ความสัมพันธ์ระหว่างความแตกต่างทางพันธุกรรมของ FcγRIIIa กับการตอบสนองต่อริทูซิแมบในประชากรไทย

นายชยพล สมบูรณ์ยศเดช

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Correlation of FcγRIIIa polymorphisms and the response to rituximab in Thai population.

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ชยพล สมบูรณ์ยศเดช : ความสัมพันธ์ระหว่างความแตกต่างทางพันธุกรรมของ FcγRIIIa ต่อการตอบสนองของยาริทูซิแมบในประชากรคนไทย. (CORRELATION OF FcγRIIIa POLYMORPHISMS AND THE RESPONSE TO RITUXIMAB IN THAI POPULATION.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. สุพีชา วิทยเลิศปัญญา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วัชรี ลิมปนสิทธิกุล, 78 หน้า.

ริทูซิแมบ (rituximab) เป็น IgG_1 chimeric monoclonal antibody ที่มีความจำเพาะเจาะจงต่อโมเลกุล CD20 ซึ่งนำมาใช้ในการรักษาเซลล์มะเร็งต่อมน้ำเหลืองชนิด B-cell โดยมีกลไกการออกฤทธิ์หลักผ่านการกระตุ้นการเกิด antibody dependent cellular cytotoxicity (ADCC) โดยผ่านตัวรับ FcγRIIIa ซึ่งมีการแสดงออกอยู่บนเซลล์ natural killer (NK) มีการศึกษาพบความแตกต่างทางพันธุกรรมของ FcγRIIIa ส่งผลให้เกิดการเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง 158 จาก Valine (V) เป็น Phenylalanine (F) ความแตกต่างทางพันธุกรรมนี้ส่งผลต่อค่า affinity ของ FcγRIIIa ในกลุ่มที่มีการแสดงออกแบบ V/V และ V/F จะมีค่า affinity ที่สูงกว่าในกลุ่มที่มีการแสดงออกเป็น F/F ในการศึกษานี้ได้ใช้วิธี RFLP-Nested PCR และ allele specific amplification ในการตรวจวิเคราะห์ความแตกต่างทางพันธุกรรมดังกล่าว เพื่อนำมาศึกษาความสัมพันธ์กับการกระตุ้นการเกิด ADCC ในหลอดทดลอง และการตอบสนองต่อยา rituximab ในผู้ป่วยมะเร็งต่อมน้ำเหลือง จากผลการศึกษาพบการกระจายตัวของความแตกต่างทางพันธุกรรมของ FcγRIIIa ในประชากรไทยดังนี้ VV 40.25%, VF 16.88% และ FF 42.85% ในการศึกษาความสัมพันธ์ต่อฤทธิ์การเหนี่ยวนำการเกิด ADCC พบว่าในอาสาสมัครที่มีการแสดงออกของยีนแบบ V/V และแบบ V/F จะเหนี่ยวนำการเกิด ADCC สูงกว่าในกลุ่มที่มีลักษณะทางพันธุกรรมแบบ F/F อย่างมีนัยสำคัญทางสถิติ (33.16%, 36.87% และ 20.07%, ตามลำดับ) นอกจากนี้จากผลการศึกษาความสัมพันธ์ของ FcγRIIIa genotype กับผลการรักษาในผู้ป่วย Non-Hodgkin's lymphoma พบแนวโน้มในการทำนายผลการรักษาในผู้ป่วยมะเร็งต่อมน้ำเหลืองที่ได้รับยา rituximab โดยผู้ป่วยที่มีลักษณะทางพันธุกรรมแบบ V/V หรือ V/F จะมีการตอบสนองต่อยา rituximab แบบ complete response ในขณะที่กลุ่มผู้ป่วยที่มีลักษณะทางพันธุกรรมแบบ F/F จะมีแนวโน้มผลการตอบสนองเป็น partial response เป็นส่วนใหญ่ เนื่องจากจำนวนผู้ป่วยที่ค่อนข้างน้อยทำให้ผลการศึกษาความสัมพันธ์ที่ได้ไม่ชัดเจน เพื่อความเข้าใจทางด้านเภสัชพันธุศาสตร์ของยา rituximab ต่อการรักษาผู้ป่วยมะเร็งต่อมน้ำเหลืองจำเป็นต้องขยายผลการวิจัยและเพิ่มขนาดจำนวนตัวอย่างการศึกษาทางคลินิกต่อไปในอนาคต อย่างไรก็ตามจากผลการศึกษานี้อาจจะเป็นข้อมูลพื้นฐานในการศึกษาถึงผลการตอบสนองต่อยา rituximab และอาจขยายผลไปถึงการศึกษายาที่เป็น IgG_1 therapeutic monoclonal antibody อื่นๆต่อไป

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : FcγRIIIa POLYMORPHISM/ NON-HODGKIN'S LYMPHOMA/ RITUXIMAB

CHAYAPOL SOMBOONYOSDECH: CORRELATION OF FcγRIIIa POLYMORPHISMS AND THE RESPONSE TO RITUXIMAB IN THAI POPULATION. THESIS ADVISOR : ASSOC. PROF. SUPEECHA WITTAYALERTPANYA, THESIS CO-ADVISOR : ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., 78 pp.

Rituximab is the chimeric IgG_1 monoclonal antibody against CD20 which has been approved for B-cell non-Hodgkins lymphomas (NHLs) treatment. Antibody dependent cellular cytotoxicity (ADCC) by rituximab-activated NK cells has been suggested to be an important mechanism of rituximab via the Fc gamma IIIa receptor (FcγRIIIa) binding on natural killer (NK) cells. FcγRIIIa has two expressed alleles that differ at amino acid position 158 in the extracellular domain; valine (V158) and phenylalanine (F158). These allelic variants have been demonstrated to differ in IgG_1 binding and ADCC. V/V homozygotes and V/F heterozygotes bind IgG high affinity than F/F homozygotes. The RFLP-Nested PCR and allele specific amplification was used to identify the FcγRIIIa polymorphism in the study. The correlation of FcγRIIIa polymorphism and rituximab response both *in vitro* and *in vivo* was also studied. The results showed the distributions of FcγRIIIa-158 polymorphism in these subjects were as followed: V/V 40.25%, V/F 16.88% and F/F 42.85%. Higher rituximab-induced Ramos cell cytotoxicity (mean rank 33.16%, 36.87%) was observed in the subjects with VV and VF genotypes, respectively; meanwhile the lower cytotoxicity (mean rank 20.07%) was determined in the subjects with FF genotype. For the *in vivo* study, the NHL patients with V/V or V/F genotypes had a primary response as complete response; meanwhile the NHL patients with F/F genotype had a primary response as partial response. The correlation of FcγRIIIa polymorphism and the primary response in NHL patients is unclear that causing the less number of subjects. The higher number of patients is necessary for the further study. However, these results may provide useful information to understand beneficial response of rituximab as well as other IgG_1 therapeutic antibody in Thai patients.

Field of Study : Pharmacology Student's Signature .....

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxiccity
ATCC	American Type Cell Culture
BCL-2	B-cell CLL/Lymphoma 2
BCL-XL	BCL-2 related gene, long isoform
CD	the Cluster of Differentiation molecules
CFSE	Carboxyfluoroscein succinimidyl ester
CO_2	Carbondioxide
CR	Complete response
CRu	Unidentified complete response
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
FasL	Fas ligand
FBS	Fetal bovine serum
FcR	Fc receptor
FcγRIIIa	Fc gamma receptor subtype IIIa
FL	Follicular lymphoma
h	Hour
HCl	Hydrochloric acid
IPI	International prognostic index
M	Molar (mole per liter)
mAb	Monoclonal antibody
MALT	Mucosa associated lymphoid tissue
mg	Milligram(s)
ml	Milliliter(s)
ng	Nanogram(s)
NaCl	Sodium chloride
NF-κB	Nuclear factor κB
NHL	Non-Hodgkin's lymphoma

NK	Natural killer cells
PBS	Phosphate buffer saline solution
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD	Progression disease
PI	Propidium Iodide
pH	the negative logarithm of hydrogen ion concentration
PR	Partial response
RFLP	Restriction long fragment length polymorphism
rpm	revolution per minute
rtx	rituximab
^{0}C	degree Celsius
µg	microgram(s)

CHAPTER I

INTRODUCTION

Background and Rationale

Non-Hodgkin's lymphoma (NHL) is the one of lymphoproliferative malignant diseases. It is the most common type of hematologic cancers which can be both B-cell and T-cell lymphomas. B-cell Non-Hodgkin's lymphoma can be occurred at the various stages of differentiation of B-lymphocytes.

There are several current strategies to treat the NHL with some considerations about side effects or problems. Patients treated by bone marrow transplantation may encounter graft versus host disease while patients treated with chemotherapy may have immunosuppression and prone to infection. Target based cancer therapy is a new strategy to treat some solid cancers as well as hematologic cancers such as NHL. Therapeutic monoclonal antibodies against tumor antigen on cancerous cells are increasingly used as target based anticancer agents for several cancers. Anti-human CD20 monoclonal antibodies are clinically approved to treat the B-cell NHL which often over-expresses CD20 molecules on the B-malignant cells. These anti-CD20 antibodies are used as naked antibody, rituximab (IgG_1), or as radiolabeled antibody, ^{90}Yttrium Ibritumomab tiuxetan.

Rituximab is the IgG1 chimeric monoclonal antibody approved for the treatment of B-cell NHL that has the over-expression of CD20 on B-cells surface such as DLBCL. Its Fab region can specifically binds to CD20 molecule on the B-cell surface while its Fc region can generate several effector mechanisms to kill target B cells. It has been reported that the main anticancer mechanisms of rituximab are complement activation

and antiobody dependent cellular cytotoxicity (ADCC). By using ADCC mechanism, the Fc region of rituximab can bind to Fc receptor on some effector cells such as NK cells and activates these cells to release cytotoxic mediators, perforin and granzyme to kill CD20 expressing target cells. Patients should have been examined the expression of CD20 molecules on their malignant B cells before receiving rituximab for the benefit of treatment. It has been noticed that not only the expression of the CD20 molecules but also the polymorphism of the FcγRIIIa on NK cells which is the receptor of IgG important for the ADCC mechanism of rituximab and other IgG1 have impact on the clinical outcome in rituximab-treated patients.

The FcγRIIIa polymorphism mainly occurs as a single nucleotide polymorphism (SNPs) at nucleotide position 559 [from thymine (T) to guanine (G)] that lead to changing amino acid at the position 158 [from valine (V) to phenylalanine (F)] of the receptor. Several studies have been reported the correlation between the FcγRIIIa polymorphisms and the clinical response in non-Hodgkin's lymphoma patients treated with rituximab. It has been suggested that patients with the V/V and V/F genotypes had higher clinical response than F/F homozygous patients. It is known that the ethnicity has an impact on the distribution of genetic polymorphisms. The distribution of each genotype of the FcγRIIIa in several countries has been investigated and has been demonstrated that the distribution is vary among races. There is no reported on the distribution of FcγRIIIa genotypes in Thai population. So, this study intended to investigate the genetic polymorphism of FcγRIIIa gene in normal Thai people and evaluate the correlation between the genotype of this gene and clinical response to rituximab in Thai NHL patients.

Research questions:

1. What are the frequencies of distribution of genotypes, V/V, F/F and V/F, of FcγRIIIa gene in Thai population?
2. Is there correlation between the FcγRIIIa polymorphism in Thai population and ADCC activity of rituximab by *in vitro* study?
3. Is there correlation between the FcγRIIIa polymorphism in rituximab treated patients with non-Hodgkin's lymphoma and clinical outcome in Thai population?

Objectives:

1. To identify the frequencies of FcγRIIIa polymorphism in Thai population.
2. To study the correlation between *in vitro* ADCC activity of rituximab and the FcγRIIIa polymorphism.
3. To investigate the correlation between FcγRIIIa polymorphism and the primary clinical outcome in rituximab-treated patients with non-Hodgkin's lymphoma in Thai population.

Hypothesis:

1. There is the correlation between the FcγRIIIa polymorphisms and the *in vitro* ADCC activity of rituximab
2. There is correlation between the FcγRIIIa polymorphisms and clinical outcome in rituximab-treated patients with non-Hodgkin's lymphoma in Thai population.

Keywords:

Anti CD20/ FcγRIIIa polymorphisms/ Non-Hodgkin's lymphoma/ Rituximab

CHAPTER II

LITERATURE REVIEWS

Non-Hodgkin's lymphoma (NHL)

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of B- and T-lymphocyte derived hematological malignancies. More than 80% of NHL is B-cell lymphoma [1]. In Thailand, NHL is the most common in hematological cancer. The average age of NHL patients is approximately 56 years old. NHL is more common in men than women. The incidence of NHL in Caucasian is more than Asian [2]. The incidence of NHL is rising over years. However, the causes of the increase of this incidence are still unclear. The enhancement may be resulted from occupational factor or the viral infection such as, human immunodeficiency virus (HIV) or Epstein-Barr virus. Post-transplant lympho-proliferative disorders can also lead to NHL. Patients with several autoimmune disorders (eg, Hashimoto's thyroiditis, coeliac disease) also have an increased risk of developing NHL [1-2]. Interestingly, in western, the incidence of the others hematological cancer, such as Hodgkin's lymphoma or chronic lymphocytic leukemia, is not increasing [3-4]. At present, NHL is the fifth most common cancer in the United States and the eighth most common in Thailand [5].

Differentiation of Lymphocytes

Most lymphoid malignancies worldwide are derived from B-lymphocytes at various stages of differentiation (figure 1) [4]. The differentiation of B-lymphocytes is occurred from immature stem cells in the bone marrow. At this early phase, B cells proliferate rapidly (antigen-independent differentiation) and differentiate into naive B lymphocytes in the bone marrow. The further phase occurs mainly in the lymph nodes, spleen and mucosa associated lymphoid tissue (MALT) (figure 2) [2]. Afterward, they migrate into peripheral lymphoid tissues and re-circulate all through the body. When they expose the foreign antigen, the re-arrangement of the variable regions of immunoglobulin genes, called somatic hypermutation, is occurred and the antigen specification is improved. Meanwhile, the memory B-cells and plasma cells are

produced and released to the peripheral blood. The isotype switching of immunoglobulin also occurs at this stage [2, 4].

Non-Hodgkin's lymphoma can be occurred in various stages of the differentiation of lymphocytes (table 1) [4]. Therefore, the malignant clonal expansion of lymphocytes at different stages might involve the different subtypes of NHL (figure 1) [2]. The appropriate staging and classification of lymphomas are necessary to make the accurate diagnosis [6].

The expression of cell surface molecules, the cluster-of-differentiation molecules (CD) and immunoglobulin proteins are depended on the type of lymphocyte and its stage of differentiation or maturation. CD molecules have several roles in the recognition, adhesion and maturation of lymphocytes. B-cell CD molecules, for example, CD19 and CD20 are involved in signal transduction [7]. Analysis of these molecules in the malignant cells is useful for the diagnosis as well as for determining tumor histogenesis. For B-cell lymphomas, CD19 and/or CD20 are over-expressed on the B-lymphocytes' surface and used to be the one of markers for B-cell lymphomas diagnosis [8-9].

Table 1: B-cell development and corresponding lymphomas derived at each stage [4].

	B cells	Immunoglobulin genes	Somatic mutation	Immunoglobulin protein	Marker	Corresponding lymphoma	Affected tissues
Foreign-antigen independent	Stem cell	Germ line	None	None	CD34		Bone-marrow
	Pro B cell	Germ line	None	None	CD19, CD79a, BSAP, CD34, CD10, TdT		
	Pre B cell	IgH rearrangement, μ-chain (cytoplasm)	None	Igμ	CD19, CD45R, CD79a, BSAP, CD34, CD10, TdT	B-LBL/ALL	
	Immature B cell	IgL/IgH rearrangements, IgM(Membrane)	None	IgM(membrane)			
Foreign-antigen dependent	Mature Naïve B cell	IgL/IgH rearrangements, IgM and IgD (membrane)	Introduction of somatic mutations	IgM/IgD	CD19, CD20, CD45R, CD79a, CD5, BSAP	B-chronic lymphocytic leukemia, Mantel cell lymphoma	Peripheral lymphoid tissues
	Germinal center (centroblastic and centrocytic)	IgL/IgH rearrangements, class switching	Somatic mutations	Immunoglobulin (minimal or absent)	CD19, CD20, CD45R, CD79a, CD10, BSAP, Bcl6	Burkitt's lymphoma, Follicle cell lymphoma, Diffuse large B-cell lymphoma	
	Memory B cell	IgL/IgH rearrangements	Somatic mutations	IgM	CD19, CD20, CD45R, CD79a, BSAP	Marginal zone lymphoma, B-chronic lymphocytic leukemia	
Terminal differentiation	Plasma cell	IgL/IgH rearrangements	Somatic mutations	IgG>IgA>IgD	CD38, Vs38c, MUM-1 CD138	Plasmacytoma/myeloma	

ALL=acute lymphoblastic leukemia; B-LBL=B-lymphoblastic lymphoma

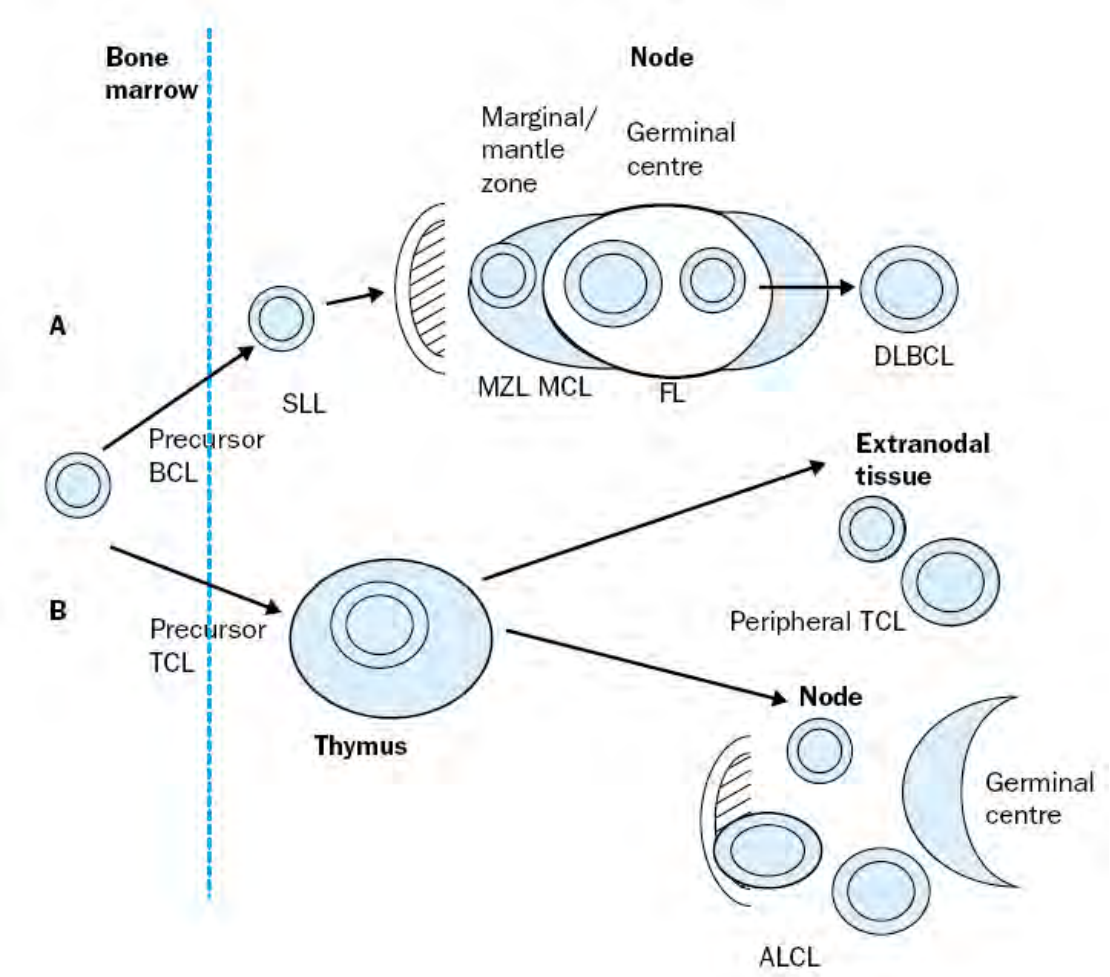


Figure 1: Cellular origins of representative non-Hodgkin's lymphomas (A) B cell; (B) T cell. ALCL=anaplastic large-cell lymphoma; BCL=B-cell lymphoma; DLBCL=diffuse large B-cell lymphoma; FL=follicular lymphoma; MCL=mantle-cell lymphoma (pre-germinal centre); MZL=marginal zone (MALT) lymphoma (post-germinal centre); SLL=small lymphocytic lymphoma; TCL=T-cell lymphoma [4].

