ARE MONOCYTES ACTIVATED IN SPLENECTOMIZED HEMOGLOBIN E/ β-THALASSEMIC PATIENTS?

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ARE MONOCYTES ACTIVATED IN HEMOGLOBIN E/ β-THALASSEMIC PATIENTS ?

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ABSTRACT

Thalassemia is a hereditary hemolytic anemia characterized by absent or decreased production of (usually) one of the globin chains of hemoglobin. One of the complications of thalassemia is thromboembolism, especially after splenectomy. Previous studies have shown a hypercoagulable state, involving phosphatidylserine (PS)-exposing red blood cells (RBCs), coagulation factors, coagulation factor inhibitors, and activation of platelets, monocytes, granulocytes and endothelial cells as contributing factors. Normally, PS-exposing RBCs are recognized and cleared by macrophages in the spleen. In the absence of the spleen, researchers have speculated that other reticuloendothelial cells, such as circulating monocytes may take over the function, resulting in monocyte activation. Furthermore, the binding between platelets and monocytes or platelet-monocyte aggregates (PMAs) may be another cause of monocyte activation. In this study, flow cytometers were used to detect the activated monocytes surface marker (CD11b) and positive intracellular cytokines (TNF-α and IL-1α), PS-exposing RBCs, and PMAs in whole blood from three groups- non-splenectomized and splenectomized Hb E/β-thalassemic patients and healthy normal subjects. Compared to the other 2 groups, the splenectomized group showed a trend towards an increased amount of circulating CD11b, TNF-α and IL-1α positive monocytes. A statistically significant higher amount of circulating annexin V-positive RBCs was, however, detected in this group ($p<0.01$). In contrast, there was no difference in the percentage of PMAs among the 3 groups. There was no correlation between annexin V-exposing RBCs and the level of monocytes expressing IL-1α or CD11b fluorescence intensity, while a slight statistical correlation was found between the percentage of annexin V-exposing RBCs and the level of monocytes expressing TNF-α ($r^2 = 0.114$, $p<0.01$). This result suggests that monocytes are activated in splenectomized Hb E/β-thalassemic patients, and not related to PMAs, but may be related to PS-exposing thalassemic RBCs.

KEY WORDS: THALASSEMIA/ SPLENECTOMY/ MONOCYTE/
TUMOR NECROSIS FACTOR α/ INTERLEUKIN 1 α.

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สภาวะของโมโนซัยท์ในผู้ป่วยฮีโมโกลบินอี/ บีตาธาลัสซีเมีย หลังตัดม้าม
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บทคัดย่อ

ธาลัสซีเมียเป็นโรคโลหิตจางโดยกรรมพันธุ์ เนื่องด้วยการสร้างฮีโมโกลบินผิดปกติ พยาธิสภาพแทรกซ้อนหนึ่งที่พบในผู้ป่วยธาลัสซีเมียคือ การอุดตันของหลอดเลือด โดยเฉพาะในผู้ป่วยหลังตัดม้าม สาเหตุมาจากภาวะ hypercoagulable ซึ่งเกี่ยวข้องกับ การปรากฏของฟอสฟาทิดิลเซอร์รินบนผนังของเซลล์เม็ดเลือดแดง, ปัจจัยการแข็งตัวของตัวเลือด, ปัจจัยต้านการแข็งตัวของเลือด และเซลล์ชนิดต่างๆ ที่ถูกกระตุ้นในกระแสเลือด เช่น เกร็ดเลือด, โมโนซัยท์, แกรนูโลซัยท์ และเซลล์บุผนังหลอดเลือด โดยปกติเม็ดเลือดแดงที่ปรากฏฟอสฟาติดิลเซอร์รินบนผิวเซลล์นั้น จะถูกจับและทำลายโดยแมคโครฟาจในม้าม ในส่วนผู้ป่วยฮีโมโกลบินอี/ บีตาธาลัสซีเมียหลังตัดม้าม เซลล์ที่ทำหน้าที่นี้คาดว่าจะเป็นโมโนซัยท์ ซึ่งเป็นสาเหตุให้โมโนซัยท์ถูกกระตุ้น นอกจากนี้ การเกาะกลุ่มของเกร็ดเลือดและโมโนซัยท์ (platelet-monocyte aggregates) ก็อาจเป็นอีกสาเหตุหนึ่งที่ทำให้โมโนซัยท์ถูกกระตุ้นได้เช่นกัน ในการศึกษาครั้งนี้ใช้เทคนิคโฟลไซโทรมิเตอร์ศึกษาโมโนซัยท์ที่ถูกกระตุ้น โดยดูจากการปรากฏของโมเลกุลที่แสดงการถูกกระตุ้น (CD11b) บนผิวของโมโนซัยท์ และค่าร้อยละของโมโนซัยท์ที่สร้างไซโตไคน์ (TNF-α และ IL-1α), ค่าร้อยละของการปรากฏฟอสฟาติดิลเซอร์รินบนผิวเม็ดเลือดแดง และ ค่าร้อยละของการเกาะกลุ่มของเกร็ดเลือดและโมโนซัยท์ ผลการศึกษาพบว่าผู้ป่วยฮีโมโกลบินอี/ บีตาธาลัสซีเมียที่ตัดม้าม มีแนวโน้มค่าความเข้มของ CD11b บนผิวโมโนซัยท์และค่าร้อยละของโมโนซัยท์ที่สามารถสร้างไซโตไคน์สูงกว่าผู้ป่วยที่ไม่ได้ตัดม้ามและคนปกติ นอกจากนี้ยังพบว่าผู้ป่วยที่ตัดม้ามจะมีค่าร้อยละของการปรากฏฟอสฟาติดิลเซอร์รินบนผิวเม็ดเลือดแดงสูงขึ้นอย่างมีนัยสำคัญด้วย ($p<0.01$) ซึ่งตรงข้ามกับค่าร้อยละของการเกาะกลุ่มของเกร็ดเลือดและโมโนซัยท์ ค่าที่ได้ไม่มีความแตกต่างกันระหว่างคนไข้ไม่ตัดม้าม คนไข้ตัดม้ามและคนปกติ จากการศึกษาพบว่าเปอร์เซ็นต์ฟอสฟาติดิลเซอร์รินบนผิวเม็ดเลือดแดงไม่มีความสัมพันธ์กับความเข้มของ CD11b บนผิวโมโนซัยท์หรือค่าร้อยละของโมโนซัยท์ที่สร้างไซโตไคน์ IL-1α ขณะเดียวกัน ก็พบว่าเปอร์เซ็นต์ฟอสฟาติดิลเซอร์รินบนผิวเม็ดเลือดแดงมีความสัมพันธ์เล็กน้อยกับค่าร้อยละของโมโนซัยท์ที่สามารถสร้างไซโตไคน์ TNF-α ($r^2 = 0.114$, $p<0.01$) จากการศึกษานี้ทำให้เราทราบว่าโมโนซัยท์ถูกกระตุ้นในผู้ป่วยที่ตัดม้าม และสาเหตุในการถูกกระตุ้นนั้นไม่เกี่ยวข้องกับการเกาะกลุ่มของเกร็ดเลือดและโมโนซัยท์ แต่อาจเกี่ยวข้องหรือไม่เกี่ยวข้องกับการฟาโกซัยท์เม็ดเลือดแดงที่ปรากฏฟอสฟาติดิลเซอร์รินในผู้ป่วยธาลัสซีเมีย

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LIST OF ABBREVIATIONS

α	=	alpha
ADCC	=	antibody-dependent cell cytotoxicity
APC	=	allophycocyanin
β	=	beta
BDB	=	Becton Dickinson Biosciences
BFA	=	brefeldin A
^{o}C	=	degree Celsius
CD	=	cluster designation
CECs	=	circulating endothelial cells
δ	=	delta
dL	=	deciliter (10^{-1} liter)
DNA	=	deoxyribonucleic acid
ε	=	epselon
ECs	=	endothelial cells
EDTA	=	ethylene diamine tetraacetic acid
ELAM-1	=	endothelial leukocyte adhesion molecule-1
et al.	=	et alii (Latin), and other
FSC	=	forward scatter
FITC	=	fluorescein isothiocyanate
FPA	=	fibrinopeptide
fl	=	femtoliter (10^{-15} liter)
γ	=	gamma
g	=	gram
G-CSF	=	granulocyte-colony stimulating factor
h	=	hour
Hb	=	hemoglobin
HbCS	=	hemoglobin Constant Spring

LIST OF ABBREVIATIONS (Continued)

HCII	=	heparin cofactor II
Hct	=	hematocrit
HPLC	=	high performance liquid chromatography
HUVEC	=	human umblilical vein endothelial cell
ICAM-1	=	intercellular adhesion molecule-1
IgG	=	immunoglobulin G
IL	=	interleukin
LPS	=	lipopolysaccharide
μl	=	microliter (10^{-6} liter)
μg	=	microgram (10^{-6} liter)
MAC-1	=	macrophage-1 antigen
MCH	=	mean corpuscular hemoglobin
MCHC	=	mean corpuacular hemoglobin concentration
M-CSF	=	macrophage-colony stimulating factor
MCV	=	mean corpuscular volume
MFI	=	mean fluorescence intensity
ml	=	milliliter (10^{-3} liter)
mRNA	=	messenger ribonucleic acid
NS	=	non-splenectomized Hb E/β-thalassemic patient
NRBC	=	nucleated red blood cell
ψ	=	psi
PBS	=	phosphate buffer saline
PE	=	phosphatidylethernolamine
PE	=	phycoerythrin
PF3	=	platelet factor 3
pg	=	picogram (10^{-12} gram)
PG	=	prostaglandin
PGI_2	=	prostacyclin
PLT	=	platelet
PMAs	=	platelet-monmcyte aggregates

LIST OF ABBREVIATIONS (Continued)

$F_{1,2}$	=	prothrombin
RBCs	=	red blood cells
R	=	region
S	=	splenectomized Hb E/β-thalassemic patients
SCD	=	sickle cell disease
SSC	=	side scatter
TAT	=	thrombin-anti thrombin
TF	=	tissue factor
TNF	=	tumor necrosis factor
TXA_2	=	thromboxane A_2
VCAM-1	=	vascular cell adhesion molecule-1
vWF	=	von Willebrand factor
WBC	=	white blood cell count
ζ	=	zeta

CHAPTER I
INTRODUCTION

Background and problems

Thalassemias are common genetic disorders of red blood cells (RBCs). They are caused by a partial or complete deficiency of α- or β-globin chain synthesis, leading to a relative excess of the other globin chains. Pathologic erythrocytes have reduced hemoglobin content and morphological abnormalities. There are two major types of thalassemia, α and β thalassemia. DNA mapping showed that gene deletion is the major cause of α-thalassemia whereas point mutations and small deletions or insertions in nucleotides sequences are mainly responsible for the molecular defects of β-thalassemia (1, 2).

In Thailand, the most common form of β-thalassemia is combination of β-thalassemia with an abnormal hemoglobin E (Hb E/β-thal) causing the public health problem. Hb E is a globin β-chain variant resulting from the substitution of glutamic acid by lysine in codon 26. Its clinical and hematological features appear to be those of thalassemia major. But normally Hb E/β-thal is less severe than β-thalassemia major (2-4). Current management of thalassemia consists of blood transfusion, iron chelation, splenectomy and bone marrow transplantation. Splenectomy is performed when patients develop hypersplenism or an increased requirement for blood transfusion (1). Many clinical manifestations have been described in thalassemia patients including infection, iron overload, endocrine dysfunction, pericarditis, cardiac failure, hypoxia, extramedullary hemopoiesis and thromboembolism (2, 5-13).

Thalassemic patients have a higher than normal incidence of thromboembolic events, including recurrent and transient cerebral ischaemic attacks, strokes as well as peripheral arterial and venous thromboses (14). Chronic pulmonary thromboembolism plays an important role in cardiac failure, which is a major cause of

death in thalassemia patients over 20 years of age (15). Thromboembolism is a well-noted phenomenon in patients with β-thalassemia major and β-thalassemia intermedia, particularly those that had been splenectomized (7, 8, 16). These patients have a life-long hypercoagulable state (7, 14, 17, 18). Multiple pulmonary thromboemboli have been found at post-mortem examination in patients with thalassemia major and Hb E/β-thalassemia (15). A cardiopulmonary assessment of thalassemia intermedia patients showed dilation of the main pulmonary artery, a dilated right ventricle with good left ventricular function, high systolic pulmonary artery pressure and normal pulmonary capillary wedge pressure, all compatible with pulmonary arterial hypertension (19). Pulmonary arterial hypertension in patients with thalassemia intermedia is probably a result of tissue hypoxia, especially in patients who undergo delayed or infrequent blood transfusion. Hypoxia-induced compensatory reactions, finally lead to high cardiac output and increased pulmonary vascular resistance (6).

The hypercoagulable state in thalassemia seem to be very complicated involving abnormal exposure of phosphatidylserine (PS) at the outer surface of erythrocytes (5, 20-22), coagulation factors (18), coagulation factor inhibitors (23), and activation of platelets (24-28), monocytes (29-33), granulocytes (29, 34) and endothelial cells (ECs) (35-37).

The presence of PS exposure on thalassemic RBCs surface is pathologically important. It provides an anchoring site for activated clotting factors interacts with other cells, such as platelets, monocytes and granulocytes alone or together and vascular endothelial cells (38, 39). They also mediate the rapid removal of these RBCs from the circulation by macrophages, further contributing to the degree of anemia (39).

There have been many reports of monocyte activation associated with thalassemia and sickle cell disease (SCD). High serum levels of monocyte colony-stimulating factor (M-CSF) and increased monocyte phagocytic activities (antibody-dependent cell cytotoxicity [ADCC]) toward RBCs were found in patients with Hemoglobin H disease and β-thalassemia major. Belcher et al have reported sickle monocytes are activated, as defined by increase CD11b surface expression and by increased intracellular expression of tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β). Inwald et al have also shown that children with SCD have

activated monocytes (29-33). In addition, increased levels of TNF-α and IL-1β in the serum of these patients have also been reported (36, 40-42).

Platelet-monocyte aggregates (PMAs) have been implicated in the pathogenesis of vascular disease (43, 44). Additionally, activated platelets bind to monocytes and activated them (45). Platelets are found to be activated in patients with Hb E/β-thalassemia (24-28). Thus, PMA formation could be one mechanism whereby monocytes become activated in patients with Hb E/β-thalassemia.

In this study, we used flow cytometer to detect the surface marker (CD11b) and intracellular cytokines (TNF-α and IL-1α) of activated monocytes in whole blood from non-splenectomized and splenectomized Hb E/β-thalassemia patients, and healthy normal subjects. Correlation between activated monocytes and hematological parameters, phosphatidylserine-exposing RBCs and PMAs were also studied. To better understand the state of monocyte in non-splenectomized and splenectomized HB E/ β-thalassemic patients.

CHAPTER II
OBJECTIVES

1. To measure the percentage of activated monocytes shown by intracellular TNFα and IL-1α and surface CD11b fluorescence expression on flow cytometric analysis.
2. To evaluate the percentage of PMAs using flow cytometric analysis
3. To determine the percentage of PS-exposing RBCs using flow cytometric analysis.

CHAPTER III
LITERATURE REVIEW

1. Thalassemias

Thalassemias are a group of disorder in which synthesis of the α, β, γ or δ globin chains is either reduced or totally absence, resulting in erythrocytes with reduced hemoglobin content and morphological abnormalities. Its name was derived from Greek roots meaning “sea” and “blood”, in the mistaken belief that it was restricted to individuals of background. The thalassemias are wide spread about 5% of the world population. It is most prevalent around the Mediterranean Sea i.e. countries like Greece, Italy, Turkey and North African countries. It is also seen in Saudi Arabia, Iran, Afghanistan, Pakistan, India and Southeast Asian like Thailand and Indonesia (1, 46).

In Thailand, there are approximately 600,000 affected individuals and more than 20 million thalassemia carriers. There are prevalence of both α-thalassemia, β-thalassemia and two hemoglobin (Hb) variants including Hb E and Hb Constant Spring (Hb CS) (Table1). The frequencies of α-thalassemia are 20% in Bangkok and 30% in Northern Thailand, while in β-thalassemia is detected only 3-9%. The frequencies of Hb E and Hb CS are 13% and 1-9%, respectively (47).

1.1 Hemoglobin

Hemoglobin is the protein of the red blood cells that allows vertebrates to transport oxygen from the lungs to the tissues and that helps the return transport of carbon dioxide from the tissues back to the lungs. The hemoglobin molecule have a tetrameric structure, consisting of two different pairs of globin chains, each attached to a heme moiety (Figure 1). Adult and fetal hemoglobins have α chains combined with β

chains (Hb A, $\alpha_2\beta 2$), δ chains (Hb A2, $\alpha 2\delta 2$) and γ chains (Hb F, $\alpha 2\gamma 2$). In embryonic life, α-like chains called ζ combine with γ chains to produce hemoglobin Portland ($\zeta 2\gamma 2$), or with ε chains to form hemoglobin Gower 1 ($\zeta 2\varepsilon 2$), and α and ε chains combine to form hemoglobin Gower 2 ($\alpha 2\varepsilon 2$) (Figure 2) (1, 2, 48).

The different globin chains are controlled by two families of globin genes The β-like globin genes are arranged in a linked cluster on chromosome 11, which is distributed over approximately 60 kb and arranged in the order 5′ to 3′ (left to right) ε-$^{G}\gamma$-$^{A}\gamma$-ψβ-δ-β. The α-like globin genes lied 40 kb upstream of the cluster on chromosome 16 and distributed in the order 5′-ζ-ψζ-ψα1-α2-α1-3′ (Figure 3) (1).

2. Pathophysiology and clinical classification of thalassemia

In the thalassemia disorders, the basic pathophysiology of premature hemolysis of red blood cells in the peripheral circulation and more extensive destruction of erytroid precursors in the bone marrow and extramedullary sites (ineffective erythropoiesis) results from the excess of α- or β-globin chains due to basic genetic defect. An excess of globin chains will aggregate and precipitate early in red blood cell formation, in bone marrow normoblast and in mature peripheral blood erythrocytes. These aggregates become oxidized and damaged the cell membrane, leading to hemolysis and ineffective erythropoiesis. In addition, The result from RBCs defect causes many clinical symptoms in thalassemia patients including iron overload, pericarditis, infections, gall stones, hypoxemia, thromboembolism, autoimmune hemolytic anemia, leg ulcer, endocrine dysfunction, extravascular hemopoiesis and post-transfusion hypertension, convulsion and cerebral hemorrhage (2, 7, 8, 13, 16, 18, 49-56).

