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Article

Estimation of cytokines in PHA-activated mononuclear cells isolated from human peripheral and cord blood

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SUMMARY

T cells or T lymphocytes are a type of white blood cell that bind to specific antigens which are found on the surface of antigen-presenting cells (APCs). T cells are activated by encountering APCs and undergoing a series of reactions which lead to immune homeostasis. Their production of multiple pro- and anti-inflammatory cytokines including IFN-γ, IL-6 and IFN-10 makes them play a vital role in immune responses and defending the body against infection. They are especially important in both progression and decline of tumor formation. T cells also have numerous clinical applications when they are improved by combining gene therapy and immunotherapy. In this study, we investigated the time-dependent cytokine secretion ability of phyto-hemagglutinin (PHA)-activated T cells derived from human peripheral (PB) and cord blood (CB). We hypothesized that the anti-inflammatory cytokine, IL-10, and pro-inflammatory cytokine, TNFα, levels would be higher in PHA-activated T cells obtained from PB as compared to the levels obtained from CB and would decrease over time. Upon PHA-activation, the IL-10 levels were relatively high while the TNFα levels decreased, making these findings applicable in therapeutic treatments e.g., rheumatoid arthritis, psoriasis, and organ transplantation. The results could potentially be used to identify, analyze, and apply cell-free systems, i.e., *in vitro* systems where biological mechanisms are observed in the absence of complex interactions which occur in whole cell studies to cancer immunotherapy.

INTRODUCTION

T cells are a major part of the adaptive immune system. They function as its primary modulators due to their ability to regulate the functions of other immune cells, such as the antigen-specific activation of B cells, CD8+ cytotoxic T cells, and macrophages (1). T cells can be found in blood, but based on their location in the body, their characteristics can differ (2). Studies have demonstrated that while cord-blood (CB) derived T cells are immature, antigen-inexperienced cells, they demonstrate higher capacities to be transformed into memory T cells with significantly higher anti-tumor effects than those derived from peripheral blood (PB) (3, 4, 5). However, PB-derived T cells have demonstrated higher clinical applicability over CB T cells due to shorter delays in immune reconstitution or CB engraftment (6). These characteristics in turn translate into lower morbidities and decreased infection risks in the patient, making PB T cells conventionally more preferred (7).

T cells are activated by a series of intracellular signals that lead to a corresponding series of reactions (8).
Antigen presenting cells (APCs) with antigens on the surface of their cells enter the lymph nodes. CD4+ helper T cells and CD8+ T cells have T cell receptors which bind the antigen-Major Histocompatibility Complexes (MHC) on the APCs. T cells receive the first signal they need to be activated through the CD4 and CD8 molecules binding to the antigen-MHC molecule. T cells require a second signal to become activated. The T cell's CD28 receptors bind to CD80 or CD86 receptors on the APC, sending a signal to the helper T cells to begin proliferating, producing millions of T cells that identify the antigen. Cytokines then provide the T cells with more specific signals, so they either become Th1 type or Th2 type (9).

This activation is often through the means of cytokines (10). Cytokines are proteins secreted by lymphocytes, monocytes, or leukocytes that affect intercellular interactions and communication. There are primarily two types of cytokines – pro-inflammatory and anti-inflammatory (11). Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the promotion of inflammatory reactions: fever production, inflammation, tissue destruction, and shock (12). On the other hand, anti-inflammatory cytokines behave as immunoregulators and generally act alongside various cytokine inhibitors and soluble cytokine receptors which curb the proinflammatory response (13). Thus, they promote healing and have therefore been studied extensively for their potential therapeutic applications (14, 15).

Tumor necrosis factor alpha (TNF-γ) is a pro-inflammatory cytokine that is a member of the tumor necrosis factor superfamily (16). It is known to play a role in inflammatory and autoimmune diseases (17). In vivo, TNF-γ kills tumor cells and has demonstrated potential to induce tumor regression. However, it also plays a role in the initiation, promotion, proliferation, invasion, angiogenesis, and metastasis of tumors; this cytokine is used in both immunomodulation and tumorigenesis (18). High levels of TNF-γ are associated with various cancers such as lymphocytic leukemia and

cervical carcinomas, a phenomenon which decreased post-chemotherapy (19, 20, 21, 22). Members of the TNF superfamily are cytokines that are potentially most important for activating co-stimulation pathways and therefore are studied while measuring T cell activation (23, 24).

