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TNFSF/TNFRSF cytokine gene expression in sickle cell anemia: Up-regulated TNF-like cytokine 1A (TL1A) and its decoy receptor (DcR3) in peripheral blood mononuclear cells and plasma

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ABSTRACT

Background: Sickle cell anemia (SCA), a disorder with an important inflammatory component, where vasoocclusion is major contributor to the disease pathophysiology. Pro-inflammatory cytokines play an important regulatory role in the process of inflammation. We investigated the expression TL1A/DR3/DcR3 cytokine signaling pathway in peripheral blood mononuclear cells (PBMC) and their corresponding plasma levels in SCA subjects who presented with acute painful episodes.

Materials and methods: PBMC were isolated from the blood of SCA subjects and normal healthy controls. RNA isolated from PBMC was used for real time gene expression of TL1A/DR3/DcR3. Gene expression was compared in subgroups within SCA subjects with co-inherited fetal hemoglobin (HbF) or alpha-globin gene deletions. Plasma prepared from blood was used for determination of TL1A/DR3/DcR3 proteins by ELISA assays.

Results: In the PBMC of SCA subjects, expression of TL1A and DcR3 is elevated, while DR3 expression is lowered in comparison to normal control PBMC. In SCA subjects with HbF > 10%, TL1A/DcR3 expression is lower, while HbF < 10% is associated with increased TL1A/DcR3 expression. Moreover, subjects with HbF > 10% appear to have significantly fewer pain episodes in comparison to those with HbF < 10%. Deletion of alpha-globin genes appears to have no significant effect on TL1A/DR3/DcR3 expression. Circulating levels of TL1A, DR3 and DcR3 in plasma were significantly elevated in SCA subjects.

Conclusions: Elevated TL1A and DcR3 expression in PBMC of SCA subjects during painful vasoocclusive crisis, suggest an altered TL1A expression may contribute to the pathophysiology of vasoocclusive crisis in SCA. HbF > 10% appears to moderate TL1A elevation, while HbF < 10% exacerbates TL1A/DcR3 responses. Furthermore, subjects with HbF > 10% have significantly lower pain episodes reported as compared to subjects with HbF < 10%.

1. Introduction

Interaction between sickle red cells and activated endothelium results in the production of cytokines and inflammatory mediators that disrupt endothelial function [1,2]. Cytokines use various signaling pathways to modulate immune response and inflammation. One such signaling pathway, TNFSF/TNFRSF, consists of TL1A (TNF-like ligand 1A, TNFSF15) and its two receptors DR3 (Death domain receptor 3, TNFRSF 25) and DcR3 (Decoy receptor 3, TNFRSF 6B). This cytokine is expressed by monocytes, macrophages, dendrite cells, and endothelial cells in response to cytokines and binds to DR3 on the surface of T cells. Binding of DR3 to TL1A activates co-stimulatory signals that differentially regulate immune response, inflammation or apoptosis. However, binding of DR3 to TL1A is blocked by DcR3, subsequently inhibiting functional signaling process [3,4]. Interaction between TL1A and DR3 in-vitro can activate both NF-κB, and caspase pathways. The former pathway being pro-inflammatory results in cytokine secretion, cell proliferation and cell activation, whereas the latter pathway activates caspases and ends with apoptosis [5,6]. In lymphocytes, TL1A/DR3 interaction preferentially induces pro-inflammatory signals and not

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apoptosis. The mechanism of pro-inflammatory environment appears to be mediated by induction of cellular inhibitor of apoptosis protein (c-IAP) by up-regulated NF-κB. Apoptosis, however, can be induced in the presence of NF-κB inhibitors, suggesting that NF-κB activation by TL1A/DR3 interaction is a reason for inhibition of apoptosis [7]. Thus, a feedback loop is created that inhibits apoptosis and favors T-cell co-stimulation [8]. DcR3 can also inhibit apoptosis by blocking FasL/Fas and LIGHT/ HVEM mediated cell death [9,10].

TL1A/DR3/DcR3 cytokine system has been implicated in several inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, mucosal inflammation, and renal disease. In the colon of patients with ulcerative colitis, levels of mRNA and protein encoded by TL1A and DcR3 are significantly elevated [11,12,13]. In addition, TNFSF14 (LIGHT), a prothrombic and pro-inflammatory TNF-superfamily cytokine, is significantly elevated in SCA platelets and is correlated with platelet activation [14].

Inflammation and endothelial damage provide a pro-adhesive milieu that play a significant role in pathophysiology of vasoocclusion in SCA [15]. Clinical complications of SCA (homozygosity for the sickle hemoglobin or HbS gene, $\alpha_2\beta^s_2$) are mainly due to vasoocclusion and hemolytic anemia. Pathogenesis of SCA is multifaceted and is initiated by polymerization of deoxygenated HbS. Polymerization induced erythrocyte injury can result in abnormal erythrocyte-endothelial interactions, oxidation induced tissue damage, inflammation and vasoocclusion. Fetal hemoglobin (HbF, $\alpha_2\gamma_2$ or $\alpha_2\beta^s\gamma$) in the sickle erythrocyte inhibits polymerization of deoxygenated HbS [16]. Inhibition of in-vivo sickling of erythrocytes has been reported in patients with α-thalassemia, an effect due to reduced mean cell HbS concentration [17,18].

