisms — highlighting the protective role Tregs play in preventing autoimmunity and maintaining peripheral tolerance. These mechanisms of immune suppression can be broadly classified as either cell contact dependent or independent^{23,24,28,37,38}.

Tregs exhibit several contact dependent mechanisms of immune suppression. Firstly, Tregs express the inhibitory protein, cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), on their surfaces (**Fig. 2A**)²⁴. CTLA-4 has a higher binding affinity for B7 molecules on APCs than CD28 expressed by naïve T cells. This prevents effective costimulation of naïve T cells from occurring, and thus inhibits T cell activation and subsequent differentiation into Teff. It is also known that Tregs express lymphocyte activation gene (LAG3), which can bind MHC class II

receptors expressed on the surface of immature DCs (**Fig. 2A**)^{24,39}. As a result, this prevents the maturation of DCs and thereby their ability to costimulate T cells. Additionally, it was discovered that Tregs can transfer cyclic adenosine monophosphate (cAMP) to Teff via gap junctions, which in turn inhibits the proliferation and IL-2 synthesis of Teff (**Fig. 2B**)³⁷.

Tregs employ multiple contact independent mechanisms of immune suppression. It has been proposed that Tregs can “starve” Teff by depleting IL-2 in the environment, needed for Teff cell survival (**Fig. 2C**)^{24,40,41}. This is a result of Tregs outcompeting Teff for IL-2 via elevated expression of the high affinity IL-2 receptor, CD25. Tregs have been found to produce cytolytic granzymes and perforins, similarly to CTLs (**Fig. 2D**)^{24,42,43}. This enables Tregs to directly kill B cells, NK cells, and CTLs, consequently suppressing their functions. However, production of TGF- β and IL-10 is predominantly responsible for the contact independent suppression mechanisms of Tregs (**Fig. 2E**). TGF- β and IL-10 inhibit the development of Th cells as well as promote the differentiation of Tregs from naïve T cells⁴⁴. Notably, Tregs are also able to induce Tregs from differentiated Teff through the secretion of TGF- β and IL-10 as well as the transfer of cAMP in a process known as infectious tolerance^{44,45}. It is known that TGF- β inhibits the development and thereby the proliferation and function of Th1 and Th2 cells¹¹. IL-10, produced by Tregs, can both downregulate the expression of costimulatory molecules and the production of proinflammatory cytokines by APCs, further preventing T cell activation and differentiation. TGF- β and IL-10, produced by Tregs, can also induce tolerogenic phenotypes in DCs and macrophages^{38,46}. In more detail, TGF- β and IL-10 drive monocyte differentiation into alternatively activated or anti-inflammatory M2 macrophages.

Another mechanism of suppression utilized by activated Tregs is the overexpression of the TGF- β activating protein, GARP.

Figure 2: Suppressive mechanisms of regulatory T cells (Tregs)

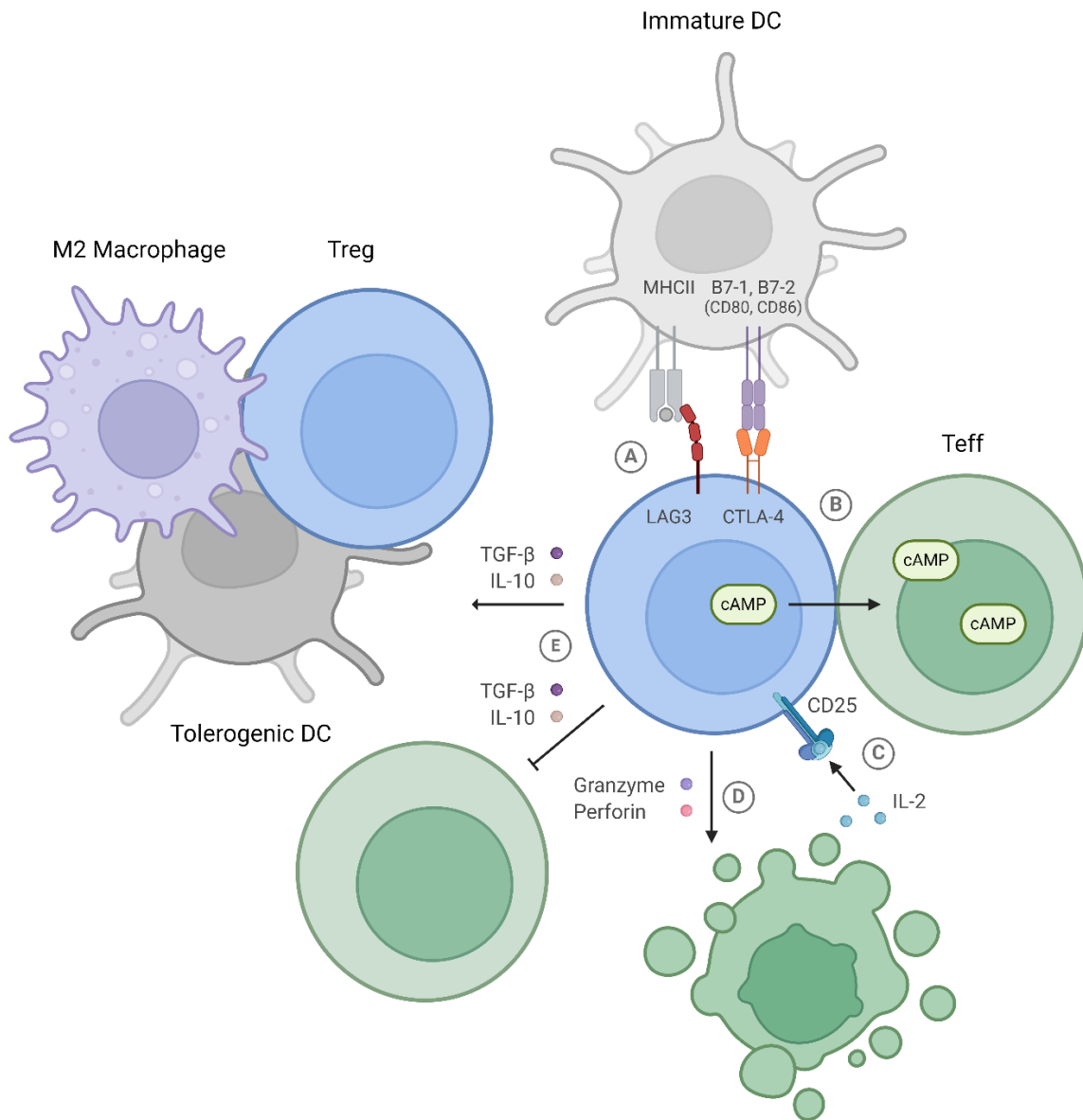


Fig. 2: Suppressive mechanisms employed by regulatory T cells (Tregs). (A-B) indicate contact dependent mechanisms, whereas (C-E) display contact independent mechanisms. Adapted from “T Cell Co-stimulation”, by BioRender.com (2024)¹⁵.

1.1.8 GLYCOPROTEIN A REPETITIONS PREDOMINANT (GARP)

GARP is a type I transmembrane protein, weighing around 78 kDa, and consists of 662 amino acids⁴⁷. In the past, GARP has been referred to by several names, including glycoprotein A repetitions predominant, garpin, leucine-rich repeat-containing protein 32, CPPRDD, and D11S833E²⁸. However, for the rest of this work, the protein will be referred to as GARP.

GARP is encoded by the LRRC32 gene (gene ID: 2615)⁴⁸. LRRC32 was first described by Ollendorff et al., 1992 as a gene amplification in breast cancer found in chromosome 11 (11q13.5-11q14)⁴⁹. LRRC32 contains two exons that encode three domains: a 15 amino acid cytoplasmic tail, a hydrophobic transmembrane domain, and an extracellular domain,

consisting of 20 leucine rich repeats (LRR) that comprise about 70% of the protein^{28,47}. Within the 7th and 12th LRR, two cysteines (Cys-192 and Cys-331) are encoded. These amino acids enable two disulfide bonds to form between GARP and its binding partner latent TGF- β (LTGF- β)^{28,50}. A signal peptide is encoded in the N-terminus of the protein. This peptide must undergo cleavage before GARP is expressed on the surface of the cell^{28,51}. The prominent LRRs and the post-translational glycosylation of the protein are responsible for its namesake.

The GARP transcript, also known as GARP mRNA, is diffusely expressed in healthy tissues found throughout the body, including but not limited to the placenta, heart, lung, liver, pancreas, kidney, skeletal muscle, and lymphoid tissues^{28,48,52}. In contrast, the expression of the GARP protein is restricted to a narrow range of cell types, namely immune cells (platelets, activated Tregs, macrophages, activated B cells), mesenchymal cells (mesenchymal stem cells, fibroblasts, hepatic stellate cells), and endothelial cells^{28–30,53–60}. In the past, it was well established that GARP is expressed on the cell surface and is secreted as a soluble factor, known as soluble GARP (sGARP) (**Fig. 3**)^{28,61–64}. However, in recent years, Zimmer et al., 2019 reported for the first time that GARP is also enriched in the nuclei (GARP^{NU}) of activated Tregs, tumor cells, and glioblastoma stem-like cells (GSCs) (**Fig. 3**)⁶⁵.

Figure 3: Localization of GARP

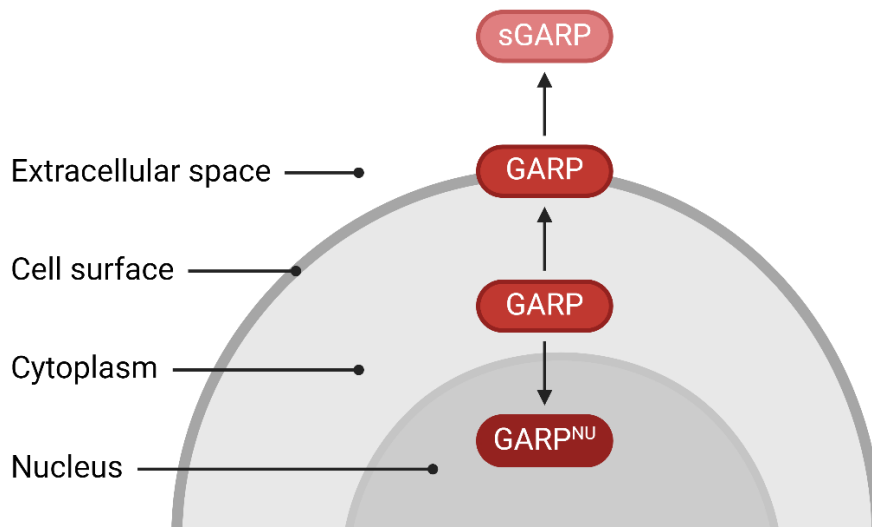


Fig. 3: GARP is differentially localized inside and outside the cell. Created with BioRender.com¹⁵.

GARP functions by binding LTGF- β , an inactive precursor form of TGF- β bound to latency associated peptide (LAP) (**Fig. 4A**)^{28,29,66,67}. In more detail, GARP binds every isoform of TGF- β (TGF- β 1-3) and aids in its activation. TGF- β is a pleiotropic cytokine that is expressed throughout the body. It mediates several fundamental biological processes, such as development, tolerance, wound healing, and cancer^{28,68}. As a result, expression of TGF- β is tightly regulated in a multi-step process²⁸. GARP plays an essential role in the final steps of TGF- β activation by presenting LTGF- β on the cell surface (**Fig. 4A**)^{28,69}. This enables LTGF- β to bind to $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins, which releases mature and biologically active TGF- β into the

surrounding environment. It is important to point out that activating LTGF- β has only been attributed to the surface and soluble forms of GARP (sGARP), whereas the functional relevance of GARP^{NU} remains unclear^{28,65,70}. This means that GARP is required for both the surface expression and activation of LTGF- β , and thereby regulates its bioavailability⁵⁰. As GARP controls the expression of TGF- β , it is no surprise that the protein has been identified as an important factor in TGF- β mediated processes.

Figure 4: Functions of GARP

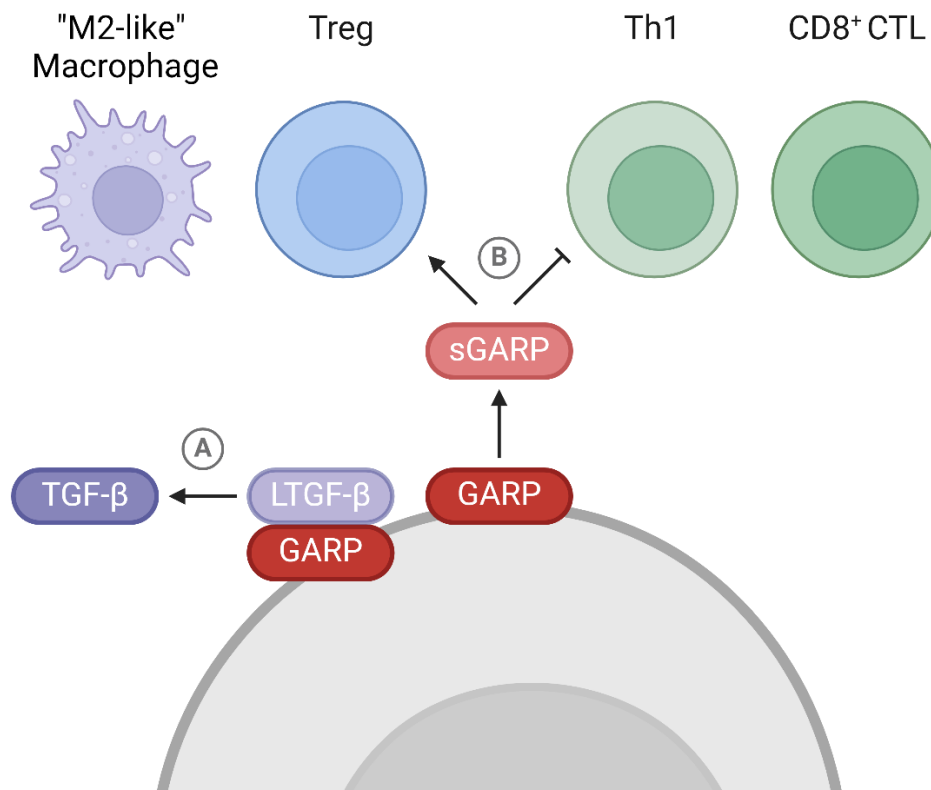


Fig. 4: Functions of GARP. GARP functions as a LTGF- β activating protein (A) and induces immunosuppressive cell types (B). Created with BioRender.com¹⁵.

GARP has been found to play an essential role in development. Wu et al., 2017 reported that knockout of GARP in mice was lethal within 24 h after birth⁶⁷. GARP knockout mice displayed developmental defects in palatogenesis (palate formation) possibly impairing their ability to feed. It was speculated that cleft palate formation resulted from the reduced production of TGF- β 3. Disruption of LRRC32 has also been linked to developmental defects in humans as shown in a case study by Harel et al., 2019⁷¹. They described three patients (2-3 years old) who had a homozygous stop gain variant of LRRC32 (c.1630C>T, p.(Arg544Ter), also known as a premature stop codon. This resulted in a truncation of both the intracellular and transmembrane domains of GARP and thus a loss of the protein's functionality. The patients exhibited a range of developmental defects, including but not limited to cleft palate formation, proliferative retinopathy (blood vessel and scar formation over the retinas), hypotonia (decreased muscle tone), and ventriculomegaly (enlargement of the cranial ventricles). Notably, Roubin et al., 1996 showed in mice that the GARP mRNA is expressed in

late organogenesis, notably in the nasal cavity, eyes, muscle, and choroid plexus, offering a possible explanation for why these developmental defects may have occurred⁷².

Dysregulation of GARP has been associated with numerous inflammatory conditions. Downregulation of the GARP transcript has been observed in the blood of patients with inflammatory bowel disease in comparison to healthy controls⁷³. LRRC32 is considered a risk locus for many inflammatory diseases (e.g., atopic dermatitis, eczema, colitis, primary immunodeficiency) and allergic conditions (e.g., asthma, eosinophilic esophagitis)^{28,74–79}. In addition, decreased numbers of CD25⁺Foxp3⁺GARP⁺ Tregs have also been inversely associated with the immune inflammatory response system, a form of chronic inflammation, and neurotoxicity in patients with major depressive disorder⁸⁰.

Similarly to mutations in FOXP3 as described in “1.1.6 Regulatory T cells (Tregs)”, disruption of LRRC32 has been connected to severe autoimmunity. It has been shown that loss of GARP expression on Tregs leads to the development of fatal autoimmunity in mice^{28,81,82}. In more detail, Zhang et al., 2015 demonstrated that knocking out the obligate chaperone of GARP, heat shock protein GP96, in Tregs resulted in the generation of dysfunctional Tregs⁸¹. These Tregs were characterized by unstable expression of Foxp3 and reduced suppressive function. Decreased suppressive capacity was attributed to loss of surface GARP expression and consequently reduced production of mature TGF- β . This led to uncontrolled inflammatory T cell activation and the development of fatal multi-organ inflammatory disease. Similar findings were also observed in patients with primary immunodeficiency, a disorder characterized by immune dysregulation, who had mutations in LRRC32⁸². Lehmkuhl et al., 2021 also demonstrated that GARP deficient mice were more susceptible to developing autoimmune disorders, including severe arthritis, experimental autoimmune encephalomyelitis (EAE), and colitis⁸². Of note, LRRC32 appears to be evolutionary conserved, with humans and mice having a 80% similarity in nucleotide sequence⁷². Collectively, these studies indicate that GARP protects against autoimmunity by activating TGF- β – a cytokine needed for the development and suppressive function of Tregs.

GARP, itself, has also been shown to display strong anti-inflammatory properties via the modulation of T cell responses. Hahn et al., 2016 reported that sGARP induced Foxp3⁺ Tregs and “M2-like” macrophages (**Fig. 4B**)⁶¹. They could also show that sGARP strongly suppresses Teff cell function. sGARP inhibited the proliferation and production of effector molecules in both Th1 cells (IL-2, IFN- γ) and CTLs (IFN- γ , granzyme B) (**Fig. 4B**). Similar GARP-mediated effects were shown by Zimmer et al., 2020 using GARP expressing platelets⁵⁶. Interestingly, blocking experiments using antibodies against TGF- β 1-3 and the TGF- β type II receptor (TGF- β RII) revealed that the suppressive effects of GARP are only partially mediated by TGF- β ^{56,83}.

Therapeutically, sGARP has been successfully applied to protect animals from developing lethal xenogeneic graft-versus-host disease (GvHD) by preventing destructive T cell mediated inflammation⁸³. Additionally, in a humanized mouse model of airway inflammation, sGARP treatment was found to reduce airway inflammation and hyperresponsiveness in a TGF- β dependent manner⁸⁴.

Altogether, these findings demonstrate the essential role of GARP in maintaining peripheral tolerance, which is mediated in part through its function in activating LTGF- β .

The immunomodulatory effects of GARP are implicated in a broad range of immunological contexts, including wound healing and cancer. GARP was further characterized in these distinct immunological settings in **Paper 1** and **Paper 2** of this work^{8,9}.

1.2 WOUND HEALING

Upon tissue injury, a complex multi-step process, known as wound healing, begins in order to repair the damage and to prevent infection^{11,85,86}. Tissue resident cells, immune cells, platelets, the vascular system, and soluble mediators, like cytokines and chemokines, work together to regenerate the tissue. Wound healing consists of four overlapping steps: (1) hemostasis, (2) inflammation, (3) proliferation, and (4) remodeling (**Fig. 5**).

Figure 5: Stages of wound healing

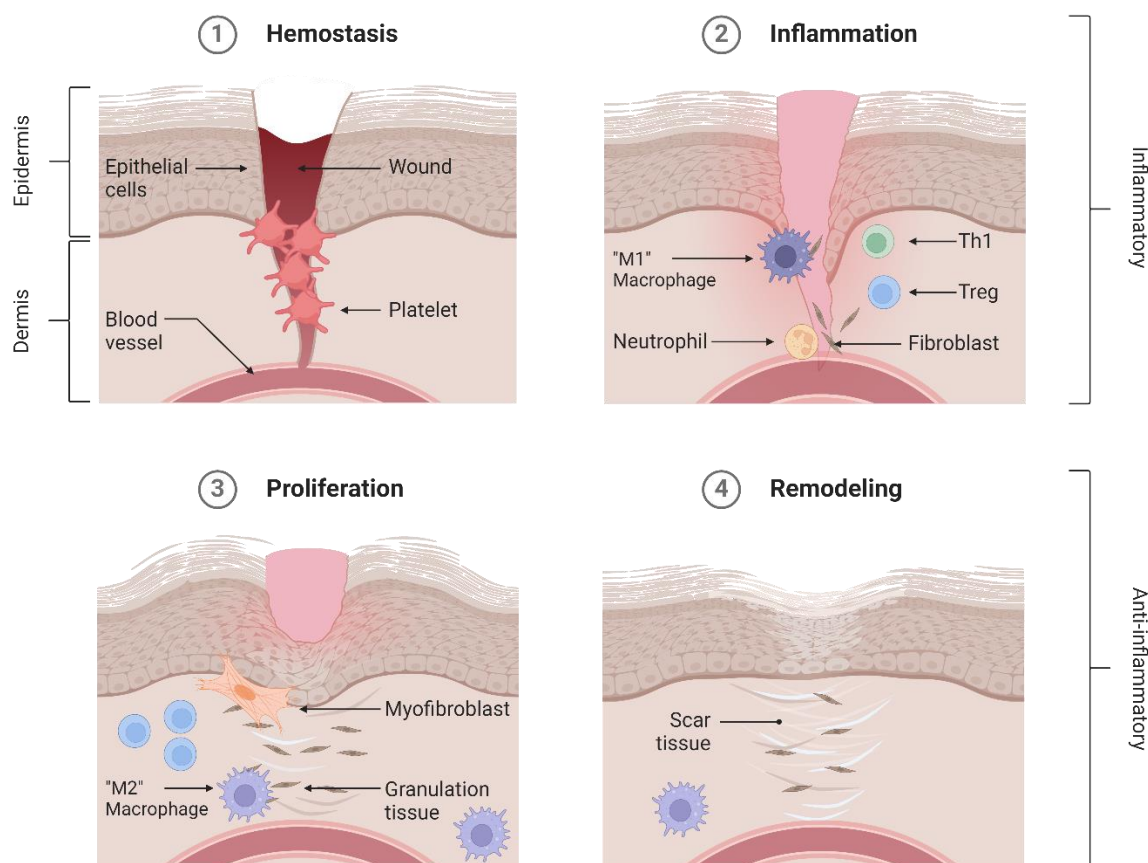


Fig. 5: Schematic representation of the four phases of wound healing in the skin. These phases can be divided into inflammatory (hemostasis, inflammation) or anti-inflammatory (proliferation, remodeling) immune responses. Adapted from "Wound Healing", by BioRender.com (2024)¹⁵.

Hemostasis occurs rapidly within minutes following damage to blood vessels^{86,87}. To stop bleeding, blood vessels near the wound site constrict, and platelets aggregate to form clots (**Fig. 5**)⁸⁶.

Inflammation begins following hemostasis (**Fig. 5**). This step is important in preventing infection and typically lasts a few days⁸⁷. Inflammation stems from the Latin word *inflammatio*, meaning to set ablaze. Fittingly, the main symptoms of inflammation are redness (*rubor*), heat (*calor*), swelling (*tumor*), and pain (*dolor*), which were correctly described by Aulus Cornelius Celsus 2000 years ago^{88,89}.

During inflammation, Th1 cells activate tissue resident macrophages to a proinflammatory M1 phenotype, which in turn secrete proinflammatory cytokines, like tumor necrosis factor alpha (TNF- α)⁹⁰⁻⁹³. These soluble mediators trigger vasodilation, the recruitment of immune cells, and the activation of endothelial cells that line blood vessels near the site of injury¹¹. Vasodilation increases blood flow to the site of injury, responsible for the redness and heat associated with inflammation. Increased blood flow brings an influx of immune cells, like neutrophils and monocytes, into the damaged tissue. Neutrophils primarily function to eliminate pathogens via phagocytosis and the secretion of cytotoxic granules^{91,94}. Recruited monocytes differentiate into M1 macrophages, which phagocytose pathogens and cellular debris, as well as secrete proinflammatory cytokines^{11,93}. Activated endothelial cells upregulate cell adhesion molecules that enable immune cells to attach to blood vessels and to enter the damaged tissue in a process known as extravasation¹¹. Blood vessels near the site of injury increase vascular permeability allowing fluid from the blood and plasma proteins, including those from the complement system, to accumulate in the damaged tissue. This acts as another layer of defense against infection and is responsible for the swelling and pain associated with inflammation. Lastly, during this stage, additional clotting in microvessels at the site of the damaged tissue occurs to prevent the spread of infection into the blood stream.

The proliferative phase represents the regrowth of tissue at the site of injury and a shift in the immune response from inflammatory to anti-inflammatory (**Fig. 5**)⁸⁷. This is mediated in part by macrophages that transition from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype, triggered by the phagocytosis of dead neutrophils at the wound site^{91,93}. These M2 macrophages support wound healing through several mechanisms^{87,91,95}. Firstly, they secrete growth factors, like vascular endothelial growth factor (VEGF) and TGF- β 1, which encourage new blood vessel formation, in a process called angiogenesis, as well as promote the proliferation of fibroblasts and epithelial cells. Fibroblasts, in turn, secrete extracellular matrix (ECM), depositing collagen and fibronectin into the wound site. M2 macrophages support the accumulation of ECM by preventing its degradation through the production of tissue inhibitor of metalloproteinases (TIMPs)⁹⁵. The newly formed vascularized ECM is known as granulation tissue, which functions to fill the wound site and serves as an attachment site for epithelial cells. Epithelial cells migrate to the wound site and cover the newly formed tissue, in a process known as reepithelization. Additionally, M2 macrophages suppress inflammatory immune responses through the production of IL-10 and TGF- β ^{93,95}. The end of the proliferative phase is marked by wound contraction and closure by myofibroblasts⁸⁷.

The remodeling stage is the longest stage of the wound healing process, lasting weeks to up to a year depending on the initial injury (**Fig. 5**)⁸⁷. In this phase, the mechanical strength of the injured tissue is restored. This is accomplished by replacing collagen fibers in the wound site from type III to more resistant type I as well as restructuring the fibers so that they align along tension lines. M2 macrophages facilitate this remodeling process by secreting matrix metalloproteinases (MMPs)^{87,95}. As the remodeling stage progresses, macrophages, myofibroblasts, and vascular cells undergo apoptosis. The resulting collagen-rich scar tissue marks the end of the wound healing process.

Disruption to any of these wound healing stages can result in infection, the development of chronic wounds, and the formation of excessive scar tissue²⁶. Chronic wounds, in particular, represent a significant and growing global healthcare burden — costing the US alone over \$25 billion each year⁹⁶. Chronic wounds often occur in the growing elderly, diabetic, and obese populations. As a result, the incidence of chronic wounds is rapidly increasing.

Chronic wounds occur when wound healing fails to proceed to the anti-inflammatory proliferation stage (**Fig. 5**)^{95,97}. Instead, the wound remains stuck in the inflammation stage, preventing it from properly healing and effectively responding to available therapies. Therefore, the wound healing process must be tightly regulated, especially as the immune response transitions from inflammatory (inflammation phase) to anti-inflammatory (proliferation phase) (**Fig. 5**)^{87,97}. Herein, Tregs have been shown to play an important role.

It is becoming increasingly recognized that Tregs orchestrate the wound healing process by controlling inflammation^{25–27}. Nosbaum et al., 2017 showed *in vivo* that the number of Tregs in the wound site increased by 20-fold seven days after the initial injury, during the transition from the inflammation to the proliferation phase²⁵. Of note, the majority of these Treg were found to be activated. Depletion of said Tregs decreased reepithelization and wound closure, essential components of the proliferation phase. Furthermore, depletion of Tregs during the inflammation phase resulted in increased numbers of inflammatory IFN- γ producing Teff and thereby an increased number of M1 macrophages, which persisted throughout the duration of wound closure (14 days). Meanwhile, control animals had nearly no M1 macrophages present after 14 days, offering a possible explanation for the observed delay in wound closure in mice depleted of Tregs. Collectively, this indicates that Tregs play an important role in facilitating the transition between the inflammation and proliferation stages of wound healing. This is achieved by the regulation of type I immunity, specifically limiting the number of IFN- γ producing Teff and M1 macrophages.

In the case of chronic wounds, which remain stuck in the inflammation phase, the induction of Tregs represents a promising therapeutic approach. As discussed in “1.1.8 Glycoprotein A repetitions predominant (GARP)”, GARP is a potent inducer of Tregs and “M2-like” macrophages, which are required for the transition to the anti-inflammatory proliferation phase (**Fig. 4B**)^{25,56,83,87,95}. GARP also inhibits IFN- γ producing Teff, which help sustain the inflammation phase. This makes modulating GARP levels a promising therapeutic approach

for the treatment of chronic wounds. However, up until now, the possible involvement of GARP in wound healing remains unclear.

It can be speculated that GARP may influence wound healing as it regulates TGF- β , a cytokine that has a well-established connection to wound healing^{28,29,50,66–68,98}. TGF- β has been found to be involved in all stages of the wound healing phase, including inflammation initiation, granulation tissue formation, angiogenesis, re-epithelization, wound contraction, and collagen organization⁹⁸. Despite this, the role of GARP in early stages of the wound healing phase has yet to be explored. Therefore, this study set out to examine how GARP may influence T cells and macrophages, key cellular mediators of wound healing, especially in the inflammation and proliferation phases (**Paper 1**)⁸. In more detail, GARP was evaluated as a possible underlying immunological mechanism responsible for the wound healing properties of the autologous platelet concentrate, injectable platelet rich fibrin (iPRF)⁸.

On the flipside, in other immunological settings, like cancer, GARP has been linked to driving disease progression and to poor patient prognosis^{28,56,60,63,99–104}.

1.3 CANCER

Cancer refers to a group of over 100 diseases, which can originate in nearly every tissue of the body¹⁰⁵. These diseases are chiefly characterized by the uncontrolled proliferation of abnormal autologous cells. Globally, cancer is one of the leading causes of death with nearly 10 million deaths attributed to the disease in 2020^{105,106}. Depending on where the cancer arises, it may manifest in the form of a solid tumor, either restrained to the primary tissue of origin (benign), or it may be capable of invading neighboring tissues and even metastasizing to distant parts of the body (malignant). Widespread metastases can interfere with the functioning of essential organs; therefore, they are attributed as the main cause of death from cancer.

Tumors do not consist solely of cancer cells. Instead, they are made-up of a dynamic and heterogeneous environment, including infiltrating immune cells, resident stromal cells, blood vessels, ECM, and secreted factors^{107,108}. Collectively, this is known as the tumor microenvironment (TME). Of note, the composition of the TME can vary greatly depending on the cancer type.

1.3.1 CANCER INITIATION

Cancer cells are derived from healthy autologous cells, which have undergone a complex malignant transformation^{105,108}. The development of cancer usually occurs slowly, most often resulting from the gradual accumulation of mutations in tumor suppressor and proto-oncogenes overtime¹⁰⁹. Tumor suppressor genes refers to a category of genes that negatively regulate the cell cycle, aid in the recognition and repair of damaged DNA, and can induce programmed cell death as a mechanism to prevent abnormal cells from further proliferating¹¹⁰. Conversely, proto-oncogenes promote cell division and survival and therefore, are tightly regulated in healthy cells. When proto-oncogenes are mutated, in which they become constitutively activated or overexpressed, they are referred to as oncogenes¹⁰⁹.

The gradual accumulation of mutations in both tumor suppressor genes and proto-oncogenes can result in uncontrolled cell division and thereby malignant transformation^{108,109}.

DNA repair genes are an important class of tumor suppressor genes, and as their name suggests, they function to ensure the genetic integrity of the cell¹¹¹. These genes aid in both the detection and the repair of damaged DNA, frequently resulting from errors in replication during the S phase of cell cycle or from exposure to carcinogens. Individuals can be genetically predisposed to develop certain cancers by inheriting mutated DNA repair genes, such as *BRCA1*, *BRCA2*, or *TP53*^{112–114}. These mutations result in a reduced efficacy in recognizing and repairing damaged DNA. This allows cells with damaged DNA to proceed through the cell cycle, when they would have normally undergone programmed cell death, and to pass down altered DNA to daughter cells.

Over time, this enables a faster acquisition and accumulation of mutations in the genome, including in tumor suppressor genes and proto-oncogenes. This slow but steady alteration of the genome is known as genomic instability and is often considered a driving force behind developing cancer¹¹⁵. This is because it results in an increased frequency of mutations, which can be either advantageous or harmful for cancer cells. Advantageous mutations that aid in the survival of cancer cells are selected for and passed down to daughter cells, increasing cellular fitness.

Mutations do not only result from ineffective DNA repair genes but also from the exposure of cells to external physical, chemical, and biological carcinogens^{105,116}. Carcinogens increase the risk of developing cancer, and they can be considered as environmental and lifestyle risk factors for developing the disease. Carcinogens can directly damage the structural integrity of DNA or bind to DNA, preventing effective DNA replication. When DNA repair mechanisms fail to recognize and correct these damages, a mutation will result, which can be inherited by daughter cells. Carcinogens can also act indirectly by resulting in conditions that support the development of the disease, such as triggering chronic inflammation (e.g., *Helicobacter pylori*) and immunosuppression (e.g., human immunodeficiency virus)^{117,118}.

Although risk factors vary depending on the cancer type, common environmental and lifestyle risk factors, include but are not limited to exposure to ultra-violet (UV) radiation, x-rays, air pollution, tobacco use, alcohol consumption, obesity, poor diet, and low physical activity^{105,116,119,120}. Aging is considered the most important risk factor for developing cancer^{105,121,122}. This is due to several factors, namely the accumulation of mutations over one's lifetime, the reduction in the efficiency of DNA repair, and the impaired functioning of the immune system.

1.3.2 IMMUNE SURVEILLANCE

In addition to keeping the body free from foreign pathogens, the immune system detects and destroys cancer cells^{123,124}. This concept, also known as the immune surveillance hypothesis, was originally formulated by Frank Macfarlane Burnet and Lewis Thomas in the 1950's^{123,125–128}. Since then, the hypothesis has been expanded to include four distinct phases: (1) cancer

initiation (described in “1.3.1 Cancer Initiation”), (2) elimination, (3) equilibrium, and (4) escape (**Fig. 6**)^{124,129}.

Figure 6: The immune surveillance hypothesis

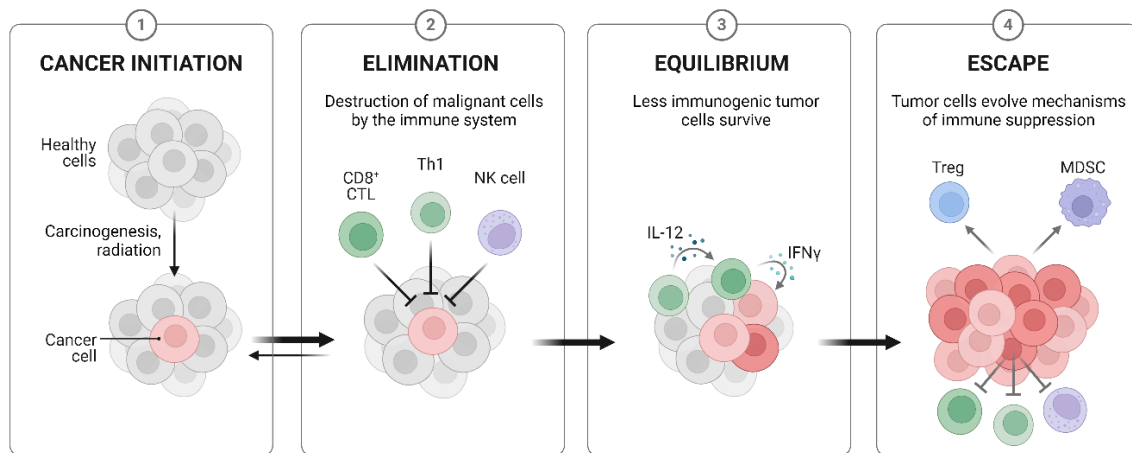


Fig. 6: Schematic representation of immune surveillance. Adapted from “Cancer Immunoediting”, by BioRender.com (2024)¹⁵.

Elimination is the second phase of immune surveillance (**Fig. 6**). During this stage, immune cells effectively recognize and destroy cancer cells^{124,130,131}. The immune system can distinguish cancer cells from healthy cells by several key differences. Due to acquired genomic instability, malignant cells accumulate mutations in genes^{131,132}. When these genes are translated, this can lead to the production of altered proteins that are not normally produced by the host organism. CTLs can recognize parts of these proteins as foreign, also known as neoantigens, when they are presented by APCs or by the tumor cell itself. Upon recognition, CTLs destroy target cancer cells through the release of cytotoxic granules described in “1.1.3 CD4⁺ and CD8⁺ T cells”^{131,133}. NK cells aid in the recognition of tumor cells, namely through the detection of abnormal ligands on the surface of tumor cells^{130,133}. For example, tumor cells can express NK cell activating ligands, like NKG2D. When bond to the respective NK cell receptor, NK cells become activated and target the malignant cell for destruction. NK cells can also recognize malignant cells as “non-self” through their downregulation of MHC class I receptors^{131,133}. In both cases, NK cells directly kill the identified cancer cell through the delivery of cytotoxic granules¹³³.

If the immune system fails to completely eliminate tumor cells, the equilibrium phase begins (**Fig. 6**)¹²⁴. During this stage, tumor cells and immune cells coexist in a balance where tumor cell growth is restrained by the immune system, also known as tumor dormancy¹³⁴. Tumor cells continue to proliferate and accumulate mutations that allow them to better adapt to their environment. Meanwhile, the immune system exerts a strong selective pressure on the cancer cells, in a process referred to as immunoediting. Herein, IFN- γ and IL-12, a stimulator of IFN- γ production, play an important role in enhancing the proliferation and cytotoxicity of Th1, CTLs, and NK cells, thereby aiding in the destruction of tumors cells^{134–136}. In response, cancer cells evolve mechanisms of immune evasion to minimize their detection by the

immune system¹³¹. Cancer cells may downregulate the expression of immunogenic neoantigens to avoid detection by T cells. They may also express coinhibitory checkpoint molecules, like programmed death ligand 1 (PD-L1), to repress T cell mediated immune responses. Additionally, tumor cells can reduce their antigen presenting capabilities. For example, tumor cells can acquire mutations in MHC molecules or downregulate their expression^{131,137}. This results in the reduced presentation of neoantigens to CTLs as well as reduced detection by NK cells.

Tumor cells employ mechanisms of immune tolerance to repress anti-tumor immune responses^{131,138}. Cancer cells secrete suppressive factors, like TGF- β and IL-10, into their local environment. These factors recruit and promote the development of suppressive immune cell types, such as Tregs, tumor-associated macrophages, and myeloid-derived suppressor cells (MDSC) as well as inhibit the maturation of DCs, necessary for T cell activation¹³⁸. These suppressive cells, coupled with factors produced by tumor cells themselves, work together to create an immunosuppressive microenvironment and to inhibit anti-tumor immune responses. In this way, tumor cells actively transform their microenvironment to evade detection by the immune system.

By the end of the equilibrium phase, cancer cells will have acquired several mechanisms to evade detection by the immune system¹³¹. The escape phase begins once tumor cells can proliferate unrestrained by the immune system (**Fig. 6**). It is in this phase that cancer becomes clinically detectable.

1.3.3 GARP IN CANCER

As described in detailed in section “1.1.8 Glycoprotein A repetitions predominant (GARP)”, GARP is a potent mechanism of immunological tolerance through its regulation of the suppressive cytokine, TGF- β ^{28-30,64,66,67,81-83}. Cancer cells frequently overexpress GARP to evade detection by the immune system. Elevated expression of the GARP mRNA transcript and protein has been reported across a broad range of cancer types, including but not limited to melanoma, glioblastoma, lung cancer, breast cancer, bone sarcoma, gastric, and papillary thyroid carcinoma^{28,49,61,65,99,100,102,104,139,140}. Secretion of sGARP into the TME by cancer cells (e.g., melanoma), activated Tregs, and platelets has also been reported^{28,56,61,63}.

GARP exhibits several suppressive effects in the TME. GARP induces tolerogenic immune cells, namely Tregs and M2 macrophages (**Fig. 4B**)^{28,55,56,61,63,83}. It is well known that these cells promote tumor survival by downregulating anti-tumor immune responses and sustaining the suppressive TME^{131,138,141}. Additionally, GARP prevents T cell mediated immune responses against the tumor from occurring. In more detail, GARP inhibits the proliferation and production of effector molecules in both Th1 cells (IL-2, IFN- γ) and CTLs (IFN- γ , granzyme B)^{61,65,83}. These cells play an essential role in detecting and destroying tumor cells¹³¹. In this way, GARP production by tumor cells results in both the accumulation of tumor promoting immune cells in the TME as well as the downregulation of anti-tumor immune responses.

Besides suppression in the TME, GARP has been linked to oncogenesis. Carrillo-Gálvez et al., 2020 showed in bone sarcoma that GARP enhances the proliferation of tumor cells and their resistance to irradiation and chemotherapy⁹⁹. Metelli et al., 2016 found that GARP promotes the malignant transformation of murine mammary cells *in vitro* via the upregulation of epithelial-mesenchymal transition (EMT) markers and by increasing the migratory ability of the cells¹⁰⁰. Furthermore, they demonstrated that GARP expression increased tumor growth and metastasis in an *in vivo* murine breast cancer model. Both Carrillo-Gálvez et al., 2020 and Metelli et al., 2016 found that the observed tumor promoting effects of GARP are mediated in a TGF- β dependent manner^{99,100}.

Collectively, these studies demonstrate that tumor cells upregulate GARP to not only avoid detection by the immune system but also to promote tumor growth. This suggests that the protein is indispensable for oncogenesis and may have multiple tumor-promoting functions in the TME. Furthermore, high expression of GARP on both tumor cells and tumor supporting immune cells (activated Tregs, platelets) makes GARP a promising therapeutic target and biomarker candidate for cancer.

Therefore, this work focused on further characterizing the role of GARP in cancer, specifically in glioblastoma biology (**Paper 2**)⁹.

1.4 GLIOBLASTOMA

Glioblastoma, previously known as glioblastoma multiforme, refers to a highly heterogeneous and malignant type of brain tumor. The WHO classifies glioblastoma as the most aggressive form of astrocytoma, grade IV¹⁴². Although it is possible for glioblastoma to occur in all parts of the brain, tumors are most often found in the supratentorial compartment, specifically the frontal and temporal lobes^{143–145}. It remains unclear from which cell type(s) glioblastoma arises. It is thought that malignant cells may originate from neural stem cells and glial precursor cells, e.g., oligodendrocyte and astrocytic precursor cells^{146–148}.

1.4.1 EPIDEMIOLOGY

Glioblastoma is the most common primary brain tumor in adults. Nearly 50% of all malignant central nervous system tumors are attributed to glioblastoma, and the disease has an incidence rate of 3.19 cases per 100,000 persons (age adjusted, United States, 2006-2010)^{119,142,147,149,150}. It is well known that men have a higher incidence of the disease and a reduced survival rate in comparison to women^{151,152}. Reasons for these sex disparities remain unclear, but studies have shown that hormonal differences may play a role^{151,153–155}.

The incidence of glioblastoma has been associated with ethnicity^{152,156}. Overall, Non-Hispanic Whites have the highest incidence of the disease. In comparison, American Indians and Alaska Natives have a 40% lower incidence. Additionally, the incidence in Whites is 1.95 times greater than in Blacks as well as 2.97 times higher than in Asian or Pacific Islanders¹⁵⁶.

Increased incidence of the disease has been linked to higher socioeconomic status; however, the underlying reasons why remain poorly understood¹⁵⁷.

From 1978 to 1992, the incidence of glioblastoma in the US increased by 2.9%. This can be explained in part by improvements in diagnostic technologies¹⁵⁸. For example, the introduction of computed tomography (CT) and magnetic resonance imaging (MRI) into the clinics enabled the improved detection of brain tumors^{158–160}. However, from 1992 to 2018, incidence slowed down, increasing only by 0.2%¹⁵⁸. Initial projections suggest that the annual incidence of glioblastoma cases will increase by 50% in the next 30 years, explained in part by the simultaneously aging and growing global population¹⁶¹.

1.4.2 RISK FACTORS

There are almost no known risk factors for developing glioblastoma. Prior exposure to mutagenic ionizing radiation is the only known environmental risk factor of the disease¹¹⁹. Lifestyle factors, including alcohol consumption and mobile phone use, are not associated with risk of developing glioblastoma^{119,162–164}. Age is the best associated risk factor^{143,165–167}. Most cases occur in adults over the age of 40, and incidence peaks between 75 to 84 years of age.

Individuals with allergic conditions, such as asthma, atopic dermatitis, and eczema, have been associated with a reduced risk of developing the disease^{119,168}. Although it is not fully understood why, it is speculated that the hyperactivity of the immune system in these conditions may have a protective anti-tumor effect.

Most glioblastoma cases are thought to arise spontaneously without a family history of disease^{169,170}. However, a fraction of these cases (~5%) is linked to hereditary cancer syndromes, in which inheritable mutations predispose an individual to developing cancer. The following genetic conditions with mutations in tumor suppressor genes have been linked to increased risk of developing glioblastoma: Li-Fraumeni syndrome (*TP53*), Neurofibromatosis type 1 (*NF1*), and Turcot syndrome type II (*APC*)¹⁶⁹. Mutations in mismatched repair genes, responsible for repairing improper DNA insertions or deletions, as seen in Turcot syndrome type I and Lynch syndrome are also associated with increased risk of developing glioblastoma. Collectively, these conditions result in either increased cell growth or genomic instability, driving oncogenesis.

1.4.3 DIAGNOSIS

There are no preventive screening methods for glioblastoma. Individuals tend to first exhibit abnormal neurological symptoms, such as persistent headaches or seizures, before being recommended by their physician for neurological imaging¹⁷¹. Some of the most common methods are by MRI and CT¹⁵⁸. These techniques provide essential diagnostic information, regarding the size, location, and presence of necrosis in the tumor, amongst other things¹⁷¹. Although imaging can reveal the presence of a brain tumor, diagnosis of the cancer type is confirmed following surgical resection where the excised tumor undergoes histological and molecular evaluation^{171,172}.

Four different types of glioblastomas can be distinguished from one and other based off their molecular characteristics¹⁷³:

- **Glioblastoma, *isocitrate dehydrogenase (IDH)* wildtype:** the majority of cases, often primary tumors that develop around 60 years of age
- **Glioblastoma, *IDH* mutant:** most commonly secondary glioblastomas that develop in younger individuals with preexisting lower grade astrocytomas (WHO grades I-III)
- **Glioblastoma not otherwise specified (NOS):** unclear *IDH* mutational status resulting from insufficient material for histological and molecular testing
- **Not elsewhere classified (NEC) glioblastoma:** glioblastomas that do not fit the categories mentioned above, resulting from inconsistent traits, not yet classified by the WHO

Patients with *IDH* wildtype glioblastomas have worse prognoses and exhibit a more aggressive form of the disease in comparison to those with *IDH* mutant glioblastomas^{174–176}. Normally, the *IDH* genes (*IDH1*, *IDH2*) encode isocitrate dehydrogenase (IDH), which catalyzes the oxidative decarboxylation of isocitrate, a reaction that is part of the Krebs cycle in the mitochondria or can alternatively take place in the cytoplasm¹⁷⁴. This reaction yields the metabolite alpha-ketoglutarate and in the process reduces the electron carrier, NADP, to NADPH. In contrast, mutated *IDH1/IDH2* produces the oncometabolite, 2-hydroxyglutarate (2-HG) that inhibits the demethylation of histones and DNA^{174,177}. The resulting widespread epigenetic remodeling induces alterations in gene expression and is considered to be a starting point in gliomagenesis^{174,175}. The improved treatment prognosis of *IDH* mutant glioblastomas results in part from their reduced metabolic fitness and thereby slower proliferation rate¹⁷⁵.

In addition to *IDH* status, glioblastomas are classified as either primary or secondary, depending on how the disease originates. Primary is the most common form of glioblastoma (~95%), arising *de novo* in older patients^{170,178}. Secondary glioblastoma is most often found in younger patients, developing from pre-existing low grade astrocytomas. New research studies show that glioblastomas can further be subtyped off their transcriptomic profiles, but this classification has yet to reach to the clinic^{179–181}.

Promotor methylation of O6-methylguanine-DNA methyltransferase gene (*MGMT*) is a prognostic and predictive biomarker in glioblastoma¹⁸². Methylation of the promotor prevents transcription from occurring. This in turn prevents production of the functional protein, MGMT. This protein catalyzes the transfer of methyl groups from O(6)-alkylguanine to itself. This results in the restoration of the nucleic acid base guanine, thereby repairing damaged DNA. Patients with *MGMT* promotor methylation respond better to treatment with the chemotherapy, temozolomide (TMZ)^{182–184}. This is because their cancer cells are less able to repair TMZ induced O(6)-alkylguanine DNA lesions, increasing therapeutic efficacy¹⁸³. Patients with *MGMT* promotor methylation exhibit improved median survival in comparison to patients with unmethylated *MGMT* promoters (2 years, 48.9% vs. 14.8%)^{174,184}.

Epidermal growth factor receptor (EGFR) is another prognostic biomarker in glioblastoma and similarly to *IDH*, it can be used to molecularly classify glioblastoma tumors^{142,185,186}. EGFR is a receptor tyrosine kinase, which becomes activated through the binding of its respective

ligands. Activation of EGFR represents the start of signal transduction for both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 (PI3K) growth pathways. In around 60% of primary glioblastoma cases, the *EGFR* gene is amplified^{187–189}. Often, amplified *EGFR* genes contain a deletion of exons 2-7, which encode a truncated receptor known as EGFRvIII^{188–190}. This mutated receptor is unable to bind its normal ligands, and instead remains constitutively active. Amplification of *EGFR* and EGFRvIII have both been found to increase the proliferation, invasiveness, resistance, and the angiogenesis of glioma cells. This explains why patients with abnormal EGFR expression have a reduced prognosis^{185,189}.

1.4.4 PATHOLOGY

Glioblastoma presents as a highly aggressive and heterogenous disease, characterized by tumor cells diffusely invading the surrounding healthy brain tissue¹⁹¹. Primary *IDH* wildtype tumors are characterized by rapid growth¹⁷⁵. Patients typically present with large hypoxic tumors containing necrotic cores^{142,192–194}.

Unlike other cancer types, glioblastomas exhibit a very low frequency of metastasis (~0.5%), and it remains unclear why¹⁹⁵. It is speculated that glioma cells may struggle to leave the central nervous system due to physical barriers, like the blood brain barrier (BBB)¹⁹⁶. Another possible explanation is that patients may die from primary tumors before secondary metastases are detected.

Glioblastoma is classified as a immunologically “cold” tumor, protected by powerful mechanisms of immune suppression^{197,198}. Glioblastoma has a low tumor mutational burden (TMB) and is known for having a TME depleted of neoantigens, impeding its detection by Teff¹⁹⁹. The TMB indicates the number of somatic mutations per mega base pair found in the genome of cancer cells and positively correlates with the number of neoantigens expressed. Detection of glioblastoma by Teff is further complicated by poor infiltration of immune cells into the glioblastoma microenvironment due to the BBB, which protects the immune privileged brain¹¹. However, it is worth noting that intracranial lymphatic vessels have been recently discovered, challenging the established paradigm that immune cells struggle to migrate to the brain^{197,200}. Approximately 50% of cells that make-up the glioblastoma microenvironment are immune cells; the majority are either microglia or glioma associated macrophages (GAMs)^{197,201,202}. Microglia, GAMs, and glioma cells secrete suppressive cytokines, e.g., IL-10 and TGF- β , that inhibit the activity of NK cells and T cells – required for effective anti-tumor immunity^{198,203}. These cytokines induce suppressive immune cell types, like M2-like GAMs that further contribute to immunosuppression in the TME. In addition, IL-10 and TGF- β downregulate MHC expression on microglia cells, preventing them from presenting antigens to CTLs^{198,204}. Elevated levels of Tregs expressing coinhibitory molecules, like CTLA-4, are also found in the TME^{198,205,206}. Of note, glioblastoma cells and GSCs innately downregulate the expression of MHC to reduce their recognition by T cells^{198,207}. Collectively, these layered mechanisms of immune suppression prevent effective anti-tumor immune responses in the glioblastoma microenvironment from occurring.

1.4.5 TREATMENT

Glioblastoma is an incurable disease with limited treatment options. Typically, patients undergo surgical resection, followed by radiotherapy and concomitant and/or adjuvant chemotherapy with the alkylating agent, TMZ²⁰⁸. With this treatment regimen, patients exhibit a mean survival of 14.6 months, whereas untreated patients have a median survival of only 3 months²⁰⁹. In comparison to other cancers, patients with glioblastoma exhibit some of the worst prognoses with a five-year overall survival rate of only 5% post-diagnosis²¹⁰.

In recent years, the recommendations for surgical excision of glioblastoma tumors have improved^{208,211,212}. Surgery can either remove all (gross total resection, GTR) or part of the tumor (subtotal resection, STR). GTR has been shown to significantly improve the survival of glioblastoma patients in comparison to STR^{211,212}. However, complete removal of the tumor is not always possible. Challenges in effective removal include but are not limited to the location, size, and number of tumors present. Additionally, glioblastoma is highly infiltrative, and it is difficult to distinguish between healthy and cancerous tissue. Although healthy tissue can be removed around the tumor, this must be performed in careful consideration of the patient's wellbeing²⁰⁸. In both cases of GTR and STR, recurrence is inevitable.

One recommended chemotherapeutic approach following surgery is the use of TMZ²⁰⁸. Nevertheless, over 50% of patients with glioblastoma who are treated with TMZ fail to respond to therapy²¹³. Despite multimodal treatment and maximal surgical resection, patients exhibit high rates of recurrence (70-80%), most commonly in the local area of the primary tumor^{193,209,214–216}. Patients with recurrent disease can undergo additional surgical resection to alleviate symptoms, but it remains unclear if resection improves overall survival¹⁹³. In some cases, additional treatment with radiation is possible, but this comes with the increased risk of radiation induced necrosis of healthy brain tissue. Altogether, patients with glioblastoma face high rates of therapy resistance and recurrence as well as limited treatment options. Characterization and development of novel therapeutic agents is urgently needed for the disease.

