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Analysis of Vγ9Vδ2 T lymphocytes response to human glioma cell lines: possible implications for therapy

Analisi della risposta dei linfociti T Vγ9Vδ2 a linee cellulari di glioma umano: possibili implicazioni per la terapia

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To myself

and to all young Italian researchers

Index

1. SUMMARY

1.1 Italian language

Analisi della risposta dei linfociti T Vγ9Vδ2 al glioma umano: possibili implicazioni per la terapia

I linfociti T γδ rappresentano un ponte di collegamento tra immunità innata ed immunità acquisita. Nell'uomo i linfociti T Vγ9Vδ2 (Vδ2) rappresentano il 90% dei linfociti γδ circolanti nel sangue periferico, e riconoscono piccoli antigeni non peptidici, come l'isopentenile pirofosfato (IPP), prodotti nel ciclo di sintesi degli isoprenoidi, con un meccanismo non ristretto dall'MHC. A seguito del riconoscimento si attivano, rilasciano grandi quantità di citochine e chemochine, e finalmente differenziano in cellule effettrici. I linfociti T Vδ2 possono essere attivati in modo indiretto dai farmaci bifosfonati mediante un meccanismo indiretto mediato dal blocco del farnesil-pirofosfato sintetasi, un enzima del ciclo di sintesi del colesterolo, inducendo così l'accumulo di intermedi fosforilati del ciclo, tra cui l'IPP, capaci di attivare i linfociti T Vδ2.

Questo tipo di meccanismo è anche responsabile del riconoscimento diretto da parte dei linfociti T Vδ2 di cellule tumorali, nelle quali una dis-regolazione del ciclo del mevalonato induce un accumulo di IPP. Diversi studi dimostrano che i linfociti T Vδ2 riconoscono e uccidono diversi tipi di cellule cancerose: cellule di linfoma, del carcinoma del colon, renale, del seno, del polmone e di tumori cerebrali. In pazienti con carcinoma renale, è stata proposta una terapia basata sull'utilizzo di linfociti T Vδ2 stimolati con farmaci sintetici fosforilati e i risultati sono stati promettenti.

Gli amminobifosfonati (Acido Zoledronico, ZOL) sono farmaci utilizzati attualmente per la cura dell'osteoporosi e di metastasi ossee del cancro alla prostata, del seno e del mieloma multiplo. In questo contesto la possibilità di attivare ed espandere *in vitro* una popolazione cellulare specifica può aprire nuove e interessanti prospettive per la immunoterapia dei tumori.

I gliomi sono tumori maligni del sistema nervoso centrale e originano dalle cellule della glia. I pazienti con gliomi hanno un' aspettativa di vita di circa un anno dalla diagnosi e a seguito del trattamento chirurgico e chemioterapico. A motivo di ciò sono necessari studi per nuove strategie immunoterapeutiche per il trattamento del glioma.

L'obiettivo del nostro lavoro è stato di analizzare *in vitro* la risposta dei linfociti T Vδ2 verso linee cellulari di glioma umano e di verificare la possibilità di utilizzare questo target cellulare per nuovi approcci

immunoterapici. A questo scopo sono state utilizzate 3 linee tumorali di glioma umano (T70, U251 e U373), e linfociti T Vδ2 ottenuti da cellule mononucleate di sangue periferico (PBMC) di donatori sani. Lo studio dell'interazione tra gliomi e linfociti T Vδ2 (fenotipo, attivazione, citotossicità ed esiti del trattamento farmacologico con lo ZOL *in vitro*) è stato studiato tramite citofluorimetria a flusso. Il fenotipo delle linee cellulari di glioma è stato analizzato tramite immunofluorescenza indiretta per la proteina GFAP (glial fibrillar acid protein), che è una proteina del citoscheletro delle cellule della glia la cui espressione è inversamente proporzionale al grado di malignità del glioma.

In un primo set di esperimenti è stato testato *in vitro* un protocollo di espansione dei linfociti T Vδ2 *ex vivo* ottenuti da donatori sani e stimolati con il fosfoantigene (IPP) e Interleuchina 2. Dopo 12 giorni di coltura, sono state ottenute linee Vδ2 short-term al 90% di purezza ed è stato analizzato il loro profilo differenziativo, come espressione dei markers fenotipici CD27 e CD45RA (markers di maturazione cellulare propri delle cellule T, B, NK) la funzionalità (come produzione di citochine pro-infiammatorie, quali IFNγ e TNFα) e la citotossicità (come rilascio naturale di perforina). I linfociti T Vδ2 espansi *in vitro* presentano un fenotipo effettore, producono citochine e rilasciano perforina a seguito di una stimolazione con il fosfoantigene. Nel contempo è stata studiata l'espressione della proteina GFAP sulle tre linee di glioma umano, confermando mediante l'espressione di GFAP la loro origine gliale.

In un secondo set di esperimenti è stata valutata la risposta delle linee T Vδ2 verso le tre linee cellulari di glioma e la loro vitalità dopo la co-coltura. Nel nostro sistema sperimentale i linfociti T Vδ2 riconoscono tutte e tre le linee di glioma umano e differenziano in cellule effettrici, rilasciano perforina e uccidono i gliomi per apoptosi e necrosi. E' stato poi valutato l'effetto del trattamento delle linee di glioma con ZOL prima della co-coltura sulla risposta delle linee T Vδ2, dimostrando che le linee T Vδ2 riconoscono tutte e tre le linee cellulari di glioma trattati con ZOL producendo una maggiore quantità di IFNγ e TNFα. E' stato inoltre valutato l'effetto diretto dello ZOL sulle linee di glioma prima/dopo co-coltura con le linee T Vδ2. Il trattamento con lo ZOL induce apoptosi nelle cellule di glioma, ma solamente la co-coltura con le linee Vδ2 induce apoptosi e necrosi evidenti in tutti i gliomi trattati.

Nel nostro modello sperimentale è stato dimostrato che i linfociti T Vδ2 sono stati in grado di riconoscere tutte le linee di glioma umano utilizzate (T70, U251 e U373), di differenziare in cellule effettrici che rilasciano perforina, e di uccidere le linee di glioma per apoptosi e necrosi, suggerendo

un meccanismo di citotossicità naturale. Il trattamento delle linee di glioma con lo ZOL si è dimostrato avere un doppio effetto: un'attività antitumorale diretta verso i gliomi e un' attività indiretta sui linfociti T Vδ2, che aumentano la propria capacità effettrice verso le linee di glioma.

Nella loro totalità questi risultati mostrano che l'induzione di una forte risposta dei linfociti T Vδ2 verso il glioma umano mediante l'utilizzo di amminobifosfonati può aprire a nuove strategie immunoterapeutiche per la cura di questa tipologia di cancro.

1.2 English language

Analysis of Vγ9Vδ2 T cells response to human glioma: possible implications for therapy.

γδ T cells link innate and acquired immunity. In humans 90% of circulating γδ T cells express the Vγ9Vδ2 TCR rearrangement and recognize non peptidic antigens in a MHC-unrestricted manner. After antigen recognition, activated Vγ9Vδ2 T cells rapidly proliferate, produce high levels of cytokines and chemokines and can differentiate in cytotoxic effector cells. Specifically, Vγ9Vδ2 T cells recognize unprocessed non-peptidic compounds such as isopentenyl pyrophosphate (IPP), which are produced through the isoprenoid biosynthesis pathway. Moreover, Vγ9Vδ2 T cells can also be activated by aminobisphosphonates drugs through an indirect mechanism: they inhibit farnesyl pyrophosphate synthase, an enzyme of cholesterol biosynthesis, acting downstream of IPP synthesis; this inhibition, in turn, leads to the accumulation of endogenous IPP, directly recognized by Vγ9Vδ2 T cells. A specific feature of Vδ2 T cell biology is their ability to recognize tumor cells presenting a dis-regulation in mevalonate pathway, resulting in an increase of phosphorilated metabolites such as isopentenyl-pyrophosphate (IPP). Thus, the increased isoprenoid metabolism in cancer cells induces Vδ2 T cell activation through cellular IPP accumulation. Mevalonate cycle is present in all eucaryotic cells and produce cholesterol and prenyl-compounds. The main two enzymes (HMG-CoA reductase and FPP synthase) in the mevalonate pathway are carefully regulated and can be farmacologically modulated by different drugs (mevastatin and aminobisphosphonates respectively). Several studies show that Vδ2 T cells recognize and kill several cancer cells, such as lymphoma, colon-, lung-, renal, breast carcinoma, and glioma. In renal cancer patients, a Vδ2-based immunotherapy with a synthetic phosphorilated compound is in course with promising results. Similarly, aminobisphosphonates (Zoledronic Acid) is currently used for bone metastases in prostate cancer patients. In this context, the possibility to massively activate and expand *in vitro* a relatively large number of cells opens new interesting prospects in the immunotherapy of cancer disease. Gliomas are tumors arising from glia or their precursors within the central nervous system. Unfortunately, the majority of patients with glioma tumors die in less then of one year; in these patients, new treatment strategies are therefore hardly needed.

Aim of this study was to analyse the activity of human Vδ2 T cells against glioma cancer cells and to verify the possibility to target these innate cells in new immunotherapeutical strategies.

In a first set of experiments, we set up an *in vitro* protocol able to expand human Vδ2 T cells by using IPP and IL-2. After 12 days the expanded Vδ2 T cell lines (80-95% of purity) were analysed for their differentiation phenotype, (as expression of CD27 and CD45RA markers of T, B, NK cells), cytokines production (IFNγ and TNFα) and natural cytotoxicity capability (Perforin). Results showed that *in vitro* expanded Vδ2 T cell lines present an effector memory phenotype and have high functionality both in terms of cytokines production and Perforin release. We then studied three different glioma cell lines: T70, U373 and U251 by analyzing GFAP expression on cell surface by direct immunofluorescence. Resulted showed that all glioma cells was positive for GFAP.

In a second set of experiments, Vδ2 T cell lines were co-cultured with glioma cells in order to analyse the activation of Vδ2 T cells and the effects on the viability of glioma cells. In our system, Vδ2 T cell lines were able to recognized glioma cells (T70, U373, U251) by specifically differentiate in effector memory cells, and release Perforin. In contrast, they did not produce cytokines. In order to verify the cytotoxic effect of Vδ2 T cells on glioma cells, we performed a viability test on glioma cells in the absence and in the presence of Vδ2 T cell lines. Briefly, glioma cells were labelled with Annexin/Propidium Iodide and were analysed by flow cytometry. Interestingly, Vδ2 T cells were able to kill glioma cells through an apoptotic mechanism, demonstrating their antitumoral activity.

We then decided to study if Zoledronic Acid (ZOL) treatment of glioma cells could improve Vδ2 T lines response. Glioma cells were treated with ZOL *in vitro* for two hours, and co-cultured with Vδ2 T cell lines, analyzing Vδ2 T cells response by flow cytometry. Results showed that Vδ2 T cell lines were able to recognize glioma cells by releasing high amount of IFNγ and TNFα. Vδ2 T cells activation was mediated by ZOL-induced IPP accumulation, since the incubation with mevastatin was able to completely block this biological effect. Finally, we studied the direct effect of different concentrations of ZOL on glioma cells viability before and after the co-culture with Vδ2 T lines. We observed that treatment with ZOL induced necrosis on glioma cells, but only the co-culture with Vδ2 T lines together to the treatment with ZOL increased both the apoptosis and necrosis of glioma cells.

Altogether, our results suggest that the induction of a strong antitumoral response of Vδ2 T cells by using aminobisphosphonates could represent a new interesting immunotherapy approach for glioma.

2. INTRODUCTION

The immune system is crucial in defending host from both microbial pathogens and autologous mutant tumor cells. Innate or natural immunity is the first line of defense against infections and tumors by using a fast non-specific response, that followed by the induction of specific, acquired immune response able to trigger very effective protection mechanisms such as cell-mediated immunity and humoral immunity and inducing immunological memory among adaptive immune cells. T lymphocytes are the principal cell-subset in cell-mediated immunity: they recognize peptidic antigens displayed by antigen presenting cells (APCs) in association with MHC complex molecules. This recognition is mediated by the T cell receptor (TCR), and induces activation and clonal expansion of specific clones of T cells. T cells activation improves also B cell differentiation into plasma cells, responsible for soluble antibody production, and may promote by soluble factors macrophages and neutrophils activation and recruitment. T lymphocytes may be divided in two major classes upon the expression on cell surface of different markers: T helper lymphocytes express CD4 molecule (fig. 1, A), while T cytotoxic lymphocytes express CD8 molecule (fig. 1, B). Natural killer T cells (NKT) are a unique cell subset that recognize glycolipid antigens presented by CD1d molecules; NKT may produce Th1 and Th2 cytokines, and are involved in several types of immune responses. Upon specific activation with alpha-Galactosylceramide (alpha-GalCer), NKT cells show strong antitumor immune responses through direct cytotoxicity and indirect activation of a cascade of antitumor effector cells such as natural killer (NK) cells and CD8+ cytotoxic T cells. In addition to alpha-GalCer, many other CD1d ligands, including self and bacterial glycolipids and modified synthetic glycolipid antigens, have also been discovered (fig. 1, C). B lymphocytes are important for humoral immune response by producing antibodies after BCR activation (B cell receptor) by antigens. B cells need two different signals for activation: BCR/antigen signal-mediated and CD40/CD40L mediated-signal between B and T cell (fig. 1, D); after triggering, B cells differentiate into mature plasma cells able to produce antibodies that eliminate pathogens.

