

COMPARITIVE STUDY OF DENDRITIC CELL VACCINE PREPARATION WITH PRESENCE AND ABSENCE OF MALPIGHIA EMARGINATA FRUIT EXTRACT USING TUMOR RNA TRANSFECTION METHOD: A PROMISING APPROACH FOR PROSTATE CANCER

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ABSTRACT

Acerola (Malpighia emarginata DC) is the richest natural source of ascorbic acid and also contains a plethora of phytonutrients such as flavonoids, anthocyanins, carotenoids, and phenolics. By using the fruits of Malpighia emarginata, are used for the treatment of cancer by inducing effective anti-tumor immunity through dendritic cells. Dendritic cells (DC) are the heterogeneous population of antigen-presenting cells that invade tumors. They play an important role in the priming and maintenance of local immunity, and their major function is diminished by some factors encountered in the local environment. For the success of cancer immunotherapy, adequate tumor-specific antigens play a very important role in inducing a tumor-specific immune response by effective delivery of these antigens. In this proposal, by using these strategies, mature and immature dendritic cells were obtained *in-vitro* by adding specific cytokines to monocyte cell culture containing Malpighia emarginata fruit extract in the presence of prostate-specific antigen (PSA), and their results were compared to those obtained without the presence of Malpighia emarginata fruit extract. In the prostate tumor lineage, the RNA is extracted into the cell by electroporation, and the transfection success was measured by immunocytochemistry of the PSA expression level in dendritic cells. For the comparative study of *in-vitro* RNA transcription, this method allows small tumors to be used for dendritic cell vaccine preparation through the activation of DC by in the presence and absence of Malpighia emarginata fruit extract and it is a promising approach for the treatment of metastatic prostate cancer.

Keywords: Malpighia emarginata DC, Dendritic cells, Cancer immunotherapy, Tumor cells, RNA transfection method.

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INTRODUCTION

In the world, the most commonly diagnosed male malignancies are prostate cancer, and it is also the fifth-leading cause of cancer death in men [1,2]. The newly diagnosed and death cases, due to prostate cancer are 1,414,249 and 375,000 worldwide yearly from this disease in 2020 [1-5]. In most of the cases, they did not show any symptoms, but lately, they produce symptoms such as fatigue due to anemia, bone pain, paralysis from spinal metastases, and renal failure appeared. Acerola (Malpighia emarginata DC) also known as Barbados cherry or West Indian Cherry, and they come under Malpighiaceae family. The fruit of this plant is one of the richest sources of ascorbic acid in the world [6]. The evergreen shrub Acerola, which flourishes in warm and tropical climates, bears a small trilobite cherry-like fruit [7]. From April to November, the tree flowers and the fruit mature in 3-4 weeks after flowering. In immature stage, the skin color is green, while at the ripening stage, it changes to an orange-red and finally to a bright red color on maturation. Malpighia emarginata not only contains an exorbitant amount of ascorbic acid but also contains several phytonutrients like carotenoids, phenolics, flavonoids, and anthocyanins [7].

DENDRITIC CELLS

Dendritic cells (DC) are said to be professional antigen-presenting cells, and they are the sentries of the immune system, which includes inducing, sustaining, and regulating T-cell responses [8,9]. During the appearance of a tumor, DC circulates through the blood and migrates to the tumor tissues, where it interacts with malignant cells and is particularly efficient in the uptake of tumor-derived material. And the tumor-derived molecules that activate dendritic cell maturation, such as heat shock proteins and high-mobility-group box 1 protein, as well as pro-inflammatory cytokines are produced by various tumor-infiltrating immune cells. After maturation, the DC migrates from tumor tissue to T-cell-rich areas of secondary lymphoid organs, and then they activate the tumor-reactive CD8⁺ cytotoxic T lymphocytes (CTLs) and

CD4⁺ cells. The CD8⁺ (CTLs) identify effectively and destroy tumors, which release peptides and are derived from tumor-associated antigen (TAA) in the complex with human leukocyte antigen (HLA) class I molecules [10]. Peptides derived from CD4⁺ T cells in the context of HLA class II molecules also play a very important role in anti-tumor immunity [11]. And also, CD4⁺ cells help maintain and regulate the expansion of CTLs by secreting cytokines such as interleukins (IL)-2 and can destroy tumor cells directly. Due to their various antitumor effects, DC is considered a promising candidate for vaccination protocols in cancer therapy [12].

