Supplementary Figures and Legends

Cancer Immunology Immunotherapy

A critical role for regulatory T cells in driving cytokine profiles of Th17 cells and their modulation of glioma microenvironment

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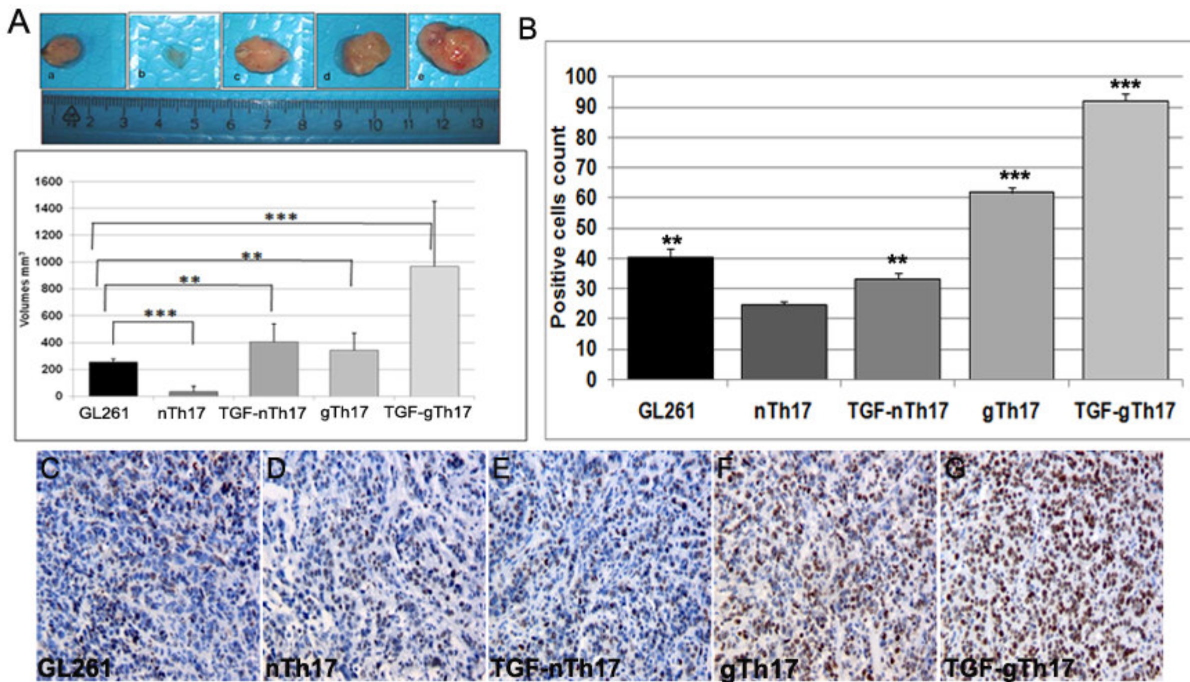
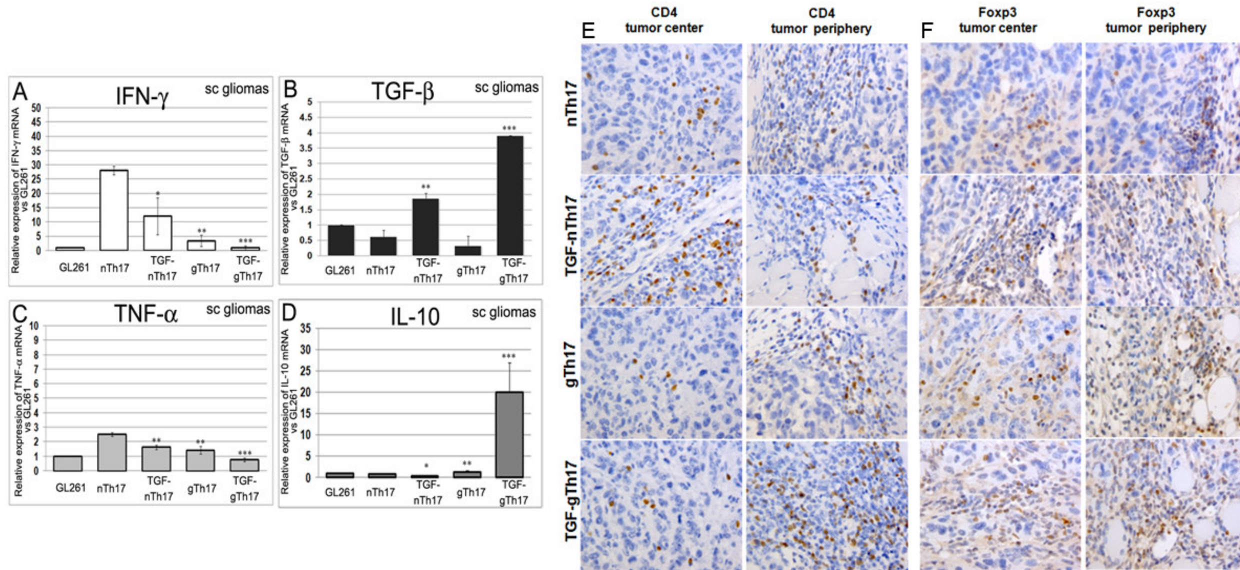
Supplementary Figure 1. Evaluation of tumor growth and proliferation index into sc glioma



