Article

Elevated proportions of activated NK cells at diagnosis predict a favourable prognosis in glioblastoma patients

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Simple Summary: Despite multimodal treatment WHO grade IV glioblastoma remains a devastating disease with dismal prognosis. The aim of this study was to identify novel biomarkers that more effectively predict outcome. Due to the high metabolic demand aggressive tumors frequently overexpress Hsp70. Depending on its intra- or extracellular localization, Hsp70 either promotes tumor progression or stimulates the immune system. All gliomas express mHsp70 and high grade gliomas show a strong nuclear and cytosolic Hsp70 expression. Large necrotic tumor areas are associated with significantly increased Hsp70 serum levels and elevated numbers of activated NK cells at diagnosis are associated with a more favorable prognosis in patients with grade IV gliomas.

Abstract: Despite rapid progress in the treatment of many cancers, glioblastoma remains a devastating disease with dismal prognosis. Recombinant heat shock protein 70 (Hsp70) and IL-2 stimulate the cytolytic activity of NK cells against a large variety of highly aggressive human tumor cells presenting Hsp70 on their cell surface. Therefore, the intra- and extracellular Hsp70 expression

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was determined in gliomas together with activatory NK cell receptors which are known to interact with membrane-bound Hsp70 (mHsp70). All gliomas are mHsp70-positive and high grade gliomas overexpress Hsp70 in the nucleus and cytosol. Significantly increased extracellular Hsp70 levels are detected predominantly in glioblastomas with large necrotic areas. Overall survival (OS) is improved in patients with low Hsp70 serum levels indicating that high Hsp70 expression levels are associated with unfavorable prognosis. Progression-free survival (PFS) in glioblastoma patients correlates with elevated proportions of activated NK cells (CD56+/CD94+, CD3-/CD69+) at diagnosis. The stimulation of these NK cells *in vivo* might be related to the presence pro-inflammatory cytokines and the Danger Associated Pattern Molecule (DAMP) Hsp70 in the circulation. Of caution, glucocorticoid therapy reduces the prevalence of activated NK cells. In summary, elevated frequencies of activated NK cells at diagnosis predict a more favorable prognosis in glioblastoma patients.

Keywords: Hsp70; biomarker; glioblastoma; NK cells; clinical study

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1. Introduction

Heat shock protein 70 (Hsp70), the major stress-inducible member of the 70-kDa heat shock protein family is evolutionary highly conserved and ubiquitously expressed in nearly all subcellular compartments [1]. Under physiological conditions Hsp70 maintains protein homeostasis by preventing protein aggregation, supporting the folding of nascent polypeptides and transporting proteins across membranes [2]. Stress such as hyperthermia, ischemia, nutrient deficiency or therapeutic interventions strongly upregulate the synthesis of Hsp70. Cytosolic Hsp70 interferes with signalling pathways that affect apoptosis, proliferation and differentiation [2] and as a result, elevated intracellular Hsp70 levels protect tumor cells from apoptosis [3]. Due to their high metabolic demand many aggressive tumor types exhibit an overexpression of Hsp70 which promotes tumorigenesis [4]. Following stress, Hsp70 rapidly translocates into the nucleus or interacts with lysosomal membranes to stabilize them [5].

We have previously shown that serum levels of extracellular Hsp70 correlate with the viable tumor mass and affect peripheral blood lymphocyte (PBL) compositions and profiles in patients with non-small lung cell cancer (NSCLC) [6]. Extracellular Hsp70 can be in the form of free protein which predominantly originates from dying tumor cells, or bound to exosomes that are actively released by viable tumor cells [7-9].

NK cells can be activated and triggered to kill mHsp70+ tumor cells by an incubation with full length Hsp70 protein, Hsp70-expressing exosomes [7,8] or a 14-mer Hsp70-peptide TKD (TKDNNLLGRFELSG) derived from the C-terminal substrate binding domain in combination with interleukin 2 (IL-2) [10,11]. A pilot [12] and clinical phase I [13] study demonstrated excellent safety profiles of *ex vivo* TKD/IL-2-activated NK cells and patients with advanced NSCLC showed promising clinical responses in a randomized clinical phase II trial after adoptive transfer of *ex vivo* TKD/IL-2 activated NK cells [14]. Apart from a study in grade IV glioblastoma/astrocytoma patients [15] insight into the expression and localization of Hsp70 in different types of brain tumors and its impact on the prevalence and activation of NK cells is limited. Therefore, intracellular, extracellular and mHsp70 levels were profiled as potential prognostic biomarkers and stimuli for NK cells. Glioblastoma the most common primary malignant tumor of the central nervous system in adults [16,17] is treated with surgery, radiation and a temozolomide-based chemotherapy [18]. However,

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despite this multimodal therapy these patients still have a dismal prognosis with median survival rates of approximately 15 months [18]. Tumor grading, age, isocitrate-dehydrogenase 1 (IDH1) and methylation status of the DNA repair gene O-6-methylguanin-DNA methyltransferase (MGMT) promotor, performance status and extent of resection are considered for predicting clinical outcome [19,20]. However, presently only the MGMT promotor status qualifies for a prediction of clinical responses to temozolomide.

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets, e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on references.

2. Results

2.1 MR imaging and Hsp70 staining of brain tumors

Clinical characteristics of all patients are summarized in Table 1A.

Table 1. WHO grade, age and diagnosis and isocitrate-dehydrogenase 1 (IDH1) wild type (wt) of patients with gliomas **(A)**.

A WHO grade	median age [years]	age range [years]	diagnosis	IDH1 WT	n subgroups	n total
II	30.5	24-52	oligodendroglioma	0	2	
			astrocytoma	0	6	8
III	49.5	29-61	anaplastic oligodendroglioma	0	8	
			anaplastic astrocytoma	1	6	15
IV	62	21-89	glioblastoma	65	66	66

Representative MR images of different brain tumors are shown in Figure 1A. Low grade tumors typically do not show contrast enhancement or necrosis and appear hyperintense on FLAIR images and hypointense on T1-weighted images, whereas glioblastomas show a thick irregular contrast enhancement, central necrosis, and perifocal FLAIR-hyperintensity that represents tumor infiltration and edema.

