THE MECHANISM OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA CLONE DOMINANT: THE ROLE OF APOPTOSIS OF NON-MUTATED CELLS IN THE EXPANSION OF MUTATED CELLS

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder with various clinical manifestations including intravascular hemolysis, deficient hematopoiesis, and thrombosis. The PIG-A gene encodes a protein essential in the early step of glycosylphosphatidylinositol (GPI) biosynthesis. The mutation of the PIG-A gene results in a deficiency of GPI-linked membrane proteins in various hematopoietic cells, especially CD55 (decay accelerating factor; DAF) and CD59 (membrane inhibitor of reactive lysis; MIRL) which protect blood cells from complement mediated lysis. The mechanism by which a PNH clone expand is the major current concern. This study investigates how PNH cells deficient in GPI-linked proteins expand and contribute substantially to hematopoiesis in PNH patients by considering the hypothesis that the autoimmune process selects and destroys non-mutated cells.

Peripheral blood samples from 21 PNH patients and 35 healthy volunteers were subjected to apoptosis assay, analysis of the number of $CD8^+$ T cells, NKT cells, and NK cells, and hemopoietic progenitor cell assay.

In PNH patients, CD59(+) granulocytes showed more apoptosis than CD59(-) granulocytes. Mononuclear cells (MNCs) were shown to affect the apoptosis of CD59(+) granulocytes as indicated by the significant increase in apoptosis of CD59(+) granulocytes compared with CD59(-) granulocytes in a culture conditioned with MNCs after 4 hours. The sensitivity to autologous MNCs of CD59(+) granulocytes from PNH patients was higher than that to CD59(+) granulocytes from normal controls. The analysis of the number of $CD8^+$ T cells, NKT cells, and NK cells in PNH patients showed no significant increase of these cells. With hemopoietic progenitor cell assay, the $CD8^+$ T cells were shown to be possibly responsible for the destruction of hematopoietic stem cells in PNH patients as demonstrated by the significant growth inhibition in the CFU-GM (but not in BFU-E).

The greater apoptosis of CD59(+) granulocytes and hemopoietic inhibition by the $CD8^+$ T cells in PNH patients suggests the sensitivity to the destruction of non-mutated cells and growth disadvantage of PNH stem cells. In addition, these findings indicate the possible role of autoreactive lymphocytes in the pathogenesis of PNH.

KEY WORDS : APOPTOSIS / AUTOIMMUNE / HEMATOPOIETIC DEFECT / PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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การศึกษากลไกการขยายตัวของ paroxysmal nocturnal hemoglobinuria clone: บทบาทของ apoptosis ของเซลล์ปรกติต่อการขยายตัวของเซลล์ PNH (THE MECHANISM OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA CLONE DOMINANT: THE ROLE OF APOPTOSIS OF NON-MUTATED CELLS IN THE EXPANSION OF MUTATED CELLS)

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บทคัดย่อ

Paroxysmal nocturnal hemoglobinuria (PNH) เป็นโรคที่มีความผิดปรกติของ hematopoietic stem cell โดยมีลักษณะทางคลินิกที่สำคัญคือ เม็ดเลือดแดงแตกในกระแสเลือด (intravascular hemolysis) ความบกพร่องในการผลิตเม็ดเลือด (deficiency hematopoiesis) และปัญหาลิ่มเลือดอุดตัน (thrombosis) PIG-A gene มีความสำคัญเกี่ยวข้องในขั้นตอนแรกของการสังเคราะห์ glycosylphosphatidylinositol (GPI) ดังนั้นการเกิด mutation ของ PIG-A gene จึงเป็นผลทำให้เกิดการขาดหายไปของ membrane proteins บนผิวเซลล์ของ hematopoietic cells โดยเฉพาะอย่างยิ่ง การขาดหายไปของ CD55 (decay accelerating factor; DAF) และ CD59 (membrane inhibition of reactive lysis; MIRL) ซึ่งมีความสำคัญในการป้องกันการแตกของเซลล์เม็ดเลือดจากการกระตุ้นของ complement กลไกการขยายตัวของ PNH clone เป็นประเด็นที่น่าสนใจในปัจจุบัน การศึกษาครั้งนี้จึงได้ทำการทดลองเพื่อหาสาเหตุว่าเหตุใด PNH cells จึงสามารถขยายตัวและทดแทนการผลิตเม็ดเลือดในผู้ป่วย PNH ได้ โดยศึกษาอิงทฤษฎีการเกิด autoimmune ในผู้ป่วย

กลุ่มศึกษาประกอบด้วยผู้ป่วย PNH จำนวน 21 ราย และคนปรกติจำนวน 35 ราย เลือดของทั้งผู้ป่วยและคนปรกติจะถูกนำไปวิเคราะห์การเกิด apoptosis ในเม็ดเลือดขาวชนิด granulocyte การวิเคราะห์จำนวน $CD8^+$ T cells NKT cells และ NK cells และการทดลองโดยการทำการเพาะเลี้ยง hemopoietic progenitor cells

จากการศึกษาในผู้ป่วย PNH พบว่า granulocyte ปรกติ (CD59+ granulocytes) มีการเกิด apoptosis มากกว่า PNH granulocytes (CD59- granulocytes) โดย mononuclear cell (MNCs) อาจมีผลต่อการเกิด apoptosis ของ CD59(+) granulocytes นี้ เนื่องจากพบการเพิ่มขึ้นของการเกิด apoptosis ของ CD59(+) granulocytes อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับการเกิด apoptosis ของ CD59(-) granulocytes หลังจากเลี้ยงเซลล์ในสภาวะที่มี MNCs เป็นระยะเวลา 4 ชั่วโมง นอกจากนี้ยังพบว่า CD59(+) granulocytes ของผู้ป่วย PNH มีการเกิด apoptosis สูงกว่า CD59(+) granulocytes ของคนปรกติ ในการวิเคราะห์จำนวนของ $CD8^+$ T cells NKT cells และ NK cells ไม่พบการเพิ่มขึ้นของเซลล์ทั้งสามชนิดในผู้ป่วย PNH และการทดลองเพาะเลี้ยง hemopoietic progenitor cells พบว่า $CD8^+$ T cells อาจมีผลต่อการทำลาย hematopoietic stem cells โดยพบการยับยั้งการเจริญของ CFU-GM แต่ไม่พบการยับยั้งการเจริญของ BFU-E

การพบการเกิด apoptosis มากใน CD59(+) granulocytes และความบกพร่องของการสร้างเม็ดเลือดในผู้ป่วย PNH แสดงให้เห็นว่าเซลล์ปรกติ (non-mutated cells) ของผู้ป่วย PNH มีแนวโน้มที่จะถูกทำลาย และแสดงให้เห็นว่า PNH stem cells เองไม่มีข้อได้เปรียบในการเจริญเติบโต การศึกษาครั้งนี้พบความเป็นไปได้ของ autoreactive lymphocytes ที่อาจเป็นสาเหตุให้เกิด PNH ได้

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CONTENTS

Pages

CONTENTS (cont.)

Pages

CONTENTS (cont.)

Pages

LIST OF TABLES

LIST OF TABLES (cont.)

Page

LIST OF FIGURES

Page

LIST OF FIGURES (Cont.)

Page

LIST OF FIGURES (Cont.)

Page

LIST OF ABBREVIATIONS

Abbreviations	Term
AA	aplastic anemia
AAA	acquired aplastic anemia
Ab	antibody
Ag	antigen
AML	acute myelogenous leukemia
ATG	antithymocyte globulin
BFU-E	burst-forming unit-Erythroid
BM	bone marrow
CAFC	cobblestone Area Forming-Cells
CB	cord blood
cDNA	complementar deoxyribonucleic acid
CDR	complementarity-determining-region
CFC	colony-forming cell
CFU-GEMM	colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	colony forming unit-granulocyte-macrophage
CFU-Mk	colony-forming unit-granulocyte – megakaryocyte
CRU	competitive repopulating unit
CTL	cytotoxic T lymphocyte
DAF	decay accelerating factor
DNA	deoxyribonucleic acid
Dol-P-Man	dolichol-phosphate-mannose
DPM	dolichol-phosphate-mannose
EAP	ethanolamine phosphate

LIST OF ABBREVIATIONS (cont.)

Abbreviations	Term
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FCS	fetal calf serum
g	gram
GlcNAc	N-acetylglucosamine
GlcN-PI	glucosaminyl- phosphatidylinositol
GPI	glycosylphosphatidylinositol
GPI-AP	glycosylphosphatidylinositol anchored Protein
GPI-GnT	glycosylphosphatidylinositol -N-acetylglucosaminyltransferase
Hb	hemoglobin
HLA	human leukocyte antigen
HPP-CFC	high Proliferative potential colony-Forming
HSC	hematopoietic stem cell
IFN	interferon
LTC-IC	long-Term Culture-Initiating Cells
MAC	membrane attack complex
MDS	myelodysplastic syndrome
MIRL	membrane inhibitor of reactive lysis
MNC	mononuclear cell
MPB	mobilized peripheral blood
mg	milligram
ml	millilitre
MoAb	monoclonal antibody
mRNA	messenger ribonucleic acid

LIST OF ABBREVIATIONS (cont.)

Abbreviations	Term
µl	microlitre
MHC	major histocompatibility complex
NK	natural killer
NKT	natural killer T
PBS	phosphase buffer saline
PCD	programmed cell death
PI	phosphatidylinositol
PIG-A	phosphatidylinositol glycan A
PIPLC	phosphatidylinositol phospholipase C
Plt	platelet
PMN	polymorphonuclear cell
PNH	Paroxysmal nocturnal hemoglobinuria
PS	phosphatidylserine
RT	room temperature
TCR	T cell receptor
T-LGL	T-cell large granular lymphocyte
TNF	tumor necrosis factor
UDP-GlcNAc	uridinediphosphate-N-acetylglucosamine
uPAR	urokinase plasminogen activator receptor
UPN	unique patient number
WBC	white blood cell
ΔΨm	mitochondrial transmembrane potential

CHAPTER I
INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder characterized by the unique triad of intravascular hemolysis, bone marrow failure, and thrombosis. PNH occurs as a consequence of somatic mutation of an X-linked gene, PIG-A, in a hematopoietic stem cell. PIG-A gene encodes a protein subunit of the a-1-6-N acetylglucosaminyltransferase complex, which is essential in the early step of glycosylphosphatidylinositol (GPI) biosynthesis, a transfer of N-acetylglucosamine from uridinediphosphaste-N-acetylglucosamine to phosphatidylinositol (3). Because GPI acts as a membrane anchor for many cell surface proteins, a somatic mutation of PIG-A gene results in the loss of several GPI-anchored proteins (GPI-APs) from the blood cell surface. The functions of these proteins are extremely varied, two of them, CD55 and CD59 are complement regulatory proteins.

CD55 (decay accelerating factor, DAF) inhibits the assembly of C3 and C5 convertase of the classical and alternative pathways, thereby regulating the complement cascade at the C3 step, while CD59 (membrane inhibitor of reactive lysis, MIRL) limits the polymerization of C9 in membrane C5b-9 complex. Thus the deficiency of these two GPI-APs leading to the clinical feature of hemoglobinuria.

Since PIG-A is located on the X-chromosome. Therefore, an inactivating mutation (one hit) will cause a loss of PIG-A function in hematopoietic cells either from male or female, because of X-inactivation in female.

PNH occurs more frequently in some Asian countries, such as Thailand, China, and Japan. It is very interesting not only that we know about the molecular biology of the disease, but also the questions of why and how PNH clones expand and contribute substantially to hematopoiesis in patients. There are two hypotheses that may explain this expansion phenomenon (4, 5).

The first hypothesis emphasizes on dominant proliferation of the PNH clone. The genetic change(s) may confer PIG-A mutant stem cells with an intrinsic growth advantage and enable this PIG-A mutant cell clone to expand similar to benign tumor. However, many experimental data did not support this hypothesis, especially *PIG-A*-mutant granulocytes are found at low frequency in most healthy people (6-8). Thus, only PIG-A mutation is not enough to support the outgrowth of PNH clone, there should be other factor(s) that mediate and select PNH clone to expand.

The second hypothesis suggests some pathological conditions that select or favor PIG-A mutant stem cells to expand. From the previous hypothesis that explain no intrinsic growth advantage in the proliferation of PNH clone, therefore, there must be and additional events occur that favor the growth of PNH clone. One of these events or factors may be the affected environment due to an autoimmune process. Since there is close association between PNH and other bone marrow (BM) failure disease with autoimmune process, such as aplastic anemia (AA) and myelodysplastic syndrome (MDS) (9-13). Thus the same mechanism may also mediate in PNH. The possibility is that autoreactive lymphocytes target and destroy stem cells via GPI-APs on the surface of the cells or GPI-anchor itself. If both GPI-APs and GPI-anchor that present on GPI-positive cells, thus GPI-negative cells would not be recognized and leading to the expansion of these PNH clones over the non-PNH counterparts.

Apart form BM transplantation, there has been no effective treatment for PNH. Therefore, trying to understand the (complicated) mechanism of the disease will become the most usefulness for patients. From the latter possibly hypothesis on the notion about the effect of autoreactive immunity on hematopoietic stem cells, many experiments demonstrated the increase of T-cell repertoire and the destruction of non-mutated cells, but there has been limited studies of co-culture of these T-cells and normal cells to see the direct effect of this immune attack. In order to prove this immune attack hypothesis, the whole laboratory design will be conducted by co-culture system in both liquid and semi-solid media. In liquid culture, co-culture between granulocytes (representative for normal and PNH cells) and mononuclear cells (MNCs) from the same individual for 0 and 4 hour and then perform apoptosis analysis by using flow cytometry to investigate the cell population in the destruction of GPI(+) and GPI(-) cells, will be done. The reasons for choosing the population of

granulocytes in the blood circulation to do the experiment depend on enough quantity and the easy assessment of the target cells. Moreover, lifespan of the PNH granulocytes is normal compared with the PNH erythrocytes, which are selectively lysed in the circulation by the lytic action of complement, whereas the abnormal granulocytes are not. Finally, analysis of granulocytes is unaffected by transfusion (14, 15). Thus, the pattern and proportion of abnormal granulocytes that is not affected by the cytolytic action of complements, can closely reflect current hematopoiesis by mutated PNH stem cells in the bone marrow. In addition, the MNCs fraction will be analysed the percentages of cell populations that may be involved in the causation of the disease (cytotoxic T lymphocytes and natural killer T cells) by flow cytometry. In semi-solid culture, co-culture between purified $CD8^+$ T cells and MNCs deprived $CD8^+$ T cells (represent $CD34^+$ cells) will be compared with the culture of purified $CD8^+$ T cells alone from the same individual. The purpose of this study is to test the hypothesis assumption and hope that it will lead to a better understanding the causative factor underlying the pathogenesis of PNH.

CHAPTER II
OBJECTIVES

To investigate how cells deficient in GPI-linked proteins (PNH cells) expand and contribute substantially to hematopoiesis in PNH patients.

CHAPTER III
LITERATURE REVIEW

1. Paroxysmal nocturnal hemoglobinuria (PNH)

1.1 PNH: The hematopoietic stem cell (HSC) disorder

PNH was firstly described as a discrete clinical entity in 1882 by Crosby (16), before the recognition that the red cells in PNH had an increased sensitivity to lysis in acidify serum. PNH is characterized as an acquired hematologic disorder in which subpopulations of peripheral blood cells especially erythrocytes are more susceptible to the lytic action of activated complement. Hemolysis is the cardinal feature. It is classically paroxysmal and most apparent in the first urine passed on waking in the morning, hence the name of the disease. It is now clear that this abnormal sensitivity is caused by the lack of two important, glycosylphosphatidylinositol (GPI)-linked cell surface complement regulatory proteins CD55 (Decay accelerating factor, DAF) and CD59 (membrane inhibitor of reactive lysis, MIRL) (Figure 1). Other GPI-linked membrane proteins (Table 2) are also deficient in PNH blood cells. A GPI-anchor deficient blood cell population has occurred initially in a $CD34^+$ stem cells from a patient with PNH, indicate that clonal dominance operates at the stem cell level leading to the deficiency of GPI-anchor in all blood cell lineages (2, 17-22).

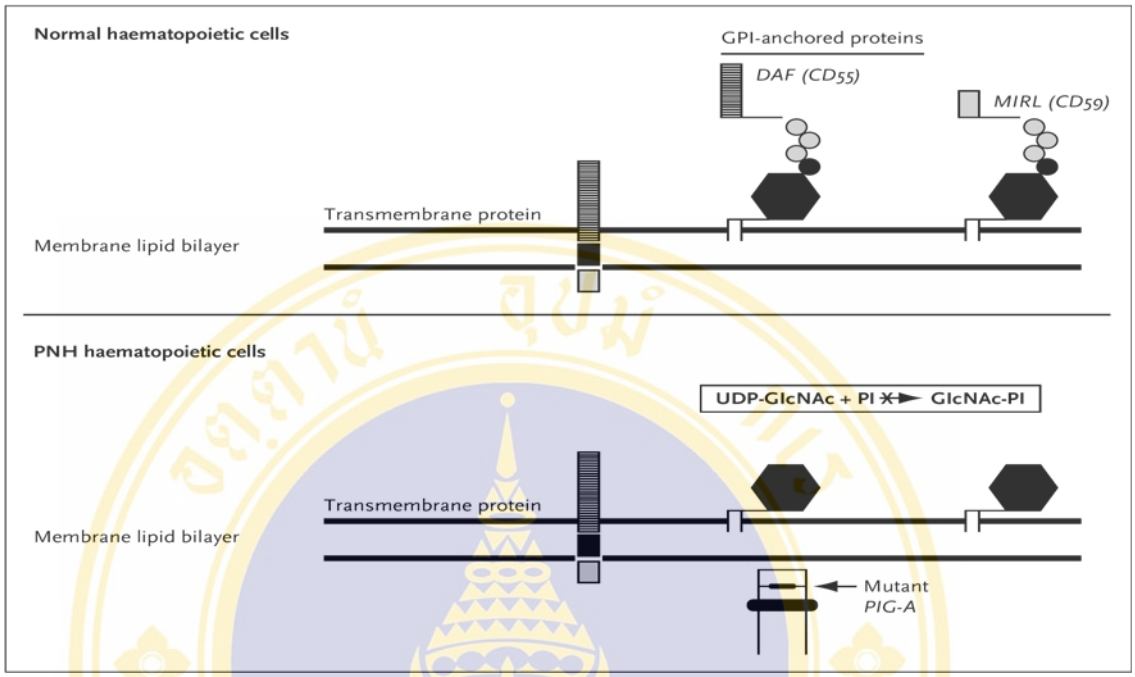


Figure 1 Normal hematopoietic cells and PNH hematopoietic cells (21).

1.2 Clinical features

1.2.1 Hemolysis

Most PNH patients have bouts of dark urine caused by the excretion of hemoglobin derived from the intravascular destruction of red cells. The chronic hemolysis with occasional crisis is caused by the activation of complement resulting in increased complement mediated lysis of erythrocytes.

Complement is the principal effector of the innate immune system. Complement activation is a complex cascade leading to the production of anaphylatoxins, chemotaxins and membrane attack complex (MAC). Three pathways activate the cascade: the classical (initiated by antigen/antibody complex), alternative (initiated by microbial membranes or immune complexes), and lectin pathways. Two of these complement pathways will be in brief described: the classical pathway and the alternative pathway (Figure 2). In the activation of the classical pathway, a molecule of C1q, two molecules of C1r, two molecules of C1s and Ca^{2+} form the C1 esterase unit, which recognizes the initial activators such as antigen-antibody (Ag-Ab) complexes. The binding of C1q to the Fc portion of IgG or IgM complexed with antigens, activates the C1 esterase unit. That is, the binding induces a conformational change that leads to the activation of C1r, resulting in its proteolytic activation of C1s. Next, activated protease C1s cleaves both C4 and C2 to yield C4b2a, which acts as a C3 convertase in the presence of Mg^{2+}. The complex cleaves C3 and produces C3b, which attaches covalently to the adjacent cell membrane. The new complex, C4b2a3b, serves as C5 convertase. The alternative pathway, on the other hand, can be activated (even in the absence of antibodies) by polysaccharides, bacterial products, rabbit erythrocytes, aggregated human IgA, and cobra venom factor. This pathway begins with the activation of C3 in the presence of Mg^{2+} by nonspecific spontaneous proteolysis or by proteolysis associated with the activation of the classical pathway. A proteolytic product, C3b, binds to the membrane and binds with factor B. Cleavage of factor B with the active protease factor D forms C3bBb, which is a C3 convertase. Similar to the formation of C4b2a3b (a C5 convertase) in the classical pathway, the addition of C3b to the C3 convertase forms C3bBb3b, which is a C5 convertase in the alternative pathway. Subsequently, the C5 convertases (C4b2aBb and C3bBb3b) cleave C5 to C5a and C5b. The serial binding of C6, C7, and C8 forms a late

complement complex, C5b-8, which has weak cytolytic activity. The most important role of the C5b-8 complex is considered to be the bond of C9 and the formation of C5b-9. This C5b-9 induces the polymerization of C9, with the resultant yield of membrane attack complex (MAC, C5b-8[C9]n). MAC cytolytically damages the biomembranes of cells and microbes.

In PNH the abnormal sensitivity to complement activation is a consequence of combined deficiency of complement control proteins, CD55 and CD59. CD55 (decay accelerating factor, DAF), a glycoprotein of about 70 kd, with function in the disruption of the formation of, or in accelerating the decay of complement complexes (C4b2a, C4b2aBb, C3bBb, and C3bBb3b) with C3/C5 convertase activity in both the classical and alternative pathways. CD59 (membrane inhibitor of reactive lysis, MIRL), a protein of 18 kd that normally controls the formation of the membrane attack complex (C5b-9) by inhibiting the binding of C9 to C5b-8 leading to blocking C9 polymerization in either the classical or alternative pathway (23-25).

Considering the hemolysis of PNH, deficiency of CD59 is more relevant than deficiency of CD55, as indicated by the PNH III cells, which are most sensitive to complement mediated lysis, completely lack CD59 and are totally or partially deficient in CD55. In contrast, PNH II cells have low level of CD59 and varying levels of CD55 (26). In addition, inherited homozygous CD59 deficiency was shown to cause a PNH-like disease, while inherited CD55 deficiency has not been observed to be associated with hemolysis.

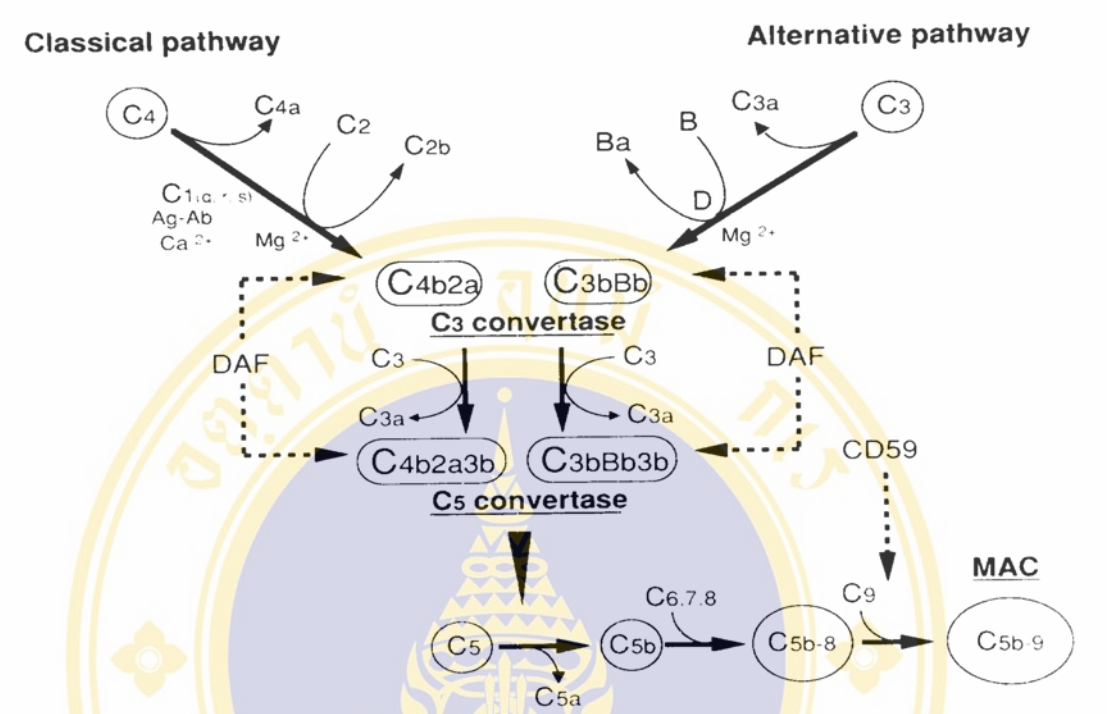


Figure 2 Complement activation pathways and the regulation sites of the complement regulatory GPI-anchored membrane proteins CD55 and CD59. CD55 inhibits the formation of, or accelerates the decay of, complement complexes with C3/C5 convertase activity, whereas CD59 blocks C9 polymerization by inhibiting the binding of C9 to C5b-8. MAC, membrane attack complex; Ag-Ab immune complex (23).

1.2.2 Thrombosis

The most serious complication of PNH is venous thrombosis. The risk or prevalence of thrombosis is greater in patients from Europe and the United States than in those from the Far East (27).

Much less is known about the mechanism that underlies the thrombophilia of PNH. The pathogenesis of venous thrombosis in PNH is probably related to platelet deficiency of CD55 and CD59. Complement-induced platelet lysis is minimized by the release from the cell surface of excess membrane attack complex (MAC) through exovesiculation. The resulting microparticles are rich in phospholipids and act as a site of generation of factor Va, factor Xa and the prothrombinase complex (28, 29). The activation of complement also mediates damage to leukocytes leading to increased leukocyte-derived tissue factor levels in plasma, which in turn may trigger thrombosis. In addition, there is some suggesting evidence that PNH platelets are activated and, therefore sensitive to thrombin-induced aggregation thrombin. The absence of urokinase plasminogen activator receptor (uPAR), one kind of GPI-APs, on PNH cells is possibly relevant to thrombosis in PNH. Its role is to bind urokinase to the surface of monocytes and granulocytes, converting plasminogen to plasmin and initiating fibrinolysis (30). Recent clinical studies support the hypothesis that the probability of a thrombotic event is directly related to the size of the PNH clone (31-33), for example one study showed a predictive thrombotic risk in patients who were found to have PNH granulocyte clone sizes larger than 50% when compared with patients with smaller clone sizes (33).

1.2.3 Association between PNH and other hematological clonal disorders

1.2.3.1 PNH associated with Aplastic anemia (AA) and myelodysplastic syndrome (MDS)

The relationship between marrow hypoplasia and PNH was firstly recognized by Dacie and Lewis (3). AA and PNH are clinically related syndromes that share a number of common biological features such as stem cells from patients with AA and PNH produce significantly lower in vitro CFU-Meg formation compared with normal donors, and have the same apoptosis resistance (34, 35).

Elevated levels of circulating microparticles, mostly from platelets, have been detected in both *de novo* PNH and AA patients with a PNH clone, but not in those with AA without a PNH clone (18, 28). During the course of PNH, pancytopenia is common and about one third of the patients will die from bone marrow (BM) failure. A minor population of blood cells deficient of GPI-anchored membrane proteins is often detected in patients with AA, in a proportion that varies among different studies between 10% and 55% (9-11, 13, 36, 37), and such PNH clones have also been found in a small percentage between 10% and 27.7% of patients with MDS (11-13, 18, 37-40). Even before the widespread use of immunosuppressive therapy, 5% of patients with aplastic anemia showed progression to clonal hemopoiesis. This evidence suggests that the increase in myelodysplastic syndrome and paroxysmal nocturnal hemoglobinuria after immunosuppressive therapy is not a direct consequence of the treatment. Instead, the longer survival after immunosuppressive therapy probably allows time for these underlying clones to develop and expand (10). PNH patients usually have an underlying aplastic process and the occurrence of BM failure during the course of PNH is a bad prognostic sign. It is suggested that PNH clones may have a relative growth or survival advantage over the residual non-PNH hematopoiesis in patients who have aplastic anemia. It also implies that although the mutation leading to the development of PNH occurs quite frequently, but in the absence of marrow hypoplasia, the clone has difficulty in establishing itself.

1.2.3.1.1 Aplastic anemia (AA)

AA is a potentially life-threatening failure of hemopoiesis characterized by pancytopenia and bone-marrow aplasia. Causes of acquired aplastic anemia (AAA) are very diverse, among the common ones are associated with many agents, including drugs, benzene exposure, insecticides, viruses, and radiation that directly cytotoxic to the BM. A population-based case-control study of aplastic anemia in Thailand found that drugs were among the commonly implicated cause, but they have recently explained only 5% of the newly diagnosed cases (41). AA is an uncommon disease with a reported incidence of approximately 2 per 1 x 10^6 annually in Europe. In Asia, it occurs three times more often with an incidence of 5 to 7 per 1 x 10^6 annually (5).

The pathophysiology of AA is believed to be immune-mediated, with active destruction of blood-forming cells by T-lymphocytes. As in other autoimmune diseases, an association with certain HLA alleles has been described in patients with AA. The over-expression of HLA-DR2 and HLA-DR15 (42-44) and the response to immunosuppressive therapy are found in these patients (9). Autoreactive T lymphocytes are thought to play an important role in the pathogenesis of AA. Studies of T-cell diversity by use of complementarity-determining-region (CDR3) spectratyping further implicated an autoimmune pathophysiology in AA. T cells from patients with the disorder showed limited heterogeneity of the T-cell-receptor β chain, which suggests the oligoclonal T-cell expansion in response to a specific antigen (45-49). Cytotoxic T lymphocytes were found to mediate the destruction of hemopoietic stem cells in the disorder (45, 50-52) by produce cytokines such as interferon (IFN)–γ and tumor necrosis factor (TNF) and increased expression of these cytokines has been shown in the bone marrow of the patients (53-55). These cytokines are direct inhibitors of hemopoiesis and seem to upregulate Fas expression on CD34-positive cells (51, 56). Fas ligand (FasL), which can be found on activated T lymphocytes, can subsequently induce apoptosis of these Fas-expressing stem cells, cause BM injury and hypoplastic stress, while BM microenvironment is not defective (57, 58).

1.2.3.2.2 Myelodysplastic syndrome (MDS)

MDS is a clonal hemopoietic stem-cell disorder characterized by multilineage hematological cytopenias and subsequently by frequent development of acute myelogenous leukemia (AML). Peripheral blood cytopenias in combination with a hypercellular bone marrow exhibiting dysplastic changes are the hallmarks of MDS. It is usually associated with heterogeneous karyotypic abnormalities, commonly involving chromosome 5, 7, or 8. Up to 15% of children and adults with AAA develop MDS after immunosuppressive therapy; monosomy 7 is the most common chromosomal abnormality (59). Precise pathogenesis of MDS is as yet unknown, but it has been generally suggested that MDS arises from a hemopoietic stem cell harboring irreversible DNA damage. While MDS is a distinct pathophysiological entity from AA, hypoplastic MDS, which is characterized by cytopenias, bone marrow dysplasia, and marrow hypocellularity, has been difficult to

distinguish from AA. The pathophysiology of the cytopenias associated with marrow failure in the two conditions, specifically the T-cell mediated immune suppression of hemopoiesis, may be similar or even identical. MDS shares some of the features of acquired AA, a disease with an established autoimmune pedigree. Both in AA and MDS, plasma TNF-α and IFN-γ levels are high and T-cell-mediated myelosuppression occurs (51, 60). Over-expressions of HLA-DR2 and HLA-DR15 are also found in MDS (44). Abnormal CD4 : CD8 ratios, increased activated cytotoxic T cells, as demonstrated by a higher percentage of $CD8^+$ $CD28^-$ and $CD8^+$ $CD28^-CD57^+$ cells, and skewing of the TCR VB CDR3 patterns have been detected in both (61, 62) diseases suggesting an autoimmune T-cell-mediated myelosuppression in both. MDS patients exhibit high percentages of $CD8^+$, $CD28^-$ $CD57^+$ cells, a phenotype characteristic of high cytotoxic cells (CTL), consistent with mature effectors' function (63, 64). The hematological response of some pancytopenic MDS patients to antithymocyte globulin (ATG), a polyclonal antibody with direct and indirect lymphocytotoxicity, led to the possibility that pancytopenia in MDS may be in part lymphocyte mediated (65, 66). A study of patients who had been treated with ATG, demonstrated that removal of $CD3^+$ or $CD8^+$ T cells from bone-marrow progenitor cultures resulted in a significant increase in CFU-GMs in responders to ATG, but not in nonresponders (67).

