RESEARCH ARTICLE
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Expression of Immune Checkpoints LAG-3, CTLA-4, TIM-3 and PD-1 in Beta Thalassemia patients Treated using HbF Augmentation Therapy and Regular Transfusions

Khan K. et al.: Immune Checkpoints Expression in Beta Thalassemia

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ABSTRACT

Introduction: Beta-thalassemia is an inherited hemoglobin disorder caused by mutations in HBB gene encoding beta globin chains. Severe anemia secondary to defective globin chains, chronic hemolysis and ineffective erythropoiesis requires transfusion support from early childhood. Recently used treatment options with promising results in resource limited countries includes drugs which augment HbF such as hydroxyurea and thalidomide. Although effective in alleviating anemia and related symptoms, these drugs particularly thalidomide has been known for its immunomodulatory role. Similarly, repeated transfusions with compromised immune system increases the risk of infections and weakened immunity. One of the key regulators of immune systems includes immune checkpoints, cell surface molecules on immune cells. Limited studies are available on immune checkpoints such as LAG-3, CTLA-4, TIM-3 and PD-1 expression in thalassemia and its treatment. **Objectives:** This study aimed to compare LAG-3, CTLA-4, TIM-3, and PD-1 expression in patients treated using HbF augmenting drugs or transfusions and with iron overload. These findings will provide an insight into the immune regulation in beta-thalassemia in response to treatment. **Methods:** In this study, the expression of LAG-3, CTLA-4, TIM-3 and PD-1 was quantified using real time PCR in patients managed on blood transfusions (n=33) or HbF augmenting drugs (n=140) and compared with healthy controls (n=27). **Results:** Our results show an increased expression of LAG-3 in patients regardless of treatment whereas increased CTLA-4 in patients on regular transfusions. On the other hand, the expressions of TIM-3 and PD-1 were higher in patients taking HbF augmentation therapy compared to patients on blood transfusions or the healthy controls. A very weak to no correlation was found between serum ferritin and immune checkpoints. **Conclusions:** These findings are suggestive of alterations in immune regulation in beta-thalassemia which could be attributed to thalassemia itself, repeated exposure to blood products, recurrent infections and the immunomodulatory drugs.

Keywords: Beta Thalassemia, HbF augmentation therapy, Hydroxyurea, Thalidomide, Immune Checkpoints

1. INTRODUCTION

Beta (β) thalassemia is one of the most common inherited hemoglobinopathies due to mutations in HBB gene encoding beta globin chains resulting in severe anemia [1]. It has a worldwide prevalence with major disease burden reported from the Mediterranean region, the middle East, Indian sub-continent, Southeast Asia and Melanesia to Pacific island [2]. Due to absence of national thalassemia registry in Pakistan, the exact prevalence remains unknown but it is estimated that approximately 5000-9000 β-thalassemia carriers are born annually [3]. Severe anemia usually manifests in the early childhood and requires regular blood transfusions in transfusion dependent β-thalassemia (TDT). Other complications including delayed growth, hepatosplenomegaly, skeletal changes and endocrinopathies are seen in both TDT and non-transfusion dependent thalassemia (NTDT) [4-7] . Blood transfusions improve the anemia and their overall development however the risk of alloimmunization, recurrent infections and iron overload remain high [8, 9]. Optimal iron chelation therapy is required to prevent from long-term cardiac, hepatic and endocrine complications [10]. Curative treatment options such as hematopoietic stem cell transplantation and gene therapy are either expensive or not available in countries like Pakistan [11, 12]. The recent use of drugs which could augment the fetal hemoglobin (HbF) such as hydroxyurea (HU) and thalidomide have shown a remarkable efficacy and safety profile [13-15].