Innate immune system is composed by non-specific mechanisms such as anatomic (epidermidis and mucosal comparts) and physiologic barriers

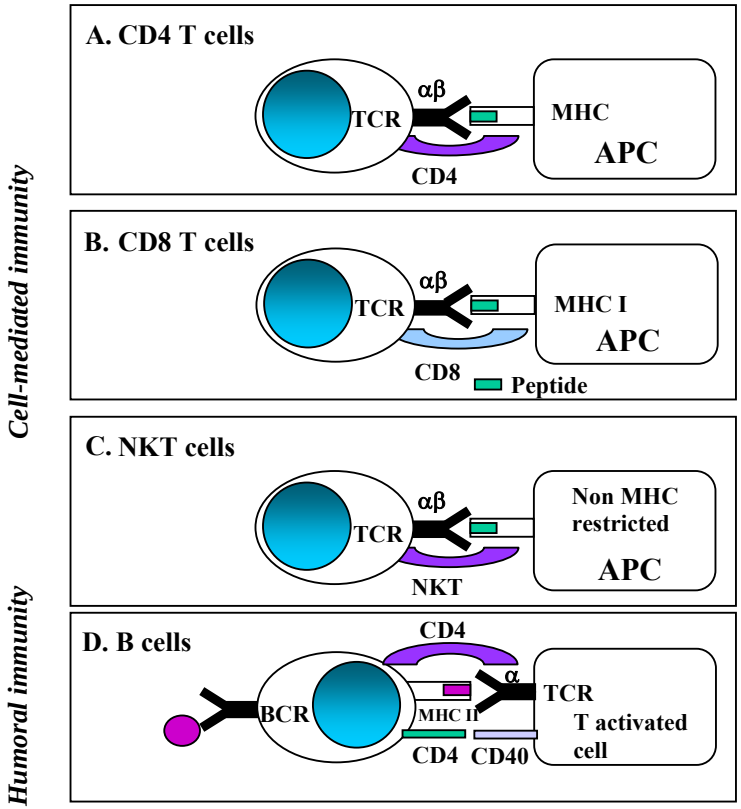

Figure 1. Acquired immunity cells and activation mechanisms. This figure shows CD4, CD8, NKT and B cells populations with their activation mechanisms.

(temperature, PH and chemical mediators), by different cellular populations with phagocytic activity (neutrophil and macrophage cells), cytotoxic activity (NK cells), and with antigen presentation functions (monocytes and dendritic cells). Effectors cells of innate immunity are neutrophils and natural killer cells (fig. 2). Phagocytosis is displayed by neutrophils (fig. 2, A) and macrophages (fig. 2, B) able to eliminate pathogens. Neutrophil cells represent about the 50-70% of circulating leukocytes and present a peculiar morphology, with granules enriched of proteolitic enzymes in cytoplasm. Macrophages (fig. 2, B) and monocytes (fig. 2, C) have an important role against microbial infections; they display cytoplasm lysosomes, vacuols and cytoscheletric filaments. Dentritic cells (fig. 2, D) are similar to monocytes but are different in morphology because they grow branched projections, called dendrites. They are localised in cutaneus epithelium, in the mucosa of respiratory and gastro-enteric comparts; after contact with antigen become mature APCs, migrate in lymph nodes and present antigens to T cells inducing specific immune response. Natural killer (NK) cells represent about the 5-20% of mononuclear cells in spleen and of the peripheral blood. They recognize infected or tumor cells by using two different types of receptors such as activatory and inhibitory receptors (NKRs) (fig. 2, E) able to modulate their effector functions.

In humans, a minor subpopulation of lymphocytes (3-6% of total circulating lymphocytes) express gamma delta TCR (fig. 3). They present some particular characteristics, acting as a bridge linking natural and acquired immunity. This subset is rare in the adult thymus, but increases with the age in the blood, suggesting a positive selection in the periphery consecutive to a sustained antigenic stimulation (1, 2). Although little is known about the physiologic significance of γδ T cells, their marked reactivity toward mycobacterial, parasitic and tumoral Ags (4-10), their accumulation in human infectious disease lesions (11) and in several autoimmune pathologies (12-17) and the strong antitumor response against different type of cancer cells (18-26) suggest that γδ T cells play an important and central role in the context of the immune response. About 90% of gamma delta T cells express the Vδ2 variable segment associated with the Vγ9 rearrangement with a disulfide link (1, 3). Remaining 10% of γδ circulating T cells express the Vδ1 rearrangement with a VγX variable segment distinct from Vγ9 region (27-28). The expression of Vδ2 or Vδ1 receptor is associated to a different anatomic distribution. Vδ1 T cells are localized in tissues, such as cutis (29-30), intestinal epithelium (31) and in the nasal

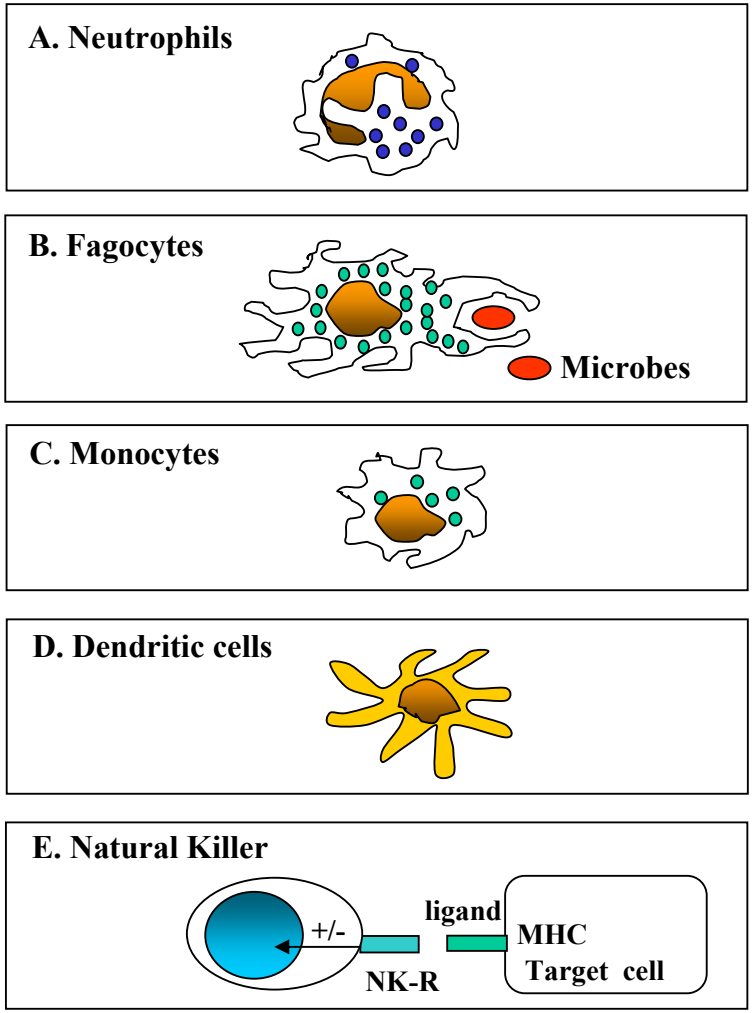

Figure 2. Innate immunity cells. The morphology of innate cells is represented (Neutrophils, Fagocytes, Monocytes, Dendritic cells, Natural Killer cells).

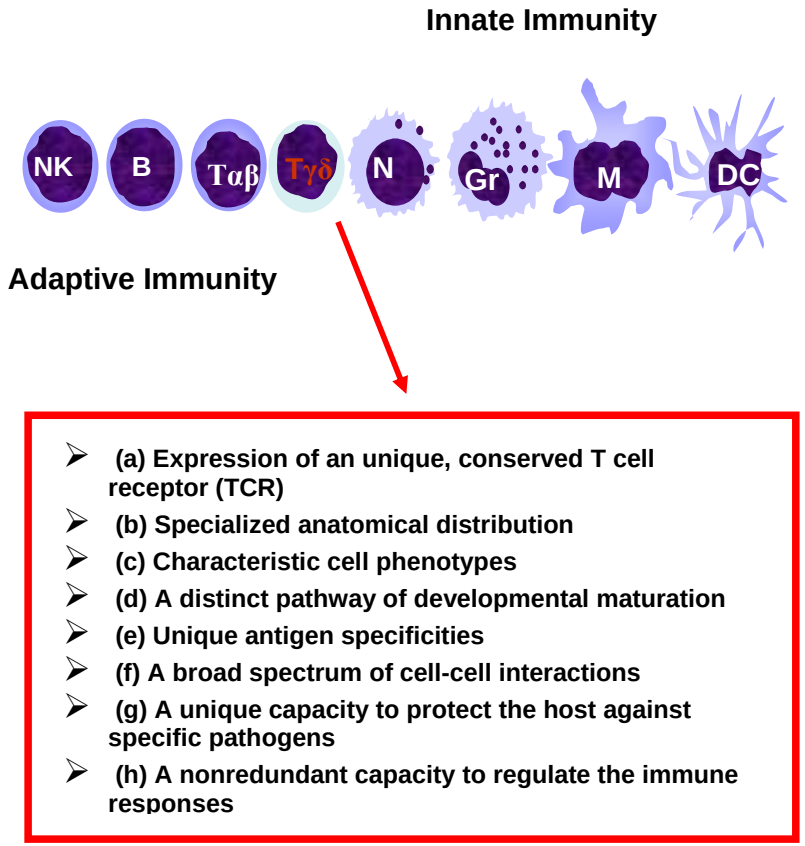

Figure 3. γδ T lymphocytes. A point of connection between innate and acquired immunity.

mucosa (32) and are thought to control pathogens entry in these sites. The mechanism of recognition of Vδ1 T cells are still scarcely known, but they have been shown to be able to recognize the CD1c molecule by TCR-mediated response, and by NKG2D receptor stress-induced proteins, such as MIC-A and MIC-B (33).

Vγ9Vδ2 T lymphocytes (also Vδ2 in literature) circulate in peripheral blood and in lymph nodes working as "sentinels" able to kill directly infected or neoplastic cells. In contrast to conventional αβ T lymphocytes, Vγ9Vδ2 T cells respond to non processed (34) and non-peptidic antigens in a HLA- unrestricted manner (3, 35- 39). These Vδ2 specific antigens include different intermediates of the mevalonate or non-mevalonate pathway of cholesterol biosynthesis (40), called phosphoantigens. Although no presenting molecule seems to be required, phosphoantigens-induced Vγ9Vδ2 T lymphocytes activation requires cell-cell contact (41, 42) and an immunological synapsis (42). Indeed, several studies have demonstrated that the components of mycobacteria responsible for the expansion of human Vγ9Vδ2 T cells are protease resistant (5), but alkaline phosphate sensitive (35, 36). Several mycobacterial Ags recognized by Vγ9Vδ2 T cells were identified, such as the isopentenyl pyrophosphate (IPP) and related prenyl pyrophosphate derivatives (38), and as a variety of other phosphorilated metabolites, including dimetyallylpyrophosphate, glycerol-3-phosphoric acid, or ribose-1-phosphate (37), which are naturally occurring metabolites in prokaryotic and eukaryotic cells (fig. 4). Four related phosphorylayed components, termed TUBAg 1-4 (fig. 5), isolated from Mycobacterium tuberculosis, were also reported to trigger the proliferative response of Vγ9Vδ2 T cell clones and to induce the selective *in vitro* amplification of peripheral blood Vγ9Vδ2 T cells from healty donors (36). These mycobaterial antigens were shown to trigger the cytotoxicity of Vγ9Vδ2 CTL against a broad set of target cells and to induce massive TNF production by these clones (41).

