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Short communication

Exosomes in the serum of Acute Myeloid Leukemia patients induce dendritic cell tolerance: Implications for immunotherapy ☆

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ABSTRACT

Exosomes may represent an interesting antigenic pulse for new forms of anti-tumor immunotherapy. We evaluated exosomes from serum of patients with acute myeloid leukemia (AML) as an antigenic source for dendritic cells (DC) and the effects upon antitumor cytotoxicity, assessed by the percentage of specific lysis of K562 leukemic cells in co-cultures. Surprisingly, incubation of exosomes with DCs decreased lysis of K562, which may correspond to a mechanism of tumor evasion *in vivo*. However, when immature DCs were pulsed with exosomes purified from K562 culture supernatants, the lysis of target cells was notably enhanced, associated with a substantial increase in the expression of the maturation marker CD83. Thus, the development of vaccines using patients' exosomes would probably add no benefits to the treatment of AML; alternately, exosomes from cultured cells may represent an effective way for maturing DCs into a cytotoxic phenotype, without the immunosuppression observed with patients' exosomes.

1. Introduction

Vaccines produced from dendritic cells (DCs) electroporated with Wilms Tumor 1 (WT1) mRNA have demonstrated good efficacy in preventing relapses in Acute Myeloid Leukemia (AML) patients [1,2]. However, the use of this therapy depends on the expression of WT1 in the blasts of the patient, which precludes the application for all individuals affected by the disease. Therefore, the search for new forms of antigenic pulse for immunotherapy with DCs is justified.

Exosomes are nanoparticles (50–200 nm) released extracellularly and bearing particular proteins, RNA and miRNAs profiles, with important functions in intercellular communication [3,4]. Due to their diverse internal content but dependent on the cells of origin [5,6], there is a growing interest in the use of these nanoparticles as personalized antigenic sources in the development of new forms of DC vaccines.

In this context, DCs pulsed with exosomes were shown to stimulate efficient cytotoxic responses and antitumor immunity in animal model [7]. In the specific case of AML, DCs pulsed with exosomes extracted from leukemia cell culture supernatants induced efficient cytotoxic responses translated into tumor size regression and longer survival of vaccinated mice [8]. Thus, the aim of this study was to evaluate the use of exosomes extracted from the serum of AML patients as an antigenic source for dendritic cell pulse and the effects upon antitumor cytotoxic responses.

2. Materials and methods

2.1. Exosome purification and characterization

Samples of venous blood (8 mL) were obtained from 8 patients newly diagnosed with AML, 2 patients with Myelodysplastic Syndromes (prior to any treatment), 1 patient with Chronic Myeloid Leukemia and 2 healthy blood donors. All subjects participating in this study signed an informed consent and the study was approved by the Hospital Ethics Committee.

Samples were processed through centrifugation at 3000 rpm for 10 min, and exosomes were isolated using the ExoQuick® exosome precipitation solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. Exosomes concentration and size distribution were characterized by Nanoparti-

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cle Tracking Analysis (NTA) (NS300 equipment, NanoSight, Amesbury, UK) equipped with a 532 nm laser. NTA is a novel *in vitro* characterization method, adequate for quantifying dispersed nanoparticles of diluted samples in real time and capable of characterizing membrane fractions, such as exosomes [9,10]. The measurements were conducted in triplicates, with approximately 2000 tracks per measure.

2.2. Generation of DCs, phenotypic analysis and antigen pulsing

Peripheral blood mononuclear cells were isolated from buffy coats of healthy blood donors by Ficoll-Paque density gradient centrifugation. Monocytes were isolated by Percoll density gradient centrifugation followed by adherence to a plastic dish for 2 h. DCs were generated from adherent monocytes in RPMI 1640 (Sigma–Aldrich Corp, St. Louis, MO, USA) with IL-4 (50 ng/mL, PeproTech Rochy Hill, NJ, USA) and GM-CSF (50 ng/mL, PeproTech, Rochy Hill, NJ, USA). Mature DCs were generated by supplementation with TNF-α (50 ng/mL PeproTech Rochy Hill, NJ, USA) and prostaglandin E2 (PGE2) (2.5μg/mL, Cayman Chemycal). Phenotypic analysis was performed using monoclonal antibodies against CD11c (BD Biosciences, California, USA), CD80, CD83, and HLA-DR (Caltag, CA, USA) and the DC population was gated in CD11c positive cells.

DCs were pulsed in co-cultures with exosomes extracted from serum of patients or healthy individuals, exosomes derived from K562 culture supernatant, or K562 cell lysates. The verification of the internalization of the exosomes in the DCs was performed by marking with CFSE tracer (Molecular Probes/Invitrogen, USA) and evaluation by immunophenotyping after 1 h of co-culture. After 48 h of incubation of the exosomes or tumor lysates with DCs, maturation, cell viability and Natural Killer phenotype were also analyzed by immunophenotyping for expression of CD83/CD86, 7AAD and CD56 markers, respectively.

