DR. SAOVAROS SVASTI (Orcid ID : 0000-0001-8334-9838)

DR. PORNTHIP CHAICHOMPOO (Orcid ID : 0000-0001-8962-7202)

Article type : Ordinary Papers

Increased ferritin levels in non-transfusion-dependent β^0 -thalassaemia/HbE are associated with reduced CXCR2 expression and neutrophil migration

Chayada Thiengtavor^{1,2,*}, Sirikwan Siriworadetkun^{1,3,*}, Kittiphong Paiboonsukwong³, Suthat Fucharoen³, Kovit Pattanapanyasat⁴, Jim Vadolas^{5,6}, Saovaros Svasti^{3,7}, Pornthip Chaichompoo^{1,**}

¹Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand;

²Faculty of Optometry, Ramkhamhaeng University, Bangkok, Thailand; ³Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand; ⁴Siriraj Center of Research Excellence for Microparticle and Exosome in Diseases, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; ⁵Centre for Cancer Research, Hudson Institute of Medical Research, Melbourne, Australia; ⁶Department of Molecular and Translational Science, Monash University, Melbourne, Australia; and ⁷Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand.

* These authors contributed equally to this work.

****Correspondence to:**

Pornthip Chaichompoo, PhD

Department of Pathobiology, Faculty of Science, Mahidol University, 272 RamaVI Rd., Ratchathewi, Bangkok 10400 Thailand.

Tel.: +66 2 201 5577, Fax: +66 2 354 7158 E-mail: pornthip.chh@mahidol.ac.th

Running title: Increased ferritin levels in NTD β^0 -thalassaemia/HbE are associated with reduced CXCR2 expression and neutrophil migration

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/BJH.16295](https://doi.org/10.1111/BJH.16295)

This article is protected by copyright. All rights reserved

Abbreviations: MBL; mannose binding lectin, NTD; non-transfusion-dependent, ROS; reactive oxygen species, Ig; immunoglobulin.

Word count: abstract 199 words, total 5,545 words

Number of table: 4

Number of figure: 3

Number of supplementary table: 3

Number of supplementary figure: 2

Funding: This work was supported by Thailand Research Fund (TRF) (MRG5980043, DPG5980001 and DPG6080003); Office of the Higher Education Commission and Mahidol University under the National Research University Initiative; and Faculty of Science, Mahidol University. SSi was supported by National Research Management System (NRMS) Year 2018, National Research Council of Thailand (NRCT).

Disclosures:

Competing interests: The authors declare no competing interests

Redundant publication: no substantial overlap with previous papers

Authors' contributions: CT and SSi performed the experiments, the analysis of the data and drafting the manuscript. KPai and SF contributed to the concept of the study and specimen collection. SSv, JV and KPat contributed to the concept of the study, design the experiments, the analysis of the data and drafting the manuscript. PC was the principal investigator and takes primary responsibility for the concept and experimental design, the analysis of the data and drafting the manuscript. All authors reviewed and approved the final version to be published.

Summary

Severe bacterial infection is a major complication causing morbidity and mortality in β -thalassaemia/HbE patients. Innate immunity constitutes the first line of defense against bacterial infection. This study aimed to comprehensively investigate the innate immune phenotype and function related to factors predisposing to infection in non-transfusion-dependent (NTD) β^0 -thalassaemia/HbE patients. Twenty-six patients and 17 healthy subjects were recruited to determine complement activity (C3, C4, MBL and CH50) and surface receptor expression including phagocytosis (CD11b, CD16 and C3bR), inflammation (C5aR) and migration (CD11b, CXCR1 and CXCR2) markers on neutrophils and monocytes. In addition, phagocytosis and oxidative burst activity of neutrophils and monocytes against

Escherichia coli and neutrophil migration were examined. Decreased C3 and surface expression of CD11b and C3bR on neutrophils were found in patients. However, phagocytosis of neutrophils in patients was still in the normal range. Interestingly, patients displayed a significant reduction of surface expression of CXCR2 ($1,705 \pm 217$ MFI) on neutrophils, leading to impaired neutrophil migration ($9.2 \pm 7.7\%$) when compared to neutrophils from healthy subjects ($2,261 \pm 627$ MFI and $27.8 \pm 9\%$, respectively). Moreover, surface expression of CXCR2 on neutrophils was associated with splenectomy status, serum ferritin and haemoglobin levels. Therefore, impaired neutrophil migration could contribute to the increased susceptibility to infection seen in NTD β° -thalassaemia/HbE patients.

Key words: iron overload, innate immunity, bacterial infection, β -thalassaemia/HbE

Introduction

The β -thalassaemias are one of the most common inherited red blood cell disorders affecting haemoglobin (Hb) production, resulting in a chronic state of severe anaemia and ineffective erythropoiesis (Taher, *et al* 2018). The β -thalassaemias can be classified into two main groups depending on their transfusion requirements. Non-transfusion-dependent thalassaemia (NTDT) includes patients who do not require lifelong regular blood transfusions, whereas with transfusion-dependent thalassaemia (TDT) patients require continual blood transfusions for survival. Haemoglobin E (HbE), an abnormal haemoglobin, is the hallmark of Southeast Asia with a frequency as high as 50-60% in certain areas of Cambodia, Laos and Thailand. Due to the high prevalence of HbE, it has been estimated that the conception of β -thalassaemia/HbE in Southeast Asia is 0.25 per 1,000 births (Fucharoen and Winichagoon 2012). β -Thalassaemia/HbE presents varying clinical forms, from mild and moderate to severe. The Hb levels range from 3 to 13 g/dL with an average level of 7.7 g/dL. The patients with severe form have clinical manifestation similar to TDT, while patients with mild and moderate forms have clinical manifestation similar to NTDT (Taher, *et al* 2018). Variation in disease progression between NTDT and TDT patients was observed. NTDT and TDT patients both share similar clinical complications including increased susceptibility to infections that lead to a cause of morbidity and mortality, particularly in developing countries. Splenectomy, high serum ferritin and abnormal immunity have been reported as factors that associated with increased susceptibility to bacterial infection in both NTDT and TDT patients (Taher and Saliba 2017).

The elevated risk of infection has been attributed to multiple immune abnormalities affecting the innate and adaptive immune systems (Vento, *et al* 2006). TDT patients have been reported to have impaired neutrophil, monocyte and natural killer cell functions (Umiel, *et al* 1984), as well as lower levels of the complement proteins C3 and C4. Moreover, TDT patients have increased cytotoxic T-cell numbers and a decreased CD4/CD8 ratio as compared to healthy subjects (Ezer, *et al* 2002). B-cell numbers were also increased, contributing to higher than normal levels of IgG, IgM and IgA (Dua, *et al* 1993).

The complement system, a key component of innate immunity, is activated via three pathways, namely the classical, lectin and alternative pathways. Following activation of the complement pathway, C5a is released into the blood circulation and binds to C5aR on neutrophils and monocytes to trigger an inflammatory response (Merle, *et al* 2015a). During inflammation, CXCR1 and CXCR2 on neutrophils and monocytes interact with interleukin-8 (IL-8) as chemotaxis agent leading leukocyte recruitment, while the β_2 integrin family members lymphocyte function-associated-1 (LFA-1) (CD11a/CD18) and macrophage-1 antigen (Mac-1) (CD11b/CD18) on neutrophils and monocytes promote adhesion on endothelial cells by interacting with the intercellular adhesion molecule-1 (ICAM-1) to promote neutrophil and monocyte migration into the site of infection (Kumar and Sharma 2010, Shi and Pamer 2011). At the site of infection, Mac-1 on neutrophils and monocytes can also bind directly to microbes resulting in engulfment and phagosome formation. Moreover, complement receptor-1 (CR-1) consisting of C3bR/C4bR also binds to complement-coated microbes leading to phagocytosis. Antibody-opsonised bacteria are taken up via the Fc γ RIII receptor (CD16). Fusion between phagosomes and lysosomes leads to microbial killing within the phagolysosome by reactive oxygen species (ROS) and nitric oxide (Dupre-Crochet, *et al* 2013).

