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Influence of the Chemotherapeutic Agent Mitomycin C on In Vitro Dendritic Cell Maturation and Interleukin-12 Production in a Colorectal Cancer Model

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ABSTRACT

Introduction: Antitumor-targeting drugs can stimulate dendritic cells (DCs) indirectly through the shedding of dying tumor cells as part of what is referred to as a "danger signal". Although chemotherapeutic agents have been shown to kill dendritic cells (DCs), the effects of low, non-cytotoxic doses on DC function have not been studied.

Objective: To investigate the impact of various concentrations of mitomycin C at low, non-cytotoxic doses on the maturation of DCs.

Methods: THP-1 monocytes were differentiated into immature dendritic cells using IL-4 and GM-CSF. HCT116 colorectal cancer cells were treated with mitomycin C at concentrations ranging from 10 to 80 nM and co-cultured with undifferentiated dendritic cells. The expression of co-stimulatory molecules (CD11c, CD86, HLA-DR, CD14) was assessed by flow cytometry, while IL-12p70 secretion was measured via Enzyme-Linked Immunosorbent Assay (ELISA). Statistical analysis was performed using GraphPad Prism 7.0, with a significance threshold set at $p<0.05$.

Results: Different dosages of Mitomycin C-treated HCT116 cells enhanced the maturation of dendritic cell markers (CD86, HLA-DR), but reduced CD14 levels ($p<0.01$). While increasing the Mitomycin C dose to 80 nM further upregulated HLA-DR and CD86 expression, the release of IL-12 was highest a 50 nM concentration of mitomycin C (686.7±125.7 pg/mL compared to 263.8±4.8 pg/mL in controls; $p<0.05$). IL-12 levels were not significantly increased even with 30 nM Mitomycin C.

Conclusion: Low concentrations of Mitomycin C contributed to an increase in dendritic cellmaturation and an increase in the expression of co-stimulatory molecules (CD86 and HLA-DR), along with the secretion of cytokines such as IL-12p70, IL-2, and GM-CSF.

Keywords: Mitomycin C, In Vitro study, Dendritic Cell maturation, Interleukin-12 secretion, colorectal cancer

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INTRODUCTION

Chemotherapy is consideredas the preferred treatment method in a selected group of patients with metastatic cancer (1). In addition to being effective in killing cancer cells through several pathways, such as inducing apoptosis, many chemotherapeutic agents are also capable of destroying a wide range of immune cell populations (2). This could, in turn, lead to lymphodepletion and inhibit the function of cell-mediated and adaptive immunity (3). However, evidence suggests that lymphocytes have an impact on the efficacy of chemotherapy. Thus, not all pharmacological therapies can suppress immune cell frequency, facilitate the immunogenic growth of tumors, invoke a tumour-specific immune response, or cause tumour cell tolerance to the immunological reaction (4). One of the most crucial steps in this process is the presentation of tumor antigens by antigen-presenting cells (APCs), and among them, the most specialized and efficient are dendritic cells (DCs) (5). Dendritic cells in their standard, steady-state form are referred to as immature dendritic cells (imDCs).

However, mature dendritic cells (mDCs) possess unique morphological and functional characteristics that facilitate efficient antigen presentation to T-lymphocytes and promote their activation (6). Specifically, imDCs are able to recognize foreign cells and internalize their antigens, triggering their maturation. Upon maturation, DCs travel to the lymph nodes to stimulate naïve T cells and elicit a potent immune reaction against the recognized antigens (7). The major functional difference between imDCs and mDCs lies in the expression of chemokine receptors, which enable DCs to respond to chemokines that mediate their migration to lymphoid tissues. In addition, DCs secret immunomodulatory cytokines, including IL-17, IL-4, and IFN-γ, as well as IL-10, IL-5, and TNF-α, thereby promoting T-lymphocyte activation.

T-cell proliferation and differentiation occur through mitosis, generating mature cells that play critical roles in immune regulation and suppression. Diverse cytoplasmic mechanisms expand the surface area of imDCs, facilitating optimal interaction with foreign cells or pathogens. This is facilitated by the presence of pattern recognition receptors (PRRs) on the surface of imDCs, enabling them to detect and respond to these foreign entities. The receptors can identify molecular patterns associated with infection or tissue damage, which are consistently expressed by either invading pathogens or damaged host cells. These molecular structures are typically referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (8).

