J Neurooncol (2013) 115:353–363
DOI 10.1007/s11060-013-1236-x

LABORATORY INVESTIGATION

Circulating T regulatory cells migration and phenotype in glioblastoma patients: an in vitro study

Chiara Vasco · Alessandra Canazza · Ambra Rizzo · Adele Mossa · Elena Corsini · Antonio Silvani · Laura Fariselli · Andrea Salmaggi · Emilio Ciusani

Received: 26 March 2013 / Accepted: 25 August 2013 / Published online: 5 September 2013

Abstract Glioblastoma multiforme (GBM) is the most aggressive primary human brain tumor. The relatively high amount of T regulatory lymphocytes present in the tumor, contributes to the establishment of an immunosuppressive microenvironment. Samples of peripheral blood were collected from GBM patients and healthy controls and a purified population of Treg ($CD4^+/CD25^{bright}$) was isolated using flow cytometric cell sorting. Treg migrating capacities toward human glioma cell line conditioned medium were evaluated through an in vitro migration test. Our data show that supernatants collected from GBM cell lines were more attractant to Treg when compared to complete standard medium. The addition of an anti-CCL2 antibody to conditioned medium decreased conditioned medium-depending Treg migration, suggesting that CCL2 (also known as Monocyte Chemoattractant Protein, MCP-1) is implicated in the process. The number of circulating $CD4^+/\mu L$ or Treg/μL was similar in GBM patients and controls. Specific Treg markers (FOXP3; CD127; Helios; GITR; CTLA4; CD95; CCR2, CCR4; CCR7) were screened in peripheral blood and no differences could be detected between the two populations. These data confirm that the tumor microenvironment is attractive to Treg, which tend to migrate toward the tumor region changing the immunological response. Though we provide evidence that CCL2 is implicated in Treg migration, other factors are needed as well to provide such effect.

Keywords Glioblastoma multiforme · Treg · CCL2 · Irradiation

Chiara Vasco and Alessandra Canazza have equally contributed to this work.

C. Vasco · A. Canazza · A. Rizzo · E. Corsini · E. Ciusani (✉)
Laboratory of Clinical Pathology and Medical Genetics, Foundation IRCCS Neurological Institute C. Besta, Via Celoria, 11, 20133 Milan, Italy
e-mail: emilio.ciusani@istituto-besta.it

Present Address:
A. Canazza
Cellular Neurobiology Laboratory, Cerebrovascular Diseases Unit, Foundation IRCCS Neurological Institute C. Besta, Via Celoria, 11, 20133 Milan, Italy

A. Mossa · A. Silvani
Department of Neuroncology, Foundation IRCCS Neurological Institute C. Besta, Via Celoria, 11, 20133 Milan, Italy

L. Fariselli
Department of Radiotherapy, Foundation IRCCS Neurological Institute C. Besta, Via Celoria, 11, 20133 Milan, Italy

A. Salmaggi
Neurologia-Stroke Unit, Manzoni Hospital, Via Dell'Eremo 9/11, 23900 Lecco, Italy

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive cerebral tumor in adults in which treatment options are very poor and recurrence occurs invariably after surgical treatment and radio-chemotherapy [1, 2]. In the last years, immunotherapy has been suggested as a powerful approach to prevent tumor recurrence in a number of neoplasms by eliminating tumor cells while sparing normal surrounding healthy cells [3].

T regulatory lymphocytes (Treg) are a subset of T-cells which play an important role in maintaining peripheral self tolerance and preventing immunopathologies [4]. They can

prevent autoimmunity through the cell surface ligand glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T-lymphocyte antigen (CTLA-4) and through the production of immunosuppressive cytokines. Recently it has been shown that tumor growth produces generation of immunosuppressor T-cells [5–8] which, residing at the tumor site, have been demonstrated to be relevant in progression of systemic diseases in peripheral blood or lymphoid organs [9–11].

The relevance of Treg in the pathogenesis of GBM has been first suggested by histological studies showing that Treg infiltrate more high-grade gliomas than low-grade oligodendrogliomas and brain tissue from non-tumoral CNS [12, 13]. The fact that the extent of Treg infiltration correlates with the WHO grade of the tumor, with GBM showing the largest infiltration, further supports the role for these cells in GBM [14]. Moreover, in a context of reduced number of circulating $CD4^+$ lymphocytes, an increased proportion of Treg has been reported in peripheral blood of patients with malignant glioma [15].

Treg are characterized by $CD4^+/CD25^{bright}/FOXP3^+/CD127^-$ markers and they are overrepresented at the tumor site when compared to the circulating ones [4]. However, the mechanism by which Treg are recruited at the tumor site is still poorly defined. Although glioblastoma-produced soluble factors may attract Treg lymphocytes in the tumor microenvironment, it is not clear whether circulating Treg in GBM patients behave abnormally or display abnormal characteristics [4].

CCL2 has been shown to be secreted by a wide variety of cancer cells in vitro, promoting tumor angiogenesis and monocyte attraction to the tumor microenvironment. Its action is mediated by two receptors CCR2 and CCR4 (C–C chemokine receptor type 2 and 4) [16]. This chemokine is one of the most important one involved in the recruitment of blood cells, especially macrophages, into the tissues [17, 18] and a recent work showed that the extravasation of lymphocytes is mediated by CCL2-expressing astrocytes [19]. Therefore, we investigated the role of CCL2 secreted by GBM cell lines in inducing Treg migration toward tumor microenvironment.

In the present work, we evaluated the effect of glioblastoma-released soluble factors on in vitro migration of purified circulating Treg lymphocytes in a group of GBM patients and healthy controls. GBM cells were in vitro exposed to ionizing radiations, routinely used in GBM treatment, and the effects of this treatment on Treg migration were also evaluated. Moreover, the expression of a number of surface markers has been evaluated in order to compare the circulating Treg phenotype in patients and controls.