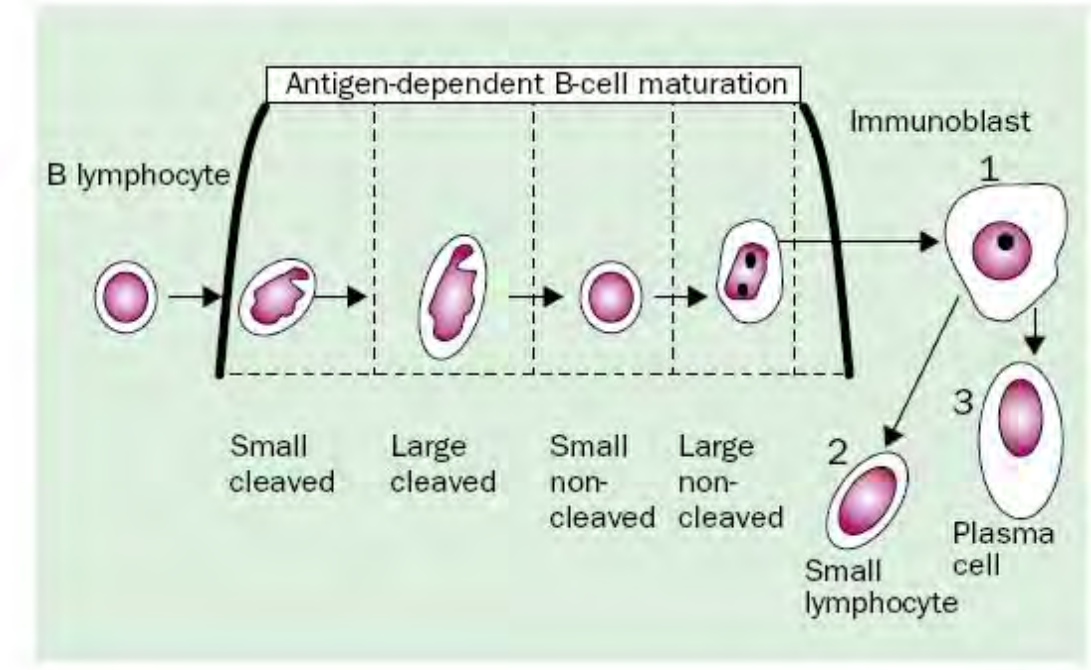


Figure 2: Antigen-dependent B lymphocyte maturation in the lymph node follicle. Neoplastic expansion of B immunoblasts (1) might lead to diffuse large B-cell lymphoma. Neoplastic expansion of small marginal-zone memory B cells (2) might lead to nodal marginal-zone B-cell lymphoma and expansion of plasma cells (3) leads to multiple myeloma [2].

Pathogenesis of Non-Hodgkin's lymphoma

B-cell lymphomas represent approximately 90% of NHLs, whereas T-cell lymphomas represent approximately 10%. Clinically, NHL can be classified as indolent (low grade, intermediate) or aggressive (high grade) lymphoma. Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive lymphoma, whereas follicular lymphoma (FL) is the most common indolent lymphoma (figure 3) [6].

- **Follicular Lymphoma (FL)**

Follicular Lymphoma (FL) is the most common type of indolent Non-Hodgkin's lymphoma. FL is more frequently founded in Caucasian than Asian [8]. The WHO Classification classified the FL into 3 grades: grade 1 (small cell follicular lymphoma),

grade 2 (mixed small and large-cell follicular lymphoma), and grade 3 (large-cell follicular lymphoma). 20% of FL patients, also progresses to diffuse large B-cell lymphoma. Many clinicians treat the grade 3 follicular lymphoma as a DLBCL [6].

FL is commonly founded in elderly patients. The average age of patients is 55 years old. The most of FL patients present the widespread large lymph nodes without the others symptoms. The size of lymph nodes in some cases can spontaneously decrease. The median survival ranges from 8 to 12 years. The progression of disease to DLBCL in FL patients can be occurred in 20-40% of cases. The regression to an aggressive type can be occurred at any stage of the disease, and causes in a more aggressive clinical performance that result to fatal. After the progression, both the response rate and relapse-free survival after treatment steadily decrease, consequential in a median survival of 4–5 years after first relapse.

FL is identified by the translocation of Bcl2 genes, t(14:18) (q32;q21). This translocation is founded in 75-90% of FL patients. Most patients are also founded the abnormality of the others chromosome, such as +7, +18 and +X [6].

- **Diffuse Large B-Cell Lymphoma (DLBCL)**

Diffuse large B-cell lymphoma (DLBCL) is the most common types of aggressive non-Hodgkin's lymphoma in Thailand and around the world. In Thailand, the DLBCL patients were founded approximately 40% of all NHL cases. From the International Lymphoma Study Project, DLBCL patients were founded 34% of all NHL cases [10].

DLBCL is a B-cell lymphoma, it expresses the B-cell CD molecules, like CD19, CD20, CD22, CD79a and surface immunoglobulin. DLBCL express these molecules in 50% to 75% of cases. In some cases, approximately 10%, the $CD10^{+}$ is founded. Because the origin of DLBCL is from germinal center, which make 25-50% of cases express bcl-2 protein and approximately 70% express bcl-6 protein, consistent with a germinal center origin. Translocation of the *bcl-2* gene, t(14;18), is founded in 30% of cases. This translocation results the over-expression of Bcl2 protein and follows by apoptosis inhibition. DLBCL patients who have the high expression of *bcl-2* and the low expression of BAX, pro-apoptotic protein, they provide a good prognostic. The c-*myc*

gene, the onco gene, is rearranged in 5% to 15%, and the *bcl-6* gene, anti-apoptotic gene, is rearranged in 20% to 40% of cases [7].

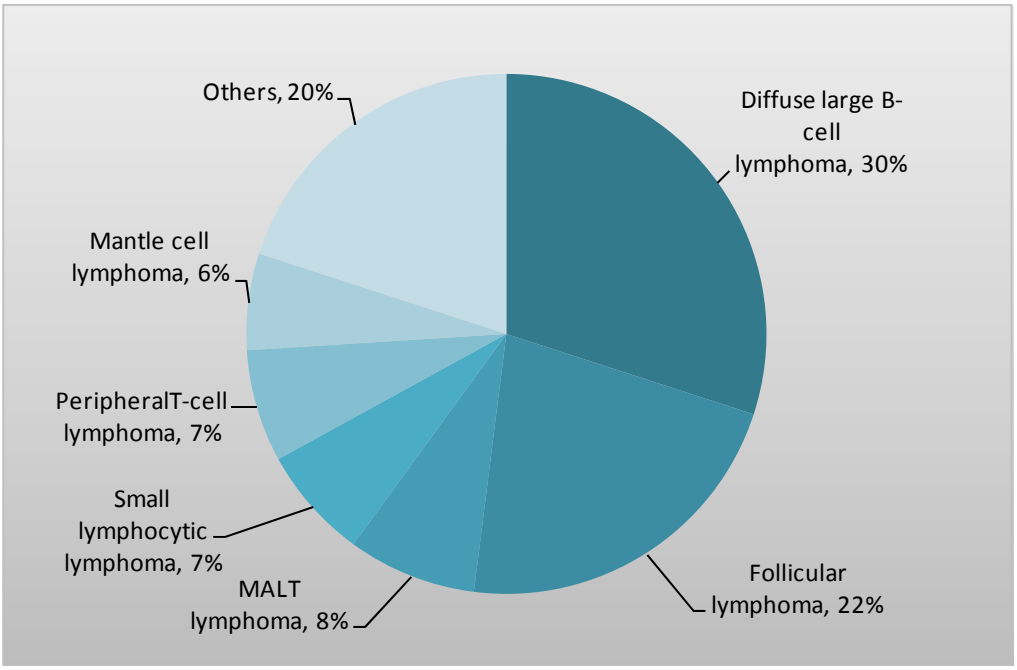


Figure 3: Distribution and frequencies of non-Hodgkin's lymphomas [6].

Staging system and Prognostic factors

The staging system is necessary for the NHL patients. This is not only aimed to provide the appropriate treatment, but also following the clinical response for NHL patients. Although, the staging system is not the only one in the prognostic factors but it is important for the evaluation of treatment [6]. The common staging system is the Ann Arbor staging system. This system was created in 1971 for Hodgkin's Lymphoma which is usually predictable. However, non-Hodgkin's lymphomas are less predictable. Nevertheless, the Ann Arbor staging system also has been applied for clinical staging of non-Hodgkin's lymphomas (table 2) [1].

In 1993, the international prognostic index (IPI) was developed by the International Non-Hodgkin's Lymphoma Prognostic Factors Project. The IPI is 5 significant risk factors related with the survival of NHL patients. It bases on age, tumor stage (the Ann Arbor staging system), lactate dehydrogenase concentration in serum, performance status and number of sites of extranodal disease (table 3) [1].

Table 2: Ann Arbor staging system [1].

Stage	Area of involvement
I	Single lymph node region
II	Multiple lymph node regions on the same side of diaphragm
III	Multiple lymph node regions on both sides of diaphragm
IV	Multiple extranodal sites or lymph nodes and extranodal disease
S	Spleen involvement
E	Extranodal extension or single isolated site of extranodal disease
A	No symptoms
B	B sympthoms: un explained fever > 101.5°F, drenching night sweats, or loss of > 10% body weight within previous 6 months

Table 3: International Prognostic Index (IPI) [1].

Factor	Adverse Prognosis
Age	≥ 60 y
Ann Arbor stage	III or IV (advance disease)
Serum LDH level	Above normal
Number of extranodal sites	≥ 2
Performance status	≥ Eastern Cooperative Onco Group (ECOG) 2 or greater

Non-Hodgkin's lymphoma therapy

In history, the treatment for non-Hodgkin's lymphoma has been chemotherapy or radiotherapy. Currently, it also has the stem cell transplantation and the immunotherapy [10].

Because of the unlimited proliferation of B-cells in NHL, these tumors tend to be more chemo-sensitive and radio-sensitive. Therefore, the chemotherapeutic drugs are used for NHL patients' treatment. The common regimens used to treat the NHL patients have included polychemotherapy regimens such as cyclophosphamide, vincristine, and prednisone. The Regimens commonly used to treat this disease are shown in table 4 [11]. For diffuse large B-cell lymphoma (DLBCL), chemotherapy like CHOP has been standardized for first-line therapy [10]. For follicular lymphoma, the most commonly regimens used for treatment have been alkylator based such as chlorambucil (as monotherapy or with prednisolone), single-agent cyclophosphamide and CVP regimen [8].

In the beginning, over than 50% of patients respond to treatment, but the response and its duration decrease with subsequent chemotherapy. However, this treatment has never been shown to expand the free-survival rates [11]. So, the intentions

to improve the disease-free survival rate of DLBCL or FL patients with the regimens of chemotherapy, the many of strategies have been investigated, including the addition of drugs with different mechanism of action, such as monoclonal antibodies, or addition of other cytotoxic drugs and high-dose therapy with stem-cell transplantation in first remission [7-8, 10]. Those strategies can increase the primary clinical outcome of the patients.

To evaluate the clinical outcome after treatment of NHL patients, the Standardize Response Criteria for Non-Hodgkin's lymphoma are used to evaluate the outcome. The standardized response criteria are necessary for the manner of clinical research. They assist data's interpretation, comparisons of the various clinical trials' results and provide an outline on which to evaluate new biological and immunological of the studied diseases. The availability of standardized guidelines ensures a reliable data to analyze and/or compare between studied patient groups. The primary clinical outcome of NHL patients' treatment is divided into 4 categories as described in table 5 [12].

For immunotherapy, because of the majority of B-cell NHL is differentiated from B-lymphocyte. The surface molecules of B-cell lymphoma, such as CD20, could be expressed similar to B-lymphocyte. The first clinically approved monoclonal antibody-based immunotherapy of lymphoma involved the anti-CD20 chimeric monoclonal antibody, called Rituximab (Rituxan®, Mabthera®) (figure 4) [13].

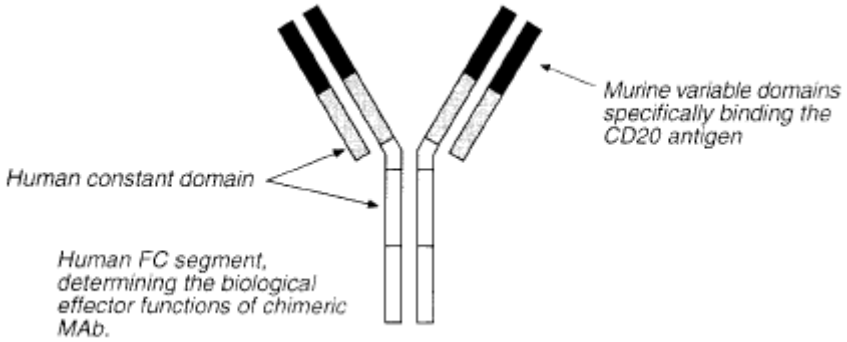


Figure 4: Structure of Rituximab, the chimeric anti CD20 mAb produced through genetic engineering [13].

Table 4: Common NHL Chemotherapy regimens [11].

Regimen	
Primary treatment	
CHOP	Cyclophosphamide/hydroxyldaunorubicin or doxorubicin/vincristine/prednisone
m-BACOD	Methotrexate/bteomycin/doxorubicin/cyclophosphamide/vincristine/ dexamethasone
ProMACE-CytaBOM	Prednisone/methotrexate/doxorubicin/cytarabine/bleomycin/vincristine/ methotrexate
MACOP-B	Methotrexate with leucovorin rescue/doxorubicin/cyclophosphamide/ vincristine/prednisone/bleomycin
ProMACE-MOPP	Prednisone/methotrexate/doxorubicirgcytarabine/rnechlorethamine/ vincristine/procarbazine/prednisone
CVP	Cyclophosphamide/etoposide/cisplatin
COPA	Cyclophosphamide/vincristine/doxorubicin/prednisone
CHVP	Cyclophosphamide/doxorubicin/teniposide/prednisone
Salvage treatment	
IMVP-I 6	Ifosfamide/methotrexate/etoposide
MIME	Methyl-gag/ifosfamide/methotrexate/etoposide
ESHAP	Etoposide/cytosine arabinoside/cisplatin/methylprednisolone

Table 5: Response Criteria for Non-Hodgkin's Lymphoma [12].

Response Category	Physical Examination	Lymph Nodes	Lymph Node Masses	Bone Marrow
CR	Normal	Normal	Normal	Normal
CRu	Normal	Normal	Normal	Indeterminate
	Normal	Normal	>75% decrease	Normal or Indeterminate
PR	Normal	Normal	Normal	Positive
	Normal	≥50% decrease	≥50% decrease	Irrelevant
	Decrease in liver/spleen	≥50% decrease	≥50% decrease	Irrelevant
Relapse/progression	Enlarging liver/spleen; new sites	New or increased	New or increased	Reappearance

Rituximab

Rituximab is a chimeric monoclonal antibody (mAb) created by fusing the light and heavy chain variable domains of 2B8, a murine monoclonal anti-CD20 antibody, and human-light chain and heavy chain constant regions (figure 4). It binds specifically to CD20 [14].

CD20 is the marker of mature B-lymphocyte which expresses on the B-cell surface. In 151 B-cell NHL patients, both follicular lymphoma and diffuse large B-cell lymphoma, 93% of tumor cells expressed the CD20 molecules. It plays an important role in the process of B-cell differentiation. Many studies suggest that the cell cycle initiation and differentiation of B-cell activation process are regulated by CD20 molecules [15]. Therefore, this molecule is the target of NHL treatment by monoclonal antibody-based immunotherapy [16].

Mechanism of action

Rituximab may affect B-cell growth and differentiation. Because of CD20 molecule is expressed during early pre-B-cell development, therefore, its mechanisms of action *in vivo* still controversy and could be different according to lymphoma sub-types [17]. However, *In vitro*, rituximab can induce antibody-dependent cellular cytotoxicity (ADCC) through the FcR/Fc binding. Rituximab also bind human C1q and induces complement-mediated lysis (CDC), apoptosis, and direct growth arrest *in vitro*. There are some evidences showed the involvement of these mechanisms *in vivo* [16-20].

- **Effect of Apoptosis and growth arrest**

In 1993, Deans et al. reported that CD20 is associated with Src family tyrosine kinases, which involved in apoptosis. PAK (phosphoprotein associated with GEMs [glycosphingolipid-enriched membrane microdomains]), as known as Csk-binding protein, normally binds the Csk and inactivates the Src-family tyrosine kinase. After the rituximab binding with CD20, it redistributes lipid rafts, consequently transactivates the Src family tyrosine kinase, and introduces downstream signaling pathways, resulting in

apoptosis. On the other hand, rituximab can also induce apoptosis via Fas molecule clustering which lead to the death-inducing signaling complex (DISC) formation and activates the death receptor (DR) pathway in Ramos B-cell NHL cells. Meanwhile, the redistribution of lipid rafts can also inhibit the p38 MAPK, ERK-1/2, NF-κB, and Akt signaling pathways, resulting in the inhibition of both transcription and expression of many genes, particularly the anti-apoptotic genes as Bcl-2, Bcl-xL, XIAP and Mcl-1, consequently making B-lymphomas susceptible to apoptosis [20-21] (figure 5).