Most of the studies on bacterial infections in β -thalassaemia patients have focused on TDT patients, while information on clinical risk factors in patients with NTDT remains limited. In order to better improve understanding of the risk factors and disease progression in NTDT patients, a comprehensive evaluation of the innate immune system including serum complement components, complement activity, expression of surface receptors involved in phagocytosis, inflammation and migration on neutrophils and monocytes, phagocytosis and oxidative burst activity against *E. coli* and neutrophil migration was undertaken in NTD β^0 -thalassaemia/HbE patients. Interestingly, we found that serum ferritin levels were associated

with decreased surface expression of CXCR2 on neutrophils that contributed to reduced neutrophil migration and effector function in NTD β^0 -thalassaemia/HbE patients.

Materials and Methods

Patients and blood samples

Twenty-six NTD β^0 -thalassaemia/HbE patients (19 non-splenectomised and 7 splenectomised) and 17 healthy subjects with an age ranged from 18 to 53 years were recruited. The study protocol was approved by the Mahidol University Institutional Review Board (approval number COA.No.2015/063.1905). Written informed consent was obtained from all individual participants included in the study. All subjects had no evidences of a concurrent infection.

Patients who were under treatment with hydroxyurea, prednisolone or antibiotics were excluded from this study, and none of the patients were hospitalised or transfused within the proceeding 4 weeks. All patients received folic acid (5 mg) daily. The history of patient infections over the past 5 years was obtained from the patient's clinical records. All blood samples were collected in citrate phosphate dextrose adenine solution (CPDA-1) anticoagulant tubes at room temperature (RT) and processed within 2-3 h. Measurement of serum ferritin (immunoenzymatic kits, DiaMetra), serum mannose-binding lectin (MBL) (MBL Oligomer ELISA Kit, BioPorto Diagnostics) and serum human 50% complement hemolysis (CH50) (CH50 ELISA Kit, MyBioSource) were undertaken as detailed in the manufacturer's instructions.

Analysis of surface receptor expression

Fifty microliters of whole blood samples were incubated with FACS lysing solution (BD Biosciences) for 10 min at RT to lysed the red cells. Then, peripheral blood leukocytes were stained with fluorochrome conjugated monoclonal antibodies specific to CD45 (BioLegend), CD11b (BioLegend), CD16 (BioLegend), C3bR (BioLegend), C5aR (BioLegend), CXCR1 (BD Pharmingen), CXCR2 (BioLegend) and glycophorin A (GPA) (BD Pharmingen) for 30 min at 4°C. Antibodies used in this study are listed in Supplementary Table I. After incubation, the stained samples were washed to remove excess monoclonal antibodies and resuspended with 1% paraformaldehyde solution. The mean fluorescent intensity (MFI) of surface receptors on 50,000 events of GPA-CD45⁺leukocytes were acquired by a BD FACS LSRII flow cytometer (BD Biosciences) and analysed by BD

Diva™ software (BD Biosciences) as shown in Supplementary Fig 1. Unstained samples and isotype control were used as the negative controls.

Measurement of phagocytosis and oxidative burst activity

Phagocytosis and oxidative burst activity of peripheral blood neutrophils and monocytes were measured by flow cytometric analysis (Bohmer, *et al* 1992). Cold-70% ethanol fixed *E. coli* strain DH5α (2×10^9 bacteria/mL) labeled with propidium iodide (PI) (Sigma) (final concentration 100 µg/mL) was used to measure phagocytosis, while 2', 7' dichlorofluorescein diacetate (DCFH-DA) (Sigma) was used as a probe to measure ROS activity. Briefly, 440 µL of whole blood samples were pre-stained with 50 µM DCFH-DA for 10 min in a 37°C water bath to determine baseline ROS levels. Then, PI-labeled *E. coli* (at 5×10^7 bacteria final volume) was added into the samples which were incubated for 90 min in a 37°C water bath. Fifty microliters of samples were collected and red cells lysed using FACS lysing solution. After washing, samples were stained with PE conjugated anti-GPA (BD Biosciences) for 30 min at 4°C. After incubation, the stained samples were washed to remove excess monoclonal antibodies and resuspended with phosphate buffer saline solution. Neutrophil and monocyte phagocytosis and ROS activity were measured within an hour. Signals were acquired from 15,000 events of GPA negative leukocytes using a BD FACScan flow cytometer (BD Biosciences) and analysed by BD CellQuest software (BD Biosciences) (Supplementary Fig 2).

Neutrophil isolation

Peripheral blood neutrophils were isolated by sequential gradient density centrifugation as described in a previous study (Siwaponanan, *et al* 2017). Briefly, the neutrophil/red blood cell fraction was separated from 10 mL of whole blood samples using gradient density centrifugation with Lymphoprep™ solution (Fresenius/Axis-Shield PoC AS) at 1,800 rpm for 20 min at RT. Then, neutrophils and red blood cells were separated by using 6% dextran (Mr ~500,000) in normal saline solution (Sigma). Samples were mix well and placed at RT for 1 h. The contaminated red blood cells were removed using cold-water, and then neutrophils were neutralized by 0.6 M potassium chloride (Sigma). Isolated neutrophils were >95% viability, as assessed by trypan blue dye (Gibco).

Blocking of surface CXCR2 and CD11b on neutrophils

Isolated neutrophils were incubated with saturating concentrations of monoclonal antibodies (final concentration 5 $\mu\text{g/mL}$ per 1.5×10^6 neutrophils) of mouse anti-human CXCR2 (BioLegend) and/or mouse anti-human CD11b (BioLegend) in RPMI1640 medium (Gibco) supplemented with 10% heated fetal bovine serum (FBS) (Sigma) for 30 min at 4°C (Slattery, *et al* 2005). Then, samples were washed and resuspended with phosphate buffer saline, following which neutrophils were used for migration assays.

Migration assay

Neutrophil migration was measured in a transwell migration assay using a 24-well plate with a 3.0 μm pore polyester membrane and 6.5 mm inserts as previously described (Henkels, *et al* 2011). Isolated neutrophils [1.5×10^6 cells/200 μL in 0.5% bovine serum albumin (BSA)-Hanks' balanced salt solution (HBSS) (Sigma-Aldrich)] were applied in duplicate to the upper chamber. The Sigmacote (Sigma-Aldrich) coated the lower chamber containing 0.5% BSA-HBSS with 20 nM IL-8. Neutrophil migration was measured in triplicate as the number of neutrophils migrating to the lower chamber following 1 hour incubation at 37°C, 5% CO_2 .

Statistical analysis

Data were analysed using SPSS version 18.0 (IBM Collaboration) and GraphPad PRISM 6.0 (GraphPad Software, Inc.) software. Comparisons between parameters were evaluated with a non-parametric Mann-Whitney U Test. The correlation coefficient between parameters including serum ferritin levels, liver function test, extracellular molecule markers and phagocytosis and oxidative burst activity were calculated with Spearman's Rho (r_s). Linear regression models were used to analyse the association between the expression of cellular receptors on neutrophils and monocytes, and the predictors including serum ferritin, haemoglobin level and splenectomy status (by scoring 1 for normal, non-splenectomised subjects, 2 for NTD β° -thalassaemia/HbE patients without splenectomy and 3 for splenectomised NTD β° -thalassaemia/HbE patients). The correlation coefficient between neutrophil migration and surface expression of CXCR2 and serum ferritin levels was calculated using Pearson's correlation coefficient (r). The threshold for statistical significance for all comparisons was $P < 0.05$.