Materials and methods

Patients

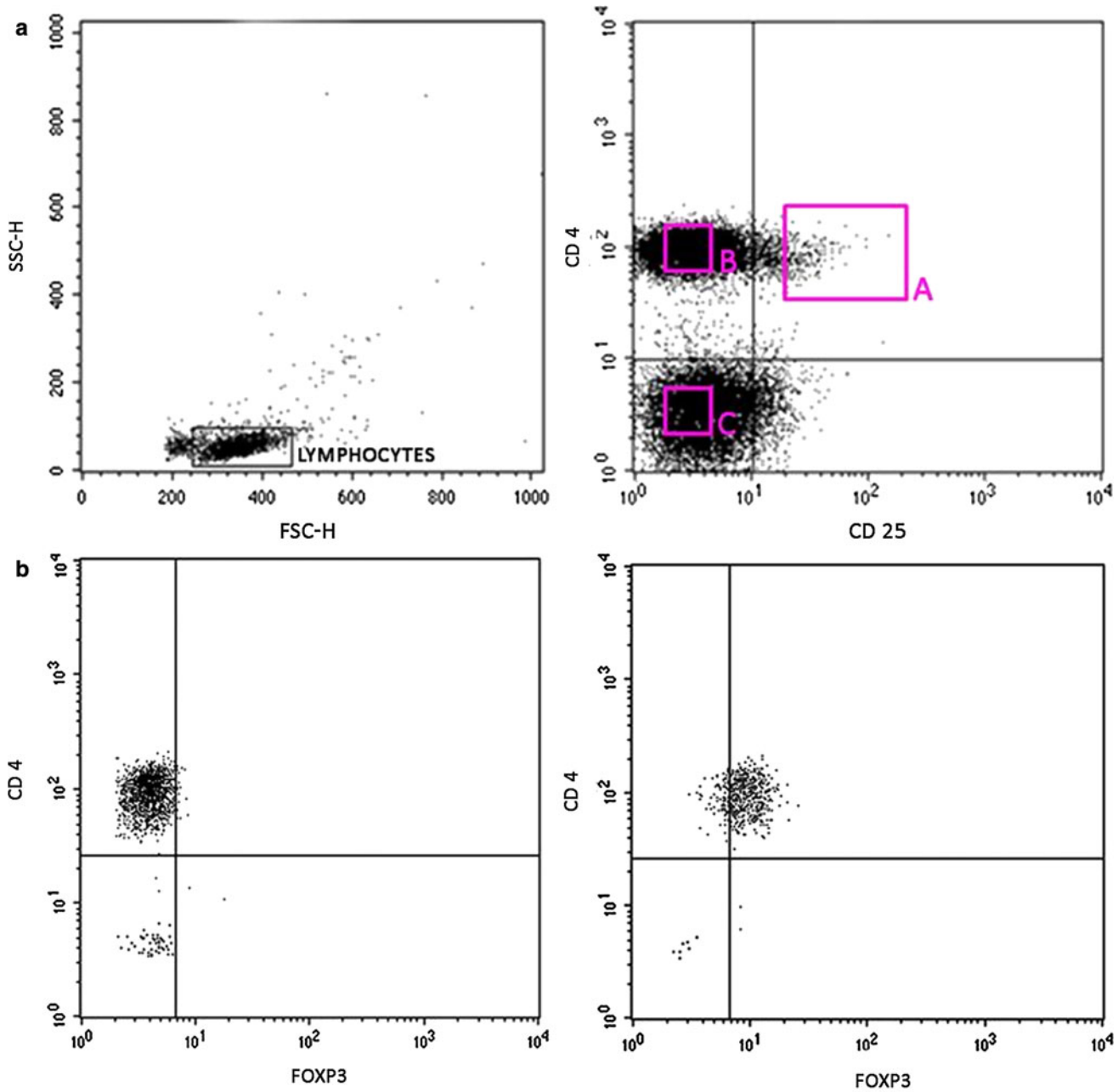
Nineteen patients affected by grade IV astrocytoma (GBM, median age: years, 54; 10 M/9F) followed at our Institution and 16 healthy individuals (CTR, median age: years, 49; 8 M/8F) were included in the study. Eleven GBM patients were sampled at first diagnosis and eight at tumor recurrence. Blood samples from patients with de novo GBM were collected before surgery. The patients with recurrent GBM were included in the study at recurrence, before surgery and were free from any pharmacological treatment except for steroids for at least 3 months. Two of first diagnosis and two of relapsing GBM patients were receiving steroids (dexamethasone 4–8 mg/day). All the eight patients with recurrent GBM had been treated with standard temozolomide therapy according to Stupp's protocol (six cycles of adjuvant temozolomide after concomitant radio/chemotherapy following first surgery) and displayed disease recurrence after 3, 3, 4, 5, 5, 6, 7 and 9 months from the last chemotherapy cycle.

This study was approved by the Ethical Committee of Fondazione IRCCS Istituto Neurologico C. Besta.

Isolation of T regulatory cell population

Peripheral blood mononuclear cells (PBMNC) were isolated from whole blood by density gradient separation technique following manufacturer's instructions (Ficoll Paque™ Plus, GE Healthcare). Briefly, whole blood was gently poured in a falcon containing the Ficoll solution at the bottom and a centrifugation at 600 g for 45 min with no brake was performed. The central ring of lymphocytes was isolated from the three separated phases in the tube, gently collected and washed twice with NaCl 0.9 % solution (400 g for 10 min and 200 g for 10 min respectively). PBMNC were resuspended in RPMI medium (3×10^6 cell/mL) and PE-conjugated anti CD4 and Cy5.5-conjugated anti CD25 antibodies (Beckman Coulter, cat. N. A07750 and BioLegend, cat. N. 302608 respectively) were added for 60 min at 4 °C.

Using cytofluorimetric sorting procedure (FACS Vantage, Becton–Dickinson), Treg ($CD4^+/CD25^{bright}$) cells were selected for migration experiments (Fig. 1a). In addition to Treg, $CD4^-/CD25^-$ and $CD4^+/CD25^-$ (non Treg, NTR, Fig. 1a) were also collected. Purity of Treg population was assessed by FOXP3 staining (eBioscience, cat N. 53-47761); purity greater than 90 % was considered acceptable for migration experiments.



c

	Treg/μL	Treg %	CD4/μL	CD4%
CTR	2.57±0.35	0.32±0.03	892.23±171.46	44.69±2.47
GBM	3.02±0.65	0.37±0.05	806.32±82.28	36.1±3.15

Fig. 1 **a** *Dot plot* showing the typical *FSC* forward scatter and *SSC* side scatter that allowed lymphocytes gating (*left*). On the *right*, a typical flow citometry *dot-plot* used for isolation of Treg and non-Treg (NTR_1 and NTR_2) populations from peripheral blood. Three cell populations were sorted: **a** Treg, $CD4^+CD25^{bright}$; **b** NTR_1, $CD4^+CD25^-$ and **c** NTR_2, $CD4^-CD25^-$. **b** *Dot-plots* showing a typical result of FOXP3 staining performed on $CD4^+CD25^{bright}$ sorted population: isotypic control staining (*left*) and anti-FOXP3 staining (*right*). **c** Absolute number and percentages of circulating Treg ($CD4^+CD25^{bright}FOXP3^+$) and $CD4^+$ lymphocytes in patients (*GBM*) and controls (*CTR*) are reported in the table. No statistically significant differences could be detected between patients and controls. Data are expressed both as percentage of circulating lymphocytes and as absolute number (cells/μL) per microliter of blood

Cell culture

Commercially available GBM cell lines U373, U251, A172, U87 and U138 (American Type Culture Collection, Manassas, VA) and primary cell culture C210BP, C220CF [20] were cultured in standard medium (SM), consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, UK) implemented with 10 % FBS and 1 % penicillin–streptomycin, at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

Conditioned media collection

Conditioned media were obtained from culturing human glioblastoma cell lines in standard medium. After 72 h the medium was collected, debris eliminated and stored at −80 °C. Two types of media were obtained: (1) conditioned medium (CM), in which GBM cells were cultured in standard conditions as mentioned above and (2) irradiated culture medium (irr-CM).

