



FULL-LENGTH ARTICLE

Basic Research

Impact of soluble tumor necrosis factor-related apoptosis-inducing ligand released by engineered adipose mesenchymal stromal cells on white blood cells



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ABSTRACT

Background aims: The proapoptotic protein tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is physiologically expressed by immune cells and performs regulatory functions in infections, autoimmune diseases and cancer, where it acts as a tumor suppressor. Adipose-derived mesenchymal stromal cells (AD-MSCs) also may play immunomodulatory roles in both primary and acquired immune responses. We have previously demonstrated the efficacy of an anticancer gene therapy based on AD-MSC engineered to secrete a soluble TRAIL variant (sTRAIL) against pancreatic cancer. However, the impact of AD-MSC sTRAIL on leukocyte subsets has been not yet considered also to predict a possible immunotoxicity profile in the clinical translation of this cell-based anticancer strategy.

Methods: Monocytes, polymorphonuclear cells and T lymphocytes were freshly isolated from the peripheral blood of healthy donors. Immunophenotype and functional (DR4 and DR5) and decoy (DcR1 and DcR2) TRAIL receptors were tested by flow cytometry. The viability of white blood cells treated with sTRAIL released by gene-modified AD-MSC or co-cultured with AD-MSC sTRAIL was then evaluated by both metabolic assays and flow cytometry. In addition, cytokine profile in co-cultures was analyzed by multiplex enzyme-linked immunosorbent assay.

Results: Monocytes and polymorphonuclear cells showed high positivity for DR5 and DcR2, respectively, whereas T cells revealed negligible expression of all TRAIL receptors. Irrespective of TRAIL receptors' presence on the cell membrane, white blood cells were refractory to the proapoptotic effect displayed by sTRAIL secreted by gene-modified AD-MSC, and direct cell-to-cell contact with AD-MSC sTRAIL had negligible impact on T-cell and monocyte viability. Cytokine crosstalk involving interleukin 10, tumor necrosis factor alpha, and interferon gamma secreted by T lymphocytes and vascular endothelial growth factor A and interleukin 6 released by AD-MSC was highlighted in T-cell and AD-MSC sTRAIL co-cultures.

Conclusions: In summary, this study demonstrates the immunological safety and thus the clinical feasibility of an anticancer approach based on AD-MSC expressing the proapoptotic molecule sTRAIL.

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Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily that can be cleaved by cysteine protease to obtain a soluble ligand [1]. It is physiologically expressed by various cells of both the innate and adaptive immune system, including macrophages, T lymphocytes, dendritic cells and natural killer (NK) and natural killer T cells, and it mediates immunoregulatory and immune-effector functions by binding to its functional death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, expressed on the target cell surface, with the consequent activation of apoptotic signaling pathways [2,3]. In particular, endogenous TRAIL acts as a modulator of events involving the immune system, such as the maintenance of physiological homeostasis and the response to viral infections and autoimmune diseases [4]. It has been reported that TRAIL induces deletion of developing autoreactive T and B lymphocytes in central tolerance mechanisms, thereby preserving T-cell homeostasis [5]; accelerates neutrophils apoptosis, inducing the resolution of inflammation [6] as well as inhibits T helper 1 and 2 (Th1 and Th2) cells [7]. TRAIL has a protective role against autoimmune diseases such as autoimmune encephalomyelitis, rheumatoid arthritis, and type I diabetes, preserving peripheral tolerance through the killing of autoreactive T lymphocytes, inhibition of their activation and promotion of T regulatory cell proliferation [8]. In addition, TRAIL expressed by immune cells plays a fundamental role in immunosurveillance against tumors and metastasis, acting as a tumor suppressor [9]. In this context, TRAIL can exert its effect directly by inducing apoptosis in tumor cells or indirectly by promoting cytokine secretion, including interleukin (IL)-8, protein urokinase and matrix metalloproteinase-7 and -9, by cancer cells stimulating immune system regulation [4]. In particular, TRAIL is a relevant part in the apoptotic activity of NK against tumor cells: NK-cell activation is followed by TRAIL expression with a proapoptotic antitumor effect even against disseminated cancer cells [10]. Moreover, monocytes, neutrophils and immune effector cytotoxic dendritic cells and T cells contribute to TRAIL-mediated apoptosis in cancer cells [11].

Based on the anticancer activity of TRAIL-expressing immune effector cells, the recombinant human (rh) TRAIL molecule has attracted significant attention as an antitumor compound because of its ability to selectively trigger apoptotic signaling in tumor cells expressing functional TRAIL receptors while sparing healthy tissues. However, because of the suboptimal pharmacokinetics of rhTRAIL, the therapeutic response in patients has been lower than expected, and its clinical use has been limited [12]. Given this scenario, different research groups explored various delivery strategies to ameliorate the bioavailability of TRAIL, including the use of genetically modified mesenchymal stromal cells (MSCs) engineered to express this proapoptotic protein. The capacity of MSCs to be manipulated by viral vectors encoding for therapeutic molecules along with their tropism for tumor sites makes them an efficient vehicle for TRAIL delivery in both preclinical and clinical settings [13,14]. In the past few years, our group has developed an anticancer gene therapy approach based on adipose-derived (AD) MSC to express TRAIL against several cancer types [15–21]. AD-MSC delivering a secreted TRAIL variant as a soluble ligand (sTRAIL) have been generated, and these cells are characterized by a greater capacity to form multimeric complexes. This increased polymerization capacity confers sTRAIL superior cytotoxic activity against different tumor cell lines compared with the wild-type form [18–21]. Considering the multiple and critical roles played by TRAIL in the immune system, both in physiological and pathological conditions, we aimed to determine the impact of the stronger cytotoxic activity by the novel sTRAIL form together with the capability of MSC to regulate immune cell activity by immunomodulatory mechanisms [22] on white blood cells (WBCs). In this regard, we investigated the viability, metabolic activity and cytokine

release of WBCs to collect exploratory *in vitro* data to inform the clinical safety of this MSC-based TRAIL delivery therapeutic approach.

Methods

Isolation and *in vitro* culture of WBCs

Human peripheral blood mononuclear cells (PBMCs) and human polymorphonuclear cells (PMNs) were separated by density gradient centrifugation (Lymphoprep; Alere Technologies AS, Oslo, Norway) from the peripheral blood of healthy donors ($n = 3$) as approved by the institutional review board of University Hospital of Modena, Modena, Italy.

Monocytes culture

PBMCs were plated in RPMI 1640 (Gibco-Life Technologies, Grand Island, NY, USA) with 0.5% heat-inactivated defined fetal bovine serum (FBS; HyClone Laboratories, UT, USA) and 1% glutamine (200 mmol/L; Euroclone, Pero, MI, Italy) for 1 h to isolate monocytes. Non-adherent cells were then removed and monocytes were cultivated for 24 h in RPMI 1640 supplemented with 10% heat-inactivated defined FBS and 1% glutamine and then used for fluorescence-activated cell sorting (FACS) analysis and cell viability assays.

Polymorphonuclear culture

PMNs, isolated after gradient density separation, were immediately tested by FACS analysis or plated in RPMI 1640 with 10% heat-inactivated defined FBS and 1% glutamine for cell viability assays.