Under specific circumstances, such as exposure to UVB rays, anthracyclines, oxaliplatin, or photodynamic therapy, calreticulin (CRT) translocates to the cell surface. Additionally, it could help facilitate intercellular communication between these cells, such as between them and antigen-presenting cells (APCs). Exposure to radiation and anthracycline increases cell surface CRT expression, which enhance the hagocytosis of tumor cells by DCs, including those derived from colon cancer. However, moderate-to-low doses of anti-cancer medications often suppress DC activation and viability.. A new study demonstrated that at low, safe doses, paclitaxel can enhance DC function in vitro.

The low levels of diverse anti-cancer chemotherapeutic agents were found to induce resistance to apoptosis in DCs. Nonetheless, the medications could elicit the activation of small Rho GTPases in DCs, which are reported to play a central role in controlling cell motility, endocytosis, and cell-to-cell interactions (9). Based on the above results, the question arises as to whether the cellular performance of DCs can be directly influenced by the administration of common chemotherapy agents provided in low doses for non-cytotoxic purposes. This study examines the impact of low, non-cytotoxic doses of the anti-cancer agent (Mitomycin C) on DCs

utilizing the HCT116 cell line of colorectal carcinoma that is aggressive and has little or no ability to fully differentiate (10).

MATERIALS AND METHODS

Co-culture of Mitomycin C–Treated HCT116 Cells with Immature Dendritic Cells to Assess Maturation Induction

A reliable *in vitro* assay of cancer stem cells (CSCs) generated by cell lines would have a number of advantages: it would permit high-throughput testing of drug and antibody activity, gene knockdown and re-expression experiments designed to test the functionality of specific CSC properties, and allow the precise identification of the genes that regulate the stem-cell state, an aspect that has so far been difficult to specify in detail (10). In this study, the HCT116 cell line of colorectal carcinoma (VH Bio, Gateshead, UK) was cultured and then incubated with mitomycin C (Sigma Aldrich, Germany) at different doses (10, 30, 50, and 80 nM) for 18 hours. After incubation, the cells were harvested and stored for subsequent co-incubation with imDCs. THP-1 cells (ATCC, USA) were cultured in RPMI 1640 medium (Lonza, UK) supplemented with 10% fetal bovine serum (FBS; Fisher Scientific, UK), 1% penicillin/streptomycin (Lonza), and 50 µM 2-mercaptoethanol (Sigma Aldrich).. Upon reaching a cell density of approximately 1 × 10^6 cells/mL, the cultures were subcultured by centrifugation and resuspended in fresh growth medium at a concentration of 2–4 × 10^4 cells/mL. The medium was refreshed every two to three days using the same centrifugation and resuspension protocol.

Regarding mitomycin C therapy, imDCs were exposed to HCT116 cells pre-treated with non-toxic concentrations (10, 30, 50, and 80 nM) of mitomycin C for 4 days in order to determine whether the compound could promote maturation. To serve as a positive control, lipopolysaccharide (LPS, Sigma Aldrich) at 1 µg/ml was used.

Assessment of dendritic cell apoptosis and necrosis by flow cytometry apoptosis and necrosis of dendritic cells were evaluated using flow cytometry. Cell death was assessed via Annexin V and propidium Iodide (PI) staining. Samples were processed on a Guava flow cytometer (Millipore/Hertfordshire, UK) and analysed using Guava software version 3.1.1. The trypsinized cells were centrifuged and resuspended in in 100 µL of an annexin V binding solution (BioLegend, USA) in a 1.5 mL Eppendorf tube. Alexa Fluor® 647-conjugated annexin V (5 µL, BioLegend) and PI (10 µL, BioLegend) were added, and the cells were incubated in the dark at room temperature for 15 minutes. Flow cytometric analysis was performed using 500 µL of 1x binding buffer. Additionally, Early apoptotic cells were characterized by Annexin V positivity and PI negativity, while late apoptotic or necrotic cells were positive for both Annexin V and PI.