Irradiation culture medium

Cells were seeded 24 h prior treatment at the density of 1.5×10^5 cells in Petri dishes in complete culture medium. After 24 h medium was substituted with serum free medium and irradiation was performed. Cells were irradiated in culture dishes at room temperature at a total dose of 8 Gy, i.e. the maximum tolerated dose delivered in single fraction in recurrent high-grade glioma patients. Conventional LINAC irradiation was performed with a 6 MV X-rays uniform beam produced by a Philips SL 75/5 (Elekta, Stockholm, Sweden) linear accelerator with a dose/rate of 2 Gy/min for field sizes of 20×20 cm^2, gantry = 180° and source-to-surface distance was fixed at 100 cm. The water–solid RW3 were used to assure the 100 % of the dose in the build-up region on cells.

After treatment, medium was replaced with fresh SM and cells were cultured for 72 h. Supernatants were then collected and stored at −80 °C.

CM and irr-CM were successively used in Treg migration assays.

Migration Assay

Purified Treg ($CD4^+/CD25^{bright}$) isolated by FACS sorting were counted and resuspended in RPMI supplemented with FBS 10 %. 10,000 purified Treg in 100 µL of medium were then seeded on each Transwell insert (Costar, 5 µm pore size) for 24 h. The polycarbonate membrane was previously coated with 1:3 diluted Matrigel (BD Biosciences) and positioned in 24 well plates. In each bottom well the appropriate conditioned medium was added (see above); all conditions were performed in duplicate. After 24 h, the Transwell insert was removed, medium from the bottom well was collected and migrated cells were counted using Fuchs-Rosenthal counting chambers. Migration is expressed as migration ratio (MR) and represents the fold increase/decrease compared to the migration measured in SM, i.e. unconditioned complete medium. Migration of cells in SM was used as control, to normalize the results obtained using CM or irr-CM.

In a different set of experiments, the same migration assay was used to evaluate the effect of CCL2 on migration; the following media were tested: (a) CCL2 (1 µg/mL) in SM; (b) CCL2 (1 µg/mL) + anti CCL2 antibody (Abcam, cat N. ab18677, 0.5 µg/mL in SM); (c) anti CCL2 antibody (0.5 µg/mL) in CM. For all migration experiments, appropriate sets of positive and negative controls were performed. Migration was also tested toward DMEM and DMEM + 10 % FBS as negative controls or DMEM + CCL2 (1 µg/mL) and DMEM + CCL2 (1 µg/mL) + anti-CCL2 antibody.

Characterization of peripheral blood Treg

Evaluation of circulating $CD4^+$ and Treg was performed. For the analysis of surface markers, 100 µL of whole blood (K_3EDTA) were triple stained for 20 min at 4 °C with PE- or FITC-conjugated anti-CD4 antibody (Beckman Coulter, cat. N. A07751/A07750), Pe/Cy5-conjugated anti CD25 antibody (BioLegend, cat. N. 302608) and one of the following antibodies: PE-conjugated anti CCR2 (0.25 µg/5×10^5 cells, R&D Systems, cat. N. FAB151P), PE-conjugated anti CCR4 (0.25 µg/5×10^5 cells, R&D Systems, cat. N. FAB1567P), FITC-conjugated anti CCR7 (0.5 µg/5×10^5 cells, eBioscience cat. N. 11-1979-71), PE-conjugated anti CD95 (0.5 µg/5×10^5 cells, BD Pharmingen, cat. N. 555674) and Alexa660-conjugated anti CD127 (0.125 µg/5×10^5 cells, eBioscience, cat. N50-1278-41). After antibody incubation, erythrocytes were lysed with BD FACS™ Lysis Solution (Becton–Dickinson, cat. N. 349202).

For cytoplasmatic markers, a specific Human Regulatory Tcell whole blood staining kit (eBioscience, cat. N. 88-8996-40) was utilized with the following antibodies: PE-conjugated anti CTLA4 (0.5 µg/5×10^5 cells, eBioscience, cat. N. 12-1529-71), AlexaFluor488-conjugated anti GITR (0.25 µg/5×10^5 cells, eBioscience, cat. N. 53-5875-41), FITC-conjugated anti FOXP3 (0.5 µg/5×10^5 cells, eBioscience, cat N. 53-47761) and FITC-conjugated anti Helios (0.25 µg/5×10^5 cells, eBioscience, cat N. 11-9883-80). Appropriate negative controls were performed for each staining using specific isotype controls (BD Simultest cat. N. 342409 or BD Tritest cat. N. 342415, Becton–Dickinson).

Samples were then analyzed by flow cytometry (FACS Vantage, BD Bioscience) using a specific software (CellQuest Pro, BD Bioscience). Treg cells were classified as $CD4^+/CD25^{bright}/FOXP3^+$ and $CD4^+/CD25^{bright}/CD127^-$. $CD4^+$ lymphocytes are expressed by absolute number (cells/μL of blood) using flow count fluorospheres. Similar to $CD4^+$ lymphocytes, Treg were also expressed as percentage of $CD4^+$ or as absolute number (Treg/μL of blood) calculated, in double platform, as: $CD4^+/CD25^{bright}/FOXP3^+$percentage × absolute number of $CD4^+$ lymphocytes × 100.

ELISA *multiple Array*

Following the manufacturer's instructions, multiple ELISA array (eBioscience) was performed to measure the concentration of the following molecules: angiogenesis factors (PDGF, HGF, FGF, VEGF), chemokines (EGF, ITAC, CCL2, SDF1ß), metalloproteases (MMP2 and TIMP2) and interleukins (IL-1, IL-4, IL-5, IL-8, IL-10-IL 12p70, IL-13, IFNγ-TNFα).

Real time PCR

Total RNA was isolated from Treg sorted from peripheral blood of three patients and three healthy controls using RNeasy® Mini Kit (Qiagen, Ge). RNA was quantified by NanoPhotometerTM Pearl (Implen GmbH, Ge) and equal amounts of all samples were reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Triplicate polymerase chain reactions were carried out on an ABI7500 Real Time PCR Detection System (Applied Biosystem) using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). Primers sequences for TGF-β1 (forward 5′-ACCTGCCACAGATCCCCTAT-3′; reverse 5′-CTCCCGGCAAAAGGTAGGAG-3′) and IL-10 (forward: 5′-TACGGCGCTGTCATCGATTT-3′; reverse 5′-TAGAGTCGCCACC-CTGATGT-3′) were designed using Primer3 software; mRNAs were amplified and relative gene expression was evaluated. ΔCt was evaluated normalising TGF-β1 and IL-10 Ct values to Ct of housekeeping gene GAPDH (forward 5′-GTCGGAGTCAAC GGATT-3′; reverse 5′-AAGCTTCCCGTTCTCAG-3′). Data are expressed by a comparative method ($2^{-\Delta\Delta C_t}$), calculating $\Delta\Delta C_t$ as $\Delta C_{t_{(GBM)}} - \Delta C_{t_{(CTR)}}$.