Particular characteristic of these little phosphoantigens (molecular weight about 500 Da) is the bond with a phosphate or aminic group in C2-C5 position in their carbonious structure. The mechanism of recognition of these compounds is thought to include a direct bond between Vγ9Vδ2 TCR and phosphoantigens (fig. 6, A) (35, 37, 38, 43). Several studies have shown that this recognition involve the residue of lysins 108 and 109 of Jγ 1.2 of CDR3 region of gamma chain and the residue of lysin 51 of CDR2 region of Vδ2 chain of γδ TCR (fig. 6, B-C) (44, 47). Moreover, mutagenesis

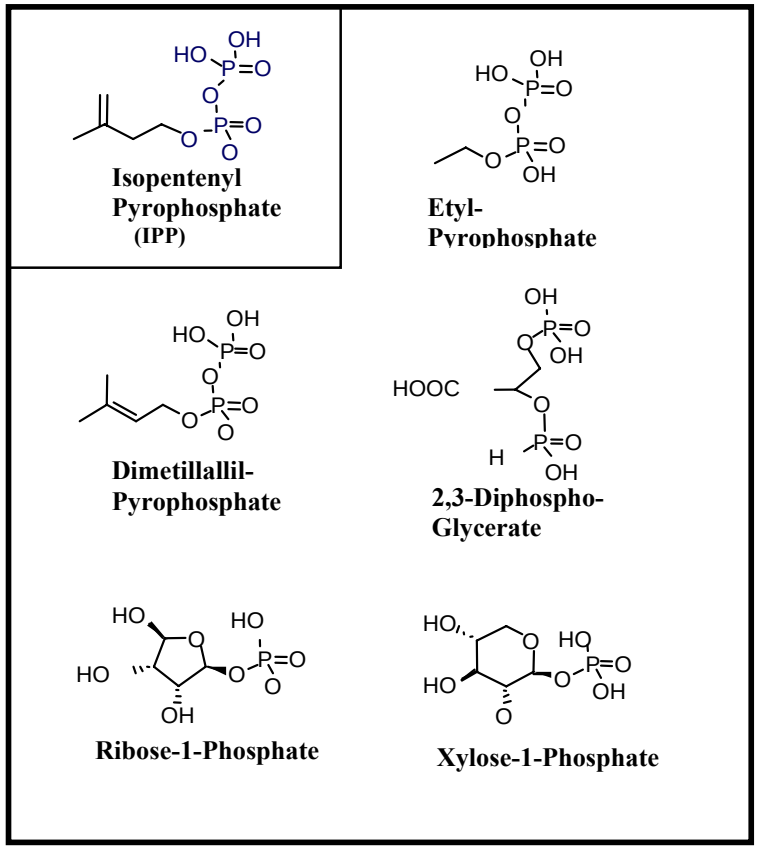

Figure 4. Non peptidic phosphorylated compounds. Chemical structures of some non peptidic compounds able to stimulate Vγ9Vδ2 T lymphocytes.

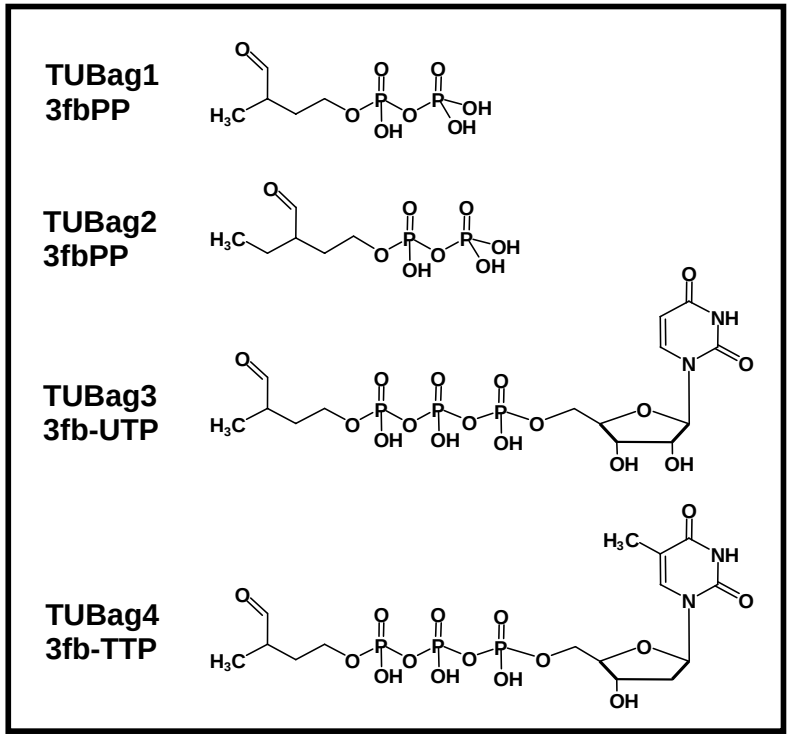

Figure 5. Some phosphorylated compounds of Mycobacterium Tuberculosis. Phosphorylayed components, termed TUBAg 1-4, isolated from Mycobacterium Tuberculosis, able to trigger the proliferative response of Vγ9Vδ2 T cells.

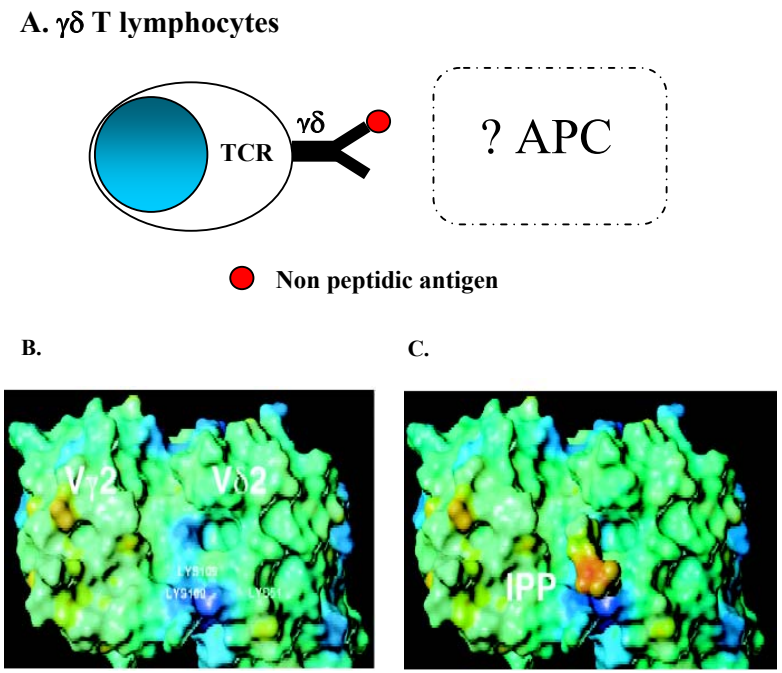

Figure 6. Vγ9δ2 T lymphocytes. A. Vγ9Vδ2 T lymphocytes phosphoantigens recognition. **B, C**. Cristallography analysis of Vγ9Vδ2 T lymphocytes phosphoantigens recognition (44, 47).

experiments have shown that this interaction involves the phosphate *core* of the antigen (45). Recently, it has been identified a new class of compounds, called bisphosphonates (fig. 7) that are able to activate Vγ9Vδ2 T cells *in vitro* and *in vivo* through an indirect mechanism (48-50). These drugs are synthetic analogs of endogenous pyrophosphates (51, 52) and are used for the bone resorption in osteoporosis (53) and in bone metastasis of breast (54, 55), prostate (56), melanoma (57), osteosarcoma (58, 59) and myeloma (60, 61) cancers. They are able to interfere with mevalonate cycle by blocking the proteic prenylation (62) and the bone resorption in the osteoclasts of bone marrow. It was observed that these compounds (risedronate, alendronate, pamidronate and zoledronate) are able to activate *in vitro* and *in vivo* peripheral Vγ9Vδ2 T cells of patients with multiple myeloma (48, 49, 63) by a monocytes-depending mechanism (44). More specific studies showed that these compounds act by blocking monocytes mevalonate cycle and inducing the accumulation of intermediate phosphorilated molecules, such as IPP, able to activate Vγ9Vδ2 T cells.

Like αβ T lymphocytes, Vγ9Vδ2 present CD27 and CD45RA differentiation markers, and their presence may be used to recognize four subsets different for their capacity to proliferate and differentiate in response to antigen or homeostatic cytokines: Naïve (CD27+/ CD45RA+), Central Memory (CD27+/ CD45RA-), Effector Memory CD27-/ CD45RA-), and Terminally Differentiated (CD27-/ CD45RA+) (64-66). Functionally, mature Effector Memory and in particular Terminally Differentiated Vγ9Vδ2 T cells display a potent cytotoxic activity against virus-infected target cells (67), and against tumor cells, such as lymphoma (68), multiple myeloma (21, 48, 62), colon carcinoma (22), renal cell carcinoma (69-71), pancreatic adenocarcinoma (72, 73), breast cancer (18), melanoma (19, 23), and brain tumors (24-26). It has been shown that in tumor cells intracellular level of IPP can be increased for disregulations in their mevalonate pathway, and Vγ9Vδ2 T cells are therefore able to recognized tumors cells on the basis of their enhanced IPP production (40, 74). It has been proposed to use bisphosphonates drugs, through Vγ9Vδ2 T cells activation, to strengthen antitumor innate and specific immune responses (75-79).

The high frequency of Vγ9Vδ2 T lymphocytes in most individuals and their ability to react towards small conserved nonpeptidic compounds, their different effector functions, and their broad reactivity against infected and tumor cells, make them a promising targets for immunotherapy. Recently, there was a broad interest for the possible application of Vγ9Vδ2 T lymphocytes in cancer immunotherapy, and clinical phase I/II studies are in

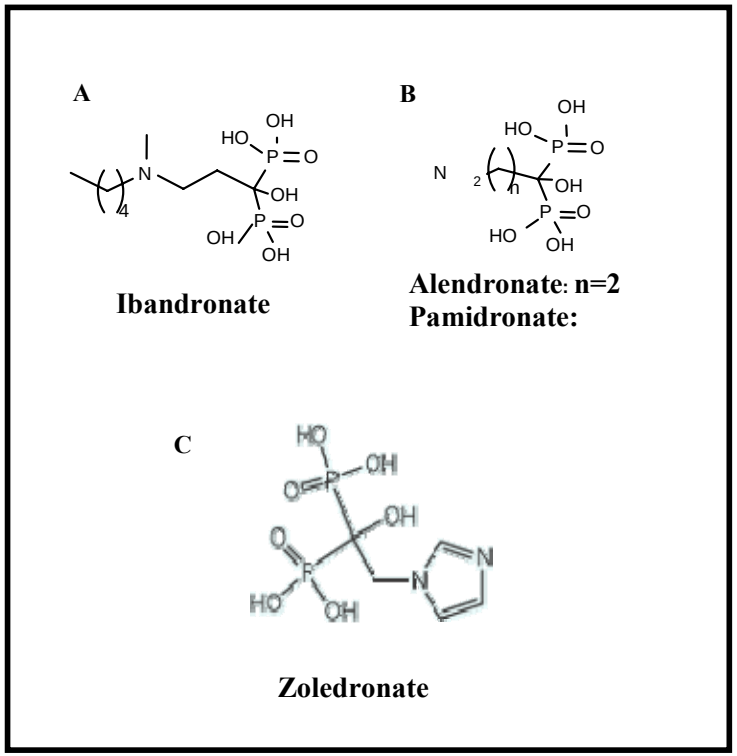

Figure 7. Bisphosphonates. Chemical structures of some bisphosphonates compounds able to activate Vγ9Vδ2 T lymphocytes.

progress (70, 71, 74, 80-84). Several studies aiming to study the *in vivo* direct activation of Vγ9Vδ2 T cells were recently completed or are ongoing. For example, increase of serum level of IFNγ and TNFα was observed in primates a few hours after injection of aminobisphosfonates (85-86) during the Phase I clinical trial of Bromohydrin Pyrophosphate, BrhPP (Phosphostim). Co-administration of both Vδ2-agonists and recombinant IL-2 led a significant expansion of Vγ9Vδ2 T cells in several cancer patients (87), in line with pre-clinical primate studies (85, 86). Interestingly, in renal cancer patients, a Vδ2-based immunotherapy with a synthetic phosphorilated compound is giving very promising results (71) and tumor stabilization or partial regression was seen in several multiple myeloma patients responding to the Vγ9Vδ2 T cells stimulation protocol (87).

Among tumors, glioma are an important cause of cancer- related deaths in children and young adults (88). Patients have a very poor prognosis, with a median survival of about one year, despite a radical treatments, including surgical resection, irradiation and chemotherapy (89). New, more targeted approaches, such as immunotherapy, antiangiogenic and gene therapy, have shown to be promising in experimental models, but their effectiveness in clinical settings remain to be proven (90-92).

The aim of this study was to analyse the activity of human Vγ9Vδ2 T cells against glioma cancer cells and to verify the possibility to target these innate cells *in vivo* in new immunotherapeutic strategies. We have analyzed Vγ9Vδ2 T cells mediated immune response (cytokines production and natural cytotoxicity) against three glioma lines, T70, U251 and U373, and the effect of Zoledronic Acid (ZOL) in glioma treatment to enhance Vδ2 T cells response. In our system, Vδ2 T cell lines were able to recognize glioma cells by specifically differentiating in effector memory cells able to release perforin. Interestingly, Vδ2 T cells were able to kill glioma cells, demonstrating their direct antitumor activity. We also studied if zoledronic acid treatment of glioma cells could improve Vδ2 T cell response; Vδ2 T cell lines were indeed able to recognize ZOL-treated glioma cells by releasing high amounts of IFNγ and TNFα. We also observed that ZOL-treatment is able to induce glioma cells apoptosis that is massively increased by the add of Vδ2 T cell lines. Altogether, our results suggest that the induction of a strong antitumor response of Vδ2 T cells lines by using Zoledronic Acid could represent a new interesting immunotherapy approach for glioma.