IL-10 is an anti-inflammatory cytokine that inhibits the proliferation of CD4+ human PB T cells (25). This inhibition results in the regulation of Th1 and Th2 type responses. IL-10 has demonstrated promotion of alternative immune responses such as those of B cells (16). Therefore, it is used for therapeutic uses as it works against pathogens and diseases. IL-10 concentrations in blood are controlled through angiogenesis inhibition and the downregulation of vascular endothelial growth factor (VEGF) (26). In the context of T cell activation, IL-10 ensures the immune response is directed solely to the pathogens and not to the body of the host. IL-10 has proven to be useful in limiting tumor growth across a range of studies by downregulating proinflammatory cytokine activity and stimulating humoral responses thus curbing tumor development, growth, and metastasis (27, 28, 29).

Phytohemagglutinin (PHA) is a plant-derived lectin used to stimulate T cells and therefore induces IL-10 production. PHA also induces a decrease in TNF-γ levels, which decreases the rate of tumor progression by promoting apoptosis and autophagy. Furthermore, PHA has proven to limit tumor growth across a range of cancers including human melanomas, breast adenocarcinomas, and human cervical cells by inhibiting inflammatory cytokine activity thus curbing tumor development, growth, and metastasis (30, 31).

We selected IL-10 and TNF-γ to study the pro-inflammatory/anti-inflammatory axis. In analyzing these cytokines, we hoped to gain a clearer understanding of the effect of PHA on T cells. We hypothesized that there would be a significant increase in anti-inflammatory cytokine and a comparative decrease in inflammatory cytokine in activated T cells of PB, which would decrease over time.

Studies comparing CB T cells to PB T cells have determined that when CB T cell-mediated enhanced tumor rejection was compared with that of PB T cells, the CB group showed a higher rate of survival (3, 30). In other words, CB T cells have been shown to be more effective in adoptive T cell therapy and thus, have a greater anti-tumor effect than PB T cells. As a secondary investigation, we aimed to contrast the potential applications of CB and PB in their anti-tumor effects. In this study, we found a significant increase in IL-10 levels and negligible amounts of TNF-γ levels.

RESULTS

We obtained the mononuclear cells (MNCs) from CB and PB using Histopaque. The cells were treated with media containing 5% PHA (PHA+) or complete media alone in the control group (PHA-) for 24h and 48h. The supernatants obtained from the respective groups were analyzed for IL-10 and TNF-γ using Enzyme-linked Immunosorbent assay (ELISA).

The cell supernatants obtained from PHA-activated MNCs of CB and PB showed a significant increase (p<0.05) in IL-10 for 24h and 48h when compared with the respective control groups (**Figure 1**). The IL-10 concentration was 0.13 pg/mL and 0.12 pg/mL in CB MNCs activated with PHA for 24 hours and 48 hours, respectively. However, the IL-10 concentration in the controls was 0.05 pg/mL at 24h and negligible at 48h, which was significantly lower than the PHA treated groups. The IL-10 concentrations were 0.33 pg/mL and 0.19 pg/mL in PHA-activated PB MNCs at 24 hours and 48 hours, respectively. However, the IL-10 concentrations were largely negligible in the respective controls, barring the 24h CB MNCs in PHA- which demonstrated IL-10 content (**Figure 1**).

The cell supernatants obtained from CB and PB had decreased TNF-γ levels at 24 hours and 48 hours as compared to the respective controls (p<0.05) (**Figure 2**). The TNF-γ concentration in CB MNCs activated with PHA