Many attempts have been made to test targeted therapies and immunotherapies for glioblastoma; however, nearly all attempts have been unsuccessful^{190,217–228}. One exception is the targeted therapy, Bevacizumab (Avastin®), which was approved for the treatment of recurrent glioblastoma in 2009^{229,230}. The monoclonal antibody, Bevacizumab, targets vascular endothelial growth factor A (VEGF-A), thereby preventing the binding of VEGF-A with its corresponding receptors, VEGFR1 and VEGFR2^{229–231}. As mentioned in “1.4.4 Pathology”, glioblastoma tumors are highly hypoxic¹⁹⁴. Glioma cells upregulate the expression of VEGF-A and VEGFRs to promote angiogenesis²³². Newly formed blood vessels in turn supply glioma cells with oxygenated blood. Bevacizumab inhibits angiogenesis, and thereby starves glioma cells of oxygen^{229,230}. Notably, Bevacizumab treatment improved progression-free survival of patients with glioblastoma, but it failed to improve overall survival²³¹.

Various EGFR inhibitors have been tested in clinical trials for treatment of glioblastoma, including Erlotinib (Tarceva®), Gefitinib (Iressa®), GC1118, Depatuxizumab-Mafodotin

(Depatux-M), and Cetuximab (Erbix®)^{190,217–226}. Overall, these inhibitors have shown limited efficacy or troubling side effects. Until now, no targeted therapy against EGFR has been approved. Significant challenges facing EGFR based targeted therapy approaches include delivering sufficient doses across the BBB, limiting side-effects, and combatting intrinsic resistance mechanisms¹⁹⁰.

Notably, almost all glioblastomas (88-100%) have been found to express PD-L1 in addition to suppressive microglia and GAMs in the TME^{198,233}. This would make glioblastoma a seemingly an ideal candidate to respond to immunotherapy. Immunotherapy, as its name suggests, refers to a class of therapies that act on the immune system. In cancer, immunotherapies are used to stimulate cells involved in generating an anti-tumor immune response, most notably T cells^{101,234,235}. Immune checkpoint inhibitors (ICIs) are a form of immunotherapy that help sustain the activation of T cells.

The process of T cell activation is tightly regulated to maintain immune homeostasis¹¹. Upon activation, T cells express inhibitory molecules, known as immune checkpoints, on their surfaces^{11,235}. The best characterized checkpoints are CTLA-4 and programmed death-1 (PD-1). CTLA-4 binds to CD80 and CD86 ligands expressed on the surface on APCs with a higher affinity than CD28, preventing costimulation and thus activation of T cells (**Fig. 7**). Like, CTLA-4, expression of PD-1 is also increased on the surface of activated T cells, but PD-1 binds to different B7 ligands, namely PD-L1 and programmed death ligand-2 (PD-L2) (**Fig. 7**). PD-L1 is widely expressed by different cell types in the body to prevent autoimmunity, whereas PD-L2 mainly limited to APCs²³⁵. Binding of PD-1 to its respective ligands, reduces TCR signaling and in turn inhibit T cell activation^{11,235}.

Figure 7: Immune checkpoint inhibitors (ICIs)

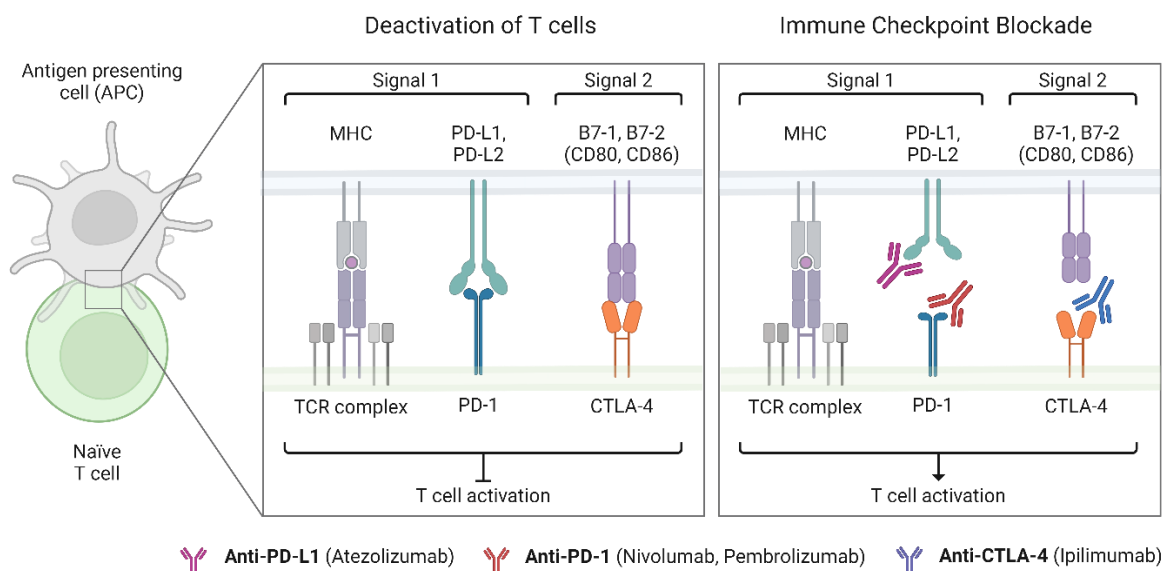


Fig. 7: Schematic representation of T cell deactivation and immune checkpoint blockade. Common immune checkpoint inhibitors (ICIs) used in the clinic and their mechanism of

action is portrayed. Adapted from “T-cell Deactivation vs. Activation”, by BioRender.com (2024)¹⁵.

There are three main classes of ICIs used in cancer treatment, namely anti-CTLA-4, anti-PD-1, and anti-PD-L1 inhibitors (**Fig. 7**)^{234,236}. These inhibitors refer to humanized monoclonal antibodies that block the interaction of immune checkpoint molecules with their respective ligands (**Fig. 7**). This “releases the brakes” on T cell activation, boosting activation, proliferation, and infiltration of Teff into the TME, where they can recognize and destroy cancer cells.

Due to the high expression of PD-L1 on glioblastoma cells as well as on suppressive microglia and GAMs, it could be expected that patients with glioblastoma may respond well to anti-PD-1 inhibitors, like Nivolumab (Opdivo®) and Pembrolizumab (Keytruda®)^{198,233,234}. In a phase III clinical study, Nivolumab was tested on patients with recurrent glioblastoma, but the treatment yielded no improvement in overall survival²²⁷. However, it was observed that a fraction of the patients who did respond to Nivolumab exhibited a more durable response. In a different phase III clinical study, Nivolumab was also tested in combination with TMZ and radiation in newly diagnosed methylated *MGMT* promotor positive patients, but it failed to improve survival^{198,228}. This failure to respond to ICIs highlights the profoundly suppressive glioblastoma microenvironment as detailed in “1.4.4 Pathology”.

1.4.6 GLIOBLASTOMA STEM-LIKE CELLS (GSCs)

In addition to overcoming suppression in the TME, targeting GSCs is a highly promising therapeutic approach.

GSCs form only a small subset of tumor cells and as their name suggests, share properties of healthy neural stem cells^{237,238}. GSCs were only recently discovered in the early 2000's^{237–240}. Therefore, it is still not completely understood on how they may originate; it is speculated from either malignant neural stem cells or from de-differentiated glioblastoma cells. However, it is agreed upon that GSCs share several common characteristics (**Fig. 8**). Firstly, GSCs are described to exist in a slow growing quiescent state, and they can undergo unlimited self-renewal (**Fig. 8**)^{238,241}. Therefore, they are attributed to sustaining the tumor mass by slowly giving rise to more differentiated, rapidly proliferating, daughter cells. These daughter cells constitute a much larger portion of the tumor and are responsible for rapid tumor growth. The ability of GSCs to differentiate and thereby produce different lineages of daughter cells is responsible in part for the remarkable heterogeneity seen in glioblastoma tumors. Of note, differentiation of GSCs is not linear – rather they can interconvert between differentiated non-GSCs and undifferentiated GSCs (**Fig. 8**)²⁴². This high plasticity is due to epigenetic reprogramming and is triggered by various environmental stimuli, such as hypoxia, nutrient deprivation, and radiation^{242–244}. One-way GSCs are distinguished from differentiated glioblastoma cells is their ability to initiate tumors (**Fig. 8**)^{239,245}. In xenograft experiments, involving the implantation of human glioblastoma cells into the brains of immunodeficient mice, only GSCs were found to be capable of forming heterogeneous tumors^{239,245,246}. These collective characteristics distinguish GSCs from their differentiated glioblastoma cell

counterparts and explain in part the profound heterogeneity observed in the glioblastoma TME.

Figure 8: Traits of glioblastoma stem-like cells (GSCs)

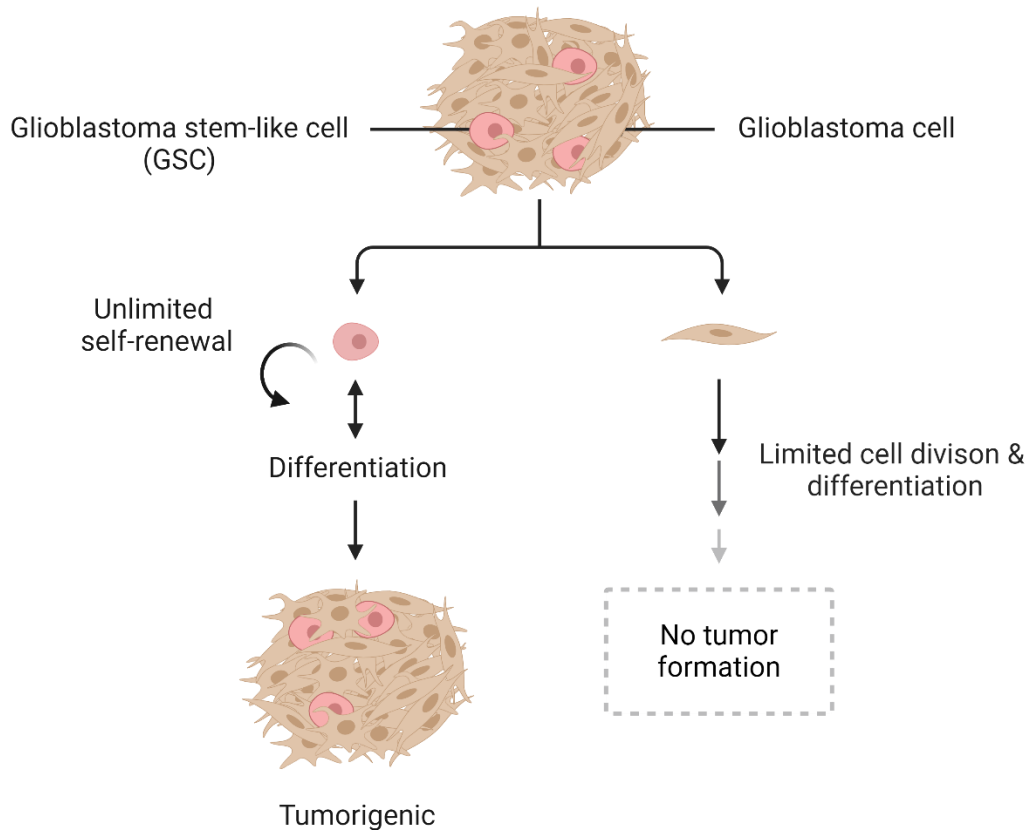


Fig. 8: Key differences between glioblastoma stem-like cells (GSCs) and glioblastoma cells.

Created with BioRender.com (2024)¹⁵.

GSCs pose a major challenge in treating glioblastoma. Due to their quiescent state, GSCs are innately more resistant to cytotoxic radiotherapy and chemotherapy, which target rapidly dividing cells^{238,247–249}. High intratumoral heterogeneity and plasticity, resulting from GSCs, further complicate treatment efficacy^{241,245}. GSCs can both initiate and propagate tumors following cytotoxic treatment, thereby driving recurrence^{239,245,246,248}. It has also been reported that GSCs promote invasion and angiogenesis in the TME^{245,250–252}. The highly invasive nature of glioblastoma limits the efficacy of surgical resection, highlighting yet another way GSCs can reduce therapeutic efficacy and thereby promote recurrence^{253,254}. Altogether, GSCs limit the efficacy of current therapies and drive recurrence – two major factors responsible for poor prognosis²⁴⁵. As a result, they are considered one of most compelling cellular targets in glioblastoma.

Despite this, there are currently no approved therapies against GSCs. This is because the field of GSC research faces several significant challenges. Firstly, GSCs are a rare cell type and are distributed unequally throughout the TME; this impairs the assessment of GSCs in tumor samples^{255,256}. In more detail, enrichment of GSCs is found in areas of the TME where GSCs have direct contact with capillaries and arterioles (perivascular niche), the invasive edges of

the tumor (invasive niche), and the hypoxic necrotic core (perinecrotic niche)²⁵⁷. Low sample availability coupled with the innately slow growth rate and complicated culture conditions of GSCs further lag research. Unlike many other cell types, there are no universal markers to identify GSCs. Instead, they only can be identified by functional traits, namely unlimited self-renewal as well as the abilities to differentiate and initiate heterogeneous tumors in immunodeficient mice²⁴⁵. In the past, many markers have been associated with GSCs, including but not limited to Prominin1 (CD133), SOX2, nestin, L1CAM, CD15, and CD44^{239,246,258–262}. However, due to the high plasticity and heterogeneity of GSCs, these markers all fail to universally identify them. For example, the best characterized GSC associated marker, CD133, is limited to a subset of GSCs, and expression of CD133 was found to be dependent on the cell cycle^{249,263–266}. Therefore, there is an urgent need to discover novel GSCs markers, which are universally expressed regardless of GSC subset or cell state.

Zimmer et al., 2019 reported for the first time that GARP is highly expressed by GSCs⁶⁵. In more detail, they found that GARP was highly expressed across several patient derived GSC cell lines. This work expanded on their study by evaluating GARP as a potential novel marker for GSCs and investigated its possible functional relevance in GSC biology for the first time (**Paper 2**)⁹.

1.5 MELANOMA

Melanoma, also known as cutaneous melanoma, is a form of skin cancer that arises from melanocytes, which reside in the basal layer of epidermis^{267,268}. Melanocytes produce the pigments eumelanin and pheomelanin. The intermix of these pigments is responsible for the coloration of skin, eyes, and hair. Melanin serves as a physical barrier against UV radiation from the sun. In more detail, melanin can both scatter and absorb UV radiation to prevent it from penetrating the epidermis, where it can induce DNA damage^{269,270}. Melanin can also scavenge, or neutralize, harmful reactive oxygen species (ROS) that can damage vital cellular structures, like DNA^{269,271}. As discussed in “1.3.1 Initiation of cancer”, accumulation of DNA damage and mutations overtime can lead to the malignant transformation of healthy cells^{108,109}.

1.5.1 EPIDEMIOLOGY

Around 20% of all skin cancer diagnoses each year are attributed to melanoma²⁷². In 2022, 331,722 cases of melanoma were diagnosed and 58,667 deaths were reported worldwide^{106,273}. The global incidence of melanoma varies greatly by geographical region and by economic status. Europe and Northern America (United States and Canada) alone reported 44.1% and 34.0% of all melanoma cases in 2022 (both sexes), whereas Africa only reported 2.3% of all cases (both sexes). High income nations (World Bank Classification) reported the majority of melanoma diagnoses, accounting for 261,662 cases in 2022 (both sexes). Collectively, this indicates that the highest incidence of melanoma cases is reported in predominantly White developed nations.

In the last decades, developed nations have implemented robust preventative skin cancer screening programs, enabling the earlier detection of melanoma^{272,274,275}. As a result, a greater number of cases were identified, thereby inflating the incidence of the disease.

The incidence of melanoma is also known to vary by sex, but the reason why remains unclear. It is well documented that older men exhibit a higher incidence of the disease than women and correspondingly exhibit worse clinical outcomes, in terms of progression, recurrence and mortality^{174,276–278}. However, this effect reverses with age with younger women, especially between the ages of 20-24, having higher incidence rates than men^{276,277}. These differences between sexes are not believed to solely originate from differing UV exposure but also in part from behavioral practices, such as tanning bed use, application of sun protectant, and self-screening for skin cancer^{277,279–281}.

The incidence of melanoma has rapidly increased from 1975 to 2018 in developed countries, rising over 320% in the US alone^{273,282}. The global incidence of melanoma (both sexes) is predicted to continue to increase by 2050^{106,283}. In addition to a rise in skin cancer screenings, this projected increase can be attributed to several factors^{106,272,274,275,283}. Firstly, the global population is predicted to continue to grow from 7.6 billion to 9.8 billion in 2050^{106,283}. Collectively, this is predicted to increase the absolute number of reported melanoma cases. The global population is also aging, a known risk factor for developing melanoma, with the percentage of individuals aged 65 and above increasing from 10% in 2022 to 16% in 2050^{284,285}. Climate change is also predicted to increase the incidence of skin cancers, due to an accumulation of several factors^{286,287}. This includes increased exposure to air pollution, depletion of the ozone layer that provides protection from UV radiation, and behavioral changes, resulting in increased sun exposure due to increased temperatures.

Despite the increasing incidence of melanoma, the overall mortality of the disease decreased by 17.9% in White Americans from 2013 to 2016^{282,288}. This dramatic improvement resulted from the approval of several targeted therapies and immunotherapies for the treatment of metastatic and inoperable melanoma²⁸⁸.

1.5.2 RISK FACTORS

Exposure to UV radiation, most often in the form of sunlight, is the biggest risk factor for developing melanoma. It is well known that high energy UV radiation is genotoxic, causing DNA damage by directly inducing structural damage and indirectly through the production of ROS¹²⁰. This leads in turn to the accumulation of somatic mutations. Excessive UV exposure that damages the skin results in sunburns, which are also a risk factor for developing melanoma²⁸⁹. Behaviors connected to UV exposure can correspondingly exacerbate (e.g., tanning bed use) or reduce (e.g., sun protectant use, avoiding sun exposure between 10 am and 4 pm) the risk of developing melanoma^{282,289}. As mentioned earlier, advanced age is also a risk factor²⁸⁵. This is due in part to the long-term exposure of the skin to mutagenic UV radiation and the gradual worsening of immune function over time.

The coloration of skin and the risk of developing melanoma are tightly connected. Individuals with darker skin tones have a reduced risk of developing melanoma^{290,291}. This is because they have a higher concentration of eumelanin in their skin. In contrast, individuals with lighter skin tones have more pheomelanin. Eumelanin is more effective at scattering UV radiation before it is absorbed into the skin, providing superior protection against DNA damage²⁸². Although individuals with darker skin tones have a lower risk of developing melanoma, melanoma can be harder to identify, which can result in later diagnosis of disease²⁹¹.

The presence and number of nevi, also known as moles, on the skin are risk factors for developing melanoma^{282,292}. Nevi are benign outgrowths of melanocytes. Although rare, nevi can undergo malignant transformation and serve as initiation sites for melanoma.

Genetic predispositions to developing melanoma are only partially understood. A family history of the disease is reported in about 10% of melanoma patients²⁸². Some of the most commonly mutated genes in familial melanoma include *MC1R* (70-90%), *CDKN2A* (20-40%), *MITF* (1-5%), and *CDK4* (<1%)²⁹³. *MC1R* and *MITF* are involved in the production of melanin and the development of melanocytes. *CDKN2A* is the most commonly associated mutation in risk of developing familial melanoma^{293,294}. This gene encodes two tumor suppressor proteins, p16 and p14, which arrest cell cycle and promote apoptosis. On the flipside, mutated *CDK4* is an oncogene that supports cell cycle progression²⁹³. Other genes found to be frequently mutated are involved in the maintenance of telomeres, like *POT1*, *ACD*, *TERF2IP*, and *TERT*, enabling enhanced proliferation of cancer cells^{293,295}. However, not all cases of familial melanoma can be explained by these mutations²⁹³.

1.5.3 DIAGNOSIS

Individuals with increased risk of developing melanoma are advised to perform monthly self-examinations and to undergo regular skin cancer screenings at their dermatologist to recognize early on new or abnormal nevi²⁹⁶. Nevi are photographed to track how they change over time. Diagnosis of melanoma typically begins with the examination of an abnormal nevi by a dermatologist using a dermatoscope²⁹⁷. The visual guidelines for distinguishing between benign and potentially cancerous nevi follow the ABCDE rule: **A**symmetry, **B**order, **C**olor, **D**iameter, and **E**levation of the nevi^{297,298}. These findings are validated by biopsy, where frequently the entire abnormal nevi and surrounding skin are removed. Biopsies undergo histological evaluation to identify the subtype of melanoma present as well as the molecular biomarkers present. The most common subtypes are superficial spreading melanoma (~70%), nodular melanoma (~20%), lentigo maligna melanoma (~5-10%), and acral lentiginous melanoma (1-2% in western countries)²⁹⁹⁻³⁰⁴.

Diagnostic staging is performed in accordance with the Eighth Edition of the American Joint Committee on Cancer Melanoma Staging^{297,305}. This system uses the TMN (**T**umor, **N**ode, and **M**etastasis) classification, which categorizes tumors based off their size and degree of spreading. In more detail, **T** indicates the tumor thickness and presence of ulceration²⁹⁷. **N** details whether local or regional metastases are present, whereas **M** indicates the presence

of distant metastases. The TMN classification, combined with information obtained from biopsies, form the foundation for the overall prognostic staging of the disease.

Stages with lower numbers (0-II) represent less advanced forms of melanoma and have better prognoses than later stages of the disease (III and IV)^{297,298,305,306}. In stages 0-II, there are no signs of spreading. These stages differ from one and other mainly by the thickness of the primary tumor and whether ulceration is present. Stage III is distinguished by the presence of cancer cells in nearby tissues, including lymph nodes, lymphatic vessels, and surrounding skin. Stage IV denotes that the cancer has spread to distant parts of the body, most commonly the skin and subcutaneous tissue, followed by the lungs, liver, bones and brain³⁰⁷.

1.5.4 PATHOLOGY

Melanoma presents as a highly heterogeneous disease. This heterogeneity can be attributed in part to its high genetic instability and exceptionally high TMB³⁰⁸. It is estimated that melanoma has up to 15 mutations per mega base, making it one of the cancers with the highest TMBs. This is connected to the classification of melanoma as an immunogenic cancer type, meaning that the immune system can induce immune responses against it^{309,310}. The elevated TMB leads to the increased prevalence of neoantigens that can be recognized as foreign by the immune system. Melanoma is often called a “hot” tumor, which describes the immunological status of the TME. The melanoma microenvironment is characterized by a high degree of tumor infiltrating lymphocytes (TILs) and expression of PD-L1 by immune cells.

Melanoma stands out from other cancer types due to its highly invasive nature, which can be explained in part by its developmental origins³¹¹. Melanocytes originate from the neural crest, a transient, highly mobile, and multipotent group of cells, arising from the ectoderm³¹². Neural crest cells migrate through the developing embryo to different tissues, where they differentiate into melanocytes, glial cells, peripheral neurons, smooth muscle cells, amongst other things. Melanoma cells express many of the same signaling molecules and factors of neural crest cells due to their shared evolutionary origin³¹¹. These traits help account for their innate plasticity and high motility, enabling them to invade neighboring tissues and to metastasize to distant organs.

1.5.5 TREATMENT

The gold standard for the treatment of melanoma is resection of the affected area(s)³¹³. This is very effective in the early stages of the disease when the tumor remains limited to the site of origin, with five-year survival rates of nearly 100% (Stages 0-II)³⁰⁶. However, when the disease becomes malignant, spreading into surrounding (Stage III) or distant (Stage IV) tissues, the efficacy of surgical removal decreases (five-year survival rates: stage III 60.3%; stage IV 16.2%).

There are several treatment options for malignant melanoma in addition to surgical resection. In some cases, radiation and chemotherapy are applied, but these therapies do not work as well in melanoma in comparison to other cancer types³¹⁴. Instead, advanced stage patients are treated with targeted therapies and ICIs.

Genetic classification of a patient's melanoma, through biopsies, is essential for deciding on a fitting targeted therapy approach. Cutaneous melanoma is categorized into four genetic subtypes based off the mutational profile, namely *BRAF* mutant (45-50%), *RAS* mutant (30%), *NF1* mutant (10-15%), and triple wildtype (5-10%)³¹⁵. Triple wildtype represents melanomas that contain no mutations in *BRAF*, *RAS*, and *NF1*³¹⁶.

Vemurafenib (Zelboraf®) was the first targeted therapy approved for the treatment of metastatic and inoperable melanoma in 2011, followed quickly by Dabrafenib (Tafinlar®) in 2013^{317,318}. Both therapies target a molecular defect in melanoma, a mutation in a kinase called BRAF, inhibiting its function, hence their colloquial name "BRAF inhibitors". Specifically, Vemurafenib and Dabrafenib target a point mutation in codon 600 of the protein, called the BRAFV600E mutation, which results in the substitution of valine for glutamic acid. Mutations in BRAF are very common in melanoma and are considered a driver of the disease^{319,320}. The most common mutation in BRAF is BRAFV600E, prevalent in 70-90% of *BRAF* mutations. Mutated BRAF is an oncogene that increases cell proliferation via the constitutive activation of the MAPK/ERK (extracellular signal-regulated kinases) (Ras-Raf-MEK-ERK) pathway. Treatment with BRAF inhibitors has been shown to effectively induce apoptosis in tumor cells, increase expression of melanoma antigens, and the number of infiltrating CTLs, favorable for anti-tumor immune responses³²¹.

The approval of Vemurafenib was highly significant as it represented one of the first improved treatment options for metastatic melanoma in decades. In comparison to the standard of care at the time, Dacarbazine, a chemotherapy approved in 1975, Vemurafenib demonstrated superior 6-month overall survival (84% vs. 64%) and response rates (48% vs. 5%)³²². About half of *BRAF* mutant melanomas respond to BRAF inhibitors; however, most patients rapidly develop resistance against the therapy after 6 months, reducing its efficacy³²³.

Currently, work on targeted therapies in melanoma is centered on testing novel combination therapy approaches as well as other drug targets, in hopes of prolonging therapeutic efficacy. To overcome resistance, BRAF inhibitors are combined with MEK inhibitors to further disable the MAPK/ERK pathway³²⁴. Combination treatment of Dabrafenib and Trametinib (Mekinist®), a MEK inhibitor, improved the overall survival of patients with metastatic melanoma in comparison to patients treated with Vemurafenib alone (72% combination vs. 65% Vemurafenib alone)³²⁵. *RAS* and its negative regulator *NF1* also form parts of the MAPK/ERK pathway, but there are no approved targeted therapies against them. Instead, advanced staged patients lacking mutations in *BRAF* undergo immunotherapy.

In 1998, high-dose systemic IL-2 treatment became the first immunotherapy to be approved to treat advanced stage melanoma patients³²⁶⁻³²⁹. As discussed in "1.1.2 T cell activation", IL-2 is required for clonal expansion of T cells, including Th1 cells and CTL, which are hallmarks of effective anti-tumor immune responses^{11,13,19,22}. Although a small fraction of patients responded to IL-2 therapy (objective response rate of 15.9%), about 1 in 3 responders exhibited durable remission, marking an important improvement in the treatment of advanced stage melanoma. A major drawback from this treatment was high toxicity, most

commonly capillary leak syndrome, which could involve multiple organ systems³²⁷. Patients required extensive observation while undergoing treatment, frequently inside an intensive care unit. Due to the low response rate and high toxicity of IL-2, other forms of immunotherapy, most notably ICIs, were later developed.

There are three main classes of ICIs approved for the treatment of melanoma: anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies (**Fig. 7**)²³⁴. The first ICI, Ipilimumab (Yervoy®), a CTLA-4 inhibitor, was approved in 2011 for late-stage melanoma³³⁰. In the groundbreaking phase III clinical study, Ipilimumab treated patients showed a drastic improvement in overall survival, with 20% of patients demonstrating an extended duration response³. In addition to boosting the activity of T cells, anti-CTLA-4 inhibitors deplete suppressive Tregs, which express a high level of CTLA-4 on their surfaces, through antibody-dependent cellular cytotoxicity (ADCC)^{11,235}. A major drawback of Ipilimumab is that it is frequently accompanied by autoimmune side effects, also known as immune related adverse events (irAE). Depending on the severity, this can lead to the secession of ICI treatment. This is thought to arise from CTLA-4's broad scale effects on activating the T cell compartment, including autoreactive T cells.

Approval of the PD-1 inhibitors, Nivolumab and Pembrolizumab, for the treatment of advanced melanoma quickly followed in 2014 (**Fig. 7**)^{4,5}. The overall response rate for both inhibitors was threefold higher than Ipilimumab (~30% in comparison to ~10%), and the inhibitors exhibited an improved safety profile^{7,331,332}. PD-1 blockade primarily results in the functional restoration and expansion of tumor infiltrating CTLs that can directly kill tumor cells^{333,334}. In 2020, Atezolizumab (Tecentriq®), a PD-L1 inhibitor, in combination with the BRAF inhibitors, Cobimetinib (Cotellic®) and Vemurafenib, was approved for the treatment of BRAFV600E positive metastatic or inoperable melanoma, prolonging median progression free survival (10.6 months Cobimetinib and Vemurafenib only; 15.1 months with Atezolizumab) (**Fig. 7**)³³⁵.

In 2016, Ipilimumab – Nivolumab combination therapy was approved for the treatment of unresectable and metastatic melanoma, regardless of *BRAF* status⁶. Combination therapy showed the highest median overall survival (+60 months, median not reached) in comparison to the individual treatments alone (Ipilimumab, 19.9 months; Nivolumab, 36.9 months)⁷. As a result, Ipilimumab – Nivolumab combination therapy are considered as the standard of care for inoperable stage III and IV melanoma patients in Europe. Like targeted therapy, patients can also develop resistance to ICIs during treatment. Loss of functional T cells and poor infiltration of T cells into the TME are amongst the factors responsible³³⁶.

Approval of CTLA-4 and PD-1 inhibitors revolutionized the treatment of melanoma. The five-year overall survival rate of advanced melanoma patients has risen dramatically from 5% to ~50% when patients are treated with Ipilimumab – Nivolumab combination therapy^{7,314,337–339}. So great was the improvement in clinical outcomes that Dr. James P. Allison and Dr. Tasuku Honjo were awarded the Nobel Prize in Physiology or Medicine in 2018 for their pivotal work in discovering the immune checkpoints, CTLA-4 and PD-1³⁴⁰.

Despite the monumental advancements made in the treatment of melanoma in the last decade, the field still faces several significant challenges. Only half of patients effectively respond to BRAF inhibitors and ICIs^{7,314,322,337–339}. Acquired resistance to these treatments reduces their efficacy^{323,336}. Currently, researchers are exploring how ICIs and targeted therapies can be combined to prolong patient survival and to avoid the development of resistance. Researchers are looking for biomarkers to optimize individualized patient treatment regimens. This is in the hopes of identifying responders and individuals at high-risk for developing severe side-effects as well as reducing therapy costs. Furthermore, novel targets and therapeutic strategies are still needed for advanced patients that fail to respond to conventional targeted therapies and ICIs.

To address this unmet need, this work sought out to characterize cold atmospheric plasma (CAP) as a promising, adjuvant, anticancer approach that could be applied in combination with existing conventional therapies (**Paper 3**)¹⁰.

2. AIMS

Optimization of immune responses has become increasingly recognized as a promising strategy to treat a wide range of clinical conditions^{1–7}. Therefore, it is important to investigate novel forms of immunomodulation, which could be applied to generate favorable immune responses. This dissertation characterized two immunomodulatory approaches, namely GARP and CAP. This work aimed to characterize the functional role of GARP in two distinct immunological settings: wound healing (**Paper 1**) and cancer (**Paper 2**)^{8,9}. In addition, the influence of CAP on cells in the melanoma microenvironment was also investigated (**Paper 3**)¹⁰.

Paper 1

GARP is a potent mechanism of immunological tolerance^{28,61,83}. Through its function in regulating and activating the pleiotropic cytokine, TGF- β , GARP is key to preventing the development of autoimmunity^{28,29,50,66,67,69,70,81,82}. As GARP controls the bioavailability of TGF- β , it is speculated that GARP plays a vital role in TGF- β mediated pathways. This has already been supported in the immunological settings of development, tolerance, and cancer^{61,67,71,83,99,100}. However, up until now, no studies have examined the possible involvement of GARP in early stages of the wound healing phase. This study investigated how GARP may influence the wound healing process by utilizing an endogenous source of GARP, injectable platelet rich fibrin (iPRF), a type of autologous platelet concentrate that is used to treat chronic wounds⁸. In more detail, this study aimed to analyze the influence of GARP, contained in iPRF, on immune cells (T cells and monocyte derived macrophages) that play key roles in early stages of the wound healing phase.

Paper 2

GSCs are a major cause of tumor initiation, therapy resistance, and recurrence in glioblastoma^{239,245,247–249}. Therefore, they are one of the top cellular targets to therapeutically address. However, there are no universal markers for GSCs due to their innately high heterogeneity and plasticity⁹. Recently, Zimmer et al., 2019 discovered that GSCs express high levels of GARP, especially in the nucleus⁶⁵. Widespread expression of GARP was present on all GSC cell lines tested, suggesting a possible utility of the protein in identifying GSCs. This study aimed to analyze the expression of GARP across different GSC cell lines that replicated key features of the disease (e.g., intratumoral heterogeneity, cellular hierarchy, and longitudinal GSC evolution)⁹. This study also investigated the potential functional relevance of GARP in GSC biology and the application of GARP as a biomarker for glioblastoma.

Paper 3

Despite recent therapeutic advancements, patients with advanced stage melanoma still face limited treatment options^{3–5,7,314,317,318,322,323,336–339}. CAP, non-thermal plasma that can be applied within physiological temperatures (<40°C), has emerged as a promising selective anti-cancer strategy^{341–343}. It is well known that CAP is toxic to cancer cells by raising exogenous RONS levels³⁴¹. However, until now, little is known regarding how immune cells react to CAP. Immune cell composition in the melanoma microenvironment is key to ICI therapy response

and can predict patient prognosis¹⁰¹. Therefore, this study aimed to examine the effects of CAP treatment on cancer cells and immune cells (T cells and monocyte derived macrophages) commonly found in the melanoma microenvironment¹⁰. In more detail, this study analyzed CAP treated cells for signs of oxidative stress and sought out to identify optimal CAP treatment strategies.

3. RESULTS

3.1 PAPER 1

GARP Regulates the Immune Capacity of a Human Autologous Platelet Concentrate

Figure 9: Graphical abstract (Paper 1)

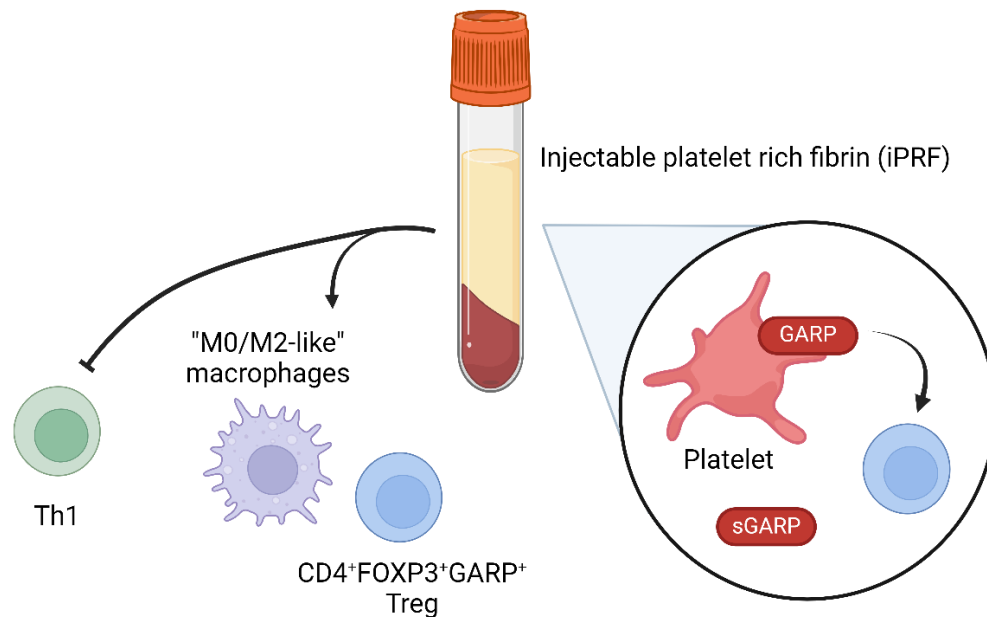


Fig. 9: Graphical abstract of **Paper 1**. Created with BioRender.com (2024)¹⁵.

3.1.1 SUMMARY

Autologous platelet concentrates represent a class of blood preparations produced from a patient's own blood^{8,344}. Autologous platelet concentrates are produced chairside via a specific centrifugation process, resulting in the separation of plasma from whole blood. This blood plasma can be applied to a patient either as a solid clot or as a liquid solution, known as injectable platelet rich fibrin (iPRF), to accelerate the healing of chronic wounds. iPRF represents an optimized form of autologous platelet concentrates, which contains an enrichment of platelets, growth factors, and white blood cells^{8,345}. Although widely used in regenerative medicine, the underlying immunological mechanisms behind the wound healing properties of iPRF remain poorly understood^{8,344,346,347}.

Platelets are the main cellular component of iPRF and highly express GARP on their surfaces^{8,56}. GARP exhibits potent immunomodulatory properties through its function in aiding in the activation of the cytokine, LTGF- β ^{28,29,50,56,61,66,67,69,83}. However, it remains unclear if GARP plays a role in the immune capacity of iPRF. Therefore, this study aimed to analyze the effects of iPRF on key cells involved in the wound healing phase, specifically human monocyte derived macrophages and CD4⁺ T cells, and the influence of GARP in these interactions⁸.

It was found that GARP is expressed on the surface of platelets isolated from iPRF, and the protein was detected as a soluble factor in iPRF⁸. Both iPRF and platelets isolated from iPRF were found to induce Foxp3⁺GARP⁺ Tregs and to inhibit the production of Th1 effector cytokines, IL-2 and IFN- γ . Treatment with an anti-GARP antibody reversed these effects. iPRF was also found to induce an anti-inflammatory "M0/M2-like" phenotype in macrophages in a GARP independent manner. Collectively, this study identified a novel immunological mechanism, namely GARP dependent induction of Tregs, which mediates the immune capacity of iPRF.

3.1.2 ZUSAMMENFASSUNG

Autologe Thrombozytenkonzentrate sind eine Klasse von Blutpräparaten, die aus dem Eigenblut des Patienten hergestellt werden^{8,344}. Autologe Thrombozytenkonzentrate werden vor Ort (am Behandlungsstuhl) durch ein spezielles Zentrifugationsverfahren hergestellt, bei dem das Blutplasma vom Vollblut getrennt wird. Dieses Blutplasma kann dem Patienten entweder als festes Zellaggregat oder als flüssige Lösung, sogenanntes injizierbares plättchenreiches Fibrin (iPRF), verabreicht werden, um die Heilung chronischer Wunden zu beschleunigen. iPRF stellt eine optimierte Form des autologen Thrombozytenkonzentrates dar, welches mit Thrombozyten, Wachstumsfaktoren und Leukozyten angereichert ist^{8,345}. Obwohl iPRF in der regenerativen Medizin weit verbreitet ist, sind die immunologischen Mechanismen, die der wundheilenden Wirkung von iPRF zugrunde liegen, noch wenig verstanden^{8,344,346,347}.

Thrombozyten sind die zelluläre Hauptkomponente von iPRF und exprimieren auf ihrer Oberfläche in hohem Maße GARP^{8,56}. GARP hat starke immunmodulatorische Eigenschaften, da es zur Aktivierung des Zytokins LTGF- β beiträgt^{28,29,50,56,61,66,67,69,83}. Es ist jedoch unklar, ob GARP eine Rolle bei der Immunkapazität von iPRF spielt. Ziel dieser Studie war es daher, die Wirkung von iPRF auf Zellen in den frühen Stadien der Wundheilung zu untersuchen, insbesondere auf aus humanen Monozyten gebildete Makrophagen und CD4⁺ T-Zellen. Außerdem wurde der Einfluss von GARP auf diese Wechselwirkung untersucht⁸.

Es zeigte sich, dass GARP auf der Oberfläche von Thrombozyten exprimiert wird aber auch als löslicher Faktor in iPRF vorhanden ist⁸. Sowohl iPRF als auch aus iPRF isolierte Thrombozyten induzieren Foxp3⁺GARP⁺ Tregs und hemmen die Produktion von Th1-Effektorzytokinen, IL-2 und IFN- γ . Diese Effekte konnten durch die Behandlung mit einem Anti-GARP-Antikörper umgekehrt werden. Unabhängig von GARP induziert iPRF auch einen anti-inflammatorischen „M0/M2-ähnlichen“ Phänotyp in Makrophagen. Somit konnte in dieser Studie ein neuer immunologischer Mechanismus identifiziert werden: die GARP-abhängige Induktion von Tregs, welche die Immunkapazität von iPRF vermittelt.

3.1.3 AUTHOR CONTRIBUTIONS

Disclaimer: First authorship was shared between E.R. Trzeciak and N. Zimmer. Both authors contributed equally to the manuscript. N. Zimmer produced the data and generated Figure 1 (A-B) and Figures 2-3. E.R. Trzeciak produced the data and generated Figure 1 (C), Figure 4, and Figure S1. E.R.T predominantly wrote the original draft of the manuscript as well as handled the reviewing and editing process.

Conceptualization	P.W. Kämmerer, N. Zimmer, S. Blatt, A. Tüttenberg
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Formal analysis	D. Thiem, N. Zimmer, E.R. Trzeciak
Investigation	N. Zimmer, E.R. Trzeciak
Resources	P.W. Kämmerer, B. Al-Nawas, A. Tüttenberg, S. Blatt
Data Curation	D. Thiem, N. Zimmer, E.R. Trzeciak , S. Blatt
Writing (Original draft preparation)	E.R. Trzeciak , N. Zimmer, S. Blatt, P.W. Kämmerer, A. Tüttenberg
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Project administration	P.W. Kämmerer, B. Al-Nawas, N. Zimmer, S. Blatt
Funding acquisition	B. Al-Nawas, A. Tüttenberg, S. Blatt

3.1.4 PUBLICATION

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biomedicines



Article

GARP Regulates the Immune Capacity of a Human Autologous Platelet Concentrate

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Abstract: Autologous platelet concentrates, like liquid platelet rich fibrin (iPRF), optimize wound healing; however, the underlying immunological mechanisms are poorly understood. Platelets, the main cellular component of iPRF, highly express the protein, Glycoprotein A repetitions predominant (GARP), on their surfaces. GARP plays a crucial role in maintaining peripheral tolerance, but its influence on the immune capacity of iPRF remains unclear. This study analyzed the interaction of iPRF with immune cells implicated in the wound healing process (human monocyte derived macrophages and CD4⁺ T cells) and evaluated the distinct influence of GARP on these mechanisms in vitro. GARP was determined to be expressed on the surface of platelets and to exist as a soluble factor in iPRF. Platelets derived from iPRF and iPRF itself induced a regulatory phenotype in CD4⁺ T cells, shown by increased expression of Foxp3 and GARP as well as decreased production of IL-2 and IFN- γ . Application of an anti-GARP antibody reversed these effects. Additionally, iPRF polarized macrophages to a “M0/M2-like” phenotype in a GARP independent manner. Altogether, this study demonstrated for the first time that the immune capacity of iPRF is mediated in part by GARP and its ability to induce regulatory CD4⁺ T cells.

Keywords: iPRF; liquid platelet rich fibrin; autologous platelet concentrate; wound healing; GARP; TGF- β ; platelets; T cells; regulatory T cells; macrophages

1. Introduction

Autologous platelet concentrates (APC) improve wound healing and tissue regeneration. APC are defined as the volume fraction of plasma in autologous whole blood that has a (up to five times) higher thrombocyte concentration than regular blood samples and contains platelet related growth factors, which promote wound healing and tissue repair [1]. APC are widely used in regenerative surgery. Clinical indications include but are not limited to orthopedic procedures as well as dentistry and oral surgery [2,3]. Important representatives of the first generation of APC are platelet rich plasma (PRP), preparation rich in growth factor (PRGF), or pure-PRP (P-PRP), that are still widely used in dental and medical reconstructive procedures [4,5]. They are produced chair side via a specific centrifugation process. After venous blood collection with respective vacutainers and tube systems, an anticoagulant is added before and/or after centrifugation. In this way, thrombocytes can be extracted within a fibrin clot and used for different medical applications [6–9].

The main disadvantages of this preparation are the usage of anticoagulants that counteract the autologous concept and the exclusion of leukocytes and other cells important

for wound healing in the (often multi-step) centrifugation process, which may lead to unfavorable outcomes [10]. To overcome these limitations, second generation APC, such as platelet rich fibrin (PRF), were developed [11]. Here, only one centrifugation step is needed without the addition of an anticoagulant [12]. Depending on the protocol, either a stable clot or a liquid fibrin solution (iPRF) can be produced, that contains potentially higher thrombocyte concentrations and growth factor levels in vitro in comparison to first generation APC [13]. Entangled components of the resulting fibrin net of PRF include different cells, like thrombocytes and leukocytes, and growth factors, which are slowly released over time that can interact with other cell types, initiate angiogenesis, and are implicated in other processes involved in the wound healing phase [11,13,14].

Although APC are recognized for their wound healing properties and widely used in regenerative medicine, little is known about the immune capacity of APC and the possibly involved cellular mechanisms [15–17]. Initial studies have provided striking data that APC directly influences host immune responses via macrophage polarization [16,18]. Recent evidence has also arisen that platelets, the principle cellular component of PRF, play essential roles in innate and adaptive immunity via their interaction with different immune cells [19].

Glycoprotein A repetitions predominant (GARP) is a surface docking receptor and activator of latent transforming growth factor beta 1 (LTGF- β 1) that is enriched on the surface of platelets [20]. Released primarily from platelets upon tissue injury, TGF- β 1 plays an essential role in the wound healing process, mediating inflammation, angiogenesis, and connective tissue regeneration, amongst other things [21–23]. GARP has been shown to exhibit anti-inflammatory properties itself through both TGF- β 1 independent and dependent mechanisms. In more detail, GARP can be cleaved from the surface of platelets as a soluble factor (sGARP), facilitating the release of active TGF- β 1 into the surrounding environment. Platelet derived GARP was also observed to exhibit potent anti-inflammatory functions, including the induction of Foxp3⁺ regulatory T cells (Treg) as well as the inhibition of T effector cells [24,25]. Despite these desirable properties, which could be used to prevent overshooting inflammatory responses during wound healing, coupled with the high expression of GARP on platelets, a major component of PRF, GARP has not yet been examined on how it might influence the wound healing properties of APC.

This study aimed to analyze the impact of iPRF on the phenotype and function of cells implicated in the wound healing response, namely human monocyte derived macrophages and CD4⁺ T cells, and to determine the distinct influences of GARP on these mechanisms in vitro. CD4⁺ T cells and macrophages were evaluated as they are known to migrate to the site of injury, where iPRF is applied to in patients, and play essential roles in mediating the wound healing response [24,26–28]. To determine the direct influence of iPRF on CD4⁺ T cells and macrophages, the respective cell types were cocultivated with iPRF/iPRF derived platelets. This study utilized iPRF as a model for second generation APC as it is known to contain an enriched amount of growth factors that are slowly released overtime and leukocytes, which may optimize wound healing [11,13,14].

GARP was found to be expressed on the surface of platelets in iPRF and to exist as a soluble factor in iPRF. iPRF derived platelets and iPRF could induce a regulatory phenotype in CD4⁺ T cells through a GARP dependent mechanism. In more detail, heightened levels of Foxp3 and GARP as well as decreased effector cytokine production was observed in CD4⁺ T cells treated with iPRF derived platelets or iPRF. These effects could be reversed with a blocking anti-GARP antibody. It was also shown that iPRF induces a wound healing “M0/M2 like” phenotype in macrophages via a GARP independent mechanism. Altogether, these results identified for the first time that the immune capacity of iPRF is mediated in part by GARP and its ability to induce Treg. These findings contribute to our understanding of the underlying immunological mechanisms that occur during the wound healing process following iPRF treatment. They may also be applied to therapeutically enhance the wound healing properties of APC via the addition of GARP to new concentrates.

2. Materials and Methods

2.1. Preparation of iPRF

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Landesärztekammer Rheinland-Palatine (no. 2019-14705_1, approved 9 September 2020). Informed consent was obtained from all participants involved in the study. In more detail, iPRF from healthy donors was manufactured as described in the literature [29,30]. Briefly, venous blood collection of 60 mL via special vacutainer systems (Process for PRF, i-PRF, Nice, France) was performed. Next, blood was immediately centrifuged according to the manufacturer's protocol (700 rpm for 3 min, relative centrifugal force (rcf-max) = $60 \times g$ at 40° rotor angulation with a radius of 88 mm at the clot and 110 mm at the max; Process for PRF, Duo Quattro centrifuge). After centrifugation, the yellow upper part (approximately 2 mL of each tube) was collected as iPRF, and the underlying parts containing red blood cells were discarded. iPRF was used as subsequently described. Healthy donors used in this study ($n = 3$) consisted of two males and a female between the ages of 30–36. Donors were on no medications, had no allergies, no severe illnesses, or history of smoking/tobacco use.

2.2. Isolation of Platelets from iPRF

iPRF was obtained from healthy donors as described above. Subsequently, platelets were isolated from iPRF as previously described [24]. To remove any potential leukocyte contamination, iPRF was collected and centrifuged for an additional 15 min at $200 \times g$ at RT. The resulting platelet rich supernatant was then centrifuged for 5 min at $2000 \times g$ at RT, after being rinsed with $1 \times$ PBS at a ratio of 1:1. Platelets from the resulting pellet were counted and used as subsequently described.

2.3. Isolation and Stimulation of Human $CD4^+$ T Cells

As described earlier, $CD4^+$ $CD25^-$ T cells were isolated from human buffy coats and stimulated with anti-CD3 and anti-CD28 antibodies [24]. Buffy coats were provided by healthy donors. This study was performed in agreement with the Declaration of Helsinki, and the Ethics Committee of the Landesärztekammer Rheinland-Palatine approved the protocol (no. 837.019.10 (7028), approved 4 March 2010). Isolated platelets or iPRF were added to $CD4^+$ T cells cultures at a ratio of 50:1 (Platelets/iPRF: $CD4^+$ T cell) as indicated at day 0 to form cocultures, meaning two different cell types were grown together as a way to examine their direct influence on each other. Cocultures were either left untreated or treated with $10 \mu\text{g/mL}$ anti-GARP antibody (Ab) (Origene, #AP17415PU-N, Rockland, MD, USA), $10 \mu\text{g/mL}$ anti-TGF- β receptor II Ab (R&D Systems, #AF-241-NA, Minneapolis, MN, USA), and $10 \mu\text{g/mL}$ anti-TGF- β I-III Ab (R&D Systems, #MAB1835R) at day 0 as indicated.

Carboxyfluorescein succinimidyl ester (CFSE) (eBioscience #65-0850-84, San Diego, CA, USA) was used to prelabel $CD4^+$ T cells to track their proliferation. Labeled $CD4^+$ T cells were plated in 48 well plates at 10^6 cells/mL in X-Vivo 15 (Lonza, #BE02-060F, Basel, Switzerland) and incubated for 3 days in the presence or absence of platelets derived from iPRF at a ratio of 1:50 as well as $10 \mu\text{g/mL}$ anti-GARP Ab (Origene) before undergoing flow cytometric analysis.

2.4. Isolation and Polarization of Human Monocyte Derived Macrophages

Buffy coats were obtained from healthy volunteers, and macrophages were isolated from them as previously described [31]. In brief, 150 million peripheral blood mononuclear cells (PBMC) were plated in RPMI1640 (Thermo Fisher Scientific, #31870, Waltham, MA, USA), containing 1% Glutamax (Thermo Fisher Scientific, #35050038) and 0.1% Primocin (InvivoGen, #ant-pm-2, San Diego, CA, USA) in cell culture Petri dishes (Corning, #430167, Corning, NY, USA) for at least 1 h in a 37°C 5% CO_2 incubator to promote attachment. Non-adherent cells were washed away with repeated pre-warmed phosphate-buffered saline (PBS) washes (Thermo Fisher Scientific, #14190-094). Adherent cells were cultured in the medium described above containing an additional 1% heat inactivated human plasma

and 50 ng/mL M-CSF (ImmunoTools, #11343113, Friesoythe, Germany). Generation of heat inactivated human plasma was prepared as previously described [31]. After 5–7 days, “M0” macrophages were collected with Accutase (Thermo Fisher Scientific, #00-4555-56) and replated at 10^6 cells/well in 6 well plates (Corning, #3506). Cells underwent polarization to an “M1-like” or a “M2-like” phenotype as previously described [31]. For polarization experiments, “M0-like” macrophages were treated with 400 μ L of iPRF/well. Cells were polarized for 2 days before undergoing flow cytometric analysis.

2.5. Flow Cytometry

Single cell suspensions were prepared and stained for flow cytometric analysis. Fibrin clots, resulting from iPRF, were manually disrupted by mashing through a 40 μ m cell strainer (VWR International, #732-2757, Radnor, PA, USA) to generate single cell suspensions. This enabled the recovery of both CD4⁺ T cells and macrophages that had become entangled in the fibrin clot. Cell suspensions were washed with prechilled PBS before undergoing further analysis.

Isolated platelets were stained with anti-CD41a (eBioscience #11-0419-42) and anti-GARP (Miltenyi Biotec, #130-103-820, Bergisch Gladbach, Germany) antibodies as well as the respective IgG1 isotype control antibody (Miltenyi Biotec, #130-113-438).

For intranuclear staining of Foxp3, CD4⁺ T cells were harvested after 3 days of co-incubation, and they were stained with fixable viability dye (Invitrogen, #65-0865-14, Waltham, MA, USA), followed by the surface staining with anti-GARP (Miltenyi Biotec, #130-103-820). Cells were fixed, and permeabilized with the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience, #00-5523-00), followed by staining with anti-Foxp3 (BioLegend, #320208, San Diego, CA, USA). After 6 days of incubation, CD4⁺ T cells were stimulated for 5 h with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, #P1585-1MG, Munich, Germany), 1 μ g/mL ionomycin (Enzo Life Sciences, #ALX-450-006-M001, Lörrach, Germany), and 1.3 μ M monensin (BD, #554724, Heidelberg, Germany). Cells were stained with fixable viability dye (Invitrogen, #65-0865-14), fixed, and permeabilized with BD Cytotfix/Cytoperm Plus (BD Biosciences, #555028, Heidelberg, Germany), followed by intracellular staining with anti-IFN- γ (BD Biosciences, #557643) or anti-IL-2 antibodies (eBioscience, #17-7049-42).

