

## Evaluation of T-cell Function after Blood Transfusion in Patients Undergoing Coronary Artery Bypass Grafting

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### ABSTRACT

Blood transfusion is associated with increased mortality and morbidity. This study aimed to determine the effect of blood transfusion on T-helper 1 (T<sub>H</sub>1), T<sub>H</sub>2, and T<sub>H</sub>17 function in patients undergoing coronary artery bypass grafting (CABG).

Two blood samples were obtained from patients undergoing CABG, before and 14 days after surgery. Production of interleukin (IL)-2, IL-4, interferon (IFN)- $\gamma$ , IL-17A, and IL-10 by CD4<sup>+</sup> T cells was measured using enzyme-linked immunosorbent assay (ELISA). mRNA expression of T-box expressed in T cells (*T-bet*), GATA binding protein 3 (*GATA3*), RAR-related orphan receptor- $\gamma$  (*ROR- $\gamma$ t*), signal transducer and activator of transcription 3 (*STAT3*), *STAT4*, and *STAT6* were measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

mRNA expression of *T-bet* and *STAT4* showed a significant decrease after blood transfusion. However, the concentration of IFN- $\gamma$  in the culture supernatant showed no significant difference after blood transfusion. mRNA expression of *GATA3* and *STAT6* showed a significant decrease after blood transfusion. However, the concentration of IL-4 in the culture supernatant showed no significant difference after blood transfusion. mRNA expression of *ROR- $\gamma$ t* showed no significant decrease after blood transfusion; however, the expression of *STAT3* and the concentration of IL-4 in the culture supernatant did significantly decrease following blood transfusion. IL-10 production increased significantly postoperatively.

Decreased T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 signaling pathway activity and increased IL-10 concentration indicate an immunomodulatory effect on the immune system after blood transfusion.

**Keywords:** Blood transfusion; Coronary artery bypass; Cytokines; Immunomodulation; T-lymphocytes; T<sub>H</sub>1 cells; T<sub>H</sub>2 cells; T<sub>H</sub>1; T<sub>H</sub>2

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## INTRODUCTION

Blood transfusion during coronary artery bypass grafting (CABG) is an unavoidable part of surgery protocol. However, blood transfusion has a lifesaving effect for patients undergoing CABG, but there are some adverse events including infection, increasing length of hospital stay, episodes of atrial fibrillation, acute renal failure, and stroke.<sup>1,2</sup> On the other side, the number of blood products negatively is associated with CABG outcome.<sup>3</sup> In this regard, attention has focused on the reduction the complications and mortality in CABG following blood transfusion.<sup>4,5</sup>

One of the main complications of blood transfusion is immune suppression. Red blood cell (RBC) products contain mediators that interact with immune cells and modulate the immune system.<sup>6</sup> Despite approaches to reducing the immunomodulatory effect of blood transfusions such as leukoreduction, and plasma depletion, modulating the immune system remains challenging, especially in CABG. The release of heme and hemoglobin during blood storage alters the macrophage from M1 to M2 phenotype. M2 phenotype is associated with interferon- $\gamma$  (INF- $\gamma$ ) secretion and nitric oxide synthesis which causes the inhibition of the immune system.<sup>8</sup> On the other side, blood transfusion inhibition of the immune system during cardiac surgery causes alteration in the helper T cell (T<sub>H</sub>)1/T<sub>H</sub>2 balance.<sup>9,10</sup> The weakened immune system following CABG is accompanied by increasing infection, impairment of pulmonary function, and mortality.<sup>11</sup> Several factors were demonstrated for immunomodulation following CABG, however, the exact mechanism of immunomodulation following blood transfusion is not fully understood. Hence, understanding the exact mechanism of the immunomodulatory effect of blood transfusion in CABG can help improve surgical outcomes.

## MATERIALS AND METHODS

### Study Population

Patients who were referred to the Fatemeh Zahra Hospital for CABG were evaluated for the current study. The main inclusion criteria were receiving a blood transfusion during the CABG procedure. Patients with immune deficiency and CABG patients without blood transfusion were excluded. Additionally, the function of

immune system cells can be evaluated by measuring cytokines. However, in the present study, we evaluated gene expression to maximize the reliability of results.