Sickle cell anemia is a multifaceted disease with an important inflammatory component and HbF is one of the major determinants of phenotype heterogeneity. Major complications of the disease include, vasoocclusive crisis leading to painful episodes, pulmonary hypertension, acute chest syndrome, sepsis and stroke. Understanding genetic basis of modifier effects, their mechanisms of action, and the gene interaction networks in which they exert their influence is going to be the defining feature of modifier genetics. Several approaches are currently being used to study modifier genes in human populations, including comparative expression profiling, genome-wide association studies and family-based association analysis.

A number of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β have been reported to be elevated in SCA subjects, suggesting activated endothelium [19,20,21]. Pro-inflammatory cytokine treatment resulted in significantly elevated TL1A/DR3/DcR3 m-RNA expression in cultured primary intestinal subepithelial myofibroblasts (SEMF's) and in other cell lines, suggest inflammatory mediators play a role in the regulation of TNFSF/TNFSFR pathway [22]. These studies and our results indicate the possibility of involvement of this pathway in development of inflammation directed vasoocclusion in SCA subjects during vasoocclusive crisis. The possibility of activation of TNFRF/TNFSRF pathway in SCA during crisis, will need further investigations on the activation of this cytokine signaling pathway in other immunocytes during vasoocclusive crisis.

2. Materials and methods

2.1. Patient selection

Blood samples were collected from SCA subjects who presented at the Hematology Clinic at King Fahad Hospital, Al-Ahsa, Saudi Arabia with an acute painful episode (pain severity required hospitalization to achieve effective pain control). The patients were homozygous for the HbS gene verified by ARMS PCR (Amplification-refractory mutation system for HbS), older than ten years of age, and not taking hydroxyurea. Blood from normal healthy controls was also collected.

2.2. Blood collection

Blood was collected in EDTA coated tubes. Each sample was divided into two portions; one was used for PBMC isolation, and the other for preparation of plasma. For isolation of PBMC, portion one of blood (diluted 1:1 with PBS) was layered over Ficol density gradient and centrifuged (400*g*) at 22 °C for 30 min. The plasma was carefully removed without disturbing the mononuclear cell layer. The PBMC collected, were diluted with PBS (1:15) and centrifuged (250*g*) at room temperature for 10 min., cellular pellet collected was washed twice with PBS, and used for isolation of RNA. The second portion of blood sample was centrifuged to recover plasma and stored at −80 °C till further use for determining levels of TL1A/DR3/DcR3 proteins by ELISA assays.

2.3. RNA isolation from PBMC

RNA was isolated from PBMC of SCA and normal subjects, using RNeasy plus kit (Qiagen, Valencia, CA. USA). To get DNA free RNA, gDNA eliminator columns and RNase free DNase were used and isolated RNA was quantitated by Nano drop method.

2.4. Real time relative quantitation of TL1A/DR3/ DcR3 in PBMC

DNA-free RNA (200 ng) isolated from PBMC from normal controls and SCA subjects was used as a template for RT-PCR to generate cDNA using TaqMan reverse transcription kit. The cDNA (20 ng) was used for real time relative quantification with the following TaqMan gene expression assay primers and probes, TL1A (TNFSF15: Hs00270802_s1), DR3 (TNFRSF25: Hs00600930_g1), DcR3 (TNFRSF6B: Hs00187070_m1). The GAPDH (Hs02786624_g1) was used as a normalizer (Thermo Fisher Scientific, USA). Real-time PCR assay was performed according to the manufacturer's instructions (Thermo Fisher 7500 real time PCR system) using TaqMan universal PCR master mix.

The cDNA samples for SCA and normal controls were run in triplicate. Amount of target gene (SCA-TL1A/DR3/DcR3), normalized to endogenous reference (GAPDH) and relative to calibrator (Normal-TL1A/DR3/DcR3) were determined by comparative C_T method ($2^{-\Delta\Delta CT}$ method). Results are expressed as a fold-change of expression in SCA, compared to normal controls. To get the true fold change, the fold-change values were converted to log base 2 values to even out the fold-change variation of up-regulated ($1 - \infty$) or down-regulated (0–1) genes. The gene expressions were compared amongst subgroups of SCA subjects, considering co-inheritance of high or low HbF, or alpha deletion (α-del).

2.5. Measurement of TL1A/DR3/DcR3 protein product in the circulating plasma

Plasma samples from SCA and normal subjects were used for determination of TL1A (Enzo Life Sciences inc., Farmingdale, NY, USA), DR3 (Cusabio and CusAb, MD, USA) and DcR3 (Biolegend, San Diego, USA) by ELISA. The assays were performed according to the manufacturer's recommendations, using undiluted plasma and each sample was run in triplicate.

