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Original Article



Autologous cervical tumor lysate pulsed dendritic cell stimulation followed by cisplatin treatment abrogates FOXP3+ cells in vitro

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ABSTRACT


Objective: Dendritic cells (DCs) are administered as immunotherapeutic adjuvants after the completion of standard treatment in most settings. However, our Phase I trial indicated that one patient out of four, who received autologous tumor lysate-pulsed dendritic cell (TLDC) also received cisplatin chemotherapy and experienced complete regression of her lung lesion, continuing to be disease free till date. Hence, the objective of our current study is to evaluate the sustenance or augmentation of immune responses when autologous human papillomavirus positive cervical tumor lysate pulsed DC- are combined with cisplatin, using co-culture assays in vitro.

Methods: Before treatment, peripheral blood and punch biopsy samples were collected from 23 cervical cancer patients after obtaining an informed consent. DC functionality was confirmed through phenotypic and functional assays using autologous peripheral blood mononuclear cells as responders. For cisplatin experiments, the drug was added at 150, 200 (clinical dose equivalent), and 400 μ M concentrations to DCs alone or DC-T cell co-cultures. Phenotypic assessment and functional characterization of DCs was done using flow cytometry. Cytokine enzyme-linked immunosorbent assay and interferon (IFN)- γ enzyme-linked immune absorbent spot assays were also performed.

Results: The functionality of TLDCs was not compromised upon cisplatin treatment in vitro even at the highest (400 μ M) concentration. Even though cisplatin treatment reduced the secretion of IFN- γ and interleukin (IL)-12p40 in co-cultures stimulated with TLDCs, this effect was not significant ($p > 0.05$). A doubling of IFN- γ secretion following cisplatin treatment was observed in at least one of three independent experiments. Additional experiments showed a reduction in both FOXP3+ regulatory T cells and IL-10 levels.

Conclusion: Our results provide evidence that cisplatin treatment may be given after autologous TLDC administration to maintain or improve a productive anti-tumor response in vaccinated patients.

Keywords: Dendritic Cells; Cisplatin; Th1 Cells; Interferon-gamma; Chemotherapy, Adjuvant; Cervical Cancer

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Data curation: S.A., J.H.; Formal analysis: D.H., P.R.; Funding acquisition: T.R.; Investigation: G.S., S.S.S.; Methodology: D.H., T.R., P.R.; Supervision: P.R.; Visualization: D.H.; Writing - original draft: D.H.; Writing - review & editing: G.S., S.S.S., T.R., P.R.

INTRODUCTION

Cervical cancer is the fourth most common cancer in women, with an estimated 570,000 new cases in 2018 [1]. Globally, India ranks second in cervical cancer burden with an estimated 96,922 new cases and 60,078 deaths from the disease every year (GLOBOCAN, 2018). The standard line of treatment for early-stage cervical cancer is either surgery or radiotherapy but for stages IIB and above, it is radiotherapy with concurrent chemotherapy with cisplatin (40 mg/kg·m²) [2,3]. Even with a concurrent chemo-radiotherapy regimen, the survival rate in locally advanced stages is less than 50% [4]. Hence there is a need for enhancing the response rate for patients with locally advanced stages specifically, stage IIIB disease by exploring adjuvant treatments including cell-based immunotherapy.

Several studies including our own [5-8], focused on the use of dendritic cells (DCs) as cancer vaccines because of their antigen-presenting potency [9]. Autologous tumor lysates prepared by a freeze thaw process release high-mobility group protein 1 (HMGB1) as well as other cellular components which may interact with the scavenger receptor A and Toll-like receptor 4 (TLR4) on DCs [10]. Additionally, cervical tumors are positive for human papillomavirus (HPV) and hence present the scope of HPV peptides or proteins being released from the necrotic cells [11]. While our in vitro studies [12] had shown that HPV positive tumor lysate-pulsed dendritic cells (TLDCs) were effective against allogenic responders, our randomized phase I clinical trial [6] established the safety of autologous TLDCs; in the same trial, one patient (1/4) received cisplatin chemotherapy after receiving 3 doses of DC vaccine following which there was complete resolution of a metastatic lung lesion in addition to complete response in the primary site. This patient is disease free till date. Currently, most clinical trials involving DC vaccines administer them after the completion of standard treatment [13]. However, based on the evidence from our phase I study, we hypothesized that when cisplatin is administered after a dose of autologous TLDCs, in some patients, it may sustain the DC based immune stimulation by inducing cytotoxic cell death causing antigen release for enhancing an effector response and additionally impact the regulatory T cell (Treg) response.

Hence, we initiated this study with the objective of evaluating-T helper 1 (Th1) responses of autologous peripheral blood mononuclear cells (PBMCs) and the effect of cisplatin treatment on DC viability, phenotype, migration and ability to stimulate proliferation of CD4/CD8+ T cells, natural killer (NK) cells and regulatory Fork head box P3 (FOXP3+) T cells from cervical cancer patients.

MATERIALS AND METHODS

1. Patients and informed consent

Cervical cancer patients presenting at the out-patient facility of Cancer Institute (WIA) were included in the study after obtaining an informed consent. The study was approved by the Institutional Ethics Committee. In all, 23 patients' blood (20 mL) and punch biopsy samples were obtained for generation of DCs and preparation of tumor cell. Samples P1-P16 were from patients with different stages of cervical cancer and were used for autologous experiments comparing TLDC and unprimed DCs. Patients 17-23 were uniformly diagnosed with stage IIIB disease.

