Immunobiology 224 (2019) 697–705

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Dexamethasone turns tumor antigen-presenting cells into tolerogenic dendritic cells with T cell inhibitory functions

Cristián Falcón-Beas[a,b,1], Andrés Tittarelli[a,b,1], Gabriela Mora-Bau[a,b], Fabián Tempio[a,b], Claudio Pérez[a,c], Daniel Hevia[a,b], Carolina Behrens[a,b], Iván Flores[a,b], Felipe Falcón-Beas[a,b], Paola Garrido[a,b], Gabriel Ascui[a,b], Cristián Pereda[a,b], Fermín E. González[b,d], Flavio Salazar-Onfray[a,b], Mercedes N. López[a,b,c,*]

[a] Disciplinary Program of Immunology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile
[b] Millennium Institute on Immunology and Immunotherapy, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile
[c] Cell Therapy Laboratory, Blood Bank Service, University of Chile Clinical Hospital, 8380453 Santiago, Chile
[d] Laboratory of Experimental Immunology & Cancer, Department of Conservative Dentistry, Faculty of Dentistry, University of Chile, 8380492 Santiago, Chile

ARTICLE INFO

Keywords:
Dendritic cells
Dexamethasone
Regulatory T cells
Tolerance
Melanoma

ABSTRACT

Background: Dendritic cells (DCs) are usually immunogenic, but they are also capable of inducing tolerance under anti-inflammatory conditions. Immunotherapy based on autologous DCs loaded with an allogeneic melanoma cell lysate (TRIMEL/DCs) induces immunological responses and increases melanoma patient survival. Glucocorticoids can suppress DC maturation and function, leading to a DC-mediated inhibition of T cell responses.

Methods: The effect of dexamethasone, a glucocorticoid extensively used in cancer therapies, on TRIMEL/DCs phenotype and immunogenicity was examined.

Results: Dexamethasone induced a semi-mature phenotype on TRIMEL/DC with low maturation surface marker expressions, decreased pro-inflammatory cytokine induction (IL-1β and IL-12) and increased release of regulatory cytokines (IL-10 and TGF-β). Dexamethasone-treated TRIMEL/DCs inhibited allogeneic $CD4^+$ T cell proliferation and cytokine release (IFNγ, TNF-α and IL-17). Co-culturing melanoma-specific memory tumor-infiltrating lymphocytes with dexamethasone-treated TRIMEL/DC inhibited proliferation and effector T cell activities, including cytokine secretion and anti-melanoma cytotoxicity.

Conclusions: These findings suggest that dexamethasone repressed melanoma cell lysate-mediated DC maturation, generating a potent tolerogenic-like DC phenotype that inhibited melanoma-specific effector T cell activities. These results suggest that dexamethasone-induced immunosuppression may interfere with the clinical efficacy of DC-based melanoma vaccines, and must be taken into account for optimal design of cellular therapy against cancer.

1. Introduction

Dendritic cells (DCs) play a pivotal role controlling immune responses and are essential mediators of innate and adaptive immunity and tolerance. While DCs are exceptionally immunogenic under inflammatory conditions, these cells are also important for self-tolerance induction and maintenance in homeostasis (Boltjes and van Wijk, 2014; Osorio et al., 2015). As professional antigen-presenting cells (APCs), DCs have the unique ability to control and orchestrate complex networks of cellular interactions that regulates the immune response, including the induction and maintenance of anti-tumor immunity (Palucka and Banchereau, 2012; Mellman, 2013). Indeed, previous studies have demonstrated the effectiveness of DC-based immunotherapy, improving long-term survival in patients with late-stage melanoma (Palucka et al., 2006; López et al., 2009; Aguilera et al., 2011; Aarntzen et al., 2012). Particularly, autologous monocyte-derived DCs stimulated with heat-shock-conditioned allogeneic melanoma cell lysate (TRIMEL) where capable to induce a powerful anti-

* Corresponding author at: Millennium Institute on Immunology and Immunotherapy, Faculty of Medicine, University of Chile. Avenida Independencia 1027, Santiago 8380453, Chile.
E-mail address: melopez@me.com (M.N. López).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.imbio.2019.05.011
Received 28 January 2019; Received in revised form 5 May 2019; Accepted 30 May 2019
Available online 06 June 2019

melanoma T cell response (Aguilera et al., 2011). TRIMEL contains pool of melanoma-associated antigens, as it is generated from a heat-shock-conditioned allogeneic melanoma cell lysate.

Glucocorticoids (GCs) are extensively used as immunosuppressive and anti-inflammatory agents in different clinical settings, with Cortisone been the first GC administered to patients with rheumatoid arthritis in 1948 (Hench et al., 1950). Currently, GCs are relevant in the treatment of asthma, dermatitis, autoimmune diseases, and inflammation (Coutinho and Chapman, 2011). Furthermore, GCs have also been used for decades in cancer therapies to reduce undesired side effects of chemotherapy (Lussier et al., 2004; Lin and Wang, 2016), protecting healthy tissues from off-target cytotoxicity, and, presumably, reducing tissue susceptibility against invasive malignant growth (Rutz, 2002; Herr and Pfitzenmaier, 2006). Moreover, GCs are included in protocols against hematological malignancies to directly deplete lymphoid cells (Schmidt et al., 2004). Furthermore, GCs are extensively used in combination with other treatments against solid malignant tumors to reduce acute toxicity of chemotherapy (Rutz, 2002).

Despite the apparent therapeutic benefits of GC, their use has also been associated with increased tumor xenograft growth in different types of cancer, such as breast (Skor et al., 2013), ovarian (Hou et al., 2013), lung (Herr et al., 2003), and prostate cancers (Zhang et al., 2007). Moreover, accumulating evidence supports that GCs increase the risk of developing skin cancer and lymphomas (Jensen et al., 2009), in addition to possibly interfering with the therapeutic efficacies of chemo- and radiotherapy (Wong et al., 2015; Surace et al., 2015).

The immunosuppressive effects of GCs are primarily evidenced as an interference in key inflammatory signalling pathways and transcriptional regulators, such as NF-κB and AP-1 (Auphan et al., 1995; Heck et al., 1994), leading to increased levels of regulatory cytokines, the inhibition of inflammatory cytokines and cellular immunity, and the induction of inhibitory DCs (Coutinho and Chapman, 2011). Inhibitory DCs are not only associated with the suppression of anti-tumor DCs activity, but also with reprogramming these cells into inhibitory DCs that can drive T cells to a state of hypo-responsiveness and induce regulatory T cell differentiation (Osorio et al., 2015; Rea et al., 2000). The induction of inhibitory DCs often require additional immune inhibitory signals in a pro-inflammatory environment. As inhibitory signals are more represented in tissues chronically exposed to microbes or tumor-associated tissues leading to an increased infiltration of APCs with tolerogenic phenotypes and functions (Grainger et al., 2010; Belkaid and Oldenhove, 2008), in the majority of cases, immune down-regulation is a consequence of the inflammation process.

Heat-shocked tumor cell lysates derived from melanoma, prostate, colorectal, and renal cancer (Aguilera et al., 2011; Brusa et al., 2009; Chen et al., 2009) can release danger signals that are detected by DCs by their toll-like receptors (TLRs) family and other pattern recognition receptors, inducing activation and increasing immunogenicity (Aguilera et al., 2011; Chen et al., 2009; Tittarelli et al., 2012). GCs such as dexamethasone are used simultaneously with cellular immunotherapy when treating several types of cancer, thus, studying the effect of this immunosuppressive agent on the cellular biology of DC vaccines becomes relevant to understand possible negative effect in clinical outcomes (Lebson et al., 2011). This study evaluated the effect of dexamethasone, an extensively used GC in cancer therapies, on the phenotype and the *in vitro* immunogenicity of clinically effective autologous DC-based vaccine against melanoma.

