



Myeloid-Derived Suppressor Cells Specifically Suppress IFN- γ Production and Antitumor Cytotoxic Activity of V δ 2 T Cells

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$\gamma\delta$ T cells represent less than 5% of circulating T cells; they exert a potent cytotoxic function against tumor or infected cells and secrete cytokines like conventional $\alpha\beta$ T cells. As $\alpha\beta$ T cells $\gamma\delta$ T cells reside in the typical T cell compartments (the lymph nodes and spleen), but are more widely distributed in tissues throughout the body. For these reasons, some investigators are exploring the possibility of immunotherapies aimed to expand and activate V δ 2 T cells, or using them as Chimeric Antigen Receptor carriers. However, the role of immunosuppressive microenvironment on V δ 2 T cells during infections and cancers has not been completely elucidated. In particular, the effects of myeloid-derived suppressor cells (MDSC), largely expanded in such pathologies, were not explored. In the present work, we demonstrated that MDSC may inhibit IFN- γ production and degranulation of phosphoantigen-activated V δ 2 T cells. Moreover, the V δ 2 T cells cytotoxic activity against the Burkitt lymphoma cell line Daudi and Jurkat cell line were impaired by MDSC. The Arginase I seems to be involved in the impairment of V δ 2 T cell function induced by both tumor cells and MDSC. These data open a key issue in the context of V δ 2-targeted immunotherapy, suggesting the need of combined strategies aimed to boost V δ 2 T cells circumventing tumor- and MDSC-induced V δ 2 T cells suppression.

Keywords: $\gamma\delta$ T cells, myeloid-derived suppressor cells, antitumoral activity, immunotherapy, IFN- γ

INTRODUCTION

$\gamma\delta$ T cells represent between 2 and 10% of total circulating CD3+ T lymphocytes (1). Among peripheral CD3+ $\gamma\delta$ T cells, those expressing a TCR formed by the V γ 9 and V δ 2 variable regions (hereafter referred to as V δ 2 T cells) constitute up to 90% of $\gamma\delta$ T cells (2). These V δ 2 T cells specifically recognize non-peptidic phosphorylated metabolites of isoprenoid biosynthesis, present in most pathogenic bacteria (3), or isopentenyl pyrophosphate, produced also by the human mevalonate biosynthesis pathway (4) that get accumulated in virus-infected and cancer cells due to alterations in the mevalonate pathway. V δ 2 T cells are characterized by high plasticity in their functions: they have the ability to produce pro-inflammatory cytokines, such as IFN- γ , TNF- α (5), and IL-17 (6) and also act as professional antigen-presenting cells (7). Further, V δ 2 T cells act as a bridge between

the innate and adaptive immune response, inducing dendritic cell (DC) maturation and shaping the Th1 and cytotoxic CD8 T cells response (8). Stimulated V δ 2 T cells also display strong cytolytic activity toward allogeneic tumors of various tissue origins. In particular, V δ 2 T cells are able to infiltrate murine and human tumors, to recognize tumor antigens, and to secrete cytotoxic molecules like granzyme and perforin (9, 10). The wide antitumor activity as well as their ability to shape both innate and adaptive immune response in the tumor microenvironment make these cells as good target in immunomodulating strategies in cancer settings. Several clinical trials of V δ 2 T cell-based immunotherapy performed for both hematological malignancies and solid tumors show promise for cellular therapy to control cancer (11); however, the effectiveness of V δ 2 T cell-based therapy is limited, probably due to suppressive function of the tumor microenvironment.

Myeloid-derived suppressor cells (MDSC) are potently immunosuppressive myeloid cells that accumulate in patients with advanced cancers (12), and in various disease settings, including chronic inflammation, viral infections (such as HIV infection), and autoimmune diseases (13–16). At present, it is well known that there are at least two main subsets of MDSC: the monocytic (Mo-MDSC) and the granulocytic (PMN-MDSC) subsets. In particular, in humans PMN-MDSC are identified as CD14[−] CD11b⁺ HLA-DR^{low/−} CD15⁺ (or CD66⁺), while Mo-MDSC as CD11b⁺ CD14⁺ HLA-DR^{low/−} CD15[−] (17), both subsets express CD33 myeloid marker. MDSC suppress T cell activation and cytotoxicity, induce the differentiation and expansion of Tregs, and inhibit NK cell activation by using different mechanisms [reviewed in Ref. (18)]. However, little is known about the suppressive activity of MDSC on V δ 2 T cells. It has been demonstrated that during HIV infection PMN-MDSC are expanded and their frequency is inversely correlated with the capacity of V δ 2 T cells to produce IFN- γ . However, differently from $\alpha\beta$ T cells, *in vitro* PMN-MDSC depletion did not completely restore IFN- γ production by V δ 2 T cells from HIV patients (13), suggesting that during HIV infection PMN-MDSC are not the unique player in dampening V δ 2 T cell response. Thus the exact role of MDSC in regulating V δ 2 T cells functions remains to be elucidated. Aim of the present work was to shed light on the effects of the suppressive capacity of MDSC on V δ 2 T cells capabilities.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMC) Separation

PBMC were obtained from buffy coats kindly provided from S. Camillo Hospital. According to NIH definition (<https://humansubjects.nih.gov>), this study does not require Ethical Committee approval. PBMC were isolated from peripheral blood by density gradient centrifugation (Lympholyte-H; Cederlane). After separation, PBMC were resuspended in RPMI 1640 (EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (EuroClone), 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) and with 2 mmol/L penicillin and 50 μ g/mL streptomycin (EuroClone), hereafter indicated as R10.