3. AIM

In human, most of our knowledge about the specificity and the biological role of γδ T lymphocytes is derived from analysis of the major peripheral subset referred to as Vγ9Vδ2 T cells. A particular importance is given by recent results that have provided new hints about the mode to activate these lymphocytes in immune response against renal and colorectal carcinomas, specifically, with drugs aminobisphosphonates following their manipulation for immunotherapeutic purposes. The aim of this study was to analyse the activity of human Vγ9Vδ2 T cells against glioma cancer cells and to verify the possibility to target these innate cells *in vivo* in new immunotherapeutic strategies for glioma cancers care.

4. RESULTS

4.1 Glioma cells (T70, U251 and U373) express GFAP glioma specific marker

Glial fibrillary acid protein (GFAP) is a 50-kD intracytoplasmic filamentous protein that constitutes a portion of the astrocytes's cytoskeleton. This protein has proved to be one of the most specific markers of cells of astrocytic origin under normal and pathological conditions. Interestingly, with increasing astrocytic malignancy, there is a progressive loss of GFA protein expression (93, 94). In order to analyse the phenotypic characteristics of three glioma cell lines used in this study (T70, U251 and U373), the expression of GFA protein was monitored by indirect immunofluorescence. All glioma cells were positive for GFAP, confirming their glial origin (fig. 8, panels A, B, C).

4.2 Short Term Vδ2 T cell lines generation

To investigate Vδ2 T cells response against glioma cells, we set up an *in vitro* model to expand Vδ2 T cells from Peripheral Blood Mononuclear Cells (PBMC) of healthy donors (HDs). Briefly, PBMC from HDs were stimulated *in vitro* with phosphoantigens (IPP 3μM) and IL-2 (100 UI/ml) for 12 days. At the end of this culture, the percentage of Vδ2+CD3+ (CD3 marker is a component of TCR complex) T cells in each culture was analysed by flow cytometry. PBMCs treated only with Interleukin-2 (100 UI/ml) was used as background control. Fig. 9 shows a representative result of Vδ2 T cell line generation. Specifically, in this example (fig. 9, panel A, I), the *ex vivo* frequency of Vδ2 T cells was 1.2% among total circulating lymphocytes; nevertheless, after 12 days of stimulation by specific antigen (IPP) and IL-2, we observed a massive Vδ2 T cells expansion (IL-2+IPP: Vδ2+CD3+: 96.5%, fig. 9 panel A, III). In contrast, the stimulation with IL-2 alone did not result in any Vδ2 T cells proliferation (IL-2: Vδ2+CD3+: 5.3%, fig. 9 panel A, II). These results showed that this protocol was able to generate quite pure (>90%) Vδ2 T cell lines.

In order to analyse the phenotype of Vδ2 T cells before and after the generation of Vδ2 T cell short-term lines, we studied the distribution of CD27 and CD45RA differentiation markers by flow cytometry in Vδ2 T cells *ex vivo* (white bars) and at 12 days after IPP-stimulation (black bars) (fig. 9, B). As known, the analysis of CD27 and CD45RA differentiation

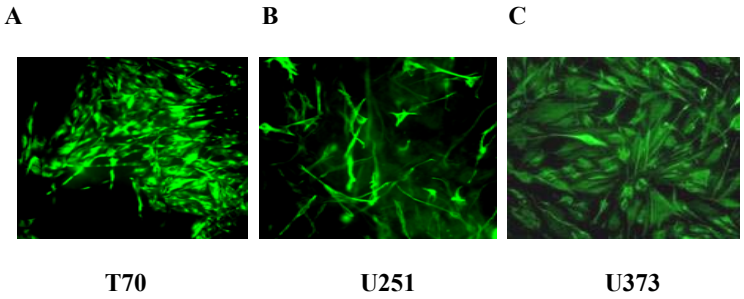

Figure 8. Expression of GFAP (Glial Fibrillary Acidic Protein) in glioma cells. The expression of GFA protein in glioma cells (T70, U251, U373) was analysed by an indirect immunofluorescence. All tumor cells are labelled with primary rabbit polyclonal antibody for GFAP and a secondary anti-rabbit antibody Fitc-coniugated (DakoCytomation). Acquisition of immunofluorescence was done with a fluorescence microscope (Olympus BX-51).

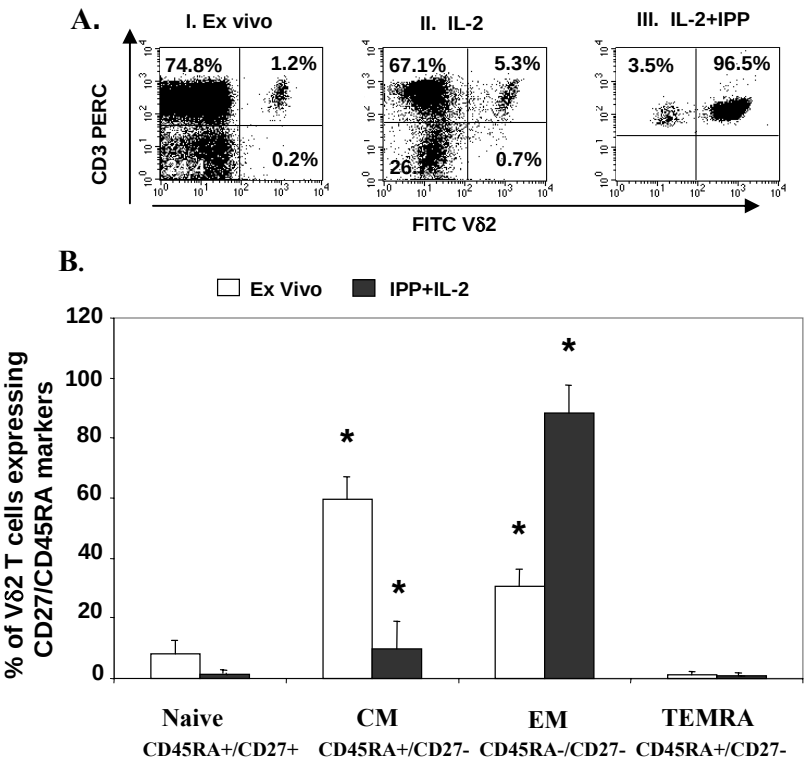

Figure 9. Short Term Vδ2 T cell lines generation. *A.* Vδ2/CD3 T cells percentage was analysed by flow cytometry by a double staining with mAbs anti-Vδ2 TCR (FITC) and anti-CD3 (PerCP) in PBMCs of HDs *ex vivo* (A. panel I: 1.2%) and after 12 days of expansion with IL-2 (100 UI), IPP (3μM) and IL-2 (A, panels II: 5.3% and III: 96,5%). We observed a massive Vδ2 T cells expansion versus the *ex vivo* condition after 12 days of IPP-stimulation. In contrast, the stimulation with IL-2 alone did not result in any Vδ2 T cells proliferation. **B.** It is represented the average and SD of phenotypic analysis (CD27 and CD45RA differentiation markers) in Vδ2 T cells *ex vivo* and after 12 days of IPP stimulation (*p<0.05). Results showed a significant increase of Vδ2 T cells Effector Memory cells respect to Central Memory cells after IPP stimulation.

markers showed the presence of four phenotypic classes: Vδ2 Naive (N) cells (CD27+/CD45RA+), Vδ2 Central Memory (CM) cells (CD27-/CD45RA+), Vδ2 Effector Memory (EM) cells (CD27-/CD45RA-) and Vδ2 terminally differentiated (TEMRA) cytotoxic cells (CD27-/CD45RA+). As shown in fig. 9 B, no significant differences were found between the Naive Vδ2 T cells percentage *ex vivo* and 12 days after IPP-expansion (*Ex vivo*: Vδ2+CD3+: 8.19%± 4.15 vs. IL-2+IPP: 1.38%±0.28). Similar results were obtained for Vδ2 Effector Cytotoxic cells (*Ex vivo*: Vδ2+CD3+: 1.23%±1.12 vs. IL-2+IPP: 0.62%± 0.29); on the contrary, the *in vitro* expansion with IPP+IL-2 induced a statistically significant decrease of Vδ2 Central Memory cells (*Ex vivo*: Vδ2+CD3+: 59.76%± 6.99 vs. IL-2+IPP: 9.77%± 9.19, *p<0.05) and a parallel increase of Vδ2 Effector Memory cells (*Ex vivo*: Vδ2+CD3+: 30.73%± 5.35 vs. IL-2+IPP: 88.23%± 8.97, *p<0.05) were found.

To investigate the functionality of Vδ2 T cell lines, we analysed cytokines production and perforin content of Vδ2 T cell lines before and after IPP stimulation (3μM) (fig. 10, Panels A-G). The cytokine analysis was performed by adding to unstimulated and IPP-stimulated Vδ2 T cell lines Brefeldin A, allowing the accumulation of newly synthesised cytokines by blocking the esocytosis pathway. After 18hs from IPP stimulation the intracellular staining for IFN-γ (fig. 10, Panels A, D) and for TNF-α (fig. 10, Panels B, E) and cytometric analysis were performed. As shown in fig. 10, stimulation is able to activate Vδ2 T cell lines inducing IFN-γ (IPP: 22.1% vs. unstimulated: 2.5%, panels A, D) and TNF-α production (IPP: 18.9% vs. unstimulated: 0.5%, panels B, E) production. The ability of Vδ2 T cell lines to promptly produce both IFN-γ (Vδ2+IFN-γ+ in IPP: 37.04%±13.8 vs. unstimulated: 2.18% ± 2.17, *p<0.05) and TNF-α (Vδ2+TNF-α+ in IPP: 42.06%± 12.4 vs. unstimulated: 1.98%± 1.66, *p<0.05) after IPP stimulation was confirmed in eight independent experiments (fig. 10, G).

Vδ2 T cells cytotoxic capability was analysed by measuring the ability of Vδ2 T cell lines to release perforin upon 18hs of IPP-stimulation (fig. 10, panels C, F). As shown in fig 10, most Vδ2 T cells (88,9%) were positive for perforin staining (fig. 10, panel C), suggesting a mechanism of cytotoxic granules accumulation during IPP-mediated expansion of Vδ2 T cells. Interestingly, when we stimulated Vδ2 T cell lines with IPP, a decrease of perforin-positive Vδ2 T cells was shown (IPP: 71.6% versus medium: 88,9%), suggesting a release of perforin induced by direct TCR triggering. The ability of Vδ2 T cell lines to promptly release perforin after IPP stimulation was confirmed in eight independent experiments (fig. 10,

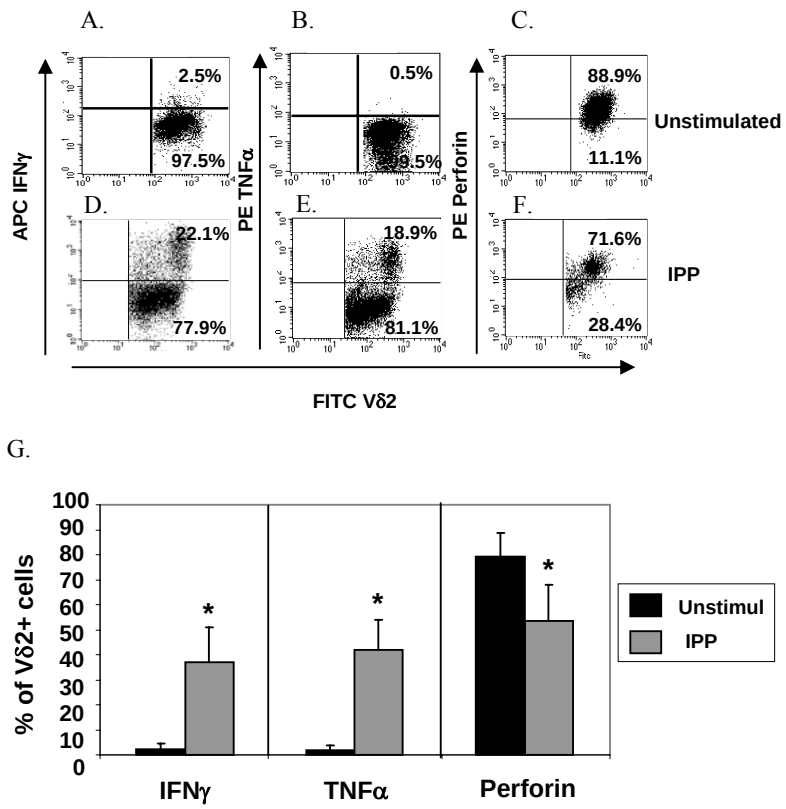

Figure 10. Short Term Vδ2 T cell lines functionality. Panels A-F. At time 12 days Vδ2 T cells were unstimulated/stimulated with IPP 3μM and after 18hs, it was analysed Vδ2 T cells IFNγ (panels A, D), TNFα (panels B, E) production and perforin release (panel C, F) by flow cytometry by using mAbs anti-Vδ2/TCR (FITC), anti-CD3 (PerCP), anti-IFNγ (APC), anti-TNFα (PE) and anti-Perforin (PE). **Panel *G.*** It is represented the average and the SD of Vδ2 T cells IFNγ and TNFα production, and perforin release after IPP-stimulation (*p<0.01) respect to CTRLs. Results showed that IPP-stimulation increase cytokines production and perforin release respect to the control by Vδ2 T cells.

panel G, Vδ2+/Perforin+: 79.28% ± 9.38 Medium vs. IPP 53.76% ± 14.13, *P<0.05).

