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Diospyros peregrina Fruit Preparation Mediated Immunomodulation of Lymphocytes Isolated from the Blood of Breast Cancer Patients

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ABSTRACT

Background: Breast cancer is an uncontrolled growth of epithelial cells. The loss of BRCA1 (Breast Cancer gene1) activity due to mutation or down-regulation of gene expression promotes tumorigenesis and increases the risk of breast cancer.

Objectives: Our focus was to pulsate lymphocytes of breast cancer patients and normal individuals, using *Diospyros peregrina* fruit preparation (DFP) to study the cancer-protective immunity, and the signal transduction processes involved with it. We also investigated the role of DFP in the release of lymphocytic nitric oxide (NO) which is a crucial tumor-destroying representative, identified to control cytokine production, cell signaling, apoptosis, and T-cell proliferation.

Methods: Using Ficoll-Hypaque gradient centrifugation, lymphocytes were isolated from the blood of 12 patients and 12 normal individuals. Cells were treated with or without DFP (2.5 µg/ml) for 48 hours. Both non-stimulated and stimulated cells were then subjected to MTT assay and NO release assay; following which qPCR was performed to estimate mRNA levels and percentage enrichment of certain genes.

Results: DFP stimulates lymphocytic proliferation (P=0.0118) and release of NO (P=0.01) significantly. DFP also noticeably enhances the manifestation of T helper (T_H) cell 1 specific interferon-gamma (IFNG), interleukin 12 (IL12), T-box transcription factor TBX21 (TBX21), and signal transducer and activator of transcription 1 (STAT1) genes. DFP treatment significantly increases tumor protective immunity by decreasing the expression levels of T_H2 network-specific GATA3 and interleukin 4 (IL4) genes but increasing the expression levels of T_H1 network-specific IFNG, IL12, TBX21, and STAT1 genes.

Conclusion: DFP increases the expression levels of TH1 specific network genes which in turn help in evoking tumor protective immunity.

Keywords: *Diospyros peregrina* fruit preparation (DFP), Nitric Oxide (NO), T helper (T_H) cell, Breast Cancer

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INTRODUCTION

The tumor arises mainly from the luminal epithelial cells in breast tissues that indicate both the channels and milk-producing lobules, and seldom from the outer portion of basal cells. It has been observed that breast cancer has been increasing at an alarming rate and would continue to do so shortly as well. BRCA1 and BRCA2 are 2 distinct genes that have been found to influence a person's chances of generating breast cancer. They are the two utmost conjoint genes that are autosomal dominant and have a great permeating form of breast cancer and ovarian cancer. Abnormally regulated and decreased expression of BRCA1 also subsidize irregular forms of breast cancer (1, 2). The key tumor defeating role of BRCA1 is related to the maintenance of genomic integrity by controlling DNA replication, repair, and transcription, furthermore to various cell cycle checkpoints that safeguard the persistence of healthy cells (3). Since BRCA1 and BRCA2 are tumor suppressor genes, women with mutations in BRCA1 and BRCA2 have a lifetime risk of developing the disease which can range as high as 87%.

The genus *Diospyros* comprises 240 species, 59 of which are dispersed in India, Thailand, Japan, Nigeria, South Africa, and the Philippines (4, 5). *D. peregrina* is a plant native to India. Apart from its traditional usage for the management of dysentery and menstrual complications, diverse parts of the plant are of different healing values (6-8). It has previously been described that *Diospyros peregrina* has antitumor action on Ehrlich Ascites Carcinoma in mice (4), and antitumor and antioxidant activities against Dalton's ascites lymphoma in rodents (9).

Cell development and differentiation are controlled by STATS (signal transducers and activators of transcription), a family of transcription factors. Until phosphorylation is finished by receptor-associated kinases, their activity remains suppressed (10). A significant figure of information from cell lines, mouse models, and human tissues presently includes these transcription factors in the oncogenesis of breast cancer (11). Signal transducer and activator of transcription-1 (STAT1) contribute a part in the DNA damage, cytokine responses, transduction of stress, and activation of B and T cell immune responses. STAT1 is a significant signaling preparation in stress responses and is the main transducer of signals from the interferon (IFN)-γ receptor (12). Th1 cells, described by secretion of IFN-γ and tumor necrosis factor (TNF)-α, are essentially in charge of enacting and managing the improvement and constancy of cytotoxic T lymphocyte (CTL). Likewise, antigen-presenting cells (APC) are activated by Th1 cells and turn on constrained production of antibodies that can ameliorate the engulfment of infected cells as well as tumor cells into APC (13). IL-12 is a hetero-dimeric cytokine that has strong effects on innate and adaptive immunity. IL-12 excites in vitro antitumor action of lymphocytes in cancer and in vivo antitumor activity in numerous murine tumor models (14).