Thalassemia syndromes are classified into three clinical groups depended on the degree of severity, namely: thalassemia major (severe thalassemia), thalassemia intermedia and thalassemia minor (thalassemia trait). Thalassemia major occurs in persons who are homozygous for thalassemia gene, such as homozygous α-thalassemia1 or Hb Bart's hydrops fetalis, homozygous β^0-thalassemia and Hb E/ β-

thalassemia. Hemoglobin levels of patients with severe thalassemia are usually 6 g/dl or lower. Untreated, the patients die early, at birth or before birth or in the first two decades of life. The second group is thalassemia intermedia which hemoglobin levels are 7 g/dl or higher at steady state, including β^+-thalassemia, Hb E/β-thalassemia, Hb H disease and homozygous Hb CS. Generally the patients have very mild or are free of symptoms, not requiring blood transfusion. Finally, thalassemia minor that occurs in all persons who are heterozygous for a thalassemia gene on the chromosome. But many homozygous and heterozygous states are also asymptomatic, such as homozygous α-thalassemia 2, homozygous Hb E, some homozygous Hb CS and α-thalassemia/β-thalassemia double heterozygous (1, 2, 47).

2.1 Alpha Thalassemia (α-thalassemia)

DNA mapping has revealed that the gene deletion is the major cause of α-thalassemia, resulting in a decrease or absent of α-globin chains production and leading to excess β-globin chains. For α-thalassemia, the homozygous state for α-thalassemia1 (--/--) or Hb Bart's hydrop fetalis is the most severe form of thalassemia syndrome. The cause of death is the absence of α-globin chain synthesis which leading to the absence of Hb F and Hb A, thus all of the fetuses die either in utero or soon after birth. The compound heterozygous state for α-thalassemia1/α-thalassemia2 (--/α-) or α-thalassemia1/ Hb CS (--/$\alpha^{CS}\alpha$) called Hb H disease, which characterized by the presence of variable amounts of Hb H that precipitate in the RBCs as inclusion bodies. Hb H is the formation of β globin tetramers (β_4), which are more stable and soluble, but under special circumstances i.e. fever or infection can lead to hemolysis. Conditions of oxidative stress cause Hb H to precipitate, interfering with membrane function and lead to RBCs damage. Patients with Hb H disease are usually mildly anemic and slighty jaundiced. Finally, silent carriers (-α/αα) and α-thalassemia trait [(--/αα) or (-α/-α)] are associated with a mild hypochromic and microcytic anemia but asymptomatic (1, 2).

2.2 Beta thalassemia (β-thalassemia)

Point mutations and small deletions or insertions in the nucleotide sequences are mainly causes of β-thalassemia, result in a decrease or absent of β-globin chains production and lead to excess α-globin chains. Excessive α-globins which are the formation of α globin tetramers (α_4) are unstable and tend to oxidized and precipitate in the red cell membrane This mechanism operates not only in peripheral blood, but also in erythroid precursor, thus the anemia of β-thalassemia reflects a combination of ineffective erythropoiesis combined with a reduced red cell survival. There are three main clinical phenotypes of β-thalassemia. The first is β-thalassemia major resulted from the inheritance of two β-thalassemia alleles (homozygous or compound heterozygous) causes a life-threatening anemia that required regular blood transfusion and extensive ongoing medical care. Iron overload is the most important consequence of transfusion in thalassemia which gets deposited in various organs like pancreas, heart, liver, thyroid, gonads etc., resulting in their dysfunction. Therefore, β-thalassemia major patients must be treated with iron-chelating therapy. Second, β-thalassemia intermedia which have a homozygous or heterozygous β-globin mutation. In this condition, the lack of β protein in the hemoglobin is great enough to cause moderately severe anemia and significant health problems, including bone deformities and enlargement of spleen. Normally patients with thalassemia intermedia do not required blood transfusion excepted when they developed infections; therefore iron chelation may not be necessary in very mild case. Finally, β-thalassemia trait which is due to a single β-thalassemia allele mutation and is usually asymptomatic (1, 2, 47).

In Thailand, Hb E/β-thalassemia is the most commom severe thalassemia syndrome in adult that is more frequence than homozygous β-thalassemia because of the high frequency of Hb E. There are two types of Hb E/ β-thalassemia, classified based on the presence or absence of Hb A, Hb E/ β^0-thalassemia and Hb E/ β^+-thalassemia. Hb F and Hb E are always present in both of patients with β-thalassemia. Hb A is only present in Hb E/ β^+-thalassemia patients, resulting in a milder clinical picture than Hb E/ β^0-thalassemia. Hemoglobin levels range from 3 to 13 g/dL with an average level of 7.7 g/dL. These clinical picture and complication are heterogeneous

ranging from a mild form of thalassemia intermedia to severe transfusion dependency. Patients with mild clinical symptoms usually have normal growth development and survive without transfusions. In contrast, patients who receive regular blood transfusions have marked anemia, growth retardation, severe bone changes, hepatosplenomegary, and heavy iron overload. Most Hb E/ β-thalassemia patients are not transfused or are transfused very little, and iron chelation is rarely administered. It is clear that variability in severity is due to the different β-thalassemia mutations present with Hb E and other modulating factors including the co-inheritance of a varying ability to produce Hb F (1, 2, 4, 47, 55).

3. Hemoglobinopathies

Abnormal hemoglobin occurs from genetic mutation including point mutations, deletions or insertions of the globin genes. Over 90% of known variants have arisen by substitution of one amino acid residue in one chain type (one point mutation) and over 60% involve the β-globin chain. Because an individual inherits only two β-globin genes, a β chain variant usually constitute about half of the total globin in the RBCs and gives rise to significant change in the function of the red cells. In contrast, most individual have four α-globin genes therefore α-chain variants usually contribute only about 25% of the total hemoglobin and the red cell function consequence to be milder than β-chain variants. In Thailand, the most common hemoglobin variants are Hb E and Hb CS (2).

3.1 Hemoglobin E (Hb E)

Hemoglobin E, the most common abnormal hemoglobin in Southeast Asians, is especially prevalent at the border of Thailand, Laos and Cambodia causing substitution of glutamic acid for lysine at position 26 of the β-globin chain (GAG → AAG), results in reduced rate of β^E chain synthesis. Hb E may be present in the heterozygous state (genotype AE or Hb E trait), the homozygous state (EE) and a variety of compound heterozygous states such as Hb E/β-thalassemia. Heterozygous

Hb E is an asymptomatic condition and no clinical relevance. In homozygous form may have normal hemoglobin levels or slight anemia. The MCV is low and many target cells are present on blood smear, but there are no significant clinical problems. Individuals who are compound heterozygotes for Hb E and β-thalassemia have a moderate to severe disease with anemia, microcytosis, splenomegaly, jaundice, and expansion of marrow space (2, 4).

3.2 Hemoglobin Constant Spring (Hb CS)

Hemoglobin Constant Sping is caused by a point mutation TAA → CAA at the terminal of the α_2-globin gene and reslts in the production of abnormally long α-globin chains. The production of the elongated chain is reduced because the α-globin mRNA transcript is unstable. Heterozygotes with Hb CS and two normal trans at genes are hematologically normal. Homozygotes for Hb CS have a mild hemolytic anemia and may have splenomegaly (2).

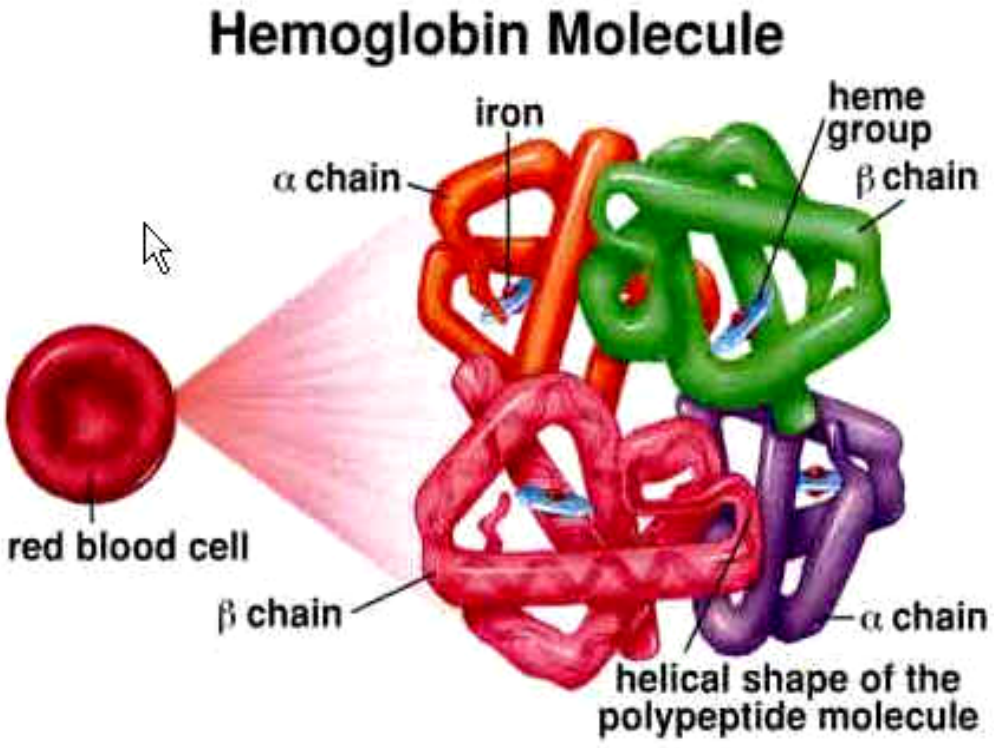

Figure 1. The hemoglobin molecule.
(From www.kacr.or.kr/img/ gene_expression/hemoglobin.jpg)

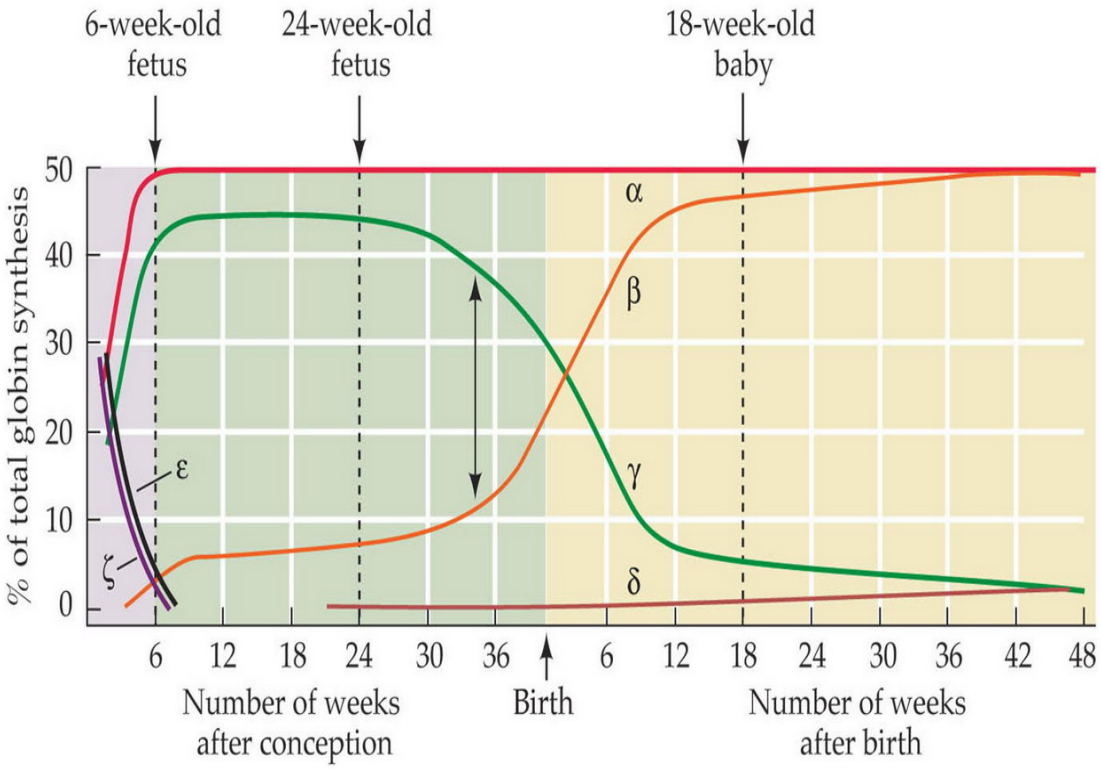

Figure 2. Differential expression in the globin gene family

(From LIFE: THE SCIENCE OF BIOLOGY, seventh edition)

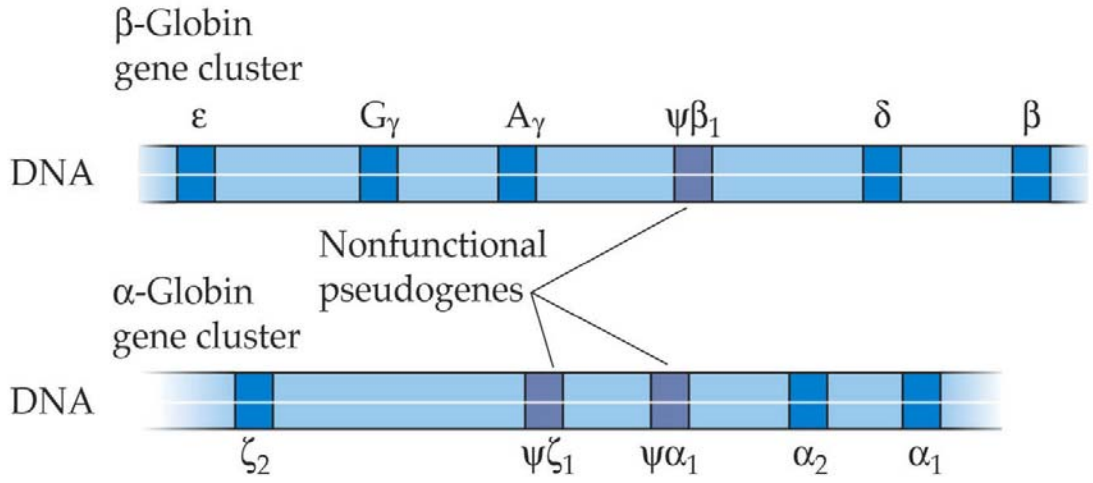

Figure 3. The globin gene family.

(From LIFE: THE SCIENCE OF BIOLOGY, seventh edition)

Table 1. Common thalassemia syndromes in Thailand.

	Genotype	Phenotype
α-thalassemia		
--/αα	Heterozygous α-thalassemia1	Thalassemia minor
-α/αα	Heterozygous α-thalassemia2	Silent carrier
--/--	Homozygous α-thalassemia1	Hb Bart's hydrops fetalis
-α/-α	Homozygous α-thalassemia2	Thalassemia minor
--/-α	α-thalassemia1/ thalassemia2	Hb H disease
--/α^{CS}α	α-thalassemia1/ Hb Constant Spring	Hb H disease
β-thalassemia		
	Heterozygous β^0-thalassemia	Thalassemia minor
	Heterozygous β^+-thalassemia	Thalassemia minor
	Homozygous β^0-thalassemia	Thalassemia major
	β^0-thalassemia/ β^+-thalassemia	Thalassemia intermedia
	Hb E/ β^0-thalassemia	Thalassemia intermedia or thalassemia major
	Hb E/ β^+-thalassemia	Thalassemia intermedia

Table 2. Normal human hemoglobin types and their subunits

Hemoglobin type	Subunits
Embryonic hemoglobin	
Hb Gower 1	$\zeta_2\varepsilon_2$
Hb Gower 2	$\alpha_2\varepsilon_2$
Hb Portland	$\zeta_2\gamma_2$
Fetal hemoglobin	
Hb F	$\alpha_2\gamma_2$
Adult hemoglobin	
Hb A	$\alpha_2\beta_2$
Hb A_2	$\alpha_2\delta_2$

4. The hypercoagulable state in thalassemias

There have been numerous reports of thromboembolic complications associated with thalassemia, many describing cerebral thrombotic events such as recurrent and transient ischemic attacks and strokes as well as deep venous thrombosis, pulmonary embolism, and recurrent arterial occlusion (6, 7, 15, 18, 39, 56-60). In many studies, hemostatic changes have been described in patients with β-thalassemia major, β-thalassemia intermedia and also in patients with Hb H disease. Many surveys showed the prevalence of thrombosis was 1.1 to 29 % in thalassemic patients. The autopsy findings in patients with thalassemia have clearly demonstrated hypercoagulability as a pathologic feature. In addition, they also revealed artherosclerotic changes, and obstructive lesion consisting of organized, recanalized thrombi in the pulmonary arteries and microvasculature (14, 39, 61).

In thalassemic patients, who have low levels of protein C and protein S show enhanced platelet consumption, and show ongoing platelet, monocyte, granulocyte, and endothelial activation. Increased plasma levels of activation peptides, thrombin-ATIII (TAT), prothrombin fragment ($F_{1,2}$), fibrinopeptide A (FPA) and D-dimer, are suggestive of continuous thrombin generation and enhanced fibrinolysis. This suggestion is associated with autopsy finding of platelet and fibrin thrombi in the microvasculature in the lungs and the brain which contribute to the pulmonary hypertension, low lung capacity, hypoxemia, and diffusion defects associated with right heart failure (cor pulmonale) and to the high frequency of ischemic brain lesions associated with asymptomatic brain damage (23, 59, 61).

Thalassemia is associated with partial or completed deficiency of α- or β-globin chain synthesis, which leads to denaturation and degradation of the remaining globin chains. This process is associated with loss of normal asymmetrical distribution of the RBC membrane phospholipids and translocation of PS to the external membrane leaflet (flip-flop). The membrane damage may be related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 protein and spectrin. The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to endothelial cells (ECs), and their capacity to enhance

thrombin generation leads to activation of platelets, monocytes, granulocytes and ECs and expression of tissue factor, which further enhances the thrombin process. The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state (Figure 4) (20, 23, 28, 38, 62-65).

5. Hemostatic changes in thalassemia

Hemostatic changes have been reported in patients with β-thalassemia major, β-thalassemia intermedia and also in Hb H disease (15, 16, 39). Defective platelet aggregation in response to adenosine diphosphate, epinephrine or collagen was found in β-thalassemia major patients, particularly those that had high platelet counts. Platelet kinetic studies showed increased circulating platelet aggregation in splenectomized and non-splenectomized Hb E/β-thalassemia patients (66). This observation is compatible with in vivo platelet activation and the existence of a hypercoagulable state. In addition, thalassemia platelet life span was also observed and found significant shorter than normal platelet (67). These data suggested that the shortened platelet life span was caused by enhanced platelet consumption, a feature usually associated with active thrombotic disease, severe atherosclerosis, diabetes mellitus, and other chronic hypercoagulable states (24, 59, 67).

Further evidence for the existence of chronic platelet activation in thalassemia was provided by the measurement of urinary metabolites of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2). Many studies showed increased 2,3-dinor-TXB_2, 11-dehydro- TXB_2 and 2,3-dinor-6-keto-prostaglandin (PG) $F_{1\alpha}$ in β-thalassemia major and β-thalassemia intermedia patients. However, these results also found in other diseases associated with in vivo platelet activation including unstable coronary disease, severe atherosclerosis and typed II diabetes mellitus (65, 68-71).

The existence of chronic platelet activation in thalassemia was confirmed by flow cytometric studies, which demonstrated the presence and increased fraction of platelets carrying the activation markers 62P (P-selectin) and CD 63. In addition, morphologic changes in thalassemia platelets, elevated plasma platelet factor 3 (PF3), and increased spontaneous whole blood platelet aggregation were previously reported.