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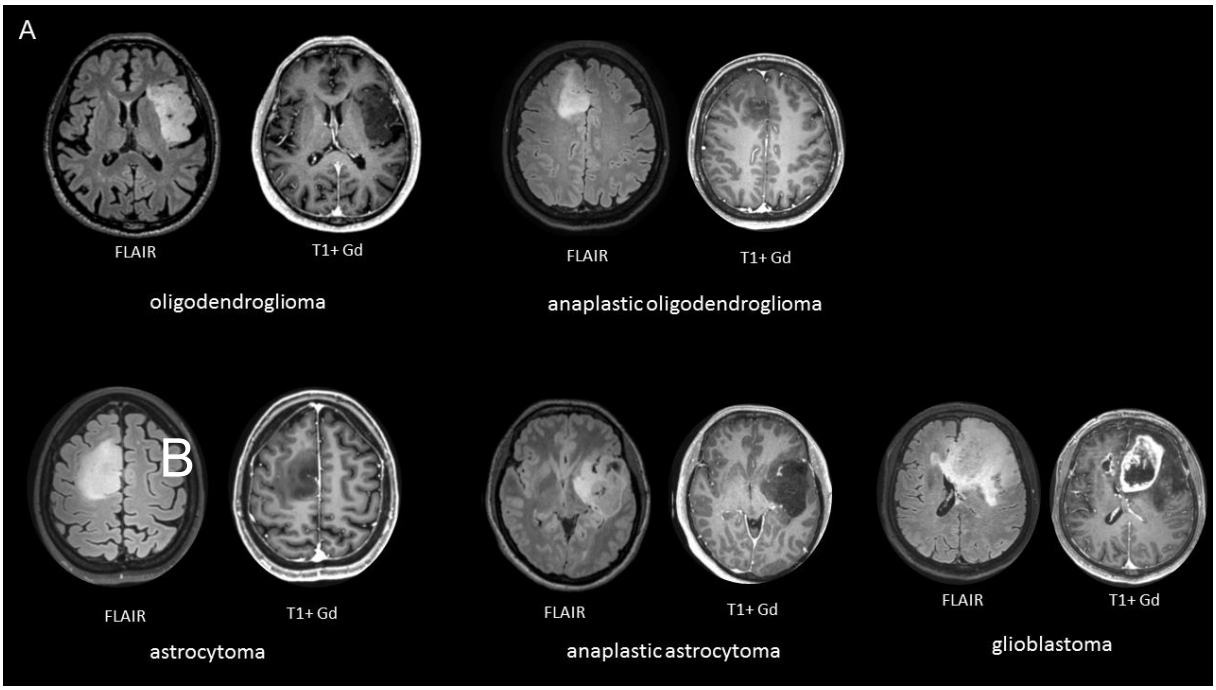

Figure 1A. Representative MR images of different glioma stages: oligodendroglioma, astrocytoma (grade II), anaplastic oligodendroglioma, anaplastic astrocytoma (grade III), glioblastoma (grade IV). Left, FLAIR images, right, T1 images with Gadolinium (Gd).

FFPE sections of tumor biopsies were stained immunohistochemically for their intracellular Hsp70 content. Only tumor areas, but not the surrounding tissue were considered for the evaluation. Figure 1B shows representative views of the different staining intensities (low/high) and localizations (nuclear/nuclear+cytosolic) of Hsp70 and Table 1B summarizes the data of grade II (n=3), grade III (n=4) and grade IV gliomas (n=17) in a four-by-four analysis. In summary, 2 of 3 grade II gliomas (67%) were scored as Hsp70 low and 1 of 3 (33%) as high, 1 of 4 grade III gliomas (25%) were scored as Hsp70 low and 3 of 4 (75%) as high, and 5 of 17 grade IV gliomas (30%) were scored as Hsp70 low and 12 of 17 (70%) as high (Table 1B). All lower grade gliomas showed a nuclear Hsp70 localization, whereas 16 of 17 (94%) grade IV gliomas exhibited a nuclear+cytosolic Hsp70 localization (Table 1C).

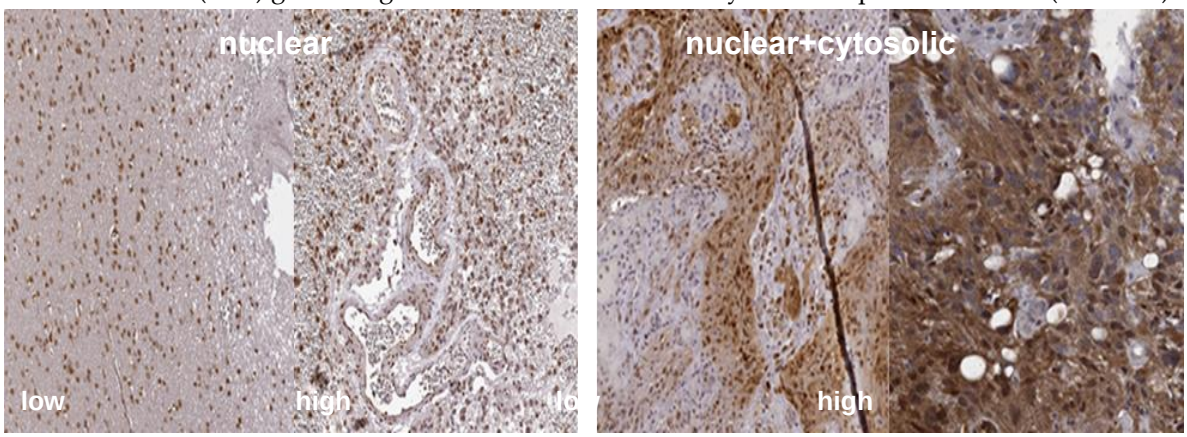

Figure 1B. Representative immunohistochemical stainings of tumor sections (3 µm) of glioma (grade II) versus high (grade IV) staining intensity.

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Table 1B. Correlation of glioma grading (grade II, III, IV) with the Hsp70 staining intensity (low versus high) and **C.** the Hsp70 localization (nuclear versus nuclear+cytosolic).