Rituximab also increases apoptosis by a caspase-independent mechanism in B-cell NHL cells, but the mechanism is still unclear [22]. Normally, apoptosis is blocked in zVAD-fmk (a caspase inhibitor)-treated cells. However, the apoptosis is still occurred in zVAD-fmk- treated cells which also treated with rituximab [23-24].

The CD 20 binding of rituximab can also induces growth arrest on B-lymphocytes. In 2004, Bezombes et al. demonstrated the direct inhibition of tumor growth by rituximab in Daudi and RL B-lymphoma cells *in vitro* [25].

- **Effect of Complement-dependent cytotoxicity** (CDC)

The complement system is the one of the innate immunity. It can be initiated by three distinct pathways known as the classical, mannose-binding lectin (MBL), and alternative pathways (Fig 6.). The classical pathway is triggered by antigen-binding antibody molecules and is initiated by the binding of the Fc region of the antibody to C1q. The MBL pathway is initiated by mannose or fucose residues on the surface of many pathogens such as bacteria. The alternative pathway is capable of spontaneous auto-activation [26-27].

All three pathways merge into the activation of C3 and then of C5 by cleaving into active fragments by highly specific enzymatic complexes, called convertases, which leads to the polymerization of C9 by C5b-8 binding and assemble into the membrane attack complex (MAC), a pore-like structure, that result the lysis of cell lysis.

The role of complement-dependent cytotoxicity (CDC) is induced after rituximab administration. *In vitro*, the Fc region of rituximab can bind human C1q and activates the classical complement pathway. This binding consequences the formation of MACs and

following by cytolysis [28-29] (figure 6). Some drugs, such as the histone de-acetylase inhibitor, can up-regulate the CD20 molecules and resulting in the sensitivity enhancement of rituximab [30].

- **Effect of Antibody-dependent cellular cytotoxicity** (ADCC)

Antibody dependent cellular cytotoxicity (ADCC) is the antibody-dependent mechanisms that occurred in several innate immune cells, such as NK cells, macrophages, monocytes, neutrophils. After the binding of rituximab with CD20, the Fc region of antibody can also bind with the Fc receptor (FcR), which expresses on the immune cell surface. Afterward, the activation of immune cells is occurred via the Fc/FcR binding and resulting ADCC. ADCC initiates a series of signaling pathways that lead to the release of inflammatory and/or cytotoxic immune modulators including cytokines, chemokines, proteases, and reactive oxygen species [31-33] (figure 7).

ADCC has involved in the antitumor activity of many mAbs. *In vitro*, mAbs can induce ADCC via several of effector cells, including NK cells, monocytes/macrophages. During the ADCC reaction, rituximab binds with CD20 on B cell surface and recruit innate immune effector cells which express Fc receptors (FcRs) [34]. The Fc region of rituximab binds to FcR on effector cells and activates the effectors cells resulting to cell lysis. Eventually, the activated monocytes/macrophages phagocytose the targeted cancer cells, whereas activated NK cells eliminate targeted lymphoma cells using the granzyme-perforin system. Perforin can form the pore structure on the target cell's membrane like the complement system. For granzyme, it is a pro-apoptotic enzyme which induced caspase-independent cell-death and resulting to apoptosis [35-37]. The antitumor activity of rituximab is greatly reduced in FcγRI/FcγRIII-deficient mice [38]. In addition, rituximab can induce the activating innate immune cells such as NK cells and macrophages, which increases the ADCC effect [39]. These results suggest that rituximab mediated ADCC is important for killing cancer cells [38-40]. Thus, the convincing evidence suggests that ADCC is a key mechanism of action.

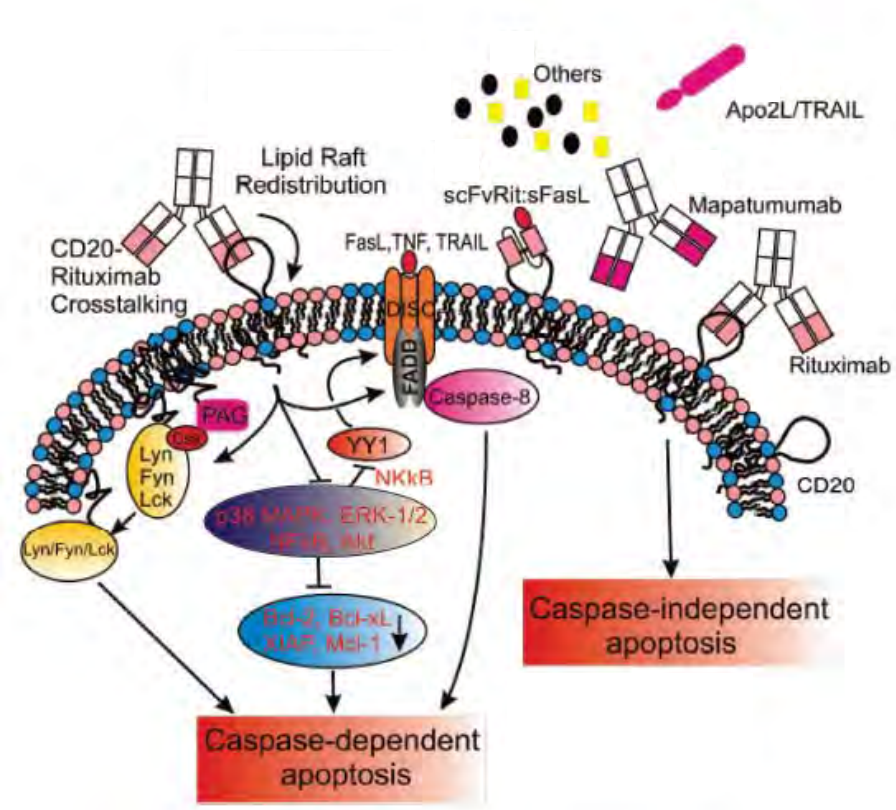


Figure 5: Rituximab-induced apoptosis in therapy [15].

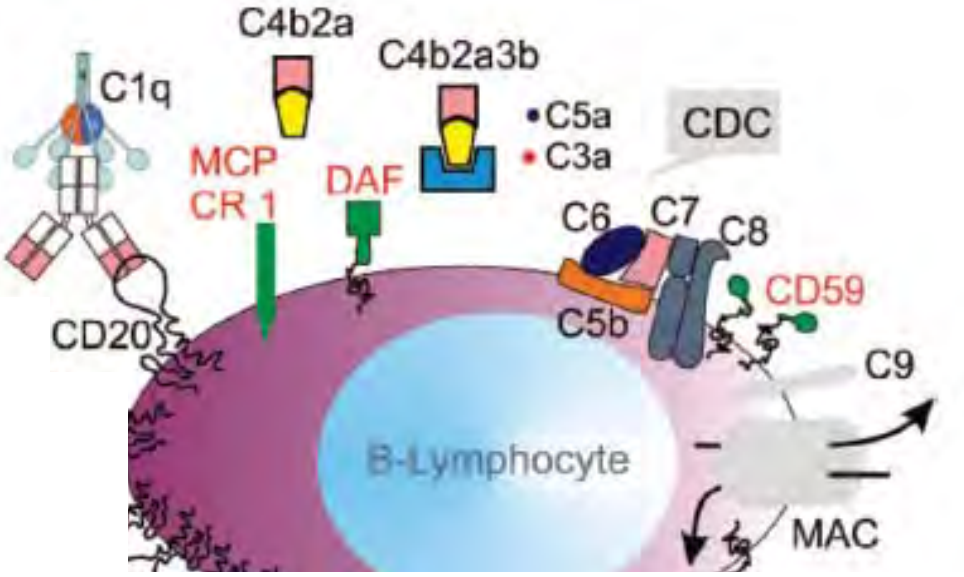


Figure 6: Rituximab-medited CDC in therapy [15].

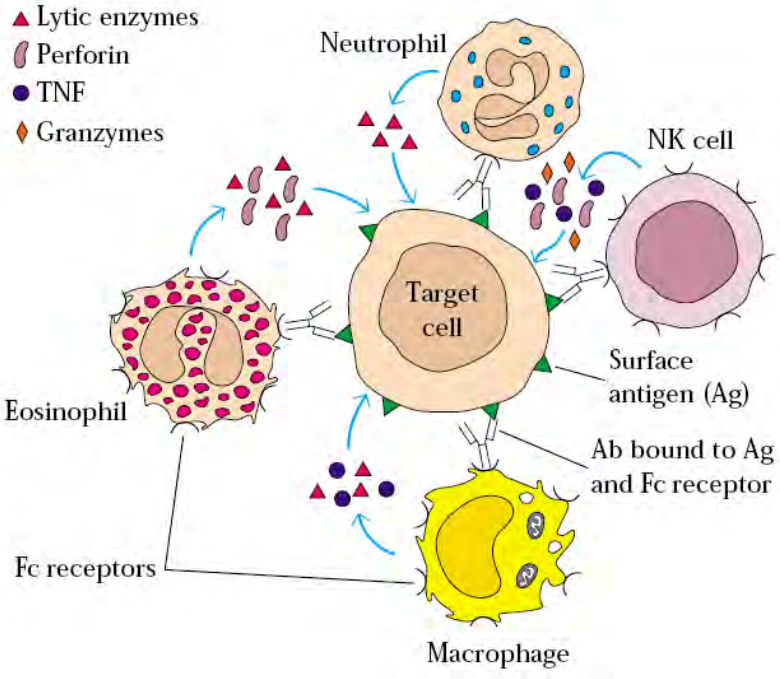


Figure 7: Rituximab-mediated ADCC in therapy [33].

Fc receptor (FcR)

The FcR could be classified into 4 different classes; FcγRI(CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV [41]. However, The FcRs can also be functionally separated into two groups; the activating and the inhibitory receptors. Whereas the activating FcRs, including the human FcγRIA, FcγIIA and FcγIIIA, promote the cell activation through the immunoreceptor tyrosine-based activation motif-dependent signaling (ITAM) pathways. On the other hand, the inhibitory receptor, FcγRIIB, recruits inhibitory signaling through the immunoreceptor tyrosine-based inhibitory motif-dependent signaling (ITIM) pathways in its cytosal domain [42-43] (figure 8).

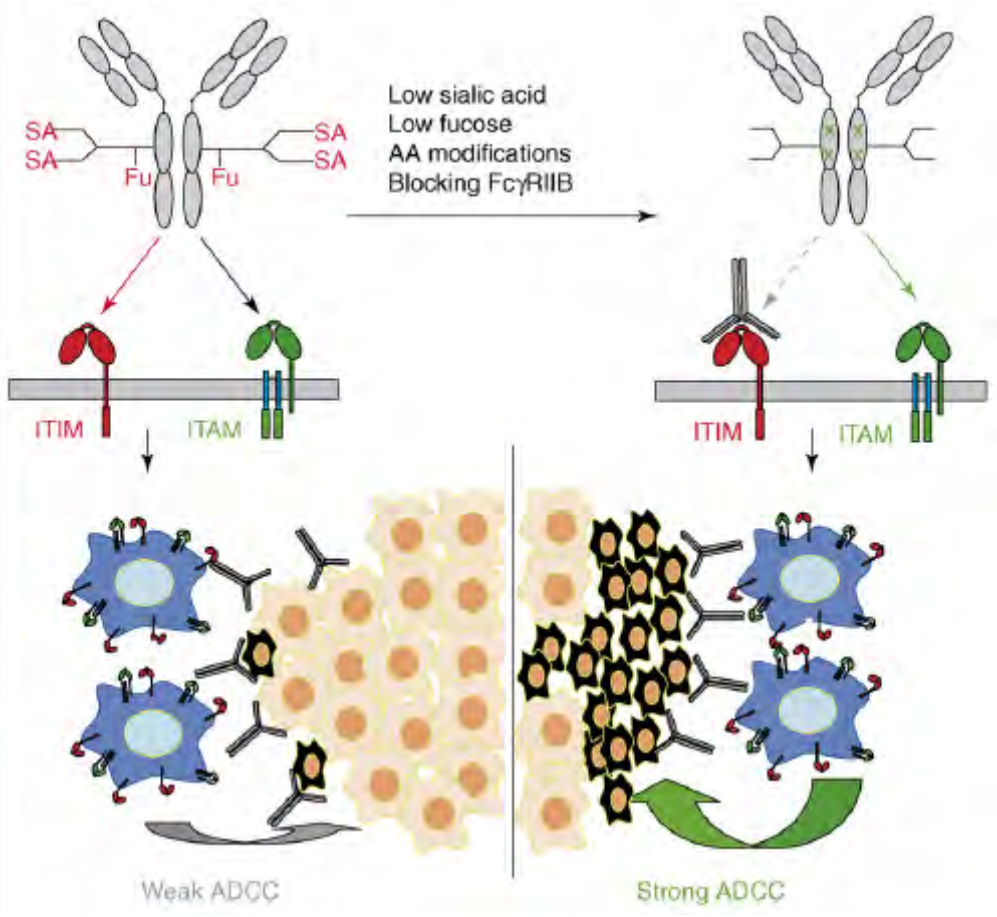


Figure 8: Optimizing Fc region of antibody/FcR interactions [44].

FcRs are generally expressed on hematopoietic lineages, such as macrophages, natural killer (NK) cells. They allow these cells to bind to antibodies that are attached to the surface of microbes, infected cells or cancer cells, processing the elimination of infected cells or cancer cells. The Fc receptors bind the Fc region of antibodies resulting to cell activation. The activation of phagocytes is the most common function attributed to Fc receptors. Another process involving Fc receptors is antibody-dependent cell-mediated cytotoxicity (ADCC). The FcR is expressed both activating and inhibitory receptors on every hematopoietic lineage cells except B-lymphocytes and Natural Killer cells (NK cells). B-lymphocytes only express the inhibitory FcR (FcγRIIb). On the other hand, NK cells only express the activating FcγRIII (table 6) [43-44].

Fc gamma receptor IIIa (FcγRIIIa)

FcγRIIIa or CD16 is the Fc receptor that is dominantly expressed on human natural killer cells (NK cells). The FcγRIIIa recognizes the IgG which bound to the surface of a target cell. Activation of FcγRIIIa by IgG causes the release of cytokines such as interferon-γ (IFN-γ) that activate the other immune cells. The other cytotoxic mediators like perforin and granzyme, promote cell death by triggering apoptosis.

The FcγRIIIa is the low affinity receptors for the Fc region of IgG. Normally, the low affinity FcRs cause the specific binding and cell activation. That makes the FcγRIIIa has more crucial for anti-tumor responses and in monoclonal antibody-based therapy than the high affinity receptors [43].

The genetic polymorphisms have been discovered in various FcγR. For FcγRIIIa, the Guanine (G) to Thymidine (T) point mutation at nucleotide 559 results the amino acid substitution at position 158 as Valine (V) to Phenylalanine (F) [45]. The FcγRIIIa-158 V allele shows higher affinity for IgG1 and IgG3 than FcγRIIIa-158F, and is able to bind IgG4. Meanwhile, FcγRIIIa-158F is not able to bind the IgG4 [46-47]. Following the incubation of NK cells from FcγRIIIa-V/V homozygous donors with IgG, the influxes of calcium and the induction of apoptosis from FcγRIIIa-V/V homozygous donors is more over than FcγRIIIa-F/F homozygous donors [48].

The impact of FcγRIIIa polymorphism

Because the majority of therapeutic antibodies are IgG1, the mechanism of monoclonal antibodies, is mostly IgG1, also involve to the FcγR. To prove this hypothesis, the study in $Fc\gamma R^{-/-}$ mice which had treated with two widely used therapeutic mAbs, trastuzumab and rituximab was investigated. The results have shown the implication of different FcγR in the *in vivo* mechanisms. These mAbs can act against tumors through both activating (FcγRIIIa) and inhibitory (FcγRIIb) receptors on myeloid cells. The FcγRIIIa-deficient mice were not capable to prevent the tumor growth in presence of therapeutic mAbs [49-51]. In humans, a recent study has shown that FcγRIIIa polymorphism is associated with the therapeutic efficacy of rituximab in non-

Hodgkin's lymphoma patients. Thus, the homozygous V/V allele patients (IgG1 higher affinity) provided a higher response to the treatment than the homozygous F/F allele patients (IgG1 lower affinity) [52-53]. Additionally, a higher response has been shown in rituximab treated follicular lymphoma patients who have the homozygous for FcγRIIIa-Val158 and FcγRIIb-His131 alleles [52]. On the other hand, some experiments have been shown that FcγRIIIa 158 V/F polymorphism is not associated to the response of R-CHOP (Rituximab combined with CHOP regimen in NHL treatment) in mostly Caucasian patients with Follicular lymphoma treated with R-CHOP [54-56], which is different from the study in Korean patients with Diffuse Large B-cell lymphoma [57]. That may cause the effects of the ethnicity background. Entirely, the role of FcγRIIIa polymorphism in the efficacy of mAbs is still controversy and might depend on the stage and type of disease or the ethnicity [57].

FcγRIIIa polymorphisms and the ethnicity

Several studies have been found the variation of ethnicity in the distribution of the FcγRIIIa genotypes (Table 7) [57-61]. From the knowledge of the genetic polymorphism, the distribution of each genotyping is involved the ethnicity and background of individual. From table 7, the distribution of homozygous V/V158 is varied from 4% to 47%. For the distribution of heterozygous V/F158 diverged from 32.1% to 50%. And the distribution of homozygous F/F158 varied from 5% to 63.2%. That has the wide range of distribution. Therefore, in Thailand, the distribution of the FcγRIIIa genotypes may be different from the others. This research is focused to study the distribution of the FcγRIIIa genotypes in Thai population, and investigate the correlation of the FcγRIIIa polymorphism and the response of rituximab, anti-CD20, both *in vitro* and *in vivo.*

Table 6: Expression of human FcγR on cells of the immune system [43].

	B Lymphocytes	Dendritic cells*	Monocytes/ Macrophages	NK cells	Neutrophils	Mast cells
FcγRI		• Antigen presentation by immune complexes • Cytokine production	• Phagocytosis • ADCC		• Superoxide production • ADCC	
FcγRIIa						
FcγRIIIa				• Cytokine production • ADCC		• Serotonin release • Cytokine production
FcγRIIIb					• Superoxide production • ADCC	
FcγRIIb	Down regulation of B-cell receptor activation	Down regulation of FcγR activation	Down regulation of FcγR activation		Down regulation of FcγR activation	Down regulation of FcγR activation

*FcγR expression depends on activation status.

Table 7: the distribution of the FcγRIIIa genotypes in several races [57-61].