Statistical analysis

Unless specified otherwise, the data are presented as mean ± standard error (SE). Differences in the levels of investigated parameters between patients (GBM) and control group (CTR) were analyzed by Student t test or Spearman's rank correlation coefficient followed by post hoc tests, where necessary. p values lower than 0.05 were considered statistically significant.

Results

$CD4^+$ and Treg in GBM patients

Circulating Treg ($CD4^+/CD25^{bright}/FOXP3^+$) and $CD4^+$ were analyzed in peripheral blood of GBM patients and controls and the results are summarized in Fig. 1c. Although no statistically significant differences could be observed, the percentage and the absolute number of circulating Treg lymphocytes was higher in GBM patients compared to controls. On the other hand, the percentage and the absolute number of $CD45^+/CD4^+$ lymphocytes was slightly lower in patients compared to controls (806 ± 82 and 892 ± 171 cells/μL of blood respectively) (Fig. 1c).

Migration experiments

Conditioned media

In our experimental conditions, purified Treg of patients and healthy controls showed a migration ratio respectively of 3.797 ± 0.545 and 2.879 ± 0.413, indicating that Treg are much more attracted from CM compared to standard medium (MR = 1) (Fig. 2a). These differences in migration were statistically significant for GBM patients ($p = 0.006$) and for healthy controls ($p = 0.009$, Fig. 2a), but no differences were found between the migration ratio of patients and healthy controls. To assess if the increased migration induced by CM was specific for Treg population, we examined the migration of a non-Treg population ($CD4^+/CD25^-$, Fig 1a). A statistically significant decrease in migration of NTR population compared to Treg was observed in both GBM patients and healthy controls (1.904 ± 0.42 vs 3.157 ± 0.8 and 1.399 ± 0.28 vs 2.055 ± 0.41; $p < 0.05$ and $p < 0.043$ respectively). As a matter of fact, the migration ratio of NTR population was not affected by CM nor in patients or in controls (Fig. 2b).

Role of CCL2

To investigate putative soluble molecules produced in vitro by GBM cells and involved in the molecular mechanisms of Treg migration, a panel of 19 soluble factors was screened by a multiple ELISA array in conditioned media derived from two human glioblastoma cell lines (A172 and U87) before and after 8 Gy irradiation. Many of the soluble factors screened were detectable in the conditioned media with some differences between glioblastoma cell lines

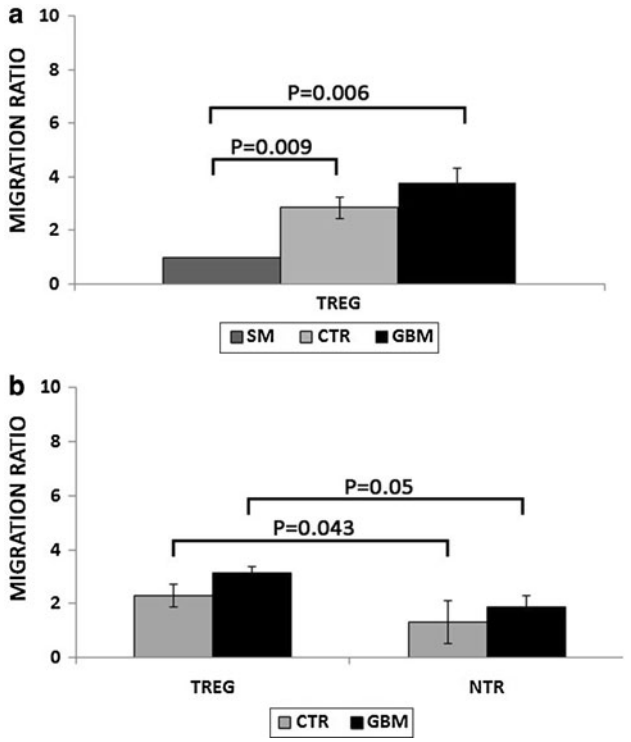


Fig. 2 **a** Mean migration ratios of Treg in patients (*GBM*) and healthy controls (*CTR*). A statistically significant increase in Treg migration toward CM was detected both in patients and controls (student's *t* test). Data are expressed as migration ratio, which represents the fold increase/decrease compared to the migration measured in unconditioned complete medium (*SM*). *Error bars* represent the standard error. **b** CM specifically induce migration of Treg lymphocytes. Migration ratios of purified Treg and NTR was measured as described above (see also methods). Migration toward CM was significantly higher in Treg compared to NTR both in *GBM* patients ($p = 0.05$ Student's *t* test) and *CTR* ($p = 0.043$ Student's *t* test). Data are expressed as mean migration ratio in *GBM* and *CTR* and represent at least two independent experiments run in duplicate. Standard error is represented by *error bars*

(data not shown). Since CCL2 has been described as a key-factor in Treg migration [21] and was abundantly present in conditioned media, its concentration was measured by conventional ELISA in CM and irr-CM (Fig. 3a).

Although not statistically significant, a trend to a correlation between CCL2 concentration in CM and Treg migration ratio was detected in healthy controls ($r^2 = 0.42$; $p = 0.2$, Spearman's rank correlation coefficient) but not in GBM patients (Fig. 3c, d). In agreement with this observation, the addition of an anti-CCL2 antibody to CM to block CCL2 activity, significantly inhibited Treg migration in both patients and controls (Fig. 3b). However, in our experimental conditions, the addition of human recombinant CCL2 (1 μg/mL) to SM did not induce a statistically significant increase in Treg migration (Fig. 3b).

The effect of irradiation on CCL2 production was not unambiguous; indeed, irradiation resulted in a statistically significant increased CCL2 levels in 3 out of the four cultures tested and in decreased levels in one cell line (Fig. 3a). However, when these conditioned media (irr-CM) were used in migration experiments, no correlation could be observed between variations in Treg migration ratio and variations in CCL2 concentration (Fig. 4a). As a matter of fact, overall irr-CM-induced Treg migration showed a trend to decrease compared to CM in both patients and controls (Fig. 4b).

Moreover, the surface expression of the CCL2 receptors, CCR2 and CCR4, has been analyzed in Treg populations. Both CCL2 receptors were highly expressed in Treg lymphocytes with no differences in expression between patients and controls (Fig. 4c–e).