Like in AA, autoreactive and clonal-involved T-cells are believed to suppress normal hematopoietic cells in MDS by secretion of inhibitory cytokines and mediated apoptosis of hematopoietic cells. Several independent groups have reported elevated levels of TNF-α mRNA and protein in the bone marrow of MDS patients, correlating with increased rates of apoptosis (68-71). Different cytokines have been shown to up-regulate Fas expression on $CD34^+$ cells, including TNF-α and IFN-γ (69). As Fas is overexpressed on MDS $CD34^+$, it has been hypothesized that TNF-α prime bone marrow $CD34^+$ cells for Fas-induced apoptosis, suggesting a Fas–Fas ligand (FasL) interaction as a possible pathogenetic mechanism contributing to immune destruction of $CD34^+$ cells in human myelodysplasia (72). Most studies propose that apoptosis is more prominent in early stages of the disease than in advanced stages during which apoptotic indices appear to be decreased (73, 74). There is now much evidence that increased hemopoietic progenitor cell apoptosis

contributes to the ineffective hemopoiesis and peripheral cytopenias, at least in the earliest stages of MDS. Apoptosis of the MDS stromal cells has been demonstrated, along with their decreased capacity to support normal hemopoiesis. Myelodysplastic syndrome-derived stromal cells were found to induce apoptosis of normal $CD34^+$ cells, while long-term bone marrow cultures of MDS patients are characterized by decreased or absent stromal growth (75). Apoptosis was significantly increased within the $CD34^+$ cells in early MDS patients. Studies in which apoptosis was assessed by annexin V binding came up with analogous results. The numbers of apoptotic $CD34^+$ cells appeared to inversely correlate with the prognostic stage, suggesting that patients with evidence of increased apoptosis may have better outcomes (76). Moreover, both *in vivo* and *in vitro* studies have demonstrated that the Fas-FasL system performs a critical function in producing apoptosis in several organ systems after triggering of Fas by FasL (77).

1.2.3.2 PNH and leukemia

In a large cohort study of natural history of PNH, leukemia did not develop in any of studied patients and only approximately 5% of patients with AA who survive the marrow aplasia develop AML (27). Thus, AA may predispose to clonal hematopoietic disorders, such as AML, PNH and MDS, but the development of a PNH clone does not increase the risk of AML/MDS, therefore, the GPI-deficient phenotype is not preleukemic. In addition, a study of cytogenetic and morphological abnormalities in PNH patients demonstrated that none of PNH patients developed excess blasts and patients with cytogenetically abnormal clones are not necessarily malignant and may not be predictive of evolution to leukemia (78).

1.3 The diagnosis of PNH

Prior to the availability of flow cytometry, PNH patients were screened with the majority characteristic of complement-mediated hemolytic feature. The Ham's, sucrose lysis, and modified Ham's tests rely on the differential sensitivity of PNH red cells to complement. Although these tests such as Ham's test, are suitable for hemolytic PNH, may give falsely positive in the rare disorder hereditary erythrocytic multinuclearity with a positive acidified-serum lysis. In addition, these assays are not

sensitive enough to detect small PNH population and do not quantitate the size of PNH clone. Moreover, the low specificity and the relatively high variability due to the different lysing potency of the used human sera, have reduced the interest in these methods, and due to the effect of transfusion and the shortened life span of PNH red cells, these tests may give falsely negative in patients requiring regular red cell transfusions (31, 79-82).

Flow cytometry with the use of monoclonal antibodies has rapidly established to be a reliable diagnostic procedure for PNH and for measuring the extent of the PNH clone within the various hematopoietic cell lineages. As a consequence, flow cytometry has now replaced the Ham's test as the "gold standard" technique for the diagnosis of PNH.

Flow cytometric analysis using antibodies directed against GPI-AP is the most sensitive and informative assay available for diagnosis of PNH. For initial studies, quantitation of at least two GPI-APs is recommended to exclude the possibility that the clinical process is consequence of an inherited, isolated deficiency of a single GPI-AP. Flow cytometric analysis can identify a population of GPI-AP deficient cells, in addition with the capacity to determine the percentage of cells that are abnormal and identify discrete populations with different degrees of deficiency that separate into PNH type III cells with complete deficiency of GPI-APs, PNH type II cells with partial deficiency of GPI-APs, and PNH type I cells with normal expression of GPI-APs (Figure 3) (32, 83).

It is clear from several studies that the proportion of GPI-deficient neutrophils, as measured by flow cytometry, gives a more accurate representation of the size of the PNH clone and reflects the bone marrow situation more accurately because of normal lifespan and unaffected by red cell transfusion (4). In addition if this flow cytometric technique is applied to other hematopoietic cell lineages, GPI deficiency can be documented on platelets, monocytes, and lymphocytes, confirming the stem cell nature of the disorder (14, 15).

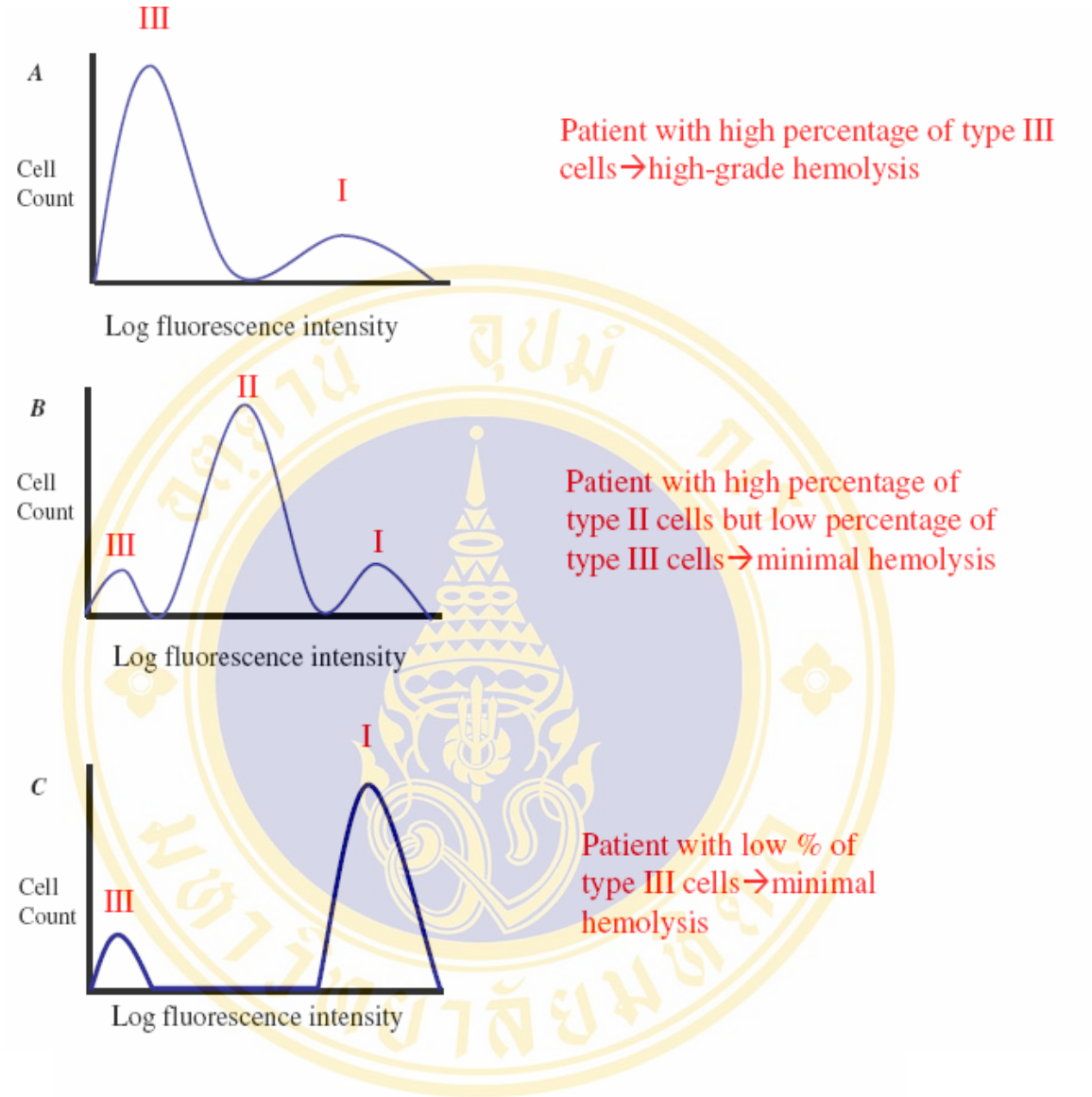


Figure 3 Phenotypic mosaicism in PNH. Hypothetical histograms of erythrocytes from patients with PNH stained with anti-CD59 are illustrated. The proportion and type of abnormal erythrocytes varies greatly among patients with PNH and these characteristics are important determinants of clinical manifestations. In general, patients with a high percentage of type III erythrocytes have clinically apparent hemolysis (panel A). If the erythrocytes are partially deficient in GPI-AP, hemolysis may be modest even if the percentage of the affected cells is high (panel B). A patient may have a diagnosis of PNH, but if the proportion of type III cells is low, only biochemical evidence of hemolysis may be observed (panel C) (32).

1.4 The GPI-anchor: structure and biosynthesis

1.4.1 Structure

The GPI is a complex molecule which binds certain proteins on the cell surface. A large number of molecules having different structures and functions. The core structure of GPI anchor is highly conserved and similar in all species and consists of three parts (Figure 4).

(I) A molecule of phosphatidylinositol (PI), which is sensitive to phosphatidylinositol phospholipase C (PIPLC) digestion. This part of GPI is responsible for membrane attachment and is achieved through the insertion of the lipid moiety into the outer leaflet of the membrane.

(II) The glycan core, consisting of a molecule of N-acetylglucosamine (GlcNAc) linked to three mannose residues.

(III) The protein attachment site is to the ethanolamine phosphate (EAP) molecule linked to the terminal mannose. The C-terminus of the relevant protein is linked to the amino group of the EAP molecule by an amide bond.

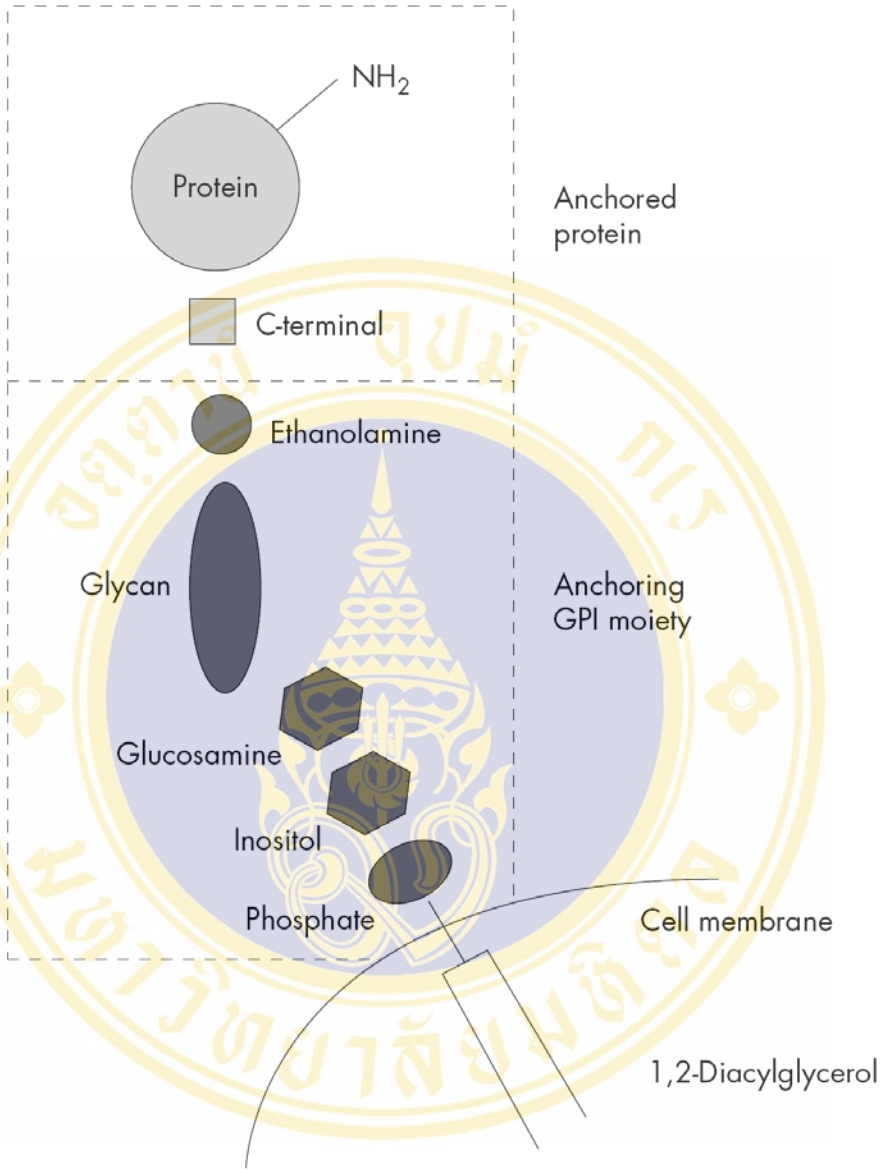


Figure 4 The glycosyl phosphatidylinositol (GPI) anchor. This is a simplified diagram of the GPI structure. The C-terminus of the anchored protein links to an ethanolamine residue on the GPI anchor. The anchor itself consists of this ethanolamine moiety attached to a glycan core. The GPI structure attaches to the cell membrane via phosphatidylinositol. The glycan core consists of a molecule of GlcNAc linked to three mannose residues. The first step in GPI synthesis is the linkage of the GlcNAc to PI. It is this reaction that fails in paroxysmal nocturnal haemoglobinuria because the genetic lesion disrupts the production of a necessary enzyme complex (81).

1.4.2 Biosynthesis

Proteins and GPI are synthesized separately on the endoplasmic reticulum (ER). The first step in GPI anchor biosynthesis is the transfer of GlcNAc from UDP-GlcNAc to PI to form GlcNAc-PI. This reaction is mediated by a complex of four gene products i.e., PIG-A, PIG-H, PIG-C, and GPI1. GPI1 is necessary for stable formation of the GPI-N-acetylglucosaminyltransferase (GPI-GnT) complex and stable expression of the PIG-C and PIG-H proteins. Class A, C, and H mutant cells, corresponding to PIG-A, PIG-C, and PIG-H mutations, do not express GPI-APs on the cell surface, and their membranes do not have GlcNAc-PI transferase activity. GlcNAc-PI is then deacetylated to form glucosaminyl-PI (GlcN-PI). This deacetylation step is mediated by a product of the PIG-L. After that the inositol residue is then acylated by palmitate to produce GlcN-PI with acylated inositol. Three mannose residues are sequentially added to the glucosamine residue from dolichol-phosphate-mannose (Dol-P-Man). This mannose donor is synthesized from dolichol phosphate and GDP-Man by the enzyme Dol-P-Man synthase. Mammalian Dol-P-Man synthase consists of at least two components, dolichol-phosphate-mannose 1 (DPM1) and dolichol-phosphate-mannose 2 (DPM2). The final step in the biosynthesis of the core backbone is the transfer of EAP to the third mannose, PIG-O and PIG-F are involved in this step. A precursor peptide of the GPI-AP is synthesized with a carboxy terminal signal sequence that directs GPI attachment. A transamidase appear to catalyze replacement of the C-terminal signal peptide and the preformed GPI anchor, generating GPI-AP. At least two proteins, GP18 and GAA1, are involved in this step. After being processed through the golgi apparatus, the GPI-APs appear on the external surface of the cell (Figure 5) (84).

If GPI is not attached due to a lack of biosynthesis, a precursor protein is degraded intracellularly or secreted into the extracellular compartment. Therefore, a lack of GPI anchor biosynthesis causes deficiency of surface expression of all GPI-APs (84, 85).

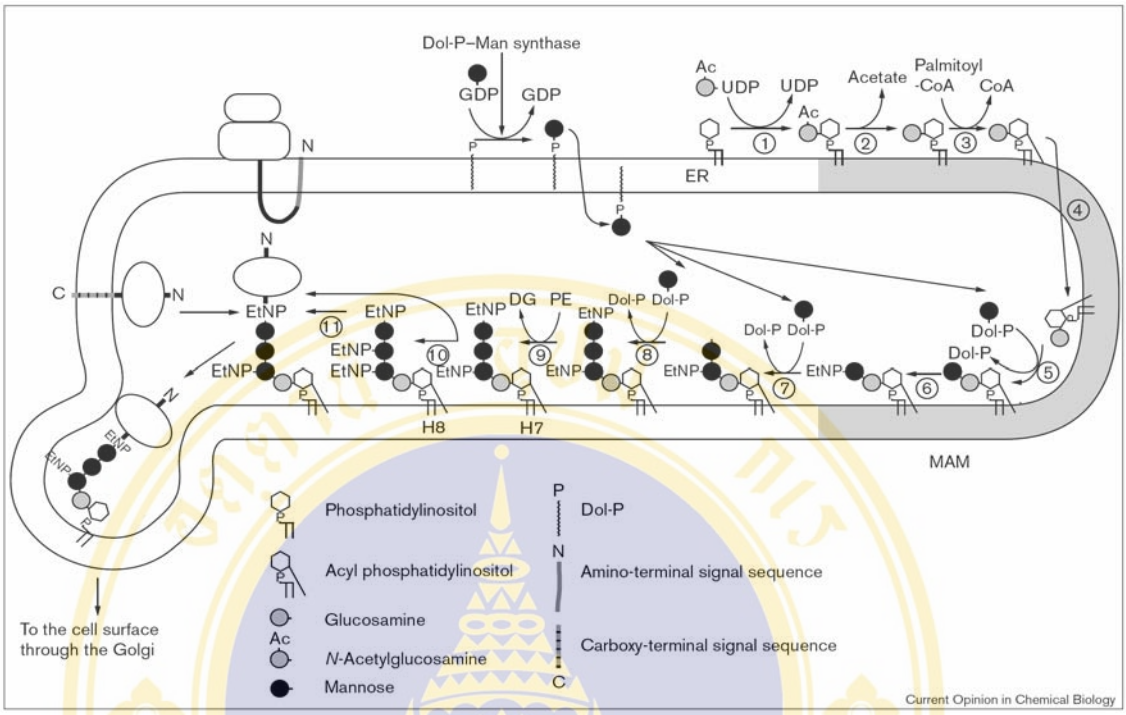


Figure 5 The biosynthesis and transfer to proteins of GPI in the ER. Biosynthesis of GPI is initiated by the transfer of *N*-acetylglucosamine to phosphatidylinositol (step 1). Steps 2 to 6 mainly occur in the mitochondria-associated membranes (MAMs). Pre-assembled GPI is transferred to proteins bearing a carboxy-terminal GPI-attachment signal sequence (step 11). GPI-anchored proteins are incorporated into vesicles and transported to the cell surface. DG, diacyglycerol; Dol-P, dolichol-phosphate; PE, phosphatidylethanolamine (86).

Table 1 Proteins involved in GPI-biosynthesis (87).

Protein (mol. wt)	Organism	Function[a]	Location[a]	Notes[a]
PIG-A (55 kDa)	Mammals	GlcNAc transferase? (Step 1)	ER membrane	Homology to a bacterial GlcNAc transferase
PIG-H (21 kDa)	Mammals	(Step 1)	ER membrane	
PIG-C (unknown)	Mammals	(Step 1)		Not cloned
GPI1 (60 kDa)	*Saccharomyces cerevisiae*	(Step 1)	ND	No homology to PIG-H
GPI2 (30 kDa)	*Saccharomyces cerevisiae*	(Step 1)	ND	No homology to PIG-H
GPI3/SPT14 (50–55 kDa)	*Saccharomyces cerevisiae*	GlcNAc transferase? (Step 1)	ND	Homology to mammalian PIG-A
PIG-J (unknown)	Mammals	GlcNAc–PtdIns deacetylase (Step 2)		Not cloned; enhancement by GTP
GPI deacetylase (45–60 kDa)	*Trypanosoma brucei*	GlcNAc–PtdIns deacetylase (Step 2)	Membrane bound	Partially purified
DPM1 (30 kDa)	*Saccharomyces cerevisiae*	Dol-P-Man synthase	ER	
PIG-B (60 kDa)	Mammals	Man 3-transferase (GPI-MT-3; transfers third Man)? (Step 7)	ER membrane	
PIG-F (25 kDa)	Mammals	EtnP transferase? (Step 8)	ND	Very hydrophobic
GAA1 (68 kDa)	*Saccharomyces cerevisiae*	Peptide/GPI anchor transamidase? (Step 10)	ER membrane	Large lumenal domain

*__Abbreviations__: GIcNAc, N.acetylglucosamine; PtdIns, phosphatidylinositol; DoI-P, dolichol phosphate; Man, mannose; Etn, ethanolamine; NO, not determined.

1.5 The GPI-anchored protein (GPI-AP)

Many Eukaryotic cell-surface proteins are anchored to the membrane by carboxy-terminal linkage to GPI. The GPI anchor is linked to proteins by amide bond formed between the carboxyl terminus and phosphoethanolamine attached to the third mannose. At least 50 GPI-APs, with a wide variety of functions, have been found in mammals. They include cell-surface hydrolytic enzymes, cell receptors, adhesion molecules, complement defence proteins, blood antigens, and others of unknown function. The GPI anchor can confer several functional properties upon proteins; these include signal transducing ability, low turnover rates, sorting to the surface of polarized cells and clathrin independent endocytosis (Table 2). GPI-APs are minor on mammalian cell surface, whereas they are abundant on the surfaces of some protozoa and yeast, that essential for the viability of these organisms (81, 87).

The unique feature of PNH blood cells is deficiency on their surface of all the proteins liked to the membrane by a GPI molecule. As a consequence of the absence of their anchor, the surface proteins are degraded within the cell leading to deficiency of all surface GPI-APs, especially the two missing proteins, CD55 and CD59, leading to the clinical manifestations of PNH.

In addition, the PNH platelets show up-regulated activation of complement that results not in lysis of the cell but in shedding C5b-9 complexes by vesiculation and promote assembly of the prothrombinase enzyme complex, leading to increasing risk of thrombosis.

The effect of the absence of GPI-APs on lymphocytes is less well understood. Several studies have suggested that deficient T cells are more naive than wild-type cells and respond less well to antigen-presenting cells in vitro. Nevertheless, no immunologic deficiency has ever been demonstrated clinically in patients with PNH (88).

In monocytes, the most characteristic deficiency is of CD14 (endotoxin-binding protein receptor) which may affect the response of monocytes to bacterial endotoxins and therefore contribute to the susceptibility to infections. However in PNH, the increased amount of soluble CD14, which may be derived from the non-attached protein functions equally well in clearing LPS (3).

Table 2 GPI-linked proteins (19).

Antigen	Cluster of differentiation	Expression/function
Complement regulatory molecules		
DAF	CD55	all haemopoietic cells; inhibitor of C3 and C5 convertases
MIRL	CD59	all haemopoietic cells; binds to C8 component of complement, preventing binding and polymerisation of complement
C8-binding protein (HRF)		complement regulation
Enzymes		
Neutrophil alkaline phosphatase		neutrophil enzyme
Red cell acetylcholinesterase		red cell enzyme
Ecto-5′-nucleotidase	CD73	B and T lymphocyte enzyme
ADP-ribosyl transferase	CD157	T lymphocyte/neutrophil enzyme
Receptors		
Fc receptor IIIa	CD16	neutrophils; involved in phagocytosis; receptor for immune complexes
uPAR	CD87	T cells, NK cells, monocytes, neutrophils; initiation of fibrinolysis
Endotoxin receptor (lipopolysaccharide receptor)	CD14	strong expression on monocytes; weakly expressed on granulocytes
Blood group antigen		
Cromer antigens (DAF)	CD55	red cell antigen
Cartwright (Yt) antigens (AchE)		red cell antigen
Holley Gregory antigen		red cell antigen
John Milton Hagen antigen (JMH)	CD108	red cell/lymphocyte/platelet antigen; possible role in adhesion
Dombrock residue		red cell antigen
Adhesion molecules		
Blast-1	CD48	lymphocytes and monocytes; receptor/ligand binding with CD224
LFA-3	CD58	all haemopoietic cells; ligand involved in T cell activation
	CD66a	neutrophil/eosinophil adhesion molecule
	CD66b	granulocytes; epithelial cells
	CD66c	
Carcinoembryonic antigen	CD66e	
Neutrophil antigens		
NA1/NA2	CD16	neutrophil antigen
NB1/NB2		neutrophil antigen
Others		
Campath-1	CD52	lymphocytes/monocytes; function unknown
	CD24	B cells and granulocytes; function unknown
GP500		platelet
GP175		platelet
Thy-1	CD90	stem cell subset; small T cell subset
GPI-80		? regulation of leucocyte trafficking
Vanin-1		expressed on thymic stromal cells and involved in thymus homing in mice

1.6 Molecular biology

1.6.1 The PIG-A gene

The PIG-A gene is at least 17 kd long and comprises 6 exons localized on the short arm of the X chromosome at Xp22.1 (Figure 6). PIG-A cDNA consists of 4,568 bp and an open reading frame of 1,452 bp and codes for a predicted protein of 484 amino acids, starting at the 86th bp. There is a short 5' non-coding region with the initiation of transcription in exon 2 and a relatively large 3' non-coding region (Figure 7) (84, 89).

The PIG-A gene product acts in the first step in GPI anchor biosynthesis by forming a complex with other proteins, PIG-H, PIG-C and GPI1. The resultant GPI-GnT is stabilised by two further components termed PIG-P and DPM2.

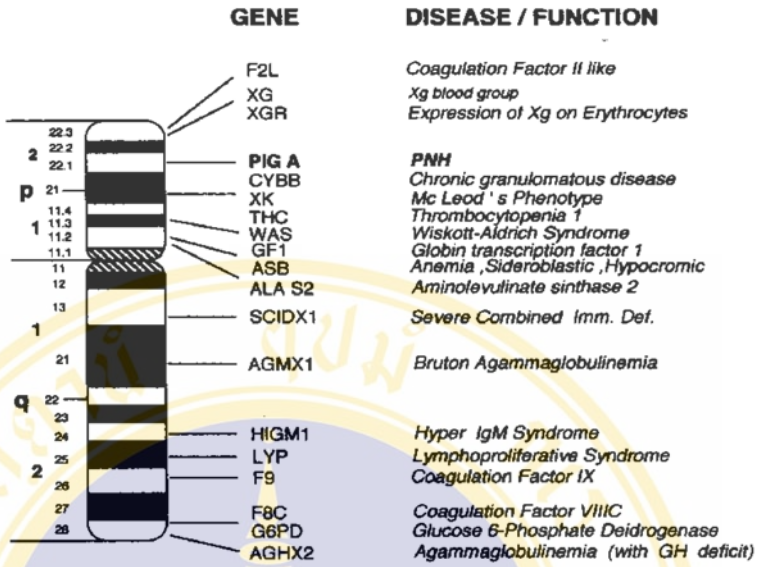


Figure 6 List of the numerous genes involved in hematological disorders that are located on the X-chromosome, to which PIG-A has been added (1).

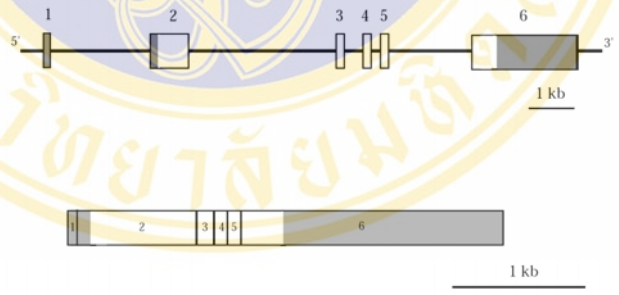


Figure 7 Schematic representation of the human PIG-A gene and its cDNA. The top part of the figure shows the structure of the PIG-A gene encoding for the PIG-A protein. Exons are represented by boxes, with gray areas indicating non-coding regions. The initiation codon lies in exon 2 and the termination codon in exon 6. The bottom part of the figure shows the structure of the PIG-A cDNA. Corresponding exon numbers are displayed. The 5' untranslated region (exon 1 and 5' end of exon 2) and the 3' untranslated region (3' end of exon 6) are represented in gray (2).

1.6.2 PIG-A mutations

The somatic mutations of the PIG-A gene occur in all PNH patients and spread throughout the entire PIG-A coding region with the highest occurrence in exon 2, probably because its largest size among all of the exons of the gene. The mutations are mainly small mutations, most are single-base substitutions, deletions, or insertions which result in a frameshift and early termination of transcription (Figure 8). Under these circumstances, no active PIG-A product is produced and the PNH cells are completely deficient in all GPI-APs. The remainders are point mutations, most of which are missense mutation, some may cause a total loss of function of protein while others may leave the product of PIG-A with residual activity. This residual activity would be the situation of those patients showing a partial deficiency of GPI-APs on the membranes of their blood cells (PNH type II cells) (90).

Up to now, more than 100 PIG-A mutations have been described and more than one different type of PIG-A mutation have been reported in the same patient, leading to the conclusion that one or more PNH clones can co-exist together with normal hematopoiesis in the same patient (91).

Since PIG-A is X-linked and because of X-chromosome inactivation, one hit of somatic mutation would cause GPI-deficiency in both male and female.

At least 12 or more genes are involved in the biosynthesis of the GPI anchor. Mutation of any one of them could result in GPI deficiency. However, only mutations of the PIG-A gene were reported in patients with PNH.

The most likely explanation why PIG-A is always responsible is that only the PIG-A gene is localized on X chromosome. Actually, nine other genes, PIG-C, PIG-H, GPI1, PIG-B, PIG-F, DPM1, SL15, GP18, and GAA1 have been proven to be autosomal. Mutation on both alleles of autosomal gene must occur to cause GPI anchor deficiency, but this event might be rare (84).

Figure 8 Mutations in the *PIG-A* gene observed in patients with PNH The cDNA structure shows the 6 exons, with the codon numbers corresponding to the exon–intron junctions given underneath. The 59 untranslated region (exon 1 and part of exon 2) and the 39 untranslated region (part of exon 6) are hatched. Below the cDNA diagram are the mutations causing complete inactivation of the gene product, including small insertion-deletions producing frame shifts (orange squares), nonsense mutations (red squares), splice-site mutations (purple lozenges), and large deletions (full black lines; the broken line indicates that the breakpoints of the large deletion removing the entire gene are not known). Above the cDNA diagram are missense mutations (blue circles) and one inframe deletion of a single codon (blue triangle). The mutation in codon 19 (green circle) is the only known *PIG-A* mutation that is germ-line rather than somatic: it entails a Arg!Trp amino acid replacement. This mutation has been observed in four unrelated families and it does not cause PNH; it is probably to be regarded as a neutral polymorphic allele. Frame shift and nonsense mutations appear to be rather randomly spread, perhaps because protein truncation will cause instability or failure of targeting almost regardless of where it takes place. By contrast, missense mutations are nearly always limited to exon 2, suggesting that this is a functionally critical domain (92).

1.7 Possible mechanisms of clonal expansion

Although it is well understood that somatic mutation of the PIG-A is responsible for the GPI-AP deficiency and the increase sensitivity to complement lysis, it is still unclear why and how PNH clones expand and contribute substantially to hematopoiesis in PNH patients. There are two hypotheses that try to explain this expansion phenomenon.

The first hypothesis emphasizes on dominant proliferation of the PNH clone. The genetic change(s) may confer PIG-A mutant stem cells with an intrinsic growth advantage and enable this PIG-A mutant cell clone to expand similar to a benign tumor. However, there are evidences which disprove this hypothesis. Using flow cytometric analysis of granulocytes, small clones (average of 0.002%) with PIG-A mutations existed commonly in normal individuals, indicating clearly that PIG-A mutations are not sufficient for the development of PNH (6, 8). When hematopoietic cell cultures were performed in patients with PNH either short term or long term culture, the numbers of colonies produced were in an order of magnitude less than in normals. In addition, if the non-PNH cells were separated from the PNH cells before culture, both components were equally poor in producing colonies (8, 58). In most patients the PNH cell population either regressed or remained stable for many years, indicated that PNH does not behave like leukemia with inexorable proliferation of abnormal cells (91). Finally, in mouse model, the generation of GPI-anchor-deficient mouse hematopoietic stem cells by disrupted PIG-A gene, the proportion of GPI-deficient cells either remains stable or decrease with time. Thus, GPI-deficient cells do not have a growth advantage over the residual normal cells in animal without BM failure (93-96). In summary, from all information above it can be concluded that the presence of PIG-A mutation alone is not enough to trigger the expansion of PNH clone, but there should be other factor(s) that mediate and select PNH clone to expand.