Cell Subsets Purification

V δ 2 T cells and PMN-MDSC frequency were analyzed before cell sorting. To obtain a better purification, when V δ 2 T cell and PMN-MDSC percentages were higher than 2 and 3%, respectively, we proceeded to cell sorting as follow: $\gamma\delta$ T cells were purified from PBMC using a magnetic negative selection (TCR $\gamma\delta$ + T cells isolation kit, Miltenyi Biotec) according to manufacturer's instruction. PMN-MDSC isolation from PBMC using CD15 microbeads (Miltenyi Biotec) according to manufacturer's protocol. The purity of sorted $\gamma\delta$ T cells and PMN-MDSC was >85 and 90%, respectively, as verified by flow cytometry (Figures 1A,B respectively). Purified cells were rested for 18 h in R10.

Cell Stimulation

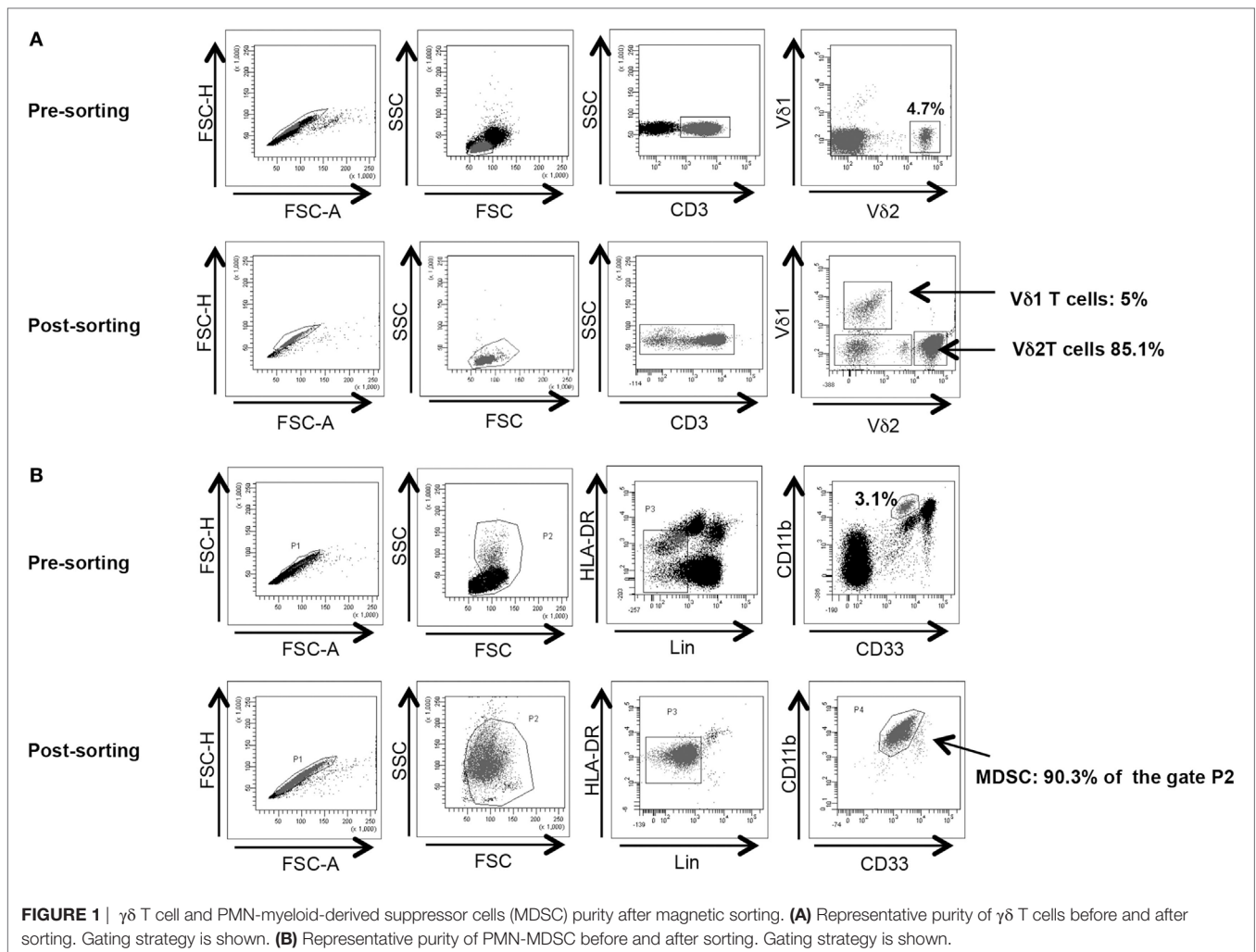
Cytokine production and degranulation by $\gamma\delta$ T cells was tested by stimulating cells with IPH11 (3 μ M, Innate Pharma) in the presence of PMN-MDSC. Brefeldin A was added after 1 h of stimulation. After 18 h, IFN- γ and CD107a were evaluated by flow cytometry. Titration of PMN-MDSC/ $\gamma\delta$ T cells ratio was performed (2:1, 1:1, 1:2, 1:5) evaluating CD107a expression.

