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Research Article

Phenotype of Exosomes Derived from Dendritic Cells Treated with Different Stimuli

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Keywords

• Exosomes; Dendritic cells; TNFα, LPS, IFNγ

Abstract

Dendritic cells (DCs) may secrete nanometricparticules named exosomes that are implicated in antigen presentation to T lymphocytes, by mean of the expression of MHC II, costimulatory and coinhibitory molecules on its surface, which may determine the type of immune response developed. Also, it has recently proposed the use of exosomes as a new strategy for the generation of cell free anti-cancer immunotherapies, so it is important to know the phenotype differences between exosomes of DCs treated with different molecules such as IFNγ, TNFα and LPS, in order to avoid the induction of immunological tolerance in immunotherapy. Hence, the aim of this study was to analyze and compare the exosomes phenotype secreted by DCs treated with different stimuli. For this, DCs were treated with 500U or 10000U of IFNγ, TNFα, and LPS to generate different phenotypes. Exosomes were coupled to α-CD11c microbeads to detect the DCs exosomes by flow cytometry. To compare the phenotype of the DCs and the exosomes, costimulatory and coinhibitory molecules were analyzed by flow cytometry. As results, immature DCs and the DCs treated with 500U IFNγ, exhibited a tolerogenic phenotype, while the exosomes produced by this DCs exhibited low levels of costimulatory and coinhibitory molecules. In contrast, DCs treated with 10,000U IFNγ, TNF-α and LPS showed an activator phenotype, and their exosomes had high levels of costimulatory and coinhibitory molecules. In conclusion, this study becomes important since disclosed not only the phenotype of DCs cultured with different treatments but also the phenotype of the exosomes produced by DCs

ABBREVIATIONS

DCs: Dendritic Cells; TNFα: Tumor Necrosis Factor Alpha; IFNγ: Interferon Gamma; LPS: Lipopolysaccharide; MIF: Mean Fluorescence Intensity

INTRODUCTION

The attempted to harness the immune system to mediate the rejection of tumors *in vivo* has led the development of cancer vaccines. Moreover, the cancer vaccines against cancer has received so much interest due to its safety and effectiveness [1]; in this point, there are different strategies of cancer vaccines that involved dendritic cells (DCs) cells adoptive therapy, antibodies (i.e. anti- CD40) or cytokine based therapy; whose objective is the induction of an effective anti-tumor immune response that may induce tumor regression or increased survival. Specifically, the immunotherapy based on DCs has been widely used in multiple murine cancer models and even in patients [1]. Furthermore, it has been observed that the use of tumor antigens promotes the development of not only an effective antitumor response but also a specific and dependent T lymphocyte. There are numerous antigens that are able to used in DCs based immunotherapy; however, it has been observed that one of the most successful is MAGE, which is a tumor antigen that has been used in DCs based immunotherapy, and it promotes the increased expression of IFNγ and the survival of mice with melanoma [2]. Nonetheless, it is necessary to improve the immunotherapy against cancer and study other alternatives that may be applied alone or in combination with DCs or even with other therapies. The free cell vaccines may be an option due to this kind of vaccines potentiate the antitumor immune responses induced by the administration of DCs treated with tumor antigens. An alternative of the free cell vaccines is the use of exosomes released by the DCs, which may ensure and maximizes the capture and the presentation of tumor antigens and the development of an effective antitumor response [3]; also, exosomes released by DCs not only contain tumor antigens but also may induce antitumor immunity *via* transfer of exosomal molecules to DCs and trigger potent CD8 T-cell dependent antitumor responses and induce antitumor immunity [4]; in addition, the exosomes may trigger the development of different immune responses, such as Th1 and Th2 depending of different factors, such as the microenvironment and the phenotype of the exosomes [5].

Exosomes are vesicles originated from the late endosomal compartment and secreted from the fusion of multivesicular bodies with the plasma membrane [6]. Exosomes have an approximate size of 40-120 nm, and unlike other vesicles such

as the apoptotic bodies which measure approximately 500-2000 nm, exosomes express an abundant variety of molecular markers, such as tetraspanins (CD63) [7]. Besides, one of the more striking attributes of DCs derived-exosomes surface membranes relates to their immunostimulatory potential due to the possession of molecules involved in antigen processing and presentation [8]. This set of proteins involved major histocompatibility complex class I and II (MHC-I and MHC-II) costimulatory (CD40, CD80, CD86) and coinhibitory molecules (CD273, CD274), and tetraspanin (CD63) [9]. So, all above suggest that phenotype of exosomes may influence the development of certain immune responses, very important to the successful of the immunotherapy against cancer.