The presence of morphologic platelet abnormalities in splenectomized patients with Hb E/β-thalassemia may also contribute to an enhanced risk of vascular complication (28).

The determination of serum and plasma of thalassemic patients showed elevated levels of endothelial adhesion proteins (intercellular adhesion molecule-1 [ICAM-1], endothelial leukocyte adhesion molecule-1 [ELAM-1], vascular cell adhesion molecule-1 [VCAM-1], von Willebrand Factor [vWF] and thrombomodulin). These results suggested that endothelial activation or injury may be a feature of the disorders (35, 72). Furthermore, RBCs from patients with β-thalassemia major and β-thalassemia intermedia showed an enhanced adhesion to cultured ECs (10 to 25-fold increased) as compared to normal RBCs (73). The adherence of RBCs to ECs which correlated to microvascular occlusions was reported in sickle cell disease (SCD) and malaria considered to be a major contributor to microcirculatory disorders in these patients (21).

Monocyte activation may also play a significant role in heightening endothelial activation or injury in thalassemia and SCD. In patients with Hb H disease and β-thalassemia major, a high serum levels of monocyte colony-stimulating factor and increased monocyte phagocytic activities (anti-dependent cell cytotoxicity [ADCC]) toward RBCs were reported (30, 32).

Activated granulocytes could also contribute to the endothelial damage and the hypercoagulable state in thalassemia. Elevated granulocyte phagocytic function was observed in β-thalassemia major patients, with greater prominence of the abnormalities (34). In patients with β-thalassemia major, leukocytes of transfused blood were removed with Leukostop filte,r resulting in improved pulmonary function tests (forced expiratory volume in 1 second/ forced vital capacity ratio) 6 months after the procedure (74). This clinical observation illustrates the deleterious effect of activated granulocytes to the lungs of thalassemic patients.

Many studies of the coagulation proteins provide strong evidence for the existence of a chronic hypercoagulable state in thalassemia. They have reported profound changes in the levels of coagulation factors, coagulation factors inhibitors, and components of the fibrinolytic system (14, 23, 30, 61).

Plasma prothrombin levels were significantly lower in patients with β-thalassemia major compared to healthy controls (61). Low levels of coagulation factor

inhibitors, protein C and protein S have been observed in β-thalassemia patients from a variety of ethnic backgrounds The decreased level of free protein S were also found in thalassemia patients (14, 23, 30, 61). In addition, Low levels of heparin cofactor II (HC II), known to be associated with increased thrombotic risk, have been also found in thalassemia patients (75).

The existence of a chronic and lifelong hypercoagulable state in thalassemia was further supported by the increased levels of TAT complexes found in β-thalassemia major whom none had any clinical signs of overt thrombosis. Significantly increased levels of $F_{1.2}$ and fibrinopeptide (FPA) were found in splenectomized patients with β-thalassemia intermedia, and these patients' also demonstrated high-level plasma D-dimer, a manifestation of enhanced fibrinolylsis. Moreover, increased TAT levels were also observed in patients with α-thalassemia (14, 61).

6. Thalassemic red blood cells and hypercoagulable state

In thalassemia patients, RBCs may provide a source of negative charged phospholipids, which can increase thrombin generation, as measured by prothrombinase assay (76, 77). The procoagulant effect of thalassemic RBCs seems to be due to an increase surface expression of anionic phospholipids (PS and PE). This was demonstrated by experiments, which showed that annexin V, which binds anionic phospholipids, could block the procoagulant effect of isolated thalassemia RBCs (78). The membrane damage may be related to lipid peroxidation mediated by free iron and increased amount of membrane-bound hemichromes and immunoglobulins and modification of membrane band 3 protein and spectrin. Exposure of PS on thalassemic RBCs increases their adherence to ECs and enhances thrombin generation via the assembly of the prothrombinase complex. The enhanced thrombin generation leads to activation of platelets, monocytes, granulocytes, ECs and expression of tissue factor, which further enhances the thrombotic process (21). The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state (23).

Moreover, thalassemic RBCs were demonstrated to enhance cohesiveness, which may contribute to the hypercoagulable state by using a novel image analysis system to measure RBC aggregation in a flow chamber. An increased cohesion of thalassemic RBCs was detected by demonstrating of the formation of large aggregates (79). It is noteworthy that RBC aggregate size was reduced to normal after patients received a blood transfusion and this observation was confirmed by *in vitro* experiments where the addition of normal RBCs to thalassemic RBCs resulted in reduced aggregation under flow (79). These *in vitro* finding could partly explain the recent clinical observation that patients with β-thalassemia intermedia who do not receive regularly transfusion had a much higher incidence of thrombotic events compared to the incidence of such events in those receiving regular by transfusions (16).

The contribution of the abnormal RBCs to the thrombotic process has also been demonstrated in animal models of congenital hemolytic anemias (80-83). A lethal hypercoagulable state manifested by large thrombotic lesions in the heart and the liver and large venous thrombi was found in mice in which the expression of erythroid band 3 had been eliminated via targeted mutagenesis (80). The abnormal RBCs from these mice significantly shortened the Russel viper venom clotting time of normal plasma in a dose-dependent fashion, whereas RBCs from normal mice had no effect. These experiments suggested that the membrane band 3 null RBCs provides a suitable surface for activation of the prothrombinase complex and, indeed, PS exposure on the outer membrane leaflet of the affected RBCs was demonstrated by increased FITC-annexin V binding. A high incidence of thrombosis in the heart and brain was also found in α-spectrin and β-spectrin-deficient mice with hereditary sperocytosis (81). Thrombosis incidence in these animals was significantly reduced following the transfusion of normal RBCs or transplantation of normal bone marrow (82). The presence of normal RBCs in the peripheral circulation of these α-spectrin and β-spectrin-deficient mice prolonged the survival of young animals and abrogated the development of thrombosis in adult animals (82).

7. Monocyte activation and platelet-monocyte aggregates (PMAs)

Multiple factors may contribute to the removal of thalassemic red cells from the circulation. Included among them are changes in cell rigidity, deformability, exposure of galactosyl residues, and the binding of autologous IgG to hemichromes and band 3. All of these factors alter the normal exposure and composition of antigens on the outer surface of the membrane and consequently cause erythrophagocytosis by macrophages (84).

Using mouse peritoneal macrophages, it was found that RBCs from splenectomized patients were phagocytized 22-fold higher than cells from normal donors. The phagocytized cells consisted of both mature and nucleated red blood cells (NRBC) (85). The cells that seem to be involved in the phagocytosis are monocytes, which attached to three-fold as many red blood cells from Hb E/β^0-thalassemia disease compound heterozygotes as to normal red blood cells. It is possible that the activation of mononuclear cell phagocytes plays a role in determining the severity of the anemia in β-thalassemia because there was also showed a three-fold increase in the percentage of leukocytes expressing the Fc-gamma receptor in Hb E/β^0-thalassemia (31).

In sera of patients with Hb H disease and Hb E/β^0-thalassemia disease there was also showed an increase in macrophage colony-stimulating factor (M-CSF), which enhance the effector function of the mononuclear phagocytes on pathologic red cells such as ADCC, phagocytosis and Fc-receptor expression (30). Exposure of monocyte/ macrophage to M-CSF causes production of plasminogen activator and stimulates the synthesis and secretion of cytokines, including IL-1, TNF-α and granulocytes colony-stimulating factor (G-CSF) (86). M-CSF is produced in most tissues including the uterus where its synthesis is greatly increased during pregnancy causing raised circulating concentrations of this growth factor (87). However, raised M-CSF levels have been found in other diseases such as idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia and hemophagocytic syndrome (87-89). In addition, membrane PS is also recognized by macrophage as a signal for attachment and phagocytosis. Normally, PS is confined to the inner surface of the membrane phospholipids bilayer and thus does not provide a signal to macrophages (90). Moreover, the elevation of TNF-α and IL-1β in the sera of these patients have reported

which as similar as the findings were described in SCD (36, 42). Belcher et al and Inwald et al have shown that sickle monocytes are activated, as defined by increased CD11b surface expression and by increased intracellular expression of IL-1β and TNF-α (32, 91). Thus, monocytes are a likely source of the elevated IL-1β and TNF-α levels found in these patients. CD11b, an α-chain integrin, is expressed on the surface of activated leukocytes in a heterodimer complex with CD18, a β-chain integrin. The complex, also known as Macrophage-1 antigen (MAC-1), is a ligand for ICAM-1 (92).

PMAs are complex formations between platelet and monocyte interaction which have been implicated in the pathogenesis of vascular disease such as unstable atherosclerosis, stable coronary disease and hypercholesterolemia (43, 93-95). The interaction between platelets and monocytes might modulate thrombosis and atherogenesis (96, 97). Wun et al have shown that a high proportion of monocytes in SCD patients had adherent platelets (33). Platelet-leukocyte complexs in circulation are markers of platelet activation. Monocytes activation in these patients could be due to the binding of activated platelets to monocytes. Activated platelets can bind to and activated monocytes through thrombospondin cross-linking of glycoproteinV on the surface of both kinds of cells (98). Thus, PMA formation could be one mechanism whereby monocytes become activated.

8. Clinical significance of activated monocytes and PMAs

In vitro, monocytes from SCD patients can activate cultured endothelial cells, as judged by the increased expression of cell adhesion molecules and tissue factor (TF) in endothelial cells. In contrast, when sickle monocytes were preincubated with blocking antibodies to IL-1β or TNF-α individually they showed a slight decreased HUVEC E-selectin and VCAM-1, but when both antibodies were incubated, HUVEC E-selectin and VCAM-1 expression were almost inhibited. These data indicated that sickle monocytes were activated and produced TNF-α and IL-1β that in turn activated endothelium. Moreover, monocyte activation of endothelial cells can be primarily attributed to TNF-α and IL-1β (32). *In vivo*, sickle patients have elevated numbers of circulating endothelial cells (CECs) with markers of activation such as adhesion

molecules and tissue factor on their surface, as well as the abnormal presence of circulating E-selectin, ICAM-1, VCAM-1 in their plasma (99, 100). In addition, elevated levels of endothelial adhesion protein, including ICAM-1, E-selectin, VCAM-1, von Willebrand factor, and thrombomodulin in serum and plasma have been also reported in thalassemic patients (29, 36). These results suggested that patients with SCD and thalassemia have endothelial activation and injury, which enhanced transendothelial migration of white blood cells and RBCs adhesion leading to an early event in inflammation and a vasoocclusion (101).

It is known that increased expressed TF in sickle CECs have been reported. Furthermore, activated monocytes also showed increased TF expression (102). TF is the principal initiator of coagulation, a transmembrane glycoprotein that forms a complex with circulating FVII (a). Upon binding to TF, zymogen FVII may become a better substrate for auto activation and perhaps activation by others serine proteases, including FIXa, FXa, FXIIa and thrombin. In SCD, abnormal expression of TF associated with thrombotic manifestations such as stroke and pulmonary thrombosis (103).

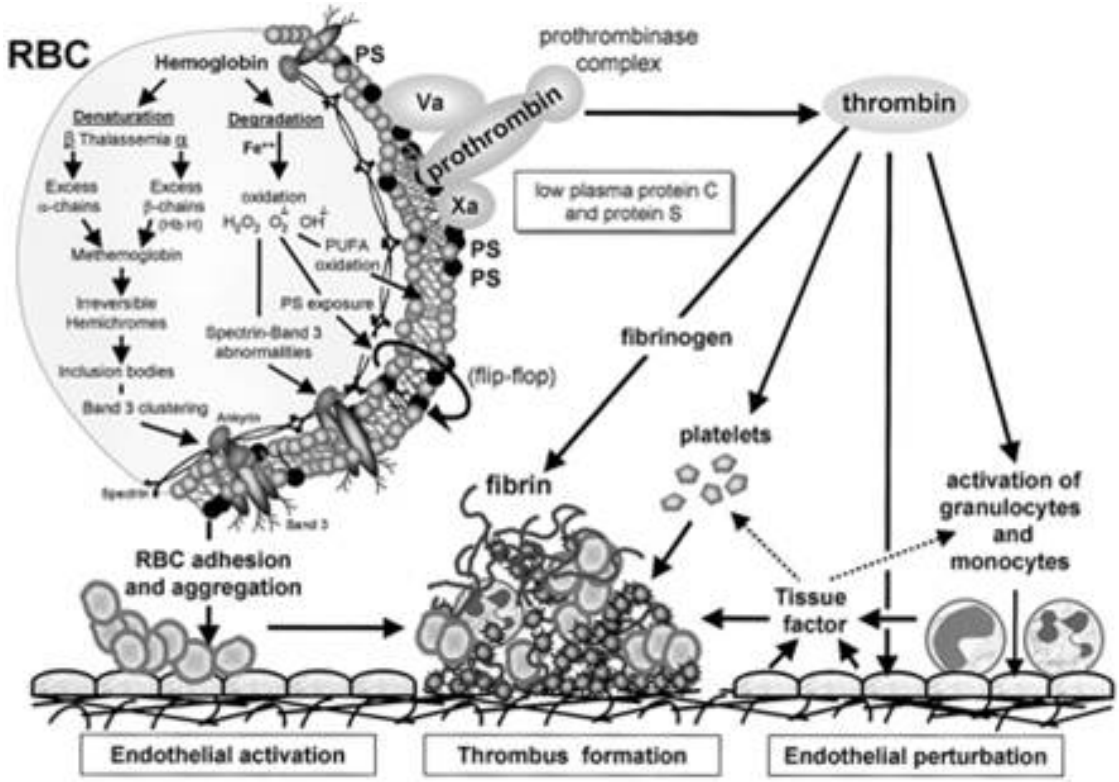

Figure 4. The hypercoagulable state in thalassemia.
(http://www.bloodjournal.org/cgi/content/full/99/1/36/F1)

CHAPTER IV
MATERIALS AND METHODS

1. Study Subjects

After having obtained written inform consent, ten milliters of venous blood samples from 20 normal healthy control subjects and 40 Hb E/ β-thalassemic patients were collected and divided in to 3 tubes. 3 ml each of blood were collected in EDTA and sodium heparin and another 4 ml were collected in 3.2% sodium citrate tube. Fourty Hb E/ β-thalassemic patients consisted of 20 non-splenectomized and 20 splenectomized patients. All subjects were free from any drug and blood transfusion for at least 4 weeks.

2. Chemical Reagents

2.1 1% Paraformaldehyde (Sigma, USA)

2.2 Annexin V-conjugated FITC (Becton Dickinson Bioscince; BDB, USA)

2.3 Glycophorin A-conjugated PE (DAKO, Germany)

2.4 Mouse Anti-Human CD14-conjugated PE (BDB, USA)

2.5 Mouse Anti-Human CD14-conjugated APC (BDB, USA)

2.6 Mouse Anti-Human CD42a-conjugated FITC (BDB, USA)

2.7 Mouse Anti-Human CD11b-conjugated PE (BDB, USA)

2.8 Mouse Anti-Human TNF-α conjugated FITC (BDB, USA)

2.9 Mouse Anti-Human IL-1α conjugated PE (BDB, USA)

2.10 Anti-Mouse IgG1 conjugated FITC (BDB, USA)

2.11 Anti-Mouse IgG1 conjugated PE (BDB, USA)

2.12 1X Binding Buffer (BDB)

2.13 1X FACS Lysing Solution (BDB, USA)

2.14 1X FACS Permeabilizing Solution (BDB, USA)
2.15 10 µg/ml Brefeldin A (Sigma, USA)
2.16 1 µg/ml Lipopolysaccharide (Sigma, USA)
2.17 Phosphate Buffer Saline pH 7.4
2.18 Fetal Bovine Serum (Sigma, USA)
2.19 0.85% Normal Saline Solution
2.20 Wright' s Stain
2.21 Steriled Distilled Water

3 Instruments

3.1 Laminar flow biological cabinet class II (BH 2000, Australia)
3.2 CO_2 incubator
3.3 Light microscope (Nikon, Japan)
3.4 FACSCalibur flow cytometer (BDB, USA)
3.5 Refrigerator 2-8 ^{o}C (Hitachi R-33 NCX, Japan)
3.6 Freezer –30°C (Sanyo Medical Freezer, Japan)
3.7 Vortex mixer
3.8 Centrifuge (Kubota 5100, Japan)
3.9 Technicon H*3 hematology analyzer (Bayer Diagnostics, USA)
3.10 Autoclave (Tomy SS-320, Japan)
3.11 Hot air oven (Venticell 55, Germany)

4 Supplies

4.1 Sodium heparin VACUTAINER® tubes (Greiner bio-one, Germany))
4.2 EDTA VACUTAINER® tubes (BDB, USA)
4.3 3.2% Sodium citrate VACUTAINER® tubes (BDB, USA)
4.4 Needles and Syringes (Terumo, USA)

4.5 Tourniquet

4.6 Automatic pipettes 10, 100, 1000 µl (Socorex, Switzerland)

4.7 Automatic pipette tips (Corning Incorporated, Mexico)

4.8 Serological pipettes 10 ml (HBG, Germany)

4.9 Pasteur pipettes (Costar®, USA)

4.10 Glass slides (Sail Brand; Cat.No.7105, China)

4.11 Immersion oil

4.12 12X75-mm capped Falcon® polypropylene test tubes (BDB, USA)

4.13 12X75-mm capped Falcon® polystyrene test tubes (BDB, USA)

5 Hematological Analysis

The complete blood count and reticulocyte count were performed after blood Sample collection using Technicon*H3 hematological analyzer. Important hematological parameters including of red blood cells (RBCs), white blood cell count (WBCs), platelet count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count were analyzed. The hemoglobin identification was defined by using high performance liquid chromatography (BIORAD) and the diagnosis is based on the chromatogram that requested the levels of Hb A (%), Hb A_2 (%), Hb E (%), Hb F (%) and abnormal hemoglobins.

6 Flow Cytometric Analysis

6.1 Determination of PS exposure on the outer membrane leaflet of RBCs

Two microliters of sodium heparinzed whole blood was incubated with 2 µl of fluorescene isothiocyanate (FITC)–conjugated annexinV and 2 µl of phycoerythrin (PE)-conjugated glycophorin A, then 94 µl of annexinV binding buffer was added and mixed. Cells were stained and incubated at room temperature for 15 minutes in the dark. Anti-mouse IgG1-FITC was used to set negative control. After incubating, 300 µl of annexinV binding buffer was added into stained blood samples and analyzed by BDB FACSCulibur flow cytometer. Acquisition and data analysis were performed using CellQuest Software (BDB). The light scatter and fluorescence channels were set at logarithmic gain. A total of 100,000 events were collected. The RBCs population was presented in region R1 and the annexinV-positive RBCs was presented in the upper right quadrant (Figure 5).