Cytotoxic ability of V δ 2 T cells was analyzed using Daudi and Jurkat cell lines as targets and measuring annexin V binding to apoptotic cells. Briefly, titration of Daudi cells and Jurkat cells/ $\gamma\delta$ T cells ratios were performed (2:1, 1:1, 1:2, 1:5, 1:10 and 1:1, 1:2, 1:5, 1:10, 1:20, respectively) testing the binding of annexin V (Annexin V-FITC Apoptosis Detection Kit, eBiosciences) to apoptotic Daudi or Jurkat cells by flow cytometry after 18 h of culture. The percentage of killing was calculated as follow: [100% – (% of living Daudi (or Jurkat) cells in the presence of $\gamma\delta$ T cells/% of living Daudi (or Jurkat) cells without $\gamma\delta$ T cells) \times 100]. Where indicated, specific inhibitors of Arginase I (*N*-Hydroxy-nor-L-arginine, nor-NOHA, 1 mM, Calbiochem), indoleamine 2,3-dioxygenase (1-Methyl-D-tryptophan, 1-MTD, 1 mM, Sigma-Aldrich) or nitric oxide synthases (*N*-Monomethyl-L-arginine, Monoacetate Salt, L-NMMA, 1 mM, Calbiochem) were used.

In order to evaluate MDSC capacity to inhibit T cell proliferation, PBMC or purified $\gamma\delta$ T cells were labeled with CFDA-SE (Vibrant CFDA SE cell tracer kit, Invitrogen) according with manufacturer's instruction. Labeled PBMC were then cultured with purified PMN-MDSC at 1:1 ratio and stimulated with *Staphylococcus enterotoxin B* (SEB, 200 ng/mL, Sigma-Aldrich). CFDA-SE labeled purified $\gamma\delta$ T cells were seeded with PMN-MDSC (1:1 ratio) and activated with IPH 11 (3 μ M, Innate Pharma) or with the Burkitt lymphoma cell line Daudi (2:1 ratio effector:target) and IL-2 (100 U/mL, Sigma-Aldrich). Cells were maintained at 37°C in humidified air with 5% CO₂. After 5 days, lymphocytes proliferation was evaluated by flow cytometry.

Flow Cytometry

The V δ 2 T cells and PMN-MDSC frequency and phenotype were evaluated utilizing the following monoclonal antibodies: anti-V δ 1 (Life technology), anti-NKG2A, anti-NKG2D (Beckman Coulter), anti-NKG2C (R&D system), anti-V δ 2, anti-CD3, anti-CD15, anti-CD33, anti-HLA-DR, cocktail of antibodies anti-CD3,



-CD56, -CD19, anti-CD14, anti-CD11b (BD Biosciences). In brief, the cells were washed twice in PBS, 1% BSA, and 0.1% sodium azide and were stained with the mAbs for 15 min at 4°C. The cells were then washed and fixed with 1% paraformaldehyde and analyzed using a FACS Canto II (Becton Dickinson). For intracellular staining, membrane staining was performed as above described. After fixation cells were incubated with anti-IFN γ (BD Biosciences) for 30 min at room temperature. CD107a detection was accomplished by antibody staining during cell stimulation. After washing cells were analyzed using a FACS Canto II (Becton Dickinson). Apoptosis induction of Daudi cells were accomplished by evaluating Annexin V ligation to Daudi (Annexin V-FITC Apoptosis Detection Kit, eBiosciences) following the manufacturer's instruction. Then cells were stained with anti-CD19, anti-V δ 2, anti-CD3, anti-CD15.

Statistical Analysis

Results were evaluated using a paired *t* test. A *p* value < 0.05 was considered statistically significant. GraphPad Prism software (version 4.00 for Windows; GraphPad) was used to perform the analysis and graphs.

RESULTS

V δ 2 T Cells Are Partially Inhibited by PMN-MDSC

It has been demonstrated that MDSC are able to inhibit T cell activity, but little is known about MDSC/V δ 2 T cell relationship. To address this issue PMN-MDSC and $\gamma\delta$ T cells were magnetically purified (purity >90 and >85%, respectively, **Figures 1A,B**) and were cocultured at different ratios. The ability of MDSC to modulate V δ 2 T cell cytotoxicity and IFN- γ production was evaluated by analyzing the expression of CD107a or IFN- γ on V δ 2 T cells after 18 h. In two preliminary experiments, we optimize the V δ 2/MDSC ratio by looking at CD107a modulation on V δ 2 T cells. As shown in **Figure 2A**, PMN-MDSC partially inhibit the capacity of V δ 2 T cells to express CD107a in response to IPH stimulation at all ratios (**Figure 2A**). Therefore, the $\gamma\delta$ T cells/PMN-MDSC 1:1 ratio has been used in subsequent five independent experiments, confirming that PMN-MDSC were able to decrease CD107a expression on V δ 2 T cells (**Figures 2B,C**). We also tested the capability of PMN-MDSC to interfere with IFN- γ production.