MATERIALS AND METHODS

Diospyros Peregrina Fruit Preparation (DFP)

Diospyros peregrina fruits (same size and color, indicative of same age) were acquired from a standard source and finally shade-dried and ground properly. 2.5 mg of *Diospyros peregrina* fruit powder was soaked in 1 ml of phosphate-buffered saline (PBS), pH 7.4 [Sisco Research Laboratories Pvt. Ltd (SRL), India] overnight. The next day it was centrifuged at 1500 rpm and the supernatant was collected which is termed as *Diospyros peregrina* fruit preparation (DFP). For in vitro uses this DFP solution was membrane (0.22 mm) (Avantor, India) filtered. In vitro treatment of 1 ml of cell culture, only 2.5 mg of DFP was considered for further experiments because of the best immunostimulation property among other doses (0.5mg/ml, 1.5mg/ml, 2.5mg/

ml) examined. Limulus Amebocyte Lysate (LAL) test was used to monitor the endotoxin content of the freshly prepared DFP as per protocol (Salesworth India, Bangalore). The endotoxin content of all the batches of DFP was discovered to be under 5pg/ml.

Screening of the Subjects and Collection of Samples

Screening of the control and patients was done in either the outpatient department (OPD) and/or ward of the SRM General Hospital. To achieve the target study population we screened 12 patients suffering from breast cancer and 12 as control subjects. The age of the patients and control subjects was more than 18 years with no comorbidity. The patients had no prior history of either radiotherapy or chemotherapy. Clinician investigators have chosen the controls and patients from the outpatient department and/or ward for the current project for documentation and sample collection purposes. The controls and the patients were gotten up outpatient office as well as a ward by clinician-examiners of the current venture for documentation and test assortment purposes. The clinician-investigators of the current research proposal collected 2-5 ml of blood from the aforementioned human subjects for molecular studies involved in the current research project only after the subjects had given their informed consent as per the Institutional Ethical Committee guidelines.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Density gradient centrifugation of heparinized venous blood was done to isolate peripheral blood mononuclear cells (PBMCs) from healthy contributors and breast cancer patients. 2 ml of blood was diluted with 2 ml of 1X phosphate buffer saline (PBS). 2 ml of Ficoll-Paque (GE Health Care, USA) was taken in a falcon tube and 4 ml of diluted blood was layered on it. It was centrifuged at 2200 rpm for 30 minutes in a fixed-angle rotor without a brake. The ring-like foggy layer of PBMCs formed at the Ficoll-plasma interface was directly withdrawn using a micropipette and transferred to a new falcon tube. It was washed with 1ml of PBS and centrifuged at 1500 rpm for 10 minutes and the supernatant discarded.

Treatment with DFP

The pellet of PBMCs derived lymphocytes were resuspended in Roswell Park Memorial Institute (RPMI 1640) (Hyclone, USA) media containing 10% fetal bovine serum (FBS) (Hyclone, USA), 10 µg/ml of penicillin , and streptomycin (Hyclone, USA) and transferred to a cell culture plate and kept at 37^0C in a humidified atmosphere with 5% CO2. 1 X 10^6 lymphocytes were cultured in complete RPMI-1640 medium in the presence or absence of DFP (2.5 µg/ml) for 48 hours.

Lymphocytic Proliferation Assay Using MTT

DFP treated and non-treated lymphocytes were used for lymphocytic proliferation assay using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (SRL, India). In each well, 20 µl of MTT solution (5 mg/ml) were mixed and incubated in dark for 4 hours at 37^0C. Purple-color formazan crystals were formed and 100 µl dimethyl sulfoxide (DMSO) (SRL, India) were added to each well. Absorbance was measured at 540 nm using a microplate reader and obtained absorbance was directly proportional to lymphocyte proliferation (15).

NO Release Assay

A colorimetric assay based on Griess reaction was used to quantify the accumulation of nitrate in culture media. In short, lymphocytic culture supernatants were aliquoted (50 µl) and incubated with an equal volume of Greiss reagent [1% sulphanilamide (SRL, India), 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (SRL, India) and 2.5% ortho-phosphoric acid (SRL, India)] at room temperature for 10 min. Based on absorbance at 550 nm concerning the sodium nitrite absorbance standard curve,

NO concentration was determined (15).

