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ORIGINAL ARTICLE

Generation and immunologic functions of Th17 cells in malignant gliomas

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Abstract Th17 cells, a recently discovered inflammatory T cell subtype, have been implicated with autoimmune disorders. However, mechanism of generation or functions of intratumoral Th17 cells are still unclear. We have been investigating the mechanism of induction and role of Th17 cells in malignant gliomas using primary tumor as well as cell lines. We report here that: (1) a higher frequency of Th17 cells in gliomas were associated with higher number of myeloid (CD11b) cells as well as the expression of TGF-β1 or IL-6; (2) conditioned medium from glioma cells (Gl CM) induced Th17 cell differentiation, which was inhibited by anti-TGF-β1 and anti-IL-6; (3) glioma-associated monocytes secreted Th17-promoting cytokines IL-1β and IL-23; (4) CM from glioma and monocyte co-culture (Gl+Mo CM) induced high frequency of Th17 cells in naïve T cell culture, which was abrogated by anti-IL-1β and anti-IL-23 antibodies; (5) In vitro Gl+Mo CM-mediated Th17 generation was associated with a decrease in IFN-γ and a concomitant increase in IL-10 secretion. Anti-TGF-β1, but not anti-IL-6, significantly reversed this cytokine profile. These results demonstrate prevalence of Th17 cells in gliomas and implicate the cytokines derived from the tumor as well as infiltrating myeloid cells in the induction of Th17 cells in glioma microenvironment. Moreover, the data also suggest that glioma-associated Th17 cells may contribute to immune-suppression via TGF-β1-induced IL-10 secretion. Further studies on the mechanism of tumor-infiltration, developmental pathways, and pro-/anti-tumor functions of Th17 cells will provide rationale for developing novel adjuvant immunotherapeutic strategies for malignant gliomas.

Keywords Th17 cells · Glioma · Myeloid cells · TGF-β · IL-1β · IL-6

Abbreviations

CM	Conditioned medium
nT	Naïve T cells
GI-CM	Conditioned medium from glioma cells
Mo-CM	Conditioned medium from monocyte culture
TGF	Transforming growth factor
CCL	Chemokine ligand

Introduction

Malignant gliomas are one of the most lethal tumors with 22,000 new cases and 13,000 deaths per year in the United States [1, 2]. In spite of aggressive surgery and radio-chemotherapeutic regimens, the median survival for malignant gliomas remains around 15 months [3]. An increasing body of evidence derived from studies in extra-cranial tumors points to a critical role of inflammation in cancer progression and recurrence [4, 5]. It is therefore important to understand the mechanism of this link in order to find ways to intervene and improve survival. The Th17 cells, a recently discovered inflammatory T cell

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subtype [6], have well described roles in auto-immune diseases, tumor immunology and may be a target for cancer therapy [7]. Th17 cells have been reported in several extracranial tumors, where they have been implicated with either pro- or anti-tumor activity, depending on the tumor type [8]. A mechanistic insight into regulation of Th17-mediated inflammation and its role in glioma progression would provide novel avenues for developing more effective therapies for this deadly disease.

The T helper type 17 cells (Th17) were recently identified as an independent subtype of inflammatory T cells with a distinct cytokine (IL-17) and transcription factor (RORγt) profile [6]. Naive $CD4^+$ T cells (nT) can be induced to differentiate towards Th1, Th2, Th17 and regulatory T cell (Treg) phenotypes according to the local cytokine milieu. Each phenotype is characterized by unique signaling pathways and expression of specific transcription factors, notably T-bet for Th1, GATA-3 for Th2, forkhead box P3 (FoxP3) for Tregs, and retinoid-related orphan receptor (ROR)α and RORγt for Th17 cells [9]. Gliomas have been known to secrete TGF-β1 and IL-6 [10–13] and in mouse models, Tregs and Th17 cells have been demonstrated to arise from common precursors in a reciprocal manner based on exposure to transforming growth factor (TGF)-β or TGF-β plus IL-6, respectively [6, 14]. A number of groups have now described conversion of Treg to the Th17 phenotype or vice versa, induced by appropriate inflammatory stimuli [15]. Unlike the rapid advances in understanding their role in inflammation and autoimmunity, there are few studies on the activity of Th17 cells in cancer, and the results are controversial [8].

Myeloid ($CD11b^+$) cells are predominant among immune cell that infiltrate malignant gliomas, constituting more than 1 % of total cells and are often the first ones to be recruited [16–18]. IL-1β and IL-23 are critical in the induction of Th17 phenotype in humans [19–22]. Tumor-associated monocytes have been shown to induce and expand Th17 cells via secretion of IL-1β and IL-23 in ovarian carcinoma and hepatocellular carcinoma [23, 24]. Tumor associated macrophages expressed higher levels of IL-1β than normal tissue macrophages and normal-monocyte derived macrophages. Thus, molecular mechanisms involved in inducing Th17 cell in patients with tumors can be different from those in patients with auto-immune diseases [7]. Therefore, it is pertinent to study the role of glioma-associated myeloid cells in the generation or expansion of tumor-promoting Th17 cells in the tumor microenvironment.

We report here that factors derived from glioma and glioma-activated myeloid cells mediate the generation of intratumoral Th17 cells. Moreover, glioma-associated Th17 cells are potentially non-cytotoxic and may also contribute to immune suppression via secretion of IL-10.

Materials and methods

Glioma tissues, primary tumor culture (pGL) and cell lines

Tumor tissues were obtained from patients with glioblastoma immediately after surgical resection, with informed consent under a protocol (#111610M1E), approved by the WSU IRB. One half of the tumor tissue was immediately frozen in O.C.T. solution (Sakura Finetek USA, Torrance, CA) for immunohistochemical analysis. The other half of the tumor was enzymatically digested to prepare pGL cell culture, as described elsewhere [25]. Briefly, tumor tissue was cut into fine pieces and incubated at 37 °C for 30 min in the presence of 14 U/ml of Liberase Blendzyme II reagent containing collagenase and dispase (Roche Applied Science, Indianapolis, IN). The dissociated tumor cells were cultured in poly-L-lysine coated tissue culture flasks in DMEM containing 10 % FBS to derive pGL. U87-MG cells were obtained from American type culture collection (ATCC, Manassas, VA) and maintained in DMEM containing 10 % FBS.