B

	Hsp70 intensity	Hsp70 intensity
WHO grade	low	high
II	2/3	1/3
	(67%)	(33%)
III	1/4	3/4
	(25%)	(75%)
IV	5/17	12/17
	(30%)	(70%)

C

	Hsp70 localization	Hsp70 localization
WHO grade	nuclear	nuclear+cytosolic
II	3/3	0/3
	(100%)	(0%)
III	4/4	0/4
	(100%)	(0%)
IV	1/17	16/17
	(6%)	(94%)

2.2 Membrane Hsp70 (mHsp70) expression on viable brain tumor cells

In addition to the cytosolic Hsp70 content, the mHsp70 expression was determined in freshly isolated, single cell suspensions of brain tumors by flow cytometry. Due to the poor viability of brain tumor cells *in vitro*, the mHsp70 status could only be determined in a total of 37 tumor samples. The proportion of mHsp70+ tumor cells was above 60% in all glioma grades, but did not differ significantly in the different tumor grades (Figure 1C).

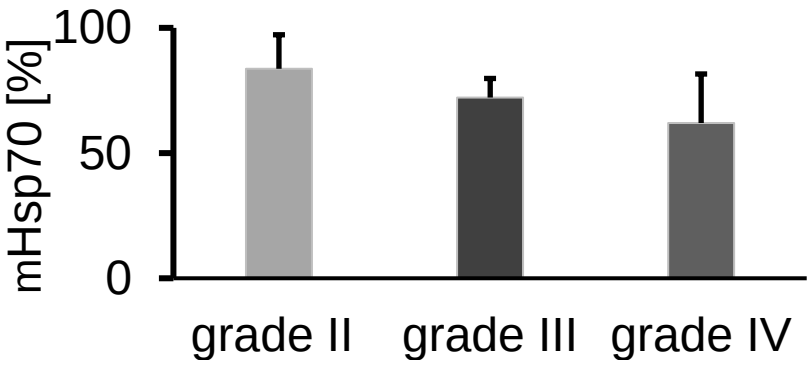

Figure 1C. Comparative analysis of the proportion of membrane Hsp70+ tumor cells in grade II (n=3), grade III (n=6) and grade IV (n=28) gliomas.

2.3 Extracellular Hsp70 concentrations

Serum Hsp70 levels were determined in grade II (n=5), grade III (n=12) and grade IV (n=51) patients at first diagnosis using the R&D ELISA which predominantly detects free Hsp70 originating from dying tumor cells and by an "in-house" Hsp70 ELISA [21] that detects both, free and liposomal Hsp70. As shown in Figure 2A, the median free Hsp70 concentrations in the circulation of patients

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with grade IV gliomas at diagnosis were significantly higher (3.48 ng/ml) than in healthy controls (n=150, 2.60 ng/ml, Tukey Test, $p<0.05$). Median serum Hsp70 concentrations in grade II and III glioma patients were 3.14 ng/ml and 3.34 ng/ml, respectively, but did not differ significantly to that of healthy control donors (Figure 2A). With respect to liposomal Hsp70 which is representing the viable tumor mass in other tumor entities [21], no significant differences were observed in all tumor grades (data not shown).

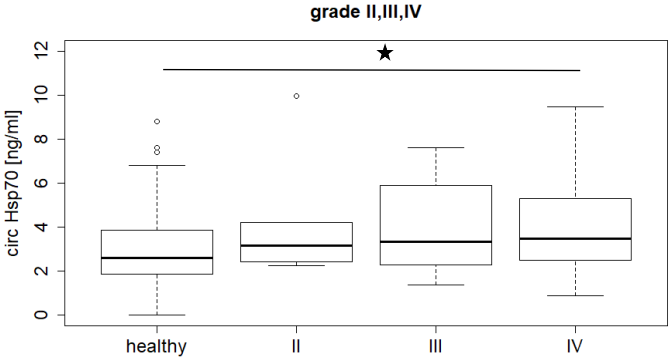

Figure 2A. Circulating Hsp70 concentrations in healthy controls (n=150) versus grade II (n=5) grade III (n=12) and grade IV (n=51) glioma patients, as determined with an ELISA

According to their tumor volumes, patients with grade IV gliomas (n=44) were subclassified into groups of patients with small (<30 cm^3), medium (30-90 cm^3) and large (>90 cm^3) tumors. The median tumor volumes in patients with small tumors (n=6) was 9.2 cm^3 (range 4.8-26 cm^3), in those with medium tumors (n=16) was 71.7 cm^3 (range 35.3-81.8 cm^3), and in those with large tumors (n=22) was 140.9 cm^3 (range 99-222 cm^3). As shown in Figure 2B, glioblastoma patients with large tumor volumes had significantly higher median Hsp70 concentrations in the serum (3.68 ng/ml, n=22) than healthy controls (2.60 ng/ml, n=150), as calculated by the Kruskal Wallis test ($p<0.05$), whereas those patients with small and medium tumor volumes did not differ significantly from the healthy control group.

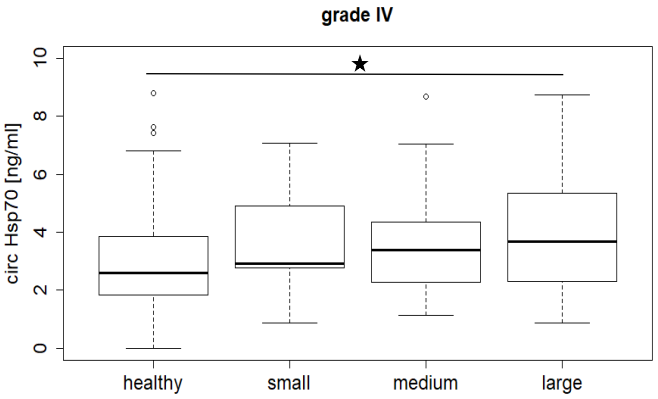

Figure 2B. Circulating Hsp70 concentrations in healthy controls (n=150) versus grade IV glioma patients (n=44) with small (<30 cm^3, n=6), medium (30-90 cm^3, n=16) and large (>90 cm^3, n=22) tumor volumes, as determined with an ELISA detecting free Hsp70 (*$p<0.05$).