2. Materials and methods

2.1. DC generation

Adherent monocytes isolated from peripheral blood mononuclear cells of healthy donors were obtained from the Blood Bank Service of the University of Chile Clinical Hospital and were cultured in serum-free AIM-V medium (Invitrogen, USA) with recombinant human IL-4 (rhIL-4: 500 U/mL; United States Biological, USA) and recombinant human GM-CSF (rhGM-CSF: 800 U/mL; Schering Plough, USA) for 3 days. The resulting cells were immature DCs (iDCs). Mature DCs (TRIMEL/DCs) were obtained by adding conditioned melanoma cell lysate (TRIMEL 100 μg/mL) at day 2, as previously reported (Aguilera et al., 2011). Dexamethasone (1μM), as a suppressor stimulus, and TRIMEL (100 μg/mL), as an inflammatory stimulus, were added to the medium at days 1 and 2 to generate DEX/TRIMEL/DCs. All adherent cells were recovered at day 3.

2.2. Cell lines

The CD40L-transfected mouse embryo fibroblast NIH/3T3 cell line was kindly provided by Dr. Eduardo Villablanca (Karolinska Institute, Stockholm, Sweden). Melanoma cell lines Mel1, Mel2, and Mel3 were used to prepare the conditioned tumor lysate, TRIMEL, in addition of isolated metastasized melanoma lymph nodes, as previously reported (Aguilera et al., 2011).

2.3. $CD4^+$ T cell isolation

Buffy coats from healthy donors were obtained from the Blood Bank Service of the Universidad de Chile Hospital. $CD4^+$ T cells were isolated utilizing the RosetteSep system (STEMCELL Technologies, Canada) in a Ficoll-Hypaque density gradient according to the manufacturer's recommendations.

2.4. Tumour infiltrating cells (TILs)

TILs obtained from a fresh pulmonary metastasis of a stage IV melanoma patient ($HLA\text{-}A2^+$) were cultured as previously described (Mendoza-Naranjo et al., 2007).

2.5. Flow cytometry

The DCs and T cells were characterized by flow cytometry using the following fluorochrome-conjugated monoclonal antibodies (Ab) for cell surface staining: anti-CD4, CD11c, HLA-ABC, HLA-DR, CD14, CD25, CD40, CD80, CD83, CD86, CD252, CD273, CD274, and CCR7 (eBioscience Inc., USA). The following Abs were used for intracellular T cell staining: anti-IFN-γ, IL-17, TNF-α, IL-2, IL-4, IL-10, TGF-β, and Foxp3 (eBioscience Inc., USA). Corresponding fluorochrome IgG control isotypes were used as well (eBioscience Inc., USA). All samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, USA) and using the FlowJo v8.7 software.

2.6. DC-TIL co-culture assays

DCs and TIL cells were co-cultured in a 1:2 ratio for three days. At day 2, the cells were stimulated with phorbol myristate acetate (50 ng/mL), ionomycin (1 μg/mL), and Brefeldin A (1 μg/mL) (eBioscience Inc., USA) for 5 h. Cells were stained with the corresponding fluorochrome-conjugated Abs and evaluated as described above.

2.7. DC-NIH/3T3 CD40L culture

DCs were cultured with NIH/3T3 CD40 L cells in a 1:1 ratio for 24 h in a serum-free AIM-V medium (Invitrogen, USA). For IL-12 measurement by ELISA, DCs and NIH/3T3 CD40 L (7.5×10^4 cells each) were co-cultured in 150 μL of AIM-V medium. For FlowCytomix (eBioscience Inc., USA) determinations, DCs and NIH/3T3 CD40 L (2×10^5 cells each) were co-cultured in 400 μL of AIM-V medium. The resulting supernatants were collected and cryopreserved for later cytokine quantification.

2.8. Cytokine release determination

For IL-12 measurements, the commercial Human IL-12p70 ELISA Ready-SET-Go! kit (eBioscience Inc., USA) was used according to the manufacturer's recommendations and was detected *via* the SUNRISE ELISA reader (Tecan Trading AG, Switzerland). For IL-1β, IL-6, IL-10, and TGF-β determinations, FlowCytomix technology (eBioscience Inc., USA) was used according to the manufacturer's recommendations. The samples were analyzed by the FACSCalibur flow cytometer (BD Biosciences, USA) and using the FlowCytomix PRO v2.3 software.

2.9. Proliferation assay

Isolated $CD4^+$ T cells or TILs pre-labelled with carboxy-fluorescein succinimidyl ester dye (CFSE, 1μM; Invitrogen, USA) were co-cultured with DCs in a 1:2 ratio for four (TILs) or five (isolated $CD4^+$ T cells) days in serum-free AIM-V medium (Invitrogen). After co-cultures, cells were stained with anti-CD4 and anti-CD8 monoclonal Abs (eBioscience Inc., USA), and samples were analyzed by flow cytometry. T-cell proliferation was determined by evaluating the dilution of the fluorescent CFSE dye. The results were represented as percentage of proliferating cells relative to maximum of an unspecific stimulation with OKT-3 (2.5 ng/mL) and rhIL-2 (150 IU/mL).

2.10. T helper differentiation assay

DCs and isolated $CD4^+$ T cells were co-cultured in a 1:10 ratio for 7 days. At day 7, $CD4^+$ T cells cultures were stimulated with phorbol myristate acetate (50 ng/mL), ionomycin (1 μg/mL), and Brefeldin A (1 μg/mL) (eBioscience, USA) for 5 h in the serum-free AIM-V medium (Invitrogen, USA). Cells were stained with the corresponding fluorochrome-conjugated Abs, and the different T helper profiles were determined by flow cytometry.

2.11. Endocytosis assay

To evaluate pinocytosis, receptor-mediated endocytosis, and phagocytosis capabilities of the DEX/TRIMEL/DCs and iDCs, the uptake of dextran-FITC (Sigma-Aldrich), ovalbumin-Alexa Fluor 488 (Invitrogen, USA), and CFSE-stained melanoma cells (Mel1) were measured. DCs were generated as mentioned above and incubated with 0.5 mg/mL of dextran-FITC, 10 μg/mL of ovalbumin-Alexa Fluor 488, or 1×10^5 melanoma cells in a 1:1 ratio. DCs were incubated at 4 °C to measure nonspecific binding and phagocytosis and at 37 °C to measure specific uptakes. After different time points (0, 1, and 2 h for endocytosis and receptor-mediated endocytosis; and 0, 6, and 12 h for the phagocytosis assay), DCs were recovered washed in cold PBS, labelled with PE-conjugated anti-CD11c Ab, and analyzed by flow cytometry. The endocytosis indexes were determined by dividing the percentage of double positive cells at 37 °C by the percentage of double positive cells at 4 °C.

2.12. Cytotoxicity assay

The cytotoxic activities of TILs previously stimulated with the different DCs for 72 h were measured by conventional 4 h chromium-51 release assays (PerkinElmer, USA) using triplicate cultures in round-bottomed 96-well plates. Effector:Target cell ratios were as indicated on 5000 target cells/well. Specific lysis was calculated according to the following formula: percent specific lysis = ([experimental release - spontaneous release]/[maximum release - spontaneous release]) x 100. Radioactivity was measured using a TopCount NXT Gamma Counter (PerkinElmer, USA).

2.13. Statistical analysis

Paired and unpaired Student's *t*-tests were used to analyze differences between each DC-generation protocol. Statistical analyses were performed using the Graph Pad Prism 5 software. Statistical differences were considered significant for values of $p < 0.05$.