A. Representative images of sc tumor mass in different group of treatment (a: GL261; b: nTh17; c: TGF-nTh17; d: gTh17; e: TGF-gTh17). Tumors derived from sc injection were excised 20 days later and measured. The highest tumor growth inhibition was observed in the group of nTh17 treated mice compared to the other groups: GL261-cells only 34±39.5mm3 vs 235.3±22.0 mm3 respectively, (p=0.003); TGF-nTh17 and gTh17 (p<0.02); TGF-gTh17 (p=0.003). Co-injection with TGF-nTh17 or gTh17 promoted tumor growth and glioma volumes were 405±134.5 mm3 (p=0.02 vs GL261; p=0.01 vs nTh17) and 341±128.7 mm3 (p=0.02 vs GL261; p=0.01 vs nTh17), respectively. Increased tumor growth was found in gliomas from mice co-injected with TGF-gTh17 (866±627 mm3, p=0.01 vs GL261, p<0.001 vs nTh17, p<0.04 vs TGF-nTh17 and gTh17). **B.** Quantitative analysis of the number of Ki67 positive tumor cells. Data are expressed as mean values ± SD and are representative for three tumors for each group (** $p<10^{-6}$, *** $p<10^{-8}$ vs nTh17). **C-G.** Tumors derived from injection of GL261 cells are poorly proliferative and (40,36±2,7) % of tumor cells express Ki67 antigen. The most significant difference in proliferating index was observed in nTh17 (24,77±1,12 % Ki67+ cells) and TGF-gTh17 (92,13±2,18%). These data correlate with the analysis of relative tumor size.

Supplementary Fig. 2. Characterization of tumor microenvironment in sc gliomas



A, C. In nTh17 we found enhanced expression of IFN-γ (28.2 folds vs TGF-gTh17 tumors; $p<0.0001$) and TNF-α (3.3 fold higher, $p<0.001$). **B, D.** On the contrary IL-10 and TGF-β strongly increased in TGF-gTh17. In particular, TGF-β 6.5 fold higher than sc nTh17 gliomas ($p<0.0001$). IL-10 25.6 fold higher ($p<0.0001$). **E, F.** CD4+ and Foxp3+ cells are detected in each sc experimental group within the tumor mass (left columns) and at the periphery (right columns). CD4+ TIL are relevant in all groups and located at tumor periphery with exception of TGF-nTh17 where CD4+ cells were preferentially distributed into the tumor mass. Foxp3+ cells appeared lower in nTh17-tumors preferentially located at tumor borders in all groups. In the centre of the tumor CD4+ lymphocytes are dispersed as single cells, except in TGF-nTh17 group where they were localized as clusters near tumor vessels. Most lymphocytes are concentrated at the interface of the tumor mass suggesting their recruitment from the periphery. There were no significant differences in CD4+ infiltrate for each experimental groups. We observed a similar distribution of Foxp3+ cells: large number of these cells were found mainly at the tumor edge. The infiltration of CD8+ cells was absent in all tumors, rare positive cells were revealed near the periphery vessels but not into the tumor mass (data not shown).

Sequences of target genes for SYBR-GREEN Chemistry:

b2m: F 5'-CACTGACCGGCCTGTATGCT-3'R 5'-AGGCGGGTGGAACTGTGTTA-3'

TGF-β: F 5'-CTAATGGTGGACCGCAACAAC-3' R 5'- GCACTGCTTCCCGAATGTCT-3'

IL-10: F 5'-CAGAGCCACATGCTCCTAGA -3' R 5'-GTCCAGCTGGTCCTTTGTTT-3'