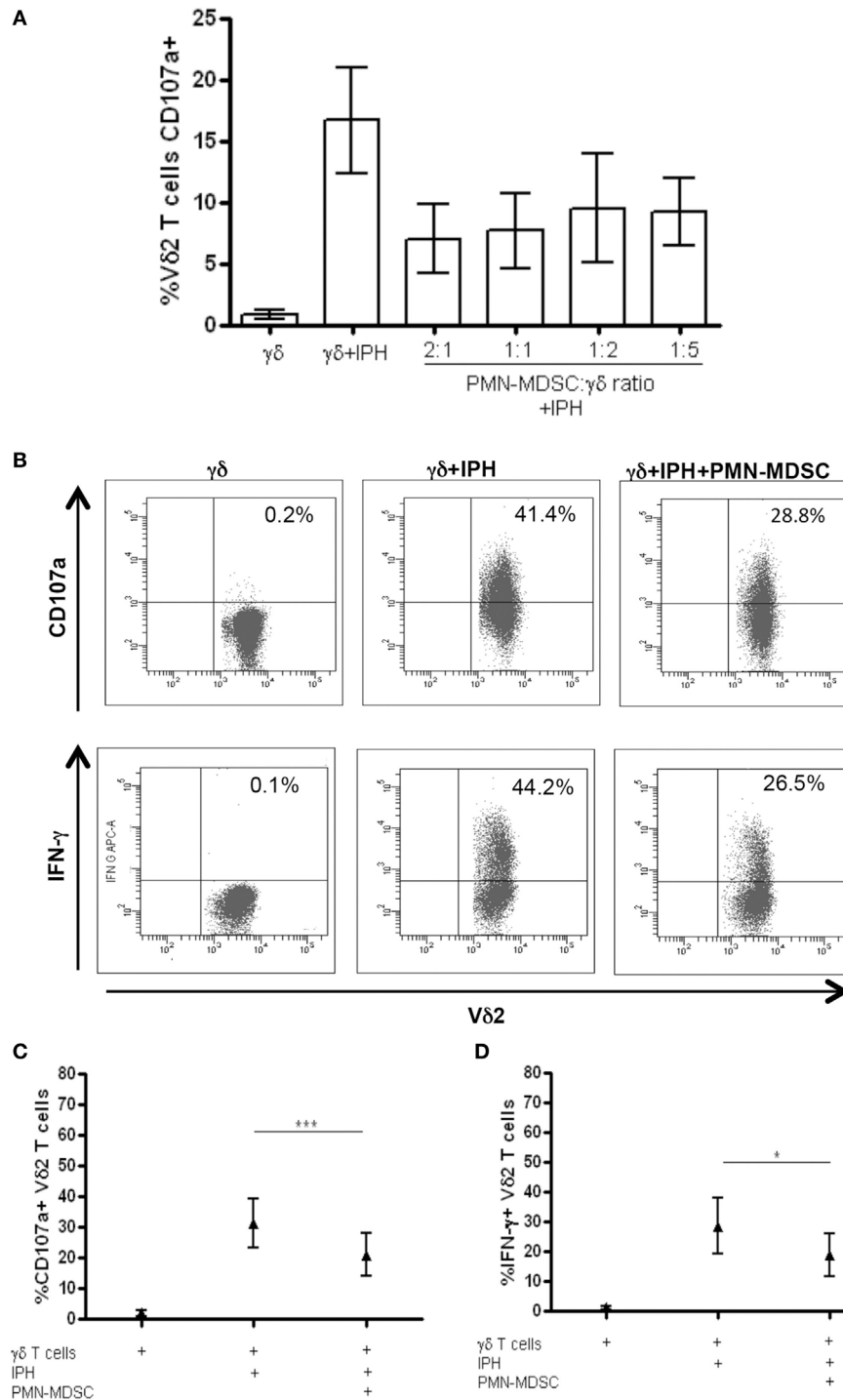


FIGURE 2 | PMN-myeloid-derived suppressor cells (MDSC) inhibit IFN- γ production and CD107a expression by V δ 2 T cells. Purified $\gamma\delta$ T cells were stimulated with IPH and IL-2 in the presence of PMN-MDSC and after 18 h IFN- γ and CD107a were evaluated by flowcytometry. **(A)** Titration of PMN-MDSC/ $\gamma\delta$ T cells ratio tested by measuring CD107a expression on V δ 2+ T cells after IPH stimulation by flowcytometry. Results are shown as mean + SEM of two independent experiments. **(B)** Representative dot plots of IFN- γ production and CD107a expression by V δ 2 T cells. **(C)** Percentage of CD107a + V δ 2 T cells and **(D)** IFN- γ + V δ 2 T cells and in the indicated conditions. Results are shown as Mean + SEM of five independent experiments (* p < 0.05, *** p < 0.0001).

To this aim, we cultured purified PMN-MDSC and $\gamma\delta$ T cells at 1:1 ratio and after 18 h of stimulation with IPH the production of IFN- γ was evaluated by flow cytometry. A decrease of

IFN- γ expression was observed in the presence of PMN-MDSC (Figures 2B,D), suggesting that PMN-MDSC may also inhibit cytokine production capacity of V δ 2 T cells.

We wondered whether PMN-MDSC may impair the proliferation capability of V δ 2 T cells. To this aim, purified CFDA-SE labeled V δ 2 T cells were stimulated with IPH and IL-2 for 5 days and the proliferation rate was evaluated by flow cytometry. We found that PMN-MDSC did not impact the proliferation of V δ 2 T cells (**Figure 3A**). On the contrary, as previously demonstrated (19) they are able to inhibit the CD3+ T cell proliferation, as SEB-induced proliferation of CD3+ T cells (evaluated using CFDA-SE labeled total PBMC) was decreased by PMN-MDSC (**Figure 3B**).