3. Results

3.1. Dexamethasone pre-treatment turns TRIMEL-mediated DC maturation into a semi-mature tolerogenic-like phenotype

Previous research demonstrated that melanoma cell lysate, TRIMEL, induces differentiation and maturation of human monocytes to mature DC-like phenotype (Aguilera et al., 2011). To study the effect of dexamethasone on TRIMEL-mediated maturation, DC-like cells were generated from monocytes in the presence or absence of dexamethasone, as previously described (Aguilera et al., 2011). The expression of human monocyte marker (CD14) and DC markers (CD11c) were evaluated on monocytes, iDCs, mature DCs (TRIMEL/DCs), and dexamethasone pretreated TRIMEL/DCs (DEX/TRIMEL/DCs). After 72 h, all DC types showed lower levels of CD14 expression than untreated monocytes, but similar levels of CD11c were expressed (Fig. 1A). Dexamethasone treatment slightly suppressed the downregulation of CD14, increasing the population of $CD14^+$ cells (58.8% DEX/TRIMEL/DC *vs*. 7.7% TRIMEL/DC). Compared with iDCs, TRIMEL/DCs presented a mature phenotype with increased surface expression of MHC-I and MHC-II, CD80, CD83, CD86, CD40, OX40-L, CCR7, PD-L1, and PD-L2 (Fig. 1B). DEX/TRIMEL/DCs also showed an increased expression of some of these surface markers, however, had lower surface expression of CD80, CD83, CD86, and CD40, than TRIMEL/DCs (Fig. 1B).

The effect of dexamethasone on the TRIMEL/DC phenotype occurred when added before TRIMEL, affecting the surface expression of CD83 and CD86 (Data not shown). In contrast, after TRIMEL-mediated DC maturation, dexamethasone did not affect surface molecule levels, indicating that TRIMEL/DCs already have a committed phenotype, in agreement with Aguilera et al. (2011). Interesting dexamethasone maintains its suppressive effect when both stimuli are given together (Fig. Supplementary 1).

Following CD40 ligation, DCs can secrete both pro- and anti-inflammatory cytokines, depending on other received stimuli (Boonstra et al., 2006). To evaluate if dexamethasone treatment affects the profile of cytokines secreted by TRIMEL/DCs, we cultured this cells and dexamethasone treated TRIMEL/DCs for 24 h with a CD40L-expressing mouse embryo fibroblast line (NIH/3T3 CD40 L). Supernatants of the cell cultures were collected and the presences of pro- (IL-1β, IL-6, and IL-12) and anti-inflammatory cytokines (IL-10 and TGF-β) were measured by ELISA or FlowCytomix. Secretion of both IL-1β and IL-12 was significantly lower for DEX/TRIMEL/DCs than TRIMEL/DCs (Fig. 1C). DEX/TRIMEL/DCs also secreted significantly higher amounts of IL-10 and TGF-β than TRIMEL/DCs (Fig. 1C).

Accumulated evidence indicates that expression of surface molecules may fail to anticipate T cell activation capacities of APCs. On the other hand, co-stimulatory-to-co-inhibitory ratio has been correlated with the ability of APCs to stimulate allogeneic T cells (Abe et al., 2005). When comparing CD86/PD-L1 and CD86/PD-L2, DEX/TRIMEL/DCs had significantly lower co-stimulatory-to-co-inhibitory ratio than TRIMEL/DCs (Fig. 2). Altogether, these results indicated that dexamethasone pre-treatment restricts the TRIMEL-mediated DC maturation process, leading to the generation of DCs with a semi-mature and tolerogenic-like phenotype.

3.2. Dexamethasone pretreatment does not affect the endocytic capability of DCs

Endocytosis is essential for antigen capturing and presentation of

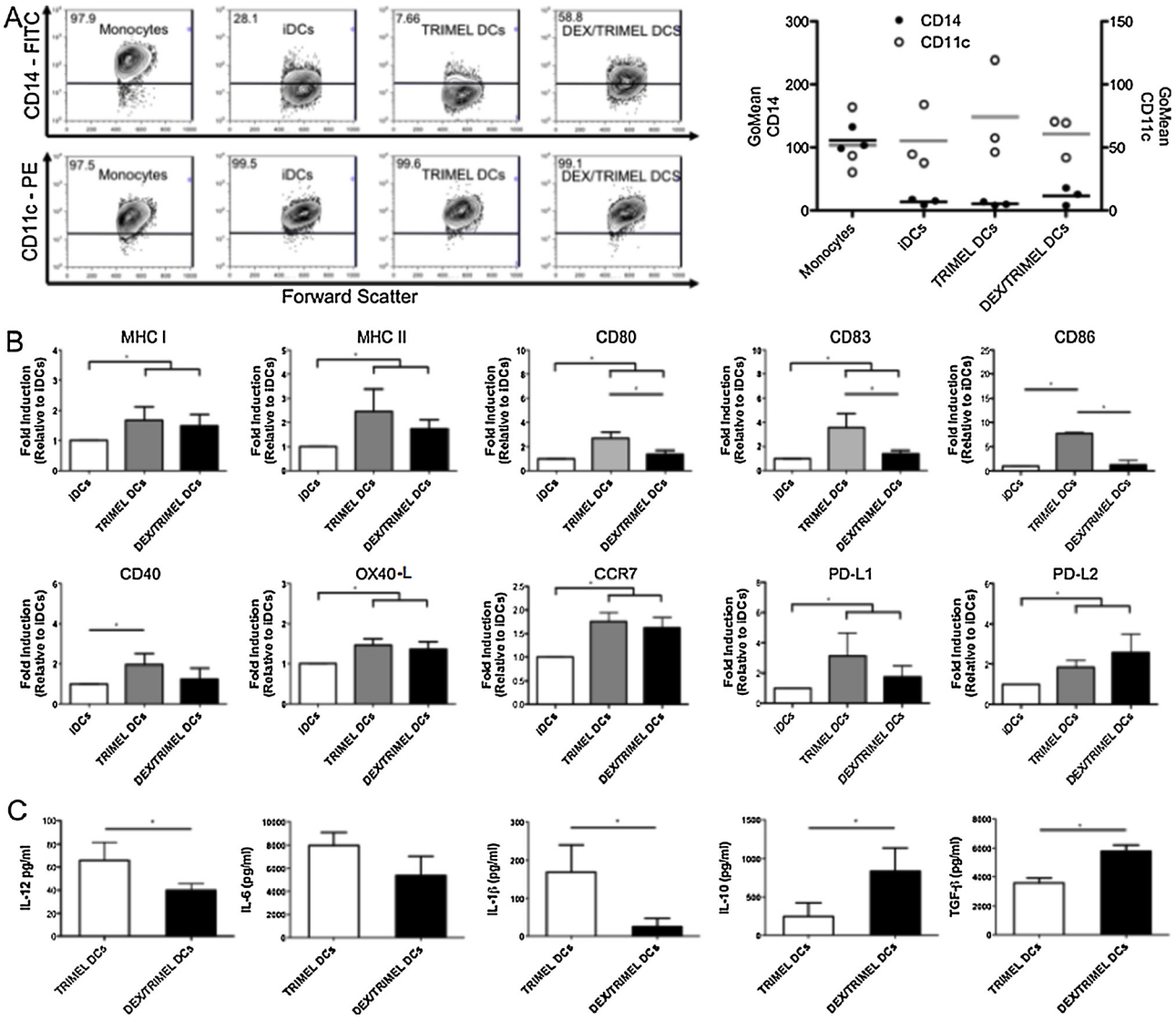

Fig. 1. Dexamethasone pre-treatment inhibited TRIMEL-mediated DC maturation producing a semi-mature DC phenotype.
Untreated monocytes (Mo), iDCs, TRIMEL-treatedDCs (TRIMEL/DC), or dexamethasone pre-treated TRIMEL/DCs (DEX/TRIMEL/DCs) were analyzed by flow cytometry for surface markers expression and cytokine production. **(A, left panels)** Representative density plots showing the expression of CD14 and CD11c. The percentages of positive cells are depicted for each density plot. (**A, right panels**) Expression levels (geometric means, GeoMean) for CD14 and CD11c. The mean values for each marker are shown (CD14: dark lines, CD11c: light lines) (n = 3 donor samples). **(B)** Expression of surface maturation markers. The results are expressed as fold induction relative to iDCs. Each bar represents mean ± SD of three independent experiments. **(C)** Cytokine release in TRIMEL/DCs and DEX/TRIMEL/DCs. DCs were cultured for 24 h with CD40L-expressing fibroblasts and supernatants collected to determine cytokine secretion. The results represent the mean ± standard deviation (SD) of at least three independent experiments. (* $p < 0.05$).