The inclusion criteria were age between 40 and 80 years, undergoing CABG with a history of coronary artery disease, use of heart and lung pumps during surgery, receiving 1 or 2 packed cell units, and 3 to 5 platelet units during or within 24 hours after surgery. Additionally, patients with a history of chronic viral infections, including hepatitis B virus, hepatitis C virus, human immunodeficiency viruses, autoimmune diseases, immune deficiencies, cancers, or the use of any immunosuppressive drugs were excluded from the study.

The study was approved by the local research ethics committee and written informed consent was obtained from all patients. Two peripheral blood samples were taken from each subject, 1 before the surgery, and another 3 weeks after surgery.

### Cell Culture and Stimulations

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood using Lymphodex (Inno-Train, Germany) density centrifugation according to the manufacturer's instructions. Next, CD4<sup>+</sup> T cells were positively isolated from the PBMCs using a magnetic beads-conjugated anti-CD4 monoclonal antibodies (mAbs) kit (Miltenyi Biotec, Germany). The purity of magnetic-activated cell sorting (MACS)-separated CD4<sup>+</sup> T cells was measured using a dual-color flow cytometric analysis with anti-CD3-phycoerythrin (PE) and anti-CD4-fluorescein isothiocyanate (FITC) (both from BioLegend, USA). To evaluate T-cell proliferation and cytokine production, isolated CD4<sup>+</sup> T cells ( $2 \times 10^5$ /well) were cultured in 200  $\mu$ L of Roswell Park Memorial Institute (RPMI)-1640 medium containing penicillin (100 IU/mL), streptomycin (100  $\mu$ g/mL), and 10% (v/v) fetal calf serum (FCS) (Biosera, Nuaille, France) and stimulated with combination of phorbol 12-myristate 13-acetate (PMA)/ionomycin cocktail (final concentration of 0.081  $\mu$ M PMA and 1.34  $\mu$ M ionomycin, eBioscience, USA) for 6 hours at 37°C with 5% CO<sub>2</sub>.

### Gene Expression Analysis

Total RNA was isolated from  $1 \times 10^6$  PBMCs using the FavorPrep Blood/Cultured Cell Total RNA Mini Kit (Favorgen, Taiwan) based on the manufacturer's

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protocol. The quantity and quality of isolated RNA were confirmed by a nano-spectrophotometer and electrophoresis, respectively. Complementary DNA (cDNA) was synthesized using the Yekta Tajhiz Azma cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the Applied Biosystems 7500 Fast Real-Time PCR instrument (ABI, Thermo Fisher Scientific, USA) using Real Q Plus 2x Master Mix (High Rox, Ampliqon, Denmark) and specific primers for T-box expressed in T cells (*T-bet*), GATA binding protein 3 (*GATA3*), RAR-related orphan receptor- $\gamma$  (*ROR- $\gamma$ t*), signal transducer and activator of transcription 3 (*STAT3*), *STAT4*, and *STAT6*, as well as  $\beta$ -actin protein, as a housekeeping gene (Table 1 in the Supplement). PCR was carried out at 95°C for initial denaturation followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and extension at 72°C for 30 seconds. All samples were done in duplicate, and the relative expression levels of these genes were calculated using the  $2^{-\Delta\Delta C_t}$  method, with  $\beta$ -actin as an internal housekeeping gene.<sup>12</sup>

### Cytokine Assay

After stimulation of CD4<sup>+</sup> T cells with PMA/ionomycin cocktail for 1 night, culture supernatants were collected and used to measure cytokines including IL-2, IL-10, IL-4, IFN- $\gamma$ , and IL-17A using Human ELISA Ready Set Go Kits (Sanquin, Amsterdam, the Netherlands), according to the manufacturer's protocol. All samples were measured in duplicate.

### Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean. All statistical analyses were performed using SPSS 20 statistical package (SPSS, Chicago, USA). Results were evaluated by the Mann-Whitney U test and Spearman correlation tests as appropriate. *p* values<0.05 were considered statistically significant. All graphs were designed using GraphPad Prism 6 software.

## RESULTS

### T<sub>H1</sub> Pathway After Blood Transfusion

Considering the role of the T<sub>H1</sub> pathway in the immune response against viral infections and malignancies, in this study, we evaluated factors related

to T<sub>H1</sub> function after blood transfusion. mRNA expression of 2 main transcription factors of the T<sub>H1</sub> pathway, *T-bet*, and *STAT4*, were evaluated using qRT-PCR and showed a significant decrease after blood transfusion (*p*<0.0001 for both). However, IFN- $\gamma$  concentrations in culture supernatant showed no significant difference after blood transfusion (*p*=0.374) (Figure 2C).