Results

Haematological and serological parameters in NTD β^0 -thalassaemia/HbE patients

Microcytic hypochromic anaemia with altered red blood cell indices, jaundice and iron overload were found in NTD β^0 -thalassaemia/HbE patients (Table I). Average haemoglobin levels in healthy subjects, non-splenectomised and splenectomised patients were 13.6 ± 1.5 , 7.5 ± 1.2 and 6.8 ± 1.4 g/dL, respectively. Reticulocyte count ($12.0 \pm 2.2\%$), nucleated red blood cells [338.3 ± 128.7 cells per 100 white blood cells (WBCs)] and platelet count ($607.7 \pm 127.5 \times 10^3$ cells/ μ L) in splenectomised patients were higher than those of non-splenectomised patients ($4.3 \pm 3.0\%$, 19.2 ± 16.0 cells per 100 WBCs and $166.3 \pm 74.2 \times 10^3$ cells/ μ L, respectively) ($P < 0.01$). Leukocytosis was presented in splenectomised patients, however, absolute neutrophil counts and differential WBC counts were in the normal reference range. Not only are the number of circulating immune cells important, but the liver and spleen are also important organs in the innate immune system. Hepatocytes are the major source of complement component production such as C3, C4 complements and MBL, while immunoglobulins are produced by plasma cells in the spleen. Both complement components and immunoglobulins play important roles in opsonisation of invading pathogens, and enhance the phagocytosis activity of neutrophils and monocytes.

A liver function test was used to monitor hepatic injury that could be related to high serum ferritin levels and iron deposit in the liver. A total of 46% (12/26) of patients had abnormal high levels of aspartate aminotransferase (AST) (range from 15 to 119 U/L, 44.4 ± 25.8 U/L) (Table I), while others liver enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) were in the normal reference range. All patients in this cohort study had normal levels of albumin and had no indications of liver damage as indicated by the liver function tests. Interestingly, decreased serum C3 complement levels were found in NTD β^0 -thalassaemia/HbE patients without splenectomy (0.68 ± 0.17 g/L) and with splenectomy (0.66 ± 0.08 g/L) as compared to healthy subjects (1.01 ± 0.18 g/L) ($P < 0.01$) (Table II). However, the levels of C4, MBL and CH50 were not significantly different between patients and healthy subjects. In addition, IgA levels were significantly increased in splenectomised patients (5.3 ± 2.0 mg/mL), while IgG levels were significantly increased in both splenectomised (20.7 ± 3.5 mg/mL) and non-splenectomised patients (17.2 ± 5.2 mg/mL) as compared with healthy subjects (2.2 ± 0.7 and 12.2 ± 2.4 mg/mL, respectively) ($P < 0.01$) (Table II).

Analysis of surface receptors on neutrophils and monocytes from NTD β^0 -thalassaemia/HbE patients

As a first line of defence against infections, phagocytes migrate to the site of infection, in response to microbial molecules and cytokines, and proceed to kill bacteria via phagocytosis and oxidative burst. Therefore, the expression of cell surface receptors that mediate phagocytosis (CD11b, CD16 and C3bR), inflammation (C5aR) and migration (CD11b, CXCR1 and CXCR2) on both neutrophils and monocytes was investigated. Our results showed that cell surface expression of CD11b, C3bR, C5aR and CXCR2 ($P < 0.05$, $P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively), but not CD16 and CXCR1, on neutrophils from both splenectomised and non-splenectomised NTD β^0 -thalassaemia/HbE patients were significantly decreased as compared to neutrophils from healthy subjects (Fig 1A and Supplementary Table II). Monocytes from splenectomised NTD β^0 -thalassaemia/HbE patients had decreased C5aR expression as compared to monocytes from healthy subjects ($P < 0.01$) (Fig 1B). Decreased expression of receptors on the cell surface could affect their functions, therefore, the phagocytosis and migration activity were further examined.

Monocytes from splenectomised NTD β^0 -thalassaemia/HbE patients exhibit impaired phagocytosis and oxidative burst activity

Decreased surface expression of CD11b, C3bR and C5aR on neutrophils and C5aR on monocytes from NTD β^0 -thalassaemia/HbE patients could affect their functions. Therefore, *ex vivo* phagocytosis and oxidative burst activity by neutrophils and monocytes were investigated. Flow cytometry was used to measure the percentage of neutrophil and monocyte phagocytosis and oxidative burst activity following incubation with PI-labeled *E. coli* (Supplementary Fig 2). The percentage of double positive neutrophils for phagocytosis and oxidative burst activity were not significantly different between splenectomised NTD β^0 -thalassaemia/HbE patients and healthy subjects ($P = 0.127$) or non-splenectomised NTD β^0 -thalassaemia/HbE patients and healthy subjects ($P = 0.880$) (Fig 2A-i and Supplementary Table III). However, neutrophils from NTD β^0 -thalassaemia/HbE patients displayed reduced oxidative burst activity (24 ± 11 MFI) as compared to neutrophils from healthy subjects (37 ± 10 MFI) ($P < 0.05$) (Fig 2A-iii).

Surprisingly, the percentage of monocytes with phagocytosis and oxidative burst activity from splenectomised patients ($9.0 \pm 7.6\%$) were decreased as compared to monocytes from non-splenectomised patients ($26.5 \pm 15.8\%$) and healthy subjects ($22.8 \pm 13.0\%$) ($P <$

0.05) (Fig 2B-i). The phagocytic activity of monocytes from splenectomised patients (85 ± 63 MFI) was significantly lower as compared to monocytes from non-splenectomised patients (160 ± 68 MFI) ($P < 0.05$) (Fig 2B-ii). Moreover, oxidative burst activity of monocytes from splenectomised NTD β^0 -thalassaemia/HbE patients (20 ± 7 MFI) was also significantly reduced when compared to monocytes from healthy subjects (33 ± 11 MFI) ($P < 0.05$) (Fig 2B-iii).

High serum ferritin levels were associated with CXCR2 expression and led to reduced neutrophil migration in NTD β^0 -thalassaemia/HbE patients

CXCR1 and CXCR2 interact with IL-8 to regulate leukocyte migration while CD11b binds to ICAM-1 on endothelial cells to regulate leukocyte rolling leading to migration into the site of infection. However, surface expression of CXCR1 on neutrophils from patients was not significantly different compared to healthy subjects. Therefore, a reduction of CXCR2 and CD11b on neutrophils could lead to a defect in neutrophil migration. To determine whether neutrophil chemotaxis is altered, neutrophil migration in response to IL-8 was investigated in a transwell migration assay.

Isolated neutrophils from healthy subjects were pre-incubated with either anti-CXCR2 or anti-CD11b or combined anti-CXCR2 and anti-CD11b to block CXCR2 and CD11b, respectively. Without CXCR2 and CD11b blocked, there was $24.9\pm 11.5\%$ neutrophil migration. Blocking of CXCR2 alone showed a slightly reduction of neutrophil migration ($15.5\pm 4\%$), but this was not statistically significant ($P = 0.082$) (Fig 3A). However, blocking CD11b alone or both CXCR2 and CD11b resulted in a significant reduction of neutrophil migration ($1.6\pm 1.1\%$ and $0.6\pm 0.5\%$, respectively) ($P < 0.01$).