6.2 Detection of monocyte activation in peripheral blood

For intracellular cytokines study, 500 µl of sodium heparinized whole blood was incubated with 10 µl of 10 µg/ml Brefeldin A (BFA) for 4 hours at 37 ^{o}C in 5% CO_2 with and without 5 µl of 1 µg/ml lipopolysaccharide (LPS). 200 µl of unstimulated and LPS-stimulated blood samples were lysed with 2 ml of FACS Lysing solution for 10 minutes. After centrifuging and decanting the supernatant, cells were washed with PBS and centrifugation at 2000 rpm for 5 minutes and stained with 2 µl of allophycocyanin (APC)-conjugated CD14 for 15 minutes at room temperature in the dark. Samples were washed and incubated with 500 µl of FACS permeabilizing solution for 10 minutes. After washing, cell were incubated with 5 µl of FITC-conjugated antihuman TNF-α and and 5 µl of PE-conjugated antihuman IL-1α for 30 minutes at room temperature in the dark. Anti-mouse IgG1 conjugated FITC and anti-mouse IgG1 conjugated PE were used as the negative markers. Before analysis, the stained cells were washed and resuspended in 350 µl of 1% paraformaldehyde and

stored at 4 ^{o}C in the dark and were analyzed within 24 hours by flow cytometer. For determination, a total of 5,000 events was collected. As shown in Figure 6a, the monocytes population was presented in region R1 (panel (A)), Monocytes in the upper right quadrant are activated, and monocytes in the lower right quadrant are resting as presented in a panel (B) and (C) for TNF-α and IL-1α, respectively.

For CD11b on monocytes cell surface study, venous blood was collected into EDTA vacutainer tube. Blood sample could be kept at 4 ^{o}C before processing, or either rapidly cooled to 4 ^{o}C (placed directly into a crushed ice/ ice water bath) after collection. 100 µl of EDTA blood was lysed with 2 ml of 4 ^{o}C FACS Lysing solution for 10 minutes. After centrifugation and discarding the supernatant, the WBCs were washed with 4 ^{o}C PBS and centrifugation at 1400 rpm for 5 minutes. After washing, these cells were incubated with 2 µl of APC-conjugated CD14 and 5 µl PE-conjugated CD11b for 20 minutes at 4 ^{o}C in the dark. The stained cells were washed in PBS at 1400 rpm for 5 minutes and resuspended in 350 µl of 1% paraformaldehyde. These samples were stored in the dark at 4 ^{o}C and analyzed within 24 hours by flow cytometer. For analysis, a total of 5,000 events were collected. Monocyte activation was measured as the mean fluorescence intensity of CD11b. The x-axis measure mean fluorescence intensity of CD11b and the y-axis measure cell count (Figure 6b).

6.3 Detection of the percentage of platelet-monocyte aggregation in peripheral blood

Fifty microliters of citrated blood was incubated immediately with 5 µl of PE-conjugated CD14 and 5 µl of FITC-conjugated CD42a at room temperature for 20 minutes in the dark. After incubation, red cells were lysed and white cells were fixed with FACS Lysing solution. The samples were analyzed immediately by flow cytometer and using mouse IgG1 conjugated FITC as isotype control. For analysis, a total of 5,000 events were collected. Cells coexpression of CD14 and CD42a were defined as PMAs as presented in Figure 7.

7 Statistical Analysis

Statistical analysis of quantitative variables was performed using non-parametric One-Sample Kolmogorov-Smirnov Test. To compare the mean of each variable using non-parametric Mann-Whitney U Test and to study the linear relationship between variables, Spearman's correlation coefficients were calculated. P-value less than 0.05 was considered significant. All statistical calculations were performed using SPSS Version 12.

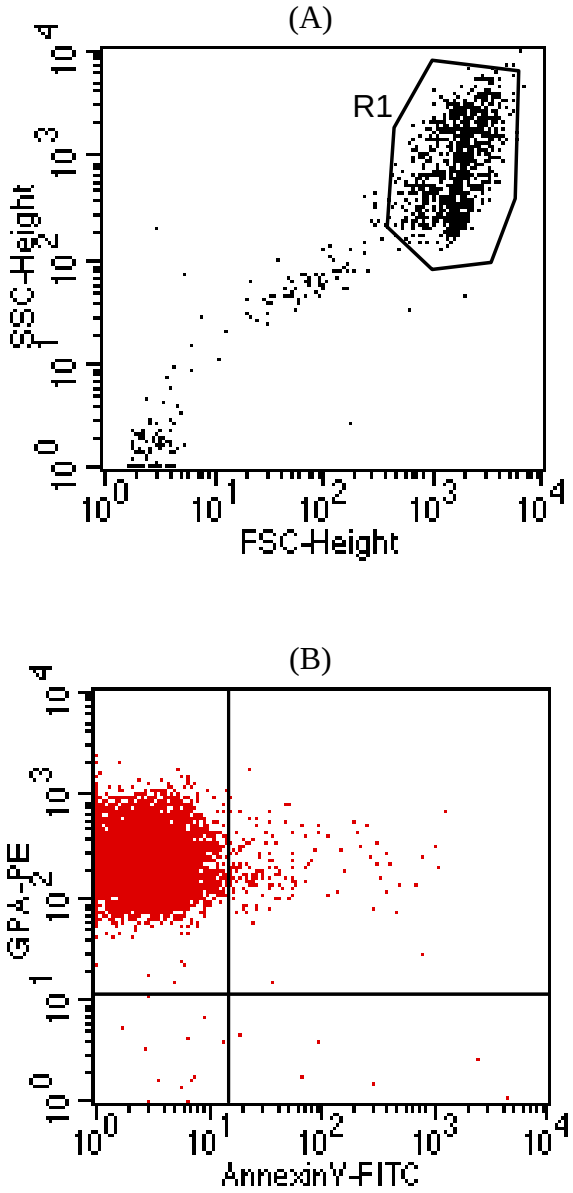

Figure 5. Typical flow cytometry analysis of glycophorin A and annexin V on the surfaces of RBCs from a normal subject. RBC gates (R1) are shown in panel (A) and the percentage of annexinV-positive RBCs are shown in panel (B).

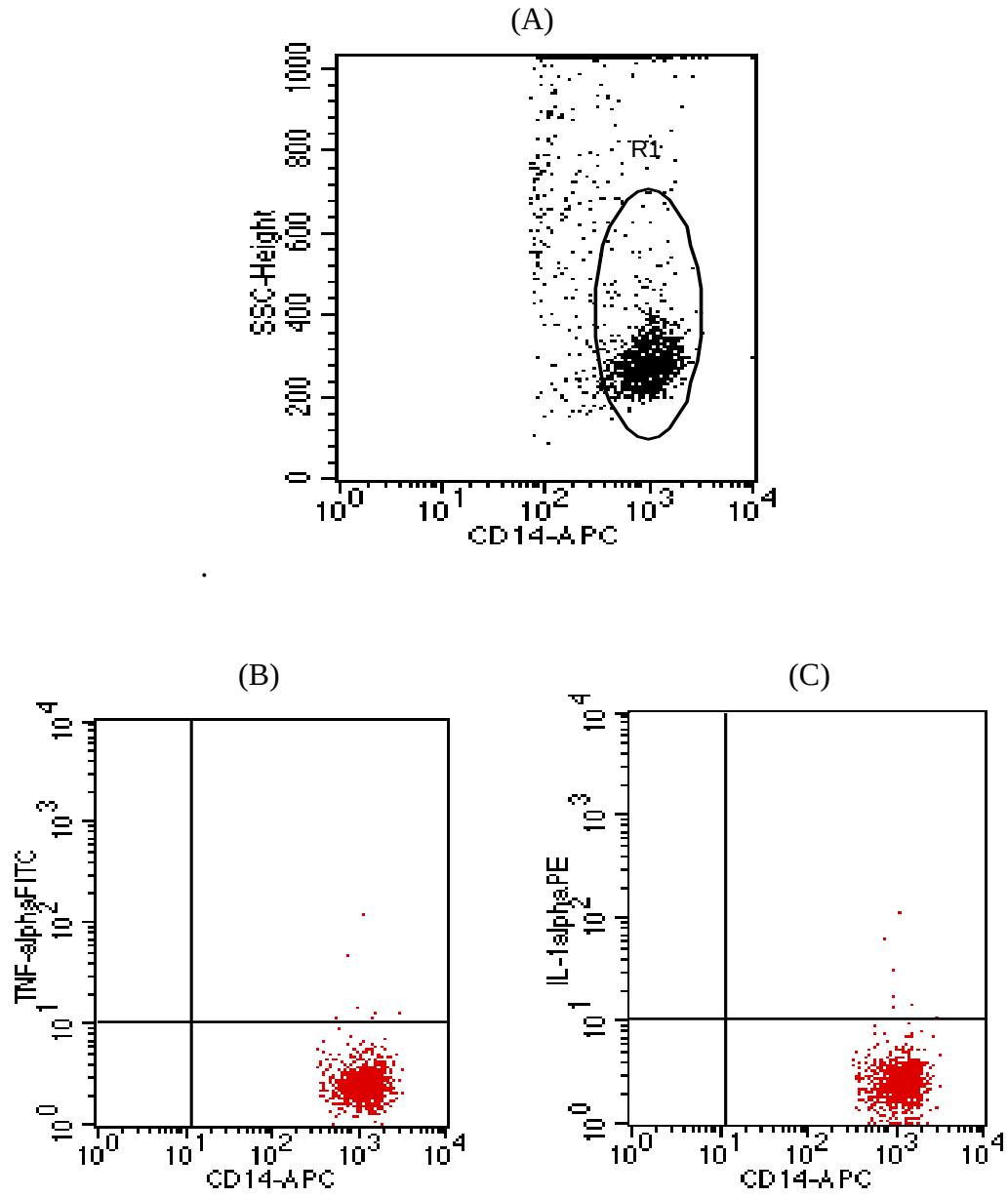

Figure 6a. Typical flow cytometry analysis of TNF-α and IL-1α in peripheral blood monocytes from a normal subject. Monocyte gates (R1) are shown in panel (A). The percentage of CD14+ cells expressed TNF-α or IL-1α are presented in panels (B) and (C), respectively.

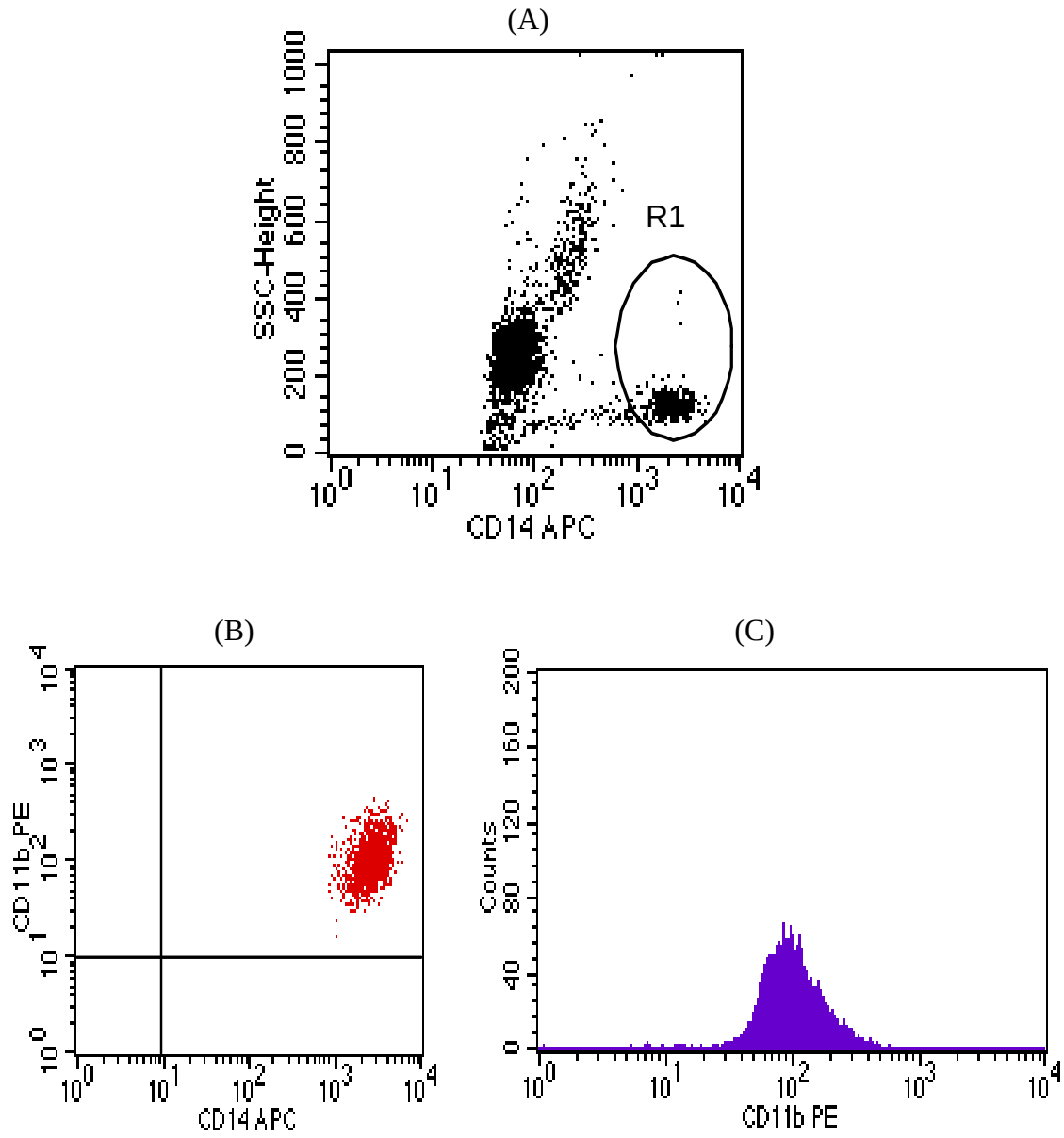

Figure 6b. Typical flow cytometry analysis of CD11b and CD14 expression on the surfaces of monocytes from a normal subject. Monocyte gates (R1) are shown in panel (A). Panel (B) shown the percentage of cells with CD11b and CD14 positive. The mean fluorescence intensity of monocyte CD11b are shown in panel (C).

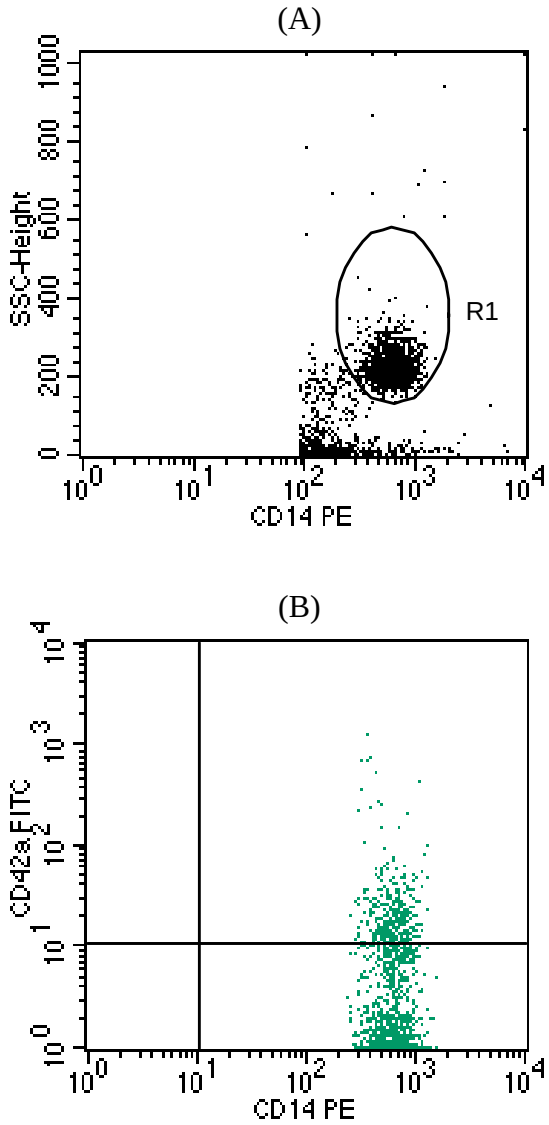

Figure 7. Typical flow cytometry analysis of CD42a and CD14 coexpression on PMAs from a normal subjects. Monocyte gates (R1) are shown in panel (A). Panel (B) shown percentage of PMAs.

CHAPTER V
RESULTS

1. Hematological Analysis

Comparison of hematological parameters and reticulocyte count determined by automated hematology analyzer Technicon H*3 and nucleated red blood cell count determined by manual technique between non-splenectomized HbE/ β-thalassemia, splenectomized HbE/ β-thalassemia and normal subjects were summarized in Table 3. The number of RBCs , hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) in both non-splenectomized and splenectomized patients were statistically significant decreased as compared with normal subjects ($p<0.001$). The splenectomized patients showed statistically significant increased in the number of white blood cells (WBC), platelets and nucleated red blood cells (NRBC) when compared with non-splenectomized patients and normal subjects ($p<0.001$). The number of reticulocyte was statistically significant increased ($P<0.001$) in non-splenectomized and splenectomized patients when compared to normal subjects, and the highest number was detected in splenectomized patients. Box plots and statistically significant of hematological parameters between non-splenectomized, splenectomized patients and normal subjects were shown in Figure 8a-i and box plot and statistically significant of reticulocyte number between non-splenectomized, splenectomized patients and normal subjects was shown in Figure 8j. The percentage of Hb F determined by HPLC was shown in Table 3. The level of Hb F in non-splenectomized and splenectomized patients was statistically significant increased when compared to normal subjects ($p<0.01$). Box plot and statistically significant of Hb F level between non-splenectomized, splenectomized patients and normal subjects was shown in Figure 8k.

Table 3. Mean ± S.D. of hematological parameters and in non-splenectomized Hb E/β-thalassemia (NS), splenectmized Hb E/β-thalassemia (S) and normal subjects determined by automated hematology analyzer Technicon H*3.