Country	V/V158 (%)	V/F158 (%)	F/F158 (%)
Japanese	4	44	52
Dutch	10	48	42
Sami	4.7	32.1	63.2
Norwegian	13.7	37.4	48.9
Croatian	28	55	17
France	20	45	35
Korean	47	48	5
Aferican-american	8	50	42
Caucasian	11	39	50

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Cells

Ramos cells

Ramos cells were Burkitt's lymphoma cells purchased from the American Type Cell Culture (ATCC). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in 100 mg/ml streptomycin and 100 units/ml penicillin condition, at 37 $^{\circ}$C with 5% CO_2. The cells were initially cultured at the density of 2 x 10^5 cells/ml and then sub-cultured when the cell reached 2 x 10^6 cells/ml.

Human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from 20-35 years old healthy male blood donors from the Red Cross society with informed consent. The whole blood was collected in 9 ml EDTA tube. One ml was separated and stored at -20°C for genetic polymorphism study. The left was freshly used to prepare to PBMCs for ADCC study.

The whole bloods from NHL patients subjected for genotyping was collected from the patient with inform consent. These patients were treated with rituximab-containing drug regimen. Three ml of the blood was removed from each patient. The blood was stored at -20°C before used

1.2 Equipments and Instruments

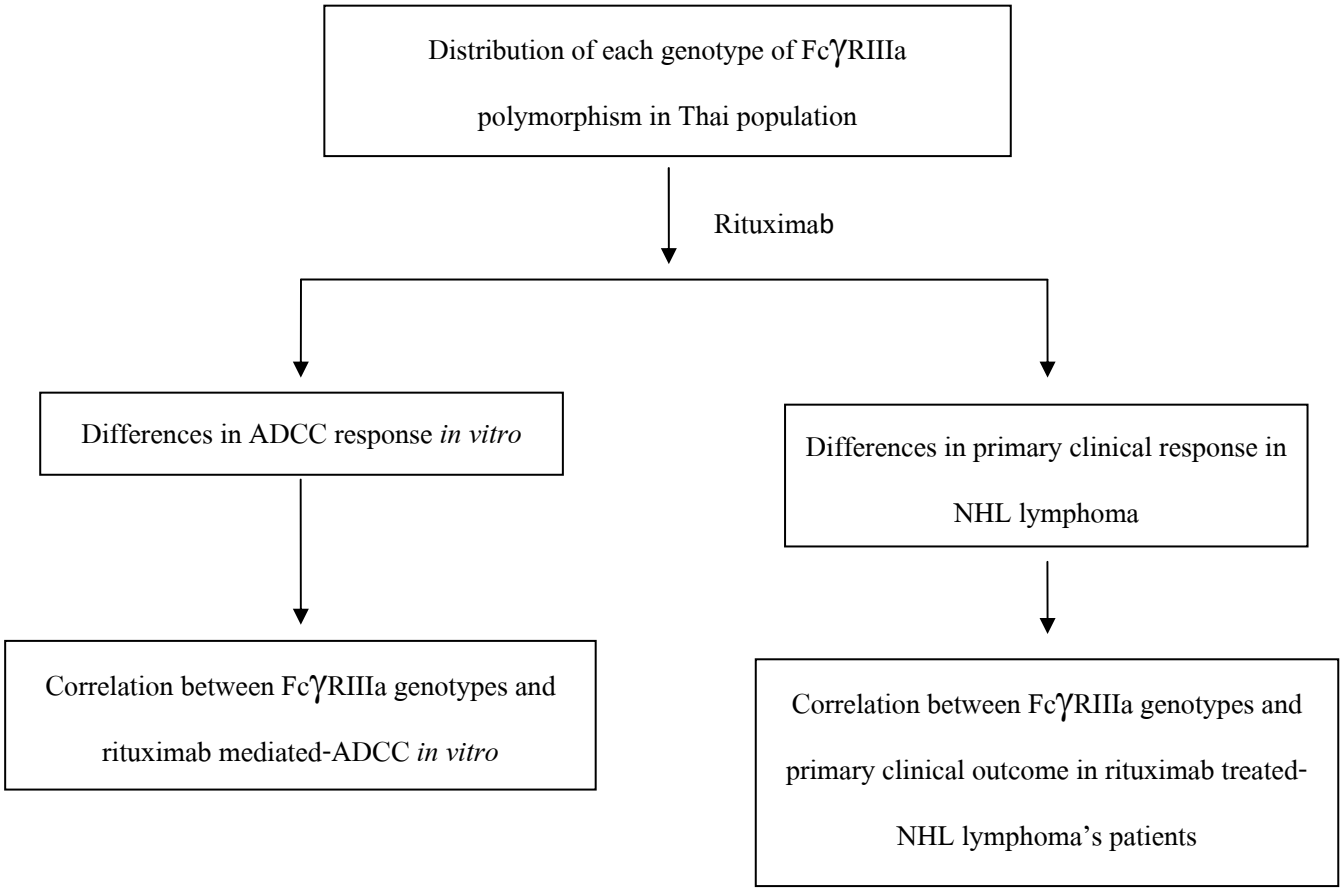
The equipments and instruments used in this study were in the following; CO_2 incubator (Thermo, USA), sterile laminar flow hood (ESSCO, USA), flow cytometer (Beckman Coulter, USA), centrifuge (Eppendorf, Germany), gel electrophoresis (Bio-Rad, USA), hemocytometer (Brand, Germany), light microscope (Nikon, USA), analytical balance (GMPH, Satorius (Germany and UMT2, Mettler Toledo, Switzerland), PCR thermocycler machine (Eppendorf, USA), autopipette (Gilson, USA), T25 tissue culture flask (Corning, USA), sterile polypropylene centrifuge tube : 15 ml, 50 ml (Corning,

USA), autoclave (Hirayama, Japan), pipette (Falcon, USA), pH meter (Mettler tuledo, Switzerland), gel documentation (Bio-Rad, USA)

1.3 Chemicals and reagents

The reagents used in this study were in the following; Rituximab, an IgG1 chimeric monoclonal antibody against human CD20 (Roche, Switzerland), carboxyfluoroscein succinimidyl ester (CFSE) (Dojindo, Japan), propidium iodide (PI) (Santa Cruz, USA), trypan blue dye (Sigma, USA), RPMI 1640 medium (sigma, USA),sodium bicarbonate (Baker, USA), fetal bovine serum (Gibco, USA), L-glutamine (Gibco, USA), penicillin/streptomycin (Gibco, USA), Histopaque®-1077 (Sigma, USA), 0.4% trypan blue dye (Sigma, USA), Taq polymerase (Invitrogen, UK), Accuprime® Taq DNA polymerase (Invitrogen, USA), agarose (Bio-Rad, USA), dNTP mix (Vivantis, Malaysia), absolute ethanol (Merck, Germany).

2. Conceptual framework



3. Methods

3.1 ADCC assay

In ADCC assay, PBMCs containing NK cells were used as effector cells, Ramos cells which express CD20 molecules on their cell surface were used as target cells. They were stained with fluorescent CFSE for separating from the effector cells, and anti-human CD20 Ab rituximab was used as an Ab for initiating ADCC. This assay was performed at E:T ratio (PBMCs:Ramos cells) 10:1.

(1) Target cell (Ramos cells) preparation

Ramos cells were stained with a fluorescent dye CFSE for separating the cells from their effector cells. Ramos cells were separated from the RPMI 1640 medium by centrifugation at 1,200 rpm for 10 minutes. The cell pellet was washed once with PBS containing 10% FBS and re-suspended in the same buffer at 1 x 10^7 cells/ml. One ml of this cell suspension was stained with 5μM CFSE for 5 minutes at room temperature in the dark. The cells were washed twice with PBS containing 10% FBS and re-suspended in RPMI 1640 complete medium. These stained cells were left overnight in an incubator at 37°C with 5% CO_2 under light protection before assay.

(2) Effector cell (human PBMCs) preparation

The whole blood of Thai male donor was centrifuged at 3,200 x g for 10 minutes. The buffy coat on the top of the pellet was collected into the 15 ml tube and re-suspended with 5 ml incomplete RPMI medium. The diluted blood was slowly overlaid on the ficoll-hypaque solution and centrifuged at 400 x g for 30 minutes. The buffy coat was carefully collected and then washed with 10 ml incomplete RPMI medium twice by centrifugation at 250 x g rpm for 10 minutes. Finally, the pellet was re-suspended in complete RPMI medium and incubated overnight at 37°C with 5% CO_2.

(3) ADCC assay

CFSE-stained Ramos cells at 2 x 10^5 cells/ml and added into 5 ml culture tube. The cells were treated with 10 μM rituximab for 1 hour at 37°C with 5% CO_2. This

allowed the antibody to specifically bind to CD20 molecules on the Ramos cell surface. After 1 hour, human PBMCs at 2 x10^6 cells/ml were added into the treated CFSE-stained Ramos cells. This made E:T ratio 10:1. The co-culture cells were thoroughly mixed and further incubated for 4 hours at 37°C with 5% CO_2. After the co-incubation, the cells were separated by centrifugation at 400g for 10 minutes. The cell pellet was re-suspended in 100 µl assay buffer, stained with 0.5 µg/ml of propidium iodide (PI) for 15 minutes at room temperature, and added 400 µl assay buffer. The cells were added with 400 µl of assay buffer and immediately analyzed by fluorescence flow cytometer.

3.2 FcγRIIIa polymorphisms analysis

Whole blood of both 60 healthy Thai male and NHL patients was subjected for genotyping. Genomic DNA was extracted from the whole blood and used for genotyping by RFLP-nested PCR method as described below:

(1) DNA extraction

Genomic DNA was extracted by using a blood DNA extraction kits (Vivantis®). The stored whole blood was thawed at room temperature. Two hundred of the diluted with 200 µl buffer solution in a 1.5 ml centrifuge tube, mixed by pulsed vortex quickly, added 20 µl proteinase K, vigorously mixed by vortex, and incubated for 15 minutes at 65°C. During incubation, the tubes were mixed by vortex every 3 minute. Two hundred µl absolute ethanol was added to precipitate the genomic DNA. The solution was quickly mixed by vortex, transferred to a column provided in the DNA extraction kit, and left for 1 minute for genomic DNA binding to membrane of the column. The column was centrifuged at 5,000 x g for 1 minute, the filtrate in a collecting tube was discard while the column was washed once with 500 µl washing buffer by centrifugation at 5,000 x g for 1 minute, and then washed with 500 µl washing buffer II twice by centrifugation at 5,000 x g for 1 minute in the first wash and at 14,000 x g for 3 minutes in the second wash. This made the column dried and prevented the ethanol contamination. A hundred µl of elution buffer, pre-treated to 65°C, was added into the washed and dried column and left for 4 minutes. The column was inserted into the new sterile collecting tube and

centrifuged at 5,000 x g for 1 minute. The genomic DNA in the collecting tube was collected, determined DNA content and contamination by Nanodrop® at 260 and 280nm, and stored at -20°c until used. All the genomic DNA samples had their OD_{260}/OD_{280} values 1.7-2.0.

(2) RFLP-Nested PCR

The FcγRIIIa genotypes of the genomic DNA samples from healthy subjects and NHL patients were identified by a nested-PCR method. There were 2 sets of primer used in this method. The first set gives a 1.2 Kb PCR product from a human genomic DNA template. The second set was used to amplify the first 1.2 Kb PCR product before digested with restriction enzyme *Nla*III. The procedure in detail was in the following;

The genomic DNA was used to be the DNA template in the first reaction. Two μl of the DNA was mixed with the master mix solution containing 0.2 mM dNTP, 1 U *Taq* polymerase, 1.5 mM $MgCl_2$ and 0.5 μM the first set FcγRIIIa primers in a 0.2 ml PCR tube. The FcγIIIa PCR product was amplified in a thermaocycler machine by using the following conditions; initial denaturation 95°c for 10 minutes, followed by 40 cycles of PCR amplification protocol (95°C for 1 minute, 56°c for 1 minute 30 seconds and 72°c for 1 minute 30 seconds), and finally the final extension at 72°c for 8 minutes. The PCR product was stored at -20°C.

The PCR product from the genomic DNA template was subjected for nested-PCR. It was used as the template of the second set of primers for FcγRIIIa gene which gives rise to a 96 bp PCR product. The nested-PCR was performed in the following procedure;

Two μl of PCR product from the first reaction was added into the master mix solution containing 0.2 mM dNTP, 1 U *Taq* polymerase, 1.5 mM $MgCl_2$ and 0.5 μM the second set FcγRIIIa primers in a 0.2 ml PCR tube. The second FcγRIIIa PCR product was amplified in a thermaocycler machine by using the following conditions; initial denaturation 95°C for 5 minutes, followed by 40 cycles of PCR amplification protocol (95°C for 1 minute, 67.5°C for 1 minute 30 seconds and 72°C for 1 minute 30 seconds), and finally the final extension at 72°C for 9 minutes 30seconds. The nested PCR product

was subjected for restriction fragment length polymorphism (RFLP) by being digested with 10 U *Nla*III in 20 μl the PCR product. The digestion was performed at 37°C for 3 hours. The PCR product was stored at -20°C before used.

(3) Allele specific amplification

To confirm the heterozygous VF genotype, the allele specific amplification method was used. The genomic DNA was used to be the DNA template in this reaction. Two μl of the DNA was mixed with the master mix solution containing 0.2 mM dNTP, 1 U Accuprime® *Taq* polymerase, and 0.5 μM the set of F allele specific primers in a 0.2 ml PCR tube. The FcγRIIIa PCR product was amplified in a thermaocycler machine by using the following conditions; initial denaturation 94°c for 2 minutes, followed by 40 cycles of PCR amplification protocol (94°C for 30 seconds, 65°c for 30 seconds and 68°c for 1 minute), and finally the final extension at 72°c for 8 minutes. The PCR product was stored at -20°C.

(4) Gel electrophoresis

The FcγRIIIa genotype of the digested PCR product was identified by agarose gel electrophoresis. A 3% agarose gel was prepared. Twenty μl of the digested product was mixed with 4 μl loading dye, and then loaded into a well on the agarose gel.

For the F allele specific product, a 1.5% gel was prepared. Six μl of the PCR product was mixed with 2 μl loading dye, and loaded into a well on the agarose gel.

The gel was run on gel electrophoresis at 100 volts for 45 minute, then stained with 0.5 μg/ml ethedium bromide in 1x TBE buffer for 5 minutes, and finally de-stained with 1x TBE buffer for 10 minutes. The digested PCR product was identified by exposing to UV light in the gel documentation.

3.3 Clinical outcome evaluation

Primary clinical outcomes of NHL patients treated with rituximab-containing drugs were assessed for correlating with their genotypes. The data of clinical outcomes recorded by the expert clinicians were collected from the patients' chart. The treatment response of each patient was evaluated based on the standardize response criteria, International Workshop Criteria for Non-hodgkin's Lymphoma, which divides the response to 3 levels as complete response (CR), partial response (PR) and progression disease (PD) as described above in chapter II.

3.4 Ethical consideration

The protocol from this study was approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University. All donor and patient in the study understood the protocol of the study and had the opportunity to question in detail before making decision to sign the informed consent.

The inclusion criteria for normal volunteer

1. Male.
2. Age between 20-30 (±2) years old.
3. Strict to the criteria of the Red Cross society for blood donor.
   - More than 45 kg bogy weight.
   - Healthy physical condition.
   - No record to be hepatitis or jaundice.
   - Do not over loss weight in the short period.
   - Do not have the sexual behavior risk or drug addiction.

The inclusion criteria for Non-hodgkin's lymphoma patient

1. Diagnosed as non-Hodgkin's lymphoma
2. CD20+ was detected
3. Received rituximab during the process of treatment

3.5 Sample size determination

Normal volunteers

The sample size used for evaluating the distribution of FcγRIIIa polymorphisms in Thai population was calculated based on the results in Japan population from Straat F.G.J. *et.al* by the following formula:

$$n = \frac{(Z_{\alpha/2})^2 \, P \, Q}{(d)^2}$$

Which: n = sample size

$Z_{\alpha/2}$ = the critical value at 95% confidence, which is 1.96 ($Z_{0.05/2}$) for two-tail analysis.

P = the percentage of evidence of the interested incidence= 0.04

Q = P-1 = 0.96

d = the maximum error of the estimation = 0.05

The sample size calculated from this formulation is as follow;

$$n = \frac{(1.96)^2 \, 0.04 \times 0.96}{(0.05)^2}$$

$$n = 59.007$$

So, the number of normal volunteers used in this study was 60.

Non-Hodgkin's lymphoma patients

The number of NHL patients used in the study was calculated based on the data of the clinical study from Carlton *et.al* by the following formula;

$$n = \frac{2(Z_{\alpha/2} + Z\beta)^2 \bar{P}\,\bar{Q}}{(P_1-P_2)^2}$$

Which: $Z_{\alpha/2}$ = the critical value at 95% confidence. which is 1.96 ($Z_{0.05/2}$) for two-tail analysis.

$Z\beta$ = the critical value at 90% power, $Z_{0.1}$ = 1.28

P_1 = the percentage of evidence of the first incident (V carrier) = 0.65

P_2 = the percentage of evidence of the second incident (FF carrier) = 0.35

The sample size calculated from this formulation is as follow;

$$n = \frac{2(1.96+1.28)^2\ 0.65 \times 0.35}{(0.65-0.35)^2}$$

$$n = 53.0712$$

So, the number of Non-Hodgkin's lymphoma patients used in this study was 54.

3.6 Statistical analysis

Data were individually presented. For the *in vitro* ADCC study, the difference of the ADCC activity in each genotyping group was compared by using the nonparametric Kruskal-Wallis test. The correlation between the genotypes and the clinical responses of NHL-patient treated with rituximab-containing regimen was assessed by using the two-tailed Fisher's exact test. The statistically significant value was considered at *p*-value < 0.05.

CHAPTER IV

RESULTS

1. The distribution of FcγRIIIa polymorphism in Thai population.

Two techniques were used to determine the FcγRIIIa genotype, RFLP-nested PCR and direct PCR using F allele-specific primer for complete results.

From RFLP-nested PCR, The nested PCR products were generated by using 2 set of primers. The first set of the primers gave rise to the 1.5 Kb PCR product whereas the second set of the primer generated the 94 bp PCR products. The restriction enzyme; *Nla*III was used to digest the second PCR product at the 158 polymorphic site of the FcγRIIIa. The restriction site of *NlaIII* is CATG. This enzyme cuts only PCR product of V allele into 61 and 33 bp products but it does not cut PCR product of F allele. *Nla*III theoretically generates one band (94 bp) for F/F genotype, two bands (61 and 33 bps) for V/V genotype and 3 bands (94, 61, and 33 bps) for V/F genotype. However, the V/F genotype could not be clarified by this method in this study because there was no three bands among *Nla*III digested products. Only V/V (2 bands) and F/F (1 band) genotypes were identified. In order to identify heterozygous V/F genotypes, F allele in the genomic DNA samples of V/V genotype identified by RFLP-nested PCR were amplified by PCR using F allele specific primer. The genomic DNA samples were counted as the heterozygous V/F genotype if they generated the 96 bp PCR product from the F allele specific primer. Three of the sixty genomic DNA samples were confirmed their genotypes by DNA sequencing (see in the Appendix B, figure 16).