PLASMACYTOID DENDRITIC CELLS (PDC)

In Bone marrow, dendritic cells are considered a multifunctional population [13,14] specializing in the production and secretion of type I interferons (IFNs). In mice, the pDC are expressed as Siglec-H, B220, and Ly6c but with a low amount of CD11c along with a variable amount of CD8 α and CD4. In mice, the periphery regions of pDC are expressed as CC-Chemokine receptor 9, LY49Q, and SCA1 [15,16]. In humans, pDC show plasma cell morphology and express CD4, HLA-DR, CD123, and blood-derived cell antigen-2 as well as Toll-like receptors (TLR) 7 and 9 within endosomal compartments, but CD11c is not expressed [15-17]. pDCs are found in smaller numbers in T cell areas of the spleen, LNs and mucosal-associated tissues, thymus, and liver when they are in a homeostatic condition. pDCs secrete a high amount upon TLR7/9 triggering, such as type I IFN, and produce interleukin-12 (IL-12), IL-6, tumor necrosis factor α (TNF- α), and also some other pro-inflammatory chemokines. pDCs are much less efficient than conventional dendritic cells and they can act as antigen-presenting cells so they can induce an immunogenic response or tolerance.

CONVENTIONAL DENDRITIC CELLS

In conventional dendritic cells they are divided into two populations in both mice and humans: cDC1 and cDC2. cDC1 of the mouse includes

most lymphoid-resident CD8⁺ dc then the tissue-resident and migratory CD103⁺ DC. CD141⁺DC are considered human cDC1 [18]. Both humans and mice have specifically expressed surface markers such as Clec9A [19,20] and XCR1 [21,22], for their development, they require transcription factors such as Basic Leucine Zipper ATF-like transcription Factor 3 (BATF3), IRF8, and ID2 [23]. In mice and humans, the cDC2 are classified as CD11b⁺ DC in mice and CD1c⁺ DC in humans [18] and they depend on some transcription factors like IRF4 and zinc finger E box binding homeobox 2 (ZEB2) [23,24]. Recently, new dendritic cell subsets have been identified, such as AXL and Singlec6, by single-cell RNA sequencing and cytometry by time-of-flight [25-27]. cDC is one of the most powerful antigen-presenting cells, and as such, they are strong inducers of T cell-mediated immune response. In mice cDC1 is highly active in antigen cross-presentation and is also a strong producer of IL-12 that drives the polarisation of activated CD8⁺ T cells into CTLs [28]. Mouse cDC2 are specialized in MHC II presentation and the stimulation of CD4⁺ T cell responses, which include Th1, Th2, and Th17 cells [18]. Human cDC1 and cDC2 are equally potent in MHC II presentation and priming CD4⁺ T cells [18], and also human Axl⁺ DC are the potent inducers of CD4⁺ and CD8⁺ T cells in allogeneic cultures [25,27].

DENDRITIC CELL-BASED IMMUNOTHERAPY FOR PROSTATE CANCER

The induction of innate and adaptive antitumor immune response dendritic cells plays a very important role, and they also act as attractive candidates for vaccination protocol in cancer therapy. An animal model proves that TAA presenting dendritic cells are able to induce protective and therapeutic antitumor response [29,30]. In clinical trials, report says that B-cell lymphoma or renal cancer patients disclose promising immunologic and clinical responses of TAA-loaded DCs which is administered as a vaccine against cancer [31-34] (Fig. 1).

