

Interleukin family (IL-2 and IL-1 β) as predictive biomarkers in Indian cancer patients: A proof of concept study

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SUMMARY

The immune system protects and defends its host against disease through immune cells. Immune cells such as T lymphocytes play a critical role in disease and health by producing key cytokines (pro-inflammatory TH1 or anti-inflammatory TH2). The inflammatory cytokines produced by immune cells such as peripheral blood mononuclear cells (PBMCs) may suppress the immune state or can lead to disease progression. In our study, we evaluated the functionality of resting PBMCs as well as phytohaemagglutinin mitogen (PHA-M) stimulated PBMCs in cancer patients and healthy controls in the Indian population. PHA-M was used as an activator of PBMCs and served as an index to assess the ability of PBMCs in both cohorts to respond to a superantigen stimulus. We hypothesized that key cytokines (TH1 and TH2 cytokines) could serve as biomarkers to assess the immune status of cancer patients based on the functionality of PBMCs primarily in resting conditions and their ability to respond to PHA-M. In our study, we found that PBMCs from cancer patients showed a marked difference in their resting and activated cytokine profiles with respect to Interleukin-2 (IL-2) and Interleukin-1 β (IL-1 β) as compared to their healthy counterparts. The study also demonstrated changes in Interleukin-10 (IL-10) profiles which require further study. These cytokines may serve as a potential predictive biomarker and provide the basis to form an immune index for each individual – a tool that could be used to prescribe treatment and therapies for cancer patients.

INTRODUCTION

Through the use of innate and adaptive immune responses, the immune system cues effective responses against antigens (1). Both immune responses rely on white blood cells (WBCs), which consist of various monocytes and lymphocytes such as T cells and B cells. T cells and B cells work to induce cell-mediated and humoral responses, respectively (2). Cytokines produced by activated T cells trigger the function of the immune system. However, in the presence of disease or infection, cytokine production may vary and be impaired (3). The secretion of these cytokines and the impairment of such secretion could be used as markers to establish the immune status of an individual and further deduce the patient's ability to produce cytokines and cue an immune response upon activation. Assessment of the immune competence of a cancer patient can be conducted

using peripheral blood mononuclear cells (PBMCs) (4). The use of PBMCs ensures a more effective quantification of cytokine presence rather than the use of serum levels where cytokines are subject to short serum half-lives (5,6).

The 3 primary cytokines studied in this investigation were IL-2, IL-10, and IL-1 β . The study focused on two populations — healthy individuals and cancer patients. PBMCs were extracted from both populations and activated using phytohaemagglutinin mitogen (PHA-M), a lectin that non-specifically activates some of the T cells present in the PBMCs of the cancer patients or healthy controls (7). PHA-M activates PBMCs by binding to T cell membranes, stimulating metabolic activity and cell division, which results in the release of cytokines (8). PHA-M was used as a tool to assess the magnitude of response of PBMCs with respect to cytokine production and not as a definitive indicator. Exposure to different lectins results in varying cytokine profiles.

Out of the three cytokines under study, IL-1 β and IL-2 are regarded as proinflammatory cytokines (9, 11). IL-1 β is a pleiotropic, proinflammatory cytokine that plays the role of cueing the inflammatory response and hematopoiesis (9). It is often produced by macrophages and allows for the activation of lymphocytes. In the context of cancer, the pleiotropy of IL-1 β impacts cancer cell proliferation, migration, and metastasis (10). IL-2 is attributed to have a proinflammatory and immunoregulatory role (11). Primarily secreted by activated T cells, IL-2 is an essential cytokine that allows for the activation of T cells and natural killer cells, cueing a cell-mediated response (12). Alternatively, IL-10 behaves as an anti-inflammatory cytokine that can trigger both immunosuppressive and immunostimulatory functions under different circumstances (13). In cancer patients, IL-10 plays a paradoxical role and can contribute to or inhibit tumor growth (14,15). Our study hypothesized that resting and PHA-M activated PBMCs in cancer patients would produce distinct cytokine profiles when compared to healthy controls and that this could provide a predictive immune index to assess the status of cancer patients during their disease and various stages of intervention. Our results suggest that resting PBMCs in cancer patients produce significantly elevated levels of IL-1 β when compared to healthy controls. IL-2 levels were observed to be varied in cancer patients and this could be attributed to the disease condition of the patient. Compared to healthy controls, more cancer patients' PBMCs produced IL-10 at resting state. Both cancer patients' and healthy controls' PBMCs produced varied concentrations of IL-10 after PHA-M stimulation. This observation could be attributed to the heterogeneity of the population and the dual role of IL-10 (16).