Preparation of peripheral blood leukocytes (PBL), isolation of $CD14^+$ monocytes and $CD4^+$ naïve T cells

A published protocol [26] was followed with some modifications. Briefly, $CD14^+$ cells were isolated by positive selection using CD14 magnetic beads (Miltenyi Biotec, Auburn, CA). $CD4^+$ naïve T cells were then isolated by negative selection using a kit (Miltenyi Biotec) following the manufacturer's instructions.

Preparation of conditioned media (CM) from glioma and monocyte (co-)cultures

U87-MG cells were either cultured alone at 1×10^6 cells/ml or were co-cultured with monocytes (U87+Mo) at 1:1 ratio. The U87-MG CM or U87+Mo CM were harvested after 72 h. The CM were frozen at −80 °C in 2 ml aliquots until use.

In vitro Th17 (IL-17) induction model

In order to determine the effect of glioma and monocyte-derived factors on the induction of Th17 cells, an in vitro model was developed by modifying, combining and appropriating two protocols, [27, 28]. Briefly, PBL or MACS-isolated $CD4^+$ naïve T (nT) cells were cultured 1×10^6 cells/well, in serum-free X-VIVO 20 medium (Lonza, Walkersville, MD), in a 6-well plate with anti-CD2/CD3-loaded MACS microbeads (Miltenyi Biotec, Auburn, CA) and low dose IL-2 (50 IU/ml). In order to

induce Th17 phenotype (IL-17), conditioned medium from glioma and monocyte cultures (as described above) or a combination of IL-6 (10 ng/ml), IL-1β (1 ng/ml), IL-23 (1 ng/ml) and TGF-β1 (10 ng/ml) were added as indicated. The human recombinant cytokines and respective neutralizing antibodies were obtained from PeproTech, Inc (Rocky Hills, NJ). The culture supernatants and the Th17 or control cells, harvested after 7 days of culture, were analyzed for cytokine secretion or phenotype, as described below. All experiments were performed at least three times using PBL obtained from three different donors.

Cytokine analysis

Cytokines were measured in the culture supernatants using a 25-plex human cytokine Luminex Array (Invitrogen, Carlsbad, CA) and Bio-Plex system (Bio-Rad Lab., Hercules, CA). The multiplex panel includes interleukin (IL)-1β, IL-6, IL-10, IL-17 and IFN-γ. The limit of detection for these assays is <10 pg/mL based on detectable signal of > twofold above background (Bio-Rad). Cytokine concentration was automatically calculated from a standard curve by the BioPlex Manager Software (Bio-Rad). TGF-β1 and IL-23 were detected using respective ELISA kits (BD Biosciences, San Jose, CA and R&D Systems, Minneapolis, MN). In order to include the latent TGF-β in the measurement, the culture supernatants were acid-treated as per the manufacturer's protocol.

Immunohistochemistry

A published protocol [29] was followed with some modifications. Briefly, the frozen or paraffinized tumor specimens were cut into 5 μm tissue sections, fixed in acetone for 5 min and stored at −80 °C until staining time. For staining, slides were brought to room temperature, blocked and hydrated in staining buffer (PBS with 5 % goat serum) and appropriate primary antibodies (anti-CD4 from B.D. Biosciences; anti-TGF-β1, anti-IL-6 and anti-IL-17 from Santa Cruz Biotechnology; anti-CD11b and anti-IL-1β from eBioscience, San Diego, CA) were applied, followed by washing and incubation with the appropriate biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstaining with Mayer hematoxylin followed by dehydration and mounting. For co-localization, appropriate secondary antibodies conjugated with FITC or PE (Santa Cruz Biotechnology) were utilized. The nucleus was stained with DAPI. Negative staining was performed with appropriate isotype control antibodies (eBioscience, San Diego, CA) instead of the specific primary antibody. The sections were then analyzed under a fluorescent microscope equipped with a digital camera (Olympus BX51) and micrographed at 200× or 400× magnification. The number of cells with respective immunostaining were recorded after counting 100 cells with distinct nuclear staining in at least three consecutive high power fields in each slide. Necrotic or thick areas and severely overlapping tumor cells were excluded. The infiltrating immune cells were calculated as percentage of the total number of nuclear-stained cells counted. Tumor tissues obtained from five patients with malignant gliomas were studied, as described below in the Results section

Flow cytometry

Fluorescein-conjugated human antigen-specific or isotype control antibodies were purchased from SantaCruz Biotechnology, Santa Cruz, CA (rabbit anti-human IL-17, goat anti-rabbit APC) and B.D. Biosciences, San Jose, CA (CD3 FITC, CD4 FITC, IFN-γ PE, and IL-10 PE). A published protocol was followed for surface and intracellular staining of the cells [26].

Statistical analysis

A Wilcoxon's log-rank test was performed to determine the statistical difference between various experimental and control groups, using the SPSS package (SPSS Inc, Chicago, IL) [30]. A 'p' value less than 0.05 was considered significant.

Results

Tumor tissues from malignant glioma patients contain Th17 ($CD4^+IL\text{-}17^+$) cells, and express TGF-β1 and IL-6

Five tumor tissues obtained from patients with malignant gliomas were analyzed by immunofluorescence microscopy (CD4 and IL-17) and IHC using DAB (IL-6 and TGF-β1). We observed co-localization of CD4 and IL-17 in glioma-infiltrating Th17 cells (Fig. 1a). Lymph node tissues obtained from a normal (non-cancer) cadaver donor was used as a positive control for Th17 staining (data not shown). Representative micrographs of a malignant glioma tissue showing about 100 total cells and a few Th17 cells as well as the negative (isotype) control staining have been shown in Supplementary Figure S1. IL-6 and TGF-β1 staining by IHC in representative glioma tissues are shown in Fig. 1b, c, respectively.

