Materials and Methods

Glioma cells

In vitro study have been performed using three adherent glioma cell lines: T70, U251 and U373. T70 were from Prof. G. M. Lauro from Laboratory of Pathology of University of "Roma Tre" in Rome (Fabrizi, C., 1994. J. Neuroimmunol.); U251 and U373 glioma cell lines were from ADCC (USA).

Glioma lines were manteined in culture medium (D-MEM supplemented with 10% of FBS, Fetal Bovine Serum, 2mM L-glutamine, Gentamycin 50 mg/ml), were washed twice in a week with sterile PBS 1X, removed with EDTA/Trypsin (Euroclone) at 37° C and washed with culture medium at 1700 rpm for 5 minutes; then were put in culture with 0.5% of CO_2 at 37° C. For co-culture with Vδ2 T cell lines, glioma cells were cultured 50,000 cells/250μl/well and maintained overnight at 37° C with 0.5% of CO_2.

GFAP Immunofluorescence

We used indirect immunofluorescence to analyse GFA protein expression in glioma cells. Briefly, 5,000-6,000 tumor cells/200μM/well were put in culture in chamber slides for 4 days at 37° C in 0,5% of CO_2. At 4^{th} day, confluent glioma cells were washed twice with PBS 1X and BSA 3% and fixed with Ethanol (-20°C) for 3 minutes. After fixation, glioma cells were washed twice with PBS 1X, and labelled with a mixture of primary antibody (polyclonal rabbit anti-GFAP (Z0334, 1:50) for one hour. Thereafter, glioma cells were washed with PBS 1X and labelled with the secondary antibody (FITC-conjugated anti-rabbit (Fab)' by DakoCytomation), 1:100 at room temperature. After one hour glioma cells were washed with PBS 1X and labelled with PBS 1X containing Glycerol. GFA protein was revelead at fluorescence microscope (Olympus BX-51).

Monoclonal Abs and FACS analysis

Phenotypic and functional analyses of Vδ2 T lymphocytes were done by flow cytometry.

The monoclonal Antibodies (mAbs) used for the study are: Vδ2 FITC (clone B6, Mouse IgG1,k, 1:50), CD3 Cy-Chrome (clone UCHT-1, Mouse IgG1,k, 1:50), CD3 APC (clone UCHT-1, Mouse IgG1,k, 1:50), CD27 PE (clone M-T271, Mouse IgG1,k, 1:50), CD45RA CY-Chrome (clone HI100, Mouse IgG2b,k, 1:50) IFNγ PE (clone 4S.B3, Mouse IgG1,k, 1:100), TNFα APC (clone Mab11, Mouse IgG1,k, 1:100), Perforin PE (clone GB12, Mouse IgG1, 1:50), TNFα PE (clone MAb11, IgG1, 1:100), IFNγ APC (clone B27, IgG1, 1:100) from BD Pharmingen; CD3 PerCP (clone SK7, Mouse IgG1,k, 1:50) from BD Biosciences (San Diego, CA, USA).

PBMCs preparation

Peripheral blood mononuclear cells (PBMCs) (obtained from residual samples from blood donors from Croce Rossa Italiana, courtesy of Dr. Girelli) were isolated from healty adults donors by gradient centrifugation on Ficoll- Isopaque (Pharmacia Biotech, Piscataway, NJ), counted by Trypan Blue (GIBCO BRL) and frozen 10^6 cells/ml in FBS (Fetal Bovine Serum, Euroclone) and 10% of Dimethyl-sulfoxide (DMSO, Euroclone,UK) at –150° C. PBMCs of healthy donors were thawed, or used freshly, then washed twice with culture medium (RPMI 1640 with fetal bovine serum 10%, 2 mM of L-glutamine and 10 UI/ml of penicillin/streptomycin), stained and counted by Trypan Blue and cultured 10^6 cells/ml in culture medium for the expansion of Vγ9Vδ2 T cells.

In vitro expansion of Vδ2 T lymphocytes

PBMCs of healthy adults donors were thawed or used freshly, washed twice with culture medium, stained and counted by Trypan Blue and cultured 10^6 cells/ml in culture medium with 100 UI/ml of recombinant Interleukin-2 (IL-2, Sigma-Aldrich). Phosphoantigen-specific stimulation of Vδ2 T cells was done by using Isopentenyl pyrophosphate (IPP, SIGMA, St. Louise, USA) 3 μg/ml. After seven days, culture medium was changed and added new medium and 100 UI of IL-2. At the 12^{th} day Vδ2 T cell lines purity was analysed by flow cytometry (FACS Calibur, BD Biosciences) as percentage of Vδ2/CD3 among total lymphocytes.