ELISA

The concentration of IL-12p70 in cell culture supernatants was determined using an enzyme-linked immunosorbent assay (ELISA) kit, in accordance with the manufacturer's instructions (BioLegend, sensitivity<1 pg/ml).

Generation of the Immature and Mature DCs

THP-1 cells were centrifuged, resuspended, and maintained at a concentration of 2 × 10^5 cells/mL in a culture medium supplemented with 10% fetal calf serum (FCS). To induce differentiation into imDCs, cells were treated with 500 IU/ml IL-4 and 800 IU/ml GM-CSF (both from PeproTech, London, UK) over a seven-day period, 50% of the culture medium was replaced every two days with fresh medium containing newly added. cytokines.

it has been reported that lipopolysaccharide (LPS) triggers the activation of antigen-presenting cells (APCs) (8). To evaluate the maturation of immature dendritic cells (imDCs), LPS was used as a positive control.

Cells were cultured for four additional days, in the presence of 1 µg/ml LPS to promote their maturation. At the end of the treatment period, the culture plate was harvested, and only non-adherent cells were collectedfor analysis. Flow cytometry was conducted to assess the surface expression of maturation markers on dendritic cells (DCs). The markers analyzed included PE anti-human CD11c (clone: Bu15), PE anti-human CD14 (clone: M5E2), PE anti-human CD86 (clone: BU63) and PE anti-human HLA-DR (clone: LN3). All antibodies utilized in this study were obtained from BioLegend.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 (Inc: San Diego CA, USA) and Microsoft Excel (Microsoft office ltsc professional plus 2021, Microsoft, USA). Comparisons between groups were conducted using the Mann-Whitney U test. Data are presented as mean±standard deviation (SD), and a p-value of<0.05 was considered statistically significant.