2.3. K562 cultures, cell lysates and exosome precipitation from supernatants

The human K562 leukemia cell line (major histocompatibility complex [MHC] class I-negative) was obtained from the American Type Culture Collection (ATCC® CCL-243™). The cells were cultured at 37 °C in 5% CO_2 and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS. K562 cells were resuspended in 1 mL of RPMI-1640 and lysed by 5 freezing (−80 °C freezer, 30 min) –thawing (room temperature, 10 min) cycles. Total cell disruption was microscopically validated using trypan blue staining. After sonication for 10 min, the lysate was centrifuged at 14,000 rpm (30 min, 4 °C). Supernatant was recovered and stored at −80 °C until use. Exosomes from K562 supernatants were precipitated using ExoQuick-TC™ according to manufacturer's instructions (System Biosciences). Briefly, 40 mL of culture supernatant was mixed with 10 mL of ExoQuick-TC™ solution by inverting the tube several times. The sample was incubated overnight at 4 °C, centrifuged twice at 1500*g* for 30 and 5 min, respectively, and the pellet was resuspended in 200 μL PBS.

2.4. Cytotoxicity assays

DCs were harvested and cocultured overnight with CFSE-labeled target cells (K562) in fresh RPMI/10% FBS medium at different effector-to-target ratios (1:1, 0.5:1, 0.25:1). To assess the lymphocyte dependent cytotoxicity of DCs, T cells were separated from PBMC by magnetic cell selection (MACS) using CD3-MicroBeads (Mitenyi Biotec, Germany) and co-cultured in 48-well plates (5×10^5 cells /well) with DCs (5×10^4 cells /well) and K562 (5×10^4 cells /well) for 5 days.

The cells were then resuspended in Annexin-V-binding buffer (BD) containing Annexin-V-APC (1 mL/100 mL buffer; BD). After 15 min incubation at room temperature, samples were stained with propidium iodide (PI; Sigma-Aldrich). Fluorescence analysis was performed with a FACScalibur flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson).

Secretion of INF-γ by lymphocytes after incubation with DCs under different antigenic pulses was assessed by immunophenotyping using monoclonal antibody against INF-γ (BD Biosciences, California, USA) in gated CD3 positive cells.

2.5. Statistical analysis

Statistical analysis was performed using R software version 3.5.1, Copyright (C) 2018, The R Foundation for Statistical Computing [11]. Wilcoxon rank sum test (non-normal) or a Student's *t*-test

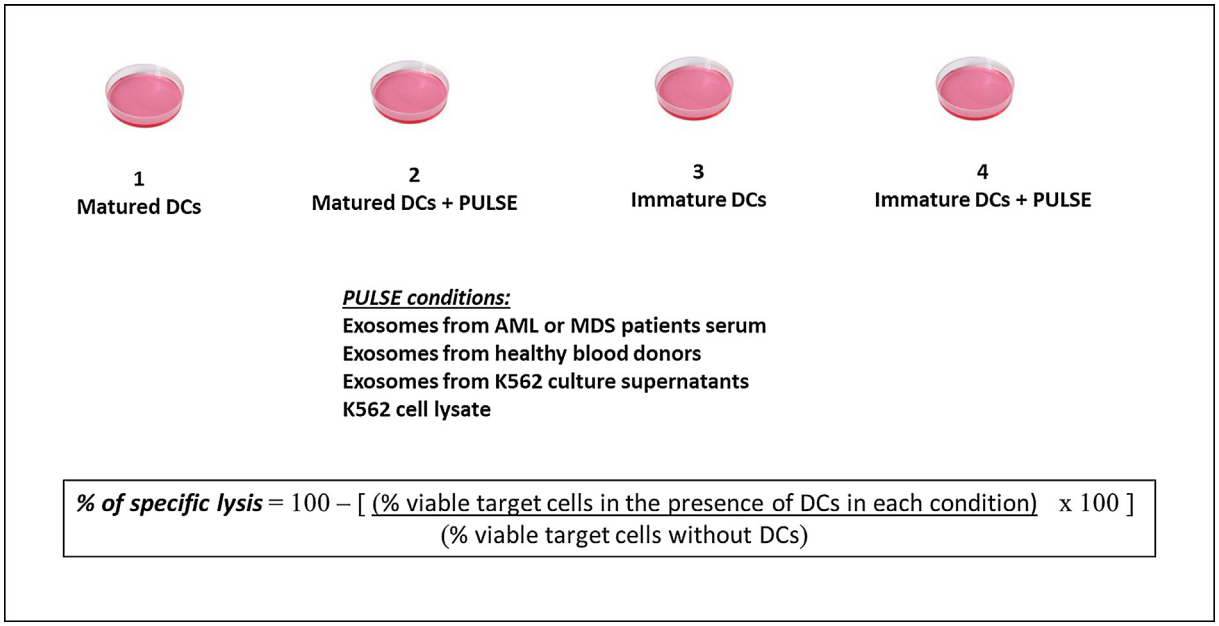

Fig. 1. Flow data for the cytotoxic studies using CFSE labeled target cells.