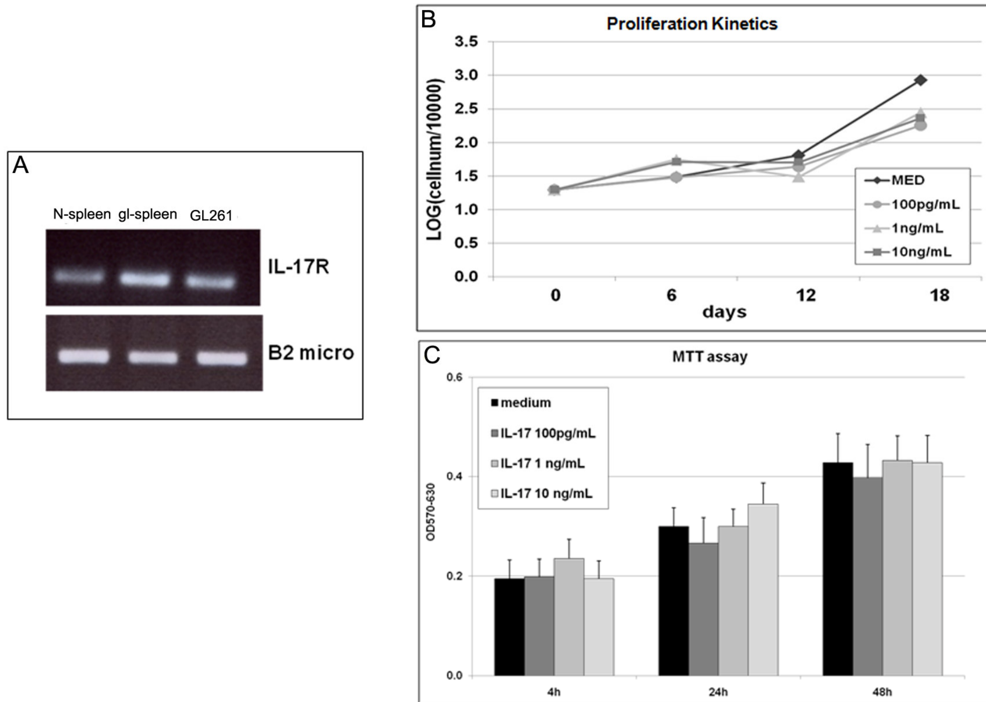
IL-17R: F 5'-TGGCCAGGAGTATGAAGTGACTG-3' R 5'-ATCTTCATCTTGCTGTCCTGACAG-3'

TNF-α: F 5'-GCCACCACGCTCTTCTGTCTA-3' R 5'-TGAGGGTCTGGGCCATAGAA-3'

Assays of target genes for TaqMan® Chemistry:

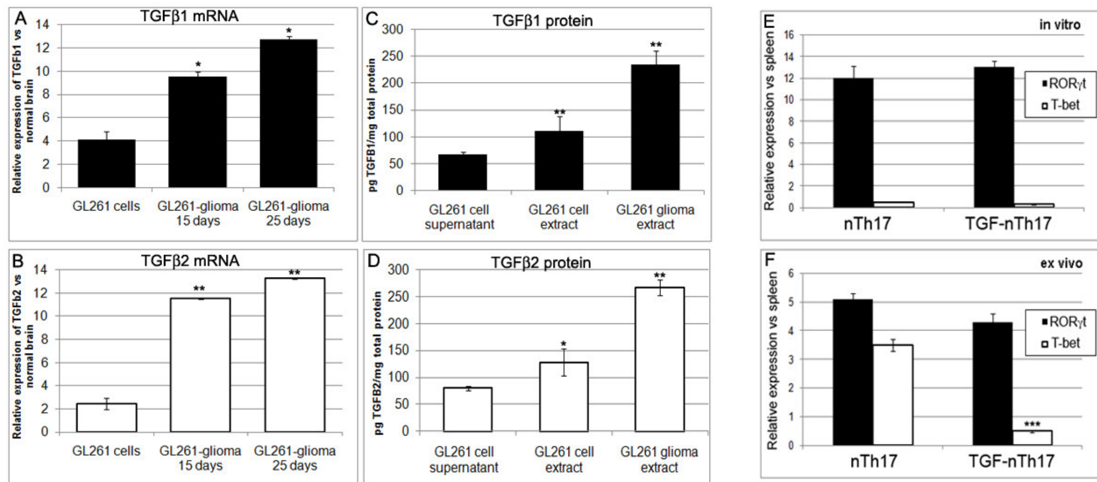
b2m: Mm00437762_m1, IFN-γ: Mm01168134_m1, IL-17A: Mm00439619_m1, Foxp3: Mm00475162_m1

Supplementary Fig. 3. ***In vitro*** **evaluation of IL-17A effects on GL261- cells proliferation**