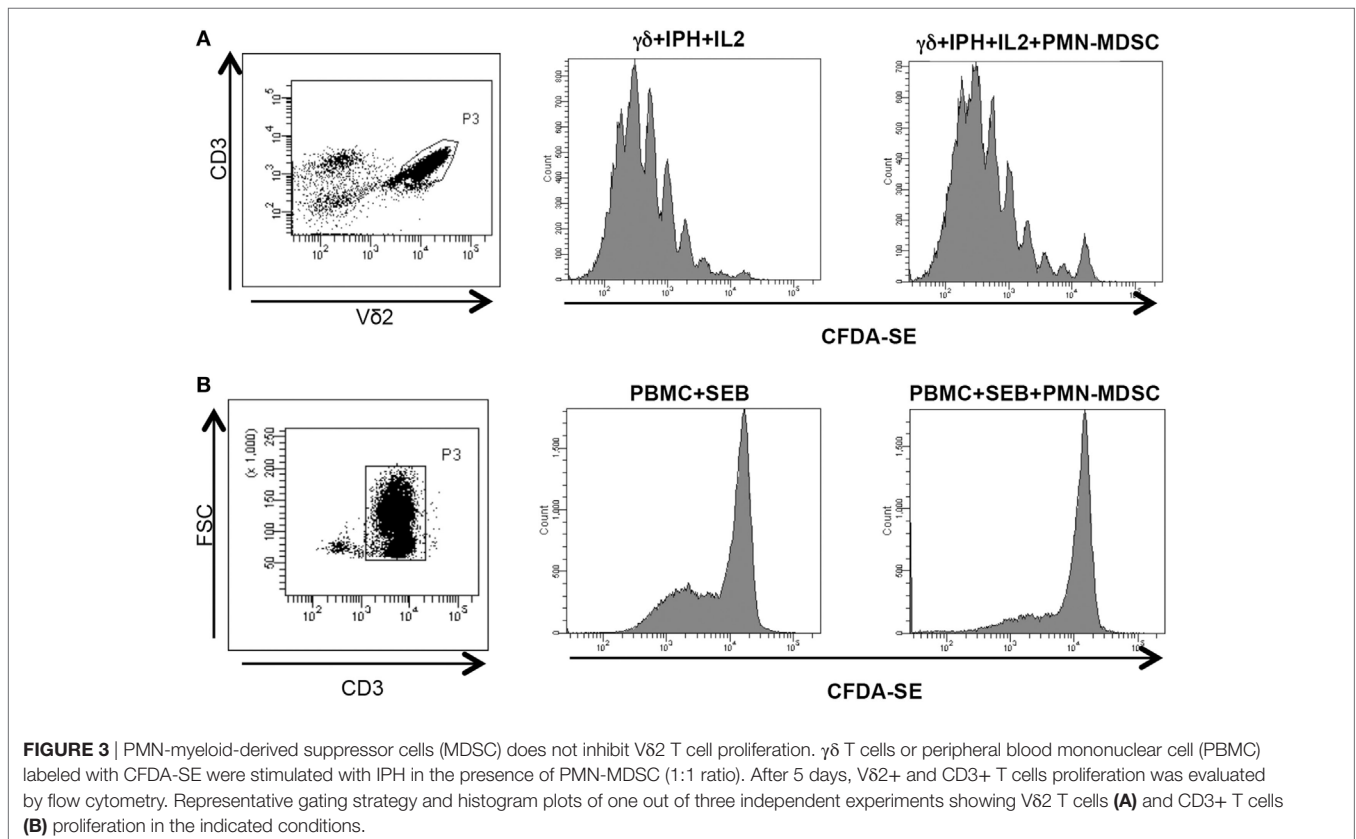
PMN-MDSC Inhibit V δ 2 T Cells Cytotoxicity Against Tumor Cell Lines

