

Poor survival in glioblastoma patients is associated with early signs of immunosenescence in the CD4 T-cell compartment after surgery

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Abbreviations: GBM, glioblastoma multiforme; HCMV, human cytomegalovirus; PBMC, peripheral blood mononuclear cell; Val-cyte, valganciclovir; WBC, white blood cell count

Patients with glioblastoma multiforme (GBM) are immunosuppressed and have a broad range of immunological defects in both innate and adaptive immune responses. GBMs are frequently infected with human cytomegalovirus (HCMV), a virus capable of causing immunosuppression. In 42 HCMV-positive GBM patients in a clinical trial (VIGAS), we investigated T-cell phenotypes in the blood and assessed their relation to survival. Blood was collected before and 3, 12, and 24 weeks after surgery, and the frequency of T-cell subsets was compared with that in 26 age-matched healthy controls. GBM patients had lower levels of CD3 cells than the controls, but had significantly higher levels of CD4⁺CD28⁻ T cells before and 3 and 12 weeks after surgery and increased levels of CD4⁺CD57⁺ and CD4⁺CD57⁺CD28⁻ T cells at all-time points. These T-cell subsets were associated with both immunosenescence and HCMV infection. GBM patients also had higher levels of $\gamma\delta$ T cells at all-times after surgery and lower levels of CD4⁺CD25⁺ cells before and 3 weeks after surgery than healthy controls. Overall survival was significantly shorter in patients with higher levels of CD4⁺CD28⁻ T cells ($p = 0.025$), CD4⁺CD57⁺ T ($p = 0.025$) cells, and CD4⁺CD28⁻CD57⁺CD28⁻ T cells ($p < 0.0004$) at 3 weeks after surgery. Our findings indicate that signs of immunosenescence in the CD4⁺ compartment are associated with poor prognosis in patients with HCMV-positive GBMs and may reflect the HCMV activity in their tumors.

Introduction

Despite advances in molecular characterization and a better understanding of cancer cell biology, GBM, the most common primary intracranial tumor in adults, has a dismal prognosis. Even with aggressive surgical and concomitant chemo- and radiotherapy, median survival is as low as 12–15 mo after diagnosis.¹ GBMs have been divided into four molecular phenotypes: proneural, neural, classical, and mesenchymal, according to genetic alterations in tumor genes, mainly EGFR, NF1, and PDGFRA/IDH1. Each subtype is enriched in gene expression signatures of distinct neural lineages, implying that the expression patterns of the different subtypes reflect the phenotype of their cells of origin.² However, all signatures seem to be represented in GBMs of different phenotypes, increasing the difficulty of understanding the heterogeneity and complexity of these tumors.³ Despite progress in understanding the genetic alterations in GBM, clinically

useful predictive molecular markers of therapeutic response and prognosis are still rare.

GBMs are often positive for HCMV,^{4–8} a herpes virus carried by 70–100% of people in populations worldwide. After an active primary infection, which is often asymptomatic or subclinical in immunocompetent persons, the virus establishes latency in myeloid cells in the bone marrow.^{9,10} The virus can be reactivated by inflammation, leading to differentiation of monocytes into macrophages or dendritic cells. HCMV may cause severe infections in immunosuppressed individuals, who are often highly viremic.^{11,12} GBM patients are rarely viremic, although about 20% are positive for HCMV IgM, which is very uncommon among healthy persons.¹³ The level of HCMV infection in GBMs appears to predict survival; patients with low amounts of HCMV in their tumors at diagnosis have a better prognosis.⁴ When added to standard therapy, anti-HCMV treatment markedly improves survival among GBM patients.⁴ Thus, HCMV may be

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important in the pathobiology of GBM and may be a novel target for therapy.

HCMV can trigger immunosenescence, which is characterized by increased frequency of CD28⁻ CD4⁺ and CD8⁺ T cells and CD57 expression.^{14,15} Loss of CD28 expression on T cells is the most consistent biological indicator of aging or replicative senescence of the immune system. Higher levels of CD28⁻ T cells in the elderly and patients with chronic heart failure and chronic kidney disease are associated with increased mortality.^{16,17} CD28⁻ T cells are also more abundant in patients with inflammatory disorders.¹⁸⁻²¹ Interestingly, CD4⁺CD28⁻ T cells are only considered to be found in HCMV-seropositive individuals.²² HCMV likely triggers differentiation of these cells *in vivo* by chronic antigen stimulation.²³ It is not known why this virus and not other pathogens expand CD4⁺CD28⁻ T cells.

CD57⁺ T cells accumulate in patients with different forms of cancer,¹⁵ perhaps as a result of constant stimulation in the absence of effective tumor clearance mediated by tumor-associated antigens.²⁴ CD57 expression on CD4⁺ and CD8⁺ T cells was once considered to indicate immune senescence in HCMV-infected patients.²⁵ However, CD57 is mainly a marker of general proliferative instability, and CD57 expression is increased in patients with defective immune responses, such as those with HIV, active HCMV infection, autoimmune disorders, and cancer.²⁶ CD57 is also a marker of natural killer cells with poor proliferation but increased cytotoxicity.²⁷

Another T cell phenotype correlated with immune modulation and cancer is the $\gamma\delta$ T cell. These cells account for only 2–5% of peripheral T-cells but are abundant in mucous tissues and different organs. In murine cancer models and *in vitro* experiments, $\gamma\delta$ T cells delay or even halt tumor growth and decrease metastatic spread.²⁸ Their role in GBM progression and patient survival has been debated. $\gamma\delta$ T cells kill GBM cells *in vitro* and reduce tumor progression *in vivo*.^{29,30} A subset of $\gamma\delta$ T cells is expanded in HCMV-infected persons.³¹

In this study, we investigated T-cell phenotypes in relation to overall survival after diagnosis in 42 patients with HCMV-positive GBMs who were enrolled in the VIGAS trial. We found that signs of immunosenescence linked to HCMV infection predict poor survival in patients with GBM.

Results

GBM patients have lower levels of CD3 T cells but higher levels of CD4⁺CD28⁻ and $\gamma\delta$ T cells than healthy controls

Forty-two GBM patients were enrolled in this study. Their clinical characteristics are shown in Table 1, and their T-cell phenotypes are shown in Figs. 1 and 2 and are summarized in Table 2. As in other studies,^{32,33} these patients had significantly lower median levels of CD3 T cells than age-matched healthy controls (37.1% at baseline, 44.6% at 3 weeks, 40.9% at 12 weeks, and 43% at 24 weeks after surgery vs. 62%, in healthy controls, Fig 1A). However, the CD4⁺ and CD8⁺ T-cell populations were similar in the two groups (Fig. 1B and C). Thus, the decreased levels of CD3 T cells reflect depletion

of other cellular subtypes. GBM patients also had significantly higher median levels of $\gamma\delta$ T cells at all-time points compared to the healthy controls (11.6% at baseline, 12.1% at 3 weeks, 10.2% at 12 weeks, and 11.8% at 24 weeks vs. 3.6% in healthy controls, Fig. 1D) and of CD4⁺CD28⁻ T cells before and 3 and 12 weeks after (9.0% at baseline, 7.0% at 3 weeks, and 7.0% at 12 weeks vs. 3.0% in healthy controls, Fig. 1E). The levels of CD8⁺CD28⁻ cells did not differ in the two groups (Fig. 1F).

GBM patients have higher levels of CD4⁺CD57⁺ and CD4⁺CD57⁺CD28⁺ T cells

In T cells, CD57 expression increases when CD28 expression is lost.¹⁵ GBM patients had significantly higher median levels of CD4⁺CD57⁺ T cells than controls at all-time points compared to healthy controls (7.7% at baseline, 7.2% at 3 weeks, 8.1% at 12 weeks, and 7.0% at 24 weeks vs. 4.5% in controls, Fig. 2A). However, the number of CD8⁺CD57⁺ T cells or CD8⁺CD57⁺/CD28⁻ T cells did not differ in GBM patients and controls (Fig. 2B).