According to MRI, the subgroup of grade IV gliomas (n=27) was subdivided into a group with a high proportion of necrosis (>10%, n=13) and a group with a low proportion of necrosis (<10%, n=14). The concentration of free Hsp70 in the circulation (3.42 ng/ml) in glioblastoma patients with highly necrotic tumors was significantly higher compared to healthy controls (2.60 ng/ml, Wilcoxon rank Test, $p<0.05$) (Figure 2C), whereas, that of patients with low tumor necrosis did not differ significantly to healthy controls (2.87 versus 2.60 ng/ml) (Figure 2C). As expected, liposomal Hsp70 levels did not differ significantly in patients with low and high proportions of tumor necrosis, but were elevated compared to that of healthy controls (data not shown). A Kaplan-Meier analysis of glioblastoma

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patients whose free Hsp70 serum levels were either below (n=18) or above (n=16) a threshold of 3.5 ng/ml which distinguishes healthy human individuals from tumor patients revealed a better OS in patients whose Hsp70 serum levels were below 3.5 ng/ml (p=0.1) (Figure 2D).

C D

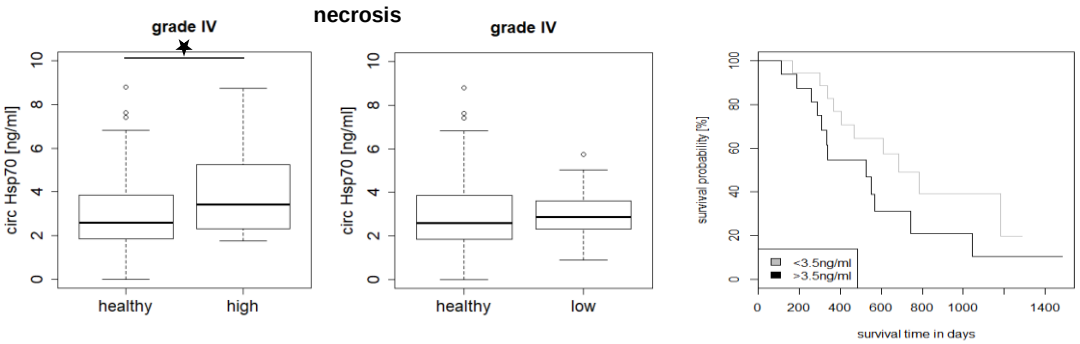

Figure 2C. Circulating Hsp70 concentrations in healthy controls (n=150) versus grade IV glioma (n=27) with a high (>10%, n=13) and low proportion of necrosis (<10%, n=14), as determined with an ELISA detecting free Hsp70 (*p<0.05). **D.** Kaplan-Meier analysis of the overall survival (OS) in glioblastoma patients with Hsp70 serum levels below (n=16) and above (n=18) a threshold of 3.5 ng/ml (p=0.1).

2.4 Composition of lymphocyte subpopulations in the peripheral blood of patients with different brain tumors

The proportions of major lymphocyte subpopulations such as CD19+ B cells, CD3+ T cells, CD3+/CD4+ helper T cells, CD3+/CD8+ cytotoxic T cells, CD4+/FoxP3+ Tregs, CD3+/CD56+, CD3+/CD94+, CD3+/NKG2D+ NK-like T cells (NKT), CD3-/CD56+ NK cells, CD3-/relevance CD56+/CD94+ NK cells, CD3-/NKG2D+ NK cells CD3-/NKp30 NK cells, CD3-/NKp40 NK cells, CD3-/CD69+ activated NK cells were assessed in the peripheral blood of 75 patients with gliomas at first diagnosis (6 grade II, 13 grade III and 56 grade IV) and healthy controls (n=15). The choice of the NK cell receptors was based on the relevance for NK cells to interact with mHsp70+ tumor cells. Neither the percentage of CD19+ B cells, CD3+/CD8+ T cells, CD4+/FoxP3+ Tregs nor any of the NK-like T cell subsets differed significantly in patients with gliomas in grade II, III, IV and healthy controls (data not shown). The proportion of CD3+ T cells appeared to be slightly reduced in glioblastoma patients at diagnosis compared to healthy controls (Figure 3A). The percentage of CD4+ T helper cells (Figure 3B) in grade IV glioma patients at first diagnosis, but not that of CD3+/CD8+ cytotoxic T cells (Figure 3C), was significantly lower than in healthy controls, (ANOVA, post-hoc Tukey test, p<0.05). Importantly, the proportion of activated NK cell subsets (CD3-/CD56+/CD94+, CD3-/CD69+) was significantly greater in glioblastoma patients at diagnosis, as compared to healthy controls (Kruskal Wallis test, p<0.05). The percentage of positively stained cells was calculated in the lymphocyte gate R1 (Figure 3F, left) and representative dot blot analysis of CD56+/CD94+, CD3-/CD69+ and CD3-/CD56+ NK cells subsets in a healthy individual and a grade IV glioblastoma patient are shown in Figure 3F, right. Additionally to an increase in the percentage of NK cells also the mean fluorescence intensity values of CD56+/CD94+ NK cells were found to be drastically increased in glioblastoma patients. A similar, albeit non statistically significant trend was observed also for other NK cell subsets such as CD3-/CD56+, CD3-/NKp30+, CD3-/NKp46+, CD3-/NKG2D+ (data not shown).