Macrophages were stained with fixable viability dye (Invitrogen, #65-0866-14) followed by surface antibody staining of anti-CD40 (Immunotools, #21270403), anti-CD36 (Miltenyi Biotec, #130-095-480), anti-CD206 (Miltenyi Biotec, #130-100-152), anti-PD-L2 (Miltenyi Biotec, #130-105-829), anti-CD163 (Life Technologies, Carlsbad, CA, USA, #A15792), anti-HLA-DR (MHC II) (BioLegend, #307650), and anti-CD14 (Invitrogen, #48-0149-42). Measurements were performed on a BD LSRII flow cytometer (BD Biosciences) and were analyzed with Cytobank software [32]. Doublets, debris, and dead cells were excluded from analysis. Example gating strategies of the different cell types examined in this study can be found in Figure S1A–C.

2.6. Western Blot

Protein lysates of iPRF were prepared by diluting iPRF twenty-fold with the following cell lysis buffer, consisting of 20 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM Na₂-EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 2 M urea, and 1x protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, #78440). Samples were incubated in lysis buffer for 10 min, followed by brief sonication. Lysates were loaded into reducing SDS page gels at 10–20 μ g/well as indicated. Human recombinant soluble GARP (R&D Systems, #6055-LR-050) was used as a positive control for antibody specificity and loaded at 1 ng/lane. Western blot was carried out using the NuPAGE system (Thermo Fisher Scientific), in accordance with the manufacturer’s recommendations. Membranes were probed with the following antibodies: anti-GARP (Cell Signaling, #83565, Danvers, MA, USA), anti- β -actin (Cell Signaling, #8457), and anti-rabbit horseradish peroxidase (Jackson ImmunoResearch, #211-032-171, West Grove, PA, USA).

2.7. GARP ELISA

iPRF was analyzed for its soluble GARP content by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (R&D Systems #DY6055). Samples were diluted 1:1 with Reagent Diluent.

2.8. Statistics

Data was analyzed by GraphPad Prism (GraphPad Software, version 8.0.0 for Windows, San Diego, CA, USA, www.graphpad.com, accessed on 1 October 2022). Results were normalized to the untreated (*w/o*) controls as indicated. Data show mean $\pm$ standard deviation (SD). Statistical significance was calculated by one-way ANOVA, two-way ANOVA, and unpaired Student's *t*-tests as listed in the figure legends. Statistical significance is indicated by asterisks as follows: * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ and not significant (n.s.) $p > 0.05$.

3. Results

3.1. Glycoprotein A Repeats Predominant (GARP) Is Expressed on the Surface of Platelets Isolated from iPRF and Exists as a Soluble Factor within iPRF

GARP was first characterized to be expressed on the surface of platelets, and activated platelets are known to upregulate GARP expression on their surfaces [20]. However, up until now, no study has shown that GARP is expressed in autologous platelet concentrates. Here, it was demonstrated for the first time that GARP is expressed on the surface of platelets found in freshly generated iPRF. By flow cytometric analysis, it was determined that approximately half the platelets expressed GARP on their surfaces, and the expression of GARP was highly elevated in comparison to the isotype control (Figure 1A,B). Previous work, using pre-activated platelets, showed comparable percentages of GARP⁺ platelets and GARP expression levels [24]. This suggests that part of the platelets found in iPRF could be partially activated, presumably resulting from their preparation process, but this should be validated in future studies.

These results could be validated by Western blot, confirming the presence of GARP in iPRF (Figure 1C). Interestingly, it was found that GARP also exists in its soluble form (sGARP) in both iPRF and peripheral blood, likely released from the surface of activated platelets (Figure 1D). However, sGARP levels were not found to be significantly increased in comparison to peripheral blood (Figure 1D). Altogether, these results indicate that GARP is expressed in iPRF, and it exists as both a surface protein on platelets as well as a soluble factor inside of iPRF.

3.2. iPRF Derived Platelets Induce a Regulatory Phenotype in CD4⁺ T Cells via a GARP Dependent Mechanism

Previously, it was shown that GARP derived from platelets exhibits potent tolerogenic properties, including the induction of peripheral Treg [24]. To determine if these effects are conserved in iPRF, platelets were isolated from iPRF and cocultured with CD4⁺ T cells. It was found that CD4⁺ T cells had a significantly increased expression of Foxp3 in the presence of platelets on day 3 of cocubation ($p < 0.05$) (Figure 2A). This effect could be reversed back to untreated CD4⁺ T cell levels with the addition of an anti-GARP antibody (no significant difference vs. *w/o*) (Figure 2A). Furthermore, on day 3 of cocubation, the addition of iPRF derived platelets increased the GARP expression of CD4⁺ T cells ($p < 0.001$); however, the proliferation of iPRF treated CD4⁺ T cells remained unaffected (no significant difference vs. *w/o*) (Figure 2B,C). Blocking with an anti-GARP antibody could not restore proliferation and GARP levels to the untreated CD4⁺ T cell control (no significant difference vs. *w/o*, $p < 0.001$) (Figure 2B,C).

On day 6 of co-incubation, production of the effector cytokines, IFN- γ and IL-2, by CD4⁺ T cells was analyzed. For both cytokines, production was significantly decreased, when CD4⁺ T cells were cocultured with iPRF derived platelets ($p < 0.01$ and $p < 0.05$) (Figure 2D,E). Once more, it was observed that the addition of an anti-GARP antibody could

completely reverse these effects to untreated CD4⁺ T cell levels (no significant difference vs. *w/o*) (Figure 2D,E). Collectively, these findings show that platelets, isolated from iPRF, induce a regulatory phenotype in CD4⁺ T cells and are capable of suppressing T effector cell function.

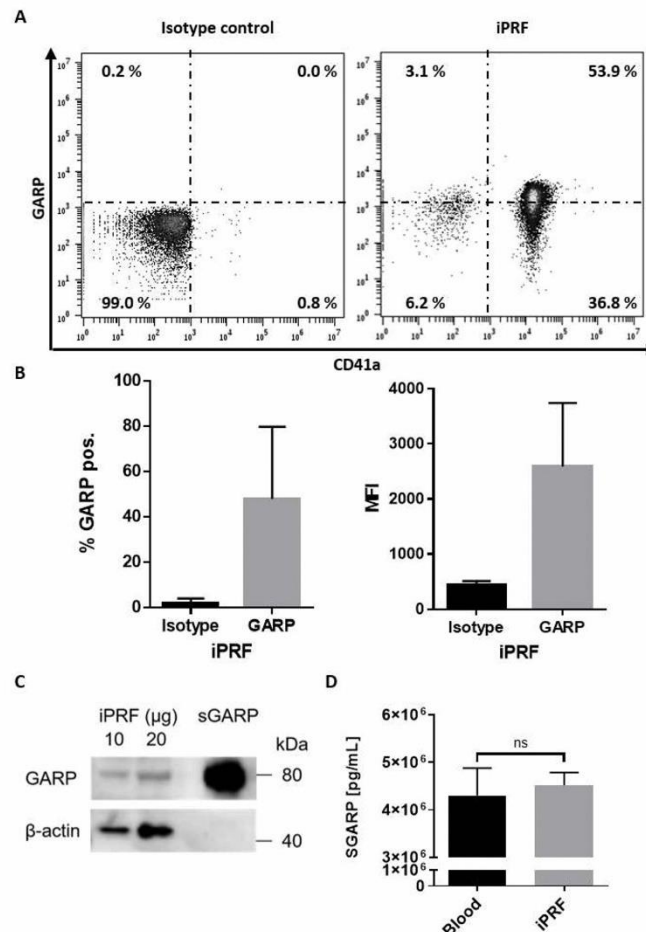


Figure 1. Glycoprotein A repetitions predominant (GARP) is expressed on the surface of platelets and exists as a soluble factor found in liquid platelet rich fibrin (iPRF). (**A,B**) iPRF was stained directly for the platelet marker, CD41a, and GARP and underwent flow cytometric analysis. Isotype antibodies were used as a control. (**A**) Dot blots show one representative result. (**B**) Bar diagrams of GARP expression show the pooled data of percentages (%) of GARP positive platelets in iPRF and the mean fluorescence intensity (MFI) of GARP ($n = 3 \pm SD$, analyzed by paired Students *T*-tests). (**C**) Expression of GARP in iPRF was analyzed via Western blot. iPRF lysate was loaded in increasing amounts. Recombinant soluble GARP (sGARP) was loaded at 1 ng/lane and served as a positive control, whereas β -actin acted as the loading control. (**D**) Bar graph shows the soluble GARP (sGARP) levels in peripheral blood and iPRF analyzed via ELISA ($n = 3 \pm SD$, ns determined by paired Students *T*-tests).

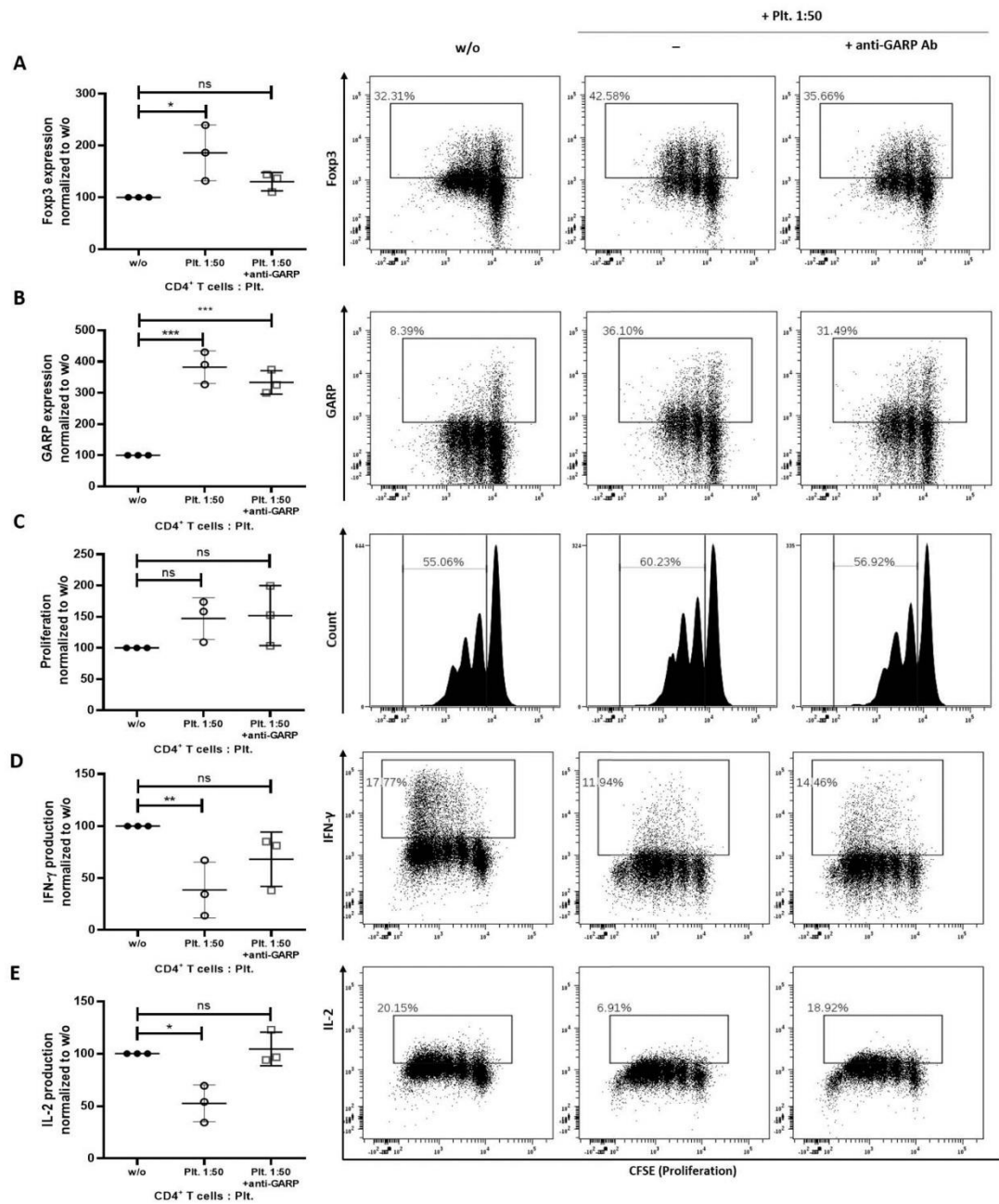


Figure 2. Isolated platelets from liquid platelet rich fibrin (iPRF) induce a regulatory phenotype in CD4⁺ T cells. CD4⁺ T cells and platelets (Plt.) were cocultured at a ratio of 1 to 50 and were treated with 10 μ g/mL anti-GARP antibody (Ab). CD4⁺ T cells were prelabeled with carboxyfluorescein succinimidyl ester (CFSE) to track their proliferation and stimulated with 0.5 μ g/mL anti-CD3 and 1.0 μ g/mL anti-CD28 Abs in the presence or absence of platelets. (A–C) The expression of

Foxp3 (A), GARP (B), and proliferation (C) of CD4⁺ T cells was measured on day 3 of cocubation via flow cytometry. (D,E) After 6 days of cocubation, cells were stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monesin for 5 h. IL-2 and IFN- γ production was quantified via intracellular flow cytometry. Representative dot plots of three independent experiments are shown. Graphs show CD4⁺ T cells cocultured with platelets normalized to untreated CD4⁺ T cells alone control (n = 3, means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns determined by one-way ANOVA).

3.3. iPRF Induces a Regulatory Phenotype into CD4⁺ T Cells

As mentioned earlier, iPRF contains a mixture of different cell types and their respective growth factors [11,13,14]. To better ascertain the collective influence of iPRF on CD4⁺ T cells, cocultures of iPRF and CD4⁺ T cells were performed. In agreement with the results above (Figure 2A), Foxp3 expression was found to be significantly increased on day 3 of cocubation ($p < 0.01$), and application of an anti-GARP antibody was able to reverse this effect ($p < 0.01$) to untreated CD4⁺ T cell levels (Figure 3A). Additionally, effector cytokine production of IL-2 and IFN- γ by CD4⁺ T cells on day 6 of cocubation revealed a significant decrease (each $p < 0.0001$), when CD4⁺ T cells were cocultured with iPRF (Figure 3B,C). Treatment with an anti-GARP antibody was able to partially restore IL-2 production ($p < 0.01$) and could completely restore IFN- γ (no significant difference vs. *w/o*) levels to the untreated CD4⁺ T cell control (Figure 3B,C).

The tolerogenic properties of GARP are known to be partially mediated through a TGF- β dependent mechanism [20,24]. To clarify whether the observed regulatory effects of iPRF on CD4⁺ T cells occur in a TGF- β independent or dependent manner, cocultures of CD4⁺ T cells and iPRF were treated with anti-TGF- β receptor II (anti-TGF- β -RII) and anti-TGF- β -I-III blocking antibodies. Interestingly, in contrast to the effects of anti-GARP antibody treatment, anti-TGF- β -RII and anti-TGF- β -I-III antibodies did not show any noteworthy influence on Foxp3 expression (no significant difference vs. *w/o*) and were unable to restore effector cytokine production of IL-2 and IFN- γ to untreated levels (all $p < 0.0001$) (Figure 3A–C). In summary, iPRF induced a regulatory phenotype in CD4⁺ T cells via GARP in a seemingly TGF- β independent manner.

3.4. iPRF Polarizes M0-Macrophages into a More “M0/M2-like” Phenotype in a GARP Independent Manner

Previously, it was shown that APC can polarize macrophages towards a wound healing “M2-like” phenotype [16–18,33]. To build on these results, by staining with an expanded surface marker panel, human monocyte derived M0 macrophages were treated with iPRF and analyzed for altered surface marker expression via flow cytometry. It was demonstrated that iPRF induces a “M0/M2-like” phenotype as shown by the significantly increased expression of the M2 surface marker, CD36 ($p < 0.0001$) and corresponding decreased expression of the M1 surface markers, CD14 ($p < 0.0001$) and CD163 ($p < 0.01$) (Figure 4), thus validating and expanding upon the results of previous studies [16–18,33].

sGARP has also been reported to induce M2 macrophage polarization, and it was confirmed to be expressed in iPRF (Figure 1D) [34]. Therefore, it was investigated if GARP may play a role in the underlying immunological mechanism behind the induction of “M0/M2-like” macrophages following iPRF treatment. To investigate this, macrophages were cocultured with iPRF and were treated with a blocking anti-GARP antibody. Interestingly, unlike CD4⁺ T cells, anti-GARP antibody treatment was unable to reverse the effects of iPRF on macrophage polarization. This indicates that a GARP independent mechanism appears to play a greater role in the influence of iPRF on macrophage polarization.

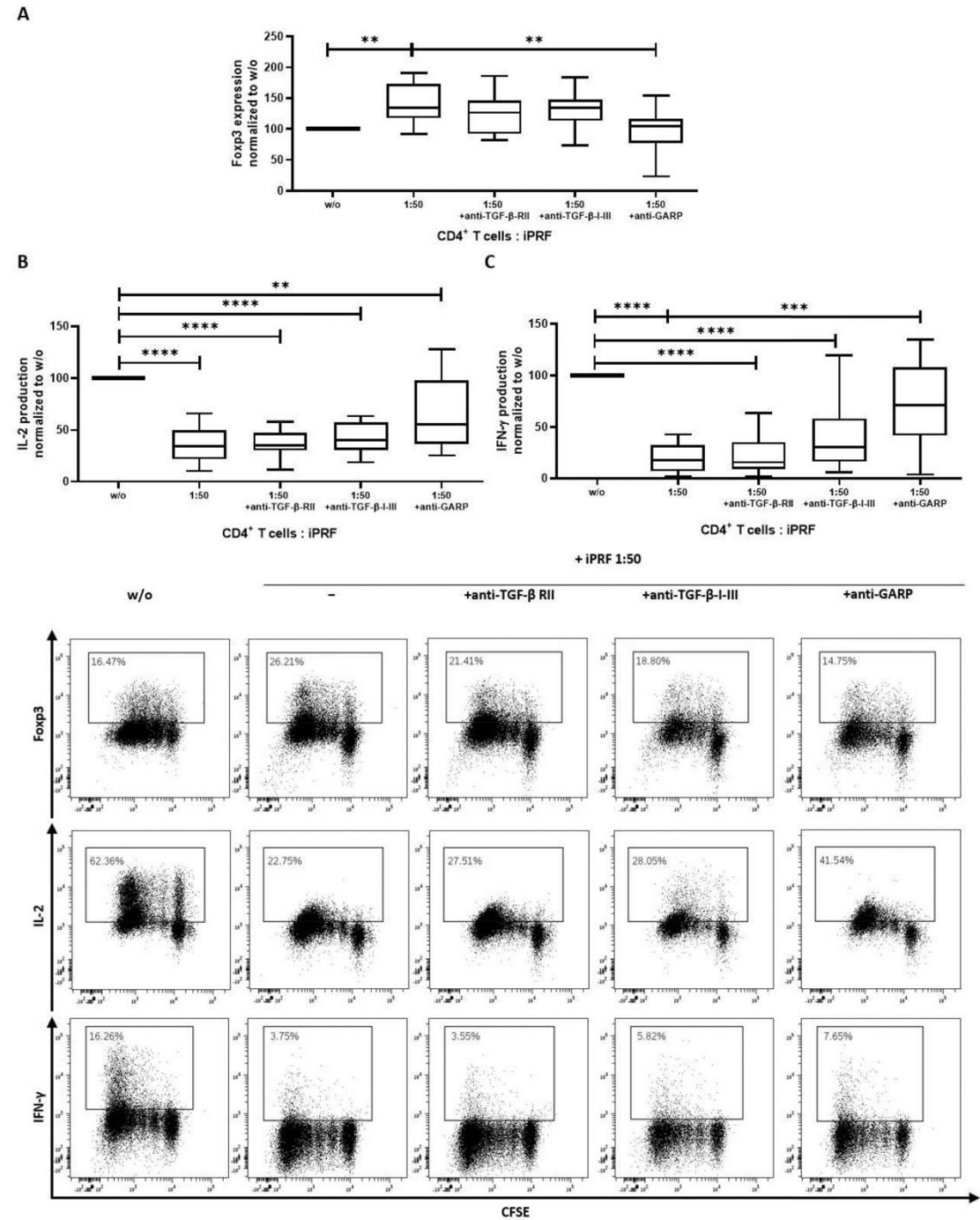


Figure 3. Liquid platelet rich fibrin (iPRF) induces a regulatory phenotype in CD4⁺ T cells. CD4⁺ T cells and iPRF were cocultured at a ratio of 1 to 50 and treated with either 10 µg/mL anti-TGF-β receptor II

(anti-TGF- β -R2) antibody (Ab), 10 μ g/mL anti-TGF- β -1-III Ab, or 10 μ g/mL anti-GARP Ab. CD4⁺ T cells were prelabeled with carboxyfluorescein succinimidyl ester (CFSE) to track their proliferation and stimulated with 0.5 μ g/mL anti-CD3 and 1.0 μ g/mL anti-CD28 Abs in the presence or absence of iPRF. (A) The expression of Foxp3 was measured on day 3 of coincubation using intranuclear flow cytometry. (B,C) After 6 days of coincubation, cells were stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin for 5 h. IL-2 and IFN- γ production was quantified via intracellular flow cytometry. Representative dot plots of 10 independent experiments are shown. Box plots show data which was normalized to the untreated CD4⁺ T cell control (n = 10, box and whiskers, medians $\pm$ min/max, ** p < 0.01, *** p < 0.001, and **** p $\leq$ 0.0001 determined by two-way ANOVA).

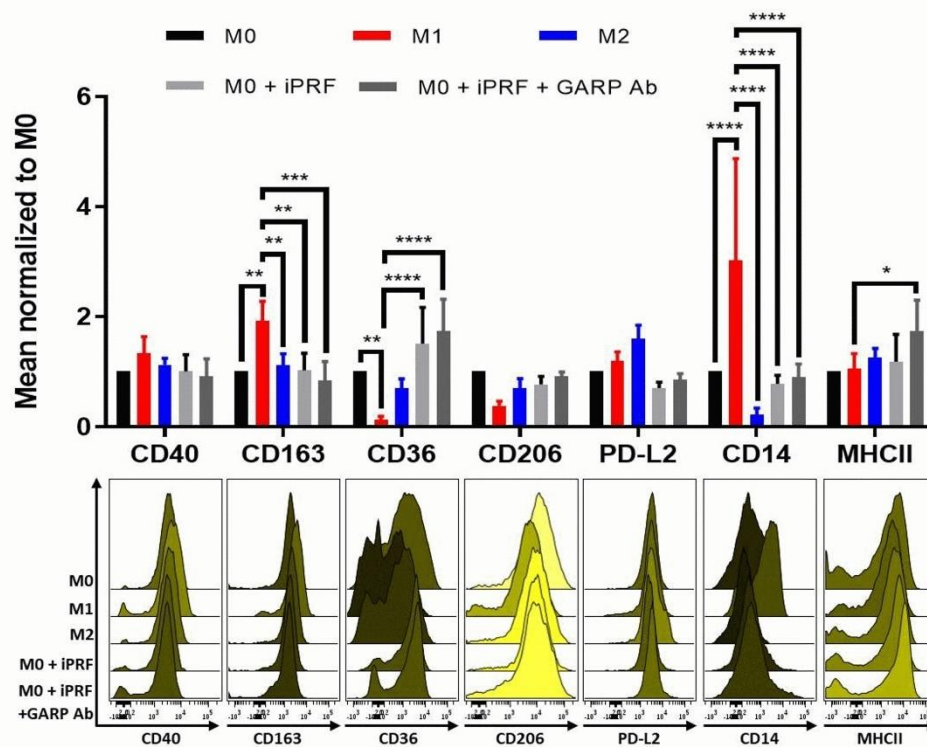


Figure 4. Liquid platelet rich fibrin (iPRF) polarizes M0-macrophages in a more “M0/M2-like” phenotype through a GARP independent mechanism. Human monocyte derived macrophages were polarized, treated with 400 μ L iPRF/well, 10 μ g/mL anti-GARP antibody (Ab) or were left untreated for 2 days as indicated. Surface marker expression was analyzed via flow cytometry. Bar diagram shows the mean fluorescence intensity (MFI) of each surface marker normalized to the respective M0 control. Representative histograms of 3 independent experiments are shown (n = 5 donors, bar means $\pm$ SD, * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 determined by two-way ANOVA).

4. Discussion

This study is the first to show the direct interaction between the APC, iPRF, and immune cells via the immunosuppressive protein, GARP. As a major result, it was shown that application of iPRF and iPRF derived platelets induced a regulatory phenotype in CD4⁺ T cells through a GARP dependent mechanism (Figures 2 and 3). Blocking GARP with an anti-GARP antibody could effectively reduce Foxp3 expression and effector cytokine

production to untreated CD4⁺ T cell levels, providing a potential targeting method. These findings are in accordance with the literature, where it was previously demonstrated that platelet derived GARP could induce peripheral Treg [24]. In addition, it was observed that the regulatory effects of iPRF and platelets derived from iPRF on CD4⁺ T cells were nearly identical—indicating that platelets are a key cell type in iPRF, which influences its immunological capacity. Lastly, platelet lysates are known to have an impact on various immune cell types, including T cells and macrophages, thus further supporting their important role in the immune capacity of APC [35].

Treg have become increasingly recognized to orchestrate tissue repair and regeneration. Recruited to the damaged site, Treg can indirectly modulate regeneration by controlling neutrophils and helper T cells, inducing M2 macrophage polarization, and directly facilitating regeneration via the activation of progenitor cells at the site of tissue damage to mediate inflammation resolution and to regulate immunity after injury [28]. Weirather et al., 2014 could show that depletion of Treg in a post-myocardial infarction model led to an inhibited production of anti-inflammatory factors essential for wound healing, like IL-10 and TGF- β 1, an increased infiltration of neutrophils and monocytes, and an induction of “M1-like” macrophages [36]. Whereas therapeutic activation of Treg via an anti-CD28 monoclonal antibody led to an accumulation of “M0/M2-like” macrophages and exhibited enhanced wound healing. The discovery that iPRF induces CD4⁺ Treg via GARP and that these Treg display heightened levels of GARP, a known marker of activated Treg, as well as increased suppressive function, help to explain in part a mechanism behind the wound healing properties of iPRF.

So far, only limited evidence exists regarding the immune capacity of APC, especially for PRF. An anti-inflammatory activity has been proven, yet the exact mechanism remained unclear [17]. In previous studies, it was shown that human PRF can interact with macrophages [16–18,33]. Here, PRF not only suppressed the M1 pro-inflammatory status of the macrophages but also supported their shift towards the M2 resolving phenotype [17,18]. These findings are encouraging as M2 macrophages have been shown to be beneficial in transplant acceptance, wound healing, and tissue remodeling, helping to explain part of the observed wound healing properties of iPRF [37]. So far, only one other known study has shown that PRP can polarize human monocyte derived macrophages to a “M2-like” phenotype [18]. The findings of this study are in accordance with our results. However, it is important to note that this work characterized the effects of iPRF, a differentially prepared APC, on the polarization of human monocyte derived macrophage for the first time and provided a more detailed analysis of macrophage surface marker alterations following iPRF treatment. Furthermore, this work also evaluated GARP as a possible underlying mechanism for the observed effects of iPRF on macrophage polarization.

Previously, it was shown by Hahn et al., 2016 that application of sGARP could polarize macrophages to a “M0/M2-like” wound healing phenotype [34]. Contrary to these findings, application of a blocking anti-GARP antibody was unable to effectively reverse the effects of iPRF on macrophage polarization (Figure 4), despite the presence of sGARP being confirmed in iPRF (Figure 1D). Only expression of the M1 marker, MHCII, was found to be significantly elevated ($p < 0.05$) on M0 macrophages treated with iPRF and a blocking anti-GARP antibody, supporting that iPRF polarizes macrophages to a “M0/M2-like” phenotype in a seemingly GARP independent manner. However, it is important to note that Hahn et al., 2016 treated macrophages with a higher concentration of sGARP (10 μ g/mL) and used a different experimental set-up than the current study (macrophages treated with sGARP vs. macrophages cocultured with iPRF +/- anti-GARP antibody) [34].

A possible explanation behind the observed effects of iPRF on macrophage polarization seen in this study may lie within the humoral composition of iPRF. Uchiyama et al., 2021 provided helpful insights into the humoral components of PRP where they determined that leukocyte rich PRP contains increased levels of the M2 macrophage polarizing agents, IL-10, TGF- β , M-CSF, and IL-1RA [18]. Varela et al., 2019 could also show an upregulation of TGF- β and IL-10 in iPRF in comparison to peripheral blood [38]. As TGF- β and IL-10 are

well known to induce M2 macrophage polarization and they are confirmed to be present in iPRF, it is reasonable to speculate that they could be responsible for the observed effects of iPRF on macrophage polarization seen in this study. A limitation of the present work is that it did not analyze the humoral composition of the iPRF applied to both CD4⁺ T cells and macrophages in detail—rather it focused instead on the phenotypic and functional alterations of immune cells following iPRF application. Future studies are needed to gain further insights into the humoral composition of iPRF and how it may alter macrophage polarization and function.

A study by Zhang et al., 2020 reported that GARP is selectively expressed on the surface of “M1-like” inflammatory macrophages, where it is thought to regulate the release of active TGF- β 1 and plays an important role in macrophage shift [39]. They found that pancreatic ductal adenocarcinoma cells directly induce DNA methylation of different genes in “M1-like” macrophages via GARP and integrin α V/ β 8 binding, which led to a functional reprogramming of macrophages from “M1-like” to “M2-like” macrophages. Blocking of either GARP or integrin α V/ β 8 could suppress this reprogramming [39]. It remains unclear if iPRF, or more specifically the platelets in iPRF, act upon macrophages in a similar manner, and this should be a topic of future studies.

As described earlier, GARP was found to exist both as a membrane bound protein enriched on the surface of platelets and as a soluble factor found in iPRF (Figure 1). However, surface GARP and sGARP levels were observed to not parallel each other. It is not well understood if sGARP levels mirror surface GARP levels on platelets, and the time it takes to accumulate significant levels of sGARP in the extracellular space. Recent work by Zimmer et al., 2020 has shown that pre-activated platelets exhibit an upregulation of surface GARP expression and an increase of sGARP in their supernatants following 16 h of incubation [24]. A limitation of this work is that sGARP levels in iPRF were only measured directly after its preparation. It could be that platelets only had enough time to upregulate GARP on their surfaces (Figure 1A,B) but not enough time to secrete sGARP in significant quantities. Others have shown that iPRF induced fibrin clots slowly reduce growth factors over time, which could also be true for sGARP, but this must be evaluated in future studies [13].

Metelli et al., 2020 have recently found that thrombin cleaves GARP from the surface of platelets, generating its soluble form (sGARP), and in turn aids in the activation of TGF- β 1 [25]. As thrombin is present in iPRF, it is reasonable to speculate that both sGARP and TGF- β 1 accumulate in iPRF through thrombin mediated proteolytic cleavage, but this should be confirmed in future studies. Of note, Treg are known to also release sGARP from their membranes into the extracellular space, which can induce Treg [34]. As iPRF derived platelets/iPRF were shown to induce a regulatory phenotype in CD4⁺ cells (Figures 2 and 3) in a GARP dependent fashion, it is reasonable to speculate that the resulting Treg are capable of inducing further Treg themselves in a paracrine sGARP dependent manner—thereby amplifying the effects of iPRF derived platelets/iPRF on CD4⁺ cells. However, a more detailed examination into the paracrine effects of GARP from Treg is needed.

The findings of this work also contribute to the understanding of the complicated and often contested interplay between GARP and Foxp3 [20,40–43]. It was observed that expression of Foxp3 appears to be dependent on GARP. Hereby, GARP, expressed by platelets in iPRF, was able to strongly induce expression of Foxp3 in CD4⁺ T cells, whereas application of anti-GARP antibodies could reverse these effects back to untreated CD4⁺ T cell levels (Figures 2A and 3A). Despite reduced expression of Foxp3 upon anti-GARP antibody treatment, GARP levels remain unaffected further supporting that the expression of GARP is not dependent on Foxp3 (Figure 2B). Notably, blocking with an anti-TGF- β -1-III antibody or an anti-TGF- β receptor II antibody were unable to significantly reduce Foxp3 expression (Figure 3A). These findings are in accordance with the literature where the role of platelets in the induction of Treg via a GARP-dependent mechanism have been already described [24]. Furthermore, it has been found that silencing of GARP partially impairs the suppressive capacity of Treg. In a murine model, lacking surface GARP on

Tregs, due to a Treg-specific deletion of the chaperone molecule GP96, mice developed fatal multi-organ inflammatory disease [44]. This is in agreement with the present findings as the expression of the Treg master regulator, Foxp3, and the associated suppressive properties of Treg, including inhibition of effector cytokine production (IL-2 and IFN- γ), were observed to be highly dependent on surface GARP expression (Figures 2 and 3). Collectively, this indicates that the expression of Foxp3 and associated suppressive properties of Treg are highly dependent on GARP.

A possible application of this work could be the addition of sGARP to APC to further enhance their wound healing properties, namely through the induction of Treg, as observed in this study. sGARP has already been shown to exhibit potent anti-inflammatory properties on human CD4⁺ T cells, including the induction of suppressive Treg and an inhibition of T effector cells [34,45]. These sGARP mediated effects are in accordance with the observed GARP dependent regulatory effects on CD4⁺ T cells in the present work. Whether the presence of sGARP, GARP expressed on the surface of platelets, or a mixture of both, are responsible for the observed regulatory effects of iPRF on CD4⁺ T cells remains unclear. Of note, Hahn et al., 2013 showed that sGARP treatment could prevent the induction of graft-versus-host disease (GVHD) in a humanized mouse model of xenogeneic GVHD via the simultaneous accumulation of Treg and the inhibition of destructive T effector cells [45]. Altogether, these potent anti-inflammatory properties of sGARP indicate its strong potential to boost the wound healing properties of APC. However, future studies are greatly needed to thoroughly evaluate the efficacy and safety of the addition of sGARP to platelet concentrates. Additionally, in the future, great care should be exercised when selecting patients that could benefit from this treatment as the observed immunological effects of iPRF/sGARP could be problematic for cancer patients.

5. Conclusions

The distinctive interplay of autologous platelet concentrates with CD4⁺ T cells and macrophages were analyzed in this study. A GARP dependent regulatory influence of platelets derived from iPRF on CD4⁺ T cells was shown for the first time. Furthermore, application of iPRF could polarize human monocyte derived macrophages to a “M0/M2-like” wound healing phenotype in a GARP independent manner. Collectively, this indicates that the immunological capacity of iPRF is partially mediated by GARP, especially in terms of its effects on CD4⁺ T cells. The findings from this work may help to better understand the immunological reactions after iPRF application during the wound healing process. Furthermore, it may be possible to therapeutically enhance the wound healing properties of APC further via the addition of a sGARP to new concentrates.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomedicines10123136/s1>, Figure S1: Flow cytometric gating strategies.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Landesärztekammer Rhineland-Palatine (no. 2019-14705_1, approved 9 September 2020 and no. 837.019.10 (7028), approved 4 March 2010).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in this article and in the Supplementary Materials.

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3.2 PAPER 2

Nuclear Glycoprotein A Repetitions Predominant (GARP) Is a Common Trait of Glioblastoma Stem-like Cells and Correlates with Poor Survival in Glioblastoma Patients

Figure 10: Graphical abstract (Paper 2)

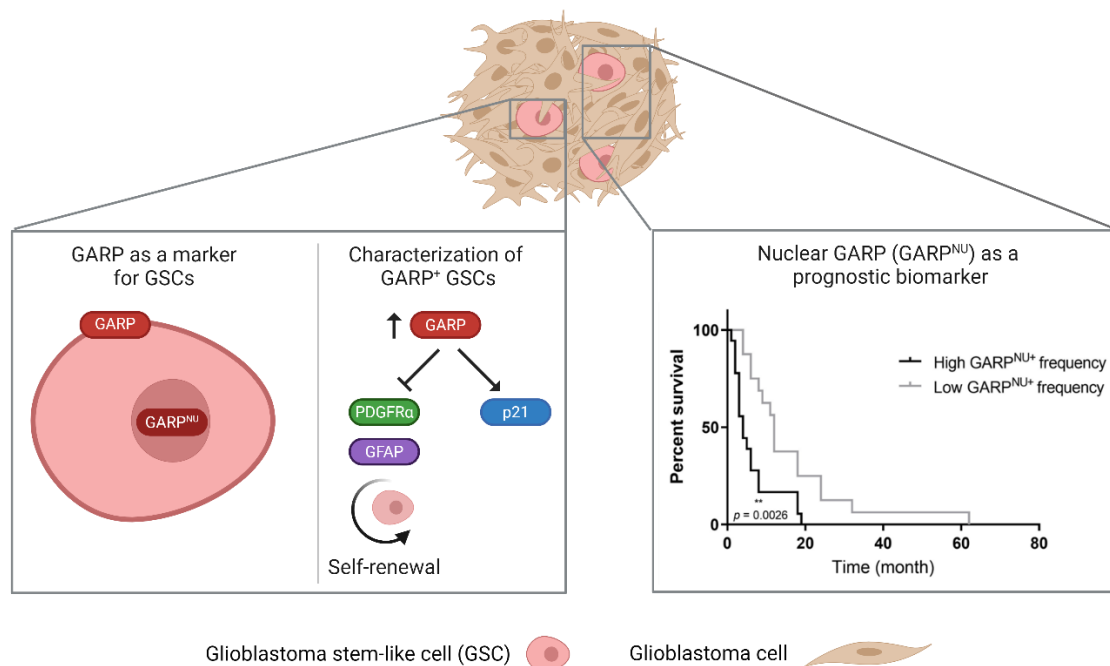


Fig. 10: Graphical abstract of **Paper 2**. Created with BioRender.com (2024)¹⁵.

3.2.1 SUMMARY

Glioblastoma, stage IV astrocytoma, is the most common malignant central nervous system tumor and is an incurable disease^{119,142,147,149,150}. Patients with glioblastoma have a five-year overall survival rate of only 5% post-diagnosis²¹⁰. This poor prognosis can be attributed in part to GSCs. Although GSCs represent only a small subset of tumor cells, they are a driving force behind tumor initiation, therapy resistance, and recurrence in glioblastoma^{239,245,247–249}. Therefore, targeting GSCs is considered a promising therapeutic strategy to improve clinical outcomes.

However, due to the innately high plasticity and heterogeneity of GSCs, there are no universal markers to identify them⁹. Recently, the tolerogenic protein GARP was reported to be highly expressed by GSCs⁶⁵. This study investigated whether GARP could be used to detect GSCs, its functional relevance in GSC biology, and evaluated the potential of GARP as a biomarker for glioblastoma⁹.

To accomplish this, several patient derived GSC cell lines were employed, and GARP expression was analyzed⁹. These cell lines recapitulated key features of the disease, including

intratumoral heterogeneity, cellular hierarchy, and recurrence. GARP was found to be expressed on GSCs, regardless of cell state and disease stage. It was determined that high levels of GARP on GSCs reduced their self-renewal capacity and increased the expression of differentiation inducing factor, p21. Interestingly, higher levels of GARP decreased the expression of differentiation commitment markers, GFAP and PDGFR- α . This suggests that GARP may play a role earlier in the differentiation process, namely regulating the self-renewal of GSCs. GARP was found to be abnormally expressed in the nucleus of GSCs, and the frequency of GARP^{NU+} cells correlated inversely with patient survival. Altogether, this study demonstrated both the promise of GARP as a marker to identify GSCs and as a biomarker to predict patient prognosis.

3.2.2 ZUSAMMENFASSUNG

Das Glioblastom, ein Astrozytom im Stadium IV, ist der häufigste bösartige Tumor des zentralen Nervensystems und eine unheilbare Krankheit^{119,142,147,149,150}. Patienten mit Glioblastom haben nach der Diagnose eine Fünfjahres-Gesamtüberlebensrate von nur 5 %²¹⁰. Diese schlechte Prognose kann zum Teil den GSCs zugeschrieben werden. Obwohl GSCs nur eine kleine Untergruppe der Tumorzellen darstellen, sind sie eine treibende Kraft hinter der Tumorentstehung, der Therapieresistenz und dem Wiederauftreten des Glioblastoms^{213,245,247–249}. Daher sind sie ein ideales therapeutisches Ziel, um die klinischen Behandlungsergebnisse zu verbessern.

Aufgrund der hohen Plastizität und Heterogenität der GSCs gibt es jedoch keine universellen Marker, um diese zu identifizieren⁹. Kürzlich wurde berichtet, dass das tolerogene Protein GARP in hohem Maße von GSCs exprimiert wird⁶⁵. In dieser Studie wurde untersucht, ob GARP zur Erkennung von GSCs verwendet werden kann, welche funktionelle Bedeutung es für die Biologie von GSCs hat und ob GARP als Biomarker für Glioblastome geeignet ist⁹.

Zu diesem Zweck wurden mehrere von Patienten stammende GSC-Zelllinien verwendet und deren die GARP-Expression analysiert⁹. Diese Zelllinien zeigen wesentliche Merkmale der Erkrankung, wie intratumorale Heterogenität, zelluläre Hierarchie und Rezidiv. Es konnte gezeigt werden, dass GARP auf GSCs unabhängig von Zellstatus und Krankheitsstadium exprimiert wird. Zudem wurde festgestellt, dass hohe GARP-Konzentrationen auf GSCs deren Selbsterneuerungskapazität reduzieren und die Expression des differenzierungsfördernden Faktors p21 erhöhen. Interessanterweise verringerte sich bei höheren GARP-Konzentrationen die Expression der Marker für die Differenzierungsbereitschaft, GFAP und PDGFR- α . Dies deutet darauf hin, dass GARP bereits früh im Differenzierungsprozess eine Rolle bei der Regulation der Selbsterneuerung von GSCs spielen könnte. Es konnte gezeigt werden, dass GARP im Zellkern von GSCs abnormal exprimiert wird und dass die Häufigkeit von GARP^{NU+} Zellen invers mit dem Überleben der Patienten korreliert. Insgesamt zeigte diese Studie, dass GARP sowohl ein vielversprechender Marker zur Identifizierung von GSCs als auch ein Biomarker zur Vorhersage der Patientenprognose ist.

3.2.3 AUTHOR CONTRIBUTIONS

Disclaimer: First authorship was shared between E.R. Trzeciak and N. Zimmer. Both authors contributed equally to the manuscript. This publication is also part of N. Zimmer's cumulative dissertation. N. Zimmer produced the data and generated Figures 1-3, 6-7, S4-5, S8-9. E.R. Trzeciak produced the data and generated Figures 3-4, S10, S2-3, S6, S10. Both authors wrote the original draft of the manuscript, and E.R.T primarily handled the review and editing process.

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3.2.4 PUBLICATION

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Article

Nuclear Glycoprotein A Repetitions Predominant (GARP) Is a Common Trait of Glioblastoma Stem-like Cells and Correlates with Poor Survival in Glioblastoma Patients

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Simple Summary: Glioblastoma (GB) is the most common primary brain tumor in adults, but it remains incurable due to its high degree of therapy resistance. Glioblastoma stem-like cells (GSCs) are believed to drive the initiation, progression, and therapy resistance of GB, making them an ideal therapeutic target to improve patient outcomes. However, due to their heterogeneity, there are no universal markers to identify GSCs. We evaluated GARP as a novel marker for GSCs and found that GARP is more stably and uniformly expressed by human GSCs, across cellular states and disease stages, than the commonly used GSC marker, CD133. Additionally, we showed that GARP is intranuclearly localized in GSCs, and we are the first to show that nuclear GARP levels (GARP^{NU+}) are associated with poor patient survival. Our findings indicate that GARP/GARP^{NU+} expression is an improved marker for GSCs and suggest a potential application of GARP as a prognostic biomarker for GB.

Abstract: Glioblastoma (GB) is notoriously resistant to therapy. GB genesis and progression are driven by glioblastoma stem-like cells (GSCs). One goal for improving treatment efficacy and patient outcomes is targeting GSCs. Currently, there are no universal markers for GSCs. Glycoprotein A repetitions predominant (GARP), an anti-inflammatory protein expressed by activated regulatory T cells, was identified as a possible marker for GSCs. This study evaluated GARP for the detection of human GSCs utilizing a multidimensional experimental design that replicated several features of GB: (1) intratumoral heterogeneity, (2) cellular hierarchy (GSCs with varied degrees of self-renewal and differentiation), and (3) longitudinal GSC evolution during GB recurrence (GSCs from patient-matched newly diagnosed and recurrent GB). Our results indicate that GARP is expressed by GSCs across various cellular states and disease stages. GSCs with an increased GARP expression had reduced self-renewal but no alterations in proliferative capacity or differentiation commitment. Rather, GARP correlated inversely with the expression of GFAP and PDGFR- α , markers of astrocyte or oligodendrocyte differentiation. GARP had an abnormal nuclear localization (GARP^{NU+}) in GSCs and was negatively associated with patient survival. The uniformity of GARP/GARP^{NU+} expression across different types of GSCs suggests a potential use of GARP as a marker to identify GSCs.

Keywords: GARP; nuclear GARP; GARP^{NU+}; LRRC32; glioblastoma; glioblastoma stem-like cells

1. Introduction

Glioblastoma (GB) is one of the most aggressive tumors, with an overall survival rate of approximately 15 months [1,2]. The current standard therapy consists of surgical removal of the primary tumor, radiation, and treatment with the chemotherapeutic agent, temozolomide (TMZ) [3]. A high degree of tumor infiltration into the surrounding tissue is a characteristic feature of GB, limiting the clinical efficacy of neurosurgical resection. Despite multi-modal therapy, GB recurrence after initial treatment is almost inevitable [4–6]. Besides the immunosuppressive properties of tumor cells, which suppress anti-tumor immune responses through microglial cells or regulatory T cells, poor prognosis is also attributed to a high degree of therapeutic resistance, either inherent or acquired by therapy, and the extraordinary intratumoral heterogeneity of GB, manifesting via the diversity of molecular and cellular subtypes/cellular states associated with GSCs [7–10].

The notorious therapeutic resistance of GB has been attributed to glioblastoma stem-like cells (GSCs), which comprise a subset of tumor cells that possess some fundamental properties of cancer stem cells, including unlimited self-renewal, aberrant differentiation response, and inherent plasticity. These characteristics enable GSCs to undergo reversible transitions between distinct cellular states in response to environmental signals [11,12].

These unique properties render GSCs capable of adapting to and surviving cytotoxic treatments that are otherwise lethal to non-stem glioma cells, thereby endowing them with the potential to reconstitute the tumor during or after therapy. GSCs are currently considered the main determinants of therapy resistance and drivers of tumor recurrence in GB. Therefore, they are arguably the most clinically relevant cellular target in gliomas. Assessments of GSCs in tumor specimens face several methodological challenges. These include: (I) the relatively low percentage of GSCs compared to the rest of the tumor cells, which are thought to be comprised primarily of non-stem glioma cells or differentiated progenies of GSCs [13,14], (II) an inhomogeneous distribution of GSCs within the tumor, which are located in specialized niches that provide a proper environment for maintaining their undifferentiated state [14], and (III) the phenotypic diversity and inherent high plasticity of GSCs, enabling dynamic transitions between different cell states accompanied by morphological alterations and changes in their phenotypic make-up [15,16]. Furthermore, GSCs possess a high degree of plasticity, which renders them capable of switching between different cellular states and distinct morphological phenotypes. Lack of definitive markers that are stably expressed on GSCs poses a further challenge to the diagnostic stratification of GB based on the evaluation of GSC content in tumor specimens [17].

Although a range of molecules like CD15, L1CAM, SOX2, and Prominin1/CD133 have been implicated as identification markers of GSCs, their diagnostic utility has been limited due to the phenotypic heterogeneity within the GSC compartment, constituted by cells in hierarchically distinct states [18–23]. For example, expression of Prominin1/CD133, historically one of the most investigated and arguably the best validated GSC marker, is sample specific, being restricted to only a subset of GSCs [24–27], and fluctuates significantly during cell cycle [28]. Furthermore, a reversible loss of CD133 expression in CD133⁺ GSCs was shown to accompany tumor propagation, as revealed in an experimental *in vivo* model of GB [24]. Considering that the tumor-propagating capacity of CD133[−] GSCs is comparable to that of CD133⁺ GSCs [24], the diagnostic utility of CD133 remains uncertain [29]. Phenotypic diversity and plasticity of GSCs as a means of adaptation to the tumor microenvironment have important implications for the continuing search for GSC markers that would be universally applicable for different subsets of GSCs and would be expressed unambiguously, regardless of cellular state.

In this regard, Glycoprotein A repetitions predominant (GARP) has recently emerged as a potential marker of human GSCs [7,30]. GARP is a type I transmembrane protein normally expressed on the surface of activated regulatory T cells, where it mediates tolerogenic functions in the tumor microenvironment of GB [7]. GARP consists of 662 amino acids and is composed of an extracellular domain with 20 leucine rich repeats, a hydrophobic transmembrane domain, and a 15 amino acid intracellular part. Recently, we have found

that GARP is also expressed by different types of GB cells, including GSCs, where it shows an atypical pattern of subcellular distribution characterized by GARP localization on both the cell surface and within the nucleus (GARP^{NU+}) [7]. Up until now, GARP expression in GSCs has only been shown in vitro, with several open-ended questions remaining. Namely, is GARP/GARP^{NU+} expression associated with a particular cellular state (self-renewal or differentiation) or a particular subtype of GSCs? Does the associated expression of GARP/GARP^{NU+} in GSCs persist during GB progression after therapy?

In the present study, these questions were addressed in vitro and in vivo by analyzing the expression of GARP/GARP^{NU+} in different subtypes of patient-derived GSCs with consideration of intratumoral heterogeneity and the longitudinal changes accompanying GB recurrence. For the first time, the present study examined the potential link of nuclear GARP expression with patient outcomes.

2. Materials and Methods

2.1. Cell Culture

The human glioblastoma cell line T98G was purchased from the ATCC (CRL-1690) and was cultured in Minimum Essential Medium Eagle supplemented with 10% FCS, 1% Glutamine, and 0.1% Primocin. The human melanoma cell lines, Mewo and Ma-Mel-19, were obtained from Dr. Daniela Kramer (Mewo, RRID:CVCL_0445, Cellosaurus) in Mainz, Germany, in 2021 and from Dr. Annette Paschen (Ma-Mel-19, RRID:CVCL_A156, Cellosaurus) in Essen, Germany, in 2014. Mewo cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FCS and 0.1% Primocin. Ma-Mel-19 cells were grown in RPMI 1640 supplemented with 10% FCS, 1% Glutamine, and 0.1% Primocin. T98G, Mewo, and Ma-Mel-19 cells were passed every 2 to 3 days by using Trypsin-EDTA. The cell lines T98G and Ma-Mel-19 were authenticated in August 2022 by PCR single locus technology. The results were compared to the online databases of the DSMZ and Cellosaurus (Eurofins Genomics Europe). Patient-derived GSC lines used in this study were established as previously described and have been well characterized in previous studies, in terms of their stem cell frequency (SCF) and expression of various GSC markers [28,31–33]. Additional information regarding their origin, SCF, predominant phenotype (Nestin^{+/–}, GFAP^{+/–}), and percentage of CD133-positive cells, as well as an exemplary analysis of the GSC markers, CD133, platelet-derived growth factor receptor alpha (PDGFR- α), and aldehyde dehydrogenase 1 family member A3 (ALDH1A3), can be found in Figure S1 [28,33,34]. In brief, excess glioblastoma tumor tissue was obtained from patients operated on at the Department of Neurosurgery of the Johannes Gutenberg University Medical Center Mainz (JG-UMC), with informed consent. The use of tumor tissue for research purposes was approved by the JG-UMC Institutional Review Board (permission 08.06.2017 #837.211.12(8312-F). For GSC isolation, a combined enzymatic and mechanical titration procedure was used as previously described [28]. To promote self-renewal, glioma cells were cultured in serum-free NeuroBasal (NB) medium supplemented with the following factors: B27 supplement (Invitrogen, Darmstadt, Germany) and the recombinant human cytokines, basic fibroblast growth factor 2 (bFGF) (10 ng/mL) and epidermal growth factor (EGF), (20 ng/mL) (Biochrom GmbH, Merck KGaA, Darmstadt, Germany). For in vitro differentiation, cells were subjected to EGF and bFGF withdrawal and assessed for the expression of neural lineage specific markers after 7 days. Self-renewal promoting conditions are hence referred to as “NB+bFGF/+EGF” whereas differentiation is indicated by “NB-bFGF/-EGF” in the manuscript.

2.2. Western Blot

Protein preparation and Western blotting were performed as previously described in Müller et al., 2023 [35]. Membranes were probed with the following antibodies: anti-CD133/1 (clone: W6B3C1), anti-PDGFR- α (D13C6) (Cell Signaling, #5241T, Danvers, MA, USA), anti-ALDH1A3 (Thermo Fischer Scientific, MA5-25528, Waltham, MA, USA), anti-p53 (DO-1) (Cell Signaling, #18032), anti-actin (C4) (Santa Cruz Biotechnology, sc-47778,

Dallas, TX, USA), anti-glia fibrillary acidic protein (GFAP) (DAKO, Z0334, Santa Clara, CA, USA), anti-p21 (Cell Signaling, #2947), anti-phosphorylated-histone H3 (Ser28) (Cell Signaling, #9713S), anti-HSP70 (Enzo Life Sciences Inc., Farmingdale, NY, USA), anti-mouse IgGκ light chain-binding protein horseradish peroxidase (Santa Cruz Biotechnology, sc-516102), goat anti-rabbit IgG H&L horseradish peroxidase (Abcam, ab205718, Cambridge, UK), goat anti-mouse IgG horseradish peroxidase (Santa Cruz Biotechnology, sc-2055), and goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, sc-2054). Signal intensity was analyzed via densitometry (<https://imagej.nih.gov>, accessed on 17 October 2023) [36].