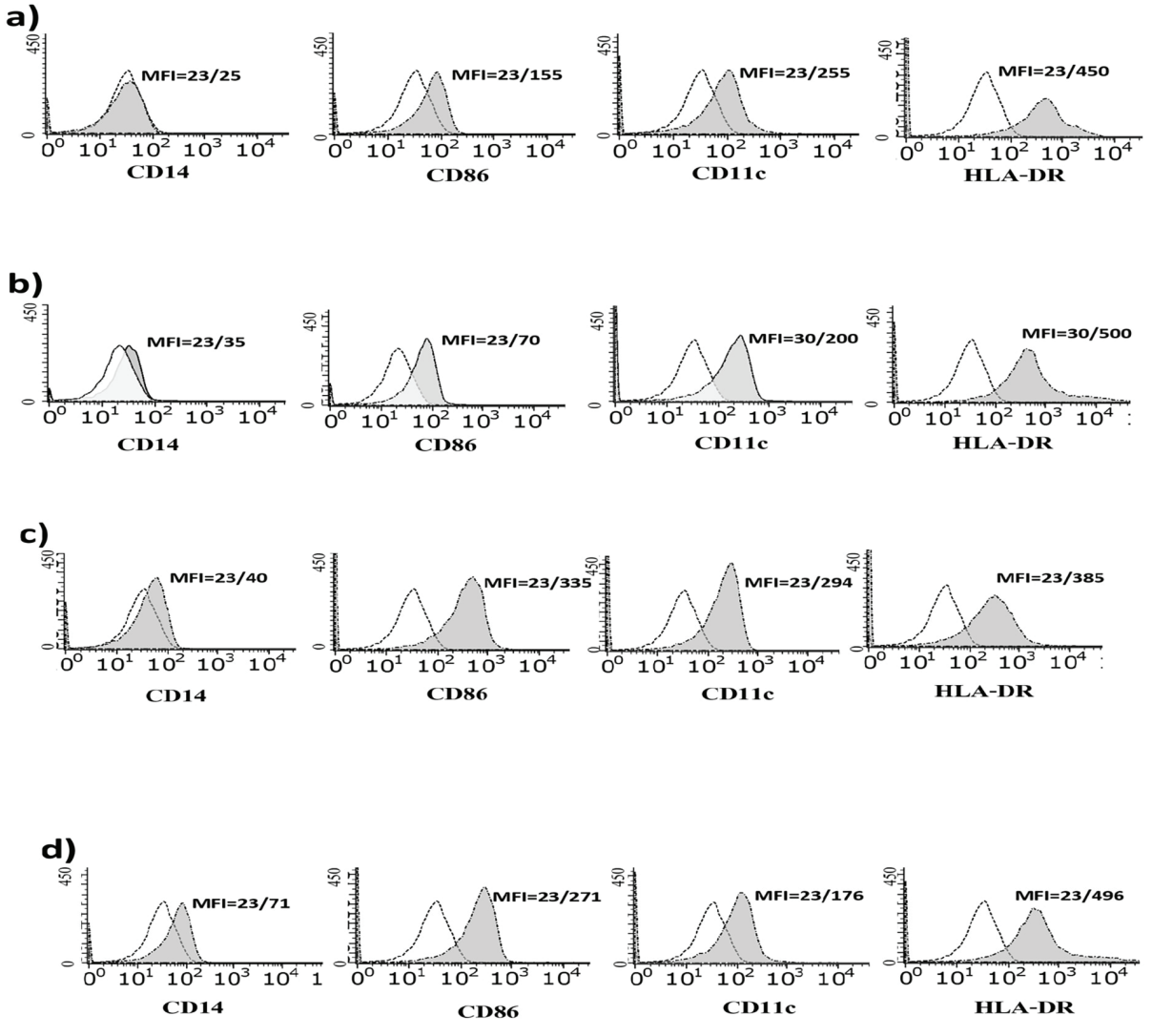

Fig. 1. HCT116 cells were treated with Mitomycin C at concentrations of 10, 30, 50, and 80 nanomolar for 16 hours. Following drug removal, the pretreated cells were co-cultured with THP-1-derived immature dendritic cells (imDCs) THP-1 for 7 days to assess their effect on dendritic cell maturation. Flow cytometry was performed to evaluate the expression of maturation markers including CD14, CD11c, CD86, and HLA-DR. Representative histograms from one of four independent experiments are shown. Each panel depicts the mean fluorescence intensity of the indicated marker, comparing unstained control cells (light grey histograms) and monocyte-derived immature dendritic cells (imDCs) on day 7 (dark grey histograms) after co-culture with Mitomycin C–treated HCT116 cells at the following concentrations : (a) 10 nM, (b) 30 nM, (c) 50 nM, and (d) 80 nM.

RESULTS

The study demonstrated that DC maturation could be induced by co-culture with colorectal carcinoma cells (specifically the HCT116 cell line) pre-treated with a non-toxic concentration of Mitomycin C. DC maturation was assessed based on the expression of surface markers (Fig. 1). The extent of maturation appeared to correlate with the concentration of mitomycin C used to treat the HCT116 cells. While all tested concentrations of mitomycin C (10, 30, 50, and 80 nM) resulted in a notable downregulation in the of CD14 expression, only HCT116 cells treated with higher concentration of mitomycin C, induced a marked expression of maturation markers (Fig. 1).

Effect of Mitomycin C concentration on the maturation of THP-1–derived immature dendritic cells Mitomycin C effectively induced the induction of maturation markers on immature dendritic cells derived fromTHP-1 cells, which was subsequently tested in monocyte-derived immature dendritic cells. Untreated monocyte-derived immature dendritic cells showed lower expression of CD11c, CD86, and HLA-DR compared to those treated with Mitomycin C (Figs. 2a-2c). Notably, the expression of HLD-DR and CD11c increased significantly at 10 nM of Mitomycin C whereas all markers were significantly elevated at the three higher concentrations of mitomycin C. For example, treatment with 80nM Mitocycin C further enhanced phenotypic maturation increasing HLA-DR from 202.3±2.062 to 443.5±9.434, and CD86 from 151.8±2.363 to 271.5±2.380 comparison to 10 nM of mitomycin C (p<0.05, n=4 for all) (Figs. 2 (a, b, c, d)). CD14 expression was markedly reduced across all tested concentrations of Mitocycin C (Fig. 2).