DCs, however, endocytosis decreases after DC maturation. To evaluate if early exposure to dexamethasone could affect this property, different endocytic assays were performed. Pinocytosis, measured by receptor-mediated dextran uptake measured by OVA-Alexa Fluor 488 uptake, and phagocytosis, measured by CSFE-stained tumor cells uptake, were tested in iDCs and dexamethasone-treated DCs (DEX/DCs). Dexamethasone did not affect the overall endocytic ability of DCs (Fig. 3), indicating that the phenotypic differences between TRIMEL/DCs and DEX/TRIMEL/DCs were not due to variances in endocytic potentials.

3.3. Dexamethasone pre-treated TRIMEL/DCs inhibit proliferation of allogeneic effector CD4+ T cells and prevent TH1 and TH17 cytokine release

Increased T_H1 and T_H17 populations have been associated with anti-tumor immune responses after a TRIMEL/DC-vaccination in melanoma patients (Durán-Aniotz et al., 2013). To obtain an optimal antitumor immune response, DCs need to prime T cells to induce proliferation and differentiation into effector T_H1/T_H17 cells. The effect of dexamethasone-treated DCs on allogeneic T cell proliferation and cytokine release was evaluated by CFSE dilution assay and intracellular cytokine staining in mixed lymphocyte reactions of allogeneic isolated $CD4^+$ T cells, respectively. Dexamethasone pre-treatment strongly decreased the T cell priming activity of TRIMEL/DCs, even at levels lower than iDCs (Fig.4A).

Accordingly, DEX/TRIMEL/DCs co-cultured $CD4^+$ T cells produced less canonical T_H1 cytokines IFN-γ and TNF-α, and T_H17 cytokine IL-17, as compared with TRIMEL/DCs (Fig. 4B). In turn, no significant differences were observed regarding IL-10 production (data not shown). Taken together, dexamethasone pre-treatment gave tolerogenic functionality to DCs, been able to both reduce allogeneic $CD4^+$ T cell proliferation and differentiation to anti-tumor T helper profiles, even in the presence of a strong, tumor-related inflammatory stimulus, such as

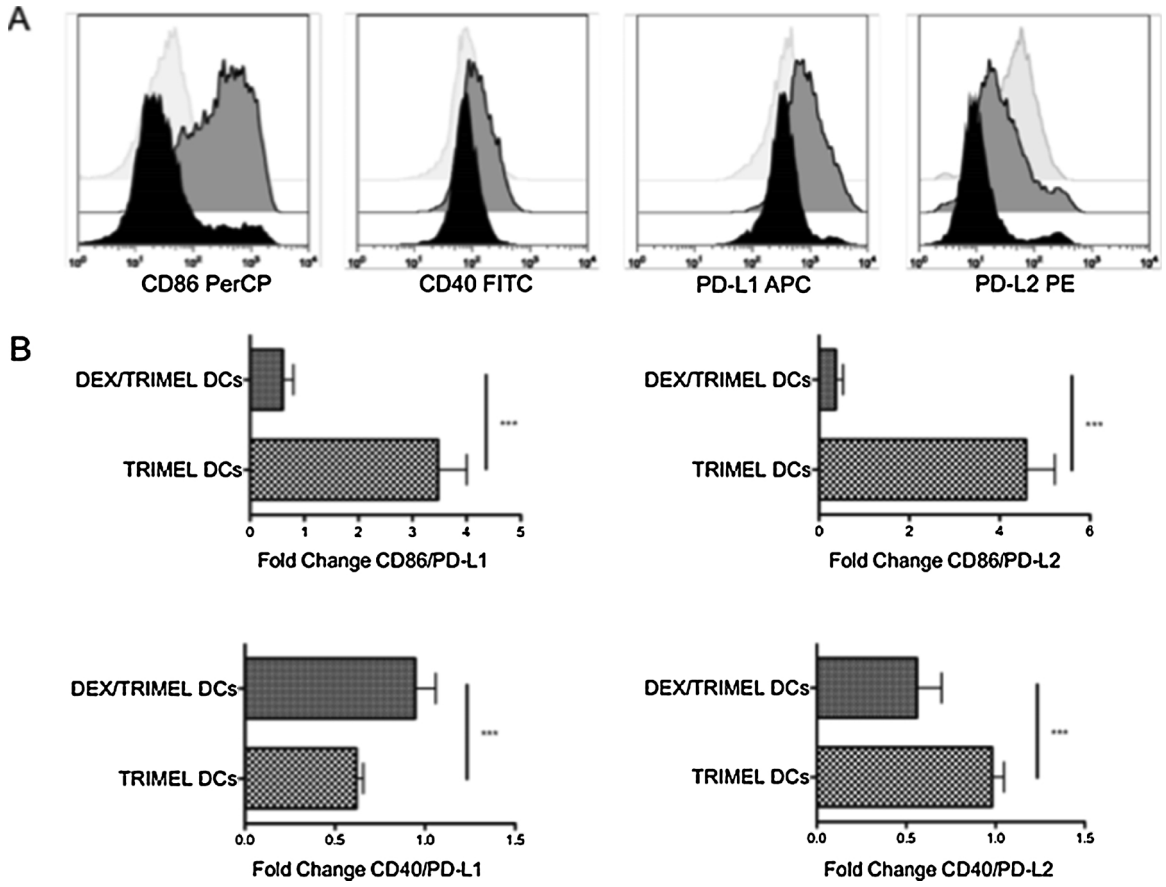

Fig. 2. Dexamethasone affects the co-stimulatory/co-inhibitory molecule expression ratio in TRIMEL/DCs.
The iDCs, TRIMEL/DCs, and DEX/TRIMEL/DCs were analyzed by flow cytometry for surface expression of markers. **(A)** Representative histograms of the expression of CD86, PD-L1, and PD-L2 (iDCs: black, TRIMEL/DCs: dark grey, DEX/TRIMEL/DCs: light grey). **(B)** Fold change of co-stimulatory/co-inhibitory molecules ratio relative to iDCs. The data represents the mean of the geometric mean fluorescence ± SD of three independent experiments. (*** $p < 0.01$).

TRIMEL.

3.4. Dexamethasone pre-treated TRIMEL/DCs inhibit the proliferation of reactivated melanoma-specific memory T cells

DCs also play a role in memory T cell reactivation (Wakim et al., 2008). A major objective of cancer immunotherapy is to reactivate TILs, mainly memory and tumor-specific T cells, to destroy cancer cells (Higham et al., 2010). To evaluate the impact of dexamethasone treatment on the activity of DCs in reactivating melanoma-specific memory T cells, TILs (HLA-A2$^+$) isolated from melanoma biopsy were co-cultured with TRIMEL/DCs or DEX/TRIMEL/DCs, and proliferation of CD4$^+$ and CD8$^+$ T cell compartments were evaluated. Notably, the isolated TILs were primarily effector memory (CD45RO$^+$CD62L$^{low/-}$CCR7$^-$) CD8$^+$ T cells and, to a lesser extent, effector memory CD4$^+$ T cells (Data not shown). DEX/TRIMEL/DCs strongly decreased proliferation of CD4$^+$ TILs as compared with TRIMEL/DCs, while no significant differences were observed in CD8$^+$ TILs (Fig. 5).

3.5. Dexamethasone pre-treated TRIMEL/DCs inhibit reactivated melanoma-specific memory T cell effector activities

To assessed if dexamethasone pre-treatment could affect cytokine production of melanoma-specific memory T cells, T cells isolated from a melanoma biopsy were co-cultured either with TRIMEL/DCs or DEX/TRIMEL/DCs. DEX/TRIMEL/DCs reduced production of IL-2, IFN-γ, and TNF-α by CD4$^+$ TILs compared to TRIMEL/DCs co-cultured TILs, with no significant differences in IL-4 or TGF-β expressions were observed (Fig. 6A). Additionally, CD4$^+$ TILs co-cultured with DEX/TRIMEL/DCs expressed significantly higher levels of IL-10 than those co-cultured with TRIMEL/DCs (Fig. 6A). On the other hand, dexamethasone pre-treated DCs decreased the production of IL-2 and IFN-γ on CD8$^+$ TILs compared to TILs co-cultured with TRIMEL/DCs, while no differences were observed for CD8$^+$ TIL expressions of TNF-α, IL-4, IL-10, or TGF-β (Fig. 6B).