2. DNA isolation and HPV detection

DNA extraction was performed from formalin fixed paraffin embedded sections and punch biopsy cancer tissue as described previously [12]. The presence of HPV was confirmed using GP5+ and GP6+/MY09 and MY011 [14,15] primers.

3. Generation of DCs pulsed with autologous tumor cell lysate

Cervical punch biopsy samples were digested with collagenase (Cat. No.: 17459, 0.07 IU/mL, SERVA Electrophoresis GmbH, Heidelberg, Germany) as described previously [12]. The percentage of tumor cells in suspension was determined by a trained cytologist. Monocyte derived dendritic cells were generated using granulocyte-macrophage colony stimulation factor (GM-CSF) (Cat. No.: 1412-050) and IL-4 (Cat. No.: 1403-050) and were pulsed with tumor lysates [12] at a 3:1 (tumor cells:DCs) ratio and matured in the presence of tumor necrosis factor- α (TNF- α) (Cat. No.: 1406-050) and IL-1 β (Cat. No.: 1411-050). Unprimed mature DCs (controls) were generated in the presence of cytokine cocktail alone. All the cytokines and Cell Gro DC media (Cat. No.: 20801-0500) were purchased from CellGenix GmbH (Breisgau, Germany). The culture supernatants were stored in aliquots at -80°C .

4. Cisplatin treatment of DCs and autologous PBMC co-cultures

Cisplatin (cis-diamminedichloridoplatinum [II] or CDDP, Kemoplat; Fresenius Kabi, Bad Homburg, Germany) was used at 3 different concentrations—150 μM (dose 1), 200 μM (dose 2), and 400 μM (dose 3) with dose 2 being approximately equal (40 $\text{mg}/\text{kg}\cdot\text{m}^2$) to the dose of cisplatin administered weekly to patients with cervical cancer, using DuBois formula. Dendritic cells alone were treated for 48 hours after which phenotypic marker expression and migratory capacity were analyzed.

Autologous TLDC: PBMC (enriched for lymphocytes after monocyte reduction by adherence) cell co-cultures were treated thrice with cisplatin. The frequency of treatment was day 1, 3 and 5 for carboxyfluorescein diacetate succinimidyl ester (CFSE) based proliferation. The cultures were maintained, as done with untreated controls, for a week. Approximately 25,000 TLDCs were added 24 hours prior to each dose and rested overnight followed by addition of the corresponding dose of cisplatin to each well. Phenotypic characterization of proliferating cells was done as mentioned in the proliferation assay.

5. Phenotypic characterisation of cisplatin treated DCs by flow cytometry

Phenotypic characterization of immature and mature DCs (unprimed and TLDCs) was done using CD14-PC5 (Cat. No.: A07765, clone:RMO52), CD80-FITC (Cat. No.: IM1853U, clone:MAB104), HLADR-ECD (Cat. No.: IM13636, clone:Immu-357), CD40-PE (Cat. No.: IM1936U, clone:MAB89) and CD86-PE (Cat. No.: IM2729U, clone:HA5.2B7) antibodies according to manufacturer's instructions. All the antibodies were purchased from Beckman Coulter Inc. (Brea, CA, USA) except the maturation marker CD83-APC (Cat. No.: 305312, clone:HB15), purchased from BioLegend (San Diego, CA, USA). All the samples were acquired in a MoFlo XDP flow cytometer/sorter and analyzed using summit 5.2 (both Beckman Coulter Inc.).

6. Migration assay

Migratory capacity of cisplatin treated and untreated DCs towards 300 ng/mL macrophage inflammatory protein 3 β (MIP-3 β) (cat.no.:361-MI-025) and 250 ng/mL 6Ckine (Cat. No.: 366-6C-025) (R&D Systems, Minneapolis, MN, USA) was assessed, as described previously [12].

7. Proliferation assay

Autologous PBMCs enriched for non-adherent lymphocytes following monocyte depletion were stained with CFSE (Cat. No.: C34554) as described previously [12]. Briefly, DCs were harvested and co-cultured with 2.5×10^5 PBMCs/well in a U bottomed 96 well plate at a ratio of 1:10 (DC: PBMCs) for 7 days. Cells were removed on day 7 to be stained with CD56-PE (Cat. No.: AO7788, clone:N901), CD4-PC5 (Cat. No.: A07752, clone:13B8.2) and CD8-APC (Cat. No.: IM2469U, clone:B9.11), CD45RA (Cat. No.: IM2711U, clone:2H4) antibodies to check for the phenotypic characterization of proliferating PBMCs upon stimulation with DCs. A fixable live dead discriminator (Fixable Live/Dead Violet kit, Cat. No.: L34961; Thermo Fisher Scientific, Waltham, MA, USA) was used in all the experiments.

8. Characterisation of Treg population

For the analysis of Tregs, non-adherent PBMCs were co-cultured, for 7 days with TLDCs (1:10 ratio), in the presence or absence of cisplatin as done for the proliferation assay, but without CFSE staining. Anti- CD4-PC5, CD8-APC, CD25-ECD (Cat. No.: 6607112, clone: B1.49.9), CD3-PC7 (Cat. No.: 6607100, clone:259D) (all from Beckman Coulter Inc.) and FOXP3-PE (Cat. No.: 320108, clone:206D from BioLegend) markers were used to stain the cells after a week for Treg analysis by flow cytometry. A fixable live dead discriminator (Thermo Fisher Scientific) was used to estimate the percentage of live/dead cells.