(normal) was then used to test the statistical significance of group differences in characteristics as specific cytotoxicity or surface marker expression. Results were considered significant at $p < 0.05$.

3. Results

3.1. AML derived exosomes are effectively internalized in DCs and suppress cytotoxicity in a dose dependent manner

CFSE-labeled exosomes from one AML patient were added to DC cultures and their internalization was observed by immunophenotyping. After 1 h of incubation, exosome incorporation was observed in approximately 80% of the DCs, proving their efficient internalization.

Co-culture assays were then performed separately under the following conditions: (i) mature or immature DCs, (ii) non-pulsed or pulsed with exosomes, (iii) incubated with K562 alone or in the presence of lymphocytes. CFSE-labeled target cells cultured without DCs were used to determine spontaneous cell death. Percentages of viable target cells (PI/Annexin-V negative) were used to quantify the cytotoxic responses according to the following formula: % of specific lysis = 100 − [(% viable target cells in the presence of DCs/% viable target cells without DCs) × 100]. Flow data for the cytotoxic studies using CFSE labeled target cells can be visualized in detail in Fig. 1.

Both non-pulsed mature and immature DCs exhibited *in vitro* cytotoxic against K562, regardless of lymphocyte activation. The addition of exosomes extracted from the serum of a patient diagnosed with AML, in both mature and immature DCs, decreased the percentage of specific lysis of K562, in the presence or not of lymphocytes. This effect was directly correlated to the number of DCs in culture: higher concentrations of DCs reduced the lysis of K562 close to zero or actually increased proliferation of leukemic cells, as observed in Fig. 2.