THP-1-derived dendritic cell maturation is accompanied by elevated IL-12 levels Dendritic cells were produced using THP-1 cells as a source in order to compare the functional aspects of dendritic cells derived fromtwo different sources with regard to the levels of IL-12 secreted. The process of differentiating THP-1 cells into immature

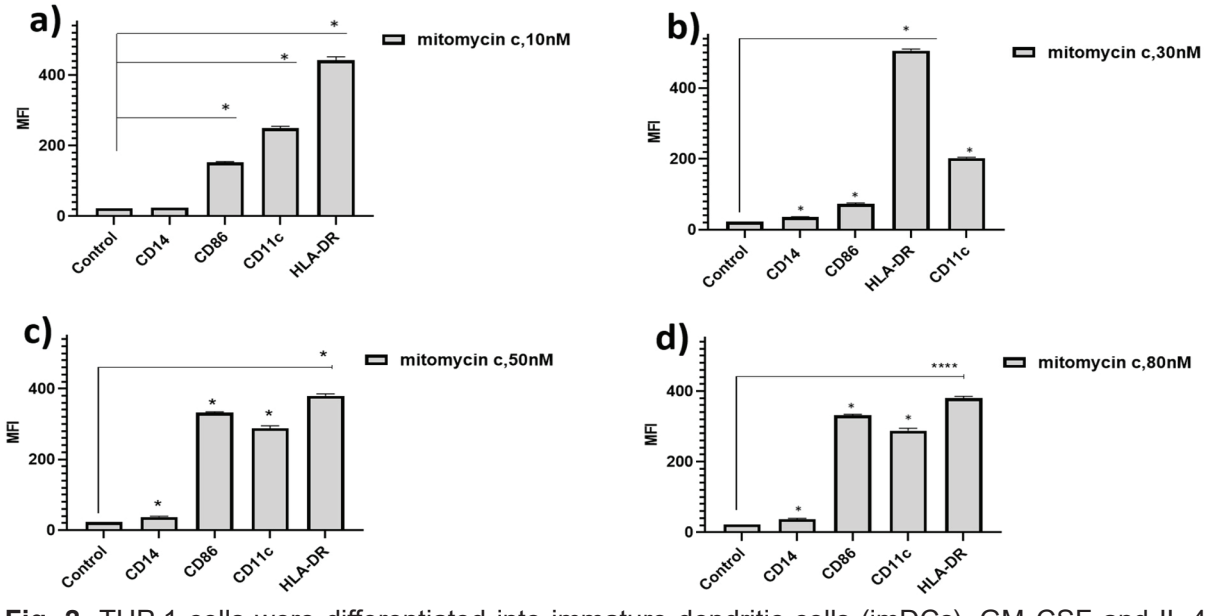

Fig. 2. THP-1 cells were differentiated into immature dendritic cells (imDCs). GM-CSF and IL-4 were added to the THP-1 cells and cells were incubated for 7 days when flow cytometry was used to evaluate the surface molecules of CD11c, HLA-DR and CD86 at baseline (day 0) and after treatment (day 7). All tests included isotype controls and were performed using four different mitomycin C concentrations (a) 10 nM, (b) 30 nM, (c) 50 nM, and (d) 80 nM. The graph displays the proportion of cells that express each marker in the various groups, presented as mean±standard deviation. Statistical analysis revealed significant increases in marker expression (p<0.01) compared to unstimulated cells at day 0 (n=4)