The second hypothesis suggests some pathological conditions that select or favor PIG-A mutant stem cells to expand (Figure 9). The basis of this hypothesis is related to the strong association between PNH and bone marrow failure especially aplastic anemia. In this hypothesis, somatic mutations in PIG-A lead to PNH only if the affected cells are in a BM under hypoplastic stress. The mutation and abnormal environment are both required. Thus, PNH cells will not prosper in a normal BM.

The low level of $CD34^+$ cells and decreased in colony-forming cells in PNH patients compared with normal volunteers, suggesting hematopoietic failure in PNH (97) and when sublethally irradiated SCID mice was transplanted with BM from either normal individuals or from patients with PNH and the mice treated with cytokines, only mice transplanted with PNH BM had detectable human blood compartments with a PNH phenotype (98). These supported that hypoplastic environment selects only PNH phenotype to proliferate at advantage. Therefore, there must be additional events that favor the growth of PNH clone (PNH clone is created by a somatic mutation and after that some conditions select this PNH clone to proliferate). One of these events or factors may be the affected environment due to an autoimmune process. Since there is close association between PNH and other BM failure disease with autoimmune process, such as AA and MDS as describe above.

If this autoimmunity occurs in AA and MDS, it is likely that the extrinsic factor that select or favor PNH clone expansion is the immunological process too. The association of PNH with immunological process could be confirm by the increase frequency of HLA-DR2 correlated with the presence of GPI(-) cells in PNH patients (42, 99), the observation of larger clonal populations of T-cell large granular lymphocytes (T-LGLs) and the higher degree of skewness in T-cell receptor (TCR) β-chain in PNH patients (100-105). However, how the immunological process selects PNH clone is now still unclear. The possibility is that autoreactive lymphocytes target and destroy stem cells via GPI-APs on the surface of the cells or GPI anchor itself (106). GPI-APs may served as antigens recognized by autoimmune cytotoxic lymphocytes. Surface GPI-APs would be internalized via the endocytic pathway, degraded into peptides in the endosomes, and presented on MHC class II molecules. Therefore, if a peptide derived from GPI-AP is an autoantigen recognized by autoreactive T lymphocytes, GPI-deficient cells would not be recognized and would be positively selected. Recent study shows that leukemic cells with PIG-A mutation survived natural killer (NK) cell-mediated cytotoxicity in vitro because the lack of GPI-APs, ULBP1 and ULBP2 which mediated the activation of NK and T cells (107). Another possible autoantigen is GPI anchor itself. A family of MHC class I-related molecules; CD1d can present glycolipid antigens such as phosphatidylinositol derivatives and glycosylceramides to natural killer T (NKT) lymphocytes. In this

assumption, CD1d binds GPI in its antigen-binding groove and present to NKT cells. If lymphocytes recognize GPI presented on CD1d molecules, GPI-negative cells would not be recognized.

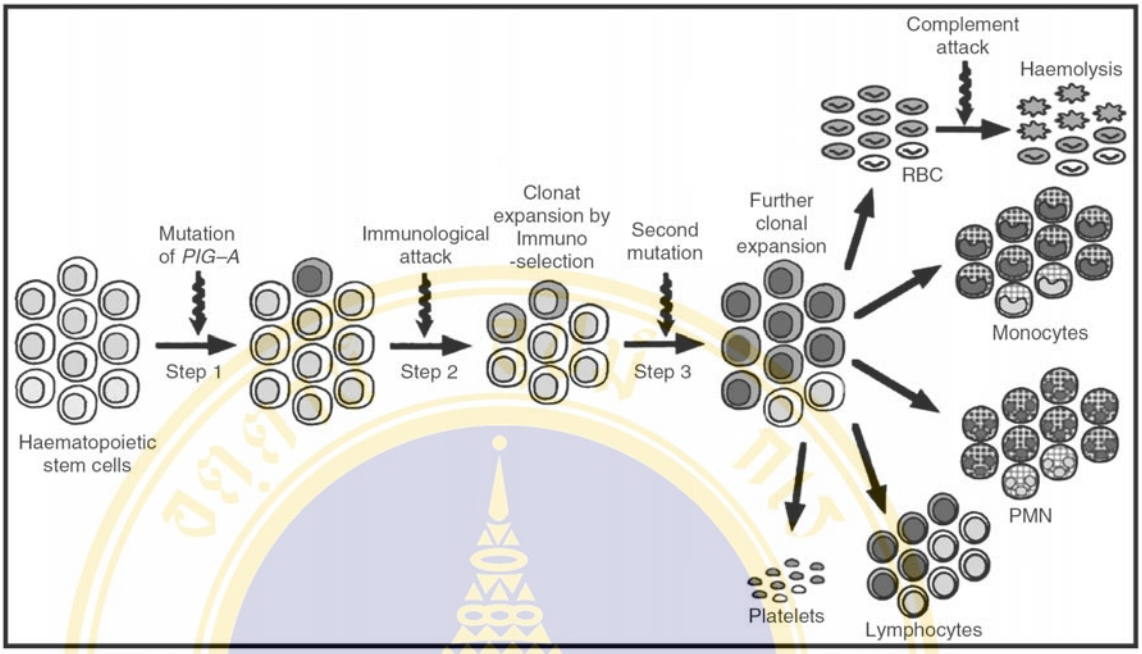


Figure 9 Three-step model for the development of paroxysmal nocturnal hemoglobinuria (PNH). Step 1, somatic mutation of phosphatidylinositolglycan (PIG-A) in a hemopoietic stem cell. Step 2, immunological attack on hemopoietic stem cells selectively decreases the number of PIG-normal stem cells, resulting in relative increase in the PIG-A mutant clone. Step 3, a second mutation occurs in the PIG-A mutant, leading to further expansion and generation of a large number of PIG-deficient blood cells (108).

1.7.1 Cytotoxic T lymphocytes (CTLs) and NKT cells mediated immunological process, inhibits non-mutated hematopoietic stem cells, leaving hypoplastic environment that support mutated cells to expand.

For the dual pathogenesis above, two kinds of cytotoxic "killer" cells that may involved in the pathogenesis of PNH i.e., CTLs and NKT cells.

Cytotoxic T lymphocytes are lymphocytes that kill other ("target") cells which may be foreign cells or autologous cells expressing foreign or mutated self proteins. Most of them belong to the $CD8^+$ subset of T cells, play role in adaptive immune responses upon recognizing the antigen epitope in the context of class I histocompatibility (MHC) molecules by using the αβTCR and receiving various signals from the antigen-presenting cells (109, 110).

Another population of lymphocytes that function as "killer" cells, these so-called NK cells and NKT cells. In the hypothesis NKT of PNH pathogenesis cells may be one of the lymphocyte subsets that would be regulated in this autoimmune reaction through CD1d-lipid/glycolipid interaction.

NKT cells constitute a unique subpopulation of T lymphocytes that is highly conserved in both human and murine species that mediated in innate immunity. NKT cells express surface markers that are commonly found on conventional T cells and NK cells. Human NKT cells express NK cell markers, such as NKR-PIA (CD161), CD56, CD57, and CD122. Since these cells express a TCR composed of an invariant Vα14-Jα281 chain together with a polyclonal Vβ8 (and to some extent Vβ7 or Vβ2) chain, NKT cells express an extremely limited T cell repertoire. In contrast to typical T cells, NKT cells respond to antigen presented by CD1d - a non-polymorphic MHC Class I–like molecule, which requires β_2-microglobulin for surface expression. CD1d is expressed by all hematopoietic cells, including immature and mature T cells, B cells, dendritic cells, macrophages, and hepatocytes. In addition, NKT cells are either single positive $CD4^+$ of approximately 60% of all NKT cells or double negative $CD4^-CD8^-$TCR α/β+, and have a highly unique antigen recognition system in that they respond to lipids, glycolipids, or highly hydrophobic peptides presented by CD1d (111-114).

After autoantigen recognition and the activation of autoreactive lymphocytes, the effector function of activated lymphocytes occurs by the secretion

of cytokines, especially IFN-γ and TNF. For CTLs and NKT cells, not only cytokine secretion but also cytotoxic function that mediated in target cell death by program cell death called apoptosis (Figure 10, Figure 11). This lethal hit can be mediated by two mechanisms of cytotoxicity. The first mechanism involves granule exocytosis and perforin released. After antigen recognition, cytoplasmic granules of the CTL or NKT cell become concentrated to the area of the cytoplasm near the contact with the target cell. Membrane fusion results in exocytosis of the granule contents onto the surface of the target cell that include two important granule proteins, perforin and granzyme. Perforin is a pore-forming protein that will form a large aqueous channel on the target cell plasma membrane, resulting in the influx of water followed by osmotic swelling of the target cell. Which is unable to exclude high concentration of calcium ion leading to apoptosis. After a pore-formation, granzyme that is a serine protease, especially granzyme B will enter target cells through perforin-created holes. Granzyme B proteolytically cleaves and thereby activates cellular enzymes called caspases, which in turn cleave several substrates, and induce target cell apoptosis. The second mechanism is mediated by interactions of membrane molecules on CTLs/NKT cells and target cells. Upon activation, CTLs/NKT cells express a membrane protein called FasL, which binds to its target protein Fas express on many cell types. The reaction results in activation of caspases and apoptosis of targets. In this case, the overproduction of both IFN-γ and TNF (115, 116) induce expression of the Fas receptor on hematopoietic stem cells. Thus, hematopoietic stem cells with elevated Fas would be sensitive to FasL-expressing cytotoxic lymphocytes or NKT cells. It likely that only normal cells (GPI(+)) will present autoantigen and activate lymphocytes, will be killed by this Fas-FasL interaction mediated apoptosis. This can be supported by the less sensitivity of GPI(-) cells to T lymphocyte activation (117, 118). Moreover, by performing pair culture, GPI(-) cells from PNH patients proliferated to the levels approaching those of normal cells, while GPI(+) cells produce fewer cells and have also shown the increasing of Fas receptor. These imply the impaired growth and more susceptible to T cells response of GPI(+) cells in PNH (119). The other similar experiments showed increased Fas expression and higher apoptosis in GPI(+) cells (120, 121). Furthermore, recent data have shown that the residual GPI(+) hematopoiesis in PNH is not normal as indicated by the presence of

short telomeres of the GPI(+) cells (122). In contrast to the impaired growth of GPI(+) stem cells, GPI(-) cells escape this immune attack (which may be) by the resistance to the inhibitory effect of IFN-γ and TNF or the resistance to apoptosis as demonstrated by the findings that IFN-γ and TNF had effected to reduce only GPI(+) colony-forming cells (123). Granulocytes from PNH patients had shown to be less susceptible to apoptosis spontaneously without ligands or induced by anti-Fas (CD95) (35). In addition PIG-A mutant cells are relatively resistant to cytotoxic attack by natural killer cells and cytotoxic lymphocytes (117, 124) and the over expression of four anti-apoptosis genes; human A1, hHr23B, Mcl-l, and Rho A (125), implies apoptosis resistance of these PIG-A mutant cells. In conclusion, these greater susceptibility to apoptosis by immune attack of the non-mutated cells, may play a major role in the pathogenesis of PNH subsidizing the mutated cells to expand.

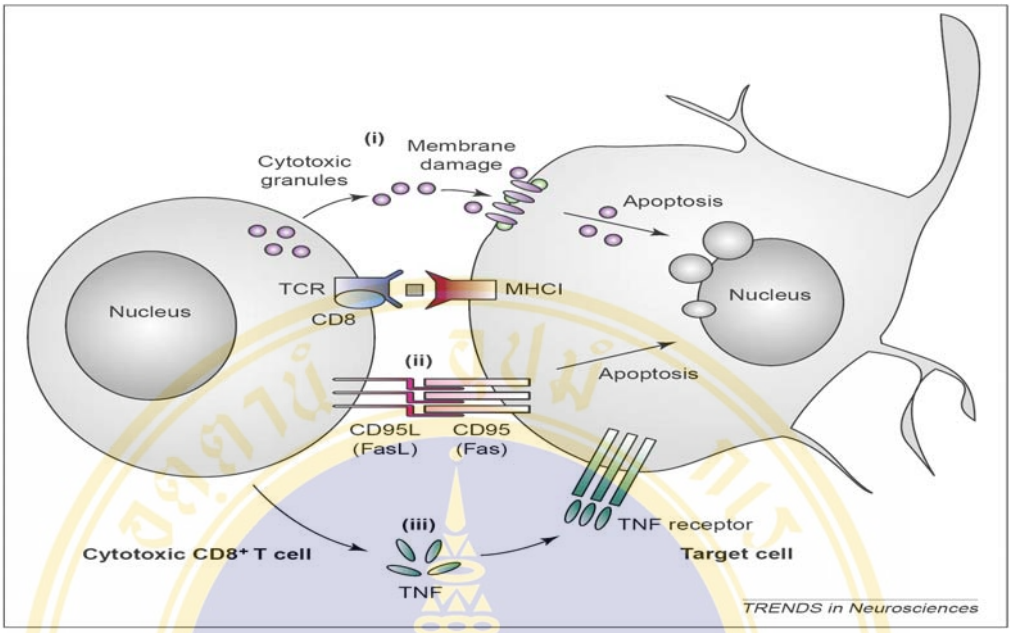


Figure 10 Three different, although not mutually exclusive, pathways used by cytotoxic T cells (CTLs) to destroy target cells. A $CD8^+$ CTL recognizes a target cell by binding of its T-cell receptor (TCR) to the appropriate combination of major histocompatability complex I (MHC I) and peptide (square). Destruction of the target cell can be performed by: (i) secretion of cytotoxic granules resulting in disintegration of the cell membrane and induction of apoptosis; (ii) activation of Fas/CD95 receptors by Fas ligand/CD95 ligand (FasL) inducing apoptosis; and (iii) release of cytokines, such as tumor necrosis factor-α (TNF) (110).

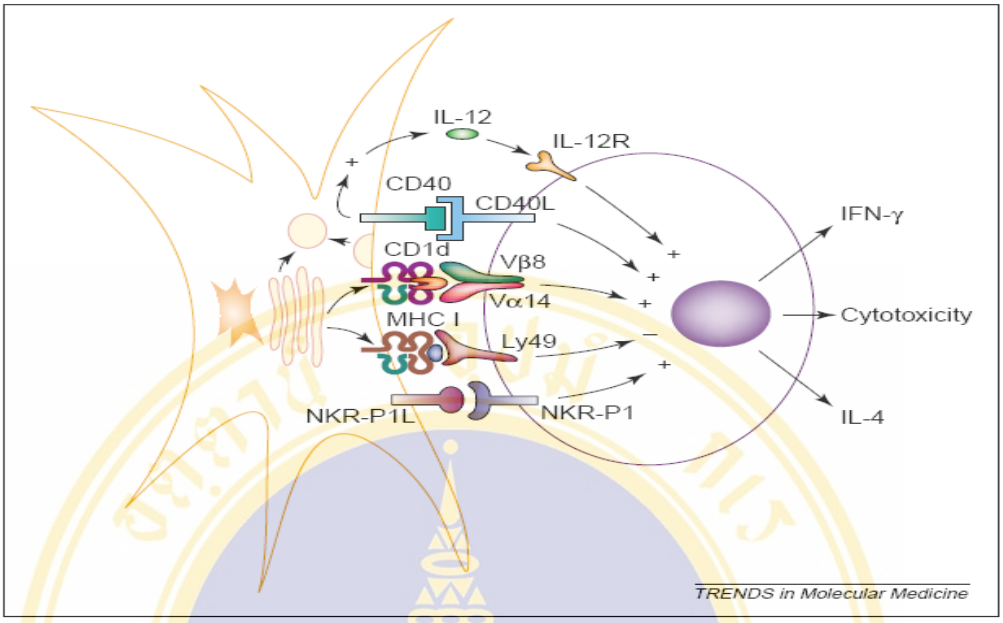


Figure 11 Specificity and effector functions of murine natural killer T (NKT) cells. NKT cells (on the right) can receive signals through a variety of receptor–ligand interactions: semi-invariant TCRs (Vα14 paired predominantly with Vβ8) of NKT cells interact with glycolipid or phospholipid antigens presented by CD1d molecules on antigen-presenting cells (APC) (on the left), NKR-P1 (also called CD161) molecules interact with their ligands (NKR-P1L), Ly49 receptors bind with major histocompatibility complex class I (MHC I) molecules, CD40 interacts with CD40 ligand (CD40L), various cytokine receptors [the interleukin (IL)-12 receptor (IL-12R) is shown as an important example] might be activated by cytokines produced by APC and other cell types. These receptor–ligand interactions induce various signal transduction pathways in NKT cells that, in turn, might induce cytokine production and cytolytic activity. Human NKT cells exhibit similar specificities and functions to murine NKT cells (113).

1.7.2 Apoptosis

Apoptosis or programmed cell death (PCD) is a process of cell removal that conserved throughout animal evolution. A unique feature of this cell suicide is the removal of the residual cell constituents without inflammation in the neighboring tissues. It plays a crucial role in normal cell homeostasis, embryogenesis, normal tissue turnover, immune development and defense, the prevention of malignancies such as tumor growth and viral infection, and many more situations.

The cell death pathway is activated when a cell is no longer needed or has sustained serious damage. Initiation of apoptosis can occur via an external stimulus such as a protein released from cytotoxic T cells (granzyme) and by corticosteroids, the withdrawal of a critical growth factor or hormone as well as through cell surface interactions with specific apoptosis receptors (eg, Fas protein expressed on the cell membrane). Alternatively, apoptosis can be induced by an internal stimulus resulting from exposure to radiation or toxins as well as in response to metabolic or cell cycle perturbations. The initiation step is followed by signalling that specifically activates the death pathway.

Apoptosis is characterized by a series of morphologic changes. Initially there is decrease or shrinkage in cell volume, the generation of a pyknotic nucleus, and condensation of the chromatin along the nuclear membrane. This is followed by nuclear collapse and the generation of small cellular fragments or apoptotic bodies. The latter may appear on the cell surface as vesicles or blebs, changes in membrane lipid distribution that result in externalization of phosphatidylserine (PS) to the outer leaflet of plasma membrane, and disruption of the inner mitochondrial transmembrane potential ($\Delta\Psi m$). The last change is phagocytosis of intact apoptotic bodies that prevents release of cellular contents into the surrounding areas and blocks any inflammatory response. Apoptosis, in contrast with those of necrosis that is an alternative cell death process associated with pathologic processes that caused by severe tissue trauma or bacterial infection, induces a robust inflammatory response by spilling their cytoplasmic contents into the surrounding extracellular space.

1.7.2.1 Apoptosis in immune function

Apoptosis plays a central role in immune function as the critical pathway in lymphoid development used to eliminate self-reactive pre-T cells in the thymus and self-reactive pre-B cells in the bone marrow. In addition, it is the mechanism that controls the number of lymphocytes participating in an immune response once the foreign antigen has been successfully eliminated. Apoptosis, under these circumstances, is primarily induced via the generation of Fas protein and FasL on the surface of activated T cells. The appearance of these surface molecules allows for the elimination of activated cells (via apoptosis) that are no longer necessary for immune protection. Thus, this pathway provides a critical means for controlling the magnitude of an immune reaction. An additional role of apoptosis in immune function centers on the cytotoxic effector function of T cells. Target cell destruction (Figure 12) appears to involve the initiation of apoptosis via two different mechanisms as well as the direct induction of cell necrosis. The secretory pathway for the induction of apoptosis depends on the release of granzyme, an enzyme that induces apoptotic death. This also involves perforin, a second granular protein which upon release inserts pores into the target cell membrane. This allows granzyme intracellular entry which increases its efficiency in causing target cell apoptosis. Perforin may also directly initiate necrosis in certain target cells by allowing the free passage of electrolytes and water across the cell membrane. An alternative pathway for inducing target cell apoptosis is through the expression of FasL on activated cytotoxic T cells. The engagement of FasL with Fas expressed on the target cell provides a death signal to the target cell.

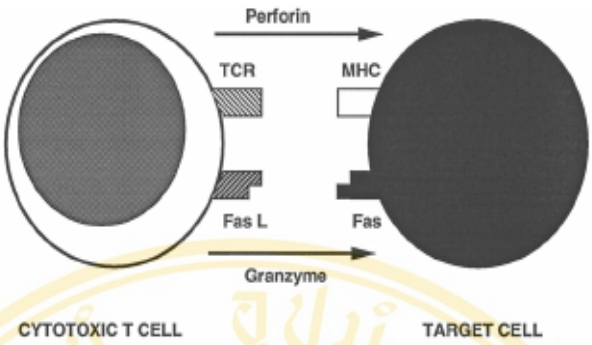


Figure 12 Cytotoxic T cells induce apoptosis in the target cells through at least three mechanisms: the induction of Fas ligand which binds to Fas on the target cell surface and the secretion of two proteins, granzyme and perforin. The former protein directly induces apoptosis while the latter inserts a pore-like structure in the target cell membrane. This alteration in the cell membrane increases the effectiveness of granzyme by allowing its entry into the cell. Perforin also can directly induce necrosis of the target cell by allowing the free movement of water and electrolytes between the intracellular and extracellular environment (126).

1.7.2.2 Apoptosis detection

The detection of apoptosis can be done at any levels of apoptosis such as quantitation of DNA degradation by the analysis of DNA fragmentation, quantitation of membrane changes by the analysis of PS externalization and mitochondrial membrane depolarization. There are many methods for studying apoptosis include the assessment of morphological changes at the cellular level by light-, electron- or time-lapse microscopy in combination with vital fluorescent dyes, the use of annexin V to monitor the loss of membrane phospholipid asymmetry during apoptosis, the use of fluorescent labeling to detect active caspase-3 form/caspase-3 activity and Fas expression, the measurement of fluorescence intensity of mitochondria specific cationic lipophilic dyes by flow cytometry to detect the loss of transmembrane potential ($\Delta\Psi m$) of mitochondria, or assays to detect DNA fragmentation by agarose gel electophoresis, by in situ nick-end labeling (TUNEL), or by using an enzyme-linked immunoabosorbent assay.

2. Methods using in thesis

2.1 Flow cytometry for apoptosis detection

For our experiment to perform apoptosis analysis, we choose to detect apoptosis of two populations of cell, normal cells (GPI-AP+) and abnormal cells (GPI-AP-), from the same individual by using annexin V labeling (apoptosis kit) and analysis by flow cytometry to separate the apoptotic and the living cells. This method is fast, easy or simple to do. It detects onset of apoptosis earlier than DNA-based assays, and can monitor apoptosis in living-unfixed cells. It is a sensitive assay because externalization of PS occurs earlier than the nuclear changes associated with apoptosis. Thus, annexin-FITC staining is an excellent indicator of the onset of apoptosis in many cell types. Annexin V, which has strong anticoagulant activity, is a member of a family of proteins that have high, Ca^{++}-dependent affinities for aminophospholipids. The assay is based on the externalization of PS, an event occurs early in apoptosis in many different cell types. In living cells, PS is predominantly located on the inner leaflet of the plasma membrane. When cells initiate apoptosis, PS is rapidly translocated to the outer leaflet. In the presence of Ca^{++}, annexin V binds PS with high affinity. Flow cytometry can detect apoptotic cell death with a signal of

decrease in forward scatter; but little change in side scatter for early apoptosis, and diminished intensity of both forward and side scatter for late apoptotic cells.

2.2 In vitro assay for detection of hemopoietic progenitor cells

Hematopoietic stem cells (HSC) are well-characterized tissue-specific stem cells that exhibit remarkable self-renewal capacity and to differentiate into progenitor cells and mature blood cells of all hematopoietic lineages (Figure 13). HSC are rare cells that reside in adult bone marrow where hematopoiesis is continuously taking place, and they can also be found in cord blood, fetal liver, adult spleen and peripheral blood. For more than a decade, CD34 has been the critical marker for HSC identification and isolation. Recent studies suggest that HSCs can also contribute to nonhematopoietic tissues including muscle, liver, vasculature and skin. The development of clinical applications of stem cells for regeneration of hematopoietic and nonhematopoietic tissues requires continued progress in the analysis of HSC phenotype and functional properties, efficient HSC isolation procedures, and experimental approaches to expand and manipulate HSCs in culture.

2.2.1 Colony assay

Colony-forming cell (CFC) assays are used to quantify multi-potential progenitors and single lineage-restricted progenitors of the erythroid, granulocytic, monocyte-macrophage and megakaryocytic pathways. The CFCs are classified and enumerated based on morphologic recognition of mature cells within the colony by light microscopy. Colony assays have been used extensively for research and clinical applications. CFC assays are used as read-out for the LTC-IC assay, and to measure progenitor cell numbers in the development of stem cell enrichment strategies and other ex vivo manipulations. Applications of CFC assays also include identification of stimulatory and inhibitory growth factors and evaluation of the hematopoietic proliferative potential of BM, cord blood (CB) and mobilized peripheral blood (MPB) samples for transplantation. The colony assay has been the benchmark functional assay to assess the ability of various hematopoietic cell types to divide and differentiate, especially following ex vivo manipulations, including T cell depletion, HSC and progenitor cell enrichment, gene therapy and cryopreservation. In addition,

CFC assays provide convenient screening assays to assess general or lineage-specific toxicity of novel drugs or other compounds on human hematopoietic cells and can be used as an alternative to drug screening in animal models and to select potential candidates for phase I clinical trials.

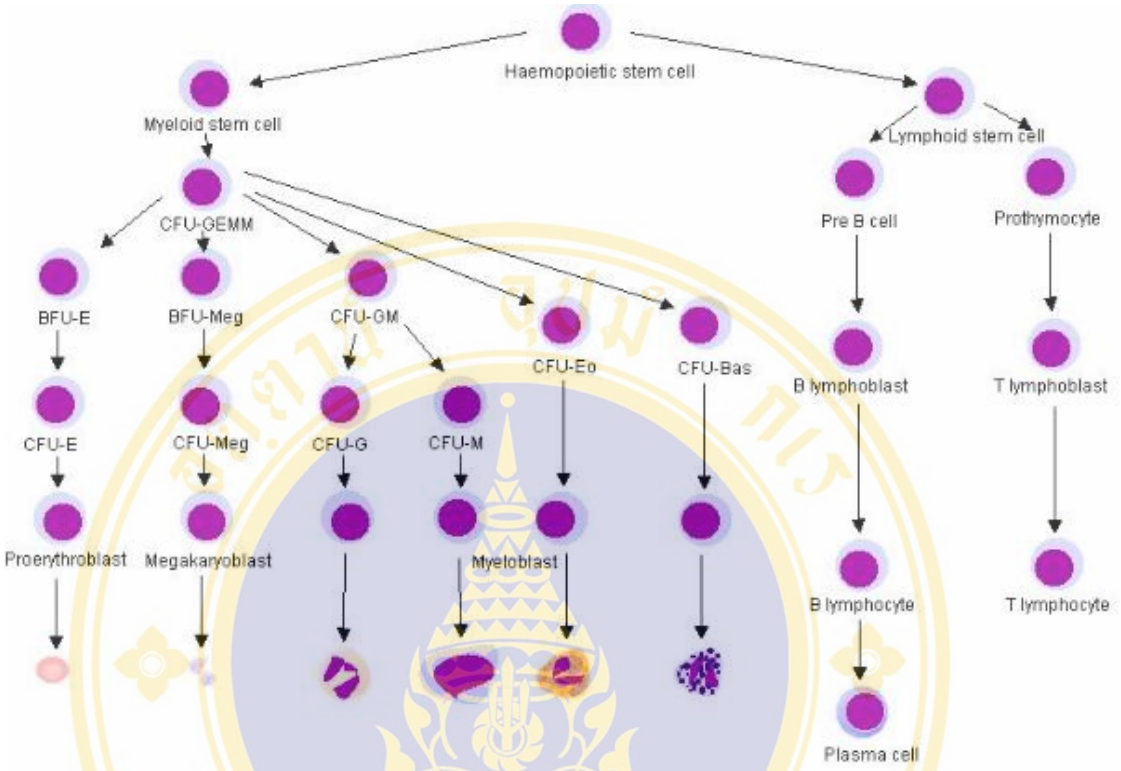


Figure 13 Haemopoiesis proliferation and differentiation pathway. (Figure from http://www.bh.rmit.edu.au/mls/subjects/haemo/resources/haemprolif.jpg)

CHAPTER IV
MATERIALS AND METHODS

1. Experimental strategy

Three experiments including apoptosis detection, CTL and NKT cell study, and hemopoietic progenitor cell culture assay were performed in this study. Heparinized blood from both PNH patients and healthy volunteers was separated into two equal parts. One part for polymorphonuclear cells (PMNs) and mononuclear cells (MNCs) isolation for apoptosis, CTL and NKT cell study. For the study of apoptosis, the fractions of PMNs were co-cultured with MNCs from the same individual or culture without MNCs as a control for 0 and 4 hour in liquid medium. After culture, cells were stained with monoclonal antibody (MoAb) reactive with GPI(+) and marker for apoptosis cells, and analyzed by flow cytometry to investigate the percentages of apoptosis in the portion of GPI(+) and GPI(-) cells. Results were compared between in the same individual and between healthy volunteers and PNH patients. Moreover, the cells from MNC fraction were used to study CTL and NKT cell by staining with MoAb for markers of CTL and NKT cell and investigated by flow cytometry. The other part of heparinized blood was prepared for hemopoietic progenitor cell culture study. MNCs were separated by using indirect immunomagnetic activated cell sorter, $CD8^+$ cell fraction and $CD8^-$ cell fraction were isolated from MNCs. The $CD8^-$ cell fraction which contained hemopoietic stem cells were culture only by methycellulose culture system or co-culture in the present of $CD8^+$ cell fraction. Number of colonies were enumerated after 14 days under inverted microscopy. Outline of the three experiments was shown in Figure 14.

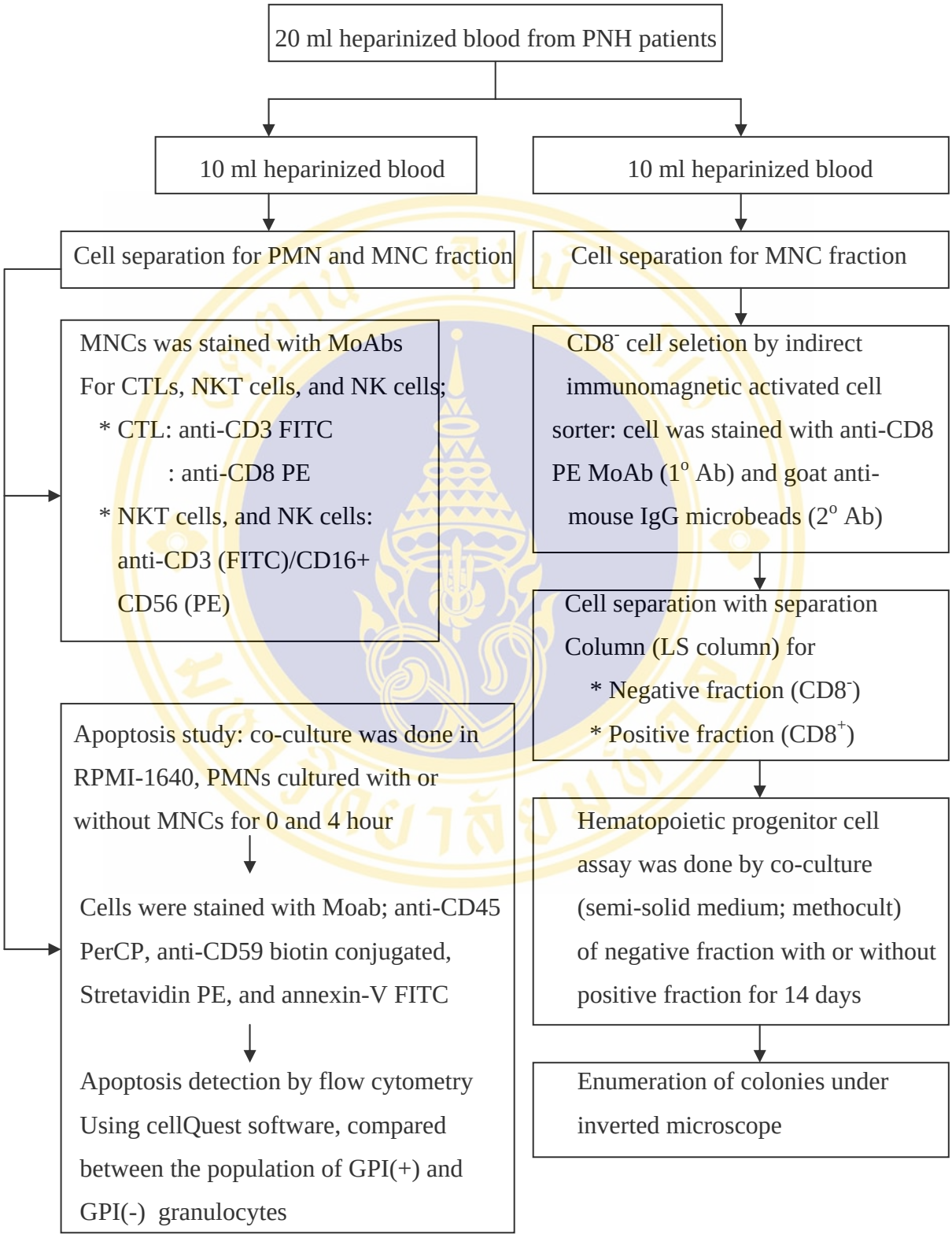


Figure 14 Experimental strategy of apoptosis study, CTL, NKT cell, and NK cell study, and hemopoietic progenitor cell study.