2.3. Flow Cytometry

For flow cytometric analysis, the following fixable viability dye and antibodies were used: FVD506 (eBioscience #65-0866-14, San Diego, CA, USA), anti-GARP (Miltenyi #130-103-820 and 130-103-890, updated ordering numbers: 130-125-511 and 130-125-532, Bergisch Gladbach, Germany), anti-CD133 (epitope AC133, Miltenyi # 130-113-111), and their respective isotype controls (Miltenyi #130-113-434 and Miltenyi #130-113-200). Cells were stained with fixable viability dye prior to surface antibody staining of anti-GARP and anti-CD133. Cells were not fixed for the analysis.

Extensive validation of the anti-GARP antibodies mentioned above and a demonstration of their specificity can be found in Figures S2 and S3 as well as in previous work by Zimmer et al., 2019 [7]. In more detail, the anti-GARP antibodies from Miltenyi were validated against two other flow cytometry certified antibodies (Biolegend, 352506, San Diego, CA, USA; Origene, TA337028, Rockland, MD, USA) (Figure S3) and against the polyclonal anti-GARP antibody used in this study (Origene, AP17415PU-N) (Figure S2). Antibody specificity was demonstrated using GARP-overexpressing Mewo cells, resulting from transient transfection using the LOX-IMVI Cell Avalanche Transfection Reagent (EZ Biosystems, EZT-LOXI-1, College Park, MD, USA) as well as a LRRC32 overexpression plasmid (Origene, SC116699) and an empty vector control plasmid (Origene, PS100001) (Figure S3). Transfection was performed in accordance with the manufacturer's recommendations. Cells were stained with fixable viability dye and for surface GARP as described above 48 h post-transfection.

Flow cytometry was performed on a BD LSRII flow cytometer (Heidelberg, Germany) and was analyzed using Cytobank [37]. Doublets, debris, and dead cells were excluded from analysis (Figure S4).

2.4. Confocal Microscopy

Confocal imaging was performed on a Leica SP8 with HyD detector (Wetzlar, Germany) at the Imaging Core Facility (ICF) of the Forschungszentrum für Immuntherapie (FZI) of the University Medical Center Mainz as described before [7]. The following antibodies were used in the study: anti-nestin (Abcam, ab22035), anti-GARP (Origene, AP17415PU-N), and secondary antibodies goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 555 (both Thermo Fisher Scientific, Waltham, MA, USA). Validation and specificity of the anti-GARP antibody (Origene AP17415PU-N) for its use in confocal microscopy can be found in Figure S5 and in previous work by Zimmer et al., 2019 [7].

2.5. Animal Experiments

Animal experiments were performed at the Translational Animal Research Facility (TARC) of the JG-UMC, Germany, in accordance with the guidelines of the European Convention for the Protection of Vertebrates Used for Scientific Purposes and under the approval of the State Office of Chemical Investigations of Rhineland-Palatinate (permission #23 177-07/G12-1-020). Immunodeficient mice (strain NMRI) were purchased from a commercial supplier (Charles River Laboratories Germany). After an adaptation period of one to two weeks, mice were subjected to intracerebral injection of GSCs using a standardized procedure as described previously [34,38]. In brief, single-cell suspensions were prepared

from glioma sphere cultures by using a combined trypsin/mechanical titration procedure. Cells were washed twice in PBS and re-suspended in PBS at 2×10^4 cells/ μ L. Cell viability was determined by trypan blue staining. Single-cell suspensions were injected at 5 μ L into the caudato-putamen of the right hemisphere using a stereotactic frame (TSE Systems, Bad Homburg, Germany) and the following stereotactic coordinates in reference to the bregma: 1 mm (anteroposterior axis), 3 mm (lateromedial axis), 2.5 mm (vertical axis). Mice were sacrificed at the first manifestation of tumor-associated neurological symptoms.

2.6. GARP Immunohistochemistry and Immunofluorescence

Tumor-bearing mouse brains were extracted and fixed in 4% paraformaldehyde in PBS for at least 24 h at 4 °C as described previously [38]. Briefly, after fixation, brains were paraffin-embedded, dissected into 1–3 μ m thick coronal sections and analyzed by immunohistochemical or immunofluorescence staining using antibodies specific to human nestin (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), GFAP (DAKO, Z0334), or GARP (Origene, AP17415PU-N). Previous work has demonstrated the specificity of the anti-GARP antibody (Origene, AP17415PU-N) for its use in immunohistochemistry and immunofluorescence [7,39]. For analysis, ImageJ2 (Available online: <https://imagej.net/ImageJ2>, accessed on 16 August 2021) was used [40].

A GB patient cohort from Zimmer et al., 2019 [7], was reanalyzed to correlate the frequency of GARP^{NU+} cells in tumor tissue to patient overall survival regardless of IDH status. Patient characteristics are described in detail in Figure 1 of Zimmer et al., 2019 [7]. In brief, the patient cohort consisted of 35 newly diagnosed (WHO stage IV) GB patients from the Department of Neurosurgery in Idar-Oberstein, Germany, between January 2009 and May 2015. The median high and low survival times were 12 and 4 months. Primary tumor tissue was resected and stained for GARP via immunohistochemistry. Description of the immunohistochemical staining process can be found in Zimmer et al., 2019 [7]. The frequency of GARP^{NU+} was semi-quantified in tumor tissue with regions of labeled nuclei (categorized as >90%, >50%, >10% GARP^{NU+} cells) at the Institute of Neuropathology, University Medical Center Mainz, Germany [7].

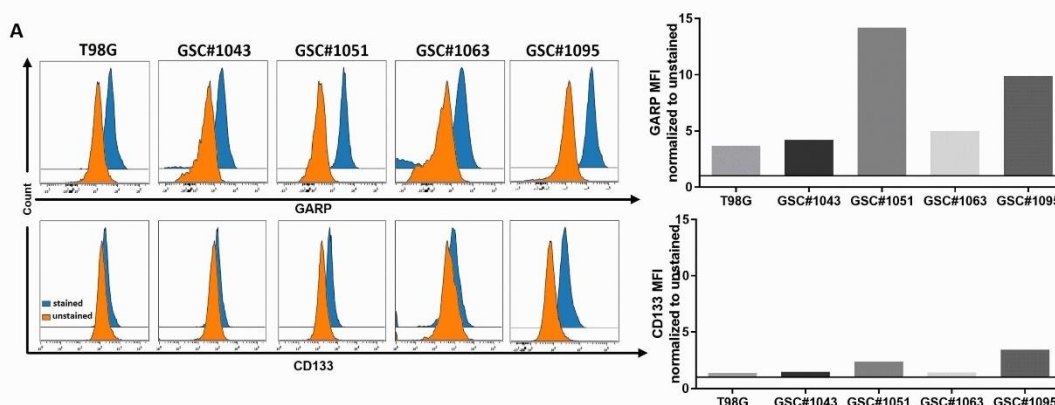


Figure 1. Cont.

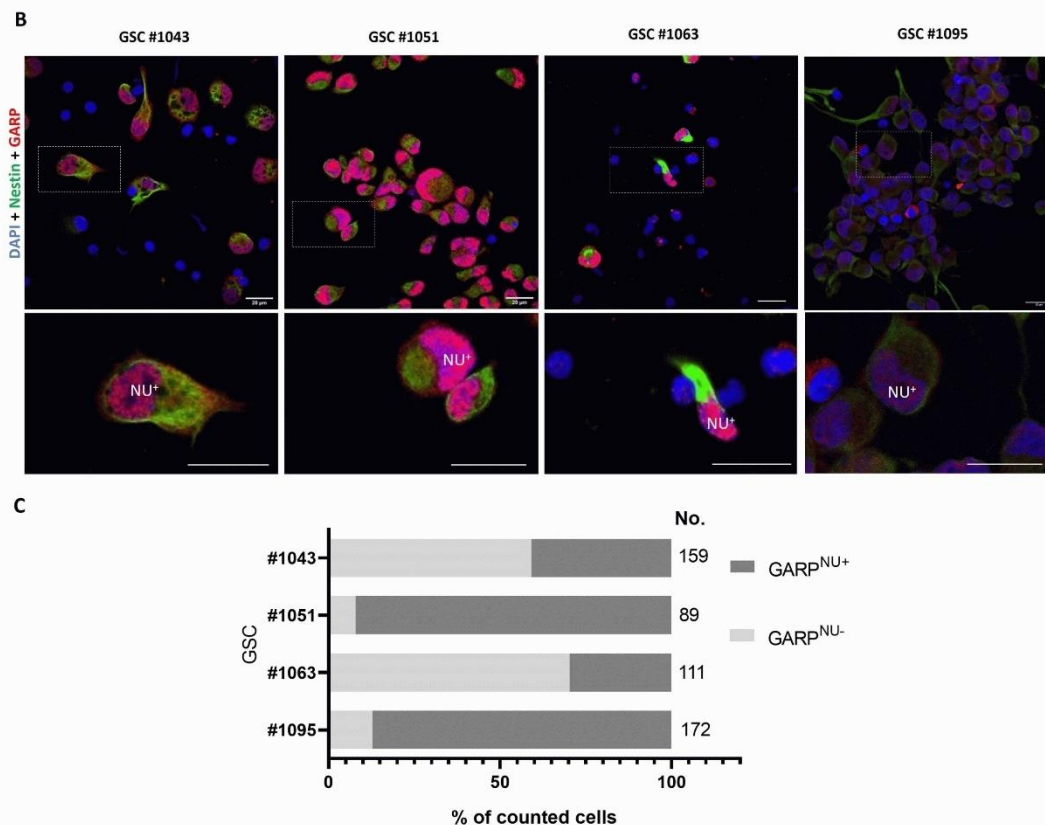


Figure 1. (A) Flow cytometric analysis of surface GARP and CD133 on different GSCs and the control, non-stem, GB cell line, T98G. Doublets, debris, and dead cells were excluded from the analysis. Mean fluorescence intensity (MFI) was normalized to the MFI of the respective unstained control. (B) Confocal images of GARP- and nestin-expressing GSCs and T98G. Cells were stained for GARP (red) and nestin (green). Cells were counterstained for their nuclei with Hoechst (blue). Note the intranuclear localization of GARP (NU⁺). Scale bar corresponds to 20 μ m. (C) Percentage of GARP^{NU+} cells were determined by counting GARP stained nuclei. “No.” indicates the number of counted cells for the analysis.

2.7. Cell Sorting

Single-cell suspensions of the GSC line, #1095, were stained sequentially with the following: fixable viability dye FVD780 (eBioscience #65-0865-14), unconjugated anti-GARP antibody (Origene, AP17415PU-N) or a control unconjugated IgG rabbit isotype antibody (R&D Systems, AB-105-C), followed by a PE-conjugated goat anti-rabbit secondary antibody (Invitrogen, P2771MP). Cells were sorted into GARP^{low} and GARP^{high} populations. Cell sorting gates were defined as the lower 10th (GARP^{low}) and upper 90th percentiles (GARP^{high}) of all cells. An example gating strategy and proof of positive GARP staining can be found in Figure S6. Debris, doublets, and dead cells were excluded from analysis. Sorting was performed using BD Aria II and III cell sorters at the Core Facility Flow Cytometry (CFFC) of the Forschungszentrum für Immuntherapie (FZI) of the University Medical Center Mainz.

2.8. Extreme Limiting Dilution Assay

The self-renewal capacity of GSC lines was analyzed by extreme limiting dilution assay (ELDA). In brief, single-cell suspensions were serially diluted in self-renewal promoting medium (NB+bFGF/+EGF) and seeded into 24 well plates. The number of replicates used for each serial dilution are indicated as follows: 12 for 100 cells/well, 18 for 50 cells/well, 24 for 25 cells/well, 58 for 12.5 cells/well, 24 for 6.25 cells/well, 18 for 3.125 cells/well, and 12 for 1.56 cells/well. Cells were incubated for three weeks to develop neurospheres. Wells were assessed for neurosphere formation; a positive result was recorded for each dose (number of seeded cells/well) if the examined well contained at least one neurosphere. Each experiment was repeated independently three times. Stem cell frequency (SCF) was calculated using the ELDA: Extreme Limiting Dilution Analysis webtool from the Walter and Eliza Hall Institute of Medical Research (<https://bioinf.wehi.edu.au/software/elda/>, accessed on 6 September 2023) [41].

2.9. Bioinformatic Pipeline

In a previous work, Kim et al., 2020, performed Illumina RNA-Sequencing on a total of 155 glioblastoma samples derived from 28 patients [32]. These consisted of primary, recurrent, and secondary recurrent tumors (128 samples) as well as GSC cultures developed from freshly resected tumor tissue (27 samples). We obtained unnormalized gene counts through the Gene Expression Omnibus database (GEO) under the accession number: GSE139533. Gene counts were normalized with DESeq2 and analyzed using the likelihood ratio test to decipher the effect of progressing tumor stages on transcript levels within the same patient [42]. Normalized counts for CD133 and GARP were plotted with GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

Survival analysis of CD133 and GARP was performed using OncoLnc (<http://www.oncolnc.org/>, accessed on 8 July 2023) which is based upon data generated by The Cancer Genome Atlas (TCGA) Research Network (<https://www.cancer.gov/tcga>, accessed on 8 July 2023) [43–45].

2.10. Statistics

Statistical analysis was performed with Student's *t*-test, the likelihood ratio test, the chi-squared test, or two-way ANOVA as indicated. Data are displayed as mean values $\pm$ SEM or $\pm$ SD as indicated. Survival curve comparison was analyzed using the log-rank (Mantel–Cox) test using GraphPad Prism. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$, and ns (not significant).

3. Results

3.1. GARP Expression Is Conserved across Different Types of GSCs In Vitro and In Vivo

We have previously shown that GARP is expressed by three human GSC lines and by the conventional human glioblastoma cell line, T98G [7]. The questions that remained were whether GARP expression is restricted to a particular type of GSCs or if it represents a common phenotypic trait shared by different subsets of GSCs. To address these questions, we analyzed the expression of GARP in a panel of heterologous GSC lines, differing in their self-renewal capacity, degree of differentiation, and expression of CD133, a proposed marker for GSCs in the past (Figure S1A). All GSCs used in this study invariably expressed nestin, a neural stem cell marker, but they varied in their expression of the astrocyte differentiation marker, GFAP, and CD133, a putative GSC marker (Figure S1A). A non-stem glioblastoma cell line, T98G, (ATCC CRL-1690) was analyzed in parallel as a control. Flow cytometry revealed that the surface expression of GARP varied across heterologous GSCs (Figure 1A). Notably, variations in GARP expression paralleled variations in CD133 levels indicating that GARP and CD133 are not mutually exclusive markers (Figure 1A). Line-dependent variations in GARP expression were also confirmed by microscopic evaluation of intracellular GARP (Figure 1B,C). Confirming our previous observations, microscopic analysis

revealed that GARP localization in GSCs is not restricted to the cell membrane, a normal localization site for GARP, but it also extends to the nuclear compartment (Figure 1B) [7,30]. The nuclear localization of GARP was evident in confocal microscopy with co-staining for nestin, an established marker of neural stem/progenitor cells expressed in the cytoplasm. The prevalence of cells with nuclear GARP (termed hereafter as “GARP^{NU+}”) varied between different GSC lines (Figure 1B,C) and mirrored the levels of surface-expressed GARP (Figure 1A), indicating a possible relationship between the two forms. For example, the GSC lines #1051 and #1095 had the highest levels of surface-membrane-associated GARP (Figure 1A), and they also exhibited a high proportion of cells with GARP^{NU+} (92.1% and 87.2%, Figure 1B,C). Vice versa, GSCs with moderate levels of surface GARP (#1043 and #1063, Figure 1A) had lower proportions of cells with GARP^{NU+} (40.9% and 29.7%, Figure 1C).

Our in vitro findings prompted us to test if GARP/GARP^{NU+} expression is sustained in vivo in GSCs involved in tumor propagation. To this end, we analyzed xenograft tumors grown from two GSC lines that express the lowest (line #1043) and highest (line #1051) levels of GARP in vitro (Figure 1). Both lines gave rise to highly invasive brain tumors as ascertained by immunohistochemical staining with an antibody specific for human nestin (Figure S7) and had comparable rates of tumor growth [34]. Immunofluorescence staining for GARP revealed its expression in both #1051 and #1043 xenografts (Figure S8). Notably, GARP expression in #1043 xenograft (low expressor in vitro, Figure 1) was comparable with that in #1051 xenograft (high expressor in vitro, Figure 1), suggesting that GARP expression in GSCs might be even more profound in the tumor context. Concordant with our in vitro findings, tumor-propagating GSCs also showed GARP localization in both the cytoplasm and nucleus (Figure S8). Additionally, GARP^{NU+} was observed to be co-expressed with nestin (Figure S8). These results further support the conclusion that GARP/GARP^{NU+} expression might be a common trait stably sustained (or even augmented) in GSCs involved in tumor propagation.

3.2. Intratumoral Heterogeneity of Subcellular Distribution Patterns of GARP

GBs are known for their high degree of intratumoral heterogeneity, which is thought to reflect the hierarchical diversity of cellular states generated by GSCs [12,46]. Our observation that heterologous GSCs vary in their levels of GARP/GARP^{NU+} (Figures 1 and S8) prompted us to check if this is a mere reflection of intertumoral diversity, GARP/GARP^{NU+} association with a particular GSC subtype or cellular state, or a hierarchical diversification taking place during tumor growth. To address these questions, we made use of isogenic GSCs (lines IT-726-#1, IT-726-#2, IT-726-#3a, IT-726-#3b, and IT-726-#4) that have been isolated from different regions of the same tumor (Figure S9—Cohort 2”—comparison line 1) and provide a unique model for analyzing the impact of intratumoral heterogeneity in an isogenic background [31,32]. Indeed, despite their identical genetic background, isogenic GSCs from the IT-726 set displayed notable morphological differences, considerable variations in their self-renewal capacity, and expression of GSC-associated markers CD133, ALDH1 A3, and PDGFR- α (Figure S1) [31,32].

Interestingly, we found no apparent correlation between CD133 expression and the degree of self-renewal activity. For example, the lines IT-726-1 and IT-726-3B had comparable degrees of self-renewal activity (Figure S1A), but they differed profoundly in the expression of surface CD133 (glycosylated epitope AC133) (Figure 2). Vice versa, the line IT-726-4 expressed similar levels of surface CD133 as the lines IT-726-2, IT-726-3A, and IT-726-3B, (Figure 2), but it stood out markedly from the other lines in terms of its extremely low self-renewal capacity (Figure S1A). In contrast to CD133, the expression of surface GARP was very similar across isogenic lines, and it did not parallel the striking difference in CD133 expression between the IT-726-1 line and its isogenic counterparts (Figure 2). In comparison to the uniform expression of surface GARP, the patterns of GARP subcellular distribution between IT-726 lines were heterogeneous, with the proportion of GARP^{NU+} cells varying across different isogenic lines (Figure 3A). The highest level of GARP^{NU+}

was found in line IT-726-2, which had a prominent expression of nuclear GARP in nearly every cell (Figure 3B, IT-726-2 upper panel). GARP expression was also examined on IT-726 cell lines grown in self-renewal-promoting (NB+bFGF/+EGF) versus differentiation-promoting (NB-bFGF/-EGF) conditions. Interestingly, IT-726-2 displayed a prominent expression of nuclear GARP in almost every cell regardless of culture condition. In contrast, other isogenic IT-726 lines exhibited a mixed pattern of GARP localization in both nuclear and cytoplasmic compartments in self-renewal-promoting conditions (NB+bFGF/+EGF) (Figure 3B, shown for IT-726-4). Notably, the nuclear localization of GARP appeared to be more profound when cells were grown in differentiation-promoting conditions (NB-bFGF/-EGF), suggesting an inverse correlation between GARP^{NU+} and self-renewal capacity. The IT-726-2 line, in which the GARP^{NU+} pattern was predominant (Figure 3B), had a lower self-renewal capacity when compared to the other isogenic counterparts (Figure S1A), consistent with this interpretation.

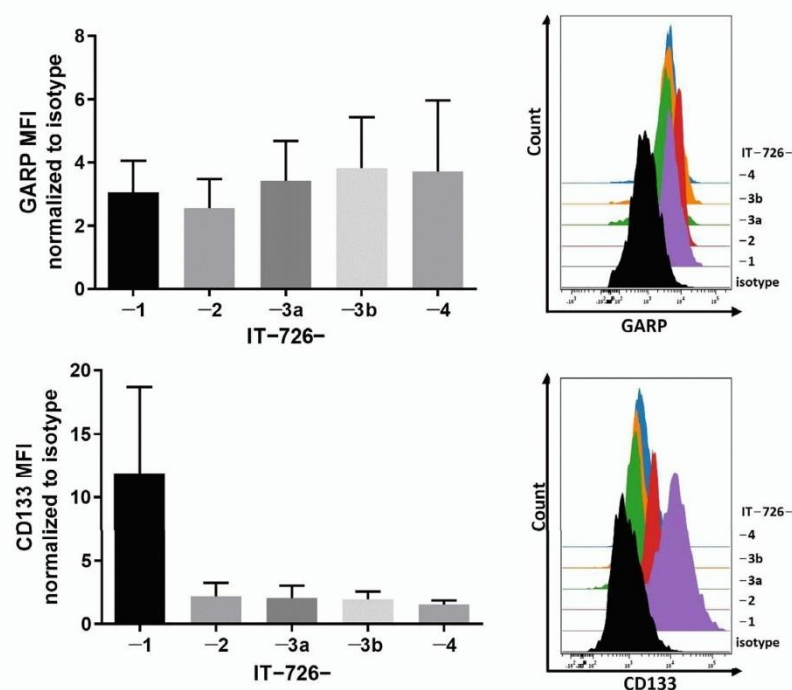


Figure 2. GARP expression in isogenic GSCs derived from newly diagnosed GB IT-726. Flow cytometric analysis of IT-726-1, -2, -3a, -3b, and -4. Doublets, debris, and dead cells were excluded from analysis. Mean fluorescence intensity (MFI) was normalized to the MFI of the unstained control. Histograms display one representative result of three independent measurements. Data are displayed as mean values $\pm$ SEM.

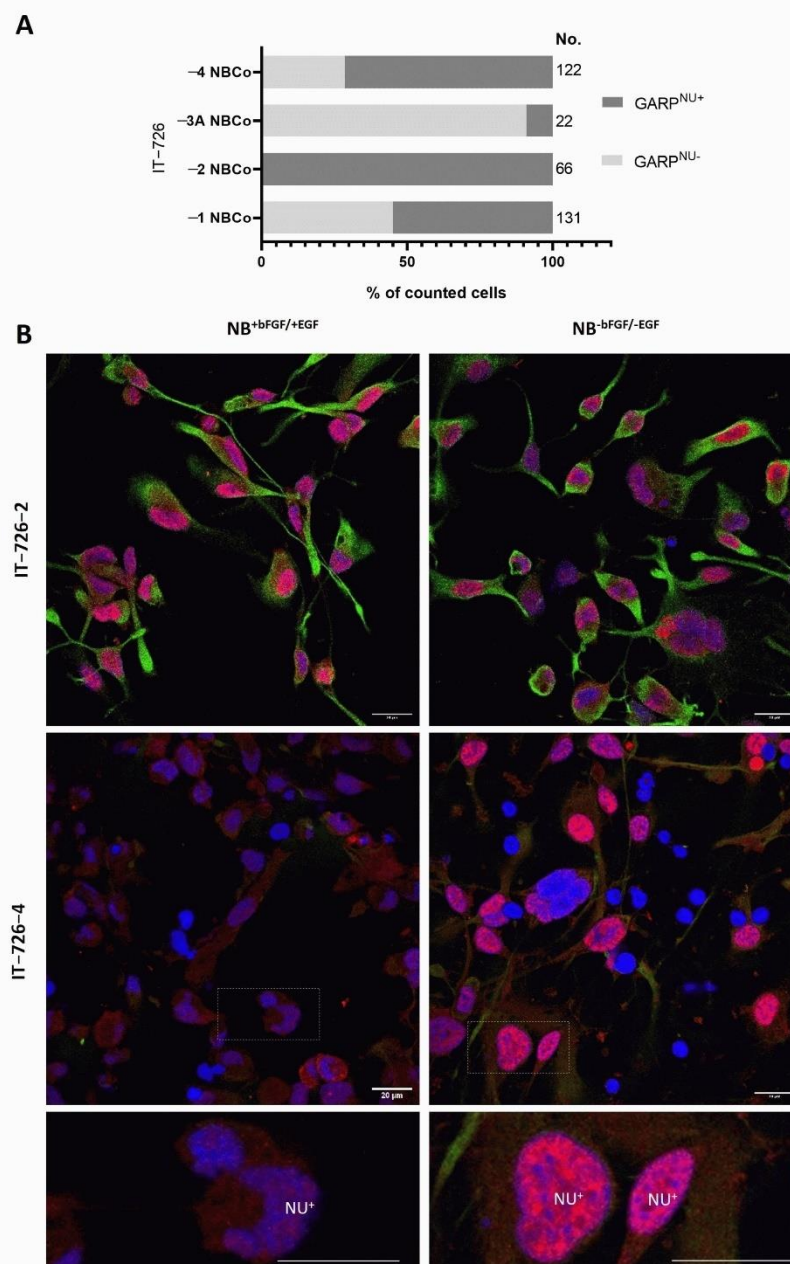


Figure 3. Analysis of expression and localization of GARP in isogenic GSC cell lines, which vary in differentiation states derived from different regions of the same tumor. (A) Number of GARP positive nuclei for GSC lines IT-726—1, -2, -3A, and -4 were analyzed by counting double positive (Hoechst and GARP) cell nuclei (NU⁺). “No.” indicates the number of counted cells for the analysis. (B) Confocal images of GARP- and nestin-expressing GSC IT-726 -2 and -4. Cells were stained for their nuclei with Hoechst (blue), GARP (red), and nestin (green). Note the intranuclear localization of GARP. Scale bar corresponds to 20 μ m.

3.3. Relationship between GARP Expression and GSCs Stemness

The dual capacity of self-renewal and differentiation are the fundamental and unique properties of stem cells. We therefore sought to determine if there is an association between GARP expression and self-renewal. To this end, cell populations differing in GARP expression (GARP^{high} or GARP^{low}) were FACS sorted from the GSC line #1095 and compared with respect to self-renewal activity by ELDA. A demonstration of the sorting efficacy and quantification of surface GARP expression on GARP^{high} vs. GARP^{low} sorted cells via flow cytometry can be found in Figure S6A. The GSC line #1095 was chosen for these investigations because of its well-established stemness attributes as well as molecular and cellular responses to clinically relevant treatments in vitro and in vivo [28,33–35]. The results of ELDA assessments revealed that GARP^{high} and GARP^{low} populations of GSCs differ in their self-renewal propensity, which was significantly ($p = 2.48 \times 10^{-16}$) lower in the GARP^{high} subpopulation compared to GARP^{low} subpopulation (Figure 4).

As loss of self-renewal is a prerequisite for stem cell differentiation, the outcome of the ELDA experiments raised the possibility that GARP expression may be related to differentiation of GSCs. To address this question, GARP^{high} and GARP^{low} GSCs were subjected to comparative assessments for the differentiation-inducing factor p21 and the differentiation-associated markers, GFAP and PDGFR α , activated during astrocyte or oligodendrocyte differentiation. The results showed that GARP^{high} GSCs had considerably higher steady-state levels of p21 compared to GARP^{low} GSCs, which seems consistent with the interpretation that increased expression of GARP is associated with a more differentiated state. However, an elevated level of p21 was unaccompanied by increased expression of GFAP or PDGFR α in GARP^{high} GSCs. Quite the contrary, the expression of either GFAP or PDGFR α was found to be lower in GARP^{high} GSCs than in GARP^{low} GSCs (Figure 5) with the difference in PDGFR α levels being especially profound (Figure 5B). Although the difference in GFAP expression between GARP^{high} and GARP^{low} GSCs was less profound, it was also confirmed by using a different approach, namely the estimation of GFAP-positive differentiating cells by immunofluorescence staining (Figure S10). A decline in proliferative activity is an important functional hallmark of normal stem cell differentiation. Deviating from this rule, GARP^{high} GSCs, which had a reduced self-renewal capacity in comparison to GARP^{low} GSCs (Figure 4), had comparable levels of the proliferation marker PHH3 (Figure 5). Collectively, our data indicate that increased expression of GARP correlates with reduced self-renewal but not with the cessation of proliferation or induction of phenotypic traits of neural differentiation.

3.4. GARP mRNA and Surface Protein Levels Do Not Predict GB Patient Survival

Having established that GARP is expressed in patient-derived GSCs, we sought to determine whether a correlation exists between GARP expression and GB patient survival. To address this question, gene expression and survival data from the TCGA database were analyzed for GARP and CD133 by using OncoLnc.org (Figure S9—Cohort 1) [43–45]. Based on the TCGA dataset, consisting of 152 patients with newly diagnosed glioblastomas, all patients analyzed were stratified by their expression levels of GARP into either “GARP-high” (upper 50%) or “GARP-low” (lower 50%) groups and were analyzed for their survival rates via the online tool OncoLnc [43]. We could not find any significant difference in survival between GARP-high and GARP-low groups (Figure 6A). Similarly, no significant correlation was found between survival and CD133 expression (Figure 6A). In a second approach, GARP and CD133 transcript levels were compared between newly diagnosed glioblastomas (ndGB) and progressed recurrent glioblastomas (recGB) as depicted in Figure S9—Cohort 2—comparison line 2. To this end, we retroactively analyzed RNAseq data from a database that compiles RNAseq data for ndGBs (23 patients) or recGBs (21 patients) as well as 27 primary cultures derived from either ndGB (ndGB-GSCs, 17 cultures) or recGB (recGB-GSCs, 10 cultures) [34]. GARP and CD133 mRNA expression were compared between ndGB samples (ndGB tissues and ndGB-GSC cultures) and recGB samples (recGB tissues and recGB-GSCs). The results showed that expression levels of GARP do not differ

significantly between ndGB and recGB samples whereas CD133 levels were found to be significantly reduced in recGB samples compared to ndGB samples (Figure 6B). In a third approach, surface GARP expression was compared between isogenic ndGB-GSCs and recGB-GSCs isolated from ndGB and recGB tumors of the same patient (Figure S9—Cohort 2—comparison line 3). Both ndGB-GSCs and recGB-GSCs showed virtually the same levels of surface GARP expression, whereas the level of CD133 was significantly lower in recGB-GSCs in comparison to ndGB-GSCs (Figure 6C). This agreed with the results of the RNAseq analysis (Figure 6B) as both GARP transcript and surface GARP (Figure 6C) levels were consistently expressed regardless of disease progression. Interestingly, in contrast to GARP transcript and surface GARP levels, it was found that the percentage of GARP^{NU+} cells were elevated in the recurrent GSC line, IT-654 (Figure 6D).

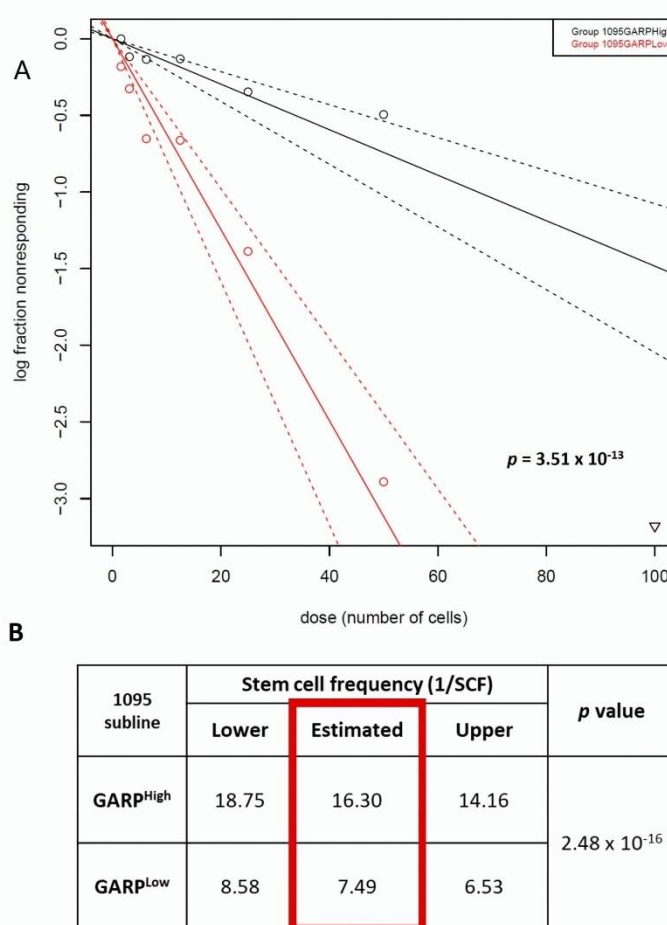


Figure 4. Quantitative assessments of self-renewal capacity by extreme limiting dilution assay (ELDA). (A) Representative results. (B) The pooled results from three independent experiments are indicated in the table. GARP^{high} and GARP^{low} correspond to isogenic GSCs differing in their GARP expression, which were FACs sorted from the GSC line #1095. Estimates of the stem cell frequency (SCF) are framed in red, while lower and upper indicate the confidence intervals for 1/SCF. Statistical significance between groups was calculated by chi-squared tests.

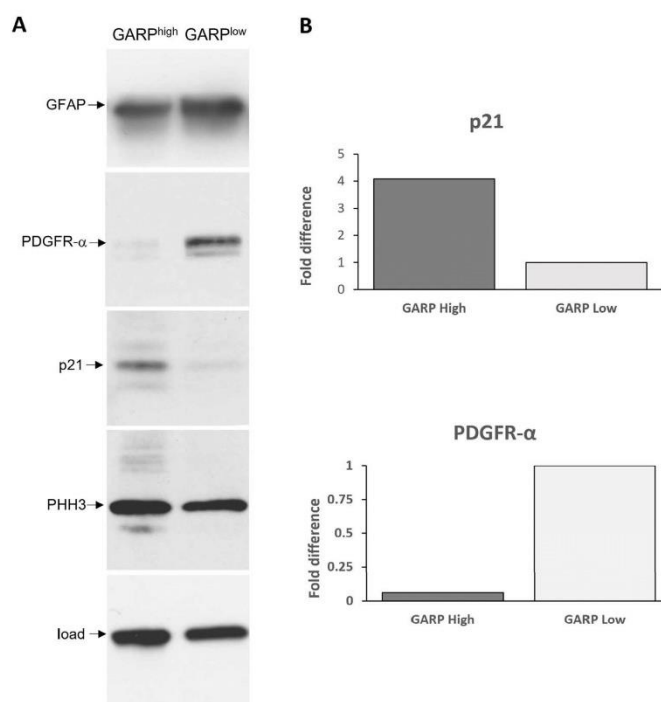


Figure 5. Comparative assessments of glial fibrillary acidic protein (GFAP), platelet-derived growth factor receptor alpha (PDGFR- α), p21, and phosphorylated histone H3 (PHH3) in FACS-sorted GARP^{high} and GARP^{low} isogenic GSCs (#1095) by Western blot. (A) Representative results. HSP70 was used as a loading control. The following antibodies were used to probe the membranes: anti-GFAP (DAKO, Z0334), anti-PDGFR- α (D13C6) (Cell Signaling, #5241T), anti-p21 (Cell Signaling, #2947), anti-phospho-histone H3 (Ser28) (Cell Signaling, #9713S), anti-HSP70 (Enzo Life Sciences Inc.), anti-mouse IgG κ light chain-binding protein horseradish peroxidase (Santa Cruz Biotechnology, sc-516102), and goat anti-rabbit IgG H&L horseradish peroxidase (Abcam, ab205718). (B) PDGFR- α and p21 bands were quantified by densitometry.

Collectively, these results obtained via different experimental approaches indicate that expression of GARP mRNA and surface protein remain at a constant level throughout GB progression and after therapy—in contrast to the fluctuating expression of CD133. This sustained expression of surface GARP and GARP transcript levels in ndGBs and recGBs suggests the potential utility of GARP as a reliable GSC biomarker, which persists at different tumor stages, possibly allowing for the detection of potential residual disease of a remarkably invasive cancer type.

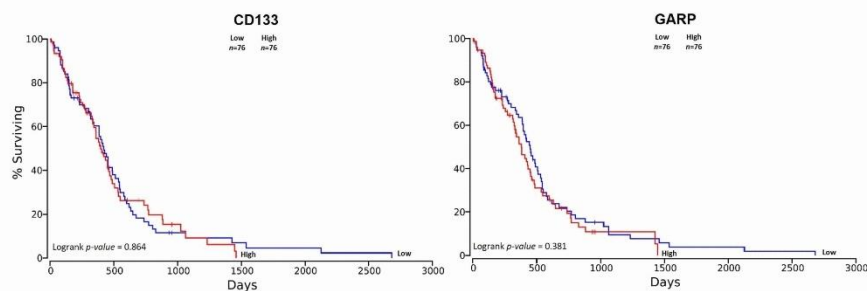
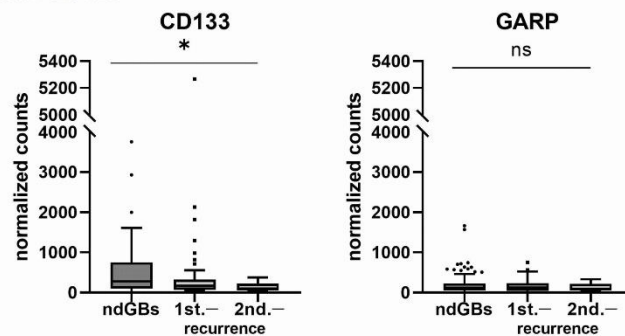
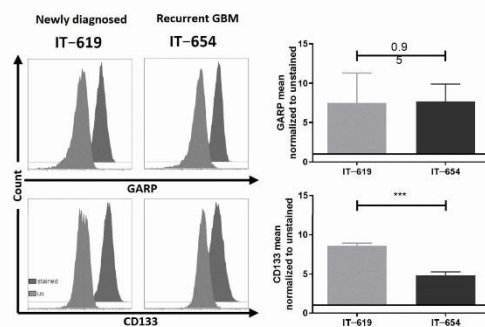
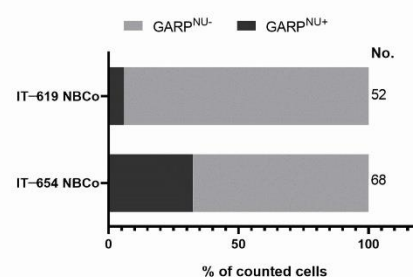
A. Newly diagnosed glioblastoma**B. ndGBs vs recurrent GBs****C.****D.**

Figure 6. GARP expression in GB is unaffected throughout therapy. **(A)** Survival analysis of GARP and CD133 based on data available through The Cancer Genome Atlas (TCGA). GARP and CD133 mRNA expression data of 152 primary glioblastomas were divided 50/50 into either “low” expression or “high” expression and were analyzed for patient survival. **(B)** Retrospective analysis of transcriptomic data of 155 GB samples from 28 patients of Kim et al., 2020 [32]. ndGBs, first, and second recurrent tumors were analyzed for their GARP and CD133 mRNA levels across tumor stages. **(C)** Flow cytometric analysis of IT-619 and IT-654. Doublets, debris, and dead cells were excluded from analysis. Recurrent IT-654 GSCs exhibited stable surface GARP levels after TMZ and radiotherapy, whereas expression of CD133 decreased after treatment. The MFIs were normalized to the unstained control. $n = 3$. Significance was calculated by Student’s t-test and is indicated as follows: * $p < 0.05$, *** $p < 0.001$, and ns (not significant). **(D)** Number of GARP-positive nuclei for GSC IT-619 and IT-654 were analyzed by counting double-positive (Hoechst and GARP) cell nuclei. “No.” indicates the number of counted cells for the analysis.

3.5. Nuclear Localization of GARP Correlates with Poor Survival in Patients with GB

As we observed an upregulation of the percentage of GARP^{NU+} cells in the recurrent GSC line, IT-654 (Figure 6D), we wanted to explore a possible link between GARP^{NU+} and the survival rate of GB patients (Figure S9—Cohort 3). Therefore, we retroactively assessed GARP^{NU+} levels in tumor tissue from a cohort of 35 newly diagnosed GB patients (WHO stage IV) and correlated them to patient overall survival (Figure 7, representative images) [7].

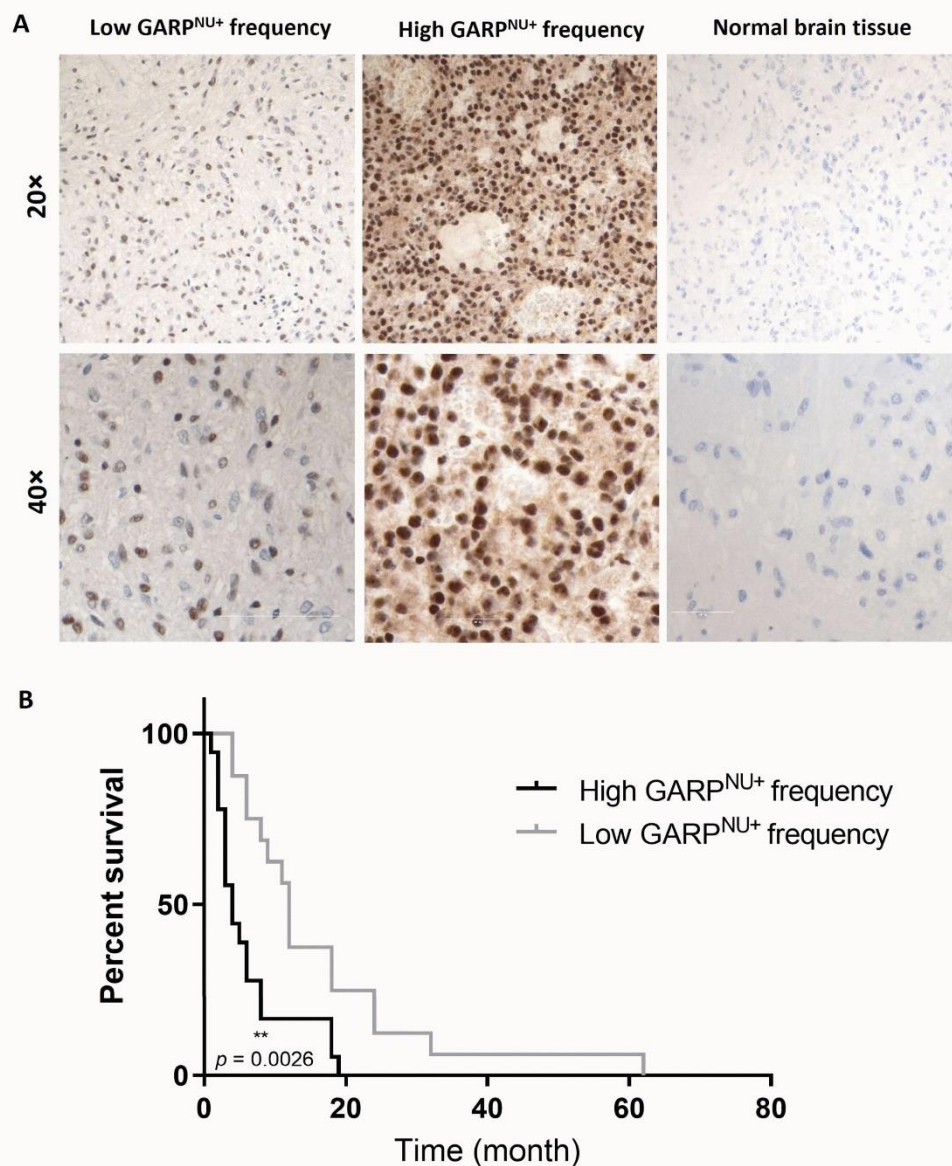


Figure 7. Nuclear GARP is a potential new prognostic biomarker for GB patient survival. (A) Immunohistochemistry of GARP in glioblastoma, GB (WHO grade IV) with low frequency of labeled

nuclei (magnification $\times 20$ and $\times 40$) and GB with palisading necroses and a high frequency of stained nuclei (magnification $\times 20$ and $\times 40$). Normal brain tissue had no detectable GARP expression. Bar corresponds to 50 μm ($40\times$) and 200 μm ($20\times$), respectively. (B) Survival analysis of 35 GB patients based on their GARP-positive nuclei counts (1: high frequency, $n = 16$ and 2: low frequency, $n = 19$). Comparison of survival curves was performed by log-rank (Mantel–Cox) test (** $p < 0.01$).

Notably, all GB patients in the cohort were found to express GARP^{NU+} but varied in their frequency of GARP^{NU+} cells. Therefore, we divided the cohort into two groups based on their frequency of GARP^{NU+} expression. The first group encompassed 19 GB patients with tumors having a low frequency ($\sim 50\%$) of GARP^{NU+} cells. The other group included 16 patients with a high frequency ($>90\%$) of GARP^{NU+} cells. In striking contrast with the transcriptomic analysis, which showed a consistent lack of correlation between GARP mRNA levels and patient survival (Figure 6A), stratification by GARP^{NU+} revealed a significant correlation between GARP^{NU+} and GB patient survival (Figure 7B). The results showed that patients with a low frequency of GARP^{NU+} had a significantly longer overall survival in comparison to patients with a high frequency of GARP^{NU+} (medians low: 12 months, high: 4 months; $p = 0.0026$, Figure 7B). These results indicate that the abundance of the GARP protein in the nuclear compartment—not GARP transcript levels—is associated with survival in patients with GB.

4. Discussion

GSCs comprise a heterogeneous and highly volatile group of cells, which can switch between different phenotypes and molecular programs in response to environmental changes. The high degree of phenotypic plasticity displayed by GSCs poses a challenge in the development of GSC-based diagnostic and GSC-targeting therapeutic strategies. The continuing search for GSC-associated markers has led to the identification of several molecules expressed in some but not all subtypes of human GSCs or associated with some but not all cellular states [11,12]. One approach to counterbalance the phenotypic diversity of GSCs is to simultaneously target multiple markers associated with different types of GSCs, in order to increase the diagnostic coverage of the GSC content in a highly heterogeneous milieu of GBs [11,28,32,47,48]. An alternative possibility is that some phenotypic traits may be conserved across heterogeneous GSCs. Our data indicate that expression of GARP/ GARP^{NU+} may be one such trait. We provide several lines of evidence that GARP/ GARP^{NU+} is expressed in otherwise phenotypically distinct GSCs (Figures 1 and 3) and persists invariably across different cellular states (Figure 3). In the past, several groups have tried to identify universal GSC markers. One challenge is that most of the previously identified putative markers of GSCs, e.g., CD133, are not universally expressed in all types of GSCs, which limits the diagnostic utility of such markers for estimating GSC content in tumors [26]. In this regard, both surface GARP and GARP^{NU+} expression appear to be invariably expressed in phenotypically distinct GSCs including CD133⁺ and CD133[−] subtypes, under in vitro and in vivo experimental conditions (Figures 1, 3 and S8) and in different stages of GB progression (ndGBs or recGBs) (Figure 6).

Our data indicate that GSC-associated expression of GARP persists in different cellular states. However, the degree of GARP expression varies between different cellular states. Interestingly, we find that expression of GARP is elevated in the state associated with a reduced self-renewal but not proliferative capacity and in conjunction with loss of differentiation-associated traits (Figures 4, 5 and S10). Such a pattern is reminiscent of the transit-amplifying state during neurogenesis whereby slow-cycling neural stem cells must first exit from the state of self-renewal and convert into more differentiated but uncommitted and fast-proliferating transit-amplifying progenitors, prior to entering the lineage-commitment stage and differentiation [49]. The simultaneous reduction in self-renewal and differentiation-associated traits without loss of the proliferation activity seen in GARP^{high} GSCs suggests that GARP may have a role in GSCs' transition from the

slow-cycling self-renewal state to a more differentiated and proliferation-competent state similar to that of transit-amplifying progenitors. It should be noted that even the complete loss of self-renewal does not lead to a loss of the tumor-propagating capacity of GSCs, and recent evidence indicates that GB propagation is driven primarily not by self-renewing GSCs but their non-self-renewing progenies [50].

It is important to note that GARP expression is not limited to GSCs alone. Cancer cells, including glioblastoma (as shown in this work with T98G in Figure 1A), activated regulatory T cells and B cells, and platelets are all known to express GARP on their surfaces [28]. Therefore, GARP alone cannot be used to identify GSCs but rather in combination with a panel of other markers to better distinguish between GSCs and other GARP-positive cells in the tumor microenvironment. In this regard, our finding that elevated GARP expression coincides with a significant increase in p21 expression suggests that dual assessments for GARP and p21 may enable a distinguishment between GSC and non-GSC cells. Considering that p21 plays important roles in the maintenance of neural stem cells and is one of the factors implicated in GB radioresistance, the concomitant elevation of p21 and GARP in GSCs further supports the potential merits of GARP as a predictive and prognostic biomarker for GB [51,52]. A limitation of this exploratory work is that it mainly analyzed the expression of GARP in comparison to one GSC reference marker, CD133. Future studies are needed to analyze in depth the association of GARP expression with an expanded panel of putative GSC markers to further evaluate how universally and stably expressed GARP is on GSCs, and on different cellular components of the tumor microenvironment, especially those that are known to express GARP, like activated regulatory T cells and platelets.

Intriguingly, whereas GARP mRNA levels are comparable between ndGBs and recGBs, the level of GARP^{NU+} protein correlated with poor survival in patients with GB (Figure 7). Notably, the critical cutoff for GARP^{NU+} was >90% (Figure 7), which is significantly higher than the 10% cutoff for CD133 expression implicated as a predictive marker for GB recurrence [53].

Although the link between a high frequency of nuclear GARP and poor outcomes of GB patients provides a novel and intriguing insight into the previously unsuspected role of GARP^{NU} in GB, it is important to also acknowledge the limitations of this exploratory study. One is the small patient cohort size ($n = 35$). The relationship between GARP^{NU+} and clinical outcome from GB must be validated in future studies using larger datasets. A further confirmation in larger follow-up studies is a prerequisite for the conclusion on the diagnostic value of GARP^{NU+} as a prognostic biomarker for GB.

Cancer stem cells are related to reduced survival in glioblastoma patients [11,12]. Therefore, it was surprising to see that a high frequency of GARP^{NU+} tumor cells was linked to reduced overall survival, despite the observed upregulation of GARP^{NU+} in differentiation-promoting conditions (NB-bFGF/-EGF) (Figure 3B). One possible explanation for this is that elevated GARP levels are linked to enhanced immunosuppression in the tumor microenvironment [7,39]. In more detail, previously, we demonstrated both in melanoma [39] and in glioblastoma [7] that tumor cells upregulate the expression of GARP and thus gain tolerogenic potential. This in turn aids in the suppression of effector CD4⁺ and CD8⁺ T cell function, required for anti-tumor immune responses, and correspondingly induces suppressive regulatory T cells, which further contribute to the suppression of effective anti-tumor immune responses. Furthermore, the upregulation of GARP, an inhibitory protein, upon the differentiation process of cancer stem cells is consistent with previous reports by Ullah et al., 2020, who similarly demonstrated that the immune checkpoints PD-L1 and HLA-G are upregulated by cancer stem cells upon differentiation [54]. The principal binding partner of GARP, TGF- β , has been shown to induce the expression of PD-L1, but it remains unclear if GARP expression can as well [55,56]. It is worth noting that the simultaneous targeting of GARP, TGF- β 1, and PD-1 has been shown to be an effective combination therapy, capable of restoring T effector cell function and overcoming resistance to PD-1/PD-L1 blockade [57,58]. Future studies are planned to clarify the relationship

between GARP, PD-L1, and differentiation to determine if their contribution to immune suppression is responsible for the observed reduction in patient survival.

Interestingly, we found a discrepancy between RNAseq data from primary tumor tissue samples (Figure 6A) and our histological analysis of the frequency of GARP^{NU+}-positive cells in GB patient samples (Figure 7). Whereas no relationship between GARP transcript levels and patient survival was detectable in the TCGA data (Figure 6A), the frequency of GARP^{NU+} GB cells seems to be a suitable prognostic marker for patient survival. It should be considered that the tissue samples used for TCGA RNAseq analysis (Figure 6A) presumably consisted of tumor lysates, which contain a multitude of cell types, ranging from tumor cells to immune cells, up to healthy tissue. As information on the cellular origin of the transcripts is missing due to the bulk sequencing, otherwise significant differences between donors can be diluted into insignificant results based on the individual composition and frequencies of cell types included in the analysis. In addition, GARP mRNA can be detected in many tissues, e.g., heart, kidney, liver, and lung, whereas surface expression of the GARP protein itself seems to be limited to only a number of cell types, e.g., activated regulatory T cells [59], platelets [60], various cancers like GB and malignant melanoma [7,39], and mesenchymal stem cells [61], further contributing to a decreasing validity. Therefore, the identification of the cell type analyzed in RNAseq is key to interpreting and understanding future datasets.

More advanced methods like spatial transcriptomics, multiplex immunofluorescence, and spatial multi-omics single-cell imaging are more fitting to further enhance our understanding of GARP transcript and protein levels in glioblastoma cells and their surrounding microenvironment, as well as their distribution within subcellular compartments [62]. The additional information gained by these techniques would enable the identification of different cell types, their localization within the tumor and relation to other cells of the tumor microenvironment, and the determination of whether a surface or intranuclear localization of the GARP protein is present in these cells. Furthermore, the exclusion of certain cell types (e.g., regulatory T cells or platelets) from the analysis would enable a better understanding of GARP and its subcellular localization on patient outcomes.

Our data suggest that nuclear localization of the GARP protein—rather than abundance of the GARP transcript—is a factor associated with GB progression after therapy. Our finding that GARP is localized to the nucleus is novel and intriguing, as GARP has previously been characterized only as a surface and secreted protein, which currently has no annotated nuclear localization signal (NLS). Interestingly, the use of nuclear localization of an otherwise surface-associated protein as a prognostic marker has been described before [63–65]. One such example is the protein Src, which plays a key role in cell morphology, motility, proliferation, and survival [66]. Urciuoli et al., 2017, was able to show in human osteosarcoma that nuclear localization of Src correlates with overall survival and therefore has relevance as a prognostic marker for osteosarcoma patients [64]. Likewise, it has also been described that PD-L1, a T cell inhibitory molecule in cancer, shows a nuclear localization as a reaction to therapy. In more detail, PD-L1 is translocated from the cell surface into the nucleus as a reaction to high-dose doxorubicin therapy regimens. The nuclear localization of PD-L1 was described as a prognostic biomarker, as patients with low PD-L1 nuclear expression had significantly fewer circulating cancer cells and exhibited a longer overall survival [63,65]. While the mechanisms of GARP nuclear localization in GSCs still have to be elucidated, the potential clinical implications of this previously unknown phenomenon are clear given the critical role of GARP in the activation of TGF- β , one of the key factors [67] contributing to GB progression particularly via the maintenance of GSCs via the induction of, e.g., Sox2 and LIF expression [68,69]. Considering that targeting TGF- β -activating ligands in GB has been intensively explored as a promising therapeutic strategy [67,70], the clarification of GARP^{NU+} activities in GSCs may provide novel insights into the interaction of GARP and TGF- β , as TGF- β activation is known to trigger the nuclear localization of proteins like Smad and Smad4 [71]. Further pointing to the potential merit of GARP as a diagnostic and therapeutic target is the dual impact of GARP on cancer

progression—via the modulation of regulatory T cells and through the direct activities of GARP exerted on cancer (stem) cells themselves.

