

# The Immunomodulatory Activity of Thymosin Alpha 1 on Tumor Cell Lines and Distinct Immune Cell Subsets

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**Background:** The immune-balancing role of thymosin alpha 1 (Tα1) is well-recognized in contexts of immune dysregulation. Within the anti-tumor context, Tα1 demonstrated to act as an immune-enhancer, with potential roles in immunotherapy-based treatments. However, Tα1 immunomodulatory potential on tumor cells is poorly understood. Additionally, Tα1 pleiotropic effects on immune cells require in-depth investigations to unravel its specific impact on different immune cell populations. Thus, we first aimed to investigate whether Tα1 treatments influenced the transcriptional immune profile of various cancer cell lines. Alongside, CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and natural killer cells from healthy donors (HDs) were treated individually with Tα1, to assess its direct effects on each immune cell population.

**Methods:** Cutaneous melanoma, glioblastoma, and pleural mesothelioma cell lines and HD immune cell subsets were treated with Tα1 for 48 hours. Total RNA was subsequently isolated, and gene expression profiles were analyzed by the nCounter<sup>®</sup> SPRINT Profiler. Genes with a log2ratio ≥0.58 and ≤-0.58 in Tα1-treated vs untreated cells were defined as differentially expressed (DEGs) and subsequently evaluated for the enrichment of Gene Ontology terms to identify biological processes potentially affected by Tα1 in tumor and immune cells.

**Results:** Tα1 minimally changed cancer cell DEGs and immune-related biological processes, suggesting a comprehensive lack of transcriptional immunomodulatory potential on the tumor counterpart. Conversely, Tα1 exhibited to directly affect the proliferation and/or transcription processes of each studied immune cell subset, with the greatest transcriptional impact observed for activated CD8<sup>+</sup> T cells, crucial players in anti-tumor immunity.

**Conclusion:** Our findings question the tumor immunomodulatory properties of Tα1, simultaneously underscoring the importance of further investigating Tα1 influence on specific immune cell subsets in the periphery or within the tumor microenvironment of cancer patients. This would contribute to understand Tα1 potential in immunotherapy-based combination strategies, within the anti-tumor setting.

**Keywords:** cancer, immune response, immunomodulatory agents, cancer immunotherapy

## Introduction

Thymosin α1 (Tα1) is a 28-aminoacid thymic peptide classified as a biological response modifier (BRM), being a natural immunomodulant agent with pleiotropic activities.<sup>1</sup> Due to these features, its drug formulation has a clinical history as immunologic adjuvant of more than 30 years, with worldwide therapeutic applications in contexts of immunity dysregulation (eg, cancer, viral infections, chronic inflammations, sepsis, immunodeficiencies, and vaccine non-responsiveness).<sup>2</sup> Among its described activities, Tα1 can promote dendritic cell (DC) maturation and antigen presentation via the toll-like receptor (TLR)-9 and TLR2 stimulation,<sup>3</sup> key linker molecules of innate and adaptive immunity. Tα1 showed to have effects also on restoring

CD4<sup>+</sup> T, CD8<sup>+</sup> T, and/or natural killer (NK) cell counts when concomitantly administered with immunosuppressive cancer therapy<sup>4,5</sup> or when administered to patients with immunocompromised conditions.<sup>6,7</sup>

Thus, its pleiotropic effects and its optimal safety profile<sup>8</sup> prompted many investigators to combine Tα1 with different therapeutic strategies.<sup>2</sup> Within the anti-tumor setting, Tα1 has given proof to ameliorate chemotherapy response when administered to metastatic melanoma patients, by improving the duration of therapeutic response and the clinical benefit, with no additional toxicity.<sup>9</sup> A retrospective study investigated whether stage I–III non-small cell lung cancer (NSCLC) patients had benefitted from Tα1 administration alone or combined with chemo(radio)therapy and/or targeted therapy.<sup>10</sup> Results showed that, except for squamous carcinoma patients and targeted therapy-treated patients, Tα1-receiving groups in the adjuvant setting exhibited an increased survival benefit, compared to non-Tα1 groups. Furthermore, in a Phase 2 study, Tα1 displayed beneficial effects for local advanced NSCLC patients by significantly reducing the immune-related adverse events derived from chemoradiotherapy treatments (eg, pneumonitis, lymphopenia), compared to the non-Tα1 control group.<sup>11</sup>

So far, despite the promising results of preclinical studies in murine models,<sup>12,13</sup> no clinical trial has demonstrated the efficacy of combining Tα1 with the more recent immune-checkpoint blockade (ICB)-based immunotherapy that entirely remodeled the cancer treatment panorama. However, Danielli et al in a retrospective study pointed out that metastatic melanoma patients treated with the anti-CTLA4 monoclonal antibody (mAb) Ipilimumab (IPI) that were previously treated with Tα1 had a prolonged median overall survival (OS) compared to those patients that were not pre-Tα1-treated, with an OS rate at 5 years being 41.2% and 13.0% ( $p=0.006$ ) for Tα1-IPI and IPI alone patients, respectively.<sup>14</sup>

Although clinical benefits from Tα1 administration in patients are evident among several tumor histotypes,<sup>15</sup> the molecular mechanisms behind Tα1 effects have not been fully elucidated and are sometimes controversial.<sup>16–19</sup> Besides, whether the clinical efficacy depends on direct effects exerted by Tα1 on immune and also on tumor cells is still unclear. Because of this gap and to better investigate the possible role of Tα1 in improving the effectiveness of immunotherapy-based strategies, the current work aimed to deepen the potential immunomodulatory effect of Tα1 on tumor cell lines of 3 different histotypes ( $n=13$  cell lines) and on distinct immune cell subsets from healthy donors (HDs;  $n=3$ ). In particular, for the first time to our knowledge, an in-depth study evaluating Tα1 ability to explicate a common modulation of the transcriptional immune landscape of tumor cells was conducted. Additionally, to better understand the role of Tα1 within the immune system, its effects on proliferation and gene expression profile of distinct immune cell subsets (ie, CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK cells) from HDs were studied by culturing and Tα1-treating cells separately. Thus, Tα1 direct stimulation of different immune populations allowed us to unravel the individual responses of each investigated cell subset to Tα1. Ultimately, whether Tα1 was able to modulate the cytolytic activity of immune effector cells (ie, Lymphokine-activated killer cells (LAK) and Cytotoxic T Lymphocytes (CTL)) was explored.

## Materials and Methods

### Reagents and Compounds

BV421-conjugated mouse anti-human CD45, FITC-conjugated mouse anti-human CD3, APC-H7-conjugated mouse anti-human CD8, PE-conjugated mouse anti-human CD4, BV650-conjugated mouse anti-human CD20, and BV421-conjugated mouse anti-human PD-L1 were purchased from BD Biosciences (NJ, USA); PE-conjugated mouse anti-human CD56 and the anti-human CD3ε antibody (clone OKT3) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Alexa fluor488-conjugated mouse anti-human HLA-ABC, PE-conjugated mouse anti-human CD54, and PE-conjugated mouse anti-human Melan-A (MART-1) were purchased from Biolegend (CA, USA), Beckman Coulter (CA, USA), and Santa Cruz Biotechnology (TX, USA), respectively. Interleukin (IL)-2 was purchased from Clinigen (PA, USA). Tα1 was kindly provided by SciClone Pharmaceuticals Inc (CA, USA).

### Tumor Cell Lines

Mel146, Mel195, Mel261, Mel275, Mel313, Mel514, Mel599, and Mel601 cutaneous melanoma (cMM), SiGBM56 and SiGBM71 glioblastoma (GBM), Meso4, Meso6, and Meso7 pleural mesothelioma (PM) cell lines originated, respectively, from patients' metastatic tissue of melanoma, primary GBM tumor lesions, and pleural effusions of mesothelioma. Patient-derived cell lines were established in our laboratory, as follows. In detail, cMM and GBM tissues were processed

within 60 min following surgical removal to be dissected into fragments by mechanical digestion (1–2 mm<sup>3</sup>), and subsequently cultured in the appropriate medium (RPMI Medium 1640; Biochrom, Berlin, Germany); whereas mesothelioma pleural effusions were collected by thoracentesis or paracentesis, centrifuged for 10 min at 150 g, and cell pellets were resuspended in the ad hoc medium (HAM's F-12; Euroclone, Milan, Italy). Culture media were supplemented with 20% heat-inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), up to the 5<sup>th</sup> passage of in vitro culture, and with 10% FBS for subsequent ones. For the experiments reported below, continuous immortalized tumor cell lines, established after at least 20 passages of in vitro culture, were used.

In addition, the LN-18 GBM cell line (American Type Culture Collection (ATCC) company) was cultured in 5% FBS-DMEM medium (Sigma-Aldrich, MO, USA); the FO-1<sup>20</sup> melanoma and the DBTRG-05MG GBM (ATCC) cell lines were cultured in 10% FBS-RPMI 1640 medium; the LoVo colorectal adenocarcinoma cell line (ATCC) was maintained in 10% FBS-HAM's F-12 medium; and the K562 erythroleukemia cells<sup>21</sup> were grown in 10% FBS-Iscove's Modified Dulbecco's Medium (Euroclone, Milan, Italy).

All cell media were supplemented with 2mM L-glutamine and 100 µg/µL penicillin/streptomycin (Biochrom, Berlin, Germany). Cells were incubated at 37°C and 5% CO<sub>2</sub> and passaged at 80–90% confluency.