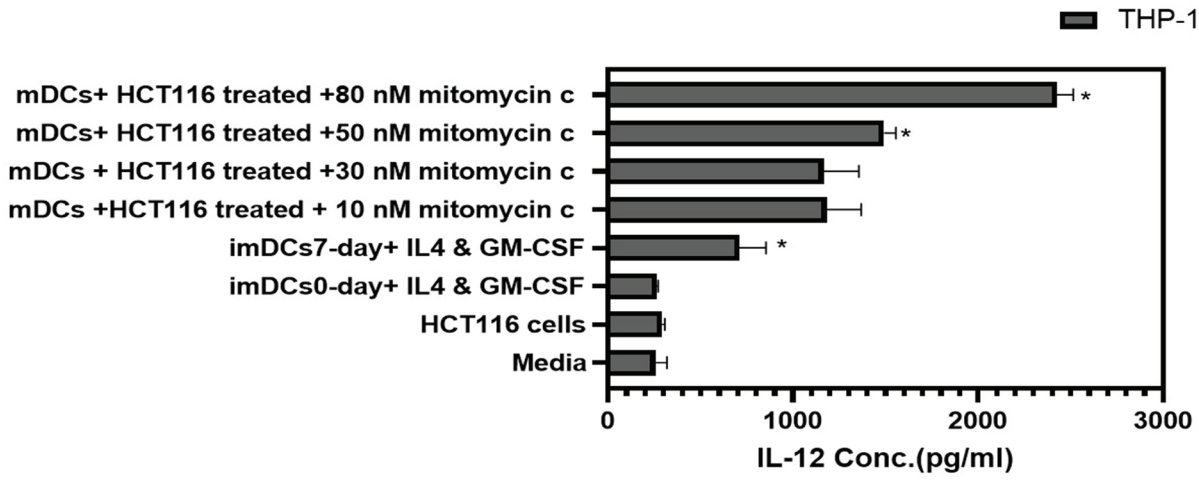

Fig. 3. The co-incubation of HCT116 cells treated with Mitomycin C at varying concentrations with immature dendritic cells (imDCs) derived from THP-1 cells induces IL-12 release. To induce the maturation of THP-1-derived DCs, they were co-incubated with HCT116 cancer cells pre-treated with Mitomycin C (10, 30, 50, and 80 nM). Cultures were incubated at 37 o C for 7 days; supernatants were collected and examined using a commercially available ELISA kit to quantify IL-12 (p40) levels . Data are presented as mean±standard deviation (SD). These values were obtained from three separate experiments, with each measurement performed three times. statistical significance (p<0.05) was observed when comparing treated conditions to both basal media and untreated cells at day 0.

dendritic cells (imDCs) was associated with a significant increase of IL-12 secretion (Fig. 3). IL-12 was found to be 686.7±125.7 pg/ml (day 7 of maturation under the influence of GM-CSF and IL-4) compared to 263.8±4.79 pg/mL (day 0). Dendritic cells derived from THP-1 and treated with mitomycin C at concentrations of 10, 30, 50 and 80 nM showed an increased production of IL-12 although this increase was notstatistically significant note, except at 80nM where there was no further stimulation of this cytokine. Additionally, IL-12 secretion was assessed in immature dendritic cells of THP-1 co-culturedwith mitomycin C-treated HCT116 cells. The HCT116 cell line treated with the highest concentration of Mitomycin C (80 nM) induced a significant cytokine production (from 686.7±125.7 pg/ml to 2445±80.21 pg/ml , p<0.05, n=3, Fig. 3). Although exposure of HCT116 cells to 10 nM Mitomycin C also increased IL-12 secretion, this change was not statistically significant. These results suggest that dendritic cells derived from different sources exhibit distinct responses (Fig. 3).

DISCUSSION

In this study, low, non-cytotoxic doses of the antineoplastic chemotherapeutic agent, Mitomycin C,were investigated for their effects on DCs using the colorectal carcinoma HCT116 cell line as a reference. The results demonstrated that Mitomycin C directly stimulated DC maturation in vitro. This was evidenced by the up-regulation of co-stimulatory molecules on DCs. Additionally, treatment with low doses of Mitomycin C enhanced the expression of IL-12p70 by DCs. Given the limited efficacy of current chemotherapeutic agents in colorectal cancer, there has been growing interest in alternative approaches such as immunotherapy, induction of immunogenic cell death (ICD) and immune-mediated tumor elimination. However, these approaches remain challenging, in part because many cancer cells exhibit low intrinsic immunogenicity (11). ICD relies on the recognition and uptake of cancer cells by professional phagocytes and antigen-presenting cells (APCs), including dendritic cells (DCs), which in turn can activate a T-cell mediated response that generates T

cells proficient in identifying and destroying cancer cells. Enhanced dendritic cell activity has been associated with improved in several cancer types (12). According to recent research, the activation of DCs may be facilitated by the release of "danger" signals (damage-associated molecular pattern, DAMP) from decaying tumour cells, thereby promotingthe engulfment and processing of tumor sntigens (4). A number of DAMP molecules have been identified, including high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), S100 proteins, uric acid, and adenosine triphosphate (ATP) (13).