5. Conclusions

The scope of the present study was to evaluate GARP as a biomarker for heterogeneous GSCs and to determine the effects of GARP on GB patient outcomes. Based on our data, we propose that GARP^{NU+} could potentially serve, in combination with existing GSC markers, as a universal and stably expressed marker for different subsets and cellular states of GSCs as well as a possible prognostic marker for patient outcomes in GB. We propose that GARP assessments may provide the means to identify not only self-renewing GSCs but also their progenies that exit from self-renewal but retain proliferative activity. Further validation of this hypothesis in future studies will require analyses of larger patient cohorts using an extended panel of markers associated with GSCs and GB progression. Future investigations should focus on addressing mechanistic questions, such as the functional significance of GARP in regulating GSC-specific functions, by employing knockdown and/or overexpressing lines, as well as further investigating the role of nuclear GARP, its nuclear retention, and functional relevance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers15245711/s1>, Figure S1: Heterologous GSC lines differing in their self-renewal capacity, Figure S2: Anti-GARP antibody validation for flow cytometry, Figure S3: Specificity demonstration and validation of anti-GARP antibodies, Figure S4: Flow cytometric gating strategy for GSCs, Figure S5: Anti-GARP antibody validation for confocal microscopy, Figure S6: Flow cytometric gating strategy for GARP^{high} and GARP^{low} sorted GSCs, Figure S7: Invasive xenograft tumors arisen from GSC lines, #1051 and #1043, Figure S8: GARP is expressed in xenograft tumors arisen from GSC lines, #1051 and #1043, Figure S9: Study design and models used for the assessment of GARP, Figure S10: Frequency of GFAP⁺ GARP^{high} and GARP^{low} GSCs.

Author Contributions: Conceptualization: N.Z., A.T., E.K., J.T., F.R., V.M. and C.S.; methodology: N.Z., E.R.T., A.M., P.L. (Philipp Licht), P.L. (Petra Leukel) and B.S.; validation: N.Z. and E.R.T.; formal analysis: N.Z., E.R.T., A.M. and P.L. (Philipp Licht); resources: A.T. and E.K.; writing—original draft: N.Z., E.R.T., A.T. and E.K.; writing—review and editing: all authors; visualization, N.Z.; project administration: N.Z.; supervision: A.T. and E.K.; funding acquisition: A.T. and E.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The use of tumor tissue for research purposes was approved by the JG-UMC Institutional Review Board (permission 08.06.2017 #837.211.12(8312-F) and patients' informed consent. Animal experiments were performed in the Translational Animal Research Facility (TARC) of the JG-UMC, Germany, in accordance with the guidelines of the European Convention for the Protection of Vertebrates Used for Scientific Purposes and under the approval from the State Office of Chemical Investigations of Rhineland-Palatinate (permission #23 177-07/G12-1-020).

Informed Consent Statement: Written informed consent of all patients was obtained for “scientific use of tumor tissue not needed for histopathological diagnosis” in the admission contract of Idar-Oberstein hospital.

Data Availability Statement: Data are available in a publicly accessible repository that does not issue DOIs. Publicly available datasets were analyzed in this study. This data can be found here: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139533> and here: <http://www.oncolnc.org/> (accessed on 9 April 2021).

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3.3 PAPER 3

Oxidative Stress Differentially Influences the Survival and Metabolism of Cells in the Melanoma Microenvironment

Figure 11: Graphical abstract (Paper 3)

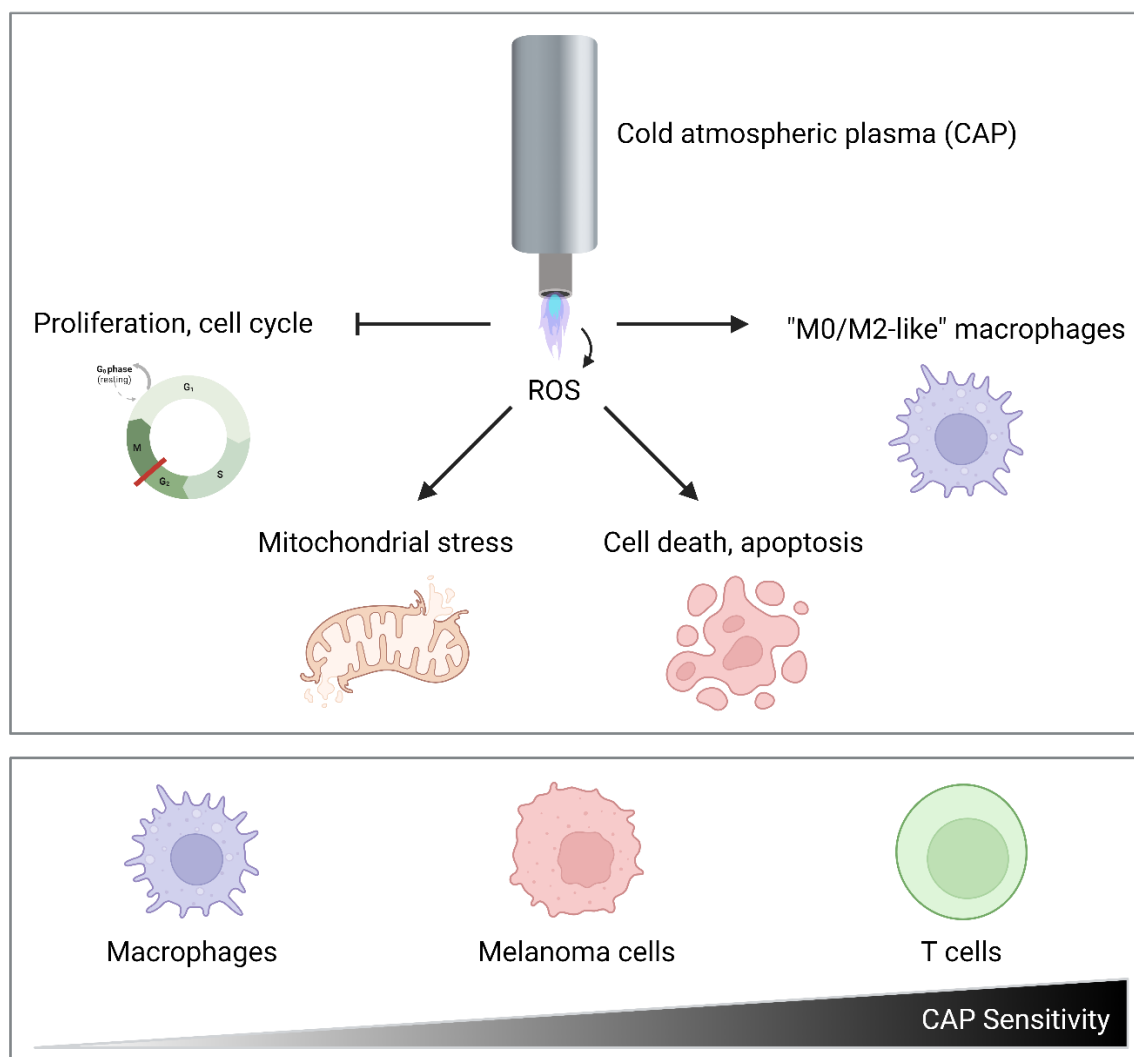


Fig. 11: Graphical abstract of **Paper 3**. Created with BioRender.com (2024)¹⁵.

3.3.1 SUMMARY

Striking progress have been made in the treatment of melanoma in the last decade. The introduction of targeted therapies and ICIs into the clinics has greatly improved the overall survival of advanced stage patients^{3–5,7,317,318,322}. Despite these improvements, a significant portion of patients fail to respond to these therapies or rapidly develop resistance against them, leaving them with limited treatment options^{7,314,323,336–339}.

CAP represents a promising anticancer approach by targeting the redox balance in cancer cells³⁴⁹. CAP is a partially ionized gas that also consists of neutral atoms, excited electrons, and

electromagnetic radiation, which can be applied within physiological temperatures. The interaction of CAP with biological material results in the production of RONS. Cancer cells are innately more vulnerable to increases in oxidative stress, and many studies have shown that CAP exhibits selective anticancer properties. However, little is known regarding how CAP influences immune cells, key determiners of immunotherapy response in the melanoma microenvironment^{10,101}. This study aimed to determine the influence of CAP on cells found in the melanoma microenvironment *in vitro*, namely human melanoma cells, T cells, and monocyte derived macrophages¹⁰.

CAP treatment was found to increase intracellular ROS levels¹⁰. CAP reduced the proliferation and viability of T cells and tumors cells (in 2D and 3D culture) in a dose dependent manner. This resulted from CAP induced G2/M cell cycle arrest, increased mitochondrial stress, and apoptosis. The anti-proliferative and cytotoxic effects of CAP could be partially reversed through the application of antioxidants. Cells displayed varying sensitivity to CAP treatment with T cells being the most affected. M1 macrophages exhibited higher levels of mitochondrial stress following CAP treatment. It was also found that CAP treatment polarizes macrophages to an anti-inflammatory “M0/M2” phenotype. Collectively, this study showed that CAP strongly induces oxidative stress in cells found in the melanoma microenvironment, with T cells displaying the highest sensitivity.

3.3.2 ZUSAMMENFASSUNG

Im letzten Jahrzehnt wurden bei der Behandlung des Melanoms beachtliche Fortschritte erzielt. Die Einführung von zielgerichteten Therapien und ICIs in die klinische Praxis hat das Gesamtüberleben von Patienten im fortgeschrittenen Stadium erheblich verbessert^{3–5,7,317,318,322}. Trotz dieser Verbesserungen spricht jedoch ein erheblicher Anteil der Patienten jedoch nicht auf diese Therapien an oder entwickelt rasch Resistenzen, was die verfügbaren Behandlungsoptionen einschränkt^{7,314,323,336–339}.

CAP ist ein vielversprechender Ansatz zur Krebsbekämpfung, da es auf das Redox-Gleichgewicht in Krebszellen abzielt³⁴⁹. CAP ist ein teilweise ionisiertes Gas, das zudem neutrale Atome, angeregte Elektronen und elektromagnetische Strahlung enthält und welches bei physiologischen Temperaturen angewendet werden kann. Die Wechselwirkung von CAP mit biologischem Material führt zur Produktion von RONS. Krebszellen sind von Natur aus anfälliger für erhöhten oxidativen Stresses, und viele Studien haben gezeigt, dass CAP selektiv krebshemmende Eigenschaften besitzt. Es ist jedoch nur wenig darüber bekannt, wie CAP Immunzellen beeinflusst, die für das Ansprechen auf eine Immuntherapie in der Mikroumgebung des Melanoms entscheidend sind^{10,101}. Ziel dieser Studie war es, den Einfluss von CAP auf Zellen zu bestimmen, die in der Mikroumgebung des Melanoms *in vitro* vorkommen, nämlich menschliche Melanomzellen, T-Zellen und von Monozyten abstammende Makrophagen¹⁰.

Es wurde festgestellt, dass die Behandlung mit CAP die intrazellulären ROS-Werte erhöht¹⁰. CAP verringerte dosisabhängig die Proliferation und Lebensfähigkeit von T-Zellen und Tumorzellen (in 2D- und 3D-Kultur). Dies resultierte aus einem CAP-induzierten G2/M-

Zellzyklus-Stillstand, erhöhtem mitochondrialen Stress und Apoptose. Die antiproliferativen und zytotoxischen Effekte von CAP konnten durch die Applikation von Antioxidantien teilweise rückgängig gemacht werden. Die Zellen reagierten unterschiedlich empfindlich auf die CAP-Behandlung, wobei die T-Zellen am stärksten betroffen waren. M1-Makrophagen wiesen nach der CAP-Behandlung einen erhöhten mitochondrialen Stress auf. Es wurde auch festgestellt, dass die CAP-Behandlung Makrophagen zu einem anti-inflammatorischen „M0/M2“-Phänotyp hin polarisiert. Insgesamt zeigte diese Studie, dass CAP starken oxidativen Stress in den Zellen des Melanom-Mikromilieus induziert, wobei T-Zellen die höchste Empfindlichkeit aufweisen.

3.3.3 AUTHOR CONTRIBUTIONS

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Visualization	E.R. Trzeciak , N. Zimmer
Supervision	A. Tüttenberg
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Funding acquisition	A. Tüttenberg

3.3.4 PUBLICATION

Disclosure: This paper was published open access and permission of re-use in this dissertation was granted by the publisher (MDPI)^{10,348}. The authors maintain all copyrights.



Article

Oxidative Stress Differentially Influences the Survival and Metabolism of Cells in the Melanoma Microenvironment

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Abstract: The cellular composition of the tumor microenvironment, including tumor, immune, stromal, and endothelial cells, significantly influences responses to cancer therapies. In this study, we analyzed the impact of oxidative stress, induced by cold atmospheric plasma (CAP), on tumor cells, T cells, and macrophages, which comprise part of the melanoma microenvironment. To accomplish this, cells were grown in different in vitro cell culture models and were treated with varying amounts of CAP. Subsequent alterations in viability, proliferation, and phenotype were analyzed via flow cytometry and metabolic alterations by Seahorse Cell Mito Stress Tests. It was found that cells generally exhibited reduced viability and proliferation, stemming from CAP induced G2/M cell cycle arrest and subsequent apoptosis, as well as increased mitochondrial stress following CAP treatment. Overall, sensitivity to CAP treatment was found to be cell type dependent with T cells being the most affected. Interestingly, CAP influenced the polarization of M0 macrophages to a “M0/M2-like” phenotype, and M1 macrophages were found to display a heightened sensitivity to CAP induced mitochondrial stress. CAP also inhibited the growth and killed melanoma cells in 2D and 3D in vitro cell culture models in a dose-dependent manner. Improving our understanding of oxidative stress, mechanisms to manipulate it, and its implications for the tumor microenvironment may help in the discovery of new therapeutic targets.

Keywords: tumor microenvironment; metabolic alterations; tumor therapy; cold atmospheric plasma; tumor spheroids; plasma jet; malignant melanoma; T cell; macrophage

1. Introduction

Skin cancer is one of the most common type of cancers, and malignant melanoma is its deadliest form [1,2]. Although the introduction of immune checkpoint inhibitors represents a great advancement in the treatment of melanoma and thus has improved patient prognosis, many patients do not respond to therapy—leaving them with limited treatment options [2–7]. Melanoma prognoses remain particularly poor in advanced metastatic disease and in mucosal forms [1,2,8]. One proposed strategy to improve therapeutic efficacy is by targeting the delicate redox balance inside cancer cells.

It is well known that healthy cells require low levels of reactive oxygen species (ROS) for signaling [9]. ROS are naturally produced intracellularly from mitochondrial oxidative phosphorylation (OXPHOS) and from environmental factors, including ultra-violet (UV) radiation and pollutants [10,11]. However, elevated and prolonged amounts of ROS have been associated with tumorigenesis and the progression of cancer, namely by activating pro-tumor signaling pathways, increasing proliferation, cell survival, and genetic instability [12–14]. As a result of these conferred advantages combined with their own increased metabolic rates, cancer cells live with increased ROS levels, and thus exist in an inherent state of oxidative stress [12]. However, when ROS levels exceed their threshold of tolerance, they damage vital cellular components, including DNA, lipids, and proteins, and can lead to eventual oxidative stress induced apoptosis [12,15].

Melanoma stands out amongst other solid tumors for having especially high oxidative stress levels, which can be explained from both external and internal factors [16,17]. Due to their physical location, melanoma cells are directly exposed to a myriad of environmental oxidative stress inducing factors, like UV radiation [16,18]. In addition, they also contain melanosomes, which raise intracellular ROS levels through the production of melanin [19,20]. The links between increased oxidative stress levels and malignant melanocyte transformation, as well as melanoma initiation, progression, and metastasis, are well established [19–22]. As melanoma cells already live with elevated amounts of oxidative stress, they are innately more vulnerable to increases in exogenous ROS levels, which can be therapeutically manipulated, such as with the use of cold atmospheric plasma (CAP).

Plasma, also called the fourth state of matter, can be formed by subjecting gas to an electromagnetic field [23]. Plasma is made of excited ionized gases, which release electromagnetic radiation, including infrared, visible, and UV radiation [23]. The discovery of CAP, non-thermal plasma consisting of neutral atoms, ions, and highly energized electrons, within mild physiological temperatures (less than 40 °C at the point of application) enabled new applications of CAP in biological settings [23,24]. Since then, CAP has been found to have diverse biological effects, including but not limited to tissue regeneration, blood circulation, cell detachment, and disinfection [23,24]. These effects have been principally linked to the increased presence of reactive oxygen and nitrogen species (RONS) resulting from the chemical reaction of CAP with biological tissues and cells [23]. In recent years, CAP has garnered much attention for its anticancer properties, such as its selective cytotoxicity and inhibition of proliferation of cancer cells [25]. These effects have been observed in a wide variety of cancer types, including but not limited to brain, skin, lung, and colorectal cancers [25].

Although the influence of CAP on tumor cells has been extensively studied, relatively little is known regarding how CAP influences the different cells that make up the tumor microenvironment, which can greatly influence therapy response and patient prognosis [26,27]. The present study aimed to address how CAP treatment affects the survival and metabolism of tumor cells, T cells, and macrophages, which, amongst others, compose the melanoma microenvironment. To accomplish this, cells were treated directly with argon and microwave-based CAP for varying treatment times, and CAP induced alterations in cell viability, proliferation, phenotype, and metabolic activity were investigated.

It was found that CAP treatment could significantly decrease the viability and proliferation of melanoma and T cells with T cells being the most affected. When these effects were further investigated, it was found that CAP induced G2/M cell cycle arrest and the consequent apoptosis of melanoma cells. Additionally, CAP treatment influenced the polarization of human monocyte derived macrophages into a “wound-healing” and rather tolerogenic M0/M2 phenotype. CAP treated cells also displayed hallmark signs of mitochondrial stress, including disrupted ATP production and reductions in cell fitness. Antioxidant treated melanoma cells and T cells could partially recover their viability and

proliferation in spite of CAP treatment—supporting that the cytotoxic and anti-proliferative effects of CAP treatment likely stem from increased ROS levels.

To better understand how CAP influences the naturally more complex melanoma microenvironment, melanoma cells were cultured to form *in vitro* 3D tumor spheroids and repeatedly treated with CAP. The resulting spheroids were significantly smaller in size and displayed reductions in cell viability and proliferation when compared to the untreated control. Nevertheless, 3D melanoma spheroids were more resistant to CAP than melanoma cells grown as *in vitro* 2D monolayers—requiring higher doses of CAP to have its desired anticancer effects.

Collectively, we could show that oxidative stress induced by CAP treatment differentially decreased the viability and proliferation as well as increased the mitochondrial stress levels of various cell types, which comprise the melanoma microenvironment in a CAP treatment time dependent manner. Improving our understanding of oxidative stress, mechanisms to manipulate it, and its implications for the distinct cells in the tumor microenvironment may help in the discovery of new and effective clinical targets.

2. Materials and Methods

2.1. Cell Culture and Cell Lines Employed

The human melanoma cell lines, Ma-Mel-19 and UKRV-Mel-15a, were cultured with RPMI-1640 (Thermo Fisher Scientific, #31870, Waltham, MA, USA) supplemented with 10% FCS (Thermo Fisher Scientific, #10500064), 1% GlutaMAX™ (Thermo Fisher Scientific, #35050038), and 0.1% Primocin (InvivoGen, #ant-pm-2, San Diego, CA, USA). The human glioblastoma cell line, T98G, was cultured with Minimum Essential Medium Eagle (Sigma-Aldrich, #M2279, St. Louis, MO, USA) supplemented with 10% FCS, 1% GlutaMAX™, and 0.1% Primocin.

These cell lines were cultured as previously described [28,29]. Cells were passed every 3–4 days using Trypsin-EDTA (Sigma-Aldrich, #T3924) to detach the cells. UKRV-Mel-15a and Ma-Mel-19 cell lines were obtained from Dr. A. Paschen, in Essen, Germany in 2014 [28,29]. T98G was purchased from the ATCC in 2015. The Cellosaurus accession numbers for each cell line are as follows: Ma-Mel-19 (CVCL_A156), UKRV-Mel-15a (CVCL_A713), and T98G (CVCL_0556). Cell lines were authenticated at Eurofins Genomics in March 2021. The resulting STR profiles matched with the online databases of the German collection of microorganisms and cell cultures (DSMZ) (Available online: <https://www.dsmz.de/services/human-and-animal-cell-lines/online-str-analysis>, accessed on 30 January 2022) and the Cellosaurus database (Available online: <https://web.expasy.org/cellosaurus/>, accessed on 30 January 2022). For viability and proliferation experiments, Ma-Mel-19 and UKRV-Mel-15a cells were prelabeled with carboxyfluorescein succinimidyl ester (CFSE) (eBioscience #65-0850-84, San Diego, CA, USA), plated at 100,000 cells per well in 24 well plates (Corning, #3527, Corning, NY, USA), and incubated overnight in a 37 °C 5% CO₂ incubator. The next day, cells were CAP treated as indicated and incubated another 3 days before analysis.

2.2. Isolation and Stimulation of Human T Cell Populations

Buffy coats were obtained from healthy volunteers, with the approval from the local ethics committee (Landesärztekammer Rheinland Palatine No. 837.019.10 (7028), approved on 4 March 2010). PBMC were isolated as described previously [30]. Either CD3⁺, CD4⁺, or CD8⁺ T cells were isolated via CD3, CD4, or CD8 microbeads (Miltenyi Biotec #130-050-101, #130-045-101, #130-045-201, Bergisch Gladbach, Germany) from the company's respective protocols. For proliferation and viability assays, CD3⁺, CD4⁺ and CD8⁺ T cells were prelabeled with CFSE and plated at 2.5 million cells per well in X-Vivo 15 (Lonza, #BE02-060F, Basel, CH) in 24 well plates, stimulated with 0.5 µg/mL anti-CD3 mAb (clone OKT3, eBioscience) plus 1 µg/mL anti-CD28 mAb (clone 28.2, eBioscience) in the presence or absence of the following antioxidants caffeic acid (Sigma-Aldrich #C0625), rutin-hydrate (rutin) (Sigma-Aldrich #E5143), and resveratrol (Sigma-Aldrich #R5010),

as indicated. Plates were incubated overnight in a 37 °C 5% CO₂ incubator. The next day, cells were CAP treated as indicated and incubated another 3 days before analysis.

2.3. Isolation and Polarization of Human Monocyte Derived Macrophages

To generate macrophages, 200 million PBMC were seeded in RPMI1640, containing 1% Glutamax, 0.1% Primocin, and 50 ng/mL M-CSF (ImmunoTools, #11343113, Friesoythe, Germany) in cell culture petri dishes (Corning, #430167) for 1 h in a 37 °C 5% CO₂ incubator. Afterwards, non-adherent cells were rinsed off by repeated washing of the petri dishes with pre-warmed to 37 °C DPBS (Thermo Fisher Scientific, #14190-094), checked by microscopy. Adherent cells were cultured in the medium described above containing an additional 1% heat inactivated human plasma. Human plasma of ten healthy donors was pooled and centrifuged for 15 min at 800× g. The resulting supernatant was centrifuged for 15 min at 1000× g to deplete the cellular contents. The pooled plasma was heat inactivated for 20 min at 56 °C. Insoluble denatured protein was removed by centrifugation at 3000× g for 15 min at room temperature. The heat inactivated plasma was stored at −20 °C until further use. Cells were harvested by incubating plates with Accutase (Thermo Fisher Scientific, #00-4555-56) for 1 h at 4 °C after 5–7 days of culture. The resulting “M0”-macrophages were polarized with 100 ng/mL LPS (InvivoGen, #tlrl-eblps), 20 ng/mL human IFN-γ (Immunotools, #11343536), and 50 ng/mL M-CSF for an M1-like phenotype and with 20 ng/mL human IL-4 (Immunotools, #11340045) plus 50 ng/mL M-CSF for an M2-like phenotype for 2 days in a 37 °C 5% CO₂ incubator. For polarization experiments, M0-like macrophages were treated with CAP, as indicated, and were incubated for 2 days before analysis.

2.4. Flow Cytometry

Single cell suspensions were stained in 10 µL/sample FACS buffer containing 0.5% human serum albumin (CSL Behring, #10530a/96, King of Prussia, PA, USA), 1 mM EDTA (AppliChem, #A3553, Darmstadt, Germany), 10 µg/mL human IgG (CSL Behring, #EU/1/08/446/001) in DPBS using the following antibodies for flow cytometric analysis. Cells were stained with fixable viability dye FVD506 or FVD780 (Thermo Fisher Scientific, #65-0866-18, #65-0865-14) prior to the surface staining of anti-CD40 (Immunotools, #21270403), anti-CD36 (Miltenyi Biotec, #130-095-480), anti-CD206 (Miltenyi Biotec, #130-100-152), anti-PD-L2 (Miltenyi Biotec, #130-105-829), anti-CD163 (Thermo Fisher Scientific, #A15792), anti-HLA-DR (BioLegend, #307650, San Diego, CA, USA), anti-CD68 (BD Biosciences, #565594, Franklin Lakes, NJ, USA), anti-CD14 (Immunotools, #21279143), anti-CD3 (Immunotools, #21620036), anti-CD4 (BD Biosciences, #555347), and anti-CD8 (BioLegend, #344734). For cell cycle analysis, single cell suspensions were washed once with DPBS and stained with Hoechst 33342 (PromoKine, #PK-CA707-40046, Heidelberg, Germany) in DPBS. Apoptotic and necrotic cells were detected by staining single cell suspensions with the FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, #640914) in accordance with the manufacturer's protocol. Data were acquired on a BD LSR II (BD Biosciences) flow cytometer and analyzed using Cytobank software [31]. Doublets, debris, and dead cell were excluded from analysis. Example gating strategies for melanoma cells, macrophages, and CD3⁺, CD4⁺, and CD8⁺ T cells can be found in Figure S1A–C.

2.5. T Cell-Tumor Cell Co-Cultures

Before plating, 24 well plates were precoated with 1 µg/mL anti-CD3 mAb antibody for 1 h in a 37 °C 5% CO₂ incubator. Ma-Mel-19 cells were either left untreated or irradiated with a dose of 10 Gy from a Gammacell 2000 irradiator (Mølsgaard Medical, Skodsborg, Denmark) to inhibit their proliferation. Ma-Mel-19 cells were then seeded at 120,000 cells per well in anti-CD3 precoated plates and incubated overnight. On the following day, isolated CD4⁺ or CD8⁺ T cells were treated with 1 µg/mL anti-CD28 and seeded with the Ma-Mel-19 cells at 3 million cells per well. On the next day, the co-cultures were treated with 30 s of CAP per well and were incubated another 3 days before analysis.

2.6. Seahorse Cell Mito Stress Assays

Seahorse Cell Mito Stress assays were performed according to the manufacturer's protocol and specifications for a Seahorse XFp Analyzer (Agilent, Santa Clara, CA, USA). The day before the assay, tumor cells (Ma-Mel-19, UKRV-Mel-15a, and T98G) and macrophages were plated at 30,000 cells per well in their standard cell culture medium in Seahorse XFp Cell Culture Miniplates (Agilent, #103025-100). CD4⁺ and CD8⁺ T cells were stimulated with 0.5 µg/mL anti-CD3 mAb and 1 µg/mL anti-CD28 mAb and plated at 500,000 cells per well in their standard cell culture medium in XFp Cell Culture Miniplates. The following day, medium was exchanged for Seahorse assay medium consisting of XF DMEM (Seahorse XF DMEM medium, Agilent, #103575-100) or XF RPMI (Seahorse XF RPMI medium, Agilent, #103576-100) supplemented with 1 mM pyruvate (Agilent, #103578-100), 2 mM glutamine (Agilent, #103579-100), and 10 mM glucose (Agilent, #103577-100). Ma-Mel-19, UKRV-Mel-15a, CD4⁺, and CD8⁺ T cells received XF RPMI medium, whereas T98G received XF DMEM medium. Cells were treated with various amounts of CAP and incubated for 1 h in a 37 °C non-CO₂ incubator. Meanwhile, Seahorse sensor cartridges (Seahorse XFp FluxPak, Agilent, #103022-100) were loaded with 1.5 µM oligomycin, 1.0 µM FCCP, and 0.5 µM rotenone/antimycin A (Seahorse XFp Cell Mito Stress Test Kit, Agilent, #103010-100). Medium was exchanged once more with the relevant supplemented assay medium for each cell type. Plates were placed into a Seahorse XFp Analyzer and the program "Seahorse XF Mito Stress Test" was ran.

2.7. Intracellular ROS Quantification

This assay was performed according to the Reactive Oxygen Species (ROS) Detection Kit protocol (PromoKine, #PK-CA577-K936). Ma-Mel-19 cells were plated at 20,000 cells per well in their standard cell culture medium in flat bottom 96 well cell culture plates (Corning, 96-well Clear Flat Bottom Polystyrene TC-treated Microplates, #3598) and incubated in a 37 °C 5% CO₂ incubator overnight. The next day, medium was removed, and cells were washed with ROS Assay Buffer, treated with ROS Label (1×), and incubated in a 37 °C 5% CO₂ incubator for 45 min. ROS Label was removed, and cells were washed once more with ROS Assay Buffer. Cells were treated with various amounts of CAP as indicated and were measured 1 h after treatment. The positive control was treated with ROS Inducer (1×) for 1 h prior to measurement. Fluorescence was recorded at Ex 485 nm/Em 535 nm wavelengths on a Sense Beta Plus microplate reader (Hidex, Turku, Finland) with Plate Reader Software Version 0.5.55.0.

2.8. Tumor Spheroid Culture

Ma-Mel-19 cells were prelabeled with CFSE and plated at 2000 cells per well in their standard pre-filtered (0.2 µm) (Pall, Acrodisc sterile filters, #4612, Port Washington, NY, USA) cell culture medium in ultra-low attachment 96 well plates (Thermo Fischer Scientific, Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate, #174925). Plates were centrifuged at 100× *g* for 5 min at 4 °C and then incubated in a 37 °C 5% CO₂ incubator to promote tumor spheroid formation. After 2 days, spheroids of ~300 µm in diameter had formed and began their different CAP treatment regimens. Medium was exchanged every 3–4 days by carefully removing 100 µL of medium from each well and replacing it with 100 µL of their standard pre-filtered cell culture medium.

2.9. Spheroid Digestion

Spheroids were digested into single cell suspensions by first pooling (*n* = 6/condition) spheroids and centrifuging them down at 400× *g* for 5 min at 4 °C to form pellets. Supernatants were discarded, and the pellets were digested in 1 U/mL Dispase II (Sigma-Aldrich, Dispase II, #D4693) in a 37 °C shaker set to 400 rpm for 30–40 min, followed by gentle pipetting to completely dissociate the remaining cells. The reaction was stopped by adding equal parts ice-cold DPBS. The cell suspension was centrifuged down at 400× *g* for 5 min

at 4 °C, and the supernatant was discarded. The remaining cell pellets were washed with DPBS and were stained for flow cytometry as indicated.

2.10. Spheroid Imaging

Spheroid growth was monitored over time through imaging. Starting at 2 days after plating, spheroids were imaged every 2 days for the duration of each experiment. Images were taken at a 4× magnification using a light inverted microscope (Invitrogen, EVOs FL Color Imaging System, #AMEFC4300, Waltham, MA, USA) at the widest field of view. Images were subsequently analyzed in the analysis program ImageJ to determine the average diameter of each spheroid [32]. The average diameter was calculated by taking the mean of three different diameter measurements per spheroid.

2.11. CAP Treatment

Cells were CAP treated with a MiniJet-R from Heuermann HF Technik (Aachen, Germany). The set-up of the device and an explanation of its components can be found in Figure S2A,B. The standard settings used throughout this study were a power level of 4, an argon flow rate of 2 L/min, and a treatment distance of 1 cm from the plasma jet to the surface of the cell culture medium. The duration and frequency of CAP treatment varied depending on the experiment. Cells and spheroids grown in 96 well plates were treated in the center of each well with the plasma jet. However, cells in 24 well plates, due to reasons of increased plate surface area, were CAP treated by manually moving the cell culture plate under the plasma jet to ensure that all cells were equally exposed to CAP.

2.12. Statistics

Statistical analysis was performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, www.graphpad.com, accessed on 30 January 2022). Results were normalized to the untreated controls as indicated. Bar diagrams display mean ± standard deviation (SD). Statistical significance was determined using ordinary one-way ANOVAs and two-way ANOVAs corrected for multiple comparisons using Dunnett or Tukey tests as well as by paired and unpaired Students *T*-tests, as indicated in the figure legends with * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p < 0.0001$. Not significant values are indicated by ns and refer to $p > 0.05$.

3. Results

3.1. CAP Treatment Differentially Affects the Viability and Proliferation of Cells That Make Up the Melanoma Microenvironment

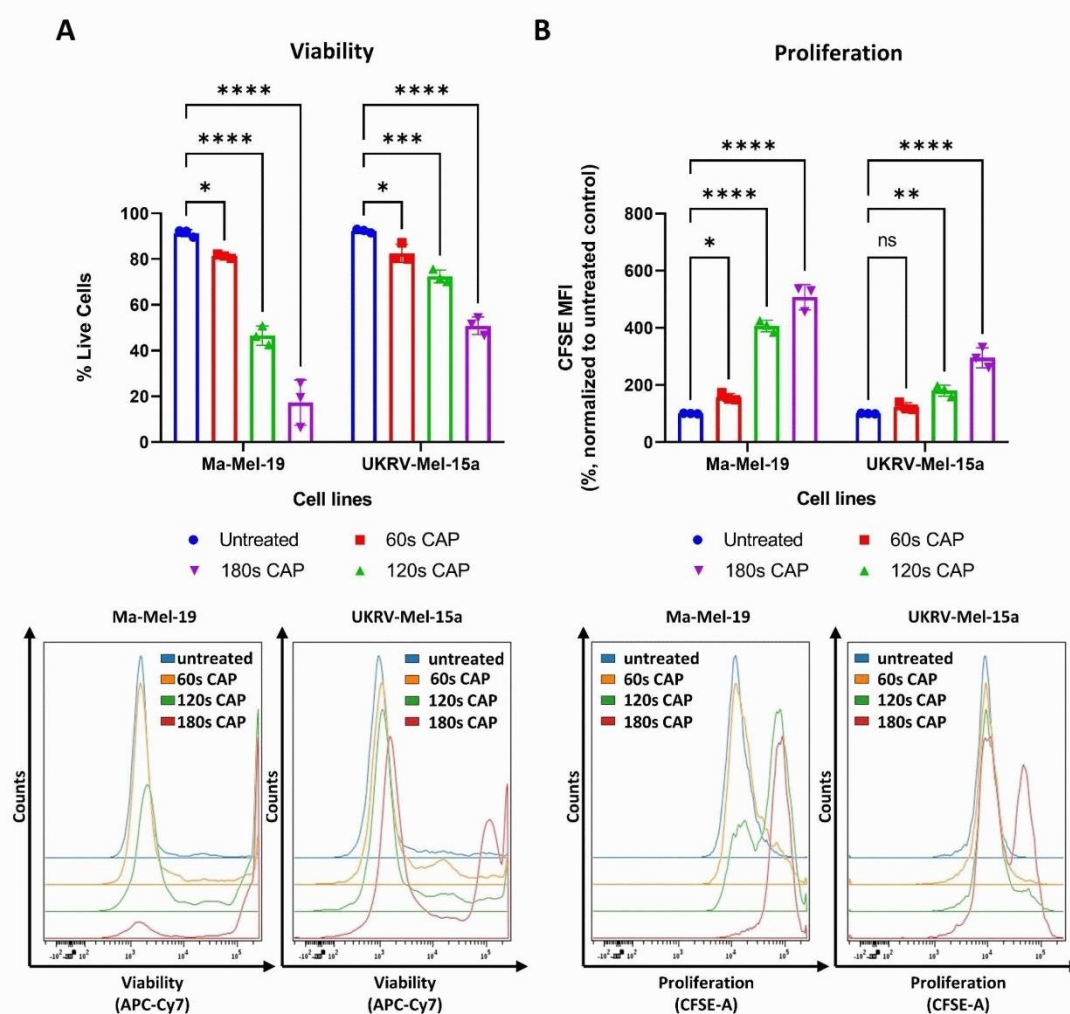
3.1.1. CAP Treatment Reduces the Viability and Proliferation of the Human Melanoma Cell Lines, Ma-Mel-19 and UKRV-Mel-15a, in a Dose-Dependent Manner

CAP has been previously shown to have potent inhibitory effects on melanoma cells, including the suppression of cell proliferation and the induction of cell death [33,34]. This study expanded on these results by using the human melanoma cell lines, Ma-Mel-19 and UKRV-Mel-15a, and by evaluating their response to direct CAP treatment by the MiniJet-R, an argon and microwave-based plasma jet. As seen in Figure 1A, significant dose-dependent reductions in cell viability were observed beginning at 60 s of CAP treatment in both cell lines.

At the highest duration of CAP treatment, 180 s, only 17% of Ma-Mel-19 cells survived, while nearly 50% of UKRV-Mel-15a cells remained alive—demonstrating that melanoma cell lines can display differing sensitivities to CAP treatment. In addition to viability, the effect of CAP on melanoma cell proliferation was also simultaneously examined using CFSE prelabeled cells. As cells divide, the amount of CFSE in the cells proportionally decreases allowing cell proliferation to be analyzed. Therefore, a high CFSE mean fluorescent intensity (MFI) value indicates that cells have proliferated less often than a lower CFSE MFI value. As seen in Figure 1B, a dose-dependent inhibition of cell proliferation was also observed in both cell lines. A significant inhibition of cell proliferation was observed in Ma-Mel-19 cells

beginning at 60 s of CAP treatment, while UKRV-Mel-15a required 120 s of CAP treatment to begin to observe a significant inhibition. This demonstrated that UKRV-Mel-15a cells were also more resistant to the anti-proliferative effects of CAP treatment than Ma-Mel-19 cells.

The observed anti-proliferative and cytotoxic effects of CAP on Ma-Mel-19 and UKRV-Mel-15a cells were further investigated to identify the location of cell cycle arrest and the mechanism of CAP induced cell death. It was found that Ma-Mel-19 and UKRV-Mel-15a cells had significantly higher percentages of cells in the G2/M phase following 180 s of CAP treatment than the untreated controls (Figures 1C and S3A). G2/M cell cycle arrest occurs following DNA damage [35]. Therefore, these findings imply that CAP treatment effectively raised the oxidative stress levels of the melanoma cells leading to DNA damage and subsequent cell cycle arrest. These findings also help explain the earlier observed anti-proliferative effects of CAP treatment on melanoma cells (Figure 1B).



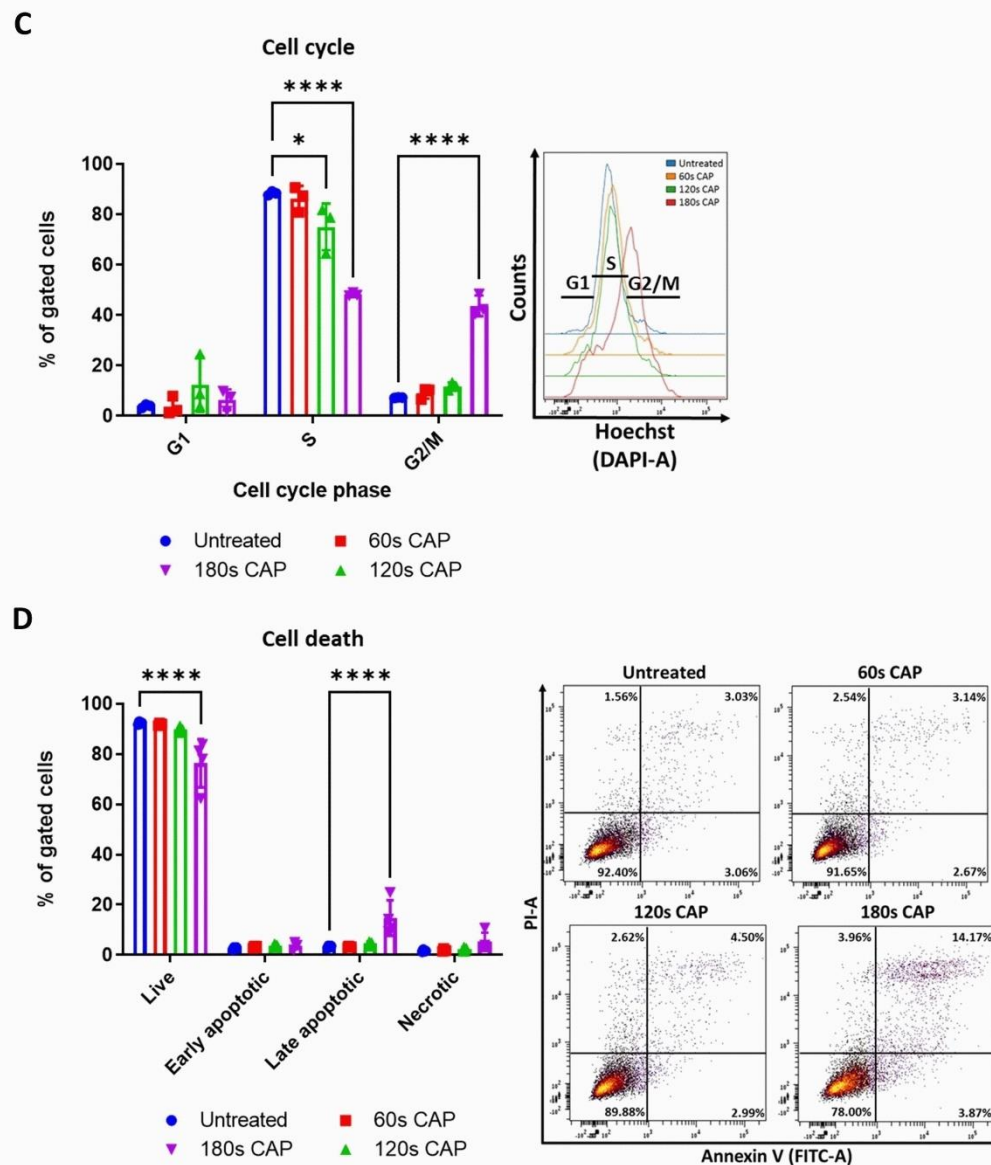


Figure 1. CAP treatment reduces the viability and proliferation of the human melanoma cell lines, Ma-Mel-19 and UKRV-Mel-15a, in a dose-dependent manner. (A,B) CFSE prelabeled Ma-Mel-19 and UKRV-Mel-15a cells were treated with varying amounts of CAP (60 s, 120 s, 180 s). After 3 days, cells were analyzed via flow cytometry; (A) cells were stained for viability. Bar diagram shows the average percentage of live cells measured ($n = 3$) $\pm$ SD; (B) bar diagram shows the average CFSE mean fluorescent intensity (MFI) measured, normalized to the untreated control ($n = 3$) $\pm$ SD; (C,D) Ma-Mel-19 cells were treated with varying amounts of CAP (60 s, 120 s, 180 s). After 3 days, cells were analyzed via flow cytometry; (C) cells were stained with Hoechst. Bar diagram shows the percentage of cells in each gate ($n = 3$) $\pm$ SD; (D) cells were stained with annexin V and propidium iodide (PI). Bar diagram shows the percentage of cells in each quadrant gate ($n = 4$) $\pm$ SD. The contents

of each gate are as follows: lower left (LL) live cells, lower right (LR) early apoptotic cells, upper right (UR) late apoptotic cells, and upper left (UL) necrotic cells; histograms and dot plots paired to bar diagrams show one representative result; statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Dunnett or Tukey tests and is indicated by the asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

To analyze the cytotoxic effects of CAP treatment in more detail, annexin V propidium iodide stainings were performed to differentiate between apoptotic and necrotic cells. It was discovered that both Ma-Mel-19 and UKRV-Mel-15a cells had significantly higher percentages of late apoptotic cells following 180 s of CAP treatment in comparison to the untreated control; however, their percentages of necrotic cells were not significantly altered (Figures 1D and S3B). This showed that the main form of cell death following CAP treatment of Ma-Mel-19 and UKRV-Mel-15a cells is apoptosis. This is well fitting with the G2/M cell cycle arrest results as it is well known that when excessive DNA damage occurs, cells undergo apoptosis [35]. Additionally, these findings of CAP induced G2/M cell cycle arrest and apoptosis are well supported by the literature [25,34].

Altogether, these results show that CAP decreases the viability and suppresses the proliferation of Ma-Mel-19 and UKRV-Mel-15a cells in a dose-dependent manner. Ma-Mel-19 cells were also found to be more sensitive to CAP treatment than UKRV-Mel-15a cells. Furthermore, it was determined that CAP treatment induces G2/M cell cycle arrest and consequent apoptosis of Ma-Mel-19 and UKRV-Mel-15a cells.

3.1.2. CD4⁺ and CD8⁺ T Cells Exhibit a Higher Sensitivity to CAP Treatment Than Melanoma Cells

CAP has been widely hailed as a promising anticancer treatment strategy due to its selective targeting of cancer cells [25]. However, relatively little is known about how CAP affects the other cells that make up the melanoma microenvironment, which can greatly influence the therapy outcome [26,27].

To begin to understand how CAP may affect T cells, which play a pivotal role in the anticancer immune response, stimulated human PBMC isolated CD3⁺ T cells were treated with varying amounts of CAP and assessed for potential changes in viability and proliferative capacity. It was discovered that even with a treatment time of only 5 s CAP, a significant reduction in viability of the whole CD3⁺ T cell compartment could be seen (Figure 2A).

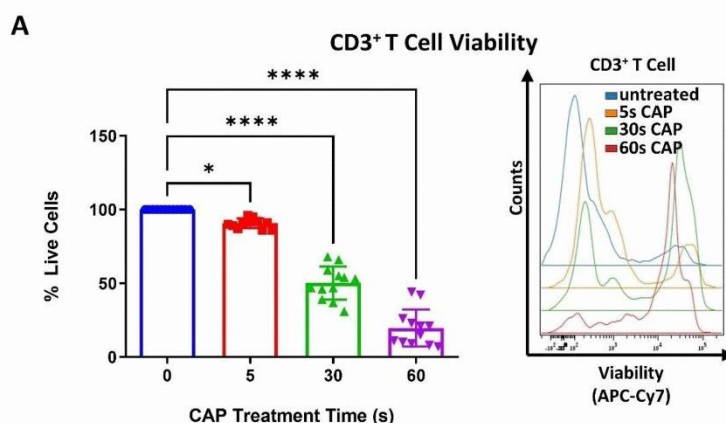


Figure 2. Cont.

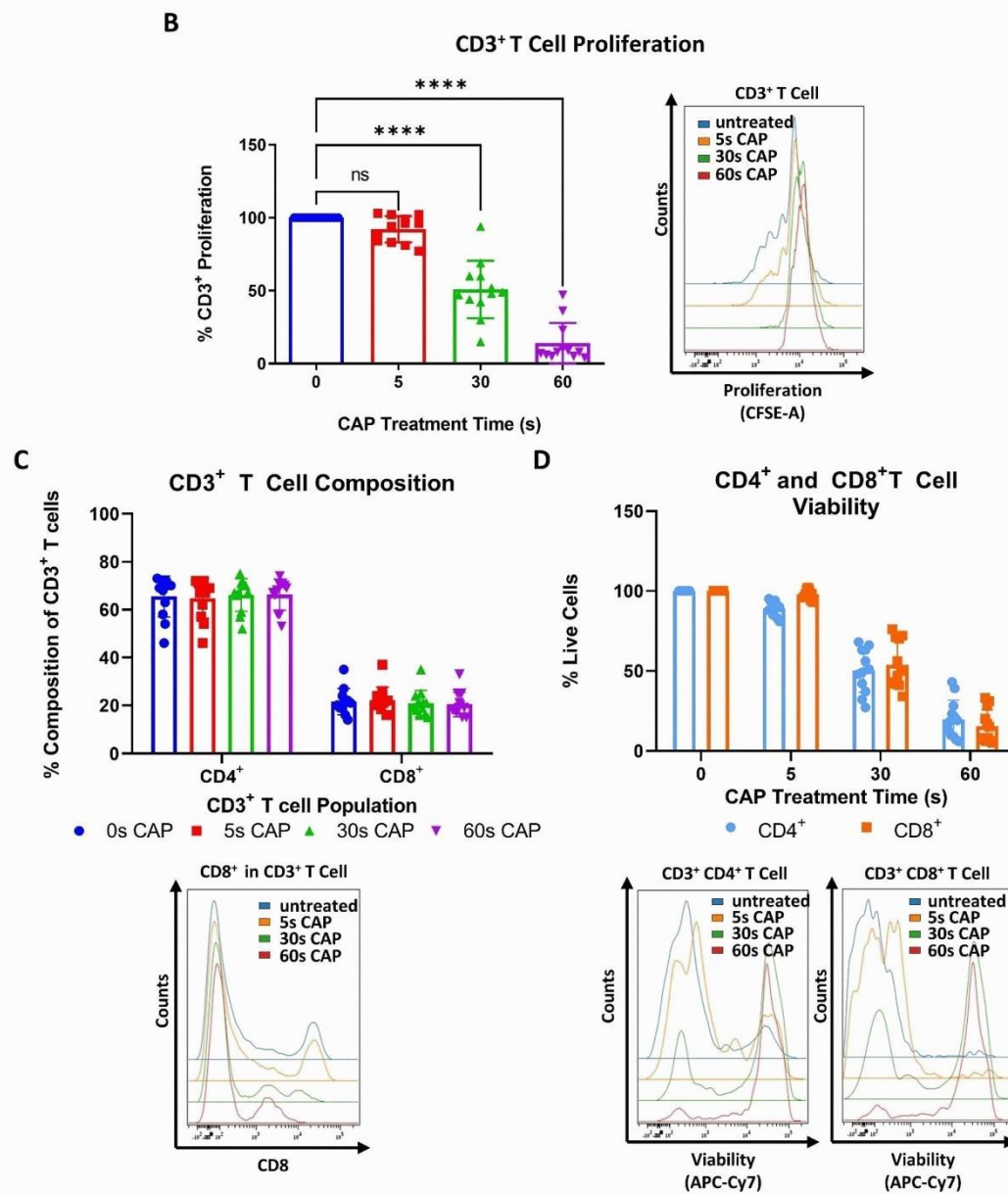


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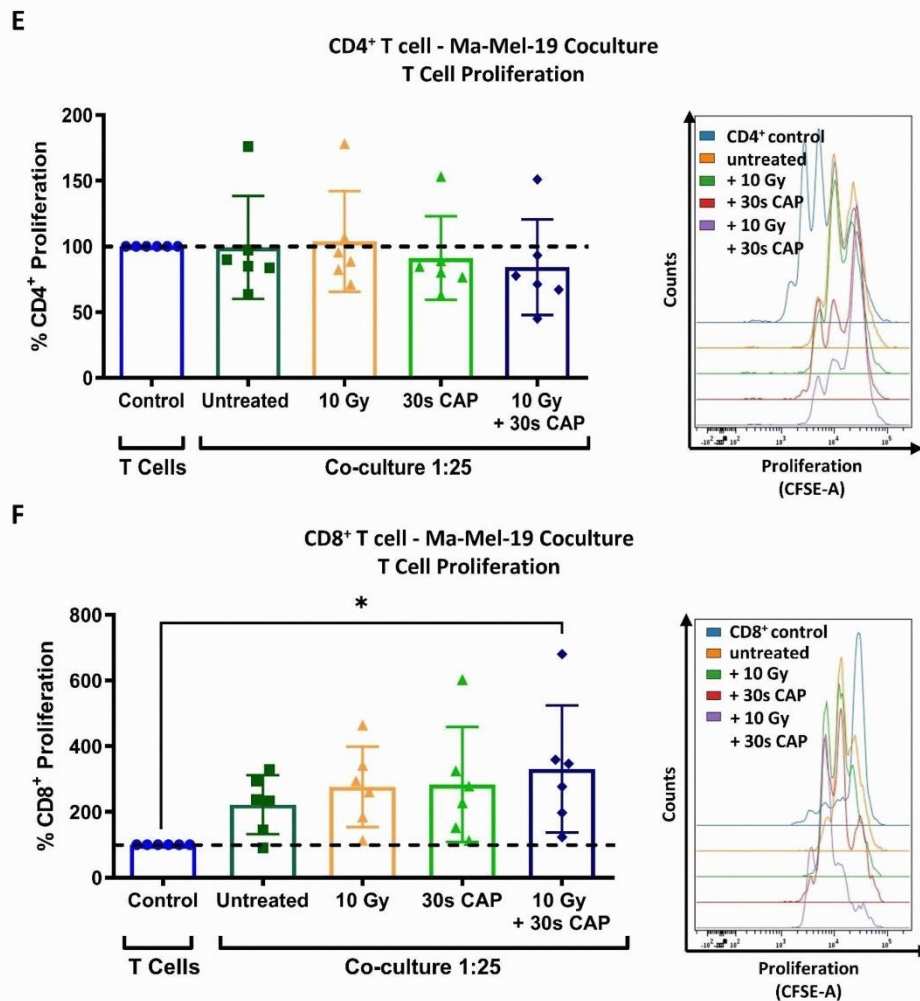


Figure 2. CD4⁺ and CD8⁺ T cells exhibit a higher sensitivity to CAP treatment than melanoma cells. (A,B) CFSE prelabeled stimulated CD3⁺ T cells were treated with varying amounts of CAP (0 s, 5 s, 30 s, and 60 s). After 3 days, cells were analyzed via flow cytometry; (A) cells were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD, normalized to the untreated control of the same donor. Data consist of the pooled results of 4 independent experiments ($n = 12$ donors); (B) bar diagram shows the average percentage of proliferating CD3⁺ T cells, $\pm$ SD normalized to the untreated control of the same donor. Data consist of the pooled results of 4 independent experiments ($n = 12$ donors); statistical significance was calculated by performing ordinary one-way ANOVA tests corrected for multiple comparisons with Dunnett tests; (C,D) stimulated CD3⁺ T cells treated with varying amounts of CAP (0 s, 5 s, 30 s, 60 s). After 3 days, cells were analyzed in flow cytometry; (C) bar diagram shows the average percentage of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in CD3⁺ T cells $\pm$ SD, normalized to the untreated control of the same donor. Data consist of the pooled results of 4 independent experiments ($n = 12$ donors); (D) CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD, normalized to the untreated control of the same donor. Data consist of the pooled results of 4 independent experiments ($n = 12$ donors); (E,F) CFSE prelabeled stimulated CD4⁺ and CD8⁺

T cells were grown in co-culture with Ma-Mel-19 cells. Before plating, Ma-Mel-19 cells were either left untreated or were irradiated with a dose of 10 Gy. Co-cultures were treated with 30 s CAP and after 3 days, T cells were analyzed via flow cytometry; (E) bar diagram shows the average percentage of proliferating CD4⁺ T cells $\pm$ SD, normalized to the T cell as only control of the same donor ($n = 6$ donors). The data consist of the pooled results of 3 independent experiments; (F) bar diagram shows the average percentage of proliferating CD8⁺ T cells $\pm$ SD, normalized to the T cell as only control of the same donor ($n = 6$ donors). The data consist of the pooled results of 3 independent experiments; histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Dunnett tests and is indicated by the asterisks as follows: * $p < 0.05$; **** $p < 0.0001$.