## Immune Cells

Human peripheral blood mononuclear cells (PBMCs) from HDs, purchased from ZenBio (NC, USA; catalog number SER-PBMC-200-F), were processed to isolate specific immune cell subsets. In detail, PBMCs went through the MACS<sup>®</sup> Column-based cell separations following the CD19, CD56, CD8, and CD4 MicroBeads human protocols (Miltenyi Biotec, Bergisch Gladbach, Germany), to isolate B, NK, CD8<sup>+</sup> T, and CD4<sup>+</sup> T cell subsets, respectively. An aliquot of 0.5×10<sup>6</sup> isolated immune cells was used to assess the purity of each cell subset by multiparametric flow cytometry, with a percentage (%) threshold of positive cells to specific subset markers (ie, CD3<sup>+</sup>CD20<sup>+</sup>, CD56<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>) >80% ([Supplementary Files 1](#) and [2](#)). The remaining immune cell subsets were cultured in vitro under the following specific conditions: B cells were activated for 14 days with 50 IU/mL IL-4 and human CD40-Ligand (CD40L) Multimer Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in the StemMACS<sup>™</sup> HSC Expansion Media XF supplemented with 5% human serum (Euroclone, Milan, Italy), according to the B Cell Expansion Kit guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany); resting CD4<sup>+</sup> and CD8<sup>+</sup> T cells were cultured for 24 hours in 10% human serum-Iscove's Modified Dulbecco's Medium with 100 U/mL IL-2; activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were maintained for 3 days in 10% human serum-Iscove's Modified Dulbecco's Medium with 100 U/mL IL-2 and 50 ng/mL anti-CD3 antibody; activated NK cells were cultured for 24 hours with 600 U/mL IL-2 in 10% FBS-RMPI 1640 medium.

LAK cells<sup>20</sup> were obtained by PBMCs from the same HDs cultured in 10% FBS-RMPI 1640 supplemented with 1000 U/mL IL-2 for 4 days. HLA-A2-restricted gp100-peptide-specific CTL, cultured in 10% human serum-Iscove's Modified Dulbecco's Medium with 1000 U/mL IL-2, were generated and characterized as previously described.<sup>22</sup>

All immune cell media were supplemented with 2mM L-glutamine and 100 µg/µL penicillin/streptomycin. B and NK cells were plated at a density of 1×10<sup>6</sup> cells/mL; LAK, CD4<sup>+</sup> T, CD8<sup>+</sup> T, and CTL cells at a density of 2×10<sup>6</sup> cells/mL. Cells were incubated at 37°C and 5% CO<sub>2</sub>.

## WST-1 Assay

Cell proliferation was assessed after treatments with scalar doses of Tα1 in tumor cMM (ie, Mel146, Mel195, Mel261, Mel514, Mel599, and Mel601), GBM (ie, LN-18, DBTRG-05MG, SiGBM56, and SiGBM71), and PM (ie, Meso4, Meso6, and Meso7) cell lines (1 µM, 10 µM, 100 µM) and in HD's immune cell subsets (30 nM, 300 nM, 3 µM), by the WST-1 assay (Roche, Molecular Biochemicals, Mannheim, Germany). Briefly, 5×10<sup>3</sup> tumor cells and 2.5×10<sup>5</sup> immune cells were seeded in triplicate or quintuplicate, according to cell availability, in a 96-well culture plate in a final volume of 200 µL/well culture medium and treated with Tα1 following the appropriate treatment schedule. After 48 hours, 1:10 WST-1 reagent was added to cell plates, and spectrophotometer lectures were performed after 3 and 4 hours for tumor cell lines and immune cell subsets, respectively. Untreated cells that underwent the same experimental conditions represented the assay controls. The blank consisted of the culture medium and an equal amount of the WST-1 reagent. Absorbance was measured using the Benchmark<sup>™</sup> Plus microplate reader (Bio-Rad, CA, USA) at 450 nm, subtracting

wavelength at 655 nm. The % of cell proliferation was calculated by optical density (OD) results, as follows:  $(\text{OD treatment} - \text{OD blank}) / (\text{OD control} - \text{OD blank}) \times 100$ .

## In vitro Cell Treatments with Tα1

Tα1 in vitro cell treatments were performed on both tumor and immune cells according to the following schedules. Tumor cell lines cMM (ie, Mel146, Mel195, Mel261, Mel514, Mel599, and Mel601), GBM (ie, SiGBM56, SiGBM71, DBTRG-05MG, and LN-18), and PM (ie, Meso4, Meso6, and Meso7) were seeded on day 0 and treated with 10 μM Tα1 on day 1.

Immune cells (ie, immune cell subsets, LAK, and CTL), cultured/activated according to their in vitro cell culture conditions, were seeded and treated with 3 μM Tα1 on day 0.

Tumor and immune cells were harvested 48 hours from drug exposure for the following in vitro experiments. Untreated cells underwent the same experimental conditions and were used as controls.

## Gene Expression Profiling After Tα1 Treatments

Cell total RNA was isolated following the Trizol reagent (Invitrogen, CA, USA) protocol and quantified by the NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, MA, USA). 80 ng of total RNA were used for gene expression analyses conducted with the nCounter® SPRINT Profiler (NanoString Technologies Inc, Seattle, WA, USA). In detail, gene expression profiles were analyzed in untreated and Tα1-treated cMM (ie, Mel146, Mel195, Mel261, Mel514, Mel599, and Mel601), GBM (ie, DBTRG-05MG, LN-18, SiGBM56, and SiGBM71), and PM (ie, Meso4, Meso6, and Meso7) cell lines, utilizing the 770 gene-PanCancer IO 360™ panel; gene expression profiles of untreated and Tα1-treated immune subsets (CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK cells) were evaluated from 3 HDs exploiting the 770 gene-PanCancer Immune Profiling™ panel. The nSolver 4.0 Analysis Software (NanoString Technologies Inc, Seattle, WA, USA) elaborated raw data into normalized log<sub>2</sub> ratios of Tα1-treated vs untreated cells.

## Flow Cytometry Analysis

Direct immunofluorescence staining was performed on  $0.5 \times 10^6$  cell aliquots to detect antigens of interest. Cells were incubated for 10 min at +4°C in 1X PBS (Thermo Scientific, MA, USA) + 2 mM EDTA (Sigma-Aldrich, MO, USA) + 0.5% BSA (Sigma-Aldrich, MO, USA), followed by a 30-minute incubation at +4°C with the appropriate fluorochrome-conjugated antibodies to detect cell surface antigens; for antigen intracellular staining, cells were processed following the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit instructions (BD Biosciences, NJ, USA) and antibody-labeled for 30 min at +4°C. Unstained cells used as the assay controls underwent the same experimental conditions without antibody. Cells were acquired by the CytoFLEX S instrument (Beckman Coulter, CA, USA). Data were analyzed with the Kaluza® Flow Analysis Software (Beckman Coulter, CA, USA) and displayed as fold-change (FC) of percentage of antigen-positive cells and as mean fluorescence intensity (MFI; *mean stained/mean unstained value*) in Tα1-treated vs untreated cells.

## Cytotoxicity Assay

The cytotoxicity of untreated and Tα1-treated effector cells (ie, LAK and CTL) was measured against specific target tumor cells. The assay was performed by flow cytometric analysis using the Annexin V-FITC Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), which allowed to distinguish early apoptotic (Annexin V +, Propidium iodide (PI) -), late apoptotic (Annexin V +, PI +), and necrotic (Annexin V -, PI +), from live cells (Annexin V -, PI -). Effector (E) cells were co-cultured with their specific target (T) cells in 96-multiwells and the following E:T ratios were used for the assays: 10:1, 5:1, and 2.5:1 for LAK:T; 12:1, 6:1, 1:1, and 0.5:1 for HLA-A2-restricted gp100-peptide positive CTL:T. E: T plates were incubated for 4 hours at 37°C in a 5% CO<sub>2</sub> incubator. To follow, both suspension and adherent cells were recovered and fluorescently stained with BV421 mouse anti-human CD45, to discriminate the CD45-negative target cells from the CD45-positive effector cells in the subsequent flow cytometric analysis. Then, cells were further processed following the Annexin V-FITC Kit protocol and ultimately read by the CytoFLEX S instrument (Beckman Coulter, CA, USA), according to the manufacturer's guidelines. Data were elaborated by the Kaluza® Flow Analysis Software (Beckman Coulter, CA, USA) and expressed as the % of necrotic+apoptotic target cells, following the subtraction of

spontaneous target cell death. Unstained cells were not antibody-labeled and were used as flow cytometry control. FO-1 and LoVo cell lines were selected as positive and negative targets for LAK-mediated lysis, according to the presence or absence of CD54 on their surface, respectively.<sup>20,23</sup> Mel195, Mel275, and Mel313 melanoma cell lines (HLA-A2-positive, gp100-positive; data not shown) were selected as positive targets for CTL-mediated lysis, whereas the K562 cell line (HLA-A2-negative, gp100-negative) represented the negative control.<sup>21</sup>

## Data Analysis

Statistical analyses were conducted by the paired or unpaired two-tailed Student's *t*-test and *p*-value (*p*) <0.05 was considered statistically significant. Differentially expressed genes (DEGs) from NanoString assays were defined as those genes with log<sub>2</sub>ratio ≥0.58 and log<sub>2</sub>ratio ≤−0.58, being up- or down-regulated respectively, in Tα1-treated vs untreated cells. DEGs that were positively or negatively modulated in at least 3 cMM, 2 GBM, and 2 PM cell lines, and with opposite modulation in no more than 1 cMM, 1 GBM, and 0 PM cell lines, were used to build the Venn diagrams, generated by the InteractiVenn web tool.<sup>24</sup> Those identified DEGs for the tumor cell lines and DEGs up- or down-regulated in at least 2/3 HDs for each immune cell subset were evaluated for the enrichment of Gene Ontology (GO) terms, considering biological processes (BPs), utilizing the Enrichr web tool.<sup>25</sup> The final sets of GO terms were ranked based on their adjusted *p*-value (<0.05) and on the number of associated genes (*n*>5). GO terms for immune cell subsets were clustered into main “GO term groups” for the similarity of the involved BPs.