Lymphocytes culture

PBMCs were plated in RPMI 1640 with 1% heat-inactivated defined FBS, 1% glutamine and 1% penicillin–streptomycin (10 000 units penicillin and 10 mg/mL streptomycin in 0.9% sodium chloride; Sigma-Aldrich, St Louis, MO, USA) for 2 h to obtain lymphocytes. Non-adherent cells were collected and pre-stimulated for 48 h in RPMI 1640 supplemented with 10% heat-inactivated defined FBS, 1% glutamine, 1% penicillin–streptomycin, 500 UI/mL rh interleukin-2 (Proleukin; Clinigen Healthcare Ltd, Staffordshire, UK) and 7 μ g/mL phytohemagglutinin (Sigma-Aldrich) at a concentration of 1×10^6 cells/mL. After 48 h, isolated cells were cultured in RPMI 1640 with 10% heat-inactivated defined FBS, 1% glutamine, 1% penicillin–streptomycin and 500 UI/mL rh interleukin-2 and used for FACS analysis, cell viability and metabolic activity assays.

FACS analyses

Monocytes were stained using fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (BD Biosciences, San Jose, CA, USA), phycoerythrin (PE)-conjugated anti-CD14 (BD Biosciences), peridinin–chlorophyll–protein–conjugated anti-CD3 (BD Biosciences) and BD Via Probe Cell Viability Solution (7-aminoactinomycin D [7-AAD]; BD Pharmingen, San Diego, CA, USA). PMNs were stained using FITC-conjugated anti-CD45, allophycocyanin (APC)-conjugated anti-CD10 (BD Biosciences) and BD Via Probe Cell Viability Solution (7-AAD). *In vitro*-expanded T lymphocytes were stained using FITC-conjugated anti-CD45, peridinin–chlorophyll–protein–conjugated anti-CD3, APC-conjugated anti-CD4 (BD Biosciences), PE-conjugated anti-CD8 (BD Biosciences) and BD Via Probe Cell Viability Solution (7-AAD). To assess TRAIL receptor expression, monocytes, PMNs and T lymphocytes were tested for PE-conjugated anti-DR4 (BioLegend, San Diego, CA, USA), APC-conjugated anti-DR5 (BioLegend), PE-conjugated anti-DcR1 (BioLegend) and APC-conjugated anti-DcR2 (R&D Systems, Minneapolis, MN, USA). Isotype control antibodies were used for all cell types and antigens analyzed. All samples were acquired using the BD FACSAria III (BD, Franklin Lakes, NJ, USA) flow cytometer and analyzed using the BD FACSDiva software.

Cell viability assays

Monocytes, PMNs and T cells were tested in a dose–response assay for sTRAIL cytotoxicity using the CellTiter-Glo Luminescent Cell Viability assay (Promega, Madison, WI, USA). In detail, cells were seeded in a 96-well plate at a density of 200 000 cells/well in cell culture medium (without IL-2 for T lymphocytes). Supernatants conditioned by AD-MSC secreting sTRAIL (AD-MSC sTRAIL) or AD-MSC EMPTY as the control were produced as previously reported [18]. The next day, the cell medium was replaced with the supernatants collected from AD-MSC sTRAIL, containing increasing doses of sTRAIL (from 50 to 5000 pg/mL). WBCs were also treated with increasing volumes of supernatants collected from AD-MSC EMPTY, corresponding to the volumes of supernatant from AD-MSC sTRAIL tested. After 24 h, treated cells were incubated with the CellTiter-Glo reagent for 10 min according to the manufacturer's instructions. The cell viability rate was quantified by measuring the bioluminescence signal using the GloMax Discover microplate reader (Promega). To assess the impact of direct cell-to-cell contact with AD-MSC sTRAIL on monocyte and T-lymphocyte death, primary immune cells were cultured over a layer of AD-MSC sTRAIL, which were previously allowed to adhere to 12-well plates for 24 h, at different effector:target (E:T) ratios (1:30, 1:15, 1:10, 1:1, and 10:1; E = AD-MSC, T = T cells) in RPMI 1640 with 10% heat-inactivated defined FBS and 1% glutamine (and 1% penicillin–streptomycin for T lymphocytes). Monocytes or T cells alone (CTL) and co-cultured with AD-MSC EMPTY at a 10:1 E:T ratio were used as controls. Monocyte and T-lymphocyte death rate was evaluated by the supravital propidium iodide (PI; Sigma-Aldrich) assay after 24 h of co-culture. To summarize, non-permeabilized cells were stained using 50 μ g/mL PI for 30 min [23] and primary immune cell death was quantified by FACS, gating on PI-positive cells.

Metabolic activity assay

The metabolic activity of T lymphocytes treated by sTRAIL was evaluated using the alamarBlue assay (Invitrogen Corporation, Carlsbad, CA, USA). T lymphocytes were incubated with increasing doses of sTRAIL (from 50 to 5000 pg/mL) following the same aforementioned protocol for the cell viability assay. After 24, 48 and 72 h of treatment, T lymphocytes were incubated with 10% alamarBlue for 4 h at 37°C. T-cell metabolic activity was quantified by measuring the fluorescence signal (λ_{ex} = 530–560 and λ_{em} = 590 nm) using the GloMax Discover microplate reader.

Multiplex enzyme-linked immunosorbent assay (ELISA)

IL-10, tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), vascular endothelial growth factor A (VEGF-A) and IL-6 levels were measured in supernatants collected from the co-culture of T cells and AD-MSC sTRAIL (or EMPTY as the control) at different E:T ratios for 24, 48 and 72 h. The evaluation was done by Labospace Srl using a magnetic Luminex assay (multiplex ELISA; R&D Systems). Data were reported as fg/mL per T cell for cytokines secreted by T lymphocytes, and fg/mL per AD-MSC for cytokines secreted by AD-MSC.

Statistical analysis

Continuous variables are expressed as absolute or percentage frequencies and are described as mean $\pm$ standard error. A multiple comparison of the observed data is obtained through the one-way ANOVA followed by Tukey's test as post hoc test. Significant results were considered when $P < 0.05$.

Results

TRAIL receptor expression is variable on WBCs from healthy donors

After density gradient centrifugation, monocytes, PMNs and T lymphocytes were isolated and grown separately *in vitro* under different culture conditions. For each leukocyte subpopulation, the immunophenotype was assessed by flow cytometry to confirm the purity and the lineage of *in vitro*–expanded cells. Cultured primary monocytes were characterized and quantified based on the co-expression of CD45 and CD14 antigens (Figure 1A, left). The analysis revealed that most cells ($63 \pm 2\%$) displayed the antigen profile typical of monocytes. Despite the selective culture conditions, a small fraction of lymphocytes ($14 \pm 10\%$) was still present within the monocyte cell culture as revealed by the co-expression of CD45 and CD3 (Figure 1A, right). Therefore, T-cell contaminants were excluded from the following analyses by gating on CD45+/CD3– cellular elements. For neutrophils, both CD45 and CD10 antigens were detected on the cell membrane in $85 \pm 5\%$ of viable PMNs, thus confirming their lineage (Figure 1B). T lymphocytes were cultured for 24 h with IL-2 *in vitro* before being used for further experiments. *In vitro*–expanded T lymphocytes were analyzed by gating on viable 7AAD– cells ($73 \pm 14\%$, data not shown). The fraction of CD4+ helper and CD8+ cytotoxic T lymphocytes within the CD45+/CD3+ population was quantified (Figure 1C, left). FACS analysis revealed that within the T-cell population, most lymphocytes were positive for the CD8 antigen ($71 \pm 9\%$), whereas only $14 \pm 1\%$ of cells expressed CD4 (Figure 1C, right).