Quantitative Real-time PCR for mRNA Estimation

PBMC originated lymphocytes were isolated from breast cancer patients and normal individuals by density gradient centrifugation followed by total RNA extraction utilizing the Quick-RNA MiniPrep Kit (Zymo Research). It was then converted to cDNA exhausting the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA Samples, as well as templates for quantitative PCR (qPCR) analysis, were done on the CFX-Connect Real-Time PCR System (Bio-Rad) using Perfecta qPCRSuperMix, carboxy-X-rhodamine (ROX) (Quanta Bioscience) , and TaqMan Gene Expression Assay (Thermo Fisher Scientific, USA) for some representative genes. The Ct values were then transformed into absolute copy numbers with a cloned DNA plasmid standard dilution curve, as formerly described (16-18).

Quantitative Chromatin Immunoprecipitation (ChIP)-qPCR Assay

Chromatin immunoprecipitation (ChIP) assays were done with micrococcal nuclease (MNase) (Active Motif, USA)-digested chromatin isolated from the breast cancer patients' and normal subjects' lymphocytes after fixing protein–DNA interactions with 1% formaldehyde (Sigma-Aldrich, USA) as described previously (16-18). In brief, ChIP-ranked antibodies and their isotype IgG control antibody were used to pull down chromatin. ChIP samples were utilized as templates for the RT-PCR examination and the obtained Ct values were transformed into absolute copy numbers with a cloned DNA plasmid standard dilution curve. Nonspecific signals obtained with control IgG-ChIP were deducted from the test samples.

RESULTS

Lymphocytic Proliferation Assay Using MTT

To determine the stimulatory effect of DFP, lymphocytic proliferation assay using MTT was performed for various concentrations of DFP with negative and positive controls (e.g. NT and PHA) (Figure 1). Optimum lymphocytic proliferation was observed with a 2.5 mg/ml concentration of DFP. MTT assay was also performed to check the effect of DFP on lymphocytic proliferation in both normal and breast cancer patient samples. The results suggest that DFP stimulates lymphocytic proliferation in breast cancer patient samples also along with the normal subjects (Figure 2). Higher proliferation is very beneficial in immune response as a greater number of lymphocytes would target the cancer cells, boosting the immune response and tumor clearance.

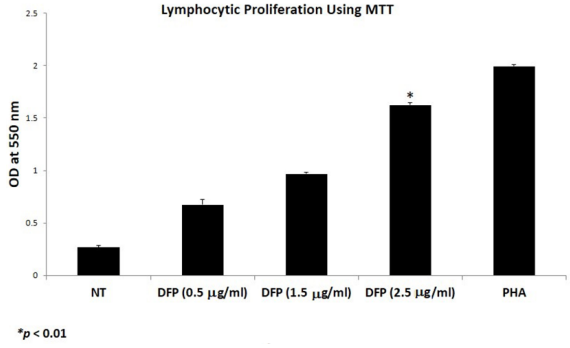

Figure 1. Peripheral blood mononuclear cells were used to isolate Lymphocytes from healthy donors (with their informed consent) and cultured in the absence or presence of different concentrations of DFP for 48h and their proliferation was assessed by MTT assay. Phytohaemagglutinin (PHA) was used as a positive control. Error bars shown as standard error (SE) are obtained from five individual experiments (*P<0.01).

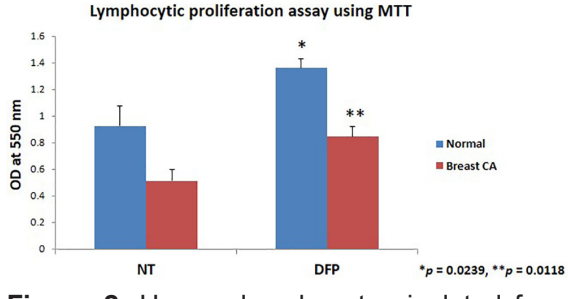

Figure 2. Human lymphocytes isolated from both breast cancer patients and normal subjects were cultured for 48h in the presence or absence of DFP (2.5 mg/ml) and their proliferation was assessed by MTT colorimetric assay.* P=0.0239 and ** P=0.0118 in comparison to NT (non-treated). MTT assay results suggest that DFP stimulates lymphocytic proliferation even in the breast cancer patients' lymphocytes.