## Results

### Effect of Tα1 on the Proliferation and Viability of Cancer Cell Lines and Immune Cell Subsets

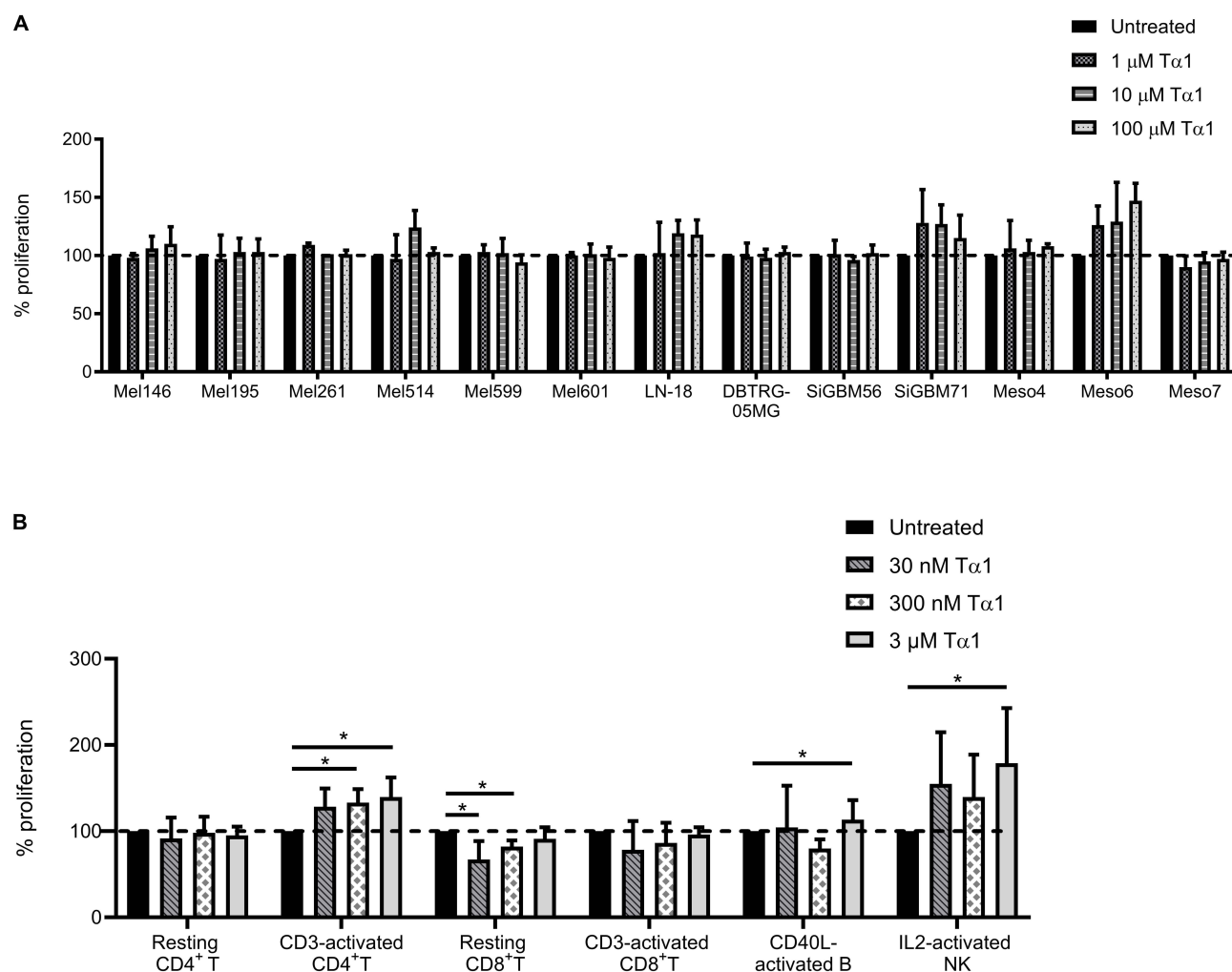
As an essential requirement for studying the effects of Tα1 on the immune profile of tumor cells, we needed to select non-cytotoxic doses of the drug, which maintained a high proliferative capacity of cells. Thus, to select the optimal Tα1 dose for cMM, GBM, and PM cell lines, the WST-1 assay was performed on different cell lines of each tumor histotype. Tα1 was tested at 1 μM, 10 μM, and 100 μM for 48 hours. No anti-proliferative/cytotoxic effects were observed at any dose analyzed, with a proliferation rate >80% for every tested cell line (Figure 1A). Based on these results, the 10 μM of Tα1 was selected for the further in vitro experiments that explored the immunomodulatory potential of Tα1 on cancer cell lines of different histotypes, also according to previous scientific evidence,<sup>17,26,27</sup> where this concentration was within the range of non-cytotoxic effect.

Simultaneously, to better understand the role of Tα1 within the immune system, the ideal drug dose for the proliferation of HD immune cell subsets (ie, CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK) was selected among 30 nM, 300 nM, and 3 μM for 48 hours (Figure 1B). The highest statistically significant (*p*<0.05) proliferation rates were induced by Tα1 at 3 μM in the activated CD4<sup>+</sup> T (140%), B (113%), and NK cells (179%) (Figure 1B). No proliferative effect was observed for resting CD4<sup>+</sup> T and resting or activated CD8<sup>+</sup> T cells, at any Tα1 dosage. The 3 μM for 48 hours was chosen as the optimal schedule for the following in vitro experiments that investigated whether the molecular and functional features of HD immune cells could be modulated by Tα1 treatments.

### Effect of Tα1 Treatment on the Transcriptional Landscape of Cancer Cells

In order to investigate whether Tα1 treatment might modulate tumor immune phenotype, gene expression profiles of cancer cell lines were evaluated by the NanoString PanCancer IO 360 gene expression panel on the nCounter SPRINT Profiler in Tα1-treated vs untreated cMM (*n*=6), GBM (*n*=4), and PM (*n*=3) cell lines.

Results showed that Tα1 treatment heterogeneously modified the gene expression profile of cell lines belonging to the same tumor histotype. Among the 770 investigated genes, a mean of 142 (range: 100–183), 163 (range: 149–175), and 136 (range: 125–147) genes were differentially expressed in Tα1-treated vs untreated cMM cells, GBM, and PM cell lines, respectively. Specifically, Tα1 treatment up-regulated or down-regulated, respectively, a mean of 42.1% (range: 17.5–74.3%) or 57.9% of genes (range: 25.7–82.5%) in the 6 cMM, 44.4% (range: 29.7–58.4%) or 55.6% (range:

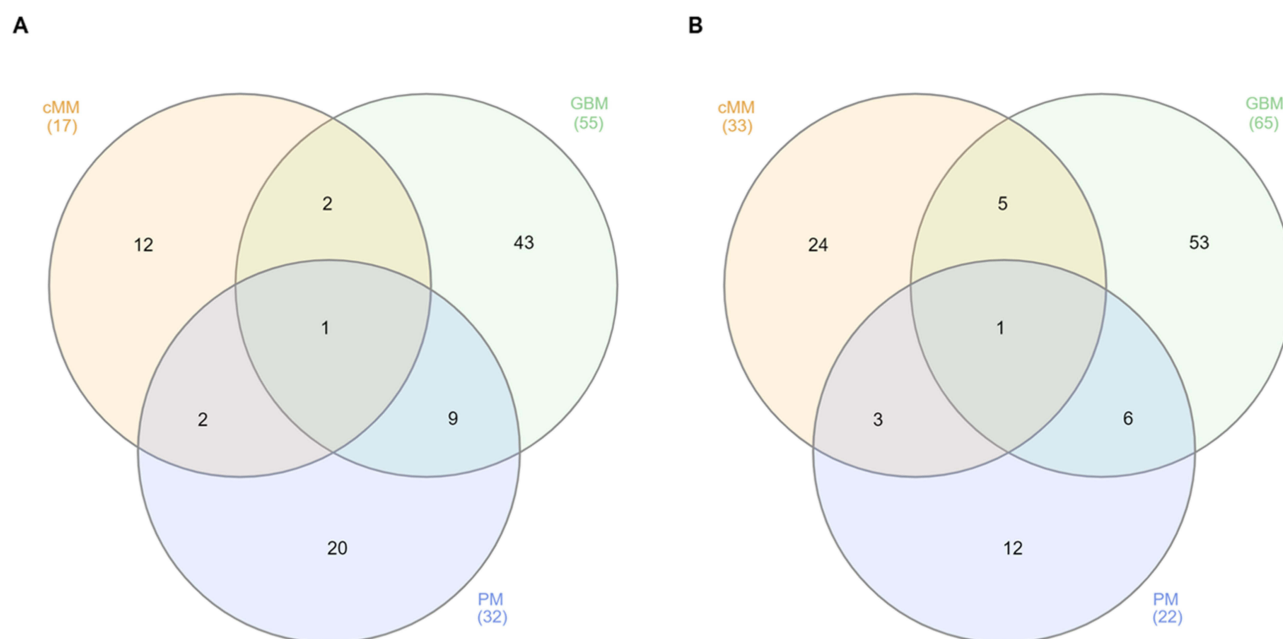


**Figure 1** Evaluation of T $\alpha$ 1 proliferative activity on tumor cell lines and healthy donor (HD) immune cell subsets. WST-1 proliferation assay was used on (A) tumor cells ( $5 \times 10^3$ ) seeded in quintuplicate in 96-multiwell plates and treated with scalar doses (1  $\mu$ M, 10  $\mu$ M, and 100  $\mu$ M) of T $\alpha$ 1 for 48 hours, and on (B) distinct immune cell subsets ( $2.5 \times 10^5$ ) cultured in triplicate or quintuplicate, according to cell availability, in 96-multiwell plates and treated with scalar doses (30 nM, 300 nM, and 3  $\mu$ M) of T $\alpha$ 1 for 48 hours. Data are represented as mean values of the % of proliferation  $\pm$ SD, calculated by optical density (OD) results in 3 independent experiments, as follows:  $(OD_{\text{treatment}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100$ . Statistical analysis was conducted using a paired two-tailed Student's *t*-test. \*, *p*-values < 0.05.

41.6–70.3%) of genes in the 4 GBM, and 50.1% (range: 45.2–56.5%) or 49.9% (range: 43.5–54.8%) of genes in the 3 PM cell lines ([Supplementary File 3](#)).

Then, focusing the analysis on sets of immune-related (IR) genes, involved in antigen processing machinery (eg, HLA class I and II antigens), tumor-associated antigens (TAA) (eg, MART-1 (MLANA), MAGE-A family genes, NY-ESO-1 (CTAG1B)), immune-stimulatory molecules (eg, ICOSLG, CD80), inhibitory immune checkpoint molecules (eg, PD-1 (PDCD1), PD-L1 (CD274), TIM-3 (HAVCR2), VISTA (VSIR)), or in interferon (IFN) signaling (eg, IFNG and IFN-stimulated genes), a very limited ability for T $\alpha$ 1 to modulate their expression in the tumor cell lines of each histotype was observed ([Supplementary File 3](#)). Flow cytometry analyses supported the gene expression results, displaying no changes in the expression levels of HLA class I, CD54, PD-L1, and MART-1, also at protein levels, by T $\alpha$ 1 in tumor cell lines ([Supplementary File 4](#)).

In addition, to investigate commonly T $\alpha$ 1-influenced changes in the transcriptional immune profiles of cancer cells of different histotypes, we analyzed only genes that were positively or negatively modulated in at least 3/6 cMM, 2/4 GBM, and 2/3 PM cell lines and with opposite modulation in no more than 1 cMM, 1 GBM, and 0 PM cell lines. Results showed that 17, 55, and 32 DEGs were up-regulated ([Figure 2A](#)), and 33, 65, and 22 DEGs were down-regulated



**Figure 2** Venn diagrams of shared differentially expressed genes (DEGs) among cutaneous melanoma (cMM), glioblastoma (GBM), and pleural mesothelioma (PM) cell lines, following TαI treatment. DEG modulation overlaps among (A) up-regulated ( $\log_2\text{ratio} \geq 0.58$ ) and (B) down-regulated ( $\log_2\text{ratio} \leq -0.58$ ) genes. Venn diagram (A) includes DEGs positively modulated in at least 3 cMM, 2 GBM, and 2 PM cell lines and negatively modulated in up to 1 cMM, 1 GBM, and 0 PM cell lines. Venn diagram (B) includes DEGs negatively modulated in at least 3 cMM, 2 GBM, and 2 PM cell lines and positively modulated in up to 1 cMM, 1 GBM, and 0 PM cell lines.

(Figure 2B) in cMM, GBM, and PM cell lines, respectively. Of note, only 1 DEG (ie, matrix metalloproteinase (MMP)-9) was commonly up-regulated (Figure 2A) and 1 DEG (ie, T-box transcription factor 21 (TBX21)) was commonly down-regulated (Figure 2B) among the 3 investigated tumor histotypes.

**Table 1** GO Terms from Up-Regulated DEGs in Tumor Cell Lines

| Tumor Histotype | GO Term                                                              | Adjusted p-value | Overlap | Combined Score | Genes                                                       |
|-----------------|----------------------------------------------------------------------|------------------|---------|----------------|-------------------------------------------------------------|
| cMM             | Cytokine-Mediated Signaling Pathway (GO:0019221)                     | 2.1E-05          | 6/257   | 723.8          | CSF3R;CCL8; IL34;LILRB2; TNF;PF4                            |
| GBM             | Cytokine-Mediated Signaling Pathway (GO:0019221)                     | 1.4E-06          | 10/257  | 356.7          | CSF1R;IL33; CSF3R;IL11RA; MX1;IRF5;TNF; LILRA3;IL18R1; CCR2 |
|                 | Positive Regulation Of Multicellular Organismal Process (GO:0051240) | 0.004            | 7/387   | 70.1           | IL10;IL33; NFAM1;CCR2; MMRN2;ITGAX; TNF                     |
|                 | Regulation Of Cell Population Proliferation (GO:0042127)             | 0.02             | 8/766   | 29.3           | IL10;CSF1R; CDKN2B;GLI1; IL11RA;LAMC2; MMRN2;ITGAX          |
|                 | Regulation Of Apoptotic Process (GO:0042981)                         | 0.03             | 7/705   | 23.3           | IL10; GAS1; TNFRSF10C; IRF5;TNF; MMP9;IER3                  |

(Continued)

**Table 1** (Continued).

| Tumor Histotype | GO Term                                                              | Adjusted p-value | Overlap | Combined Score | Genes                                           |
|-----------------|----------------------------------------------------------------------|------------------|---------|----------------|-------------------------------------------------|
| PM              | Lymphocyte Differentiation (GO:0030098)                              | 6.2E-08          | 7/91    | 1527.6         | IL10;CD79A; IL11;PTPRC; JAK3;MS4A1; IL2         |
|                 | B Cell Differentiation (GO:0030183)                                  | 3.3E-07          | 6/68    | 1532.4         | IL10;CD79A; IL11;PTPRC; JAK3;MS4A1              |
|                 | B Cell Activation (GO:0042113)                                       | 1.0E-06          | 6/92    | 1004.9         | IL10;CD79A; IL11;PTPRC; JAK3;MS4A1              |
|                 | Positive Regulation Of Cell Population Proliferation (GO:0008284)    | 5.6E-05          | 8/483   | 194.1          | CCL14;IL11; PTPRC;EPCAM;IL11RA; ITGAX;GLI1;IL2  |
|                 | Inflammatory Response (GO:0006954)                                   | 0.0001           | 6/236   | 262.2          | CCL14;CD96; PTGER4;LYZ; TNFAIP6; PIK3CG         |
|                 | Positive Regulation Of Multicellular Organismal Process (GO:0051240) | 0.0001           | 7/387   | 188.5          | PTGER4;IL10; ITGAX;TREM2; LILRB2;IL2; PIK3CG    |
|                 | Cytokine-Mediated Signaling Pathway (GO:0019221)                     | 0.0001           | 6/257   | 231.1          | CCL14;IL11; IL11RA;LILRB2;JAK3;IL2              |
|                 | Positive Regulation Of Cellular Process (GO:0048522)                 | 0.0001           | 8/594   | 139.4          | CCL14;IL11; EPCAM;IL11RA;ITGAX;GLI1; IL2;MAGEA4 |
|                 | Positive Regulation Of Cytokine Production (GO:0001819)              | 0.0004           | 6/320   | 166.0          | PTGER4;IL10; PTPRC;LILRB2; PIK3CG;IL2           |
|                 | Negative Regulation Of Programmed Cell Death (GO:0043069)            | 0.0007           | 6/381   | 126.7          | IL10;CD38; TREM2;MMP9; IL2;MAGEA4               |
|                 | Negative Regulation Of Apoptotic Process (GO:0043066)                | 0.002            | 6/482   | 86.9           | IL10;CD38; TREM2;MMP9; IL2;MAGEA4               |

**Abbreviations:** GO, Gene Ontology; DEGs, differentially expressed genes; cMM, cutaneous melanoma; GBM, glioblastoma; PM, pleural mesothelioma.

Enrichment analysis was then performed to elucidate the comprehensive biological significance of identified DEGs. Among significant BPs, only 1/1, 1/4, and 6/11 from positively modulated DEGs (Table 1), and 0/1, 9/22, and 0/2 from negatively modulated DEGs (Table 2) resulted in IR GO terms enriched in cMM, GBM, and PM cells, respectively.