Treg characterization

Expression of CD127 and HELIOS (Treg-related markers) and other functional markers has been evaluated in circulating Treg lymphocytes (Fig. 5). Treg population was also characterized by the screening of surface expression of molecules involved in autoimmunity, like GITR and CTLA-4, an apoptosis mediator CD95 and CCR7, an activator of T and B lymphocytes which can control the migration of memory T cells to secondary lymphoid organs. As expected, most of Treg did not express CD127, while were positive for HELIOS (a marker of naïve Treg) with no differences between patients and controls (Fig. 5). CD95 was also highly expressed, while CTLA4, GITR and CCR7 were expressed in a lower percentage of Treg without statistically significant differences between patients and controls, supporting the idea that Treg lymphocytes in glioblastoma patients are phenotypically similar to that of healthy individuals.

To verify the function of circulating Treg in GBM patients, we analyzed the production of Treg-specific transcripts such as IL-10 and TFG-β1 in purified populations. Compared to healthy controls, GBM patients displayed similar amounts of transcripts for IL10 ($2^{-\Delta\Delta C_t} = 0.86 \pm 0.06$). Whereas, TGF-β1 transcripts were slightly higher in GBM patients compared to controls ($2^{-\Delta\Delta C_t} = 1.64 \pm 0.18$), but no statistically significant differences were detected.

Discussion

Although the role of various factors involved in the immune response on clinical evolution of glioma patients is suggested by a number of studies, the specific role of Treg in GBM progression requires further investigations [13, 14, 22]. In fact, while the study by Kim did not show a predictive value of FOXP3 expression in tumor specimens on progression and survival in a group of 67 GBM patients [22], the study from

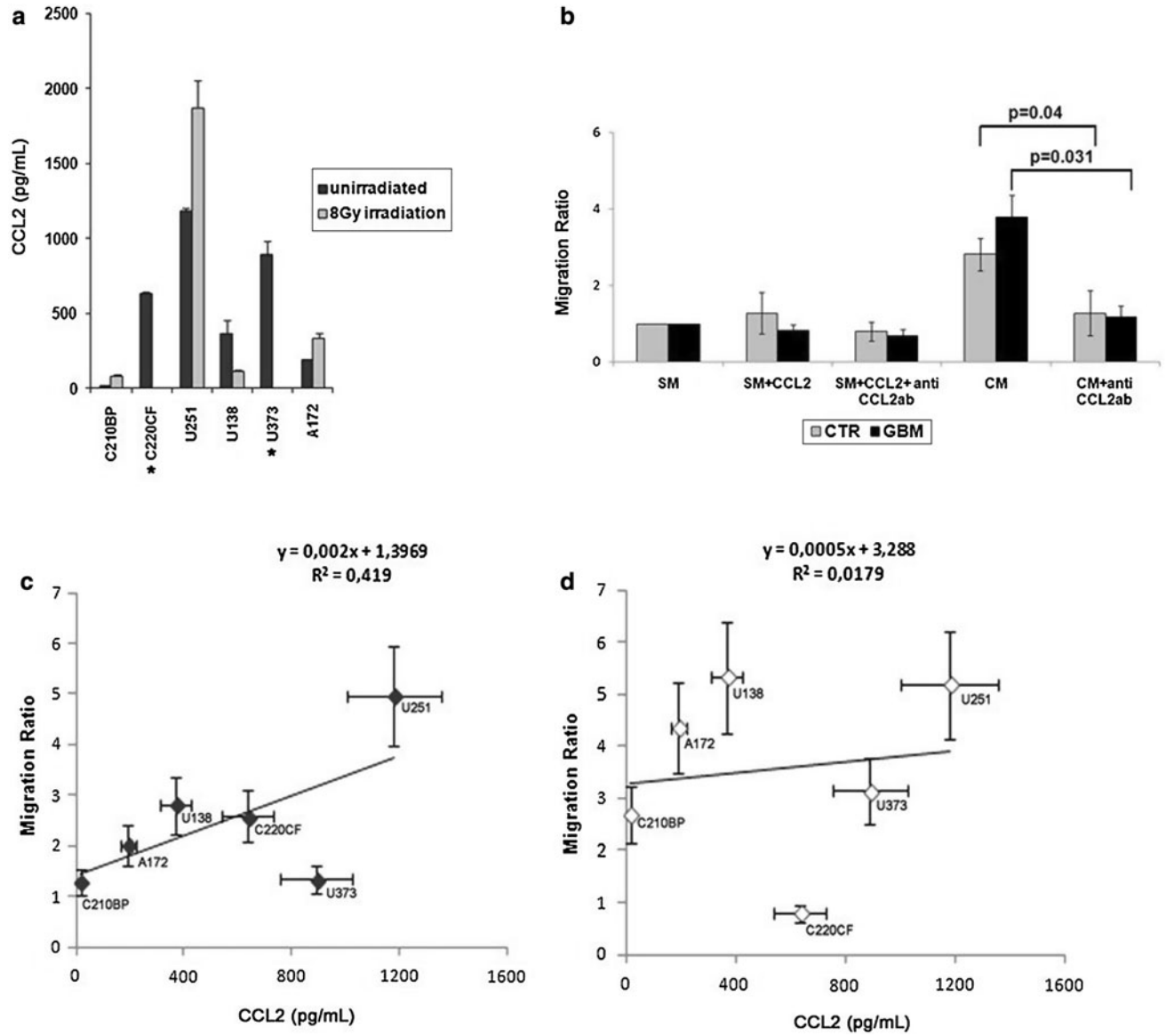


Fig. 3 **a** CCL2 concentration (pg/mL) in various *CM* from glioblastoma-derived primary cultures (*C210BP* and *C220CF*) and *GBM* cell lines (*U251*, *U373* and *A172*). Cells were either untreated or were irradiated (*C210BP*, *U251*, *U138* and *A172*) by a single dose of 8 Gy. Data are expressed as mean concentration of at least two different *CM* for each cell line or primary culture obtained in independent experiments. Standard deviations are represented by *error bars*. * for these cell lines experiments with irradiated *CM* were not performed. **b** Effect of *anti-CCL2* antibodies on Treg migration. Migration of purified Treg from *GBM* patients (*black histograms*) and healthy controls (*gray histograms*) has been measured using both glioblastoma cell lines *CM* conditioned media and, in parallel, the same *CM* added with a blocking *anti-CCL2* antibody (0,5 μg/mL) (*CM* + *anti-CCL2ab*). All data are represented as mean ± standard error (*error bars*). **c**, **d** Correlation between *CCL2* concentration (pg/mL) and migration ratio is represented for *CTR* **c** and *GBM* patients **d**. The mean value (*error bars* represent standard deviations) of all migration ratios obtained using each *CM* (the specific cell line or primary culture is indicated), were correlated with mean *CCL2* concentration measured by ELISA assay in each conditioned medium (see Fig. 2a). Linear correlation has been evaluated and R^2 values are indicated

Jacobs suggested a moderate increase in survival of GBM patients displaying less Treg infiltration, compared to patients with a high proportion of intratumoral Treg [14]. Efforts to improve immunotherapy, supported by the work of Donson 2012, confirm the importance of the expression of local immune-related genes on the survival of patients with high-grade glioma [23].