Viability and proliferation were found to decrease in a dose-dependent manner as CAP treatment times increased (Figure 2A,B). It was also investigated if CAP could influence the composition of CD4⁺ and CD8⁺ T cell sub-populations. However, no significant differences were found (Figure 2C). Additionally, CD4⁺ and CD8⁺ T cells were treated with varying amounts of CAP to see if these T cell sub-populations differentially respond to the cytotoxic effects of CAP. Herein, no significant differences in viability between CD4⁺ and CD8⁺ T cells were observed at any time point (Figure 2D). It was also observed that CD4⁺ and CD8⁺ T cells had a nearly identical response to CAP treatment as the tested whole CD3⁺ T cell compartment (Figure 2A,D).

To further examine how CAP affects T cells in the melanoma microenvironment, co-cultures of CD4⁺ and CD8⁺ T cells cultured with Ma-Mel-19 cells were performed. Interestingly, the proliferation of CD4⁺ T cells grown in co-culture could not be inhibited by 30 s of CAP treatment (Figure 2E) although the proliferation of T cells grown in single cell culture was significantly inhibited following 30 s of CAP treatment (Figure 2B). CD8⁺ T cells also responded differently to 30 s of CAP treatment depending on how they were cultured. When they were co-cultured with Ma-Mel-19 cells, they appeared to exhibit a non-significant increase in proliferation when treated with 30 s of CAP alone. Of note, they showed a significant increase in proliferation when treated with 30 s of CAP and when the Ma-Mel-19 cells were pre-irradiated with 10 Gy to inhibit their proliferation (Figure 2F). Previous studies have supported that CAP can induce immunogenic cell death in cancer cells [36–38]. This observed increase in the proliferation of CD8⁺ T cells seems to be suggestive of their potential activation by tumor cell antigens. These antigens were likely released from an increased number of dying tumor cells following combined radiation and CAP treatment, but this must be validated in future studies.

Collectively, these results show that CD3⁺, CD4⁺, and CD8⁺ exhibit a higher sensitivity to CAP treatment than melanoma cells as they required lower doses of CAP to see its cytotoxic and anti-proliferative effects. Additionally, these results suggest that CD4⁺ and CD8⁺ T cells may exhibit differential sensitivities to CAP treatment depending on the way they are cultured—highlighting the large influence an experimental model can have on results.

3.1.3. Macrophages Are Highly Resistant to CAP and Can Be Polarized into a “M0/M2-like” Phenotype

This study also examined the effect of CAP on human monocyte-derived macrophages, another important immune cell type found in the melanoma microenvironment [27]. Resting M0 macrophages were treated with varying amounts of CAP to determine if CAP treatment could affect their viability.

Notably, it was found that even at the highest treatment duration of 120 s CAP, M0 macrophages did not significantly differ in their viability when compared to the untreated M0 control (Figure 3A).

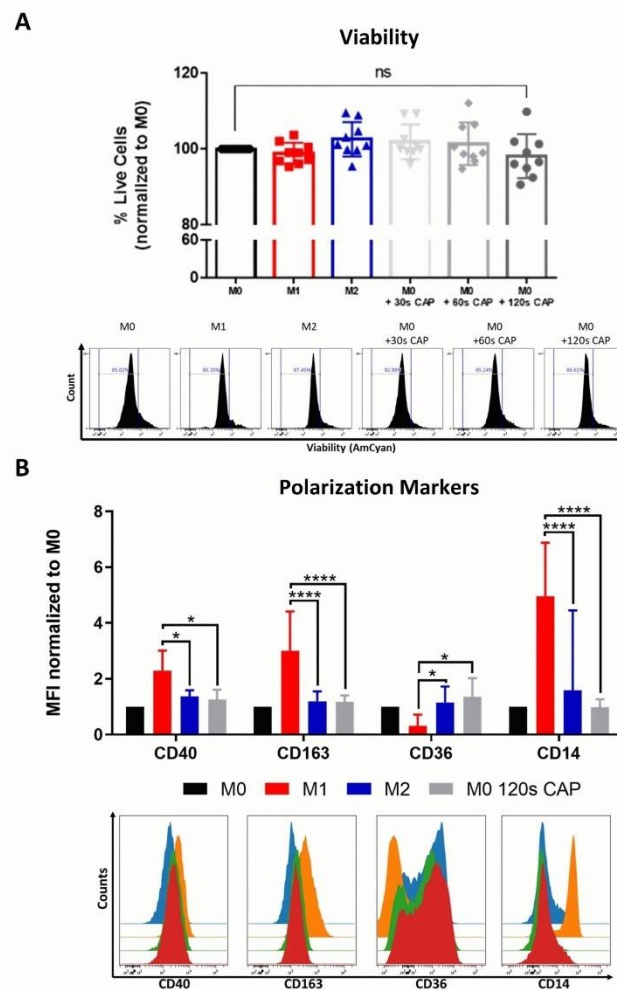


Figure 3. Macrophages are highly resistant to CAP and can be polarized into a “M0/M2-like” phenotype. **(A,B)** M0 macrophages were treated with varying amounts of CAP (30 s, 60 s, 120 s). M1 and M2 polarized macrophages served as a control. After 2 days, cells were analyzed via flow cytometry; **(A)** cells were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD, normalized to untreated M0 macrophages, and represents the pooled results of twelve independent experiments ($n = 12$ donors); **(B)** cells were stained with different macrophage polarization markers. Bar diagram shows the average mean fluorescent intensity (MFI) of each measured marker normalized to untreated M0 macrophages and represents the pooled results of twelve independent experiments ($n = 12$ donors); histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Tukey tests and is indicated by the asterisks as follows: * $p < 0.05$; **** $p < 0.0001$.

This indicates that, in this study, they were the cell type tested which was most resistant to the cytotoxic effects of CAP, followed by melanoma cells (Figure 1A) and then by T cells (Figure 2A,D), which were most affected.

Macrophage polarization state, or phenotype, can influence tumor progression, but relatively little is known about the influence of CAP on macrophage polarization. This study aimed to clarify this question by CAP treating M0 macrophages and examining their following polarization state with an extensive macrophage polarization marker panel. M0 macrophages appeared to adopt a rather “M0/M2-like” phenotype following 120 s CAP treatment (Figure 3B). This was supported by the significantly higher expression of the M2 marker, CD36, and significantly lower expression of the M1 markers, CD40, CD163, and CD14. No significant differences in the markers CD206, PD-L2, CD86, and MHCII were observed (Figure S4).

In summary, these results indicate that M0 macrophages are highly resistant to CAP treatment and can be polarized into a more “M0/M2-like” phenotype following CAP treatment.

3.2. Cells of the Melanoma Environment Display Varying Signs of Mitochondrial Stress Following CAP Treatment

3.2.1. CAP Induces Mild Metabolic Stress in Cancer Cells

Mitochondrial OXPHOS is an important metabolic process, which is sensitive to the cellular redox balance [10]. Therefore, this study aimed to explore how CAP might influence the mitochondrial functioning of different cells that make up the melanoma microenvironment. This was accomplished by performing Seahorse Cell Mito Stress Tests. This assay provides information on aspects of mitochondrial respiration by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as various electron transport chain inhibitors (oligomycin, rotenone A, antimycin) and the uncoupling reagent, FCCP, are added to the sample. Example OCR and ECAR graphs can be found in Figure S5A,B. Some of the parameters that this assay measures are ATP-production coupled respiration, coupling efficiency, proton leak, and spare respiratory capacity. ATP-production coupled respiration represents the amount of ATP produced by the mitochondria. Coupling efficiency refers to how well the linked reactions of electron carrier oxidation and ADP phosphorylation generate ATP. A proton leak, when protons cross from the inner mitochondrial membrane into the matrix without traveling through the ATP synthase, lowers coupling efficiency and can indicate mitochondrial damage [39,40]. Spare respiratory capacity represents the ability of a cell to respond to increased energetic needs and can be used as an indicator of cell fitness [40].

To determine how CAP acutely affects the mitochondrial functioning of cancer cells, two human melanoma cell lines, Ma-Mel-19 and UKRV-Mel-15a, and a human glioblastoma cell line, T98G, were CAP treated and underwent Seahorse Cell Mito Stress Tests. T98G was tested to see if different cancer types differ in their metabolic response to CAP treatment. UKRV-Mel-15a and T98G were CAP treated for 60s, whereas Ma-Mel-19 cells required 120 s of CAP treatment to begin to observe signs of mitochondrial stress. It was found that ATP-production coupled respiration was significantly reduced in T98G cells, while the melanoma cell lines had only slight but non-significant reductions (Figure 4A).

All cell lines displayed significant reductions in coupling efficiency following CAP treatment, likely due to an increased proton leak (Figure 4B). It is believed that proton leakage across the inner mitochondrial membrane into the matrix is one of the main causes of incomplete coupling [39]. The proton leak was significantly increased in CAP treated T98G cells, and it appeared to be slightly, but not significantly, elevated in both melanoma cell lines (Figure 4C). Significant reductions in spare respiratory capacity were also observed in Ma-Mel-19 and T98G, likely resulting from disrupted ATP production (Figure 4D). Notably, different cancer cell lines and cancer types tested in this study also displayed varying sensitivities to CAP—highlighting the need to test other cancer entities against CAP in future studies.

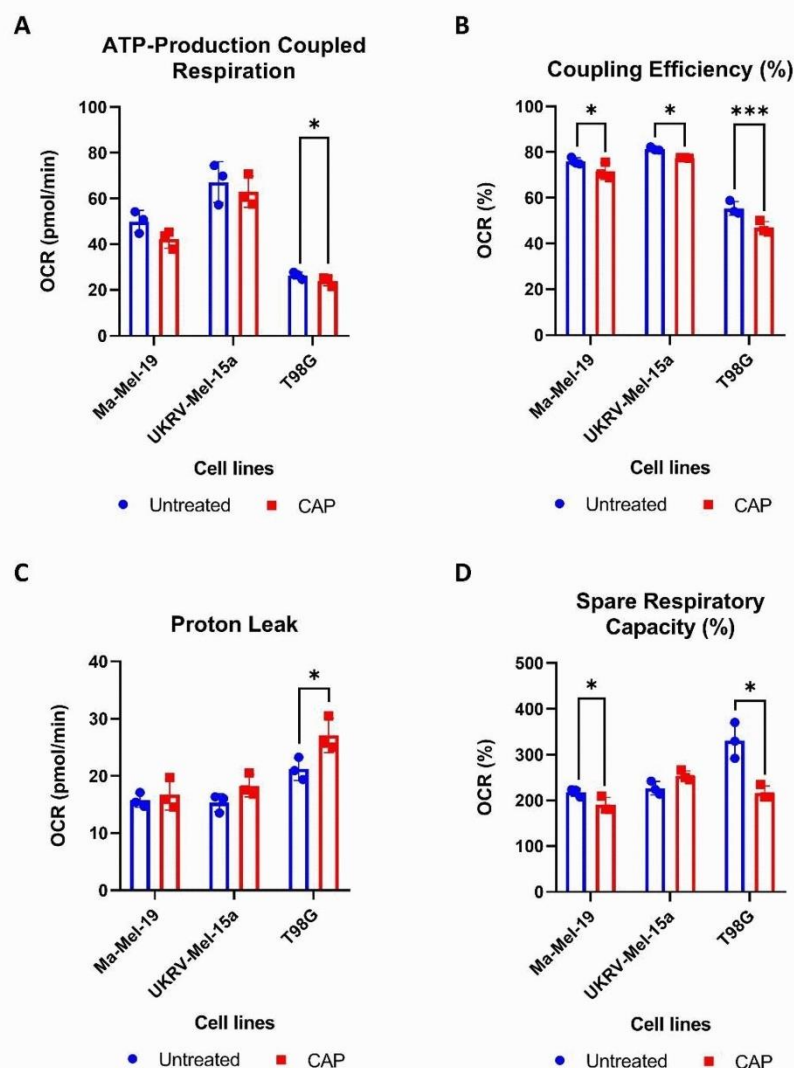


Figure 4. CAP induces mild metabolic stress in cancer cells. (A–D) Cancer cells were differentially treated with CAP (Ma-Mel-19—120 s CAP, UKRV-Mel-15a—60 s CAP, T98G—60 s CAP) 1 h before undergoing Seahorse Cell Mito Stress Tests. The data from each of the following parameters, (A) ATP-production coupled respiration, (B) coupling efficiency (%), (C) proton leak, and (D) spare respiratory capacity (%), show the mean $\pm$ SD ($n = 3$); statistical significance was calculated by performing paired Student's *t*-tests and is indicated by the asterisks as follows: * $p < 0.05$; *** $p < 0.001$.

Altogether, these results show that CAP induces mitochondrial stress in both melanoma and glioblastoma cells through the disruption of ATP production as evinced from the observed decreases in ATP-production coupled respiration and coupling efficiency as well as the increased proton leak following CAP treatment. It was also determined that cancer cell lines and types exhibit differing sensitivities to CAP induced mitochondrial stress.

3.2.2. CD4⁺ and CD8⁺ T Cells Are More Sensitive to CAP Induced Mitochondrial Stress Than Macrophages

The acute influence of CAP on the mitochondrial respiration of CD4⁺ and CD8⁺ T cells and differentially polarized macrophages was also examined. As CD4⁺ and CD8⁺ T cells were found to be highly sensitive to CAP treatment, in terms of viability (Figure 2D), a CAP treatment time of 30 s was selected to be used for the Seahorse Cell Mito Stress experiments. Macrophages were also treated with 30 s of CAP to directly compare how the two different immune cell types respond to CAP treatment.

Like cancer cells (Figure 4A,B), CAP treated CD4⁺ T cells also displayed significant reductions in ATP-production coupled respiration and coupling efficiency (Figure 5A,B).

However, both CD4⁺ and CD8⁺ T cells did not show any significant increases in proton leaking (Figure 5C). Interestingly, only CD8⁺ T cells had a significant decrease in spare respiratory capacity following CAP treatment (Figure 5D). Collectively, these results show that, although CD4⁺ and CD8⁺ T cells display signs of mitochondrial stress following CAP treatment, they appear to respond to these effects differently. For example, CD4⁺ T cells exhibit a greater disruption of ATP production than CD8⁺ T cells, but only the spare respiratory capacity, or fitness, of CD8⁺ T cells was reduced following CAP treatment (Figure 5A,B,D).

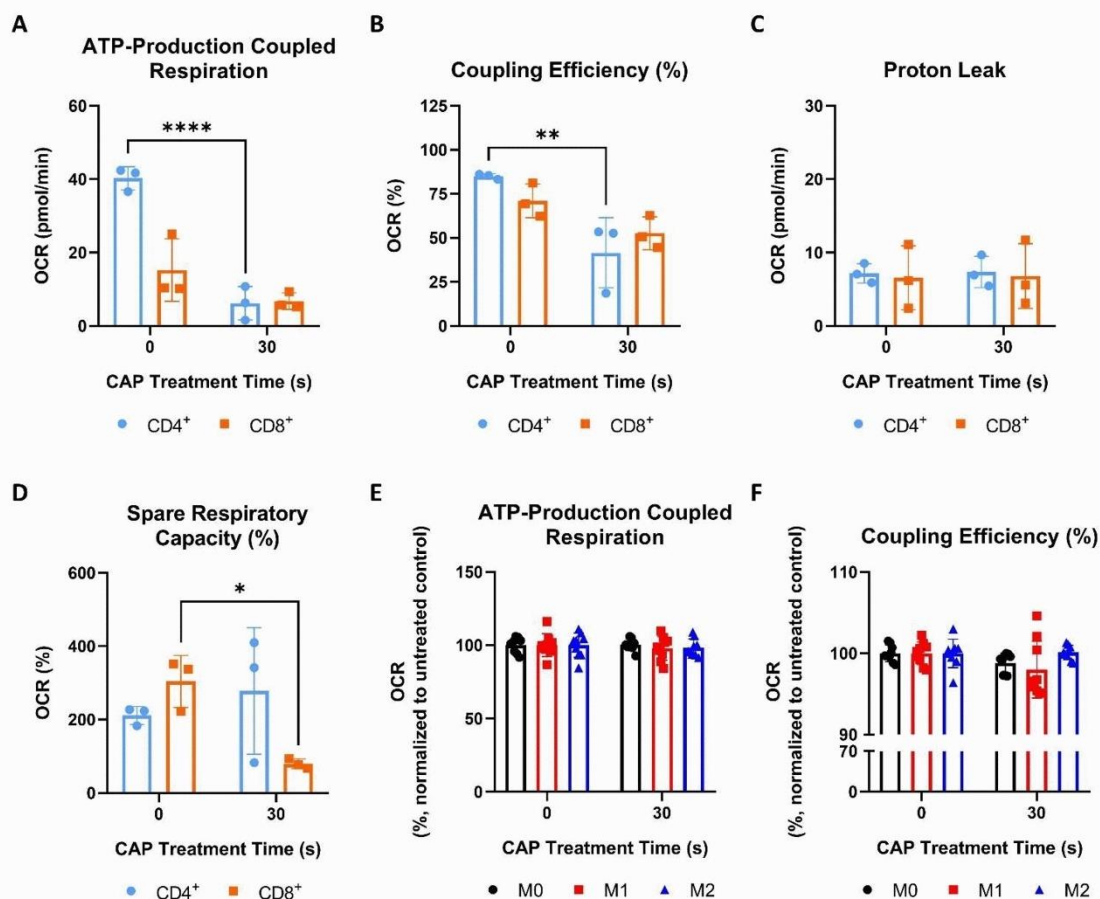


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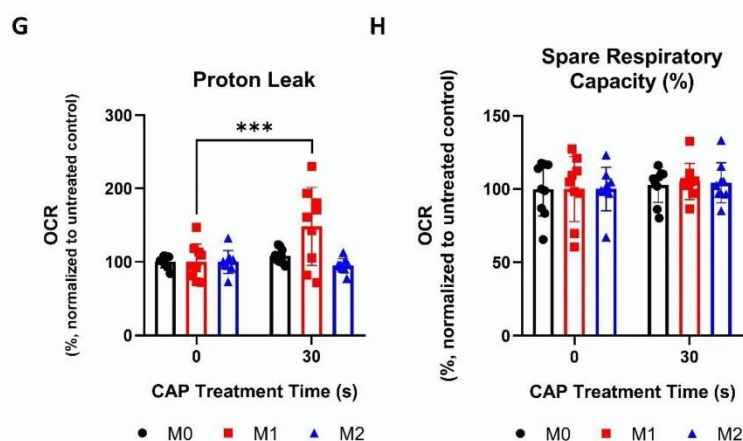


Figure 5. CD4⁺ and CD8⁺ T cells are more sensitive to CAP-induced mitochondrial stress than macrophages. (A–D) CD4⁺ and CD8⁺ T cells were treated with 30 s of CAP 1 h before undergoing Seahorse Cell Mito Stress Tests. The data from each of the following parameters, (A) ATP-production coupled respiration, (B) coupling efficiency (%), (C) proton leak, and (D) spare respiratory capacity (%), show the mean $\pm$ SD ($n = 3$ donors); statistical significance was calculated by performing unpaired Student's *t*-tests; (E–H) macrophages were treated with 30 s of CAP 1 h before undergoing Seahorse Cell Mito Stress Tests. The data from each of the following parameters, (E) ATP-production coupled respiration, (F) coupling efficiency (%), (G) proton leak, and (H) spare respiratory capacity (%), show the mean normalized to the untreated control $\pm$ SD ($n = 9$, 3 donors with 3 replicates each); statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Dunnett tests and is indicated by the asterisks as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

In contrast to T cells (Figure 5A–D), M0, M1, and M2 macrophages were found to be highly resistant to CAP induced mitochondrial stress—exhibiting no significant reductions in ATP-production coupled respiration, coupling efficiency, or spare respiratory capacity following CAP treatment (Figure 5E,H). Surprisingly, it was discovered that only M1 macrophages exhibited a significantly increased proton leak and a slight but non-significant reduction in coupling efficiency following CAP treatment (Figure 5F,G).

In summary, these results show that CD4⁺ and CD8⁺ T cells displayed greater signs of acute mitochondrial stress following CAP treatment than macrophages and that subpopulations of T cells and macrophages metabolically respond to CAP treatment differently.

3.3. Antioxidant Treatment Mitigates the Effects of CAP on Melanoma and CD3⁺ T Cells

One of the objectives of this study was to see if the cellular response to CAP treatment and thus to CAP induced metabolic alterations could be manipulated. This was especially interesting in the case of T cells to determine if the earlier observed adverse effects of CAP treatment might be reversible. One of the ways CAP is believed to exert its cytotoxic and anti-proliferative effects is through the production of ROS [25,41]. To begin, it was first verified that CAP treatment, from the device used in this study, could increase intracellular ROS levels. This was confirmed by treating Ma-Mel-19 cells with varying amounts of CAP and quantifying the resulting intracellular ROS levels. As seen in Figure S2C, Ma-Mel-19 cells exhibited a dose-dependent increase in intracellular ROS levels following CAP treatment.

Previous studies in cervical cancer cells have shown that the cellular effects of CAP can be combated with various antioxidants [41,42]. To expand upon these results, this study treated cells found in the melanoma microenvironment with a range of easily accessible and naturally occurring antioxidants, belonging to the polyphenol group, alone or in

combination with CAP. In more detail, this study tested antioxidants from three different subclasses of polyphenols: phenolic acid (caffeic acid), flavonoids (rutin), and stilbenes (resveratrol) [43]. This was done to provide a more comprehensive understanding of how polyphenol derived antioxidants might be used to provide protection against the potentially undesired effects of CAP treatment.

As described earlier, CAP partially consists of ROS, which are believed to be the main cause of CAP induced cytotoxicity in vitro [25]. Polyphenol derived antioxidants are potent free radical scavengers, which sequester free radicals, such as those found in and produced by ROS. Therefore, it was speculated that they could be used to counteract ROS produced by CAP, and thus reduce the effects of CAP induced oxidative stress. To the best of our knowledge, none of the antioxidants listed above have been tested for their influence on the cellular effects of CAP in the context of the tumor microenvironment, and this combined CAP-antioxidant treatment approach has not been tested before on cells, which specifically make up the melanoma microenvironment. More specifically, this study tested CD3⁺ T cells and melanoma cells (Ma-Mel-19 and UKRV-Mel-15a) with this CAP-antioxidant combination therapy approach as they were found to be the most sensitive to CAP treatment in this study.

It was found that the tested concentrations of resveratrol, caffeic acid, and rutin alone had no effect on the viability and proliferation of Ma-Mel-19 cells (Figure 6A,B).

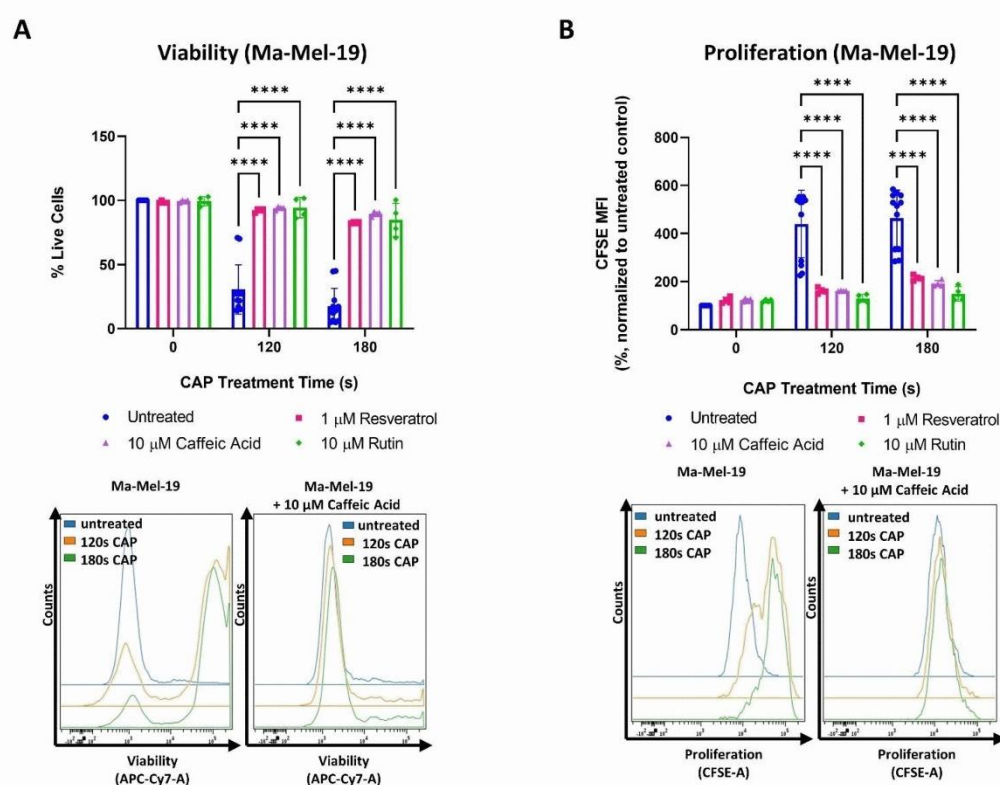


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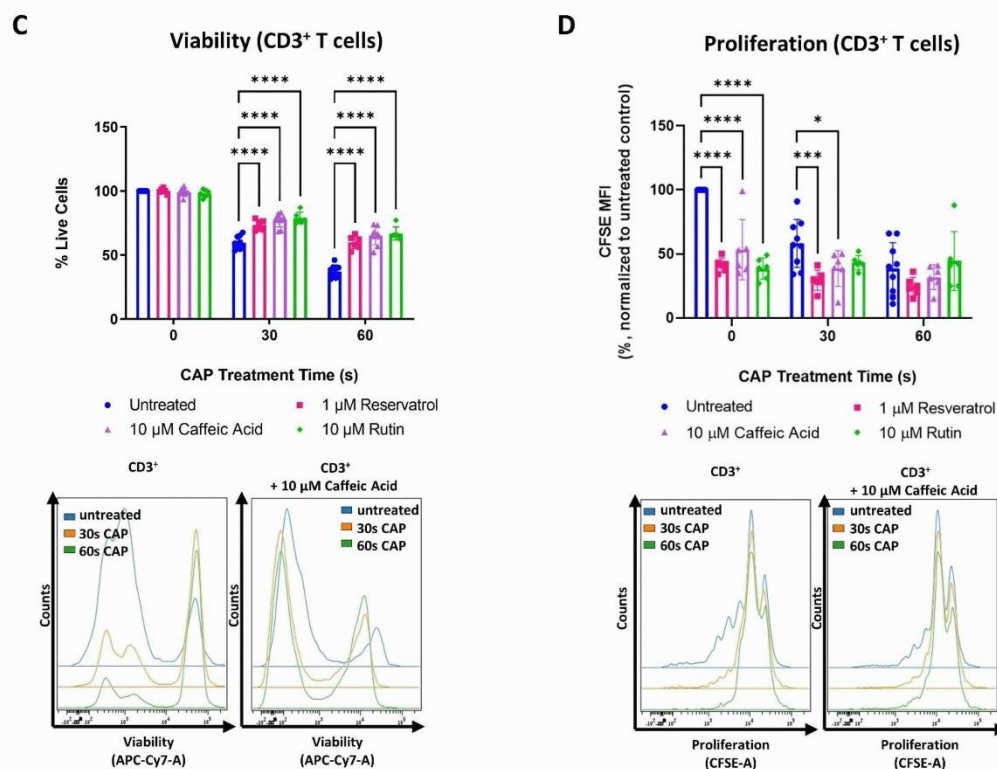


Figure 6. Antioxidant treatment mitigates the effects of CAP on melanoma and CD3⁺ T cells. (A,B) CFSE prelabeled Ma-Mel-19 cells were plated in the presence of various antioxidants and differentially treated with CAP (0 s, 120 s, 180 s). After 3 days, cells were analyzed via flow cytometry; (A) Ma-Mel-19 cells were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD and represents the pooled results of 3 independent experiments ($n = 12$ /untreated, $n = 4$ /antioxidant); (B) bar diagram shows the average CFSE mean fluorescent intensity (MFI) measured, normalized to the untreated control $\pm$ SD and represents the pooled results of 3 independent experiments ($n = 12$ /untreated, $n = 4$ /antioxidant); (C,D) CFSE prelabeled stimulated CD3⁺ T cells were plated in the presence of various antioxidants, and differentially treated with CAP (0 s, 30 s, 60 s). After 3 days, cells were analyzed via flow cytometry; (C) CD3⁺ T cells were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD and represents the pooled results from 3 independent experiments (total replicate size: $n = 9$ /untreated, $n = 6$ /antioxidant); (D) bar diagram shows the average percentage of proliferating CD3⁺ T cells normalized to the untreated control from the same donor ($n = 9$ /untreated, $n = 6$ /antioxidant) $\pm$ SD and represents the pooled results from 3 independent experiments; histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Dunnett tests and is indicated by the asterisks as follows: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

However, when Ma-Mel-19 cells were treated with a combination of CAP and antioxidants, they were found to be significantly more viable and proliferative than when treated with CAP alone. In fact, CAP-antioxidant combination treated cells nearly recovered their viability and proliferation to untreated levels.

Similar effects were also observed with the cell line, UKRV-Mel-15a. None of the tested antioxidants had a significant effect on cell viability or proliferation when compared

to the untreated control (Figure S6A,B). However, with the application of antioxidants, the viability and proliferation of CAP treated UKRV-Mel-15a cells could nearly be restored to untreated levels. When the different antioxidants tested were compared to each other, no significant differences in their effects on Ma-Mel-19 and UKRV-Mel-15a cells, in terms of viability and proliferation, were observed at any time point (Figures 6A,B and S6A,B).

The same tested concentrations of resveratrol, caffeic acid, and rutin were also found to have no effect on the viability of CD3⁺ T cells (Figure 6C), but CAP-antioxidant combination treatment could only partially restore the viability of CD3⁺ T cells. In contrast to the Ma-Mel-19 and UKRV-Mel-15a cells, every antioxidant tested was found to inhibit the proliferation CD3⁺ T cells (Figure 6D). When CD3⁺ T cells received a combination of CAP-antioxidant therapy, their proliferation was not only unable to be recovered but significantly decreased, as in the cases of 30 s CAP-resveratrol and 30 s CAP-caffeic acid combination therapy.

Collectively, these results support that CAP partially mediates its cytotoxic and anti-proliferative effects through its generation of ROS as viability (melanoma and CD3⁺ T cells) and proliferation (melanoma) of CAP treated cells could be recovered following treatment with various polyphenol derived antioxidants.

3.4. Ma-Mel-19 Cells Are More Resistant to Repeated CAP Treatment When Grown as 3D Tumor Spheroids

As shown in Figure 2B,E,F, T cells responded differently to CAP depending on whether they were grown in single cell culture or were co-cultured with Ma-Mel-19 cells. To investigate differential responses to CAP treatment depending on the experimental model utilized further, Ma-Mel-19 cells were cultured in both 2D in vitro monolayers and as 3D in vitro tumor spheroids to better model the more complex melanoma microenvironment. Normally, in vitro studies almost exclusively use cells cultured as homogenous adherent 2D monolayers or as free-floating suspension cells [44]. Although these models are rightfully widely used, cells are grown in highly artificial conditions, such as having minimal contact to each other and the surrounding extracellular matrix. Three-dimensional tumor spheroids offer a more physiologically relevant in vitro test system as spheroids share many of the characteristics of in vivo tumors, such as gradients in oxygen and nutrient availability and a resulting hypoxic and necrotic core [44,45]. These test systems were directly compared to each other by treating Ma-Mel-19 cells grown in 2D monolayers and as 3D tumor spheroids with an identical CAP treatment regime, consisting of 60 s of CAP every 24 h for 3 consecutive days. This repeated CAP treatment plan was implemented to enhance the effects of CAP treatment on Ma-Mel-19 cells. It was found that Ma-Mel-19 cells grown in 2D were significantly more sensitive to repeated CAP treatment, as shown by their significant reductions in viability and proliferation (Figure 7A,B).

However, 3D Ma-Mel-19 spheroids did not display a significant reduction in viability following the same repeated CAP treatment, but a significant reduction of cell proliferation was observed, albeit to a lesser extent, as seen in 2D (Figure 7C,D). In summary, this demonstrates that Ma-Mel-19 cells grown as 3D tumor spheroids are more resistant to CAP therapy than when grown as 2D monolayers.

Due to this observed increased resistance of Ma-Mel-19 spheroids to CAP, the duration of CAP therapy was extended to 8 days to see if the spheroids could be made more susceptible to CAP treatment. Two different CAP treatment approaches were used, where spheroids were treated every day (60 s CAP/24 h) or every other day (60 s CAP/48 h) with CAP, to explore how the frequency of CAP application could affect the spheroids. Spheroids began to receive CAP treatment starting at 2 days after plating, once they were completely formed and had an average diameter of ~300 µm. It is important to note that all conditions had no significant differences in their average diameters on day 2 before the start of CAP treatment (Figure 7E). At day 4, spheroids treated with either CAP treatment regime already exhibited significantly smaller average diameters than the untreated control. It was also seen that spheroids treated with CAP daily were significantly smaller than

spheroids treated with CAP every other day. Differences in average diameter between untreated versus CAP treated spheroids only became larger with time, as did the differences between spheroids treated with CAP daily versus every other day (Figure 7E). Although CAP treated spheroids grew slower than the untreated control, CAP treatment was not successful in halting the growth of the spheroids completely. It is interesting to note that CAP treated spheroids also exhibited noticeable alterations in their morphology, namely an accumulation of surrounding loosely attached cells and the cores of the spheroids were visibly less dense and compact (Figure 7E). These features became more pronounced with more frequent applications of CAP (60 s CAP/24 h). This suggests that prolonged CAP treatment is seemingly able to damage the structure of Ma-Mel-19 spheroids.

On day 10, spheroids were digested and analyzed for alterations in their viability and proliferation. Only spheroids treated every day with CAP, displayed significant reductions in viability (Figure 7F). However, proliferation in both CAP treatment regimens was significantly inhibited in comparison to the untreated control (Figure 7G). It is important to note that although increasing the duration and frequency of CAP treatment resulted in less viable and proliferative cells than spheroids treated only 3 times with CAP (Figure 7C,D), the effects of extended CAP treatment on 3D spheroids (Figure 7F,G) were markedly less effective, in terms of reducing viability and proliferation, than those observed in 2D culture (Figure 7A,B).

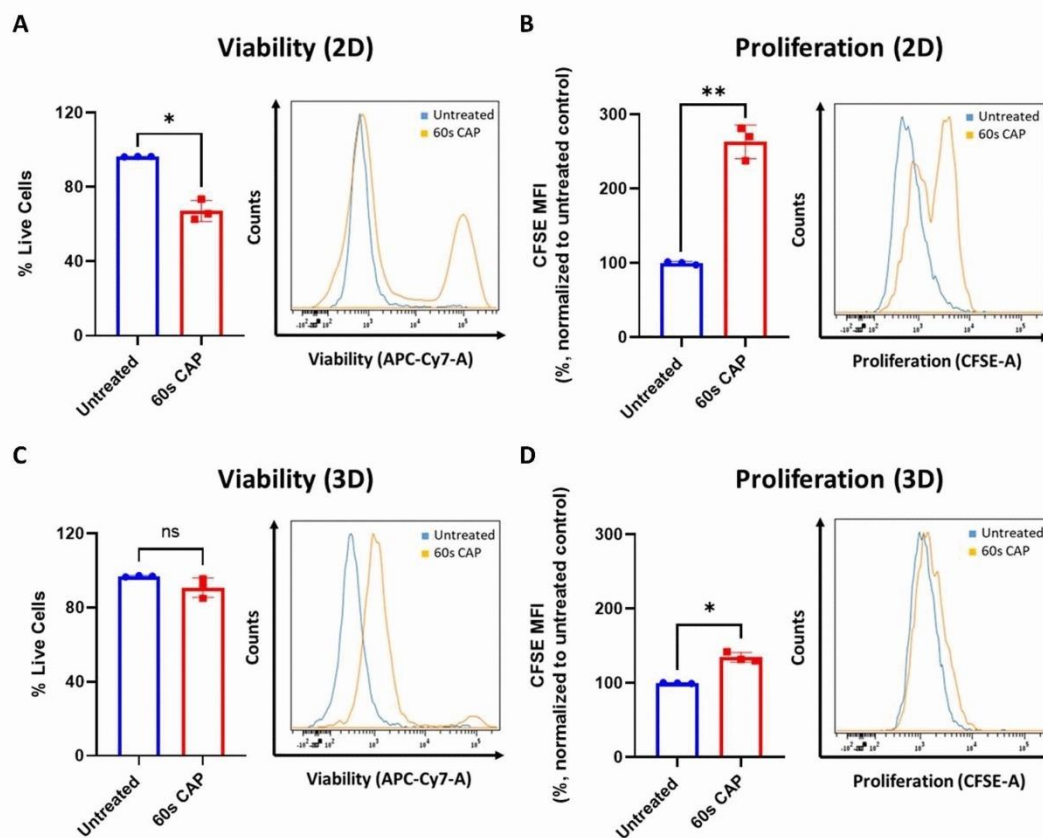


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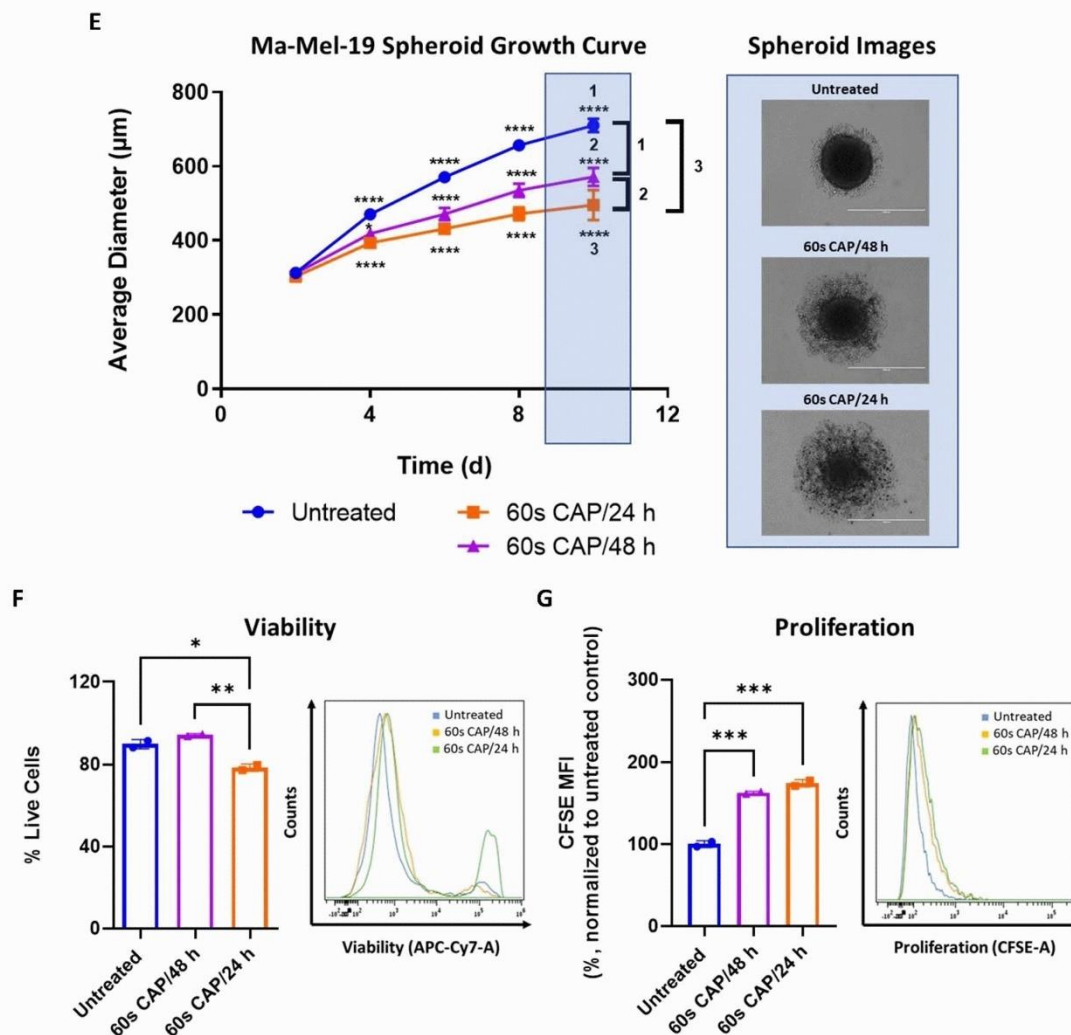


Figure 7. Ma-Mel-19 cells are more resistant to repeated CAP treatment when grown as 3D tumor spheroids. (A,B) CFSE prelabeled Ma-Mel-19 cells were grown in 2D in vitro monolayers and treated with 60 s CAP for 3 consecutive days. On the day after, cells were analyzed in flow cytometry; (A) cells were stained for viability. Bar diagram shows the average percentage of live cells measured ($n = 3$) $\pm$ SD; (B) bar diagram shows the average CFSE mean fluorescent intensity (MFI) normalized to the untreated control ($n = 3$) $\pm$ SD; statistical significance was calculated by performing paired Student's *t*-tests; (C,D) CFSE prelabeled Ma-Mel-19 cells were grown as 3D in vitro tumor spheroids and were treated with 60 s CAP for 3 consecutive days. On the day after, spheroids were pooled ($n = 6$ spheroids/replicate of each condition, $n = 3$), digested, and analyzed in flow cytometry; (C) spheroids were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD; (D) bar diagram shows the average CFSE MFI measured, normalized to the untreated control $\pm$ SD; statistical significance was calculated by performing paired Student's *t*-tests; (E–G) CFSE prelabeled Ma-Mel-19 cells were grown as 3D in vitro tumor spheroids. Spheroids were treated with 60 s of CAP every day (60 s CAP/24 h) or every other day (60 s CAP/48 h) for 8 consecutive days. On the day after, spheroids were pooled ($n = 6$ spheroids/replicate of each condition, $n = 2$), digested, and analyzed

in flow cytometry; (E) spheroids were imaged ($n = 9$) every other day to monitor their growth. Images were analyzed to determine spheroid diameter. Data shown represents the average spheroid diameter of each condition overtime $\pm$ SDs. Representative images of spheroids with or without CAP treatment at day 10 are shown; (F) spheroids were stained for viability. Bar diagram shows the average percentage of live cells measured $\pm$ SD; (G) bar diagram shows the average CFSE MFI measured, normalized to the untreated control $\pm$ SD; histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing ordinary one-way ANOVA tests corrected for multiple comparisons with Tukey tests and is indicated by the asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Taken together, these results demonstrate that Ma-Mel-19 cells are more resistant to CAP when grown as 3D tumor spheroids. However, by increasing the duration and frequency of CAP treatment, it was possible to reduce the size of Ma-Mel-19 spheroids and the viability and proliferation of the cells that make it up.

4. Discussion

The discovery of cold atmospheric plasma (CAP), or plasma within physiological temperatures ($<40^\circ\text{C}$), enabled new applications of CAP in biological settings. When CAP is applied to biological tissues, the main reactive components it generates are RONS and UV radiation [23]. These reactive components lead to complex biochemical interactions with cells and result in a diverse range of biological effects, including blood coagulation, sterilization, cell detachment, and wound healing [24]. In recent years, CAP has attracted much attention as an innovative therapeutic option for cancer treatment. CAP has been shown to have multiple anticancer effects, such as selectively inhibiting proliferation, increasing DNA damage, and subsequently inducing apoptosis of cancer cells [13,25,46]. This selective effect of CAP on tumor cells is not yet completely understood, but one theory is that they are more sensitive to CAP induced oxidative stress than other cells in the body [25].

Normally cells require low levels of ROS for signaling, and ROS are naturally generated as by-products of OXPHOS [10]. However, prolonged elevated ROS levels have been linked to cancer promoting effects, including activating pro-tumor signaling pathways, increasing proliferation, cell survival, and genetic instability [12,13]. Due to these advantages and their own increased rates of metabolism, cancer cells constantly live with elevated ROS levels and thus exist in an inherent state of oxidative stress. Cancer cells cope with these elevated levels by increasing their production of antioxidant proteins [12]. However, when ROS levels rise above their tolerance threshold, ROS can damage cellular components, including lipids, DNA, and proteins, and lead to oxidative stress induced apoptosis [12,15]. This makes tumors cells innately more vulnerable to increases in exogenous ROS levels, which can be therapeutically manipulated, such as through the use of CAP.

CAP can be applied directly administered to cells of the tumor microenvironment or be indirectly administered as plasma treated liquids [47]. A drawback of direct CAP application is that it remains physically limited to superficial tissues; however, it is possible to use it as a post-operative treatment for the surgical field to eliminate remaining tumor cells [24]. Indirect treatment through the application of plasma pretreated liquids allows the potential systemic application to larger tumors and metastases. However, concerns exist regarding the safety of this method of application [24]. Nevertheless, CAP has shown promising results in a wide range of cancer types in *in vitro* studies, in several *in vivo* studies, and in a few clinical studies [24,25,36,48].

The present study addressed how CAP treatment can affect the survival and metabolism of cells that compose the melanoma microenvironment, including tumor cells, T cells, and macrophages. It was found that with increasing CAP treatment times, viability and proliferation of melanoma and T cells decreased in a dose-dependent manner. When these CAP induced cytotoxic and anti-proliferative effects were further investigated, it was found that CAP treatment led to G2/M cell cycle arrest and apoptosis of melanoma cells

(Figures 1C,D and S3A,B). This is highly suggestive that CAP treatment led to extensive DNA damage, resulting from increased oxidative stress levels, and subsequent apoptosis of melanoma cells. Although this study did not confirm the presence of DNA damage, it is well established that CAP can increase DNA damage, inhibit cell proliferation, and induce apoptosis of cancer cells, making these collective findings in agreement with previous works [13,25,46].

The findings of this study also showed that T cells were more sensitive to CAP than tumor cells. Previous studies have shown that T cells require low levels of ROS for activation, proliferation, and differentiation; however, with too high levels, they inhibit proliferation, damage DNA, and induce apoptosis [49]. The results of this study are in agreement with these tell-tale signs of too high ROS exposure. The CAP treatment times used in this study seem to have consistently produced too high ROS levels to be tolerated by CD3⁺, CD4⁺, and CD8⁺ T cells, as seen by the decreases in viability and proliferation following 30 s of CAP exposure (Figure 2A,B,D). This indicates that the minimum CAP treatment time (60 s) required to have noticeable effects on the tested melanoma cell lines (Figure 1A,B) already would result in the severe cytotoxic and anti-proliferative effects on CD3⁺, CD4⁺, and CD8⁺ T cells grown in a single cell culture (Figure 2A,B,D). CD4⁺ and CD8⁺ T cells also exhibited various signs of mitochondrial stress following 30 s of CAP treatment, whereas the different melanoma cell lines tested required double (UKRV-Mel-15a) or even quadruple (Ma-Mel-19) the dose of CAP to begin to show signs of mitochondrial stress. These results collectively challenge the established field of thought that CAP selectively targets tumor cells as CD3⁺, CD4⁺, and CD8⁺ T cells were consistently more sensitive to CAP treatment in this study.

This difference in observed selectivity likely results from the chosen cell comparison. In the past, many studies have compared the sensitivity of cancer cells to CAP with their corresponding healthy cell type [25,33,46]. Although these comparisons provide helpful information, it is important to also consider how CAP influences the immune cell compartment in the tumor microenvironment, which can greatly influence therapy response and patient prognosis [26,27]. The discovery that CD3⁺, CD4⁺, and CD8⁺ T cells were more sensitive to CAP treatment than tumor cells in this study is disconcerting. It is worth noting that differences in resistance to oxidative stress have been observed amongst different T cell subpopulations. For instance, regulatory T cells are believed to be more resistant to oxidative stress due to their elevated production of antioxidants and their capability to persist in high numbers in the tumor microenvironment [50,51]. Future studies should take care to examine how CAP influences the different T cell sub-populations, especially Tregs, and their implications for therapy response.

To see if the undesired effects of CAP treatment on CD3⁺ T cells could be counteracted, CD3⁺ T cells and melanoma cells were treated with various polyphenol derived antioxidants to attempt to neutralize CAP induced ROS. As a brief background, polyphenols are generally easily oxidized by free radicals, especially when they contain ortho or para diphenol functional groups. Delocalized electrons in their aromatic phenol rings then allow for the formation of more stable phenol radicals, which help end the radical transfer chain [43]. This leads to the sequestration of highly reactive free radicals, which can readily react with and damage vital cellular components. Due to the well-known free radical reducing function of polyphenol derived antioxidants, it was tested in this study if they could be used to neutralize ROS produced by CAP. Although this approach could partially recover the viability of CD3⁺ T cells, all the tested antioxidants inhibited their proliferation with and without CAP treatment (Figure 6C,D). It was also found that both of the melanoma cell lines tested responded significantly better to antioxidant treatment than CD3⁺ T cells—nearly recovering their viability and proliferation to untreated levels (Figures 6A,B and S6A,B). These collective results indicate that this antioxidant intervention strategy to CAP treatment was only partially successfully. Overall, these results agreed with other studies as they showed that one of the ways CAP exerts its cytotoxic and anti-proliferative effects is through its generation of ROS as these effects could be directly negated with the application

of various antioxidants [25,41]. Future studies should test alternative antioxidants to see if the anti-proliferative effects on CD3⁺ T cells observed in this study can be avoided.

This study also provided valuable information on the influence of CAP on the viability and polarization on macrophages. Out of all the cell types tested in this study, M0 macrophages were found to be the most resistant to the cytotoxic effects of CAP, displaying no significant decreases in viability even after 120 s of CAP treatment (Figure 3A). These results agree with a previous study, which showed that the survival of monocyte derived macrophages was unaffected following 30 s of CAP treatment [52]. However, macrophages still exhibited potentially detrimental effects from CAP treatment, in terms of their polarization state and mitochondrial stress levels. Our study demonstrated that M0 macrophages could be effectively polarized into a more “M0/M2-like” phenotype following CAP treatment. Previous studies have examined the effects of CAP on macrophage polarization with conflicting results, likely stemming from their different macrophage sources and analyzed polarization markers [52,53]. Our study provided much needed clarity by staining with a more expansive macrophage polarization panel and by utilizing more physiologically relevant human monocyte derived macrophages in place of THP-1 differentiated macrophages [53]. Tumor associated macrophages, which aid in cancer progression, are generally described to exhibit more of a M2-like phenotype, making the implications of CAP treatment on the tumor microenvironment concerning [26]. Nevertheless, CAP induced M2 polarization is in accordance with the well-known wound healing and skin regeneration effects of CAP described in the literature [23,24].

This study reported for the first time that M1 macrophages exhibited a greater sensitivity to CAP induced oxidative stress as indicated by their increased proton leak, suggestive of mitochondrial damage. This finding is in alignment with the results of Griess et al., who found that M1 macrophages have higher amounts of intracellular ROS and are more sensitive to increases in ROS levels than M2 macrophages [54]. Usually, M1 macrophages are considered to be desirable in cancer treatment as they can help mediate inflammatory anti-tumor immune responses [55]. Therefore, future studies should investigate the long-term effects of CAP treatment on macrophages, especially in more complex experimental models, such as 2D and 3D macrophage-tumor cell co-cultures and in *in vivo* studies, to determine whether CAP may lead to an unwanted increase in M2 macrophages in the tumor microenvironment.

Although the results of this study showed potentially concerning effects of CAP on T cells and macrophages *in vitro*, initial studies using murine melanoma models have shown promising effects of CAP *in vivo*. Depending on the different study designs and treatment modalities, significant inhibition of tumor growth, reduction of tumor burden, increased survival rates, and even tumor eradication have been observed [56–59]. In addition, it was reported that CAP did not damage the skin at the point of application [56,57]. Further analysis of the *in vivo* murine melanoma microenvironment has remained limited to introductory histology stainings. Although they have revealed the presence of necrotic tumor tissue following CAP treatment, in-depth studies regarding the effect of CAP on the cells in the *in vivo* murine melanoma microenvironment have yet to be performed [57,58]. However, the importance of the immune cell compartment in *in vivo* murine melanoma tumor clearance following CAP treatment has already been supported. In more detail, initial studies have shown an increase in tumor immunogenicity following CAP treatment in *in vivo* murine melanoma models, including the upregulation of MHC-I and calreticulin [60,61]. Altogether, this suggests that CAP could be used to induce immunogenic cell death of *in vivo* murine melanoma cells.

The results of this study remain difficult to reconcile when compared to the findings of *in vivo* murine melanoma models as an in-depth analysis of the immune cell compartments found in the *in vivo* melanoma microenvironment has yet to be performed. However, it is clear to see that CAP plays a highly complex role in the melanoma microenvironment. On one hand, it can seemingly support tolerogenic immune responses, such as the induction of M2 macrophages, as observed in this study. On the other hand, it is also linked to

inducing inflammatory immunogenic cell death of melanoma cells [60,61]. Unraveling how these seemingly conflicting immunological effects play out in more complex in vivo experimental models, including the identification of favorable immune cell populations and their implications for anti-tumor immune responses, should be an essential focal point for future studies.