# Effect of Tα1 Treatment on the Transcriptional Landscape of Immune Cell Subsets

To investigate the effects of Tα1 in the immune cell compartment, transcriptional changes between Tα1-treated and untreated CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK cells isolated from 3 HDs were analyzed by the NanoString PanCancer Immune Profiling gene expression panel. Among 770 investigated genes, a mean of 179 (range: 152–215), 186 (range: 167–217), 172 (range: 165–178), 253 (range: 167–390), 184 (range: 152–231), and 188 (range: 181–193) DEGs were identified in Tα1-treated vs untreated resting or activated CD4<sup>+</sup> T, resting or activated CD8<sup>+</sup> T, B, and NK cells, respectively. Among

**Table 2** GO Terms from Down-Regulated DEGs in Tumor Cell Lines

| Tumor Histotype | GO Term                                                           | Adjusted p-value | Overlap | Combined Score | Genes                                                                      |
|-----------------|-------------------------------------------------------------------|------------------|---------|----------------|----------------------------------------------------------------------------|
| cMM             | Positive Regulation Of Cellular Process (GO:0048522)              | 0.01             | 6/594   | 57.8           | BMP2;CCL21;<br>IFNG;IL11RA;<br>LAMC2;IL2                                   |
| GBM             | Positive Regulation Of MAPK Cascade (GO:0043410)                  | 1.1E-06          | 11/310  | 257.3          | GHR;CCL14;<br>MARCO;CD40;<br>PTPRC;IL1B;PLA2G2A;<br>FGF18;TREM2;NOD2;ICAM1 |
|                 | Regulation Of T Cell Proliferation (GO:0042129)                   | 1.1E-06          | 7/77    | 651.2          | HLA-DMB;<br>PTPRC;IL1B;<br>PLA2G2A;IDO1;<br>CD28;CD3E                      |
|                 | Positive Regulation Of Lymphocyte Proliferation (GO:0050671)      | 2.0E-05          | 6/74    | 467.8          | CD40;CD28;<br>HLA-DMB;<br>PTPRC;IL1B;<br>CD3E                              |
|                 | Positive Regulation Of Interleukin-6 Production (GO:0032755)      | 2.1E-05          | 6/76    | 449.8          | TLR1;IFNG;<br>IL1B;IL16;<br>NOD2;TLR2                                      |
|                 | Positive Regulation Of Immune Response (GO:0050778)               | 2.4E-05          | 6/80    | 417.0          | HLA-DMB;<br>PTPRC;IFNG;<br>C7;NLRP3;<br>NOD2                               |
|                 | Regulation Of Interleukin-8 Production (GO:0032677)               | 2.4E-05          | 6/81    | 409.4          | TLR1;PTPRC;<br>IL1B;NOD2;<br>CD244;TLR2                                    |
|                 | Cellular Response To Cytokine Stimulus (GO:0071345)               | 5.7E-05          | 9/308   | 150.4          | GHR;CCL14;<br>CD40;CXCL12;<br>IL1B;STAT4;<br>LILRA1;IFIT1;<br>TLR2         |
|                 | Positive Regulation Of Response To External Stimulus (GO:0032103) | 5.7E-05          | 7/155   | 228.7          | IFNG;IL1B;<br>PLA2G2A;<br>NLRP3;IL16;<br>TREM2;TLR2                        |
|                 | Positive Regulation Of Inflammatory Response (GO:0050729)         | 7.9E-05          | 6/104   | 282.4          | IFNG;IL1B;<br>PLA2G2A;TLR2;<br>NLRP3;IL16                                  |
|                 | Positive Regulation Of T Cell Activation (GO:0050870)             | 7.9E-05          | 6/107   | 270.6          | HLA-DMB;<br>PTPRC;IL1B;<br>CD28;NOD2;<br>CD3E                              |
|                 | Positive Regulation Of ERK1 And ERK2 Cascade (GO:0070374)         | 9.9E-05          | 7/179   | 183.1          | CCL14;MARCO;<br>PTPRC;ICAM1;<br>PLA2G2A;<br>TREM2;NOD2                     |

(Continued)

**Table 2** (Continued).

| Tumor Histotype | GO Term                                                                      | Adjusted p-value | Overlap | Combined Score | Genes                                                    |
|-----------------|------------------------------------------------------------------------------|------------------|---------|----------------|----------------------------------------------------------|
| GBM             | Positive Regulation Of Protein Serine/Threonine Kinase Activity (GO:0071902) | 0.0001           | 6/117   | 236.6          | GHR;CD40; IFNG;IL1B; FGF18;NOD2                          |
|                 | Positive Regulation Of Defense Response (GO:0031349)                         | 0.0001           | 6/124   | 216.7          | IFNG;IL1B; PLA2G2A; NLRP3;IL16; TLR2                     |
|                 | Positive Regulation Of Peptidyl-Tyrosine Phosphorylation (GO:0050731)        | 0.0002           | 6/130   | 201.7          | GHR;CD40; PTPRC;IFNG; TNFRSF18; TREM2                    |
|                 | Positive Regulation Of Protein Modification Process (GO:0031401)             | 0.0003           | 7/225   | 127.7          | CDH5;MARCO; CD40;IFNG; IL1B;FGF18; TREM2                 |
|                 | Positive Regulation Of Macromolecule Metabolic Process (GO:0010604)          | 0.0006           | 8/364   | 82.5           | CDH5;IFNG; IL1B;FGF18; CD28;TREM2; CD3E;TLR2             |
|                 | Positive Regulation Of Protein Phosphorylation (GO:0001934)                  | 0.0007           | 8/377   | 77.7           | GHR;MARCO; CD40;PTPRC; TGFB3;IL1B; FGF18;TREM2           |
|                 | Apoptotic Process (GO:0006915)                                               | 0.002            | 6/228   | 83.5           | IFNG;PDCD1; TLR2;NLRP3; ADORA2A;IL1B                     |
|                 | Positive Regulation Of Cell Migration (GO:0030335)                           | 0.003            | 6/272   | 62.3           | CDH5;CXCL12; PTPRC;IFNG; TNFRSF18;IL1B                   |
|                 | Positive Regulation Of Intracellular Signal Transduction (GO:1902533)        | 0.003            | 8/525   | 42.9           | CD40;PTPRC; TGFB3;IL1B; NLRP3;TREM2;NOD2;TLR2            |
|                 | Regulation Of Gene Expression (GO:0010468)                                   | 0.02             | 10/1127 | 17.5           | CDH5;PTPRC; IFNG;IL1B;BCL6B;FGF18; CD28;TREM2;CD3E; TLR2 |
|                 | Positive Regulation Of Transcription By RNA Polymerase II (GO:0045944)       | 0.05             | 8/938   | 13.0           | EOMES;CD40; TGFB3;TBX21; STAT4;NLRP3; NOD2;TLR2          |
| PM              | Positive Regulation Of DNA-templated Transcription (GO:0045893)              | 0.001            | 8/1243  | 89.8           | SPIB;NGFR; TBX21;STAT4; NFATC2;PF4; NLRP3;CX3CL1         |
|                 | Positive Regulation Of Transcription By RNA Polymerase II (GO:0045944)       | 0.007            | 6/938   | 59.8           | SPIB;TBX21; STAT4;NLRP3;CX3CL1;PF4                       |

**Abbreviations:** GO, Gene Ontology; DEGs, differentially expressed genes; cMM, cutaneous melanoma; GBM, glioblastoma; PM, pleural mesothelioma.

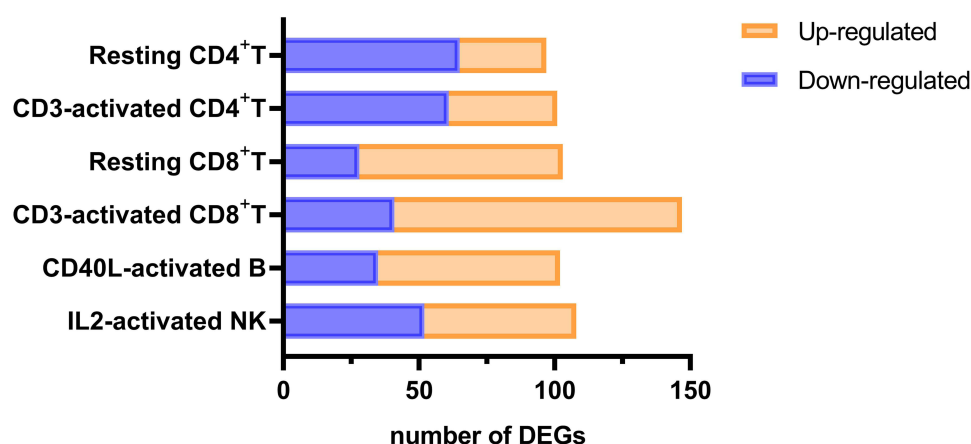
DEGs, Tα1 treatment up- and down-regulated the expression of a mean of 39.5% (range: 34.3–44.7%) and 60.5% (range: 55.3–65.7%) genes in resting CD4<sup>+</sup> T cells; 40.8% (range: 34.7–50.3%) and 59.2% (range: 49.7–65.3%) in activated CD4<sup>+</sup> T cells; 61.9% (range: 48.8–75.8%) and 38.1% (range: 24.2–51.2%) in resting CD8<sup>+</sup> T cells; 55.6% (range: 27.4–82.6%) and 44.4% (range: 17.4–72.6%) in activated CD8<sup>+</sup> T cells; 53.3% (range: 31–73.2%) and 46.7% (range: 26.8–69%) in B cells; 50.4% (range: 25.9–68.4%) and 49.6% (range: 31.6–74.1%) in NK cells ([Supplementary File 5](#)).