4.3 Vδ2 T lines recognized glioma cells and differentiate in Effector Memory cells

In order to analyse the ability of Vδ2 T cell lines to recognize glioma cell lines, we set up an *in vitro* model of Vδ2/glioma cells interaction by performing co-cultures between Vδ2 T cell lines and glioma cells at 1:1 ratio. As control we analysed Vδ2 T cell lines activity in the absence of glioma cells (Vδ2 lines-ctrl). After 18 hours, effector/memory phenotype of Vδ2 T cell lines was analyzed by flow cytometry (fig. 11). The percentage of naïve (N), central memory (CM), effector memory (EM) and terminally differentiated (TEMRA) cytotoxic Vδ2 T cells was analysed in the absence of glioma cells (black bars) and in co-culture with T70 (white bars), U251 (hatched bars), and U373 cells (grey bars). As shown in fig. 11, glioma cells did not modify the frequency of Naïve Vδ2 T cells (Vδ2 lines-ctrl: 1.64% ± 1.51 vs. Vδ2/T70: 1.66% ± 1.39 vs. Vδ2/U251: 1.65% ± 1.67 vs. Vδ2/U373: 1.04% ±0.68) and of TEMRA Vδ2 T cells (Vδ2 lines-ctrl: 1.05% ± 1.16 vs. Vδ2/T70: 2.04% ± 1.39 vs. Vδ2/U251: 1.25%±1.32; Vδ2/U373: 1.71%±1.75). In contrast, glioma cells induced a statistically significant decrease in the frequency of CM Vδ2 T cells when compared with Vδ2 T lines in the absence of glioma cells (Vδ2 lines-ctrl: 44.80% ± 10.29 vs. Vδ2/T70: 29.73% ± 8.45 vs. Vδ2/U251: 27.37% ± 7.15 vs. Vδ2/U373: 14.08% ± 12.21, *p<0.05), and a parallel increase in EM Vδ2 T cells (Vδ2 lines-ctrl: 52.49% ± 8.05 vs. Vδ2/T70: 66.23% ± 6.51 vs. Vδ2/U251: 69.71% ± 7.79 vs. Vδ2/U373: 83.14% ± 10.84, *p<0.05). These results were confirmed in six independent experiments, and suggest that Vδ2 T cell lines are able to recognize tumor cells of glial origin and that, after recognition, they differentiate in effector memory cells.

4.4 Vδ2 T cell lines release perforin after encountering glioma cells

To investigate the effector functions of Vδ2 T cell lines against to glioma cells, the functional activation of Vδ2 T cell lines induced by glioma was analysed in terms of pro-inflammatory cytokines production (fig. 12, panel A), and perforin release (fig. 12, panel B). These results were compared with the functional activity of Vδ2 T cell lines in the absence of glioma cells (Vδ2 lines-ctrl). No statistically significant differences were

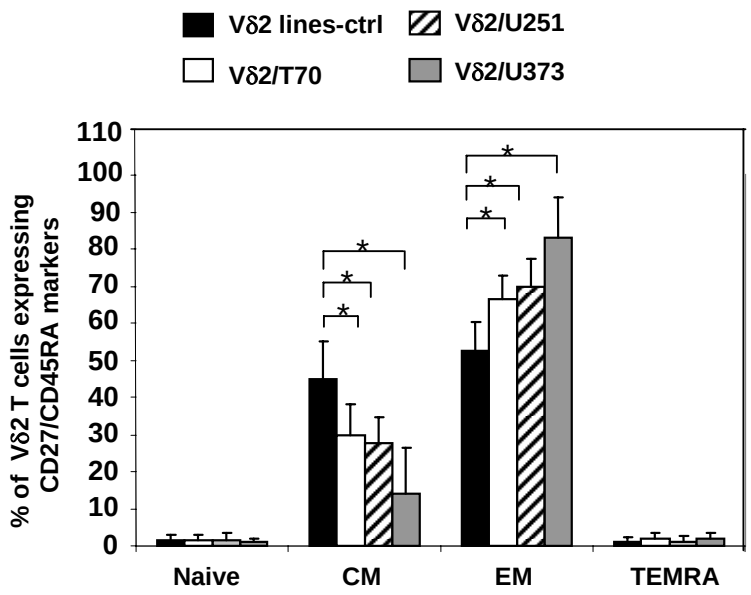

Figure 11. Vδ2 T cell lines recognize glioma cells and differentiate in effector/memory cells. It was analysed Vδ2 T cell lines differentiation markers expression, such as CD27 and CD45RA, by flow cytometry before/after the co-culture with glioma cells. CD27 and CD45RA markers on Vδ2 T cells gate were analysed by a double staining with mAbs anti-CD27 (APC) and anti-CD45RA (CY-Chrome) Results are expressed as the average ± SD (* $p<0.05$) and showed that Vδ2 T lines are able to differentiate from Central memory cells (CM) into Effector Memory cells (EM) after culture with all glioma cells.

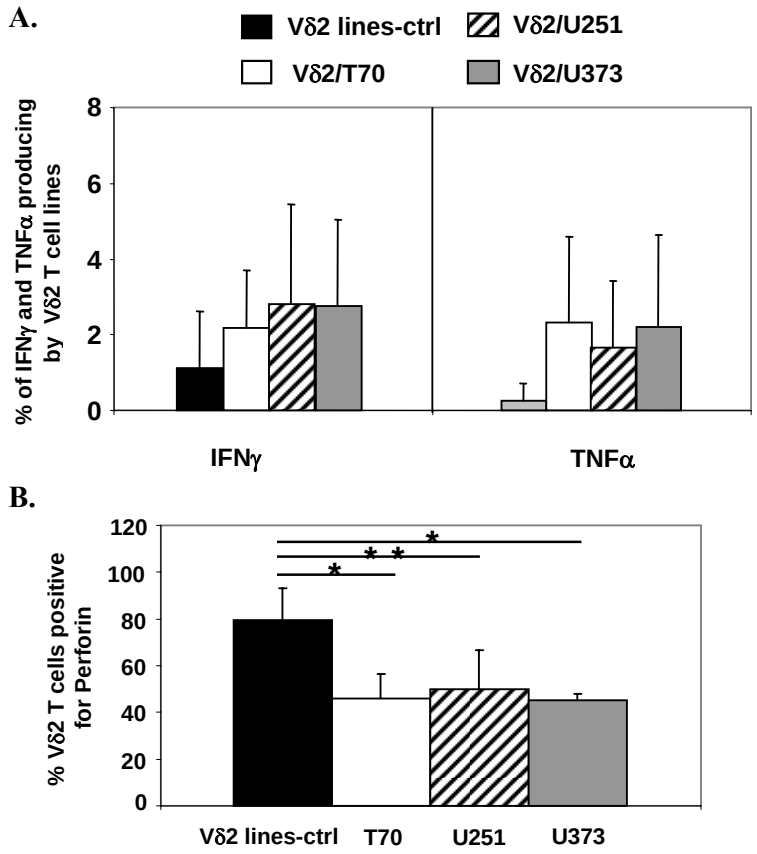

Figure 12. Vδ2 T cell lines release perforin after encountering glioma cells. A. IFNγ and TNFα production was analysed in Vδ2 T cells by flow cytometry with a double staining with mAbs anti-IFNγ (APC) and anti-TNFα (PE), before/after the co-culture with glioma cells and It wasn't statistically significant versus CTRLs. **B.** Vδ2 T cells perforin content was analysed by flow cytometry by using mAb anti-perforin (PE) before/after co-culture with glioma cells. Results are expressed as the average ± SD (* $p<0.05$) and showed a significant decrease of Vδ2 T lines perforin content after culture with glioma cells respect to Vδ2 T cells CTRL.

found on cytokines production (fig. 12, panel A) between the frequency of IFN-γ producing by Vδ2 T cells in Vδ2 cell lines-ctrl (Vδ2+IFNγ+: 1.11% ± 1.45, black bars) and those after co-cultures with T70 (Vδ2+IFNγ+: 2.17±1.53, white bars), U251 (Vδ2+IFNγ+: 2.81% ± 3.33, hatched bars) and U373 (Vδ2+IFNγ+: 2.74% ± 2.27, grey bars). Similar results were obtained by analysing TNFα production in Vδ2 lines-ctrl (Vδ2+TNFα+: 0.26% ± 0.26, black bars) and in Vδ2/T70 co-cultures (Vδ2+TNFα+: 2.33% ± 2.24, white bars), in Vδ2/U251 co-cultures (Vδ2+TNFα+: 1.67% ± 1.70, hatched bars) and in Vδ2/U373 co-cultures (Vδ2+TNFα+: 2.20% ± 2.39, grey bars).

To analyse the ability of Vδ2 T cell lines to kill glioma cells, we studied the content of perforin in Vδ2 T cell lines before and after co-culture with glioma cells (fig 12, panel B). As described in the previous paragraph, a large amount of Vδ2 T cell lines express perforin (79.46% ± 13.57, black bar). Nevertheless, when Vδ2 T cells were co-cultured with glioma cells, a decrease in the percentage of perforin-positive Vδ2 T cells was shown in Vδ2/T70 (Vδ2 lines-ctrl: 79.46% ± 13.57 vs. Vδ2/T70: 46.08% ± 10.43, * $p<0.05$) in Vδ2/U251 co-cultures (Vδ2 lines-ctrl: 79.46% ± 13.57 vs. Vδ2/U251: 49.88% ± 16.84, **$p<0.01$) and in Vδ2/U373 co-cultures (Vδ2 lines-ctrl: 79.46% ± 13.57 vs. Vδ2/U373: 45.38% ± 2.6, **$p<0.01$), suggesting that after tumor recognition, Vδ2 T cells release perforin in the culture medium. The release of perforin usually results in the death of target cells. To verify the death/viability of glioma cells after co-cultures, we performed a microscopic analysis of T70, U251 and U373 cultures in the absence (fig 13, Panels A, C, E) or in the presence of Vδ2 T cell lines (fig 13, Panels B, D, F). This analysis showed that the presence of Vδ2 T cell lines on glioma cells determined a loss of T70, U251 and of U373 cells, suggesting that the perforin release by Vδ2 T cell lines may result in killing of tumor cells.

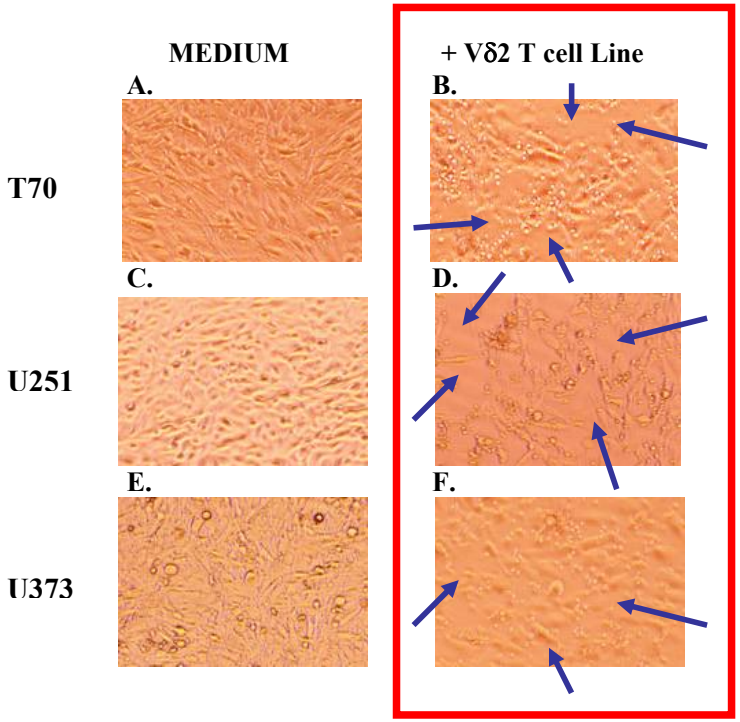

Figure 13. Cytotoxic effect of Vδ2 T cell lines on glioma cells (microscopic analysis). Vδ2 T cell lines cytotoxic effect on glioma cells was evaluated by microscope analysis (model Nikon Eclipse TE200- and Nikon Digital Camera XM1200F, 20X) before/after co-culture with glioma cells. We observed that Vδ2 T cell lines displayed their cytotoxic activity killing glioma cells.