Immune checkpoints (ICPs) are cell surface molecules, critical for immune regulation by maintainingb a balance between T cell expansion and survival as well as to limit the tissue damage [16]. This equilibrium is essential to protect healthy tissues from damage during insults such as infections, sepsis and cancers [17]. ICPs such as Lymphocyte Activation Gene-3 (LAG-3), Cytotoxic T Lymphocyte Associated protien-4 (CTLA-4), T cell Immunoglobulin and Mucin-domain containing-3 (TIM-3) and Programmed cell death protein-1(PD-1) are important regulators of immune homeostasis [18]. These inhibitory ICPs are expressed on T cells, dendritic cells, natural killer cells, and other immune cells, and maintain self-tolerance under normal conditions [19]. Their dysregulated function contributes to pathological states including cancers, autoimmunity and chronic inflammatory disorders [19].

The role of ICPs is well studied in cancers and immune mediated disorders but their role in thalassemia remains least explored. LAG-3, PD-1 and TIM-3 are known to play an important role in T cell exhaustion which leads to tumor escape from the immune system [20]. LAG-3 works by binding to the MHC class II molecules with high affinity, suppressing T cell proliferation and enhancing regulatory T cell (Treg) functions [21, 22]. Similarly, PD-1 inhibits TCR signaling by binding to its ligands PD-L1/PD-L2, leading to T cell apoptosis, reduced cytokine secretion and increased Treg proliferation, thus suppressing normal immune responses [23]. Both PD-1 and LAG-3 also effect the interferon-gamma (IFN-γ) regulation either separately or together and are upregulated in tumor infiltrating lymphocytes [24]. Although TIM-3 interacts with several ligands, its interaction with Galectin-9 is associated with T cell inhibition and cell death, critical in autoimmune disorders and cancer related immune suppression [25]. CTLA-4, a FoxP3 target gene, competes with CD28 for CD80/86 binding on antigen-presenting cells, reinforcing Treg-mediated immune suppression [26, 27]. In addition, PD-1 interaction and function with its ligands is also controlled by CTLA-4 via CD80 [28].

In TDT, chronic immune activation, systemic inflammation and iron overload disrupts the immune homeostasis, potentially altering ICPs expression and function. This study aims to determine the changes in the expression of LAG-3, CTLA-4, TIM-3, and PD-1 genes in TDT with iron overload and NTDT following treatment with HbF augmenting drugs. Our findings will provide a useful insight in understanding of the immune regulation in β-thalassemia patients treated with blood transfusions with potential to iron overload and treatment with HbF augmenting drugs particularly thalidomide.

2. METHODS

2.1 Participants

This was a cross-sectional study with a total of 200 study participants including 173 TDT patients and 27 healthy controls (HCs), conducted over a period of 6 months. Among 173 TDT patients, 33 patients were on regular blood transfusions (Tx group) while 140 were using HbF augmenting drugs (HbF group), HU and thalidomide in combination. Our inclusion criteria included patients with confirmed diagnosis of TDT, over 1 year of age and on same treatment protocol for at least 6 months. Patients on HbF augmenting drugs (HbF group) were transfusion independent and were reclassified as NTDT. Patients with active infections and positive for Hepatitis B, C or HIV were excluded. Individuals with no known history of any acute or chronic illness, normal hematological and biochemical profiles were considered as HCs.

2.2 Sample collection

Blood samples were taken from each participant in EDTA and gel tubes after taking complete clinical history. Complete blood counts (CBCs) were done on automated hematology analyzer (Sysmex XP-300, 5-part analyzer, Sysmex Corporation, Japan). Biochemical profiles including total bilirubin, Alkaline transferase (ALT), creatinine and serum ferritin were also performed on serum samples of each participant.