2.6. Statistical analysis

The real-time gene expression fold-change was calculated using the comparative C_T method ($\Delta\Delta C_T$ method) as has been described in Dataassit tools (http://tools.thermofisher.com/content/sfs/manuals/cms_040980.pdf). SPSS software package (Version 17-SPSS Inc. Chicago) was used for statistical analysis. Comparison of mean values for plasma TL1A/DR3/DcR3 (ELISA) and frequency of pain episodes were performed by two-sample student *t*-test and presented by box and whisker plots. Correlation between pain episodes and HbF and between

Table 1
Clinical, laboratory and genetic characteristics of SCA and healthy control subjects. The values are expressed as Mean ± SD and differences were considered significant ($p < 0.05$), (a). In HbF < 10%, pain episodes for 1-alpha deletion are significantly higher than 2-alpha deletions ($p < 0.05$), (b) HbS levels are significantly lower in SCA (HbF > 10%) as compared to SCA (HbF < 10%), $p < 0.05$), (c). In SCA subjects HbF > 10% is significantly higher than subjects with HbF < 10%, ($p < 0.05$), (d). In SCA subjects with HbF > 10%, HBA2 levels are significantly lower than subjects with HbF < 10% ($p < 0.05$), (e). In SCA subjects, lymphocyte count is significantly lower than normal control count ($p < 0.05$), (f). In SCA subjects, reticulocyte count is significantly higher than normal control count ($p < 0.05$), (g). In SCA subjects, Ferritin levels are significantly higher than in normal control subjects ($p < 0.05$).

Parameter	Normal	SCA	SCA (HbF > 10%)	SCA (HbF > 10%) Normal α-genes	SCA (HbF > 10%) 1-α-del	SCA (HbF > 10%) 2-α-del	SCA (HbF < 10%)	SCA (HbF < 10%) 1-α-del	SCA (HbF < 10%) 2-α-del	
Number of subjects	15	27	(17/27)	7	7	3	(10/27)	5	5	
Age (years)	22.6 ± 5.92	29.83 ± 8.96	31.9 ± 9.65	30.57 ± 12.9	34.16 ± 7.70	31.6 ± 5.03	26.62 ± 4.98	25.75 ± 6.9	23.75 ± 1.25	
Acute chest syndrome	0	(12/27)	7	3	4	0	5	3	2	
Osteonecrosis		(15/27)	7	1	4	2	8	4	4	
Pain episode/1 year		8.5 ± 7.4 (22/27)	4.8 ± 2.5 (13/17)	4.1 ± 0.8 (5/7)	4.6 ± 1.5 (6/7)	4.5 ± 2.1 (2/3)	12.7 ± 9.7 (9/10)	19.7 ± 9.5 (4/5)*	12.5 ± 5.9 (5/5)*	a
Hemoglobin (gm/dl)	13.5 ± 1.5	9.1 ± 1.8	8.9 ± 1.8	9.2 ± 1.9	9.2 ± 2.4	9.2 ± 2.1	8.9 ± 1.3	7.8 ± 1.1	9.7 ± 1.1	
HbS (%)	0	77.5 ± 16.4	75.9 ± 6.5	72.3 ± 6.5*	78.2 ± 7.6	79.5 ± 11.1	88.1 ± 2.8*	87.5 ± 3.2	87.7 ± 1.5	b
HbF (%)	< 1%	16.3 ± 9.2	20.6 ± 6.9*	24.8 ± 6.9	18.2 ± 8.0	22.7 ± 1.5	6.4 ± 2.6*	7.8 ± 1.9	5.9 ± 2.3	c
HbA2 (%)	< 2.0%	3.5 ± 1.4	2.9 ± 1.1*	2.4 ± 0.51	3.5 ± 1.2	2.4 ± 0.06	4.1 ± 1.3*	3.5 ± 0.42	5.1 ± 1.4	d
HCT (%)	36.9 ± 12.9	27.5 ± 5.8	27.3 ± 6.3	26.1 ± 5.6	29.3 ± 7.3	28.4 ± 6.8	27.9 ± 4.8	24.1 ± 4.7	29.5 ± 4.3	
RBC (X 10^8 mm^3)	5.2 ± 0.45	3.7 ± 1.1	3.4 ± 1.1	3.12 ± 0.95	3.9 ± 1.1	3.6 ± 1.3	4.1 ± 0.8	3.4 ± 0.9	4.2 ± 0.7	
WBC(10^9/L)	6.4 ± 2.5	8.7 ± 3.9	8.4 ± 3.8	8.5 ± 4.2	7.6 ± 3.7	10.2 ± 2.1	9.3 ± 4.1	9.1 ± 6.1	11.6 ± 2.7	
Monocytes (%)	8.3 ± 4.6	9.2 ± 4.6	8.6 ± 3.9	7.7 ± 4.9	8.7 ± 3.2	9.2 ± 3.8	10.3 ± 5.5	10.1 ± 2.9	9.9 ± 4.5	
Lymphocytes (%)	44.1 ± 11.5*	32.2 ± 14.7*	31.6 ± 11.5	31.2 ± 15.8	28.3 ± 9.2	27.2 ± 21.8	33.3 ± 19.1	21.6 ± 8.2	46.7 ± 15.7	e
Neutrophils (%)	43.2 ± 11.8	53.8 ± 18.6	54.9 ± 14.8	56.5 ± 19.8	58.4 ± 11.3	58.2 ± 28.3	52.2 ± 23.9	66.5 ± 10.5	38.3 ± 20.3	
Eosinophils (%)	3.1 ± 1.6	3.4 ± 2.6	3.5 ± 2.3	2.8 ± 2.5	3.7 ± 2.5	2.1 ± 1.9	3.2 ± 3.1	1.5 ± 1.7	4.1 ± 4.2	
Basophils (%)	1.2 ± 0.6	1.1 ± 0.8	1.1 ± 1.9	0.39 ± 0.16	0.81 ± 0.56	3.2 ± 2.8	1.1 ± 1.2	0.3 ± 0.12	0.9 ± 0.7	
Platelets (10^9/L)	274.8 ± 54.5	240.2 ± 145.7	226.7 ± 165.2	215.7 ± 191.2	221.5 ± 154.5	247.8 ± 214.4	261.2 ± 95.2	204.5 ± 118.3	320.8 ± 29.7	
Reticulocytes (%)	1.8 ± 0.25*	6.5 ± 4.5*	7.7 ± 4.5	8.7 ± 5.6	5.6 ± 2.7	6.8 ± 6.1	4.8 ± 3.8	6.6 ± 4.8	3.5 ± 3.1	f
MCV (fL)	79.9 ± 6.8	74.7 ± 13.2	78.2 ± 14.2	78.8 ± 19.9	75.2 ± 6.8	81.6 ± 15.2	69.5 ± 7.7	71.5 ± 6.8	69.9 ± 7.5	
MCH (pg.)	26.6 ± 2.8	25.6 ± 5.4	27.9 ± 5.1	30.4 ± 5.3	24.9 ± 2.9	27.2 ± 8.8	22.2 ± 3.2	23.4 ± 3.8	22.9 ± 1.5	
MCHC (g.DL)	33.3 ± 0.9	33.8 ± 2.6	34.2 ± 2.2	35.2 ± 1.7	33.2 ± 2.2	32.6 ± 4.9	31.8 ± 2.5	32.5 ± 2.3	32.9 ± 1.9	
AST (units/ml)	22.7 ± 8.7	36.18 ± 14.12	38.6 ± 15.7	42.8 ± 22.1	32.21 ± 13.0	41.3 ± 8.02	31.11 ± 7.16	28.25 ± 10.25	31.25 ± 5.82	
ALT (units/ml)	29.5 ± 15.9	29.8 ± 14.5	26.2 ± 9.4	30.3 ± 15.9	26.28 ± 9.6	25.46 ± 4.9	31.6 ± 19.2	33.25 ± 11.8	25.25 ± 8.13	
LDH (units/ml)	328.5 ± 52.2	406 ± 177.9	427.4 ± 133.6	484.2 ± 250.3	392.5 ± 73.4	341.6 ± 139.6	320.5 ± 62.3	366.7 ± 69.7	317.3 ± 81.1	
Creatnine (mg/dl)	0.64 ± 0.17	0.59 ± 0.19	0.62 ± 0.21	0.55 ± 0.15	0.61 ± 0.14	0.7 ± 0.18	0.57 ± 0.25	0.45 ± 0.11	0.53 ± 0.04	
Bilirubin (mg/dl)	0.36 ± 0.21	0.47 ± 0.27	0.5 ± 0.25	0.64 ± 0.39	0.55 ± 0.26	0.47 ± 0.27	0.34 ± 0.11	0.34 ± 0.09	0.37 ± 0.06	
Alkaline Phosphatase(U/L)	78.3 ± 30.7	124.4 ± 64.2	140.8 ± 66.3	145.3 ± 66.3	138.3 ± 74.9	157.6 ± 111.4	105.8 ± 57.8	81.35 ± 35.9	1450.3 ± 66.6	
Ferritin (mg/ml)	46.6 ± 29.5*	911.8 ± 804.6*	1053.8 ± 819.9	1022.4 ± 858.8	783.5 ± 729.43	1353.7 ± 1119.9	846.2 ± 755.3	898.8 ± 851.9	952.1 ± 801.0	g