At the present time it is possible to generate exosomes derived from DCs by different strategies, for instance DCs may be treated with molecules such as IDO, IL-10, LPS or even antigens, in order to obtain exosomes with pro or anti-inflammatory properties [10]. According to this, exosomes secreted by LPS-treated DCs exhibited superior priming abilities than exosomes obtained from untreated DCs [11] and exosomes produced by IL-10 treated bone marrow-DCs (BMDCs), showed immunosuppressive properties *in vivo* [12]. Alternatively, it has been found that exosomes from monocyte derived-DCs (MDDCs) did not show the same levels of expression in all molecules as DCs [10], so it is still uncertain the phenotype of the exosomes obtained from DCs treated with different molecules, especially if exosomes would be used in immunotherapy against cancer. All things considered and, in order to avoid the development of a deficient immune response by the use of exosomes with an inappropriate phenotype in immunotherapy, the aim of the investigation was to compare the phenotype of the exosomes obtained from DCs treated with different stimuli, so this information may be employed when exosomes are used in immunotherapy.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Ethics and Research Commission of the Faculty of Medicine (Comisiones de Investigación y de Ética, Oficio No. FMED/CI/SPLR/094/2014, dictamen 078/2014), National Autonomous of Mexico Univertity (UNAM). The present investigation was also performed in accordance with the Mexican Official Norm NOM 062-ZOO-1999.

Animals

Males C57BL/6 mice aged eight to twelve weeks old were purchased from Harlan, Laboratories, UNAM. Also, mice were bred in controlled light-dark and temperature conditions, and fed *ad libitum* at the Cell Biology and Tissue department animal facilities, Faculty of Medicine, UNAM.

Generation of bone marrow DC

Generation of BMDCs was performed as described by Inaba et al., 1992 with some modifications and Piñon-Zárate et al. 2014 [13,2]. First, mice were sacrificed by cervical dislocation, then, bone marrow from femurs and tibias were flushed. $3x10^6$ bone marrow cell precursors were seeded in $75cm^2$ cell culture flask with 10 ml of complete culture medium containing RPMI 1640 (Sigma, USA) supplemented with 10% fetal bovine serum (GIPCO™, USA), and antibiotic. In order to induce differentiation, cultures were added with 20% GM-CSF-containing supernatant from culture of X-63 cell line transfected with the murine GM-CSF gene, dose equivalent to 400 U/ml of GM-CSF. The X-63 cell line was kindly donated by Prof. Dr. Laila Gutiérrez-Kobeh (Experimental Medicine Department, Faculty of Medicine UNAM, Mexico). At day 4, GM-CSF-containing medium was added to the culture and on day 6, 10 ml of the culture medium were replaced with fresh GM-CSF-containing medium, and harvested on day 7. Immature 4 days-DCs were harvested on the fourth day culture after flushed.

DCs treatment

After differentiation, BMDCs were seeded in 6 well plaques at $5x10^6$ per well on RPMI medium. Afterwards, BMDCs were incubated for 24 hours with MAGE-AX peptide (LGITYDGM) (synthesized by Research Genetics, Invitrogen, Leiden, Netherlands with 90% pure). DCs treated with MAGE-AX were also incubated with 1μg/ml of TNF-α (Sigma, USA); or 1μg/ml of LPS (Sigma, USA); or 500U/ml of murine IFN-γ (PeproTech, Inc, 315-05-100UG) according the protocol used by Rojas and Krishnan, 2010 [14] or 10, 000U/ml of murine IFN-γ in order to obtain different types of DCs. As control groups, immature BMDCs were obtained by 4 and 7 days of precursor bone marrow cells cultures supplemented with GM-CSF.

Exosomes-bead coupling

Exosomes secreted into the supernatant of BMDCs cultures were filtered with a 0.2 μm filter (TPP®) in order to eliminate the debris and microvesicles. The clarified supernatant was concentrated by ultrafiltration through a 10,000 MWCO hollow fiber membrane. The supernatant was concentrated to 100 μl and 10 μl of mouse anti-CD11c magnetic beads (MACS® MiltenyiBiotec) was incubated within the samples for at least 24 h. After this time the magnetic beads couple with exosomes derived from dendritic cells (positive o CD11c) were retained and wash with PBS. The rest of the supernatant was discarded. In order to discard other kind of vesicules, the presence of CD63 on the vesicules couple to the magnetic beads determined by flow cytometry.