Neutrophils isolated from splenectomised and non-splenectomised NTD β^0 -thalassaemia/HbE patients showed a significant decrease in IL-8-induced chemotaxis ($6.8\pm 5.8\%$ and $11.6\pm 9.2\%$, respectively) when compared to neutrophils from healthy subjects ($27.8\pm 9.0\%$) ($P < 0.01$ and $P < 0.05$, respectively) (Fig 3B). Interestingly, the percentage of neutrophil migration correlated with surface expression of CXCR2 on the isolated neutrophils ($r = 0.726$, $P = 0.005$) (Fig 3C). However, there was no correlation between neutrophil migration and surface expression of CD11b on the isolated neutrophils ($r = -0.366$, $P = 0.219$). In NTD β^0 -thalassaemia/HbE, serum ferritin is widely used to assess body iron status. We next investigated whether serum ferritin levels are associated with reduced neutrophil effector functions. Importantly, serum ferritin levels were inversely correlated with

neutrophil migration ($r = -0.617$, $P = 0.014$) (Fig 3D). In addition, serum ferritin levels were also inversely correlated with surface expression of CXCR2 on peripheral neutrophils ($r_s = -0.718$, $P < 0.001$) (Fig 3E). This could indicate that iron status is one of the factors which affects neutrophil function.

Several factors have been proposed to be associated with neutrophil function. Therefore, a multivariate analysis was used to adjust for clinical parameters related to the surface expression of CXCR2 on neutrophils. Clinical parameters such as splenectomy status, serum ferritin and haemoglobin levels as well as anaemic status were considered as independent variables in linear regression models (Table III). Serum ferritin levels and splenectomy were found as predictors for neutrophil heterogeneity, with 57.4 % variation in the surface expression of CXCR2 ($R = 0.757$, $P < 0.001$). Combined serum ferritin levels and haemoglobin levels were associated with 65.8% of the variation of the surface expression of CXCR2 on neutrophils ($R = 0.811$, $P < 0.001$). Moreover, combined serum ferritin levels, splenectomy status and haemoglobin levels were associated with 66.0% of the variation of the surface expression of CXCR2 on neutrophils ($R = 0.812$, $P < 0.001$).

High serum ferritin levels could be a key factor that is associated with infection. A history of infection for the five previous years of patients in the cohort was undertaken and analysed (Table IV). Patients were divided into two groups based on serum ferritin levels. All NTD β° -thalassaemia/HbE patients who had undergone splenectomy and had serum ferritin $> 1,000$ ng/mL showed 2.8 ± 2.0 episodes of infection per year, with infection including the common cold, acute pharyngitis, diarrhea, dermatitis, chronic leg ulcer and herpes zoster, while the 38% of non-splenectomised patients who had serum ferritin $> 1,000$ ng/mL showed 1.8 ± 2.0 episodes of infection per year including the common cold and diarrhea. The number of infection per year in NTD β° -thalassaemia/HbE patients who had low levels of serum ferritin ($\leq 1,000$ ng/mL) was 0.1 ± 0.3 in non-splenectomised patients and 0.5 ± 0.7 in splenectomised patients.

Discussion

Infectious complications such as pneumoniae and sepsis represent a leading cause of morbidity and mortality, particularly in developing countries where such complications represent the primary cause of death amongst β -haemoglobinopathy patients where access to regular blood transfusions, vaccination and/or antibiotic therapy may not be readily available (Ricerca, *et al* 2009, Wanachiwanawin 2000). β -Thalassaemia/HbE patients have a high risk of susceptibility to severe bacterial infections (Aswapokee, *et al* 1987, Teawtrakul, *et al*

2015, Wanachiwanawin 2000), which can lead to death, and several reports have proposed that abnormal innate immunity in thalassaemia is associated with a high risk of susceptibility to infection (Pattanapanyasat, *et al* 2007, Sari, *et al* 2014, Sinniah and Yadav 1981, Siwaponanan, *et al* 2017). However, those findings were fragmented investigations on isolated parts of innate immunity. There has been no comprehensive study to observe the overall innate immune response in thalassaemia patients, and also to discern any relationship with clinical features. Moreover, the innate immune abnormality in NTD β° -thalassaemia/HbE patients differs from TDT patients (Vento, *et al* 2006). Therefore, this study comprehensively analysed innate immunity including the complement system and the phenotypic and functional properties of neutrophils and monocytes as related to factors predisposing to infection in NTD β° -thalassaemia/HbE patients.

Splenectomy, the conventional treatment for hypersplenism, is one of factors that is reported to be related to an increased risk of bacterial infection (Fucharoen and Winichagoon 2000, Taher, *et al* 2018). Neutrophils from healthy subjects in serum from patients who had undergone splenectomy caused by trauma, hereditary spherocytosis, splenic cysts and idiopathic thrombocytopenic purpura were reduced in phagocytosis and migration activity compared to neutrophils from healthy subjects in autologous serum. *Vice versa*, neutrophils from these splenectomised patients in serum from healthy subjects were showed normal phagocytosis and migration functions (Foster, *et al* 1985). This suggests that some factors in serum from splenectomised patients could be a key component that reduced neutrophil activity. However, we did not find significant difference in serological parameters, expression of surface receptors on neutrophils and monocytes and activity of phagocytosis and migration of neutrophils from non-splenectomised NTD β° -thalassaemia/HbE patients compared to splenectomised NTD β° -thalassaemia/HbE patients. Therefore, the pathology of β -thalassaemia itself could affect neutrophils more than the effect of splenectomy in NTD β° -thalassaemia/HbE patients.

As the majority of iron accumulation in NTD β -thalassaemia/HbE patients (Shuler, *et al* 1990) and β -thalassaemia intermedia (Musallam, *et al* 2013, Musallam, *et al* 2011) occurs in the liver, patients are at risk of developing liver disease. Hepatocytes produce several proteins in the complement system, which are affected by liver iron levels (Glargaard, *et al* 2017, Walker and Walker 2000). The complement system represents a major part of the innate immune system, triggering both antibody-mediated and cell-mediated immune responses against pathogens (Merle, *et al* 2015b). The classical complement activation

pathway is mediated by antibodies bound to the bacterial surface to activate C1, while the lectin pathway is initiated by MBL or ficolin binding to carbohydrate structures on the surface of microbes or apoptotic cells. Binding of MBL activates MBL-associated serine proteases (MASPs) that cleaves C4 and C2 to form the C3 convertase (C4bC2a). The alternative pathway of complement activation can be activated by spontaneous C3 hydrolysis, which also serve to amplify the classical and lectin pathways (Merle, *et al* 2015a). Intriguingly, decreased C3 and C4 complement levels have previously been reported in TDT patients (Sinniah and Yadav 1981, Vento, *et al* 2006), however, in this study only decreased C3 complement levels were found in NTD β^0 -thalassaemia/HbE patients, while C4, MBL and CH50 levels remained in the normal reference range. Moreover, all patients in this study cohort had no apparent liver disease as measured by serum albumin, ALT, ALP and GGT levels, while AST levels were elevated in patients with high serum ferritin levels, signifying an underling non-hepatic injury.

Neutrophil migration to site of infection is a critical early step in host immunity and the process of pathogen elimination involves a wide variety of surface receptors such as CD11b, CD16 and C3bR (Karsten and Kohl 2012, Nauseef and Borregaard 2014). Interestingly, the expression of CD11b and C3bR was found to be significantly reduced on neutrophils from NTD β^0 -thalassaemia/HbE patients, while patients' neutrophil phagocytosis and oxidative burst parameters were not significantly different from those for neutrophils from healthy subjects. Our finding is similar to a previous study that reported that opsonophagocytosis and intracellular killing by neutrophils from β -thalassaemia/HbE patients against to *Candida albicans* yeast were not significantly different as compared neutrophils from healthy subjects (Pattanapanyasat, *et al* 2007). It is clear that neutrophils from NTD β^0 -thalassaemia/HbE patients differ from those from TDT patients (Vento, *et al* 2006) but that the decreased CD11b and C3bR expression of neutrophils does not affect the efficiency of phagocytosis in NTD β^0 -thalassaemia/HbE patients. However, null expression of these protein on neutrophils can affect their function, as seen with β_2 integrin deficient expression such as LFA-1 and Mac-1 in patients with leukocytes adhesion deficiency type 1, a rare inherited autosomal recessive disorder resulting in defective leukocyte adherence to the endothelium, neutrophil aggregation and chemotaxis, phagocytosis and cytotoxicity mediated by neutrophils, NK cells and T-cells (van de Vijver, *et al* 2013). Additionally, Mac-1 deficiency in mice (Carter, *et al* 2009, Morrison, *et al* 2008) and in humans (Lukacsi, *et al* 2017, van de Vijver, *et al* 2013) were shown to have a relationship to increased susceptibility to infections. Therefore,

the possible association between CD11b expression levels and susceptibility to infection in NTD β° -thalassaemia/HbE patients should be further studied.