To predict the potential sensitivity of WBCs to the cytotoxic effects of sTRAIL, the presence of both functional (DR4 and DR5) and decoy (DcR1 and DcR2) TRAIL receptors was investigated on the cell membrane of all three WBC subpopulations (Figure 2A–C, left panels). Monocytes showed high positivity for DR5 ($77 \pm 4\%$), whereas the presence of DR4 and decoy receptors was negligible (DR4: $1 \pm 0.5\%$; DcR1: $4 \pm 2\%$; DcR2: $1 \pm 0.1\%$; Figure 2A, right). After isolation, PMNs revealed weak expression of both functional and DcR1 receptors (less than 25%); in contrast, the presence of decoy receptor DcR2 was detected in $62 \pm 14\%$ of neutrophils (Figure 2B, right). T cells showed a low positivity for all TRAIL receptors (DR4: $7 \pm 4\%$; DR5: $13 \pm 5\%$; DcR1: $3 \pm 2\%$; DcR2: $1 \pm 1\%$) after *in vitro* culture (Figure 2C, right).

sTRAIL secreted by AD-MSC does not alter WBC viability

To evaluate whether sTRAIL variant secreted by AD-MSC with an improved trimerization capacity could be associated with an increased cytotoxic profile on healthy WBCs, the viability of monocytes, PMNs and T cells was assessed in a dose–response assay testing AD-MSC sTRAIL-conditioned medium (CM) containing increasing doses of sTRAIL (from 0 to 5000 pg/mL; Figure 3). The analysis revealed the number of living monocytes and PMNs was not reduced by sTRAIL treatment; rather, after 24 h, cell viability increased in parallel with sTRAIL concentrations in the CM compared to the control (up to 2-fold increase; Figure 3A,B, $P \leq 0.0001$ for both monocytes and PMNs). In particular, monocyte viability seems to be significantly improved by the greatest doses of our sTRAIL variant, considering that at 2500 pg/mL the number of living monocytes is doubled compared with untreated control. For this cell type, the booster effect seems to be directly related to the sTRAIL concentrations contained in CM, since the corresponding amount of CM from AD-MSC EMPTY did not enhance monocyte viability with the same extent (see supplementary Figure 1A). Regarding PMNs, sTRAIL did not provoke any cytotoxic effect and, on contrary, we registered a marked increase in living cells after sTRAIL treatment (Figure 3B). The analysis of PMN viability revealed the same growth trend after the addition of CM from AD-MSC sTRAIL or EMPTY; therefore, we could assume that this cellular behavior may be the result of cytokines and soluble factors

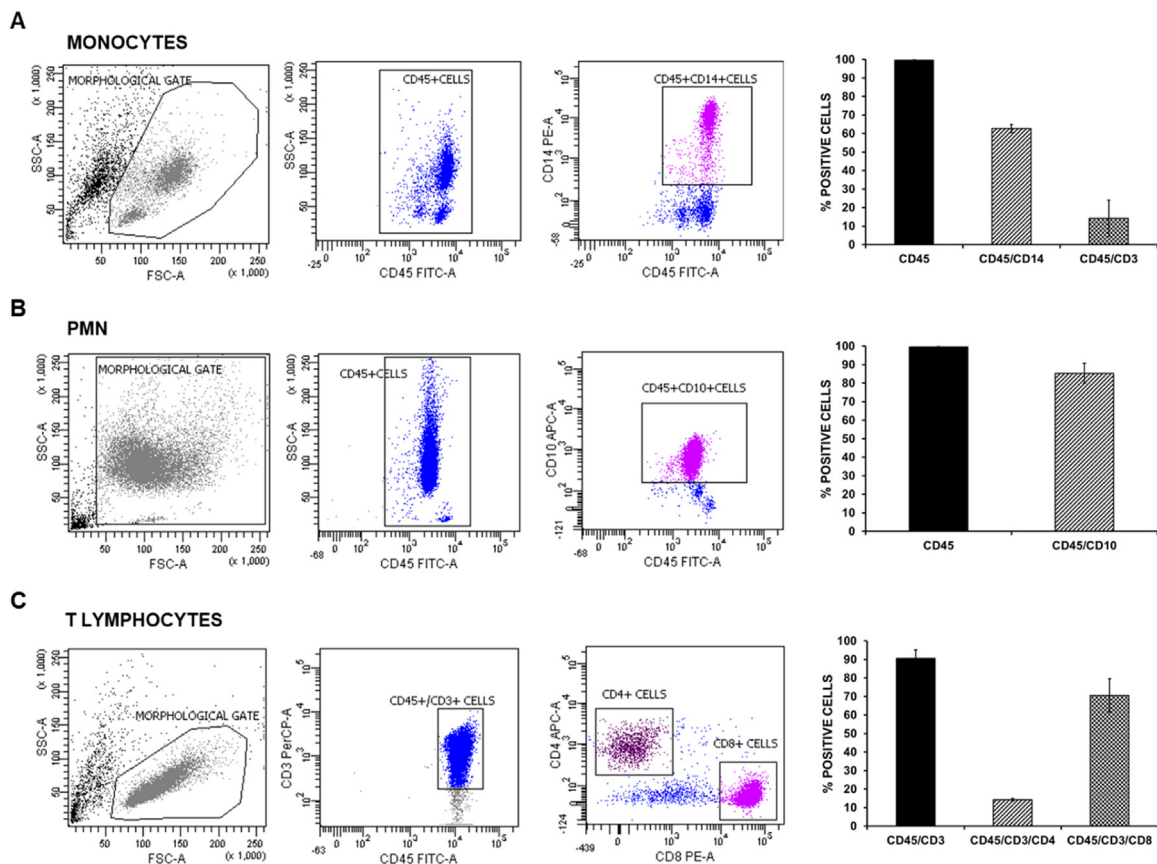


Fig. 1. Immunophenotype of monocytes, polymorphonuclear cells, and T lymphocytes. Gating strategies of representative FACS analysis for each cell type are shown on the left and histograms of mean percentage of the positive cells for their respective antibodies are reported on the right. Error bars denote standard error of the mean. Viable single-cell populations were selected based on morphology using side scatter (SSC) and forward scatter (FSC), excluding debris. (A) Monocytes were identified based on the expression of CD45+ and the co-expression of CD14+ in the CD45+ cells. Contaminant T lymphocytes were detected by the co-expression of CD45 and CD3 antigens, quantified and excluded from the analysis. (B) PMNs were identified based on the identification the expression of CD45+ and the co-expression of CD10+ in CD45+ cells. (C) T lymphocytes were detected by the co-expression of CD45 and CD3 antigens. The expression of both CD4+ helper and CD8+ cytotoxic T cells was quantified within the CD45+/CD3+ subpopulation. (Color version of figure is available online.)