The FcγRIIIa genotypes of 60 healthy subjects were 23 V/V, 10 V/F, and 27 F/F, respectively. The genotypes of 17 NHL patients treated with anticancer drug regimen containing rituximab were 8 V/V, 3 V/F, and 6 F/F (Table 8).

Table 8: The distribution of FcγRIIIa polymorphism in Thai population (see detail in Appendix B, table 11).

Genotype	VV	VF	FF	Total
Normal Volunteers	23	10	27	60
NHL patients	8	3	6	17
Total	31 (40.25%)	13 (16.88%)	33 (42.85%)	77

Figure 9: Analysis of the FcγRIIIa polymorphism. (a) Representative results from RFLP nested-PCR technique 1) undigested PCR product, 2) and 3) *Nla*III digested PCR products as V/V (2) and F/F (3) genotypes, respectively. (b) A representative results of determination of heterozygous V/F genotype from V/V genotype identified by RFLP nested-PCR by using F allele-specific primer; 1) V/V genotype 2) V/F genotypes. All data were in the Appendix B, figure14-15. (n=60 for healthy volunteers, n=17 for Non-Hodgkin's lymphoma patients.)

2. Correlation of the FcγRIIIa genotype and rituximab-mediated ADCC *in vitro.*

The correlation between FcγRIIIa genotype and in vitro ADCC activity of NK cells from 60 healthy subjects was also evaluated in this study. ADCC of NK cells was induced by using rituximab-bound Ramos cells (CD20 positive cells) as the target cells. Cytotoxicity on Ramos cells was identified by staining these cells with fluorescent CFSE to separate them from PBMCs and by staining them with PI to detect cell death. Ramos cell death was determined as CFSE+/PI+ cells by fluorescence flow cytometer (Fig. 10, see detail in Appendix B, table 11). PMBCs from each subject with known genotype induced Ramos cell death by ADCC was presented in Table 9. The mean values of the percentage of rituximab induced Ramos cell cytotoxicity were 31.16% in V/V genotype subjects, 36.87% in heterozygous V/F genotype subjects, and 20.07% in F/F genotype subjects (Fig.11). Both V/V and V/F genotypes had significantly higher ADCC activity than F/F genotype at $p<0.001$.

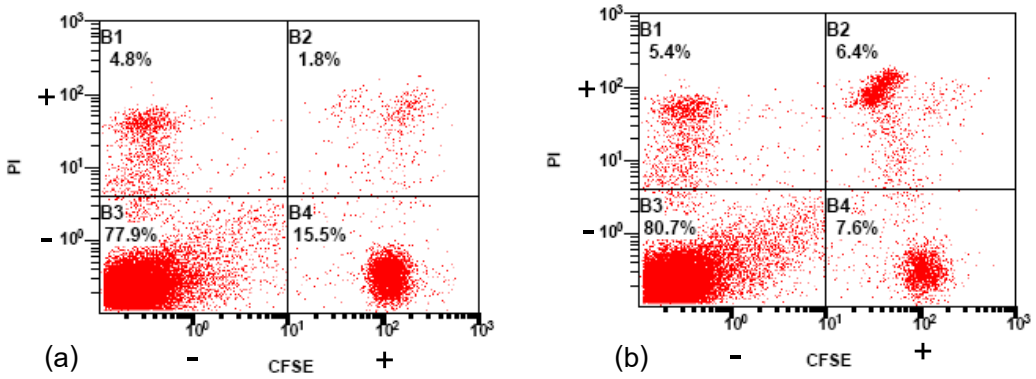


Figure 10: The representative results of rituximab-mediated Ramos cell death by ADCC at 10:1 PBMCs:$CFSE^+$ Ramos cells ratio using fluorescence flow cytometer.(a) without rituximab and (b) with rituximab. The amount of Ramos cell death is identified in B2 quadrant. The percentage of Ramos cell death was calculated from the percentage of cells in quadrant B2 and B4. All of data were in the Appendix B, figure 13 and table 11 (n=60).

B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$).

B2: The percentage of Ramos cells death ($PI^+/CFSE^+$).

B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$).

B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

Table 9: *In vitro* rituximab-mediated Ramos cell cytotoxicity by ADCC using PBMCs from 60 healthy volunteers as effector cells.

Sample No.	Average % cytotoxicity	Genotype
1	18.06	FF
2	33.55	FF
3	33.05	FF
4	41.61	VV
5	58.46	VV
6	27.50	VV
7	12.27	VV
8	6.07	FF
9	35.37	FF
10	34.04	VF
11	25.44	FF
12	26.89	VV
13	8.92	FF
14	50.82	VV
15	16.32	FF
16	12.84	FF
17	54.05	VF
18	30.26	VV
19	18.10	FF
20	13.15	FF
21	28.71	VF
22	29.40	VV
23	16.48	FF
24	58.43	VF
25	30.81	VF
26	46.31	VV
27	34.42	FF

Table 9 (cont.): *In vitro* rituximab-mediated Ramos cell cytotoxicity by ADCC using PBMCs from 60 healthy volunteers as effector cells.

Sample No.	Average % cytotoxicity	Genotype
28	31.06	FF
29	22.74	FF
30	34.31	FF
31	7.05	FF
32	18.43	VV
33	7.94	FF
34	34.95	VF
35	25.63	VV
36	7.59	FF
37	25.23	VV
38	47.15	VV
39	17.44	VV
40	16.14	FF
41	16.60	FF
42	26.07	FF
43	8.46	VF
44	26.46	VV
45	32.01	VV
46	19.71	FF
47	14.01	FF
48	44.54	FF
49	3.54	VF
50	38.65	VV
51	5.78	FF
52	51.71	VF
53	64.02	VF

Table 9 (cont.): *In vitro* rituximab-mediated Ramos cell cytotoxicity by ADCC using PBMCs from 60 healthy volunteers as effector cells.

Sample No.	Average % cytotoxicity	Genotype
54	47.23	VV
55	43.44	VV
56	36.97	VV
57	32.94	VV
58	34.28	VV
59	13.39	VV
60	16.62	FF

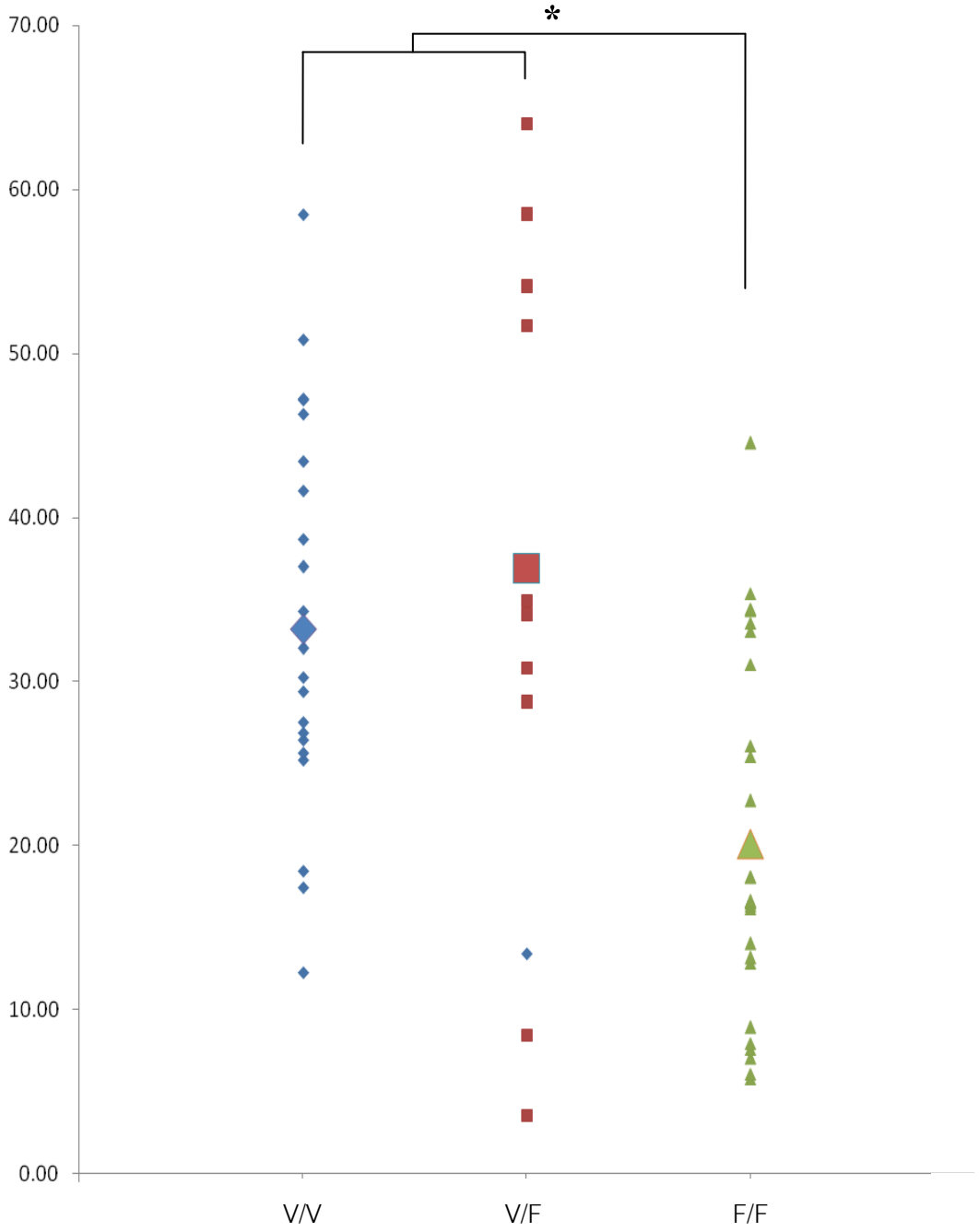


Figure 11: The correlation between FcγRIIIa genotypes and rituximab-mediated ADCC *in vitro* by using PBMCs from 60 healthy subjects as target cells.

* statitistically significance at $p<0.001$

3. Correlation of the FcγRIIIa polymorphism and the primary clinical outcome of rituximab-treated NHL patients.

This study also investigated the correlation of FcγRIIIa genotypes and clinical outcomes of 17 NHL patients treated with anticancer drug regimens containing rituximab. The genotypes of theses patients were 8 patients with V/V, 3 patients with V/F and 6 patients with F/F. The anticancer drug regimens received by these patients were in the Appendix B, table 12. The primary outcomes of these patients were evaluated according to the standardized criteria as described in methods by specialist physicians in hematologic oncology unit of King Chulalongkorn Memorial hospital.

As in the Table 10 and Fig. 12, all of 8 patients with V/V genotype had complete response. Two of three patients with V/F genotype had complete response. The other one could not be assessed due to rituximab complications. This antibody was withdrawn in this patient. Only one of six patients with F/F genotype had complete response. Three of them had partial response and the other two patients could not be assessed because of rituximab complications.

Table 10: The primary clinical outcomes of 17 NHL patients treated with anticancer drug regimens containing rituximab.

		Genotype		
		VV	VF	FF
Primary clinical assessment	Complete Response (CR, CRu)	8	2	1
	Partial Response (PR) or Progression Disease (PD)			3
	No assessment		1	2
	Total	8	3	6

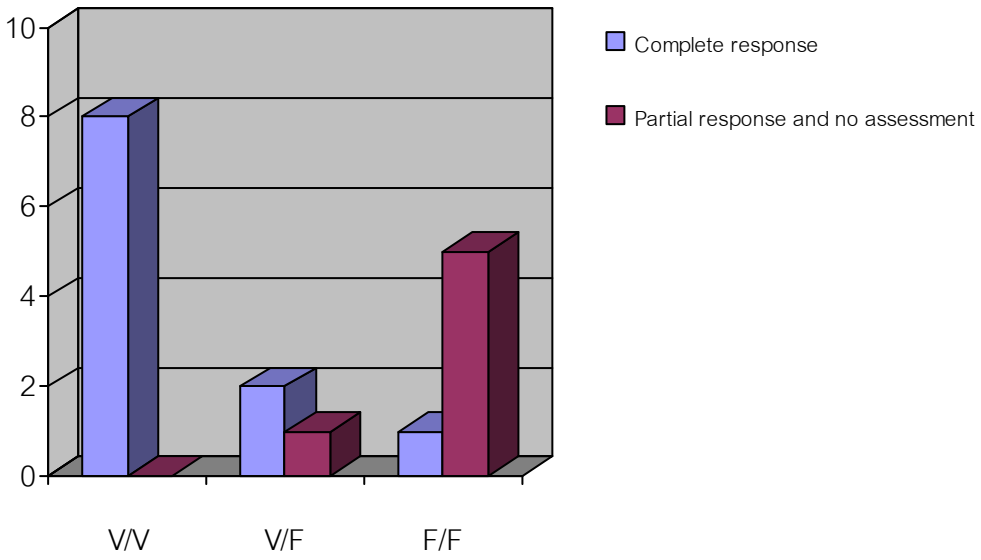


Figure 12: The correlation between FcγRIIIa genotype and primary clinical outcome of rituximab-treated NHL patients (n=17).

CHAPTER V

DISCUSSION AND CONCLUSION

This study intended to investigate the distribution of FcγRIIIa polymorphism in Thai population and its impact on the response to rituximab both in vitro and in NHL patients received rituximab-containing regimens. Several studies have been reported that ADCC is the major mechanism of action of rituximab to destroy B-lymphoma cells in NHL (REFs). Furthermore, it has been demonstrated that the FcγRIIIa polymorphisms also involves in the response to rituximab in NHL patients [52]. FcγRIIIa is a receptor of IgG expressed on macrophages and NK cells and plays role in ADCC of rituximab. The FcγRIIIa genetic polymorphism from G to T at nucleotide position 559 corresponds to phenotype expression of valine (V) to phenylalanine (F) at amino acid 158 in the extracellular domain of the receptor. These polymorphisms influence on the affinity of IgG1 to the FcγRIIIa.

This study revealed the distribution of FcγRIIIa polymorphism in Thai population. The frequencies of FcγRIIIa-158 V/V, V/F and F/F genotype are 40.25%, 16.88% and 42.85%, respectively. Distributions in several genetic polymorphisms are usually influenced by race and ethnicity. In Caucasian population, the frequency of homozygous V/V genotype is 11% which is lower than in this study whereas the frequency of the homozygous F/F genotype is 50% similar to this study [58-59]. The frequencies of FcγRIIIa genotypes in Thai population here are different from population in other countries in Asia. The frequencies of FcγRIIIa-158 V/V, V/F and F/F genotype in Korean are 47%, 48% and 5%, respectively [57]. These frequencies in Japanese are 4%, 44% and 52%, respectively [60].

It has been shown that homozygous FcγRIIIa 158V/V on NK cells bind to IgG stronger than FcγRIIIa 158F/F [62-63]. Recent studies have been suggested that healthy individuals expressing V/V and V/F genotypes increase of FcγRIIIa expression on NK cell surface, enhance the rituximab binding, and demonstrate higher levels of ADCC activity in response to rituximab [46]. This study also investigated the correlation

between FcγRIIIa polymorphism and the response to rituximab *in vitro*. As the major mechanism of action of rituximab is ADCC, NK cells which express FcγRIIIa on their cell surface are involved in the action of rituximab. It is known that IgG binds to FcγRIIIa on NK cells and activates these cells to release granzyme which induce caspase activation and apoptotic induction of the IgG-recognized target cells. This study used human B-lymphoma Ramos cells as the target cells recognized by rituximab and NK cells in human PBMCs as the effector cells. The results demonstrated that effector cells from healthy individuals with V/V and V/F genotype induced higher rituximab-mediated Ramos cell cytotoxicity than effector cells from F/F allele individuals. The percentage of Ramos cell death was 33.16%, 36.87% and 20.07% when the effector cells were from V/V, V/F and F/F individuals, respectively. These results support the correlation of FcγRIIIa polymorphism and the response to rituximab *in vitro.* The FcγRIIIa 158V/V and V/F NK cells, which FcγRIIIa binds to IgG stronger, induced higher ADCC than FcγRIIIa 158F/F NK cells which FcγRIIIa binds to IgG weaker.

Several studies revealed the influence of FcγRIIIa polymorphisms on the response to rituximab containing anticancer regimen in different types of NHL [52-58]. They have been reported that NHL patients with FcγRIIIa 158 V/V genotype response better than F/F genotype either to only rituximab monotherapy but not to rituximab-containing regimen or to both types of therapy depend on types of lymphoma. However, there are also some studies in Caucasian population which demonstrated no correlation between FcγRIIIa polymorphisms and the clinical response to rituximab [54-56]. The correlation between the FcγRIIIa polymorphism and the primary clinical outcomes of rituximab-treated NHL patients was also investigated in this study. The NHL patients recruited in the study were either DLBCL or FL patients treated with rituximab containing anticancer regimens. The primary clinical outcomes of these patients were assessed by expert clinicians during the follow up after the courses of treatment. The results demonstrated that the patients with FcγRIIIa-158 V/V and V/F genotypes responded higher than FcγRIIIa-158 F/F genotype to riruximab containing regimens. Complete response was assessed in all patients with V/V genotype (8/8), in 2 of 3 patients (2/3)

with V/F genotype and only in 1 of 6 patients (1/6) with F/F genotype. One patient with V/F genotype could not be assessed because of the drug complication. Half of patients with F/F genotype (3/6) had partial response where as 2 cases could not be assessed due to the drug complication. However, the correlation of FcγRIIIa polymorphism and survival rate in these patients was not investigated. The long term clinical response to rituximab and FcγRIIIa polyporphism in Thai NHL patients should be investigated in the future because there are a lot of contradictory results on this issue reported from previous studies in other populations.

This study demonstrated the correlation between FcγRIIIa polymorphism and both in vitro ADCC and primary clinical response to rituximab in NHL patients in Thailand. These results may have impact on the important of pharmacogenetic evaluation of FcγRIIIa in Thai patients for clinically using rituximab as well as other therapeutic IgG1 antibodies with ADCC as their major mechanism of action.

In conclusion the results in this study reveal the distribution of FcγRIIIa-158 V/V, V/F and F/F genotypes in Thai population. Data here support the previous reported that ADCC is one of the mechanisms of rituximab action on B lymphoma cells as well as there is correlation between FcγRIIIa polymorphism and the response of rituximab both *in vitro* and in Thai NHL patients. Genetic polymorphism of FcγRIIIa may have influence on clinically use of rituximab as well as other IgG_1 therapeutic antibodies in Thailand.

REFERENCES

[1] Zhong Y. Non-Hogkin's lymphoma: What Primary Care Professionals Need to Know. The Journal for Nurse Practitioners. (2006) : 309-15.

[2] Hennessy BT, Hanrahan EO, Daly PA. Non-Hodgkin lymphoma: an update. The Lancet Oncology. 5 (2004) : 341-53.