The main goal of DC-based cancer therapies is to induce tumor cell death *via* cytotoxic T lymphocytes activation. To evaluate the effect of dexamethasone pre-treatment of TRIMEL/DCs on anti-melanoma TILs cytotoxic activity, HLA-A2$^+$ melanoma cell line (Mel1) or K562 cells, as a negative control, were co-cultured with TILs and TRILMEL/DCs or DEX/TRIMEL/DCs. TRIMEL/DCs induced cytotoxic activity of the TILs, however, TILs co-cultured with DEX/TRIMEL/DCs displayed similar cytotoxic activity as unstimulated TILs (Fig. 6C), suggesting that the dexamethasone pre-treatment of TRIMEL/DCs completely inhibited their ability to increase the cytotoxic killing potential of anti-melanoma memory T cells.

Overall, these results suggest that dexamethasone reverts TRIMEL-mediated DC pro-inflammatory functions, producing a potent tolerogenic-like DC capable of inhibiting anti-tumor effector T cells activities.

4. Discussion

Activation of the immune system is a fundamental matter not only in immunotherapeutic approaches but also in all anti-cancer treatments strategies. Indeed, there is accumulating evidence supporting that immune activation is an integral part of the clinical efficacy of radiotherapy and chemotherapy protocols (Surace et al., 2015; Apetoh et al., 2007; Formenti and Demaria, 2012; Sharma et al., 2013; Pfirschke

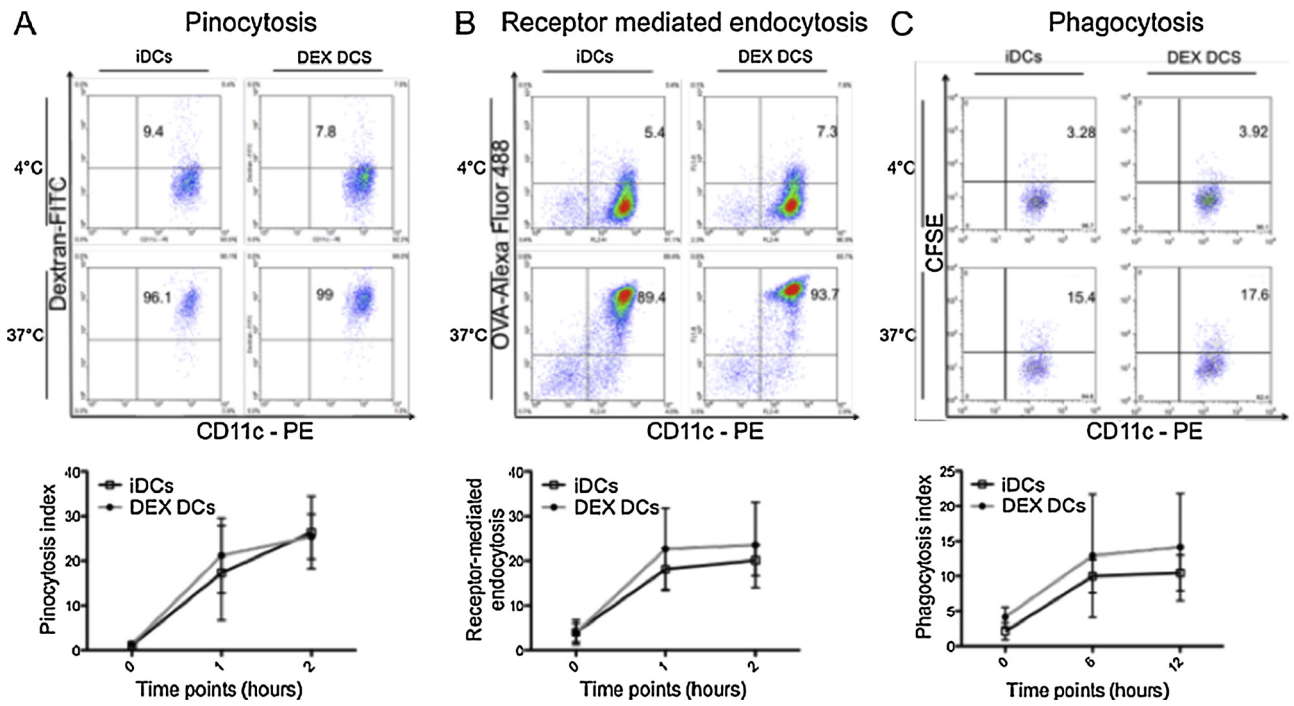

Fig. 3. Dexamethasone pretreatment does not affect pinocytosis, receptor-mediated endocytosis, or phagocytosis capability of DCs.
DCs were treated with dexamethasone (DEX/DCs) or only medium (iDCs). At day 2, FITC-conjugated dextran was added to the media to measure pinocytosis **(A)**, AlexaFluor 488-conjugated Ovalbumin (OVA) for receptor-mediated endocytosis detection **(B)** or CFSE-stained melanoma cells for phagocytosis quantification **(C)**. The activities were evaluated by flow cytometry at different time points. Representative density plots of $CD11c^+$ cells of dye uptake, 1 h after treatment at 4 °C or 37 °C (upper panels). The endocytosis indexes were determined by dividing the percentage of double positive cells at 37 °C by the percentage of double positive cells at 4 °C (lower panels). The results are showed as mean ± SD at different time points for three independent experiments.

et al., 2016). Therefore, it is notable that despite immunosuppressive activities, GCs, and particularly dexamethasone, have been extensively used in cancer therapies. The aim of the present study was to examine the effect of dexamethasone on the differentiation and functionality of DC-like APCs treated with a GC therapeutic used in patients with advanced melanoma (López et al., 2009; Aguilera et al., 2011; Tittarelli et al., 2012).

Autologous DCs loaded with tumor-associated antigens are among the most promising immunological cancer therapy available. These therapies are able to naturally stimulate the anti-tumor immune response while also generating immunological memory (Palucka and Banchereau, 2012). While clinical studies have successfully induced tumor immunity, so far, only minimal clinical impact has been reported (Constantino et al., 2016). Optimal tumor antigen delivery is one of the