The levels of CD57⁺CD28⁻ CD4⁺ and CD8⁺ T cells did not differ in GBM patients and controls (Fig. 2C and D). However, median levels of CD57⁺CD28⁺ CD4 cells (Fig. 2E) were higher in GBM patients (6.2% at baseline, 5.0% at 3 weeks, 6.0% at 12 weeks, and 5.7% at 24 weeks vs. 2.0% in controls). CD8⁺ T-cell levels did not differ significantly (Fig. 2F). There was a trend toward lower levels of CD25 positive CD4⁺ and CD8⁺ cells in GBM patients. CD4⁺CD25⁺ T cells were significantly less abundant in GBM patients than controls at baseline (1.3% vs. 4.5%) and at 3 weeks (1.8% vs. 4.5%) (Fig. 2G), as were levels of CD8⁺CD25⁺ cells at 24 weeks (1.0% vs. 4.0%, Fig. 2H). This was unexpected, as these cells are expected to serve as regulatory T cells, and GBM patients are considered to have an immunosuppressive phenotype. Unfortunately, Foxp3 — which together with CD25 is considered to be a more accurate marker for regulatory T cells — was not among the markers analyzed in blood cells from VIGAS patients. Therefore, we analyzed Foxp3 levels in cells from 15 other GBM patients. Interestingly, Foxp3-positive CD4⁺ and CD8⁺ T-cell subsets showed a trend toward greater abundance in GBM patients than healthy controls (Fig. S1). Thus, CD25 and Foxp3 subsets may be different in GBM patients, and these patients may also have higher levels of Foxp3-positive regulatory T cells.

Long-term survival in GBM patients is associated with higher levels of CD3 T cells

GBM patients are known to be immunosuppressed. Therefore, to determine whether overall survival correlates with T-cell subset levels in GBM patients, we analyzed T-cell levels at different times in long-term survivors (≥ 20 mo) and short-term survivors (< 20 mo). CD3 T-cell levels were significantly lower in short-term survivors compared to long-term survivors of GBM patients at 3 ($p = 0.002$) and 12 weeks ($p = 0.0367$) after surgery (Fig. 3A). However, no survival differences were found

Table 1. Characteristics of glioblastoma patient cohort

Patient nr	Age at diagnosis	TTP (Months)	Treatment at tumor recurrence	Overall survival (Months)	Preoperative steroid intake (days)	Post-operative steroid discontinuation (days)	Surgical tumor reduction more than 90% (y/n)
1	64	6	none	14	10	12	y
2	58	3	none	17	1	10	y
3	64	12	Surgery	16	6	12	y
4	55	73	Temodal	75	3	8	y
5	74	16	Temodal	18	5	7	y
6	68	12	Surgery + Lomustine	30	18	8	y
7	59	3	none	8	13	9	y
8	68	3	Surgery + Radiotherapy	77	12	21	y
9	59	11	Lomustine	18	4	17	y
10	71	12	Lomustine	17	5	8	y
11	75	6	Lomustine	18	14	60	y
12	61	68	Surgery	70	8	9	y
13	52	6	Gammaknife	41	4	6	y
14	60	6	Radiotherapy	17	5	8	n (50% to 90% tumor reduction)
15	75	52	Gammaknife	59	2	long term (> 6 mo)	y
16	64	6	Temodal	14	11	19	y
17	73	3	none	8	16	9	y
18	62	3	Gammaknife	18	6	9	y
19	60	3	Gammaknife	55	10	8	y
20	49	3	none	5	2	long-term (> 6 mo)	y
21	34	3	Gammaknife + Avastin	24	3	9	y
22	65	3	Temodal + Avastin	19	7	16	y
23	64	3	Lomustine	10	14	11	y
24	51	3	Lomustine	12	3	12	y
25	77	3	Temodal	10	14	25	y
26	37	6	none	Lost to follow-up	9	8	y
27	60	3	Lomustine	19	13	10	y
28	52	4	Lomustine	13	4	60	y
29	59	9	Gammaknife	22	1	42	y
30	60	3	none	10	21	long term (> 6 mo)	y
31	33	3	Surgery + Gammaknife + Lomustine	8	3	9	y
32	57	12	none	15	2	30	y
33	65	24	Temodal + Avastin	38	20	14	y
34	69	6	Gammaknife	22	1	9	y
35	46	9	Surgery + Lomustine	18	5	9	y
36	46	3	Temodal + Avastin	16	1	26	y
37	49	6	Gammaknife	18	2	12	y
38	68	17	Gammaknife	31	1	8	y
39	64	6	Gammaknife	68	7	9	y
40	61	1	Gammaknife	25	7	9	y
41	66	1	none	4	3	9	y
42	65	6	Gammaknife	24	6	8	y
Median	61	6		18	5.5	9	

when analyzed with Kaplan–Meier graphs using the median value of CD3 T-cell level as a cut-off for low or high T-cell levels (Fig. 3B and C).

We also observed trends for lower levels of CD4⁺ T cells and higher levels of CD8⁺ T cells among short-term survivors (Fig. 3D and G). Survival was not related to baseline levels of CD4⁺ (Fig. 3E and F) or CD8⁺ cells (Fig. 3H) but was significantly longer among GBM patients with lower levels of CD8⁺ T cells at 3 weeks ($p = 0.029$) (Fig. 3I). The level of $\gamma\delta$ T cells did not differ in short- vs. long-term survivors and was not associated with overall survival (Fig. 4A–C).

Higher levels of CD4⁺CD28⁻ T cells are associated with poor overall survival in GBM patients

Interestingly, among GBM patients, long-term survivors generally had lower levels of CD4⁺CD28⁻ cells than short-term survivors; the difference was significant at 3 weeks after surgery ($p = 0.009$) (Fig. 4D). Survival was also significantly longer in GBM patients with lower levels of CD4⁺CD28⁻ cells at 3 weeks than in those with higher levels ($p = 0.024$) (Fig. 4E and F). CD8⁺CD28⁻ T-cell levels were similar in short- and long-term survivors, and survival did not differ in patients with higher or lower CD8⁺CD28⁻ T cell levels (Fig. 4G–I).