[3] ROGERS BB. Overview of Non-Hogkin's Lymphoma. Seminars in Oncology Nursing. 22,2 (2006) : 67-72.

[4] Evans LS, Hancock BW. Non-Hodgkin lymphoma. Lancet. 362 (2003) : 139–46.

[5] Food and Drug Administration; 2008. Available from: http://elib.fda.moph.go.th/library/default.asp?page2=subdetail&id=21037

[6] Lu P. Staging and Classification of Lymphoma. Seminars in Nuclear Medicine. 35 (2005) : 160-4.

[7] Perosa F, Favoino E, Caragnano MA, Prete M, Dammacco F. CD20: A target antigen for immunotherapy of autoimmune diseases. Autoimmunity Reviews. 4 (2005) : 526– 531.

[8] Gandhi MK, Marcus RE. Follicular lymphoma: time for a re-think? Blood Reviews. 19,3 (2005) : 165–178.

[9] Andrea K. Diffuse Large B-Cell Lymphoma. Seminars in Radiation Oncology. 17 (2007) : 169-175.

[10] Michallet AS, Coiffier B. Recent developments in the treatment of aggressive non-Hodgkin lymphoma. Blood Reviews. 23 (2009) : 11–23.

[11] Bilodeau BA, Fessele KL. Non-Hodgkin's Lymphoma. Seminars in Ontology Nursing, 14,4 (1998) : 273-83.

[12] Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM,et.al. Report of an International Workshop to Standardize Response Criteria for Non-Hodgkin's Lymphomas. Journal of Clinical Oncology. 17,4 (1999) : 1244-53.

[13] Sacchi S, Federico M, Dastoli G, Fiorani C, Vinci G, Clo` V, et.al. Treatment of B-cell non-Hodgkin's lymphoma with anti CD 20 monoclonal antibody Rituximab. Critical Reviews in Oncology: Hematology. 37 (2001) : 13–25.

[14] Chinn P, Braslawsky G, White C, Hanna N. Antibody therapy of non-Hodgkin's B-cell lymphoma. Cancer Immunology Immunotherapy. 52,5 (2003) : 257-80.

[15] Zhou X, Hu W, Qin X. The Role of Complement in the Mechanism of Action of Rituximab for B-Cell Lymphoma: Implications for Therapy. The Oncologist. 13 (2008) : 954–66.

[16] Coiffier B. Monoclonal antibody as therapy for malignant lymphomas. C. R. Biologies. 329 (2006) : 241–54.

[17] Foran JM. Antibody-based therapy of Non-Hodgkin's lymphoma. Best Practice & Research Clinical Hematology. 15,3 (2002) : 449-65.

[18] Marcus R, Hagenbeek A. The therapeutic use of rituximab in non-Hodgkin's lymphoma. European journal of haematology. 67 (2007) : 5-14.

[19] Weiner GJ, Link BK. Antibody Therapy of Lymphoma. Advances in Pharmacology. 51 (2004) : 229-53.

[20] Cheson BD, Leonard JP. Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma. The New England journal of medicine. 359,6 (2008) : 613-26.

[21] Deans JP, Li H, Polyak MJ. CD20-mediated apoptosis: Signalling through lipid rafts. Immunology. 107 (2002) : 176-82.

[22] van der Kolk LE, Evers LM, Omene C, Lens SM, Lederman S, van Lier RA et al. CD20-induced B cell death can bypass mitochondria and caspase activation. Leukemia. 16 (2002) : 1735–44.

[23] Chan HT, Hughes D, French RR, Tutt AL, Walshe CA, Teeling JL, et al. CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. Cancer Research. 63 (2003) : 5480–89.

[24] Daniels I, Abulayha AM, Thomson BJ, Haynes AP. Caspase-independent killing of Burkitt lymphoma cell lines by rituximab. Apoptosis. 11 (2006) : 1013–23.

[25] Bezombes C, Grazide S, Garret C, Fabre C, Quillet-Mary A, Muller S. Rituximab antiproliferative effect in B-lymphoma cells is associated with acid-sphingomyelinase activation in raft microdomains. Blood. 104 (2004) : 1166-73.

[26] Morgan BP. Regulation of the complement membrane attack pathway. Critical Review Immunology. 19 (1999) : 173–98.

[27] Walport MJ. Complement. First of two parts. New England Journal Medicine. 344 (2001) : 1058–66.

[28] Bellosillo B, Villamor N, Lopez-Guillermo A, Marce S, Esteve J, Campo E, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species. Blood. 98 (2001) : 2771–77.

[29] Cragg MS, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. Blood. 103 (2004) : 2738-43.

[30] Zhao WL, Wang L, Liu YH, Yan JS, Leboeuf C, Liu YY, et al. Combined effects of histone deacetylase inhibitor and rituximab on non-Hodgkin's B-lymphoma cells apoptosis. Experimental Hematology. 35 (2007) : 1801–11.

[31] Duesberg U, Schneiders AM, Flieger D, Inchauspé G, Sauerbruch T, Spengler U. Natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) is not impaired in patients suffering from chronic hepatitis C. Journal of Hepatology. 35 (2001) : 650–57.

[32] Hatjiharissi E, Xu L, Santos DD, Hunter ZR, Ciccarelli BT, Verselis S, et.al. Increased natural killer cell expression of CD16, augmented binding andADCC activity to rituximab among individuals expressing the FcgRIIIa-158 V/V and V/F polymorphism. Blood. 110,7 (2007) : 2561-64.

[33] P Richard A. Goldsby., et al. 2003. Immunology. 5th ed. W.H. Freeman and company. P344.

[34] Campbell P, Marcus R. Monoclonal antibody therapy for lymphoma. Blood Reviews. 17 (2003) : 143–52.

[35] Moretta L, Biassoni R, Bottino C, Cantoni C, Pende D, Mingari MC, et.al. Human NK cells and their receptors. Microbes and Infection. 4 (2002) : 1539–44.

[36] Smytha MJ, Cretneya E, Kellya JM, Westwood JA, Street SEA, Yagita H, et.al. Activation of NK cell cytotoxicity. Molecular Immunology. 42 (2005) : 501-10.

[37] Lettau M, Schmidt H, Kabelitz D, Janssen O. Secretory lysosomes and their cargo in T and NK cells. Immunology Letters. 108 (2007) : 10–9.

[38] Clynes RA, Towers TL, Presta LG, Ravetch GV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nature Medicine. 6 (2000) : 443–6.

[39] Golay J, Cittera E, Di Gaetano N, Manganini M, Mosca M, Nebuloni M, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. Haematologica. 91 (2006) : 176–83.

40] Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, Oflazoglu E, Huberman A, Repasky E, et al. Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. Clinical Cancer Research. 9 (2003) : 5866-73.

[41] Nimmerjahn F, Ravetch JV. Fcg Receptors: Old Friends Review and New Family Members. Immunity. 24 (2006) : 19–28.

[42] Siberil S, Dutertre CA, Fridman WH, Teillaud J. FcγR: The key to optimize therapeutic antibodies? Critical Reviews in Oncology/Hematology. 62 (2007) : 26–33.

[43] Cohen-Solal JFG, Cassard L, Fridman WH, Sautès-Fridman C. Fcγ receptors. Immunology Letters. 92 (2004) : 199–205.

[44] Nimmerjahn F, Ravetch JV. Antibodies, Fc receptors and cancer. Current Opinion in Immunology. 19 (2007) : 239–45.

[45] Ravetch JV, Perussia B. Alternative membrane forms of FcγRIII (CD16) on human natural killer cells and neutrophils. Journal Experimental Medicine. 170 (1989) : 481-97

[46] Bowles JA, Weiner GJ. CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. Journal of immunological methods. 304,1-2 (2005) : 88-99.

[47] Bowles JA, Wang SY, Link BK, Allan B, Beuerlein G, Campbell M, et.al. Anti-CD20 monoclonal antibody with enhanced affinity for CD16 activates NK cells at lower concentrations and more effectively than rituximab. Blood. 108 (2006) : 2648-54.

[48] Perosa F, Favoino E, Caragnano MA, Prete M, Dammacco F. CD20: A target antigen for immunotherapy of autoimmune diseases. Autoimmunity Reviews. 4 (2005) : 526–31.

[49] Zhang M, Zhang Z, Garmestani K, Goldman CK, Ravetch JV, Brechbiel MW, et.al. Activating Fc receptors are required for antitumor efficacy of the antibodies directed toward CD25 in a murine model of adult T-cell leukemia. Cancer Research. 64 (2004) : 5825-29.

[50] Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific engagement of Fcg receptors regulates B lymphocyte depletion during CD20 immunotherapy. Journal Experimental Medicine. 203 (2006) : 743-53.

[51] Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nature Medicine. 6 (2000) : 443-6.

[52] Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et.al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. Blood. 99 (2002) : 754-8.

[53] Dall'Ozzo S, Tartas S, Paintaud G, Cartron G, Colombat P, Bardos P, et.al. Rituximab-Dependent Cytotoxicity by Natural Killer Cells: Influence of FCGR3A Polymorphism on the Concentration-Effect Relationship. Cancer Research. 64 (2004) : 4664–9.

[54] Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. Journal Clinical Oncology. 21,21 (2003) : 3940-7.

[55] Weng WK, Weng WK, Levy R. Immunoglobulin G Fc receptor polymorphisms do not correlate with response to chemotherapy or clinical course in patients with follicular lymphoma. Leukemia & lymphoma. 50,9 (2009) : 1494-500.

[56] Mitrovic Z, Aurer I, Radman I, Ajdukovic R, Sertic J, Labar B. FcgammaRIIIA and FcgammaRIIA polymorphisms are not associated with response to rituximab and CHOP in patients with diffuse large B-cell lymphoma. Haematologica. 92,7 (2007) : 998-9.

[57] Kim DH, Jung HD, Kim JG, Lee JJ, Yang DH, Park YH, et al. FCGR3A gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. Blood. 108,8 (2006) : 2720-5.

[58] Lin TS, Flinn IW, Modali R, Lehman TA, Webb J, Waymer S, et.al. FCGR3A and FCGR2A polymorphisms may not correlate with response to alemtuzumab in chronic lymphocytic leukemia. Blood. 105 (2005) : 289-91.

[59] Farag SS, Flinn IW, Modali R, Tibullo D, Salmoiraghi S, Rossi A, et.al. Fc gamma RIIIa and Fc gamma RIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. Blood. 103,4 (2004) : 1472-4.

[60] Leppers-van de Straat FG, van der Pol WL, Jansen MD, Sugita N, Yoshie H, Kobayashi T et.al. A novel PCR-based method for direct Fc gamma receptor IIIa (CD16) allotyping. Journal of immunological methods. 242,1-2 (2000) : 127-32.

[61] Torkildsen O, Utsi E, Mellgren SI, Harbo HF, Vedeler CA, Myhr KM. Ethnic variation of Fcγ receptor polymorphism in Sami and Norwegian populations. Immunology, 115 (2005) : 416–21.

[62] Wu J, Edberg JC, Radecha PB, Bansal V, Guyre PM, Coleman K, et al. A novel polymorphism of FcγRIIIa (CD16) alters receptorfunction and predisposes to autoimmune disease. Journal Clinical Investigation. 100 (1997) : 1059-70.

[63] Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AEG, de Haas M. FcγRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell FcγRIIIa, independently of the FcγRIIIa-48L/R/H phenotype. Blood. 90 (1997) : 1109-14.

APPENDICES

APPENDIX A

Buffers and Reagents

1. RPMI 1640 stock solution 1 liter

RPMI powder	10.4	g
$NaHCO_3$	1.5	g
Glucose	4.5	g
Sodium pyruvate	0.11	g
HEPES (1M)	10	ml
Penicillin/Streptomycin	10	ml
ddH_2O	900	ml

Adjust pH to 7.2 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by filtering through a 0.45 membrane filter

2. Complete RPMI 1640 medium 200 ml

RPMI stock	180	ml
Fetal Bovine Serum	20	ml

3. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80.65	g
KCl	2	g
KH_2PO_4	2	g
Na_2HPO_4	11.5	g
ddH_2O	900	ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by autoclaving

4. 10x Assay Buffered 100 ml

HEPES (1M)	10	ml
$CaCl_2$ (0.1M)	28	ml
NaCl (5M)	25	ml
ddH_2O	37	ml

5. Tris-HCl 1M pH 8.0 100 ml

Tris-base	12.114	g
ddH_2O	80	ml

Adjust pH to 8.0 with conc. HCl

Add ddH_2O to 100 ml and Sterilized by autoclaving

6. EDTA 0.5M pH 8.0 100 ml

EDTA	18.612	g
ddH_2O	80	ml

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 ml and Sterilized by autoclaving

7. 1x TE Buffered 100 ml

Tris-HCl 1M pH 8.0	1	ml
EDTA 0.5M pH 8.0	0.2	ml
ddH_2O	98.8	ml

Sterilized by autoclaving

8. 5x TBE Buffered 1 liter

Tris-base	54	g
Boric acid	27.5	g
EDTA 0.5M pH 8.0	20	ml

Sterilized by autoclaving

APPENDIX B

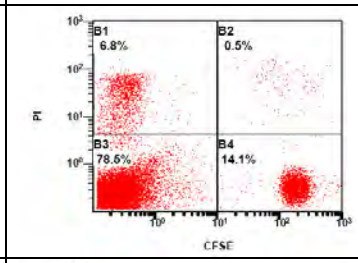
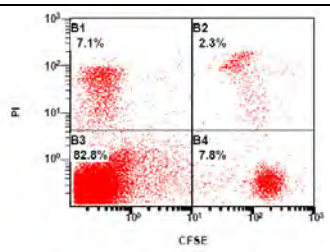
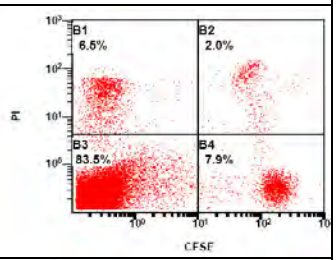
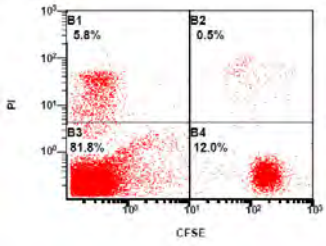
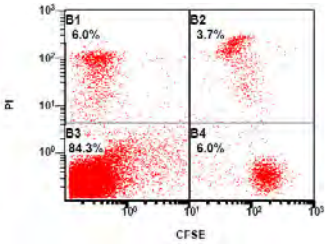
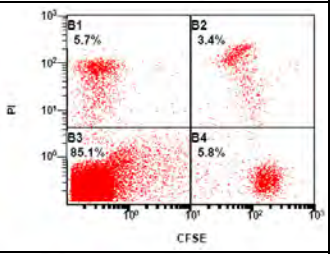
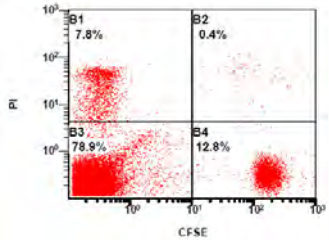
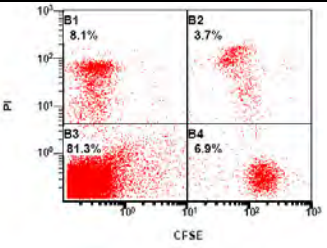
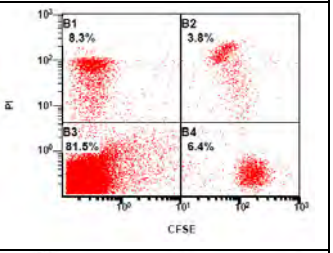
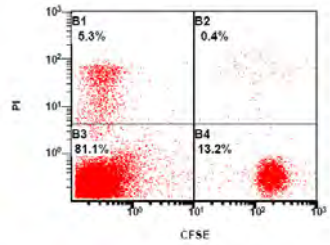
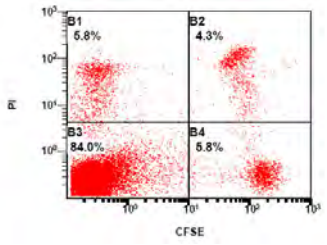
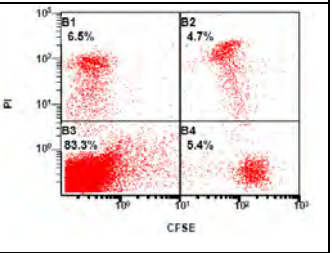
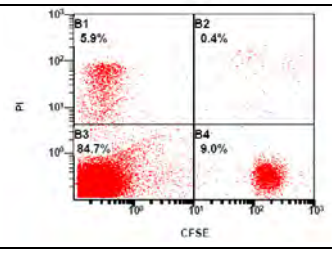
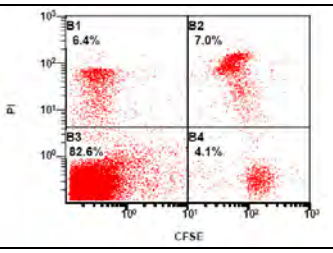
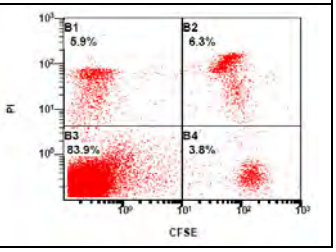
No.	Control condition	Rituximab-treated condition	
1			
2			
3			
4			
5			