Nevertheless, chemotherapy has the capacity to mobilize danger signals via dead tumor cells, the cytotoxic agents' doses applied might not be optimal to maintaining dendritic cell viability and functionality. Dendritic cells exhibit sensitivity to doxorubicin within a concentration range of 0.1–50 μM and to vinblastine within 0.025–250 μM (4, 14, 15). Cisplatin and vincristine may induce apoptosis in dendritic cells across various concentrations (16). Our previous work demonstrated that low, non-toxic levels of mitomycin C in a colon cancer model enhanced the antitumor activity of DC vaccines, protected DCs against tumor induced immunosuppression, and promoted DC maturation (17). The immune-stimulating effects of low-dose chemotherapy in vivo may be significantly influenced by the direct actions such as paclitaxel on DCs (18). To investigate this further, the present study aimed to determine whether similar immune-enhancing effects, often referred to as chemo-modulation, classes of chemotherapeutic agents in a colon cancer model. could be observed in vitro on dendritic cells using various classes of chemotherapeutics agents-in a colon cancer model. Similar studies indicated no association between low concentrations of chemotherapeutic agents with dendritic cell toxicity. We built upon these data and evaluated the outcomes of numerous antineoplastic chemotherapy medications on the Ag-presenting capabilities of DCs (19). The THP-1 cell line has been demonstrated to have the potential to be transformed into exceptionally pure monotypic dendritic cells that exhibit the morphological and functional characteristics of embryonic dendritic cells, such as the expression of co-stimulatory maturation markers on the cell surface, in previous research (20).

The THP-1 cell line was used in this study to generate imDCs. There was a dramatic increase in cell surface expression of markers such as CD11c, CD86, and HLA-DR during incubation of 7 days. The findings are consistent with previous reports. As an instance, two cell types, namely the THP-1 cells and the CD34+ leukemia cell line KG-1 have been found to have boosted the expression of CD11c, CD80, CD86, and surface receptors CD40 and CD209 (DC-SIGN) following a 5-day incubation of both cell types at different levels of recombinant human GM-CSF (rhGM-CSF) and IL-4 (rhIL-4). Nevertheless, they did not express CD83 in such conditions (21). This cell line was chosen due to its standardization and reproducibility, though we acknowledge it does not fully replicate primary DC function.

Immature dendritic cells derived from the THP-1 were treated with varying concentrations of Mitomycin C during four days to induce their maturation into mature dendritic cells (mDCs). This treatment resulted in a substantial upregulation of CD11c, HLA-DR and CD86, along with a marked downregulation of CD14. These changes in marker expression were observed on the surface of the resulting mature dendritic cells (14), confirming that dendritic cell maturation can be generated in the in vitro system used in this study. Mitomycin C induced dose-dependent effects that facilitated the maturation of THP-1-derived imDCs. Four-day exposures to 50 and 80 nM Mitomycin C led to significant overexpression of CD11c and HLA-DR whereas treatment with 10 nM Mitomycin C id not induce full expression of all maturation markers.