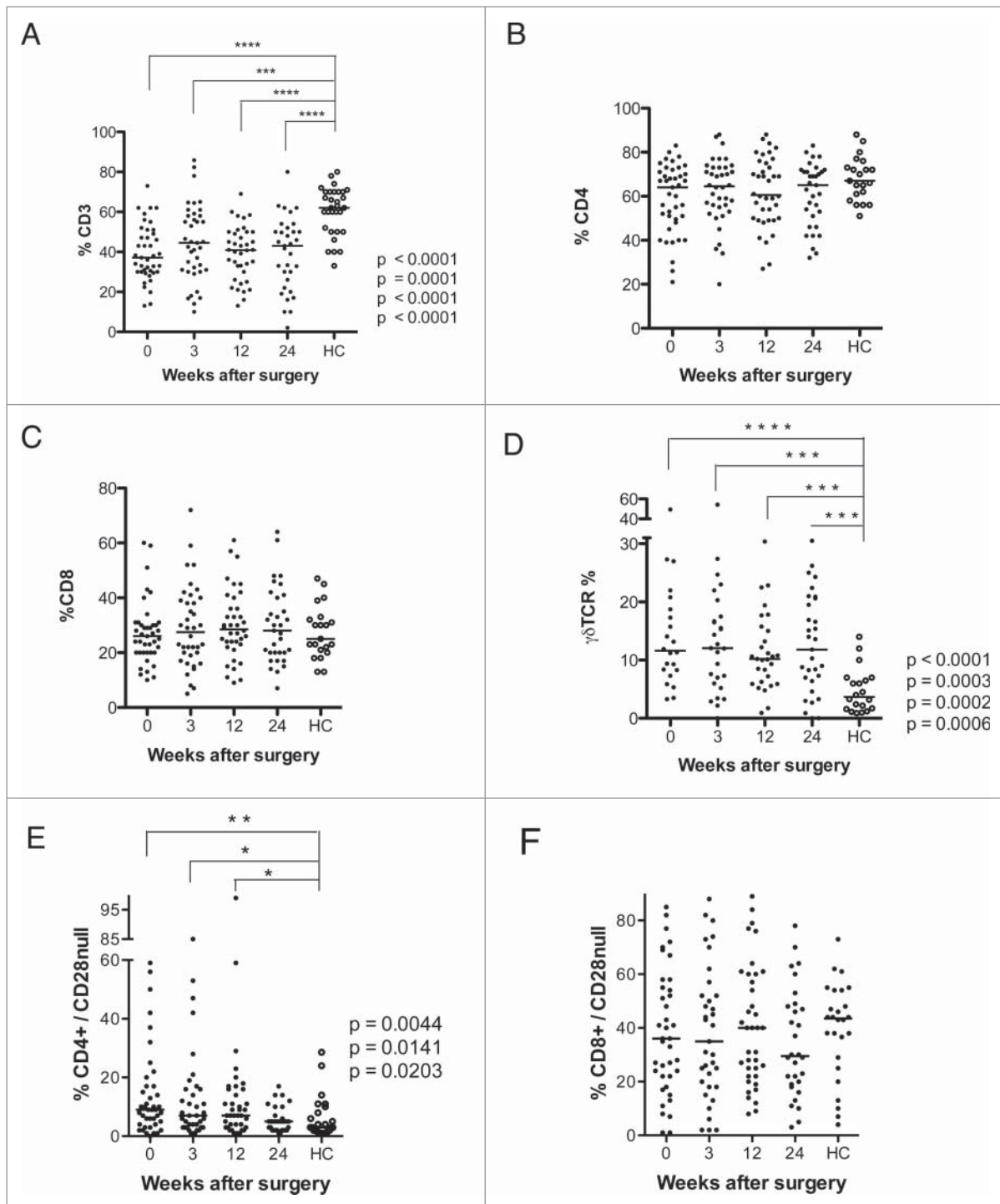


Figure 1. Levels of CD3⁺ T cells, $\gamma\delta$ T cells, and CD4⁺CD28⁻ T cells are lower after surgery in GBM patients than in healthy controls (HC). T-cell phenotype was analyzed in PBMCs from GBM patients before and 3, 12, and 24 weeks after surgery. (A) CD3⁺ T cells were less abundant than in controls at all-time points (B and C) Levels of CD4⁺ and CD8⁺ T cells did not differ. (D) Levels of $\gamma\delta$ T cells were higher in GBM patients than controls at all-time points. (E) Levels of CD4⁺CD28⁻ T cells were higher before and 3 and 12 weeks after surgery. (F) Levels of CD8⁺CD28⁻ T cells did not differ.

High levels of CD4⁺CD57⁺, especially CD4⁺CD57⁺CD28⁻ T cells, are associated with shorter overall survival in GBM patients

Further examination revealed a trend toward higher levels of both CD57⁺ CD4⁺ and CD8⁺ T cells among short-term

survivors; CD4⁺CD57⁺ T-cell levels were significantly higher at 3 and 24 weeks ($p = 0.036$ and $p = 0.019$, respectively) (Fig. 5A) in short-term survivors. Overall survival was significantly longer in GBM patients with lower levels of CD4⁺CD57⁺ cells at 3 weeks ($p = 0.025$) (Fig. 5C);

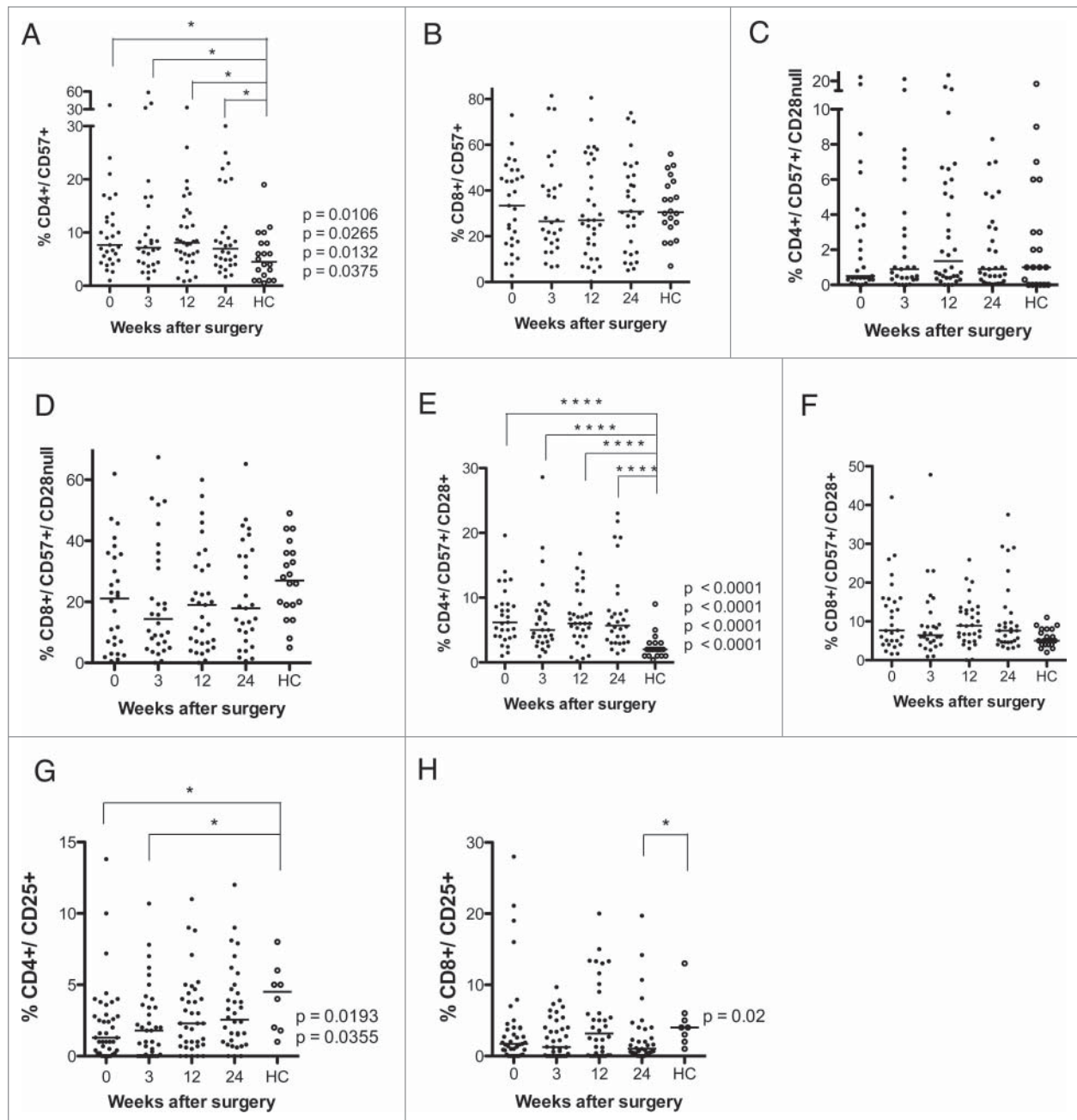


Figure 2. Levels of CD4⁺CD57⁺, CD4⁺CD57⁺CD28⁺, CD4⁺CD25⁺ and CD8⁺CD25⁺ T cells are higher after surgery in GBM patients than in healthy controls (HC). T-cell phenotype was analyzed in PBMCs from GBM patients before and 3, 12, and 24 weeks after surgery. (A) Levels of CD4⁺CD57⁺ T cells were higher in GBM patients at all-time points compared to HC. (B–D) Levels of CD8⁺CD57⁺, CD4⁺CD57⁺CD28⁻, and CD8⁺CD57⁺CD28⁻ T cells did not differ. (E) Levels of CD4⁺CD57⁺CD28⁺ T cells were higher in GBM patients than in controls at all-time points. (F) Levels of CD8⁺CD57⁺CD28⁺ T did not differ. (G) Levels of CD4⁺CD25⁺ T cells were lower at baseline and 3 weeks after surgery. (H) Levels of CD8⁺CD25⁺ T cells were lower at 24 weeks after surgery.

baseline levels were not associated with survival (Fig. 5B). The levels of CD8⁺CD57⁺ T cells were also higher among short-term survivors. The difference was significant at 24 weeks after surgery ($p = 0.03$) (Fig. 5D), but Kaplan–Meier analysis showed no difference in survival (Fig. 5D and E).