DCs have a capacity to induce and regulate T-cell responses and have been revealed as promising candidates for vaccination strategies in prostate cancer therapy. DC is loaded with the tumor-associated antigen are prostate cancer-associated antigen derived peptides, RNA or protein and with their high surface expression of HLA-peptide-complexes and costimulatory molecules, the dendritic cells activate and expand CD8⁺ CTLs and CD4⁺T cells effectively. From this, CD8⁺CTLs are able to recognize and also destroy tumors. CD4⁺T cells also increase the capacity of DCs to stimulate CTLs through the interaction between CD40 on DCs and CD40 ligand on activated CD4⁺T cells, and they also maintain and regulate the expansion of CTLs by secreting cytokines and are able to eradicate tumor cells directly [35].

FLAVONOIDS EXTRACTION FROM MALPHIGIA EMARGINATA

Flavonoids are an important secondary metabolite that is a natural organic compound that is produced during the long process of natural

selection. They are mostly found in the root, stems, leaves, flowers, and fruits. Malphigia Emerginata fruit contains several important phytoconstituents such as carotenoids, phenolics, flavonoids, and anthocyanins [7] and possesses numerous biofunctional properties. Flavonoids possess various pharmacological activities such as antitumor, antibiosis, antiviral, anti-inflammatory, and so on.

EXTRACTION

For the extraction of flavonoids from Malphigia Emarginata fruit, ethanol and methanol are widely used, and the common methods of extraction include dipping, percolation, reflux, and so on. An alcohol of high concentration (90-95%) is applied to extract free flavonoids.

VACCINATION WITH EX-VIVO PULSED DENDRITIC CELLS (DC) AND THEIR HISTORY

Although DCs are general presence in most tissues, their total number is low. An *ex vivo* derivation of dendritic cells improves, and multiple preclusion cells can be used to prepare dendritic cells, such as nonproliferative and proliferative in this nonproliferative CD14⁺ monocytes from Peripheral blood and the proliferative CD34⁺ cells from bone marrow and umbilical blood [36,37]. CD14⁺ monocytes develop ~10% of peripheral blood mononuclear cells, and the dendritic cells derived from peripheral blood monocytes (MoDCs) have been broadly studied and applied. In 1994, Sallusto and Romani developed a method for the induction of dendritic cells from monocytes by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. GM-CSF helps to promote the subsequent development of dendritic cells, while IL-4 suppresses the rapid increase of macrophages and granulocytes and also prevents the differentiation of monocytes towards macrophages [38,39]. After 2 years later, Romani and Zhou made an improved protocol study by in which they obtained the immature dendritic cells after induction for 6-7 days with GM-CSF and IL-4 and then developed the mature dendritic cells after induction for 3 days with an activating factor such as TNF- α . This method is one of the first successful efforts to replace Bovine Serum with human plasma in culture, which laid the foundation for the clinical application of *ex vivo*-derived dendritic cells [40,41]. But now-a-days they have explored more *ex vivo* derivation protocols of MoDCs, such as replacing IL-4 with IL-15 r IFN- α in the presence of GM-CSF to support the activation potency of DCs [42-44]. MoDCs cannot be generated *ex vivo* as a result, their application is something limited. CD34⁺ Hematopoietic Stem Progenitor Cells (HSPC) can be used to synthesize DCs in large amounts *ex vivo*. This CD34⁺ is superior to MoDCs in that it stimulates a more potent T-cell immune response against cancer, and these are produced by upregulating the expression of tumor necrosis factor-relevant apoptosis-stimulating ligand and enhancing cytotoxicity [45,46]. Derivation of strong CD34⁺ was typically successfully achieved in a cytokine milieu but it differs as compared to MoDC derivation. The first documented combinations of formulations include as fm-relevant tyrosine kinase 3ligand (flt3L), thrombopoietin (TPO), and stem cell factor (SCF). Before switching to culture in the presence of Flt3L, TPO, and SCF for 1 week, they were alternatively culturing in the presence of Flt3L, SCF, IL-3, and IL-6 for 3 weeks, which worked similarly well [47]. The inclusion of Notch ligand Delta-like 1 (DLL1) in the original combination of formulation that is GM-CSF, Flt3L, SCF significantly improved the stimulation of type 1 conventional cDC1s because they specialize in priming CD8⁺ CTLs from CD34⁺HSPC is recently reported [48].