Hematological parameters	Normal subjects	Hb E/ β-thalassemia	
		NS	S
No. of patients	20	20	20
RBC (x10^6/µl)	4.71±0.50	3.83±1.02[a]	2.83±0.40[a,c]
Hemoglobin (g/dl)	13.20±1.73	7.58±1.56[a]	6.17±0.97[a,c]
Hct (%)	40.68±5.75	24.49±4.59[a]	21.24±4.55[a,c]
MCV (fl)	86.50±5.88	65.30±7.77[a]	75.41±15.95[a,c]
MCH (pg)	28.27±3.28	20.18±2.46[a]	21.87±4.69[a.b]
MCHC (g/dl)	32.65±2.98	30.96±1.99[a]	29.07±6.32[a,b]
NRBC/100 WBC	0	8.65±15.28[a]	653.50±417.02[a,c]
WBC (/µl)	5,711.50± 1,204.43	7,545.50± 2,223.12[a]	10,212.00± 2,175.15[a,c]
Platelet (/µl)	286,050± 57,653.66	245,600± 97,652.66	736,400± 184,304.50[a,c]
Reticulocyte (/µl)	39,210.50± 17,743.14	83,021.10± 30,445.54[a]	274,417.50± 114,246.86[a,c]
Hb F (%)	0.39±0.39	35.29±12.51[a]	29.50±9.64[a]

a = significantly difference from normal subjects ($p<0.001$)

b = significantly difference from NS subjects ($p<0.05$)

c = significantly difference from NS subjects ($p<0.01$)

MCV = mean corpuscular volume

MCH = mean corpuscular hemoglobin

MCHC = mean corpuscular hemoglobin concentration

NRBC = nucleated red blood cell

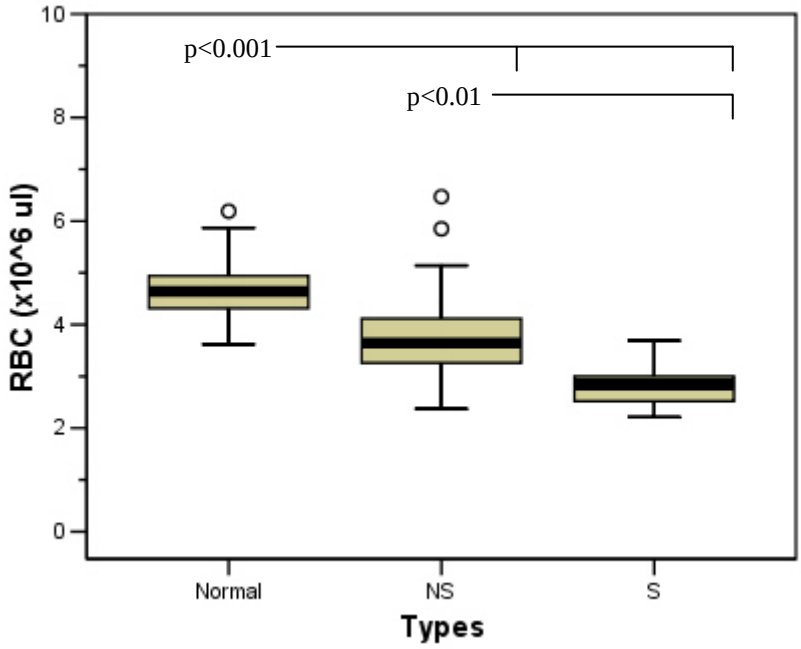

Figure 8a. Box plot and statistically significant in RBC numbers ($x10^6$/µl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/ β-thalassemia (S) and normal subjects.

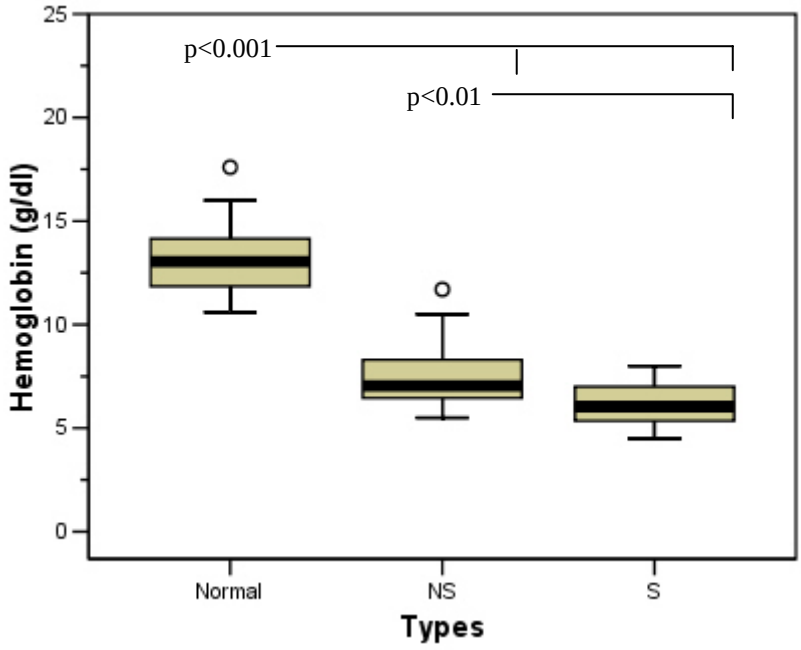

Figure 8b. Box plot and statistically significant in hemoglobin concentration (g/dl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/ β-thalassemia (S) and normal subjects.

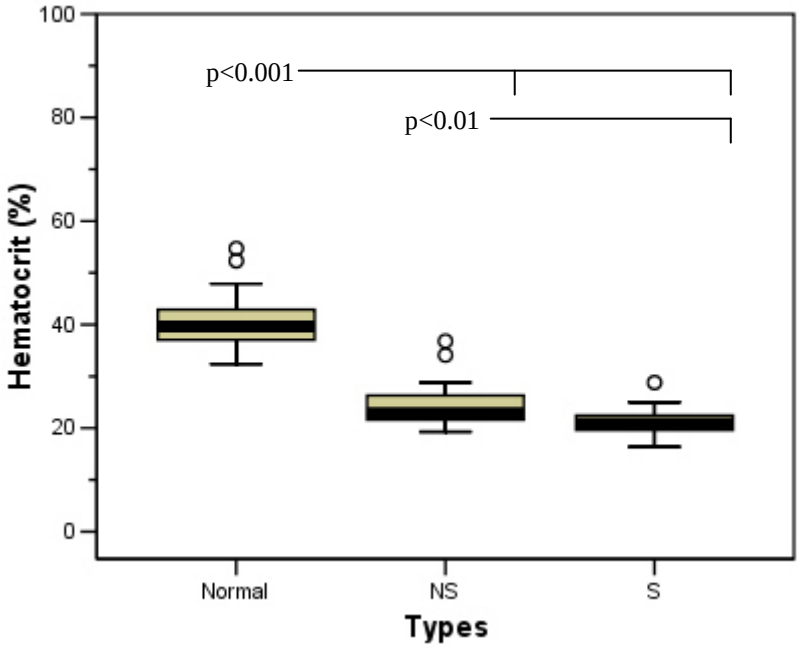

Figure 8c. Box plot and statistically significant in hematocrit (%) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

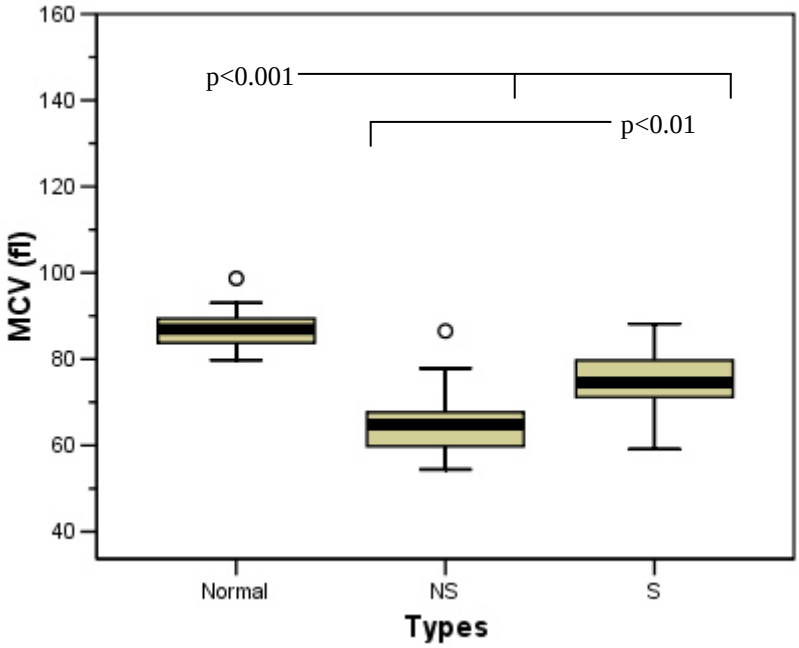

Figure 8d. Box plot and statistically significant in mean corpuscular vqlume (MCV; fl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

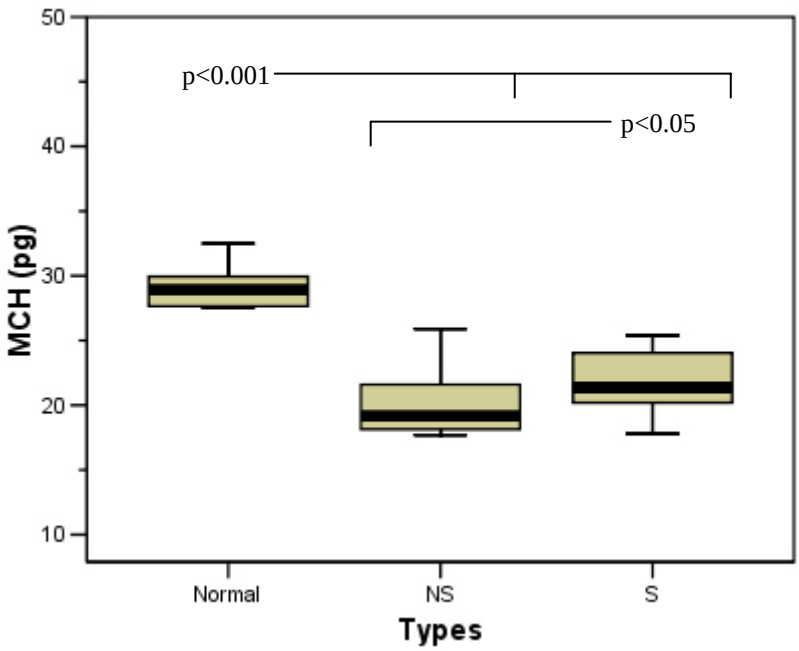

Figure 8e. Box plot and statistically significant in mean corpuscular hematocrit (MCH; pg) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

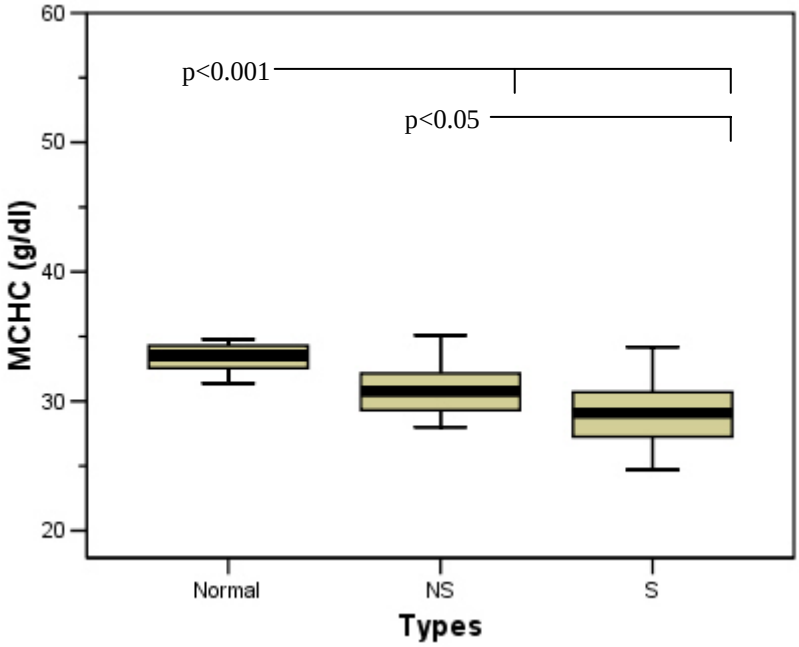

Figure 8f. Box plot and statistically significant in mean corpuscular hemoglobin concentration (MCHC; g/dl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

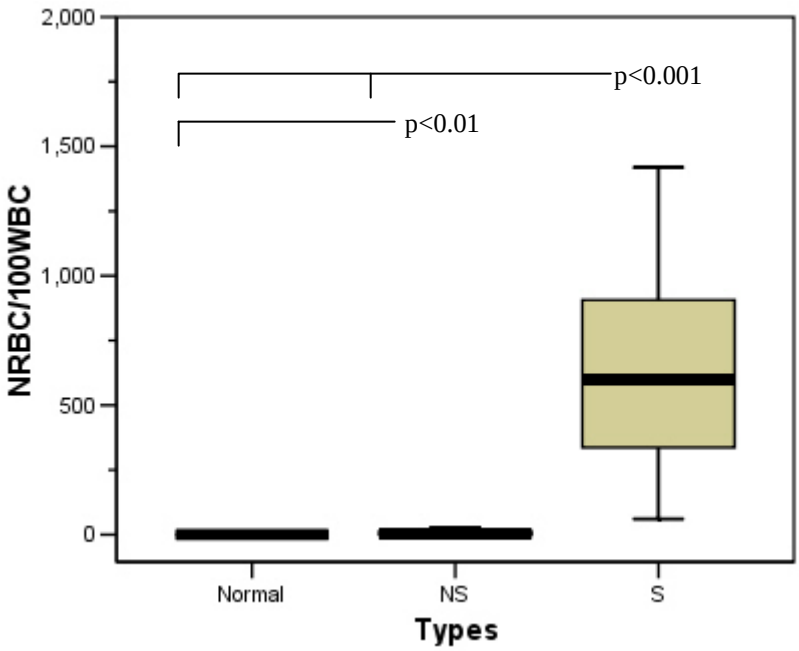

Figure 8g. Box plot and statistically significant in nucleated red blood cell (NRBC; /100 WBC) among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

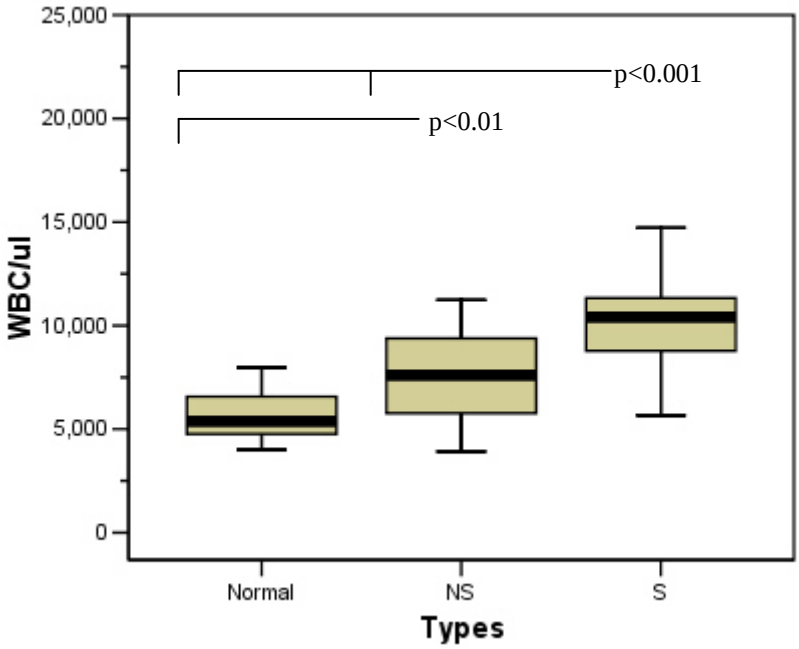

Figure 8h. Box plot and statistically significant in WBC numbers (/μl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

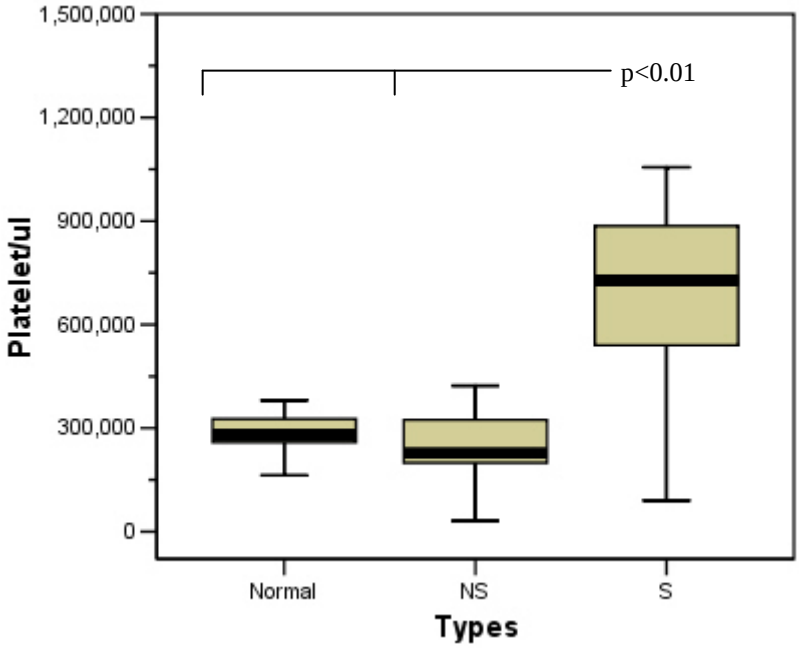

Figure 8i. Box plot and statistically significant in platelet (/µl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

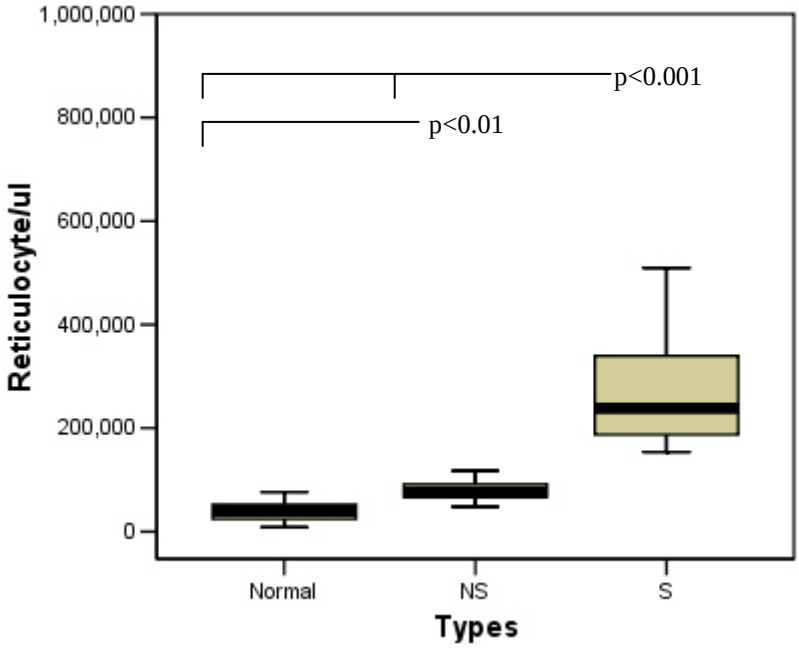

Figure 8j. Box plot and statistically significant in reticulocyte concentration (/µl) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

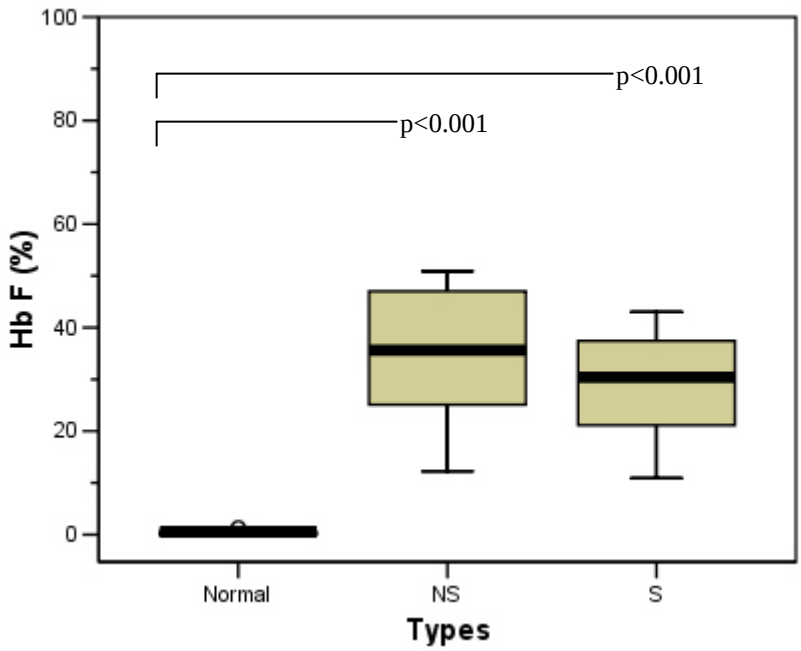

Figure 8k. Box plot and statistically significant in hemoglobin F level (%) among patients with non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

2. Percentage of Annexin V-positive in red blood cell populationin peripheral blood.

The percentage of annexin V-positive in RBCs population staining with glycophorin A and annexin V determined by flow cytometry was summarized in Table 4. Two types of Hb E/ β-thalassemia patients and normal subjects showed annexin V-positive in RBC population. Box plot and statistical analysis using non-parametric Mann-Whitney U Test shown that splenectomized patients have statistically significant higher level of annexin V-positive in RBCs population compared with non-splenectomized patients and normal subjects ($p<0.01$). In addition, non-splenectomized also showed a statistically significant higher level of annexin V-positive in RBC population when compared with normal subjects ($p<0.05$) as shown in Figure 9.