Figure 13: The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

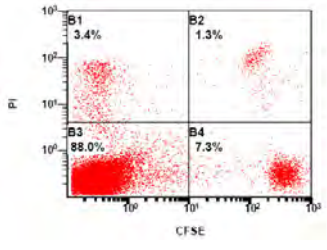
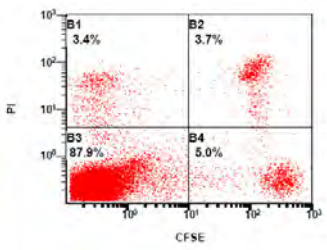
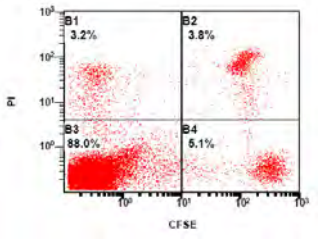
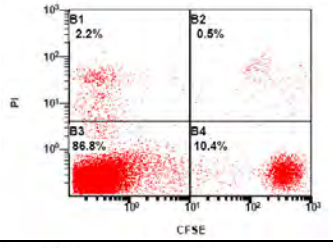
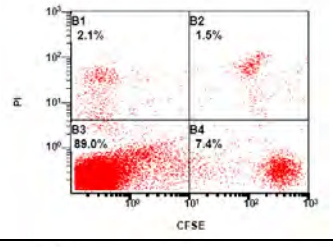
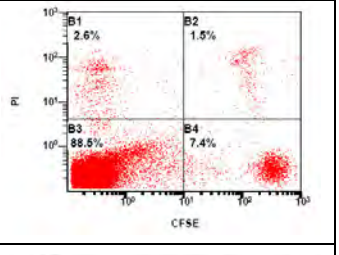
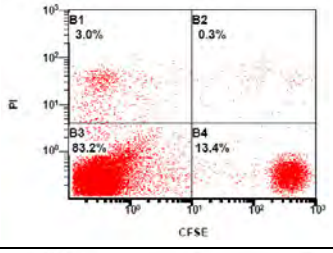
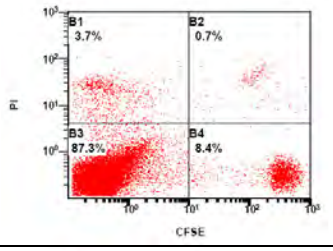
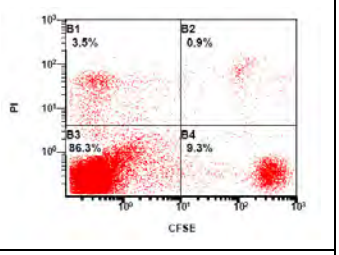
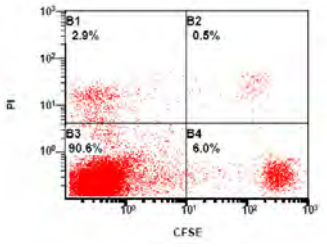
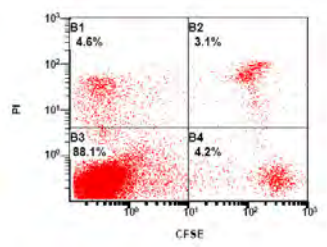
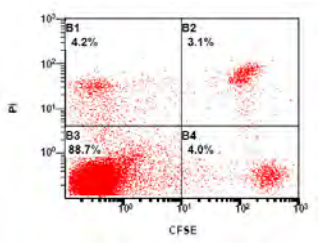
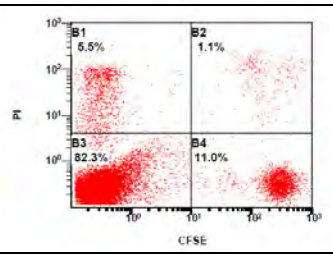
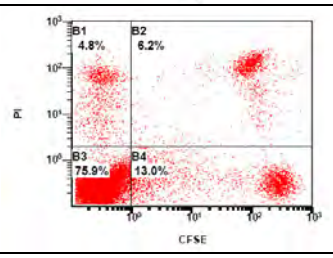
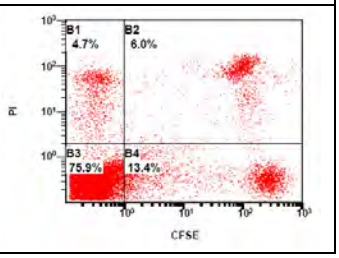
No.	Control condition	Rituximab-treated condition	
6			
7			
8			
9			
10			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

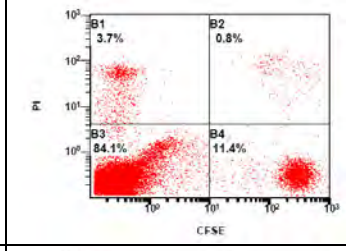
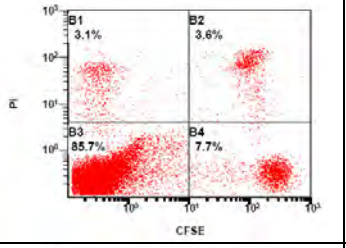
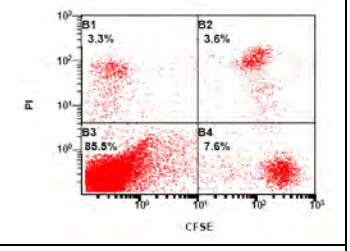
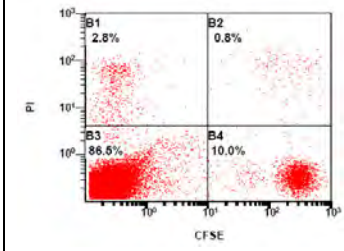
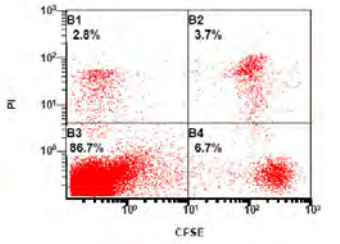
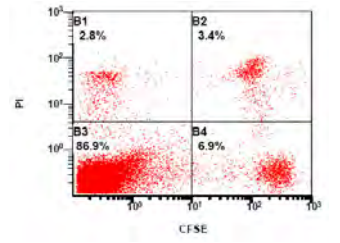
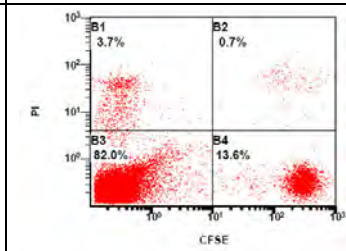
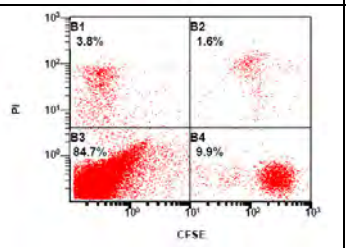
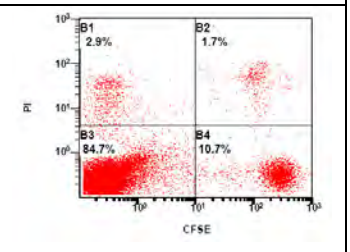
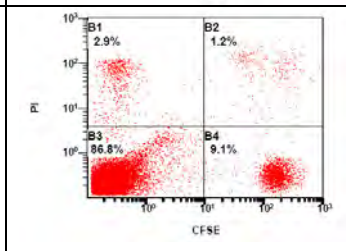
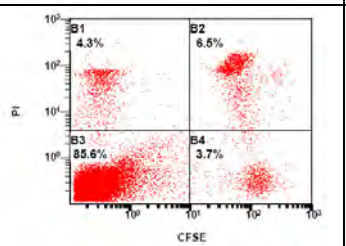
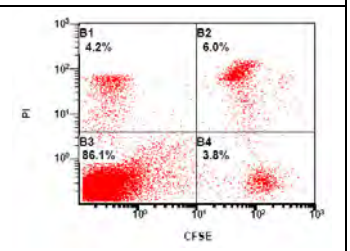
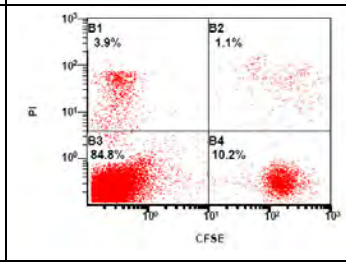
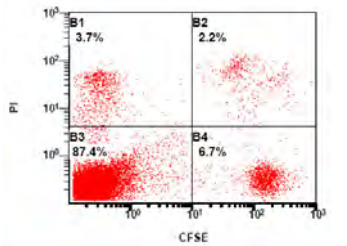
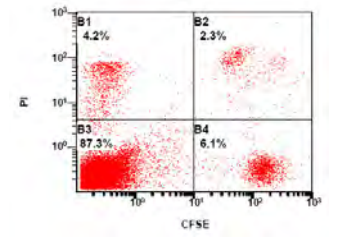
No.	Control condition	Rituximab-treated condition	
11			
12			
13			
14			
15			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

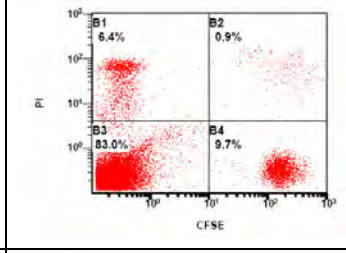
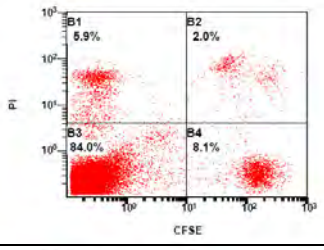
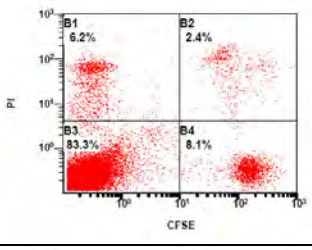
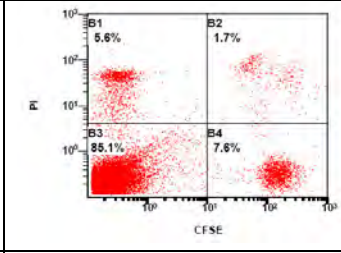
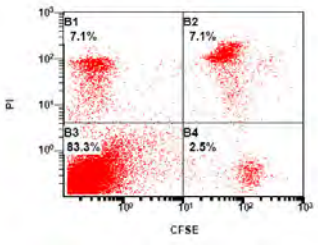
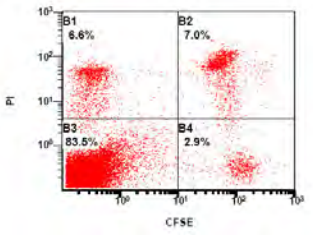
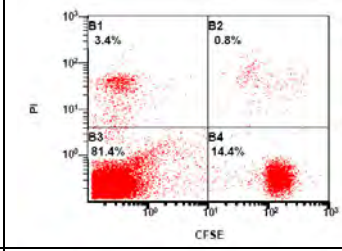
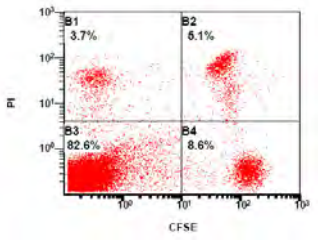
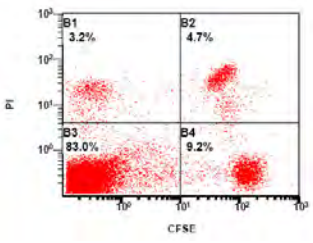
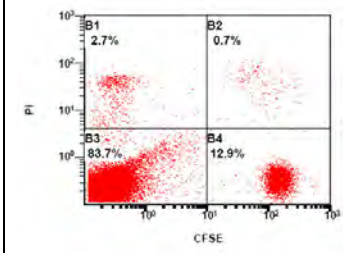
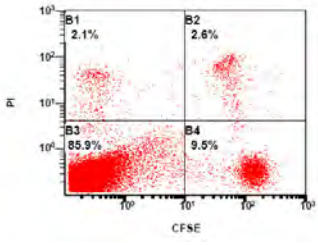
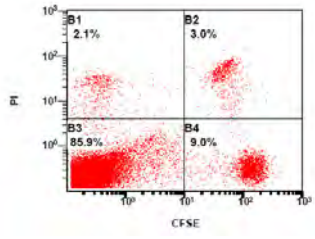
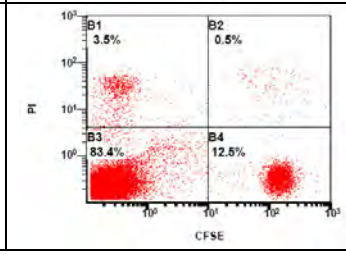
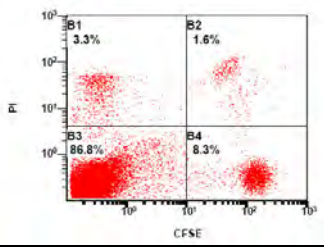
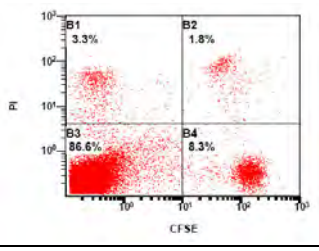
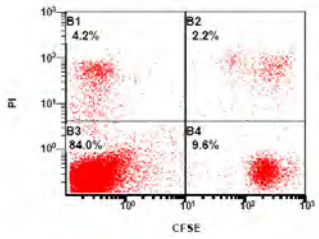
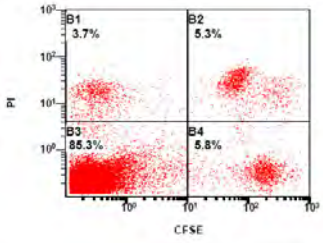
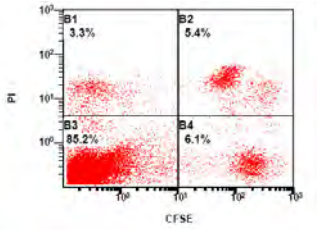
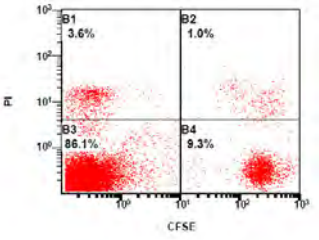
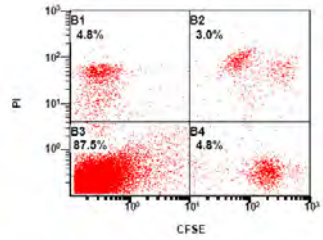
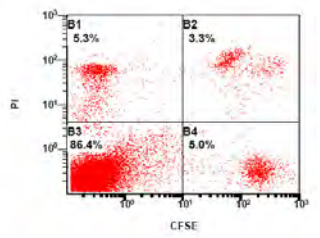
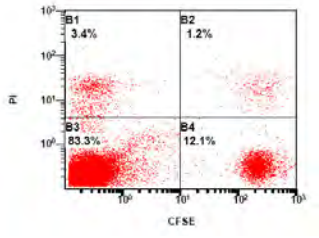
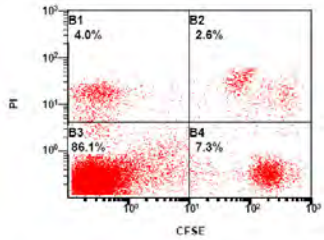
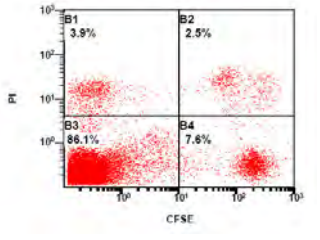
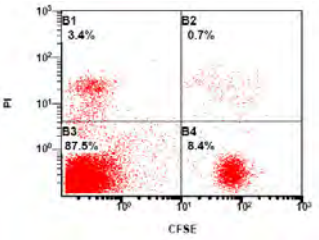
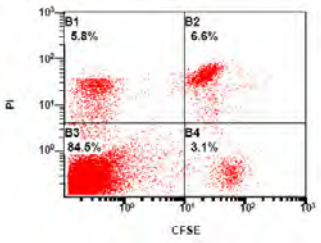
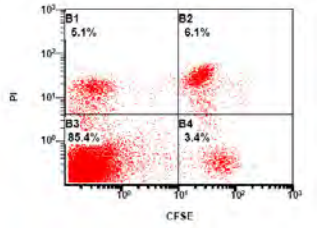
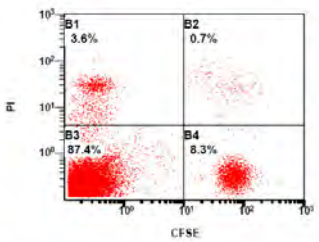
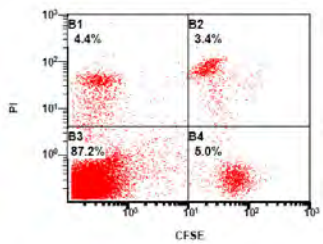
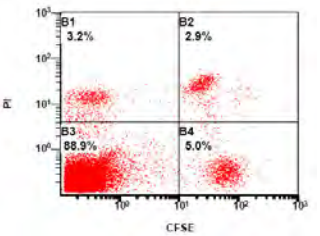
No.	Control condition	Rituximab-treated condition	
16			
17			
18			
19			
20			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

No.	Control condition	Rituximab-treated condition	
21			
22			
23			
24			
25			

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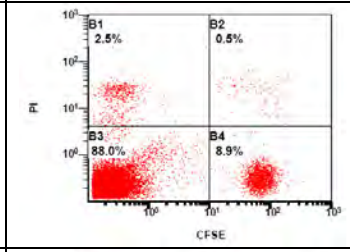
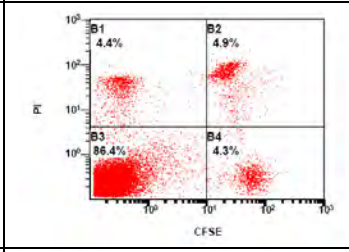
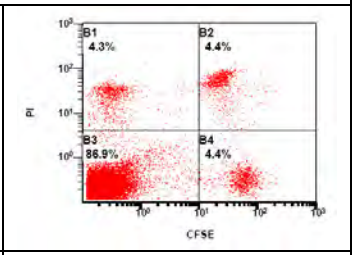
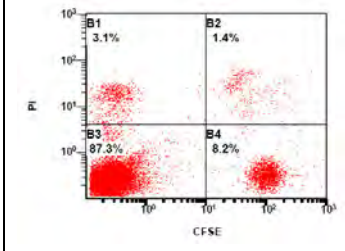
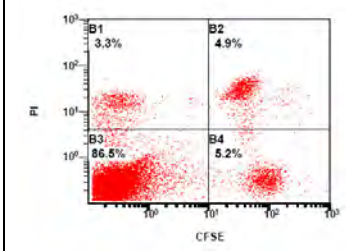
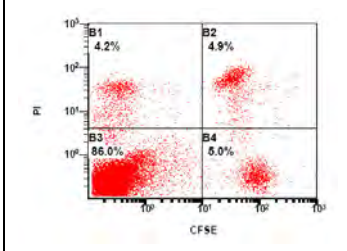
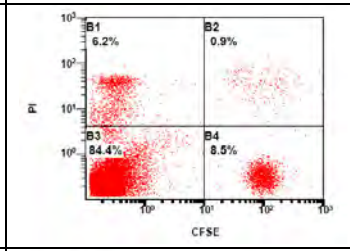
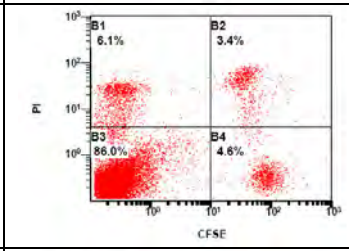
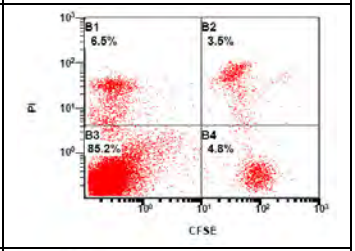
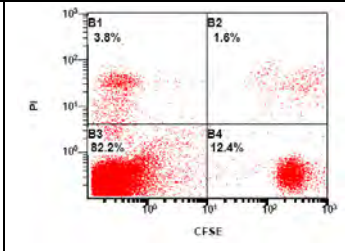
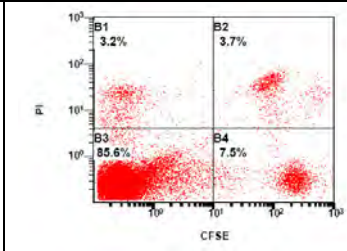
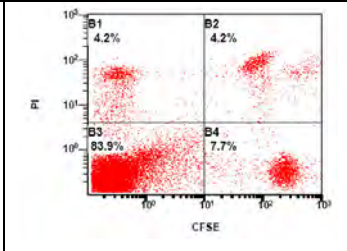
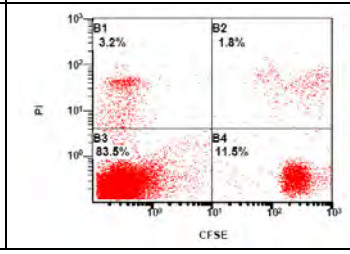
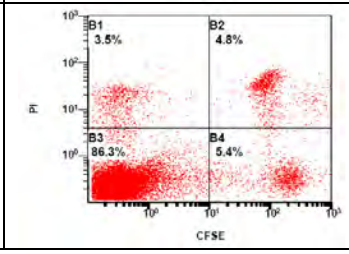
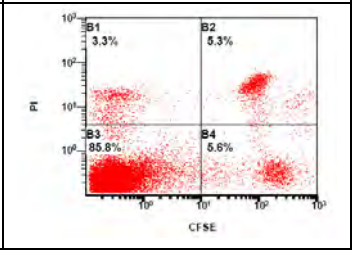
No.	Control condition	Rituximab-treated condition	
26			
27			
28			
29			
30			