RESULTS

We analyzed the cytokines such as IL-1 β , IL-2 and IL-10 from PBMCs of 10 healthy controls and 12 cancer patient samples produced in 2 different conditions: resting PBMCs and PHA-M stimulated PBMCs. We quantitatively analyzed the cytokine levels present in the supernatants from PBMCs activated with PHA-M for 48 hours and resting PBMCs (Table 1, Table 2).

Cytokine levels of IL-1 β in PBMCs of healthy controls and cancer patients (resting and activated)

PBMCs from 90% of healthy controls produced significantly lower levels of IL-1 β in resting conditions ($p < .001$) in comparison to 92% of the patient population (Figure 1, Table 1, Table 2). Following PHA-M treatment PBMCs from healthy controls displayed a significant increase in IL-1 β values from its resting state (mean of 4.56 pg/mL) to its activated state (mean of 56.39 pg/mL) ($p < 0.001$). Whereas patient PBMCs displayed only a marginal increase in IL-1 β values (from a mean of 61.36 pg/mL to a mean of 73.17 pg/mL) (Figure 1).

Cytokine levels of IL-2 in PBMCs of healthy controls and cancer patients (resting and activated)

We analyzed the IL-2 levels in the healthy and patient PBMCs at resting and activated conditions. >95% of the healthy controls' PBMCs produced significantly lower levels of IL-2 in resting conditions (less than 25 pg/mL). Following PHA-M activation, PBMCs from healthy controls produced IL-2, but the response was very varied and subject-specific (4/10 responded with IL-2 concentrations ranging between 900-7400 pg/mL) (Table 1). In patient PBMCs, 75% (9/12) of the patient population did not produce IL-2 or produced small amounts (average of 1 pg/mL) in resting conditions. The remaining 25% (3/12) showed an increased IL-2 level in the

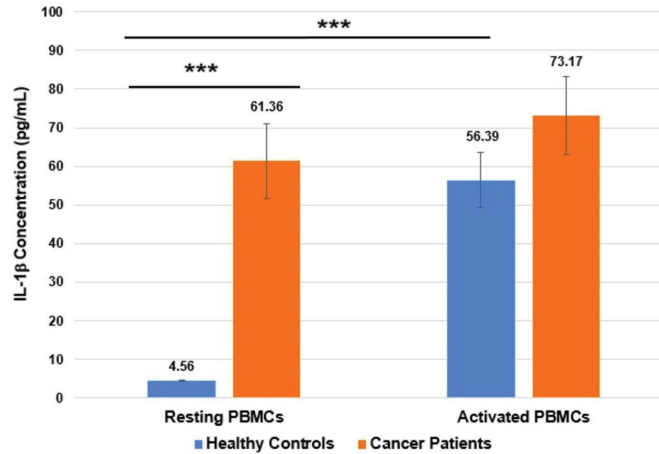


Figure 1: Mean IL-1 β levels in resting and activated PBMCs of healthy controls and cancer patients. Graphical representation of IL-1 β levels in resting and activated PBMCs of healthy controls (n=10) and cancer patients (n=12). Data values are represented as mean \pm SD. Statistical significance was analyzed by unpaired t-test. *** $p < 0.001$ is considered to be statistically significant. All samples were run in duplicates. Concentration is represented in pg/mL.

resting condition (400 to 700 pg/mL) but did not mount a high response post activation (Figure 2).