Considering only DEGs positively or negatively modulated in at least 2/3 HDs for each immune cell subset, a transcriptional modulation by Tα1 was observed in all investigated immune subgroups, resting and activated, with a greater tendency towards gene up-regulation rather than a down-regulation, with the exception of CD4<sup>+</sup> T subset ([Figure 3](#); [Supplementary File 5](#)). Furthermore, comparing the number of DEGs modulated by Tα1 in resting vs activated CD8<sup>+</sup> T cells, a higher up- and down-modulation pattern was observed in the activated T cell population ([Figure 3](#)).

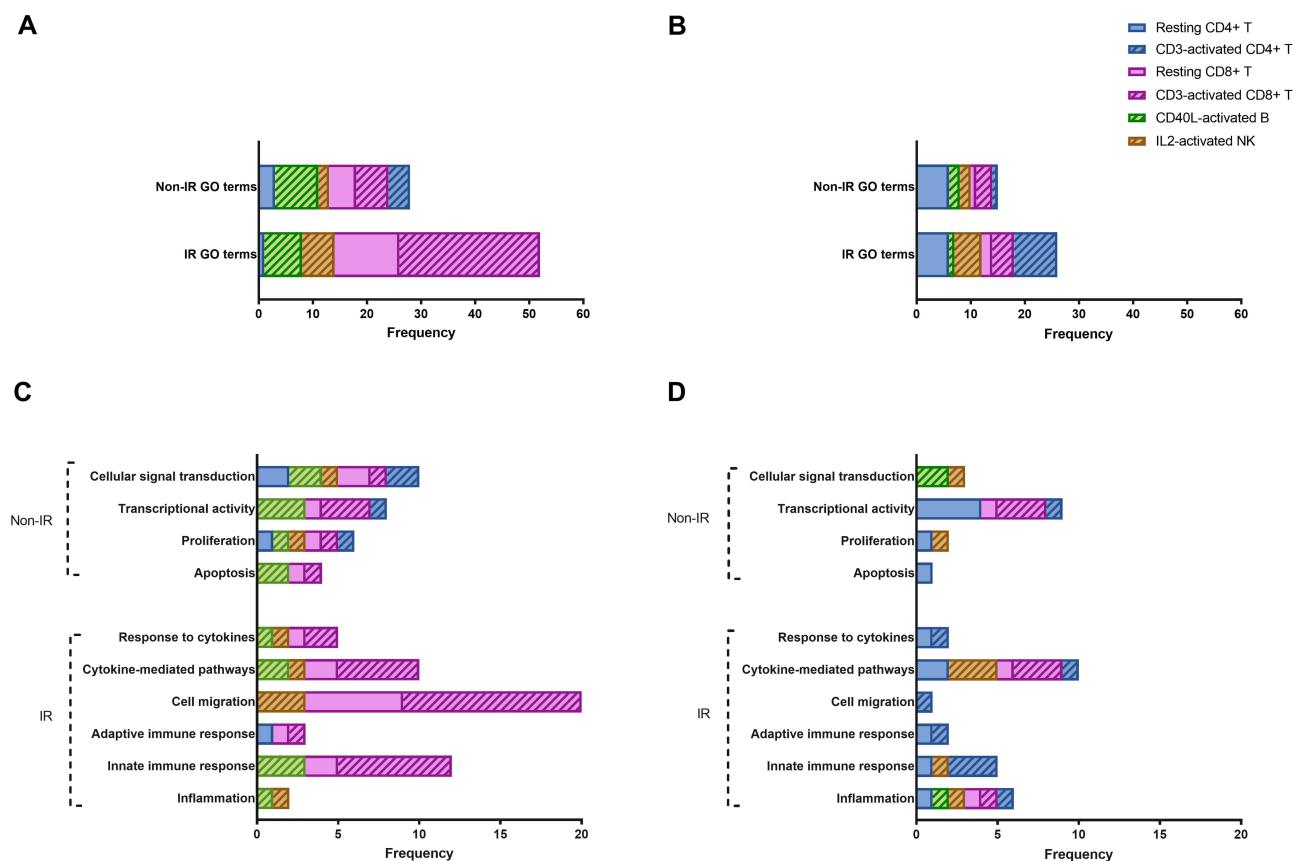
Comprehensively, enrichment analysis, showing BPs significantly affected by positively or negatively Tα1-modulated DEGs in immune subsets, highlighted a major impact on GO terms enriched by up-regulated ([Figure 4A and C](#)) rather than by down-regulated genes ([Figure 4B and D](#)). Furthermore, in BPs enriched by either up- or down-regulated DEGs, the IR terms were collectively more frequent compared to the non-IR terms. Details on IR and non-IR GO terms are listed in [Supplementary File 6](#). Among the immune cell subsets, Tα1 treatment exerted the greatest impact on CD8<sup>+</sup> T cells ([Figure 4](#)). Interestingly, Tα1-treated CD8<sup>+</sup> T cells displayed the highest frequency of IR BPs enriched by up-regulated genes, with a stronger effect observed for the activated compared to resting cells ([Figure 4C](#)). Furthermore, IR-GO term groups more enriched by up-regulated DEGs were the *immune cell migration*, the *innate immune response*, and the *cytokine-mediated pathways* ([Figure 4C](#); [Supplementary File 6](#)), with the highest contribution made by activated CD8<sup>+</sup> T, followed by resting CD8<sup>+</sup> T, B and NK cells.

## Assessment of Tα1 Activity on the Cytolytic Function of Specific Immune Cells

The Tα1 treatment schedule selected from the WST-1 data of immune cells was also tested to evaluate whether the cytolytic activity of functionally competent immune effector cells, LAK and HLA-A2-restricted gp100-peptide-specific CTL, may be affected by Tα1. Therefore, untreated and Tα1-treated effector cells were co-cultured with tumor cell lines selected as positive or negative targets for LAK- or CTL-mediated lysis. Results showed that, despite a minimal decrease (<5% necrotic+apoptotic cells), no significant difference between the cytotoxic activity of Tα1-treated vs untreated LAK cells was observed against the CD54-positive FO-1 cell line, at any E:T ratio ([Figure 5A](#)) ([Supplementary File 7](#)). Furthermore, no significant changes (<6.5% necrotic+apoptotic cells) in the cytotoxic activity of Tα1-treated vs untreated



**Figure 3** Number of genes modulated by Tα1 in different immune cell subsets. Horizontal stacked bar chart represents the number of positively ( $\log_2\text{ratio} \geq 0.58$ ) and negatively ( $\log_2\text{ratio} \leq -0.58$ ) modulated differentially expressed genes (DEGs) in different immune cell subsets. Peripheral CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK cells, isolated from 3 healthy donors (HDs), were treated with 3 μM Tα1 for 48 hours. Total RNA was extracted from treated and untreated cells and analyzed by the nCounter<sup>®</sup> SPRINT Profiler. Genes with a  $\log_2\text{ratio} \geq 0.58$  and  $\leq -0.58$  in Tα1-treated vs untreated cell subsets were defined as differentially expressed, being up- or down-regulated, respectively. Only DEGs equally modulated in at least 2/3 HDs were investigated for each immune cell subset. Up- (Orange) and down-regulated (blue) genes by Tα1 treatment are represented separately for each immune subset.



**Figure 4** Bar plots of gene ontology (GO) terms enriched by positively and negatively differentially expressed genes (DEGs) modulated by T $\alpha$ 1, in each immune cell subset. Overall visualization of representative immune-related (IR) and non-IR GO terms enriched in each immune cell subset by T $\alpha$ 1-up-regulated (**A**) and -down-regulated (**B**) DEGs equally modulated in at least 2/3 healthy donors. The enrichment analysis was conducted by the Enrichr web-based tool considering the biological processes (BPs). The sets of GO terms analyzed included those with adjusted *p*-values < 0.05 and a number of associated genes > 5. (**C** and **D**) represent a detailed focus on the GO terms enriched by T $\alpha$ 1-up-regulated and down-regulated DEGs, respectively, clustered together into main “GO term groups” by the similarity of the involved BPs. The frequency represents the number of GO terms that were enriched in each immune cell subset, in the specific analyzed GO category.