In order to confirm the secretion of IL-6 and TGF-β1 by gliomas, a primary glioma and U87-MG cells were cultured (1×10^6 cells/ml) for 72 h and conditioned media (CM) were harvested for cytokine analysis as described in

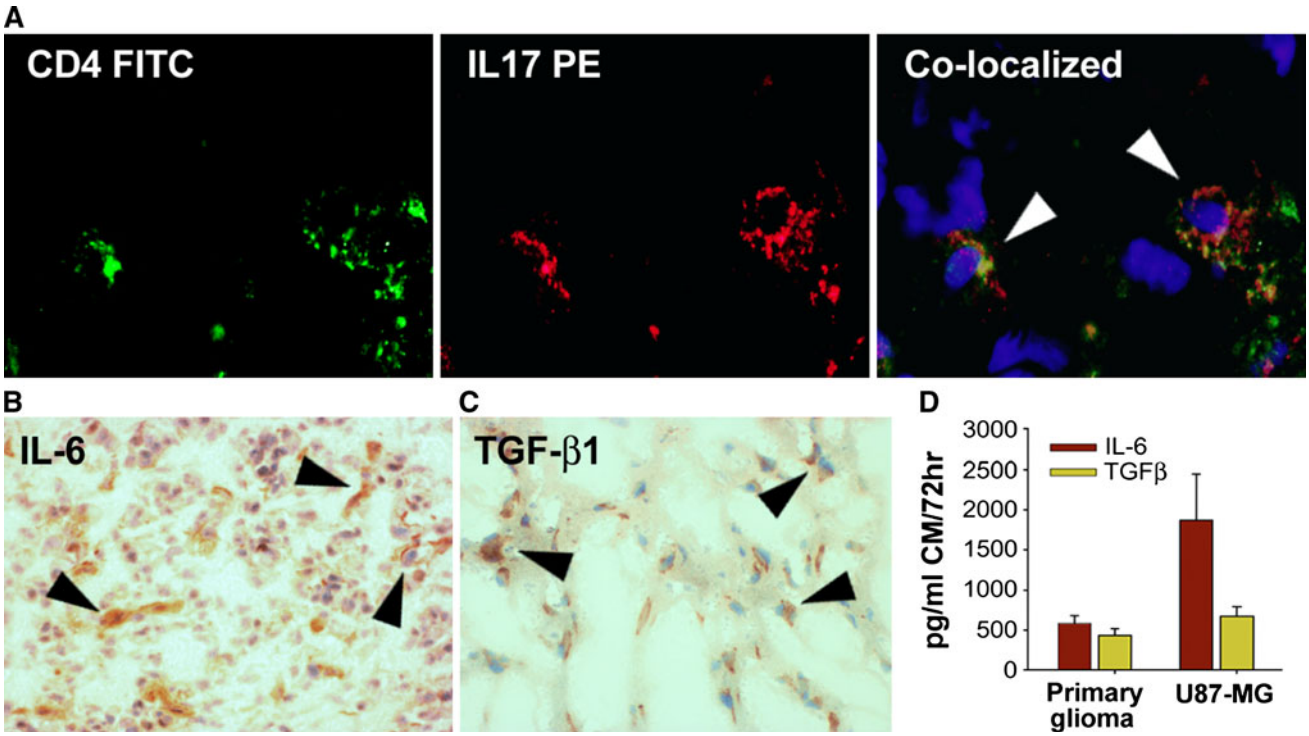

Fig. 1 Prevalence of Th17 cells and the expression of IL-6 and TGF-β1 in gliomas. Frozen or paraffinized tumor sections, obtained from patients with malignant glioma were analyzed by IHC to determine the presence of CD4+IL-17+ Th17 cells (**a**) as well as expression of TGF-b1 (**b**) and IL-6 (**c**). Result shown is from one representative paraffinized glioma specimen out of five studied. The micrographs were imaged at ×400 (**a**) and ×200 (**b**, **c**) magnifications. Conditioned media (CM) from 72 h culture of a primary glioma and U87-MG glioma cell line were analyzed for the secretion of IL-6 and TGF-β1, by Bioplex assay and ELISA, respectively, as described in the Sect. "Materials and Methods". The data are expressed in pg/ml of CM (**d**)

Table 1 Percentage of cells expressing Th17 (IL-17) and Myeloid (CD11b) cell markers, and Th17-inducing cytokines (TGF-β1 and IL-6) in malignant gliomas

Gliomas	IL-17	CD11b	TGF-β1	IL-6
#1 Grade IV	1	3	9	12
#2 Grade IV	0.8	1.6	8	9
#3 Grade III	0.5	2	0	6
#4 Grade III	0.6	1.8	4	4
#5 Grade II	1	2	8	10

Five tumor tissues obtained from patients with gliomas were analyzed by immunofluorescence microscopy (IL-17 and CD11b) and IHC using DAB (IL-6 and TGF-β1). Data is expressed as percentage positive cells per total cells counted

the Materials and Methods section. Consistent with the IHC data, nearly 500 pg/ml each of IL-6 and TGF-β1 were detected in the CM of primary glioma, and 1,800 pg/ml IL-6 and 700 pg/ml TGF-β1 from U87-MG cells (Fig. 1d).

All five glioma tissues analyzed were positive for IL-17, CD11b and IL-6, while 4 out of 5 tumors were positive for TGF-β1, at varying degrees of percent positivity (Table 1). The number of samples analyzed was not sufficient to examine any possible correlation between the level of Th17 infiltration and tumor grade. Notably, one of the tumors that was negative for TGF-β1 expression was still positive for IL-17 (Table 1) and also showed presence of Th17 by IHC, prompting further investigations into possible TGFβ1-independent mechanism of Th17 induction in gliomas.

Glioma CM induces IL-17 expression in T lymphocytes, which is inhibited by antibodies to TGF-β1 and IL-6

In order to examine the role of glioma-derived factors in the induction of Th17 phenotype (IL-17), non-adherent (monocyte depleted) PBL, obtained from normal donors, were cultured in the Th17 (IL-17) induction model, in the presence or absence of U87-MG CM (50 % v/v) as described in the Materials and Methods section. The naïve T cell cultures used serum-free X-VIVO 20 medium; complete DMEM (50 % v/v) was added in the 'control' cultures. After 6 days of culture, the cells were analyzed by flow cytometry. PBL cultured in presence of U87-MG CM contained significantly higher frequency of Th17 cells (4.76 %) compared to the control cultures (0.19 %) (Fig. 2b vs. a). Anti-IL-6 or anti-TGF-β1 completely inhibited the U87-MG CM mediated induction of Th17 cells (Fig. 2c, d, respectively). The results show that