9. Cytokine enzyme-linked immunosorbent assay (ELISA)

Dendritic cells were removed after termination of their culture period (7 days), washed and suspended in plain Cell Gro Medium alone for a period of 48 hours. This medium was considered as cytokine withdrawn (CW). DC:PBMC co-culture supernatants were centrifuged and stored separately after termination of proliferation assays. CW and co-culture supernatants were stored at -80°C and used for the analysis of the cytokines IL-12p40 (Cat. No.: 431708) and p70 (Cat. No.: 430701). A sandwich ELISA was performed as per the manufacturer's instructions (BioLegend). The intensity of colour developed was read at 450 nm using Multiskan Ascent (Thermo Electron Corporation, Waltham, MA, USA) ELISA reader.

10. Quantibody array

A multiplex sandwich ELISA (Quantibody[®] Human Custom Cytokine Antibody Array, RayBiotech, Peachtree Corners, GA, USA) was customized and procured to detect—IL-4, IL-15, IL-10, transforming growth factor (TGF)- β , IL-12p40, and IL-12p70 in CW and co-culture supernatants of 6 patients. The experiments were carried out as per the manufacturer's instructions. The slides were scanned using GenePix[®] 4100A microarray scanner (Molecular Devices, Inc., San Jose, CA, USA) and the intensity of cytokine secretion was calculated using the Q-analyzer software (RayBiotech).

11. Interferon (IFN)- γ ELISPOT assay

The monocyte depleted enriched lymphocyte fraction was cultured with TLDCs for a period of 14 days with intermittent stimulation on day 7. At the end of 13th day, the cells were transferred to plates coated with IFN- γ capture antibody. An additional DC and IL-2 (100 IU) stimulation was performed. Unstimulated cells, were, treated with IL-2 alone and used as controls. IFN- γ ELISPOT assay (CTL Technologies, Cleveland, OH, USA) was performed according to the manufacturer's instructions. The spots were scanned and counted using ImmunoSpot Versa analyzer (CTL Technologies) with Immuno-Capture software version 6.4.

12. Statistical analysis

Student's t-test was performed to compare unprimed and TL primed cultures. Pearson's correlation was done to check the correlation between the secreted cytokine levels and respective cell types. One-way analysis of variance (ANOVA) was used to compare the phenotypic and functional characteristics of untreated and cultures treated with three different doses of CDDP. All the analyses were done using GraphPad prism 7 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

1. HPV status and pathological staging of cervical cancer patients

Information about age, histo-pathological grading, clinical staging and tumor cell percentage from punch biopsy samples of patients included in the study are mentioned in **Table 1**. Out of 23 patients only 15 opted for treatment at our Institute after confirmed diagnosis.

2. Phenotypic and migratory capacity of tumor lysate primed DCs and the effect of cisplatin on DC functionality

We performed routine phenotypic analysis of the TLDCs by flow cytometry. We observed significant costimulatory marker (CD80 and 83) expression and migratory capacity in TLDCs (**Supplementary Fig. 1A-F**). Next, we evaluated the effect of cisplatin on the viability of TLDCs (**Fig. 1A**). Our results showed that, although significant cell death was induced in treated DCs at dose 3 ($p=0.001$) and 2 ($p=0.020$) of cisplatin, there was no change in the surface marker expression or their mean fluorescence intensity (MFI), even at the highest dose (**Supplementary Table 1** and **Fig. 1B**). A comparison of migratory capacity of the treated

Table 1. Age, histopathological grading, tumor cell percentage, HPV status and clinical staging details of patients included in the study

Patient	Age	Pathological, grade	Clinical stage*	% of tumor cells Mean=70% (SD±16)
1	45	SCC, grade III	IIB2	40
2	38	SCC, grade II-III	IIIB	80
3	47	Adenocarcinoma, grade II	IIA2	60
4	52	SCC, grade III	-	70
5	51	Invasive SCC, grade III	-	80
6	45	SCC, grade III	-	80
7	55	SCC, grade III	-	70
8	65	SCC, grade III	IIIA	40
9	50	SCC, grade III	-	90
10	52	SCC, grade III	-	80
11	40	SCC, grade III	-	50
12	39	SCC, grade III	IIA2	90
13	59	SCC, grade III	IB	50
14	76	SCC, grade III	IIB	70
15	55	SCC, grade II-III	IIB	80
16	57	SCC, grade III	IIB	75
17	56	SCC, grade II-III	IIIB	50
18	53	SCC, grade II-III	IIIB	70
19	50	SCC, grade II	IIIB	90
20	47	SCC, grade III	IIIB	75
21	43	SCC, grade II	IIIB	90
22	53	SCC, grade III	IIIB	70
23	47	SCC, grade III	IIIB	50

HPV, human papillomavirus; SCC, squamous cell carcinoma; SD, standard deviation.

*Available only for 15 patients who continued treatment after diagnosis at the Cancer Institute (WIA).