It was also found that the different cancer cell lines and types tested in this study exhibited varying sensitivities to CAP induced oxidative stress in terms of their respective viability, proliferation, and mitochondrial stress levels. Between the two melanoma cell lines tested, Ma-Mel-19 was found to be more sensitive to CAP treatment, in terms of viability and proliferation (Figure 1A,B), while the glioblastoma cell line T98G displayed greater signs of mitochondrial stress than both the tested melanoma cell lines following CAP treatment (Figure 4A–D). Nonetheless, differences between other cancer cell lines and types and their sensitivity to CAP treatment have been reported previously [62,63]. The underlying mechanisms for differential sensitivities of cancer cells to CAP must be investigated in future studies.

Only two other known studies have examined the effects of CAP on melanoma spheroids [64,65]. The findings of this study validated the cytotoxic and the inhibitory effects of CAP on melanoma spheroid size, as observed by Hasse et al. [65]. However, this study differed from Hasse et al. and Sagwal et al. as it examined the effects of repeated long-term CAP treatment on melanoma spheroids [64,65]. Whereas Hasse et al. and Sagwal et al. only treated their spheroids once with CAP and incubated them for up to 1 day before analysis, this study explored how extended and repeated CAP treatment could affect the long-term growth, viability, and proliferation of melanoma spheroids [64,65]. This study also tested different CAP treatment regimens. It was found that increasing the frequency of CAP application (60 s/24 h) and the duration of treatment (8 consecutive days) led to the most significant reductions in spheroid size, viability, and proliferation, providing valuable treatment information to inform future in vivo studies (Figure 7E–G). Additionally, by using a prolonged treatment duration, CAP induced morphological alterations on melanoma spheroids could be uncovered, demonstrating that long-term CAP application can greatly disrupt the structure of melanoma spheroids (Figure 7E). Future work aims to characterize these morphological changes further and to examine the effects of CAP on 3D immune and tumor cell co-cultures.

Previous studies have shown that 3D tumor spheroids can exhibit increased drug resistance when compared to cells grown in 2D monolayers [45]. Herein, the inherent structure of spheroids themselves appears to play an important role. For instance, spheroids naturally develop gradients in oxygen and nutrient availability leading to a size dependent formation of hypoxic and necrotic cores [45,66–68]. This hypoxic environment leads to the activation of genes associated with drug resistance and cell survival and a decrease in ROS formation, thus reducing therapeutic efficacy [45,69–72]. This study was one of the first of its kind to evaluate if melanoma spheroids are more resistant to CAP induced oxidative stress than melanoma cells grown in 2D monolayers. By using an identical CAP treatment regime, it was discovered that cells grown as 3D tumor spheroids were much more resistant to the cytotoxic and anti-proliferative effects of CAP (Figure 7A–D).

The choice of experimental model was also seen to affect the proliferation of CAP treated CD4⁺ and CD8⁺ T cells. When T cells were grown in a single cell culture, their proliferation was greatly inhibited following 30 s of CAP treatment (Figure 2B). However, when they were co-cultured with melanoma cells and treated with the exact same dose of CAP, their proliferation was seemingly unaffected (Figure 2E,F). Taken together, it becomes evident that cells grown in 2D single cell cultures, the least physiologically relevant experimental model tested in this study, were the most sensitive to CAP treatment. This observed decrease in the therapy effectiveness as the experimental models increased in their complexity has been attributed to one of the reasons many promising in vitro drug candidates fail in vivo studies [45,73]. Future studies should evaluate potential drug candidates and therapeutic strategies in more complex in vitro cell culture models, such

as tumor spheroids, before proceeding to in vivo studies to limit the unnecessary use of experimental animals and to increase translational efficacy [44,45,74].

The results from this study also suggest that CAP may be suitable for other treatment applications. In more detail, the findings, that CAP could polarize M0 macrophages towards a tolerogenic M0/M2 phenotype and the observed heightened sensitivity of inflammatory M1 macrophages to CAP induced mitochondrial stress, all seem to suggest that CAP may enrich M2 macrophages and in turn support wound healing and anti-inflammatory responses. As mentioned earlier, CAP is already used in clinical dermatology for wound healing and skin regeneration therapies [24,25]. However, alternative applications of CAP for other inflammatory diseases are much less characterized. Due to the inherent physical limits of CAP application, superficial inflammatory skin conditions, such as psoriasis, eczema, and atopic dermatitis, represent particularly promising diseases that may profit from CAP treatment. Evidence of the beneficial effects of CAP for the treatment of psoriasis and atopic dermatitis has already been shown. Initial case studies in psoriasis have shown a beneficial effect of CAP treatment in clearing plaques and preventing recurrence [75,76]. Additionally, an initial pilot study with atopic dermatitis patients found that CAP could reduce the disease severity [77]. Altogether, these results support the promise of CAP treatment for inflammatory conditions, which should be an important focus of future studies.

Collectively, this study provided a comprehensive insight into how CAP induced oxidative stress affects the viability, proliferation, phenotype, and metabolism of different cells that comprise the melanoma microenvironment. Herein, the sensitivity to CAP treatment differed between T cells, macrophages, and tumor cells. In general, T cells and tumors cells were more sensitive to oxidative stress than macrophages. However, we could also show that CAP influenced the polarization of M0 macrophages to a “M0/M2-like” phenotype and that M1 macrophages exhibit a heightened sensitivity to CAP induced mitochondrial stress. Additionally, we could also demonstrate that CAP treatment could effectively inhibit the growth and kill melanoma cells in both 2D and 3D in vitro cell culture models in a dose-dependent manner. Improving our understanding of oxidative stress, ways we can manipulate it, and its implications on the cells that comprise the tumor microenvironment may help in the discovery of new and effective clinical targets.

5. Conclusions

In conclusion, we could show that oxidative stress, induced by CAP, could significantly influence viability, proliferation, and mitochondrial respiration of cells, which comprise the melanoma microenvironment. This study contributed to our understanding of oxidative stress, its implications on the survival and metabolism of cells in the tumor microenvironment, and how it can be manipulated in the hopes of contributing to the identification of new and effective therapeutic strategies.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cells11060930/s1>, Figure S1: Gating strategies, Figure S2: MiniJet-R and intracellular ROS quantification, Figure S3: UKRV-Mel-15a cell cycle and cell death data, Figure S4: Non-significant macrophage polarization markers, Figure S5: Example Seahorse OCR and ECAR data, Figure S6: UKRV-Mel-15a antioxidant data.

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4. DISCUSSION

Disclaimer: In-depth discussions of the results in **Paper 1**, **Paper 2**, and **Paper 3** can be found in their respective publications in the “3. Results” section^{8–10}. The following “4. Discussion” places the collective findings of this work into a greater context and explores outstanding questions facing the respective research fields.

Immunotherapy has emerged as an increasingly promising method to treat a diverse range of clinical conditions, ranging from cancer to autoimmunity^{1–7}. Herein, immune responses are optimized using immunomodulatory agents. Depending on the condition, immune responses may be amplified (e.g., cancer) or attenuated (e.g., wound healing). Cancer and deficiencies in wound healing represent significant global healthcare burdens^{106,350}. Globally, there are more than 40 million patients suffering from chronic wounds³⁵¹. Additionally, 20 million cases of cancer were diagnosed, and 9.7 million deaths were attributed to the disease in 2022¹⁰⁶. These numbers are expected to increase as the global population continues to age^{106,350}. Despite this, many patients lack effective treatment options. To address this unmet need, this work characterized two novel immunomodulatory approaches, namely GARP and CAP^{8–10}. In the future, these mechanisms could be used as possible therapeutic approaches for the treatment of cancer and the optimization of wound healing.

GARP is a tolerogenic protein, expressed by activated Tregs and platelets, that is essential for the maintenance of peripheral tolerance^{28,30,61–64,81,82}. Cancer cells upregulate GARP to evade detection by the immune system and to drive tumor progression^{55,61,65,99,100}. Therefore, depletion of GARP in the TME has emerged as a highly promising anti-cancer approach²⁸. Currently, there are ten registered clinical studies, ranging from phase I to 2/3, testing GARP as a therapeutic target^{352–361}. Nine studies are examining anti-GARP antibodies as possible treatment options for advanced or metastatic solid tumors as well as lymphomas^{352,353,355–361}. In more detail, companies are testing monoclonal antibodies that either target GARP alone (ClinicalTrials.gov IDs: NCT05606380, NCT05483530, NCT04419532, NCT05821595), GARP bound to LTGF- β 1 (ClinicalTrials.gov IDs: NCT03821935, NCT06109272, NCT05822752, NCT06236438, NCT06310746), or are bispecific against GARP and PD-L1 (ClinicalTrials.gov IDs: NCT05869240)^{352–361}. Although all antibodies address GARP in some form, they vary in their method of action. Many prevent the release of active TGF- β into the TME, whereas others deplete GARP⁺ cells, like activated Tregs and tumor cells, via ADCC^{28,352–366}. Most studies are examining the efficacy of anti-GARP antibodies in combination with anti-PD-1 antibodies, which has previously shown compelling synergy in preclinical *in vivo* experiments^{353,355–358,362,367–369}.

These preclinical studies showed that this combination approach (anti-GARP/anti-PD-1 antibodies) enhanced the function of Teff, increased the infiltration of CTLs into the TME, depleted GARP⁺ Tregs in the TME, and resensitized PD-1 resistant tumors to therapy^{367,369}. This combined treatment approach would be particularly interesting to test in glioblastoma as the disease is characterized by low immune cell infiltration, high resistance to PD-1/PD-L1 ICIs, and the fact that glioblastoma cells use GARP as a mechanism of immune

evasion^{65,198,227,228,370}. **Paper 2** showed that GARP is universally expressed by GSCs, which are known to drive tumor initiation, therapy resistance, and recurrence⁹. Targeting of GARP⁺ glioblastoma cells, GSCs, and Tregs offers a compelling approach to overcome suppression in the glioblastoma microenvironment, but this must be evaluated in future *in vivo* studies.

It is important to mention that there is a pressing concern that anti-GARP antibody therapy may deplete platelets and thereby increase the risk of bleeding related complications. Initial *in vivo* experiments, utilizing the anti-GARP monoclonal antibody, PIIO-1, which specifically recognizes GARP unbound to LTGF- β , have demonstrated that this approach can be safely tolerated in mice³⁶⁹. It is important to note that platelets only express GARP-LTGF- β complexes, which prevented PIIO-1 from depleting GARP⁺ platelets. Future studies are needed to determine if anti-GARP antibodies with differing modes of action, especially those that target GARP-LTGF- β complexes or act by depleting GARP⁺ cells with ADCC, pose a risk for humans by excessively depleting platelets^{28,352–359,362}.

So far, the first results from clinical studies have been promising. AbbVie reported the results of their phase I clinical trial (ClinicalTrials.gov ID: NCT03821935) of an anti-GARP/LTGF- β 1 antibody (ABBV-151) alone and in combination with an anti-PD-1 monoclonal antibody (Budigalimab)^{355,371}. They observed a manageable safety profile and, in some patients, even durable responses following combination therapy. In the upcoming years, results from the other clinical studies will be made available. Only then, can the safety and efficacy of anti-GARP monoclonal antibodies for the treatment of cancer be determined.

Modulation of GARP levels may also serve as a promising therapeutic strategy to enhance wound healing. **Paper 1** reported for the first time that GARP may play an important role in early stages of wound healing through the induction of Tregs⁸. It is known that Tregs promote tissue repair and regeneration by regulating inflammation^{25–27}. For example, Tregs suppress the production of proinflammatory cytokines by neutrophils as well as by CD4⁺ and CD8⁺ Teff, which sustain the inflammation phase. Similarly, **Paper 1** demonstrated that GARP, derived from iPRF and iPRF derived platelets, inhibits the production of the proinflammatory Th1 cytokines, IFN- γ and IL-2⁸. **Paper 1** also showed that iPRF and iPRF derived platelets induce suppressive GARP⁺Foxp3⁺ Tregs in a GARP dependent manner⁸. This opens-up the possibility of modulating GARP levels to enhance wound healing, e.g., the addition of sGARP to autologous platelet concentrates. It is known that sGARP displays strong biological potency in inducing anti-inflammatory immune responses, making this approach promising for wound healing^{56,70,83,84}. This approach is particularly attractive for the treatment of chronic wounds, which fail to transition from the inflammation stage to the anti-inflammatory proliferation stage^{95,97}. However, the efficacy of this approach must be evaluated in future studies.

It should be noted that GARP has been previously reported to be dysregulated in wound healing, specifically in the later remodeling stage of wound healing^{372–374}. GARP has been implicated in the development of fibrosis (the formation of excessive scar tissue)^{372–376}. Fibrosis occurs when there is an excessive accumulation of ECM. Over time, this increases tissue rigidity and can alter the functionality of the affected organ. GARP has been found to

enhance fibrosis through its function in activating the profibrotic cytokine, LTGF- β ³⁷⁴. TGF- β acts as a driving force behind the development of fibrosis by simultaneously activating cells, like hepatic stellate cells and myofibroblasts, to produce excessive ECM and by inhibiting the degradation of ECM^{374–376}. Zhang et al., 2023 demonstrated that selective deletion of GARP on hepatic stellate cells in an *in vivo* model of liver fibrosis decreased collagen deposition³⁷⁴. Additionally, they found that GARP mRNA levels are upregulated in patients with fibrosis in comparison to healthy donor controls. Similarly, activated GARP⁺ Tregs were also reported in patients with chronic hepatitis C, a disease characterized by liver fibrosis, and correlated with fibrosis staging^{372,373}. Therefore, in the case of fibrosis, it would not be recommended to increase GARP levels to promote wound healing — but rather to deplete them. This approach, specifically using anti-GARP antibodies, has already shown promise as a novel treatment approach for fibrosis^{354,364,377}.

Shanghai Henlius Biotech, Inc. has developed HLX6018, an anti-GARP/TGF- β 1 antibody with the aim of treating idiopathic pulmonary fibrosis^{354,364}. The company recently gained approval for a phase I clinical trial, examining the safety and immunogenicity of HLX6018 in healthy participants³⁵⁴.

Collectively, these studies support the promise of modulating GARP levels to optimize immune responses in cancer (depletion) as well as in the early (enhancement) and late (depletion) stages of wound healing.

Upregulation of GARP in the tumor microenvironment, coupled with its dual role in immune evasion and tumor progression, makes GARP a promising biomarker candidate for cancer^{28,49,55,61,65,99,100,102,104,139,140}. Previous studies have analyzed different forms of GARP (sGARP, GARP⁺ cells, and GARP mRNA levels) as predictive, prognostic, and diagnostic biomarkers for different tumor entities^{28,56,60,63,99–104}. **Paper 2** stands out from them as it was the first study to analyze GARP^{NU} as a biomarker⁹. In more detail, a high frequency of GARP^{NU+} cells in the glioblastoma microenvironment correlated inversely with overall survival of patients. Similarly, studies have reported that other abnormally localized nuclear proteins, like CD55 and PD-L1, exhibit promise as prognostic biomarkers for cancer^{378–381}. However, future studies are needed to expand sample sizes and to optimize the cut-off before it can be determined if GARP^{NU} can be used as a prognostic biomarker for glioblastoma.

The strong potential of GARP as a therapeutic target and biomarker makes it important to better understand the biology of the protein. **Paper 2** reported for the first time a possible regulatory relationship between GARP and cyclin-dependent kinase inhibitor 1 (p21)⁹. In more detail, it was found that GARP^{high} GSCs express a higher level of p21 than GARP^{low} GSCs. It is well known that p21 promotes cell cycle arrest by inhibiting CDK4,6/cyclin-D and CDK2/cyclin-E³⁸². Therefore, higher levels of p21 are associated with decreased progression through the cell cycle. It has been reported that expression of p21 can be induced by TGF- β ^{382,383}. The results of **Paper 2** suggest a possible upstream regulation of p21 expression by GARP, in a seemingly TGF- β dependent manner⁹. It is important to point out that a significant difference in the expression of the proliferation marker, phosphorylated histone H3, between GARP^{high}

and GARP^{low} GSCs was not observed. This could be attributed to the innate slow growing nature of GSCs, making differences in the proliferation marker marginal. Future experiments, including the inhibition of TGF- β and extended functional proliferation assays of GSCs, are required to verify this potential regulatory relationship.

It has been found that p21 is essential for maintaining the quiescence of hematopoietic stem cells (HSCs)³⁸⁴. By inhibiting the cell cycle, p21 limits cell division of HSCs, which puts them at risk for developing DNA damage^{385,386}. Over time, the accumulation of DNA damage reduces the long-term ability of HSCs to self-renew and subsequently leads to their functional exhaustion. Similarly, p21 has been found to maintain quiescence in cancer stem cells^{386,387}. p21 inhibits the hyperproliferation of cancer stem cells, and thus safeguards the long-term self-renewal capacity of cancer stem cells. The findings of **Paper 2** suggest that GARP may play an important role in maintaining quiescence in GSCs by regulating p21⁹. This is further supported by the discovery that GARP^{high} GSCs exhibit significantly less self-renewal than GARP^{low} GSCs. This makes sense as the process of self-renewal requires cell division to generate daughter cells, whereas cells in a quiescent state do not replicate. Analysis of additional quiescence and self-renewal markers on GARP^{high/low} GSCs are needed to test this hypothesis.

Paper 2 reported the abnormal nuclear localization of GARP in GSCs and the glioblastoma microenvironment⁹. Previously, it was widely accepted that GARP is located on surface of cancer cells and can be released as a soluble factor into the TME (sGARP)^{28,56,61,65}. Zimmer et al., 2019 reported for the first time that GARP is highly expressed in the nuclei of glioblastoma cells and GSCs⁶⁵. **Paper 2** demonstrated for the first time that a high frequency of GARP^{NU+} cells in glioblastoma tumor tissue is linked to poor prognosis⁹. These factors coupled with knowledge that GARP's only known binding partner, LTGF- β , has not been reported to be localized to the nucleus, compelling support the potential of GARP acting as a moonlighting protein³⁸⁸.

Moonlighting proteins refers to a subset of proteins, in which a single protein exhibits more than one function^{389–391}. The function of a moonlighting protein can vary depending on where it is localized in the cell or by what cell type expresses it. Graeme Wistow and Joram Piatigorsky first described the concept in 1987, where they reported that crystallins exhibit dual structural and enzymatic functions³⁹². For example, in *Anas platyrhynchos* (Mallard ducks), ϵ -crystallin acts as both a structural component of the lens as well as the enzyme, lactate dehydrogenase^{393,394}. Since the initial discovery of moonlighting proteins, more than 500 proteins have been identified^{390,393,395}. Many of these proteins are highly conserved enzymes and exhibit a diverse range of functions (e.g., acting as a transcription factor, DNA binding protein, cytokine, receptor, chaperone, proteasomal subunit, etc.)³⁹⁰. GARP has structural characteristics of a moonlighting protein as it is highly conserved, is rich in LRRs that are known to facilitate protein-protein interactions, and is differentially localized in and outside of the cell (**Fig. 3**)^{28,47,49,72,396,397}.

Currently, the search for an alternative function of GARP in the nuclei of cancer cells is underway. Initial *in silico* predictions have identified two possible interaction partners, namely the proteins signal transducer and activator of transcription 3 (STAT3) and decapping MRNA 2 (DCP2) (**Fig. S18-20**). Across several protein interaction prediction platforms, STAT3 and DCP2 were found have a high likelihood of interacting with GARP; they are also known to be localized to the nucleus (**Fig. S18-20**)^{398–408}. STAT3 is a transcription factor that translocates to the nucleus upon phosphorylation. DCP2 removes the 7-methyl guanine cap from mRNA molecules and is required for mRNA degradation. Notably, STAT3 and DCP2 are upregulated in the glioblastoma microenvironment, where they promote tumor growth and immune evasion, similar to the known functions of GARP^{65,99,100,409,410}.

Preliminary work has biochemically confirmed for the first time the presence of GARP in the nucleus and its enrichment in the chromatin of tumor cells (**Fig. S21A**). Furthermore, co-immunoprecipitation of GARP and STAT3 has confirmed the direct interaction of the proteins (**Fig. S21B**). Two independently performed high-throughput affinity capture mass spectrometry experiments also reported purification of GARP and STAT3 from tumor cells, providing further evidence for the binding interaction^{411,412}. These same studies reported purification of GARP and DCP2, but this binding interaction must still be biochemically confirmed on a smaller scale. These results indicate the possibility of GARP acting as moonlighting protein, most promisingly by binding STAT3, but there is much to be understood.

Future studies must determine how the proteins interact with each other. It remains unclear what domains are essential for the binding interaction. GARP has 20 LRRs that contain conserved domains, including a protein phosphatase 1 regulatory subunit 42 (PPP1R42) domain and an another domain implicated in transcription^{28,47,49,72,397,397,413}. The transcription factor, STAT3 contains a SH2 domain that can be phosphorylated by tyrosine kinases, leading to its translocation to the nucleus^{399,400}. Of note, LRRs are reported to interact with SH2 domains⁴¹⁴. Therefore, it can be speculated that GARP and STAT3 may interact with each other via binding of their respective LRR and SH2 domains; however, this must be evaluated *in vitro* by mutating suspected amino acids involved in the binding interaction. The localization of the GARP-STAT3 binding interaction must also be determined to evaluate whether this can be attributed as a function of GARP^{NU}. This could be accomplished by performing subcellular fractionation, followed by co-immunoprecipitation.

A possible regulatory relationship between GARP and STAT3 as well as the functional implications of the interaction must also be clarified. Kuhn et al., 2017 reported that IL-6 treated murine naïve CD4⁺ T cells inhibited the expression of the GARP mRNA in a STAT3 dependent manner⁴¹⁵. Furthermore, Walton et al., 2020 showed that human Tregs treated with a pSTAT3 inhibitor had significantly higher levels of GARP⁺ Tregs⁴¹⁶. It remains unclear whether a similar regulatory relationship of GARP by STAT3 exists in human malignant cells. Future studies will investigate both the regulatory and functional relationships of the proteins, specifically using GARP overexpression and knockdown cell lines. RNA sequencing

experiments are also planned to examine if GARP may influence transcription, especially in the case of STAT3 regulated genes.

Paper 2 reported the abnormal localization of GARP in the nuclei of GSCs and cells in the glioblastoma microenvironment⁹. It remains unclear how GARP translocates into the nucleus. There are no annotated nuclear localization sequences (NLS) for the GARP protein. NLS are often conserved and consist of basic amino acids, like arginine (R) and lysine (K)^{417,418}. Preliminary *in silico* predictions identified a possible NLS candidate for the GARP protein (**Fig. S22A**). The identified bipartite NLS candidate (KR 517-518, RR 564-565) was found to be evolutionary conserved (**Fig. S22A**). The identified candidate is located outside the LTGF- β binding cleft on the surface of the protein, enabling it to be theoretically accessed by nuclear importin proteins (**Fig. S22B-C**). Selective mutation of the amino acids in the identified NLS candidate are planned to determine whether nuclear translocation of the protein can be prevented.

Lastly, it remains unclear from where GARP^{NU} originates. Traditionally, nuclear bound proteins are translated in the cytoplasm before being trafficked into the nucleus⁴¹⁹. Bharti et al., 2024 recently challenged this paradigm by demonstrating that the moonlighting protein CD55 can be trafficked from the cell surface into the nucleus³⁸¹. Future experiments are planned to see if this is also the case for GARP. In more detail, tumor cells will be treated with the GARP cleaving protease thrombin to remove GARP from the cell surface⁶³. Following cleavage, cells will be assessed for their expression of cytoplasmic and GARP^{NU} over time to determine if they are dependent on surface GARP expression.

Besides GARP, this work analyzed the effects of CAP on cells found in the melanoma microenvironment (**Paper 3**)¹⁰. It is not fully understood how CAP interacts with the cell. It is known that CAP treatment induces the production of diverse and highly reactive RONS³⁴⁹. These include but not limited to hydrogen peroxide (H₂O₂), hydroxyl radical (OH^{*}), superoxide (O₂⁻), ozone (O₃), singlet oxygen (¹O₂), nitric oxide (NO), nitrogen dioxide (NO₂), nitrous oxide (N₂O), nitrogen trioxide (NO₃), and dinitrogen tetroxide (N₂O₄)^{349,420,421}. RONS indiscriminately interact with fundamental components of the cell (e.g., DNA, protein, lipids), which leads to a plethora of biological effects⁴²².

The findings of **Paper 3** indicate several distinct biological effects of CAP¹⁰. Firstly, it was observed that CAP treatment triggered G2/M cell cycle arrest, inhibited cell proliferation, and induced apoptosis in melanoma cells — characteristic signs of excessive DNA damage^{10,423}. Although DNA damage was not evaluated in **Paper 3**, it has already been shown to result from CAP treatment in the literature^{349,424–428}. **Paper 3** found that CAP reduced the proliferation and viability of cancer cells and T cells; this could be reversed in part with the application of antioxidants¹⁰. This indicates that excessive RONS are responsible in part for these observed biological effects. Additionally, CAP treated cells showed signs of mitochondrial stress. This may be explained by possible damage to mitochondrial membranes, leading to metabolic dysfunction. This is supported by recent studies that have demonstrated that CAP disrupts mitochondrial membrane potential as well as induces mitochondrial swelling and the intrinsic

apoptosis pathway^{429,430,430}. Excessive lipid peroxidation induced by CAP in cancer cells has also been linked to ferroptosis, indicating yet another biological effect of CAP⁴³¹. Although the biological effects of CAP are still far from being understood, the clinical promise of CAP has already been supported.

Currently, CAP is predominantly used in clinical dermatology to treat chronic wounds, such as ulcers, to accelerate wound healing^{432,433}. In 2010, a phase II clinical trial reported that CAP treated wounds exhibited a significant reduction in bacterial loads, regardless of the bacteria species present at the wound site⁴³². They also reported no associated side effects of CAP treatment. These anti-bacterial effects have been attributed to innate characteristics of CAP, such as the emission of UV radiation as well as the generation of RONS and electrical current⁴³³. Besides disinfection, CAP promotes wound healing by promoting angiogenesis as well as increasing the proliferation and migration of keratinocytes^{433–438}.

The first clinical trial investigating the safety of CAP for cancer treatment was conducted from 2020 to 2021 (ClinicalTrials.gov ID: NCT04267575)⁴³⁹. The phase I clinical trial examined CAP as a post-operative treatment for the surgical margins of advanced (IV) and recurrent solid tumors. In more detail, this study aimed to target residual microscopic tumor cells at the surgical margin to prevent local recurrence of the disease. It was found that CAP exhibited a selectivity for cancer cells in comparison to healthy cells. Strikingly, the study reported no adverse effects related to CAP treatment. Although the authors did not analyze alterations in immune cell composition in the TME, like **Paper 3**, assurance that CAP exhibits a strong safety profile is highly encouraging¹⁰.

Recent studies have shown that CAP is not limited to the treatment of superficial tumors. Rather, it shows strong promise as a post-operative adjuvant treatment^{440,441}. Yoon et al., 2018 demonstrated that in an *in vivo* model of vestibular schwannoma, post-operative CAP treatment following primary tumor resection significantly reduced recurrent tumor growth⁴⁴⁰. Chen et al., 2021 showed similar results in a *in vivo* model of recurrent breast cancer⁴⁴¹. These results are in alignment with **Paper 3**, which showed CAP exerts cytotoxic and anti-proliferative effects on melanoma cells *in vitro*¹⁰. Of note, the duration of CAP as a post-operative treatment varied widely by cancer type (vestibular schwannoma, 10 min; breast cancer, 1-4 min)^{440,441}. **Paper 3**, in contrast, utilized a repeated treatment approach (1 min CAP/24h, 1 min CAP/48 h), which is more suitable for easily accessible superficial tumors¹⁰. This implies that CAP treatment should be optimized to the respective clinical application (post-operative treatment or direct treatment of tumors) as well as tumor type.

CAP can also induce anti-tumor immune responses when applied as an adjuvant post-operative treatment strategy^{441,442}. Chen et al., 2021 found that CAP treatment induced anti-tumor T cell mediated immunity in a model of recurrent breast cancer *in vivo*⁴⁴¹. In more detail, CAP treatment resulted in the upregulation of the immunogenic cell death (ICD) marker, surface calreticulin, on residual tumor tissue. It is known that when cancer cells undergo ICD, they release tumor associated antigens; this leads to the activation and maturation of DCs⁴⁴³. The findings from Chen et al., 2021 supported this as they found a higher

frequency of mature DCs in the tumor draining lymph nodes of CAP treated animals⁴⁴¹. Mature DCs aid in the activation of T cells⁴⁴³. They also found a significant increase in the infiltration of CD3⁺CD4⁺ and proliferating CD3⁺CD8⁺ T cells in CAP treated recurrent tumors⁴⁴¹. Similarly, **Paper 3** demonstrated increased proliferation of CD8⁺ T cells *in vitro* when in co-culture with irradiated CAP treated melanoma cells, suggestive of ICD¹⁰. Chen et al., 2021 also showed an upregulation of pro-inflammatory cytokines, like IFN- γ , TNF- α , IL-2, and IL-12, in the serum of mice increased in a dose-dependent manner following CAP treatment⁴⁴¹. Anti-tumor immunity can be potentially further enhanced through the combination of CAP and anti-PD-L1 antibody therapy⁴⁴². Han et al., 2020 showed using a murine melanoma model (B16F10) *in vivo* that the combination treatment synergistically inhibited the tumor growth rate as well as increased the infiltration of CD4⁺ and CD8⁺ T cells into tumor tissue⁴⁴².

Although **Paper 3** reported that CD3⁺, CD4⁺, and CD8⁺ T cells were highly sensitive to CAP treatment *in vitro*, the described works above indicate that a robust infiltration of functional CD4⁺ and CD8⁺ T cells into the TME *in vivo* is indeed possible^{10,441,442}. This does not rule out the possibility of initial T cell death in the TME following CAP treatment as the findings of **Paper 3** suggest¹⁰. Rather, it suggests that ICD induced infiltration of T cells into the TME seemingly plays a larger role in influencing T cell numbers in the TME *in vivo*. Collectively, these studies demonstrate that CAP exhibits a dual role in both inhibiting tumor growth as well as promoting anti-tumor T cell mediated immunity in the TME.

Altogether, these findings show that CAP exhibits diverse immunomodulatory effects that are desirable in distinct immunological settings. CAP has been clinically proven to optimize wound healing and exhibits strong promise as a post-operative adjuvant anti-cancer therapy^{432,440,441}. Furthermore, CAP has been reported to have an excellent safety profile in both immunological settings, encouraging therapeutic development and application of CAP to other clinical conditions^{432,439}.

5. CONCLUSIONS

This work contributed to the understanding of two promising immunomodulatory approaches, namely GARP and CAP. In more detail, the functional relevance of GARP in wound healing (**Paper 1**) and GSC biology (**Paper 2**) was described for the first time^{8,9}. Furthermore, the biological effects of CAP on cells found in the melanoma microenvironment were elucidated (**Paper 3**)¹⁰. In the future, these immunomodulatory mechanisms might be applied to elicit favorable immune responses in the treatment of cancer and the optimization of wound healing.

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APPENDIX

SUPPORTING INFORMATION

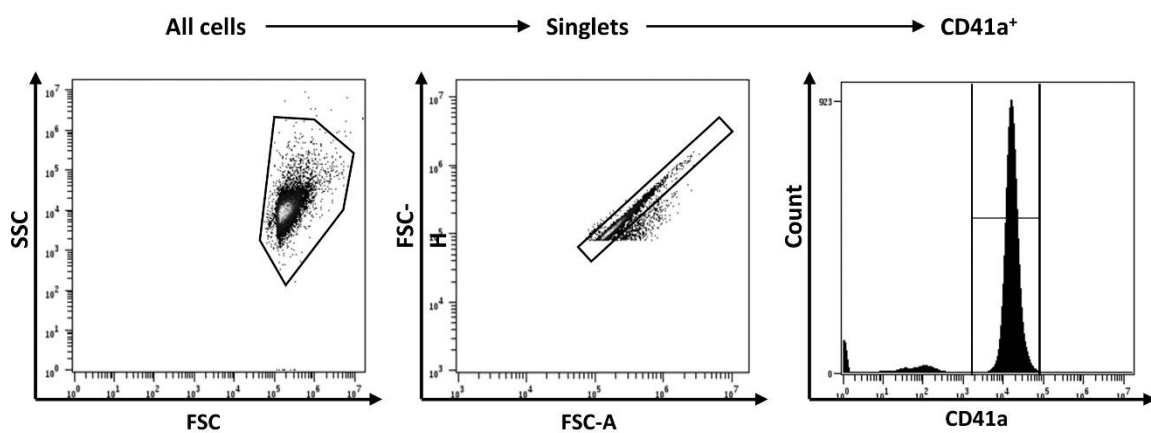
PAPER 1

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Figure S1 (Paper 1): Flow cytometric gating strategy

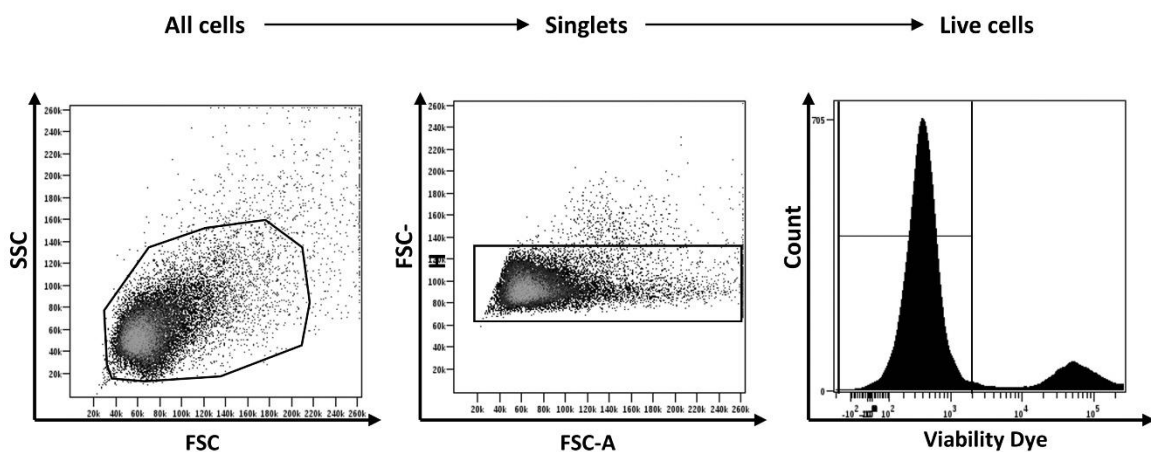
A

iPRF-Platelets:



B

CD4⁺ T cells:



C

Macrophages:

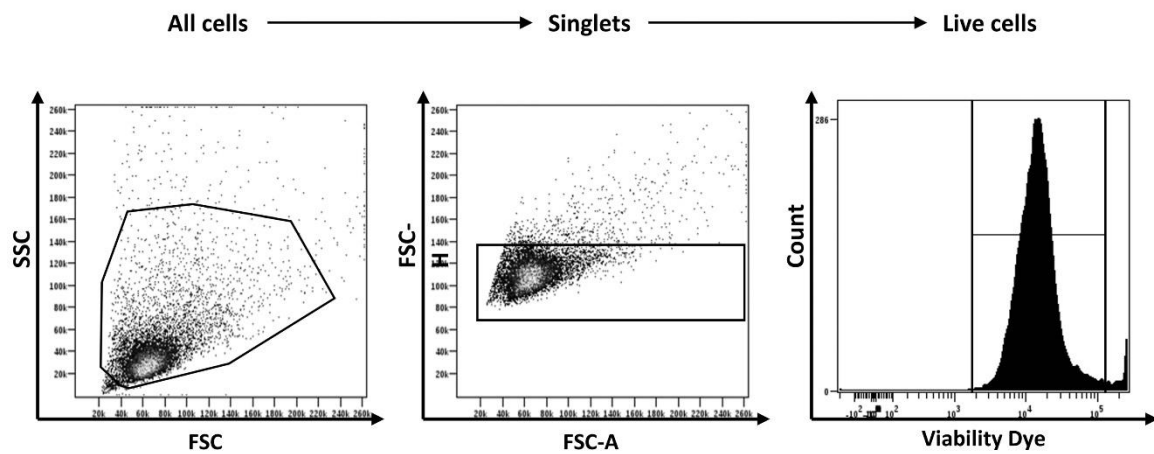


Fig. S1 (Paper 1): Representative flow cytometric gating strategies used for iPRF derived platelets (A), CD4⁺ T cells (B), and monocyte derived macrophages (C). Debris, doublets, and dead cells were excluded from analysis.

PAPER 2

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Figure S2 (Paper 2, prev. S1): Characterization of patient derived GSC cell lines

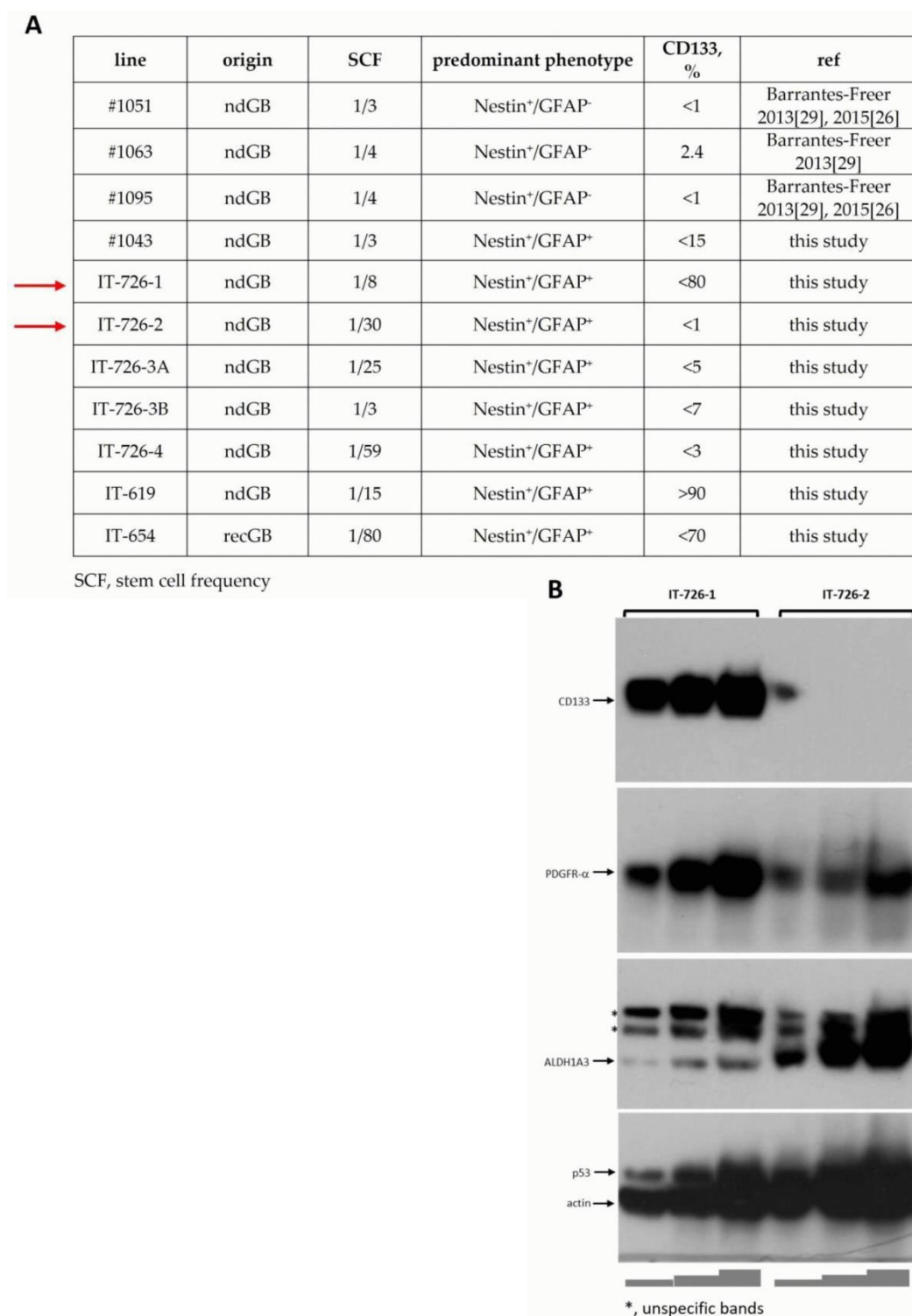


Fig. S2 (Paper 2, prev. S1): Heterologous GSC cell lines differing in their self-renewal capacity. Previous characterization of the heterologous patient derived GSC cell lines used in this work (**A, B**)^{266,444–446}. Considerable variation in the expression of several GSC markers, including glial fibrillary acidic protein (GFAP), CD133, platelet-derived growth factor receptor alpha (PDGFR- α), and aldehyde dehydrogenase 1 family member A3 (ALDH1A3), was observed in both heterologous and isogenic GSC cell lines. (**B**) Example analysis of several GSC markers, including CD133, PDGFR- α , and ALDH1A3, in the isogenic GSC cell lines, IT-726-1 and IT-726-2 (GSC cell lines indicated by the red arrows, featured in **A**), via western blot using the following antibodies: anti-CD133/1 (clone: W6B3C1), anti-PDGFR- α (D13C6) (Cell Signaling, #5341), anti-ALDH1A3 (Thermo Fisher Scientific, MA5-25528), anti-p53 (DO-1) (Cell Signaling, #18032), anti-actin (C4) (Santa Cruz Biotechnology, sc-47778), goat anti-mouse IgG horseradish peroxidase (Santa Cruz Biotechnology, sc-2055), goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, sc-2054). Cell lysates were loaded in increasing volumes, and actin was used as a loading control.

Figure S3 (Paper 2, prev. S2): Anti-GARP antibody validation for flow cytometry

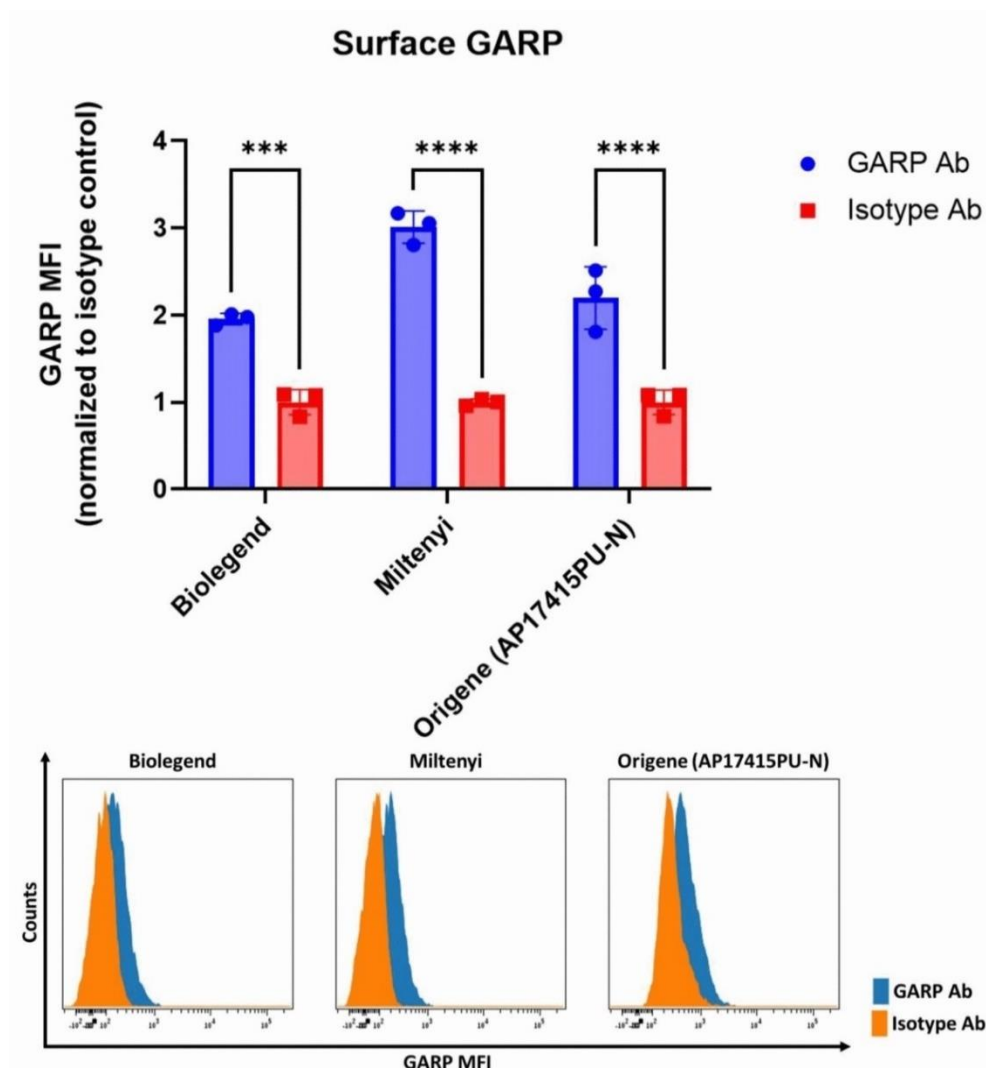


Fig. S3 (Paper 2, prev. S2): Anti-GARP antibody (Ab) validation for flow cytometry. Comparative flow cytometric analysis of surface GARP levels on a control human melanoma cell line, Mewo, using three different human anti-GARP antibodies. The following antibodies were analyzed: Miltenyi (130-103-890), Biologend (352502), Origene (AP17415PU-N). Doublets, debris, and dead cells were excluded from the analysis. Graph shows the mean fluorescence intensity (MFI) normalized to the MFI of the respective isotype control, whereas histograms display one representative result ($n=3$, $\pm$ SD, *** $p < 0.001$, and **** $p < 0.0001$ determined by two-way ANOVA).

Figure S4 (Paper 2, prev. S3): Specificity demonstration of anti-GARP antibodies

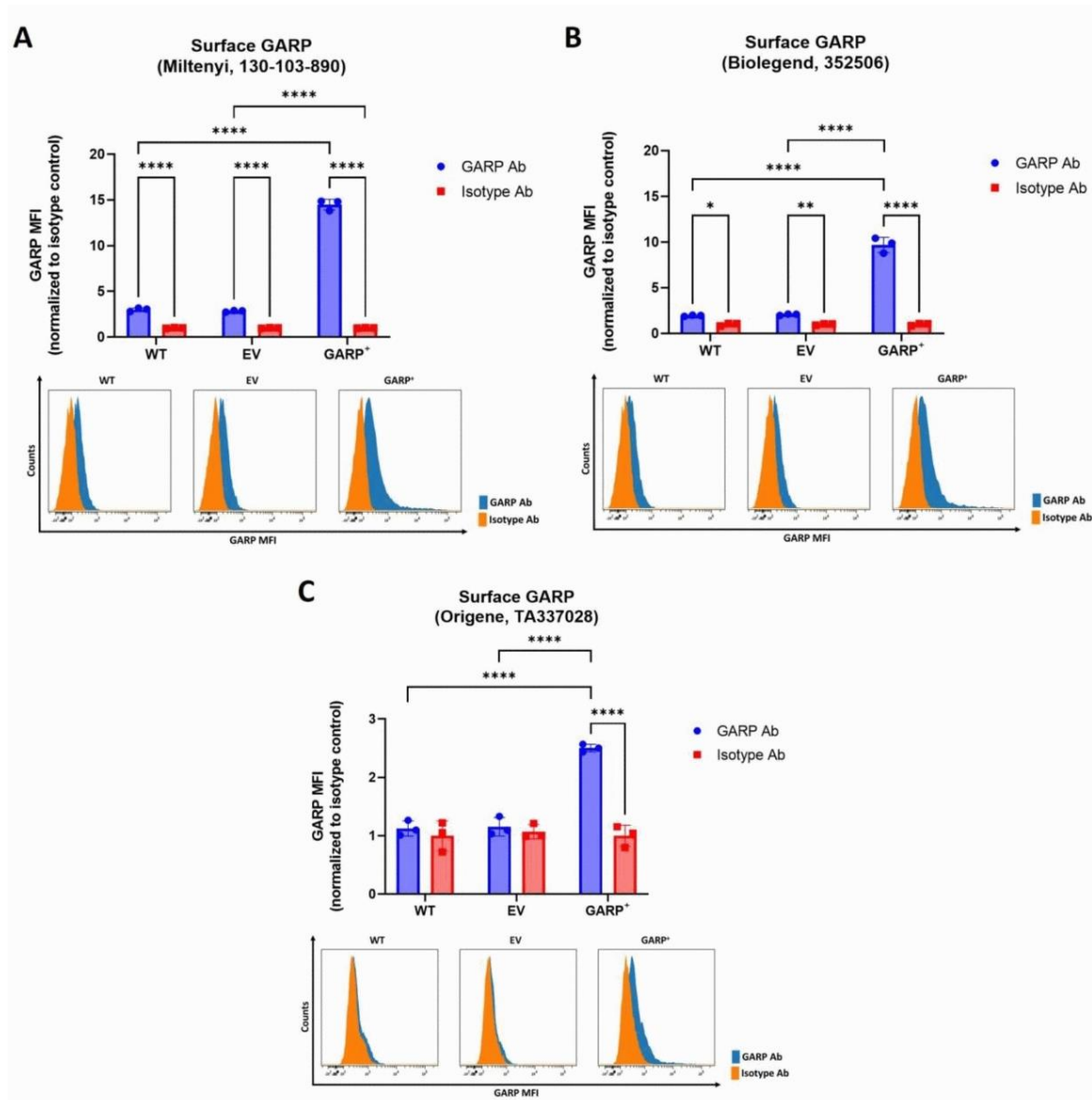


Fig. S4 (Paper 2, prev. S3): Specificity demonstration and validation of anti-GARP antibodies (Ab). Comparative flow cytometric analysis of surface GARP levels on wildtype (WT) and transfected (GARP overexpression (GARP⁺), empty vector control (EV)) Mewo cells using three different human anti-GARP Abs. The following antibodies were analyzed: Miltenyi (130-103-890) (**A**), Biolegend (352502) (**B**), Origene (TA337028) (**C**). Doublets, debris, and dead cells were excluded from the analysis. Graph shows the mean fluorescence intensity (MFI) normalized to the MFI of the respective isotype control, whereas histograms display one representative result (n=3, $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ determined by two-way ANOVA).

Figure S5 (Paper 2, prev. S4): Flow cytometric gating strategy for GSCs

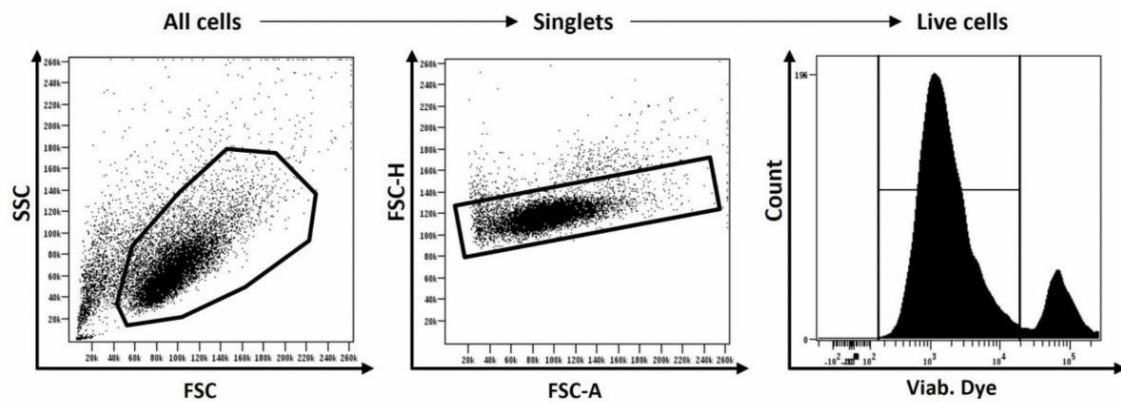


Fig. S5 (Paper 2, prev. S4): Flow cytometric gating strategy for GSCs. Representative flow cytometric gating strategy used for GSCs. Debris, doublets, and dead cells were excluded from analysis.

Figure S6 (Paper 2, prev. S5): Validation of anti-GARP antibodies for confocal microscopy

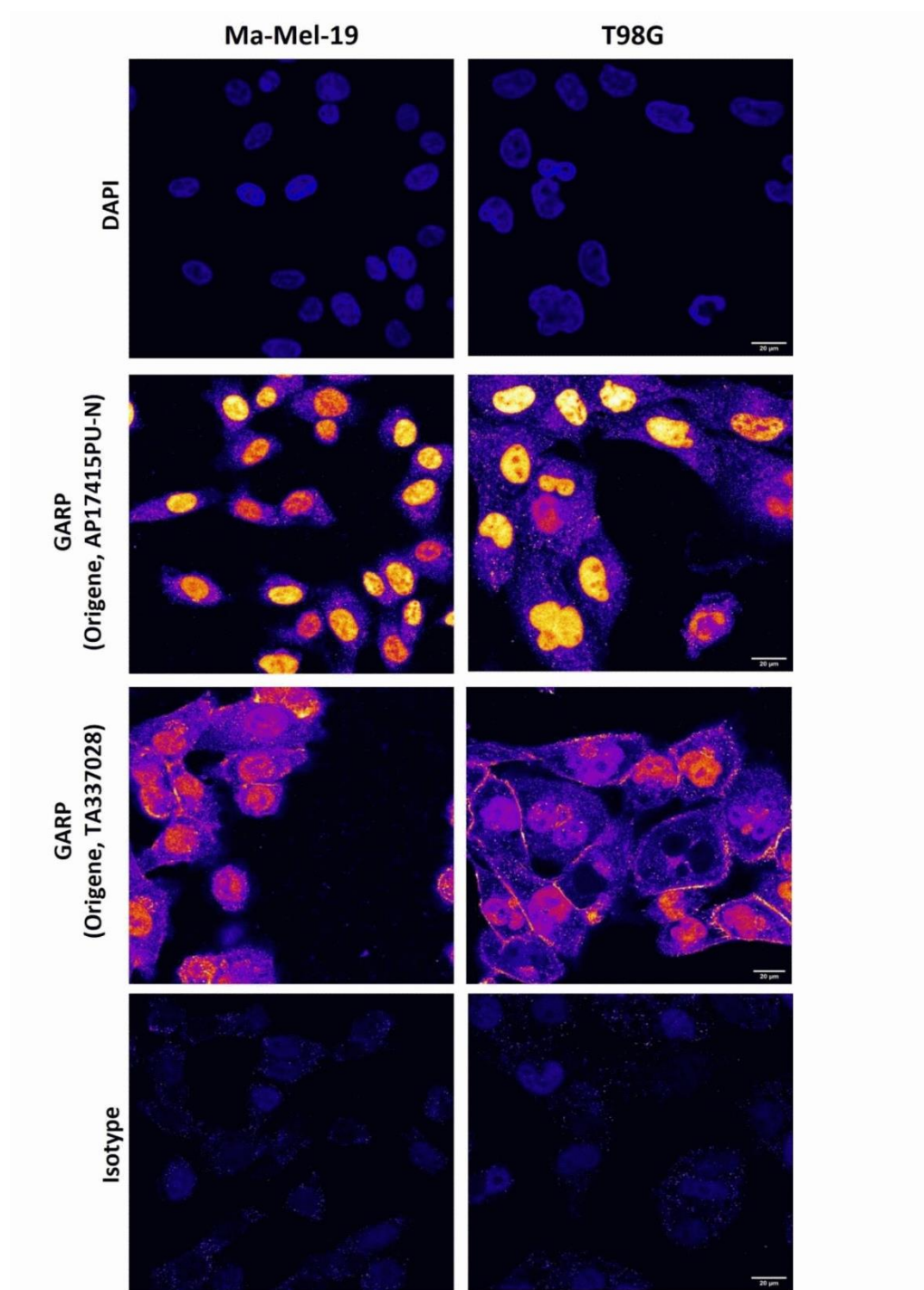


Fig. S6 (Paper 2, prev. S5): Anti-GARP antibody validation for confocal microscopy. Confocal images of the human GARP expressing cell lines, Ma-Mel-19 and T98G. Cells were stained for GARP using two different antibodies (Origene, AP17415PU-N; Origene, TA337028) as seen in orange. Cells were counterstained for their nuclei with Hoechst (blue). Note the intranuclear localization of GARP (GARP^{NU+}) detectable with both antibodies. Scale bar corresponds to 20 μm.