Antibodies

The following fluoresce in isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) or biotin-conjugated mouse monoclonal antibodies were used for flow cytometry: anti-CD11c APC, anti-CD80 Biotin, anti-CD86 PE, anti-CD40 Biotin, anti-CD273 PE, anti-CD274 PE, anti-MHCI Biotin and anti-MHCII FITC (all from BioLegend, San Diego, CA). The biotin antibodies were conjugated with streptavidin-PE (BioLegend).

Flow cytometry analysis

BMDCs and exosomes-coated magnetic beads were labeled with fluorescent monoclonal antibodies according to the manufacturer's instructions. The analysis was performed on a FACS calibur flow cytometer (Becton Dickinson, San José, CA) using CELLQUEST software (Becton Dickinson) and data were analyzed with the software FlowJo 8.7

Statistical analysis

Percentages data were expressed as mean ± standard deviation of mean and intensity mean fluorescence are expressed as geometric mean ± standard deviation of geometric mean. Statistical evaluation of data was performed by ANOVA test and Tukey test using GraphPad Prism 5. $P<0.05$ values were considered statistically significant.

RESULTS

BMDCs were treated with different stimuli, an inhibitory (IFN 500U) and three usual maturation treatments (10,000U, TNF and LPS). The Figure 1 shows the BMDCs phenotype characteristics by its SSC, FSC and the expression of CD11c and MHCII; here is possible to observe that 94.5% of the cells analyzed resulting in BMDCs cells. Afterwards, exosomes produced by BMDCs were isolated. Figure 2 shows that the exosomes obtained were rich in CD63, which is commonly found in this kind of vesicles and not in apoptotic bodies or microvesicles. Subsequently levels of MHCII molecules costimulatory (CD40, CD80 and CD86) (Figures 3-5) and coinhibitory molecules (CD273 and CD274) (Figures 6,7) in BMDCs and exosomes secreted, were analyzed; also the percentage of BMDCs and positive microbeads to the molecules mentioned above was analyzed. According to the expression of MHC II (Figure 8B), it was observed that only BMDCs increased its expression in TNFα-treated group, while the percentage of MHCII positive BMDCs also increased in the group treated with IFNγ 10,000U, TNFα and LPS group. Regarding MHCII expression in exosomes (Figure 8A), just the group obtained from BMDCs treated with 500U IFNγ showed increased expression of this molecule, nonetheless the percentage of positive microbeads decreased compared to control group. While, the group of exosomes obtained from BMDCs treated with IFNγ 10,000U showed an increase in the percentage of positive microbeads to MHCII molecules compared with exosomes obtained from the BMDCs group cultured for 7 days (Figure 8A).

In case of costimulatory molecules, it was noticed that the BMDCs treated with the stimulatory treatments10,000U IFNγ, TNFα or LPS, showed increase in the number of positive cells and in the expression of CD40, CD80 and CD86 (Figures 3-5), when compared to the control group, especially the BMDCs treated with IFNγ. While the dose of 500U IFNγ induced decrease in both cases, the percentage of CD86+ (Figure 4A) and CD80+

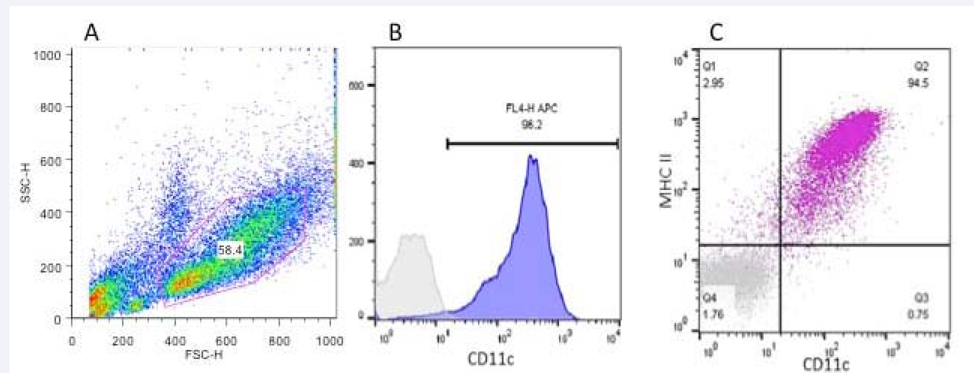

Figure 1 DCs detection. (A) SSC and FSC dot blot of bone marrow cells precursors after treatment with GM-CSF. (B) CD11c expression of cells obtained after seven days of culture with GM-CSF. (C) Percentage of CD11c and MHCII+ cells obtained from cultures of bone marrow dendritic cells precursors treated with GM-CSF.