Cytokine levels of IL-10 in PBMCs of healthy controls and cancer patients (resting and activated)

Our study found that 50% of the healthy controls' PBMCs produced IL-10 in their resting state, with their IL-10 concentration ranging from 90-966 pg/mL (Table 1). However, 70% of the patient population's PBMCs produced IL-10 in their resting state, with an IL-10 concentration of 26-966 pg/mL (Table 2). PBMCs from the healthy population when

Healthy Control Number	Mean Conc. Of IL-2 In pg/mL		Mean Conc. Of IL1- β In pg/mL		Mean Conc. Of IL-10 In pg/mL	
	Resting	Activated	Resting	Activated	Resting	Activated
H1	0.69	1077.8	0	38.04	251.725	597.52
H2	23.6	969.78	0	21.34	225.146	437.02
H3	0	7372.72	45.62	33.92	966.42	2679.38
H4	0	121.31	0	99.42	350.3	1283.48
H5	0	18.67	0	30.68	90.24	427.03
H6	0	161.92	0	23.47	0	225.14
H7	0	6778.32	0	102.12	0	14.93
H8	0	60.58	0	89.34	0	1258.7
H9	0	33.2	0	23.47	0	125.51
H10	7.32	6.75	0	102.11	0	195.16

Table 1: Mean concentration (pg/mL) of IL-2, IL1- β and IL-10 in resting and activated PBMCs of healthy controls.

Patient Number	Disease Condition	Mean Conc. Of IL-2 In pg/mL		Mean Conc. Of IL1- β In pg/mL		Mean Conc. Of IL-10 In pg/mL	
		Resting	Activated	Resting	Activated	Resting	Activated
P1	Oral cancer	0	3663.34	97.32	81.17	85.79	400.36
P2	Not known	0	0	0	5.6	966.42	2679.38
P3	Head and neck cancer	0	48625.67	85.94	102.02	77.03	504.88
P4	Oral cancer	416.05	3198.67	78.67	102.32	35.96	254.08
P5	Head and neck cancer	731.94	3802.9	82.08	51.92	0.79	4278.18
P6	Oral cancer	440.93	4215.98	64.68	101.57	0	10.61
P7	Not known	1.87	12681.52	101.05	101.98	174.86	783.43
P8	Oral cancer	2.42	6626.98	69.01	98.6	26.77	378.41
P9	Oral cancer	0.83	0	54.94	37.03	77.03	110.34
P10	Head and neck cancer	0	0	19.47	74.18	0	90.24
P11	Head and neck cancer	2.42	0	66.52	60.57	0	0
P12	Oral cancer	0.59	103.70	16.59	100.01	27.53	198.43

Table 2: Mean concentration (pg/mL) of IL-2, IL1- β and IL-10 in resting and activated PBMCs of cancer patients.