released by AD-MSC in culture media (Figure 3B and supplementary Figure 1B). In addition, after 24 h and for all tested doses of sTRAIL, one-way ANOVA analysis did not reveal significant variations in T-cell viability compared with the negative controls (T lymphocytes untreated or treated with CM from AD-MSC EMPTY $P > 0.05$; Figure 3C and supplementary Figure 1C). Collectively, these results indicate that irrespective of the expression of functional TRAIL receptors on the different cell types, WBCs are refractory to the proapoptotic effect displayed by sTRAIL secreted by AD-MSC. Moreover, the impact of sTRAIL on the health condition of T cells was further investigated by the alamarBlue assay. alamarBlue is a nontoxic viability compound that exploits the natural reducing power of healthy and metabolically active cells to convert a non-fluorescent substrate to a highly fluorescent molecule that can be evaluated quantitatively based on absorbance or fluorescence signals, returning a snapshot of cellular metabolic activity. Hence, a reduction in the alamarBlue signal may signify an impairment of cellular metabolism or mitochondrial dysfunction provoked by cytotoxic stimuli [24]. At 24 h, starting from the lowest sTRAIL dose (50 pg/mL), a slight decrease in T-cell metabolism was recorded compared with untreated cells. This trend was completely reversed at later time points of 48 and 72 h, at which statistically significant variations in alamarBlue reduction were observed compared with the control. In addition, at the greatest sTRAIL dose (5000 pg/mL) and for 48 and 72 h, CM from both AD-MSC sTRAIL and EMPTY significantly increased T-cell metabolic activity compared with that of untreated cells (Figure 3D). Collectively, these data indicate that both the viability and health condition of T

cells, which are characterized by the negligible expression of TRAIL receptors, are not affected by sTRAIL. Rather, trophic factors released by AD-MSC in the CM may favor lymphocyte growth in culture.

Direct cell-to-cell contact with AD-MSC expressing sTRAIL does not negatively impact monocyte and T-cell viability in vitro

To investigate whether direct cell-to-cell contact with AD-MSC sTRAIL compromises the number of living WBCs, monocytes as well as T cells were further cultured in the presence of AD-MSC sTRAIL at a variety E:T ratios (1:30, 1:15, 1:10, 1:1 and 10:1) for 24 h (Figure 4). Leukocytes alone (CTL) or co-cultured with AD-MSC EMPTY at the greatest E:T ratio were used as the control. No statistically significant variations in monocyte viability were recorded at any of the tested E:T ratios compared with CTL ($P \geq 0.05$), confirming that direct cell-to-cell contact with AD-MSC sTRAIL did not provoke cytotoxicity in primary monocytes (Figure 4A) despite the high expression of DR5 on the cell membrane. In this light, we could assume that the slight but significant differences in monocyte death registered at the higher E:T ratios (1:30; 1:15; 1:10) of treatment compared with the lower ones (1:1; 10:1 or 10:1 EMPTY) may be due to the greater cell seeding density occurring at the 1:30; 1:15; 1:10 E:T ratios, which better support monocyte survival.

From E:T ratios of 1:30 to 1:10, T-cell viability was not affected by the presence of AD-MSC sTRAIL, whereas a progressive slight increase in T-cell mortality (less than 20% over CTL) was registered at greater doses (E:T ratios of 1:1 and 10:1; Figure 4B). The modest but

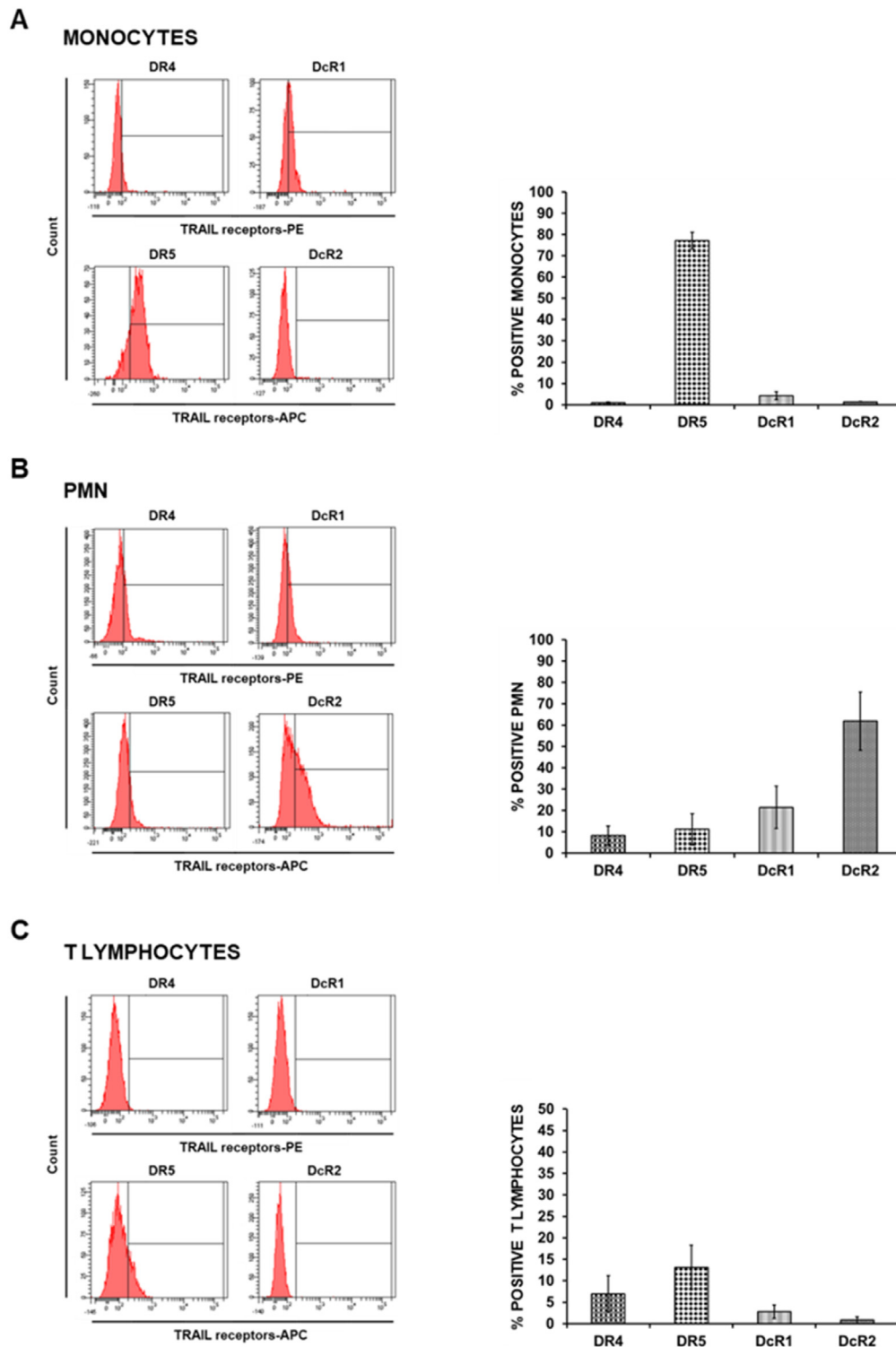


Fig. 2. Expression of TRAIL receptors on monocytes, PMN and T lymphocytes. Expression of both functional (DR4 and DR5) and decoy (DcR1 and DcR2) TRAIL receptors evaluated by FACS on (A) monocytes, (B) PMN and (C) T lymphocytes. Representative FACS analysis of one donor is depicted on the left; the histograms on the right present the mean values of the three donors. Error bars denote standard error of the mean. (Color version of figure is available online.)