Since CD57 expression increases after loss of CD28 on CD4⁺ T cells in HCMV-infected patients, we further investigated whether T cells that express CD57 but had lost CD28 expression were correlated to survival. There was a clear trend toward increased levels of these T cells at 3 weeks in short-term survivors ($p = 0.01$) (Fig. 5G). Interestingly, median

Table 2. Summary of the analysis of immunological cell types (%) in blood in glioblastoma patients after surgery (weeks). Data shown in the table: median (significant *p* values)

	0 weeks	3 weeks	12 weeks	24 weeks	Healthy controls
CD3 cells	37.1**** (<i>p</i> < 0.0001)	44.6 *** (<i>p</i> = 0.0001)	40.9 ****(<i>p</i> < 0.0001)	43****(<i>p</i> < 0.0001)	62
CD4 ⁺ cells	64	64.5	60.5	65	67
CD4CD28null cells	9** (<i>p</i> = 0.0044)	7* (<i>p</i> = 0.0141)	7* (<i>p</i> = 0.0203)	5	3
CD4CD57 cells	7.7 * (0.0106)	7.15 * (<i>p</i> = 0.0265)	8.1 * (<i>p</i> = 0.0132)	7* (<i>p</i> = 0.0375)	4.5
CD4CD57CD28 cells	6.2 **** (<i>p</i> < 0.0001)	5 **** (<i>p</i> < 0.0001)	6 **** (<i>p</i> < 0.0001)	5.7 **** (<i>p</i> < 0.0001)	2
CD4CD57CD28null cells	0.5	0.9	1.35	0.9	1
CD4CD25 cells	1.3* (<i>p</i> = 0.0193)	1.8 * (<i>p</i> = 0.0355)	2.3	2.5	4.5
CD8 ⁺ cells	26	27.5	28.5	28	25
CD8CD28null cells	36	35	40	29.5	43.5
CD8CD57 cells	33.4	26.5	27	30.8	30.5
CD8CD57CD28 cells	7.7	6.5	8.9	7.6	5
CD8CD57CD28null cells	21.1	14.4	19	17.9	27
CD8CD25 cells	1.7	1.2	3.1	1.1 * (<i>p</i> = 0.02)	4
gd T cells	11.6****(<i>p</i> < 0.0001)	12.1***(<i>p</i> = 0.0003)	10.2***(<i>p</i> = 0.0002)	11.8***(<i>p</i> = 0.0006)	3.6

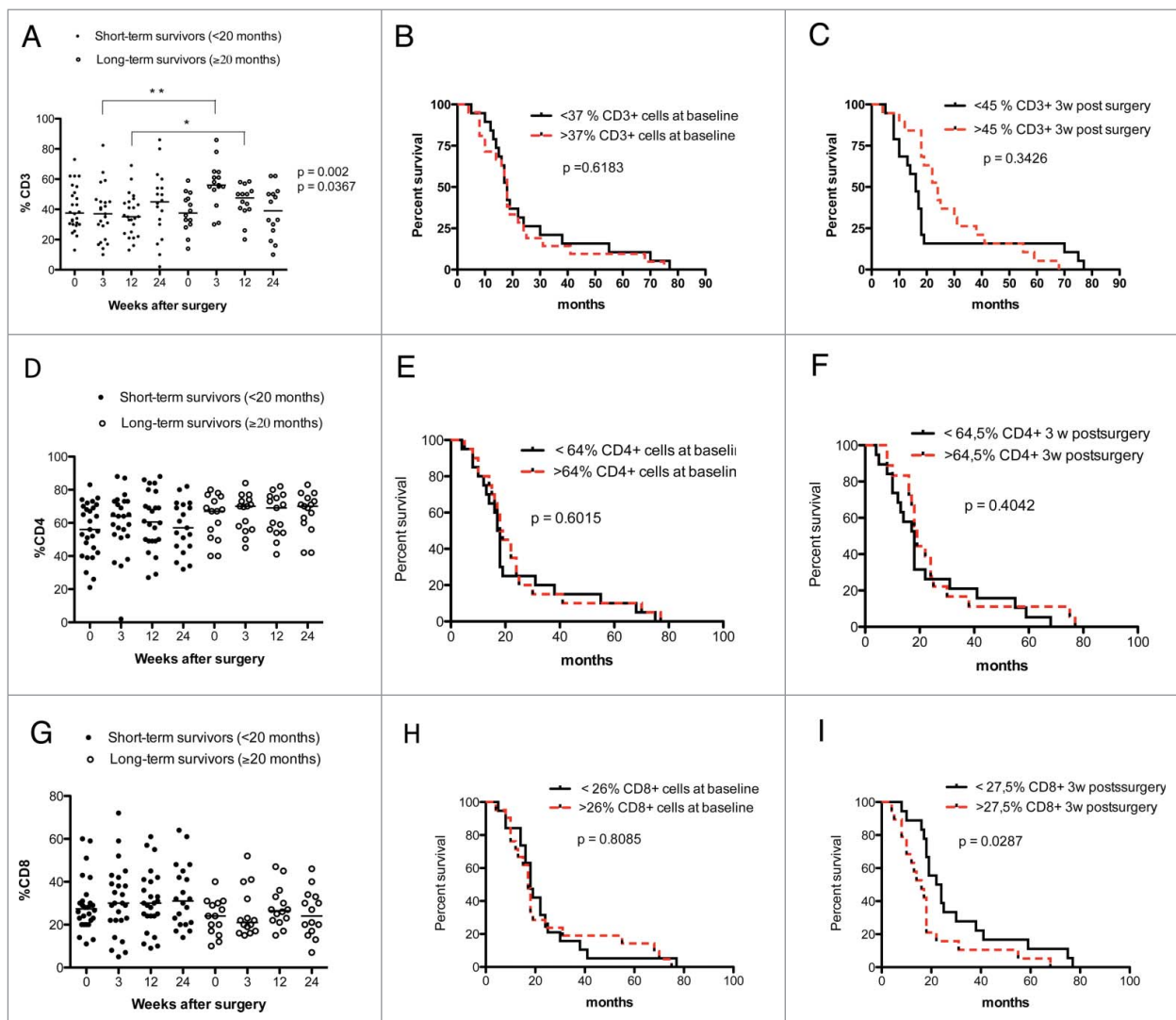


Figure 3. GBM patients with longer survival time (≥ 20 mo) have higher levels of CD3⁺ but not CD4⁺ and CD8⁺ T cells. Survival was analyzed with respect to CD3, CD4⁺, and CD8⁺ expression (median values were used as a cut-off for Kaplan–Meier graphs). (A) Patients who survived longer had higher levels of CD3⁺ T cells at 3 and 12 weeks after surgery. (B and C) Survival did not differ in patients with high vs. low levels of CD3⁺ cells before and 3 weeks after surgery (D and G). No significant differences in CD4⁺ and CD8⁺ T-cell levels were observed in GBM patients with long-term survival. (E–H) Survival did not differ in patients with high vs. low levels of CD4⁺ cells at baseline and 3 weeks after surgery and low levels CD8⁺ cells at baseline. (I) GBM patients with higher levels of CD8⁺ T cells have shorter survival than patients with lower levels of these cells.