2. Patients and normal controls

Twenty-one PNH patients who were attended at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital were recruited in this study. The patients consisted of 6 females and 15 males, age ranged from 28-68 years. The diagnosis of PNH was confirmed by positive Ham's test and/or the presentation of CD59 negative cells population by flow cytometry determination. Nine patients (43%) had pre-existing aplastic anemia. The hematological parameters of the patients were shown in table 3.

Thirty-five healthy volunteers were included in this study as normal controls.

Table 3. Clinical details of PNH patients.

UPN	Sex	Age at onset (years)	Initial diagnosis	Hb (g/dl)	WBC (10^9/L)	Plt. (10^9/L)
1	F	28	AA/PNH	5.0	4.8	92
2	F	55	PNH	12.6	5.00	355
3	F	38	PNH	11.2	6.67	142
4	F	31	AA/PNH	12.7	3.14	71
5	F	60	PNH	10.2	7.41	178
6	F	45	PNH	11.9	6.06	100
7	M	38	PNH	7.8	6.08	198
8	M	50	PNH	8.9	5.73	49
9	M	68	PNH	9.4	3.66	32
10	M	47	AA/PNH	11.1	11.64	172
11	M	39	AA/PNH	9.6	4.18	170
12	M	35	AA/PNH	10.6	2.69	43
13	M	39	AA/PNH	14.2	4.22	91
14	M	34	AA/PNH	11.0	4.40	41
15	M	32	AA/PNH	10.4	6.04	230
16	M	36	PNH	11.3	6.00	206
17	M	43	PNH	7.6	3.39	168
18	M	33	PNH	9.2	5.06	111
19	M	29	PNH	10.4	7.46	273
20	M	42	PNH	11.1	5.49	141
21	M	45	AA/PNH	12.0	4.48	68

UPN, unique patient number; Hb, hemoglobin; WBC, white blood cell; Plt., platelet; F, female; M, male; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia.

3. Blood samples and cell separation

20 ml heparinized blood was obtained from the patients and healthy volunteers after obtaining their informed consent. Heparinized blood were separated into two parts. The first part of 10 ml heparinized blood were firstly separated for leukocyte fraction by 6% dextran sedimentation. Leukocyte fraction were separated again by lymphoprep centrifugation gradient to separate mononuclear cell (MNC) fraction and polymorphonuclear (PMN) cell fraction. After washing with phosphase buffered saline (PBS), separated cells were resuspended in Rosewell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, N.Y., U.S.A.) supplemented with 1% L-glutamine, 10% fetal calf serum (FCS), 50 ug/ml streptomycin, and 50 IU/ml penicillin for apoptosis and CTL, NKT cell study.

The remainder of the second part of 10 ml heparinized blood were separated for MNC fraction by lymphoprep centrifugation. Separated cells were washed 2 times with PBS/EDTA buffer. 10^7 total cells were resuspended in 100 ul PBS/EDTA buffer for hemopoietic progenitor cell co-culture study.

4. Apoptosis and flow cytometry analysis

1 x 10^6 PMNs were cultured alone as a control and co-cultured with 1 x 10^6 MNCs in 1 ml of RPMI-1640 supplemented with 1% L-glutamine, 10% FCS, 50 ug/ml streptomycin, and 50 IU/ml penicillin (Figure 15).

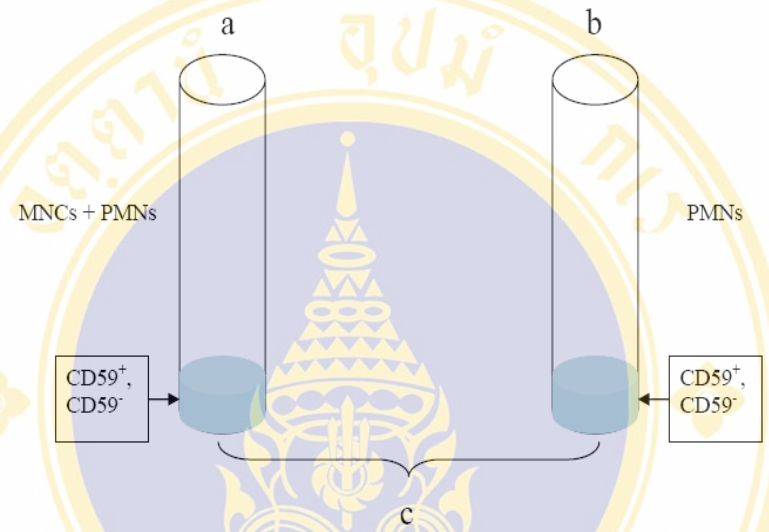
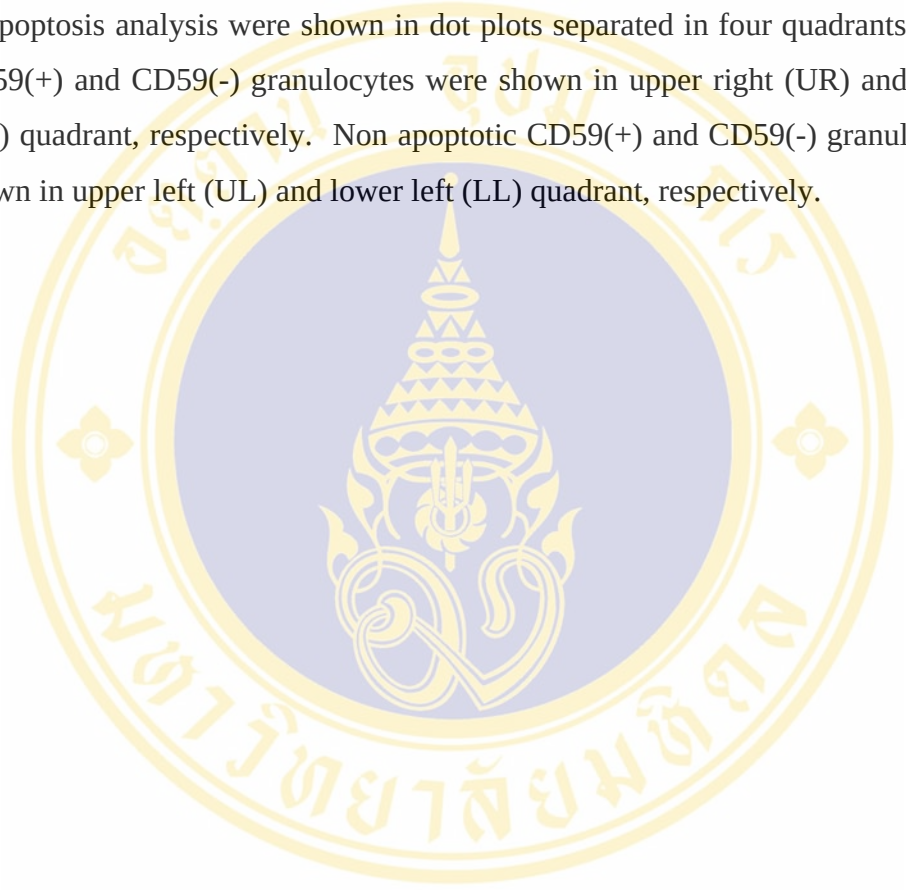


Figure 15 The study was performed in two sample tubes. The first tube was the co-culture of PMN fraction with MNC fraction from the same individual. The second tube was the culture of PMN fraction alone. (a) and (b); The study performed in the same sample tube. (a) The co-culture of PMN fraction with MNC fraction from the same individual. (b) The culture of PMN fraction. (c) The study to compare between the sample tube i.e., (a) and (b).

Firstly, Pilot study had been done in 13 normal controls to choose the suitable period of time for co-culture by doing kinetic culture at 0, 2, 4, 6, 12, 18, and 24 hours. Apoptosis were analysed by flow cytometry based on three colour analysis (Figure 16). Granulocytes within a gate set based on side and forward scatter properties and expression of CD45 were analysed for annexin-V positive. The result of apoptosis analysis were shown in dot plots separated in four quadrants. Apoptotic CD59(+) and CD59(-) granulocytes were shown in upper right (UR) and lower right (LR) quadrant, respectively. Non apoptotic CD59(+) and CD59(-) granulocytes were shown in upper left (UL) and lower left (LL) quadrant, respectively.



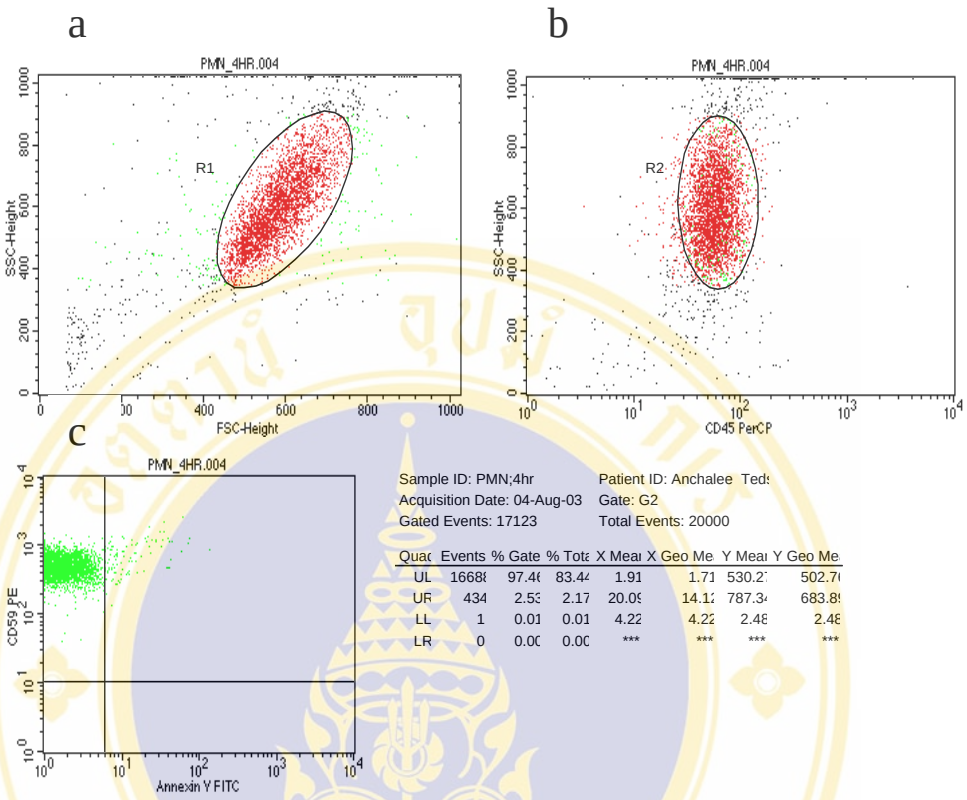


Figure 16 Granulocyte analysis: gating strategies and apoptosis analysis of peripheral blood granulocytes in normal controls for pilot study i.e., pilot study for apoptosis of granulocytes measured at 4 hour. Dot plots showed apoptosis analysis based on three colour analysis. (a) Granulocytes gate based on forward and side scatter (FSC/SSC) (R1). Only CD45 (leukocyte antigen) positive granulocytes (R2) were further analysed to examine expression of GPI-anchored CD59 positive to separate normal and PNH phenotype and identified annexin-V positive granulocytes. (c) Plot showed only granulocytes with normal phenotype (CD59+ granulocytes) in upper left (UL) and upper right quadrant and granulocytes with annexin-V positive which indicated cell apoptosis were shown in UR quadrant.

Figure 17 showed spontaneous apoptosis of granulocytes in the culture with or without MNC fraction, and indicated the best period of time that showed the least number of apoptotic granulocytes for the baseline of spontaneous apoptosis *in vitro* is 4 hours.

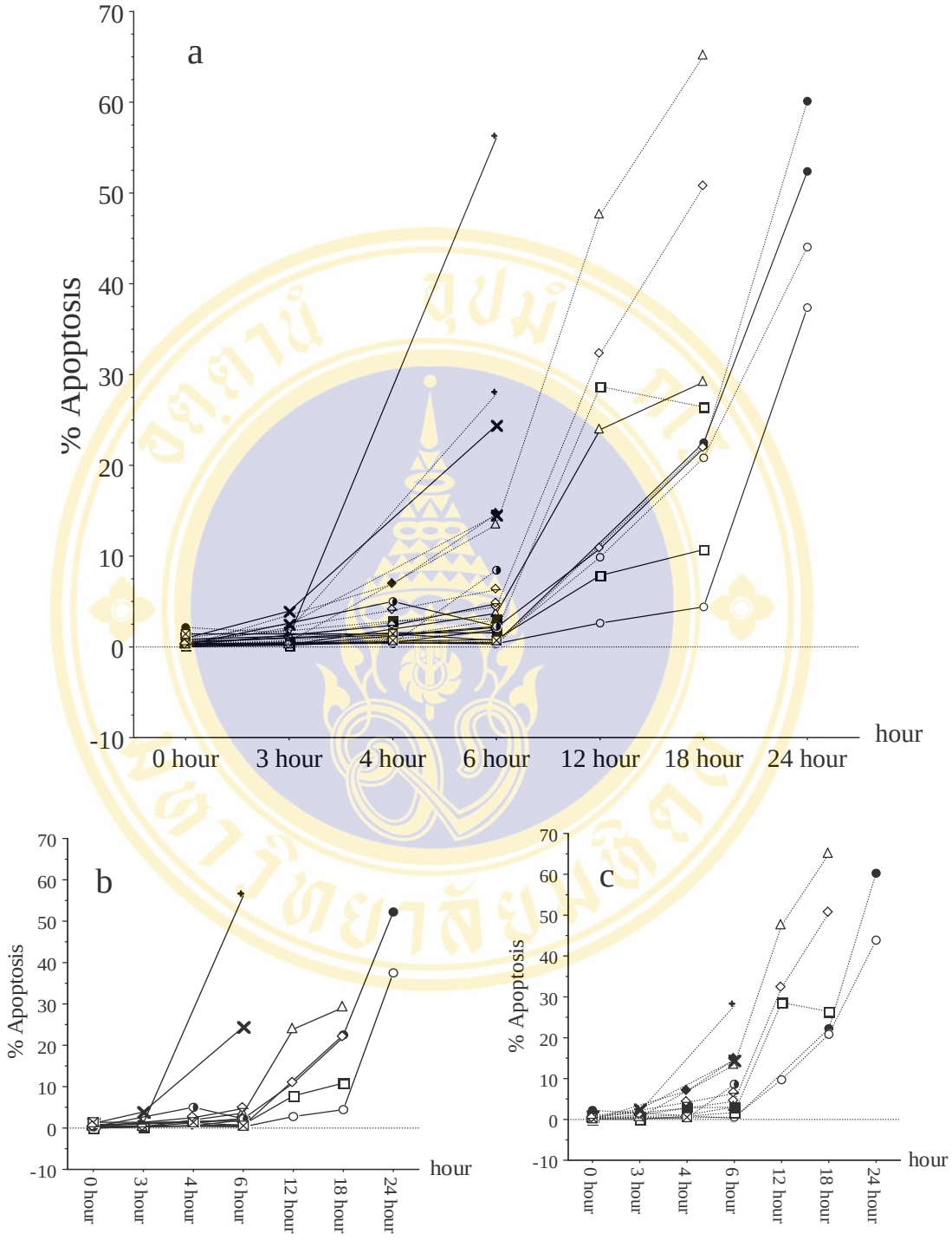


Figure 17 Pilot study for suitable period of time for co-culture. Apoptosis analysis by flow cytometry had been done in normal controls (n = 13) to choose the suitable period of time for co-culture by doing kinetic culture at 0, 2, 4, 6, 12, 18, and 24 hours. The result showed that the least number of granulocytes apoptosis for the baseline of spontaneous apoptosis *in vitro* is

4 hours. each symbol represented for each normal control with the study of the co-culture with MNC fraction (solid line) and the culture without MNC fraction (dotted line). (a) Granulocytes apoptosis by incubation up to 24 hours in 13 normal controls in the study of the culture with (solid lines) and without (dotted lines) MNC fraction. (b) and (c) Granulocytes apoptosis by incubation up to 24 hours in 13 normal controls only in the study of the co-culture with MNC fraction or the culture without MNC fraction, respectively.

After culture for 0 and 4 hours, 0.5 ml of cells were incubated with 50 µl of anti-CD59 biotin conjugated, 7 µl of anti-CD45 PerCP, 2 µl of annexin-V FITC, and 100 µl of binding buffer and incubated at 2^{o} to 8^{o}C for 15 minutes. Annexin-V binding in the CD59(+) and CD59(-) cell populations were determined by flow cytometry based on three colour analysis (Figure 18). Granulocytes within a gate set based on side and forward scatter properties and expression of CD45 were analysed for annexin-V positive. The result of apoptosis analysis were shown in dot plots separated in four quadrants. Apoptotic CD59(+) and CD59(-) granulocytes were shown in upper right (UR) and lower right (LR) quadrant, respectively. Non apoptotic CD59(+) and CD59(-) granulocytes were shown in upper left (UL) and lower left (LL) quadrant, respectively.

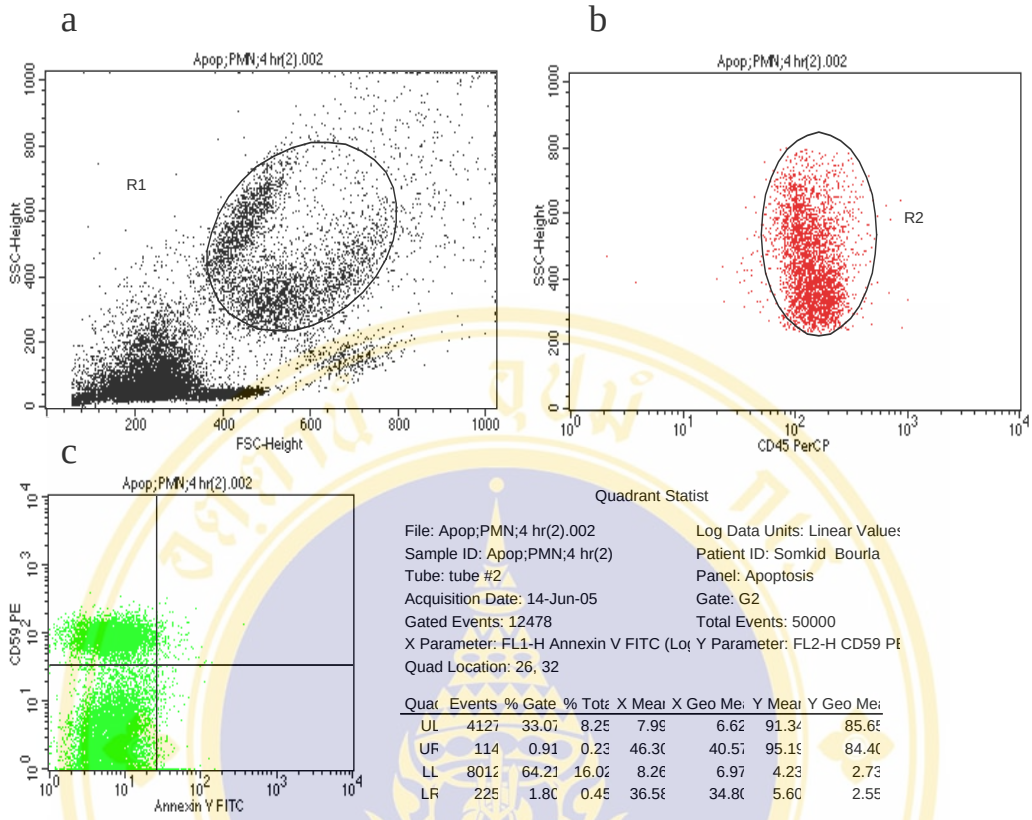


Figure 18 Granulocyte analysis: gating strategies and apoptosis analysis of peripheral blood granulocytes i.e., apoptosis of granulocytes measured at 4 hour. Dot plots showed apoptosis analysis based on three colour analysis. (a) Granulocytes gate based on forward and side scatter (FSC/SSC) (R1). Only CD45 (leukocyte antigen) positive granulocytes (R2) were further analysed to examine expression of GPI-anchored CD59 positive to separate normal and PNH phenotype and identified annexin-V positive granulocytes. (c) Plot showed granulocytes with normal phenotype (CD59+ granulocytes) in upper left (UL) and upper right quadrant. Granulocytes with PNH phenotype (CD59- granulocytes) were shown in lower left (LL) and lower right (LR) quadrant. Granulocytes with annexin-V positive which indicated cell apoptosis were shown in UR and LR quadrant, non-apoptotic granulocytes with annexin-V negative were shown in UL and LL quadrant.

The percentages of apoptosis were evaluated by the formula below.

$$\text{The percentages of apoptosis} = \left(\frac{\text{The number of apoptotic granulocytes (UR or LR)}}{\text{Total number of each granulocyte population}} \right) \times 100$$

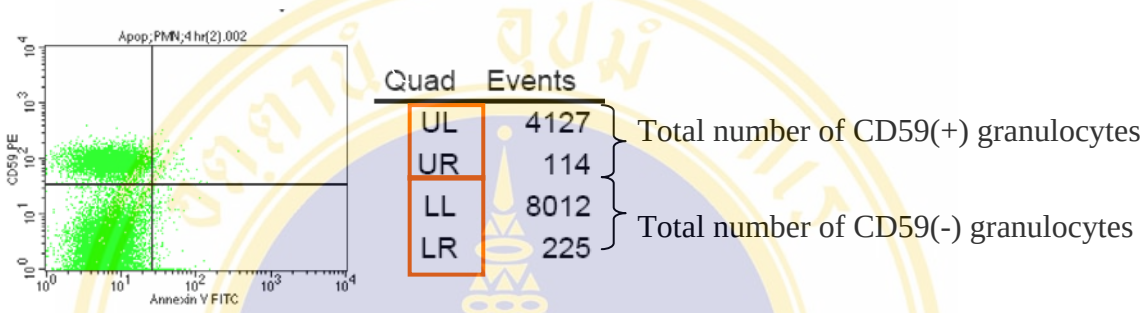


Figure 19 Dot plots showed granulocytes population. UL and UR represented CD59(+) granulocyte population and LL and LR represented CD59(-) granulocyte population.

5. Cytotoxic T cell (CTL), natural killer T (NKT) cell, and natural killer (NK) cell flow cytometry analysis

CTL, NKT cell, and NK cell flow cytometry determination were performed in a separate tube. 1 x 10^6 MNCs were washed with 2 ml of PBS, centrifuged at 2,000 rpm for 5 minutes. After washing, cell pellet was stained with MoAb for CTL profile; 5 ul of anti-CD3 FITC and 5 ul of anti-CD8 PE, or NKT cell, NK cell profile; 5 ul of anti-CD3 FITC/CD16+CD56 PE at 2^o to 8^oC for 15 minutes. Erythrocytes were lysed by incubation with 1 ml of 1/10 dilution of fluorescence activated cell sorting (FACS) lysing solution (BD, San Jose, CA, U.S.A.) for 10 minutes, and subsequently centrifuged at 2,000 rpm for 5 minutes. The supernatant was discarded and cell pellet was washed with 2 ml of PBS at 2,000 rpm for 5 minutes, then cell pellet fixed with 0.5 ml 1% paraformaldehyde. The cells were determined by a FACSCalibur flow cytometer and analysed by cellQuest software (BD, San Jose, CA, U.S.A.) (Figure 20). Lymphocytes were gated based on FSC/SSC (R1) and further analysed to examine expression of anti-CD3 FITC positive, anti-CD8 PE positive for CTLs in UR quadrant. For NKT cells and NK cells, lymphocytes were further analysed for the expression of anti-CD3 FITC positive, anti-CD16/56 PE positive in UR quadrant, and expression of anti-CD3 FITC negative, anti-CD15/56 PE positive in UL quadrant, respectively.

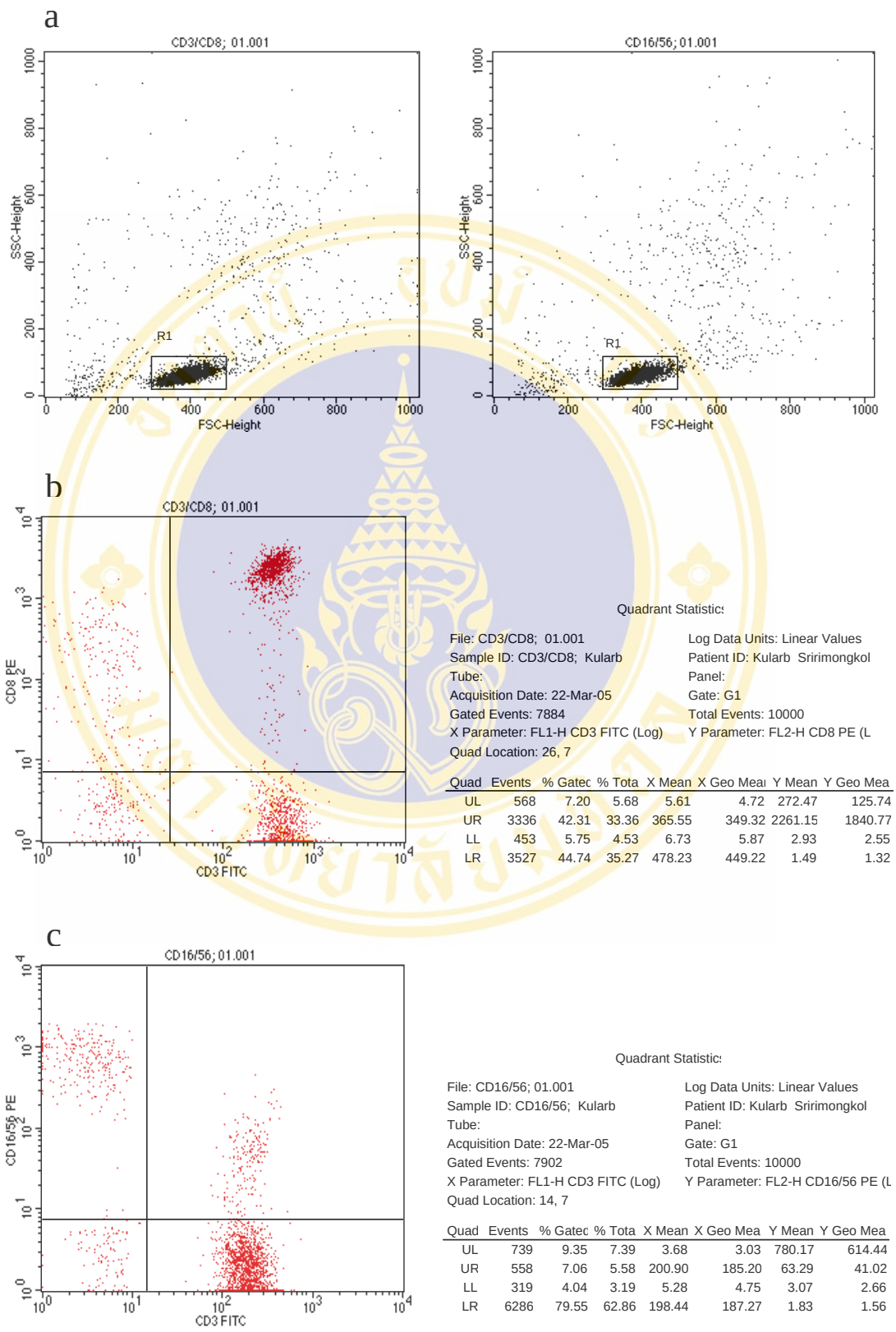


Figure 20 Flow cytometry analysis for CTLs, NKT cells, and NK cells. Lymphocytes were gated based on FSC/SSC (R1) (a). Cells were further analysed for the

the expression of anti-CD3 FITC, and anti-CD8 PE or anti-CD16/56 PE. Dot plots showed the population of CTLs in UR quadrant for anti-CD3 FITC positive, anti-CD8 PE positive (b). NKT cells and NK cells were shown in UR and UL quadrant for anti-CD3 FITC positive, anti-CD16/56PE positive, and anti-CD3 negative, anti-CD16/56PE positive, respectively (c).

6. Hemopoietic progenitor cell assay

6.1. MNCs staining for indirect immunomagnetic activated cell sorter.

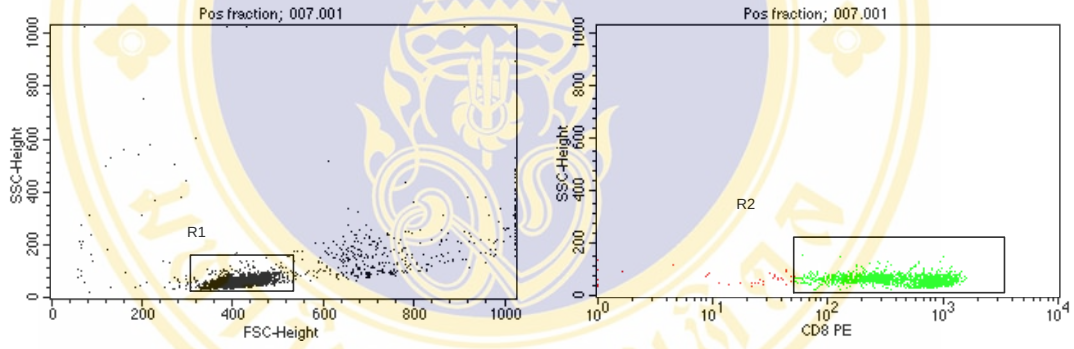
The $CD8^-$ cells were purified by using indirect immunomagnetic activated cell sorter. MNCs were resuspended into a final concentration of 10^7/100 ul of PBS/EDTA (buffer). 70 ul of anti-CD8 PE antibody were added to each 100 ul of MNCs and incubated for 15 minutes at room temperature (RT). Stained cells was washed with 50 ml of buffer, centrifuged at 2,000 rpm for 5 minutes. Cell pellet were resuspended in a final concentration of 10^7/80 ul of buffer, and subsequently each 10^7 cells were incubated with 20 ul of goat anti-mouse IgG microbeads for 15 minutes at 2^o to 8^oC. 10^7 Cells were further washed with 50 ml of buffer centrifuged at 2,000 rpm for 5 minutes. Supernatant was discarded and 10^8 cells were resuspended with 0.5 ml of buffer. The $CD8^+$ and $CD8^-$ fraction were separated by magnetic separation with LS column (Miltenyi Biotec, Earhart Avenue Auburn, CA, U.S.A.).

6.2. Magnetic separation with LS column

The column was prepared by rinsing with 3 ml of buffer, then apply cell suspension onto the column. Then the column was washed 3 times with 3 ml of buffer each time. Cells from this step were $CD8^-$ cell fraction (negative fraction) which contained hemopoietic progenitor cells. After removal of column from the separator and placed it on suitable collection tube and immediately flush out $CD8^+$ cell fraction (positive fraction) with 5 ml of buffer by firmly applying the plunger supplied with the column.

6.3. Negative and positive fraction flow cytometry analysis for $CD8^+$ marker

0.5 ml of negative and positive fraction were centrifuged at 2,000 rpm for 5 minutes. Cell pellet was stained with 5 ul of anti-CD45 FITC and 5 ul of anti-CD8 PE at 2 to 8^{o}C for 15 minutes. After MoAb staining, cells were washed with 2 ml of PBS at 2,000 rpm for 5 minutes and subsequently fixed with 0.3 ml of 1% paraformaldehyde. Fixed cells were analyzed to investigate the number of $CD8^+$ cells by FACSCalibur flow cytometry (Figure 21). Lymphocytes were gated according to FSC/SSC and further analysed for the expression of anti-CD8 positive within gate generated by combining SSC with anti-CD8 PE.