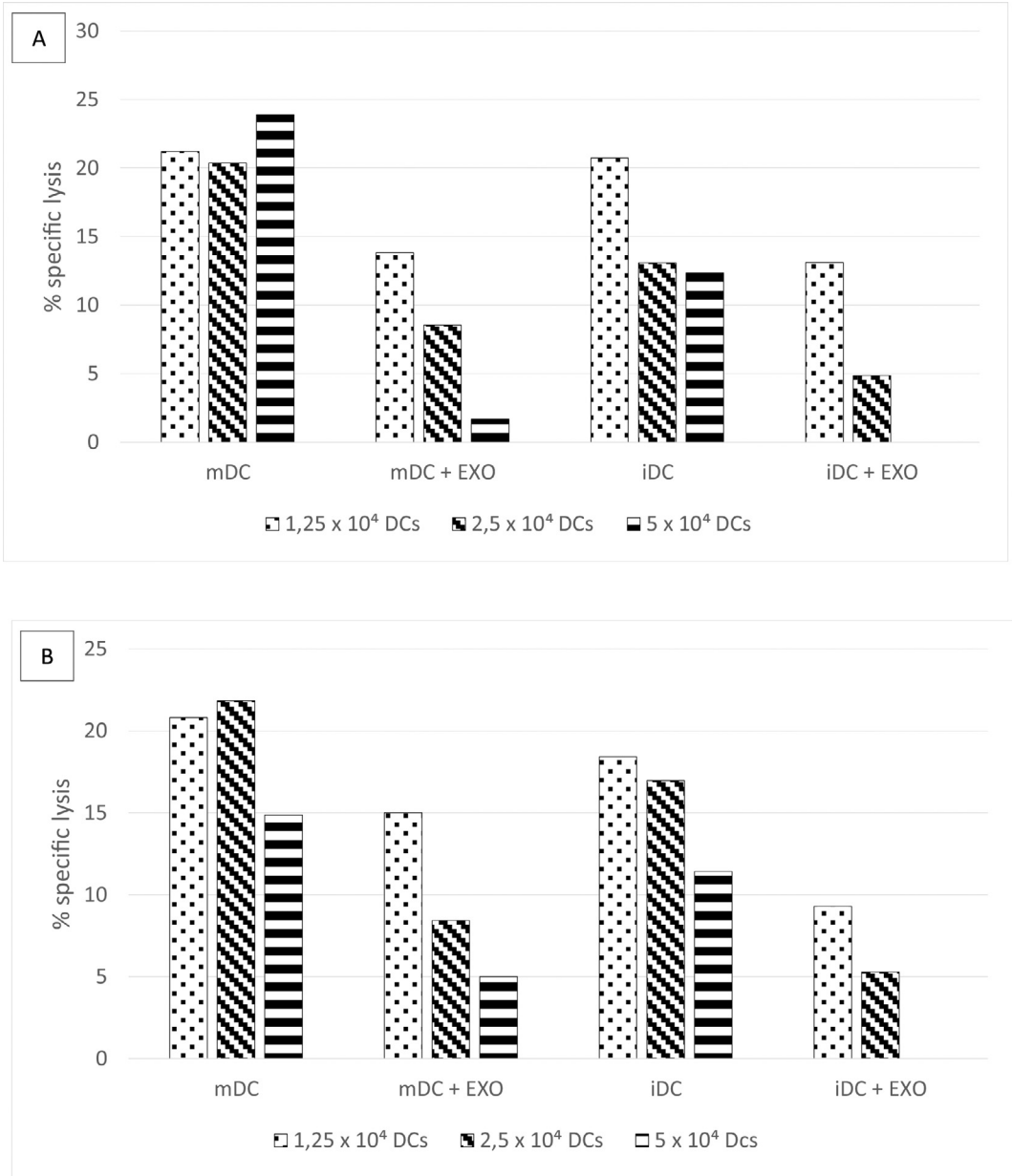

Fig. 2. Percentage of specific lysis of K562 leukemic cells upon contact with dendritic cells in different pulsing conditions. A – in the presence of lymphocytes, B – DCs direct cytotoxicity.

3.2. AML derived exosomes suppress DCs mediated cytotoxicity against K562 cells, contrary to K562 lysates and exosomes from K562 culture supernatants

We performed the same experimental procedures with samples of 8 AML patients, 2 patients diagnosed with MDS, and 1 patient with Chronic Myeloid Leukemia (patients characteristics are detailed in Table 1). The experiments were also performed with DCs incubated with exosomes from serum of 2 healthy individuals, to exclude the possibility of artifacts by simply adding the nanoparticles into the cultures. Assessment by Nanoparticle Tracking Analysis (NTA) showed size distribution in the exosome range: median size 145.7 (95–229.2) nm. All samples were diluted to contain approx. 10^{15} nanoparticles in 100 μl suspension. In addition, these experiments were also repeated pulsing mature and immature DCs with K562-exo and K562-lysates (K562-lysates would be representative of higher antigenic load and considered as positive control).

For better comparison of the cytotoxic potential of DCs under different pulse conditions, we calculated the difference between the percentages of specific lysis of K562 cells, observed in each situation, in relation to that observed in non-pulsed DCs, which was considered as baseline cytotoxicity. These results are shown in Fig. 3.

In the case of DCs pulsed with patients' exosomes, the calculated difference resulted in negative values, evidencing a suppres-

Table 1
Patients clinical and laboratory data.

	Gender	Age	FAB classification	Cytogenetics	% blasts (BM)	% blasts (PB)	NP1 mutant	FLT3 mutant
AML 1	M	61	M4	46, XY	75	77	Yes	No
AML 2	M	51	M4	46, XY	28	6	No	No
AML 3	M	34	M1	46, XY	98	86	Yes	Yes
AML 4	M	47	M4	46, XY	57	77	Yes	Yes
AML 5	F	41	M3	46, XX,t(15;17)(q22;q11)	93	0	No	No
AML 6	M	57	M6	47, XY,+2/46,XY	26	3	No	No
AML 7	M	65	M2	46, XY	63	23	No	No
AML 8	M	72	M2	n/a	59	37	No	No
MDS 1	F	81	RAEB-2	46, XX	11	0	n/a	n/a
MDS 2	M	79	t-MDS	46, XY	3	1	n/a	n/a
CML	F	51	Chronic phase	46, XX,t(9;22)(q34;q11)	1	1	n/a	n/a

AML: Acute Myeloid Leukemia, CML: Chronic Myeloid Leukemia, MDS: Myelodysplastic Syndrome, RAEB-2: Refractory cytopenia wih excess blasts -2, t-MDS: therapy-related Myelodysplastic Syndrome, BM: bone marrow, PB: peripheral blood, NP1: Nucleophosmin 1, FLT3: FMS-like tyrosin kinase 3, n/a: not available.