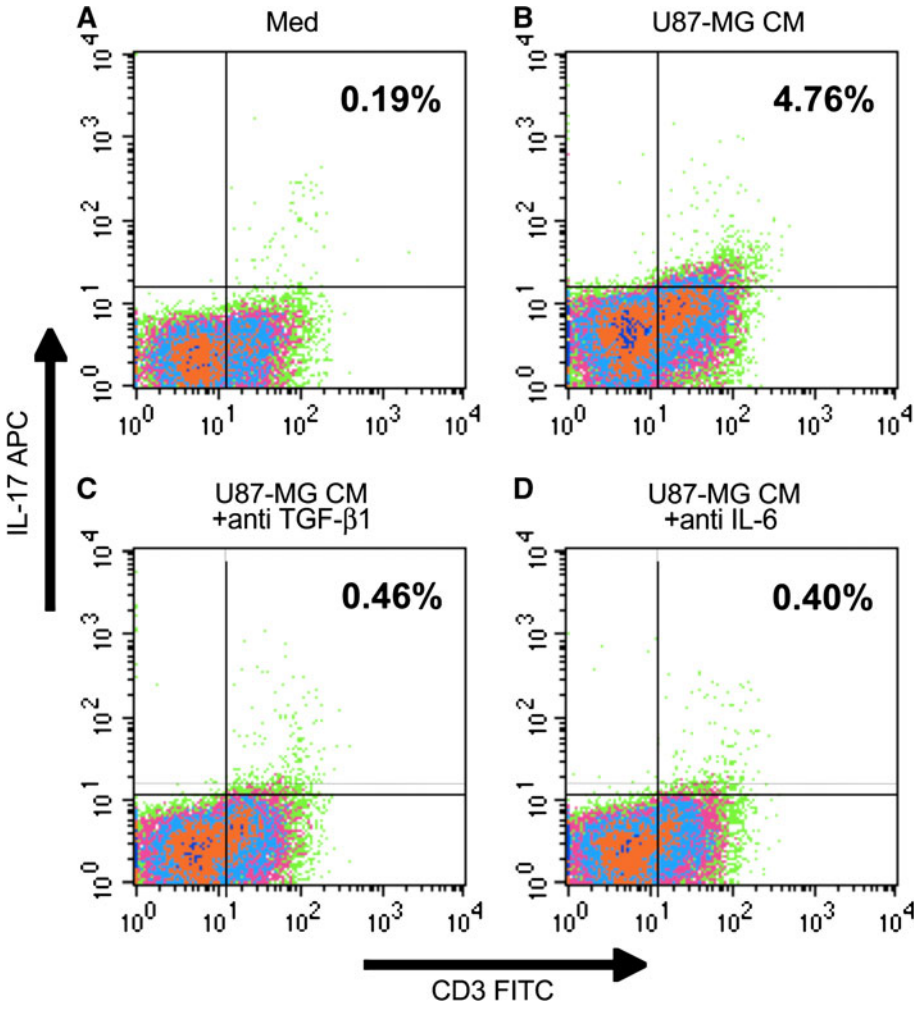

Fig. 2 Glioma CM induces Th17 cells, which is inhibited by anti-TGF-β1 and anti-IL-6. Non-adherent (monocyte depleted) PBL, obtained from normal donors, were cultured in the Th17 generation model in the presence of Gl-CM, as described in the Sect. "Materials and Methods". After 6 days of culture, the cells were analyzed by flow cytometry. The data are from one representative experiment out of three experiments performed with similar results

gliomas can induce Th17 phenotype, albeit at low levels, via secretion of TGF-β1 and IL-6.

Glioma-activated monocytes secrete high levels of Th17 promoting cytokines IL-1β and IL-23

Myeloid cells are known to comprise a predominant infiltrating immune cell population in gliomas [16]. We investigated the role of glioma-associated cytokines in the induction of Th17 cells in gliomas. We observed prevalence of IL-1β co-expressing CD11b$^+$ myeloid cells in glioma tissues (Fig. 3a). Next, we investigated if glioma cells or glioma-derived factors induced secretion of IL-1β and IL-23 in monocytes. Monocytes were cultured either in presence of U87-MG CM or were co-cultured with U87-MG cells at 1:1 ratio for 72 h. cytokines were analyzed as described in the Materials and Methods section. Low levels of IL-1β and IL-23 were detectable in the medium control group (Fig. 3b, c, respectively). U87-MG CM itself contained negligible amount of IL-1β or IL-23, but significantly upregulated the secretion of these cytokines by monocytes. Levels of IL-1β (Fig. 3b) as well as IL-23 (Fig. 3c) were significantly higher in the U87+Mo co-culture groups compared to any other group. Moreover, the quantities of cytokines detected in Mo+U87-CM groups were only marginally higher than the values obtained by adding the quantities of respective cytokines detected in individual cell cultures. On the other hand, cytokine levels in Mo+U87-MG co-culture groups were significantly higher than the Mo+U87CM or Mo+U87 (insert) group. These results suggest that secretion of Th17-promoting cytokines by glioma-associated monocytes may involve some degree of cell-to-cell interaction between gliomas and monocytes.

Role of glioma-activated monocyte-derived factors in the induction of Th17 cells

These sets of experiments examined whether glioma-associated monocytes and related cytokines were necessary during induction of Th17 cells in a simulated glioma microenvironment. CD4$^+$ nT cells were cultured in the Th17 (IL-17) induction model for 6 days in the presence of