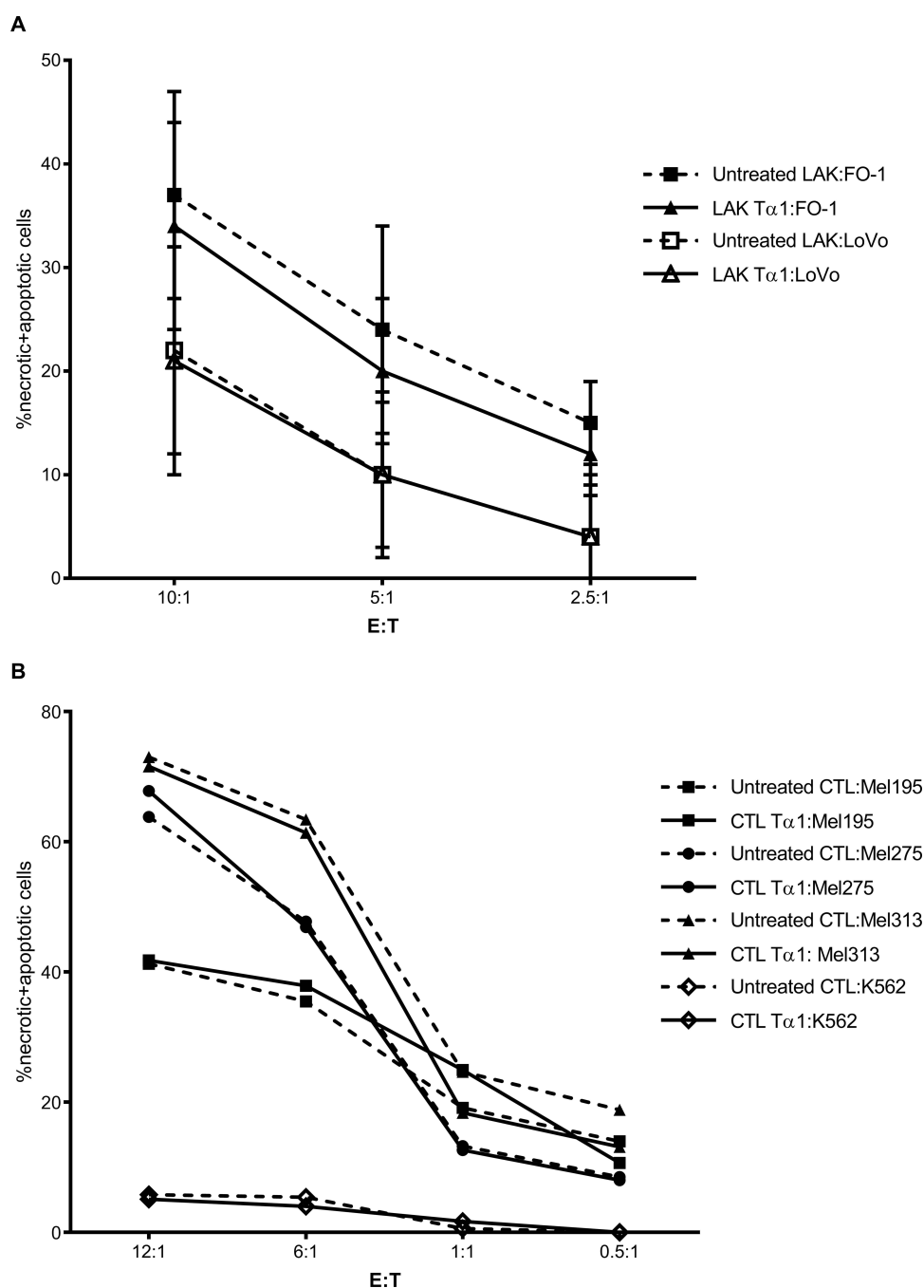
HLA-A2-restricted gp100-positive CTL were detectable against all the tested target melanoma cells, at any E:T ratios (Figure 5B) (Supplementary File 7).

## Discussion

Deeply understanding the entangled interplay between the immune system and the tumor is one of the main challenges faced by the scientific community.<sup>28</sup> Indeed, during cancer development, the host’s immune system is challenged by the progressive immunosuppressive tumor microenvironment (TME), the acquisition of strategies to evade immunosurveillance by cancer cells, and an increasingly immune-exhausted state.<sup>29</sup> Therefore, studying molecules with immunostimulatory activity is one crucial aspect that could favor the design of new therapeutic strategies for cancer patients.<sup>30</sup>

In this perspective, T $\alpha$ 1 is a natural enhancer of the immune system that has a long history of broad clinical applications in stimulating patients’ immune repertoire, mainly acting in immunocompromised contexts.<sup>2</sup> In the clinic, the most common administration of T $\alpha$ 1 exploits its role as an immunologic adjuvant, followed by its use in combination with disease-specific therapies, as with chemo/radio-therapy for melanoma and NSCLC,<sup>9,10</sup> or with IFN and nucleoside analogs for chronic hepatitis B and C.<sup>31</sup> Furthermore, encouraging results were reported from T $\alpha$ 1 application in cancer clinical contexts,<sup>15</sup> with promising perspectives to combine the most recent anti-tumor frontier of ICB with T $\alpha$ 1.<sup>12–14</sup>

However, whether T $\alpha$ 1 benefits derive from effects on the immune and also tumor compartment still needs to be deeper characterized to fully unleash a better and more accurate applicative potential. Contextually, our study investigated the ability of T $\alpha$ 1 to increase the immunogenicity of tumor cells and, thus, its potential to enhance tumor sensitivity



**Figure 5** Tα1 effects on the cytolytic activity of effector cells by the flow cytometric Annexin V/PI assay. **(A)** LAK cells were treated with 3 μM of Tα1 for 48 hours. Following Tα1 treatment, LAK cell cytotoxicity was tested against FO-1 cell line, in a co-culture of 4 hours, at effector:target (E:T) ratios of 10:1, 5:1, and 2.5:1. LoVo cell line was used as negative control target cells. **(B)** HLA-A2-restricted gp100-specific CTL cells were treated with 3 μM of Tα1 for 48 hours. Following Tα1 treatment, CTL function was tested against Mel195, Mel275, and Mel313 cell lines, in a co-culture of 4 hours, at E:T ratios of 12:1, 6:1, 1:1, and 0.5:1. K562 leukemia cell line was used as negative control target cells. Data are shown as mean values of % of necrotic+apoptotic cells ±SD in three independent experiments, where performed in replicates.

to the immune response induced by ICB. To this end, we investigated the Tα1 immunomodulatory effect on cell lines of three tumor histotypes—melanoma, GBM, and mesothelioma—where numerous pre-clinical and clinical studies are exploring combination strategies that could improve the efficacy of ICB-based therapies.<sup>32–36</sup> Importantly, the unmet medical needs in cancer, particularly high for GBM and mesothelioma, highlight the urgency of developing innovative immunotherapeutic approaches, which could include Tα1 administration. Thus, despite the unfeasibility to explore Tα1 effects on tumor cells within the complexity of the TME, our performed in vitro experiments were well-suited to

determine whether Tα1 exerted a direct effect on the immune profile of cancer cells. In this regard, the immunomodulatory effect of Tα1 on tumor cell lines, that we explored through expression analyses of key transcripts involved in tumor, immunity, and TME pathways included in the Nanostring panel, was comprehensively limited for each histotype. Along this line, analyzing specific sets of IR genes, involved in enhancing tumor immunogenicity (ie, antigen processing machinery, TAAs, immune-stimulatory molecules), in immune signaling pathways (eg, IFN pathways), or genes belonging to key inhibitory immune checkpoint molecules, Tα1 exhibited a minimal ability to influence their expression in the tumor cell lines of each histotype ([Supplementary File 3](#)). The low impact of Tα1 assessed on the immunomodulation of cancer cells in our experiments was further supported by the few commonly Tα1-modulated DEGs in cell lines of the different tumor histotypes ([Figure 2](#)). Additionally, the absence of immunostimulatory effects by Tα1 on tumor cells was reinforced by the scarcity of IR BPs influenced by Tα1 in each tumor histotype ([Tables 1 and 2](#)). Then, our transcriptional results were validated by flow cytometry analyses assessing minimal changes in the expression of proteins with key immunomodulatory roles (ie, HLA class I, CD54, PD-L1, and MART-1) in our Tα1-treated vs untreated tumor cell lines ([Supplementary File 4](#)). However, our findings, suggesting that Tα1 administered at a non-cytotoxic dose is unable to modify the immunologic profile of different cancer cells, contrast with available literature data. Indeed, previous evidence reported the tumor immunomodulatory ability of Tα1 by assessing the up-regulation of specific immune antigens (eg, HLA class I, MART-1, CD80, ICOSLG) in tumor cells.<sup>37–39</sup> Nevertheless, the observed effects in these prior studies may not represent a broadly generalizable phenomenon, as they were identified for limited immune targets in a small number of tumor cell lines. Moreover, these discrepancies might be attributed to a transient effect of Tα1, which usually started to decay after 12 hours from treatment interruption. Along this line, the differences in the timing of our analyses, conducted after 48 hours of Tα1 treatment, as previously tested,<sup>38</sup> allowed us to question Tα1 ability to induce stable modifications of the immune profile of tumor cells.