Region Statistics

File: Pos fraction; 007.001
Log Data Units: Linear Values
Sample ID: Pos fraction; Narongrid
Patient ID: CD8+
Tube:
Panel:
Acquisition Date: 11-May-05
Gate: G1
Gated Events: 8376
Total Events: 10000
X Parameter: FL2-H CD8 PE (Log)
Y Parameter: SSC-H SSC-Height (Linear)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	8376	100.00	83.76	604.04	453.36	59.53	57.69	1, 2
R2	8204	97.95	82.04	616.15	486.00	59.45	57.63	4, 2

Figure 21 Flow cytometry analysis for $CD8^+$ cells i.e., $CD8^+$ analysis in positive fraction. Lymphocytes were gated base on FSC/SSC (R1). Cells were further analysed for the expression of anti-CD8 PE positive within gate generated by combining SSC with anti-CD8 PE (R2).

6.4. Hemopoietic cell culture

After negative selection by indirect immunomagnetic activated cell sorter, 1.2 x 10^6 cells from $CD8^-$ cell fraction and 0.3 x 10^6 cells from $CD8^+$ cell fraction resuspended in Iscove's modified Dulbecco's medium (IMDM; Gibco) for final volume equal to 0.3 ml for the co-culture with $CD8^+$ cells, and 1.2 x 10^6 from $CD8^+$ cell fraction resuspended in IMDM for final volume equal to 0.3 ml for the culture without $CD8^+$ cells were added in 3 ml of methocult (Stem Cell Technologies, Vancouver, Canada) containing 1% methylcellulose in IMDM, 30% FBS, 1% BSA, 104 M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml stem cell factor, 10 ng/ml GM-CSF, 10 ng/ml interleukin (IL)-3, and 3 U/ml erythropoietin. 1.1 ml of the mixture of cells and methocult was plated into 35 mm culture dish in duplicate; 4 x 10^5 of $CD8^-$ cells/dish and 1 x 10^5 of $CD8^+$ cells/dish for the co-culture with $CD8^+$ cells and 4 x 10^5 of $CD8^-$ cells/dish for the culture without $CD8^+$ cells. Incubated the cells for 14 days at 37° C and 5% CO_2 (Figure 22). Colonies consisting of at least 50 cells were enumerated and characterized according to their morphology (Figure 23 and 24) with an inverted microscope

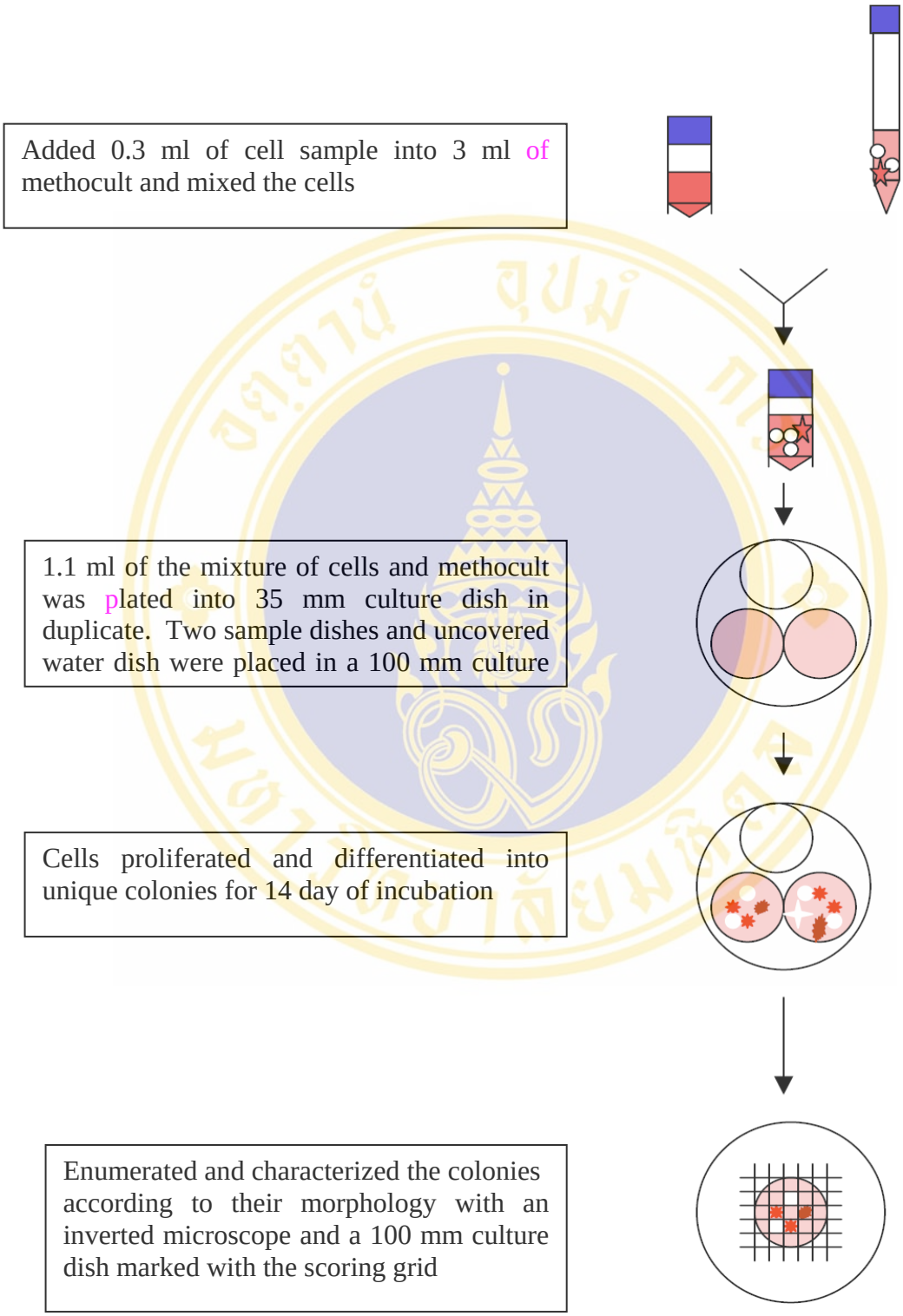


Figure 22 Procedure outline for hemopoietic cell culture. (Applied from http://www.rndsystems.com/stem_cell_protocol_detail_objectname_CFC.aspx)

6.4.1 Colony types

6.4.1.1 BFU-E (Burst forming unit-erythroid)

The size of the colony can be described as small (3 to 8 clusters), intermediate (9 to 16 clusters), or large (more than 16 clusters) according to the number of clusters present. A single large cluster is occasionally observed. They are the primitive erythroid progenitors that have high proliferative capacity. Due to the variation in the differentiation stage, some colonies may not be completely hemoglobinized and mature until days 18 to 20.

Figure 23 Morphology of BFU-E (Burst forming unit-erythroid). (Figure from http://www.rndsystems.com/stem_cell_protocol_detail_objectname_CFC.aspx)

6.4.1.2 CFU-GM (Colony forming unit-granulocyte, macrophage)

Progenitors that give rise to colonies containing a heterogeneous population of macrophages and granulocytes. Produces a colony containing at least 20 granulocyte cells (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM). CFU-GM colonies arising from primitive progenitors may contain thousands of cells in single or multiple clusters. The developed colony is colorless, unlike the reddish color displayed by CFU-E and BFU-E colonies.

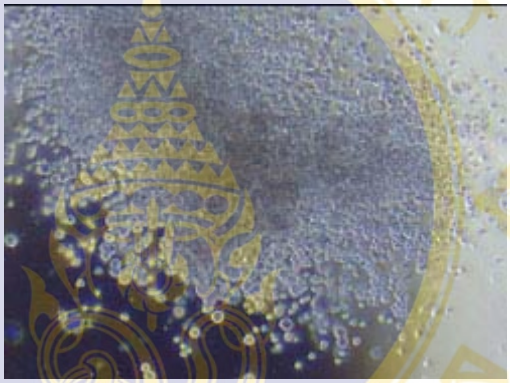


Figure 24 Morphology of CFU-GM (Colony forming unit-granulocyte, macrophage). (Figure from http://www.rndsystems.com/stem_cell_protocol_ detail_ objectname_CFC.aspx)

CHAPTER V
RESULTS

1. Apoptosis assay

To test the assumption of autoimmunity that may be responsible for the selection of PNH clone, we investigated the effect of MNCs which represented for lymphocyte subsets, $CD8^+$, NKT cells, and NK cells, on the apoptosis of target cells (CD59(+) and CD59(-) granulocytes), *in vitro*.

CD59 GPI-anchored protein expression and apoptosis of granulocytes were analysed by flow cytometry based on three colour analysis. The percentages of CD59(+) and CD59(-) granulocytes in normal controls and PNH patients were shown in Table 4 and 5.

Table 4 represents the percentages of CD59(+) and CD59(-) granulocytes in the sample tube with and without MNC fraction measured at 0 and 4 hour of 33 normal controls. In the sample tube with MNC fraction measured at 0 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 99.56 ± 0.11, median; 99.85, minimum; 97.16, maximum; 100.00. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 0.44 ± 0.11, median; 0.15, minimum; 0.00, maximum; 2.84.

In the sample tube without MNC fraction measured at 0 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 99.77 ± 0.05, median; 99.89, minimum; 98.76, maximum; 100.00. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 0.24 ± 0.05, median; 0.12, minimum; 0.00, maximum; 1.24.

In the sample tube with MNC fraction measured at 4 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 99.73 ± 0.06, median; 99.87, minimum; 98.31, maximum; 100.00. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 0.27 ± 0.06, median; 0.14, minimum; 0.00, maximum; 1.69.

In the sample tube without MNC fraction measured at 4 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 99.80 ± 0.04, median; 99.89,

minimum; 99.11, maximum; 100.00. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 0.20 ± 0.04, median; 0.11, minimum; 0.00, maximum; 0.89.

Table 4 % CD59 population of normal controls.

UPN	% CD59 population							
	0 hr				4 hr			
	With MNCs		Without MNCs		With MNCs		Without MNCs	
	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)
1	100.00	0.00	99.90	0.10	99.99	0.01	100.00	0.00
2	100.00	0.00	99.98	0.02	99.91	0.09	99.99	0.01
3	99.85	0.15	99.76	0.24	99.09	0.91	99.85	0.15
4	99.99	0.01	99.98	0.02	99.98	0.02	100.00	0.00
5	99.95	0.05	99.94	0.06	99.96	0.14	100.00	0.00
6	99.96	0.04	99.97	0.03	99.98	0.02	99.99	0.01
7	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
8	99.99	0.01	100.00	0.00	100.00	0.00	100.00	0.00
9	100.00	0.00	100.00	0.00	99.99	0.01	99.99	0.01
10	100.00	0.00	100.00	0.00	99.99	0.01	100.00	0.00
11	99.99	0.01	100.00	0.00	99.99	0.01	100.00	0.00
12	99.88	0.12	99.85	0.15	99.88	0.12	99.93	0.07
13	99.97	0.03	99.90	0.10	99.22	0.78	99.77	0.23
14	99.18	0.82	99.59	0.41	99.85	0.15	99.81	0.19
15	99.14	0.86	99.50	0.50	99.65	0.35	99.56	0.44
16	99.28	0.72	99.25	0.75	99.54	0.46	99.51	0.49
17	99.82	0.19	99.86	0.14	99.72	0.29	99.74	0.26
18	99.95	0.05	99.89	0.12	99.56	0.44	99.89	0.11
19	99.54	0.47	99.68	0.32	99.96	0.04	99.95	0.06
20	98.61	1.39	99.33	0.67	99.93	0.07	99.32	0.68
21	98.63	1.36	99.62	0.39	99.94	0.06	99.87	0.13
22	98.55	1.45	99.48	0.52	99.98	0.02	99.11	0.89
23	99.75	0.25	99.92	0.08	99.91	0.09	99.94	0.06

Table 4 % CD59 population of normal controls (Continued).

UPN	% CD59 population							
	0 hr				4 hr			
	With MNCs		Without MNCs		With MNCs		Without MNCs	
	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)
24	99.78	0.22	99.94	0.06	99.78	0.22	99.30	0.70
25	99.77	0.23	99.68	0.32	99.87	0.13	99.71	0.29
26	99.86	0.14	99.90	0.10	99.71	0.29	99.89	0.11
27	99.96	0.04	99.97	0.03	99.69	0.31	99.83	0.17
28	99.73	0.27	99.87	0.13	99.74	0.26	99.67	0.33
29	99.96	0.04	99.98	0.02	99.86	0.14	99.90	0.10
30	97.16	2.84	99.77	0.23	99.48	0.52	99.89	0.11
31	99.59	0.41	99.46	0.54	99.34	0.66	99.59	0.41
32	98.96	1.03	99.53	0.47	99.40	0.60	99.88	0.12
33	98.68	1.31	98.76	1.24	98.31	1.69	99.52	0.48
Mean	99.56	0.44	99.77	0.24	99.73	0.27	99.80	0.20
SEM	0.11	0.11	0.05	0.05	0.06	0.06	0.04	0.04
MD	99.85	0.15	99.89	0.12	99.87	0.14	99.89	0.11
Min	97.16	0.00	98.76	0.00	98.31	0.00	99.11	0.00
Max	100.00	2.84	100.00	1.24	100.00	1.69	100.00	0.89

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

Table 5 represents the percentages of CD59(+) and CD59(-) granulocytes in the sample tube with and without MNC fraction measured at 0 and 4 hour of 15 PNH patients. In the sample tube with MNC fraction measured at 0 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 32.85 ± 6.64, median; 26.29, minimum; 1.35, maximum; 76.86. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 67.15 ± 64, median; 73.71, minimum; 23.14, maximum; 98.65.

In the sample tube without MNC fraction measured at 0 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 31.83 ± 6.58, median; 24.44, minimum; 1.70, maximum; 74.13. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 68.11 ± 6.58, median; 75.57, minimum; 25.87, maximum; 98.30.

In the sample tube with MNC fraction measured at 4 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 32.13 ± 6.28, median; 24.63, minimum; 1.67, maximum; 72.91. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 67.86 ± 6.28, median; 75.37, minimum; 27.09, maximum; 98.33.

In the sample tube without MNC fraction measured at 4 hour, the percentages of CD59(+) granulocytes indicated, mean ± SEM; 31.54 ± 6.45, median; 24.76, minimum; 0.89, maximum; 72.58. The percentages of CD59(-) granulocytes indicated, mean ± SEM; 65.13 ± 7.03, median; 70.32, minimum; 25.24, maximum; 99.11.

Table 5 % CD59 population of PNH patients.

UPN	% CD59 population							
	0 hr				4 hr			
	With MNCs		Without MNCs		With MNCs		Without MNCs	
	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)
1	1.35	98.65	1.70	98.30	1.67	98.33	0.89	99.11
2	8.54	91.46	8.96	91.04	9.21	90.79	8.63	91.37
3	21.78	78.22	24.44	75.57	23.93	76.07	24.39	75.61
4	62.31	37.69	63.26	36.74	61.55	38.46	63.74	36.26
5	6.61	93.39	5.30	94.70	7.14	92.86	6.83	93.17
6	26.29	73.71	24.16	75.84	24.63	75.37	24.76	25.24
7	21.60	78.40	20.35	79.65	19.30	80.70	18.76	81.24
8	74.11	25.89	72.93	27.07	72.91	27.09	71.55	28.45
9	76.86	23.14	74.13	25.87	72.59	27.41	72.58	27.42
10	35.68	64.32	34.84	65.16	35.81	64.19	35.95	64.05
11	67.65	32.35	65.51	34.49	63.21	36.69	63.12	36.88
12	34.74	65.26	33.60	66.40	30.52	69.48	34.04	65.96
13	6.45	93.55	3.00	97.00	6.56	93.44	4.40	95.60
14	16.69	83.31	15.27	84.73	21.33	78.64	13.75	86.25
15	32.05	67.95	30.04	69.06	31.61	68.39	29.68	70.32
Mean	32.85	67.15	31.83	68.11	32.13	67.86	31.54	65.13
SEM	6.64	6.64	6.58	6.58	6.28	6.28	6.45	7.03
MD	26.29	73.71	24.44	75.57	24.63	75.37	24.76	70.32
Min	1.35	23.14	1.70	25.87	1.67	27.09	0.89	25.24
Max	76.86	98.65	74.13	98.30	72.91	98.33	72.58	99.11

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

The percentages of apoptosis in each target population were shown in Table 6 and 7. The variation of the percentages of apoptosis was pronounced among patients and normal controls.

Table 6 represents the percentages of apoptotsis of CD59(+) granulocytes in the sample tube with and without MNC fraction measured at 0 and 4 hour of 33 normal controls. In the sample tube with MNC fraction measured at 0 hour indicated, mean ± SEM; 0.68 ± 0.07, median; 0.60, minimum; 0.04, maximum; 1.55.

In the sample without MNC fraction measured at 0 hour indicated, mean ± SEM; 0.74 ± 0.11, median; 0.47, minimum; 0.12, maximum; 2.80.

In the sample with MNC fraction measured at 4 hour indicated, mean ± SEM; 2.10 ± 0.37, median; 1.36, minimum; 0.25, maximum; 9.86.

In the sample without MNC fraction measured at 4 hour indicated, mean ± SEM; 3.24 ± 0.52, median; 2.44, minimum; 0.23, maximum; 9.43.

Table 6 % Apoptosis of normal controls.

UPN	% Apoptosis			
	CD59(+)			
	0 hr		4 hr	
	With MNCs	Without MNCs	With MNCs	Without MNCs
1	0.46	0.69	1.53	2.88
2	0.60	0.26	1.36	1.17
3	0.04	0.12	0.49	6.90
4	0.28	0.41	5.53	6.84
5	0.34	1.45	0.66	1.43
6	0.29	0.25	4.10	2.44
7	0.59	0.45	3.55	5.94
8	0.68	0.58	4.03	5.06
9	1.20	0.47	2.99	6.02
10	0.33	0.35	3.82	7.54
11	0.66	0.22	3.53	8.31
12	0.50	1.64	6.47	9.41
13	0.96	2.80	2.46	2.70
14	1.21	0.19	0.69	0.30
15	1.55	2.00	9.86	9.43
16	0.22	0.33	1.41	3.02
17	0.81	0.81	2.71	6.93
18	1.25	2.26	0.48	3.99
19	0.53	1.59	1.53	2.70
20	1.46	0.99	1.53	1.32
21	0.69	0.55	1.18	4.06
22	1.48	0.44	0.98	0.45
23	0.54	0.36	0.47	0.41
24	0.64	0.40	0.44	0.64
25	0.30	0.17	1.10	0.23

Table 6 % Apoptosis of normal controls (Continued).

UPN	% Apoptosis			
	CD59(+)			
	0 hr		4 hr	
	With MNCs	Without MNCs	With MNCs	Without MNCs
26	0.94	1.08	0.95	0.32
27	0.43	0.43	1.50	0.66
28	0.22	0.53	0.43	1.01
29	0.23	0.35	0.25	0.94
30	1.19	0.74	1.33	1.25
31	0.79	0.66	0.62	0.81
32	0.93	0.16	0.50	0.35
33	0.23	0.61	0.89	1.36
Mean	0.68	0.74	2.10	3.24
SEM	0.07	0.11	0.37	0.52
MD	0.60	0.47	1.36	2.44
Min	0.04	0.12	0.25	0.23
Max	1.55	2.80	9.86	9.43

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

Table 7 represents the percentages of apoptosis of CD59(+) granulocytes and CD59(-) granulocytes in the sample tube with and without MNC fraction measured at 0 and 4 hour of 15 PNH patients. In the sample tube with MNC fraction measured at 0 hour, the percentages of apoptosis of CD59(+) granulocytes indicated, mean ± SEM; 1.78 ± 0.33, median; 1.28, minimum; 0.35, maximum; 4.30. The percentages of apoptosis of CD59(-) granulocytes indicated, mean ± SEM; 1.31 ± 0.27, median; 0.95, minimum; 0.22, maximum; 3.81.

In the sample tube without MNC fraction measured at 0 hour, the percentages of apoptosis of CD59(+) granulocytes indicated, mean ± SEM; 2.12 ± 0.47, median; 1.59, minimum; 0.59, maximum; 6.58. The percentages of apoptosis of CD59(-) granulocytes indicated, mean ± SEM; 1.20 ± 0.20, median; 0.96, minimum; 0.34, maximum; 3.57.

In the sample tube with MNC fraction measured at 4 hour, the percentages of apoptosis of CD59(+) granulocytes indicated, mean ± SEM; 3.35 ± 0.66, median; 2.25, minimum; 0.58, maximum; 10.12. The percentages of apoptosis of CD59(-) granulocytes indicated, mean ± SEM; 1.67 ± 0.29, median; 1.60, minimum; 0.34, maximum; 3.89.

In the sample tube without MNC fraction measured at 4 hour, the percentages of apoptosis of CD59(+) granulocytes indicated, mean ± SEM; 3.35 ± 0.67, median; 2.95, minimum; 0.38, maximum; 8.92. The percentages of apoptosis of CD59(-) granulocytes indicated, mean ± SEM; 1.82 ± 0.26, median; 1.62, minimum; 0.36, maximum; 3.51.

Table 7 % Apoptosis of PNH patients.

UPN	% Apoptosis							
	0 hr				4 hr			
	With MNCs		Without MNCs		With MNCs		Without MNCs	
	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)	CD59 (+)	CD59 (-)
1	1.89	0.76	3.25	0.71	4.35	1.65	8.06	3.51
2	1.17	0.44	1.59	0.94	2.41	0.47	1.71	0.93
3	3.34	2.63	1.71	1.07	1.88	2.00	1.29	0.87
4	0.77	0.95	0.64	0.74	0.58	0.34	0.38	0.36
5	1.18	0.78	1.11	0.34	5.99	1.25	6.39	2.59
6	2.14	1.14	2.63	1.45	1.31	0.58	3.02	2.67
7	4.30	3.01	1.18	0.96	4.73	3.89	4.60	2.95
8	0.35	0.59	0.63	0.79	1.25	1.74	1.57	2.31
9	1.28	0.57	2.13	1.14	1.76	1.42	1.59	1.62
10	0.35	0.60	1.06	1.37	2.12	2.27	1.20	1.24
11	3.16	3.81	0.66	0.78	1.85	1.60	1.87	1.91
12	0.96	1.18	0.59	0.90	3.24	2.81	3.20	3.28
13	0.40	0.22	6.58	1.31	10.12	0.87	8.92	0.90
14	3.48	1.32	5.58	3.57	6.48	3.64	2.95	1.00
15	1.92	1.72	2.44	1.93	2.25	0.51	3.53	1.18
Mean	1.78	1.31	2.12	1.20	3.35	1.67	3.35	1.82
SEM	0.33	0.27	0.47	0.20	0.66	0.29	0.67	0.26
MD	1.28	0.95	1.59	0.96	2.25	1.60	2.95	1.62
Min	0.35	0.22	0.59	0.34	0.58	0.34	0.38	0.36
Max	4.30	3.81	6.58	3.57	10.12	3.89	8.92	3.51

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

1.1. The relationship between hemoglobin (Hb) concentration and the percentages of apoptosis of granulocytes of PNH patients.

Correlation coefficient was determined to find out the relationship between Hb concentration and the percentages of apoptotic granulocytes of PNH patients by Pearson's correlation.

Table 8 represents Hb concentration and the percentages of apoptotic CD59(+) granulocytes of 15 PNH patients including 12 male patients and 3 female patients. Eleven of the twelve male patients and one of the three female patients had anemia (normal male Hb; 13.0-16.0 g/dl, normal female Hb; 12.0-16.0 g/dl).

Table 8 Hemoglobin (Hb) concentration and the percentages of apoptosis of CD59(+) granulocytes of PNH patients.

UPN	Sex	Hb concentration (g/dl)	% Apoptosis			
			CD59(+)			
			0 hour		4 hour	
			With MNCs	Without MNCs	With MNCs	Without MNCs
1	M	8.9	1.89	3.25	4.35	8.06
2	M	9.4	1.17	1.59	2.41	1.71
3	M	11.1	3.34	1.71	1.88	1.29
4	F	12.6	0.77	0.64	0.58	0.38
5	M	10.4	1.18	1.11	5.99	6.39
6	M	11.1	2.14	2.63	1.31	3.02
7	M	9.6	4.30	1.18	4.73	4.60
8	M	14.2	0.35	0.63	1.25	1.57
9	M	10.6	1.28	2.13	1.76	1.59
10	M	12.0	0.35	1.06	2.12	1.20
11	F	12.7	3.16	0.66	1.85	1.87
12	M	9.2	0.96	0.59	3.24	3.20
13	M	7.6	0.40	6.58	10.12	8.92
14	M	11.3	3.48	5.58	6.48	2.95
15	F	10.2	1.92	2.44	2.25	3.53

UPN; unique patient number, Hb; hemoglobin, g; gram, dl; decilitre, M; male, F; female

The relationship between Hb concentration and the percentages of apoptotic granulocytes measured up to 4 hours of PNH patients is shown in Figure 25. Only the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction and without MNC fraction measured at 4 hour had shown negative correlation with the Hb concentration of patients ($r = -0.649$, $P = 0.009$, and $r = -0.722$, $P = 0.002$, respectively). The percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction and without MNC fraction measured at 0 hour did not correlated with Hb concentration ($r = -0.025$, $P = 0.93$, and $r = -0.508$, $P = 0.053$, respectively).

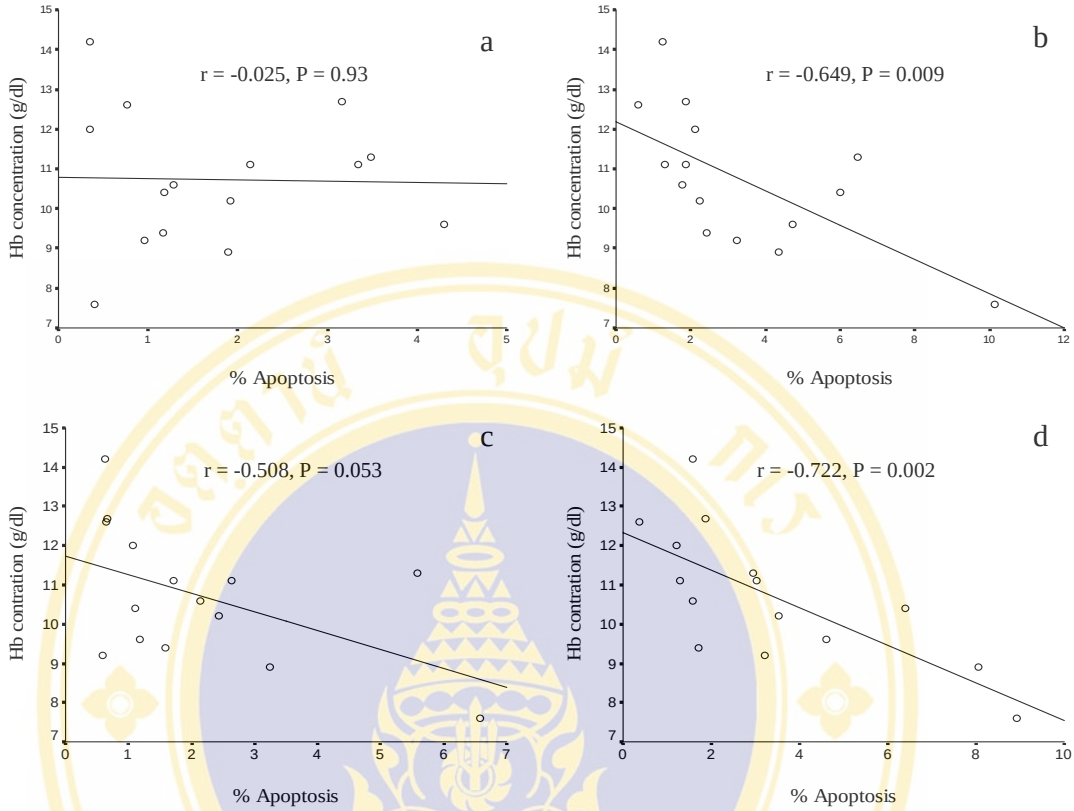


Figure 25 The relationship between hemoglobin (Hb) concentration (vertical axis) and the percentages of apoptosis of CD59(+) granulocytes (horizontal axis) of PNH patients (n = 15). The relationship between Hb concentrations and the percentages of apoptotic granulocytes of PNH patients was determined by Pearson's correlation. Scattergram demonstrated no correlation between Hb concentration and the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction measured at 0 hour (r = -0.025, P = 0.93) (a). (b) Hb concentration of patients demonstrated negative correlation with the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction measured at 4 hour (r = -0.649, P = 0.009). (c) Hb concentration of patients demonstrated no correlation with the percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction measured at 0 hour (r = -0.508, P = 0.053). (d) Hb concentration of patients demonstrated negative correlation with the percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction

measured at 4 hour, (r = -0.722, P = 0.002). Each dot indicates an individual patient.

Table 9 represents the Hb concentration and the percentages of apoptosis of CD59(-) granulocytes of 15 PNH patients.

Table 9 Hemoglobin (Hb) concentration and the percentages of apoptosis of CD59(-) granulocytes of PNH patients.

UPN	Sex	Hb concentration (g/dl)	% Apoptosis CD59(-)			
			0 hour		4 hour	
			With MNCs	Without MNCs	With MNCs	Without MNCs
1	M	8.90	0.76	0.71	1.65	3.51
2	M	9.40	0.44	0.94	0.47	0.93
3	M	11.10	2.63	1.07	2.00	0.87
4	F	12.60	0.95	0.74	0.34	0.36
5	M	10.40	0.78	0.34	1.25	2.59
6	M	11.10	1.14	1.45	0.58	2.67
7	M	9.60	3.01	0.96	3.89	2.95
8	M	14.20	0.59	0.79	1.74	2.31
9	M	10.60	0.57	1.14	1.42	1.62
10	M	12.00	0.60	1.37	2.27	1.24
11	F	12.70	3.81	0.78	1.60	1.91
12	M	9.20	1.18	0.90	2.81	3.28
13	M	7.60	0.22	1.31	0.87	0.90
14	M	11.30	1.32	3.57	3.64	1.00
15	F	10.20	1.72	1.93	0.51	1.18

UPN; unique patient number, Hb; hemoglobin, g; gram, dl; decilitre, M; male, F; female

There was no correlation between the percentages of apoptosis of CD59(-) granulocytes in the sample tube with MNC fraction or without MNC fraction measured at 0 hour and Hb concentration of the patients ($r = 0.208$, $P = 0.46$, and $r = 0.002$, $P = 1.0$, respectively). Finally, the percentages of apoptosis of CD59(-) granulocytes in the sample tube with MNC fraction and without MNC fraction measured at 4 hour also showed no correlation with Hb concentration of the patients ($r = -0.004$, $P = 0.99$, and $r = -0.190$, $P = 0.50$, respectively) (Figure 26).