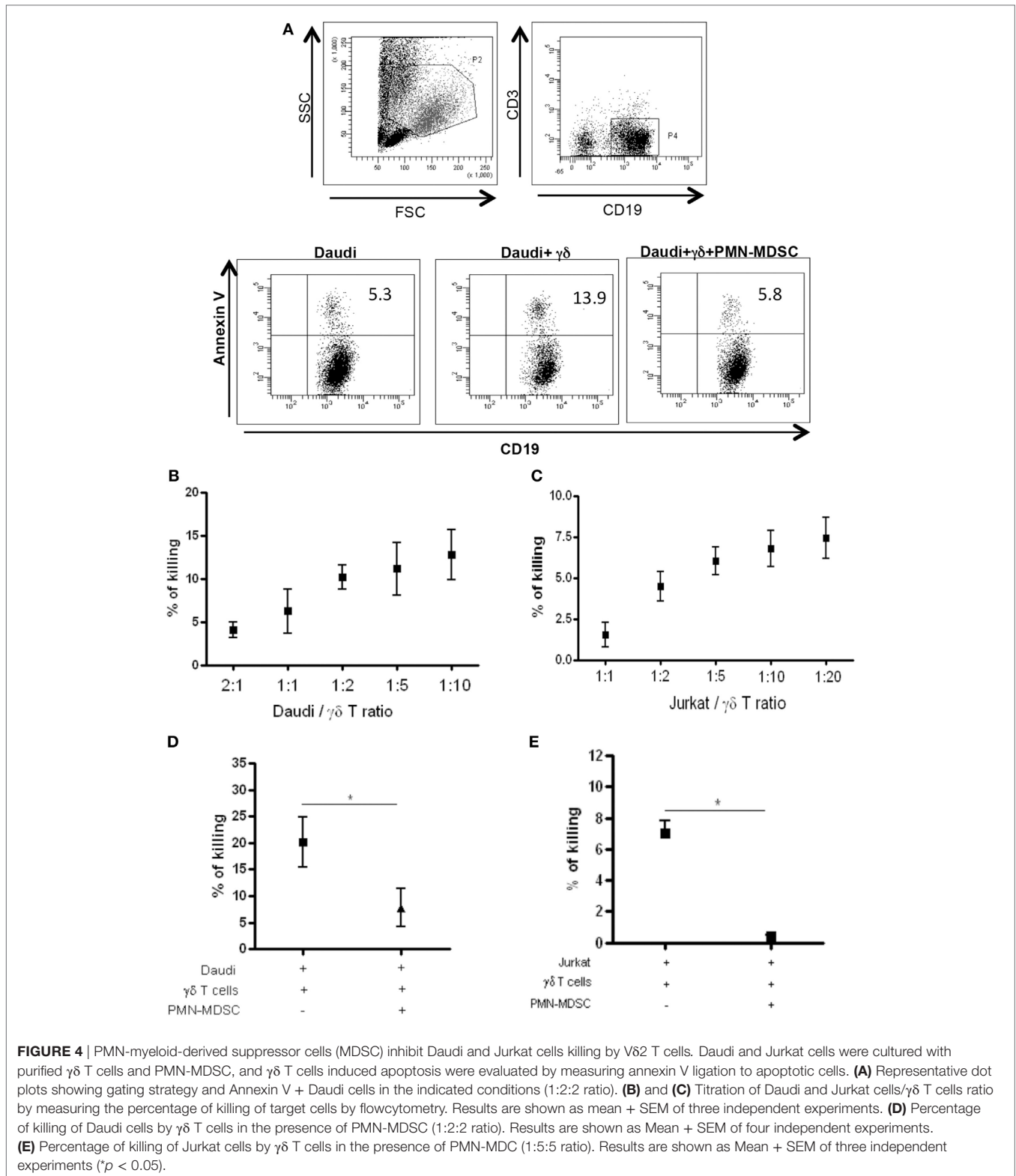
V δ 2 T cells play a pivotal role in controlling tumor cells exerting a potent cytotoxic function. We wondered if the PMN-MDSC-induced down-modulation of CD107a was correlated to a decrease cytolytic capability towards tumor cells. To answer this question, we first cultured Daudi cells, a Burkitt's lymphoma known to be a target for V δ 2 T cell cytotoxicity, with purified $\gamma\delta$ T cells at different ratios (to select the best ratio) and the cytotoxic activity of V δ 2 T cells was evaluated by analyzing membrane Annexin V ligation on apoptotic Daudi cells by flow cytometry. We found that 1:2 ratio was sufficient to induce Daudi cell apoptosis (**Figures 4A,B**). Next, Daudi cells were cultured with purified $\gamma\delta$ T cells and PMN-MDSC (1:2:2 ratio) for 18 h and apoptotic Daudi cells were analyzed. As expected, we found that V δ 2 T cells are able to kill Daudi cells; however, the presence of PMN-MDSC strongly inhibit their cytotoxic function (**Figure 4C**). We performed the

same experiments with a different tumor cells line (Jurkat), and after assessing the ratio sufficient to induce Jurkat cell apoptosis (1:5, **Figure 4C**), a decreased capacity of V δ 2 T cells to kill Jurkat were also observed when PMN-MDSC (1:5:5) were added to the culture (**Figure 4E**). These data indicate that PMN-MDSC inhibition of cytotoxic activity of V δ 2 T cells is not restricted towards Daudi cells. V δ 2 T cell cytotoxicity was deeply controlled by NK receptors expression (20). To evaluate whether MDSC-induced inhibition of V δ 2 T cell cytotoxicity could be mediated by NKR modulation, the expression of NKG2A, NKG2C, and NKG2D on V δ 2 T cells was evaluated in the absence and in the presence of PMN-MDSC. Results showed that PMN-MDSC did not alter the expression of NKG2A (66.2 ± 8.6 vs $66.3 \pm 8.4\%$), NKG2C (mfi 772 ± 82 vs 748 ± 101), and NKG2D (mfi $1,467 \pm 414$ vs $1,548 \pm 560$) on V δ 2 T cells. Finally, Daudi cells were able to induce V δ 2 T cell proliferation in the presence of IL-2, and the proliferation rate of V δ 2 T cells was not affected by PMN-MDSC (**Figure 5**), confirming that PMN-MDSC do not impair V δ 2 T cells proliferating capacity.

Specific Inhibitor of Arg1 Restore $\gamma\delta$ T Cells Cytotoxic Activity Against Daudi Cells