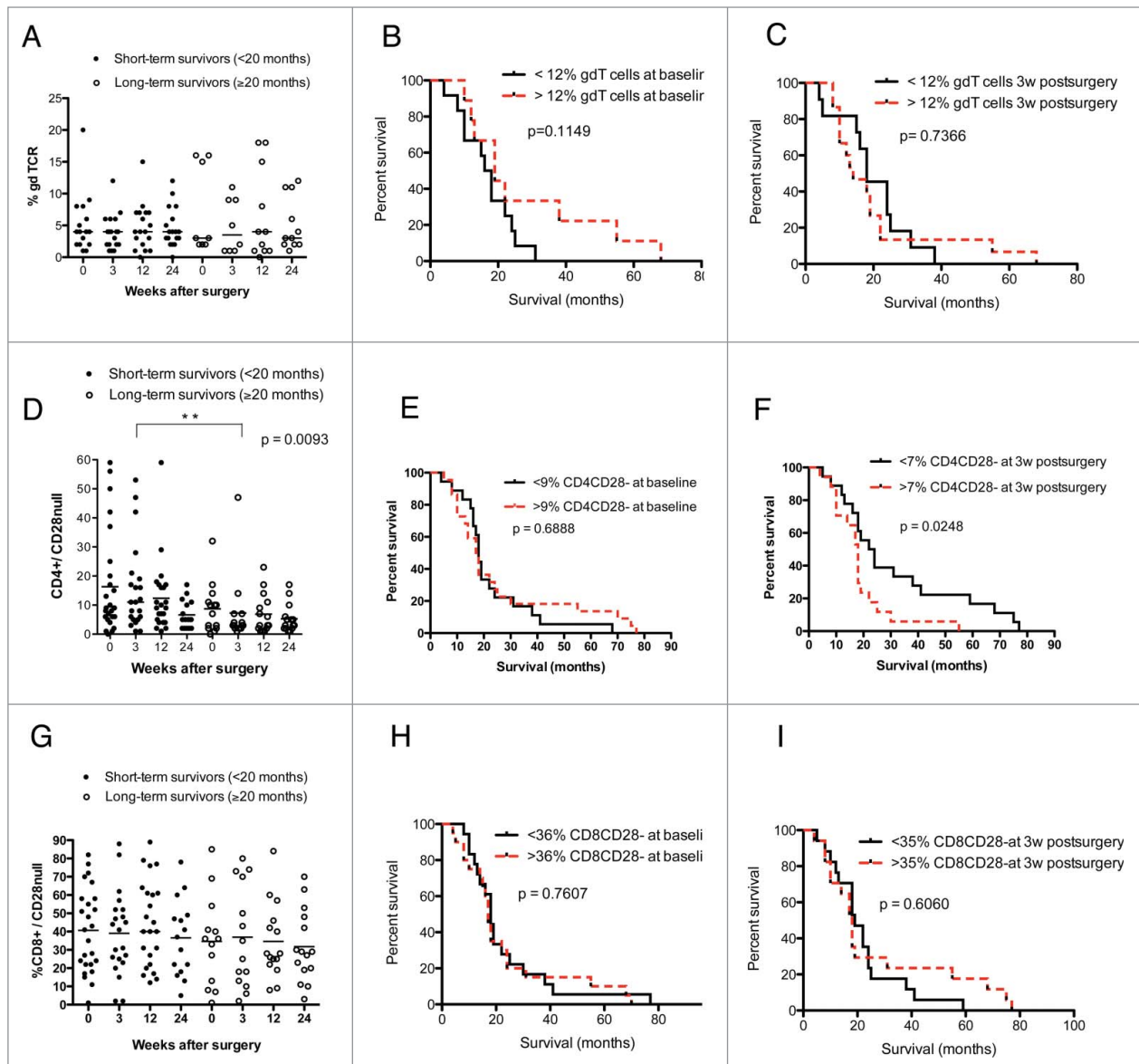


Figure 4. GBM patients with shorter overall survival have higher levels of CD4⁺CD28⁻ cells. (Median values were used as a cut-off for Kaplan–Meier analysis). Long-term GBM survivors had lower levels of CD4⁺CD28⁻ at 3 weeks after surgery. (A) $\gamma\delta$ T-cell levels did not differ in GBM patients with longer or shorter survival. (B and C) Survival did not differ in patients with high vs. low levels of $\gamma\delta$ T cells before and at 3 weeks after surgery. (D) Among GBM patients, short-term survivors had significantly higher levels CD4⁺CD28⁻ cells 3 weeks after surgery than long-term survivors. (E) Survival did not differ in patients with high vs. low levels of CD4⁺CD28⁻ T cell at baseline. (F) GBM patients with higher levels of CD4⁺CD28⁻ cells have shorter overall survival than those with lower levels of these T cells. (G) CD8⁺CD28⁻ T-cell levels did not differ in GBM patients with longer vs. shorter survival. (H and I) Survival did not differ in patients with high vs. low levels of CD8⁺CD28⁻ cells at baseline and 3 weeks after surgery.

overall survival was longer in GBM patients with lower levels of CD57⁺CD28⁻ CD4⁺ T cells than in those with higher levels (24 vs. 17.5 mo) (Fig. 5I). Loss of CD28 expression correlated with increased expression of CD57 ($r^2 = 0.40$, $p = 0.0002$) (Fig. 5J).

The levels of CD4⁺ and CD8⁺ T cells expressing CD57 with or without CD28 expression and the survival rate did not differ in short-term and long-term survivors (Fig. 6A–I). Furthermore, the number and percentage of CD25⁺ CD4⁺ T cells were unrelated to survival among short-term or long-term survivors (Fig. 7A–C).

Neither corticosteroid treatment nor antiviral treatment affects T-cell subsets

Steroids have immunological effects on numerous immune cell types, and most GBM patients receive corticosteroids before and for a short time after surgery. In the VIGAS cohort, all patients had short-term corticosteroid treatment. A high dose was given before and directly after surgery; postoperatively, the corticosteroid dose was rapidly decreased to the minimum effective dose to control cerebral edema. We therefore analyzed whether T-cell subsets were affected by steroid intake longer than

3 weeks. T-cell subsets did not differ in the patients with longer vs. shorter steroid treatment in the cohorts with significant T cell findings (Table 3). Since VIGAS patients were given Valcyte or placebo during the study, we next examined whether antiviral

treatment affected T-cell phenotypes. Antiviral treatment did not influence T cells phenotypes over time in the cohorts with significant T cell findings. (Table 4). We also analyzed total white blood cell counts (WBCs) in these patients. As expected,