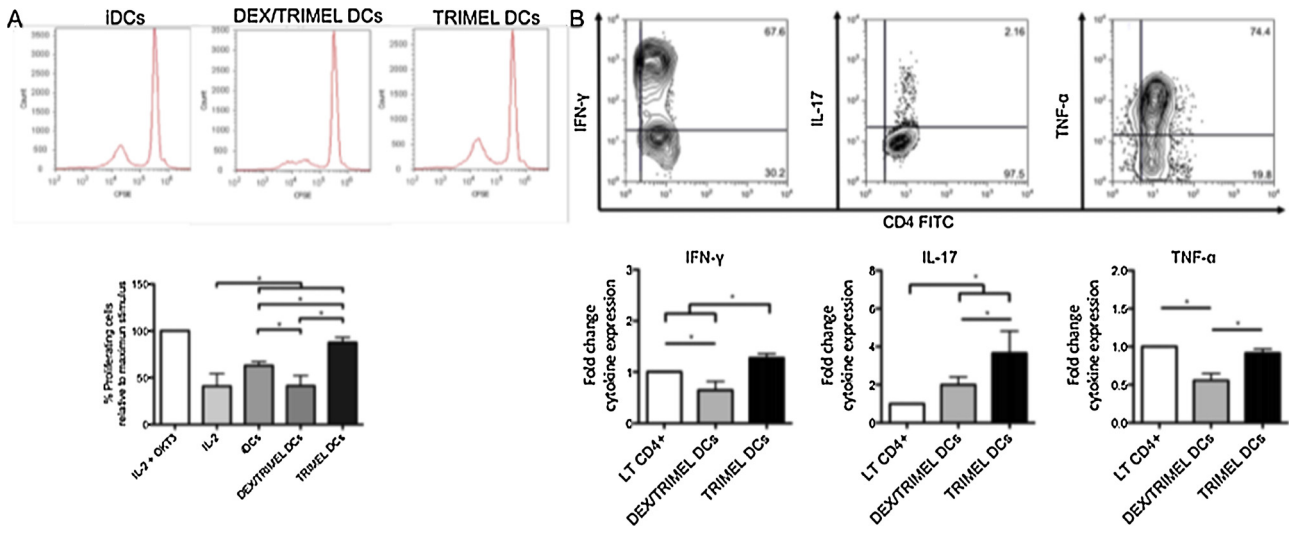

Fig. 4. Dexamethasone pretreated TRIMEL/DCs inhibit proliferation and cytokine production of allogeneic $CD4^+$ T cells.
(A) iDCs, TRIMEL/DCs, and DEX/TRIMEL/DCs, were cultured with CFSE-loaded isolated allogeneic $CD4^+$ T cells for 5 days in a 1:2 (DC:T cell) ratio. T cells stimulated with IL-2 and anti-CD3 antibodies (OKT3) were used as positive controls. The data represents the mean ± SD of the percentage of proliferating T cells (relative to the maximum, *i.e.* IL-2 + OKT3-stimulated T cells) for three independent experiments. **(B)** Isolated allogeneic $CD4^+$ T cells were cultured in the absence or presence of the different DCs for 7 days in a 1:2 (DC:T cell) ratio, and treated for 5 h with phorbol myristate acetate and ionomycin in the presence of Brefeldin A. The production of IFN-γ, TNF-α, and IL-17 in $CD4^+$ T cells was determined by flow cytometry. The results show the relative cytokine expression means ± SD for three independent experiments and the fold change relative to the T cells cultured without DCs. (* $p < 0.05$).

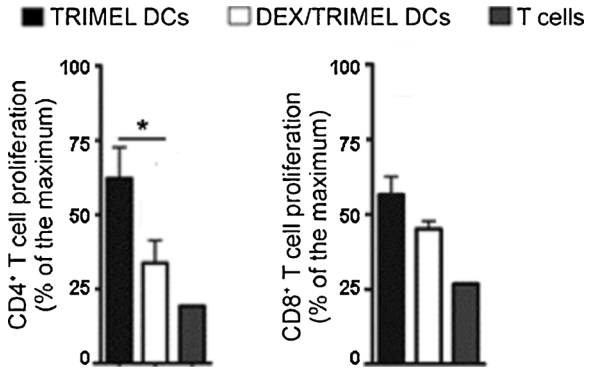

Fig. 5. Dexamethasone pre-treatment inhibits the proliferation of DEX/TRIMEL/DC-reactivated $CD4^+$ TILs.
$CD4^+$ (left panel) and $CD8^+$ (right panel) TILs were labelled with CFSE and cultured in the absence or presence of the different DCs. As positive control, TILs were stimulated with IL-2 and anti-CD3 (OKT3). Lymphocyte proliferation was analyzed after 4 days. The data represent the average ± SD of three independent experiments and are shown as a percentage of the maximum proliferation (IL-2 + OKT3 stimulus). (** $p < 0.01$; * $p < 0.05$).

most important factors for the success of cancer vaccines. Accordingly, DCs loaded with the autologous tumor lysates or allogeneic tumor lysates can induce potent and long-lasting immune responses *in vivo* (Neller et al., 2008). Unfortunately, these approaches are limited to a small population of patients with resectable tumors, leaving this kind of therapy out of range from patients with unresectable metastases, micrometastases, and high recurrence risks.

To overcome this limitation, our laboratory has spent the last ten years developing a therapeutic, DC-based immunization approach to improve long-term survival in patients with advanced melanoma (López et al., 2009; Aguilera et al., 2011). Specifically, this approach derives a lysate from heat-conditioned, allogeneic melanoma cells, termed TRIMEL, and has been used as a tumor-associated antigen source, thus providing a unique strategy for obtaining efficient tumor APCs with a mature DC-like phenotype (Aguilera et al., 2011; González et al., 2014). Worth highlighting, the produced TRIMEL contains some damage-associated molecular patterns, including the endogenous TLR4 ligand HMGB1, which mediate optimal APC maturation and Ag cross-presentation (Aguilera et al., 2011).

In vitro dexamethasone pre-treatment inhibited the expression of differentiation and maturation markers in monocyte-derived DCs generated with TRIMEL. DEX/TRIMEL/DCs had higher $CD14^+$ cells percentages and lower surface levels of the co-stimulatory molecules CD80 and CD86 when compared to TRIMEL/DCs, suggesting that dexamethasone treatment restricts the TRIMEL-induced differentiation and maturation of DCs (Fig. 1). This observation is in concordance with