### T<sub>H2</sub> Pathway After Blood Transfusion

T<sub>H2</sub> cells can be a key factor in altering the immune response after blood transfusion by producing anti-inflammatory responses and suppressing several immune pathways, including T<sub>H1</sub>. Therefore, we evaluated the mRNA expression of 2 main transcription factors of the T<sub>H2</sub> pathway, *GATA3* and *STAT6*, using qRT-PCR. The results showed that the mRNA expression of both *GATA3* (*p*<0.001) and *STAT6* (*p*<0.001) significantly decreased after blood transfusion. However, IL-4 concentrations in the culture supernatant showed no significant change after blood transfusion (*p*=0.774).

### T<sub>H17</sub> Pathway after Blood Transfusion

T<sub>H17</sub> cells, characterized by the secretion of IL-17A, are now identified as essential cells in the clearance of extracellular pathogens and the development of many autoimmune responses. Therefore, changes in their function after blood transfusion can be used to predict immune responses. qRT-PCR results showed that mRNA expression of *ROR- $\gamma$ t*, the main transcription factor of the T<sub>H17</sub> pathway, did not change significantly after blood transfusion (*p*=0.054); however, expression of *STAT3*, another transcription factor of the T<sub>H17</sub> pathway, decreased significantly (*p*=0.025). IL-17A concentrations in culture supernatant also decreased significantly after blood transfusion (*p*=0.007).

### Immunomodulatory Pathway after Blood Transfusion

Since IL-10, as an anti-inflammatory cytokine, enhances the immune response of T<sub>H2</sub> cells and has been described as a major inhibitor of T<sub>H1</sub> cell function, we measured the concentration of this cytokine in culture supernatant before and after blood transfusion. The results showed that IL-10 production significantly increased after blood transfusion.

## DISCUSSION

Blood transfusions during and after surgery are associated with changes in the patient's immune system. These changes can be detected by evaluation of immune cell frequencies, cytokine concentrations, changes in the expression of surface markers, and transcription factors.<sup>13</sup> CD4<sup>+</sup> T cells, together with cell-mediated immunity, play a pivotal role in the immune response after surgery.<sup>14</sup>

Although several studies have evaluated the influence of blood transfusion on immune system function, they investigated immune response within 48 to 72 hours following surgery.<sup>15-17</sup> Since the inflammatory cascade of cytokines is highly complicated following CABG, determining the exact shift of the T<sub>H</sub>1/T<sub>H</sub>2 balance requires more time. To reduce this interpretation factor, we evaluated patients 2 weeks after CABG.

Stored whole blood has an immunosuppressive effect attributed mainly to storage-related factors. The inhibitory effects of blood transfusions are reduced but not eliminated by prestorage leukofiltration. However, the severity of immunomodulation from blood transfusion is highly dependent on the leukocyte count. Khan et al investigated the consequences of different amounts of leukocyte transfusion on the outcome of patients undergoing cardiac surgery. They found that patients who received leukofiltered blood had shorter intensive care unit and hospital stays, and shorter duration of mechanical ventilation duration compared with patients who received buffy coat-depleted, and non-leukoreduced packed cells. They also indicated that 7 days after surgery, IL-10 was significantly higher in the buffy coat-depleted, and non-leukoreduced groups.<sup>18</sup> In our study, we observed high IL-10, 14 days postoperatively. These findings demonstrate that the immune systems of patients who underwent CABG are at their weakest 7 to 14 days after the operation. In this regard, Martinez et al revealed that intraoperative cell-saver blood transfusion is accompanied by IL-10 reduction postoperatively.<sup>19</sup> Blood transfusion alters the function of helper T cells, including a decreased ratio of helper to suppressor lymphocytes, and reduced production of inflammatory cytokines,<sup>20</sup> while autologous blood transfusion activates the T<sub>H</sub>1 pathway by decreasing IL-10 and increasing IL-2.<sup>21</sup> Additionally, the immune response to major surgery is anti-inflammatory.<sup>22</sup> The results of our study showed a decrease in the expression of *T-bet* and *STAT4*, both involved in the T<sub>H</sub>1 pathway and IFN- $\gamma$  production, after