Vδ2 T cells surface and intracellular staining

The expression of differentiation markers (CD27 and CD45RA) were analyzed in Vδ2 T cell population by using specific monoclonal antibodies (mAbs). Phenotypic analysis of Vδ2 T cell lines was done at 12^{th} day before and after the co-culture with glioma cells. Brefly, Vδ2 T lines from healty donors were used freshly and washed in culture medium (RPMI supplemented with 10% of FBS, Fetal Bovine Serum, 2mM L-glutamine, 50 IU/ml Penicillin and 50 μg/ml Streptomycin) and put in co-culture with glioma cells (1:1 ratio) for 18hs. For cell staining, Vδ2 T lines were incubated for 10 minutes at room temperature in a mixture of mAbs and then washed in PBS 1X. Samples were then fixed in Paraformaldeyde (PFA) 1% (Sigma) for 5 minutes at room temperature, washed in buffer of PBS 1X, NaN_3 0,1%, BSA 1% and acquired by cytometer (FACS Calibur, BD Biosciences).

In order to evaluate the ability of Vδ2 T cell lines to produce cytokines, thawed or freshly Vδ2 T lines were adjusted to final concentration of $1x10^6$ cells/ml in culture medium, was added IL-2 (100 UI) and were harvested overnight at 37° C in presence of 0,5% CO_2. The next day, samples were cultured for 18hs with glioma lines (1:1 ratio) untreated and treated with Zoledronic Acid (150

μM). Vδ2 T cells positive control was done IPP (30μg) stimulation. For intracellular cytokines analysis, after 2hs from the beginning of culture, Brefeldine A (BFA) (10 μg) (Serva, Electrophoresis) was added to block Golgi transport and allow cytokines accumulation. At the end of stimulation, samples were stained with monoclonal antibodies specific for surface markers, as described in the previous paragraph, and fixed with PFA 1% (e-Bioscience) for 5 minutes at room temperature. Then, intracellular staining was performed by incubating cells for 10 minutes at room temperature with cytokine- (IFN-γ and TNF-α) specific antibodies in permeabilizing solution (PBS1X, NaN_3 0.1%, BSA 1%, saponin 0.5%). After washing with 100 μl of wash-solution (PBS1X, NaN_3 0.1%, BSA 1%, 0.1% of saponin) cells were acquired by cytometer (FACS Calibur, BD Biosciences). Sample were analyzed with Cell Quest program and significance was evaluated by Mann-Whitney and Student's T tests (* $p<0.05$).

In vivo Perforin release by Vδ2 T cells

To analyze the content of perforin in Vδ2 T cell lines before and after the co-culture with glioma cells. Glioma cells and Vδ2 T cell lines were co-cultured for 18hs in the absence of BFA, in order to monitor perforin release by Vδ2 T cell lines. Vδ2 T cell lines were stained with anti-perforin monoclonal antibody in intracellular staining as described above, and analyzed by cytometer (FACS Calibur, BD Biosciences). Perforin release was evaluated in Vδ2 T cells as percentage of Vδ2 T cells positive for perforin in respect to Vδ2 T cell lines CTRLs.

Cytotoxicity assay

In order to evaluate the ability of Vδ2 T cell lines to kill glioma cells and in order to analyse glioma cells death, freshly Vδ2 T lines were adjusted to final concentration of $1x10^6$ cells/ml in culture medium and were cultured overnight at 37° C in 0.5% CO_2. Glioma cell lines were cultured at 50,000 cells /well. The next day, Vδ2 T cell lines were co-cultured for 18 hs with glioma lines (1:1 ratio) untreated/treated with different concentration of Zoledronic Acid (6 μM, 150 μM, 400 μM). In order to analyse glioma cells viability before and after co-culture with Vδ2 T cell lines after 18hs co-culture, glioma cells were labelled with Annexin and Propidium Iodide (Annexin V/Fitc Kit, Bender MedSystems, CA, USA). Brefly, glioma cells were treated with a solution of Trypsin-EDTA 1X (Cambrex, Belgium) for 5 minutes at 37° C in 0.5% CO_2. After washing with PBS 1X at 1800 rpm for 5 minutes, samples were labelled in Binding Buffer 1X (Annexin V/Fitc Kit, Bender MedSystems, CA, USA) with anti-Annexin V mAb for 10 minutes at room temperature. After washing with Binding Buffer solution, cells were labelled with Propidium Iodide and analyzed by

cytometer (FACS Calibur, BD Biosciences). Percentage of glioma cells death was calculated among gated glioma cells.

Drug treatment

To analyse Vδ2 T cell lines response to glioma cells after drug-treatment, we used Zoledronic Acid (Zol) (Zometa 4mg, Novartis, UK) to block FPP Syntase of Mevalonate cycle of glioma cells, known to be able to induce IPP accumulation and consequent activation of Vδ2 T cells. Zol was used 150μM in order to study Vδ2 T cell lines activation, and in different concentrations (6μM, 150μM, 400μM) for cytotoxicity Annexin and Propidium Iodide test on glioma cells.

Microscopy

To analyze GFA protein expression on glioma cells was used a fluorescence-microscope (Olympus BX-51) and to evaluate morphologic changes in glioma culture before/after co-culture with Vδ2 T cell lines a fluorescence microscope was used (Nikon Eclipse TE200- and Nikon Digital Camera XM1200F).