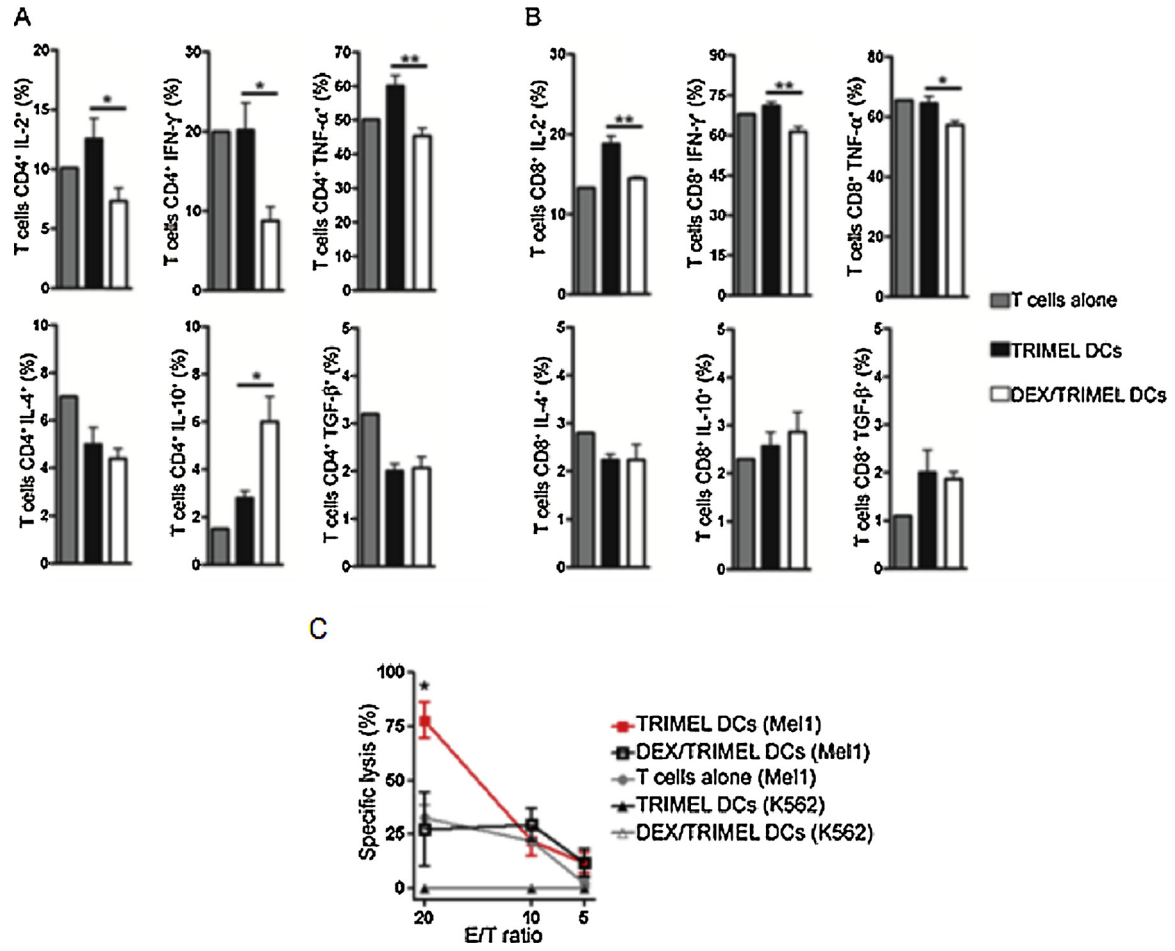

Fig. 6. Dexamethasone pre-treatment impacts the effector activities of DEX/TRIMEL/DC-reactivated melanoma-specific memory T cells.
(A, B) TILs were cultured in the absence or presence of the different DCs for 3 days in a 1:2 (DC:T cell) ratio, and treated for 5 h with phorbol myristate acetate and ionomycin in the presence of Brefeldin A. The percentages of $CD4^+$ **(A)** or $CD8^+$ **(B)** TILs positive for IL-2, IFN-γ, TNF-α, IL-4, IL-10, and TGF-β were determined by flow cytometry. The data represent the average ± SD of three independent experiments. **(C)** TILs were cultured in the absence or presence of the different DCs for 3 days in a 1:2 (DC:T cell) ratio. Cytotoxicity was assessed by chromium-51 release assays, targeting HLA-$A2^+$ melanoma cell line (Mel1) or K562 cells. The results are plotted as mean ± SD for the percentage of specific lysis for different effector/target (E/T) ratios and are representative of three independent experiments. (** $p < 0.01$; * $p < 0.05$).

previous reports on human monocyte-derived DCs differentiated/maturated with cytokines (Hu et al., 2013) or cytokines and lipopolysaccharides (Spallanzani et al., 2015). Mechanistically, Lebson et al. (2011), as dexamethasone treatment showed to up-regulates the *glucocorticoid-induced leucine zipper* gene in murine DCs, and that this gene was involved in the dexamethasone-mediated down-regulation of CD86 and DC vaccines efficacy. The role of the *glucocorticoid-induced leucine zipper* gene on the human DC vaccine phenotype and functionality remains as an open question.

In experimental settings, once DCs are activated with TRIMEL, these cells acquire a committed mature phenotype (Data not shown) (Aguilera et al., 2011) that is unaffected by subsequent dexamethasone exposure. Therefore, these TRIMEL/DC vaccines could be irresponsive to GCs in cancer patients. However, injected DC vaccines may present antigens directly to T cells or transfer the antigens to endogenous DCs (Mendoza-Naranjo et al., 2007; Petersen et al., 2011; Yewdall et al., 2010), which are fundamental in mounting a protective anti-tumor immune response. Accordingly, it is tempting to speculate that GC treatments could affect the phenotype and functional status of endogenous DCs, thereby repressing anti-tumor immunity and affecting the clinical efficacy of TRIMEL/DCs. Given the known immunosuppressive effects of GCs, the phase III IMPACT study for prostate cancer DC-therapy sipuleucel-T required a minimum 28-day washout period of all GCs before treatment (Kantoff et al., 2010). Therefore, the influences of timing and GC-containing sequences on treatment regimens relative to autologous DC immunotherapies remain a clinically relevant question (Dorff and Crawford, 2013).

During immunological synapsis, DC antigen presentation to T cells requires the combined action of signal 1 (MHC-cognate antigen), signal 2 (co-stimulatory/inhibitory molecules), and signal 3 (cytokines). In this context, our observations that DEX/TRIMEL/DCs had similar surface levels of MHC I and MHC II as TRIMEL/DCs (Fig. 1) and that dexamethasone did not affect the endocytic activity of the DEX/DCs (Fig. 3), suggest that both DEX/TRIMEL/DCs and TRIMEL/DCs had similar antigen-presenting capacities. Therefore, the differential outcomes of T cells co-cultured with different DCs (Fig. 4–6) could be due to the higher levels of the regulatory cytokines IL-10 and TGF-β and to the lower levels of the pro-inflammatory cytokines as IL-12 and IL-1β. These results could also be influenced by the expressional balance between inhibitory (PD-L1, PD-L2) and co-stimulatory (CD86) molecules (signal 2) on DEX/TRIMEL/DCs and TRIMEL/DCs.

Overall, our findings suggest that dexamethasone represses melanoma cell lysate-mediated DC maturation, generating a potent tolerogenic-like DC phenotype that is capable of inhibiting melanoma-specific effector T cell activities. Consequently, these results indicate that dexamethasone-induced immunosuppression may interfere with the clinical efficacy of DC vaccines against melanoma or other cancers. Finally, the results exposed here promote that future clinical trials using DC vaccines for cancer treatment must take into account the influence of dexamethasone and other GCs on their therapeutic outcome.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was funded by grants from the Millennium Science Initiative of the Ministry of Economy, Development and Tourism (P09/016-F); the Chilean National Fund for Scientific and Technological Development (FONDECYT 1130324 M.L.; and 1130320 F.S-O. and funding for the Promotion of Scientific and Technological Development (FONDEF D11I1036 F.S.-O. and ML).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.05.011.

References

Aarntzen, E.H., Bol, K., Schreibelt, G., Jacobs, J.F., Lesterhuis, W.J., Van Rossum, M.M., Adema, G.J., Figdor, C.G., Punt, C.J., De Vries, I.J., 2012. Skin-test infiltrating lymphocytes early predict clinical outcome of dendritic cell-based vaccination in metastatic melanoma. Cancer Res. 72, 6102–6110.
Abe, M., Wang, Z., de Creus, A., Thomson, A.W., 2005. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. Am. J. Transplant. 5, 1808–1819.
Aguilera, R., Saffie, C., Tittarelli, A., González, F.E., Ramírez, M., Reyes, D., et al., 2011. Heat-shock induction of tumour-derived danger signals mediates rapid monocyte differentiation into clinically effective dendritic cells. Clin. Cancer Res. 17, 2474–2483.
Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., et al., 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat. Med. 13, 1050–1059.
Auphan, N., DiDonato, J.A., Rosette, C., Helmberg, A., Karin, M., 1995. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 270, 286–290.
Belkaid, Y., Oldenhove, G., 2008. Tuning microenvironments: induction of regulatory T cells by dendritic cells. Immunity 29, 362–371.
Boltjes, A., van Wijk, F., 2014. Human dendritic cell functional specialization in steady state and inflammation. Front. Immunol. 5, 131.
Boonstra, A., Rajsbaum, R., Holman, M., Marques, R., Asselin-Paturel, C., Pereira, J.P., et al., 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J. Immunol. 177, 7551–7558.
Brusa, D., Migliore, E., Garetto, S., Simone, M., Matera, L., 2009. Immunogenicity of 56 degrees C and UVC-treated prostate cancer is associated with release of HSP70 and HMGB1 from necrotic cells. Prostate 69, 1343–1352.
Chen, T., Guo, J., Han, C., Yang, M., Cao, X., 2009. Heat shock protein 70, released from heat-stressed tumour cells, initiates antitumor immunity by inducing tumour cell chemokine production and activating dendritic cells via TLR4 pathway. J. Immunol. 182, 1449–1459.
Constantino, J., Gomes, C., Falcão, A., Cruz, M.T., Neves, B.M., 2016. Antitumor dendritic cell-based vaccines: lessons from 20 years of clinical trials and future perspectives. Transl. Res. 168, 74–95.
Coutinho, A.E., Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol. Cell. Endocrinol. 335, 2–13.
Dorff, T.B., Crawford, E.D., 2013. Management and challenges of corticosteroid therapy in men with metastatic castrate-resistant prostate cancer. Ann. Oncol. 24, 31–38.
Durán-Aniotz, C., Segal, G., Salazar, L., Pereda, C., Falcón, C., Tempio, F., et al., 2013. The immunological response and post-treatment survival of DC-vaccinated melanoma patients are associated with increased Th1/Th17 and reduced Th3 cytokine responses. Cancer Immunol. Immunother. 62, 761–772.
Formenti, S.C., Demaria, S., 2012. Radiation therapy to convert the tumour into an *in situ* vaccine. Int. J. Radiat. Oncol. Biol. Phys. 84, 879–880.
González, F.E., Gleisner, A., Falcón-Beas, F., Osorio, F., López, M.N., Salazar-Onfray, F., 2014. Tumor cell lysates as immunogenic sources for cancer vaccine design. Hum. Vaccin. Immunother. 10, 3261–3269.
Grainger, J.R., Hall, J.A., Bouladoux, N., Oldenhove, G., Belkaid, Y., 2010. Microbe-dendritic cell dialog controls regulatory T-cell fate. Immunol. Rev. 234, 305–316.
Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H.J., Herrlich, P., Cato, A.C.B., 1994. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J. 13, 4087–4095.
Hench, P.S., Kendal, E.C., Slocumb, C.H., Polley, H.F., 1950. Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions. Arch. Intern. Med. 85, 545–666.
Herr, I., Ucur, E., Herzer, K., Okouoyo, S., Ridder, R., Krammer, P.H., von Knebel Doeberitz, M., Debatin, K.M., 2003. Glucocorticoid cotreatment induces apoptosis resistance toward cancer therapy in carcinomas. Cancer Res. 63, 3112–3120.
Herr, I., Pfitzenmaier, J., 2006. Glucocorticoid use in prostate cancer and other solid tumours: implications for effectiveness of cytotoxic treatment and metastases. Lancet Oncol. 7, 425–430.
Higham, E.M., Shen, C.H., Wittrup, K.D., Chen, J., 2010. Cutting edge: delay and reversal of T cell tolerance by intratumoral injection of antigen-loaded dendritic cells in an autochthonous tumour model. J. Immunol. 184, 5954–5958.
Hou, W.J., Guan, J.H., Dong, Q., Han, Y.H., Zhang, R., 2013. Dexamethasone inhibits the effect of paclitaxel on human ovarian carcinoma xenografts in nude mice. Eur. Rev. Med. Pharmacol. Sci. 17, 2902–2908.
Hu, J., Kinn, J., Zirakzadeh, A.A., Sherif, A., Norstedt, G., Wikström, A.C., Winqvist, O., 2013. The effects of chemotherapeutic drugs on human monocyte-derived dendritic cell differentiation and antigen presentation. Clin. Exp. Immunol. 172, 490–499.
Jensen, A.Ø, Thomsen, H.F., Engebjerg, M.C., Olesen, A.B., Friis, S., Karagas, M.R., Sørensen, H.T., 2009. Use of oral glucocorticoids and risk of skin cancer and non-Hodgkin's lymphoma: a population-based case-control study. Br. J. Cancer 100, 200–205.
Kantoff, P.W., Higano, C.S., Shore, N.D., for the IMPACT Study Investigators, et al., 2010.