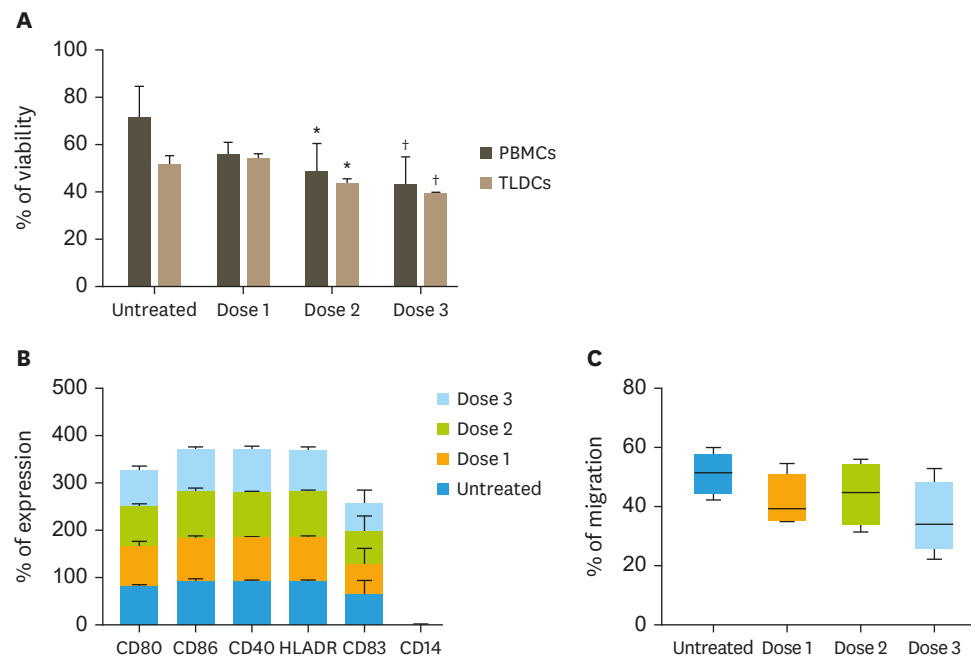


Fig. 1. Cell viability, phenotypic and migratory capacity of TLDCs upon cisplatin treatment (dose 1: 150 μ M, dose2: 200 μ M, and dose 3: 400 μ M).

(A) Difference in the viability of TLDCs and PBMCs in untreated and cisplatin treated cultures in different doses (n=3). (B) Phenotypic characterization of untreated and cisplatin treated TLDCs (n=3). (C) Migratory ability of untreated and cisplatin treated TLDCs upon chemotactic stimuli (n=3). Statistical method used one-way ANOVA with multiple comparison.

ANOVA, analysis of variance; PBMC, peripheral blood mononuclear cell; TLDC, tumor lysate-pulsed dendritic cell. * $p \leq 0.05$; † $p \leq 0.01$.

and un treated DCs, showed a reduction following cisplatin treatment at dose 3 which was however not statistically significant ($p=0.190$) (**Fig. 1C**). Although our results showed that TLDC retained their phenotypic and migratory characteristics, it was necessary to ascertain their ability to function efficiently in the presence of the drug.

3. Autologous tumor lysate primed DCs were capable of inducing CD4, CD8 and NK cell proliferation in the presence of cisplatin

First, we established the ability of untreated DCs to stimulate autologous lymphocyte enriched PBMC proliferation by flow cytometry which helped us profile the dividing cell phenotype. As expected, on an average, the proliferation index of PBMC was higher when stimulated with TLDCs (1.35 ± 0.2) indicating that the dividing percentage of cells was significantly higher ($p=0.020$) in TLDC co-cultures (**Supplementary Fig. 2A**).

We also found that an increase in CD8+ T cell proliferation correlated significantly with CD56+ NK cell and CD4+ T cell proliferation (**Supplementary Table 2**), indicating that both cell types were stimulated by the TLDC to potent CTL response. We also observed a reduction in CD45RA+ cells in TLDC co-cultures ($53.3\% \pm 13.5\%$) compared to unstimulated controls ($64.11\% \pm 13.9\%$).

As a standard protocol, cervical cancer patients treated at Cancer Institute (WIA), are given cisplatin (CDDP) weekly for up to 8 weeks. In three independent experiments with sufficient DCs for all the experiments DC-PBMC co-cultures were treated with 150, 200, and 400 μ M (dose 1, 2, and 3, respectively) of CDDP thrice a week to reach a cumulative

weekly dose of 450, 600, and 1,200 μM . The first dose was given 24 hours after the addition of DCs to autologous PBMC cultures *in vitro*, in line with our objective to introduce immune stimulation before beginning chemotherapy. DC:PBMC co-cultures were maintained for a week and DCs were also added at the same frequency (thrice) preceding the cisplatin dosage. Although this amount of cisplatin is much higher than the weekly dosage, we wanted to gain an understanding of the maximum dose up to which cells could proliferate without compromised functional ability.

Our results showed that, upon cisplatin treatment, (**Fig. 1A**, PBMCs), the viability of cells in co-culture dropped by approximately 20% at dose 1, 31% at dose 2, and 39% at dose 3. Although the average proliferation index (PI) was reduced in treated cultures (**Supplementary Fig. 3A**), the number of dividing generations in dose 1 and 2 treated cultures were the same as observed in untreated TLDCs (data not shown). **Supplementary Fig. 2B** is a representative histogram overlay of proliferating cells. In all three doses, the overall percentage of CD4, CD8, and CD56+ cells decreased after cisplatin treatment. In two of three experiments, we found an average $23\% \pm 13\%$ reduction in CD4+ and $31\% \pm 11\%$ drop in CD8+ cells while CD56+ cells were reduced by $40\% \pm 2\%$ when treated with dose 2 of CDDP. It is however noteworthy that the cells received cisplatin thrice within a week. In one patient (1/3) however, at dose 2, although the overall viability was reduced, in the CD8+ T cell compartment alone, we saw a 12% increase in the no. of cells undergoing proliferation accompanied by almost no change in the CD56+ T cell numbers and a 6.4% drop in CD4+ T cells compared to untreated cells (**Supplementary Fig. 3C**).