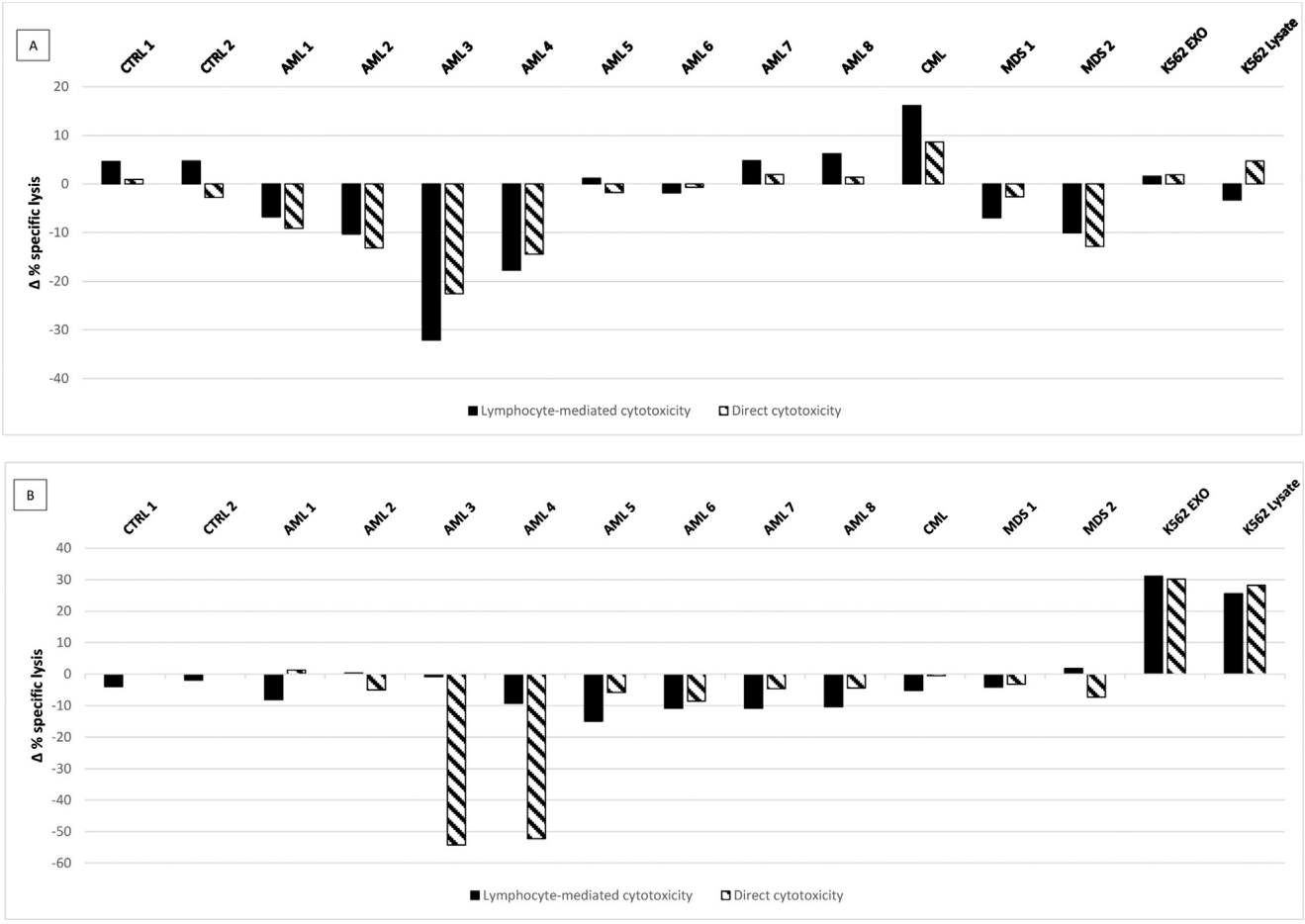

Fig. 3. Differences of DCs mediated cytotoxicity in diverse pulsing conditions in relation to non-pulsed DCs, in the presence or absence of lymphocytes. A – DCs matured in culture, B – DCs cultures without maturation factors.

sion in cytotoxicity, contrarily to DCs pulsed with exosomes from normal individuals, in which the differences in % of specific lysis of K562 varied around zero. The only exceptions to this pattern were 2 patients with M2 type AML, with similar results to those using the exosomes of controls, and the case of CML, where there was a considerable increase in cytotoxicity compared to the other patients. However, this was observed only in matured DCs; immature DCs had their cytotoxic activity suppressed after pulsing with exosomes from all of the tested patients.

In matured DCs pulsed with K562 exosomes or lysates, little difference in cytotoxicity was observed in relation to non-pulsed DCs. In the case of immature DCs, we observed that pulsing with K562 exosomes or lysates notably enhanced the lysis of target cells, both in the presence or absence of lymphocytes (Fig. 3B).