IDEAL DENDRITIC CELL VACCINATION

Preclinical and clinical studies have shown that for optimal stimulation of tumor-specific T cells, DC Vaccine should express three major qualities. First is the ability to migrate to lymph nodes; which organize an optimal environment for T-cell activation, second is longevity; for activation and expansion of a tumor-specific T-cell response, the DC must maintain its nature phenotype in the lymph node for sufficient time, and this response is able to eliminate tumor, third is that dendritic cell must steadily present TAA, and this process may be easier to achieve with vector-transduced or transfected DC.

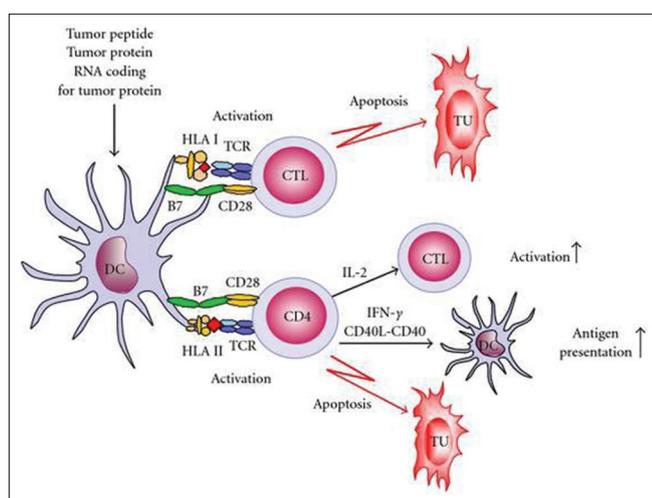


Fig. 1: DC-based immunotherapy strategies for prostate cancer

MATURATION

During DC maturation, they are characterized phenotypically by the high expression of membrane-bound co-inducing molecules: CD80, CD86, CD83, and MHC class II molecules and half-lives of peptide-MHC complexes, secretions of T-cell inducing chemokines and cytokines [49] and these complex processes occur at a time when proinflammatory cytokines and pathogen-associated molecular patterns are produced based on microbial structure or cellular stress. If the dendritic cell is not appropriately matured, their activity may induce tolerance rather than immunity. DCs in their immature state are highly phagocytic, and they are retained in their tissues in a resting state, where they present only healthy self-proteins and are tolerogenic. When the immature DCs are administered by subcutaneous injection, they elicit antigen-specific suppressive responses [50].

MIGRATION

When stimulation occurs, such as with TLR ligands or cytokine cocktails, the maturation process is started, and the DC downregulate the expression of chemokine receptors such as CCR6 and CCR2 which direct them to sites of inflammation, and rapidly up-regulate the regulation of CCR7 [51,52] which direct the migration of mature DCs into the lymph node through its ligands CCL19 and CCL21 [53]. Hence, mature antigen-presenting DC encounter resting nave and memory T-cells that also produce CCR7 and circulate through lymph nodes, where they replace CCL19-producing mature DCs. These results produce specific T-cell proliferation and the initiation of that adaptive immune response [54,55]. Both clinical and preclinical studies report that the magnitude of T-cell response relates with the ability of DC to migrate to the lymph node and to the maturation state [56-58].

DENDRITIC CELL VACCINE PREPARATION

Clinical data from monocyte-derived DC vaccines

For dendritic cell vaccination, Mo-DCs are the important source of DC, which are generated from *ex vivo* allogeneic CD14+ monocytes [59]. Leukemia-associated antigens (LAAs) are loaded in Mo-DCs [60]. For LAAs loading in Mo-DC, there are three antigens used, such as Wilms Tumor 1 (WT1), Preferentially Expressed Antigen of Melanoma (PRAME), and Human Tolerance Reverse Transcriptase (HTERT) [59,61]. They are loaded with whole apoptotic leukemic cells, leukemia lysates or leukemic cell-derived mRNAs [59,62,63]. These antigen-loading Mo-DCs are re-administered to acute myeloid leukemia (AML) patients in intradermal or intravenous DC vaccination [59,61]. Due to several blood draws occurring some variability within the same individual, and this can be avoided by using cryopreservation of Mo-DC can be a good method to preserve the cells before use in immunotherapy [64] (Fig. 2).