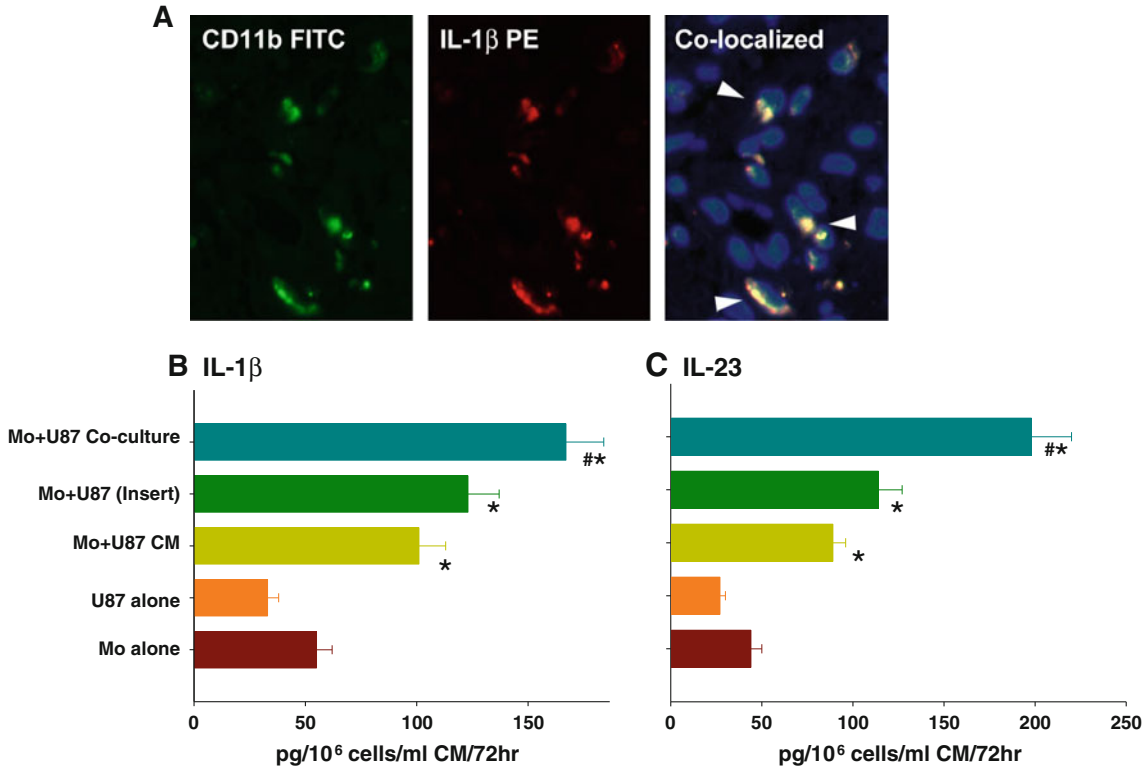

Fig. 3 Prevalence of $CD11b^{+}IL\text{-}1\beta^{+}$ Myeloid cells in gliomas **a** tumor sections, obtained from patients with glioblastoma were analyzed by IHC to determine the presence of $CD11b^{+}IL\text{-}1\beta^{+}$ myeloid cells as described in the Sect. "Materials and Methods". Result shown is from one representative frozen glioma specimen out of five studied. The micrographs were imaged at ×400 magnification. Secretion of IL-1β and IL-23 by Glioma-activated monocytes **b**, **c** peripheral blood monocytes (Mo) were either cultured in presence of U87-MG CM or with U87-MG cells (either in an insert or in a co-culture) at 1:1 ratio for 72 h. Cytokine levels in the CM were then analyzed using Bioplex cytokine array (Invitrogen) as described in the Sect. "Materials and Methods". The data, expressed in pg/ml of CM, represents mean ± S.D. of three independent experiments. '*' $p < 0.05$ versus monocytes cultured alone; '#' $p < 0.05$ versus all other groups

CM from U87+Mo co-cultures (Mo+U87 CM) as described above. The cells were then analyzed by 3-color flow cytometry. All cells were gated on CD4-FITC. The medium control group contained less than 1 % $CD4^{+}IL\text{-}17^{+}$ (Th17) cells, but with a higher frequency of 5.6 % $IL\text{-}17^{-}$ IFN-γ^{+} cells (Fig. 4a). Addition of U87+Mo CM significantly increased the frequency of total Th17 cells (29.9 + 1.1 %, upper two quadrants), while decreasing the frequency of IFN-γ^{+} T cells to 1.2 % (Fig. 4b). Addition of anti-IL-1β + anti-IL-23 significantly inhibited U87+Mo CM mediated induction of Th17 cells (10.4 vs. 29.9 %) (Fig. 4c). Together with data presented in Fig. 3, these results strongly implicate glioma-associated monocytes in the induction of Th17 cells in the tumor milieu.

Glioma-activated monocyte derived factors switches differentiation of nT cells from Th1 to immune-suppressive Th17 phenotype

Data presented in Fig. 4 suggested a decrease in IFN-γ^{+} T cell population upon addition of Gl+Mo CM into the nT cell culture. In order to further investigate this phenomenon, and to examine the role of glioma-derived cytokines in the modulation of T cell phenotype, nT cells were cultured in Th17 (IL-17) induction model in the presence of U87-MG CM or U87+Mo CM and antibodies to IL-6 and TGF-β1 for 6 days. nT cells cultured with anti-CD2/3-loaded beads and IL-2 only (Medium control) secreted large amount of IFN-γ (721 ± 234 pg/ml; Fig. 5a) and very little IL-17 (50 pg/ml; Fig. 5b) or IL-10 (50 pg/ml; Fig. 5c). Addition of U87-MG CM significantly decreased the secretion of IFN-γ while the secretion of IL-17 as well as IL-10 was significantly enhanced. Addition of U87+Mo CM further enhanced the secretion of IL-17 and IL-10 by T cells with concomitant inhibition of IFN-γ, indicating a switch from Th1 to Th17 type T cell phenotype. Addition of anti-IL-6 along with U87+Mo CM did not significantly alter the cytokine profile, while anti-TGF-β1 inhibited IL-17 by about 40 %, restored IFN-γ secretion to almost 70 % of the control level, and inhibited IL-10 secretion by more than 50 % in U87+Mo CM-treated nT cell culture (Fig. 5). The results, once again indicated that glioma-activated

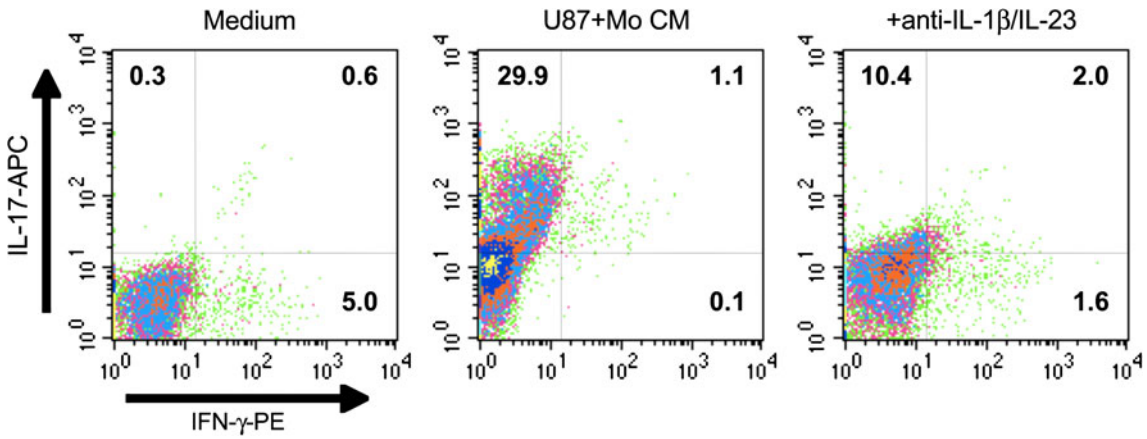

Fig. 4 Role of glioma-activated monocyte-derived factors in TH17 generation. $CD4^+$ nT cells were cultured in the Th17 (IL-17) induction model for 6 days in the presence of culture supernatants from various monocyte cultures as indicated. The cells were then analyzed by 3-color flow cytometry as described in the Sect. "Materials and Methods". All cells were gated on CD4-FITC. The data are from one representative experiment out of three experiments performed with similar results