Unlike the situation with neutrophils, impaired phagocytosis and oxidative burst of monocytes from splenectomised NTD β° -thalassaemia/HbE patients was shown in this study. However, phagocytic markers such as CD11b, CD16 and C3bR expression on monocytes from NTD β° -thalassaemia/HbE patients were in the reference range. The mechanism of impaired phagocytosis and oxidative burst of monocytes in these patients is therefore still unclear. The mechanism of monocyte phagocytosis and killing process in splenectomised NTD β° -thalassaemia/HbE patients should be further studied. It is important to note that the measurement of oxidative burst in neutrophils and monocytes is a qualitative measurement of ROS levels by using flow cytometry. The oxidative burst activity of neutrophils and monocytes is mediated by NADPH oxidase, although in addition to NADPH oxidase, xanthine oxidase can also generate ROS which could interfere with the measurement and leading to a high background. Therefore, a cell permeable inhibitor of the NADPH oxidase such as diphenyleneiodonium (DPI) could be used as measurement of ROS levels for background control.

A key effector function of neutrophils and monocytes involves the migration from the blood towards the site of infection. In response to bacterial infection, tissue-resident macrophages and other cell types such as endothelial cells produce IL-8, and IL-8 is recognised as the primary chemokine involved in neutrophil recruitment to the site of infection or inflammation. Then, neutrophils and monocytes adhering to endothelial cells via LFA-1 or Mac-1 with ICAM-1 is necessary to promote leukocyte rolling and migration. Here we showed that neutrophils from NTD β° -thalassaemia/HbE patients displayed a significant decrease in IL-8-induced chemotaxis when compared to neutrophils from healthy subjects. Reduced surface expression of CXCR2 but not CXCR1 on neutrophils from NTD β° -thalassaemia/HbE patients was clearly demonstrated, suggesting that the impaired migratory ability of NTD β° -thalassaemia/HbE neutrophils was associated with reduced expression of the IL-8 receptor, CXCR2. Notably, using a mouse model of NTD β -thalassaemia ($Hbb^{th3/+}$), we previously demonstrated $Hbb^{th3/+}$ neutrophils also displayed a significant reduction in surface expression of CXCR2 (Siwaponanan, *et al* 2017), thus adding further support to the impairment of the CXCR2-IL-8 axis in NTD β° -thalassaemia/HbE patients. In patients with sepsis, a two-fold reduction of CXCR2 surface expression on neutrophils has been reported, while CXCR1 surface expression persisted when compared to neutrophils from healthy

subjects. Reduced surface expression of CXCR2, but not CXCR1, on neutrophils from patients with sepsis was clearly demonstrated as impaired migratory response to chemotaxis agents such as IL-8, the epithelial cell-derived neutrophil activator (ENA-78) and the growth-related oncogene (GRO) subfamily of proteins (GRO- α , GRO- β and GRO- γ) (Cummings, *et al* 1999).

In this study, blocking CXCR2 of neutrophils from healthy subjects has no effect on migration, while an antibody specific to CD11b completely blocked migration of neutrophils from healthy subjects. This study was consistent with previous studies which showed that antibodies to CXCR1 reduced neutrophil migration by up to 44.8% of an unblocked neutrophil control, but anti-CXCR2 blocked neutrophils showed no significant difference (95.2%) (Godaly, *et al* 2000). While CXCR1 and CXCR2 are the main IL-8 receptor, CXCR1 displays greater ligand specificity than CXCR2 and binds with a high affinity to IL-8. However, only surface expression of CXCR2, but not CXCR1, on neutrophils from NTD β^0 -thalassaemia/HbE patients was reduced. Firm adhesion of neutrophils plays an important role on neutrophil migration as neutrophil migration is completely blocked by anti-Mac-1 and anti-CD18 antibodies, but not by anti-LFA-1 antibodies (Blake, *et al* 2004, Gao and Issekutz 1996). Therefore, the reduction of neutrophil migration in NTD β^0 -thalassaemia/HbE patients might not solely resulted from decreased CXCR2 surface expression alone, but possibly by a combination of abnormal surface receptor expression including CD11b. To understand the mechanism of impaired neutrophil migration in NTD β^0 -thalassaemia/HbE patients, further study on the effect of these receptors on neutrophil migration is needed.

Serum ferritin is one of the markers used to assess iron overload. Iron overload is a serious complication that is commonly found in both TDT and NTDT patients. The toxic effects of iron overload on cellular oxidative stress, lipid oxidation, adipocyte biology and vascular inflammation have been reported in TDT patients (Chaliasos, *et al* 2010, Walter, *et al* 2006) and NTD β -thalassaemia/HbE patients (Atichartakarn, *et al* 2014). Elevated levels of plasma malondialdehyde (MDA) as a lipid peroxidation marker, serum high-sensitive C-reactive protein (hs-CRP) and IL-6 as inflammatory markers, serum adiponectin as an adipocyte tissue dysfunction marker, and serum E-selectin, VCAM-1 and ET-1 as endothelial cell injury markers were significantly higher in TDT and NTDT patients than in healthy subjects. Additionally, decreased level of leptin, an adipocytokine that induces immune function, in TDT patients have been observed and were shown to be inversely correlated with serum ferritin levels (Chaliasos, *et al* 2010). Therefore, long term iron overload could induce

oxidative damages and cellular injury to induce inflammatory responses in both TDT and NTDT patients. Importantly, we also demonstrated that elevated serum ferritin levels were inversely correlated with reduced neutrophil CXCR2 surface expression levels, suggesting that the iron status in NTD β^0 -thalassaemia/HbE patients either directly or indirectly influences surface expression of CXCR2.

In conclusion, NTD β^0 -thalassaemia/HbE patients with high serum ferritin levels displayed an impaired CXCR2-IL-8 axis leading to reduced neutrophil migration. Splenectomy is another key factor that affects monocyte phagocytosis and oxidative burst in NTD β^0 -thalassaemia/HbE patients. Together, this study further demonstrates that high serum ferritin levels and splenectomy are associated with aberrant innate immune effector functions in NTD β^0 -thalassaemia/HbE patients. Furthermore, we propose that in a clinical laboratory setting the levels of surface expression of CXCR2 on neutrophils may represent an additional marker to screen for disease progression in NTD β^0 -thalassaemia/HbE patients, and to identify patients that may benefit from more intensive therapy. The suggested interventions particularly in NTD β^0 -thalassaemia/HbE patients with high serum ferritin levels ($>1,000$ ng/mL) include iron chelator therapy, prophylactic antibiotic treatment and vaccination.

Acknowledgements

The authors give special thanks to Prof. Dr. Duncan R. Smith for his valuable comments. This work was supported by Thailand Research Fund (TRF) (MRG5980043, DPG5980001 and DPG6080003); Office of the Higher Education Commission and Mahidol University under the National Research University Initiative; and Faculty of Science, Mahidol University. SSi was supported by National Research Management System (NRMS) Year 2018, National Research Council of Thailand (NRCT).