The data presented here further confirm that in vitro cultured glioblastoma cells release factors able to attract Treg lymphocytes. Overall, these results are in agreement with the long recognized idea that glioblastoma may induce an immunosuppressive microenvironment which contributes to the development and the survival of the tumor [24]. In addition, our data indicate that circulating Treg

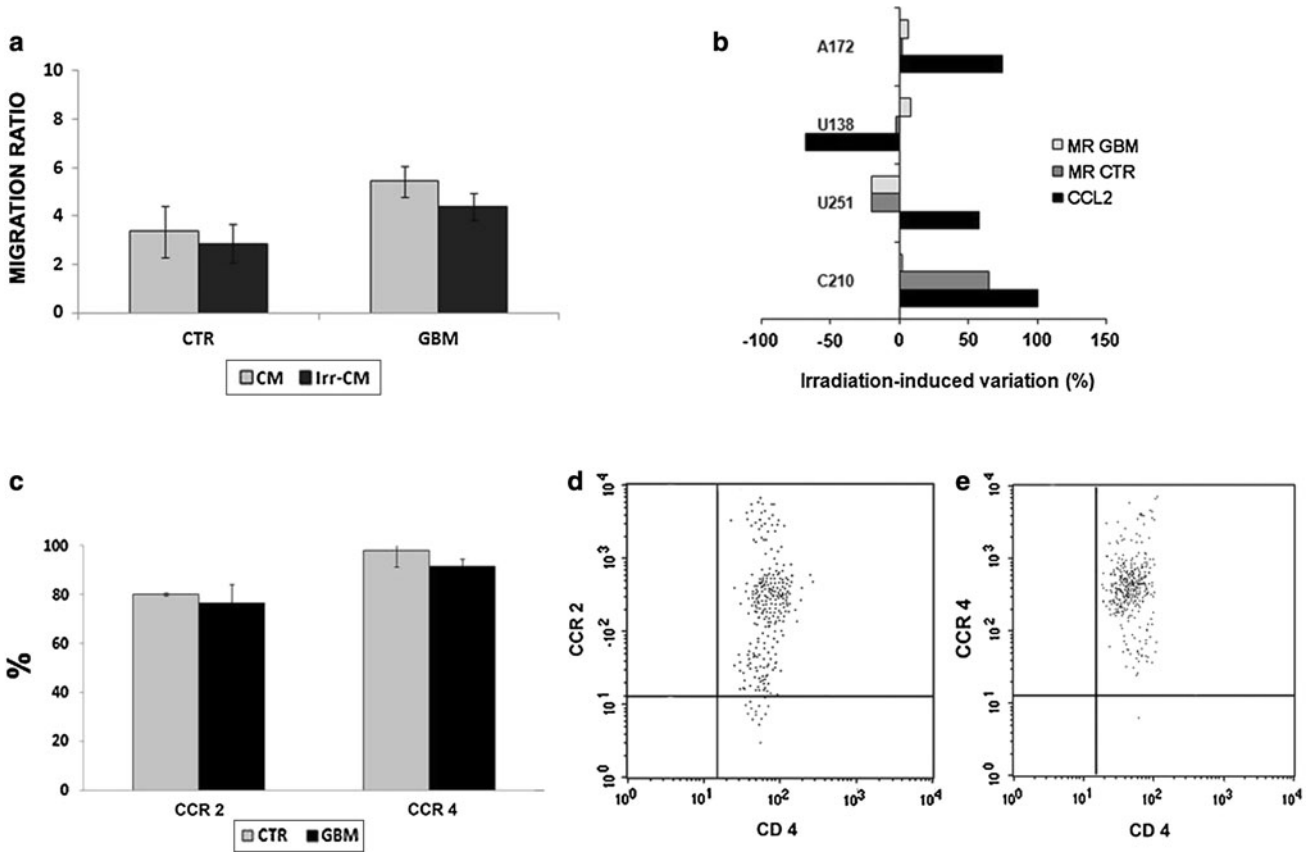


Fig. 4 **a** Effect of irradiation on Treg migration. Conditioned media were used in Treg migration experiments in patients (*GBM*) and controls (*CTR*). No statistically significant differences were detected comparing Treg migration induced by *CM* or *irr-CM*. Data represent media of all experiments (*error bars* represent standard deviations). **b** Correlation between irradiation-induced *CCL2* release and variations in Treg *MR* migration ratio. Irradiation-induced changes in Treg migration seemed to be independent on irradiation-induced variations in *CCL2* release in the conditioned medium. Histograms represent the irradiation-induced variation, in the specific cell lines tested, of Treg migration ratios in *GBM* patients (*empty histograms*) or controls (*gray histograms*) and *CCL2* concentrations in conditioned media (*black histograms*). Data refers to a minimum of two independent experiments and are expressed as mean percentage increase/decrease detected comparing *irr-CM* with *CM*. **c** Expression of *CCL2* receptors in circulating Treg. Percentage of $CD4^{+}CD25^{bright}$ $CCR2^{+}$ and $CD4^{+}CD25^{bright}$ $CCR4^{+}$ lymphocytes in *GBM* patients and in controls. No statistically significant differences were detected. Data are expressed as mean (*error bars* represent standard deviations) of all individuals included in the study. **d** Expression of *CCR2* and *CCR4* as detected by flow cytometry

lymphocytes isolated from patients with GBM display similar characteristics compared to Treg of healthy subject, further suggesting that the migration of Treg is mainly due to tumor-released factors rather than to an altered function of circulating Treg [4, 25].

Indeed, our results showed that the attractant effect exerted by GBM-conditioned media was greater in Treg subpopulation than in non-Treg. This was in line with previously reported data showing that glioblastoma have relatively few infiltrating lymphocytes, even if T cell infiltration in GBM has been shown to increase in a WHO-grade-dependent fashion [26, 27].

However, our data are partially in contrast with data reported by Crane showing that general T-lymphocytes are also attracted by CM [4]. Differences in T-lymphocytes populations may explain this discrepancy since in our migration test, as non-Treg cells, only $CD25^{-}$ populations were tested. Although the migration rate was very similar in both experimental sets, differences in glioma cell lines used should also be considered.