appreciable reduction of T-cell viability can be specifically ascribed to the apoptotic effect mediated by sTRAIL released in the supernatant by AD-MSC sTRAIL, since it was not observed in co-culture with AD-MSC EMPTY. The amount of sTRAIL detected by ELISA in culture media collected from samples having E:T ratios of 1:1 and 10:1 was 75 ± 2 pg/mL and 251 ± 2 pg/mL, respectively. Both these concentrations are sufficient to induce relevant cytotoxic effects in TRAIL-sensitive

tumor cells (data not shown). In particular, we have reported that a 250 pg/mL dose of sTRAIL can provoke apoptosis in more than 50% of Ewing sarcoma and pancreatic tumor cell lines [18,21]. Lastly, to evaluate whether AD-MSC sTRAIL equally targeted both CD4⁺ and CD8⁺ T lymphocytes, T-cell immunophenotype after co-culture with AD-MSC sTRAIL at E:T ratios of 1:1 and 10:1 was assessed, and no variations were detected compared with T cells alone (Figure 4C, $P > 0.05$).

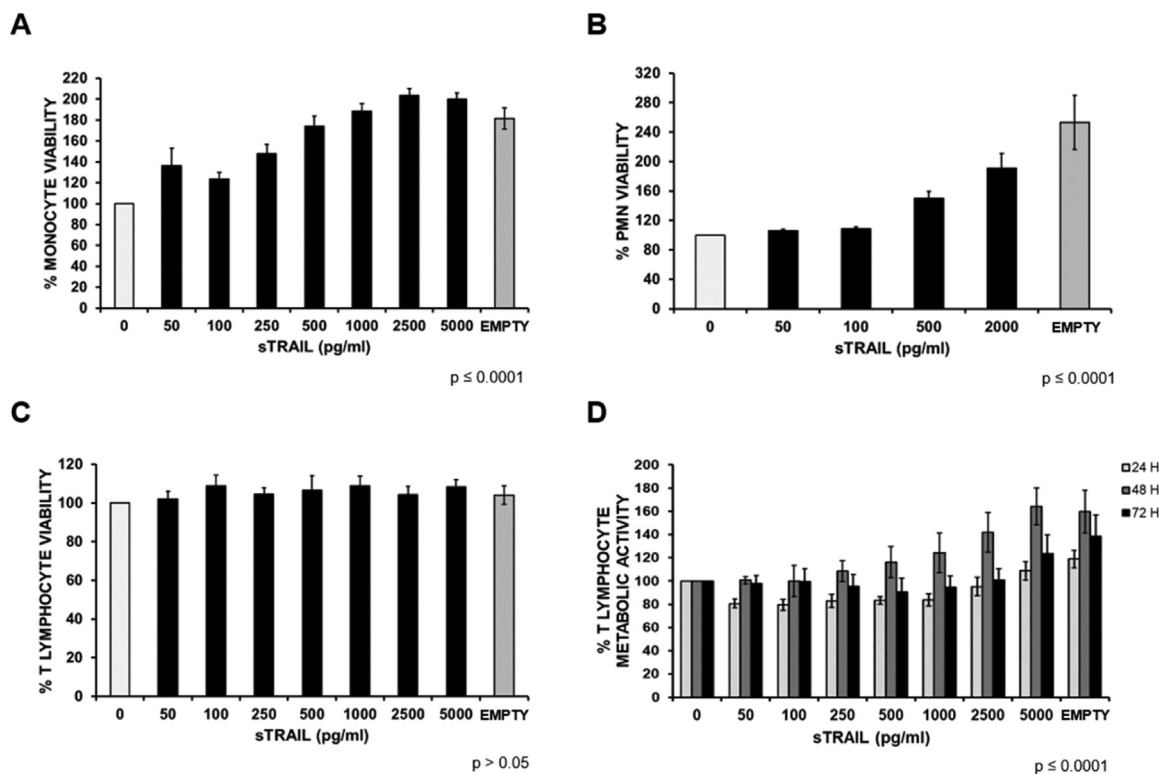


Fig. 3. Quantification of the cytotoxic effect of different doses of sTRAIL on monocytes, PMN and T lymphocytes from three donors. (A) Monocyte, (B) PMN and (C) T-lymphocyte viability evaluated by the CellTiter-Glo Luminescent Cell Viability assay after 24 h of treatment with CM from AD-MSC sTRAIL with increasing sTRAIL doses (0 to 5000 pg/mL for monocytes and T cells; 0 to 2000 pg/mL for PMN). CM from AD-MSC EMPTY (EMPTY) was used as a control. Mean values of the three donors are reported. Monocytes statistical analysis revealed significant differences starting from 250 up to 5000 pg/mL of sTRAIL compared with control and EMPTY CM. Consistent with these statistical analyses, Turkey multiple comparison also demonstrated a statistically significant increase in monocyte viability at the increasing doses of sTRAIL, starting from 500 pg/mL, but not registered significant differences in 5000 pg/mL of sTRAIL compared with EMPTY CM. PMN Tukey's multiple comparison results reported a significant difference from 500 pg/mL of sTRAIL compared with control. *P* value by ANOVA. Tukey's multiple comparison was used as a post-hoc Test Error bars = SEM. (D) T lymphocyte metabolic activity percentage as a function of sTRAIL doses (0–5000 pg/mL) after 24, 48 and 72 h, evaluated by the alamarBlue assay. CM from AD-MSC EMPTY (EMPTY) was used as a control. Tukey's multiple comparison revealed that 24 h there is a significant increase in T-cell metabolic activity when treated with 5000 pg/mL of sTRAIL or CM from EMPTY compared to control. At 48 and 72 h, 5000 pg/mL of sTRAIL provoked a significant increase of alamarBlue reduction compared with lower doses of sTRAIL, and this effect was comparable with the one obtained by CM from EMPTY. *P*-value by ANOVA. Tukey's multiple comparison was used as a post-hoc. Mean values of the three donors are shown. Error bars denote standard error of the mean.

Impact of sTRAIL-producing AD-MSC on T-cell cytokine release

To investigate cellular crosstalk between T lymphocytes and AD-MSC sTRAIL, the secretion of several anti-inflammatory and tumor-related cytokines was investigated in supernatants collected from T-cell–MSC co-cultures at different E:T ratios (1:30, 1:15, 1:10, 1:1 and 10:1) for 24, 48 and 72 h. Five cytokines secreted by either T lymphocytes (IL-10, TNF α and IFN γ) or AD-MSC (VEGF-A and IL-6) were analyzed. T lymphocytes alone or AD-MSC alone were used as controls. The evaluation was performed by multiplex ELISA. Since different T-cell–MSC ratios were tested in co-culture, thus creating differences in the absolute number of T cells in each culture condition, the amount of IL-10, TNF α and IFN γ was divided by the real number of T cells in the plate to make all conditions comparable with each other; a similar approach was used to normalize the amount of VEGF-A and IL-6 produced by MSC. Data were reported as fg/mL per T cell for cytokines secreted by T lymphocytes and fg/mL per AD-MSC for cytokines secreted by AD-MSC.