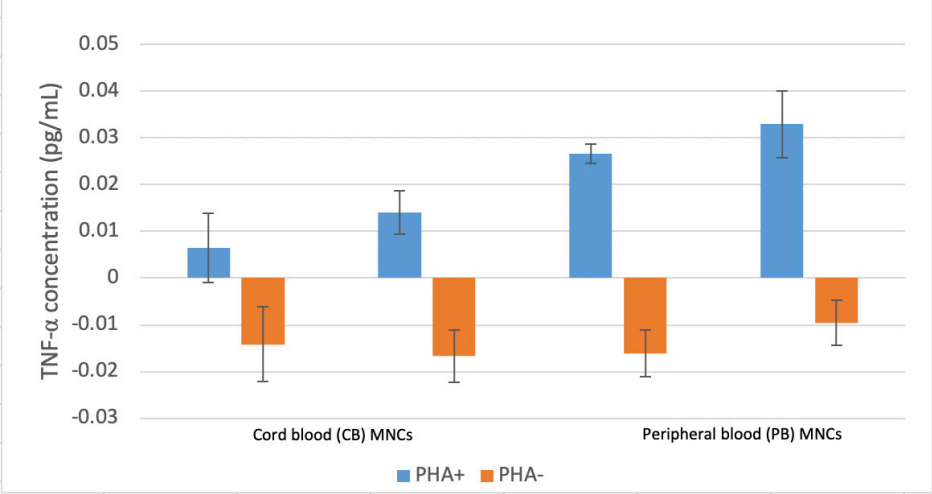

Figure 1: TNF-α concentrations (pg/mL) in PHA-activated MNCs isolated from cord and peripheral blood at 24 hours and 48 hours. Error bars were calculated using standard deviation. p-value = 0.04. As p value < 0.05, results are statistically significant.

was 0.006 pg/mL at 24 hours and 0.014 pg/mL at 48 hours. Furthermore, it was found to be 0.026 pg/mL at 24 hours and increased further to 0.032 pg/mL at 48 hours. However, the TNF-γ concentrations were negligible in the respective controls (**Figure 2**).

DISCUSSION

We aimed to compare the concentrations of IL-10 and TNF-γ between PHA-activated and untreated T cells derived from human CB and PB. While there was little to no expression of either cytokine in PHA-, we found that the concentrations of IL-10 and TNF-γ were consistently higher in peripheral MNC than in cord MNC.

The concentration of TNF-γ was highest in PHA+ 48 hour peripheral blood (PB), at around 0.33 pg/mL, and lowest in PHA+ 24 hour CB, at 0.005 pg/mL. No concentration of TNF-γ was measured in PHA-, which was the control experiment. There was a significant increase in the concentration of TNF-γ in both CB and PB from 24 hours and 48 hours.

However, the highest concentration of IL-10 was observed in PHA+ 24 hour PB at 0.33 pg/mL, where the lowest concentration was measured at 0.13 pg/mL. Unlike the trend observed in TNF-γ, IL-10 demonstrated a decrease in concentration between PHA+ PB 24 hour and 48 hour. This result suggests that activated PB-isolated T cells secrete higher levels of IL-10 than CB-isolated T cells, which decreases after 24 hours. However, T cells isolated from both sources produce negligible amounts of TNF-γ after activation by PHA.

T cell therapy consists of genetically engineered T cells that express a chimeric antigen receptor (CAR), which can be used to treat both hematological and solid tumor malignancies (20). Lymphocytes that inhibit the growth and implantation of tumor cells are ideal for adoptive immunotherapy (32). Since our study found that PHA stimulates the production of anti-inflammatory cytokine IL-10 and reduces the production of pro-inflammatory cytokine TNF-γ, transfusion therapy with T cells treated with PHA may have a net anti-tumor effect, a finding echoed in Hiwarkar et. al's study (20).

However, in direct contradiction to the secondary hypothesis, PHA+ PB T cells were shown to have higher levels of all cytokines under study than PHA+ CB T cells. Thus, while CB may not truly be superior to PB in employment in T cell based anti-cancer therapies, it is possible that other anti-inflammatory cytokines make CB T cells more effective against tumors.

The investigation of the causes behind the fluctuations of cytokine concentrations within CB- and PB- batches could potentially comprise the aim of our future studies.

MATERIALS AND METHODS

Collection of blood

The human umbilical CB was collected within 48 hours of delivery from the maternity ward of V-Care Hospital, Bangalore. The blood samples were screened for infectious diseases using ELISA. The fresh unused white blood cell (WBC) pellet from human PB was collected from the blood bank in HCG Hospital, Bangalore. Both the blood samples were biological discards. The screened samples were used for isolation of MNCs.