The correlation between percentage of annexin V-positive RBCs of Hb E/β-thalassemic patients and their hemoglobin concentration was detected ($r^2 = 0.111$, $p<0.05$) as shown in Figure 10a.

The correlation between percentage of annexin V-positive RBCs of Hb E/β-thalassemic patients and their hematocrit was found ($r^2 = 0.157$, $p<0.01$) as shown in Figure 10b.

Table 4. Mean ± S.D. of percentage of annexin V-positive in RBCs population in peripheral blood of non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/ β-thalassemia (S) and normal subjects.

	Normal subjects	**Hb E/ β-thalassemia**	
		NS	**S**
	n = 20	n = 20	n = 20
Percent annexin V-positive	0.88±0.50	1.26±0.59[a]	3.47±1.77[b,c]

a = significantly difference from normal subjects ($p<0.05$)

b = significantly difference from normal subjects ($p<0.01$)

c = significantly difference from NS ($p<0.01$)

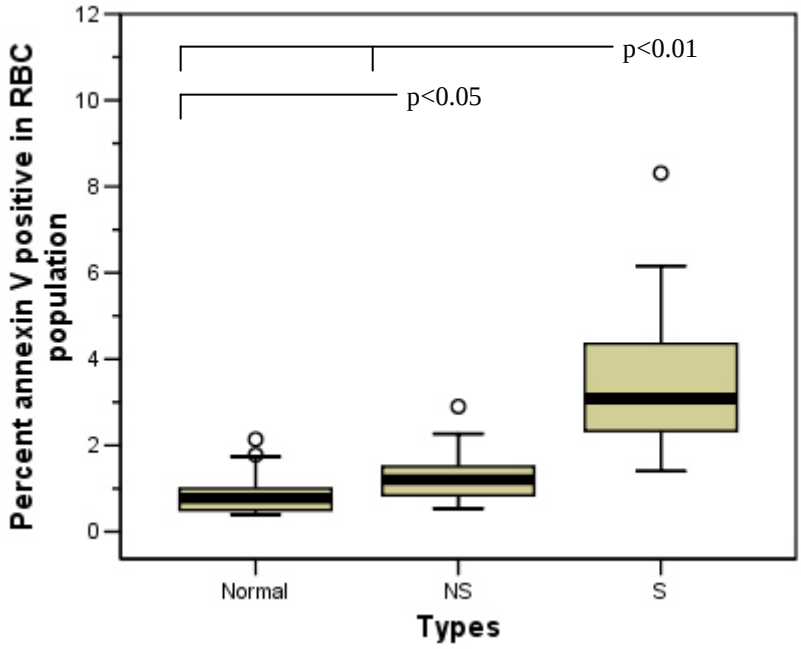

Figure 9. Box plot and statistically significant in the percentage of annexin V-positive in RBCs of non-splenectomized Hb E/ β-thalassemia (NS), splenectomized Hb E/ β-thalassemia (S) and normal subjects.

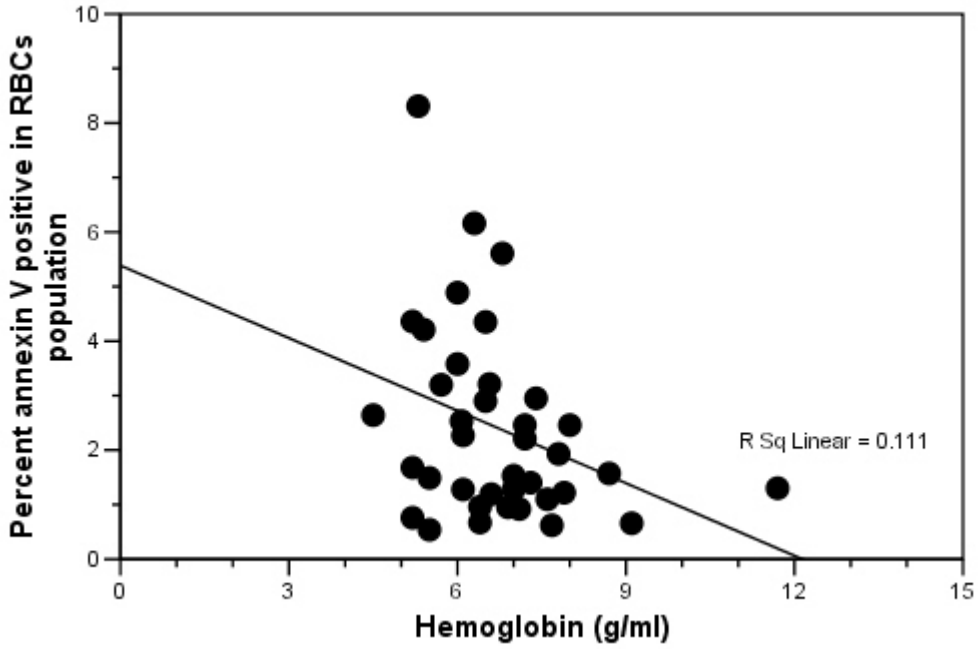

Figure 10a. Correlation between percentage of annexin V-positive RBCs of Hb E/β-thalassemic patients and their hemoglobin concentration.

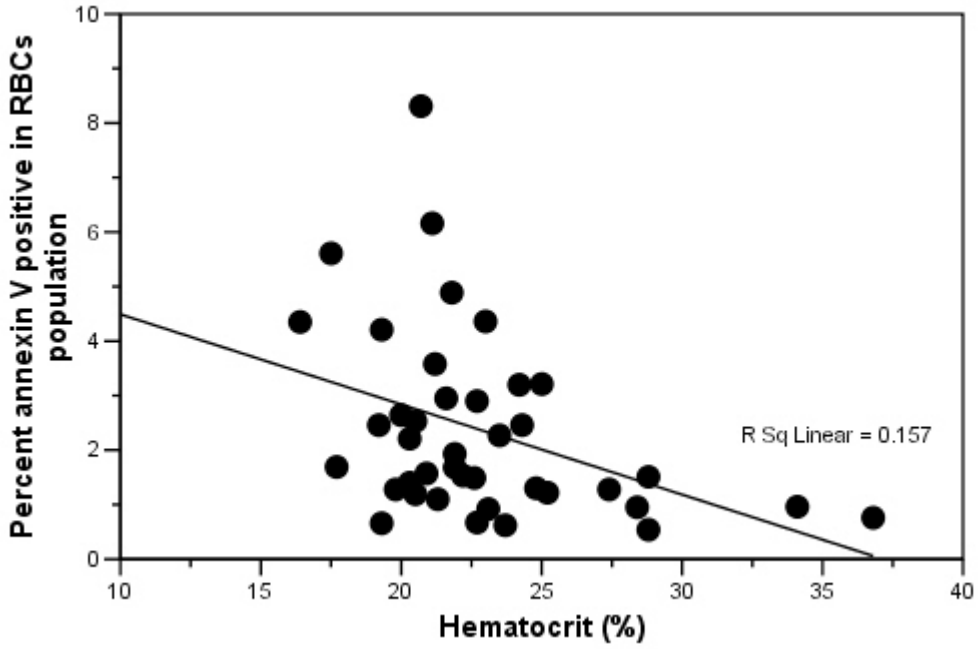

Figure 10b. Correlation between percentage of annexin V-positive RBCs of Hb E/β-thalassemic patients and their hematocrit.

3. The percentage of TNF-α and IL-1α expression in peripheral blood monocyte

TNF-α and IL-1α intracellular cytokines expression in peripheral blood monocytes were determined by flow cytometry and defined as monocyte activation. As shown in Table 5, Unstimulated monocytes from splenectomized patients expressed TNF-α and IL-1α higher than non-splenectomized patients and normal subjects. The statistically significant was found in TNF-α expression between splenectomized patients and normal subjects only ($p<0.05$). In contrast, LPS-stimulated monocytes from splenectomized patients showed statistically significant decreased production of TNF-α and IL-1α as compared with non-splenectomized patients ($p<0.01$) and normal subjects ($p<0.01$). There was no difference between non-splenectomized patients and normal subjects after LPS stimulation. Box plot and statistically significants of percentage of TNF-α and IL-1α expression in unstimulated and LPS-stimulated monocytes were shown in Figure 11a-d.

Correlations between percentage of annexin V-positive RBCs and percentage of TNF-α and IL-1α expression in unstimulated peripheral blood monocytes were detected ($r^2=0.114$, $p<0.01$ and $r^2=0.003$, $p<0.01$, respectively) as shown in Figure 12a-b.

Table 5. Mean ± S.D. of percentage of intracellular TNF-α and IL-α expression in peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

Groups	Unstimulated CD14+ cells		LPS-stimulated CD14+ cells	
	TNF-α+	IL-1α+	TNF-α+	IL-1α+
Normal subjects	0.36±0.34	0.79±0.50	78.38±10.31	84.72±14.25
NS	0.44±0.76	0.46±0.33[b]	82.59±11.36	82.06±8.97
S	0.69±0.78[a]	0.96±1.14	37.22±28.34[b,c]	31.99±27.03[b,c]

a = significantly difference from normal subjects ($p<0.05$)

b = significantly difference from normal subjects ($p<0.01$)

c = significantly difference from NS ($p<0.01$)

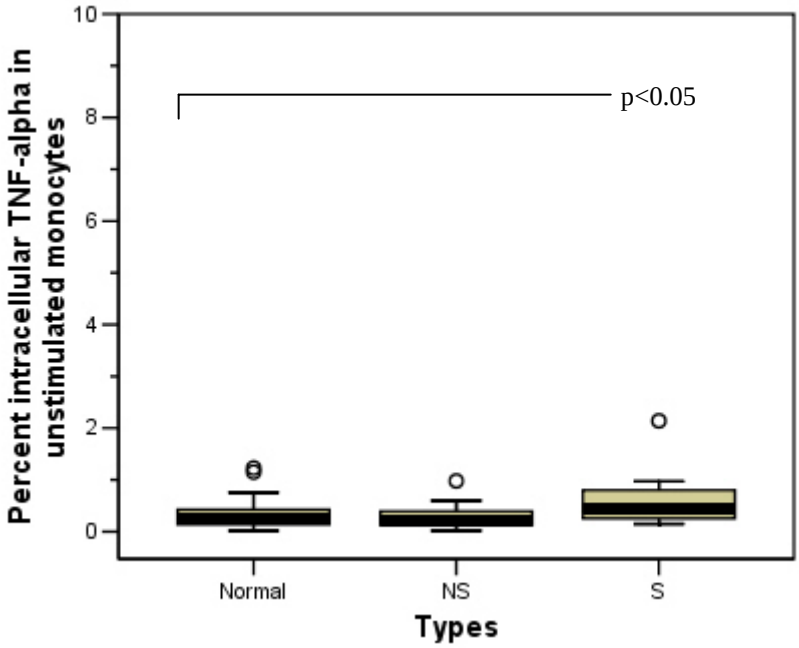

Figure 11a. Box plot and statistically significant in the percentage of intracellular TNF-α in unstimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

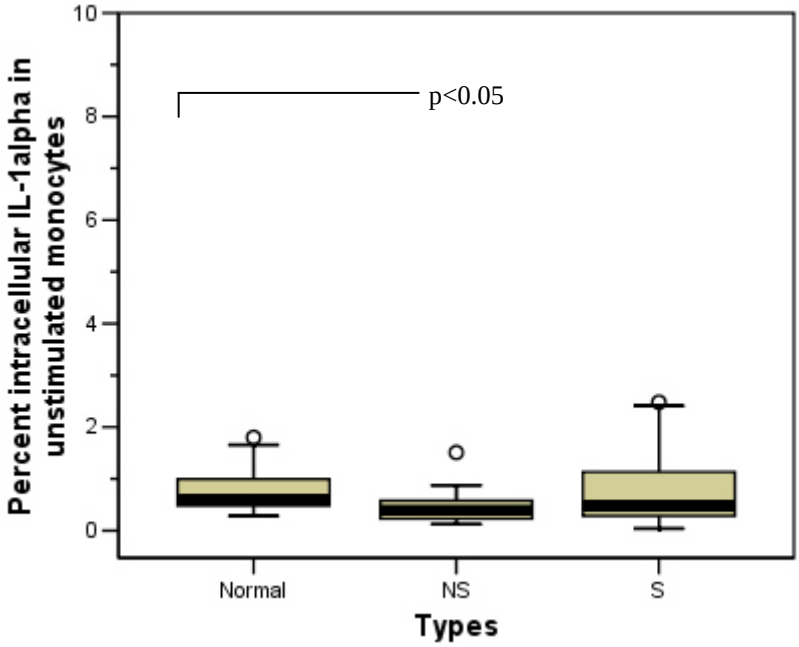

Figure 11b. Box plot and statistically significant in the percentage of intracellular IL-1α in unstimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

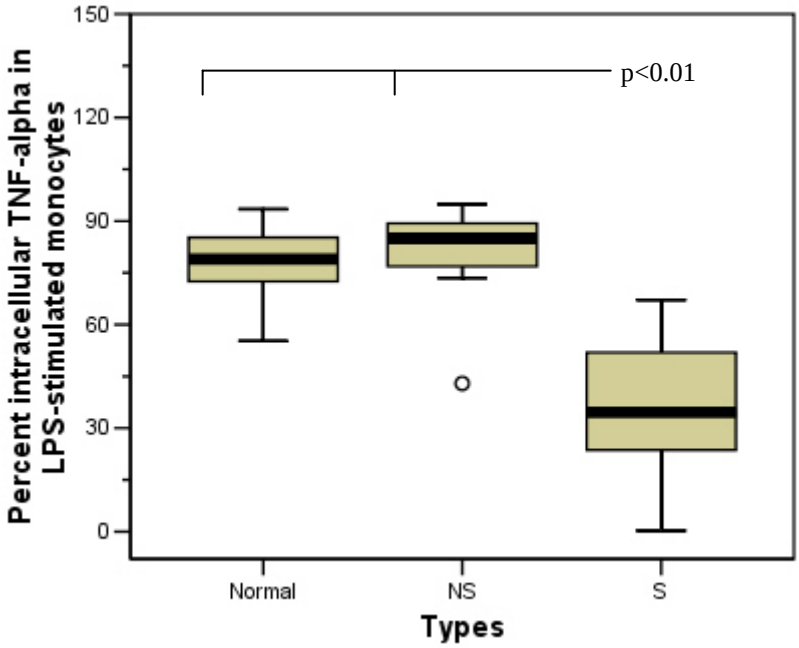

Figure 11c. Box plot and statistically significant in the percentage of intracellular TNF-α in LPS-stimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

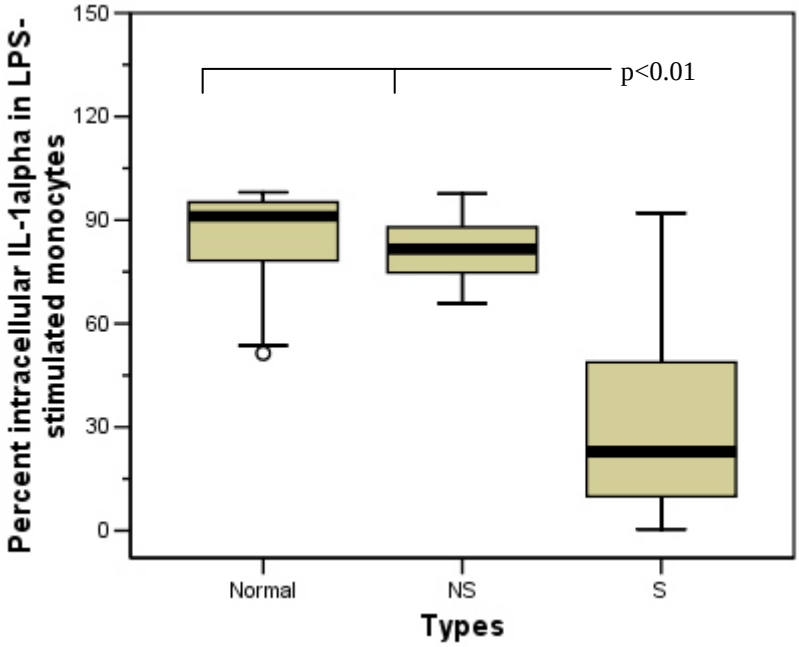

Figure 11d. Box plot and statistically significant in the percentage of intracellular IL-1α in LPS-stimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

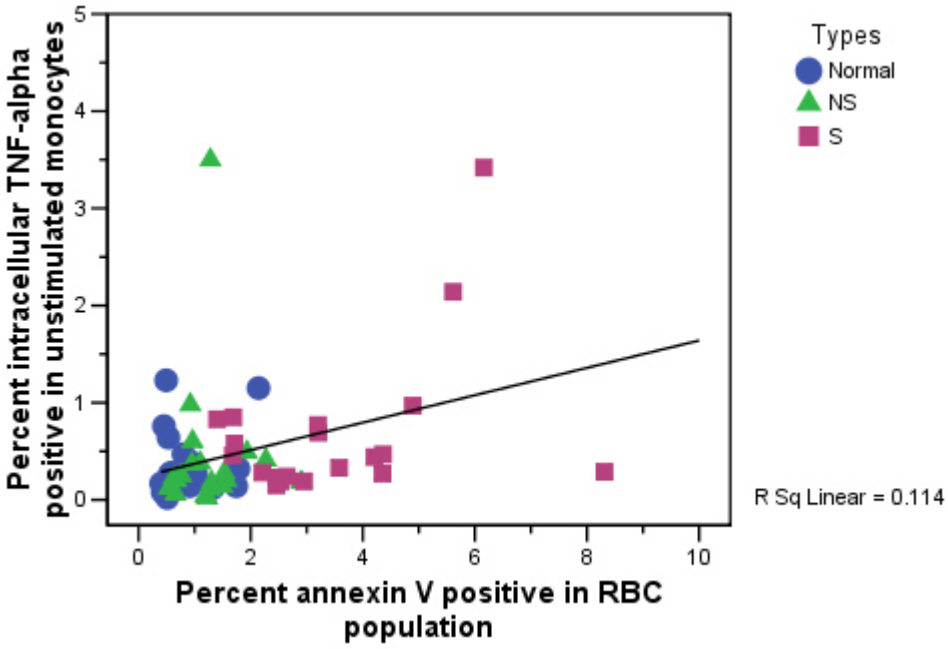

Figure 12a. Correlation between percent intracellular TNF-α positive in unstimulated peripheral blood monocytes and percent annexin V positive in RBCs population among non-splenectomized Hb E/ β-thalassemia (NS), splenectomized HbE/ β-thalassemia (S) and normal subjects.