The collected results revealed the greatest statistically significant increase in IL-10 (Figure 5A, $P < 0.0003$), TNF α (Figure 5B, $P < 0.05$) and IFN γ (Figure 5C, $P < 0.007$) secretion by T cells when co-cultured with AD-MSC sTRAIL (or EMPTY) at a 10:1 E:T ratio. T lymphocytes, in turn, induced a peak of VEGF-A (Figure 5D) and IL-6 (Figure 5E) secretion by AD-MSC after 72 h of co-culture at a 1:30 E:T ratio, an effect probably mediated by TNF α and IFN γ released by the T cells themselves. No difference in VEGF-A and IL-6 was detected between AD-MSC sTRAIL (or EMPTY) co-cultured with T lymphocytes

at a 10:1 E:T ratio and AD-MSC sTRAIL (or EMPTY) alone (data not shown). Collectively, these data highlight the cytokine crosstalk between T lymphocytes and AD-MSC sTRAIL in co-culture: AD-MSC sTRAIL stimulate IL-10, TNF α and IFN γ secretion by T cells, and T lymphocytes in turn induce VEGF-A and IL-6 secretion by AD-MSC, probably mediated by TNF α and IFN γ .

Discussion

For years, the potential use of TRAIL as an anticancer agent has aroused great interest in oncology research. The tumor-selective activity of TRAIL, which triggers the apoptotic signaling cascade preferentially in cancer cells with little-to-no effect in normal cells (e.g., lung fibroblasts, skeletal muscle cells, epidermal keratinocytes and melanocytes) [12], could be of value to the clinical use of this molecule. Nevertheless, the issues concerning its poor pharmacokinetics and biodistribution have considerably limited its use in oncologic patients [12,25]. For this reason, many technological improvements have been made to ameliorate TRAIL delivery and effectiveness against solid tumors, including the use of gene-modified MSC as a cellular vehicle [14,15]. Thanks to their ability to be attracted to tumor sites and their resistance to the proapoptotic stimuli mediated by TRAIL, MSCs represent a good cellular carrier for the delivery of this antitumor molecule in a clinical scenario. Recently, we have developed an anticancer cell therapy approach based on gene-modified AD-MSC expressing a novel secreted variant of TRAIL characterized by the presence of an isoleucine zipper domain that confers the

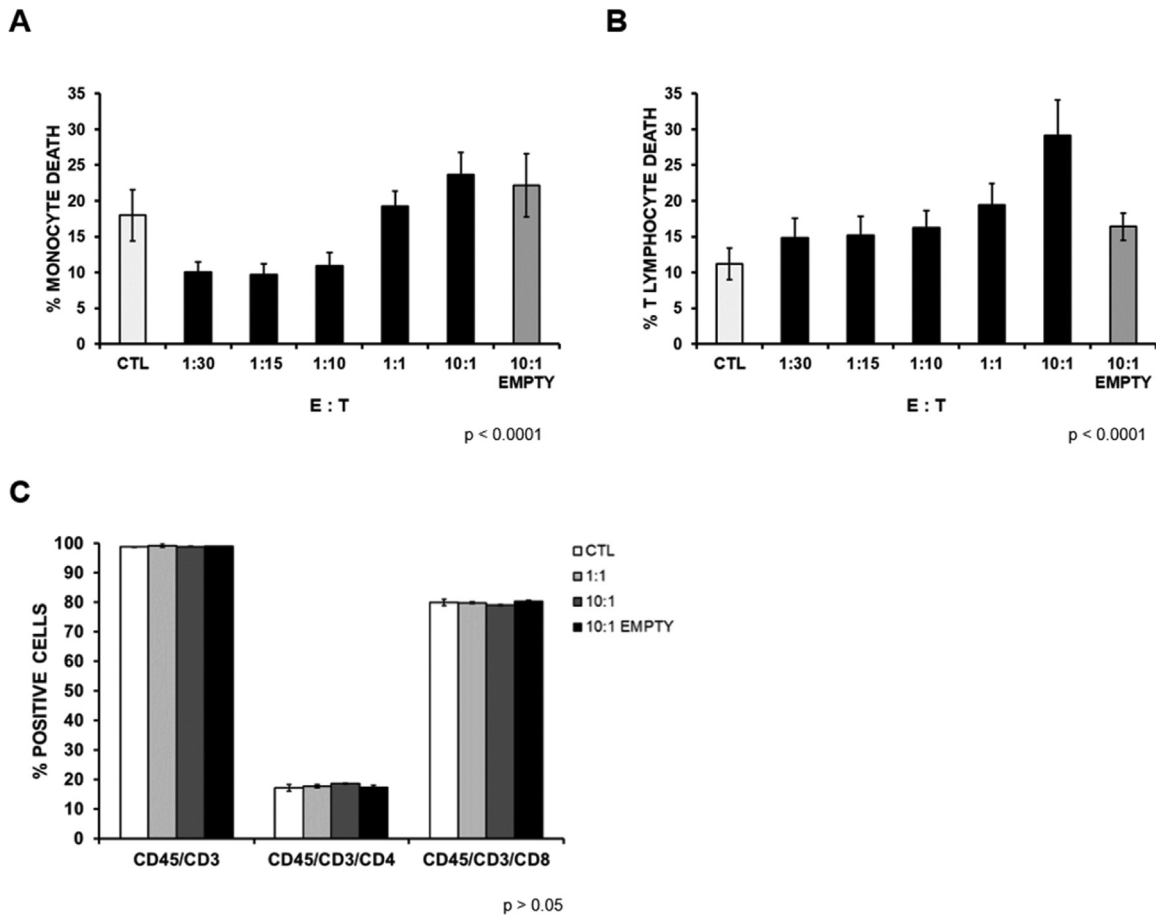


Fig. 4. Quantification of the cytotoxic impact of direct cell-to-cell contact with AD-MSC sTRAIL on monocytes and T lymphocytes from three donors. AD-MSC sTRAIL and EMPTY co-culture with (A) monocytes or (B) T lymphocytes at different effector:target ratios (1:30, 1:15, 1:10, 1:1, and 10:1; E:T, E = AD-MSC sTRAIL or EMPTY as a control, T = target cells, i.e., monocytes or T cells). After 24 h, leukocyte death was quantified by supravital PI staining and FACS analysis. Statistical analysis for monocytes revealed a slight but significant increase of cell mortality at the greater E:T ratios (1:1, 10:1, and 10:1 EMPTY) compared with lower ones (1:30; 1:15 and 1:10). Conversely, no statistical differences were reported comparing CTL group with all different treatment conditions, thus suggesting that AD-MSC sTRAIL did not alter monocyte viability in respect to the standard culture condition. *P*-value by one-way ANOVA followed by Tukey's multiple comparison results as a post-hoc. Error bars denote standard deviation. CTL = T cells alone. (C) The expression of both CD4+ and CD8+ T cells after co-culture with AD-MSC sTRAIL and EMPTY at E:T ratios of 1:1 and 10:1 was quantified within the CD45+/CD3+ subpopulation by FACS. *P*-value by one-way ANOVA followed by Tukey's multiple comparison results as a post-hoc. Error bars denote standard deviation. CTL = T cells alone.

capacity to form more stable multimeric complexes of TRAIL, thus resulting in greater cytotoxic activity on tumor cells compared with the recombinant human molecule [18]. The improvement in the protein structure and the MSC-based delivery may represent the needed strategies to enhance the use of TRAIL in a clinical setting.