plasma TL1A/DR3/DcR3 and HbF or pain episodes, were analyzed by using Spearman's test for rank correlation coefficient (r^s) and probability (p value). Results were considered significant at $p \leq 0.05$.

3. Results

3.1. Population characteristics

Out of 27 SCA subjects, 17 had HbF > 10% and 10 had HbF < 10%. Among the SCA subjects, 20 had α-globin deletions (13 with a single gene deleted and 7 with two genes deleted). Twelve subjects experienced acute chest syndrome (ACS) and fifteen had osteonecrosis; five had ACS only and eight had osteonecrosis only. Furthermore, seven had both ACS and osteonecrosis, while five of the subjects had neither. In addition, twenty two subjects reported pain episodes and five reported none. Some of the laboratory and hematological indices in SCA patients compared to normal subjects are elevated and are significant ($p < 0.05$). Furthermore, in SCA subjects, co-inherited α-thalassemia (1-α deletion) appears to improve some of the laboratory and hematological indices (Table 1).

3.2. Expression of TL1A/DR3/DcR3 in PBMC

In the PBMC of SCA subjects, expression of TL1A and DcR3 at mRNA level were elevated, while DR3 was lowered, as compared to normal controls. The fold change in expression of TL1A was elevated by 3.34-fold (log base2 of 10.18-fold). However, DR3 expression exhibited a positive $\Delta\Delta C_T$ value and thus lowered by −1.78-fold (log base2 of 0.29-fold). DcR3 expression was again elevated by 3.64-fold (log base2 of 12.5-fold). This group of SCA subjects included both with high or low HbF levels. The results show that TL1A and DcR3 are elevated while DR3 level is lowered (Fig. 1).