4. Cisplatin concentration affects the level of Th1 cytokines secreted in co-culture supernatants

Before checking for the effect of cisplatin on the DC-PBMC co-cultures, we confirmed the secretion of the Th1 cytokine IL-12. Both the homo(p40) and heterodimeric (p70) forms of IL-12 were measured based on our previous findings that HPV positive tumor lysates induced IL-12p40 at levels in excess of the p70 heterodimer. We found that the secretion of IL-12p40 was higher in CW supernatants of TLDCs, uniformly in all cultures (n=10) compared to DC:PBMC co-cultures on day 7 (**Supplementary Fig. 2E**). In 6 patients, additionally, day 14 supernatants were also assessed which showed significant reduction in the level of IL-12p40 ($p=0.001$), compared to CW TLDCs (**Fig. 2A**). Although IL-12p40 levels were lower in day 7 supernatants, we observed a positive correlation (**Fig. 2B**) with IFN- γ secretion.

The p40 homodimer although considered inhibitory, precedes p35 synthesis and leads to the formation of IL-12p70 secretion. Hence, in CW supernatants, negligible levels of the cytokine were seen, which increased significantly on Day 7 ($p=0.002$) and again dropped by day14 (**Fig. 2C**). Our results also indicated an increased secretion of IL-15 (**Fig. 2D**) by the same untreated TLDC-PBMC co-cultures compared to unstimulated PBMC, in line with our findings of increased NK cell proliferation in TLDC co-cultures. A correlation matrix showed that the percentage of CD8+ and NK cells in the proliferating compartment correlated significantly with the levels of IFN- γ and IL-12p40 indicating a Th1 polarization of the immune response (**Supplementary Table 2**). It is well known that the IL-12 molecule may induce IFN- γ secretion which in turn acts as the primer for a positive feedback loop for more IL-12 synthesis [16] since we terminated the cultures on day 14, we were unable to verify the same.

In cisplatin treated CW and co-culture supernatants of TLDC, the amount of IL-12 cytokine secreted was inversely proportional to increasing drug concentration, with a reduction in

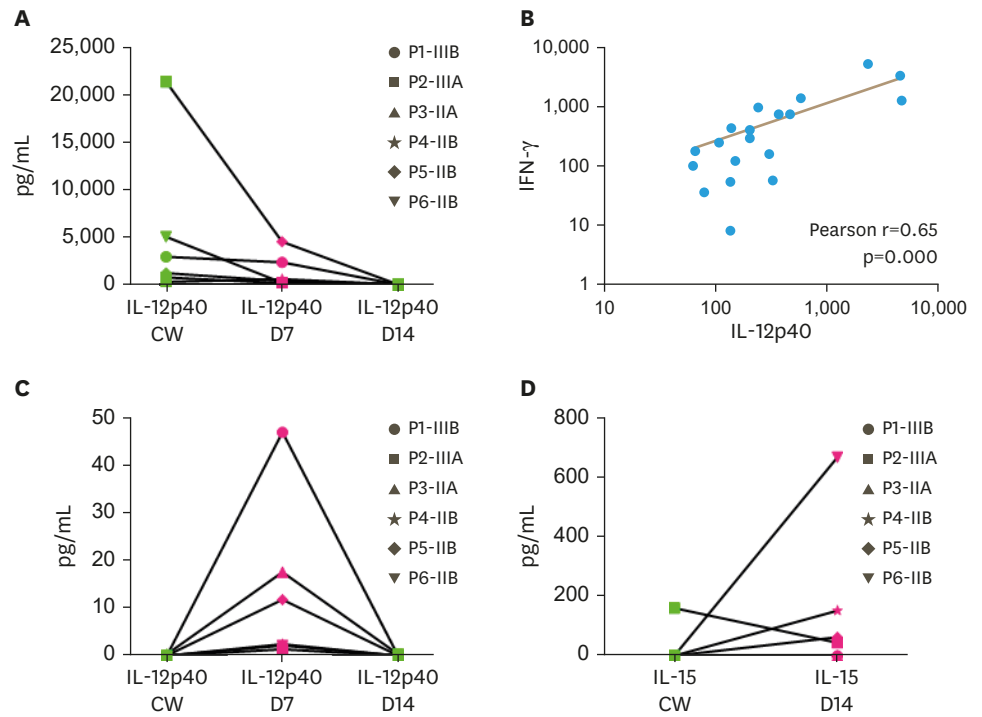


Fig. 2. Secretion of Th1 cytokines in untreated TLDCs CW supernatants and co-culture supernatants. Quantification of (A) IL-12p40 and (C) IL-12p70 secretion in CW and co-culture supernatants (day 7 & day 14) (n=6). (B) Correlation between IFN- γ and IL-12p40 (day 7) secretion from co-culture supernatants (n=11). (D) Quantification of IL-15 secretion in CW and co-culture supernatants (day 14) (n=6). Statistical method used unpaired 2-tailed Student's t-test and Pearson correlation analysis. CW, cytokine withdrawn; IL, interleukin; Th1, T helper 1; TLDC, tumor lysate-pulsed dendritic cell.

both IL-12p40 and p70 levels (**Fig. 3A and B**). IL-15, though, was not detected in any of the treated cultures (data not shown).

5. Cisplatin treatment abrogated Treg proliferation in the presence of TLDCs and improved IFN- γ response

Evaluation of IFN- γ secretion in cisplatin treated and untreated cultures showed that despite the reduction in Th1 cytokines, the secretion of IFN- γ was not completely inhibited. In 2/3 independent experiments with patient samples P18 and 19, as expected, the number of IFN- γ spots were reduced in the cisplatin treated co-cultures alone (all three doses) compared to untreated cultures. However, in one patient (P20) we found that the number of spots had actually doubled in the same co-cultures (**Fig. 3C and D**).

