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Dual Expression of GARP in Immune and Glioma Cells: Yet Another Mechanism of Cancer Immune Escape

Emily Trzeciak[1#], Niklas Zimmer[1#], Ella Kim[2,3], Jonathan Schupp[1], Bettina Sprang[2,3], Petra Leukel[4], Fatemeh Khafaji[5], Florian Ringel[2], Clemens Sommer[4], Jochen Tuettenberg[5], Andrea Tuettenberg[1*]

[1]Department of Dermatology, University Medical Center Mainz, 55131 Mainz, Germany

[2]Department of Neurosurgery, University Medical Center Mainz, 55131 Mainz, Germany

[3]Clinic for Neurosurgery, Laboratory of Experimental Neurooncology, Johannes Gutenberg University, 55131 Mainz, Germany

[4]Institute of Neuropathology, University Medical Center Mainz, 55131 Mainz, Germany

[5]Department of Neurosurgery, SHG-Klinikum Idar-Oberstein, 55743 Idar-Oberstein, Germany

[#]Contributed equally

*Correspondence should be addressed to Andrea Tuettenberg; antuette@uni-mainz.de

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Glioblastomas (GB) are amongst the most lethal human tumors exhibiting a highly aggressive behavior manifested by tumor cell infiltration into surrounding tissue. Furthermore, GBs are notorious for their high degree of resistance to cytotoxic treatments [1-3]. Despite extensive clinical and experimental research, the prognosis for patients diagnosed with GB is grim; GB has a final mortality rate of nearly 100%, less than a 10% five-year survival rate, and a median overall survival of 15 months [4]. The current standard of care for GB patients consists of surgical resection, radiation, and chemotherapy. However, despite these therapeutic options, the recurrence rate of GB is high [5,6].

One of the main reasons for the difficulty in cancer treatment is that malignant tumors, in general, possess strong capabilities to suppress anti-tumor immune responses [7]. The number of immunosuppressive strategies utilized by tumor cells is expansive. An example of a general mechanism used is the recruitment of tolerance inducing immune cells to the tumor microenvironment (TME), such as tolerogenic dendritic cells, tumor associated macrophages, regulatory T cells (Treg), and in case of GB, microglial cells [8,9]. Additionally, the innate genetic instability of tumor cells leads to alterations of surface proteins and antigenic structures as well as the downregulation of human leukocyte antigens (HLA) molecules, which significantly impairs recognition of tumor cells by the immune system [10]. Malignant tumors also produce a range of soluble factors that aid in the suppression of immune responses, including IL-10 and TGF-β, and proangiogenic factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which promote tumor angiogenesis [11,12]. These mechanisms collectively contribute to the formation of an inhibitory TME, which works to suppress and prevent antitumor immune responses.

Immunotherapies present as a promising alternative to traditional therapeutic approaches, which are often characterized by their severe cytotoxic side effects. Due to the systemic nature of circulating immune cells, tumor

cells dispersed throughout the body can be targeted. However, there is little known about the factors that influence immune cell migration and effective immune responses in the TME of GBs, both in the context of a newly diagnosed tumor and at the stage of post-treatment tumor recurrence. Tumor immune evasion strategies, whether active or general, all contribute to the formation of an inhibitory and immunosuppressive TME [7]. Overcoming this immunosuppressive TME represents one of the greatest challenges to achieve clinically meaningful outcomes of immunotherapies.

Therefore, delineation of regulatory molecules and pathways determining the ability of tumor cells to affect the tolerogenic TME in order to find novel immunotherapeutic targets is a matter of the utmost importance. GARP (glycoprotein A repetition predominant) is a transmembrane protein with an extracellular 21 leucine-rich domain (LRR) and a specific marker of activated Treg [13,14]. GARP is required for the formation, binding, and surface expression of latent TGF-β [13,15]. Production of TGF-β is an immune escape mechanism to induce tolerance in a variety of immune cells. In particular, TGF-β1 has also been associated with poor prognosis of patients with malignant tumors [16]. We have previously demonstrated that GARP contributes significantly to immunological tolerance in humans through the induction of peripheral Treg, regulatory M2 macrophages, and the inhibition of antigen specific T effector cells [17]. Furthermore, we have determined that GARP is also expressed on primary malignant melanoma and melanoma cerebral metastases [18]. In the present study, we evaluated GARP as potential biomarker, factor for immunoregulation, and its relevance as a therapeutic target in GB [19].

To determine in situ GARP expression, its relevance in the TME of primary brain tumors, and its comparative expression levels to astrocytomas grade II and III, 37 patients with histologically confirmed astrocytomas were recruited for this study [19]. Immunohistochemistry was performed on excised tumor tissue and stained for GARP. Notably, all astrocytomas grade II and III and GBs, except for one GB sample, exhibited high levels of GARP expression (Figure 1a, b, d, e, f). Altogether, the prevalent expression of the inhibitory molecule GARP in brain tumor tissue (Figure 1a, b, d, e, f), such as GB and low-grade glioma, and relatively low abundance in healthy brain tissue (Figure 1c), suggest potential important roles of GARP in the immunosuppressive TME and as a biomarker.