3.3. Effect of HbF on TL1A/DR3/DcR3 expression in PBMC

Both high HbF and α-thalassemia are prevalent in the SCA population from the Eastern Province of Saudi Arabia. In our SCA subjects, 17 had HbF > 10% and 10 had HbF < 10%. Seven of subjects with HbF > 10%, with normal compliment of four α-globin genes, seven with 1- α-globin deletion and three with two α-globin deletions. Among subjects with HbF < 10%, five each have 1- and 2- α-globin deletions. Accordingly, a total of 20 subjects co-inherited alpha thalassemia (13 with 1 α-globin gene deletion, seven with 2 α-globin gene deletion) and seven subjects with no α-globin gene deletions (Table 1).

We investigated whether co-inheritance of high HbF, a genetic modifier of SCA affected TL1A/DR3/DcR3 expression in PBMC of SCA as compared to PBMC from normal control subjects. In SCA subjects with HbF > 10%, TL1A/DR3/DcR3 fold-change in expressions were 5.4/0.22/9.88 fold and with HbF < 10%, fold changes were 24.9/0.5/21.78 fold in comparison to normal. From above results, it is apparent that there is an inverse relation between HbF levels and TL1A/DR3/DcR3 fold changes (Fig. 1).

3.4. Effect of α-deletions on TL1A/DR3/DcR3 expression

Alpha deletions appear to have no significant effect on TL1A expression in SCA subjects with α-deletions (1 or 2 α-globin deletions) as one half had HbF > 10% and other half HbF < 10%. Furthermore, seven subjects with 4α-globin genes, all having HbF > 10%, had TL1A/DcR3 fold change expression comparable to high HbF group (Fig. 1).

3.5. Effect of HbF levels and alpha deletions on frequency of pain episodes in SCA subjects

We analyzed the frequency of occurrences of pain episodes as reported by SCA subjects over a period of one year. The subjects with HbF < 10% had significantly higher pain episode occurrences as compared to subjects with HbF > 10% ($p < 0.01$). Furthermore, in HbF < 10%, subjects having 2-alpha gene deletions reported significantly lower pain episodes as compared to 1-alpha deletion, ($p < 0.05$). This observation suggests that HbF > 10% may ameliorate the frequency of pain episodes, as compared to HbF < 10% (Table 1, Fig. 2).

3.6. Plasma levels of TL1A, DR3 and DcR3

TL1A/DR3/DcR3 were measured in the plasma of SCA and normal controls. Plasma levels of TL1A ($p < 0.008$), DR3 ($p < 0.03$) and DcR3 ($p < 0.006$) were significantly elevated in comparison to normal control plasma. Data from these assays were also compared between individuals with high or low HbF. In SCA subjects with either high or low HbF, TL1A or DcR3 are significantly elevated in comparison to normal plasma controls. In subjects with HbF > 10%, DR3 level was not significantly different from normal control plasma ($p < 0.23$). However, in group with HbF < 10%, DR3 elevation was significant compared to normal circulating plasma levels ($p < 0.04$). Our results show significant elevation of circulating plasma levels of TL1A, DR3 or DcR3 in SCA subjects. The presence of high HbF appears to moderate circulating DR3 but not TL1A or DcR3 levels (Fig. 3).

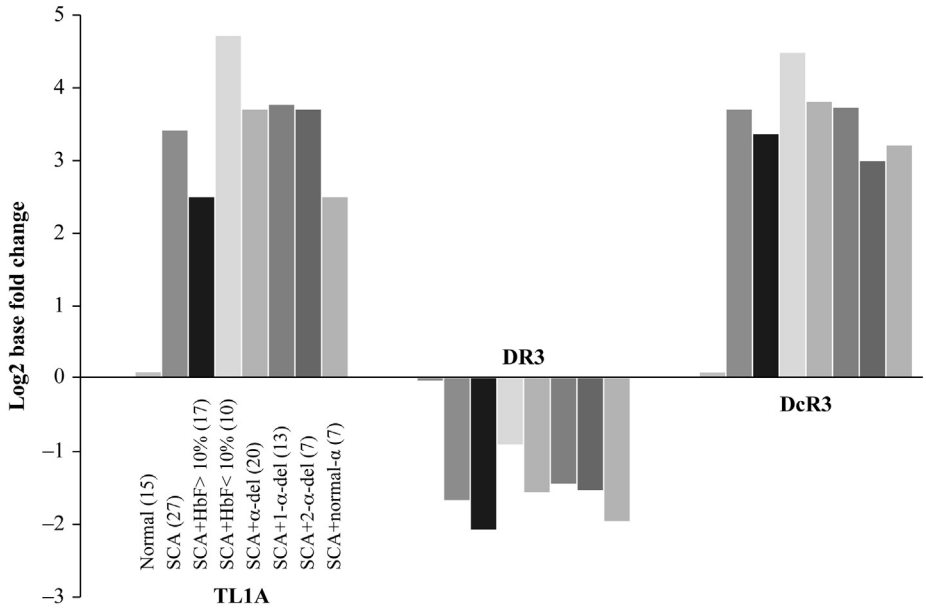
3.7. Effect of HbF and pain episodes on circulating levels of TL1A/DR3/DcR3

There was no significant effect of either HbF levels or number of pain episodes on the circulating plasma levels of TL1A/DR3/DcR3. Also, no correlation was identified between number of pain episodes and HbF levels. However correlation was slightly better for TL1A and HbF levels, though not significant. It may be because of a small sample size and heterogeneity in our SCA subjects (Table 2).