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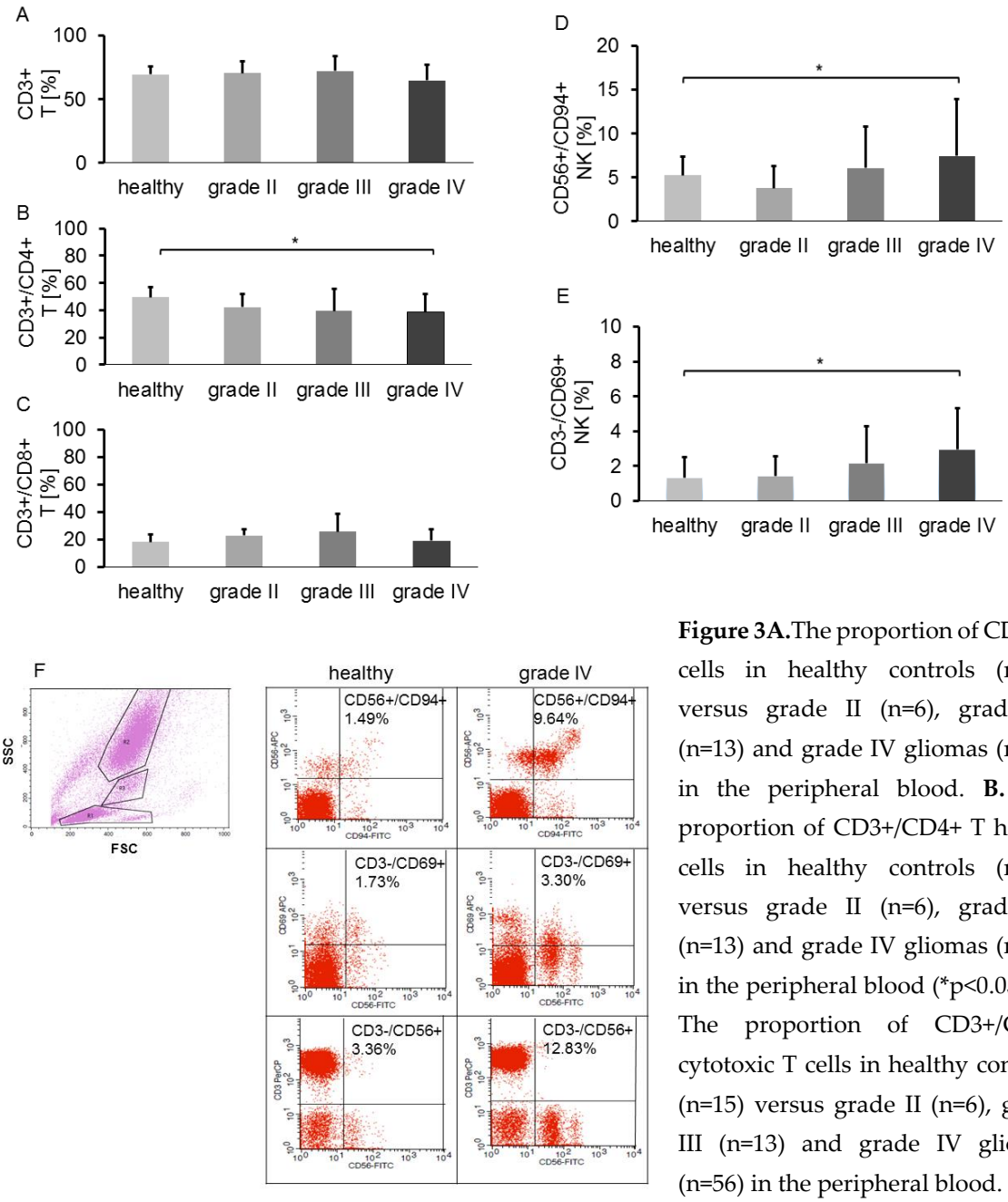

Figure 3A. The proportion of CD3+ T cells in healthy controls (n=15) versus grade II (n=6), grade III (n=13) and grade IV gliomas (n=56) in the peripheral blood. **B.** The proportion of CD3+/CD4+ T helper cells in healthy controls (n=15) versus grade II (n=6), grade III (n=13) and grade IV gliomas (n=56) in the peripheral blood (*p<0.05). **C.** The proportion of CD3+/CD8+ cytotoxic T cells in healthy controls (n=15) versus grade II (n=6), grade III (n=13) and grade IV gliomas (n=56) in the peripheral blood. **D.** The proportion of CD3-/CD94+ NK cells in healthy controls (n=15) versus grade II (n=6), grade III (n=13) and grade IV gliomas (n=56) in the peripheral blood (*p<0.05). **E.** The proportion of CD3-/CD69+ NK cells in healthy controls (n=15) versus grade II (n=5), grade III (n=13) and grade IV gliomas (n=56) in the peripheral blood (*p<0.05).

2.5 Composition of lymphocyte subpopulations in the peripheral blood of healthy donors after ex vivo stimulation with Hsp70/IL-2 or TKD/IL-2

To mimic potential immunostimulatory effects of circulating Hsp70 in glioblastoma patients, PBL of healthy human donors (n=4) were stimulated *in vitro* either with TKD/IL-2 (TKD) or Hsp70/IL-2 (Hsp70) [8,10]. Like glioblastoma patients, no significant differences in the composition of CD19+ B cells, CD3+/CD4+ and CD3+/CD8+ T cells, NK-like T cells, and Tregs were detected after this stimulation (data not shown). However, the proportion of CD56+/CD94+ NK cells, as represented in

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Figure 4A, was significantly increased after stimulation with TKD/IL-2 (6.6±2.08%, $p<0.05$) as compared to unstimulated PBL controls (2.27±0.24%), a similar trend was observed after stimulation with Hsp70/IL-2 (5.81±1.49%). The percentage of CD3-/CD69+ activated NK cells increased significantly after a stimulation with TKD/IL-2 (9.48±3.85%, $p<0.05$) and Hsp70/IL-2 (10.69±4.09%, $p<0.01$) as compared to unstimulated PBL controls (0.27±0.24%) (Figure 4B).

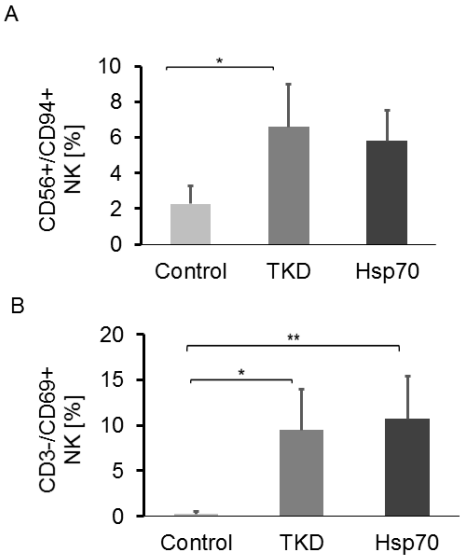

Figure 4A. The proportion of CD3-/CD94+ NK cells in PBL of unstimulated healthy controls versus Hsp70-peptide TKD/IL-2 or Hsp70/IL-2 stimulated PBL (n=4) ($p<0.05$, $^{**}p<0.01$). **B.** The proportion of CD3-/CD69+ NK cells in in PBL of unstimulated healthy controls (n=4) versus Hsp70-peptide TKD/IL-2 or Hsp70/IL-2 stimulated PBL (n=4).