Significant differences in CCL2 release were detected among the various glioblastoma cell lines tested (range 10–1,200 ng/mL). However, primary glioblastoma cell cultures seemed not to behave differently compared to long-term cultured cell lines, suggesting that both may be used in this kind of experiments [4].

The mechanisms and molecules that are responsible for T lymphocytes recruitment at the tumor site are still poorly known [28]. Investigating some of the molecules that are relevant as chemoattractant for Treg, we found that CCL2 showed to play a pivotal role. Moreover, the addition of an anti-CCL2 antibody to the conditioned media almost completely abrogated the increase of in vitro Treg migration, indicating that CCL2 has a necessary, but not

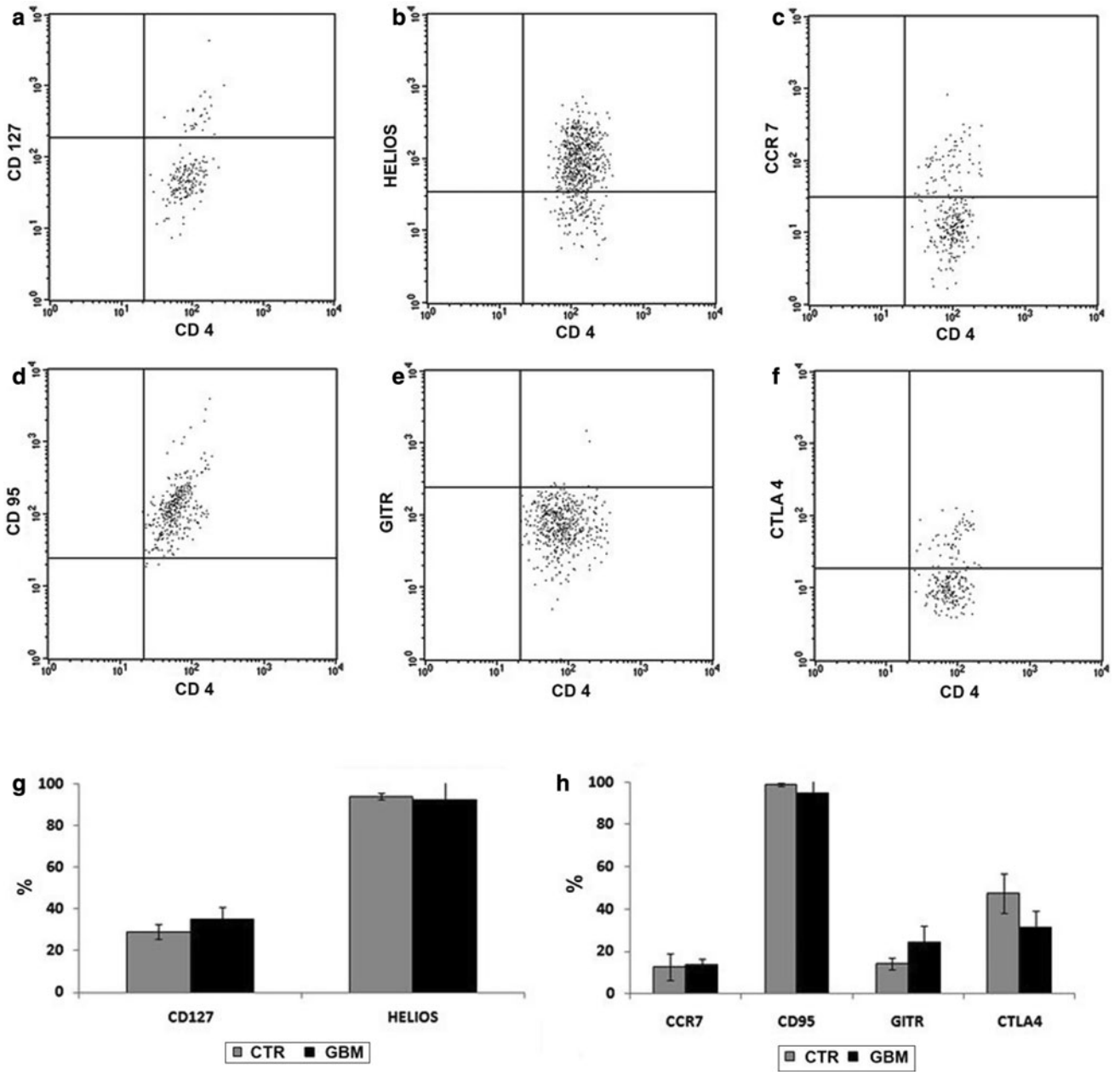


Fig. 5 **a**–**f** Treg markers expression in FOXP3$^+$CD4$^+$CD25$^+$ circulating lymphocytes as detected by flow cytometry. FOXP3$^+$CD4$^+$CD25$^+$ were first gated and the expression of each marker was calculated: **a** *CD127*; **b** *Helios*; **c** *CCR7*; **d** *CD95*; **e** *GITR*; **f** *CTLA4*. **g** *Histogram* that represent the percentage of Treg specific markers *CD127* and *Helios* and **h** proteins involved in activation (*CCR7*), apoptosis (*CD95*) and in autoimmunity (*GITR* and *CTLA4*). Data are expressed as mean ± SD of percentages detected in patients and controls

exclusive, role in attracting Treg at the tumor site [29, 30]. Similar results have been shown by Crane for CCL2 and commented by Weller [4, 31].

GBM cell lines and primary cultures seemed to respond to irradiation increasing CCL2 release in the conditioned media, similarly to what previously observed in two meningioma cell lines [32], even if the different behavior observed in one cell line suggests heterogeneity in the response. However, in our experimental conditions, this increase did not result in a rise of Treg migration, nor in patients or in controls, and no statistically significant differences in CCL2 receptors were detected, suggesting a "normal" phenotype of circulating Treg in GBM patients. Taken together our data suggest that, although CCL2 plays a crucial role in the first phases of Treg attraction, a synergy of protein interactions is likely needed to drive effective migration to the tumor site in GBM [4, 33].

Data regarding phenotypic and functional analysis of circulating Treg further suggest that this immunosuppressive subpopulation display similar characteristics in GBM patients and in healthy controls [4]. Among the molecules studied, GITR upregulation in GBM-infiltrating lymphocytes has been reported to be particularly pronounced on infiltrating Treg [34]. In our patients, expression of GITR on circulating Treg was almost doubled compared to healthy controls; however, due to the high variability observed in patients, this increase was not statistically significant. Since GITR-targeted immunotherapy has been shown to positively impact tumor immunity, this preliminary observation may deserve further attention [35, 36]. The role of other Treg-associated molecules such as ICOS (inducible costimulatory molecules) in immune escape of glioma, remains to be elucidated [37].

A further and deeper knowledge of the complex interactions between systemic immunity, locoregional immunosuppressive and chemoattractant factors and tumor-versus therapy-induced alterations is mandatory for the development of effective immunotherapeutic protocols in glioblastoma, in which the standard of cure contemplates immunosuppressive therapy.