blood transfusion. Also, the expression of *GATA3* and *STAT6*, both involved in the T<sub>H</sub>2 pathway and IL-4 production, decreased. However, production of IFN- $\gamma$  and IL-4 by CD4<sup>+</sup> T cells did not show significant changes. Moreover, both expression of *STAT3* and production of IL-17A by CD4<sup>+</sup> T cells significantly decreased following blood transfusion, indicating a suppressed T<sub>H</sub>17 function following blood transfusion. Based on these findings, it can be said that allogeneic blood transfusion in patients undergoing surgery may lead to suppression of the immune system.

In contrast with our findings Spinella et al., have shown that the levels of IL-2, IL-17, IL-10, and IFN- $\gamma$  did not increase significantly after cardiac surgery. They also demonstrated that fresh transfusion versus aged RBCs did not result in substantial changes in hemostasis, immune, or nitric oxide parameters.<sup>23</sup> Additionally, the type of cardiac surgery influences the cytokines secretion postoperative. Saracevic et al. indicated that off-pump surgery is accompanied by having higher IL-6 and IL-10 levels than on-pump surgery,<sup>24</sup> this finding can explain the heterogeneity between kinds of literature.

In the study by Tárnok et al,<sup>14</sup> it was reported that increased IL-10 secretion postoperatively is a negative risk factor for outcome. In the present study, a significant increase in IL-10 production by CD4<sup>+</sup> T cells indicate suppression of the immune response. It can be hypothesized that an increase in IL-10 might be associated with increased regulatory T-cell function and its transcription factors, which requires investigation in future studies.

The results of our study also showed a decline in the T<sub>H</sub>17 pathway. Decreasing IL-17A, a pleiotropic inflammatory cytokine that is considered a multiple-target cytokine in inflammation<sup>25</sup> after blood transfusion can enhance the imbalance of immune factors postoperative and weaken the patient's immune system. The published guidelines for the management of cardiac patients requiring surgery and without surgery are being updated to reduce the mortality rate, postoperative complications, and length of hospitalization, and improve surgical outcomes.<sup>5,26</sup> In this regard, the published information from patients with the same goals as the present study can be useful.

The disruption in the molecular pathways observed in our study is probably due to the presence of leukocyte DNA. In general, the intracellular response of blood receptor T cells to donor DNA can cause this reversal of molecular pathways and be a defense mechanism to

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prevent the immune system from overreacting. However, some studies showed that fresh allogeneic blood counts with normal leukocyte counts did not significantly affect IL-10 and tumor necrosis factor- $\alpha$  production and leukocytes do not appear to be responsible for that. Leukocytes degenerate and become apoptotic during storage in the refrigerator, and apoptotic cells in transfused blood cannot suppress the immune system by producing immunologically active substances.<sup>27</sup> Major histocompatibility complex-peptide complexes that remain on apoptotic cells have been suggested to act as T-cell receptor antagonists and inhibit T-cell function.<sup>28</sup> On the other hand, evidence suggests that by degrading leukocytes, leukocyte-derived bioactive substances such as histamine, eosinophil cationic protein, eosinophil protein X, and myeloperoxidase accumulate extracellularly, all of which play an essential role in suppressing the immune system.<sup>29,30</sup> It has been shown that RBCs may also be involved in suppressing storage-dependent immune systems because erythrocyte membrane phospholipids activate macrophage-derived prostaglandins, which are potent immune regulators.<sup>31,32</sup>

In summary, the results of this study showed that blood transfusions alter the balance of helper T cells and reduce their activity. A decline in the expression of T<sub>H1</sub>, T<sub>H2</sub>, and T<sub>H17</sub> transcription factors and an increase in IL-10 production indicate the immunomodulatory effect of blood transfusion. Our findings demonstrated that 14 days postoperatively the immune systems of patients are weak and susceptible to further complications such as infection and impaired pulmonary function. Further studies are needed to determine the duration of the immunomodulated immune system following CABG.

### STATEMENT OF ETHICS

All study subjects gave their written informed consent. The study protocol was approved by the Ethics Committee of Mazandaran University of Medical Sciences.

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### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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