4.5. Cytotoxic effect of Vδ2 T cell lines on glioma cells

To study the cytotoxic activity of Vδ2 T cell lines on glioma cells, we performed a viability test using Annexin V/Propidium Iodide markers on glioma cells before and after co-culture with Vδ2 T cell lines (fig 14), allowing the analysis of four different subsets: viable cells (Annexin-/PI-), early apoptotic cells (Annexin+/PI-), late apoptotic cells (Annexin+/PI+) and necrotic cells (Annexin-/PI+). These analysis was performed on T70 (fig. 14), U251 (fig. 15) and on U373 glioma cells (fig. 16). Briefly, glioma cells were co-cultured with Vδ2 T cell lines in ratio 1:1 for 18 hours and the percentage of glioma cells expressing early apoptosis markers (A+/PI-), late apoptosis markers (A+/PI+) and necrosis markers (A-/PI+) was analysed by flow cytometry. As a control, we analysed the viability of glioma cells in the absence of Vδ2 T cell lines (T70-ctrl, U251-ctrl, U373-ctrl). As shown in Fig.14, activated Vδ2 T cell lines were able to kill all glioma cells. Specifically, we observed a cytotoxic effects of Vδ2 T cell lines on T70 cells (fig. 14) resulting in an increased frequency of early apoptotic T70 (T70-ctrl: 3.1% + 2.8 vs Vδ2/T70: 9.6% + 5.4, *$p<0.05$), late apoptotic T70 (T70 ctrl: 1.2% + 0.6 vs Vδ2/T70: 9.17% + 7.7, *$p<0.05$), necrotic T70 (T70-ctrl: 4.2% + 2 vs Vδ2/T70: 24.4% + 13.4, *$p<0.05$). Similarly, Vδ2 T cell lines induced an increased frequency of early apoptotic U251 cells (fig. 15; U251-ctrl: 2.5+0.6 vs Vδ2/U251: 12.6% + 6.6, *$p<0.05$) late apoptotic U251 cells (U251-ctrl: 0.7% + 0.6 vs Vδ2/U251: 22% + 5.2, *$p<0.05$) and necrotic U251 cells (U251-ctrl: 10.5% + 7 vs Vδ2/U251: 24.9% + 6.1, *$p<0.05$). Finally, the same results were obtained on U373: early apoptotic U373 cells (fig. 16; U373-ctrl: 12.3% + 3.6 vs Vδ2/U373: 43.23% + 22, $p<0.05$), late apoptotic U373 cells (U373-ctrl: 3.4% + 1.7 vs Vδ2/U373: 16.5% + 3.6, $p<0.05$), and necrotic U373 cells (U373-ctrl: 12.9% + 4.2 vs Vδ2/U373: 38.7% + 15.7, *$p<0.05$). Overall, these results demonstrated that Vδ2 T cell lines were able to kill glioma cells.

Finally, we performed a dose dependent viability assay by co-culturing T70 glioma cells with an increasing number of Vδ2 T cells, resulting in a different effector/target ratio (1:1, 10:1 and 20:1) (fig. 17). The results showed that Vδ2 T cell lines increased the frequency of early apoptotic T70 cells in a ratio dependent manner, confirming an antitumor cytotoxic activity of Vδ2 T cell lines.

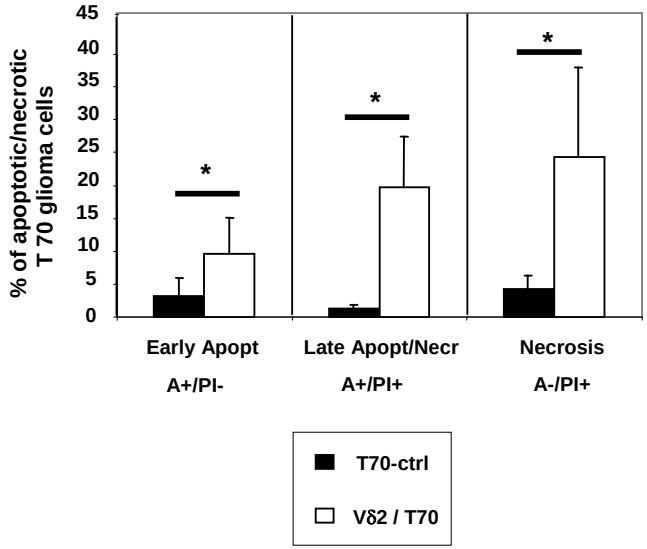

Figure 14. Cytotoxic effect of Vδ2 T cell lines on T70 glioma cells. Vδ2 T cell lines cytotoxic effect on glioma cells was evaluated by flow cytometry by using Annexin V/Propidium Iodide viability test. Results are expressed as the average ± SD in T70 glioma cells (* $p<0.05$) and showed a statistically significant increase of apoptotic and necrotic T70 glioma cells after co-culture with Vδ2 T cell lines.

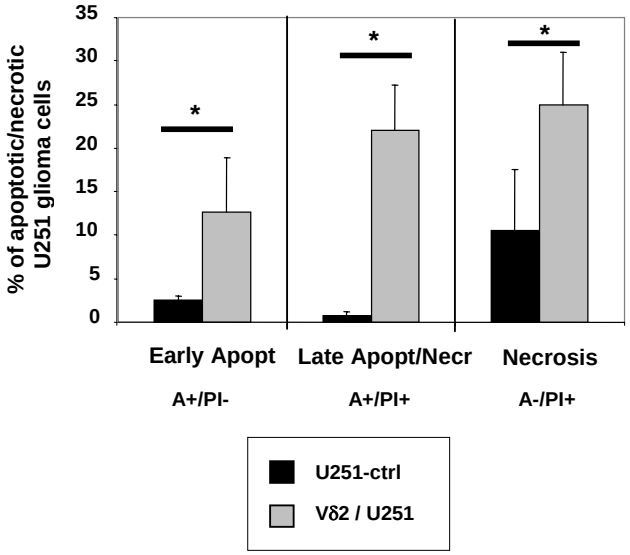

Figure 15. Cytotoxic effect of Vδ2 T cell lines on U251 glioma cells. Vδ2 T cell lines cytotoxic effect on glioma cells was evaluated by flow cytometry by using Annexin V/Propidium Iodide viability test. Results are expressed as the average ± SD in U251 glioma cells (* $p<0.05$) and showed a statistically significant increase of apoptotic and necrotic U251 glioma cells after co-culture with Vδ2 T cell lines.

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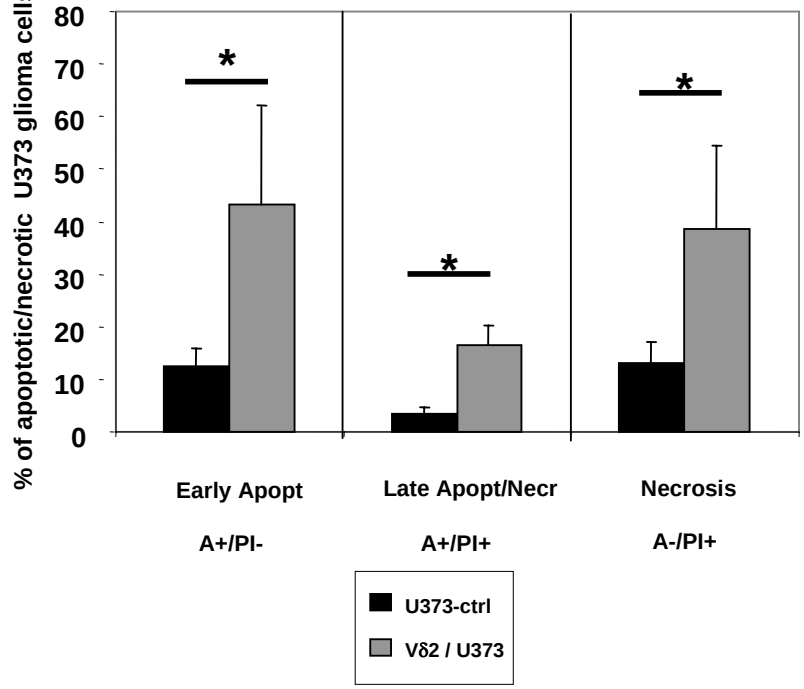

Figure 16. Cytotoxic effect of Vδ2 T cell lines on U373 glioma cells. Vδ2 T cell lines cytotoxic effect on glioma cells was evaluated by flow cytometry by using Annexin V/Propidium Iodide viability test. Results are expressed as the average ± SD in U373 glioma cells (* $p<0.05$) and showed a statistically significant increase of apoptotic and necrotic U373 glioma cells after co-culture with Vδ2 T cell lines.

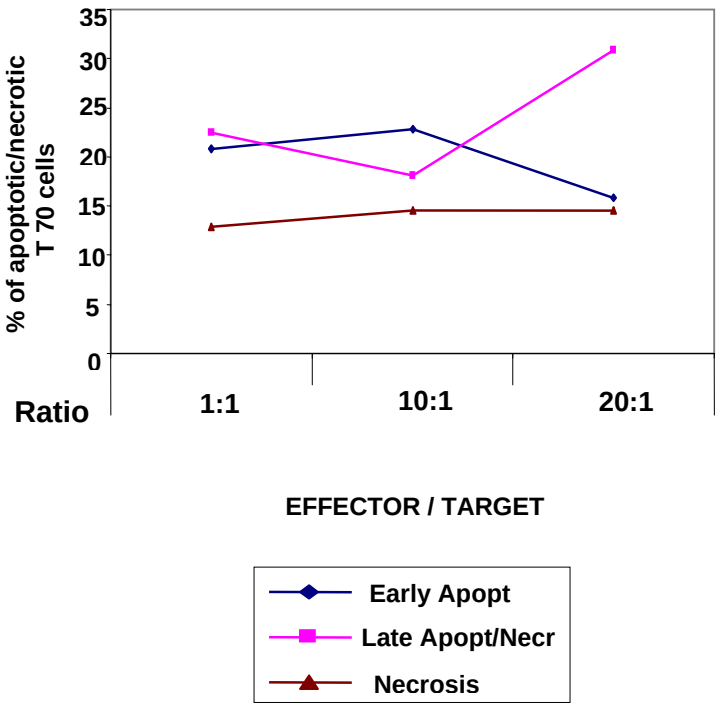

Figure 17. Cytotoxic effect of Vδ2 T cell lines on T70 glioma cells. Vδ2 T cell lines cytotoxic effect on T70 glioma cells was evaluated by flow cytometry by using Annexin V/Propidium Iodide viability test. T70 glioma cells were cultured at different effector/target ratio (1:1, 10:1, 20:1) for 18hs and after incubated with Annexin V mAb for 10 minutes, washed with buffer, labelled with anti-Propidium Iodide mAb and analysed by flow cytometry. Results are expressed as the average in T70 and showed that Vδ2 T cells at 10:1 and 20:1 ratio increased T70 glioma cells death.

4.6 Cytokines production by Vδ2 T cell lines on ZOL-treated glioma cells

Since zoledronic acid (ZOL) is able to activate Vδ2 T cells through an indirect mechanism involving the accumulation of intracellular IPP in target cells (40), we tested the possibility to induce Vδ2 T cells activation by treating glioma cells with ZOL (fig. 18). Briefly, glioma cells were treated for 2 hours with ZOL (150 μM), washed and co-cultured overnight (18 hours) with Vδ2 T cell lines in the presence of BFA (10μg/ml). Fig. 16 shows the frequency of IFN-γ producing by Vδ2 T cells in the presence of untreated glioma cells (black bars) or of ZOL-treated glioma cells (white bars). ZOL treatment of T70 cells induced an increased frequency of IFN-γ production (untreated-T70: 2.29% ±1.84 vs. ZOL-treated-T70: 67.43% ± 12.69, *p<0.01) and of TNF-α producing by Vδ2 T cells (untreated-T70: 3.11% ± 1.97 vs. ZOL-treated-T70 vs. 64.48% ± 16.94, *p<0.01) by Vδ2 T cells. Similar results were obtained by using U251 cells both in terms of IFN-γ production (untreated-U251: 3.12% + 4.43 vs. ZOL-treated-U251: 54.37% ± 11.42, *p<0.01) and of TNF-α production (untreated-U251: 2.22% ± 1.57 vs. ZOL-treated-U251: 45.02% ± 11.18, *p<0.01). Finally, we confirmed the results on U373 cells in terms of IFN-γ production (untreated-U373: 1.38% ± 0.94 vs. ZOL-treated-U373: 85.17% ± 8.28, *p<0.01) and of TNF-α production (untreated-U373: 2.94% ± 2.31 vs. ZOL-treated-U373: 89.19% ± 3.9, *p<0.01). These results demonstrated that, accordingly to literature, all glioma cells treated with ZOL induced the activation of Vδ2 T cells, presumably by the accumulation of intermediate metabolites of mevalonate cycle able to trigger these cells.