2.6 Glioblastoma patients without progress exhibit elevated proportions of NK cells at diagnosis

A comparison of CD19+ B cells, CD3+/CD4+ and CD3+/CD8+ T cells, Tregs and NK-like T cells in glioma patients at diagnosis revealed no significant differences in the prevalence of these cells to that of healthy controls, irrespective of tumor progression at a later time point (data not shown). However, differences were observed with respect to CD3-/CD56+/CD94+ and CD3-/CD69+ NK cells. Patients with grade III gliomas whose tumors had not progressed after 18 months (n=9) had elevated proportions of CD3-/CD56+ NK cells (8.5±5.3% versus 6.3±2.8%, data not shown), CD56+/CD94+ NK cells (6.5±5.5% versus 5.3±3%) and CD3-/CD69+ NK cells (2.4±2.4% versus 1.6±1.6%) compared to patients whose tumors had progressed (n=3) within 18 months (Figure 5A). Similar results were observed in grade IV glioma patients that had not relapsed within 6 months. The proportion of CD3-/CD56+, CD56+/CD94+ and activated CD3-/CD69+ NK cells in patients without progression compared to those who progressed within 6 months was 11.9±8.9% versus 9.7±5.1%, 7.1±5.6% versus 6.9±5.2% and 3.8±3.3% versus 2.5±1.4%, respectively (Figure 5B). Compared to healthy individuals (n=15) the proportion of activated CD3-/CD69+ NK cells at diagnosis was significantly higher in glioblastoma patients who remained progression-free for 6 months ($p<0.01$) (Figure 5B). As demonstrated in Figure 5C, a glucocorticoid therapy which was given to glioblastoma patients before surgery drastically decreases the proportion of CD3-/CD56+ NK cells (13.84±9.79%, n=33 to 8.87±6.53%, n=12, data not shown), CD3-/CD94+ NK cells (8.25±6.25%, n=33 to 5.47±4.41%, n=12) and CD3-/CD69+ NK cells (4.56±5.37%, n=33 to 2.86±2.22%, n=12) (Figure 5C).

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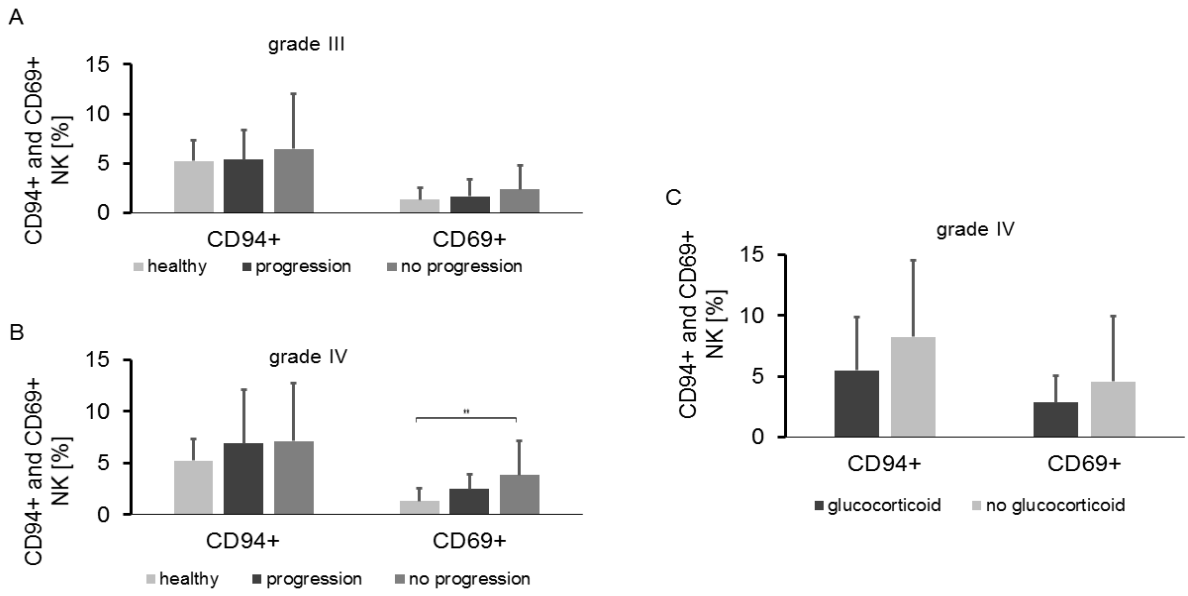

Figure 5A. Percentages of CD3-/CD94+, CD3-/CD69+ NK cells in glioma patients grade III with (n=3) and without tumor progression (n=9) within 18 months after diagnosis compared to healthy controls. **B.** Percentages of CD3-/CD94+ and CD3-/CD69+ NK cells in glioma patients grade IV with (n=10) and without tumor progression (n=27) within 6 months after diagnosis compared to healthy controls. **C.** Percentages of CD3-/CD94+ and CD3-/CD69+ NK cells in glioma patients grade IV with (n=12) and without (n=33) glucocorticoid treatment.

3. Discussion

Since the Nobel price in Physiology and Medicine was awarded to James P. Allison and Tasuku Honjo (2018) for their discovery of immunoregulatory mechanisms mediated by immune checkpoint inhibitors [22] the number of immunotherapeutic clinical approaches including immune checkpoint inhibitor blockades [23] and genetically engineered chimeric antigen receptor (CAR)-T/NK cell therapies has increased tremendously [24-27]. However, despite promising results in a number of cancer entities, a significant and relevant proportion of patients, including those with brain tumors, do not profit from these immunotherapies [28,29]. Herein, we wanted to obtain a better understanding of immune-related effects in patients with gliomas by profiling the composition of PBLs and by measuring the expression of intra- and extracellular Hsp70 levels. The concepts were based on the facts that Hsp70 exerts immunostimulatory or anti-apoptotic activities, depending on its extra- or intracellular localization [30]. Since the C-type lectin receptors CD94/NKG2C, NKG2D together with the activation marker CD69 on NK cells were found to be responsible for the recognition of mHsp70+ tumor cells [31], we decided to focus on these markers on NK cells together with the intra-, extracellular and membrane-bound Hsp70 levels.

In contrast to normal cells, nearly all highly malignant tumor cells exhibit an upregulated cytosolic Hsp70 expression and a mHsp70-positivity that mediate anti-apoptotic activities and therapy resistance [32,33]. In line with previous results, an upregulated intracellular Hsp70 expression was predominantly found in high grade gliomas [34,35]. Compared to healthy controls, significantly elevated serum levels of free Hsp70 were observed in glioblastoma patients with large necrotic tumor

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areas, but not in low grade gliomas. From this we conclude that extracellular Hsp70 predominantly originates from dying tumor cells [21]. An intact blood-brain-barrier in patients with low grade gliomas might hinder the release of Hsp70 into the circulation of these patients.