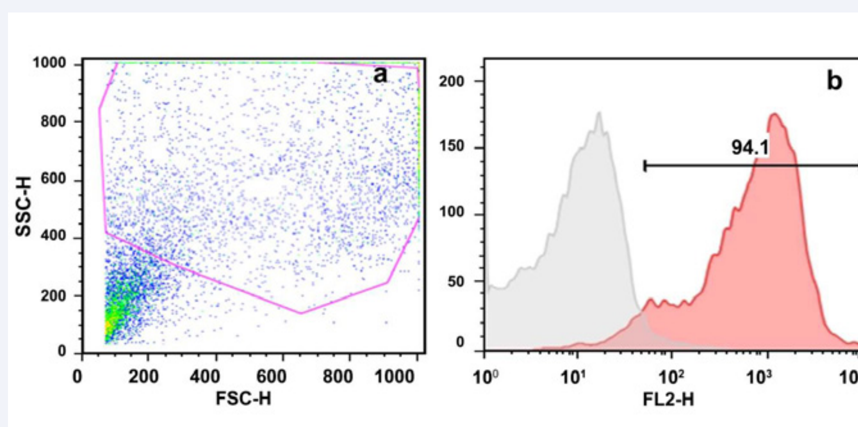

Figure 2 Exosome detection. (A) SSC and FSC dot blot of α-CD11c coated microbeads, used for exosome recognizing and detected by flow cytometry. The α-CD11c coated microbeads were selected according to their SSC and FSC characteristics, the smallest particles were not considered. (B) Percentage of microbeads coated by exosomes CD63+..

(Figure 3A) BMDCs, in contrast, this treatment increased the expression of CD40 in BMDCs (Figure 5B) and the percentage of CD40+ BMDCs (Figure 5A). Regarding the percentages of microbeads coupled to positive exosomes to CD80 (Figure 3A), CD86 (Figure 4A) and CD40 (Figure 5A), they were similar to the percentages of positive BMDCs to the same markers, therefore, groups of exosomes obtained from DCs treated with 10,000U of IFNγ, TNFα or LPS showed an increase in the percentage of positive microbeads to CD80, CD86 and CD40. In addition, in the expression of costimulatory molecules, CD40 expression increased in exosomes obtained from BMDCs treated with 10,000U IFNγ, TNFα and LPS; while, increased expression of CD80 was observed in exosomes from DCs treated with TNFα. CD86 expression increased only in exosomes obtained from DCs treated with IFNγ 10,000U (Figure 4B). It is essential to note that the expression of all the costimulatory molecules were higher than the expression of the same molecules in DCs.

On the other hand, we studied the effect of 500 U of IFNγ in coinhibitory molecules of BMDCs, it was observed that the percentage of CD273 positive cells decreased (Figure 6A) in comparison with BMDCs differentiated for 7 days. In contrast, groups of BMDCs treated with 10,000U IFNγ, TNFα and LPS increased the percentage of positive BMDCs to CD273 (Figure 6A). Analyzing the expression of CD273 in exosomes, those obtained from BMDCs treated with 500U IFNγ, TNFα or LPS reflected increased expression of the molecule mention above (Figure 6B).