Isolation of mononuclear cells

The blood sample was diluted with normal saline in a 1:1 ratio. The diluted sample was then added to Histopaque (Sigma) in a 3:1 ratio that was then centrifuged at 1500rpm for 20 minutes. Histopaque centrifugation separates the blood into different layers based on the density of its components. The buffy coat (the fraction of blood sample that contains WBCs) was then moved to a separate tube and washed twice with saline in a 1:10 ratio. This solution was centrifuged for 10 minutes at 2000rpm. The supernatant was discarded. The pellet was re-suspended in complete medium containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen). The cells were

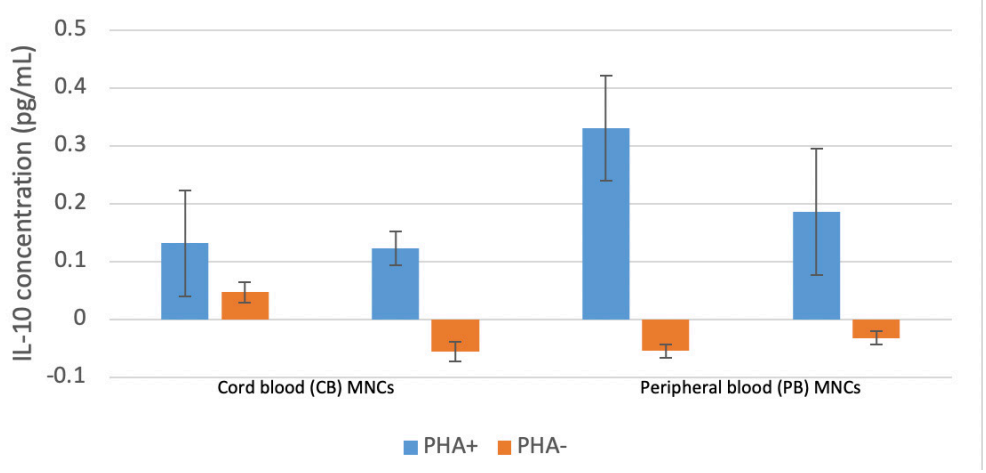

Figure 2: IL-10 concentrations (pg/mL) in PHA-activated MNCs isolated from cord and peripheral blood at 24 hours and 48 hours. Error bars were calculated using standard deviation. *p*-value = 0.03. As *p* value < 0.05, results are statistically significant

counted using WBC diluting fluid (Himedia) under a compound microscope. The viability was checked using trypan blue dye (Himedia).

Culture conditions

The cells were cultured in a 24-well plate at 37ºC for 24 and 48 hours. Each well containing 1 million cells was cultured in media containing 5% PHA or without PHA (control). PHA allows only T cells to proliferate, inhibiting the growth of the remaining cell population (33). However, cells will not respond in control group due to lack of stimuli. The following groups were formed: PHA-activated CB MNCs (24 hours), PHA-activated CB MNCs (48 hours), PHA-activated PB MNCs (24 hours), PHA-activated PB MNCs (48 hours), CB MNCs cultured in complete medium without PHA (24 hours), CB MNCs cultured in complete medium without PHA (48 hours), PB MNCs cultured in complete medium without PHA (24 hours), PB MNCs cultured in complete medium without PHA (48 hours).

After incubation, the culture medium was collected from each group in respective tubes. After centrifugation at 1500rpm for 10 minutes, the clear supernatant was collected in several aliquots and stored at -80˚C until further use.

Protein estimation and Enzyme-linked Immunosorbent Assay (ELISA)

The total protein of each cell supernatant was analyzed using Biuret method (Sigma) (34). ELISA is used to determine the concentration of protein present in a sample by the occurrence of antigen-antibody reaction. The substrate is catalyzed by the enzyme to result in a colored product. The stored supernatants were subjected to ELISA for the identification of IL-10 and TNF-γ concentrations. The ELISA kits were obtained from Ray Biotech and the procedure was performed as provided in the manual guide. The ELISA values were normalized with the respective protein concentration.

Statistical Analysis

All the values were normalized with respective protein concentrations. The data were analyzed using GraphPad Prism software. Different groups were compared using ANOVA and Tukey's test. A value of $p<0.05$ was considered as significant and was shown as '*' and '#'. The experiment was independently repeated three times. Bar graphs representing the results were plotted using Microsoft Excel with standard deviation as error bars (**Figures 1, 2**).

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