An improved OS of glioblastoma patients was associated with Hsp70 serum levels below a threshold of 3.5 ng/ml which is typically found in healthy individuals. These findings support the hypothesis that the aggressiveness of glioblastomas is associated with a high Hsp70 content.

On the other hand, extracellular Hsp70 and other danger associated molecular patterns (DAMPs) derived from necrotic tumor cells can stimulate the release of pro-inflammatory cytokines such as IL-2. We have previously established that Hsp70 in combination with IL-2 stimulates NK cell-mediated immune responses which are mediated by activatory receptors such as CD94/NKG2C [31,36] and CD69. To test the hypothesis that Hsp70-mediated immunostimulation of NK cells occurs in patients with high grade gliomas, PBLs from healthy donors were stimulated with recombinant Hsp70 or the TKD Hsp70-peptide together with low dose IL-2. This *in vitro* stimulation resulted in a similar upregulation of the frequency and density of these activatory NK cell receptors such as CD94+ and CD69+ on NK cells to that observed in glioblastoma patients. An upregulated CD94 expression on NK cells correlates with an elevated cytolytic activity against mHsp70+ tumor cells *in vitro* and in clinical trials [8,12-14].

In line with the literature, patients with high grade gliomas in our study exhibit a decreased frequency of CD3+/CD4+ T cells [37,38]. However, the value of tumor-infiltrating NK cells as a biomarker for gliomas is not completely understood. Although one study denies an involvement, another study indicates that NK cell infiltration into the tumor microenvironment is more common in high grade than low grade gliomas [39,40]. Due to a certain NK cell receptor allele being associated with the capacity to kill glioblastoma cells, NK cell-based immunotherapies have been discussed as an additional treatment option in combination with surgery and radiochemotherapy [41,42]. Moreover, targeted CAR-NK cell-based immunotherapies are presently tested in preclinical and clinical studies of glioblastoma [43-45].

A pre-surgical treatment with glucocorticoids reduces the prevalence of activated CD94+ and CD69+ activated NK cells. Since glucocorticoids also exert immunosuppressive functions in combination with immune checkpoint inhibitor therapies [46] their use should be considered with caution.

4. Materials and Methods

4.1 Patients

The study was approved by the local ethical committee of the medical faculty of the Technical University Munich (TUM, #2403/09) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before the start of therapy. EDTA-anticoagulated blood (for flow cytometry) and non-coagulated blood (for ELISA) was collected from patients with different types of brain tumors attending in the Department of Neurosurgery. A total of 88 patients were included into the study. According to the WHO classification of Tumors of the Central Nervous System [47] and histopathological analysis (Dpt. Neuropathology), 2 patients were diagnosed as having grade II oligodendroglioma, 6 as having grade II astrocytoma, 8 as having grade III anaplastic oligodendroglioma, 6 as having grade III anaplastic astrocytoma, and 66 patients as having grade IV gliomas. The samples were reviewed for this study. All patients were treated with

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radiotherapy concomitant with temozolomide after surgery according to the Stupp-regimen [18]. Clinical outcome and extracellular Hsp70 levels below and above a cut-off value of 3.5 ng/ml which was determined by using the Youden-index, at diagnosis were associated with OS in grade IV glioblastoma patients (n=34) by Kaplan-Meier analysis.

4.2 Tumor volumetry by magnetic resonance imaging (MRI)

Pre-operative MRI scans were performed on a 3Tesla MRI scanner (Philips Achieva, Philips Ingenia (Philips Medical Systems, The Netherlands B.V.) or Siemens Verio (Siemens Healthcare, Erlangen, Germany) and analysed as 3DT2-FLAIR and 3DT1 pre- and post-contrast sequences. Tumor volumes were determined by 2 independent neuroradiologists. MRI sequences (3DT2-FLAIR, 3DT1 post-contrast) were co-registered using a 3D Slicer (www.slicer.org) [48] and tumor volumes were segmented semi-automatically using a freely available software ITK-SNAP (www.itksnap.org) [49] in contrast-enhancing and FLAIR-hyperintense tumor areas.

4.3 Immunohistochemistry (IHC) and scoring of Hsp70 in brain tumor sections

A total of 24 formalin-fixed paraffin-embedded (FFPE) sections (3 µm) of different brain tumors (Dpt. Neuropathology) were stained for the expression of intracellular Hsp70 content. Briefly, the sections were dewaxed and hydrated in xylol (9713.3, Roth) and descending concentrations of ethanol (ethanol absolute, 27694, Fischar GmbH, ethanol 96% v/v, 27687, Fischar GmbH). After heating at 50-60°C for 30 min in citrate buffer (target retrieval solution, S1699, Dako, 1:10 dilution in ddH_2O), sections were washed in ddH_2O, H_2O_2 (3% v/v) and sodium azide (0.1% v/v) in phosphate buffered saline (PBS, D8537-500ML, Sigma Life Science) to block the activity of endogenous peroxidase. Unspecific binding was blocked by rabbit serum (5% v/v in antibody diluent, S2022, Dako). The sections were incubated with the Hsp70 monoclonal antibody cmHsp70.1 [50] (multimmune GmbH, Munich, Germany) or a respective IgG1 isotype-matched control antibody (1:500 in antibody diluent, S2022, Dako) at 4°C overnight. Antibody binding was detected by incubating horseradish peroxidase (HRP) labelled polymer conjugated with secondary antibodies (Envision+System HRP labelled polymer anti-mouse, K4001, Dako) for 20 min, followed by a Diaminobenzidine chromogen (DAB+substrate buffer, K3468, Dako, DAB+chromogen, K3468, Dako) for exactly 5 min and nucleus was counterstained with haematoxylin and eosin (T865.2, Roth). Sections were embedded and coverslipped in Eukitt quick-hardening mounting medium (Roti-Histokitt 100ml, 6638.1, Roth). Staining of Hsp70-negative (fibroblasts) and Hsp70+ (FaDu tumor cells) was included in each immunohistochemical staining for a better inter-assay comparability of the staining patterns. Samples were analysed on an Axio Vison microscope (Carl Zeiss, Jena) using the software ImageScope version 12.3.2.8013 and Hsp70 expression was classified into low and high intensity and nuclear versus nuclear and cytosolic localization. Scoring of the Hsp70 staining was performed blindly by 2 researchers and 2 neuropathologists.