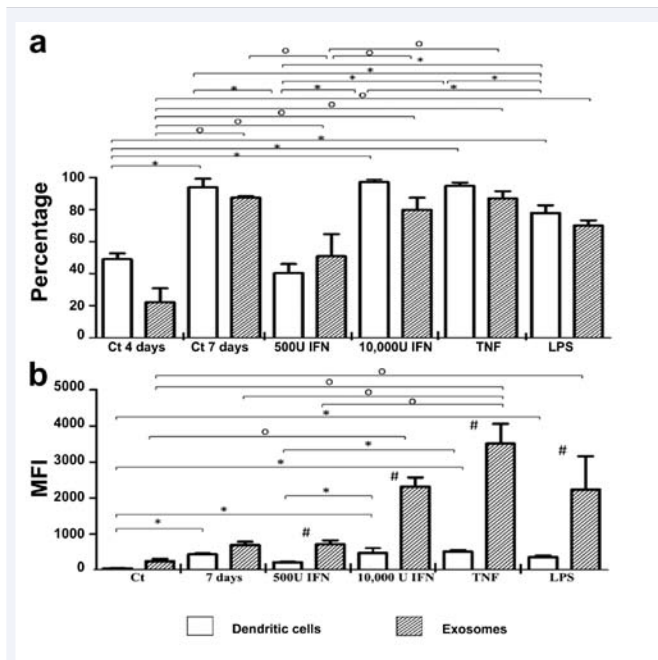

Figure 3 CD80 analysis in DCs and exosomes. (A) Percentage of CD80+ DCs and exosomes. (B) Expression of CD80 in DCs and exosomes, CD80 expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

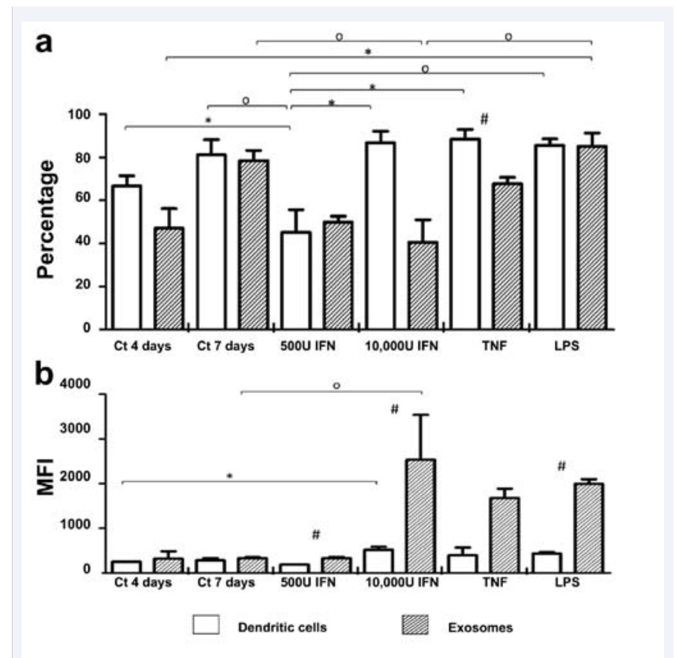

Figure 4 CD86 analysis in DCs and exosomes. (A) Percentage of CD86+ DCs and exosomes. (B) Expression of CD86 in DCs and exosomes, CD86 expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

Regarding to CD274, BMDCs treated with 500U IFNγ and TNFα 10,000U showed increased expression of this molecule (Figure 7B). With respect to the percentage of microbeads positive to CD274, all groups showed increased percentage in comparison with exosomes from control groups. In case of CD274 expression in exosomes (Figure 7A), those obtained from BMDCs treated with IFNγ showed increased expression of CD274 in comparison with control group, while exosomes from BMDCs treated with LPS or TNFα showed decreased expression of CD274.

DISCUSSION

In the present investigation, exosomes from DCs with inhibitory or stimulatory doses of IFNγ, TNFα or LPS were obtained, in order to examine the phenotype of the exosomes that may be used in immunotherapy. Here, the expression of MHCII, costimulatory and coinhibitory molecules in exosomes were analyzed and compared with the expression of the molecules in DCs, so, it is essential to know the possible immunological response that the exosomes may induce when applied as a therapy.

First, the phenotype of the DCs differentiated for 4 and 7 days and then treated with different stimuli was analyzed (Figure 1). When the bone marrow cells that were cultured in the presence of GM-CSF were observed, a phenotype characteristic of dendritic cells was identified on day 4 of culture. However, only 61.4% of these cells were positive for MHC II. This suggests that on day

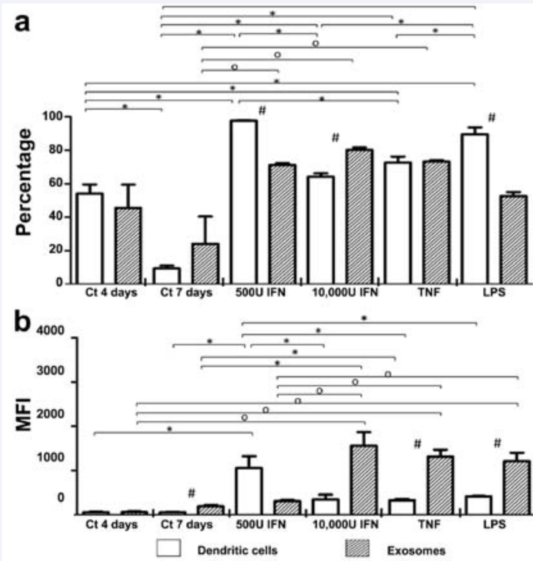

Figure 5 CD40 analysis in DCs and exosomes. (A) Percentage of CD40+ DCs and exosomes. (B) Expression of CD40 in DCs and exosomes, CD40 expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