CLINICAL DATA FROM LEUKEMIA-DERIVED DC VACCINES

In AML and MDS (Myelodysplastic Syndrome), the DC can be produced directly from DCleu (leukemia-derived DC) after culture with different combinations of modifiers [65-67]. For the generation of DCleu different protocols have been developed [68], and their morphology is similar to that of typical DCs. Unique characteristics of DCleu include stronger antigen-presenting capability, stronger *ex vivo* antileukemia immune response, and increased costimulatory molecule expression [69]. The *ex vivo* production of DCleu and Mo-DC from leukemic blood cells for vaccination is a challenging process. In the generated DCleu, their confirmation methods include western blot, immunophenotyping, and fluorescence in situ hybridization with chromosome-specific DNA probes to detect leukemia-specific numeric or structural chromosomal abbreviations [70,71], and also a special method such as flow cytometric gating strategy has been developed. After DCleu production can be detected, patient-specific blast staining antibodies are incorporated with some specific dendritic cell staining antibodies, and some specific antigens that are expressed on leukemic blasts, and this is only applicable when DCleu generation can be detected. After the DC populations are cultured, they are further divided into different subpopulations, such as leukemia-derived dendritic cells, nonleukemia-derived DC and nonconverted blasts [71]. This report demonstrated that only mature DCleu can activate immune reactive cells, and these express chemokine receptor 7 (CCR7), which is important for the migratory capacity of DCleu [69,72], and also that the mature DCleu also express CD83 and secrete IL-12 [73].

DC VACCINES IN NONLEUKEMIA MALIGNANCIES

Prostate cancer

Patients who have high-risk prostate cancer can experience relapse, which produces a noncurative disease. In clinical trials, vaccines targeting TAA have been applied for prostate cancer treatment. There are different types of vaccines, including DC based (e.g. Sipuleucel-T), and peptide or gene-based (e.g. DNA/RNA) that have been applied as auxiliary therapy in patients with prostate cancer [74]. Although the initial success with Sipuleucel-T and further dendritic cells vaccines failed to progress. To improve the efficacy of vaccination, developing antigen loading and presentation technologies, such as nanoparticles, antibody-antigen conjugates, and virus codelivery systems [75]. In the phase I trial it was shown that an antigen-loading autologous dendritic cell-based vaccine for advanced prostate cancer produced by *in vivo* activation of inducible CD40 that produces immune upregulation and antitumor activity decreases the prostate-specific antigen (PSA), objective tumor regression, and has strong efficacy for post-trial therapy [76].

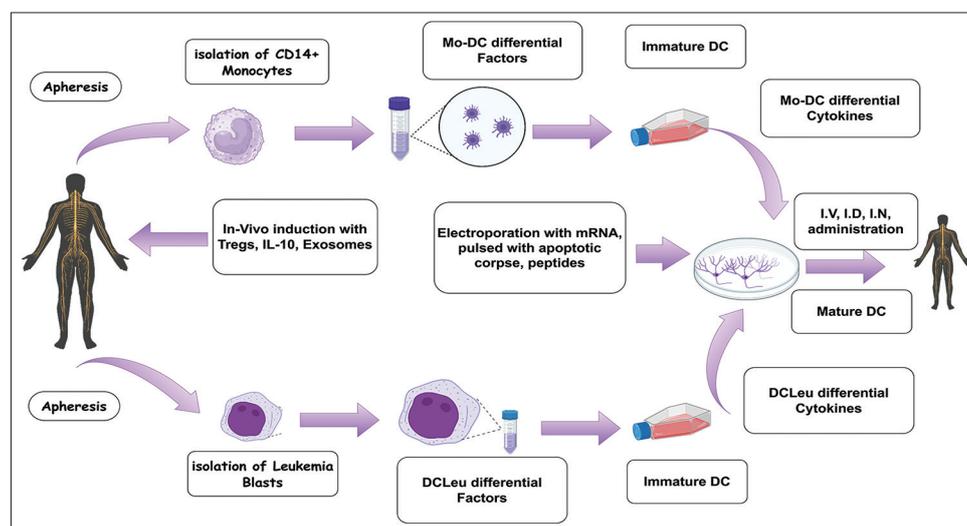


Fig. 2: Procedure for dendritic cell vaccine preparation for hematological malignancy