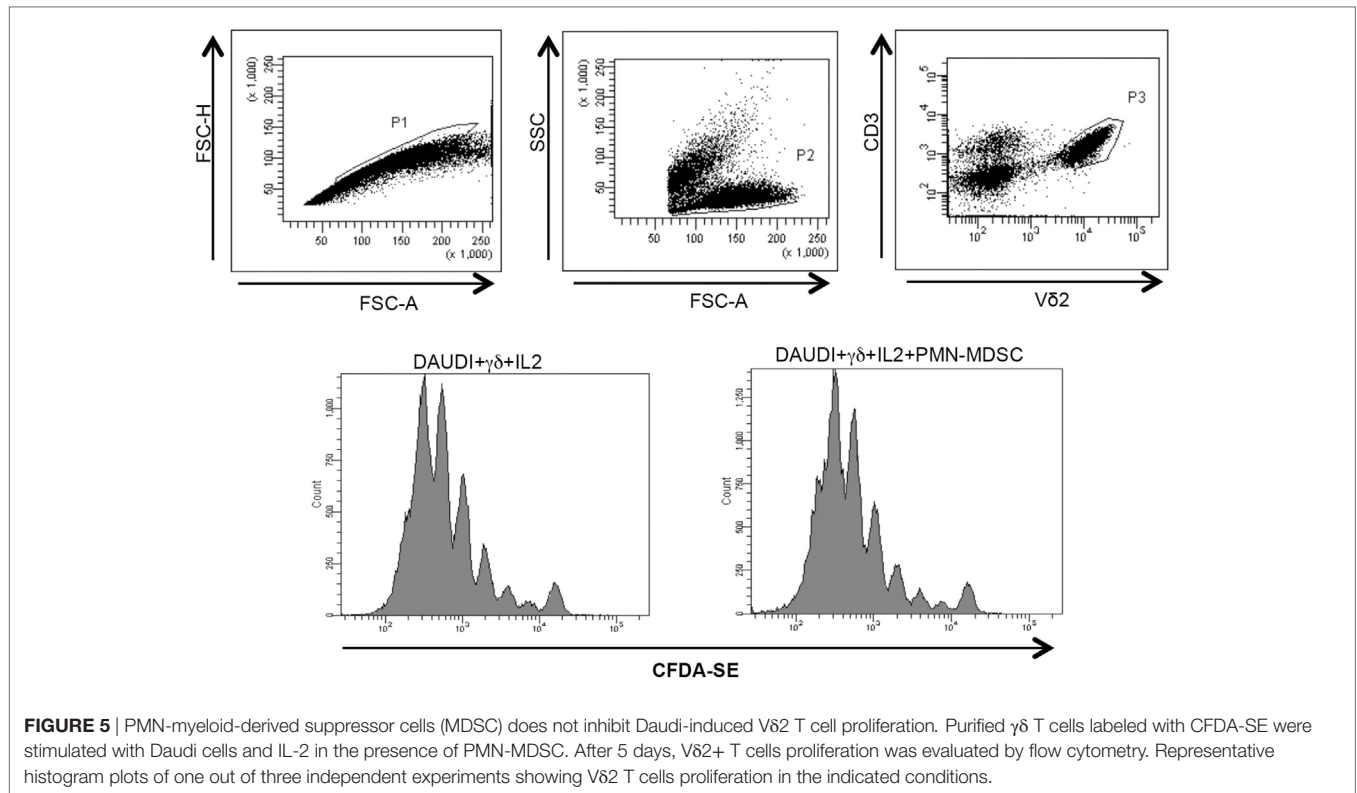
To understand the mechanisms used by PMN-MDSC to inhibit $\gamma\delta$ T cells cytotoxic activity, we cultured Daudi cells with purified $\gamma\delta$ T cells and PMN-MDSC (1:2:2 ratio) in the presence of specific inhibitors of Arginase I (nor-NOHA), indoleamine





2,3-dioxygenase (1-MTD) or nitric oxide synthases (L-NMMA). We found that 1-MTD and L-NMMA did not affect the capacity of PMN-MDSC to decrease cytotoxic function of $\gamma\delta$ T cells (data not shown), suggesting that indoleamine 2,3-dioxygenase

and nitric oxide synthases are not involved. On the contrary, nor-NOHA abrogated the inhibition of cytotoxic function of $\gamma\delta$ T cells performed by PMN-MDSC (**Figures 6A,B**). However, an enhancement of $\gamma\delta$ T cells cytotoxicity was also observed in the



absence of PMN-MDSC (**Figures 6A,B**), suggesting that Daudi cells may produce ArgI. However, since the addition of PMN-MDSC to nor-NOHA treated $\gamma\delta$ T cells cultured with Daudi cells did not decrease the capacity of $\gamma\delta$ T cells to kill their targets may suggest that PMN-MDSC used ArgI as well.