monocyte derived factors induce/expand Th17 cells via a TGF-β1-independent mechanism, while presence of TGF-β appears to play a vital role in rendering Th17 cells immunosuppressive via inhibition of IFN-γ and enhancement of IL-10 secretion.

Neutralization of TGFβ1 resulted in an observed increase in IFNγ^+ cells and a concomitant decrease in IL-10^+ Th17 cells

In the next experiment, nT cells were cultured in Th17 (IL-17) induction model under the same conditions as described in the previous experiment, and then analyzed by flow cytometry. In agreement with the results shown in Fig. 5, we observed an increased frequency of IFN-γ^+ Th17 cells (Fig. 6b vs. a) with a concomitant decrease in IL-10^+ Th17 cells (Fig. 6d vs. e) following neutralization of TGF-β1 in U87+Mo CM. On the other hand, neutralization of IL-6 did not have a significant effect in the Th17 phenotype (Fig. 6c, f). Interestingly, unlike the U87-MG CM-induced Th17 culture (see Fig. 2, above), anti-TGF-β1 or anti-IL-6 alone did not alter the frequency of U87+Mo CM-induced Th17 cells, suggesting that, in the presence of myeloid-derived factors, TGF-β1 and IL-6 may regulate more than one redundant pathways for the induction of Th17 cells in glioma microenvironment.

These results, together with results shown in Figs. 3 and 4, clearly demonstrate that gliomas induce secretion of IL-1β and IL-23 from monocytes, which can lead to induction of Th17 cells independent of TGF-β1. However, presence of TGF-β1 enhances the frequency of Th17 cells with an immune-suppressive (low IFN-γ, high IL-10) phenotype.

Discussion

The Th17 cells, a recently discovered inflammatory T cell subtype, have been reported in several extracranial tumors, where they have been implicated with either pro- or anti-tumor activity, depending on the tumor type. Th17 cells with anti-tumor activities have been reported in head and neck squamous cell carcinoma (HNSCC) [31] and ovarian cancer [32]. On the other hand, IL-17 promoted multiple myeloma (MM) cell growth and colony formation via IL-17 receptor, adhesion to bone marrow stromal cells (BMSCs) as well as increased growth in vivo in murine xenograft model of human MM [32]. Similarly, Zhang et al. have reported increased prevalence of Th17 cells in patients with gastric cancer, which was associated with poor prognosis [33]. The frequency of Th17 cells also showed negative correlation with time to disease progression in 23 prostate cancer patients receiving a dendritic cell based vaccine [34]. In another study, 23 patients with medulloblastoma had a considerable population of Th17 cells in tumor-infiltrating T cells, along with high mRNA levels for Th17-related factors (IL-17, IL-23 and ROR-γt) in tumor tissues. Moreover, the serum concentrations of IL-17 and IL-23 protein were significantly increased in patients with medulloblastoma compared to normal controls, indicating that Th17 cells may contribute to medulloblastoma pathogenesis [35]. Recently, Wainwright et al. have demonstrated mRNA expression of IL-17A in human glioma. In the same study, they also demonstrated Th17 cells in mouse brain tumors [36]. However, the prevalence of Th17 cells in human gliomas, mechanism of their induction or expansion and their immunologic functions have not been studied.