Sipuleucel-T immunotherapy for castration-resistant prostate cancer. New Engl. J. Med. 363, 411–422.
Lebson, L., Wang, T., Jiang, Q., Whartenby, K.A., 2011. Induction of the glucocorticoid-induced leucine zipper gene limits the efficacy of dendritic cell vaccines. Cancer Gene Ther. 18, 563–570.
Lin, K.T., Wang, L.H., 2016. New dimension of glucocorticoids in cancer treatment. Steroids pii: S0039-128X(16)00057-X.
López, M.N., Pereda, C., Segal, G., Muñoz, L., Aguilera, R., González, F.E., Escobar, A., Ginesta, A., Reyes, D., González, R., et al., 2009. Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumour-specific delayed type IV hypersensitivity response and reduction of tumour growth factor β-expressing T cells. J. Clin. Oncol. 27, 945–952.
Lussier, D., Huskey, A.G., Portenoy, R.K., 2004. Adjuvant analgesics in cancer pain management. Oncologist 9, 571–591.
Mellman, I., 2013. Dendritic cells: master regulators of the immune response. Cancer Immunol. Res. 1, 145–149.
Mendoza-Naranjo, A., Sáez, P.J., Johansson, C.C., Ramírez, M., Mandakovic, D., Pereda, C., et al., 2007. Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. J. Immunol. 178, 6949–6957.
Neller, M.A., López, J.A., Schmidt, C.W., 2008. Antigens for cancer immunotherapy. Semin. Immunol. 20, 286–295.
Osorio, F., Fuentes, C., López, M.N., Salazar-Onfray, F., González, F.E., 2015. Role of dendritic cells in the induction of lymphocyte tolerance. Front. Immunol. 6, 535.
Palucka, K., Banchereau, J., 2012. Cancer immunotherapy via dendritic cells. Nat. Rev. Cancer 12, 265–277.
Palucka, A.K., Ueno, H., Connolly, J., Kerneis-Norvell, F., Blanck, J.P., Johnston, D.A., Fay, J., Banchereau, J., 2006. Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. J. Immunother. 29, 545–557.
Petersen, T.R., Sika-Paotonu, D., Knight, D.A., Simkins, H.M., Hermans, I.F., 2011. Exploiting the role of endogenous lymphoid-resident dendritic cells in the priming of NKT cells and CD8+ T cells to dendritic cell-based vaccines. PLoS One 6, e17657.
Pfirschke, C., Engblom, C., Rickelt, S., Cortez-Retamozo, V., Garris, C., Pucci, F., et al., 2016. Immunogenic chemotherapy sensitizes tumours to checkpoint blockade therapy. Immunity 44, 343–354.
Rea, D., van Kooten, C., van Meijgaarden, K.E., Ottenhoff, T.H., Melief, C.J., Offringa, R., 2000. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. Blood 95, 3162–3167.
Rutz, H.P., 2002. Effects of corticosteroid use on treatment of solid tumours. Lancet 360, 1969–1970.
Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S., Kofler, R., 2004. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ. 11 (Suppl. 1), S45–55.
Sharma, A., Bode, B., Studer, G., Moch, H., Okoniewski, M., Knuth, A., von Boehmer, L., van den Broek, M., 2013. Radiotherapy of human sarcoma promotes an intratumoral immune effector signature. Clin. Cancer Res. 19, 4843–4853.
Skor, M.N., Wonder, E.L., Kocherginsky, M., Goyal, A., Hall, B.A., Cai, Y., Conzen, S.D., 2013. Glucocorticoid receptor antagonism as a novel therapy for triple-negative breast cancer. Clin. Cancer Res. 19, 6163–6172.
Spallanzani, R.G., Torres, N.I., Avila, D.E., Ziblat, A., Iraolagoitia, X.L., Rossi, L.E., et al., 2015. Regulatory dendritic cells restrain NK cell IFN-γ production through mechanisms involving NKp46, IL-10, and MHC class I-specific inhibitory receptors. J. Immunol. 195, 2141–2148.
Surace, L., Lysenko, V., Fontana, A.O., Cecconi, V., Janssen, H., Bicvic, A., et al., 2015. Complement is a central mediator of radiotherapy-induced tumour-specific immunity and clinical response. Immunity 42, 767–777.
Tittarelli, A., González, F.E., Pereda, C., Mora, G., Muñoz, L., Saffie, C., et al., 2012. Toll-like receptor 4 gene polymorphism influences dendritic cell *in vitro* function and clinical outcomes in vaccinated melanoma patients. Cancer Immunol. Immunother. 61, 2067–2077.
Wakim, L.M., Waithman, J., van Rooijen, N., Heath, W.R., Carbone, F.R., 2008. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. Science 319, 198–202.
Wong, E.T., Lok, E., Gautam, S., Swanson, K.D., 2015. Dexamethasone exerts profound immunologic interference on treatment efficacy for recurrent glioblastoma. Br. J. Cancer 113, 1642.
Yewdall, A.W., Drutman, S.B., Jinwala, F., Bahjat, K.S., Bhardwaj, N., 2010. CD8+ T cell priming by dendritic cell vaccines requires antigen transfer to endogenous antigen presenting cells. PLoS One 5, e11144.
Zhang, C., Wenger, T., Mattern, J., Ilea, S., Frey, C., Gutwein, P., et al., 2007. Clinical and mechanistic aspects of glucocorticoid-induced chemotherapy resistance in the majority of solid tumours. Cancer Biol. Ther. 6, 278–287.