2.3 RNA extraction and cDNA synthesis

Manual RNA extraction was performed for each sample followed by conversion into cDNA. Briefly, 250µl of blood and 750µl of TRIzol reagent (Thermo Fisher Scientific) were taken in the Eppendorf tube and vortexed till the appearance of homogenous brown color. Chloroform (250µl) was added followed by vortexing and incubation at room temperature (RT) for 5mins. The samples were then centrifuged (Eppendorf Centrifuge 5417R) at 13000 rpm for 15mins at 4°C. The top clear aqueous layer was transferred to another Eppendorf tube with addition of 700µl of 2-Propnaol and incubation for 5mins at RT followed by centrifugation at 12000rpm for 10mins at 4°C. The supernatant was discarded, 700µl Ethanol (70%) was added and centrifuged at 10000rpm for 8 mins at 4°C. The process was repeated followed by removal of Ethanol and addition of 20µl RNase/Nuclease free water to persevere the RNA from degradation. RNA quality and quantity was determined using Nanodrop (Colibri Microvolume Spectrometer, Germany) (29, 30). RNA was stored at -80°C until ready for cDNA synthesis. The synthesis of cDNA was done using OneScript® Plus cDNA synthesis kit (abm, Canada) on BIO-RAD T100 ThermoCycler (California, US) as per manufacturers guidelines. The newly synthesized cDNA was stored at -20°C until further use.

2.4 RNA expression analysis

The mRNA expressions of *LAG-3, CTLA-4, TIM-3,* and *PD-1* were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using BlasTaq™ 2X qPCR Master Mix (abm, Canada) and SYBR Green dye for fluorescence detection. The Primer sequences were designed using Primer3Plus, are given in Table 1.
The qRT-PCR was done using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) under the following cycling conditions: 95°C for 3mins followed by 40 cycles of 95°C for 15 second and 60°C for 60 seconds. Data were analyzed using the QuantStudio Design and Analysis software (Thermo Fisher Scientific). The mRNA expression levels of *LAG-3, CTLA-4, TIM-3,* and *PD-1* were normalized to the GAPDH housekeeping gene by using ΔΔCt calculations. Relative expression levels between groups were calculated using $2^{-\Delta\Delta Ct.}$

Table 1: Primer sequences for *LAG-3, CTLA-4, TIM-3, PD-1* and *GAPDH*

Primers	Forward	Reverse
GAPDH	5′-GTCTCCTCTGACTTCAACAGG-3′	5′-ACCACCCTGTTGCTGTAGCCAA-3′
LAG-3	5′-CTTCTTGGAGCAGCAGTG-3′	5′-AAAGGAGCAGAAAGGAC-3′
CTLA-4	5′- ACGGGACTCTACATCTGCAAGG-3′	5′- GGAGGAAGTCAGAATCTGGGCA-3′
TIM-3	5′-GTCATCAAACCAGCCAAGG-3′	5′-AGTGTCTGTGTCTCTGCT-3′
PD-1	5′-CTCAGGGTGACAGAGAGAAG-3′	5′-GACACCAACCACCAGGGTTT-3′

2.5 Statistical analysis

Data were collected in Microsoft Excel sheets and statistically analysed using GraphPad Prism version 10.2.3 software (GraphPad Software, USA). Categorical data are summarised as frequency (n) and percentages (%). Numerical data were checked for normality using Shapiro Wilk test and non-parametric data are represented as median and interquartile range. Intergroup comparisons of gene expression data among three groups; HbF group, Tx group and HC group were done using Kruskal-Wallis H test followed by post-Hoc Dunn's test. Spearman's correlation analysis was performed to determine an association between serum ferritin and ICPs relative expression

in each group. A p-value of ≤ 0.05 was considered as statistically significant and denoted as ns; non-significant, *; < 0.05, **; < 0.01, ***; < 0.001, ****; < 0.0001.

3. RESULTS

3.1 Participant characteristics

In this study, the overall median age of β-thalassemia patients was 7 years, with 6 years in HbF group and 10 years in Tx group. Majority of the patients in HbF group were females (n=86, 61.43%) while 60.6% (n=20) of Tx group comprised of male patients. This study included patients with splenomegaly, hepatomegaly and history of splenectomy. The summarized data are provided in the Table 2.