Next, we investigated GARP expression on the surface of GB tumor cell cultures. This was addressed in a commercially available GB cell line (T98G) and three GB cell lines (#1043, #1051, #1063) established in our group. Resting and activated Treg as well as one melanoma cell line (MaMel-19) served as positive controls for GARP expression. Flow cytometry and confocal microscopy were performed to assess GARP expression. Flow cytometry confirmed that all GB cell lines used in our study express GARP on their surfaces and further supported our earlier in situ results showing GARP expression in brain tumor tissues [19]. Intriguingly, we also observed intracellular and intranuclear localization of GARP in resting and activated Treg, T98G, the three established GB cell lines (#1043, #1051, #1063), and in MaMel-19 cells (Figure 1g, h). This finding is intriguing because intracellular expression of GARP has not been previously reported. Moreover, while intracellular GARP expression was significantly higher than surface GARP expression, the intranuclear levels were even greater (Figure 1g, h). Our data demonstrates for the first time that GARP is expressed intracellularly in Treg and in GB and melanoma cell lines.

Co-culture experiments were then performed to elucidate the GARP-dependent modulatory effect of GB cells on T effector cells. Our results showed GARP-dependent inhibition of T effector cell proliferation and cytokine production, in concordance with our previous findings of T effector cell suppression by melanoma tumor cells [17-19]. Collectively, these findings support the hypothesis that GARP has a role in the suppression of T effector cells in GB.

As described earlier, GARP is a transmembrane protein characterized by its extracellular 21 LRR domain [13,14]. A variety of proteins have been discovered containing LRRs with many different functions, including signal transduction, cell differentiation, and migration. Such proteins are frequently membrane bound, but they also can be secreted or localized to the cytoplasm or nucleus [20]. Examples of molecules containing LRRs are adhesion molecules, enzymes, and tyrosine kinase receptors (RTK). Although RTKs are usually localized to the plasma membrane, they have also been observed in the nucleus where they can interact with transcription factors which regulate cell proliferation, survival, and migration [21]. In the light of our findings of intranuclear GARP in Treg and in tumor cells and the structural similarities between GARP and other LRRs-containing proteins, it is tempting to speculate that GARP may have some previously unrecognized functions that may or may not be related to the canonical TGF-β dependent mechanism of tumor immunosuppression mediated by GARP. However, further studies are needed to confirm or rule out this assumption.