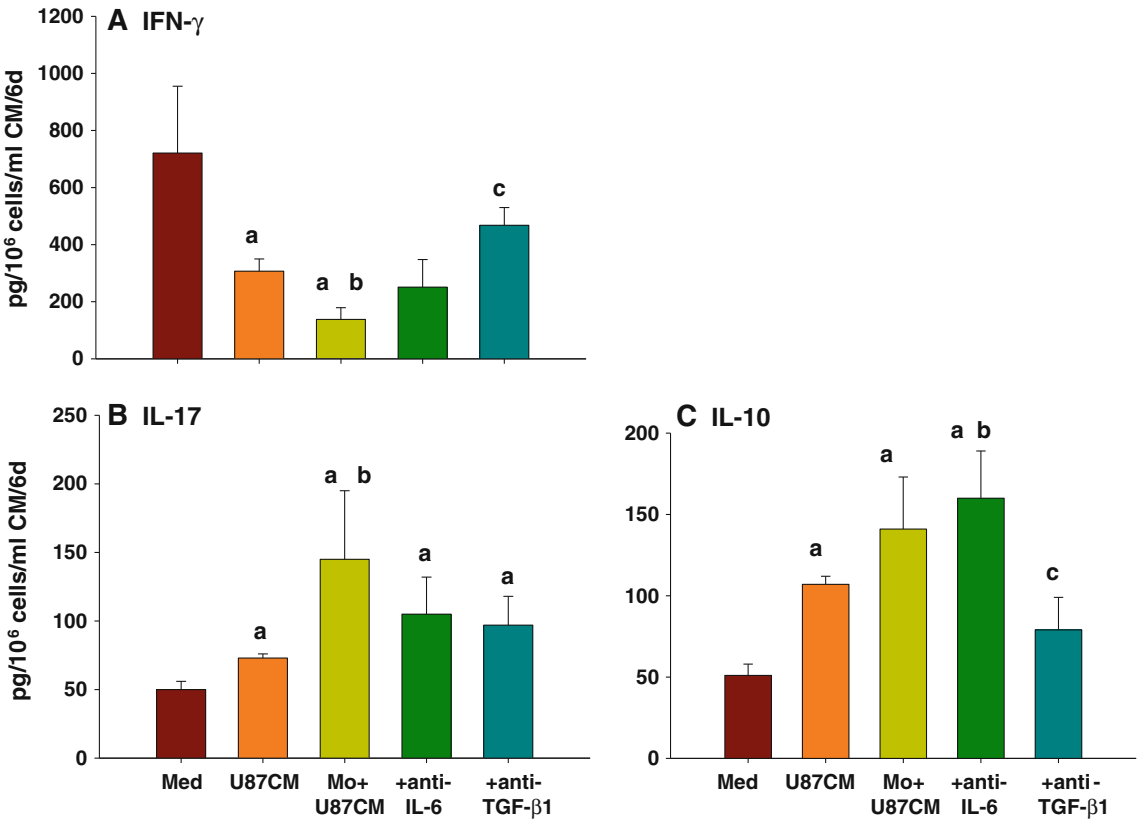

Fig. 5 Glioma-monocyte interaction switches differentiation of nT cells from Th1 to immune-suppressive Th17 phenotype. nT cells were cultured in the Th17 (IL-17) induction model for 6 days in presence of U87-MG CM or Mo+U87 CM with anti-TGF-β1 or anti-IL-6, as indicated. Cytokine levels in the CM were then analyzed using Bioplex cytokine array (Invitrogen) or ELISA, as described in the Sect. "Materials and Methods". The data, expressed in pg/ml of CM, represents mean ± S.D. of three independent experiments. 'a'-$p <$ 0.05 versus Med; 'b'-$p < 0.05$ versus U87-MG CM; 'c'-$p < 0.05$ versus Mo+U87 CM

This study demonstrates, for the first time, prevalence of Th17 cell in human malignant glioma tissues. The results also showed the presence of CD11b$^+$ myeloid cells along with Th17 promoting cytokines TGF-β, IL-6, IL-1β and IL-23 in gliomas, strongly implicating the infiltrating myeloid cells in the induction of Th17 cells in glioma microenvironment. We observed significant but small enhancement of Th17 frequency (about 5 %, Fig. 2b) by glioma-derived factors in the in vitro simulation of glioma microenvironment. However, supernatants from glioma-monocyte co-culture (U87+Mo CM) exponentially enhanced the frequency of Th17 cells to about 30 % in the in vitro simulation model, which was almost completely abrogated (10 %) by neutralization of glioma-activated monocyte derived factors IL-1β and IL-23 (Fig. 4). These data clearly suggested that glioma-monocyte interaction is required for effective induction of Th17 cells in the glioma microenvironment.

Th17 cells constitute a minor population in human peripheral blood and lymph nodes with no major frequency changes in cancer patients compared with healthy donors [24]. There is, however, a strikingly high frequency of tumor-infiltrating IL-17$^+$ T cells in patients with diverse cancer types, including ovarian and pancreatic cancer [24, 37], suggesting that Th17 cells may be generated or expanded in the tumor microenvironment. We observed expression of Th17 inducing cytokines TGF-β1 and IL-6 in most glioma tissues as well as in malignant glioma cell lines and primary culture. However, our IHC studies also revealed prevalence of Th17 cells in one glioma tissue that did not express TGF-β1, but was positive for IL-6. This result, combined with results of our in vitro simulation studies, implicates glioma-stimulated myeloid cell-derived IL-1β and IL-23 as a possible TGF-β-independent mechanism of Th17 cell generation in gliomas. We are analyzing tumor tissues from more patients as well as conducting further in vitro studies to confirm the same.

IL-6 seems to regulate induction of Th17 cells in synergy with TGF-β (TGF-β-dependent mechanism) or in synergy with IL-1β (TGF-β-independent mechanism) [38]. A synergistic role of IL-1β and IL-6 in the induction of Th17 cells have also been demonstrated in some other models [39, 40]. IL-23 is required for maintenance and expansion of Th17 cells [41]. Our results are in agreement