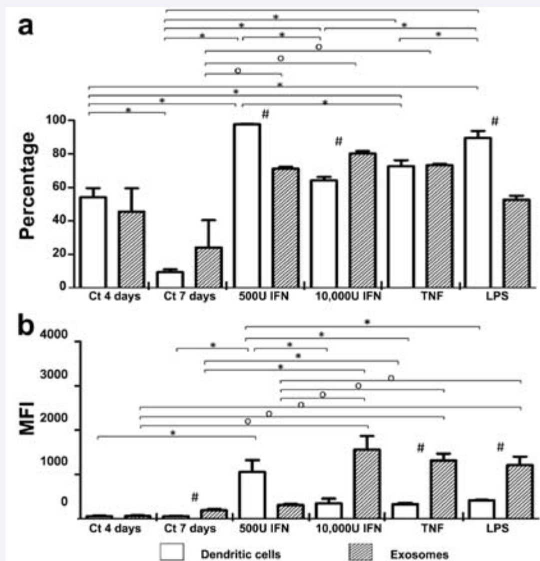

Figure 7 CD274 analysis in DCs and exosomes. (A) Percentage of CD274+ DCs and exosomes. (B) Expression of CD274 in DCs and exosomes, CD274 expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

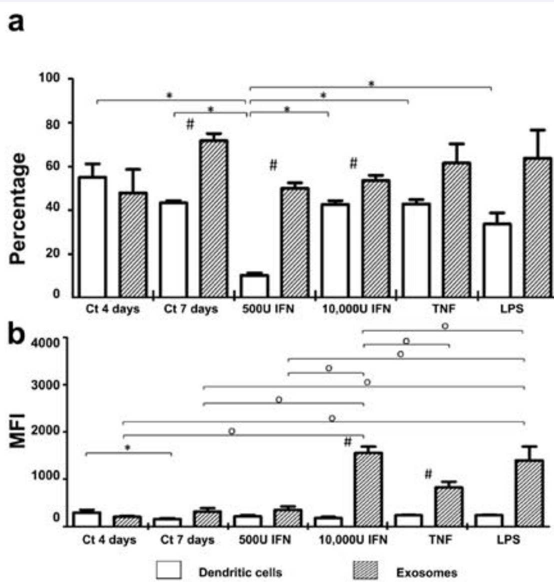

Figure 6 CD273 analysis in DCs and exosomes. (A) Percentage of CD273+ DCs and exosomes. (B) Expression of CD273 in DCs and exosomes, CD273 expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

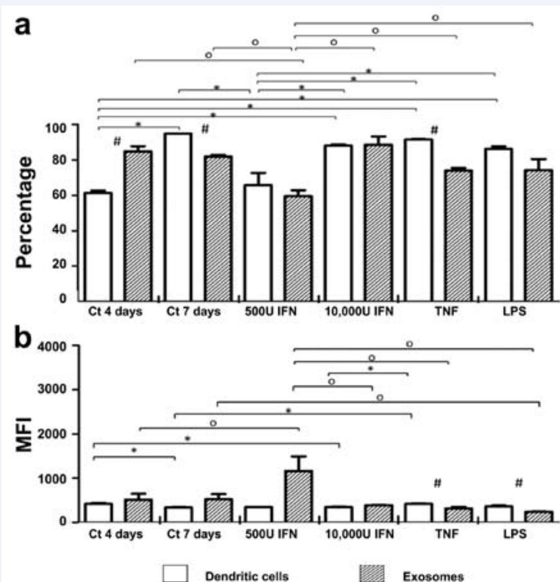

Figure 8 MHCII analysis in DCs and exosomes. (A) Percentage of MHCII+ DCs and exosomes. (B) Expression of MHCII in DCs and exosomes, MHCII expression is expressed as Mean fluorescence intensity (MIF). In both panels, DCs were treated with inhibitory or stimulatory doses of IFNγ, TNFα or LPS, then the exosomes obtained were analyzed. (*) Significant differences between groups of DCs ($p<0.05$). (o) Significant differences between groups of microbeads coupled to exosomes. (#) Significant differences between groups of DCs and microbeads coupled to exosomes produced by DCs.