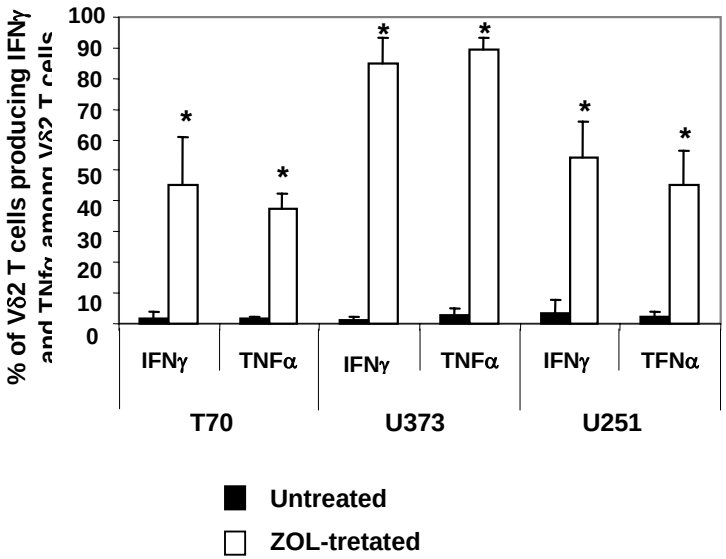

Figure 18. Cytokines production by Vδ2 T cell lines on untreated/ZOL-treated glioma cells. Glioma cells were treated with ZOL for 2 hs, washed with culture medium and co-cultured with Vδ2 T lines. After 18hs was done a cytofluorimetryc analysis of IFNγ and TNFα producing Vδ2 T lines by using mAbs anti-IFNγ (PE) and anti-TNFα (APC). Results are expressed as the average ± SD (* $p<0.05$) and showed that the treatment of all glioma lines with ZOL enhanced the IFNγ and TNFα production by Vδ2 T cells respect to the untreated glioma cells.

4.6 Zoledronic acid enhanced Vδ2 T cells killing of treated glioma cells

To investigate whether the ZOL treatment of glioma cells can induce an increase in the cytolytic activity of Vδ2 T cell lines, we performed both a microscopic analysis (fig. 19-21) and a viability tests by staining glioma cells with Annexin V/Propidium Iodide (fig 22) of glioma cells. The microscopic analysis of untreated-T70 cells (fig 19, panel A) and of ZOL (150μm)-treated-T70 cells (fig 19, panel B), ZOL (400μm)-treated-T70 cells (fig 19, panel C) showed that an high concentration of ZOL (400μm) on T70 cells is able to inhibit cell growth. Nevertheless, as previously described, the addition of Vδ2 T cell lines on untreated T70 cells resulted in an evident tumor cell death (fig. 19, panels D). Moreover, ZOL treatment of glioma cells enhanced the susceptibility of these tumor cells to Vδ2 T cell mediated killing (fig. 19, panels E, F). Similar results were obtained for U251 glioma cells untreated/ZOL-treated (fig. 20, panels A, B, C) and cultured with Vδ2 T cell lines (fig. 20, panels D, E, F). These results were confirmed also for untreated and ZOL-treated U373 glioma cells (fig. 22, panels A, B, C) and cultured with Vδ2 T cell lines (fig. 22, panels D, E, F).

To confirm these findings, and to verify the dose dependency of cytotoxic mechanism on ZOL-treatment concentration, the effect of ZOL-treatment on T70 glioma cells viability was analysed (fig. 22). As shown in fig 22, panel A, ZOL treatment (white bars) resulted a statistically significant increase of tumor cells apoptosis when compared with untreated T70 cells (black bars, fig. 22, panel A). This antitumoral effect was shown using ZOL 6 μM (T70 untreated: 2.77%±1 vs. ZOL 6 μM 5.61%± 3.5, *$P<0.05$), ZOL 150 μM (T70 untreated: 2.77%±1 vs. ZOL 150 μM 10.9%± 6.54, *$P<0.05$), ZOL 400 μM (T70 untreated: 2.77%±1 vs. ZOL 400 μM 15.4%± 10.1, *$P<0.05$).

Moreover, we performed co-cultures between Vδ2 T cell lines with untreated-T70 cells (fig 22, white bars) or with ZOL-treated T70 cells (fig 22, hatched bars) at different concentrations (ZOL: 6μM, 150μM, 400μM). After 18 hours from the beginning of the co-cultures, the percentage of T70 annexin+ apoptotic cells (both early and late apoptotic cells) was analysed by flow cytometry.

Interestingly, the addition of Vδ2 T cell lines both on untreated-T70 and on ZOL-treated T70 cells (hatched bars) induced a strong cytotoxic effect when compared with T70 cells cultured in the absence of Vδ2 T cell lines (white bars), suggesting a key role of Vδ2 T cell lines immunity against glioma tumors.

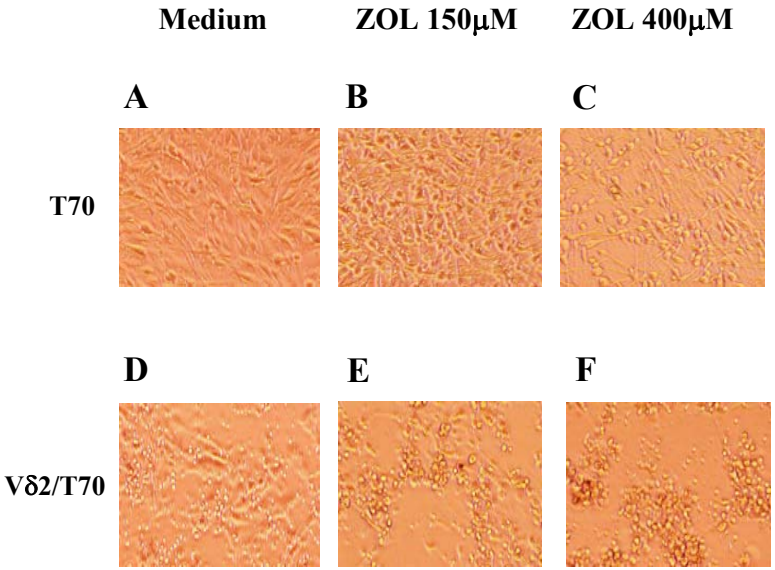

Fig. 19. Microscopic analysis of T70 glioma cells untreated/ZOL-treated in culture with Vδ2 T lines. We have performed a microscopic analysis (model Nikon Eclipse TE 200- and Nikon Digital Camera XM1200F, 20X) of T70 glioma cells untreated/treated with two different concentrations of ZOL (150μM, 400μM) and before/after culture with Vδ2 T cell lines. Results showed a great lost of T70 glioma cells in culture after ZOL/treatment and co-culture with Vδ2 T cell lines.

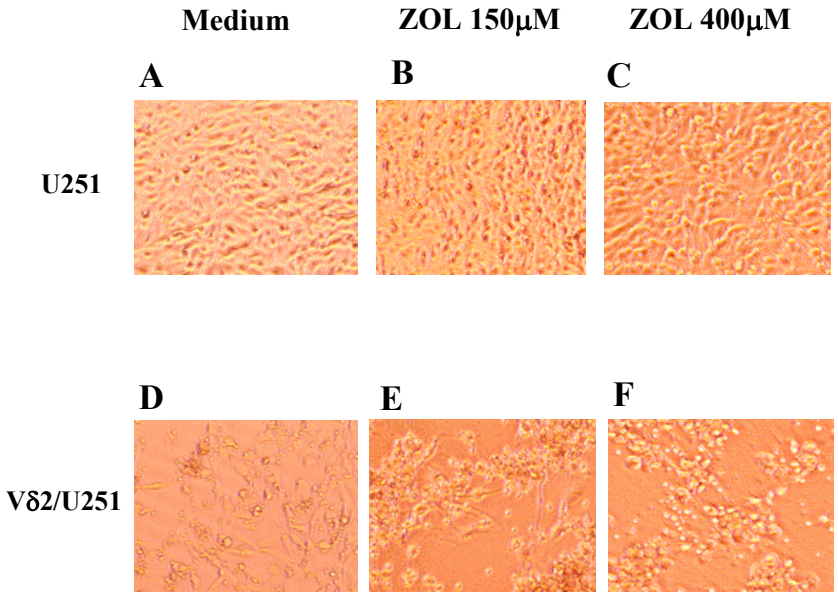

Fig. 20. Microscopic analysis of U251 glioma cells untreated/ZOL-treated in culture with Vδ2 T lines. We have performed a microscopic analysis (model Nikon Eclipse TE 200- and Nikon Digital Camera XM1200F, 20X) of U251 glioma cells untreated/treated with two different concentrations of ZOL (150μM, 400μM) and before/after culture with Vδ2 T cell lines. We observed a great lost of U251 in culture after ZOL/treatment and co-culture with Vδ2 T cell lines.

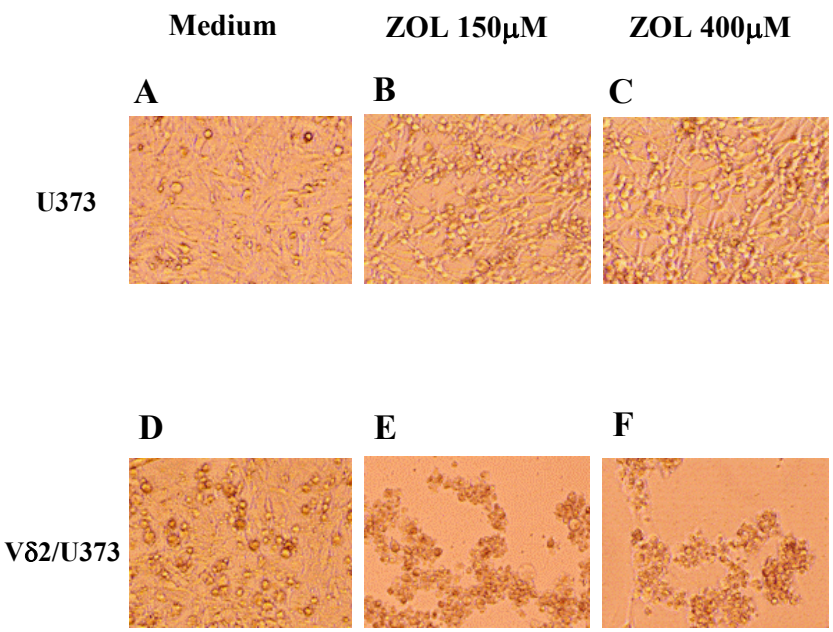

Figure 21. Microscopic analysis of U373 glioma cells untreated/ZOL-treated in culture with Vδ2 T lines. We have performed a microscopic analysis (model Nikon Eclipse TE 200- and Nikon Digital Camera XM1200F, 20X) of U373 glioma cells untreated/treated with two different concentrations of ZOL (150μM, 400μM) and before/after culture with Vδ2 T cell lines. We observed a great lost of U373 in culture after ZOL/treatment and co-culture with Vδ2 T cell lines.

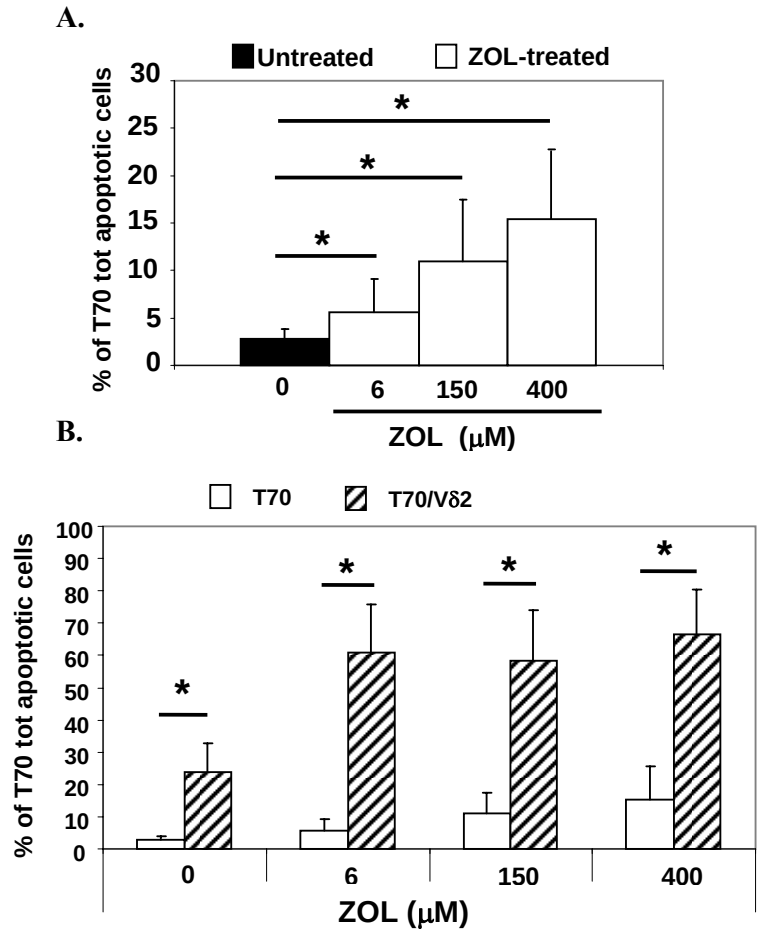

Figure 22. Zoledronic Acid treatment of glioma cells enhanced Vδ2 T cell lines killing. A. The apoptotic effect of different concentrations of ZOL (6μM, 150μM, 400μM) on T70 glioma cells was analysed by flow cytometry. **B.** The role of Vδ2-T cell lines mediated apoptosis on untreated/ ZOL-treated T70 glioma cells was monitored by flow cytometry. Results are expressed as the average ± SD (* $p<0.05$) and showed that ZOL treatment and culture with Vd2 T lines increased apoptosis of all glioma cells.