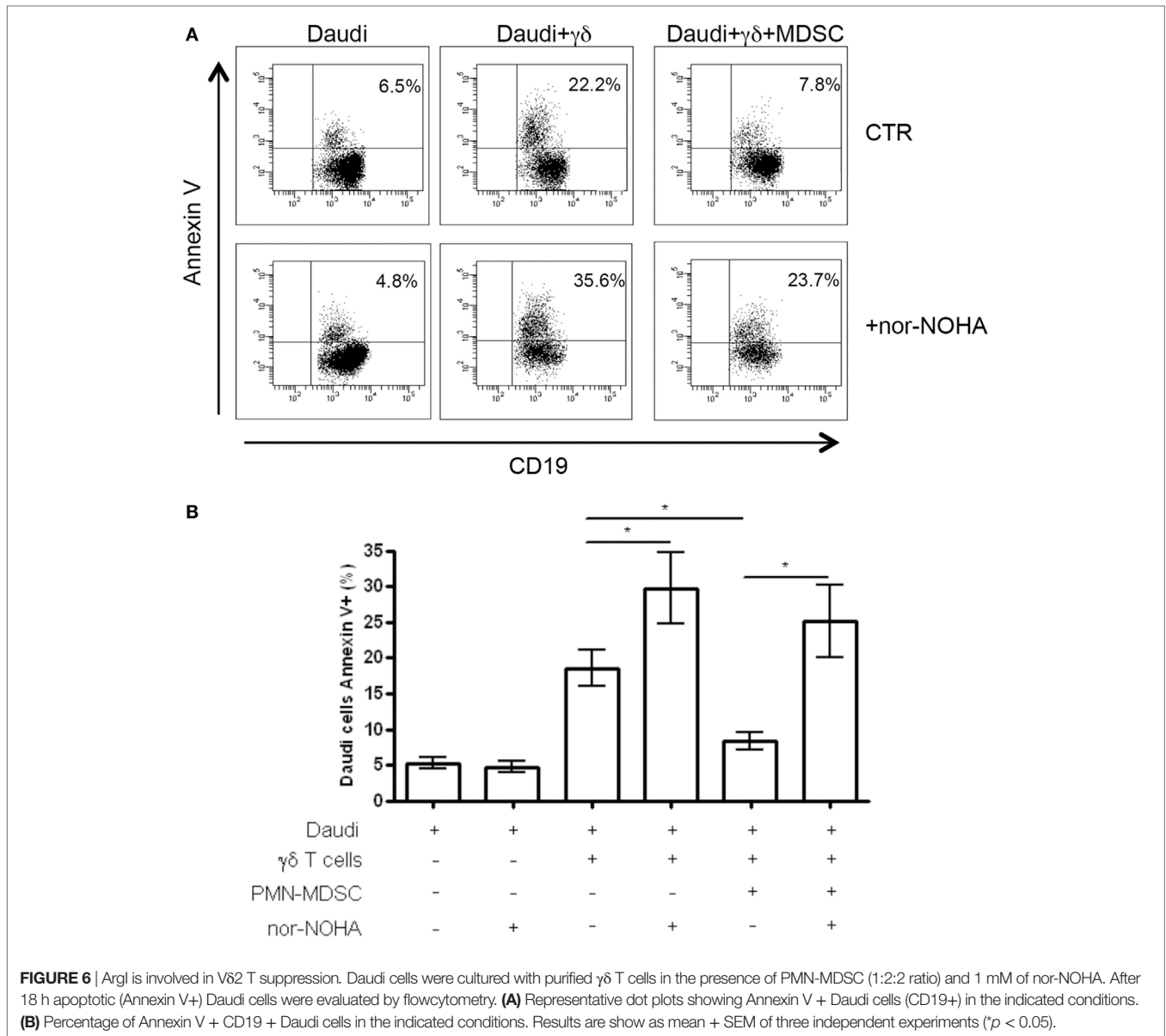
DISCUSSION

V δ 2 T cells participate in the early stages of the immune response, in fact they are able to recognize antigens displayed following infection or other forms of stress, and to respond to stress without requiring extensive clonal expansion. Moreover, they are able to shape the subsequent adaptive immunity, representing a bridge between innate and adaptive immune response. Furthermore, V δ 2 T cells crosstalk with different cell types of the innate and adaptive immunity potentiating their activity. V δ 2 T cells are able to give help to B cells in producing antibodies (21), and to induce maturation of DC (22) thus influencing $\alpha\beta$ T cell priming. Activated V δ 2 T cells shortly produce huge amount of IFN- γ , TNF- α (5) that play a central role in controlling tumor and infections. A pivotal feature of V δ 2 T cells is the cytotoxic potential against tumor cells (9, 10) and infected cells (23, 24), that participate to control the diseases. However, tumors and microbial pathogens are able to evade the immune system by means different mechanisms. It has been clearly demonstrated that MDSC play a detrimental role during tumor pathologies, and their expansion has been shown during several infections (13, 25, 26). MDSC are able to suppress different cell types [reviewed in Ref. (27)]; however, data

on V δ 2 T cells are still lacking. We found that, similarly to $\alpha\beta$ T cells (19), PMN-MDSC are able to inhibit IFN γ production by phosphoantigen activated V δ 2 T cells. Differently from $\alpha\beta$ T cells, the proliferation of V δ 2 T cells was not affected. It has been suggested that IL-2 may inhibit MDSC functions (28), then we cannot exclude that the use of IL-2 necessary to induce *in vitro* V δ 2 T cell proliferation could alter MDSC suppressive capability. PMN-MDSC induced a decrease of CD107a expression on V δ 2 T cells, indeed the cytotoxic activity against Daudi and Jurkat cells was reduced. The impaired cytotoxicity was independent of the modulation of the major inhibitory and activating NK receptors, suggesting a possible TCR-mediated mechanism.

Myeloid-derived suppressor cell are able to produce different immunosuppressive mediators such as ArgI, iNOS, and IDO, that are all able to induce T cell anergy through different pathways (29). Our data suggest that ArgI may be involved in the inhibition of V δ 2 T cells by PMN-MDSC. Interestingly, ArgI inhibition increased $\gamma\delta$ T cells cytotoxicity also in the absence of PMN-MDSC, indicating that Daudi cells are able to produce ArgI.

The immunomodulating strategies represent one of the main exciting approaches in the battle against cancer and V δ 2 T cells have been tested as a good target for their wide and potent antitumor activity. Nevertheless, the suppressive tumor environment could affect these strategies. We showed for the first time that PMN-MDSC inhibit the antitumor activity of V δ 2 T cells, opening a key issue in the context of V δ 2-targeted immunotherapy. Other studies are mandatory in order to better clarify the molecular mechanisms of these suppression and to propose combined



strategies aimed to boost V δ 2T cells circumventing tumor- and MDSC-induced V δ 2 T cells inhibition.

AUTHOR CONTRIBUTIONS

ASacchi and CA designed the study and wrote the paper. NT and ASabatini performed the experiments. ASacchi analyzed the

data. EC, VB, RC, and GG contributed to analyze data and revise the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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