4 of differentiation the BMDCs showed an immature phenotype. Likewise, BMDCs differentiated for 7 days showed a mature or semi-mature phenotype. Thus, it is important to note that the BMDCs treated with 500U of IFNγ showed a similar phenotype to the BMDCs differentiated for 4 days and that the other treatments generated cells with a mature cell phenotype, characterized for the higher expression of costimulatory molecules such as CD40 (Figures 3-5,8). Even though all above has already proven, it was important to know the phenotype of the DCs treated with various stimuli, in order to be compared with the phenotype of the exosomes produced by them.

After DCs treatment, exosomes were isolated by αCD11c coated microbeads, subsequently phenotype of the exosomes were measured. It is noteworthy to note that unlike other investigations where western blot analysis was used for evaluating the phenotype of total secreted exosomes, the procedure used in this study allowed us to evaluate selectively phenotype CD11c-labeled vesicles, i.e. vesicles secreted by DCs specifically. Nonetheless, it is significant to consider that is not entirely known if the vesicles are homogeneous in terms of content. For instance, it is known, that exosomes of smaller size (30-50 nm) are enriched with CD63 tetraspanin, while larger vesicles (100-200 nm) are enriched by MHC II and only 20% of these exosomes containing both proteins [15], by comparison, in this investigation, it was found that exosomes obtained were highly positive for CD63, although the expression of MHC II was similar to control groups (immature BMDCs). The procedure used in this work may not discriminate between different vesicle sizes, so it was not possible to know the vesicle size with major content of molecules. This indicates that although this method is effective to determine the phenotype of exosomes, it is important to find other procedure to isolate vesicles of different sizes, in order to get more homogeneous exosomes with specific phenotypes to be used in immunotherapies and to induce more accurate responses.

On the other hand, although it was not possible to analyze the size of the vesicles secreted by the BMDCs, it is very possible that the vesicles obtained were exosomes for two reasons. First, by the double expression of molecules CD11c and CD63 evaluated by flow cytometry and second, because the ultracentrifugation of the supernatant of BMDCs was carried out in tubes provided with a membrane with pores of 200 nm. The exosomes measure from 40 to 120 nm while larges vesicles as apoptotic bodies measure from 500 to 2,000 nm, so that only vesicles measuring less than 200 nm could be obtained.

According to the exosomes phenotype, there were analyzed the expression of the MHCII, costimulatory and coinhibitory molecules on exosomes derived from BMDCs treated with different stimuli (Figure 8). Regarding MHCII, there were a few differences in the expression of MHCII between the groups of BMDCs, although the most notable difference was presented in terms of percentage of positive BMDCs to this molecule, having a clear resemblance between the cells treated with 500U IFNγ and the control group, indicating that BMDCs treated with 500 IFNγ showed an immature phenotype. It is noteworthy that MHCII did not showed significant differences between the exosomes obtained from the BMDCs treated with different stimuli, with the exception of 500U of IFNγ (Figure 8). This result appears to contradict the results reported by Clayton et al., 2001 [16] and Zitvogel in 1998 [3], who reported the increased expression of MHCII in exosomes from immature BMDCs; however, it is important to remember that in this study were only tested exosomes CD11c+, and an exosome may or may not express both proteins simultaneously, so it is possible that many of the exosomes expressing MHCII may not express CD11c and therefore had not been recognized by microbeads α-CD11c. Interestingly, it has also been observed that deletion of the ALIX protein induces an increase of 30-400% in the fluorescence intensity of MHC II surface without affecting the level of CD63 on immature BMDCs [15]. This confirms the hypothesis that the regulation of the MHCII expression in exosomes is not directly related to CD11c protein, so its presence in the positive CD11c exosomes may or may not vary in the exosomes.

Regarding the costimulatory molecules located in exosomes on groups treated with cytokines (IFNγ and TNFα) was found that exosomes showed similar phenotype to the mature DCs phenotype (Figures 3,6,8), besides it is important to note that exosomes that were released contained higher concentration of costimulatory and coinhibitory receptors (Figures 3-6,8), and according to Johansson et al., 2007 [10], the increased MFI for a molecule may reflect an enrichment of that molecule on each exosome. This fact is noted since it is possible that the exosomes obtained from BMDCs treated with IFNγ or TNFα would be successful during the antigen presentation when used in immunotherapy and it is even possible that the exosomes released during DCs-based immunotherapy would be importantly involved in the development of an antitumor immune response, since a high expression of costimulatory molecules is directly implicated in a successful T lymphocyte response.