Then, to deepen whether Tα1 could enhance the activity of those immune cells with a key role for an effective ICB-therapeutic response, we focused on the proliferative and transcriptional changes exerted by Tα1 on the immune counterpart of HDs. More in detail, we investigated whether Tα1 was able to induce direct effects on distinct immune cell subsets (ie, CD4<sup>+</sup> T, CD8<sup>+</sup> T, B, and NK cells) isolated from the same HD, by culturing and treating them separately, rather than studying the effects of Tα1 on the whole PBMC population. Indeed, several works, that explored the effects of Tα1 on immune cells, conducted their experiments mainly on Tα1-in vitro-treated PBMCs or PBMCs collected from Tα1-clinically treated patients, leaving the issue of direct stimulation on the different immune cell subsets only partially assessed. It is well-established the role of Tα1 in restoring NK, CD4<sup>+</sup> T, and CD8<sup>+</sup> T cell counts in immunosuppressing therapy-treated patients or in contexts of immunodeficiency.<sup>4,5,7</sup> In accordance with this immunostimulatory effect, we observed a significant proliferative effect on activated CD4<sup>+</sup> T, B, and NK cells from HDs ([Figure 1](#)), following Tα1 exposure. Of note, we are not aware of previously reported proliferative effects on B cells from Tα1 and works that explored its role as a vaccine enhancer did not provide information about Tα1 direct effects on the antibody-producing B cell subsets.<sup>8,40,41</sup> Thus, because of the newly rediscovered role of B cell-based tertiary lymphoid structures (TLS) presence in TME,<sup>42</sup> associated with positive prognostic outcome and immunotherapy response, it could be of struggling importance to deepen the stimulatory effect of Tα1 that we observed in this immune cell subset also in cancer patients. Regarding CD8<sup>+</sup> T cells, it resulted to be the most Tα1-transcriptionally influenced immune cell subset, with a greater effect in the CD3-activated condition, compared to the other investigated immune subgroups ([Figures 3 and 4](#)). Our results reinforce what was previously observed by Matteucci et al that, although in the different anti-viral setting, demonstrated the ability for Tα1 to modulate the gene expression profile of activated CD8<sup>+</sup> T cells isolated from HDs, impacting the expression of chemokines, cytokines, and their receptors, and increasing their ability to produce soluble anti-viral factors.<sup>43</sup> According to those results, we observed that up-regulated DEGs of activated CD8<sup>+</sup> T cells preferentially enriched BPs involved in the immune cell migration, innate immunity, and cytokine-mediated pathways. These observations were further supported by previous works, where PBMCs treated with Tα1 showed an up-regulation of genes belonging to these same IR pathways.<sup>44,45</sup> The enrichment of migrative, innate immunity, and cytokine-mediated pathways, for the CD8<sup>+</sup> T, B, and NK cells reflect a multi-faced stimulatory activity of Tα1 on both the adaptive and innate immune response ([Figure 4](#)). In a tumor murine model, Tα1, alone or combined with anti-CTLA4 mAb, demonstrated the ability to favor the migration of CD8<sup>+</sup> T cells at the tumor site, with the upregulation of mRNA levels of *Cxcl9* and *Cxcl10* in the TME.<sup>13</sup> Accordingly, we found the gene expression of CXCL10 to be up-regulated by Tα1 treatment in activated CD8<sup>+</sup> T cells and in activated CD4<sup>+</sup> T as well ([Supplementary File 5](#)). However, our results

([Supplementary File 5](#)) did not show the reported effects of Tα1 on unsorted immune cells from HDs, such as the up-regulation of HLA class I and II genes, or molecules involved in the NK effector functions.<sup>44</sup> In this regard, it is worth mentioning that Tα1-mediated effects on the whole PBMC population could be influenced by modulatory interaction(s) among different immune cell subsets.<sup>46</sup> Therefore, the discrepancies with our results may be attributed to our different analysis focus, which aimed to obtain an innovative exploration of the Tα1 effects on single immune cell subsets. Collectively, the enriched BPs by Tα1-modulated DEGs, in immune cells, propose an influence of Tα1 on cellular pathways at a global level (IR and non-IR pathways) ([Figure 4](#) and [Supplementary File 6](#)), consistent with its pleiotropic features<sup>30</sup> and as previously suggested by Matteucci et al.<sup>44</sup> However, the major impact exerted by Tα1 was yielded on IR pathways rather than the non-IR among those investigated in our enrichment analysis ([Figure 4](#)). Interestingly, we observed that the lower activating transcriptional effect promoted by Tα1 on CD4<sup>+</sup> T and NK cells was accompanied by a more pronounced proliferation effect on those same subsets; *vice versa* for B and CD8<sup>+</sup> T cells, where a richer activating transcriptional impact was coupled with the lower or absent proliferative effect ([Supplementary File 6](#) and [Figure 1](#)). Thus, we hypothesized that the Tα1 inverse relation between proliferation and transcriptional promotion could be in accordance with the *context-dependent* mode of action of Tα1, described by Renga et al.,<sup>13</sup> which implies the ability for Tα1 to stimulate the immune system and at the same time modulate its homeostasis, by preventing an excessive activation. Along this line, and consistent with preliminary data,<sup>47</sup> we did not find an increase in the cytolytic activity of already functionally competent immune effector cells (ie, LAK and CTL) when treated with Tα1, against their appropriate target cell lines ([Figure 5](#)). However, this absence of improved effector function could be justified by Tα1 ability to preserve the physiologic activity of competent immune cells, whereas its immune-enhancing role is mainly observed within a context of general immune suppression/down-modulation.<sup>44,47,48</sup> In this regard, Tα1 promoted an interesting, yet not significant, augment of CTL-mediated cytotoxicity against the target cell line with the lowest susceptibility to CTL lysis among those investigated (ie, Mel195; [Figure 5B](#)), suggesting a Tα1 preference of action in cases of immune reduced activity.

Comprehensively, our results suggest the ability for Tα1 to exert a direct effect, proliferative and/or transcriptional, on each investigated immune cell subset, broadening its range of actions over the already well-known direct effect mainly pursued on DCs.<sup>31,46</sup> Furthermore, its greater transcriptional effect exerted on activated CD8<sup>+</sup> T cells, key players in anti-tumor immunity, sustains the importance of further studying the impact of Tα1 on this immune subgroup also in cancer patients. Indeed, the use of immune cell subsets from a small number of HDs was a crucial approach for this study, intended to be a preliminary work aimed to provide the biological background on Tα1 effects under physiological conditions. As a result, it sets the rationale for a subsequent larger study to be conducted on the transcriptional and functional Tα1 impact on distinct immune cell subsets of cancer patients. Collectively, these perspectives underscore the necessity of complementing our findings with *in vivo* studies for more accurate results.

## Conclusions

With the aim to deepen the rationale for combining Tα1 with immunotherapy strategies for the treatment of solid tumors, we explored the role of Tα1 in cancer immunomodulation and its direct effect on some of the main subsets of anti-tumor immune cells. Although Tα1 proved ineffective in modulating the immunologic profile of tumor cells, prompting us to question its global immunomodulatory potential on cancer, our study highlights its direct stimulatory effect on distinct HD immune cell populations. Specifically, data from the current work support Tα1 pleiotropic role, immune proliferative features, and activating transcriptional effects, mainly observed in activated CD8<sup>+</sup> T cells. Thus, our evidence might suggest a favorable combination of Tα1 with ICB-based therapies within anti-tumor settings, as already sustained by previous promising studies,<sup>12,13</sup> since Tα1 could amplify the effector response of T cells whose activities are enhanced by anti-CTLA4/PD1 mAb. However, we underline the need to translate our findings in a larger follow-up study in immune subsets of cancer patients, where Tα1 impact will be investigated with *in vitro* and *in vivo* functional assays, to support the findings of this preliminary work.

## Abbreviations

Tα1, Thymosin alpha 1; HDs, healthy donors; DEGs, differentially expressed genes; BRM, biological response modifier; DC, dendritic cell; TLR, toll-like receptor; NK, natural killer; NSCLC, non-small cell lung cancer; ICB, immune-checkpoint blockade; mAb, monoclonal antibody; IPI, ipilimumab; OS, overall survival; LAK, Lymphokine-activated killer cells; CTL, Cytotoxic T Lymphocytes; IL, Interleukin; cMM, cutaneous melanoma; GBM, glioblastoma; PM,

pleural mesothelioma; FBS, fetal bovine serum; ATCC, American Type Culture Collection; PBMCs, peripheral blood mononuclear cells; CD40L, CD40-Ligand; OD, optical density; FC, fold-change; MFI, mean fluorescence intensity; PI, propidium iodide; E, effector; T, target; GO, Gene Ontology; BPs, biological processes; IR, immune-related; TAA, tumor-associated antigens; IFN, interferon; MMP, matrix metalloproteinase; TBX21, T-box; TME, tumor microenvironment; TLS, tertiary lymphoid structures.

## Data Sharing Statement

The authors state that data generated from this study will be available upon request. Availability of such data will begin upon publication and will end 36 months following article publication, from the corresponding author.

## Ethics Approval and Informed Consent

Cancer patients, from whom cell lines originated, signed an institutional informed consent for research purposes only and without commercial interests. We confirm that patients' data were anonymized or maintained with confidentiality. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and all the data and procedures collected in the study were carried out according to good clinical practice. The protocol was approved by the independent ethics committee of the University Hospital of Siena (Siena, Italy).

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

## Funding

This work was sponsored by SciClone Pharmaceuticals Inc.

## Disclosure

MM has served as a consultant and/or advisor to Roche, Bristol-Myers Squibb, Merck Sharp Dohme, Incyte, AstraZeneca, Amgen, Novartis, Iontura, Pierre Fabre, Eli Lilly, Glaxo Smith Kline, SciClone, Sanofi, Alfasigma, and Merck Serono. AMDG has served as a consultant and/or advisor to Incyte, Pierre Fabre, Bristol-Myers Squibb, Merck Sharp Dohme, Sanofi, Novartis, Glaxo Smith Kline, Sunpharma, and Immunocore. MM, SC, and AC own shares in Epigen Therapeutics. XW is employed by SciClone. No potential conflicts of interest were disclosed by the other authors.

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