3.3. Evaluation of the effect of different pulse conditions on DC maturation, lymphocyte proliferation and INF-γ secretion

Phenotypic analysis of dendritic cells generated *in vitro* from peripheral blood mononuclear leukocytes can be visualized in detail in Fig. 4. In all pulse situations tested, differentiation in DCs was evidenced through high expression of CD11c, CD80 and HLA- DR associated with low CD14 expression. Immunophenotyping for CD56 was always positive in less than 1% of the cells, excluding the possibility of the observed effect being the action of contaminating Natural Killer cells. In addition, under all conditions studied, more than 90% of the cells expressed high levels of HLA-DR, also excluding the possibility that the immunosuppressive effect observed was due to myeloid derived suppressor cells.

The expression of the maturation marker CD83 remained stable in matured DCs, regardless of pulsing. However, there was a substantial and unexpected increase in CD83 expression in immature DCs pulsed with K562 exosomes or lysates (Fig. 5). Contrarily, CD86 expression increased after pulsing of dendritic cells with AML patient exosomes, relative to non-pulsed DCs, which did not occur with cells pulsed with K562 tumor cell lysate (see Fig. 5).

Regarding the effects of pulsed DCs over effector cells, we observed that different pulsing conditions did not influence lymphocyte proliferation rates, since the lymphocyte counts (evalu-

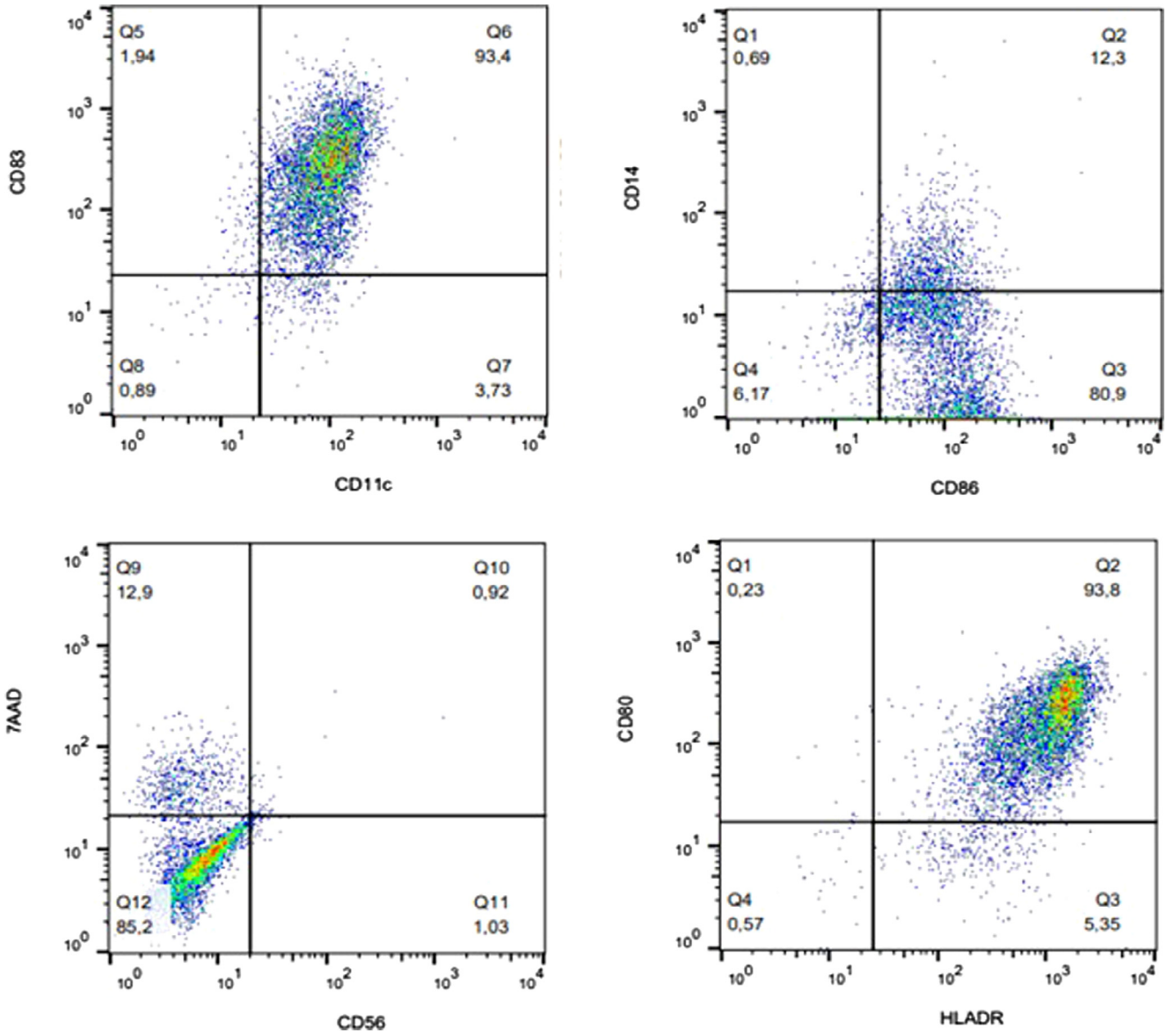

Fig. 4. Phenotypic analysis of dendritic cells generated *in vitro* after addition of maturation cytokines. This same panel was used for analysis and comparison of DCs generated under different pulse conditions.