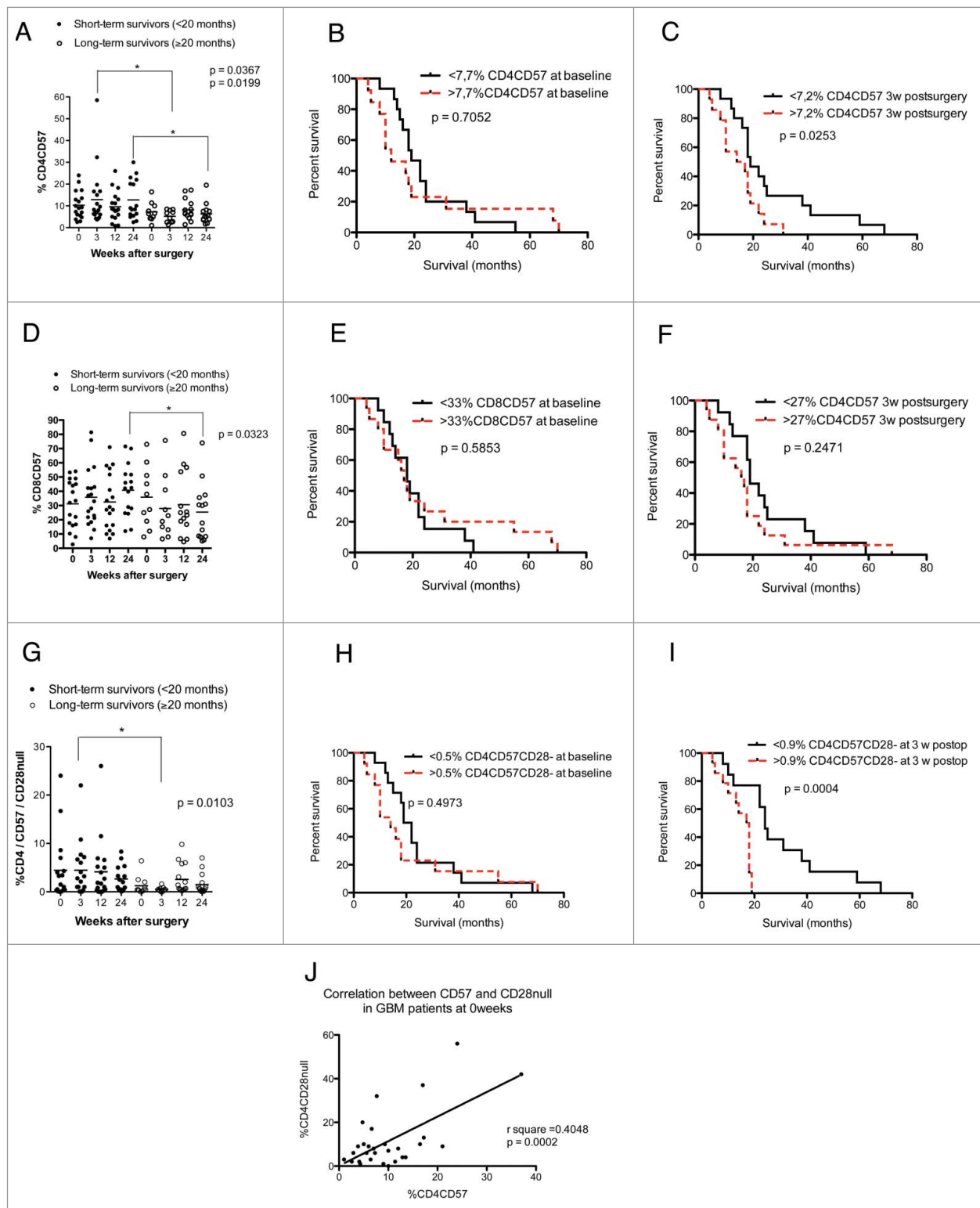


Figure 5. For figure legend, see next page.

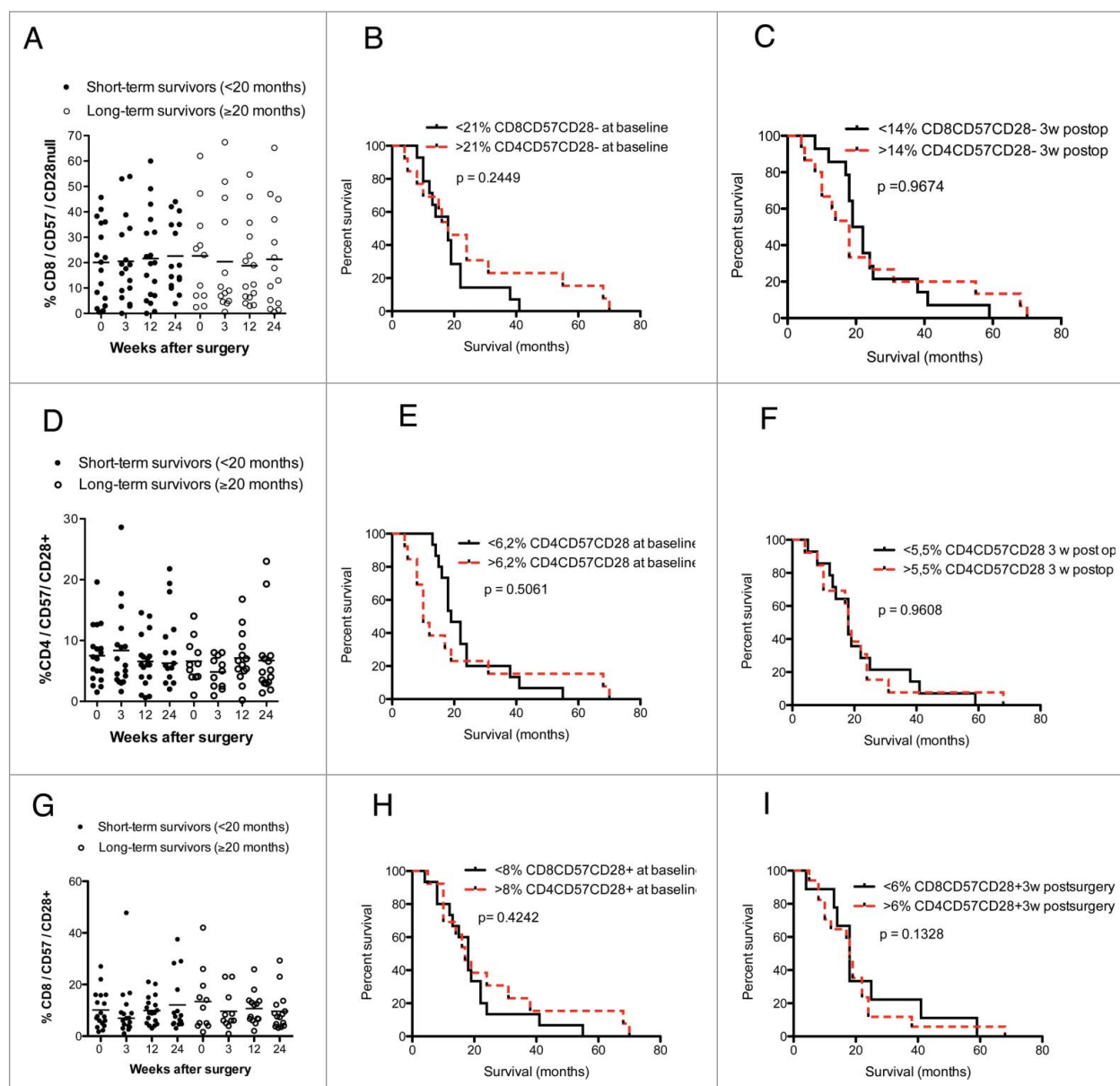


Figure 6. In GBM patients, levels of CD8⁺CD57⁺CD28⁻, CD4⁺CD57⁺CD28⁺ and CD8⁺CD57⁺CD28⁺ T cells do not correlate with survival (median values were used as a cut-off for Kaplan–Meier analysis) and did not differ in long-term vs. short-term survivors. **(A–C)** Survival did not differ in patients with high vs. low levels of CD8⁺CD57⁺CD28⁻ T-cells as well as in short-term vs. long-term GBM survivors. **(D–I)** Survival did not differ in patients with high vs. low CD4⁺CD57⁺CD28⁺ or CD8⁺CD57⁺CD28⁺ T-cell levels as well as in short-term vs. long-term GBM survivors.

Figure 5 (See previous page). Higher levels of CD4⁺CD57⁺ and CD4⁺CD57⁺CD28⁻ T cells are associated with poor survival in GBM patients using median values as a cut-off for Kaplan–Meier analysis. Direct correlation between expression of CD57 and loss of CD28 on T cells was observed. **(A)** GBM patients with short-term survival time had significantly higher levels of CD4⁺CD57⁺ cells at 3 and 24 weeks after surgery than long-term survivors. **(B)** Survival did not differ in patients with high vs. low levels of CD4⁺CD57⁺ T cell at baseline. **(C)** GBM patients with higher levels of CD4⁺CD57⁺ T cells have shorter overall survival than those with lower levels of these cells. **(D)** Among GBM patients, short-term survivors had significantly higher levels of CD8⁺CD57⁺ cells at 24 weeks after surgery than long-term survivors. **(E and F)** Survival did not differ in patients with high vs. low levels of CD8⁺CD57⁺ cells before and 3 weeks after surgery. **(G)** GBM patients with shorter survival times (<20 mo) had significantly higher levels CD4⁺CD57⁺CD28⁻ cells 3 weeks after surgery than those with long-term survival. **(H)** Survival did not differ in patients with high vs. low levels of CD4⁺CD57⁺CD28⁻ cells at baseline. **(I)** GBM patients with higher levels of CD4⁺CD57⁺CD28⁻ cells have shorter overall survival than those with lower levels of these cells. **(J)** Linear regression analysis of direct correlation between expression of CD57 and loss of CD28 on CD4⁺ cells.

WBCs were reduced in patients treated with valganciclovir; however, this change was not significant (Fig. S1). This drug affects the number of leukocytes and platelets, but does not affect T-cell numbers according to the manufacturer and our clinical experience. Thus, we conclude that neither corticosteroid nor valganciclovir treatment significantly affected WBCs during the study phase.