In order to verify if the increase in IFN- γ response was due to a fall in the suppressive Treg proliferation due to cisplatin treatment, we evaluated the same patient's co-cultures (P20) along with three other independent samples (P21–23) without CFSE staining as FOXP3 is an intra-nuclear marker. **Fig. 4A** shows the abrogation of FOXP3+ cells in P20. In all 4 independent experiments, when compared to untreated cells, there was an increase in the ratio of CD8/CD4+ T ratio (p=0.030) (**Fig. 4B**) in the lowest dose (dose 1) treated co-cultures. When we further analyzed the FOXP3+ Treg compartment, we observed a lower CD8/FOXP3+ ratio in the untreated co-cultures while a significant increase in the ratio (p=0.020 and p=0.012) was seen at dose 1 and dose 2 (**Fig. 4C**).

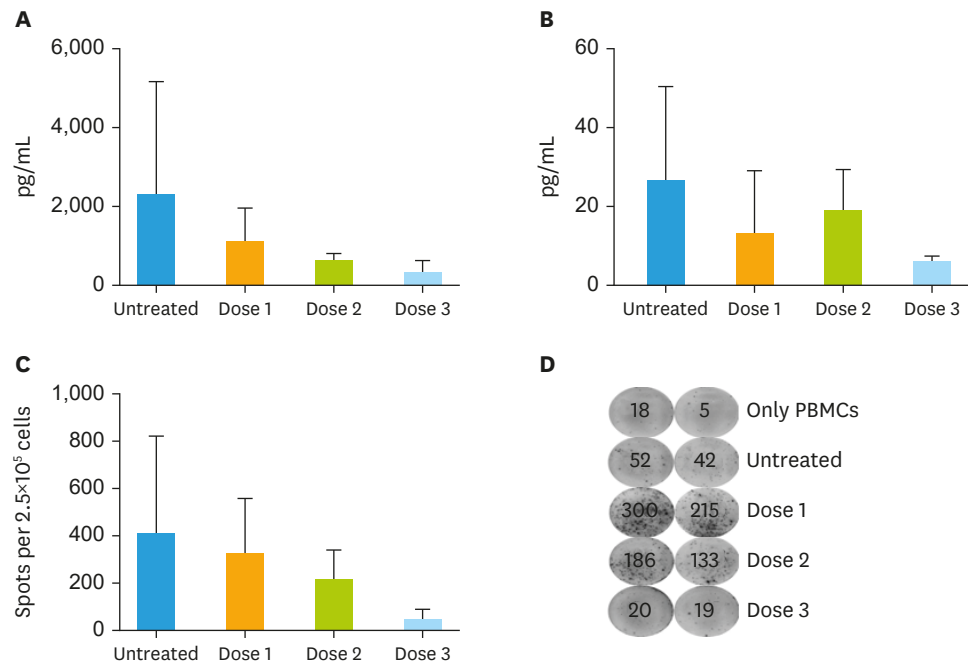


Fig. 3. Secretion of Th1 cytokines in co-culture supernatants upon cisplatin treatment (dose 1: 150 μ M, dose 2: 200 μ M, and dose 3: 400 μ M).

Secretion of (A) IL-12p40, (B) IL-12p70, and (C) IFN- γ by PBMCs upon TLDCs stimulation in the presence or absence of cisplatin (n=3). (D) Representative ELISpot image showing an increase in IFN- γ upon cisplatin treatment (dose 1 and 2) compared to untreated co-cultures. Statistical method used one-way ANOVA with multiple comparisons.

ANOVA, analysis of variance; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; Th1, T helper 1; TLDC, tumor lysate-pulsed dendritic cell.

6. Cisplatin improves IFN- γ secretion by reducing the level of IL-10 and enhancing the effector/Treg ratio

Based on the improved effector/Treg ratio observed in TLDC co-cultures treated with cisplatin, we speculated that reduction in TGF- β or IL-10 levels may be key to the increase in IFN- γ levels. Hence, we first evaluated the levels of both cytokines in the untreated TLDC (CW) and stimulated co-cultures on day 14. Although we could detect TGF- β secretion in CW (**Fig. 5A**) as well as day 14 (co-culture) supernatants, the difference when compared with unprimed DC controls in both was not high (<1.5-fold). Despite this, TLDC showed superior phenotypic and migratory characteristics and Th1 response. Hence, we speculated that TGF- β may play a homeostatic role [17,18] in balancing autoimmunity since autologous lysates used for priming may carry non mutated self-antigens also without altering mature DC functionality.

We next examined the level of IL-10 in the same supernatants (untreated TLDC-CW and day 14 supernatants). In almost all co-cultures (n=6), its level was negligible in the CW supernatants but on day 14 it was elevated by more than ten-fold (**Fig. 5B**). Subsequently, when we checked unprimed DC controls, we found nearly 6-fold difference between them. Hence, we hypothesised that IL-10 secreted by proliferating FOXP3+ cells may mediate a suppressive response and may be abrogated by cisplatin treatment. In order to verify the same, we utilized the supernatants from untreated and cisplatin treated TLDC-PBMC co-cultures of three patients (P21-23). We again observed that, both FOXP3+ cells and IL-10 were found to be higher in the untreated co-cultures alone which, were reduced drastically in the CDDP treated cultures even at the lowest concentration (**Fig. 5C**). This leads us to speculate