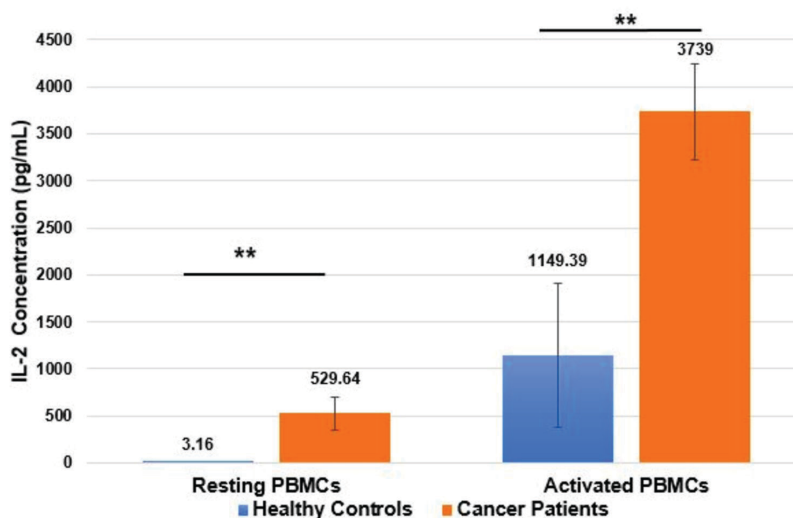


Figure 2: Mean IL-2 levels in resting and activated PBMCs of healthy controls and cancer patients. Graphical representation of IL-2 levels in resting and activated PBMCs of healthy controls (n=10) and cancer patients (n=12). Data values are represented as mean \pm SD. Statistical significance was analyzed by unpaired *t*-test. *******p*<0.01 is considered to be statistically significant. All samples were run in duplicates. Concentration is represented in pg/mL.

activated produced 14-2679 pg/mL of IL-10 (**Table 1**) while PBMCs from the patient population produced 10-4278 pg/mL (**Table 2**).

DISCUSSION

We undertook a study to evaluate the cytokine production of PBMCs derived from cancer patients in the resting state and after non-specific stimulation with PHA-M. This method, as employed in previous studies, investigated the potency of PBMCs in producing cytokines (17). Results from this previous study suggested that the intrinsic ability of resting PBMCs to produce IL-6 was higher in cancer patients than in healthy controls. Furthermore, the study reported that nearly 20% of the patients failed to produce interferon-gamma (IFN- γ) following PHA-M stimulation, suggesting that the cancer patients were unable to mount a robust anti-cancer immune response mediated by IFN- γ (17). In continuation, we chose to study and further investigate IL-1 β , IL-2, and IL-10.

PBMCs of cancer patients in the resting state produced higher levels of IL-1 β when compared to healthy controls, which suggested a basal inflamed phenotype in patients resulting from disease or other contributing factors presented during the course of the disease. In a previous study, PBMCs from post-surgery cancer patients produced slightly elevated levels of IL-1 β when compared to PBMCs of pre-surgery patients (18). Surgery leads to an acute response involving exaggerated production of proinflammatory cytokines such as IL-1 β by macrophages and monocytes in the injury or surgery site (19). Furthermore, anti-cancer treatments such as surgery promote the secretion of IL-1 β produced by immune or cancer cells which can minimize cancer progression and tumor development (20). Hence, increases of IL-1 β in patients often indicates an inflammatory response due to external surgical intervention. The behavior of IL-1 β as a proinflammatory marker in tandem with its tendency to increase upon surgical intervention could be utilized to assess the recovery of the patient. The marginal increase in IL-1 β levels in activated PBMCs of cancer patients could suggest that the IL-1 β levels have reached maximal activation in the patient population and cannot be further enhanced by *in vitro* activation, suggesting a chronically inflamed condition (**Figure 1**).

IL-2 is an essential cytokine attributed to the sustenance of T cells. The pleiotropic cytokine mediates T-cell growth and its activation-induced cell death (21). It also augments natural killer cell activity while maintaining the Th1 and Th2 differentiation pattern (22). IL-2 is attributed to have several immunostimulatory and immunoregulatory functions (23). In our study, majority of the healthy controls displayed a minimal concentration of IL-2 in resting state. 75% of the cancer patients displayed a minimal concentration of IL-2 and 25% of the same population displayed a high concentration of IL-2 in resting condition. These high IL-2 levels at resting state could be a result of T-cell anergy, which is characterized by cell proliferation and differentiation (24).

IL-2 presence upon activation signifies responsiveness and immunocompetence in patients (25). But the supposedly anergic patients were not able to mount a high response post activation because of downregulation of some functions in spite of persistent activation. PBMCs from healthy controls displayed a 10-fold increase in IL-2 compared to cancer patients after activation. The varied responses in healthy controls activated PBMCs could be attributed to several

reasons including age, general health conditions, and other factors.

Since these are initial results, we still need to understand the implications of the magnitude of the responses following activation. The heterogeneity of the patients caused varied responses to PHA-M ranging from no response to large fold increases in comparison to resting conditions. The extreme elevation of IL-2 upon activation could imply chronic inflammation in the individual.