METHODS FOR VACCINE DELIVERY

A kind variety of methods to deliver DC-based vaccines to patients such as intravenous [77-79], Intradermal [80,81], and less usage of Intranodal [77,82] and Intratumoral routes [83-86] as well as dendritic cell induction [69] by *in vivo*. For effective sensitizing T cells, currently, there is no consistency as to which route of administration is best. Depending upon the route of administration, the antigen-loaded DC can prime T cell immunity, but the quality of the response and induction of antigen-specific antibodies may be different [84]. Antigen-pulsed DC are administered intravenously, and subcutaneous administration of immature DC has been considered an effective method for generating sensitized T cells [87,88]. In the mouse model, the comparisons of subcutaneous or intravenous immunization, with Intranodal injection of peptide-pulsed DCs showed that the latter effectively induced greater expansion of antigen-specific T lymphocytes in the spleen and also a stronger antigen-specific Th1-type response, as a result, Intranodal administration of DC vaccination was an effective and feasible method [89]. In advanced melanoma patients, the vaccination by Intranodal administration of semimature DCs produces a strong, long-lasting CD4+ T cell response with a Th1-type cytokine profile [90].

CHALLENGES OF DC VACCINES

Despite the fact that much improvement has been made in the field of DC vaccines, there are still several challenges to a wider application of leukemic DC vaccines. In a previous experiment report, it was noted that failure to generate enough qualified AML-DCs was the most common reason [91], and another reason was the high cost of stimulants required to differentiate leukemic DC [92]. A critical lesson was learned, however, due to the immunosuppressive effect of GM-CSF, which produces insufficient therapeutic efficacy of vaccination using genetically modified GM-CSF-secreting leukemia DC [93]. Due to the immunosuppressive effect of phosphatidylserine produces a lack of immunogenicity of the whole leukemia cell vaccine causing inactivated immune-responsive T cells [94]. Immunosuppressive effects from malignant cells can inhibit the function of both DC and T cells and inhibit the vaccine-generated protective immune response. These factors bring additional challenges and highlight the systemic immunosuppression and malfunction of DC [95]. Many studies reported that different mechanisms of weak immunogenicity of DCs, including failure to stimulate or activate the NK cells [42,96-98], failure to inhibit the immunosuppressive action, and undesirable effects of Tregs and MDSCs [99,100].

CONCLUSIONS AND FUTURE DIRECTIONS

The development and success of DC-based immunotherapies have been affected by several factors, such as the immunosuppressive effect produced in the tumor microenvironment, the limited capacity of systemically administered DC, and the low avidity of TAA-specific T cells. The rapidly enhancing knowledge about dendritic cell subsets and the tumor-stimulated suppressive effect must be exploited to design novel and improved vaccine therapy in cancer. These limitations also include weak cellular immune response, not economic and also time-consuming process. The DC vaccine will certainly confide on combination therapies. In addition to DC vaccination will combined with Malpighia Emarginata fruit extract of flavonoids compound, systemic monoclonal antibody, and immune checkpoint blockades can enhance DC-mediated activation of NK cells and prevent the stimulation of Tregs. In this study, we believed that the use of carefully designed dendritic cell vaccination with Malpighia Emarginata fruit extract of flavonoids compound can enhance the DC activation because these fruits are rich in antioxidants and these adjuvant antioxidants can further improve DC vaccination and this strategy may produce a promising approach in patient have prostate cancer.

COMPETING INTEREST

The author declare that there is no competing interest

AUTHOR'S CONTRIBUTION

The corresponding author collected all the study material, analyzed it, and prepared the complete manuscript.

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Nil

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I hereby acknowledge that I prepared the project. And all the details are correct to the best of my knowledge.

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