4. Discussion

The mechanism of TL1A up-regulation in the PBMC of SCA subjects in the presence of elevated DcR3 and decreased DR3 expression is not clear. Elevated expression of TL1A and DcR3 in the PBMC, are suggestive of both an inflammatory and anti-apoptotic environment during vasoocclusive crisis. This anti-apoptotic and pro-inflammatory state may promote a favorable environment for survival and maintenance of chronic inflammation during vasoocclusive crisis. It has been suggested that initial up-regulation of DcR3 may be secondary to an initial ineffective response to the inflammatory effects of TL1A. The ability of DcR3 to bind Fas and LIGHT ligands and its ineffective response to elevated TL1A, could lead to blockade of apoptosis, thus rendering PBMC resistant to apoptosis. The reason for elevated DcR3, considering its primary role is to inhibit TL1A signaling (a possible protective role during inflammation) is not known. It appears that there is a deficiency of functional DcR3 in an inflammatory environment. Most of the reports on TL1A involvement in several inflammatory diseases involved measuring TL1A/DcR3 in the plasma. However, in one of the studies, using inflammatory cytokines to activate TNFSF/TNFSRF cytokine family in cultured primary intestinal SEMF's and other colonic epthelial cell lines, significant differences in the fold change expression of TL1A/DcR3 compared to DR3 were observed [22]. These results are similar to our observations in PBMC of SCA subjects. TL1A levels have also been reported to be elevated in PBMC of Turkish SCA subjects [23]. Our results on activation of TNFRF/TNFSRF pathway in SCA subjects during crisis and above mentioned in-vitro studies, suggests further investigations into the activation of this signaling pathway in other immunocytes during vasoocclusion crisis in SCA subjects.

The sickle mutation in all sickle populations produce heterogeneous phenotypes. This observation demonstrates the importance of modifier



	TL1A		DR3		DcR3	
PBMC	Fold change	log2	Fold change	log2	Fold change	log2
Normal (15)	1	0	1	0	1	0
SCA (27)	10.18	3.34	0.29	−1.78	12.5	3.64
SCA+HbF>10% (17)	5.4	2.43	0.22	−2.18	9.88	3.30
SCA+HbF<10% (10)	24.9	4.63	0.5	−1.0	21.78	4.4
SCA+α-deletion (20)	12.23	3.61	0.31	−1.68	13.29	3.73
SCA+1α-deletion(13)	12.9	3.68	0.34	−1.55	12.61	3.65
SCA+2α-deletions (7)	12.38	3.62	0.32	−1.64	7.45	2.91
SCA+normal alpha genes (7)	5.35	2.41	0.24	−2.05	8.75	3.12

Fig. 1. RNA expression of TL1A/DR3/DcR3 in the PBMC of normal and SCA subjects. Gene expression of TL1A, DR3 and DcR3 was determined by real time PCR using comparative C_T method ($\Delta\Delta C_T$ method), normalized to endogenous reference (GAPDH) and relative to calibrator (normal TL1A/DR3/DcR3). The calculated fold change data is presented as log base2 fold change in expression. The figure also shows fold change levels in SCA subjects with HbF levels (> 10% or < 10%) or deleted α-globin genes. The fold change and their corresponding logbase 2 values are also presented.

genes and other regulatory elements in determining phenotype heterogeneity. Modifier effects may afford an opportunity to understand functional gene interactions and processes involved in their regulation. Modifier genes may also provide promising targets for therapeutic intervention. Majority of SCA patients in the Eastern Province of Saudi Arabia have elevated HbF and co-inherited α-thalassemia. Elevated HbF inhibits polymerization of deoxygenated HbS and co-inherited α-thalassemia reduce HbS polymerization in sickle erythrocytes [16,17,24]. However, high HbF does not always ameliorate the severity of the disease, as unevenly distributed HbF in F-cells results in sickle erythrocytes not being equally protected from polymerization induced damage [25]. Furthermore, an inverse relationship between HbF levels and TL1A/DR3/DcR3 fold change is evident. In HbF > 10%, TL1A/DR3/DcR3 fold change is 5.5/ 0.22/9.9, and with HbF < 10%, the fold changes are 25/0.5/22 as compared to normal, indicative of exacerbation of this pathway in SCA subjects with HbF < 10%. The reason for this inverse relation may be attributed to role of high HbF in inhibiting polymerization of deoxygenated HbS. Higher the HbF level, greater the inhibition and lower the HbF level, lesser or no inhibition of polymerization of HbS. Also depending upon high HbF distribution (evenly or unevenly) in F-cells, may determine either no response or mild to severe inflammatory cytokine surge and activation of this signaling pathways.

In our SCA patients with high HbF and still experiencing pain episodes, again reinforces that it is the distribution of HbF in F-cells rather than overall percentage of HbF, as previously suggested. However, attenuated capability of high HbF to thwart HbS polymer in SCA subjects may still provide an improved inflammatory environment compared to low HbF. Lowered TL1A expression and decreased occurrence of pain episodes in the presence of HbF > 10% may be a result of an improved inflammatory environment.