4.4 Flow cytometric analysis of mHsp70 expression on viable brain tumor cells

Freshly aspirated tumor material (Dpt. Neurosurgery) was cut in 1 mm^3 pieces, incubated with trypsin for 8 min and forced through a sterile mesh (70 µm strainer). After resuspension of the single cell suspension in PBS/10% v/v FCS, $5x10^5$ cells were incubated with the following fluorescence-labelled antibodies for 30 min on ice: IgG1-FITC (BD Biosciences), CD45-APC (Thermo Fisher), cmHsp70.1-FITC (multimmune GmbH), pan-HLA class I-FITC (F5662, Sigma). After two washing steps, 7AAD (BD Biosciences) was added. Only viable, 7AAD-negative cells were gated upon and analysed using a FACSCalibur™ flow cytometer (BD Biosciences).

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4.5 Measurement of circulating Hsp70 levels

Serum (S-Monovette 7.5 ml Z, Sarstedt, Nürnbrecht, Germany) was obtained after centrifugation of peripheral blood (10 min, 5000 rpm). Aliquots (300 µl) were stored at -80°C. Hsp70 serum concentrations were determined according to the manufacturer`s instructions using the commercial Duo Set Hsp70 ELISA-kit (R&D Systems, Wiesbaden, Germany) or our "in-house" Hsp70 ELISA [21] which predominantly detects exosomal Hsp70 derived from viable tumor cells.

4.6 Immune phenotyping of peripheral blood lymphocytes (PBLs)

Lymphocyte subpopulations in the peripheral blood were profiled by multicolour flow cytometry on a FACSCalibur™ instrument (BD Biosciences) using the following fluorescence-labelled antibodies: T cell antibodies CD3-PerCP (345766-BD Biosciences), CD4-FITC (555346-BD Biosciences), CD8-PE (555366-BD Biosciences), FoxP3-PE (560046-BD Biosciences), NK cell antibodies CD56-FITC (345811-BD Biosciences), CD94-FITC (555888-BD Biosciences), NKG2D-PE (FAB139P-R&D Systems), NKp30-PE (PNIM3709-Beckman Coulter), NKp46-PE (PNIM3711-Beckman Coulter), and B cell antibody CD19-PE (555413-BD Biosciences). All stainings included appropriate isotype- and fluorescence-matched control antibodies. After incubation (15 min) in the dark at room temperature, cells were washed in PBS/10% v/v FCS. Red blood cells were lysed by incubating in BD FACS™ lysing solution (1:9 dilution in $_{dd}H_2O$, 349202-BD Biosciences, 10 min). In brief, lymphocytes were selected based on FSC/SSC characteristics, doublets were excluded and live cells were gated (PI/7-AAD-negative). NK and T cells were discriminated based on their CD56 and CD3 expression together with additional NK and T cell markers as mentioned above.

For regulatory T cell (Treg) analysis selected CD3+ T cells were subdivided into CD4+ and CD8+ T cells and the percentage of CD25+ and FoxP3+ cells were determined in the CD4+ subpopulation after fixing (buffer A, 1:10 in $_{dd}H_2O$, 51-9005451-BD Biosciences) and permeabilizing the cells (buffer C, 1:50 in buffer A, 51-9005450-BD, 30 min). The percentage of positively stained cells was determined within a defined lymphocyte gate.

4.7 Ex vivo stimulation of peripheral blood lymphocytes with Hsp70/IL-2 or Hsp70-peptide TKD/IL-2

To mimic potential immunostimulatory effects of circulating Hsp70 in glioblastoma patients, PBL of healthy individuals (n=4) were stimulated either with Hsp70 or Hsp70-peptide TKD together with IL-2. EDTA-blood (7.5 ml) was collected from healthy volunteers with a median age of 23.5 (range: 22-24 years) from which PBL were separated by Ficoll density gradient centrifugation. After counting, trypan blue-negative, viable cells ($5x10^6$) were resuspended in RPMI-1640 with L-glutamine, 10% v/v FCS, penicillin G (100 IU/ml) and streptomycin (100 µg/ml) and stimulated either with recombinant Hsp70 (10 µg/ml) and IL-2 (100 IU/ml) or Hsp70-peptide TKD (2 µg/ml, TKDNNLLGRFELSG, Bachem AG, Bubendorf) and IL-2 (100 IU/ml) for 5 days [8,10]. After stimulation, cells were counted and analysed by flow cytometry.

4.8 Statistics

The statistical analysis was performed using the programming language R, R studio version 3.5.2. Normal distribution was tested by the Shapiro-Wilk normality test. Parametric data were analysed by ANOVA and post-hoc Tukey tests, non-parametric were analysed by using the Kruskal Wallis test and Wilcoxon signed-rank test, as appropriate. A value of $p<0.05$ was considered as representing statistically significant differences.

5. Conclusions

Hsp70, together with IL-2 activate CD94+ NK cells to recognize and kill mHsp70+ tumor cells. The potential of Hsp70-targeting NK cells to exert beneficial anti-tumor immune responses has been confirmed in preclinical models of glioblastoma using Hsp70-targeting theranostic nanoparticles [36], and in a phase II clinical trial in patients with advanced, mHsp70+ NSCLC [14]. The present study provides first evidence that elevated proportions of activated NK cells in glioblastoma patients at diagnosis might be predictive for a more favourable clinical outcome. However, due to the relatively low number of patients the results need to be repeated in a larger patient cohort.

Author Contributions: DL performed experiments, analyzed data, wrote the manuscript; JG, MB, IR, JS treated patients, provided clinical material and data; WS, SSch, HTN, StS, CW, FW, ZW, HZ, MS, MP, MSch performed experiments, analyzed data; GM conceived and designed the study, analyzed and interpreted the data and reviewed/edited the manuscript. All authors approved the submitted version.

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Conflicts of Interest: Gabriele Multhoff declares a conflict of interest with respect to multimmune GmbH. The company did not have a role in the results or the interpretation of the results but provided the antibody cmHsp70.1. All other authors declare no conflict of interest.

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