According to that, the assessment of the immunological safety of AD-MSC sTRAIL is crucial considering both the immunomodulatory activity displayed by MSC on immune cells and the role played by TRAIL in the regulation of innate and adaptive immune responses [2,22]. Indeed, TRAIL can be expressed by immune cells based on their activation status, with the upregulation of TRAIL on both innate and adaptive immune cells, such as monocytes, neutrophils, T and B lymphocytes and NK cells, when stimulated with type I IFN. In addition to TRAIL expression, activated immune cells also can express decoy TRAIL receptors, thereby creating resistance to TRAIL-mediated apoptotic signaling [26]. TRAIL has been referred to as a guardian against pathogen infections, autoimmunity and cancer onset and progression, playing a crucial role in immunosurveillance mechanisms [4]. Moreover, it has been reported that TRAIL expressed by neutrophils, CD8+ T lymphocytes and NK cells promotes the elimination of pathogen-infected cells expressing functional TRAIL receptors, thus containing infections [26]. In autoimmunity settings, TRAIL inhibits the activation of autoreactive T cells but promotes T regulatory proliferation in diabetes, rheumatoid arthritis, and autoimmune

encephalomyelitis [8]. Moreover, monocytes stimulated with IFN γ , CD4+ T cells and NK cells expressing TRAIL can recognize and then neutralize transformed cells, inhibiting tumor growth and preventing tumor metastases within the immunosurveillance process [27].

For the first time, we also have evaluated the impact of a novel form of sTRAIL released by AD-MSC on the viability, metabolic activity and cytokine release of WBCs. At first, we evaluated by flow cytometry the expression of both functional and decoy TRAIL receptors responsible for inducing or inhibiting TRAIL signaling, respectively, on freshly isolated purified blood leukocytes from healthy donors. As reported by Liguori *et al.* [28], we observed heterogeneous expression of TRAIL receptors on resting WBCs: DR5 was mainly expressed on monocytes, whereas the decoy receptors (Dcr1 and Dcr2) were essentially expressed on neutrophils. On the contrary, resting T cells revealed weak expression of all TRAIL receptors.

Since our MSC-based cell product releases multimeric sTRAIL complexes into the cell culture medium [18], we first investigated the biological impact of supernatant collected from AD-MSC sTRAIL on primary resting leukocytes. For all considered immune cell subtypes, we did not observe any toxicity even at the greatest tested sTRAIL concentration (5000 pg/mL). Particularly for monocytes, despite the high expression of DR5, we did not observe any cytotoxic effect mediated by sTRAIL but rather we registered an unexpected significant increase of cell viability directly related to the sTRAIL

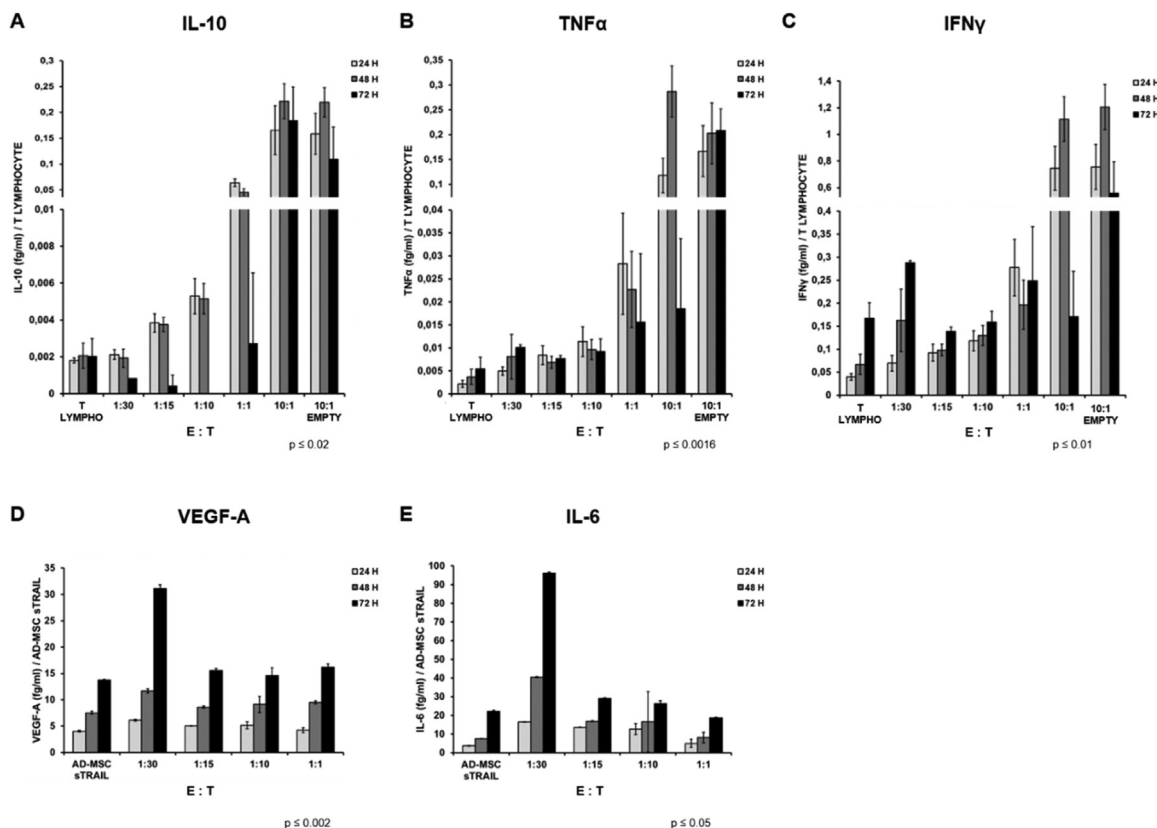


Fig. 5. Cytokine profile in co-cultures of T lymphocytes and AD-MSC sTRAIL. Evaluation of (A) IL-10, (B) TNF α and (C) IFN γ protein levels (fg/mL) per T lymphocyte in co-culture supernatants of T lymphocytes with AD-MSC sTRAIL at different E:T ratios (1:30, 1:15, 1:10, 1:1 and 10:1; E = AD-MSC sTRAIL or EMPTY as a control, T = T cells) after 24, 48 and 72 hours by multiplex ELISA. Results from three donors are presented together. (D) VEGF-A and (E) IL-6 protein levels (fg/mL) per AD-MSC sTRAIL in co-culture supernatants of T lymphocytes with AD-MSC sTRAIL at different E:T ratios (1:30, 1:15, 1:10 and 1:1; E = AD-MSC sTRAIL, T = T cells) after 24, 48 and 72 h by multiplex ELISA. Results from three donors are presented together. The significance results of one-way ANOVA followed by Tukey's multiple comparison results as a post-hoc test, are reported for all panels. Error bars denote standard error of the mean.