The role of co-inherited α-thalassemia is not that well established. In the Cameroonian SCA population with co-inherited α-thalassemia is associated with improved hematological indices and that may improve their survival [26]. Co-inherited α-thalassemia has been reported to increase in complications like painful episodes, acute chest syndrome or osteonecrosis, and this has been ascribed to increased blood viscosity that results from the higher PCV [24]. We also did observe some improvement in hematological parameters in SCA subjects with 1-α deletion as compared to 2-α deletion both in HbF (< 10% and > 10%). However, 2-α deletion subjects with HbF < 10% fared slightly better compared to 1-α deletion in frequency of pain episodes. The reason for this observation is not clear.

In SCA subjects, PBMC are not the only source of TL1A, DR3 or DcR3 in circulation, as activated macrophages, dendrite cells and damaged endothelium can also release these molecules into circulation during a vasoocclusive crisis. The plasma levels of TL1A, DR3 and DcR3 proteins in SCA subjects were significantly elevated in comparison to normal controls. HbF levels, had no effect on the circulating plasma levels of TL1A and DcR3. However, in subjects with HbF < 10%, DR3

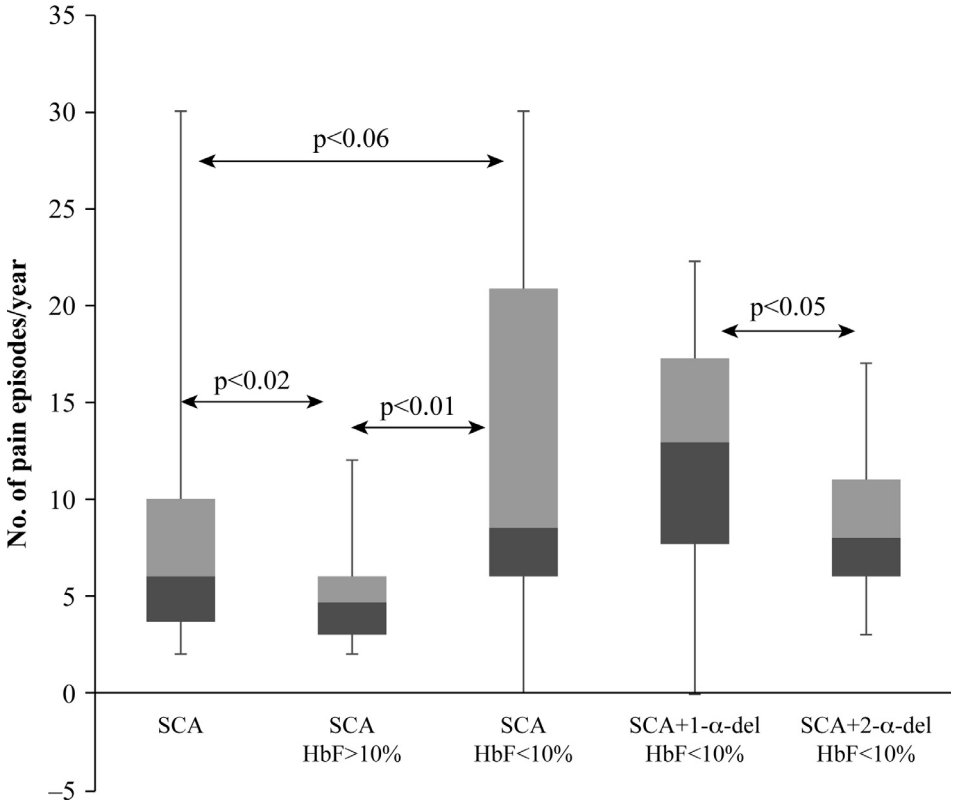


Fig. 2. Frequency of occurence of pain episodes. In the SCA subjects, the occurence of pain episodes over a period of one year was analyzed by two-sample student *t*-test and presented as box and whiskers plots. Comparison made within the SCA subjects with with HbF > 10% reported significantly lesser pain episodes as compared to those with HbF < 10% ($p < 0.01$).

levels were significantly elevated from normal control levels. We found no significant correlation between HbF levels and number of pain episodes and between circulating levels of TL1A/ DR3/DcR3 and HbF or number of pain episodes. This lack of correlation may be due to small sample size and phenotype heterogeneity.

Elevated expression of both the mRNA and protein of TL1A or DcR3 has been observed in the intestinal mucosa during active episodes of Crohn's disease or in ulcerative colitis. In such patients, elevated levels of circulating TL1A or DcR3 correlate with the severity of the disease and tend to return to normal after successful anti-inflammatory therapy