Table 2: Participant characteristics of β-thalassemia patients

Characteristics	Known β-thalassemia patients	
	HbF group (n=140)	**Tx group (n=33)**
Median age (Range)	6 (1-22) years	10 (1.5-22) years
Females (n, %)	86 (61.43%)	13 (39.4%)
Male (n, %)	54 (38.57%)	20 (60.6%)
Splenomegaly		
Yes (n, %)	48 (34.28%)	24 (72.72%)
No (n, %)	92 (65.72%)	9 (27.28%)
Splenectomy done		
Yes (n, %)	02 (1.43%)	13 (39.4%)
No (n, %)	138 (98.57%)	20 (60.6%)
Hepatomegaly		
Yes (n, %)	16 (11.43%)	12 (36.37%)
No (n, %)	124 (88.57%)	21 (63.63%)

3.2 Hematological and biochemical profiles

CBCs and biochemical profiles of β-thalassemia patients in both groups were done. Patients in HbF group were less anemic (median Hb 8.3 g/dl) compared to Tx group (median Hb 6.3 g/dl) while rest of the hematological parameters were comparable in both groups. The median of total bilirubin, ALT and creatinine were comparable in both groups with slight variations in the ranges. The median serum ferritin was higher in Tx group (1546 ng/mL) compared to HbF group (1295 ng/mL). The median serum ferritin in HCs was 185 ng/mL (Range: 112-234).
Data summarized in Table 3.

Table: 3 Hematological and biochemical profiles of study participants

Parameters	HbF group (n=140) Median (Range)	Tx group (n=33) Median (Range)
Hb (g/dL)	8.3 (4.5-11.9)	6.3 (5.1-12.2)

RBC count ($x10^6/\mu L$)	3.4 (1.2-4.2)	3 (1.8-5)
WBCs ($x10^3/\mu L$)	4.4 (1.4-7.9)	6.5 (2.8-10)
MCV (fL)	73 (49-85)	73.3 (45.3-77)
MCH (pg)	25.2 (17.4-28)	24.9 (20-31.5)
MCHC (g/dL)	33 (31.2-35.6)	31.4 (30-34.4)
PLT ($x10^3/\mu L$)	192 (119-339)	266 (148-520)
Total Bilirubin (mg/dL)	1.7 (0.1-3.7)	1.6 (0.5-2.5)
ALT (IU/L)	29 (10-112)	29 (11-66)
Creatinine (mg/dL)	0.5 (0.4-0.6)	0.5 (0.3-0.8)
Serum Ferritin (ng/mL)	1295 (17-7556)	1546 (166-8944)

3.3 Expression of *LAG-3, CTLA-4, TIM-3* and *PD-1*

The relative expression of *LAG-3, CTLA-4, TIM-3* and *PD-1* genes was measured in β-thalassemia patients in HbF group, Tx group and compared with HC group. Overall, the expression of these four ICPs was higher in β-thalassemia patients compared to HC group.
However, in subgroup analysis there was an increased expression of *LAG-3* in patients on HbF drugs or Tx group compared to HC ($p < 0.01$) group (**Fig: 1a**). *CTLA-4* expression was raised in Tx group compared to both HbF ($p < 0.0001$) and HC ($p < 0.001$) groups **(Fig: 1b)**. On the other hand, *PD-1* and *TIM-3* expressions were increased in HbF group compared to Tx ($p < 0.0001$) and HC ($p < 0.001$) groups (**Fig: 1c,d).**
No to weak correlation was found between serum ferritin and ICPs expression in either group as shown in Table 4.