Discussion

In this study, we investigated the frequency of T-cell subsets in the blood and their association with survival of GBM patients. We found that GBM patients had lower levels of CD3⁺ T cells than controls, as in other reports,³⁴ and lower levels of CD4⁺CD25⁺T cells, but a trend toward higher numbers of Foxp3-positive T cells. GBM patients also had increased levels of CD4⁺ T cells that expressed CD57 but had lost CD28 expression, which may indicate increased proliferative instability and senescence. Survival times were shorter among GBM patients with lower levels of CD3⁺ T cells, higher levels of CD28⁻ T cells, or higher levels of CD57⁺ T cells. These subsets are highly associated with HCMV infection, which was present in all the GBM patients and can cause immunosuppression and immunosenescence.³⁵ Furthermore, $\gamma\delta$ T cells were more abundant in GBM patients than controls. These cells have potent antitumor effects, and a subset of them is highly associated with HCMV infection.³¹ Thus, our results indicate that GBM patients have greater immunosuppression and immunosenescence, possibly reflected by higher levels of HCMV activity in these patients than controls. Both phenotypes were associated with a poor outcome.

For unknown reasons, GBM patients exhibit multiple immunosuppressive features affecting different cellular processes of the host. HCMV proteins are frequently detected in GBMs, and this virus induces immunosuppression both directly and indirectly. Earlier studies implied that GBM patients have an impaired cell-mediated immune response (reviewed in ref.³³). Likewise, HCMV-infected patients show clear signs of

immunosuppression, impaired cell-mediated immune responses, and higher rates of bacterial and fungal infections.³⁶ Biologically, HCMV suppresses antigen presentation, impairs the activation of T cells, natural killer cells, and B cells, and has many other immunomodulatory functions.^{35,37} The virus enhances production of the immunosuppressive factors TGF- β and IL-10 and can produce a functional virus-encoded IL-10 homolog (cmvIL-10) during active or latent infection.³⁸ HCMVIL-10, produced by CD133⁺ cells in the tumor of GBM patients, has been suggested to mediate immunosuppressive effects on tumor macrophages.³⁹

In patients with GBM or other cancers, HCMV proteins are readily detected in tumor tissue by immunohistochemistry and western blot; however, only low levels of HCMV DNA and RNA are detectable, and the virus does not seem to replicate its DNA in tumors. These observations sparked controversy about whether the virus is truly present in GBMs. However, GBM lysates used for dendritic cell vaccination result in expansion of HCMV-specific T cells in patients, and HCMV pp65-specific T cells recognize and kill autologous GBM cells.⁴⁰⁻⁴² These observations provide indisputable immunological evidence that GBM cells express HCMV peptides. HCMV peptides expressed in GBM cells may thus in theory provide a chronic antigen stimulation, which may trigger expansion of CD4⁺T cells that express CD57 and later lose CD28 expression; this T cell phenotype was highly associated with HCMV infection.

Since CD28 is important in T-cell activation and mediates proliferative stability, loss of its expression on CD4⁺ T cells in GBM patients may severely impair immune responses. CD28 has a crucial role during T-cell activation: it mediates induced cytokine production (IL-2), procession and generation of cytotoxic T lymphocytes, activation of B cells, and promotion of cell proliferation.⁴³ CD28⁻ T cells are therefore believed to inhibit the immune response through various suppressive functions,⁴⁴ and lack of this co-stimulatory signal reduces the immune response to pathogens.⁴⁵ Loss of CD28 expression on T cells is also one of the most consistent biological indicators of human immune senescence, and the frequency of CD28⁻ T cells predicts immune incompetence in the elderly.⁴⁶ Interestingly,

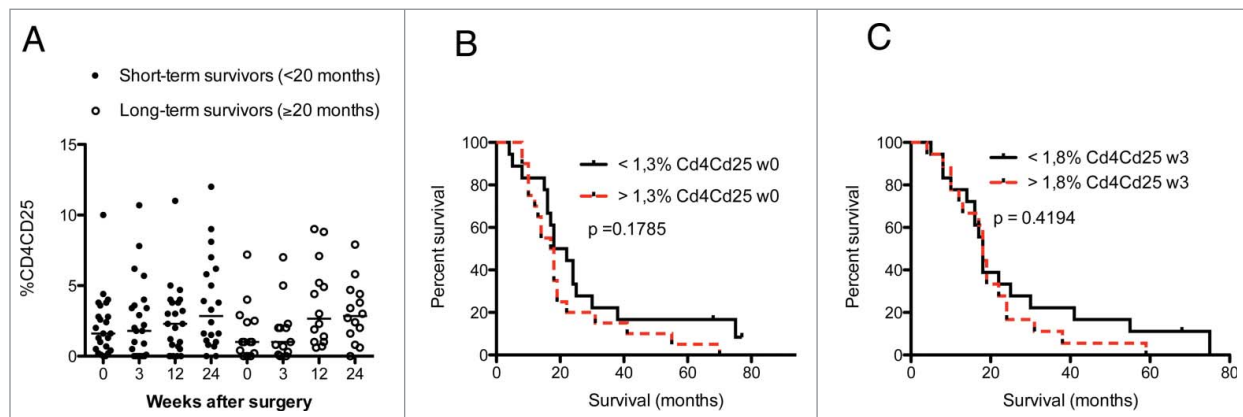


Figure 7. (A–C) Levels of CD4⁺CD25⁺ T cells did not correlate with GBM patient survival.

Table 3. Linear regression analysis of patient cohorts with steroid treatment longer than 3 weeks versus shorter than 3 weeks post surgery

CD3 cells	Regression coefficient	p value
0 weeks	-0.2345511	0.1099
3 weeks	-0.4374638	0.1845
12 weeks	-0.3675055	0.2356
24 weeks	0.1472976	0.6469
CD4⁺ cells		
0 weeks	0.2300614	0.05
3 weeks	-0.0851683	0.4607
12 weeks	0.0847497	0.4321
24 weeks	-0.0672685	0.5645
CD8⁺ cells		
0 weeks	-0.1520693	0.3401
3 weeks	0.077754	0.736
12 weeks	-0.0734131	0.6772
24 weeks	-0.1384975	0.5219
γδ T cells		
0 weeks	0.1478178	0.652
3 weeks	-0.0623596	0.8675
12 weeks	0.3344783	0.2845
24 weeks	0.2441888	0.4758
CD4/CD57 cells		
0 weeks	0.1852333	0.5615
3 weeks	0.0553747	0.88
12 weeks	0.4497213	0.1782
24 weeks	-0.182609	0.5779
CD8/CD57 cells		
0 weeks	-0.0922303	0.7747
3 weeks	0.0430214	0.8816
12 weeks	-0.0638975	0.8847
24 weeks	-0.1975558	0.5477
CD4/CD28null cells		
0 weeks	0.4659717	0.2652
3 weeks	-0.5119411	0.2396
12 weeks	0.4751103	0.251
24 weeks	-0.5901584	0.0863
CD8/CD28null cells		
0 weeks	0.3325712	0.3999
3 weeks	-0.8171889	0.0453
12 weeks	0.0879745	0.7117
24 weeks	-0.8251942	0.0114
CD4/ CD57/CD28null cells		
0 weeks	-0.1180299	0.8643
3 weeks	0.9674673	0.1709
12 weeks	0.0219018	0.9694
24 weeks	0.4697549	0.5238
CD8/CD57/CD28null cells		
0 weeks	-0.0862766	0.8732
3 weeks	-0.3726477	0.3934
12 weeks	0.0554057	0.8842
24 weeks	0.7204616	0.1374
CD4/CD25 cells		
0 weeks	-0.6599094	0.1663
3 weeks	0.2641433	0.6468
12 weeks	0.2000327	0.6008
24 weeks	0.3657016	0.2899
CD8/CD25 cells		
0 weeks	-0.9189226	0.0857
3 weeks	0.4819171	0.356
12 weeks	0.3546721	0.6073
24 weeks	0.1461828	0.7551

Table 4. Linear regression analysis comparing patient cohorts with Valcyte treatment versus placebo