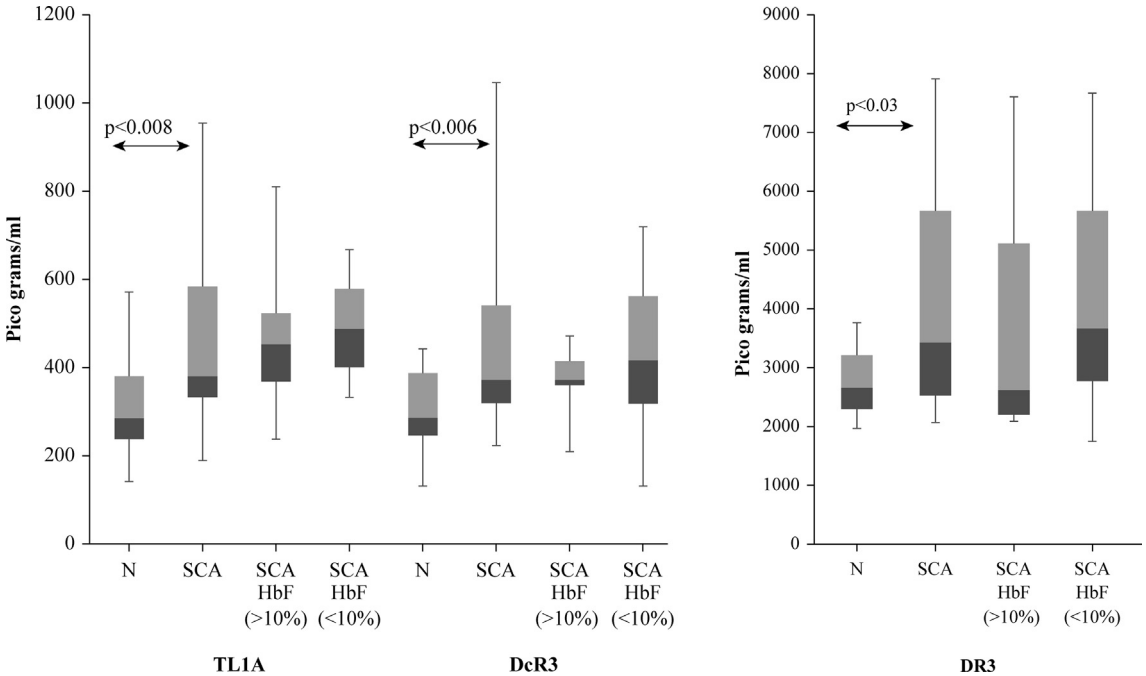


Fig. 3. Plasma levels of TL1A, DR3 and DcR3 in SCA and Normal subjects. In the SCA (n = 27) and normal control subjects (n = 15) plasma levels of TL1A/DR3/DcR3 were determined by ELISA and expressed as Pico gram/ml of plasma. Results were analyzed by two-sample student *t*-test and presented as box and whisker plots. The plasma levels of TL1A ($p < 0.$ 0.008), DR3 ($p < 0.03$) and DcR3 ($p < 0.006$) were significantly elevated in SCA plasma as compared to normal control plasma. The SCA subjects were further grouped into SCA with HbF > 10% and HbF < 10% and TL1A and DcR3 plasma levels were again significantly elevated as compared to normal plasma. However, DR3 in SCA group with HbF > 10% was not significantly different from normal control DR3 levels ($p < 0.27$).

Table 2
Correlation of HbF levels and pain episodes with plasma TL1A/DR3/DcR3 levels. The HbF and pain episodes were compared and no significant correlation was found. Circulating levels of TL1A/DR3/DcR3 were also compared with HbF levels or frequency of pain episodes in SCA subjects and again no significant correlation was observed. Correlations were determined by using Spearman's test of rank correlation and considered significant ($p < 0.05$).

(n = 27)	Pain	TL1A	DR3	DcR3
HbF	$r^s = 0.185$	$r^s = 0.300$	$r^s = 0.168$	$r^s = 0.149$
	p = 0.343	p = 0.133	p = 0.400	p = 0.455
Pain episodes		$r^s = 0.144$	$r^s = 0.0839$	$r^s = 0.092$
		p = 0.464	p = 0.677	p = 0.647
HbF < 10%	$r^s = 0.101$	$r^s = 0.408$	$r^s = 0.150$	$r^s = 0.0808$
	p = 0.781	p = 0.241	p = 0.677	p = 0.824
HbF > 10%	$r^s = 0.103$	$r^s = 0.279$	$r^s = 0.295$	$r^s = 0.338$
	p = 0.681	p = 0.246	p = 0.249	p = 0.183
Pain (HbF < 10%)		r^s-0.350	$r^s = 0.046$	$r^s = 0.103$
		p = 0.321	p = 0.897	p = 0.775
Pain (HbF > 10%)		$r^s = 0.033$	$r^s = 0.166$	$r^s = 0.090$
		p = 0.895	p = 0.656	p = 0.730

[27,28,29]. Elevated levels of of TL1A and DcR3 protein have also been reported in serum and synovial fluid of rheumatoid arthritis patients. Our results are in agreement with these studies and point to the possibility of a common signaling system during an inflammatory response [30,31]. DcR3 protein has been shown to be a reliable plasma marker of inflammation in ulcerative colitis and systemic lupus erythematosus [32,33].

The limitation of our study is reflected in a small sample size, and our inability to study same patients when in steady-state due to the fact that these patients experienced infrequent periods of steady stable states. In conclusion, up-regulated TL1A and DcR3 in the PBMC accompanied with elevated TL1A, DR3 and DcR3 proteins in plasma of SCA subjects suggests that this cytokine signaling pathway may be an important mediator of the inflammatory process contributing to vasoocclusion in SCA. Furthermore, high HbF may indirectly modulate TL1A expression and reduce the frequency of pain episodes by modifying inflammatory environment in SCA subjects.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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