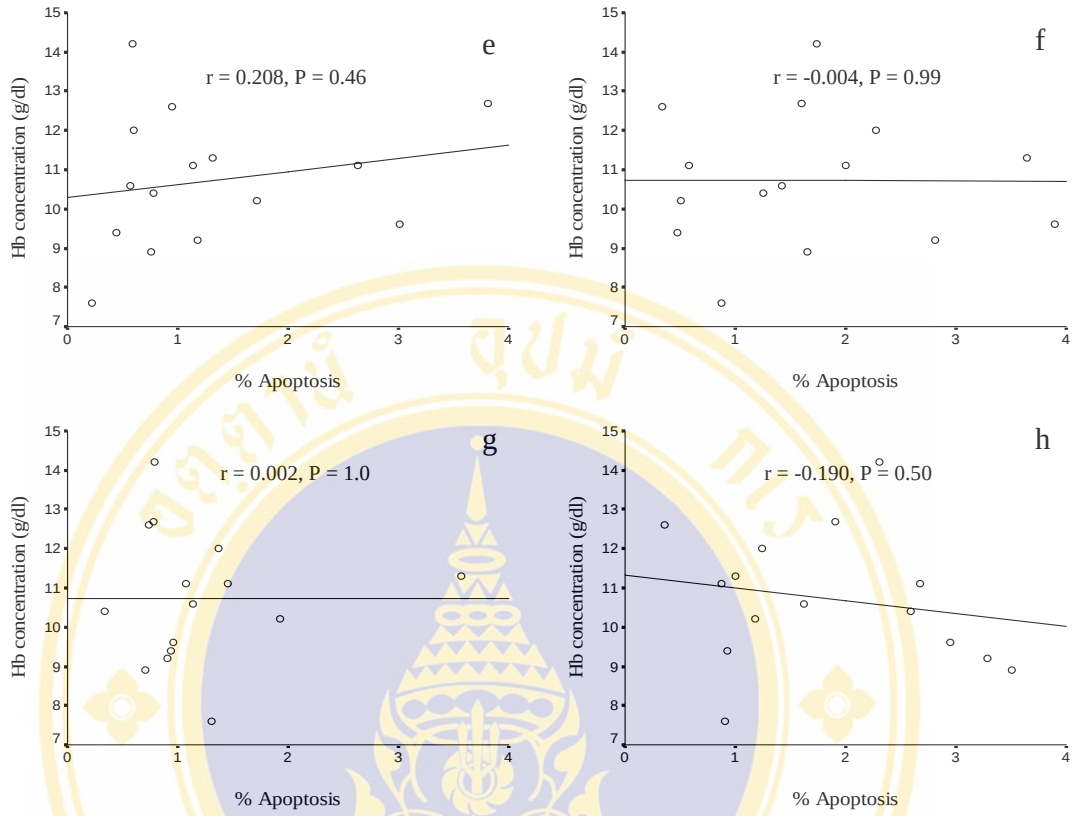


Figure 26 The relationship between hemoglobin (Hb) concentration (vertical axis) and the percentages of apoptosis of CD59(-) granulocytes (horizontal axis) of PNH patients (n = 15). The relationship between Hb concentrations and the percentages of apoptotic granulocytes of PNH patients was determined by Pearson's correlation. Scattergram demonstrated no correlation between Hb concentration and the percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction measured at 0 hour, (r = 0.208, P = 0.46) (e). (f) Hb concentration of patients demonstrated no correlation with the percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction measured at 4 hour, (r = -0.004, P = 0.99). (g) Hb concentration of patients demonstrated no correlation with the percentages of apoptotic CD59(-) granulocytes in the sample tube without MNC fraction measured at 0 hour, (r = 0.002, P = 1.0). (h) Hb concentration of patients demonstrated no correlation with the percentages of apoptotic CD59(-) granulocytes in the sample tube

without MNC fraction measured at 4 hour, (r = -0.190, P = 0.50). Each dot indicates an individual patient.

1.2. The effect of time to apoptosis of granulocytes.

We assessed the percentages of apoptosis of granulocytes after culture these cells in liquid growth system for 0 and 4 hours. The observation of the effect of time in 33 normal controls demonstrated an increase in the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction when incubated the culture up to 4 hours in 27 normal controls (81.82%). The comparison of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction between 0 hour and 4 hour indicated significant difference (mean ± SEM; 0.68 ± 0.07, median; 0.60, for 0 hour vs mean ± SEM; 2.10 ± 0.37, median; 1.36, for 4 hour, $P = 0.0002$) (Figure 27a). In the sample tube without MNC fraction, 30 normal controls (90.91%) showed an increase in the percentages of apoptotic CD59(+) granulocytes when incubated the culture up to 4 hours. The comparison of the percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction between 0 hour and 4 hour indicated the significant difference (mean ± SEM; 0.74 ± 0.11, median; 0.47, for 0 hour vs mean ± SEM; 3.24 ± 0.52, median; 2.44, for 4 hour, $P < 0.0001$) (Figure 27b).

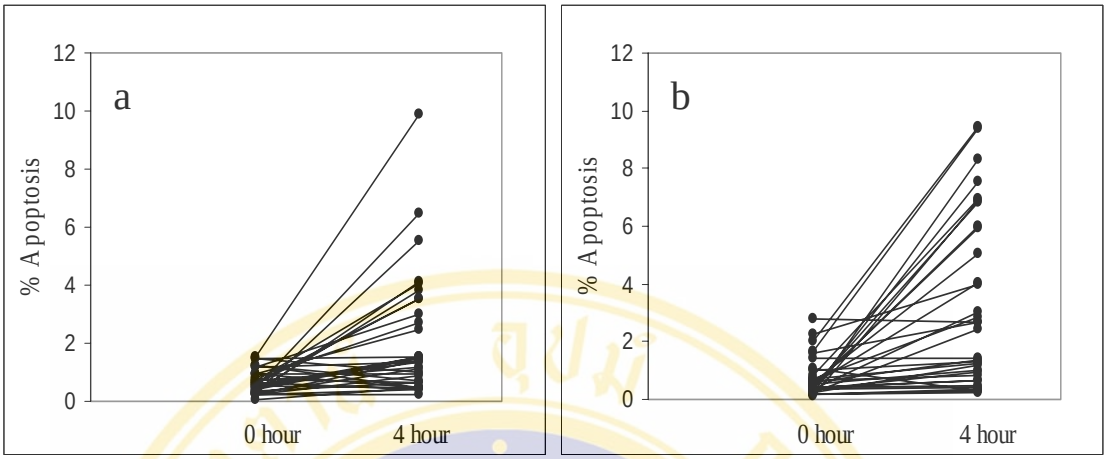


Figure 27 The effect of time to apoptosis of granulocytes in normal controls. Scattergram demonstrated flow cytometry analysis for apoptosis of granulocytes of normal controls (n = 33) measured at 0 and 4 hour. (a) The percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction compared between 0 and 4 hour in normal controls (mean ± SEM; 0.68 ± 0.07, median; 0.60, for 0 hour vs mean ± SEM; 2.10 ± 0.37, median; 1.36, for 4 hour, P = 0.0002). (b) The percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction compared between 0 and 4 hour in normal controls (mean ± SEM; 0.74 ± 0.11, median; 0.47, for 0 hour vs mean ± SEM; 3.24 ± 0.52, median; 2.44, for 4 hour, P < 0.0001). The comparison was determined by Wilcoxon signed-rank test. Each pair of dots indicate an individual patient.

The observation of the effect of time in the 15 PNH patients demonstrated that 11 PNH patients (73.33%) had an increase in the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction when incubated the culture up to 4 hours. The comparison of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction between 0 hour and 4 hour indicated significant difference (mean ± SEM; 1.78 ± 0.33, median; 1.28, for 0 hour vs mean ± SEM; 3.35 ± 0.66, median; 2.25, for 4 hour, P = 0.04) (Figure 28c). In the sample tube without MNC fraction, CD59(+) granulocytes from 11 PNH patients (73.33%) showed a tendency of increased apoptosis after culture for 4 hours. However, this difference was not observed in the other four patients. The comparison of the percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction between 0 hour and 4 hour indicated no significant difference (mean ± SEM; 2.12 ± 0.47, median; 1.59, for 0 hour vs mean ± SEM; 3.35 ± 0.67, median; 2.95, for 4 hour, P = 0.053) (Figure 28d).

Moreover, in 10 PNH patients (66.67%), the percentages of apoptotic CD59(-) granulocytes in the sample tubes with MNC fraction when incubated the cultures up to 4 hours were increased. However, comparison of the percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction between 0 hour and 4 hour indicated no significant difference (mean ± SEM; 1.31 ± 0.27, median; 0.95, for 0 hour vs mean ± SEM; 1.67 ± 0.29, median; 1.60, for 4 hour, P = 0.19) (Figure 28e). Finally, in the sample tubes without MNC fraction, 8 PNH patients (53.33%) had an increase in the percentages of apoptotic of CD59(-) granulocytes after incubation of the culture for 4 hours. While the other 7 PNH patients (46.67%) did not shown. Comparison of the percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction between 0 hour and 4 hour did not show this phenomenon. (mean ± SEM; 1.20 ± 0.20, median; 0.96, for 0 hour vs mean ± SEM; 1.82 ± 0.26, median; 1.62, for 4 hour, P = 0.17) (Figure 28f).

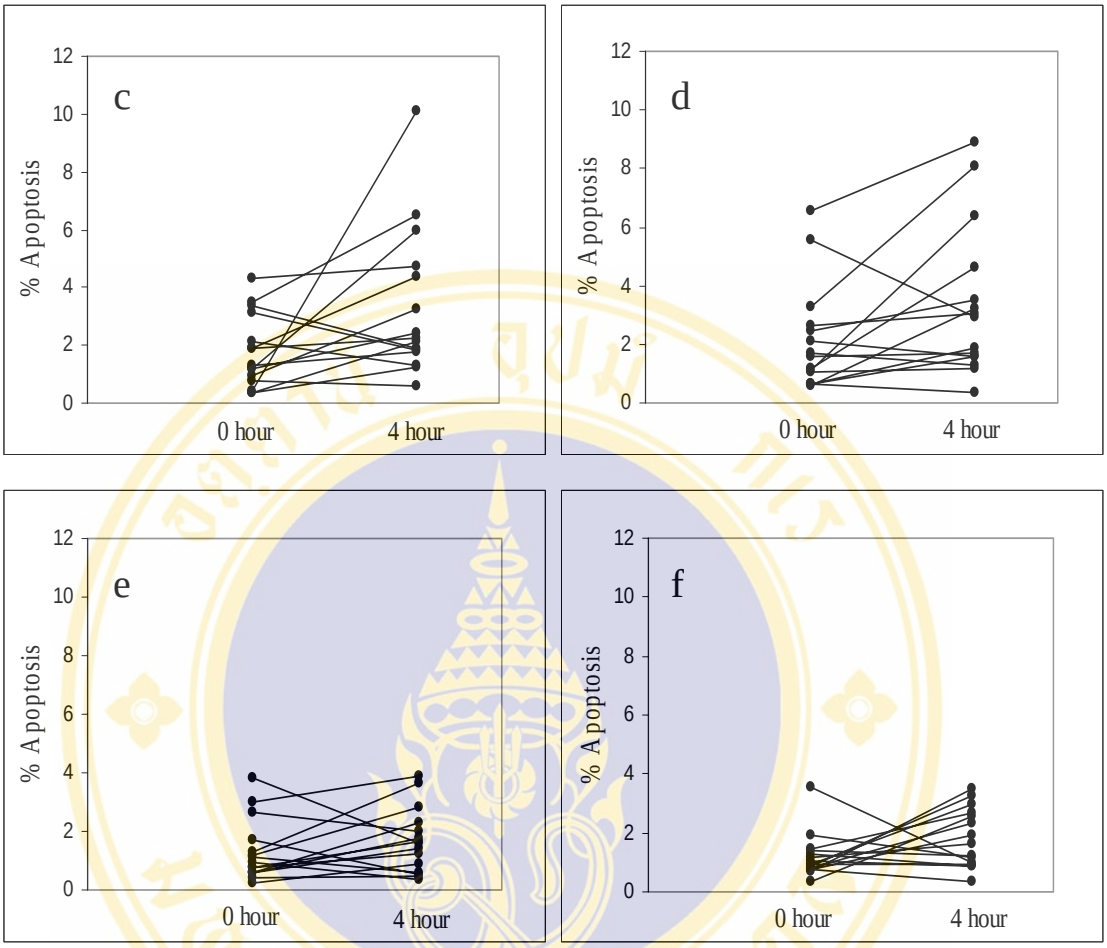


Figure 28 The effect of time to apoptosis of granulocytes in PNH patients. Scattergram demonstrated flow cytometry analysis for apoptosis of granulocytes of PNH patients (n = 15) measured at 0 and 4 hour. (c) The percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction compared between 0 and 4 hour in PNH patients (mean ± SEM; 1.78 ± 0.33, median; 1.28, for 0 hour vs mean ± SEM; 3.35 ± 0.66, median; 2.25, for 4 hour, P = 0.04). (d) The percentages of apoptotic CD59(+) granulocytes in the sample tube without MNC fraction compared between 0 and 4 hour in PNH patients (mean ± SEM; 2.12 ± 0.47, median; 1.59, for 0 hour vs mean ± SEM; 3.35 ± 0.67, median; 2.95, for 4 hour, P = 0.053). (e) The percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction compared between 0 and 4 hour in PNH patients (mean ± SEM; 1.31 ± 0.27, median; 0.95, for 0 hour vs mean ± SEM; 1.67 ± 0.29, median; 1.60, for 4 hour, P = 0.19).

(f) The percentages of apoptotic CD59(-) granulocytes in the sample tube without MNC fraction compared between 0 and 4 hour in PNH patients (mean ± SEM; 1.20 ± 0.20, median; 0.96, for 0 hour vs mean ± SEM; 1.82 ± 0.26, median; 1.62, for 4 hour, P = 0.17). The comparison was determined by Wilcoxon signed-rank test. Each pair of dots indicate an individual patient.

1.3 Comparison of apoptosis of CD59(+) and CD59(-) granulocytes in PNH patients.

To investigate and prove that only CD59(+) cells are selected to destroy, apoptosis of CD59(+) granulocytes and CD59(-) granulocytes in 15 PNH patients were also compared in the same sample tube. The study in the sample tube with MNC fraction measured at 0 hour demonstrated that 5 PNH patients (33.33%) were increased in apoptosis of CD59(+) granulocytes than CD59(-) granulocytes. The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes indicated significant difference (mean ± SEM; 1.78 ± 0.33, median; 1.28, for CD59(+) granulocytes vs mean ± SEM; 1.31 ± 0.27, median; 0.95, for CD59(-) granulocytes, P = 0.04) (Figure 29a). In the sample tube with MNC fraction measured at 4 hour, 3 PNH patients (20%) were increased in apoptosis of CD59(+) granulocytes than CD59(-) granulocytes. The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes indicated significant difference (mean ± SEM; 3.35 ± 0.66, median; 2.25, for CD59(+) granulocytes vs mean ± SEM; 1.67 ± 0.29, median; 1.60, for CD59(-) granulocytes, P = 0.005) (Figure 29b).

The study in the sample tube without MNC fraction measured at 0 hour demonstrated that 5 PNH patients (33.33%) were increased in apoptosis of CD59(+) granulocytes than CD59(-) granulocytes. The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes indicated significant difference (mean ± SEM; 2.12 ± 0.47, median; 1.59, for CD59(+) granulocytes vs mean ± SEM; 1.20 ± 0.20, median; 0.96, for CD59(-) granulocytes, P = 0.01) (Figure 29c). In the sample tube without MNC fraction measured at 4 hour, 5 PNH patients (33.33%) were increased in apoptosis of CD59(+) granulocytes than CD59(-) granulocytes. The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes indicated significant difference (mean ± SEM; 3.35 ± 0.67, median; 2.95, for CD59(+) granulocytes vs mean ± SEM; 1.82 ± 0.26, median; 1.62, for CD59(-) granulocytes, P = 0.03) (Figure 29d).

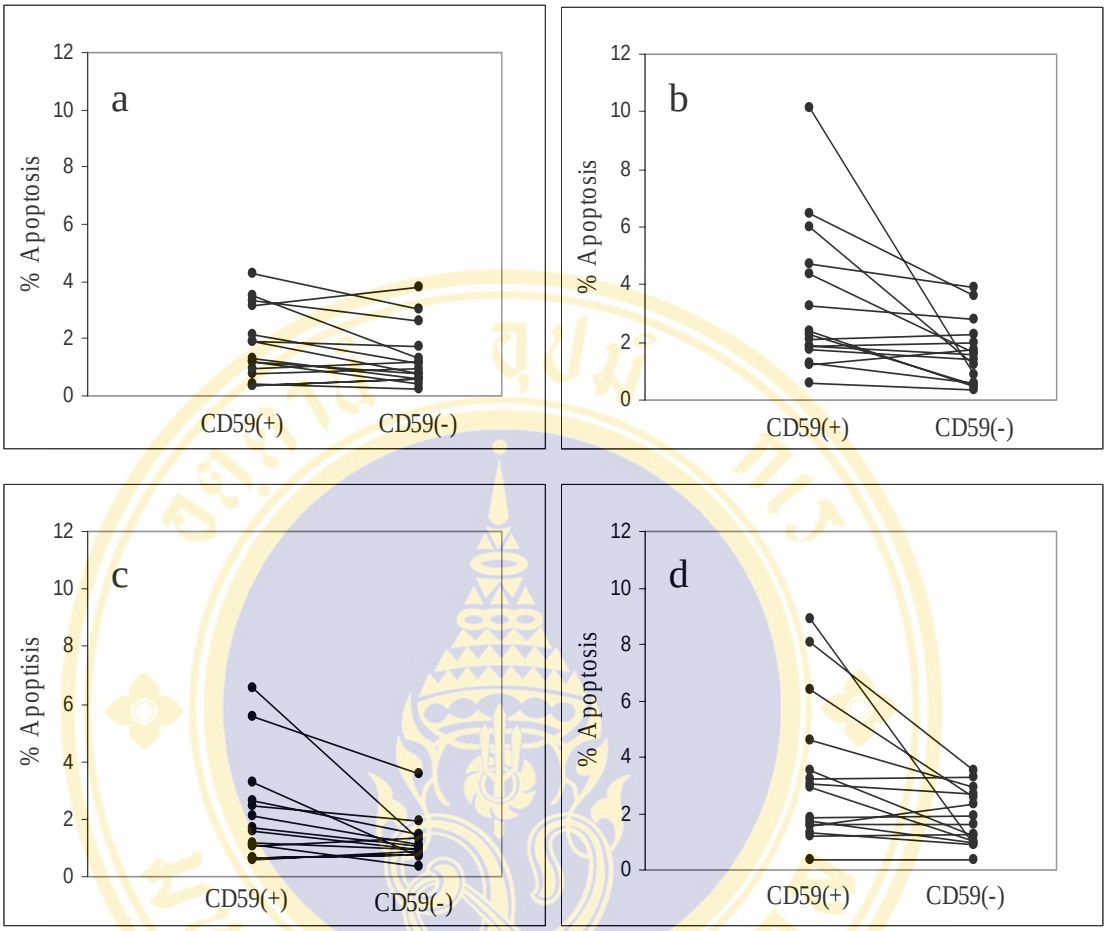


Figure 29 Comparison of apoptosis of CD59(+) and CD59(-) granulocytes in PNH patients. Scattergrams demonstrated flow cytometry analysis of the percentages of apoptotic of CD59(+) and CD59(-) granulocytes after cultured in liquid growth culture system up to 4 hours in PNH patients (n = 15). The percentages of apoptotic of CD59(+) granulocytes and CD59(-) granulocytes was compared by Wilcoxon signed-rank test.

(a) The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes in the sample tube with MNC fraction measured at 0 hour (mean ± SEM; 1.78 ± 0.33, median; 1.28, for CD59(+) granulocytes vs mean ± SEM; 1.31 ± 0.27, median; 0.95, for CD59(-) granulocytes, P = 0.04). (b) The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes in the sample tube with MNC fraction measured at 4 hour (mean ± SEM; 3.35 ± 0.66, median; 2.25, for CD59(+) granulocytes vs mean ± SEM; 1.67 ± 0.29, median; 1.60, for CD59(-) granulocytes, P = 0.005). (c) The

comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes in the sample tube without MNC fraction measured at 0 hour (mean ± SEM; 2.12 ± 0.47, median; 1.59, for CD59(+) granulocytes vs mean ± SEM; 1.20 ± 0.20, median; 0.96, for CD59(-) granulocytes, P = 0.01). (d) The comparison of the percentages of apoptosis between CD59(+) granulocytes and CD59(-) granulocytes in the sample tube without MNC fraction measured at 4 hour (mean ± SEM; 3.35 ± 0.67, median; 2.95, for CD59(+) granulocytes vs mean ± SEM; 1.82 ± 0.26, median; 1.62, for CD59(-) granulocytes, P = 0.03). Each pair of dots indicate an individual patient.

1.4. The effect of mononuclear cells (MNCs)

As in method, the study was performed in two different sample tube, with or without MNC fraction to point out cell mediated apoptosis. We investigated the effect of MNCs mediated granulocyte apoptosis in both in the different sample tube and in the same sample tube in 33 normal controls and 15 PNH patients.

In normal controls, 15 normal controls (45.45%) indicated the higher of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction than in the sample tube without MNC fraction measured at 0 hour. The comparison of the percentages of apoptotic of CD59(+) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 0 hour indicated no significant difference (mean ± SEM; 0.68 ± 0.07, median; 0.60, for the sample tube with MNC fraction vs mean ± SEM; 0.74 ± 0.11, median; 0.47, for the sample tube without MNC fraction, P = 0.96) (Figure 30a). The percentages of apoptotic of CD59(+) granulocytes measured at 4 hour demonstrated that 11 normal controls (33.33%) indicated the higher of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction than in the sample tube without MNC fraction. The comparison of the percentages of apoptosis of CD59(+) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 4 hour indicated significant difference (mean ± SEM; 2.10 ± 0.37, median; 1.36, for the sample tube with MNC fraction vs mean ± SEM; 3.24 ± 0.52, median; 2.44, for the sample tube without MNC fraction, P = 0.005) (Figure 30b).

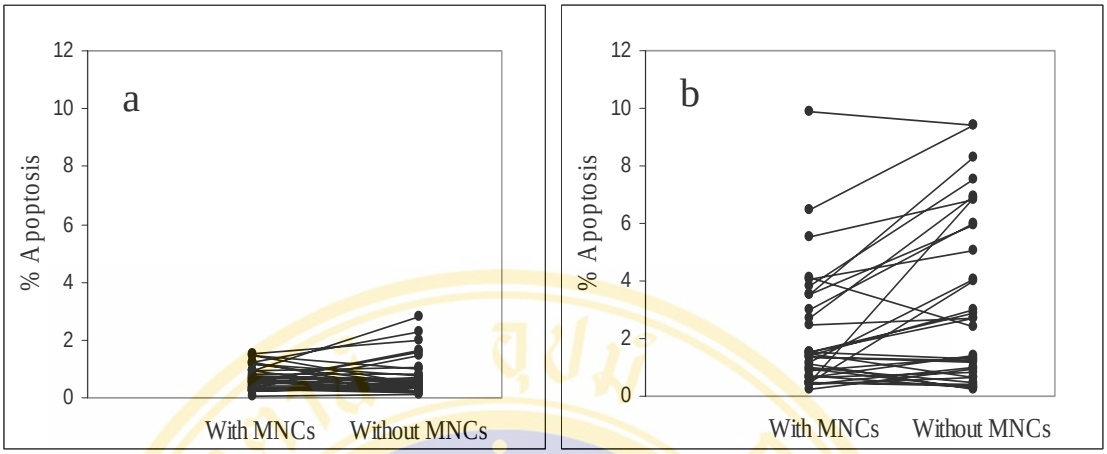


Figure 30 The effect of mononuclear cells (MNCs) in normal controls. Scattergram demonstrated flow cytometry analysis of the percentages of apoptosis of granulocytes in the sample tube with and without MNC fraction after cultured in liquid growth culture system up to 4 hours in normal controls (n = 33). The percentages of apoptosis of granulocytes in the sample tube with MNC fraction and without MNC fraction was compared by Wilcoxon signed-rank test. (a) The comparison of the percentages of apoptotic CD59(+) granulocytes between in the sample tube with and without MNC fraction measured at 0 hour (mean ± SEM; 0.68 ± 0.07, median; 0.60, for the sample tube with MNC fraction vs mean ± SEM; 0.74 ± 0.11, median; 0.47, for the sample tube without MNC fraction, P = 0.96). (b) The comparison of the percentages of apoptotic CD59(+) granulocytes between in the sample tube with and without MNC fraction measured at 4 hour (mean ± SEM; 2.10 ± 0.37, median; 1.36, for the sample tube with MNC fraction vs mean ± SEM; 3.24 ± 0.52, median; 2.44, for the sample tube without MNC fraction, P = 0.005). Each pair of dots indicate an individual patient.

In PNH patients, 6 PNH patients (40%) indicated the higher of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction than in the sample tube without MNC fraction measured at 0 hour. The comparison of the percentages of apoptosis of CD59(+) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 0 hour indicated no significant difference (mean ± SEM; 1.78 ± 0.33, median; 1.28 for the sample tube with MNC fraction vs mean ± SEM; 2.12 ± 0.47, median; 1.59, for the sample tube without MNC fraction, P = 0.39) (Figure 31c). The percentages of apoptosis of CD59(+) granulocytes measured at 4 hour demonstrated that 9 PNH patients (60%) indicated the higher of the percentages of apoptotic CD59(+) granulocytes in the sample tube with MNC fraction than in the sample tube without MNC fraction. The comparison of the percentages of apoptosis of CD59(+) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 4 hour indicated no significant difference (mean ± SEM; 3.35 ± 0.66, median; 2.25, for sample tube with MNC fraction vs mean ± SEM; 3.35 ± 0.67, median; 2.95, for sample tube without MNC fraction, P = 0.73) (Figure 31d).

In addition, the percentages of apoptosis of CD59(-) granulocytes in the sample tube with MNC fraction measured at 0 hour in 7 PNH patients (46.67%) indicated higher than in the sample tube without MNC fraction. The comparison of the percentages of apoptosis of CD59(-) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 0 hour indicated no significant difference (mean ± SEM; 1.31 ± 0.27, median; 0.95, for sample tube with MNC fraction vs mean ± SEM; 1.20 ± 0.20, median; 0.96, for sample tube without MNC fraction, P = 0.84) (Figure 31e). The percentages of apoptosis of CD59(-) granulocytes measured at 4 hour demonstrated that 4 PNH patients (26.67%) indicated the higher of the percentages of apoptotic CD59(-) granulocytes in the sample tube with MNC fraction than in the sample tube without MNC fraction. The comparison of the percentages of apoptosis of CD59(-) granulocytes between in the sample tube with MNC fraction and in the sample tube without MNC fraction measured at 4 hour indicated no significant difference (mean ± SEM; 1.67 ± 0.29, median; 1.60, for sample tube with MNC fraction vs mean ± SEM;

1.82 ± 0.26, median; 1.62, for sample tube without MNC fraction, P = 0.39) (Figure 31f).

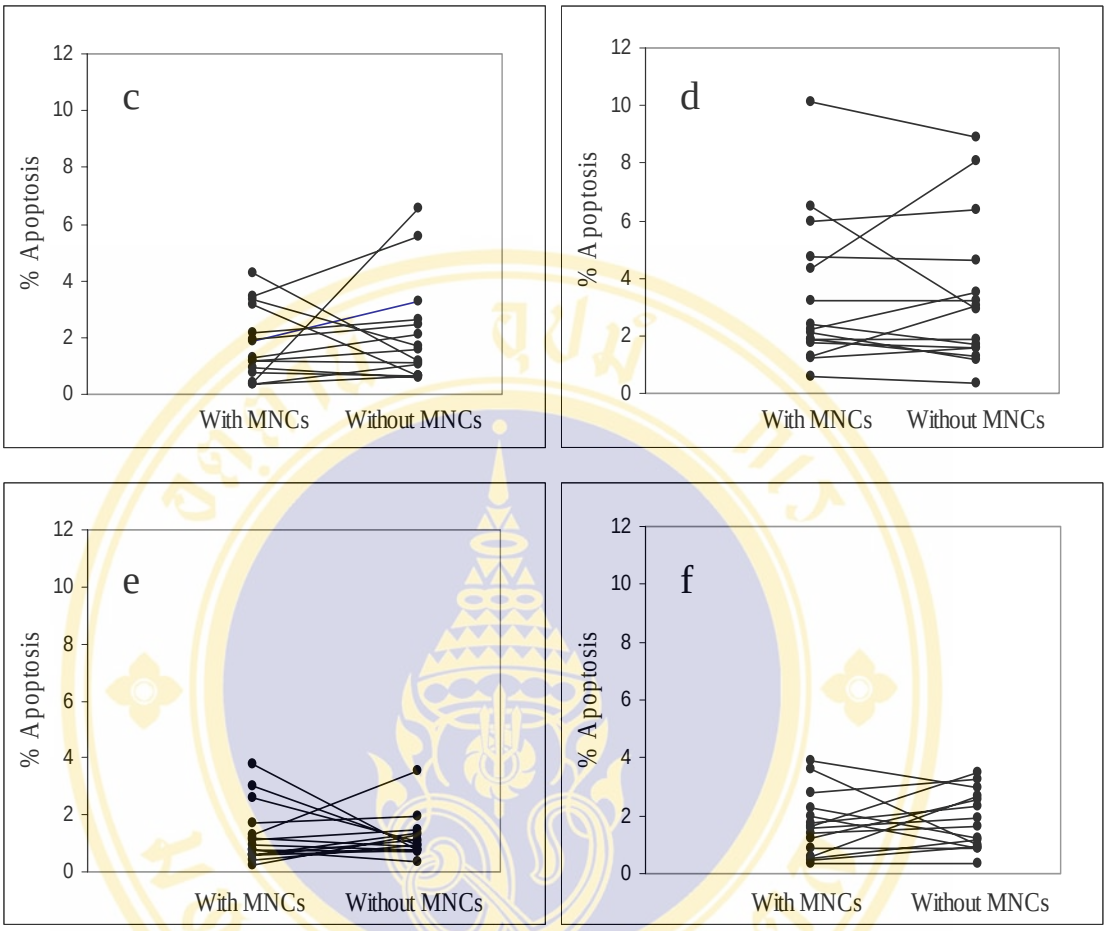


Figure 31 The effect of mononuclear cells (MNCs) in PNH patients. Scattergram demonstrated flow cytometry analysis of the percentages of apoptosis of granulocytes in the sample tube with and without MNC fraction after cultured in liquid growth culture system up to 4 hours in PNH patients (n = 15). The percentages of apoptosis of granulocytes in the sample tube with MNC fraction and without MNC fraction was compared by Wilcoxon signed-rank test. (c) The comparison of the percentages of apoptotic CD59(+) granulocytes between in the sample tube with and without MNC fraction measured at 0 hour (mean ± SEM; 1.78 ± 0.33, median; 1.28 for the sample tube with MNC fraction vs mean ± SEM; 2.12 ± 0.47, median; 1.59, for the sample tube without MNC fraction, P = 0.39). (d) The comparison of the percentages of apoptotic CD59(+) granulocytes between in the sample tube with and without MNC fraction measured at 4 hour (mean ± SEM; 3.35 ± 0.66, median; 2.25, for sample

tube with MNC fraction vs mean ± SEM; 3.35 ± 0.67, median; 2.95, for sample tube without MNC fraction, P = 0.73). (e) The comparison of the percentages of apoptotic CD59(-) granulocytes between in the sample tube with and without MNC fraction measured at 0 hour (mean ± SEM; 1.31 ± 0.27, median; 0.95, for sample tube with MNC fraction vs mean ± SEM; 1.20 ± 0.20, median; 0.96, for sample tube without MNC fraction, P = 0.84). (f) The comparison of the percentages of apoptotic CD59(-) granulocytes between in the sample tube with and without MNC fraction measured at 4 hour (mean ± SEM; 1.67 ± 0.29, median; 1.60, for sample tube with MNC fraction vs mean ± SEM; 1.82 ± 0.26, median; 1.62, for sample tube without MNC fraction, P = 0.39). Each pair of dots indicate an individual patient.

When explored apoptosis in the same sample tube as indicated above, only CD59(+) granulocytes in the sample tube with MNC fraction were shown to increased in apoptosis with time (Figure 28c). Furthermore, although in both sample tube with MNC fraction or without MNC fraction, CD59(+) granulocytes showed significant increase in apoptosis over CD59(-) granulocytes with time (Figure 29), but at 4 hour, apoptosis of CD59(+) granulocytes compared with CD59(-) granulocytes in the sample tube with MNC fraction showed more statistically significant than in the sample tube without MNC fraction (in the sample tube with MNC fraction; P = 0.005, in the sample tube without MNC fraction; P = 0.03, at 4 hour, respectively).

1.5 Comparison between PNH patients and normal controls.

The percentages of apoptosis of CD59(+) granulocytes in PNH patients were found to be statistically higher than that of normal controls when compared in the sample tube with MNC fraction measured at 0 hour (mean ± SEM; 1.78 ± 0.33, median; 1.28; for PNH patients vs mean ± SEM; 0.68 ± 0.07, median; 0.60; for normal controls; P = 0.002) (Figure 32a) and in the sample tube with MNC fraction measured at 4 hour (mean ± SEM; 3.35 ± 0.66, median; 2.25; for PNH patients vs mean ± SEM; 2.10 ± 0.37, median; 1.36; for normal controls; P = 0.02) (Figure 32b). Moreover, The percentages of apoptosis of CD59(+) granulocytes in PNH patients were also found to be statistically higher than that of normal controls when compared in the sample tube without MNC fraction measured at 0 hour (mean ± SEM; 2.12 ± 0.47, median; 2.12; for PNH patients vs mean ± SEM; 0.74 ± 0.11, median; 0.74; for normal controls; P = 0.0002) (Figure 32c). However, the percentages of apoptosis of CD59(+) granulocytes of PNH patients at 4 hour showed no difference from normal controls when compared in the sample tube without MNC (mean ± SEM; 3.24 ± 0.52, median; 2.44; for PNH patients vs mean ± SEM; 0.74 ± 0.11, median; 0.74; for normal controls; P = 0.40) (Figure 32d).