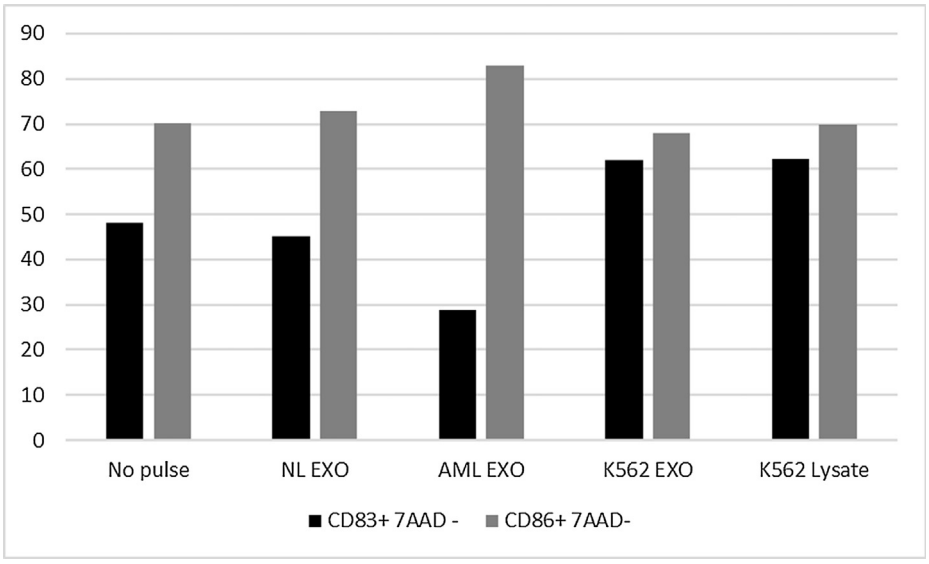

Fig. 5. Expression of the maturation markers CD83 and CD86 in immature DCs after different pulse conditions.

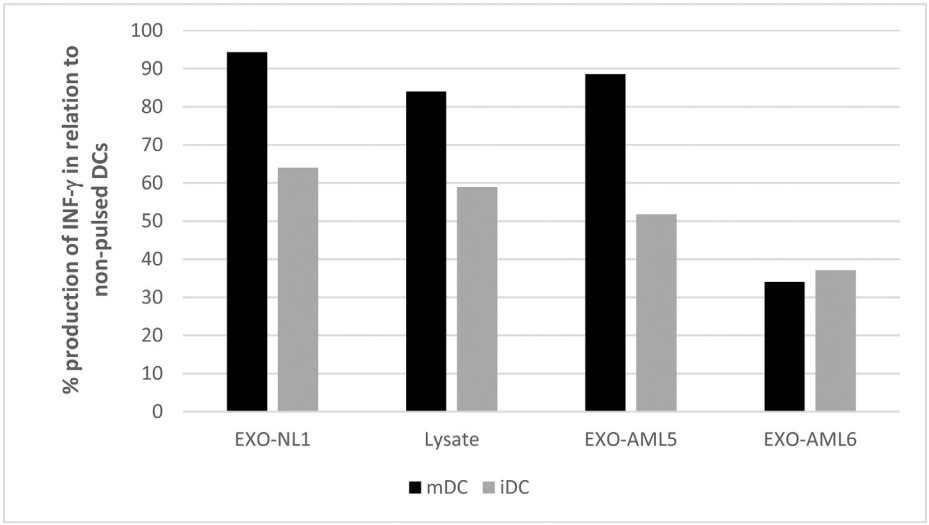

Fig. 6. % production of INF-γ by lymphocytes incubated with DCs under different pulse conditions (in relation to lymphocytes incubated with non-pulsed DCs).

ated by immunophenotyping through CD3 labeling) remained stable under different conditions.