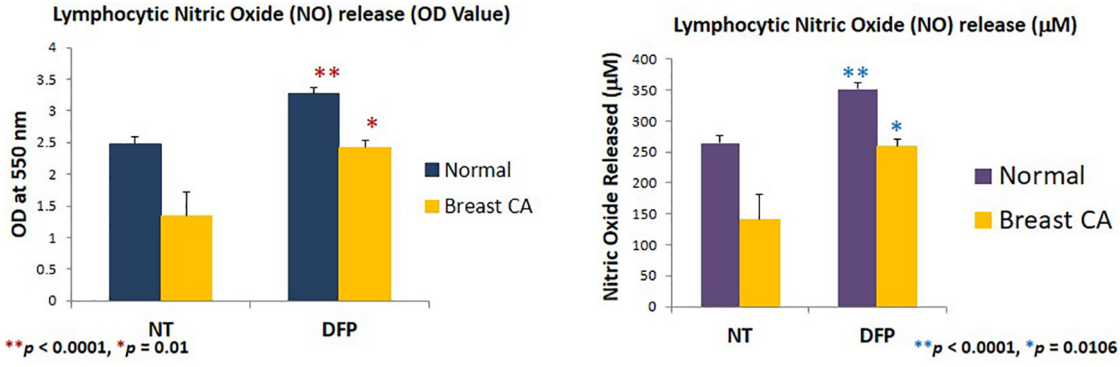

Figure 3. Nitric oxide released by human lymphocytes isolated from both breast cancer patients and normal subjects were cultured for 48h in the presence or absence of DFP (2.5 mg/ml) and nitric oxide (NO) release was accessed by a colorimetric assay based on Griess reaction. ** P<0.0001 and ** P=0.01 in comparison to NT (non-treated). The NO release assay results suggest that the production of NO is high when the lymphocytes were treated with DFP even in the breast cancer patients' lymphocytes.

Table 1. RT-qPCRPrimers and Probes for mRNA estimation

Gene (AssayID)	Exon Boundary	Chromosomelocation
IFNG (Hs00989290_g1)	Ex2-3	Chr.12: 68548550 - 68553521
IL12RB2 (Hs02558264_s1)	Ex16-16	Chr.1: 67773047 - 67862583
TBX21 (Hs00894392_m1)	Ex3-4	Chr.17: 45810610 - 45823485
STAT1 (Hs01013996_m1)	Ex 17 - 18	Chr.2: 190969036 - 191014250
GATA3 (Hs00166001_m1)	Ex4-5	Chr.10: 8045421 - 8075201
IL4 (Hs00929861_g1)	Ex1-2	Chr.5: 132673986 - 132682678
GAPDH (Hs00266705_g1)	Ex2-3	Chr.12: 6643657 - 6647536

NO Release Assay

To check the activation of the lymphocytes, their ability to produce nitric oxide was tested as it is characteristic of activated lymphocytes to release nitric oxide. As nitric oxide has a short life expectancy and is promptly oxidized to nitrite, it is by implication estimated by assessing the convergence of nitrite utilizing Griess reagent. NO release assay results suggest that nitric oxide production is highest in the case of DFP treatment both in the case of normal as well as breast cancer patient samples (Figure 3). Our data suggest that the DFP treatment increases the release of lymphocytic nitric oxide (NO) which is a crucial tumor-destroying representative, identified to control cytokine production, cell signaling, apoptosis and T-cell proliferation.

Estimation of mRNA Level of Candidate Genes Using RT-qPCR

For RT-PCR assays, total RNA prepared using Quick-RNA^{TM}MiniPrep (Zymo), was utilized to synthesize cDNA, and the samples were used as templates for qPCR analysis for the specific genes (Table 1). Normalized gene expression [2^(- ΔCt)] in the test sample is divided by the normalized gene expression [2^(- ΔCt)] in the control sample to calculate fold changes [2^(- ΔΔCt)]. Normalization was performed using Ct values derived for GAPDH from the corresponding test sample. Our data suggest that DFP treatment in the lymphocytes of both the normal subjects' and breast cancer patients' samples decreases the expression levels of GATA3 and IL4 genes but increases the expression levels of IFNG and IL12, TBX21 ,and STAT1 genes which are mainly associated with tumor protective immune response (Figure 4).