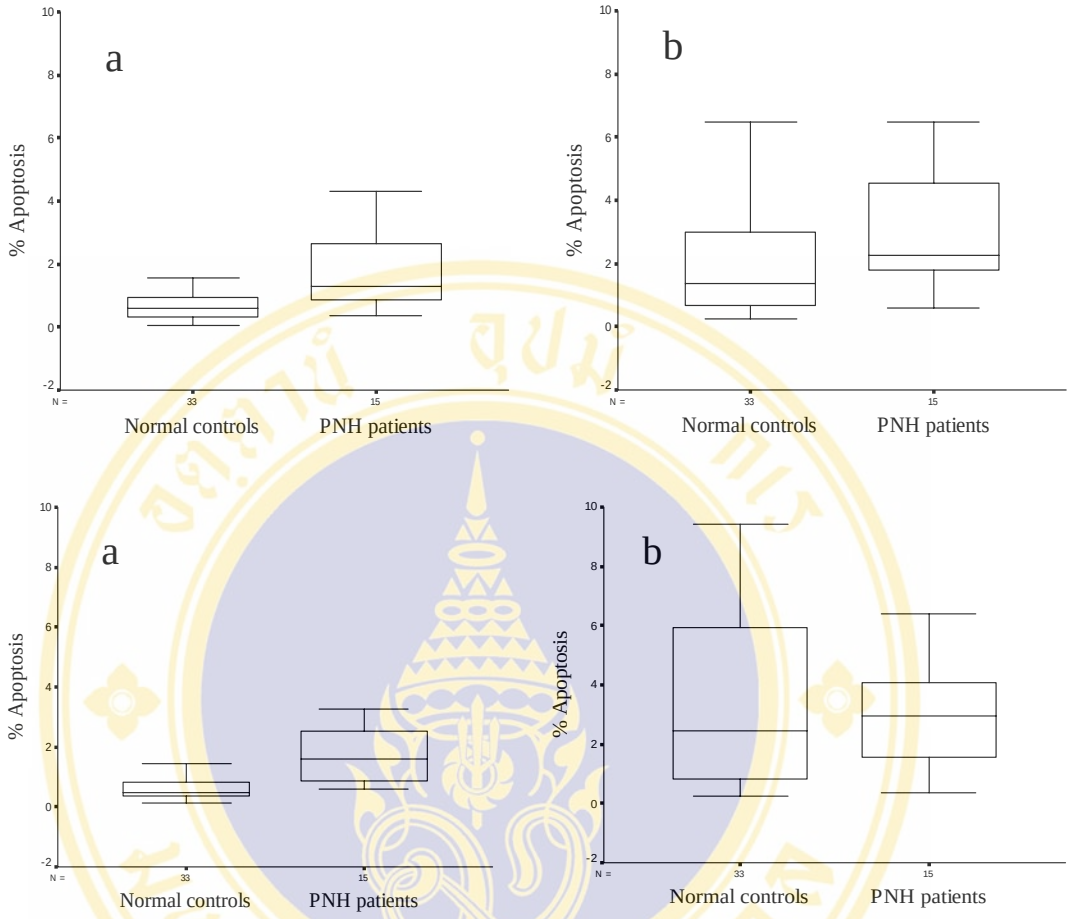


Figure 32 The percentages of apoptosis of CD59(+) granulocytes compared between PNH patients and normal controls. The comparison of apoptosis of CD59(+) granulocytes between PNH patients (n = 15) and normal controls (n = 33) was determined by Mann-Whitney test. (a) The comparison of apoptosis of CD59(+) granulocytes in the sample tube with MNC fraction measured at 0 hour (mean ± SEM; 1.78 ± 0.33, median; 1.28; for PNH patients vs mean ± SEM; 0.68 ± 0.07, median; 0.60; for normal controls; P = 0.002). (b) The comparison of apoptosis of CD59(+) granulocytes in the sample tube with MNC fraction measured at 4 hour (mean ± SEM; 3.35 ± 0.66, median; 2.25; for PNH patients vs mean ± SEM; 2.10 ± 0.37, median; 1.36; for normal controls; P = 0.02). (c) The comparison of apoptosis of CD59(+) granulocytes in the sample tube without MNC fraction measured at 0 hour (mean ± SEM; 2.12 ± 0.47, median; 2.12; for PNH patients vs mean ± SEM; 0.74 ± 0.11, median; 0.74; for normal controls; P = 0.0002).

(d) The comparison of apoptosis of CD59(+) granulocytes in the sample tube without MNC fraction measured at 4 hour (mean ± SEM; 3.24 ± 0.52, median; 2.44; for PNH patients vs mean ± SEM; 0.74 ± 0.11, median; 0.74; for normal controls; P = 0.40). Box plot represents; median, and 25th-75th percentile.

2. Flow cytometry study of CTLs, NKT cells and NK cells.

Because of the closed association between PNH and other BM failure disease with cell mediated autoimmune process, CTLs, NKT cells or NK cells were possible candidates to be an extrinsic factor that involved in CD59(+) cells selection resulting in the expansion of PNH clone. Using flow cytometry based immunocytometric method, we analysed the percentages of $CD8^+$ T cells (cytotoxic T lymphocytes; CTLs), NKT cells and NK cells of 15 normal controls and 26 PNH patients.

Table 8 represents the percentages of $CD8^+$ T cells compared between 15 normal controls and 26 PNH patients: the percentages of $CD8^+$ T cells of normal controls, mean ± SEM; 30.65 ± 1.86, median; 29.16, minimum; 21.80, maximum; 47.74, the percentages of $CD8^+$ T cells of PNH patients, mean ± SEM; 34.96 ± 1.56, median; 36.19, minimum; 20.11, maximum; 50.99. The comparison of the percentages of $CD8^+$ T cells was determined by Mann-Whitney test, P = 0.07.

Table 10 The percentages of CTLs of normal controls and PNH patients.

UPN	% CTLs	
	Normal controls	PNH patients
1	33.20	28.92
2	33.36	20.11
3	29.11	31.11
4	26.26	50.48
5	21.80	32.75
6	35.04	31.71
7	23.45	42.01
8	25.54	26.34
9	47.74	30.97
10	21.82	41.84
11	29.36	28.24
12	28.26	43.96
13	29.16	50.99
14	33.93	38.90
15	41.78	23.28
16		37.19
17		37.10
18		41.47
19		36.96
20		29.52
21		42.24
22		24.21
23		26.93
24		35.41
25		38.59
26		37.70

Table 10 The percentages of CTLs of normal controls and PNH patients (Continued).

UPN	% CTLs	
	Normal controls	PNH patients
Mean	30.65	34.96
SEM	1.86	1.56
MD	29.16	36.19
Min	21.80	20.11
Max	47.74	50.99
P-value	0.07	

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

We found that the percentages of CTLs were not different when compared PNH patients with normal controls (mean ± SEM; 34.96 ± 1.56, median; 36.19, for PNH patients vs mean ± SEM; 30.65 ± 1.86, median; 29.16, for normal controls; P = 0.07) (Figure 33).

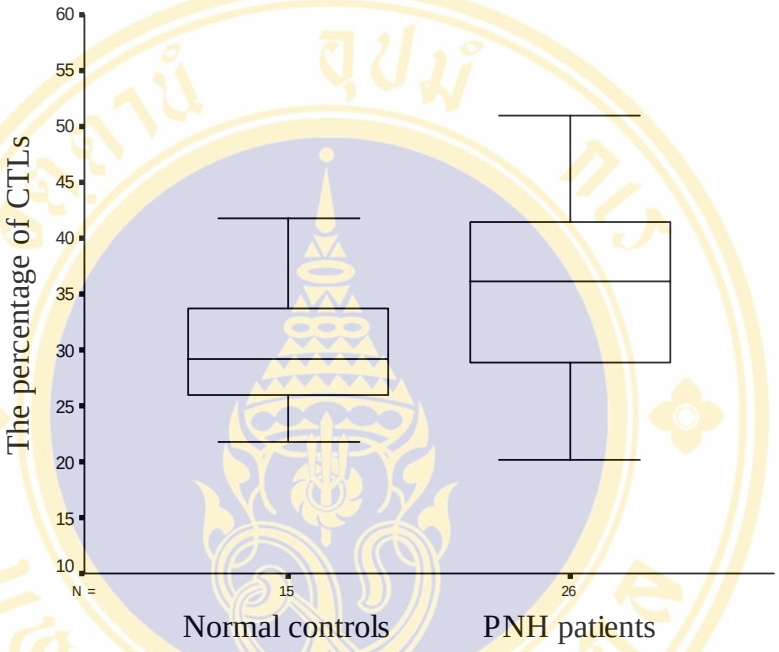


Figure 33 Comparison of the percentages of CTLs between normal controls (mean ± SEM; 30.65 ± 1.86, median; 29.16, n = 15) and PNH patients (mean ± SEM; 34.96 ± 1.56, median; 36.19; 8.83, n = 26) by Mann-Whitney test, P = 0.07. Box plot represents; median, and 25th-75th percentile.

We further assessed the percentages of NKT cells (Table 9) in both PNH patients and normal controls.

Table 9 represents the percentages of NKT cells compared between 15 normal controls and 26 PNH patients: the percentages of NKT cells of normal controls, mean ± SEM; 7.24 ± 0.87, median; 6.35, minimum; 2.39, maximum; 15.34, the percentages of NKT cells of PNH patients, mean ± SEM; 10.18 ± 1.08, median; 8.83, minimum; 3.64, maximum; 26.79. The comparison of the percentages of NKT cells was determined by Mann-Whitney test, P = 0.08.

Table 11 The percentages of NKT cells of normal controls and PNH patients.

UPN	% NKT cells	
	Normal controls	PNH patients
1	15.34	4.98
2	3.58	19.1
3	9.45	20.67
4	4.09	12.53
5	6.35	6.63
6	7.47	9.26
7	5.69	6.89
8	6.14	14.07
9	11.61	5.52
10	8.67	26.79
11	7.72	8.73
12	9.65	12.55
13	2.39	9.14
14	6.34	11.29
15	4.11	8.78
16		7.19
17		9.65
18		6.07
19		4.63
20		16.33
21		12.01
22		3.64
23		6.99
24		8.88
25		6.56
26		5.89

Table 11 The percentages of NKT cells of normal controls and PNH patients (Continued).

UPN	% NKT cells	
	Normal controls	PNH patients
Mean	7.24	10.18
SEM	0.87	1.08
MD	6.35	8.83
Min	2.39	3.64
Max	15.34	26.79
P-value	0.08	

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

The similar result was shown in the comparison of the percentages of NKT cells between PNH patients and normal controls, that there was no significant different (mean ± SEM; 10.18 ± 1.08, median; 8.83, for PNH patients vs mean ± SEM; 7.24 ± 0.87, median; 6.35, for normal controls; P = 0.08) (Figure 34).

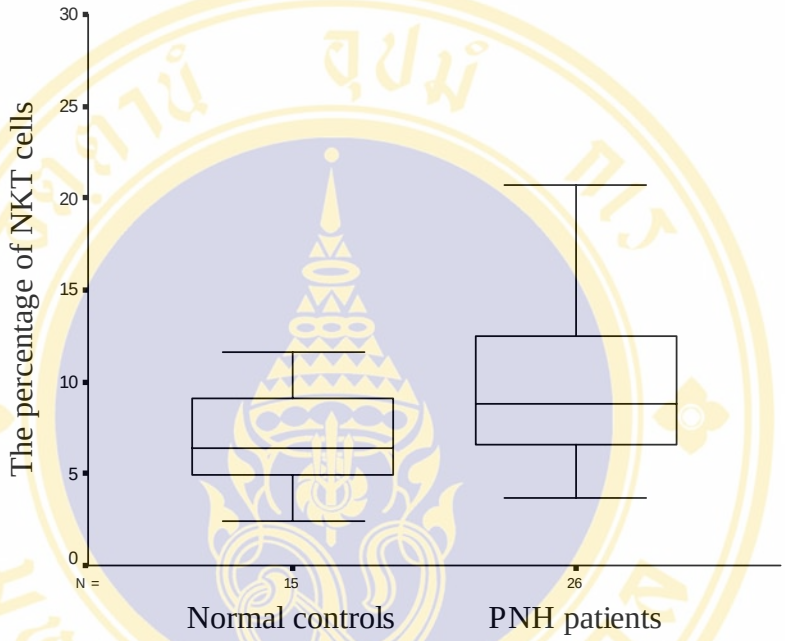


Figure 34 Comparison of the percentages of NKT cells between normal controls (mean ± SEM; 7.24 ± 0.87, median; 6.35, n = 15) and PNH patients (mean ± SEM; 10.18 ± 1.08, median; 8.83, n = 26) by Mann-Whitney test, P = 0.08. Box plot represents; median, and 25th-75th percentile.

Moreover, we also investigated the percentages of NK cells (Table 10) in both PNH patients and normal controls.

Table 10 represents the percentages of NK cells compared between 15 normal controls and 26 PNH patients: the percentages of NK cells of normal controls, mean ± SEM; 18.37 ± 2.80, median; 15.75, minimum; 7.70, maximum; 39.63, the percentages of NK cells of PNH patients, mean ± SEM; 15.81 ± 1.64, median; 13.94, minimum; 4.98, maximum; 39.35. The comparison of the percentages of NK cells was determined by Mann-Whitney test, P = 0.61.

Table 12 The percentages of NK cells of normal controls and PNH patients.

UPN	% NK cells	
	Normal controls	PNH patients
1	22.91	18.61
2	34.36	16.11
3	7.70	5.03
4	15.50	9.36
5	8.05	22.15
6	8.60	15.76
7	9.75	9.28
8	16.37	22.44
9	15.75	10.80
10	38.18	10.25
11	39.63	28.16
12	13.10	12.27
13	19.83	24.50
14	8.87	11.42
15	16.99	39.35
16		9.72
17		23.39
18		14.43
19		11.01
20		4.98
21		15.07
22		13.45
23		31.61
24		9.61
25		14.54
26		7.87

Table 12 The percentages of NK cells of normal controls and PNH patients (Continued).

UPN	% NK cells	
	Normal controls	PNH patients
Mean	18.37	15.81
SEM	2.80	1.64
MD	15.75	13.94
Min	7.70	4.98
Max	39.63	39.35
P-value	0.61	

UPN, unique patient number; SEM, standard error of mean; MD, median; Min, minimum; Max, maximum

The result demonstrated that there were no significant difference of the percentages of NK cell between PNH patients and normal controls (mean ± SEM; 18.51 ± 1.64, median; 13.94, for PNH patients vs mean ± SEM; 18.37 ± 2.80, median; 15.75, for normal controls; P = 0.61) (Figure 35).

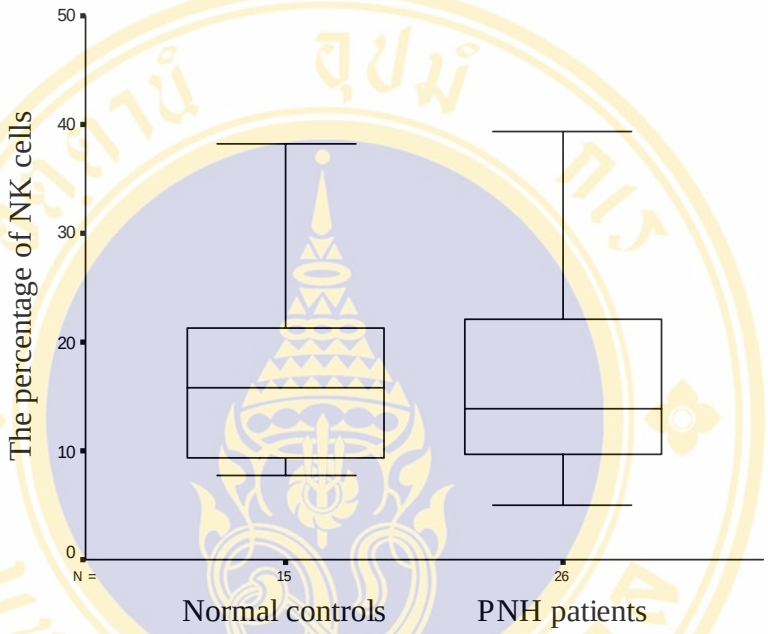


Figure 35 Comparison of the percentages of NK cells between normal controls (mean ± SEM; 18.37 ± 2.80, median; 15.75, n = 15) and PNH patients (mean ± SEM; 18.51 ± 1.64, median; 13.94, n = 26) by Mann-Whitney test, P = 0.61. Box plot represents; median, and 25th-75th percentile.

3. Hemopoietic progenitor cell assay.

3.1 The comparison of hemopoietic progenitor growth between PNH patients and normal controls.

Hemopoietic progenitor cells assay were studied in 5 PNH patients and 5 normal controls. The study separated into the co-cultured of $CD8^-$ cell fraction, which contained hemopoietic progenitor cells, with $CD8^+$ cell fraction (the co-culture with $CD8^+$ fraction) and the cultured of only $CD8^-$ cell fraction (the culture without $CD8^+$ fraction). After cultured for 14 days, hemopoietic progenitor colonies consisting of at least 50 cells were enumerated and characterized according to their morphology (Figure 36).

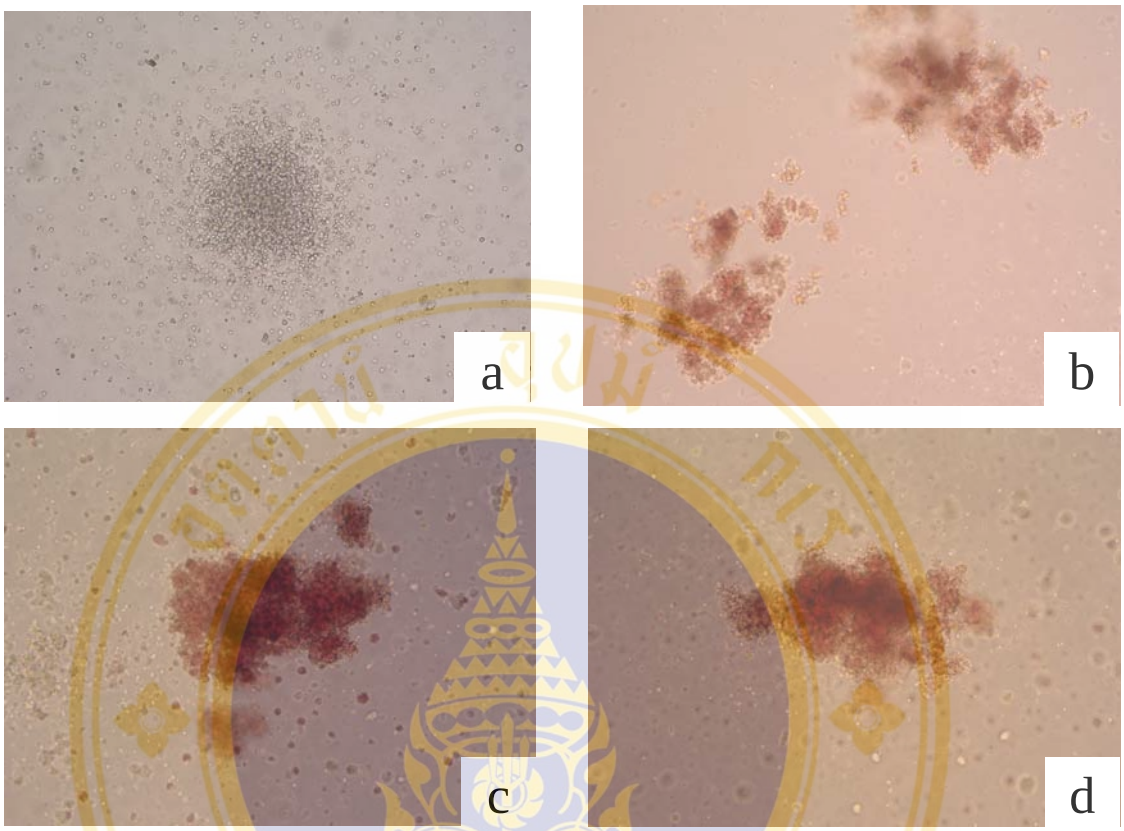


Figure 36 Morphology of hemopoietic progenitor colonies. After hemopoietic cultured for 14 days, colonies consisting at least 50 cells were enumerated and characterized according to their morphology. (a) and (b) Colonies of CFU-GM and BFU-E from normal control; one colony of CFU-GM (a) and two colonies of BFU-E (b). (c) and (d) Colonies of BFU-E from PNH patient; one colony of BFU-E (c) and the other one colony of BFU-E (d).

We found that the number of burst-forming unit-erythroid (BFU-E) showed significant decrease in PNH patients compared with normal controls whether in the co-culture with $CD8^+$ fraction (mean ± SEM; 28.80 ± 8.96, median = 28.5, for PNH patients vs mean ± SEM; 223.30 ± 20.85, median; 232.5, for normal controls, P = 0.009) (Table 11, Figure 37), or in the culture without $CD8^+$ fraction (mean ± SEM; 30.20 ± 10.06, median; 24.5, for PNH patients vs mean ± SEM; 234.60 ± 23.36, median; 238.5, for normal controls, P = 0.009) (Table 12, Figure 37).

Table 13 Data of the number of BFU-E hemopoietic progenitor colonies from co-culture with $CD8^+$ fraction compare between normal controls and PNH patients.

Number of BFU-E colonies 4 x 10^5 MNC from co-culture with $CD8^+$ fraction*	
Normal controls (5)	PNH patients (5)**
266.0	28.5
160.0	57.0
217.5	35.0
266.5	21.5
232.5	2.0

Statistical values of the number of BFU-E colonies of normal controls represented by mean ± SEM = 223.30 ± 20.85, median = 232.5; statistical values of the number of BFU-E colonies of PNH patients represented by mean ± SEM = 28.80 ± 8.96, median = 28.5; * Mean of duplicate experiments; **, significant compared with normal controls, P=0.009.

Table 14 Data of the number of BFU-E hemopoietic progenitor colonies from the culture without $CD8^+$ fraction compare between normal controls and PNH patients.

Number of BFU-E colonies 4 x 10^5 MNC from the culture without $CD8^+$ fraction*	
Normal controls (5)	PNH patients (5)**
285.0	43.5
167.0	61.0
210.5	24.5
289.0	19.0
238.5	3.0

Statistical values of the number of BFU-E colonies of normal controls represented by mean ± SEM = 234.60 ± 23.36, median = 238.5; statistical values of the number of BFU-E colonies of PNH patients represented by mean ± SEM = 30.20 ± 10.06, median = 24.5; *, Mean of duplicate experiments; **, significant compared with normal controls, P=0.009.

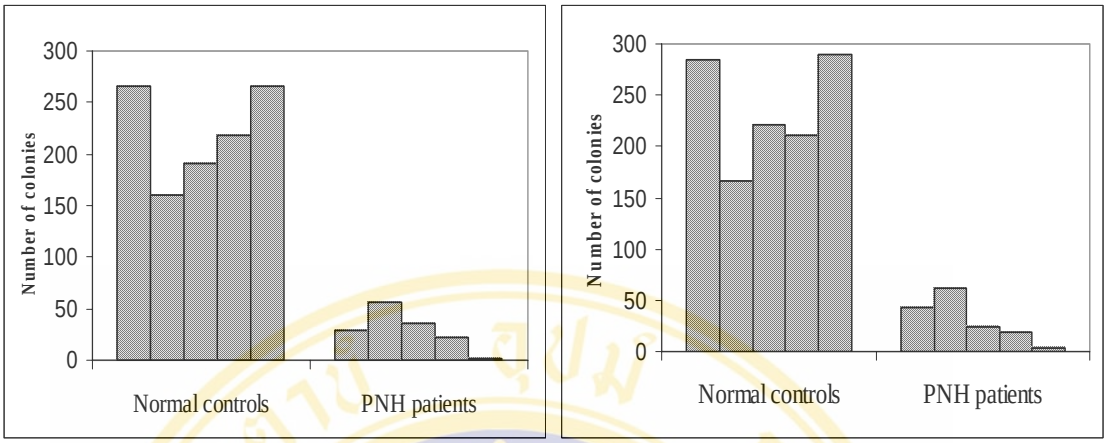


Figure 37 Comparison of the number of BFU-E colonies between normal controls (n =5) and PNH patients (n = 5). The comparison was determined by Mann-Whitney test. Left; Comparison of the number of BFU-E colonies between normal controls (mean ± SEM; 223.30 ± 20.85, median; 232.5) and PNH patients (mean ± SEM; 28.80 ± 8.96, median = 28.5) in the co-culture with $CD8^+$ fraction, P = 0.009. Right; Comparison of the number of BFU-E colonies between normal controls (mean ± SEM; 234.60 ± 23.36, median; 238.5) and PNH patients (mean ± SEM; 30.20 ± 10.06, median; 24.5) in the culture without $CD8^+$ fraction, P = 0.009. Each bar indicates an individual normal control or patient.

The number of colony forming unit-granulocyte-macrophage (CFU-GM) also showed significant decrease in PNH patients compared with normal controls whether in the co-culture with $CD8^+$ fraction (mean ± SEM; 1.50 ± 0.71, median; 1.0, for PNH patients vs mean ± SEM; 15.70 ± 2.65, median; 16.0, for normal controls, P = 0.009) (Table 13, Figure 38), or in the culture without $CD8^+$ fraction (mean ± SEM; 3.10 ± 1.16, median; 2.5, for PNH patients vs mean ± SEM; 16.90 ± 3.69, median; 18.5, for normal controls, P = 0.009) (Table 14, Figure 38).

Table 15 Data of the number of CFU-GM hemopoietic progenitor colonies from co-culture with $CD8^+$ fraction compare between normal controls and PNH patients.

Number of CFU-GM colonies 4 x 10^5 MNC from co-culture with $CD8^+$ fraction*	
Normal controls (5)	PNH patients (5)**
16.0	2.0
12.5	4.0
10.0	0.0
25.5	1.0
24.5	0.5

Statistical values of the number of CFU-GM colonies of normal controls represented by mean ± SEM = 15.70 ± 2.65, median = 16.0; statistical values of the number of CFU-GM colonies of PNH patients represented by mean ± SEM = 1.50 ± 0.71, median = 1.0; *, Mean of duplicate experiments; **, significant compared with normal controls, P=0.009.

Table 16 Data of the number of CFU-GM hemopoietic progenitor colonies from the culture without $CD8^+$ fraction compare between normal controls and PNH patients.

Number of CFU-GM colonies 4 x 10^5 MNC from the culture without $CD8^+$ fraction*	
Normal controls (5)	PNH patients (5)**
18.5	5.0
11.0	6.5
10.5	0.5
30.5	2.5
22.0	1.0

Statistical values of the number of CFU-GM colonies of normal controls represented by mean ± SEM = 16.90 ± 3.69, median = 18.5; statistical values of the number of CFU-GM colonies of PNH patients represented by mean ± SEM = 3.10 ± 1.16, median = 2.5; *, Mean of duplicate experiments; **, significant compared with normal controls, P=0.009.

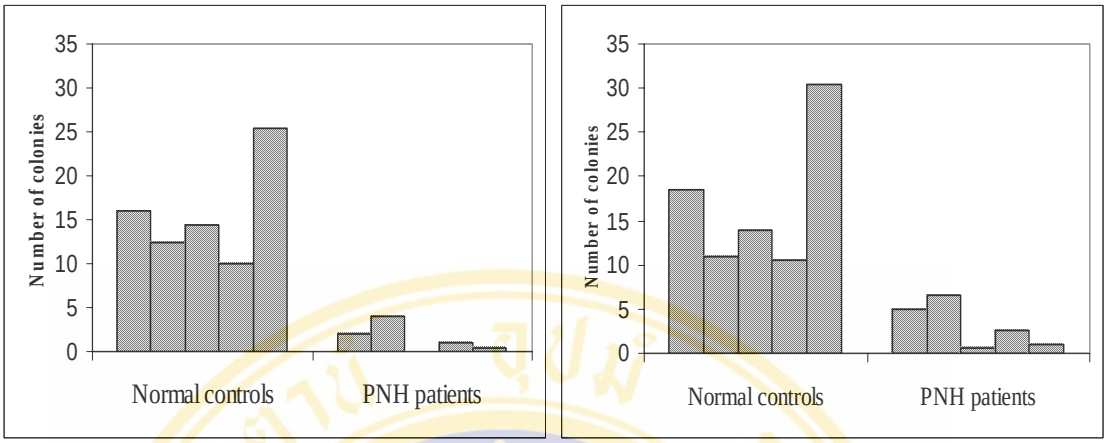


Figure 38 Comparison of the number of CFU-GM colonies between normal controls (n =5) and PNH patients (n = 5). The comparison was determined by Mann-Whitney test. Left; Comparison of the number of CFU-GM colonies between normal controls (mean ± SEM; 15.70 ± 2.65, median; 16.0) and PNH patients (mean ± SEM; 1.50 ± 0.71, median; 1.0) in the co-culture with $CD8^+$ fraction, P = 0.009. Right; Comparison of the number of CFU-GM colonies between normal controls (mean ± SEM; 16.90 ± 3.69, median; 18.5) and PNH patients (mean ± SEM; 3.10 ± 1.16, median; 2.5) in the culture without $CD8^+$ fraction, P = 0.009. Each bar indicates an individual normal control or patient.

In addition, the number of CFU-GM of PNH patients showed growth inhibition when compared with normal controls (mean ± SEM; 74.28 ± 6.67, median; 71.4, for PNH patients vs mean ± SEM; 50.68 ± 1.58, median; 51.2, for normal controls, P = 0.009) (Table 15, Figure 39a), in contrast to the growth of BFU-E (mean ± SEM; 52.04 ± 3.72, median; 51.7, for PNH patients vs mean ± SEM; 50.92 ± 0.49, median; 51.1, for normal controls, P = 0.83) (Table 16, Figure 39b).

Table 17 % Inhibition of hemopoietic progenitor cell (BFU-E) culture in normal controls compared with PNH patients.

% Inhibition* of BFU-E	
Normal controls (5)	PNH patients (5)**
51.7	60.4
51.1	51.7
49.2	41.2
52.0	46.9
50.6	60.0

Statistical values of the percentages of inhibition of normal controls were represented by mean ± SEM = 50.92 ± 0.49, median = 51.1; statistical values of the percentages of inhibition of PNH patients represented by mean ± SEM = 52.04 ± 3.72, median = 51.7; *, [The number of culture colonies from the culture without $CD8^+$ fraction / (the number of colonies from the culture without $CD8^+$ fraction plus the number of colonies from the co-culture with $CD8^+$ fraction)] x 100; **, not significant compared with normal controls, P=0.83.

Table 18 % Inhibition of hemopoietic progenitor cell (CFU-GM) culture in normal controls compared with PNH patients.

% Inhibition*** of CFU-GM	
Normal controls (5)	PNH patients (5)
53.6	71.4
46.8	61.9
51.2	100.0
54.5	71.4
47.3	66.7

Statistical values of the percentages of inhibition of normal controls were represented by mean ± SEM = 50.68 ± 1.58, median = 51.2; statistical values of the percentages of inhibition of PNH patients represented by mean ± SEM = 74.28 ± 6.67, median = 71.4; *, [The number of culture colonies from the culture without $CD8^+$ fraction / (the number of colonies from the culture without $CD8^+$ fraction plus the number of colonies from the co-culture with $CD8^+$ fraction)] x 100; **, significant compared with normal controls, P=0.009.