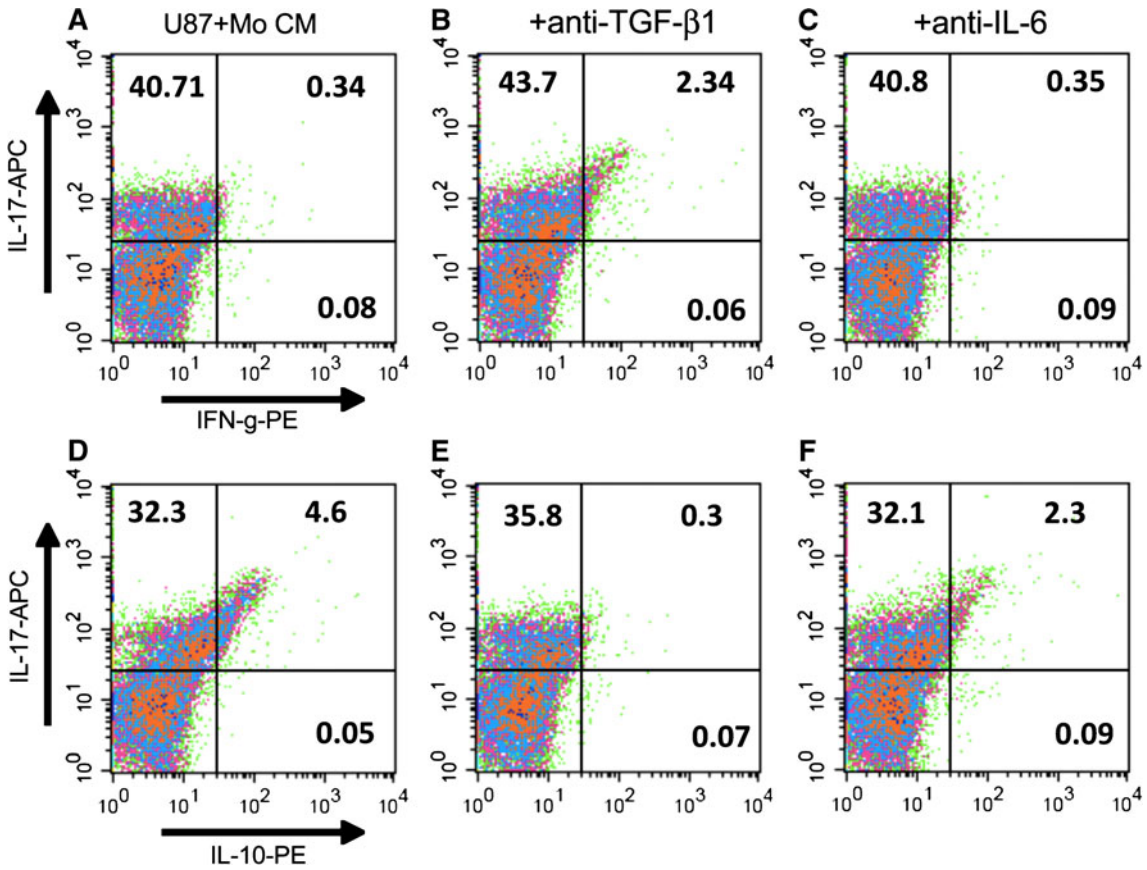

Fig. 6 Role of TGF-β1 in tumor-mediated generation of IFN-γ^{-}IL10^{+} TH17 cells. nT cells were cultured in the Th17 (IL-17) induction model for 6 days in presence of Mo+U87 CM with anti-TGF-β1 or anti-IL-6, as indicated. The cells were then analyzed by 3-color flow cytometry. All cells were gated on CD4-FITC. The data are from one representative experiment out of three experiments performed with similar results. The data for the isotype control staining are presented in supplementary Figure S2

with some reported studies showing that IL-6 and IL-23 in combination with IL-1β effectively induced IL-17 production in naive precursors, independent of TGF-β [42]. We observed that U87+Mo CM induced significantly higher frequency of Th17 cells compared to U87 CM, which was almost completely inhibited by anti-IL-1β and anti-IL-23 antibodies, suggesting that myeloid cell derived IL-1β and IL-23 may be critical in the induction of Th17 cells in gliomas. Studies in non-cranial tumors have also demonstrated that tumor-activated monocytes secrete high levels of IL-1β and IL-23, promoting induction/expansion of IL-17 secreting T cells [23, 43]. Notably, anti-TGF-β1 or anti-IL-6 alone could not inhibit U87+Mo CM mediated Th17 generation, clearly suggesting that induction of Th17 in gliomas employ redundant mechanisms which may or may not involve TGF-β1 or IL-6. These results also corroborate with published literature showing that, unlike in mice, TGF-β+IL-6 may not be strong inducers of Th17 in humans, where myeloid-derived cytokines such as IL-1β and IL-23 are required [19–22].

Th17 cells associated with autoimmune disorders often convert to Th1-like phenotype via co-expression of IFN-γ and are implicated in cytotoxicity [44, 45]. In terms of intratumoral Th17 cells, they might not mediate direct anti-tumor activity but can promote anti-tumor immunity indirectly through the recruitment of dendritic cells and cytotoxic effector cells via production of CCL20 [46, 47]. However, anti-tumor activity of a novel population of IL17+/IFN-g+ CD8 T cells (termed as Tc17 cells) have been reported in some cancers [48, 49] and, interestingly, non-cytotoxic Tc17 cells could be rendered cytotoxic upon treatment with IL-12 [50]. These studies demonstrate that the polarity and functions of tumor-infiltrating immune cell subsets clearly depends on the cytokine milieu in the tumor microenvironment. The precise mechanism of polarization and resulting immunologic functions of Th17 cells remain to be defined in tumor types where they are associated with pro-tumor activity. Exogenous IL-17 could promote tumor growth by inducing tumor vascularization particularly in immune deficient nude mice [51]. However, effect of IL-17

on tumor development and growth might be different in immune competent hosts [52]. Moreover, as result of differences in local concentrations, bioavailability and potential targets, the biological activities of endogenous IL-17 such as IL-17 derived from Th17 cells and exogenously administered IL-17 might differ [53]. Th17 cells are very plastic and can switch to non-cytotoxic (low IFN-γ, high IL-10) functional phenotype depending on the cytokines present in the milieu [15, 54, 55]. Induction of Th17 cells in the presence of TGF-β1 may lead to generation of non-cytotoxic (low IFN-γ, high IL-10) Th17 cells [56]. These studies suggest that the pro- or anti-tumor activity of Th17 cells, as reported in several tumor types, may be dictated by the relative co-expression of IL-10 or IFN-γ in these cells, which in turn depends on the cytokine milieu of the tumor microenvironment. The exact mechanism for generation and functions of such Th17 phenotype needs to be clarified. Our results concurred with these published studies. We observed that induction of IL-17 in T cell cultures by U87+Mo CM was associated with high IL-10 co-expression and concomitant inhibition of IFN-γ secretion. The cytokine profile was reversed upon neutralization of TGF-β1 in the culture. Flow cytometric analysis also revealed about 5 % IL-10 co-expressing Th17 cells in U87+Mo CM group, which was completely obliterated by anti-TGF-β1. These results strongly suggest that glioma-associated Th17 cells are potentially non-cytotoxic (low IFN-γ) and may become immune suppressive (high IL-10 expression) upon exposure to TGF-β1. These observations are in agreement with a recent report by Zhao et al. [57], who also observed an inhibition of autologous CD8+ T cell response by $CCR4^{+}CCR6^{+}$ Th17 cells in colorectal carcinoma, and the inhibition of CD8+ T cell response by Th17 was partially dependent on TGF-β1. Further studies in this direction have shown that glioma-induced Th17 cells can in fact enhance proliferation of a small population of glioma cells via IL-17/IL-17R interaction (Parajuli, unpublished observation). We are also performing correlation studies in more patient samples in order to determine a link between the level of TGF-β1 and frequency of immune suppressive Th17 cells in gliomas.

Immunotherapy has potential to prevent recurrence of malignant gliomas by specifically targeting residual or invasive tumor cells without affecting the normal tissue. However, immunotherapeutic strategies in high-grade gliomas have met with limited success in the clinic because of glioma-mediated active immune suppression [11, 58]. Development of novel adjuvant strategies to relieve tumor-mediated immune suppression is not only desirable but necessary for combination therapy of malignant gliomas. Our data provides preliminary insight into how conditions in glioma microenvironment could regulate induction of Th17 cells with an immunosuppressive phenotype and cytokine profile. Further systematic studies of tumor-infiltrating Th17 cells, their developmental pathways and their anti/pro-tumor functions, will likely identify novel mechanisms for immune intervention in gliomas by targeting the immune suppression-inflammation axis.

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

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