References

- Aswapokee, N., Aswapokee, P., Fucharoen, S. & Wasi, P. (1987) A study of infective episodes in patients with beta-thalassemia/Hb E disease in Thailand. *Birth Defects Orig Artic Ser*, **23**, 513-520.
- Atichartakarn, V., Chuncharunee, S., Archararit, N., Udomsubpayakul, U. & Aryurachai, K. (2014) Intravascular hemolysis, vascular endothelial cell activation and thrombophilia

- in splenectomized patients with hemoglobin E/beta-thalassemia disease. *Acta Haematol*, **132**, 100-107.
- Blake, K.M., Carrigan, S.O., Issekutz, A.C. & Stadnyk, A.W. (2004) Neutrophils migrate across intestinal epithelium using beta2 integrin (CD11b/CD18)-independent mechanisms. *Clin Exp Immunol*, **136**, 262-268.
- Bohmer, R.H., Trinkle, L.S. & Staneck, J.L. (1992) Dose effects of LPS on neutrophils in a whole blood flow cytometric assay of phagocytosis and oxidative burst. *Cytometry*, **13**, 525-531.
- Carter, C.R., Whitcomb, J.P., Campbell, J.A., Mukbel, R.M. & McDowell, M.A. (2009) Complement receptor 3 deficiency influences lesion progression during *Leishmania* major infection in BALB/c mice. *Infect Immun*, **77**, 5668-5675.
- Chaliasos, N., Challa, A., Hatzimichael, E., Koutsouka, F., Bourantas, D.K., Vlahos, A.P., Siamopoulou, A., Bourantas, K.L. & Makis, A. (2010) Serum adipocytokine and vascular inflammation marker levels in Beta-thalassaemia major patients. *Acta Haematol*, **124**, 191-196.
- Cummings, C.J., Martin, T.R., Frevert, C.W., Quan, J.M., Wong, V.A., Mongovin, S.M., Hagen, T.R., Steinberg, K.P. & Goodman, R.B. (1999) Expression and function of the chemokine receptors CXCR1 and CXCR2 in sepsis. *J Immunol*, **162**, 2341-2346.
- Dua, D., Choudhury, M. & Prakash, K. (1993) Altered T and B lymphocytes in multitransfused patients of thalassemia major. *Indian Pediatr*, **30**, 893-896.
- Dupre-Crochet, S., Erard, M. & Nubetae, O. (2013) ROS production in phagocytes: why, when, and where? *J Leukoc Biol*, **94**, 657-670.
- Ezer, U., Gulderen, F., Culha, V.K., Akgul, N. & Gurbuz, O. (2002) Immunological status of thalassemia syndrome. *Pediatr Hematol Oncol*, **19**, 51-58.
- Foster, P.N., Bolton, R.P., Cotter, K.L. & Losowsky, M.S. (1985) Defective activation of neutrophils after splenectomy. *J Clin Pathol*, **38**, 1175-1178.
- Fucharoen, S. & Winichagoon, P. (2000) Clinical and hematologic aspects of hemoglobin E beta-thalassemia. *Curr Opin Hematol*, **7**, 106-112.
- Fucharoen, S. & Winichagoon, P. (2012) New updating into hemoglobinopathies. *Int J Lab Hematol*, **34**, 559-565.
- Gao, J.X. & Issekutz, A.C. (1996) Mac-1 (CD11b/CD18) is the predominant beta 2 (CD18) integrin mediating human neutrophil migration through synovial and dermal fibroblast barriers. *Immunology*, **88**, 463-470.

- Glargaard, S., Boysen, T., Pilely, K., Garred, P. & Ytting, H. (2017) Prognostic value of lectin pathway molecules and complement proteins in ascitic fluid and blood in patients with liver cirrhosis. *Scand J Gastroenterol*, 1-6.
- Godaly, G., Hang, L., Frendeus, B. & Svanborg, C. (2000) Transepithelial neutrophil migration is CXCR1 dependent in vitro and is defective in IL-8 receptor knockout mice. *J Immunol*, **165**, 5287-5294.
- Henkels, K.M., Frondorf, K., Gonzalez-Mejia, M.E., Doseff, A.L. & Gomez-Cambronero, J. (2011) IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3). *FEBS Lett*, **585**, 159-166.
- Karsten, C.M. & Kohl, J. (2012) The immunoglobulin, IgG Fc receptor and complement triangle in autoimmune diseases. *Immunobiology*, **217**, 1067-1079.
- Kumar, V. & Sharma, A. (2010) Neutrophils: Cinderella of innate immune system. *Int Immunopharmacol*, **10**, 1325-1334.
- Lukacsi, S., Nagy-Balo, Z., Erdei, A., Sandor, N. & Bajtay, Z. (2017) The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes. *Immunol Lett*, **189**, 64-72.
- Merle, N.S., Church, S.E., Fremeaux-Bacchi, V. & Roumenina, L.T. (2015a) Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol*, **6**, 262.
- Merle, N.S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V. & Roumenina, L.T. (2015b) Complement System Part II: Role in Immunity. *Front Immunol*, **6**, 257.
- Morrison, T.E., Simmons, J.D. & Heise, M.T. (2008) Complement receptor 3 promotes severe Ross River virus-induced disease. *J Virol*, **82**, 11263-11272.
- Musallam, K.M., Cappellini, M.D. & Taher, A.T. (2013) Iron overload in beta-thalassemia intermedia: an emerging concern. *Curr Opin Hematol*, **20**, 187-192.
- Musallam, K.M., Cappellini, M.D., Wood, J.C., Motta, I., Graziadei, G., Tamim, H. & Taher, A.T. (2011) Elevated liver iron concentration is a marker of increased morbidity in patients with beta thalassemia intermedia. *Haematologica*, **96**, 1605-1612.
- Nauseef, W.M. & Borregaard, N. (2014) Neutrophils at work. *Nat Immunol*, **15**, 602-611.
- Pattanapanyasat, K., Sukapirom, K., Tachavanich, K. & Kaewmoon, S. (2007) Flow cytometric quantitation of opsonophagocytosis and intracellular killing of *Candida albicans* using a whole blood microassay. *Cytometry A*, **71**, 1027-1033.