Our findings may also further enhance the

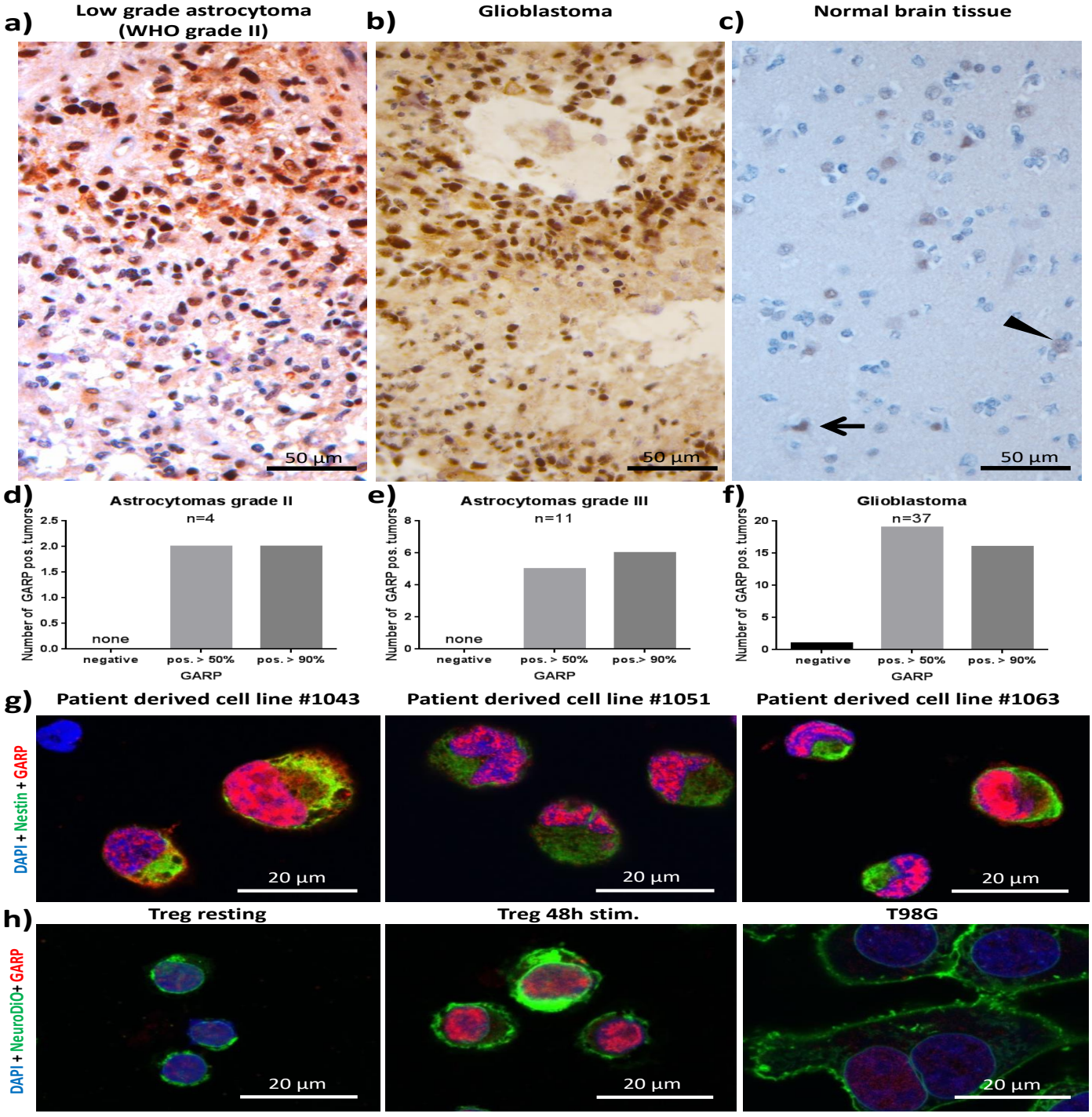

Figure 1: Immunohistochemistry and confocal imaging of stained glycoprotein, a repetition predominant (GARP) in astrocytomas grade II and III, glioblastomas (GB), healthy brain tissue, and regulatory T cells (Treg). (a, d, e) Low-grade astrocytomas (WHO grade II and III) with more than 50% GARP positive stained nuclei. (b, f) GB with palisading necroses and more than 90% GARP positive stained tumor cells (400x). (c) Largely normal brain tissue in the vicinity of a glioma with some labeled neurons (arrow) while others were unstained (arrowhead). Analysis of GARP localization in stimulated Treg, T98G (a GB cell line), and three patient derived GB cell lines. (g) The patient derived cell lines #1043, #1051, and #1063 show a strong GARP expression on the surface, intracellularly, and intranuclearly on confocal images. (h) Comparison of resting Treg, activated Treg, and T98G in confocal microscopy. The stimulated Treg show a strong increase in GARP expression on the surface, intracellularly, and intranuclearly when compared to resting Treg. Treg stimulation for 48h via 1 µg/mL anti-CD3 mAb, 1 µg/mL anti-CD28 mAb, and 10 IU/mL IL-2.

understanding of tumor immune escape mechanisms used by GB, especially in the context of GB progression and therapy resistance. Specifically, GARP is known to suppress antitumor responses via the TGF-β pathway, which is involved in the progression of GB and in the maintenance of self-renewal in glioma stem cells [17,22]. Targeting the factors involved in the formation of an immunosuppressive TME, like GARP, has become increasingly recognized as a promising strategy to improve the efficacy of immunotherapies for GB [23,24]. However, the unequivocal evidence to support this line of thought is still missing and further studies involving larger groups of patients must be conducted to confirm this hypothesis.

The need for individualized targeted therapies and immunotherapies for the treatment of malignant tumors has become widely recognized. Individual differences in response rates, side effects, and outcomes from different therapies can be attributed in part to differences in patients' unique TMEs. Characterization of TMEs through the use of biomarkers is a promising approach to individually describe patients' TMEs and to better predict possible prognoses, therapy options, side effects, response rates, and costs for each patient. The discovery of new biomarkers is a prerequisite to the development of novel immunotherapeutic approaches [25-27]. This study reveals for the first time, the expression of GARP inside glioma cells suggesting that the impact of this immunoregulatory molecule in GBs may extend beyond the canonical role of GARP in contributing to the immunosuppressive TME by modulating immune cells activities. We found a correlation between GARP expression in GB cells and decreased T effector cell function, similar to the relationship between GARP expression in melanoma cells and T effector cell suppression reported in our recent study [18,19]. It is tempting to speculate that GARP may have a conserved role in different types of cancer cells. The expression of GARP on GB cells in conjunction with its relatively low abundance in non-neoplastic brain underscores the potential diagnostic merit of GARP as a tumor cell biomarker and a prognostic marker for GB.

In summary, our present findings have described for the first time that GARP is expressed in the TME of primary brain tumors, including GB and astrocytomas grade II and III [19]. The presence of GARP on the surface of primary brain tumors, malignant melanoma, and activated Treg, is all suggestive of GARP being an interesting novel target for antibody based immunotherapeutic targeting. The results from this study provide novel insights that may have an impact on the development of new immunotherapeutic strategies for GB diagnostics and treatment.

Author Contributions

Conceptualization, A.T., E.K., J.T., and C.S.; methodology, N.Z., J.S., P.L., F.K., and B.S.; validation, N.Z. and B.S.; formal analysis, F.K.; resources, A.T.; writing—original draft preparation, A.T., N.Z., and E.K.; writing—commentary preparation, E.T., N.Z., A.T.; writing—review and editing of original draft, C.S., J.T., and F.R.; writing—review and editing of commentary, E.K., J.T.; draft supervision, A.T.; project administration, N.Z.; funding acquisition, A.T.

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