Specifically, the addition of Vδ2 T cells on untreated T70 glioma cells induce a higher apoptosis of glioma cells (untreated-T70: 2.77%±1 vs. untreated-T70/Vδ2: 23.78%± 8.9, *p<0.05). Similar results were obtained by comparing the antitumoral effects of Vδ2 T cells on ZOL-treated T70 glioma cells at any ZOL concentrations: ZOL 6 μM (ZOL 6 μM-treated-T70: 5.61%± 3.5 vs. ZOL 6 μM-treated-T70/Vδ2: 60.68%± 15.2, *P<0.05), ZOL 150 μM (ZOL 150 μM-treated-T70: 10.9%±6.54 vs. ZOL 150 μM-treated-T70/Vδ2: 58.3%± 15.8, *P<0.05) and ZOL 400 μM (ZOL 400 μM-treated-T70: 15.4%± 10.1, ZOL 400 μM-treated-T70/Vδ2: 66.4%±13.8, *P<0.05). Moreover, the treatment of glioma cells with ZOL, even at a very low concentration (6μM), strongly enhanced the Vδ2 T cell-mediated killing (untreated-T70/Vδ2: 23.78%± 8.9 vs. ZOL-treated-T70/Vδ2: 60.68%± 15.2,*p<0.05, fig. 22, panel B).

Altogether, these results suggest a dual role of ZOL: i) is able to directly induce glioma cells death and ii) is able to indirectly enhance Vδ2 T cell-mediated killing of tumor cells.

DISCUSSION

γδ T lymphocytes are able to perform a broad immune response against bacteria, viruses and parasites infections and against tumor cells by producing high amounts of pro-inflammatory cytokines and chemokines ans by exerting a potent cytotoxic activity (95-100). In antitumor response, γδ T cells are found *in situ* as tumor-infiltrating lymphocytes (22, 69, 101, 102), where recognize and kill several cancer cells *in vitro* (21, 25, 48, 63, 70-73, 103, 104) suggesting their role in the innate antitumor immune-surveillance (97, 105-107). Among γδ T cells, Vδ2 T lymphocytes represent a specific subpopulation with particular characteristics linking innate and acquired immunity. They recognize phosphorilated antigens in HLA unrestricted manner with a specific interaction between γδ TCR and the antigen (35-39). They are considered as "sentinels" in peripheral blood and tissues of adults, and display their immune function against many different pathogens (95-97). Vδ2 T lymphocytes display a strong antitumor immune activity *in vitro* and *in vivo* by recognizing and killing different types of cancer cells (18-26) with cytokinic/cytotoxicity mechanisms.

There are many evidences that it is possible to expand *in vitro* with synthetic drugs (Phosphostim) Vδ2 T cells from peripheral γδ T lymphocytes of normal healthy donors, as well as from patients with Myeloma Multiple (MM) and metastatic renal cell carcinoma (MRCC), with the aim to improve antitumor cytotoxicity of Phosphostim-triggered Vδ2 T cells from cancer patients. Results are encouraging, and confirm the possibility to expand cytotoxic Vδ2 T cells from patients with MM and MRCC aimed to kill autologous cancer cells (112, 69). Similar data were shown about Vδ2 T cells isolated from ascites of metastatic colon cancer patients; these γδ clones killed *in vitro* a large fraction of allogeneic colon carcinoma and melanoma cell lines, but did not affect a normal colon cell line, colon fibroblasts, or melanocytes. Tumor cells recognition was TCR and NKG2D mediated and induced TNFα and IFNγ secretion. These results provide a strong rationale for the use of Vδ2 T cell agonists in immunotherapy targeting cancer cells. (22).

Zoledronic acid is a bisphosphonate used for the treatment of osteoporosis and bone metastasis of prostate, breast, melanoma and myeloma multiple cancers (53-61). Bisphosphonates exhibit direct antitumor activity by both inhibiting proliferation and inducing apoptosis in tumor cells (113). They are potent inhibitors of the IPP-processing enzyme farnesyl pyrophosphate synthase, thereby leading to the intracellular accumulation of IPP in tumor cells and in consequence exerting a strong

activation effect on Vδ2 T cells. Their unique ability to render tumor cells susceptible to Vδ2 T cells attack makes these drugs particularly interesting candidates for use in γδ T cell-mediate therapy (13, 17, 24, 47). As it has been shown that bisphosphonates directly augment the antitumor effects of chemotherapy in a range of malignancies (37, 41, 42, 51), combining bisphosphonates and chemotherapy may substantially enhance the inherent susceptibility of tumors to Vδ2 T cells. Mattarollo et al. demonstrated an high cytotoxicity against solid tumor-derived cell lines with a combination treatment utilizing Vδ2 T cells, chemotherapeutic agents and zoledronic acid. They observed that the pre-treatment with low concentrations of chemotherapeutic agents or zoledronic acid sensitized tumor cells to rapid killing by Vδ2 T cells with levels of cytotoxicity approaching 90%. In addition, zoledronic acid enhanced the chemotherapy-induced sensitization of tumor cells to Vδ2 T cell cytotoxicity resulting in almost 100% lysis of tumor target cells in some cases (78). Sato K. et al. focused the interest on the importance of pre-treatment of tumor cells, such as small cells lung cancer and fibro sarcoma cells, with low concentration of zoledronic acid (5μM). Results showed that tumor cells pre-treated with ZOL presented a marked increase sensitivity to lysis by γδ T cells, while untreated tumor cells were much less sensitive to γδ T cell-mediated lysis (79).

Altogether these data confirm the importance of Vδ2 T cells in tumor response and the possibility to use bisphosphonates to enhance this antitumor activity.

In the last few years a high interest developed in order to enhance this innate subpopulation for the immunotherapy of renal, colon, breast, myeloma multiple and prostate cancers (55, 56, 63, 69-71, 76-79). Recently, Dieli et al., initiated a phase I clinical trial in metastatic hormone-refractory prostate cancer to examine the feasibility and the consequences of using the γδ T-cell agonist zoledronic acid, either alone or in combination with low-dose of interleukin 2, to activate peripheral blood γδ T cells. Results showed that the treatment of ZOL displayed a significant long-term shift of peripheral γδ T cells toward an effector-memory-like state (EM), producing IFNγ and perforin. These patients also maintained serum levels of TNFα-related to TRAIL. Moreover, the numbers of EM γδ cell showed a statistically significant correlation with declining prostate-specific antigen levels and objective clinical outcomes that comprised three instances of partial remission and five of stable disease. By contrast, patients treated with only ZOL showed progressive clinical deterioration (114).

Moreover, a pilot study of adoptive immunotherapy using *in vitro* activated γδ T cells against *in vivo* advanced RCC (renal carcinoma cells) was designed in order to evaluate the safety profile and possible anti-tumor effects of *in vivo* adoptive immunotherapy using Vδ2 T cells against a solid tumor. Patients with advanced RCC after a radical nephrectomy were administered via intravenous infusion of *in vitro*-activated autologous γδ T cells. Three out of seven patients showed an increase of γδ T cells in peripheral blood and also a high response to the antigen *in vitro*. Results showed that this cell-mediated therapy with *in vitro*-activated autologous γδ T cells was well-tolerated from RCC patients and could induce anti-tumor effect (71).

Gliomas are malignant brain cancers with devastating outcomes (115-117). Despite considerable advances in surgery, radiation, and chemotherapy, the prognosis for glioma tumors has not been substantially improved (118-122). Recently, gene therapy, stem cell therapy, and nanoparticle vector have emerged as new therapeutic modalities, and were shown able to overcome some of the problems of the current treatment strategies for glioma (123-125). In spite of these advances, whole brain radiation therapy remains the treament of choice for highly invasive and diffusive brain tumors with no other clear alternatives (118-122). Thus, the prognosis for glioma patients has been poor and the median survival is still in the range of one year; since chemotherapy alone has not been proven totally efficacious as first line treatment for glioma patients, new and innovative therapeutic strategies must be developed (110, 111). The lack of effective immune responses to glial tumors inside the central nervous system has been attributed to the immune-privileged status of the brain conferred by the blood-brain barrier, to the lack of conventional lymphatic vessels, and to the local release of immunosuppressive factors (2-4). However, both lymphocytes and macrophages infiltrate malignant gliomas, indicating the potential for lymphocyte homing and presentation of processed tumor antigens (2).

Little is known about glioma tumors and γδ T cells. Yamaguchi et al. showed a simple method for the propagation and purification of γδ T cells from a low quantity of peripheral blood of glioblastoma patients. The stimulation protocol provided the use of a solid-phase anti-CD3 and soluble interleukin 2. The incubation of this purified γδ T cells with IL-2 or IL-12 increased their cytotoxic properties against autologous tumor cells (126).

The aim of our project was to best understand the Vδ2 T cells immune response to glioma cancer cells, and to study if this immune subpopulation

could be a good target for new immunotherapeutic approaches for glioma treatment.

In order to analyse the interaction between glioma tumors and Vδ2 T cells we set up an *in vitro* experimental model of co-culture between Vδ2 T cells from PBMCs of healthy donors and three glioma cell lines: T70, U251 and U373.

Interestingly, we observed that Vδ2 T cells differentiated into effector memory cells from central memory cells after 18 hours of culture with all glioma cells, suggesting a direct mechanism of recognition able to improve their differentation in effector cells. We have studied cytokines production, such as IFNγ and TNFα, by Vδ2 T cell lines before/after the co-culture with glioma cells, but we observed that Vδ2 T cell lines were unable to produce significant quantities of cytokines. We then analysed the cytotoxicity of Vδ2 T cell lines against glioma cells as content of perforin before/after the co-culture. Our data have shown that Vδ2 T cell lines were able to release high quantities of perforin directly after the interaction with glioma cells both after three hours (data not shown) and after 18 hours of co-culture, suggesting a natural cytotoxicity mechanism of defense against glioma cancer cells. This effect was confirmed from morphological microscopy (fig. 13).

In order to study which type of glioma cells death was caused by Vδ2 T cell lines, we analysed the viability of glioma cancer cells with/withouth Vδ2 T cell lines by using Annexin V and Propidium Iodide markers. Interestingly, in all glioma cells we found improved apoptosis (Early and Late apoptosis) and necrosis after the co-culture with Vδ2 T cell lines, confirming the Vδ2 T cell lines may perform a strong innate immune response against these malignant tumors *in vitro* (fig. 14, 15, 16).

Zoledronic acid (ZOL) is an important tool for the treatment of cancer patients (62), and there are many evidences that ZOL expands and activates *in vitro* and *in vivo* Vδ2 T cells from healty donors or cancer patients (63, 69, 75-77, 79, 112) and can be therefore used to improve Vδ2-like innate response against tumors. Results are promising, and propose its use for immunotherapy against cancer cells (75).

In order to analyse the possibility to induce Vδ2 T cells activation by treating glioma cells with ZOL, we tested the effect of ZOL-treated glioma cells to activate cytokines production by Vδ2 T cell lines (fig. 16). Results showed that Vδ2 T cell lines produced high amounts of IFNγ and TNFα. Accordingly to Gober et al., the treatment with ZOL enhanced Vδ2 T cells response and cytotoxic capability against T70, U251 and U373 glioma cells

as determined by microscopic analysis (fig. 19, 20, 21 respectively). Several studies showed that TNFα alone (113) and IFNγ alone or in combination with Retinoic Acid (114) are indeed able to induce apoptosis of glioma cells. Accordingly, the cytokines produced by Vδ2 T cell lines in response to ZOL-treated glioma cells induced glioma cells death as shown in figure 18. Moreover, since ZOL has been used as anti-tumoral drug in several experimental protocols (55, 76, 78), we analysed the antitumor effect of ZOL on glioma cells by treating T70 glioma cell line with different concentration of ZOL, and by analyzing their viability by Annexin/Propidium Iodide test. Our results showed that ZOL displays an apoptosis-driven direct anti-tumor activity on T70 glioma cells, and that this effect increased with ZOL concentrations (fig. 22, panel A). We then investigated the role of Vδ2 T cells on ZOL treated glioma cells death induced by different concentrations of ZOL. Our results showed that Vδ2 T cell lines displayed a strong antitumor effector response against T70 glioma cells by inducing glioma death by both apoptosis and necrosis mechanisms (fig. 22, panel B), and open new interesting prospects on the chemotherapy/immunotherapy combined treatment.

CONCLUSIONS

Our studies show that Vδ2 T cells are able *in vitro* to recognize and kill glioma cells by releasing perforin, and by inducing apoptosis and necrosis cell death mechanisms. This underlines the possibility to improve antitumor response with Zoledronic Acid, by obtaining a dual effect: a direct antitumoral effect of ZOL against glioma cells, and an indirect effect of ZOL on Vδ2 T cells by enhancing both their killing anti-tumor function on glioma cells by apoptosis and necrosis and the release of high amounts of proinflammatory cytokines that could be important to drive adaptive response. This work is the first aimed to specifically study Vδ2 T cells response to glioma; our data support the use of this innate subpopulation as a promising target for future immunotherapy of glioma tumors. Our ongoing project will be to analyse function and functionality of γδ T cell from glioma patients.

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