- Ricerca, B.M., Di Girolamo, A. & Rund, D. (2009) Infections in thalassemia and hemoglobinopathies: focus on therapy-related complications. *Mediterr J Hematol Infect Dis*, **1**, e2009028.
- Sari, T.T., Gatot, D., Akib, A.A., Bardosono, S., Hadinegoro, S.R., Harahap, A.R. & Idjradinata, P.S. (2014) Immune response of thalassemia major patients in Indonesia with and without splenectomy. *Acta Med Indones*, **46**, 217-225.
- Shi, C. & Pamer, E.G. (2011) Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*, **11**, 762-774.
- Shuler, T.R., Pootrakul, P., Yarnsukon, P. & Nielsen, F.H. (1990) Effect of thalassemia/hemoglobin E disease on macro, trace, and ultratrace element concentrations in human tissue. *J. Trace Elem. Exp. Med.*, **3**, 31-43.
- Sinniah, D. & Yadav, M. (1981) Elevated IgG and decreased complement component C3 and factor B in B-thalassaemia major. *Acta Paediatr Scand*, **70**, 547-550.
- Siwaponanan, P., Siegers, J.Y., Ghazali, R., Ng, T., McColl, B., Ng, G.Z., Sutton, P., Wang, N., Ooi, I., Thiengtavor, C., Fucharoen, S., Chaichompoo, P., Svasti, S., Wijburg, O. & Vadolas, J. (2017) Reduced PU.1 expression underlies aberrant neutrophil maturation and function in beta-thalassemia mice and patients. *Blood*, **129**, 3087-3099.
- Slattery, M.J., Liang, S. & Dong, C. (2005) Distinct role of hydrodynamic shear in leukocyte-facilitated tumor cell extravasation. *Am J Physiol Cell Physiol*, **288**, C831-839.
- Taher, A.T. & Saliba, A.N. (2017) Iron overload in thalassemia: different organs at different rates. *Hematology Am Soc Hematol Educ Program*, **2017**, 265-271.
- Taher, A.T., Weatherall, D.J. & Cappellini, M.D. (2018) Thalassaemia. *Lancet*, **391**, 155-167.
- Teawtrakul, N., Jetsrisuparb, A., Siriijerachai, C., Chansung, K. & Wanitpongpun, C. (2015) Severe bacterial infections in patients with non-transfusion-dependent thalassemia: prevalence and clinical risk factors. *Int J Infect Dis*, **39**, 53-56.
- Umiel, T., Friedman, E., Luria, D., Cohen, I.J., Kaplinsky, H., Netzer, L., Pecht, M., Trainin, N. & Zaizov, R. (1984) Impaired immune regulation in children and adolescents with hemophilia and thalassemia in Israel. *Am J Pediatr Hematol Oncol*, **6**, 371-378.
- van de Vijver, E., van den Berg, T.K. & Kuijpers, T.W. (2013) Leukocyte adhesion deficiencies. *Hematol Oncol Clin North Am*, **27**, 101-116, viii.
- Vento, S., Cainelli, F. & Cesario, F. (2006) Infections and thalassaemia. *Lancet Infect Dis*, **6**, 226-233.

- Walker, E.M., Jr. & Walker, S.M. (2000) Effects of iron overload on the immune system. *Ann Clin Lab Sci*, **30**, 354-365.
- Walter, P.B., Fung, E.B., Killilea, D.W., Jiang, Q., Hudes, M., Madden, J., Porter, J., Evans, P., Vichinsky, E. & Harmatz, P. (2006) Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. *Br J Haematol*, **135**, 254-263.
- Wanachiwanawin, W. (2000) Infections in E-beta thalassemia. *J Pediatr Hematol Oncol*, **22**, 581-587.

Figure Legends

Fig 1. Expression of surface receptors on (A) neutrophils and (B) monocytes were determined by as mean fluorescent intensity (MFI) of (i) CD11b, (ii) CD16, (iii) C3bR, (iv) C5aR, (v) CXCR1 and (vi) CXCR2 from 13 healthy subjects (Healthy), 13 non-splenectomised NTD β° -thalassaemia/HbE patients (NS) and 6 splenectomised NTD β° -thalassaemia/HbE patients (S) using flow cytometry. Data are presented as mean $\pm$ S.D. *Significant difference when compared between groups ($P < 0.01$). **Significant difference when compared between groups ($P < 0.001$).

Fig 2. Phagocytosis and oxidative burst activity of neutrophils and monocytes. Blood samples from 13 healthy subjects (Healthy), 13 non-splenectomised NTD β° -thalassaemia/HbE patients (NS) and 6 splenectomised NTD β° -thalassaemia/HbE patients (S) were co-incubated with PI-labeled *E. coli* for 90 min and (A) neutrophils and (B) monocytes were analysed for (i) the percentage of phagocytosis and oxidative burst activity, and the mean fluorescent intensity (MFI) of (ii) phagocytosis and (iii) oxidative burst using flow cytometry. *Significant difference when compared between groups ($P < 0.05$).

Fig 3. Neutrophil migration in NTD β° -thalassaemia/HbE patients. (A) Untreated and monoclonal antibody-treated neutrophils from healthy subjects ($n = 4$) were used to determine the effects of blocking CXCR2 and CD11b on neutrophil migration. (B) A transwell assay was performed to determine the percentages of neutrophil migration from 5 healthy subjects (Healthy), 5 non-splenectomised NTD β° -thalassaemia/HbE patients (NS) and 5 splenectomised NTD β° -thalassaemia/HbE patients (S). Data are presented as mean $\pm$ S.D. Pearson's correlation coefficient (r) between the percentages of neutrophil

migration and (C) surface expression of CXCR2 on isolated neutrophils or (D) serum ferritin levels. (E) Correlation analysis of the relationship between surface expression of CXCR2 on neutrophils from whole blood staining and serum ferritin levels were calculated using Spearman's Rho correlation coefficient (r_s). *Significant difference when compared between groups ($P < 0.05$). **Significant difference when compared between groups ($P < 0.01$).

Table I. Haematological parameters.

Description	Healthy subjects	NTD β^0 -Thalassaemia/HbE patients		Reference range
		Non-splenectomy	Splenectomy	
Number (Male: Female)	17 (3: 14)	19 (13: 6)	7 (4: 3)	
Age (years) (range)	26 $\pm$ 4 (18-36)	33 $\pm$ 13 (18-53)	36 $\pm$ 11 (24-51)	
Haemoglobin typing				
HbA ₂ /HbE (%)	2.9 $\pm$ 0.2	54.3 $\pm$ 13.6 ^b	54.6 $\pm$ 8.6 ^b	2-3
HbF (%)	0.4 $\pm$ 0.3	31.7 $\pm$ 15.6 ^b	37.5 $\pm$ 8.2 ^b	0.8-2
HbA (%)	86.9 $\pm$ 0.4			85-90
Red blood cell count ($\times 10^6/\mu\text{L}$)	4.8 $\pm$ 0.5	4.1 $\pm$ 0.9	3.2 $\pm$ 0.8 ^{b,c}	4.2-5.4
Haemoglobin (g/dL)	13.6 $\pm$ 1.5	7.5 $\pm$ 1.2 ^b	6.8 $\pm$ 1.4 ^b	12-18
Haematocrit (%)	41.1 $\pm$ 4.0	24.7 $\pm$ 3.6 ^b	23.5 $\pm$ 4.0 ^b	37-52
MCV (fL)	86.5 $\pm$ 6.0	61.0 $\pm$ 7.1 ^b	74.7 $\pm$ 6.8 ^{a,e}	80-99
MCH (pg)	28.6 $\pm$ 2.5	18.5 $\pm$ 2.5 ^b	21.6 $\pm$ 1.9 ^b	27-31
MCHC (g/dL)	33.0 $\pm$ 0.8	30.4 $\pm$ 1.9 ^b	28.5 $\pm$ 0.8 ^{b,c}	31-35
Red cell distribution width (%)	13.4 $\pm$ 1.3	24.3 $\pm$ 3.5 ^b	23.7 $\pm$ 1.1 ^b	11.5-14.5
Reticulocytes (%)	1.2 $\pm$ 0.4	4.3 $\pm$ 3.0 ^b	12.0 $\pm$ 2.2 ^{b,e}	0.5-1.5
Absolute reticulocytes ($\times 10^9/\mu\text{L}$)	55.6 $\pm$ 21.9	158.6 $\pm$ 91.3 ^b	384.3 $\pm$ 114.6 ^{b,e}	25-100
NRBCs (cells/100WBCs)	0 $\pm$ 0	19.2 $\pm$ 16.0 ^b	338.3 $\pm$ 128.7 ^{b,e}	None
WBC count ($\times 10^3/\mu\text{L}$)	6.4 $\pm$ 1.5	5.1 $\pm$ 1.9	12.9 $\pm$ 3.3 ^{b,e}	4-11
Absolute neutrophils ($\times 10^3/\mu\text{L}$)	3.7 $\pm$ 0.9	3.2 $\pm$ 1.3	5.1 $\pm$ 1.6	1.5-8.0
WBC differential count				
Band form (%)	0 $\pm$ 0	0 $\pm$ 1	0 $\pm$ 0	0-1
Neutrophils (%)	57 $\pm$ 5	58 $\pm$ 11	41 $\pm$ 7	40-74
Eosinophils (%)	2 $\pm$ 2	2 $\pm$ 2	2 $\pm$ 2	0-7
Basophils (%)	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0-1.5
Lymphocytes (%)	35 $\pm$ 7	31 $\pm$ 12	47 $\pm$ 8	20-48
Monocytes (%)	7 $\pm$ 5	8 $\pm$ 4	9 $\pm$ 8	2-10
Platelet count ($\times 10^3/\mu\text{L}$)	248.7 $\pm$ 49.1	166.3 $\pm$ 74.2 ^b	607.7 $\pm$ 127.5 ^{b,e}	150-450
Serum ferritin (ng/mL)	55.6 $\pm$ 40.0	1,363.3 $\pm$ 967.9 ^b	1,414.6 $\pm$ 754.0 ^b	32-100
Liver function test				
Alkaline phosphatase (U/L)	54.9 $\pm$ 12.3	77.7 $\pm$ 20.7	79.9 $\pm$ 20.4	40-150
Aspartate aminotransferase (U/L)	20.5 $\pm$ 2.9	43.1 $\pm$ 27.3	47.9 $\pm$ 22.6 ^b	5-34
Alanine aminotransferase (U/L)	16.6 $\pm$ 7.5	32.3 $\pm$ 27.8	33.9 $\pm$ 20.2	0-55
Gamma glutamyl transferase (U/L)	16.3 $\pm$ 5.4	25.4 $\pm$ 21.3	25.7 $\pm$ 15.6	9-36
Total protein (g/L)	75.1 $\pm$ 2.8	78.1 $\pm$ 5.5	84.1 $\pm$ 4.4 ^{b,d}	64-83
Albumin (g/L)	40.5 $\pm$ 2.3	41.4 $\pm$ 2.9	40.2 $\pm$ 2.9	35-50
Globulin (g/L)	34.7 $\pm$ 2.7	36.4 $\pm$ 6.9	43.9 $\pm$ 5.8 ^{b,c}	20-35
Total bilirubin (mg/dL)	0.7 $\pm$ 0.3	3.6 $\pm$ 1.4 ^b	4.4 $\pm$ 1.5 ^b	0.2-1.2
Direct bilirubin (mg/dL)	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.2 ^b	0-0.5
Indirect bilirubin (mg/dL)	0.4 $\pm$ 0.2	3 $\pm$ 1.4 ^b	3.8 $\pm$ 1.6 ^b	0.2-0.7