DC-cisplatin effect on effector/suppressor T cells

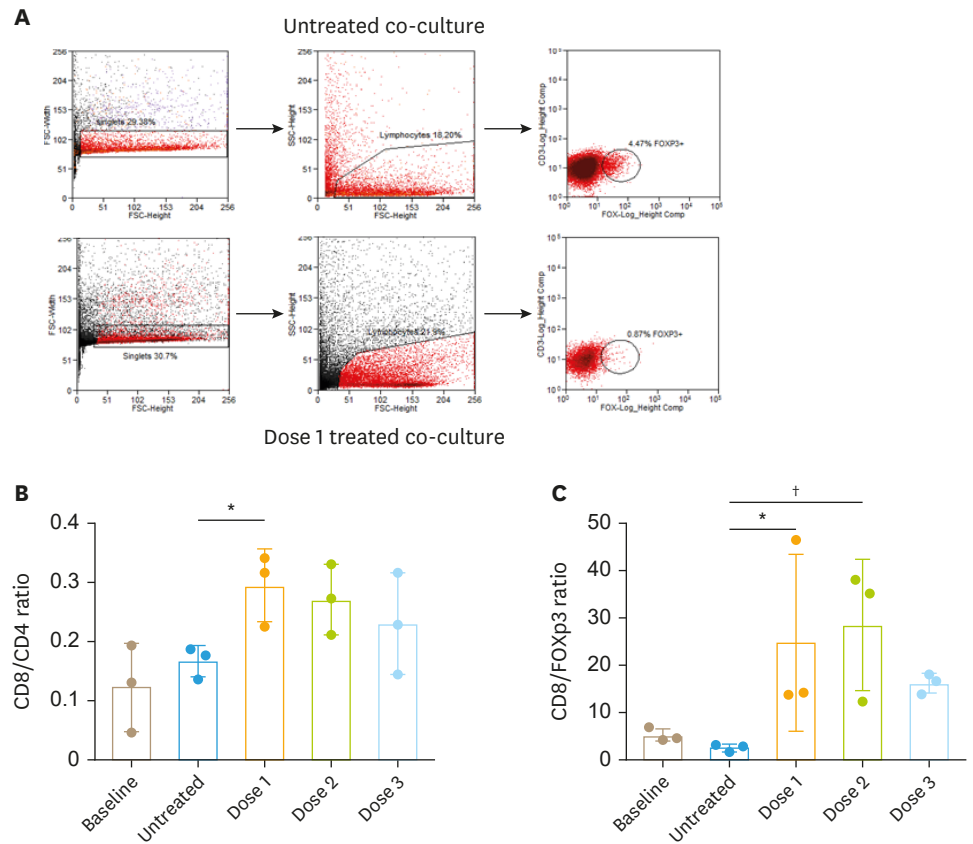


Fig. 4. FOXP3+ Tregs analysis in untreated and cisplatin treated co-cultures (dose 1: 150 μ M, dose2: 200 μ M, and dose 3: 400 μ M). (A) FOXP3+ T cell analysis by flow cytometry- representative image from one experiment (P20). (B and C) Analysis of CD8/CD4+ and CD8/FOXP3+ T cell ratio in baseline, untreated and cisplatin treated co-cultures (n=4). Statistical method used one-way ANOVA with multiple comparisons. ANOVA, analysis of variance; FOXP3+, Fork head box P3; Treg, regulatory T cell. *p \leq 0.05; †p \leq 0.01.

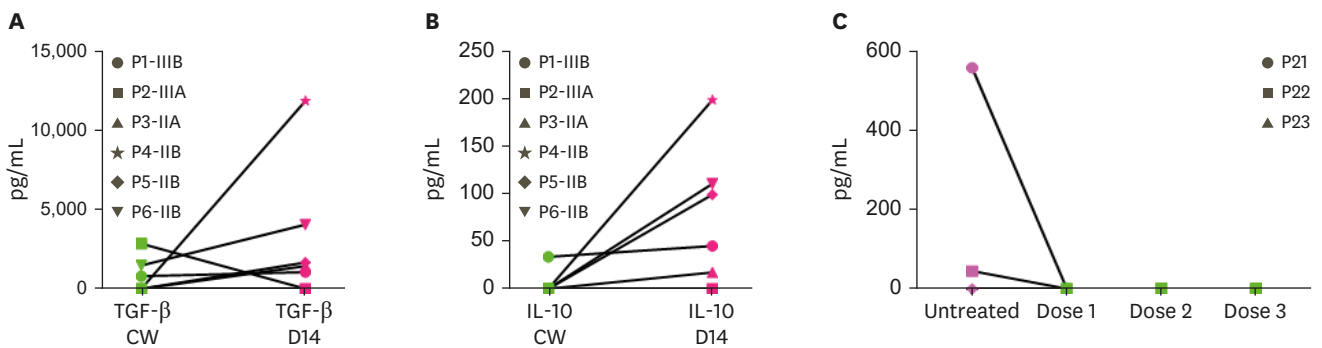


Fig. 5. Secretion of suppressive cytokines in CW and co-culture supernatant in the presence or absence of cisplatin treatment. Quantification of (A) TGF- β (B) IL-10 secretion in CW and co-culture supernatant (day 14) obtained from untreated DCs (n=6). (C) Quantification of IL-10 secretion in co-culture supernatants of three independent samples in the presence and absence of cisplatin treatment (dose 1: 150 μ M, dose2: 200 μ M, and dose 3: 400 μ M). CW, cytokine withdrawn; DC, dendritic cell; IL, interleukin; TGF, transforming growth factor.

that administering DC vaccines primed with tumor lysates prior to chemotherapy may improve IFN- γ -responses at least in some patients who may have an elevated IL10/FOXP3+ T cell response when stimulated with TLDC. Cisplatin does compromise DC functionality albeit minimally and hence may be effectively combined with the chemotherapeutic agent.