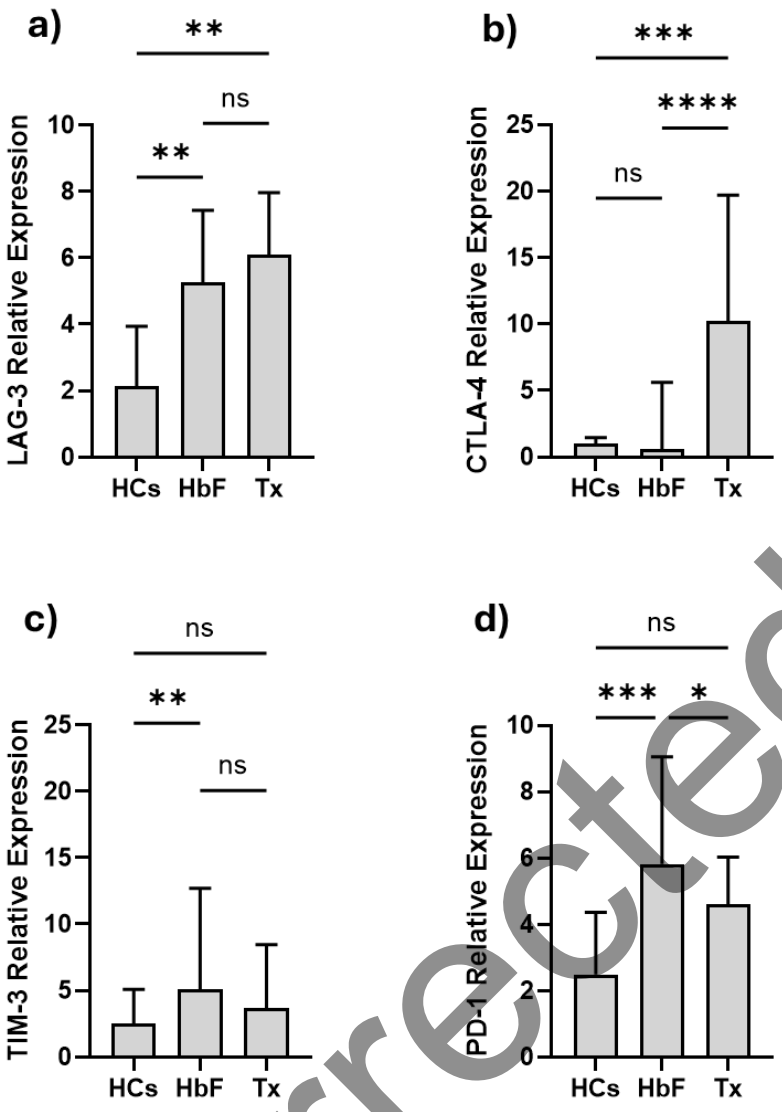

Fig 1: Immune Checkpoints Expression in β-thalassemia patients on different treatment protocols. Relative expressive of a) *LAG-3, b) CTLA-4, c) TIM-3* and d) *PD-1* β-thalassemia patients treated using HbF augmenting drugs (HbF, n=140) and regular transfusions (Tx, n=33) compared with healthy controls (HCs, n=27). Data are statistically analyzed using Kruskal Wallis with post-hoc Dunn's test comparing three groups and expressed as median with interquartile range. P value is denoted as ns; non-significant, *; < 0.05, **; < 0.01, ***; < 0.001, ****; < 0.0001.

Table: 4 Correlation between Serum Ferritin and ICPs expression

Groups	ICPs	Spearman's Correlation (r)	p-value
Serum ferritin in HCs	LAG-3	0.151	0.45
	CTLA-4	-0.066	0.74

	TIM-3	0.059	0.76
	PD-1	0.283	0.15
Serum ferritin in HbF group	LAG-3	0.185	0.02
	CTLA-4	-0.116	0.17
	TIM-3	0.091	0.28
	PD-1	-0.194	0.02
Serum ferritin in Tx group	LAG-3	0.379	0.02
	CTLA-4	-0.076	0.67
	TIM-3	-0.097	0.58
	PD-1	-0.206	0.25

4. Discussion

β-thalassemia represents a complex clinical condition with severe anemia, iron overload, extramedullary hematopoiesis and compromised immune system. Our study included TDT patients who were on regular blood transfusions or transfusion independent on HbF augmenting drugs at the time of inclusion. Similar to published studies on the efficacy on HbF augmenting drugs particularly thalidomide, our patients were transfusion independent with no clinically significant anemia or related complications [13, 31]. In this study, patients taking HbF drugs were reclassified as NTDT due to transfusion independency and had a stable hemoglobin (Hb 8.5 g/dl). A study by Ansari S.H. *et al*. showed a median Hb of 9.35 g/dl in patients taking HU and thalidomide in combination for 6 months [15]. No significant difference in the WBCs and platelet counts were noted between two groups in our study however a wide variation was observed by another study after thalidomide use [32]. The median Hb in transfusion group was lower than HbF group with comparable range in both groups likely because the transfusion frequency and last transfusion varies among TDT patients [33]. Similar to previous studies, raised serum ferritin was noted in the TDT group due to repeated transfusions.