IL-10 is a pleiotropic cytokine with anti-inflammatory properties (13). The cytokine behaves in a paradoxical manner, playing a dual role in contributing to and inhibiting tumorigenesis in cancer patients via immunosuppressive and anti-angiogenic functions (16). IL-10 plays a subjective role in different contexts (14). The observed trends amongst the subject and patient populations may be due to the diversity and heterogeneity in our study and the patient-dependent response of IL-10. Therefore, our preliminary results in terms of IL-10 are inconclusive and further studies with larger cohorts need to be undertaken to determine the role of IL-10 and its levels in resting T cells.

Thus, these preliminary results show that the functionality of PBMCs could serve as an important tool to understand the immune status of cancer patients - one that could be done throughout any stage of the patient's illness. The data also illustrate that the immune status of a patient may be formulated primarily in terms of high IL-2 production, which suggests an underlying immune suppression, inflammatory state (which could also be contributed to by IL-6 (JEI-S-22-00201)). Therefore, these data serve as a starting point for further treatment with regard to the patient's specific immune profile.

MATERIALS AND METHODS

Study Population

10 healthy controls and 12 cancer patients were randomly selected from cancer patient population irrespective of their gender, age, and type and stage of cancer. The cancer patient population were selected under the framework of the research regulations of the Central Ethics Committee (CEC) of Health Care Global (HCG) Hospitals in Bangalore, India. All the healthy controls and the patients whose blood samples were used for the study gave informed consent prior to their enrollment in the investigation.

Collection of Blood

10 mL blood was collected from cancer patients and healthy controls by venous puncture using the standard blood bank procedure in EDTA vacutainers (26). All blood samples were screened for infectious diseases before use in experiments.

Isolation of Peripheral Blood Mononuclear Cells

Density gradient centrifugation was performed to isolate PBMCs from whole blood using Ficoll – hypaque. Blood diluted with saline in the ratio of 1:3 was layered on Ficoll. Centrifugation was performed at 1500 rpm for 20 min. The buffy coat was collected and washed with saline. Cell counting of isolated cells was performed using a hemocytometer. 1×10^6 cells/mL in CTS Optimizer T-Cell Expansion Basal Medium (Gibco) with 100 ng/mL PHA-M (Gibco), and 1x glutamine (Gibco) were cultured and incubated for 48 hrs in sterile culture conditions (37°C, 5% CO₂). PBMCs were

also cultured without PHA-M for the control groups. The supernatants were collected and stored in -80°C freezer until further evaluation.

Cytokine Detection by ELISA

The presence of cytokines was determined quantitatively using ELISA, and the concentration of IL- 2, IL-1 β and IL-10 was evaluated using the manufacturer's instructions (BD Bioscience). The 96-well microtiter plates coated with capture antibody were incubated at 4°C overnight. The next day, the plates were washed with a wash buffer (20X). 200 μ L of assay diluent was added and incubated for 1 hr at room temperature (RT). The washing step was carried out, standards and samples (100 μ L) were added and kept for incubation at RT for 2 hrs. The plates were washed and the enzyme conjugate antibody and detection antibody (1:250) were added. The plates were incubated at RT for 1 hr. The plates were washed, the substrate (100 μ L) was added, and the plate was incubated at RT for 30 minutes. Stop solution (50 μ L) was added to the wells to stop the reaction. Optical density was measured at 450 nm using Lisaquant-IT Microplate ELISA Reader. The concentrations of cytokines were evaluated using the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using an Excel Spreadsheet. Healthy control group (n=10) and cancer patients (n=12). Data values were expressed as mean values mean \pm SD. Statistical significance was analyzed by unpaired *t*-test for IL-1 β and IL-2. ***p* < 0.01 and ****p* < 0.001 were considered statistically significant. n=2, where n=2 means that the cancer patients and healthy controls samples were run in duplicates.

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