MCH; mean corpuscular haemoglobin, MCHC; mean corpuscular haemoglobin concentration, MCV; mean corpuscular volume, NRBCs; nucleated red blood cells and WBC; white blood cells. ^aSignificant different when compared to healthy subjects at $P < 0.01$. ^bSignificant different when compared to healthy subjects at $P < 0.001$.

^cSignificant different when compared to non-splenectomised NTD β^0 -thalassaemia/HbE patients at $P < 0.05$.

^dSignificant different when compared to non-splenectomised NTD β^0 -thalassaemia/HbE patients at $P < 0.01$.

^eSignificant different when compared to non-splenectomised NTD β^0 -thalassaemia/HbE patients at $P < 0.001$.

Author Manuscript

Table II. Serological parameters.

Description	Healthy subjects	NTD β^0 -Thalassaemia/HbE patients		Reference range
		Non-splenectomy	Splenectomy	
Number	17	19	7	
Complements				
C3 (g/L)	1.01 $\pm$ 0.18	0.68 $\pm$ 0.17 ^a	0.66 $\pm$ 0.08 ^a	0.9-1.8
C4 (g/L)	0.23 $\pm$ 0.07	0.15 $\pm$ 0.07	0.13 $\pm$ 0.04	0.1-0.4
MBL (ng/mL)	1,958.70 $\pm$ 1,599.5	2,682.1 $\pm$ 1,681.7	2,622.5 $\pm$ 1,255.4	>500
CH50 (U/mL)	48.2 $\pm$ 15.8	49.0 $\pm$ 11.0	43.4 $\pm$ 13.2	25-110
Immunoglobulins (Ig)				
IgA (mg/mL)	2.2 $\pm$ 0.7	3.1 $\pm$ 1.5	5.3 $\pm$ 2.0 ^a	0.7-4.0
IgG (mg/mL)	12.2 $\pm$ 2.4	17.2 $\pm$ 5.2 ^a	20.7 $\pm$ 3.5 ^a	7-16
IgM (mg/mL)	1.3 $\pm$ 0.6	1.7 $\pm$ 1.1	1.5 $\pm$ 1.0	0.4-2.3

CH50; human 50% complement hemolysis and MBL; mannose binding lectin. ^aSignificant different when compared to healthy subjects at $P < 0.001$.

Table III. A multivariate analysis of surface expression of CXCR2 on neutrophils and clinical parameters.

Dependent (Y)	Independent (X)	R	P-value	R ² (%)	Regression equation $\hat{y} = b_0 + b_1x$
Neutrophils					
CXCR2	Serum ferritin Splenectomy	0.757	<0.001	57.4	$Y = 7428.446 - 1.070SF - 990.439Sp$
	Serum ferritin Haemoglobin	0.811	<0.001	65.8	$Y = 2141.270 - 0.694SF + 329.569Hb$
	Splenectomy Haemoglobin	0.780	<0.001	60.8	$Y = -495.947 + 232.115Sp + 499.272Hb$
	Serum ferritin Splenectomy Haemoglobin	0.812	<0.001	66.0	$Y = 1189.408 - 0.698SF + 270.323Sp + 378.395Hb$

Hb; haemoglobin, SF; serum ferritin, and Sp; splenectomy. The splenectomy was scoring by 1 for healthy subjects (non-splenectomised), 2 for non-splenectomised NTD β^0 -thalassaemia/HbE patients and 3 for splenectomised NTD β^0 -thalassaemia/HbE patients.

Table IV. A history of infections in NTD β^0 -thalassaemia/HbE patients.

Phenotypes	Number of	Number of	Averages of episodes of		Types of infections
	subjects	infected patients	infection in 5 years		
	[n]	[n (%)]	mean±S.D.	median	
Serum ferritin ≤ 1,000 ng/mL (n = 13)					
Non-splenectomy	11	1 (9%)	0.1±0.3	0	Conjunctivitis
Splenectomy	2	1 (50%)	0.5±0.7	0.5	Pneumonia
Serum ferritin > 1,000 ng/mL (n = 13)					
Non-splenectomy	8	3 (38%)	1.8±2.0	1.5	Common cold
					Diarrhea
Splenectomy	5	5 (100%)	2.8±2.0	3	Common cold
					Acute pharyngitis
					Diarrhea
					Dermatitis
					Chronic leg ulcer

bjh_16295_f2.eps

bjh_16295_f3.eps

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Thiengtavor, C;Siriworadetskun, S;Paiboonsukwong, K;Fucharoen, S;Pattanapanyasat, K;Vadolas, J;Svasti, S;Chaichompoo, P

Title:

Increased ferritin levels in non-transfusion-dependent β^0 -thalassaemia/HbE are associated with reduced CXCR2 expression and neutrophil migration.

Date:

2020-04

Citation:

Thiengtavor, C., Siriworadetskun, S., Paiboonsukwong, K., Fucharoen, S., Pattanapanyasat, K., Vadolas, J., Svasti, S. & Chaichompoo, P. (2020). Increased ferritin levels in non-transfusion-dependent β^0 -thalassaemia/HbE are associated with reduced CXCR2 expression and neutrophil migration.. Br J Haematol, 189 (1), pp.187-198. <https://doi.org/10.1111/bjh.16295>.

Persistent Link:

<http://hdl.handle.net/11343/286795>