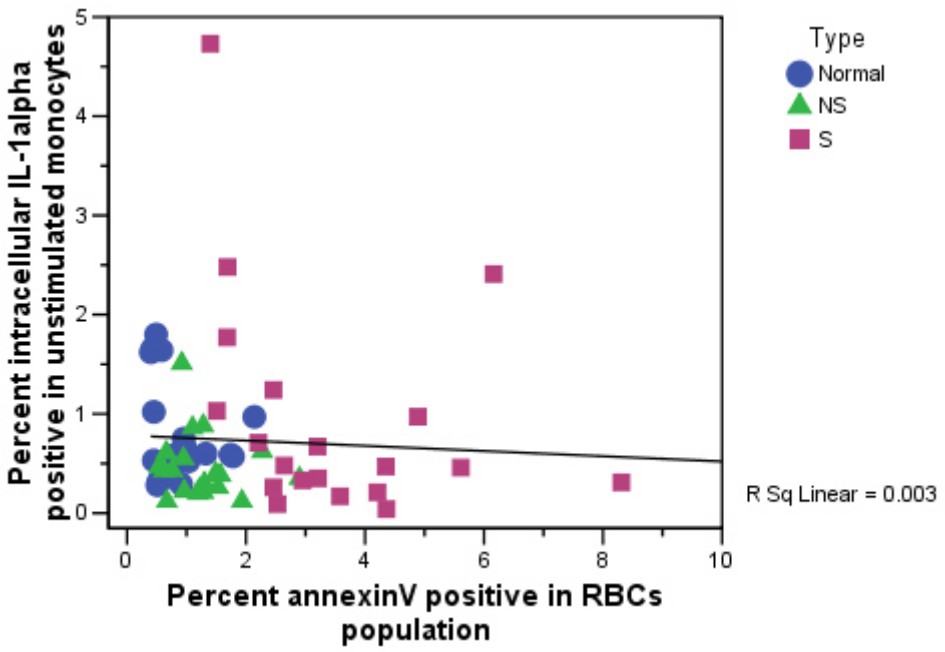

Figure 12b. Correlation between percent intracellular IL-1α positive in unstimulated peripheral blood monocytes and percent annexin V positive in RBCs population among non-splenectomized Hb E/ β-thalassemia (NS), splenectomized HbE/ β-thalassemia (S) and normal subjects.

4. The absolute number of peripheral blood monocyte with TNF-α and IL-1α production.

The absolute number of monocytes that produced intracellular TNF-α and IL-1α were determined by calculation from the following formular :

Absolute number of monocytes expressed TNF-α or IL-1α	=	Percent of TNF-α or IL-1α expression on monocyte	X	Absolute monocytes count

The absolute monocyte count in splenectomized patients was statistically significant higher than non-splenectomized patients ($p<0.01$) and normal subjects ($p<0.01$) and was no statistically significant between non-splenectomized patients and normal subjects (Table 6).

In splenectomized patients, the absolute number of monocytes expressed TNF-α and IL-1α were statistically significant higher than non-splenectomized patients ($p<0.01$) and normal subjects ($p<0.01$ and $p=0.134$, respectively) as shown in Table 6. Box plots and statistically significant of absolute monocyte count, absolute number of TNF-α positive and IL-1α positive were shown in Figure 13, 14a and 14b, respectively.

Correlation between percentage of annexin V-positive RBCs and absolute number of TNF-α positive monocytes was shown in Figure 15a ($r^2=0.174$, $p<0.01$) and absolute number of IL-1α positive monocytes was shown in Figure 15b ($r^2=0.016$, $p<0.01$).

Table 6. Mean ± S.D. of absolute monocyte numbers with TNF-α and IL-1α production among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

Group	Absolute monocyte count (/µl)	CD14+ cells	
		TNF-α+	IL-1α+
Normal subjects	440.60±220.31	125.46±73.24	344.27±294.26
NS	341.20±190.20	148.92±225.69	172.09±219.74[a]
S	1515.60±1849.33[a,b]	897.68±1481.70[a,b]	1390.58±1907.38[b]

a = significantly difference from normal subjects (p<0.01)

b = significantly difference from NS (p<0.01)

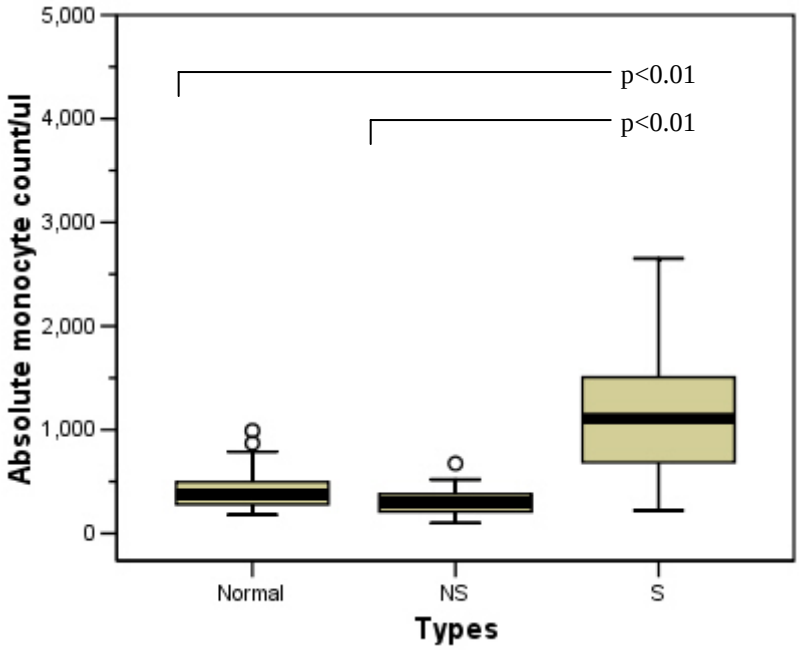

Figure 13. Box plot and statistically significant in absolute monocyte count (/µl) among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

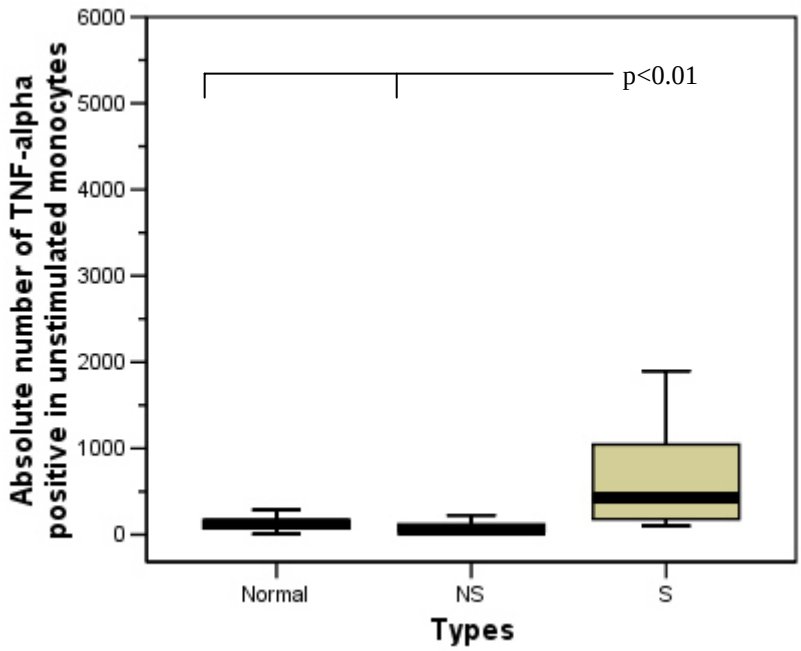

Figure 14a. Box plot and statistically significant in absolute number of TNF-α positive in unstimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

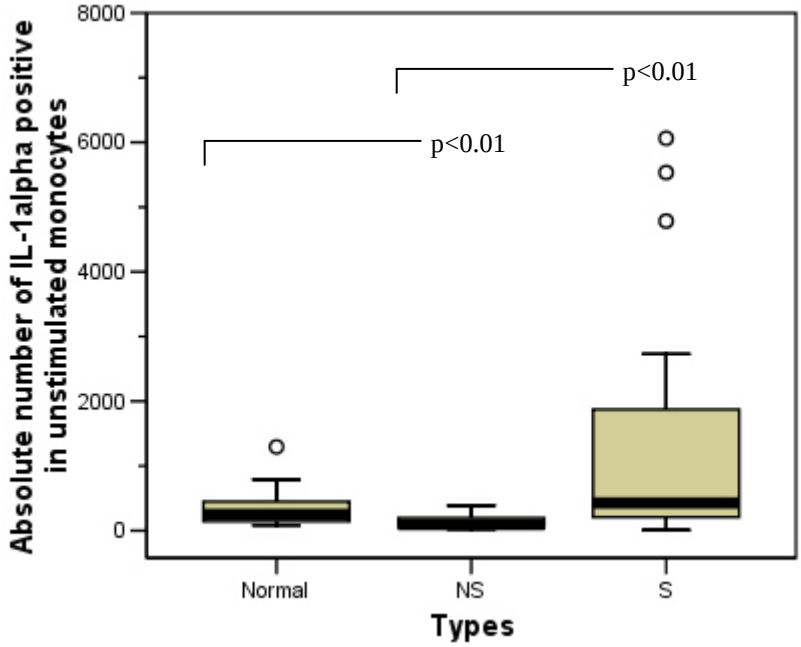

Figure 14b. Box plot and statistically significant in absolute number of IL-1α in unstimulated peripheral blood monocytes among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

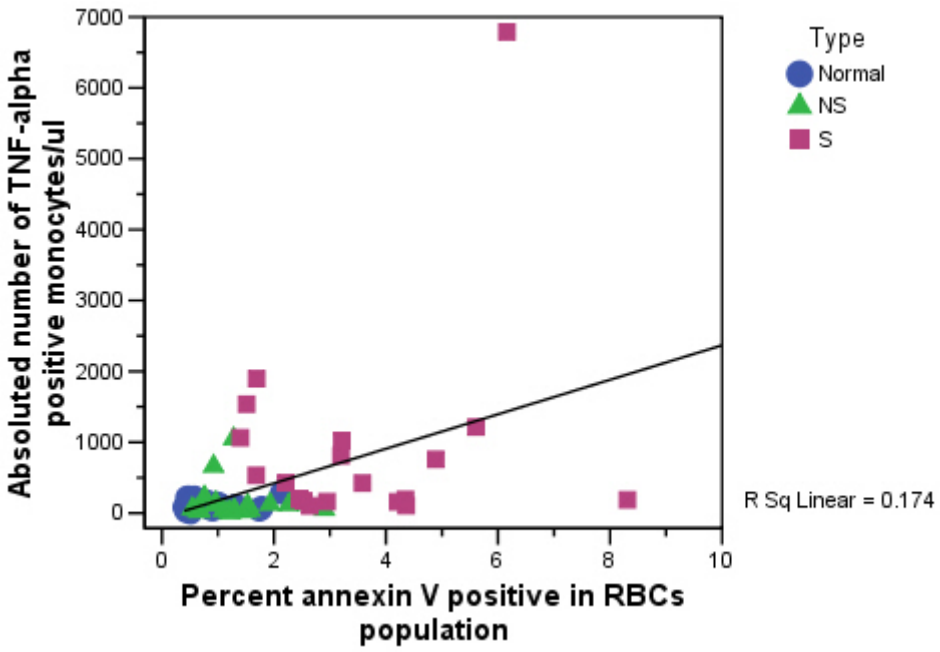

Figure 15a. Correlation between absolute number of TNF-α positive monocytes and percentage of annexin V positive RBCs among non-splenectomized Hb E/ β-thalassemia (NS), splenectomized HbE/ β-thalassemia (S) and normal subjects.

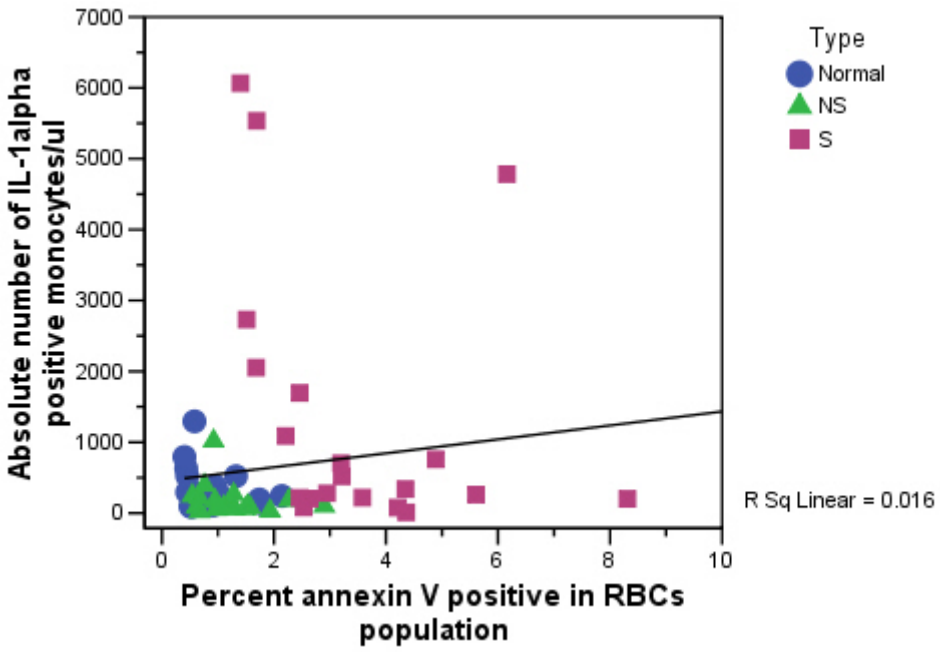

Figure 15b. Correlation between absolute number of IL-1α positive monocytes and percentage of annexin V positive RBCs among non-splenectomized Hb E/ β-thalassemia (NS), splenectomized HbE/ β-thalassemia (S) and normal subjects.

5. The mean fluorescence intensity (MFI) of CD11b on peripheral blood monocytes surface.

CD11b fluorescence intensity on monocyte surface was determined by using flow cytometry. Mean ± S.D. of CD11b fluorescence intensity in non-splenectomized patients, splenectomized patients and normal subjects was summarized in Table 7. Mean CD11b fluorescence intensity in splenectomized patients were higher than non-splenectmized patients and normal subjects ($p<0.01$). Box plot and statistically significant in CD11b fluorescence intensity among various types was shown in Figure 16.

Figure 17a showed the correlation between percentage of IL-1α positive in unstimulated monocytes and CD11b MFI ($r^2 = 0.394$, $p<0.05$) and Figure 17b showed the correlation between percentage of absolute monocyte and CD11b MFI ($r^2 = 0.271$, $p<0.01$). In contrast, the negative correlation was detected between the mean CD11b fluorescence intensity and the percentage of annexin V-positive RBCs ($r^2 = 0.080$, $p<0.05$) and percentage of TNF-α positive in unstimulatred monocytes ($r^2 = 0.045$, $p=0.653$) as shown in Figures 17c and 17d, respectively.

Table 7. Mean ± S.D. of peripheral blood monocyte CD11b fluorescence intensity among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

	Normal subjects	Hb E/β-thalassemia	
		NS	S
CD11b fluorescence intensity	51.37±10.78	44.07±11.99[a]	65.71±28.20[b]

a = significantly difference from normal subjects ($p<0.05$)

b = significantly difference from NS ($p<0.01$)

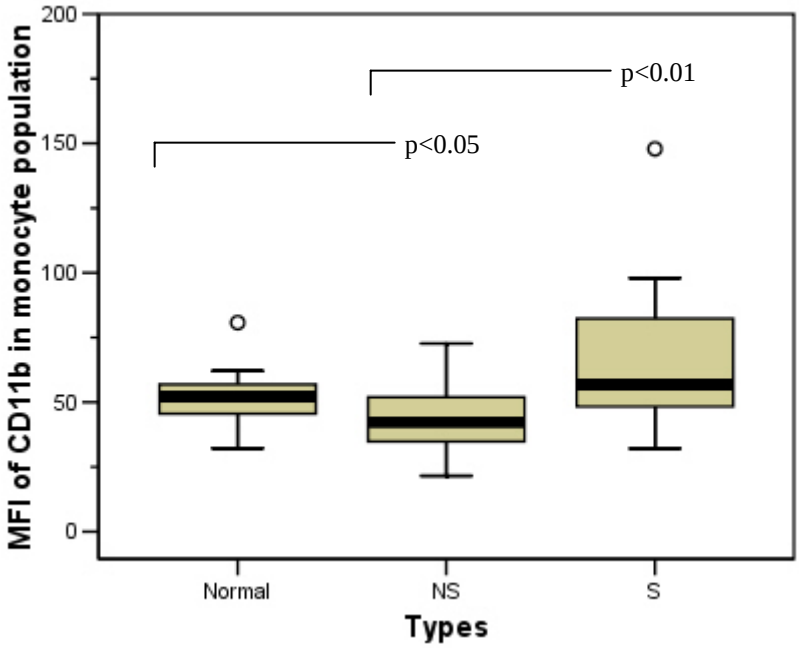

Figure 16. Box plot and statistically significant in CD11b fluorescence intensity on peripheral blood monocyte cell surface among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

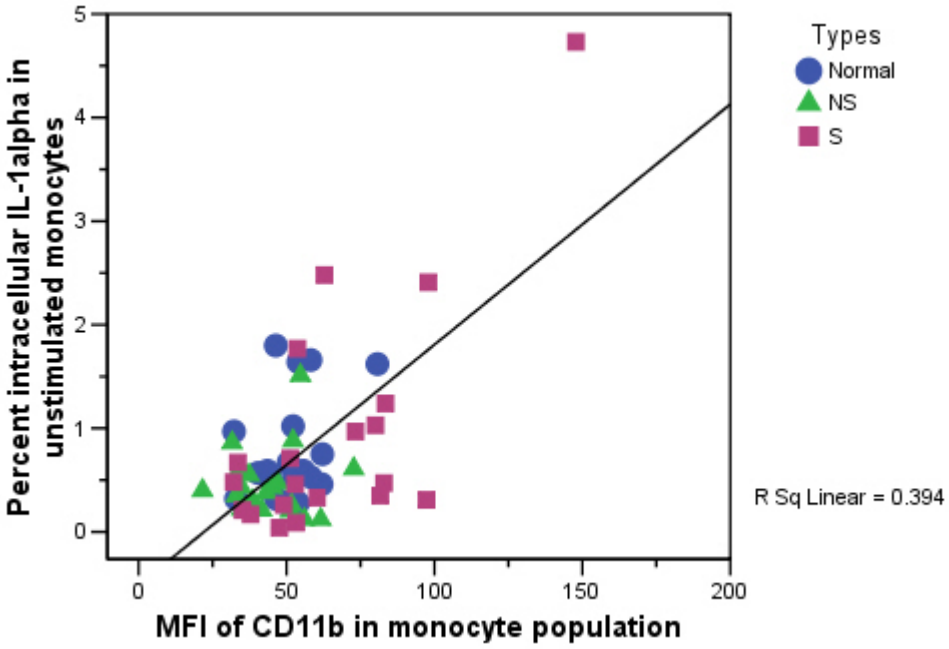

Figure 17a. Correlation between percentage of IL-1α positive in unstimulated monocytes and mean CD11b fluorescence intensity among non-splenectomized Hb E/β-thlassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