We then questioned whether pulsed DCs decreased the cytotoxic capacity of lymphocytes by suppressing their capacity to produce INF-γ. For comparison with non-pulsed DCs, patients AML5 and AML6 were chosen since the largest decreases in lymphocyte-dependent cytotoxicity in immature DCs were observed in these two cases, and therefore we were more likely to find measurable differences indicating the effect of pulsed DCs on the lymphocytes. DCs pulsed with K562 lysate and with exosomes from normal control were also compared. In fact, we observed a significant reduction in the production of INF-γ by lymphocytes incubated with DCs pulsed with exosomes of patients AML5 and AML6, especially in the case of immature DCs (Fig. 6 shows the percentage of production of INF-γ in each situation in relation to that observed in non-pulsed DCs). Interestingly, there was a statistically significant inverse relationship between the levels of INF-γ and CD86 expression considering all analyzed situations ($p = 0.042$).

4. Discussion

Dendritic cell immunotherapy has been shown to be an interesting approach as maintenance therapy for Acute Myeloid Leukemias, decreasing relapse rates and increasing patient survival [1,2]. The use of exosomes as an antigenic pulse for DCs seems particularly interesting due to the possibility of containing a pool of antigens specific to the disease, and material easy to obtain and manipulate.

However, our results surprisingly demonstrated that AML-derived exosomes might exert an immune tolerance effect on DCs. This could correspond to a mechanism of immunological tumor evasion *in vivo* since, interestingly, K562 cell lysates or K562 derived exosomes notably enhanced DCs cytotoxicity against target cells. Moreover, K562 lysates and exosomes enhanced expression of CD83 in immature DCs. CD83 is well recognized to be important for stimulation of T cell proliferation and priming of CD8+ T lymphocytes [12,13]. Thus, taking both findings together, we can speculate that both K562 exosomes and lysates have the

ability to enhance maturation of DCs and, simultaneously, enhance the immune response against target cells.

Dendritic cells are a fundamental element in the effectiveness of innate and adaptive immune responses, due to their role as professional antigen presenting cells. Recent studies however, have also explored their direct cytotoxic potential, observed in response to tumor cells, yielding the term "killer DCs" [14]. Anguille et al. demonstrated that these "killer DCs" stimulated with IL-15 exhibited CD56 upregulation, constituting a NK phenotype with high cytotoxic potential [15]. We also observed a direct cytotoxic effect of DCs against K562 cells, regardless of the presence of lymphocytes in co-cultures. However, in our study, the direct cytotoxic effect of DCs generated by K562 cell lysates and K562 exosomes did not depend on CD56 expression; possibly signifying that other pathways are also involved in the generation of this cytotoxic phenotype and may directly affect the maturation state of DCs, represented by higher positivity of CD83.

As they are responsible for the activation of T lymphocytes, DCs also play an important role in the sensitive balance between immune response and tolerance. Previous studies have shown that mature DCs can limit effector T cell responses and promote immune tolerance in response to different signaling molecules, such as IL-27 and IL-10 [16,17]. In the case of cancer patients, circulating exosomes could possibly be generated in the tumor microenvironment also containing immunosuppressive molecules, constituting an effective mechanism of paracrine induction of tolerance to tumor cells and therefore tumor escape. In fact, recent studies have also demonstrated the immunosuppressive potential of extracellular vesicles in gliomas [18], and even in the case of AML, exosomes obtained at diagnosis from patients' serum have been demonstrated to decrease cytotoxic activity of NK cells isolated from normal individuals [19].

In our study, DCs pulsed with AML patients exosomes, despite of not having altered lymphocyte proliferation, led to a marked decrease in the production of INF-γ by these effector cells, and INF-γ expression levels were inversely related to the expression of CD86 in co-cultured DCs. In fact, there are descriptions of anomalous expression of CD86 in situations of dysfunctional immune response, such as inadequate response of CD86 expression in response to inflammation in systemic lupus erythematosus [20] and an increased risk of relapse after discontinuation of treatment in patients with CML and high counts of DCs expressing CD86 [21]. There is evidence that increased induction of CD86 in antigen-presenting cells may polarize the immune response to a Th2 profile, with lower Th1 response and consequently less effective cytotoxicity [22]. Therefore, we may speculate that in AML, exosome-induced suppression of cytotoxicity may, at least in part, be a result of dysregulation in costimulatory molecules in DCs such as CD86, leading to decreased lymphocyte activation with impaired production of INF-γ.

In reality, what we conclude is that vaccines using other sources of exosomes for pulsing (such as exosomes from cultured cells) may represent a feasible and effective way for maturing DCs into a cytotoxic phenotype, without the immunosuppressive effects observed in patient exosomes, and therefore deserve further studies for their use in immunotherapy. One limitation of our study was that nor the internal contents of patients' exosomes were investigated, nor which components would be directly involved in this immunosuppressive response. Therefore, new studies specifically addressing this issue should be conducted, and will enable the manipulation of these nanoparticles to optimize anti-tumor effect.

Conflicts of interest

The authors declare no conflicts of interest.

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