A. Detection of IL-17 receptor (IL-17R) in GL261 cells was evaluated by a semi-quantitative PCR. GL261 cells express IL-17R compared with the positive controls naïve and gl-splenocytes. **B.** Proliferation Kinetic assay: to analyze the effect of IL-17 on GL261 proliferation *in vitro*, $2x10^5$ cells/flask were cultured *in vitro* for 18 days at different concentration of IL-17 and counted every six days. Cells number is reported as LOG (cell num/10000) and growth medium was used as negative control. Different concentration of IL-17 induce no significant change in the GL261 cells proliferation kinetic compared with negative control. **C.** MTT Assay (Millipore, USA) was used for quantification of GL261 proliferation using the protocol suggested by the manufacturer. Growth medium was used as blank. A standard curve was defined considering the absorbance in the MTT test (570-630 nm) and the number of cells and relative data were reported as histograms. GL261 cells were plated on 96-well flat-bottomed at a density of $5x10^5$ cells per well and cultured for 4, 24 and 48 hours at different concentration of IL-17. Values are mean results from triplicate cultures and are representative of three independent experiments. For each experimental condition, IL-17 has no effects *in vitro* on GL261 cells growth.

Supplementary Figure 4. TGF-β production by GL261 cells and the effect on RORγt and T-bet expression.



A, B. Total mRNA was isolated from GL261 cells and from GL261 gliomas explanted at two different time points (15 and 25 days after tumor implantation). The relative levels of each RNA was calculated by the $2^{-\Delta\Delta CT}$ method with beta-2-microglobulin as housekeeping gene control. Both TGF-β1 and TGF-β2 isoforms are expressed at higher levels by GL261-glioma compared to cultured cells (p=0.001). Gliomas explanted on day 25 day expressed higher level of both isoforms than gliomas explanted on day 15 after implantation (* p=0.01; ** p=0.001 cells and glioma day 15 vs glioma day 25). Data are representative of three different experiments. **C, D.** Production of TGF-β was measured by ELISA (R&D Systems, Minneapolis MN), in cell culture supernatant and in extracts obtained from GL261 cells and from intracranial gliomas. GL261-cells and explanted GL261-gliomas were homogenized in isotonic buffer. TGF-β levels were normalized to total protein content determined by MICROBCA assay (Pierce, Thermo Scientific, Rockford, IL). The data are expressed as **pg/mg total protein** and show mean ± SD of three independent evaluations.

Increasing TGF-β1 and TGF-β2 protein is prominent in extract obtained directly from explanted glioma (* p=0.01; ** p=0.001 supernatant and cell extract vs glioma extract) showing that the production of TGF-β isoforms increases *in vivo*. **E, F.** Both *in vitro* and *ex vivo* isolated cells express the transcription factor RORγt. T-bet expression in *in vitro* nTh17 and TGF-nTh17 cells was very low but detectable. When we evaluated *ex vivo* IL-17+ cells obtained from gliomas on day 20, we found increased expression of T-bet in Th17 cells from gliomas derived from co-injection with nTh17, supporting an *in vivo* generation of Th1/Th17 cells. IL-17+ cells from TGF-nTh17 gliomas showed decreased T-bet expression compared to nTh17 not exposed to TGF-β (*** p=0.0002).

Supplementary Table 1. Flow cytometry analysis of positive and negative fraction obtained from splenocytes

Positive fraction (68.0±1.5%)	**CD3/CD4 %**	**CD4/CD25/Foxp3 %**	**CD3/CD8 %**
n-splenocytes	71.6±14.8	0.5±0.3	3.3±1.6
TGF-n-splenocytes	82.8±3.9	0.4±0.1	3.5±0.1
gl-splenocytes	68.5±3.5	0.8±0.3	3.7±0.7
TGF- gl splenocytes	69.5±12.0	0.1±0.09	3.0±1.2
Mean±SD	**73.1±6.6**	**0.4±0.3**	**3.4±0.3**
Negative Fraction	**CD3/CD4 %**	**CD4/CD25/Foxp3 %**	**CD3/CD8 %**
n-splenocytes	11.2±4.8	6.2±1.2	9.3±1.9
TGF-n-splenocytes	17.7±3.3	12.6±2.7	7.4±2.6
gl-splenocytes	15.3±2.4	19.0±3.5	5.3±1.1
TGF-gl splenocytes	5.7±2.3	25.3±2.4	4.8±3.3
Mean±SD	**12.5±5.3**	**15.8±8.2**	**6.7±2.1**

T-test positive vs negative fraction	**CD3/CD4 %**	**CD4/CD25/Foxp3 %**	**CD3/CD8 %**
	<0.00001	0.003	0.02

IL-17 positive fraction is mostly enriched for CD3/CD4 + T cells (p<0.00001), depleted for CD4+ CD25+ Foxp3+ cells (p= 0.003) and for CD3/CD8+ T cells (p=0.02). *p* value was obtained comparing the positive fraction versus negative fraction. Data are presented as mean values ± SD obtained combining percentage obtained at two different time points of splenocyte pre-stimulation (24 and 48 hours).