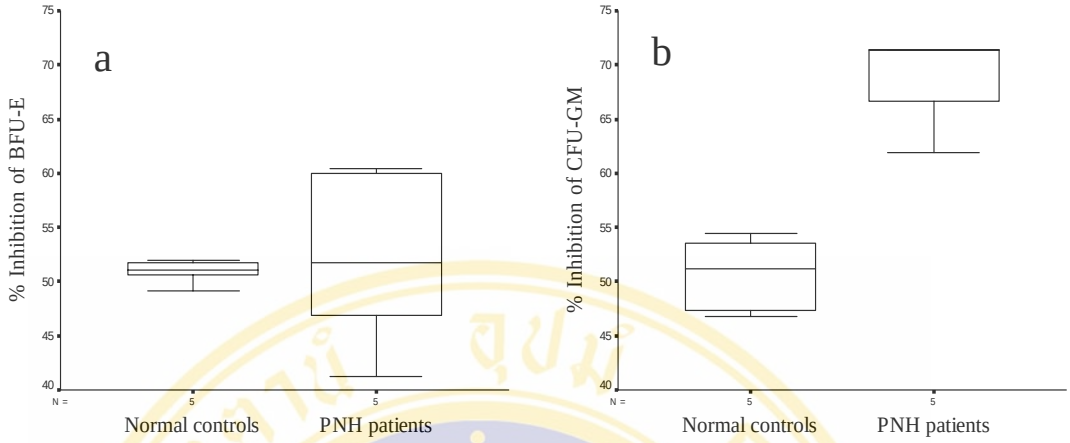


Figure 39 Comparison of the percentages of inhibition of hemopoietic progenitor colonies between normal controls (n = 5) and PNH patients (n = 5). The comparison was determined by Mann-Whitney test. (a) Comparison of the percentages of inhibition of BFU-E between normal controls (mean ± SEM; 50.92 ± 0.49, median; 51.1) and PNH patients (mean ± SEM; 52.04 ± 3.72, median; 51.7), P = 0.83. (b) Comparison of the percentages of inhibition of BFU-E between normal controls (mean ± SEM; 50.68 ± 1.58, median; 51.2) and PNH patients (mean ± SEM; 74.28 ± 6.67, median; 71.4), P = 0.009. Box plots indicate; median, and 25th-75th percentile.

3.2 The effect of $CD8^+$ T cells.

To assessed the effect of CTLs ($CD8^+$ T cells) on hematopoietic progenitor cell assay, enumeration of the number of BFU-E and CFU-GM demonstrated that in normal controls the number of BFU-E were not different compared between the number of colonies in the co-culture with $CD8^+$ fraction and in the cultured without $CD8^+$ fraction which contained hematopoietic stem cells (mean ± SEM; 220.30 ± 20.85; median; 232.5, for the co-culture with $CD8^+$ fraction vs mean ± SEM; 234.60 ± 23.26, median; 238.5, for the culture without $CD8^+$ fraction, P = 0.18) (Table 17, Figure 40).

Table 19 Data of the number of BFU-E hemopoietic progenitor colonies of normal controls compare between the number of colonies from the co-culture with $CD8^+$ fraction and the culture without $CD8^+$ fraction.

Number of BFU-E colonies 4 x 10^5 MNC from normal controls*	
Co-culture with $CD8^+$ fraction (5)	Culture without $CD8^+$ fraction (5)**
266.0	285.0
160.0	167.0
217.5	210.5
266.5	289.0
232.5	238.5

Statistical values of the number of BFU-E colonies co-culture with $CD8^+$ fraction represented by mean ± SEM = 220.30 ± 20.85, median = 232.5; statistical values of the number of BFU-E colonies from the culture without $CD8^+$ fraction represented by mean ± SEM = 234.60 ± 23.26, median = 238.5; *, mean of duplicate experiments; **, not significant compared with co-culture with CD8 positive fraction, P = 0.18.

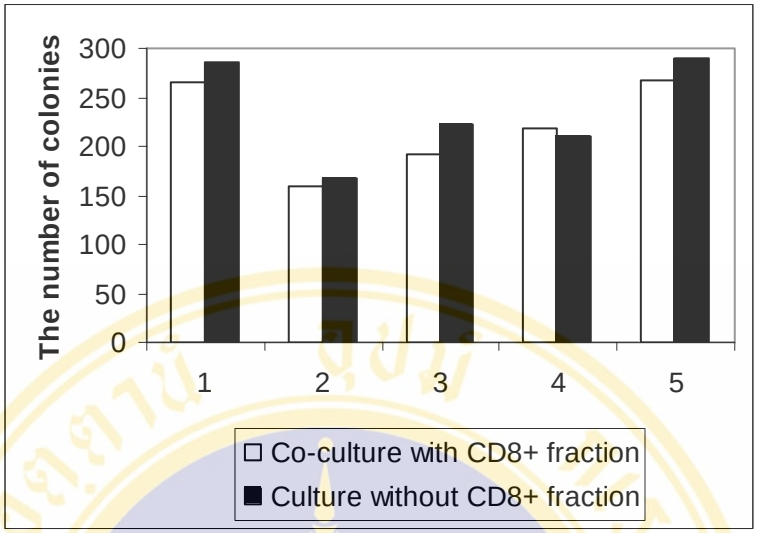


Table 40 The of number of BFU-E hemopoietic progenitor colonies derived from normal controls compared between the number of colonies from the co-culture with $CD8^+$ fraction (mean ± SEM; 220.30 ± 20.85; median; 232.5) and the culture without $CD8^+$ fraction (mean ± SEM; 234.60 ± 23.26, median; 238.5). The number of colonies were compared by Wilcoxon signed-rank test, P = 0.18. White bars indicate the number of BFU-E in the co-culture with $CD8^+$ fraction, Black bars indicate the number of BFU-E in the culture without $CD8^+$ fraction.

Enumeration of the number of CFU-GM derived from normal controls also demonstrated no significant different in the number of colonies compared between the number of colonies in the co-culture with $CD8^+$ fraction and in the cultured without $CD8^+$ fraction (mean ± SEM; 15.70 ± 2.65; median; 16.0, for the co-culture with $CD8^+$ fraction vs mean ± SEM; 16.90 ± 3.69, median; 18.5, for the culture without $CD8^+$ fraction, P = 0.59) (Table 18, Figure 41).

Table 20 Data of the number of CFU-GM hemopoietic progenitor colonies of normal controls compare between number of colonies from the co-culture with $CD8^+$ fraction and the culture without $CD8^+$ fraction.

Number of CFU-GM colonies 4 x 10^5 MNC from normal controls*	
Co-culture with $CD8^+$ fraction (5)	Culture without $CD8^+$ fraction (5)**
16.0	18.5
12.5	11.0
10.0	10.5
25.5	30.5
24.5	22.0

Statistical values of the number of CFU-GM colonies in the co-culture with $CD8^+$ fraction represented by mean ± SEM = 15.70 ± 2.65, median = 16.0; statistical values of the number of CFU-GM colonies from the culture without $CD8^+$ fraction represented by mean ± SEM = 16.90 ± 3.69, median = 18.5; *, Mean of duplicate experiments; ** not significant compared with co-culture with CD8 positive fraction, P = 0.59.

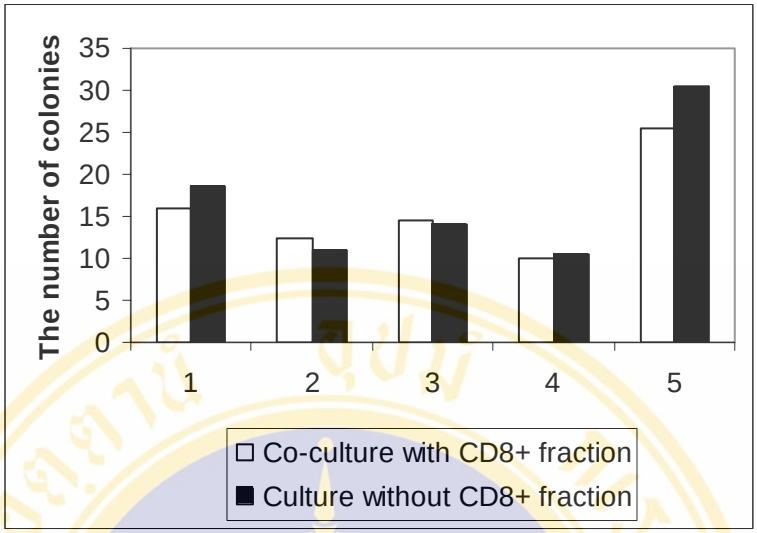


Figure 41 The of number of CFU-GM hemopoietic progenitor colonies derived from normal controls compared between the number of colonies from the co-culture with $CD8^+$ fraction (mean ± SEM; 15.70 ± 2.65; median; 16.0) and the culture without $CD8^+$ fraction (mean ± SEM; 16.90 ± 3.69, median; 18.5). The number of colonies were compared by Wilcoxon signed-rank test, P = 0.59. White bars indicate the number of CFU-GM in the co-culture with $CD8^+$ fraction, Black bars indicate the number of CFU-GM in the culture without $CD8^+$ fraction.

In PNH patients, there was no significant different in the number of BFU-E compared between the number of colonies in the co-culture with $CD8^+$ fraction and in the culture without $CD8^+$ fraction (only the number of colonies of CFU-GM were found statistically different compared between the culture with and without the present of $CD8^+$ fraction) (mean ± SEM; 28.80 ± 8.96; median; 28.5, for the co-culture with $CD8^+$ fraction vs mean ± SEM; 30.20 ± 10.06, median; 24.5, for the culture without $CD8^+$ fraction, P = 0.67) (Table 19, Figure 42).

Table 21 Data of the number of BFU-E hemopoietic progenitor colonies of PNH patients compare between number of colonies from co-culture with $CD8^+$ fraction and the culture without $CD8^+$ fraction.

Number of BFU-E colonies 4 x 10^5 MNC from PNH patients*	
Co-culture with $CD8^+$ fraction (5)	Culture without $CD8^+$ fraction (5)**
28.5	43.5
57.0	61.0
35.0	24.5
21.5	19.0
2.0	3.0

Statistical values of the number of BFU-E colonies co-culture with $CD8^+$ fraction represented by mean ± SEM = 28.80 ± 8.96, median = 28.5; statistical values of the number of BFU-E colonies from the culture without $CD8^+$ fraction represented by mean ± SEM = 30.20 ± 10.06, median = 24.5; *, Mean of duplicate experiments; **, not significant compared with co-culture with CD8 positive fraction, P = 0.67.

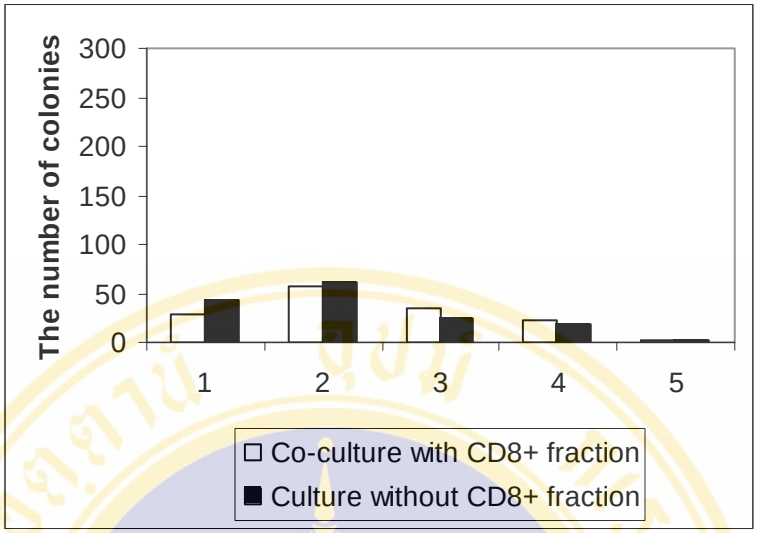


Figure 42 The of number of BFU-E hemopoietic progenitor colonies derived from normal controls compared between the number of colonies from the co-culture with $CD8^+$ fraction (mean ± SEM; 28.80 ± 8.96; median; 28.5) and the culture without $CD8^+$ fraction (mean ± SEM; 30.20 ± 10.06, median; 24.5). The number of colonies were compared by Wilcoxon signed-rank test, P = 0.67. White bars indicate the number of BFU-E in the co-culture with $CD8^+$ fraction, Black bars indicate the number of BFU-E in the culture without $CD8^+$ fraction.

In contrast, the number of CFU-GM of PNH patients demonstrated significant different in the number of colonies when compared between the number of colonies in the co-culture with $CD8^+$ fraction and the culture without $CD8^+$ fraction (mean ± SEM; 1.50 ± 0.71; median; 1.0, for the co-culture with $CD8^+$ fraction vs mean ± SEM; 3.10 ± 1.16, median; 2.5, for the culture without $CD8^+$ fraction, P = 0.04) (Table 20, Figure 43).

Table 22 Data of the number of CFU-GM hemopoietic progenitor colonies of PNH patients compare between number of colonies from co-culture with $CD8^+$ fraction and the culture without $CD8^+$ fraction.

Number of CFU-GM colonies 4 x 10^5 MNC from PNH patients*	
Co-culture with $CD8^+$ fraction (5)	Culture without $CD8^+$ fraction (5)**
2.0	5.0
4.0	6.5
0.0	0.5
1.0	2.5
0.5	1.0

Statistical values of the number of CFU-GM colonies co-culture with $CD8^+$ fraction represented by mean ± SEM = 1.50 ± 0.71, median = 1.0; statistical values of the number of CFU-GM colonies from the culture without $CD8^+$ fraction represented by mean ± SEM = 3.10 ± 1.16, median = 2.5; *, Mean of duplicate experiments; **, not significant compared with co-culture with CD8 positive fraction, P = 0.04.

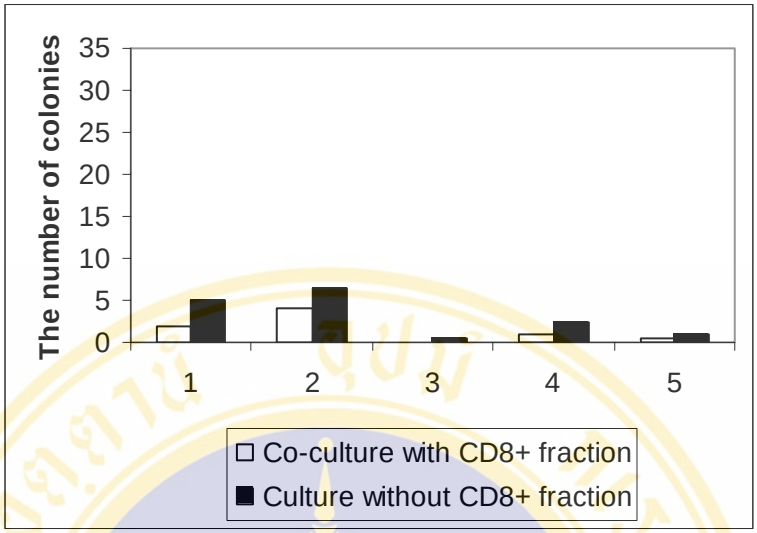


Figure 43 The of number of CFU-GM hemopoietic progenitor colonies derived from normal controls compared between the number of colonies from the co-culture with $CD8^+$ fraction (mean ± SEM; 1.50 ± 0.71; median; 1.0) and the culture without $CD8^+$ fraction (mean ± SEM; 3.10 ± 1.16, median; 2.5). The number of colonies were compared by Wilcoxon signed-rank test, P = 0.04. White bars indicate the number of CFU-GM in the co-culture with $CD8^+$ fraction, Black bars indicate the number of CFU-GM in the culture without $CD8^+$ fraction.

CHAPTER VI
DISCUSSION

PNH is an interesting haematological disorder with various clinical manifestations including hemolysis, venous thrombosis, and defective hematopoiesis. A PIG-A gene abnormality at the level of hematopoietic precursors result in the occurrence of PNH phenotype with the deficiency of GPI-APs in various hematopoietic cells in the patients and a major consequence to complement mediated hemolysis.

PNH does not behave like a leukaemia with inexorable proliferation of the abnormal cells or clones, in contrast the proportion of PNH cells is stable over years in most patients. Moreover, a tiny PNH clones can occur in normal individuals and importantly PNH stem cells do not confer a growth advantage because they rarely undergo rapid expansion and never completely replace non-mutated hemopoiesis as indicated by the studies in chimeric knockout mice with demonstrated a minor, static PNH cells in the marrow or circulating hematopoietic compartments (35, 127, 128).

On the other hand, the possible mechanism that is responsible for clonal expansion in PNH may be combined with some extrinsic factor (s), consistent with the emergence of PNH clones under selective pressure. Lymphocytes of PNH phenotype and PIG-A^- genotype appeared in lymphoma patients treated with Campath-1, a monoclonal antibody that recognized a GPI-APs (6, 129). It is considered that PNH, AA and MDS result from a common abnormality in BM with hypoplastic and decreased proliferative capacity of their precursor cells.

As we know, autoimmune process occurs in AA. There is evidence in associated aplasia for participation of cytotoxic $CD8^+$ cells and proinflammatory cytokines, IFN-γ and TNF-α have been shown to be overexpressed (53, 55, 130) and induced the expression of Fas on the surface of $CD34^+$ cells in AA patients are tightly regulated with apoptosis-associated hematopoietic suppression (69, 131, 132).

The proportions of AA with PNH clones varies from 13% to 57% in different studies (36, 133, 134). PNH has been considered as a late clonal hematologic disorder that occur in AA especially after immunosuppressive therapy (135, 136). PNH patients usually have an underlying aplastic process and the onset of BM failure during the course of PNH, suggested that PNH clones may have a relative growth or survival advantage over the residual non-PNH hematopoiesis in patients who have aplastic anemia. These findings stress a close linkage between AA and PNH that not only BM failure is primary to both disease, but also a common mechanism operates in the pathogenesis of their marrow hypoplasia. It is likely, therefore, that autoreactive T lymphocytes mediate the BM suppression in PNH similar to the hematopoietic injury that occurs in AA. In support of this idea, immunosuppressive therapy was shown to improve BM hypoplasia of patients with AA, PNH, and AA/PNH syndrome (137). The recent finding of increased frequency of expand T cell clones in PNH patients, support a pathogenetic model based on autoreactive T cells (102).

In PNH, perhaps autoreactive T lymphocytes mediate suppression of hematopoiesis through direct contact with certain GPI-linked surface proteins or GPI-anchor itself (138) and the consequence of cell-mediated mechanisms involving perforin and Fas pathways as well as the effects of cytokines such and IFN-γ have been shown to play a role in stem cell destruction (45, 139). This process, should it occur, will allow the GPI-deficiency PNH cells to escape immunosuppression. According to this principle, it is of great interest to study whether autoimmune process is involved in negative selection that is responsible for expansion of PNH clones leading to clinical PNH.

In this study, we explored the effect of autoimmune process to GPI-AP(+) and GPI-AP(-) cells in PNH patients. The study was designed to observe the effect of mononuclear cells (MNCs) which represented for lymphocyte population to apoptosis of CD59(+) and CD59(-) peripheral blood granulocytes from PNH patients. We found that there was no effect of MNCs to the sensitivity of apoptosis of both CD59(+) and CD59(-) granulocytes in PNH patients when performed the experiment in the different sample tubes, with or without MNC fraction. However, the experiment in the same sample tube showed that only CD59(+) granulocytes in the sample tube with MNC fraction had increased apoptosis with time, while CD59(+) granulocytes in the sample

tube without MNC fraction and CD59(-) granulocytes both in the sample tube with and without MNC fraction were not different in apoptotic rate. Moreover, our study demonstrated that CD59(+) granulocytes were more sensitive to apoptosis than CD59(-) granulocytes and CD59(+) granulocytes were tend to be effected by MNCs as indicated by apoptosis of CD59(+) granulocytes compared with CD59(-) granulocytes in the sample tube with MNC fraction were more statistically significant than in the sample tube without MNC fraction after 4 hour. Thus, MNCs may be regulated in negative selection to destroy non-mutated (CD59+) cells via apoptosis and suggested the role of these cells in expansion of PNH clone by autoimmune-mediated apoptotic process.

The finding of negative correlation between Hb concentration and the percentages of apoptosis of only CD59(+) granulocytes in the sample tube with and without MNC fraction measured 4 hour of PNH patients, demonstrated that the apoptosis of CD59(+) granulocytes were increased according to the severity of anemia of the patients. These finding further suggested that the apoptosis of only CD59(+) cells have a role in the pathogenesis of PNH. The selection could be referred to the destruction of stem cells in the marrow resulted in reducing their number so that normal levels of circulating blood cells are not maintain and finally leading to the severity of anemia in PNH patients.

According to the apoptosis study, the effect of MNCs on each cell population was not clearly demonstrated in different experimental tubes, this may be due to the different condition in the different sample tubes. In addition, the sensitivity of flow cytometry method used, might not be enough and the remaining of MNCs in the sample tube without MNC fraction may interfere the analysis of apoptosis of granulocytes. Cell sorting technique may resolve the problem of MNCs interference.

In addition, the observation of the percentages of apoptosis of granulocytes demonstrated spontaneous apoptosis of CD59(+) granulocytes in both PNH patients and normal controls. Three reports addressed the apoptosis of granulocytes and BM $CD34^+$ cells compared between PNH patients and normal controls. Peripheral blood (PB) granulocytes from PNH patients showed reduced susceptibility to spontaneous apoptosis ligand-induced (anti-Fas) apoptosis and apoptosis induced by serum starvation compared with healthy volunteers (35, 127, 128). BM $CD34^+$ cells from

PNH patients also revealed resistance to apoptosis compared to normal controls (35). Two reports indicated that in PNH patients, BM affected $CD34^+CD59^-$ cells were survived longer than unaffected $CD34^+CD59^+$ cells (128), and $CD34^+CD59^+$ cells demonstrated hypersensitivity to apoptosis resulted in less proliferative capacity compared to $CD34^+CD59^-$ cells (119). A study of the growth of $CD34^+$ cells in PNH patients showed that mutant $CD34^+$ cell had higher progeny than non-mutated $CD34^+$ cells. However, the proliferation rate did not exceed the $CD34^+$ cells from normal controls. Moreover, non-mutated $CD34^+$ cells from PNH patients showed higher Fas expression and higher rate of apoptosis while mutant $CD34^+$ cells and normal control $CD34^+$ cells had low rate of apoptosis (120).

Our experiment which investigated apoptosis in granulocytes instead of BM or $CD34^+$ cells, also revealed the increased apoptosis of granulocytes with normal phenotype (CD59(+) granulocytes) than PNH phenotype (CD59(-) granulocytes). Moreover, the sensitivity to apoptosis of CD59(+) granulocytes from PNH patients were highly than that of CD59(+) granulocytes from healthy people.

. Our study did not show any statistical difference in the percentages of $CD8^+$ T cells, NKT cells, and NK cells from PNH patients and healthy individuals. Since, these lymphocytes are the likely effectors that destroy or induce apoptosis of PNH hemopoietic cells, our study may merely indicate the inadequate sensitity of the test to demonstrate the roles of these cells at quantitative level.

For hemopoietic progenitor cells assay, instead of studying colony growth of hemopoietic stem cells from BM, we decided to use peripheral blood (PB) mononuclear cells (MNCs) because of their easy accessibility and accepted representation for hemopoietic stem cells in BM. Our results were similar with other reports which addressed the hemopoietic defect in PNH patients. When compared with normal volunteers, PNH patients had markedly lower clonogenicity in both the assay for committed progenitors and the long-term culture-initiating cells (LTC-IC) assay which measured hemopoietic activity in BM (8, 140) and peripheral blood cells (140). Our study also investigated the effect of $CD8^+$ T cells on the growth of progenitor cells by comparing the number of committed progenitor between the culture of MNCs with and without $CD8^+$ T cells. The result showed the effect of $CD8^+$ T cells only on the growth of colony-forming unit-granulocyte-macrophage

(CFU-GM) but not on the growth of burst-forming unit erythroid (BFU-E). A previous report did not show any significant difference in the number of both BFU-E and CFU-GM when co-cultured patient's T large granular lymphocytes (T-LGLs; $CD8^+CD57^+$ or $CD8^+CD57^-$ T cells) with purified cord blood $CD34^+$ cells (100).

In principle, an autoimmune process might selectively damage normal hematopoietic stem cells more than PNH hemopoietic stem cells because of more greater sensitivity to cytotoxicity. The nature of antigen target of this autoreactive T cells is unknown. As our co-culture experiment demonstrated only their effectes in the growth of CFU-GM. It is possible that CFU-GM may confer more sensitivity to be damaged than BFU-E. However, as we did not evaluate the viability of $CD8^+$ cells in positive fraction before culture, we could not exclude the unequal viability of $CD8^+$ cells on each experiment leading to the finding of only growth inhibition in CFU-GM. Another possibility is that the other autoreactive T cells such as NKT cells or NK cells may consider to be the other effector cells that regulate in this immune process to various progenitor cells.

As we know that PIG-A mutation occurs at the stage of hematopoietic stem cells (HSCs) and a highly frequent overlap of PNH and AA indicated that cytotoxic lymphocyte suspected to play a role in the pathogenesis of PNH as in AA. From our study and the other previous reports, a model of this suspect of the interaction between immune system and hematopoietic cells may be that cytotoxic lymphocytes recognized HSCs via GPI-APs or GPI-anchor itself that serve as target antigen.

Some GPI-APs on HSCs are important for the interaction of immune cells or are ligands of co-stimulatory molecules on effector T lymphocytes for example CD58 which is a ligand of CD2 on T lymphocytes (106). If these GPI-APs are necessary for the stimulation of T lymphocytes, GPI-APs-deficients HSCs should be survived. However, in contrast to this thesis, some reports demonstrated equal sensitivity to cytotoxic lymphocytes of both GPI-positive and GPI-negative cells. They tested the hypothesis that one or more GPI-linked proteins are crucial for the effective interaction of antigen-specific T cells, normal and PNH Epstein-Barr virus (EBV)-transformed B-cell lymphoblastoid cell lines (BLCLs) were investigated for the sensitivity to the cytotoxic effect of autologous EBV-specific T-cell lines and clones from a patient with PNH in an *in vitro* experimental system. The PNH BLCLs were

equally sensitive to T-cell-mediated cytotoxicity than non-PNH isogenic BLCLs (141). The other report indicated that after introducing the PIG-A cDNA sequence into the JY5 GPI-negative B-lymphoblastoid cell line and then measured the rate of apoptosis induced by either by Fas antibody, serum starvation, or γ-irradiation. There were no difference in the rate of apoptosis between the parent JY5 cell line or the JY25 (GPI-positive) cell line. These data suggest that other events influence the rate of apoptosis in PNH rather than only PIG-A mutation (127).

On the other hand, GPI-APs may serve as antigens recognized by autoimmune cytotoxic lymphocytes. GPI-APs would be internalized via the endocytic pathway, degraded into peptides in the endosomes, and presented on MHC class II molecule (106). Therefore, only GPI-positive cells would be selected to be destroyed.

GPI-anchor itself could be considered as an autoantigen. NKT cells can recognize lipid or glycolipid binding to CD1d, a family of MHC class I-related molecules, and if autoimmune cytotoxic T lymphocytes recognized GPI presented on CD1d molecule, only GPI-positive cells would be recognized.

After exposure to antigen, cells and cytokine such as IFN-γ and TNF act to induce the expression of Fas receptor on the surface of these target HSCs and finally mediated apoptosis of these target cell via perforin-granzyme of Fas-FasL interaction and reduce their number.

From the previous hypothesis, NK and NKT cells are the other interesting effector lymphocytes. One report explored the sensitivity of PIG-A mutant cells prepared from myeloid and lymphoid leukemic cell lines to killing by NK cells by using ^{51}Cr-release assay *in vitro*. Cytotoxicity assay demonstrated that both PIG-A mutant cell lines were less susceptible to killing than their normal counterparts and NK cells used only the perforin/granzyme pathway to mediated the lysis of cells. Therefore, PIG-A mutant cells lack molecules needed for NK activation or to trigger perforin-mediated killing, contributing to selective expansion of PNH cells in the setting of the destruction of non-PNH cells by cytotoxic lymphocytes (124).

Although our study that could not clearly mention the effect of autoimmune process in PNH patients by $CD8^+$ lymphocytes, but the other effector cells, for example NKT cells and NK cells, would be the candidates in the regulation of autoimmune process which can support by many previous data. Without

demonstration of the abnormal numbers of all $CD8^+$ T cells, NKT cells and NK cells, it may be possible that the target-effector cell interaction plays and important role in the autoimmune selection of PNH clone. Therefore, the expansion of PNH clones could still consider by immune regulation which mediated BM injury and result in the pathogenesis of PNH.

CHAPTER VII
SUMMARY

Nowadays, the mechanism of the expansion of PNH clones in PNH patients is still unclear. Our experimental study addressed the greater sensitivity to apoptosis of non-mutated granulocytes than PNH granulocytes, and indicated that this negative selection may be regulated by MNCs. The impaired growth of hematopoietic stem cells that we found in our patients is the general characteristic of PNH. Although there were no significant increase in the numbers of CD8+ T cells, NKT cells and NK cells, they are still likely effectors which mediate PNH clone selection probably bia apoptosis pathway.

This study could not clearly demonstrate the direct effect of MNCs and $CD8^+$ T cells on the destruction of normal granulocytes and hematopoietic progenitor cells respectively. In the future, the identification of target antigen is needed and we hope that with the new advance methods and technologies will make us find out which lymphocyte subpopulation is mediated the effector function and which target antigen is specific for this immune process, to explain why and how cells deficient in GPI-linked proteins can expand in PNH patients.

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APPENDIX

APPENDIX

1. Chemicals

Chemicals	Molecular weight (g/ml)	Source
Absolute ethanol (C_2H_2OH)	46.07	E.Merck, Darmstadtt, F.A., Germany
Ethylenediaminetetra-acetic acid disodium salt or EDTA disodium salt $[CH_2N(CH_2COOH)CH_2COONa]_2 2H_2O$	372.24	BDH, Poole, BH, England
Dextran $(C_6H_{10}O_5)_n$	25,00.00	Sigma, St. Louis, MO, U.S.A.
L-glutamine $(C_5H_{10}N_2O_3)_n$	146.00	Gibco, Grand Island, N.Y., U.S.A.
Paraformaldehyde $(CH_2O)_n$	30.03	Fisher scientific, Fair Lawn, New Jersey, U.S.A.
Potassium chloride (KCl)	74.55	Sigma, St. Louis, MO, U.S.A.
Potassium phosphate (KH_2PO_4)	174.20	Sigma, St. Louis, MO, U.S.A.
Sodium chloride (NaCl)	58.44	Sigma, St. Louis, MO, U.S.A.
Sodium phosphate (Na_2HPO_4)	142.00	Sigma, St. Louis, MO, U.S.A.

2. Instruments

Class II biological safety carbinets, NuAire, Plymouth, U.S.A.

Water-Jacketed Co_2 incubator, Forma Scientific, Marietta, U.S.A.

MACs multi strand, Miltenyi Biotec, Earhart Avenue Auburn, CA, U.S.A.

Flow cytometer, BD FACSCalibur, BD, San Jose, CA, U.S.A

Inverted microscope, Olympus CKX41, Olympus, Southall Middlesex, UK

Standard light microscope, Olympus CX31, Olympus, Southall Middlesex, UK

3. Media

Methocult	StemCell Technologies, Vancoluver, Canada
RPMI medium 1640	Gibco, Grand Island, N.Y., U.S.A.

4. Monoclonal antibodies

Monoclonal antibodies	**Source**
Annexin V-FITC apoptosis detection kit I	BD Phamingen, San Jose, CA, U.S.A.
Anti-CD3 FITC	BD, San Jose, CA, U.S.A.
Anti-CD45 PerCP	BD, San Jose, CA, U.S.A.
Anti-human CD8 PE	BD Phamingen, San Jose, CA, U.S.A.
Biotin anti-human CD59	BD Phamingen, San Jose, CA, U.S.A.
Simultest CD3/CD16+CD56	BD, San Jose, CA, U.S.A.
Streptavidin PE	BD Phamingen, San Jose, CA, U.S.A.

5. Reagents

5.1 10x Phosphate buffer saline (PBS)

NaCl	40	g
KCl	1	g
Na_2HPO_4	5.75	g
KH_2PO_4	1	g
Distilled water to	500	ml

The solution was sterile by autoclaving for 15 minutes at 121^{o}C, 15lb/square inches and stored at room temperature (RT). It was diluted to 1x before use.

5.2 1x PBS

10x PBS	50	ml
Distilled water to	500	ml

The solution was sterile by autoclaving for 15 minutes at 121^{o}C, 15lb/square inches and stored at RT.

5.3 PBS/EDTA buffer

NaCl	800	mg
KCl	19	mg
Na2HPO4	290	mg
KH2PO4	19	mg
EDTA disodium salt	37	mg
Distilled water to	100	ml

The solution was sterile by autoclaving for 15 minutes at 121^{o}C, 15lb/square inches and stored at RT.

5.4 70% Ethanol

Absolute ethanol	70	ml
Sterile distilled water to	100	ml

The solution was mixed and stored at RT.

5.5 6% Dextran

Dextran	6	g
0.9% Normal saline (NSS) to	100	ml

The solution was sterile by filter membrane and stored at RT.

5.6 1x FACS lysing solution

10x FACS lysing solution	10	ml
Sterile distilled water to	100	ml

The solution was mixed and stored at RT.

5.7 1% Paraformaldehyde

Paraformaldehyde	5	g
1x PBS to	500	ml

Stored at $4^{o}C$

BIOGRAPHY

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