ELISA measurements of IL-12 further

supported these findings, showing elevated secretion in THP-1 cells stimulated with 50 nM Mitomycin C. These results are consistent with previous findings (15), which confirmed IL-12 secretion through ELISA measurements.. Elevated cytokine secretion commonly seen in mDCs, was similarly observed in this study. The maturation of THP-1- derived imDCs was associated with enhanced IL-12 release, particularly when stimulated with increasing, non-cytotoxic concentrations of Mitocycine C. These findings suggest, for the first time, that Mitomycin C facilitates the differentiation of human dendritic cells with THP-1-derived dendritic cells exhibiting enhanced IL-12 cytokine production. Furthermore, GM-CSF and IL-4 effectively induced differentiation of imDCs from isolated monocytes, resulting in cells with high expression of HLA-DR and CD86, comparable to those generated from the THP-1 cells. These findings are consistent with existing literature. For example, one study reported significant increases in CD14 and CD86 expression in monocyte-derived dendritic cells following treatment with 500 IU/ml IL-4 and 800 IU/ml GM-CSF for five days (16). Changes in the surface expression of key maturation markers were used to assess the maturation of DCs in monocyte-derived imDCs. HLA-DR expression showed a significant increase specifically at a mitomycin C concentration of 30nM. At higher concentrations (50 and 80 nM), the maturation of dendritic cells appeared less effective based solely on HLA-DR expression. However, treatment of THP-1-derived imDCs with 80 nM Mitomycin C resulted in significant changes in the expression of all examined markers (CD11c, HLA-DR, CD86, and CD14), along with increased release of IL-12, a key pro-inflammatory cytokine. These findings support the conclusion that Mitomycin C is an effective inducer of DC maturation. The observation that both THP-1 derived and monocyte-derived dendritic cells exhibit enhanced expression of maturation markers in response to higher doses of mitomycin C suggests the existence of a threshold concentration requiredto effectively induce DC maturation. Interestingly, other studies using lower concentrations,, such as 10 nM, may have underestimated the effective range, as 80 nM was found to be equally or more potent in promoting DC maturation.

During classical immune responses, injured or distressed cells can exhibit both pathogen-associated molecular patterns (PAMPs) derived from microorganisms and damage-associated molecular patterns (DAMPs) originating from the host cells themselves. The initiation of phagocytosis is facilitated by the recognition of these molecular patterns by specific pattern recognition receptors (PRRs) on the surface of imDCs. Dendritic cells then engulf target cells and enter a maturation phase, during which phagocytosed cellular antigens are processed and presented on the cell surface in complex with MHC class I and II. Subsequently, T cell receptors on both CD4+ and CD8+ T cells recognize these antigen-MHC complexes, initiating the differentiation of naïve CD8+ T cells into activated cytotoxic CD8+ T cells. The hallmark features of dendritic cell maturation include the upregulation of co-stimulatory surface molecule both of which are essential for effective T cell activation. A better understanding of the molecular regulation of dendritic cells by chemotherapeutic agents will facilitate the optimization of DC-based immunotherapies either through individual agents or combination treatments.

For example, our recent findings indicate that the conversion of tolerogenic DCs into immunostimulatory DCs may be facilitated by chemomodulation, which enhances their resistance to tumor-induced immunosuppression (17). Chemomodulation of dendritic cell (DC) is a new, a powerful strategy for enhancing the effectiveness of DC-based vaccines and involves modulating DC using non-cytotoxic chemotherapy. Although chemotherapy followed by immunotherapy has been used, the new and highly promising direction is the development

of chemoimmunotherapy, which leverages the direct immunostimulatory propertiesof certain anti-cancer drugs in low, non-toxic doses. In summary, the use of low-dose chemotherapeutic agents with strong immune-stimulating potential represents a promising strategy in cancer treatment.

Limitation of the Study

The strength of this study lies in its methodology specificallythe use of standard laboratory practice cancer stem cell culturing and dendritic cell growth. However, only IL-12 was analyzed which limits the scope of cytokine profiling. and future studies should investigate additional cytokines such as IL-10 and TNF-α. Additionally, the study lacks direct evidence of T-cell activation, which is important when evaluating the immunostimulatory potential of dendritic cells.

CONCLUSION

In vitro Mitomycin C was demonstrated to have a direct effect in stimulating the maturation of DCs. This was associated with the up-regulation of co-stimulatory molecules on DCs. Non-cytotoxic chemotherapy, or chemomodulationrepresents a promising strategy by which the efficacy of DC vaccines can be augmented in cancer patients. In spite of the fact that the concept of combining chemotherapy and immunotherapy is not new, one of the innovative directions involves the development of combinatorial chemo-immunotherapy strategies, which focus on using specific antineoplastic agents with DC-stimulating activity at very low, non-cytotoxic doses. Further research involving other anti-cancer agents may be needed to substantiate this approach.

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AUTHORS' CONTRIBUTION

Trefa Mohammed Abdullah conceived and designed the study, collected and analysed the data, interpreted the results, and drafted the manuscript and approved the final version of the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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