DISCUSSION

In this study, we established that in vitro matured DCs are resistant to the effect of cisplatin and retained their phenotype and migratory capacity. Further, at higher doses (dose 2 and 3) although cisplatin induced the death of proliferating cells in DC:T cell co-cultures, thereby reducing proliferating cell numbers, this effect was not significant. While a strong Th1 response was ascertained by the secretion of the cytokines IL-12 and IFN- γ in untreated autologous TLDC co-culture supernatants, we observed that cisplatin did not abrogate peripheral T cell proliferation. On the contrary, reduced IL-10 secretion, suppressed FOXP3+ T cells along with improved IFN- γ secretion in 1 of 3 patients indicated that immune stimulation with DCs followed by standard cisplatin treatment may improve response rates in patients with advanced cervical cancer (stage III and above).

Conflicting evidence on the immune-stimulatory capacity of cisplatin has been reported. While some studies have shown that tumor cells exposed to cisplatin alone [19] or a combination of cisplatin and vinorelbine upregulate major histocompatibility complex class I expression leading to tumor rejection when injected into mice [20], others argue that cisplatin does not induce immunogenic cell death as it fails to cause the redistribution of calreticulin on the surface of dying cells [21]. Another study by Kim et al. [22] showed that bone marrow DCs when exposed to CDDP during maturation, led to differentiation of DCs to a tolerogenic phenotype. However, our results clearly show that the activation status of DCs is very important in determining their ability to stimulate anti-tumor responses. Hence DC maturation ex vivo serves to circumvent the development of tolerance in vivo. Median survival in cervical cancer patients with lung metastasis, treated with cisplatin chemotherapy alone is 18 months [23]. Cisplatin treatment in DC vaccine administered patients may release tumor antigens leading to re-stimulation of the primed immune system. However, cisplatin has not been shown to cause immunogenic cell death in all cell types [24] hence this may not be the only factor contributing to a lasting immune response.

Antonia et al. found objective clinical responses in 61.9% patients with extensive stage small cell lung cancer when DC vaccine with p53 candidate vaccine was administered followed by platinum-based chemotherapy. It is noteworthy that all the patients recruited for this study had failed platinum therapy previously and had progressed [25]. Our current in vitro study provides evidence that the DCs combined with cisplatin even at a lower concentration (dose 1) are effective at mediating a better Th1 cytokine response by curtailing FOXP3+ suppressor function.

IL-12p40 is often considered inhibitory due to the formation of the p40 homodimer [26]. However, Ing and Stevenson established the requirements for reciprocal activation of DCs and NK cells leading to the expression of IL-12p40 which was shown to have favorable disease-specific survival in cervical carcinoma patients [27]. Our results too showed a strong correlation between IL-12p40 secretion and NK cell proliferation following DC stimulation, a pattern also observed in other infectious diseases like *Mycobacterium tuberculosis*, indicating that the presence of HPV and its peptides may play a unique role in shaping the immune response [28-32].

Our study also showed that IL-15 secretion may be involved in mediating enhanced NK cell activity upon TLDC stimulation [33]. Takai et al. [34] revealed that CD8+ T cells themselves produce endogenous TGF- β upon activation and despite the presence of the cytokine, naïve T cells still differentiate into effector memory cells. The likelihood of auto antigens in the

tumor lysate stimulating the release of TGF β , however, cannot be ignored [16,17] indicating that this may be a feature linked to suppressing auto-immunity.

The role of FOXP3⁺ Tregs as suppressors is well established in the tumor microenvironment. FOXP3⁺ Tregs have also been found to be higher in the circulation of cervical cancer patients [35]. However, our results with cisplatin are in line with a recent study [36] which showed that neo adjuvant combination of cisplatin along with paclitaxel but not cisplatin alone was able to decrease the FOXP3⁺ positive population in tumor stroma of cervical cancer samples. In our experiments, cisplatin toxicity in proliferating cells did not show cell type preference however in the dose 1 treated co-cultures, we observed a significant reduction in FOXP3⁺ T cells. Although this needs to be validated in vivo using appropriate preclinical models, those effectively mimicking HPV induced cervical cancers are not robust enough to replicate tumor-immune interactions. Malignancies such as the cervical cancer grow much more slowly and are often accompanied by immune suppression/ tolerance. Previous reports using transgenic mice expressing the HPV 16 E6 & E7 proteins have also reported that cancers did not develop even though papillomatosis and dysplasia could be seen [37]. Newer strategies are focussing on creating potential humanised models which mimic the tumor microenvironment [38-40] and studies like the current one will need to be validated in such models for better understanding. In addition, randomized clinical trials will need to be performed with DC vaccination preceding or given simultaneously with concurrent cisplatin-chemotherapy and radiation treatment. To address this, we are evaluating the efficacy of such a combinatorial regime in a phase II clinical trial which is underway, with stage IIIB cervical cancer patients. We hope that results from this trial will provide more evidence on the efficacy of the DC-vaccine/concurrent chemo-radiation regime.

In conclusion, our study shows that tumor lysate pulsed DCs in combination with cisplatin were capable of decreasing the FOXP3⁺ Tregs sustaining a prudent Th1 response and may be an explored as an effective combinatorial strategy without compromising dendritic cell functionality.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Mean fluorescence intensity of phenotypic markers of TLDCs in the presence and absence of cisplatin

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Supplementary Table 2

Correlation matrix of Th1 cytokines and immune cell subtypes

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Supplementary Fig. 1

Phenotypic and migratory characteristics of primed and unprimed DCs.

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Supplementary Fig. 2

Proliferation and Th1 cytokine profiles of primed and unprimed DCs.

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Supplementary Fig. 3

Evaluating the functionality of DC: T cell co-cultures in the presence of cisplatin (dose 1: 150 μ M, dose2: 200 μ M, and dose 3: 400 μ M).

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