CD 3 cells	Regression coefficient	p value
0 weeks	-0.1510336	0.2173
3 weeks	0.139136	0.4
12 weeks	-0.1315498	0.3092
24 weeks	-0.0630347	0.8129
CD 4⁺ cells		
0 weeks	-0.0385455	0.6983
3 weeks	-0.0523832	0.5933
12 weeks	0.0612635	0.5189
24 weeks	-0.0246153	0.7993
CD 8⁺ cells		
0 weeks	0.1092555	0.4089
3 weeks	0.3792077	0.0462
12 weeks	0.00146	0.9925
24 weeks	0.205801	0.2462
GD T cells		
0 weeks	-0.4969697	0.0891
3 weeks	-0.3266768	0.3045
12 weeks	0.0294959	0.9175
24 weeks	-0.0437197	0.8812
CD 4/CD 57 cells		
0 weeks	-0.2710945	0.3597
3 weeks	-0.1967002	0.5725
12 weeks	0.2718693	0.3706
24 weeks	-0.4021137	0.1652
CD 8/CD 57 cells		
0 weeks	0.1518069	0.6128
3 weeks	-0.1124162	0.6826
12 weeks	-0.0695953	0.8608
24 weeks	-0.2129092	0.4692
CD 4/CD 28null cells		
0 weeks	0.312091	0.3743
3 weeks	-0.5047389	0.1493
12 weeks	-0.0089941	0.9803
24 weeks	-0.4274857	0.1648
CD 8/CD 28null cells		
0 weeks	0.1306234	0.6972
3 weeks	0.0858196	0.8044
12 weeks	-0.0895841	0.6666
24 weeks	-0.4305437	0.1522
CD 4/ CD57/CD 28null cells		
0 weeks	1.110387	0.0673
3 weeks	1.203756	0.058
12 weeks	0.1420544	0.7847
24 weeks	0.5643537	0.3595
CD 8/ CD57/CD 28null cells		
0 weeks	-0.0155809	0.9756
3 weeks	-0.6119404	0.137
12 weeks	0.3224287	0.3278
24 weeks	-0.6235189	0.1541
CD 4/CD 25 cells		
0 weeks	-0.21233	0.6179
3 weeks	0.7711318	0.0947
12 weeks	0.4433576	0.1565
24 weeks	0.1041078	0.7244
CD 8/CD 25 cells		
0 weeks	0.1311385	0.7944
3 weeks	0.2672356	0.5469
12 weeks	0.6383185	0.2732
24 weeks	0.0276734	0.9453

CD4⁺ T cells lacking CD28 expression exist only in HCMV-seropositive individuals, and HCMV activity is strongly correlated with enhanced expansion of CD4⁺CD28⁻ T cells in humans,²² a phenomenon related to immunosenescence.^{14,23} Expansion of this T-cell subset is believed to be dependent on chronic antigen stimulation, and most of these T cells recognize HCMV peptides.⁴⁷ Nevertheless, although they have limited proliferative capacity, CD28⁻ T cells are functionally active and can produce both IFN γ and TNF- α .⁴⁸ These cells are abundant in patients with chronic inflammatory diseases.¹⁴

CD4⁺ T cells that had lost CD28 expression were first identified as a unique cell type that expressed CD57 in HCMV-infected patients; they were later shown to have lost expression of CD28, CD2, or both. Patients with a history of infection with HCMV, Epstein–Barr virus, or HIV are today known to have an increased frequency of CD57⁺ T cells.⁴⁹ T cells are not considered to express CD57 at birth; CD57 expression increases with age and is found in 20–30% of young adults and 50–60% of the elderly (above 80 years old).²⁷ Levels of CD4⁺, CD8⁺, and CD57⁺ cells are also frequently increased in many cancers.^{26,27,50} Expanded subsets of T cells expressing CD57 but not CD28 were highly associated with survival in our GBM patient cohort. All these patients were HCMV positive, as this was an inclusion criterion for the study. Since CD28⁻ T cells are expanded in HCMV-infected individuals; the expansion of these cells may reflect greater activity of HCMV in GBM patients. These observations would confirm our previous report of the prognostic value of HCMV activity in the GBMs⁴ and explain why control of HCMV infection with antiviral treatment improves the survival of GBM patients.⁴ Several previous studies also found increased levels of CD28⁻ T cells in various malignancies (reviewed in ref.¹⁵). Expansion of late-differentiated T cells was associated with the tumor in patients with head and neck cancer; expanded CD8⁺CD28⁻ T-cell populations returned to normal levels after tumor removal.⁵¹ Taken together, our observations suggest that HCMV affects GBM progression and that expansion of certain T-cell phenotypes reflects viral activity in GBM patients. We provide further indirect evidence that higher levels of viral activity in GBMs is associated with poor survival, although it cannot be excluded that the functional immune status may be linked directly to survival.

In summary, we found that GBM patients have lower levels of CD3 T cells and CD25 T cells than healthy controls, as well as higher levels of $\gamma\delta$ T cells, CD4⁺CD28⁻ T cells, and CD4⁺CD57⁺ and CD4⁺CD57⁺CD28⁺ T cells. GBM patients with higher grade of immunosuppression — and thus lower levels of CD3 T cells and higher levels of CD28⁻ T cells, CD57⁺ T cells, and CD28⁻CD57⁺ T cells — had significantly shorter overall survival. As CD28 loss and CD57 expression are strongly associated with HCMV infection, our findings suggest that GBM patients show signs of immunosenescence that may be linked to HCMV infection and predict poor survival. Extended antiviral treatment to control HCMV infection markedly improves the survival of patients with GBM.⁴ Our results should encourage additional studies to understand the direct biological link between HCMV and immunosenescence and its impact in GBM patients.

Materials and Methods

Patient data

Peripheral blood mononuclear cells (PBMCs) were obtained from 42 patients (13 women and 29 men; mean age 60 \pm 10 years) with GBM treated surgically at Karolinska University Hospital between 20 December 2006 and 3 June 2008. All 42 patients had World Health Organization grade IV GBM and received standard treatment with concomitant radio- and chemotherapy. PBMCs were obtained before and 3, 12, and 24 weeks after surgery. PBMCs were also obtained from 26 healthy controls (18 women and 8 men; mean age 55 \pm 5 years). The control cohort included age-matched, healthy individuals without a history of cancer. These GBM patients were also part of a randomized, double-blinded, placebo-controlled phase I study to evaluate the efficacy and safety of the antiviral drug valganciclovir for 24 weeks in combination with temozolomide and/or radiation therapy in patients with HCMV-positive GBMs (VIGAS study). HCMV positivity was determined by immunohistochemistry and was an inclusion criterion. The study was registered at the Swedish Medical Agency (Eudra number 2006-002022-29) and at ClinicalTrials.gov and was approved by the Karolinska ethics committee (2006/755-31).

Flow cytometry analysis

PBMCs were isolated from blood samples with Lymphoprep (Medinor, Cat. No. 30066.03) according to the manufacturers instructions. Fresh PBMCs were stained with the following antibodies as recommended by the manufacturers: anti-CD3-CY, anti-CD8-Pacific Blue (both from Dako, Cat. Nos. CA696 and PB984, respectively) anti-CD4-PerCP, anti-CD28-PE (both from BD Biosciences, Cat. Nos. 345770 and 556622, respectively), anti-CD25-PE/Cy7, anti-FoxP3 (Biolegend, Cat. No. 320216) and anti-CD57-APC (both from Biolegend, Cat. Nos. 302612 and 322314, respectively), anti-TCR $\gamma\delta$ -PE (Beckman Coulter, Cat. No. pnim1418u). Cells were analyzed by fluorescence-activated cell sorting on a Cyan (Beckman Coulter) with Summit v4.3.02 software.⁵² Cells were initially gated as CD3⁺. This population was thereafter separately gated for CD4⁺ and CD8⁺. The CD4⁺ and CD8⁺ cells were further gated according to expression of CD25, CD 28, and CD57 and $\gamma\delta$ expression.

Statistical analysis

Protein expression levels were analyzed by the nonparametric Mann–Whitney *U*-test, yielding two-tailed *p* values. Survival was analyzed with descriptive statistic and Kaplan–Meier survival curves with median values of protein expression as a cut-off. Patient survival was analyzed by log-rank test. Long-term survivors were defined as patients who lived \geq 20 mo and short-term survivors as those who lived <20 mo; all calculations were made on this definition. The effects of steroid or antiviral therapy on T-cell phenotypes were determined by linear regression analysis, adjusting for the patient age using STATA 11.2 (StataCorp LP) and Prism 5, which were also used to correlate expression of

CD57 and loss of CD28 expression in CD4⁺ T cells. All differences were considered significant at the 5% level.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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