Estimation of Percentage Enrichment of BRCA1 at IFNG Locus Using ChIP-qPCR

ChIP assays were performed with Micrococcal nuclease (MNase)-digested chromatin (Figure 5). ChIP-grade BRCA1,

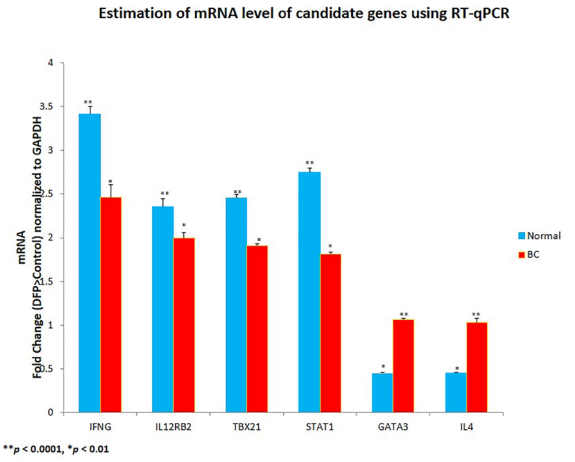

Figure 4. Real-time PCR (RT-PCR) quantification of candidate genes in Normal subjects' and Breast Cancer patients' Lymphocytes in the presence or absence of DFP (2.5 mg/ml) treatment for 48h. For RT-PCR assays, total RNA prepared using Quick-RNA^{TM}MiniPrep (Zymo), was utilized to synthesize cDNA, and the samples were used as templates for qPCR analysis for the specific genes. Normalized gene expression [2^(- ΔCt)] in the test sample is divided by the normalized gene expression [2^(- ΔCt)] in the control sample to calculate fold changes [2^(- ΔΔCt)]. Normalization was performed using Ct values derived for GAPDH from the corresponding test sample.

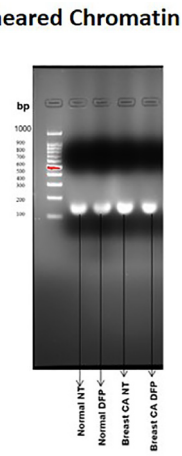

Figure 5. Micrococcal nuclease (MNase)-digested sheared chromatin. Expected bands were observed at around ~160bp. ChIP-quantitative real-time polymerase chain reaction (PCR) assay on micrococcal nuclease-digested ChIP (MNase-ChIP) to obtain a mononucleosomal resolution of the protein-DNA interactome demonstrates high efficiency of chromatin. The efficiency of chromatin digestion by MNase was >90%.

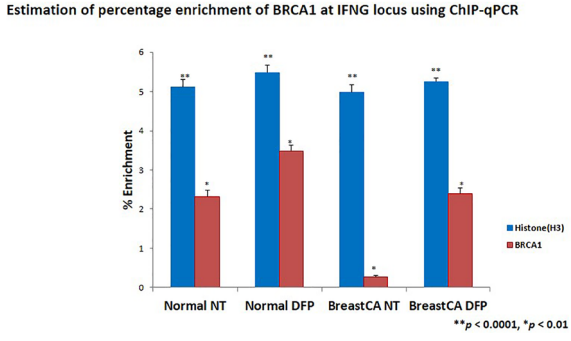

Figure 6. ChIP-qPCR enrichment profiles of the indicated antibodies [BRCA1 and Histone (H3)] across the IFNG locus in normal donor and breast cancer patients' lymphocytes. ChIP assays were performed with Micrococcal nuclease (MNase)-digested chromatin. ChIP-grade BRCA1, Histone (H3), and isotype control IgG antibodies were used to bring down DNA: protein complexes as per the ChIP protocol.

Histone (H3), and isotype control IgG antibodies were used to bring down DNA: protein complexes as per the ChIP protocol. ChIP-qPCR results suggested that DFP treatment significantly increases the enrichment of BRCA1 at the IFNG locus (Table 2) in the lymphocytes of breast cancer patients' samples along with the normal subjects (Figure 6) and our data support one of the well-established facts that the changes affecting IFN-γ responses are associated with BRCA1 mutations. However, the enrichment of Histone (H3) at the IFNG locus in the lymphocytes of breast cancer patients' samples along with the normal subjects remained almost unaltered in the presence or absence of DFP (Figure 6), this suggests well integrity of the sheared chromatin.