In addition, it was observed that the exosomes secreted by tolerogenic DCs (500U IFNγ) showed low levels of costimulatory molecules (CD80 and CD86) and coinhibitory molecules (CD273 and CD274). This behavior coincides with results reported by Ruffner et al., 2009 who observed low levels of costimulatory and inhibitory molecules in exosomes secreted by DCs treated with IL-10, which had tolerogenic phenotype [17]. According to the idea mentioned above, this pattern of expression of molecules indicates that the regulation of protein traffic in DCs is able to change depending on the treatment used, reflecting physiological changes in exosomes and DCs *in vivo* [17]. All above is significant since it reinforces the importance of knowing the phenotype of DCs and exosomes secreted, especially if exosomes would be used in clinical immunotherapy trials. Hence, this work contributes to the understanding of how the exosomes phenotype may influence a certain type of target cell or the development of a specific immune response, i.e., it has been observed that exosomes are able to interact with tumor cells and others DCs, allowing the incorporation of costimulatory, inhibitory molecules and even MHCII, primary molecules for a successful antigen presentation to T lymphocytes [18].

Moreover, in this work, it was found that in all groups costimulatory molecules were expressed in exosomes; nonetheless, in groups of exosomes obtained from BMDCs treated with 10,000U of IFNγ, TNFα and LPS, expression levels of costimulatory molecules remained higher than in control groups

(Figures 3-5,8). In addition, when the expression of inhibitory proteins was compared with those of costimulatory proteins, we observed that the expression of costimulatory molecules was always higher, so we may suggest that these exosomes may potentiate a specific immune response in immunotherapy. Importantly, BMDCs treated with LPS, IFNγ or TNFα did not generate exosomes with exactly the same phenotype, indicating that the protein regulation in exosomes was also influenced by the treatment that were exposed the BMDCs. Although exosomes showed high levels of costimulatory molecules, depending the treatment used were the levels observed. Thus, treatment with TNFα induced the increased expression of costimulatory molecules in exosomes, phenotype characterized by the expression of high levels of CD80, CD86 and CD40, and a high percentage of CD86 positive magnetic beads, as well as a low percentage of CD273 or CD274 positive magnetic beads both coupled to the exosomes produced by BMDCs. Whereas exosomes from BMDCs treated with 10,000U IFNγ or LPS although expressed high levels of costimulatory molecules, CD86 in case of LPS and CD40 in IFNγ treated DCs, they also showed higher levels of coinhibitory molecules than exosomes from DCs treated with TNFα, i.e. CD274 in exosomes from BMDCs treated with IFN and CD273 in exosomes from DCs treated with LPS. All above is relevant, due to multiple immunotherapy trials employs LPS or IFNγ as maturation inducers [19,20], while others used TNFα [2]; however, in past clinical trials the exosome phenotype were not studied. For this reason, it would be inappropriate the use of BMDCs treated with indistinct stimuli in immunotherapy, due to the secretion of exosomes with different phenotype in comparison to the DCs, resulting in the induction of undesirable responses, so it is necessary to know the exact phenotype of the exosomes in order to predict the immune response that will be developed. In particular, the knowledge generated in this work helps to promote the use of specific treatments to BMDCs in order to obtain exosomes from a particular phenotype. In addition, in the present investigation, the exosomes obtained from TNFα treated BMDCs showed the best phenotype required for an effective antigen presentation, which is relevant in the development of an effective antitumor response, however it is still required to study the analysis of the interaction between DCs, T lymphocytes, tumor cells and exosomes obtained from the various groups of DCs, in order to verify the exosomes immunotherapeutic potential.

CONCLUSION

In conclusion, exosomes derived from mature BMDCs showed high levels of costimulatory molecules, even more than those expressed in BMDCs, especially TNFα-treated group. Also, depending on the process employed to induce maturation were the phenotype found in exosomes. Therefore, because BMDCs are commonly used in immunotherapy, this study becomes important since disclosed not only the phenotype of BMDCs cultured with different treatments but also the phenotype of the exosomes produced by these cells.

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