Acknowledgments This work was supported by research Grant of the Ministero della Salute (LR9 IRCCS Foundation Neurological Institute "C. Besta", Milan Italy). The authors are grateful to Luisa Fumagalli and Francesco Ghielmetti from Radiotherapy Unit (Foundation IRCCS Neurological Institute C. Besta) for the skillful technical help with irradiation experiments. We are also thankful to Marta Dossena from Cellular Neurobiology Laboratory, Cerebrovascular Diseases Unit (Foundation IRCCS Neurological Institute C. Besta) for her contribution on data analysis.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Wainwright DA, Nigam P, Thaci B et al (2012) Recent developments on immunotherapy for brain cancer. Expert Opin Emerg Drugs 17(2):181–202
2. Behin A, Hoang-Xuan K, Carpentier AF et al (2003) Primary brain tumors in adults. Lancet 361:323–331
3. Curtin JF, Candolfi M, Fakhouri TM et al (2008) Treg depletion inhibits efficacy of cancer immunotherapy: implications for clinical trials. PLoS ONE 3(4):e1983
4. Crane CA, Ahn BJ, Han SJ et al (2012) Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. Neuro Oncol 14(5):584–595
5. Fujimoto S, Greene MI, Sehon AH (1976) Regulation of the immuneresponse to tumor antigens. II. The nature of immunosuppressor cells in tumor-bearing hosts. J Immunol 116:800–806
6. Ha TY, Park HJ, On KK et al (1995) Potentiation of anti-tumor immunity by modulation of suppressor T cells. II. Regression of tumor by modulation of suppressor T cells. Korean J Immunol 17:101–118
7. Ha TY (2009) The role of regulatory T cells in cancer. Immune Netw 9(6):209–235
8. Chen X, Oppenheim JJ (2011) Resolving the identity myth: key markers of functional $CD4^+$ $FoxP3^+$ regulatory T cells. Int Immunopharmacol 11(10):1489–1496
9. Wang RF (2006) Regulatory T cells and innate immune regulation in tumor immunity. Springer Semin Imunopathol 28:17–23
10. Fietta AM, Morosini M, Passadore I et al (2009) Systemic inflammatory response and downmodulation of peripheral $CD25^+$ $Foxp3^+$ T-regulatory cells in patients undergoing radiofrequency thermal ablation for lung cancer. Human Immunol 70:477–486
11. Liu Z, Kim JH, Falo LD et al (2009) Tumor regulatory T cells potently abrogate antitimor immunity. J Immunol 182:6160–6167
12. El Andaloussi A, Lesniak MS (2006) An increase in $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in tumor infiltrating lymphocytes of human glioblastoma multiforme. Neuro Oncol 8:234–243
13. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C et al (2008) Incidence and prognostic impact of $FoxP3^+$ regulatory T cells in human gliomas. Clin Cancer Res 14:5166–5172
14. Jacobs JF, Idema AJ, Bol KF et al (2010) Prognostic significance and mechanism of Treg infiltration in human brain tumors. J Neuroimmunol 225:195–199
15. Fecci PE, Mitchell DA, Whitesides JF et al (2006) Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. Cancer Res 66(6):3294–3302
16. Zhang J, Patel L, Pienta KJ (2010) Targeting chemokine (C–C motif) ligand 2 (CCL2) as an example of translation of cancer molecular biology to the clinic. Prog Mol Biol Transl Sci 95:31–53
17. Deshmane SL, Kremlev S, Amini S et al (2009) Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 29:313–326
18. Del Pozo MA, Cabanas C, Montoya MC et al (1997) ICAMs redistributed by chemokines to cellular uropods as a mechanism for recruitment of T lymphocytes. J Cell Biol 137:493–508
19. Carrillo-de Sauvage MA, Gómez A, Ros CM (2012) CCL2-expressing astrocytes mediate the extravasation of T lymphocytes in the brain. Evidence from patients with glioma and experimental models in vivo. PLoS ONE 7:30762
20. Calatozzolo C, Canazza A, Pollo B et al (2011) Expression of the new CXCL12 receptor, CXCR7, in gliomas. Cancer Biol Ther 11(242–53):17
21. Conductier G, Blondeau N, Guyon A et al (2010) The role of monocyte chemoattractant protein CCL2/CCL2 in neuroinflammatory diseases. J Neuroimmunol 224:93–100
22. Kim YH, Jung TY, Jung S et al (2012) Tumor-infiltrating T-cell subpopulations in glioblastomas. Br J Neurosurg 26:21–27
23. Donson AM, Birks DK, Schittone SA et al (2012) Increased immune gene expression and immune cell infiltration in high grade astrocytoma distinguish long-term from short-term survivors. J Immunol 189:1920–1927
24. Lowther DE, Hafler DA (2012) Regulatory T cells in the central nervous system. Immunol Rev 248(1):156–169
25. Jordan JT, Sun W, Hussain SF et al (2008) Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. Cancer Immunol Immunother 57(1):123–131
26. Parney IF, Waldron J, Parsa AT (2009) Flow citometry and in vitro analysis of human glioma associated macrophages. J Neurosurg 110(3):572–582
27. Lohr J, Ratliff T, Huppertz A et al (2011) Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-β. Clin Cancer Res 17(13): 4296–4308

28. de Chaisemartin L, Goc J, Damotte D et al (2011) Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Res 71(20):6391–6399
29. Wei S, Kryczek I, Zou W (2006) Regulatory T-cell compartmentalization and trafficking. Blood 108(2):426–431
30. Ménétrier-Caux C, Faget J, Biota C, Gobert M, Blay JY, Caux C (2012) Innate immune recognition of breast tumor cells mediates CCL2 secretion favoring Treg recruitment within tumorenvironment. Oncoimmunology 1(5):759–761
31. Weller M (2012) More on the immune privilege of glioblastoma. Neuro Oncol 14(5):527–528
32. Nalla AK, Gogineni VR, Gupta R et al (2011) Suppression of uPA and uPAR blocks radiation-induced CCL2 mediated recruitment of endothelial cells in meningioma. Cell Signal 23(8):1299–1310
33. Yan M, Jene N, Byrne D et al (2011) Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers. Breast Cancer Res 13(2):R47
34. Wainwright DA, Balyasnikova IV, Chang AL et al (2012) IDO Expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. Clin Cancer Res 18(22):6110–6121
35. Coe D, Begom S, Addey C et al (2010) Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy. Cancer Immunol Immunother 59:1367–1377
36. Cohen AD, Schaer DA, Liu C et al (2010) Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. PLoS ONE 5:e10436
37. Faget J, Bendriss-Vermare N, Gobert M et al (2012) ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive $CD4^+$ T cells. Cancer Res 72:6130–6141