concentrations. If on the one hand the mechanisms of TRAIL resistance in transformed cells are largely investigated, since they represent a fundamental key to improve the anticancer potential of TRAIL based therapies, on the other hand the cellular processes leading TRAIL resistance in non-transformed cells are not so explored. Along with the expression of decoy receptors, it has been demonstrated recently that non-transformed cells, including monocyte from healthy donors, escape TRAIL-mediated apoptosis by the activation of multiple and redundant antiapoptotic molecular mechanisms, including the up-regulation of c-FLIP, IAP and Bcl-2, which prevents the activation of apoptotic signals [29–32]. The removal of one of these inhibitors is not sufficient to sensitize non-transformed cells to TRAIL and only the simultaneous loss of two or more of these proteins (i.e., cellular FLICE-like inhibitory protein [cFLIP] and XIAP expression or loss of cFLIP and antiapoptotic Bcl-2 protein-function) could induce TRAIL sensitivity [31]. Surprisingly, we registered a relevant and significant increase in cell viability for PMNs after exposure to CM collected from engineered AD-MSC. This effect was visible in cells treated with supernatant collected from both AD-MSC sTRAIL and AD-MSC EMPTY, suggesting that soluble trophic factors and cytokines secreted by MSC within the supernatant, such as granulocyte-macrophage colony-stimulating factor, IL-6 and IL-8, could support PMN's *in vitro* performance [32,33]. In addition, no significant variations were revealed in the viability of T cells treated with CM from AD-MSC sTRAIL compared with the control. It has been reported that resting peripheral T cells are resistant to TRAIL-mediated apoptosis, in contrast to T lymphocytes activated by antigenic stimulation or within autoreactive processes, as a mechanism to prevent potentially dangerous autoimmune damage [7,34,35]. This result is not

unexpected, considering both the low expression of DR4 and DR5 revealed by FACS analysis and the data reported by Clancy *et al.* [36] indicating that in T lymphocytes, decoy receptors mediate resistance to TRAIL-induced apoptosis by creating mixed functional-decoy receptor complexes that do not efficiently trigger TRAIL signaling. The health status of T cells also was assessed by evaluating their metabolic activity after exposure to increasing doses of sTRAIL released by AD-MSC in CM at different time points (24, 48 and 72 hours). A significant but slight reduction in T-cell metabolism was observed at 24 h with 50–2500 pg/mL sTRAIL, but this trend was completely reversed with both CM from AD-MSC sTRAIL with the greatest sTRAIL concentration (containing 5000 pg/mL of sTRAIL) and from AD-MSC EMPTY and at later time points (48 and 72 h). This suggests that, similar to what was observed for T-cell proliferation, lymphocyte metabolism may be more influenced by trophic factors secreted by MSC than by the toxic impact of sTRAIL. More, these data suggest that non-activated T cells would be resistant to sTRAIL released by engineered AD-MSC thanks to the high expression of TRAIL-mediated apoptosis inhibitors, such as cFLIP, as reported for the wild-type form of TRAIL [36–38].

To further investigate whether the cellular crosstalk between MSC and WBCs could modify TRAIL sensitivity in leucocytes, we established co-culture experiments. Monocyte viability was confirmed as not being influenced by cell-to-cell interactions with engineered AD-MSC secreting sTRAIL despite the high expression of DR5 registered by FACS analysis. On T lymphocytes, we instead observed a slight increase in cell death at greater E:T doses (E:T ratios of 1:1 and 10:1) compared with CTL and co-culture with AD-MSC EMPTY. This modest (lower than 20%) but significant reduction in T-cell viability

registered at the 10:1 E:T ratio is likely due to sTRAIL secreted by AD-MSC, since it was not observed in co-culture with AD-MSC EMPTY. In addition, TRAIL-mediated apoptosis did not selectively affect T-cell subtypes, since no significant variations in CD4⁺ versus CD8⁺ cell ratios were observed in co-culture with gene-modified AD-MSC and lymphocytes alone. The reason for the fact that the same amount of sTRAIL is not having any cytotoxic impact on T cells when used as CM from AD-MSC sTRAIL versus coculture may be in the presence of AD-MSC cytokines able to sensitize T cells to TRAIL apoptosis during cell-to-cell interaction. In this sense, it has been reported that IFN γ and TNF α enhance TRAIL sensitivity on target cells [39–41]. Therefore, cytokine crosstalk accounting for MSC immunomodulatory properties was investigated in co-cultures of T cells and AD-MSC sTRAIL. The levels of the IL-10, TNF α and IFN γ cytokines released by T cells in co-culture with AD-MSC (both sTRAIL and EMPTY) were massively increased at the 10:1 E:T ratio, suggesting that the secretion of these molecules could be indirectly stimulated by MSC, as already reported [42]. Therefore, the relevant increase in TNF α and IFN γ amounts registered in the culture medium at the greatest E:T ratios may be responsible for the increased sensitivity of lymphocytes to the apoptotic impact of TRAIL, justifying the enhanced T-cell death after direct exposure to AD-MSC sTRAIL. Similarly, cytokines secreted by MSC, such as VEGF-A and IL-6, also were evaluated. VEGF-A is a key promoter of blood vessel growth and vascular permeability as well as an inhibitor of lymphocyte activation, thus stimulating tumor neoangiogenesis, progression and metastasis [43]. We observed the most relevant increase in both VEGF-A and IL-6 secretion per AD-MSC at the 1:30 E:T ratio after 72 h of co-culture with T lymphocytes, which is probably related to the release of TNF α and IFN γ by T cells, as shown by Nagineni *et al.* [44–46]. Moreover, it is known that IL-6 (alone or in combination with TNF α) can induce VEGF-A secretion by AD-MSC through the ERK, JNK and PI3K pathways [46]. Therefore, we highlighted the cytokine crosstalk between T cells and AD-MSC sTRAIL in co-culture. In particular, AD-MSC induced IL-10, TNF α and IFN γ secretion by T lymphocytes and TNF α and IFN γ , in turn, promoted VEGF-A and IL-6 release by AD-MSC [47].

To conclude, these data demonstrate that healthy WBCs are refractory to the proapoptotic effect displayed by sTRAIL, confirming an immunological safety and a clinical feasibility of an anticancer gene therapy strategy based on AD-MSC expressing sTRAIL.

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Declaration of Competing Interest

MD and GG hold patents in the field of cell and gene therapy and declare a consultancy role, research funding, and stock ownership with Rigenerand srl, now EVOTEC (Modena) srl. MCS declares stock ownership with Rigenerand Srl. GC, MDa and MCS are employees of Rigenerand srl, now EVOTEC (Modena) srl. The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: GC, MDa, GG and MD. Acquisition of data: GC, MDa, VM, GG, MP, CC, GN, MCS, AM and AD. Analysis and interpretation of data: GC, MDa, GG, MP and CC. Drafting or revising the manuscript: GC, MDa, GG and MD. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2023.02.008.

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