DISCUSSION

Diospyros peregrina fruit preparation

Table 2. ChIPqPCR Primer and Probe based on NCBI36/hg18 (Mar. 2006)

IFNGForward Primer	5'- TCGCCCTGGTAAAATGTTGAC-3'
IFNGReverse Primer	5'- CCAACCACAAGCAAATGATCA-3'
IFNGProbe	5'-6FAM-CTTCATTCAACAAAGCAC-3'

(DFP) stimulates lymphocytic proliferation even in breast cancer patient's lymphocytes. The NO release assay results suggest that the production of NO is high when the lymphocytes were treated with DFP even in breast cancer patient's lymphocytes. Also, the result of the MTT assay suggests that the DFP stimulates lymphocytic proliferation in breast cancer patient samples along with the normal subjects. DFP treatment in the lymphocytes of both the normal subjects and breast cancer patient's samples decreases the expression levels of GATA3 and IL4 genes but increases the expression levels of IFNG, IL12, TBX21 and STAT1 genes which are mainly associated with tumor protective immune response. IFNG induces partial remission and shows an increase in class I and II MHC expression and activation of macrophages, CTLs, and natural killer (NK) cells. As of late, expanding significance is being given to the incitement of CD4+ T helper cell (Th) reaction in malignant growth immunotherapy. After stimulating CD4+ T cells, they can differentiate into T helper (Th) type 1 (Th1) or Th2 which are the two main types of helper effector cells (19). Th1 cells release mainly IL-12, IFN-γ, and TNF and thus support stimulated CD8+ T cells to differentiate into CTL and of monocytes into macrophages (13). IL-4 is a pleiotropic cytokine established by T lymphocytes that follows up on a few cells of, for example, T and B lymphocytes, fibroblast, monocytes, endothelial cells, macrophages, and some others. IL-4 was at first portrayed as a B cell development cytokine and now is perceived to offer solid anti-tumor activity against a few tumors, including breast cancer. Th1 cell differentiation and both T cells and NK cells activation were stimulated by IL12 to provide cell-mediated immunity. In peripheral tissues, dendritic cells and macrophages act as antigen-presenting cells and release IL12 as one component of the antigen response, Th1 differentiation. The function of IL-12 in cellular immunity is mainly intervened by the STAT4 transcription factor. It has already been recorded from others studies additionally that IL-12 aided in increment of IFNG synthesis, though, IL-4 hinders the production of IFN-γ by the activated Th1 cells. Our ChIP-qPCR data suggest that DFP treatment significantly increases the enrichment of BRCA1 in the lymphocytes of breast cancer patient's samples along with the normal subjects. However, the enhancement of Histone (H3) in the lymphocytes of breast cancer patient's samples alongside the normal subjects remained practically unaltered in the presence/absence of DFP, this proposes well integrity of the sheared chromatin. DFP plays a major role in cancer-protective immunity, and the signal transduction processes involved in the lymphocytes of breast cancer patients. Hence, it can be concluded that DFP significantly increases tumor protective immune response by increasing the expression levels of Th1 specific network genes (e.g., IFNG, IL12, TBX21) and enhancing the expression of signal transducer and activator of transcription 1 (STAT1). IFN-γ, a vital mediator of immunity and inflammation exploits the Jak-STAT pathway to activate STAT1. Several purposes of IFN-γ have been credited to coordinate STAT1-mediated stimulation of immune effector genes, however, as of late it has become certain that significant IFN-γ functions are mediated by cross-reacting with other cytokines and inflammatory factors to drive cellular responses. The release of NO is primarily dependent on the type-1 immune milieu, favoring the releasing of IFN-γ and IL-12, and downregulating the secretion of type-2 cytokines, IL-4, and IL-10 that support type-2 immune environment. In addition to IFN-γ and IL-12; IL-17 plays a pivotal role in the release of NO. The arrival of IL-17 is represented by the Th17 cells in the milieu of cytokines, for example, TGFβ, IL-6, IL-23. Furthermore, cytokines IFN-γ, IL-4 that stimulate type-1 and type-2 immunity correspondingly have been revealed to negatively control Th17 differentiation. Our current study chiefly shows how DFP facilitates lymphocytic proliferation and

induction of type 1 cytokines (IL-12, IFN-γ) coupled with a tumoricidal agent, NO production.

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AUTHORS CONTRIBUTIONS

Study design/planning: Koustav Sarkar; *Data collection/entry:* Ahana Guha Roy†, Aitijhya Ghosh†, Kasturi Sinha, Basundhara Mitra, *Data analysis/statistics:* Sudeshna Rakshit, *Arrangement of Patients samples:* Melvin George, *Data interpretation:* Koustav Sarkar; *Manuscript preparation:* Koustav Sarkar; *Literature search/analysis:* Ahana Guha Roy†, Aitijhya Ghosh†, Kasturi Sinha
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Conflicts of Interest: None declared.

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