Figure S7 (Paper 2, prev. S6): Flow cytometric gating strategy for GARP sorted GSCs

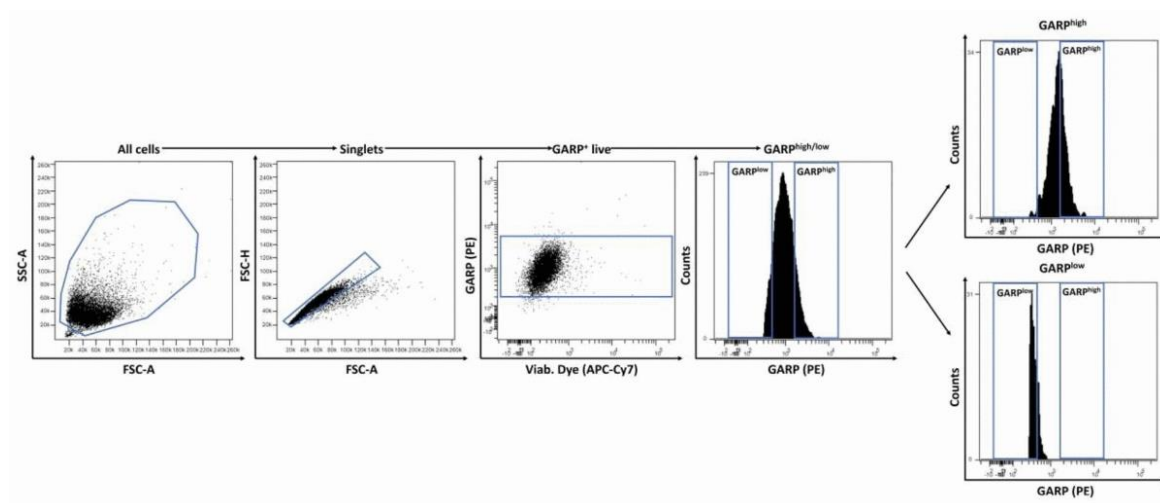


Fig. S7 (Paper 2, prev. S6): (A) Representative flow cytometric gating strategy used for sorting GARP^{high} and GARP^{low} GSCs. Sorted cells were re-measured via flow cytometry to confirm sorting efficacy. **(B)** Example GARP staining of GSCs (mean fluorescence intensity shown) compared to its respective isotype and unstained controls. Debris, doublets, and dead cells were excluded from analysis.

Figure S8 (Paper 2, prev. S7): Representative immunohistochemical stainings of xenograft tumors derived from GSC cell lines, #1051 and #1043

#1051



#1043

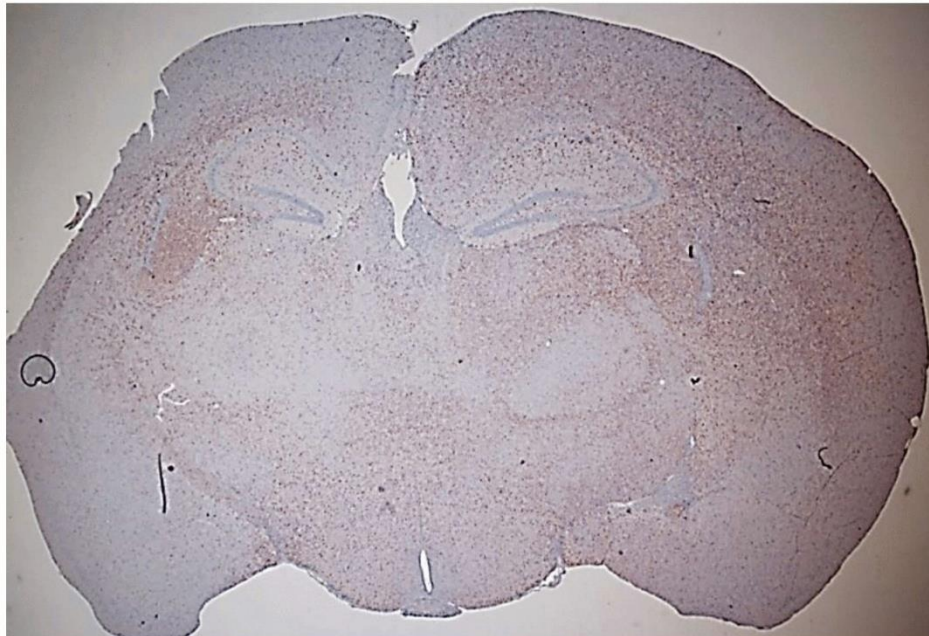


Fig. S8 (Paper 2, prev. S7): Invasive xenograft tumors arisen from GSC cell lines, #1051 and #1043. Representative images of xenograft tumors grown from human GSCs in an orthotopic mouse model for brain tumors. Immunohistochemistry stainings for human nestin (anti-human nestin antibody PA5-82905, 1:100, Life Technologies).

Figure S9 (Paper 2, prev. S8): Representative immunofluorescence stainings of xenograft tumors derived from GSC cell lines, #1051 and #1043

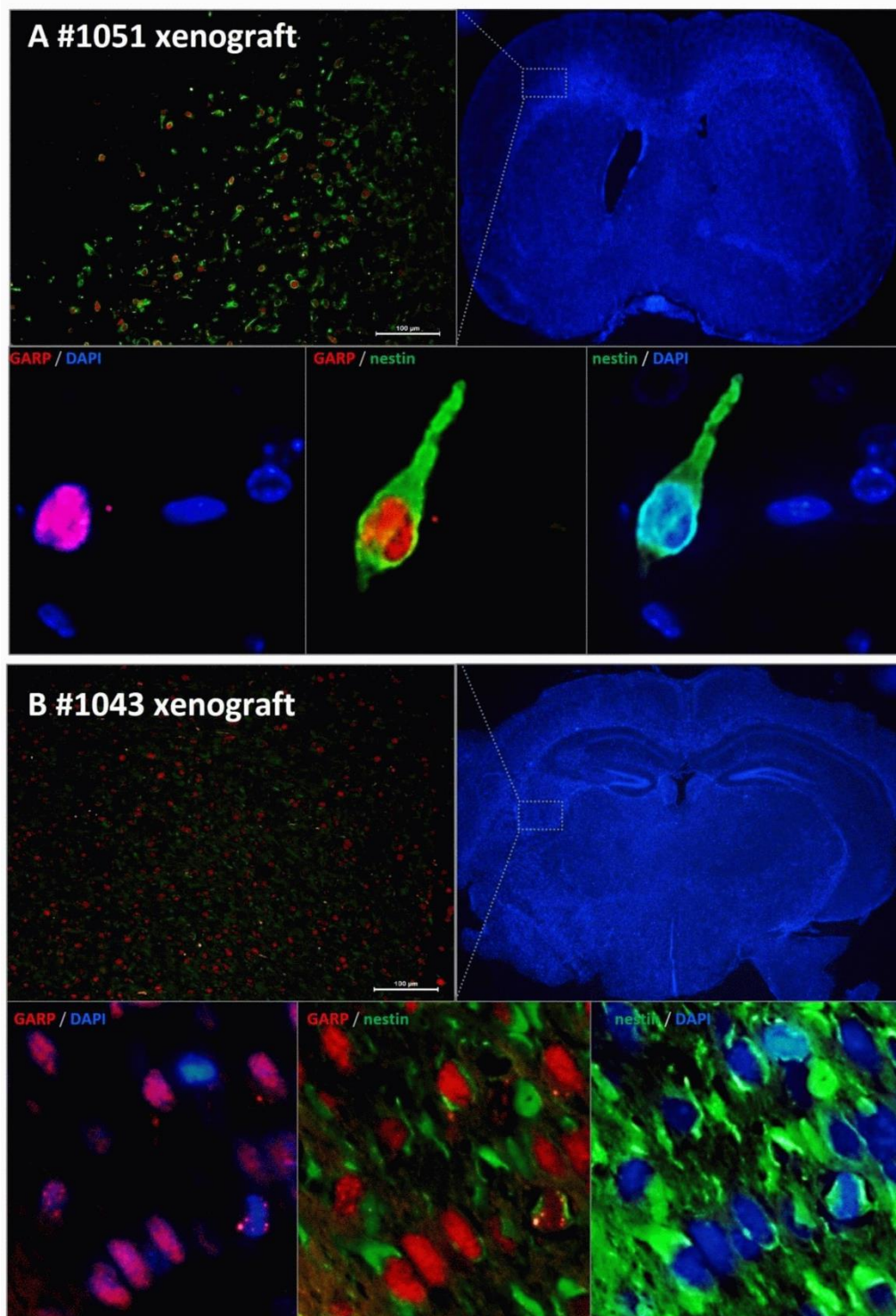


Fig. S9 (Paper 2, prev. S8): GARP is expressed in xenograft tumors arisen from GSC cell lines, #1051 and #1043. Immunofluorescence of GARP and nestin of (A) #1051 and (B) #1043 xenograft tumors. GARP seems to be exclusively expressed on GSC cells. Confocal images of GARP and nestin expressing GSCs stained for GARP and nestin. Cells were stained for their

nuclei. Nuclear counterstaining with Hoechst (blue), GARP (red), and nestin (green). Scale bar corresponds to 100 μm .

Figure S10 (Paper 2, prev. S9): Study design and models used for the assessment of GARP

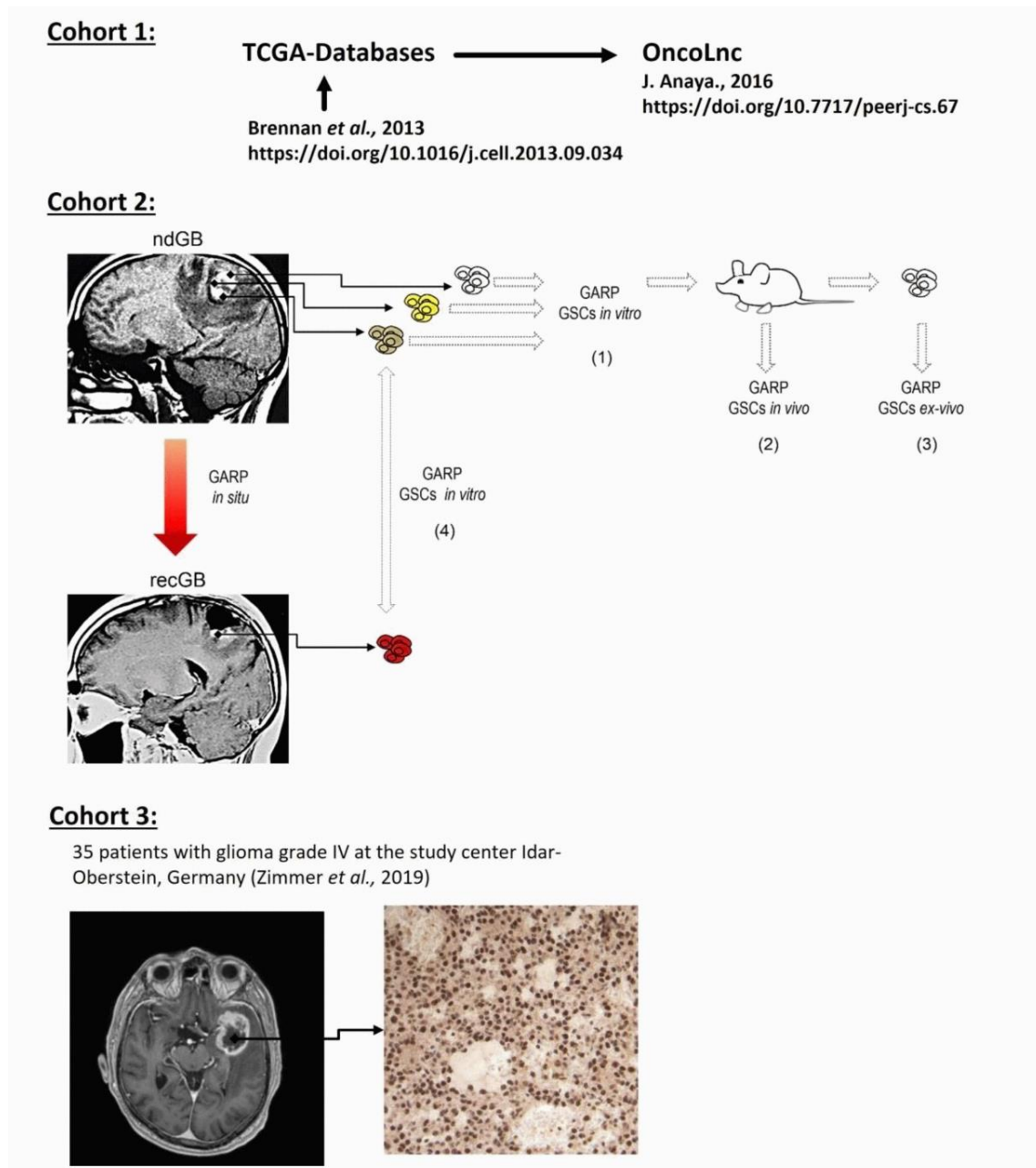


Fig. S10 (Paper 2, prev. S9): Study design and models used for the assessment of GARP.

Cohort 1: For the analysis of GARP and CD133 expression in GB, the online tool OncoLnc was used. Based on 152 complete data sets, including complete survival data, patients were divided 50/50 into “low” or “high” groups based off their mRNA expression of GARP and CD133 and were analyzed for their survival. The results shown are in whole or part based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga> and were analyzed using OncoLnc⁴⁴⁷.

Cohort 2: „GARP *in situ*“ corresponds to GARP assessments in tumor specimens from newly diagnosed or recurrent GBs. Investigation track (1) corresponds to *in vitro* assessments in GSCs either isogenic or heterogenic originating from ndGBs. Track (2) corresponds to *in vivo* assessments of GARP in tumor xenografts grown

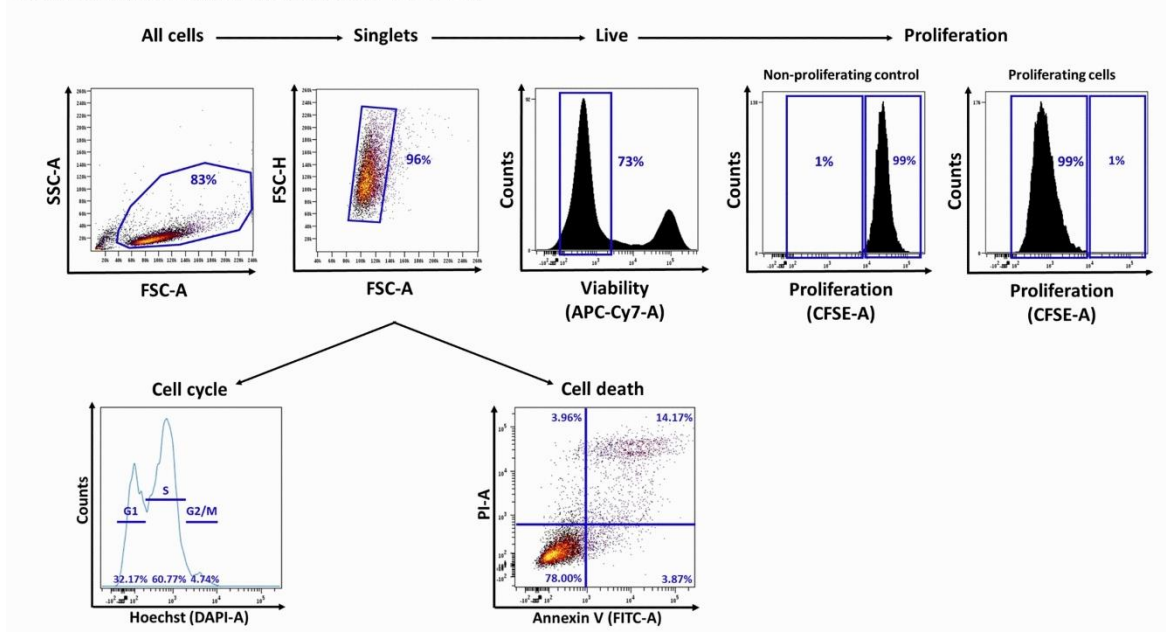
from orthotopically implanted GSCs. Track (3) corresponds to GARP assessments in GSCs explanted from tumor xenografts. Track (4) corresponds to tumor-matched GSCs isolated from the same patient at the ndGB or recGB stage. Furthermore, retrospective analysis of transcriptome data of 155 GB samples from 28 patients of Kim et al., 2020. ndGBs, first and second recurrent tumors were analyzed for their GARP and CD133 expression levels across tumor stages⁴⁴⁵. Cohort 3: A cohort of 35 patients with (WHO grade IV) glioblastoma (Zimmer et al., 2019) were analyzed for their GARP expression by immunohistochemistry and analyzed for their survival⁶⁵.

PAPER 3

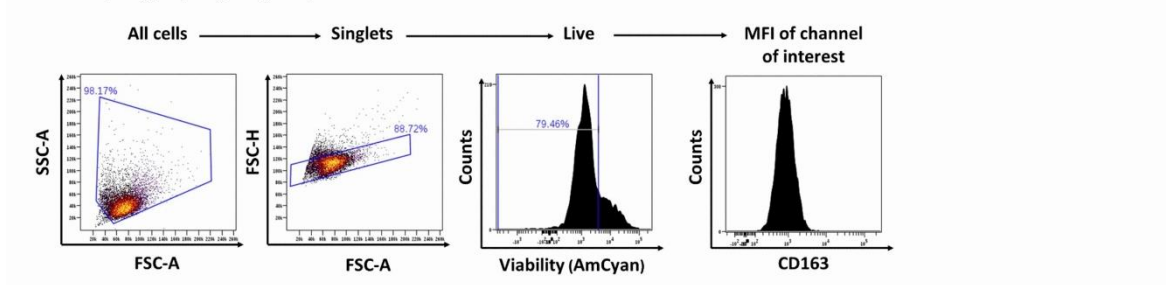
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Figure S12 (Paper 3, prev. S1): Flow cytometric gating strategies

A: Melanoma cells (Ma-Mel-19, UKRV-Mel-15a)



B: Macrophages (M0, M1, M2)



C: CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells

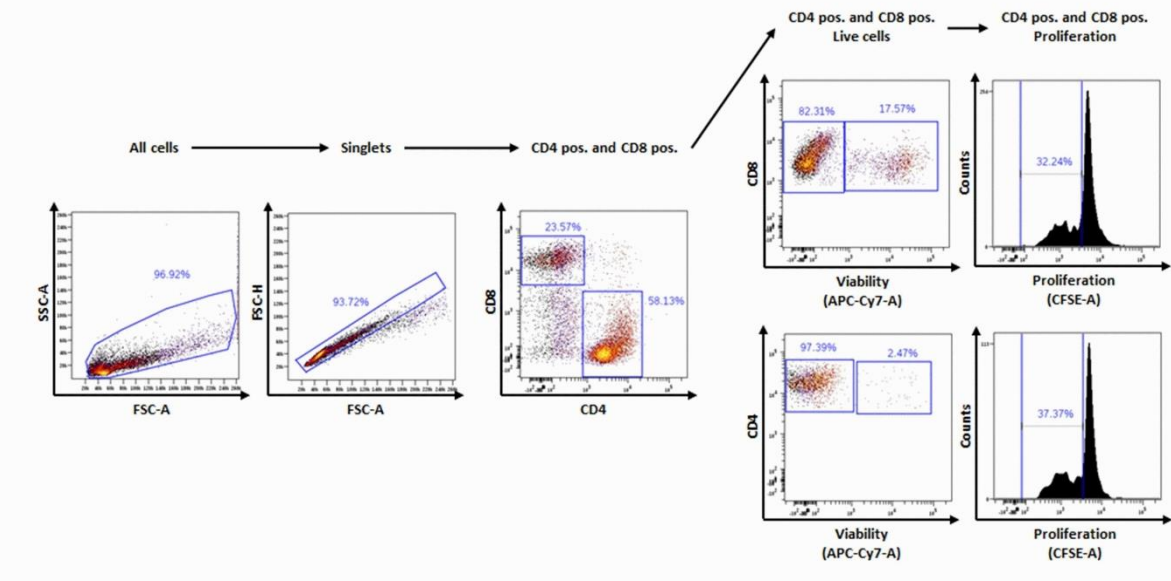


Fig. S12 (Paper 3, prev. S1): Model gating strategies used for melanoma (A), macrophages (B), and CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells (C). For cell death analysis using annexin V and propidium iodide (A), the gating contents of each quadrant are as follows: lower left (LL) live cells, lower right (LR) early apoptotic cells, upper right (UR) late apoptotic cells, and upper left (UL) necrotic cells. In addition, the macrophage gating strategy (B) shows one gated polarization marker, CD163, as an example. This same gating strategy was applied for other measured macrophage polarization markers; however, the channel of interest changed depending on the measured marker. Debris, doublets, and dead cells were excluded from analysis.

Figure S13 (Paper 3, prev. S2): MiniJet-R and intracellular ROS quantification

A: MiniJet-R device



B: MiniJet-R in operation



C: Intracellular ROS

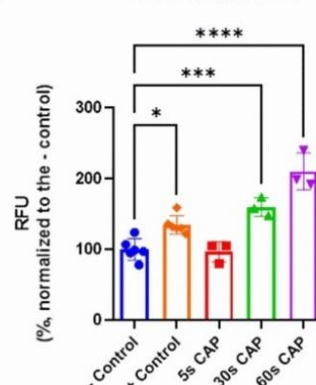


Fig. S13 (Paper 3, prev. S2): (A) The MiniJet-R, from Heuermann HF Technik, used to produce CAP in this study, is comprised of a microwave generator and handpiece. In the feed line, the process gas argon and electromagnetic waves with a frequency of 2.45 GHz are transported to the handpiece. Gas flow can be monitored with the gas flow meter, and the starter box is necessary to ignite the plasma⁴⁴⁸. (B) Example image of the MiniJet-R in use. A beam of CAP applied to the tissue of a human hand⁴⁴⁸. (C) Ma-Mel-19 cells were treated with varying amounts of CAP (5s, 30s, 60s) and their intracellular ROS levels were measured 1 h after treatment. The positive control indicates cells treated with a control ROS Inducer, while the negative control represents untreated cells. Bar diagram shows the average intracellular ROS levels in relative fluorescence units (RFU) normalized to the untreated control ($n=6/\text{control}$, $n=3/\text{CAP}$) $\pm$ SD; Statistical significance was calculated by performing ordinary one-way ANOVAs corrected for multiple comparisons with Tukey tests and is indicated by the asterisks as follows: *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure S14 (Paper 3, prev. S3): UKRV-Mel-15a cell cycle and cell death results

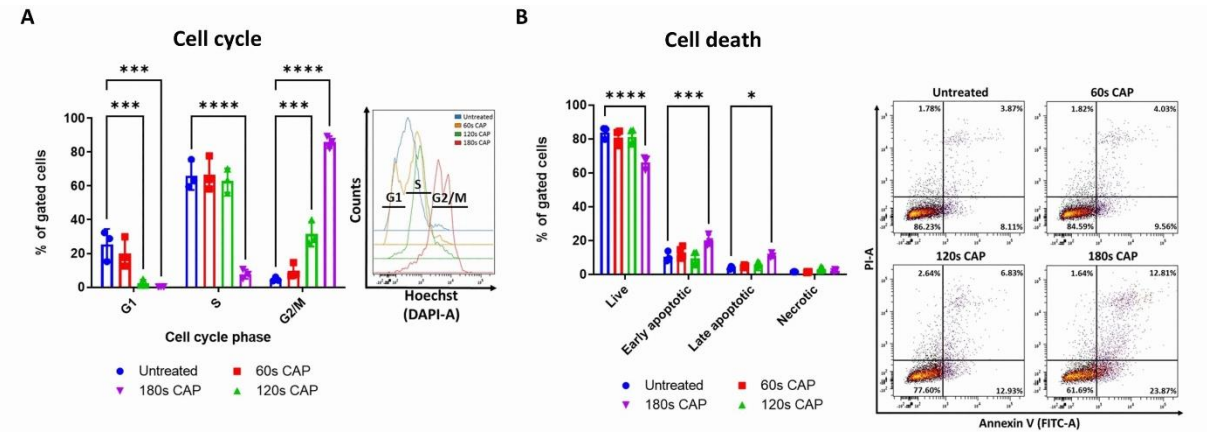


Fig. S14 (Paper 3, prev. S3): (A-B) UKRV-Mel-15a cells were treated with varying amounts of CAP (60s, 120s, 180s). After 3 days, cells were analyzed via flow cytometry. **(A)** Cells were stained with Hoechst. Bar diagram shows the percentage of cells in each gate ($n=3$) $\pm$ SD. **(B)** Cells were stained with annexin V and propidium iodide (PI). Bar diagram shows the percentage of cells in each quadrant gate ($n=4$) $\pm$ SD. The contents of each gate are as follows: lower left (LL) live cells, lower right (LR) early apoptotic cells, upper right (UR) late apoptotic cells, and upper left (UL) necrotic cells. Histograms and dot plots paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Tukey tests and is indicated by asterisks as follows: *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure S15 (Paper 3, prev. S4): Non-significant macrophage polarization markers

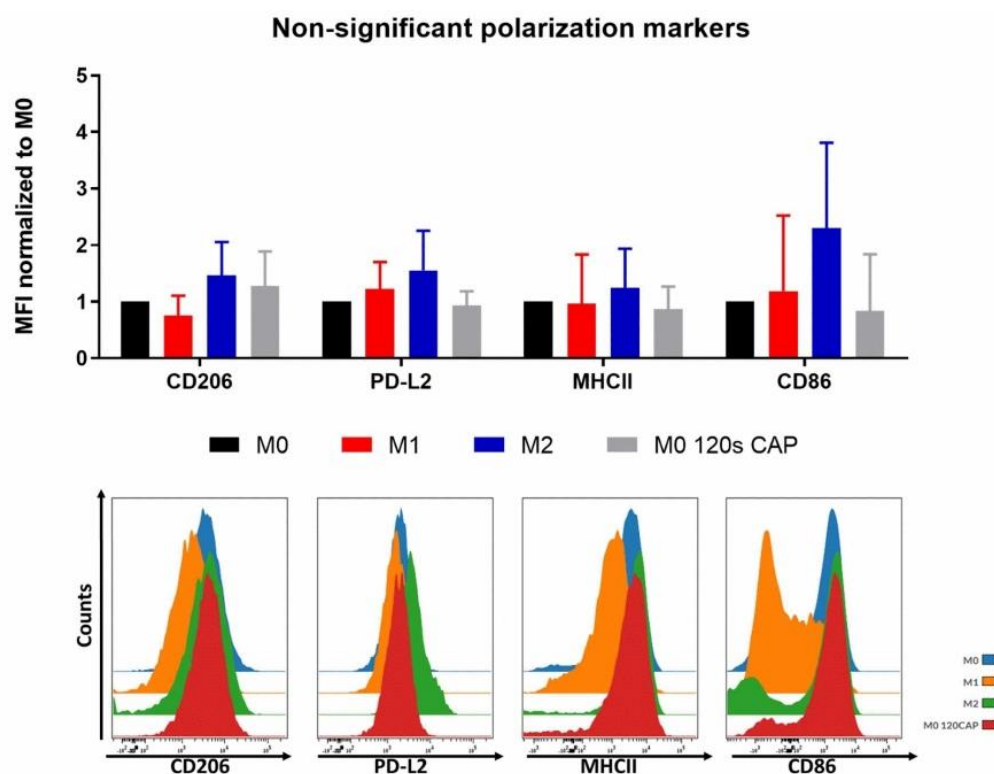


Fig. S15 (Paper 3, prev. S4): M0 macrophages were treated with 120s of CAP. M1 and M2 polarized macrophages served as controls. After 2 days, cells were analyzed via flow cytometry. Cells were stained with different macrophage polarization markers. Bar diagram shows the average mean fluorescent intensity (MFI) of each measured marker normalized to untreated M0 macrophages and represents the pooled results of twelve independent experiments (n=12 donors). Histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Tukey tests.

Figure S16 (Paper 3, prev. S5): Example Seahorse OCR and ECAR results

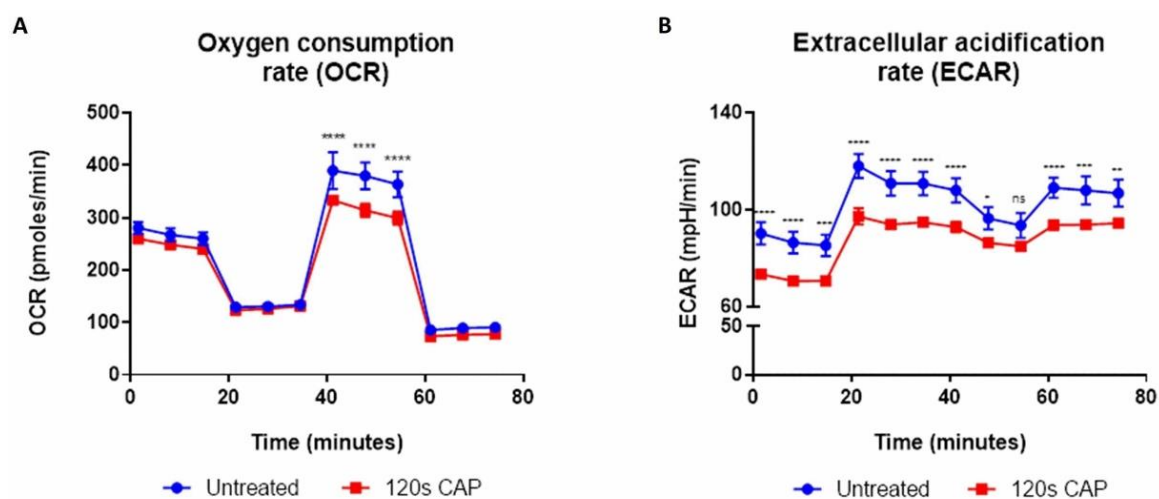


Fig. S16 (Paper 3, prev. S5): Representative (A) oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) graphs produced from a Seahorse Cell Mito Stress test are shown. Line graphs show means $\pm$ SD ($n=3$) of untreated and 120s CAP treated Ma-Mel-19 cells. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Tukey tests and is indicated by the asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure S17 (Paper 3, prev. S6): UKRV-Mel-15a antioxidant treatment results

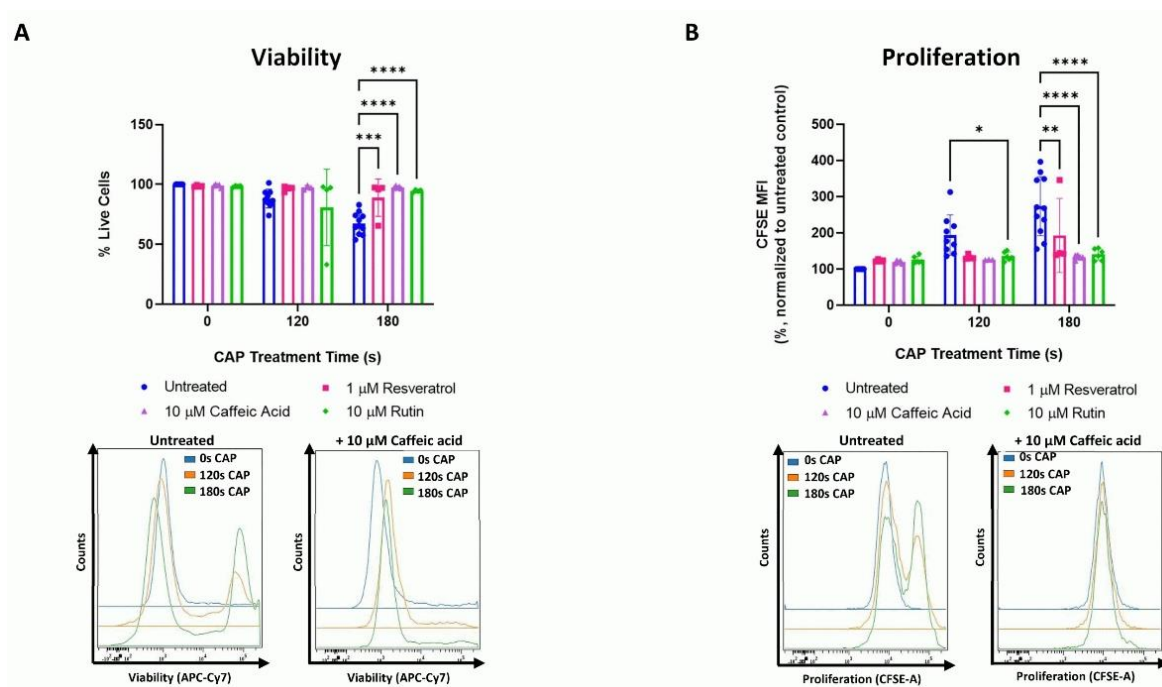


Fig. S17 (Paper 3, prev. S6): CFSE prelabeled UKRV-Mel-15a cells were plated in the presence of various antioxidants and differentially treated with CAP (0s, 120s, 180s). After 3 days, cells were analyzed via flow cytometry. **(A)** UKRV-Mel-15a cells were stained for viability. Bar diagram shows the average percentage of live cells measured normalized to the untreated control (0s) ± SD (n=12/untreated, n=4/antioxidant). **(B)** Bar diagram shows the average CFSE MFI measured normalized to the untreated control (n=12/untreated, n=4/antioxidant) ± SD. Histograms paired to bar diagrams show one representative result. Statistical significance was calculated by performing two-way ANOVAs corrected for multiple comparisons with Dunnett tests and is indicated by the asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

DISCUSSION

Figure S18: *In silico* predictions of GARP protein interactors utilizing the Human Integrated Protein-Protein Interaction rEference (HIPPIE) database

Interactor UniProt ID	Interactor ID	Interactor Gene Symbol	Confidence Score
TGFB1_HUMAN	7040	TGFB1	0.87
DCP2_HUMAN	167227	DCP2	0.82
BIP_HUMAN	3309	HSPA5	0.82
MOT10_HUMAN	117247	SLC16A10	0.82
NSMA_HUMAN	6610	SMPD2	0.82
S11IP_HUMAN	114790	STK11IP	0.82
STAT3_HUMAN	6774	STAT3	0.82
ZDHC6_HUMAN	64429	ZDHC6	0.82
MAN1_HUMAN	23592	LEMD3	0.72
TMM11_HUMAN	8834	TMEM11	0.72
1C01_HUMAN	3107	HLA-C	0.63
CANT1_HUMAN	124583	CANT1	0.63
CNPY3_HUMAN	10695	CNPY3	0.63
DAD1_HUMAN	1603	DAD1	0.63
ECE1_HUMAN	1889	ECE1	0.63
FMOD_HUMAN	2331	FMOD	0.63
ITGB8_HUMAN	3696	ITGB8	0.63
L9056_HUMAN	100288413, 100290734, 100293101	ERVMER34-1	0.63
MAGT1_HUMAN	84061	MAGT1	0.63
NAGPA_HUMAN	51172	NAGPA	0.63
PEBB_HUMAN	865	CBFB	0.63
PPM1B_HUMAN	5495	PPM1B	0.63
RPN1_HUMAN	6184	RPN1	0.63
RTN4R_HUMAN	65078	RTN4R	0.63
S39AA_HUMAN	57181	SLC39A10	0.63
TR10D_HUMAN	8793	TNFRSF10D	0.63

Fig. S18: *In silico* predictions of possible protein interaction partners of human glycoprotein A repetitions predominant (GARP). Results were obtained by querying the Human Integrated Protein-Protein Interaction rEference (HIPPIE) database for “LRC32_HUMAN”⁴⁰⁵. A total of 26 hits were found. The confidence score, ranging from 0 (very low) to 1 (very high), indicates the likelihood of interaction. Notably, TGF-B1, the best-known interaction partner of GARP has the highest confidence score of 0.87. Confidence scores were calculated as the weighted sum of several parameters: the number of works reporting the interaction, the number and quality of experimental methods used to detect the interaction, and the number of times the interaction was shown in non-human organisms.

Figure S19: *In silico* predictions of protein interactors with GARP utilizing the Physical Protein-Protein Interactions with Reality Scores (HitPredict) database

Interactor UniProt ID	Interaction ID	Interactor Gene Symbol	Interaction Score
TGFB1_HUMAN	331548	TGFB1	0.847
STAT3_HUMAN	709279	STAT3	0.789
1C01_HUMAN	456987	HLA-C	0.618
FMOD_HUMAN	906368	FMOD	0.618
RTN4R_HUMAN	967209	RTN4R	0.618
BIP_HUMAN	469173	HSPA5	0.611
MOT10_HUMAN	967206	SLC16A10	0.611
NSMA_HUMAN	231315	SMPD2	0.585
DCP2_HUMAN	967204	DCP2	0.585
S11IP_HUMAN	967205	STK11IP	0.558
TMM11_HUMAN	525113	TMEM11	0.513
MAN1_HUMAN	967216	LEMD3	0.489
ITB8_HUMAN	592026	ITGB8	0.479
ECE1_HUMAN	723848	ECE1	0.479
TR10D_HUMAN	967213	TNFRSF10D	0.479
S39AA_HUMAN	967215	SLC39A10	0.479
PEBB_HUMAN	961478	CBFB	0.458
CANT1_HUMAN	967207	CANT1	0.458
MAGT1_HUMAN	967210	MAGT1	0.458
L9056_HUMAN	967212	ERVMER34-1	0.458
RPN1_HUMAN	367476	RPN1	0.437
CNPY3_HUMAN	967214	CNPY3	0.437
NAGPA_HUMAN	967214	NAGPA	0.437
ZDHC6_HUMAN	967211	ZDHC6	0.318
PPM1B_HUMAN	257969	PPM1B	0.249
DAD1_HUMAN	825574	DAD1	0.249

Fig. S19: *In silico* predictions of possible protein interaction partners of human glycoprotein A repetitions predominant (GARP). Results were obtained by querying the Physical Protein-Protein Interactions With Reality Scores (HitPredict) database for “H. sapiens” > “LRRC32” (Protein ID: Q14392)^{406–408}. A total of 26 hits were found. The interaction score, ranging from 0 (very low) to 1 (very high), indicates the reliability of the interaction. Notably, TGF-B1, the best-known interaction partner of GARP has the highest confidence score of 0.847. Interaction scores were calculated by taking the geometric mean of the annotation-based and methods-based scores. Annotation-based scores are based on the properties of the interacting proteins, e.g., structure of interaction domains, gene ontology, and homologous interactions. The methods-based score is calculated from experimental evidence of the interaction, e.g., number of publications or experiments showing support of the interaction, experimental methods used to detect the interaction, and the type of the interaction. High confidence interaction scores are over 0.28.

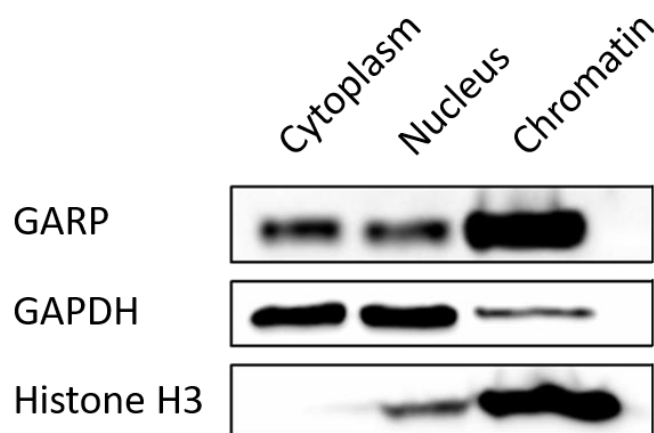
Figure S20: *In silico* predictions of protein interactors with GARP utilizing the BioGRID^{4.4} database

Interactor UniProt ID	Interactor Gene Symbol	Evidence
BIP_HUMAN	HSPA5	3
DCP2_HUMAN	DCP2	2
MOT10_HUMAN	SLC16A10	2
NSMA_HUMAN	SMPD2	2
STAT3_HUMAN	STAT3	2
S11IP_HUMAN	STK11IP	2
TGFB1_HUMAN	TGFB1	2
ZDHC6_HUMAN	ZDHHC6	2
B4GT5_HUMAN	B4GALT5	1
CANT1_HUMAN	CANT1	1
PEBB_HUMAN	CBFB	1
CNPY3_HUMAN	CNPY3	1
DAD1_HUMAN	DAD1	1
ECE1_HUMAN	ECE1	1
L9056_HUMAN	ERVMER34-1	1
FMOD_HUMAN	FMOD	1
1C01_HUMAN	HLA-C	1
ITB8_HUMAN	ITGB8	1
MAN1_HUMAN	LEMD3	1
MAGT1_HUMAN	MAGT1	1
NAGPA_HUMAN	NAGPA	1
PPM1B_HUMAN	PPM1B	1
RPN1_HUMAN	RPN1	1
RTN4R_HUMAN	RTN4R	1
S39AA_HUMAN	SLC39A10	1
STT3A_HUMAN	STT3A	1
TMM11_HUMAN	TMEM11	1
TR10D_HUMAN	TNFRSF10D	1

Fig. S20: *In silico* predictions of possible protein interaction partners of human glycoprotein A repetitions predominant (GARP). Results were obtained by querying the BioGRID^{4.4} database for “Homo sapiens” > “LRRC32”^{403,404}. A total of 28 hits were found. The interaction score, ranging from 1 (low) to 3 (high), indicating the evidence of the interaction. Notably, TGF-B1, the best-known interaction partner of GARP has an evidence score of 2. Evidence scores were calculated from experimental evidence codes designed by the developers. These codes describe the experimental evidence of the interaction, e.g., affinity capture western blot. Experimental results were derived from peer-reviewed publications.

Figure S21: Biochemical confirmation of nuclear localization of GARP and its interaction with STAT3

A



B

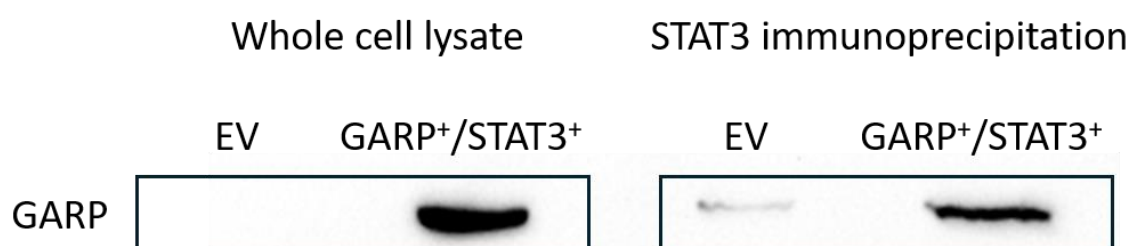
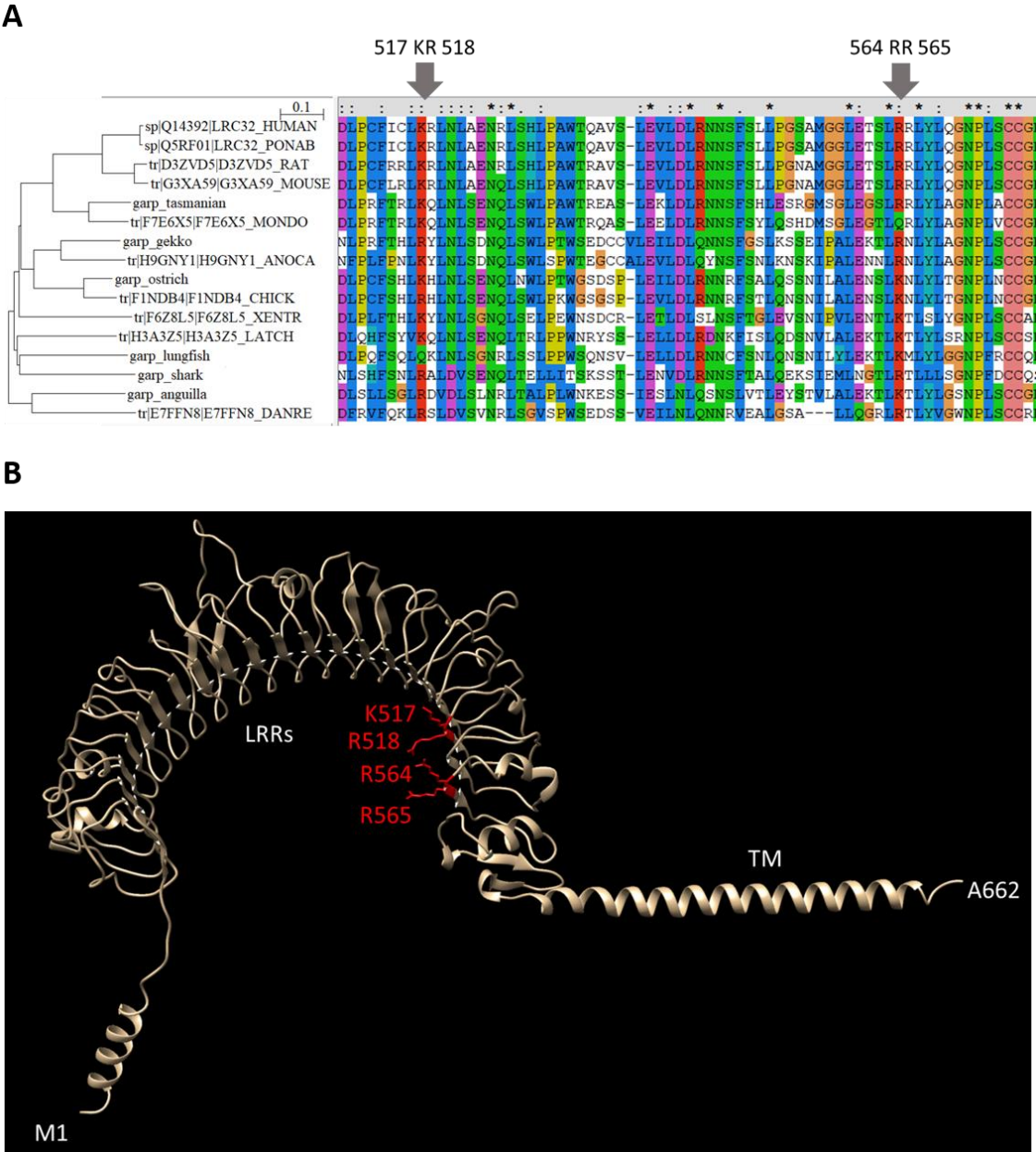


Fig. S21: (A) Chromatin fractionation of the human melanoma cell line, Mewo, was performed as described in Méndez and Stillman, 2000⁴⁴⁹. Subcellular fractions were analyzed via western blot as described in Müller et al., 2018⁴⁵⁰. GAPDH was used as a positive control for the cytoplasmic and nuclear fractions and as a negative control for the chromatin fraction. Histone H3 was used as a positive control for nuclear and chromatin fractions and a negative control for the cytoplasmic fraction. Chromatin fractionation and western blotting was performed by [REDACTED]. (B) Immortalized human embryonic kidney cells (HEK293T) were cotransfected with five µg of GARP and STAT3 overexpression plasmids as well as an empty vector (EV) control plasmid using CaCl₂ and HBS buffer. After 48 h, cells were lysed, and co-immunoprecipitation was performed utilizing an anti-STAT3 antibody (MA1-13042, Thermo Fisher Scientific) as described in Müller et al., 2020⁴⁵¹. Purified and control whole cell lysates were detected via western blot as described above using an anti-GARP antibody (#83565, Cell Signaling). Co-immunoprecipitation and western blot were performed together with [REDACTED].

Figure S22: *In silico* predictions of a nuclear localization sequence in the GARP protein



C

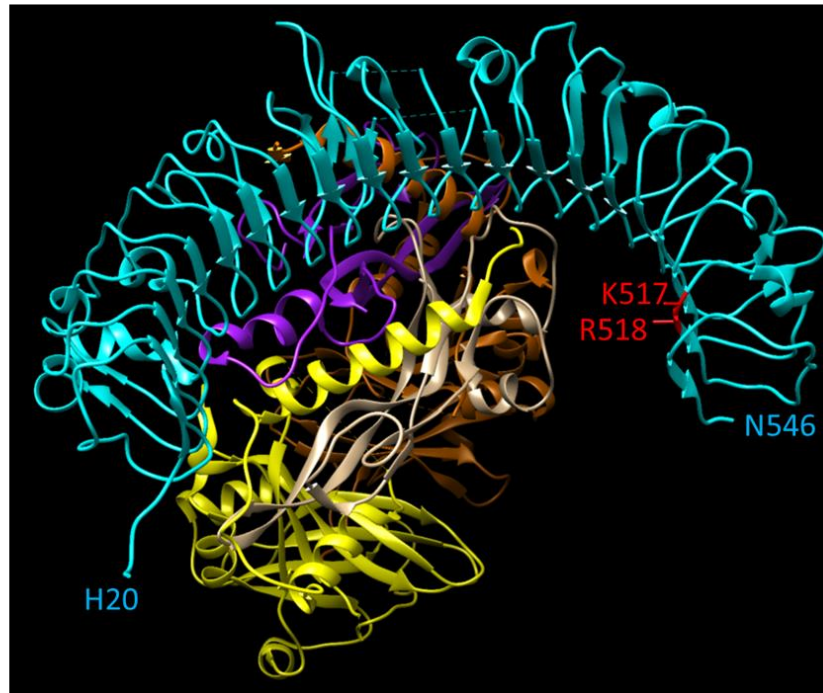


Fig. S22: (A) Multiple sequence alignment of GARP orthologs. GARP was found to be conserved in the vertebrate clade, Gnathostomata, which includes humans and bony fish (Teleostomi) as well as rays and sharks (Chondrichthyes). Conserved amino acids across species are highlighted. A possible bipartite nuclear localization sequence (NLS) was identified at K517, R518 and R564, R565 (arrows). **(B)** Location of the identified NLS candidate (red) in the tertiary structure of the GARP protein was predicted *in silico* using the AlphaFold model (AF-Q14392-F1)^{452,453}. Leucine rich repeats (LRR) and the transmembrane (TM) domain are indicated as a reference. **(C)** Location of the identified NLS candidate (red) in relationship to the latent TGF- β 1 complex (purple, yellow, and brown) in the tertiary structure of the GARP protein (blue) using the PDB6GFF model⁴⁵⁴. Multiple sequence alignment analysis and prediction of the location of the identified NLS in the tertiary structure of the GARP protein was performed by [REDACTED].

CURRICULUM VITAE

CONFERENCE LIST

- 09/2023 33rd German Skin Cancer Conference (ADO)
Hamburg, Germany
Presentation: "Nuclear Glycoprotein A repetitions predominant (GARP) as a novel potential prognostic biomarker for melanoma and glioblastoma"
- 03/2023 Gutenberg Academy Fellows Program Networking Weekend
Osthofen, Germany
Presentation: "Uncovering the function of nuclear Glycoprotein A repetitions predominant (GARP)"
- 02/2023 49th Annual Meeting of the *Arbeitsgemeinschaft Dermatologische Forschung* (ADF)
Innsbruck, Austria
Poster presentation: "Uncovering the moonlighting function of nuclear GARP in melanoma"
- 02/2022 48th Annual Meeting of the *Arbeitsgemeinschaft Dermatologische Forschung* (ADF)
Virtual
Poster presentation: "GARP, a novel driver of proliferation and growth in 2D and 3D cultured melanoma cells"
- 09/2021 31st German Skin Cancer Conference (ADO)
Virtual
Short talk: "Journey to another dimension: the development and application of a 3D melanoma spheroid model"
- 03/2021 47th Annual Meeting of the *Arbeitsgemeinschaft Dermatologische Forschung* (ADF)
Virtual
Poster presentation: "Evaluation of miR-142-3p and GARP as novel diagnostic biomarkers in melanoma patients"
- 01/2020-02/2020 *Arbeitsgemeinschaft Dermatologische Forschung* (ADF) Winter School
Zugspitze, Germany
Poster presentation: "Post-Transcriptional Regulation of GARP Expression in Melanoma and Glioblastoma by miR-142-3p"
- 04/2019 Ohio University Student Research and Creative Activity Expo
Athens, USA
Poster presentation: "Targeting *Staphylococcus aureus* infections from the inside-out"
- 06/2018 62nd Annual Wind River Conference on Prokaryotic Biology
Estes Park, USA
Poster presentation: "Investigating the contribution of the small RNA Teg41 to *Staphylococcus aureus* infection-related phenotypes"
- 03/2017 Ohio University Student Research and Creative Activity Expo
Athens, USA
Poster presentation: "ATPase, Ectonucleoside Triphosphate Diphosphohydrolase 2, as a Potential Novel Anticancer Therapeutic"