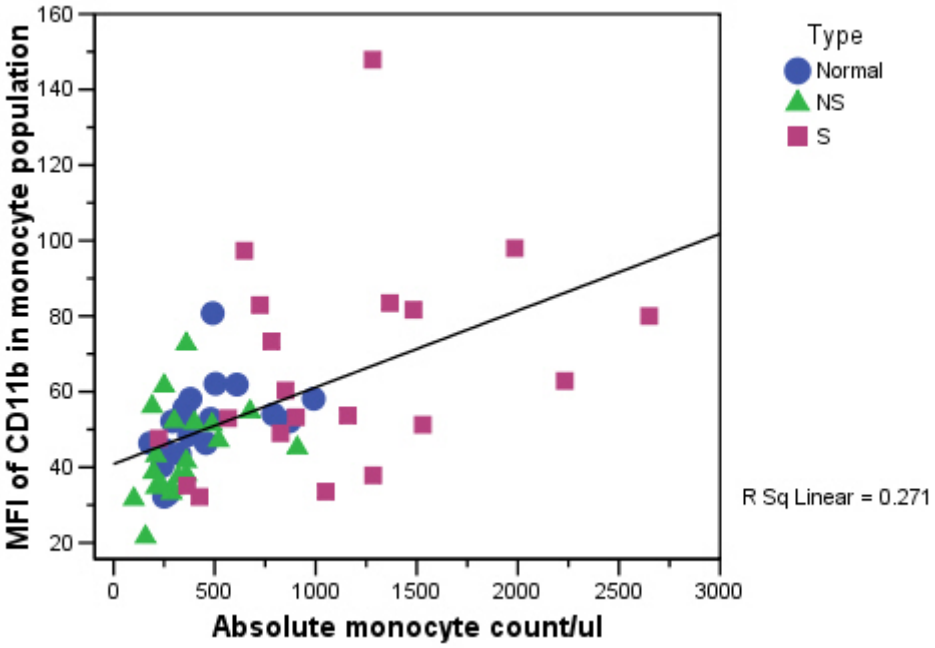

Figure 17b. Correlation between percentage of absolute monocyte count and mean CD11b fluorescence intensity among non-splenectomized Hb E/β-thlassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

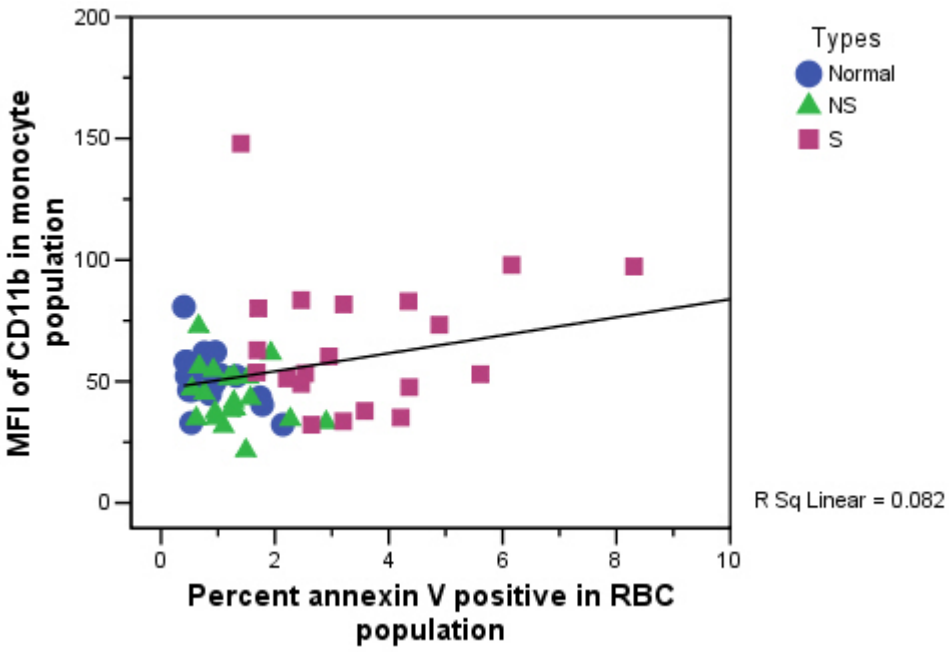

Figure 17c. Correlation between mean CD11b fluorescence intensity and percentage of annexin V-positive RBCs among non-splenectomized Hb E/β-thlassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

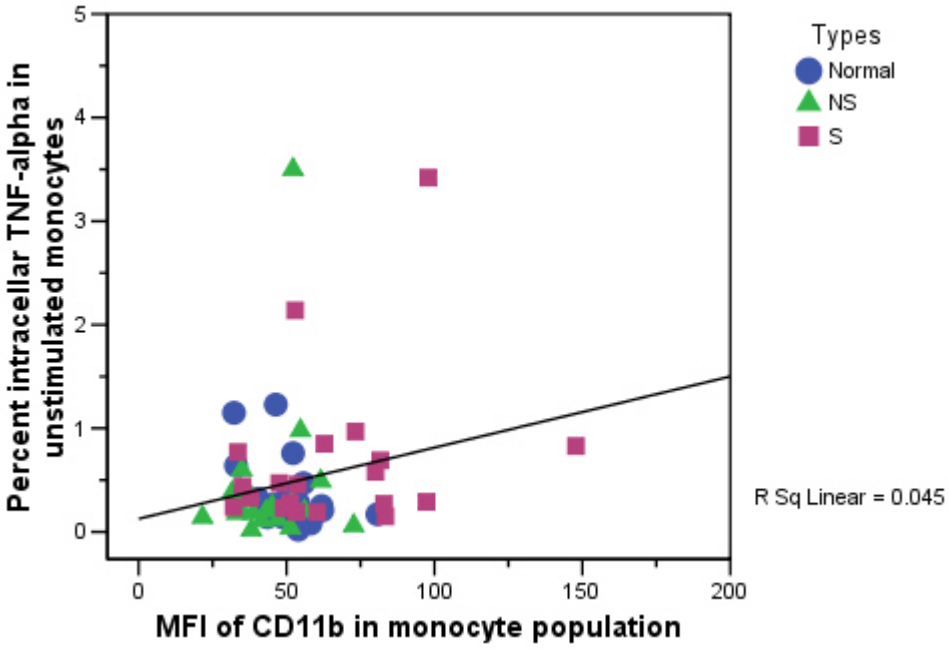

Figure 17d. Correlation between percentage of TNF-α positive in unstimulated monocytes and mean CD11b fluorescence intensity among non-splenectomized Hb E/β-thlassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

6. The percentage of platelet-monocyte aggregates (PMAs).

The percentage of platelet-monocyte aggregates in peripheral blood of Hb E/β-thalassemia patients and normal subjects was determined by detecting the coexpression of CD14 and CD42a and defined as PMAs. As shown in Table 8 and Figure 18, there was no difference in percentage of PMAs among three groups of non-splenectomized, splenectomized patients and normal subjects

Correlation between percentage of PMAs with total leukocytes count , monocyte count and platelet count were also not detected.

Table 8. Mean ± S.D. of percentage of platelet-monocyte aggregates (PMAs) among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

	Normal subjects	Hb E/β-thalassemia	
		NS	S
Percentage of PMAs	20.40±8.49	16.35±6.96*	22.30±21.25*

* = not significantly difference from normal subjects and NS

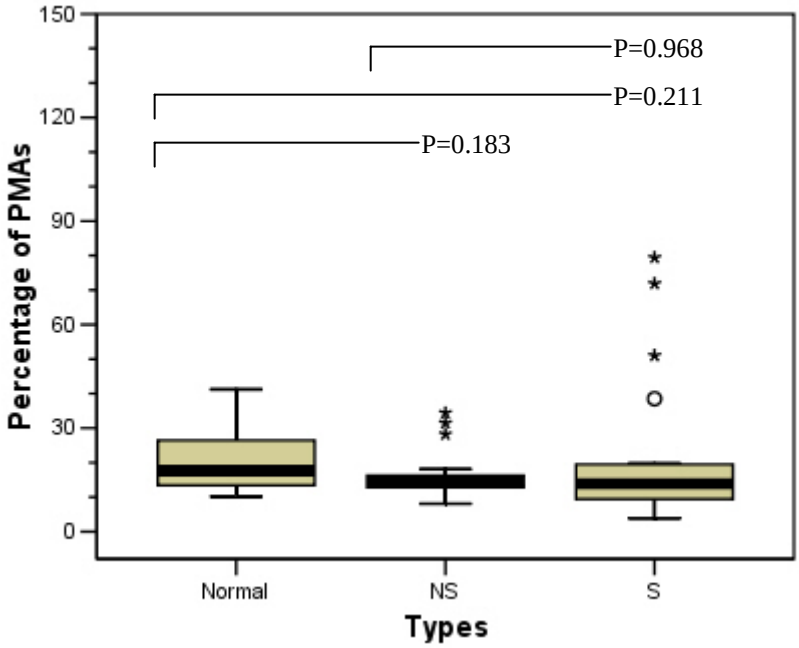

Figure 18. Box plot and statistically significant in PMAs among non-splenectomized Hb E/β-thalassemia (NS), splenectomized Hb E/β-thalassemia (S) and normal subjects.

CHAPTER VI
DISCUSSION

Thalassemias are inherited disorders of hemoglobin synthesis. The patients have various clinical severity caused by an oxidation of excess globin chain on RBCs. Both non-splenectomized and splenectomized Hb E/β-thalassemic patients showed abnormal hematological parameters such as decreasing in RBC numbers, hemoglobin concentration, hematocrit, MCV, MCH and MCHC especially patients with splenectomy. The reason for its severity is because of the excess α-globin chains are highly unstable, rapid precipitates and becomes associated with the membrane of the red blood cell precursors and red cells, causing membrane damage. This damaged RBCs are eliminated by phagocytic cells leading to an ineffective erytropoiesis in bone marrow. Furthermore, ineffective erythrocytes will be eliminated by reticuloendothelial system (RE system) after passage through the spleen and liver, leading to an anemia in β-thalassemic patients (1, 2, 38, 104).

Increase Hb F level, reticulocyte number and NRBC number were observed in Hb E/β-thalassemic patients. High level of Hb F could result from bone marrow expansion and intramedullary selection of F-cell (105). Marked increase in numbers of reticulocyte and NRBC were found in splenectomized patients, which may caused by the release of immature RBCs from the spleen pools (106).

In this study, the percentage of PS-exposing on RBCs was determined. The result supports other previous studies that thalassemic patients have statistically significant increased percentage of PS-exposing RBCs when compared with normal individual, especially in splenectomized Hb E/β-thalassemia patients (5, 20-22). This is because of thalassemic RBCs membrane is damaged by excess globin chains that results in the occurrence of inclusion body and loss of normal asymmetrical distribution of membrane phospholipids. These PS-exposing RBCs can be recognized by macrophages and eliminated by RE system in spleen, which is the one cause of anemia in thalassemia patients. Therefore, removal of spleen could cause a decrease eliminate of these cells

which consequently lead to elevated number of PS exposure RBCs in the circulation of splenectomized Hb E/β-thalassemia patients (5, 20, 76).

An intracellular cytokine assay, unstimulated monocytes were incubated for 4 h in the presence of BFA, which prevented the secretion of any intracellular cytokine that had accumulated in the cytoplasm (107). We showed that circulating unstimulated monocytes from patients with splenectomy trends to produce more TNF-α and IL-1α than monocytes from non- splenectomized patients and normal subjects. These findings are similar to the reports of Belcher et al and Wun et al which have studied in patients with SCD (32, 33). In addition, we showed that splenectomized patients have leukocytosis and monocytosis. Thus, there are greater absolute numbers of circulating monocytes that produce intracellular TNF-α and IL-1α in these patients. Activated monocytes are probably served as a source of the increased serum levels of TNF-α and IL-1α in thalassemic patients by other investigators (36, 42).

It is interesting to note that the proportion of monocytes from splenectomized patients that are able to produce TNF-α and IL-1α in response to LPS *ex vivo* were diminished when compared to monocytes from non-splenectomized patients and normal subjects. In contrast, Wun et al have shown that sickle monocytes were only decreased produced TNF-α in response to LPS. The complication of these finding is controversial, we may suggest that, the continuous activation of monocytes might deplete the capacity of the cells to produce cytokines in response to physiological agonists. Another may caused by the inherently diminished of the synthetic capacity for TNF-α and IL-1α in the monocytes from these patients. Besides, Gordeuk et al have shown a decreased concentration of TNF-α in supernatants of monocytes in response to LPS from homozygous hereditary hemochromatosis patients (108). These data suggested that monocyte and cytokines production may be impaired in chronic iron overload. However, other investigators have demonstrated that monocytes from splenectomized β-thalassemic patients function normally or had minimally reduced phagocytic activity (109-111).

CD11b expression on the circulating monocyte cell surface was measure as an additional marker of activation. Previous studies have shown a higher level of CD11b expression on thalassemic and sickle monocytes (29, 32). In our studies, CD11b fluorescence intensity trends to increase in splenectomized patients when compared

with non-splenectomized patients and normal subjects. This result supports that monocytes from splenectomized Hb E/β-thalassemic patients were activated.

Monocytosis and monocyte activation may be effectd by infection in these patients. Therefore, subclinical infection could be one explanation for our findings. However, none of our patients had clinically obvious infections. There are multiple pathways to describe for monocyte activation. One possibility is that increased erythrophagocytosis of thalassemic RBCs by monocytes stimulates monocyte activation (31, 112, 113) Another pathway could be RBC microparticles; human RBCs shed plasma membrane-derived exocytic microvesicles or microparticles *in vivo*. Increased numbers of RBCs microparticles have been found in several vascular diseases, including SCD and β-thalassemia (114). These microparticles activated inflammatory responses in cultured monocytes/macrophages (J.B.D. and G.M.V., unpublished data) In addition, Sultana et al have recently reported that adherence/contact of sickle RBCs to HUVEC in the presence of von Willebrand-containing media induces oxidant stress, leading to NF-κB nuclear translocation and transendothelial migration of HL 60 and normal peripheral blood monocytes (115). These finding suggested that activated endothelium primes monocytes.

In platelet-monocyte aggregation study, we used coexpression of CD14 and CD42a to define platelet-monocyte aggregation, this technique is similar to the study of Wun et al. The results from the studied showed no difference in the percentage of platelet-monocyte aggregates among splenectomized patients, non-splenectomized patients and normal subjects. Our results are not concordant to the results of Wun et al, which showed a high percentage of platelet-monocyte aggregates in SCD patients. This is because of CD42a is a receptors for von Willebrand factor that is critical for platelet adhesion to damaged blood vessel walls (116). We suggest that SCD patients have more vascular damage than splenectomized Hb E/ β-thalassemic patients. Thus, SCD patients may be have a high level of platelets expressing CD42a, leading to a high level of PMAs. However, other investigators have reported that anti-42a monoclonal antibody (MoAb) could bind to resting platelets and activated platelets (117). Thus, the technique in this study did not allow us to determine the effect of activated platelets and resting platelet on monocyte activation. To solve this problem the other monoclonal antibodies

that bind to activated platelets but not resting platelets such as CD41, CD61 and CD62b should be used to define platelet-monocyte aggregates.

In this study, we found that monocytes are activated in splenectomized Hb E/β-thalassemia patients, wheareas many investigators have reported activated platelets, endothelial cells, circulating cytokines and abnormal presence of adhesion molecules in serum such as ICAM-1, VCAM-1 and E-selectin in thalassemia (14, 29, 35, 36, 42). These results are also indicated of vascular injury in thalassemic patients.

CHAPTER VII
CONCLUSION

Thalassemia is a hereditary hemolytic anemia, resulting from absent or decreased production of (usually) one of the globin chains of hemoglobin. One of the complication of thalassemia is thromboembolism, especially after splenectomy. Previous studies have shown hypercoagulable state, involving PS-exposing RBCs, coagulation factors, coagulation inhibitors; and activation of platelet, monocytes, granulocytes and endothelial cells to be contributing factors. Normally, PS-exposing RBCs were recognized and eliminated by macrophage in spleen. Thus, senescent RBCs in non-splenectomized patients and normal subjects were recognized and damaged by macrophages in spleen. In contrast, in splenectomized patients, the cells that seem to be involved in the phagocytosis are monocytes. Thus, we hypothesized that senescent RBCs were erythrophagocytosed by monocytes in circulation, leading to stimulate monocyte activation. In addition, the binding of activated platelets to monocytes or platelet-monocyte aggregates may be another cause of monocytes activation. Therefore, we may be suggest that the monocytes are activated in splenectomized Hb E/ β-thalassemic patients.

Our studies shown that monocytes of splenectomized Hb E/ β-thalassemia patients are activated, as defined by production of intracellular cytokines. Splenectomized patients show an increase of the percentage and absolute numbers of monocytes expressing TNF-α and IL-1α when compared to non-splenectomized patients and normal subjects. In addition, a slight increase CD11b fluorescence intensity was also found in these patients. This result supports that monocytes from splenectomized Hb E/β-thalassemic patients were activated. Both non-splenectomized and splenectomized patients show a higher level of annexin V-exposing RBCs than normal subjects, especially patients with splenectomy. The correlation between percentage of annexin V-positive RBCs of Hb E/β-thalassemic patients and their hemoglobin concentration or hematocrit were found ($r^2 = 0.111$, $p<0.05$ and $r^2 = 0.157$,

$p<0.01$). This result suggests that the level of PS-exposing RBCs in Hb E/β-thalassemic patients seem to be related with the severity of anemia. In PMAs study, there was no difference in the percentage of PMAs among non-splenectomized, splenectomized and normal subjects. There was no correlation between the percentage of annexin V-exposing RBCs and the levels of monocytes expressing IL-1α or CD11b fluorescence intensity. However, a slight statistical correlation between the percentage of annexin V-exposing RBCs and the level of monocytes expressing TNF-α was found ($r^2 = 0.114$, $p<0.01$). This result suggests that monocytes are activated in splenectomized Hb E/β-thalassemic patients, and not related to PMAs, but may relate to PS-exposing RBCs.

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