ICPs are cell surface molecules on immune cells responsible for immune regulation and are well-studied in infections, inflammatory conditions and cancers with prognostic significance [34-39]. Limited studies are available on their role in β-thalassemia and particularly in response to treatment used [9]. HbF augmenting drugs with a potential to increase production of HbF and hence ameliorate anemia, are in use in various centers throughout world with satisfactory results. In particular, thalidomide which has been in use for other clinical indications including multiple myeloma is an immunomodulatory drug with HbF augmentation properties [15]. Apart from thalidomide, other HbF augmenting drug such as HU, used in combination in our study, have been shown to impact the immune system. A study by Siriworadetkun S. *et al*. on β-thalassemia patients using HU showed a restoration of CD8 and CD4 T cells in the peripheral circulation [40]. ICPs are critical regulators of T cells functions associated with prognosis of various malignant conditions and their inhibitors as treatment options. In this study, four ICPs namely LAG-3, CTLA-4, TIM-3 and PD-1 were selected for determination of their relative gene expression in β-thalassemia patients. An increased LAG-3 expression was seen in β-thalassemia patients regardless of the treatment used which is consistent with the results of the study done on β-thalassemia patients on regular blood transfusions in Iran by Shokrgozar N. *et al*. [9]. In our study, CTLA-4 expression was increased in patients on regular blood transfusions,

but increased TIM-3 and PD-1 expression was observed in patients taking HbF drugs. No change in the expression of CTLA-4 and TIM-3 in transfused β-thalassemia patients was reported in Shokrgozar N. *et al.* study [9]. Although PD-1 expression remains the least explored in β-thalassemia, its role in other hematological disorders including acute myeloid leukemia, lymphomas and anemia secondary to cancers has been studied. ICPs do not only have an important role in regulation of immune response but also in erythroid cells proliferation [41]. The differences in the expression of ICPs in β-thalassemia patients could be attributed to immunomodulatory effect of HbF augmenting drugs, alloimmunization and autoimmunization secondary to repeated blood transfusions or immune dysregulation due to β-thalassemia or splenic changes.

Furthermore, a very weak to no correlation was found between the serum ferritin and ICPs expression in this study which is consistent with the findings of Shokrgozar N. *et al.* study [9]. Although previous studies have shown the role of iron in immune regulation, its association with ICPs in healthy individuals or patients with iron overload is yet to be fully understood [42]. The findings of our study suggest the role of HbF augmenting drugs: HU and thalidomide either used alone or in combination, have the capacity to alter immune responses. Alterations in the immune responses whether due to chronic exposure to blood transfusions, repeated infections or the use of immunomodulatory drugs, results in a compromised immune system. This study has certain limitations such as imbalanced group sizes, not comparing the ICPs expressions in patients taking either HU or Thalidomide alone and the presence of autoimmune or alloimmune antibodies. Furthermore, another limitation includes the ICPs expression in patients with splenomegaly and splenectomized patients. It was not compared due to disparities in the number of patients in each group, it would have given an insight into the role of spleen in immune regulation in β-thalassemia. Further studies to explore the relationship between the treatment used and immune system alterations while taking the above-mentioned limitations into account and also a pre- and post-treatment comparison are needed to provide a deeper understanding of the immunoregulation in β-thalassemia.

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Ethics approval statement: This study was approved by the Ethical Committee of Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar (REF no: KMU/IPDM/IEC/2023/17-18,21,24)
Patient consent statement: Informed consent was taken from all the study participants or their parents/guardians prior to inclusion in this study.
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