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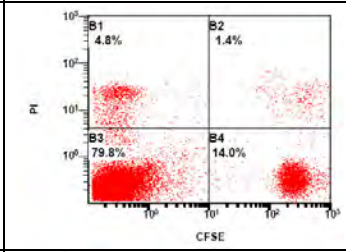
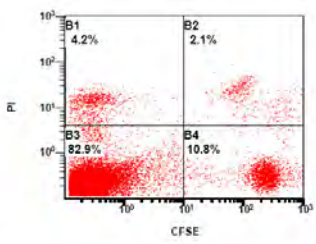
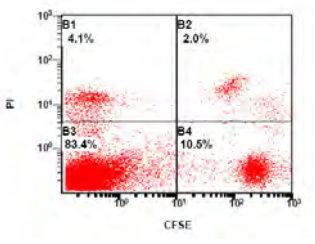
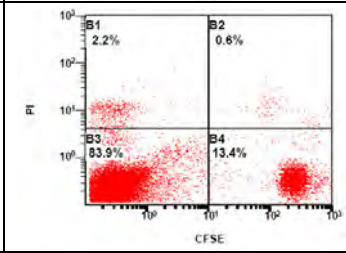
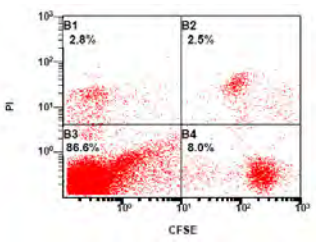
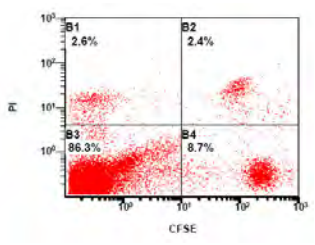
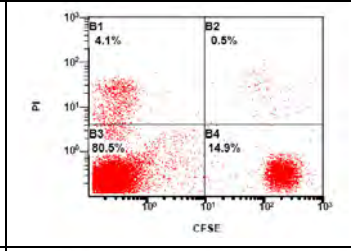
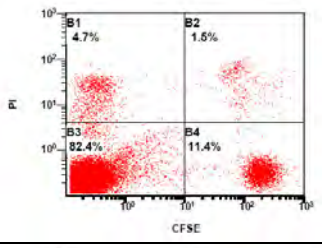
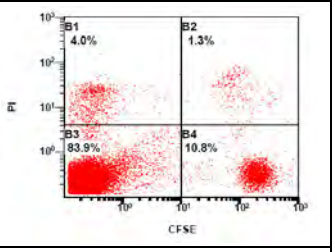
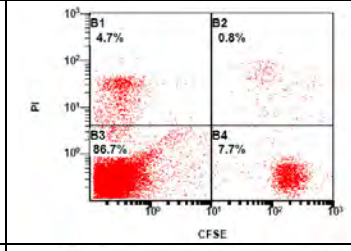
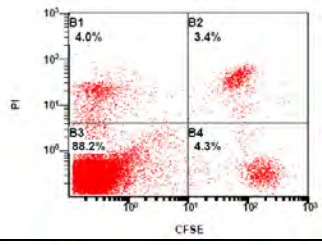
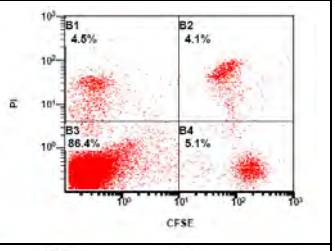
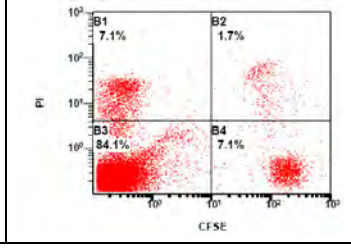
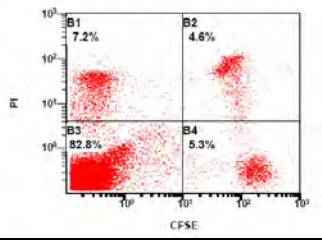
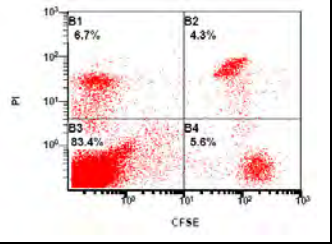
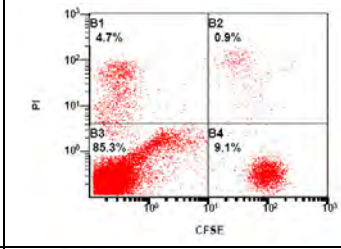
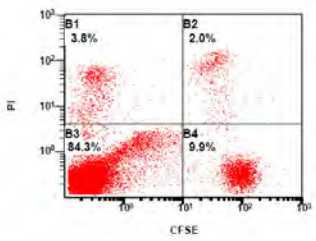
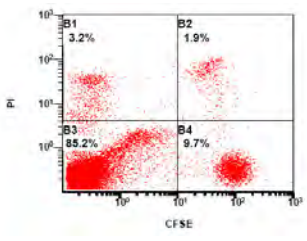
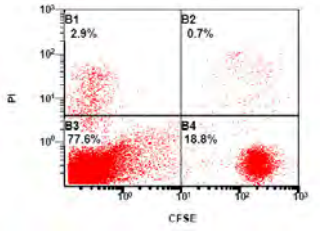
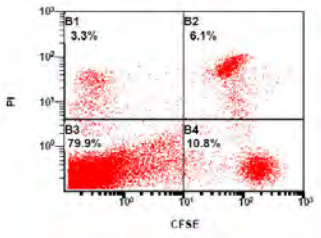
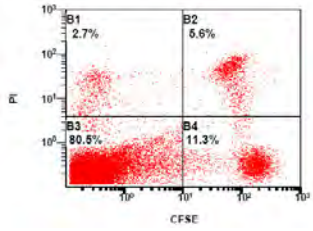
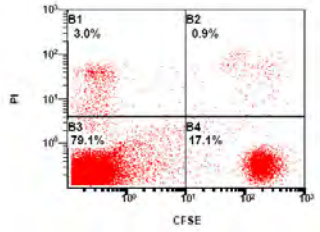
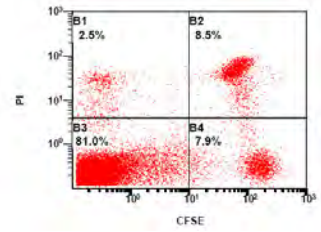
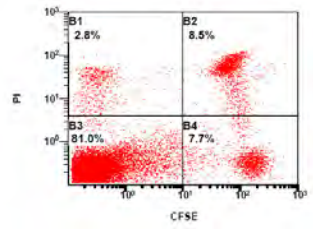
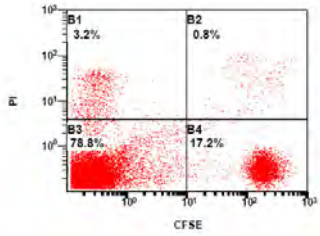
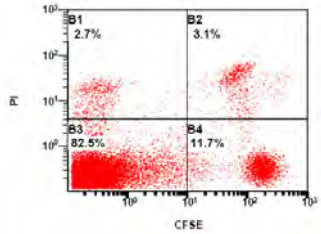
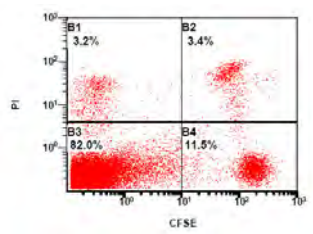
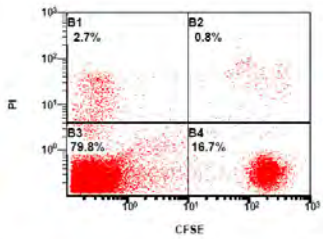
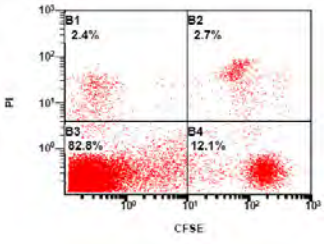
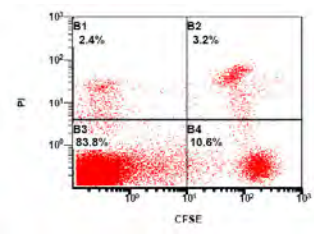
No.	Control condition	Rituximab-treated condition	
31			
32			
33			
34			
35			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro,* data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

No.	Control condition	Rituximab-treated condition	
36			
37			
38			
39			
40			

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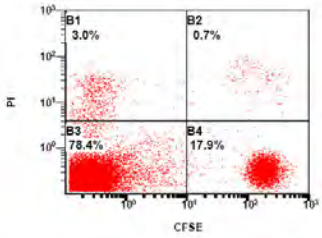
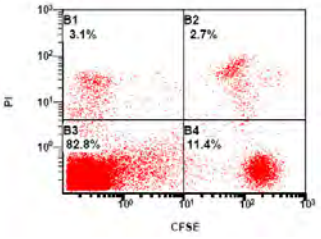
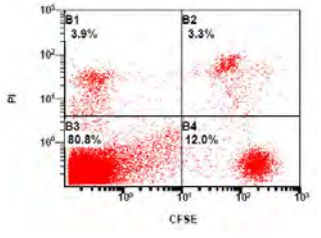
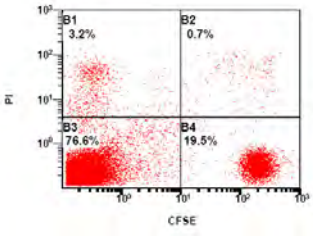
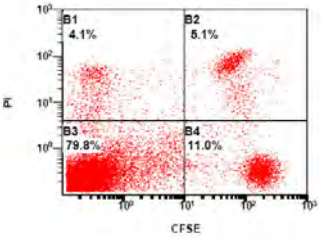
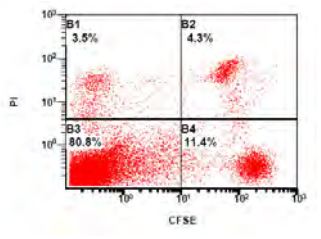
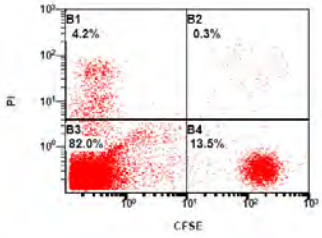
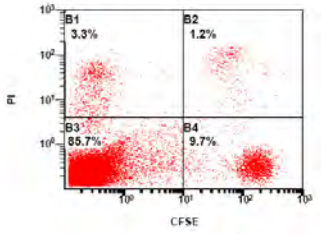
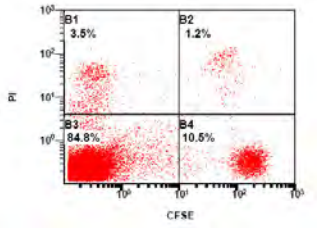
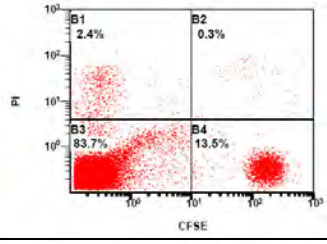
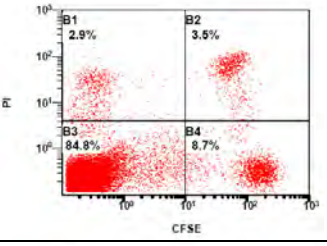
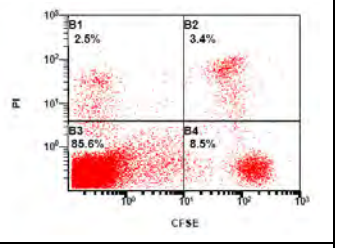
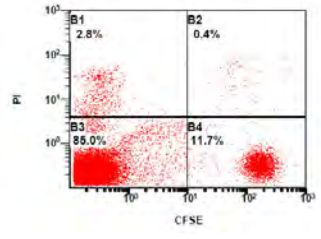
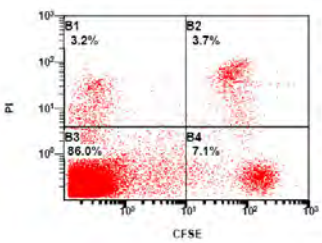
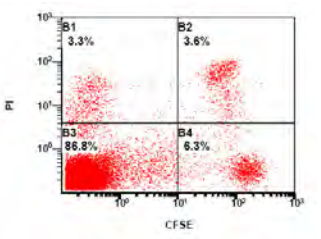
No.	Control condition	Rituximab-treated condition	
41			
42			
43			
44			
45			

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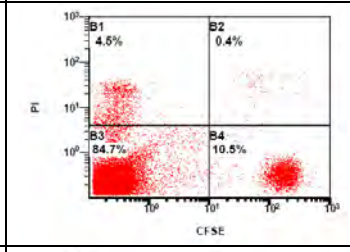
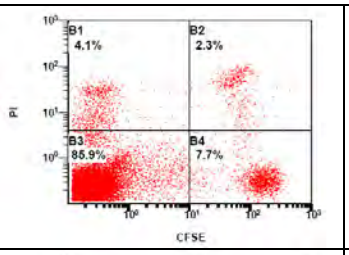
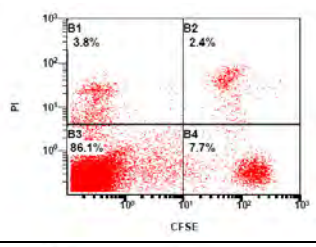
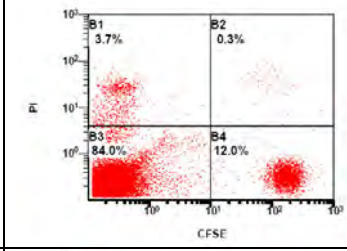
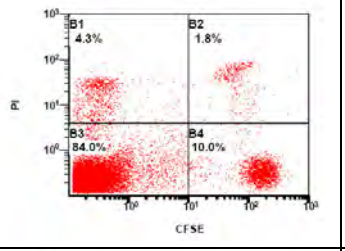
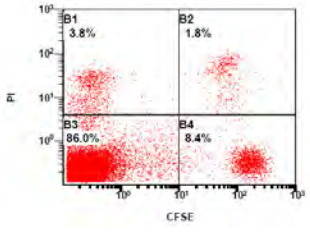
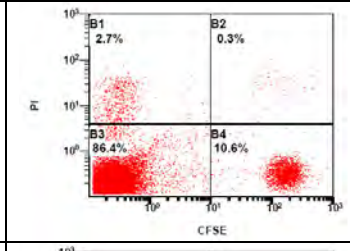
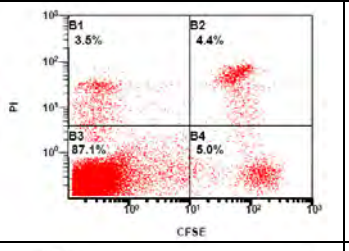
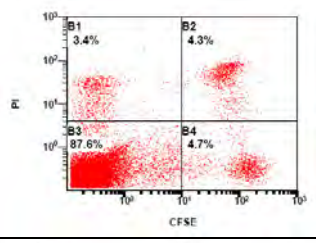
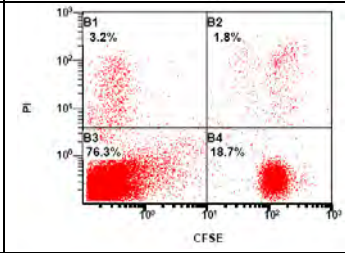
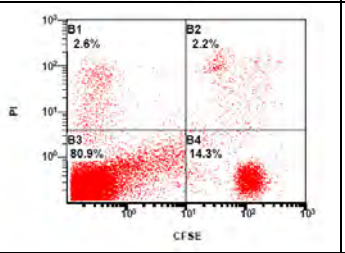
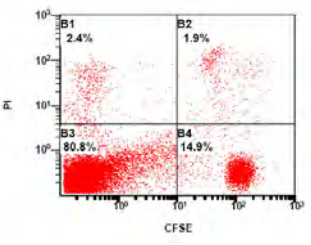
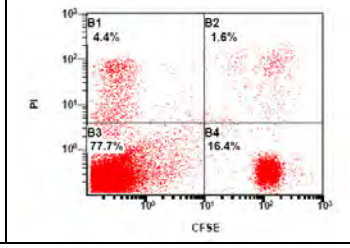
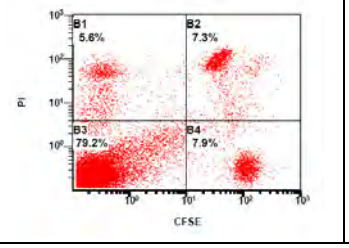
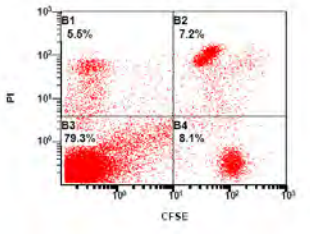
No.	Control condition	Rituximab-treated condition	
46			
47			
48			
49			
50			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro,* data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

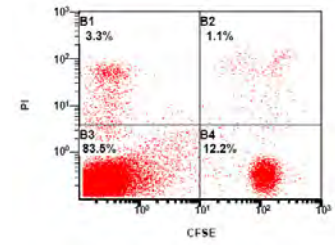
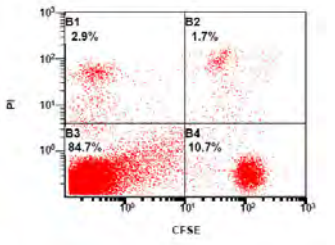
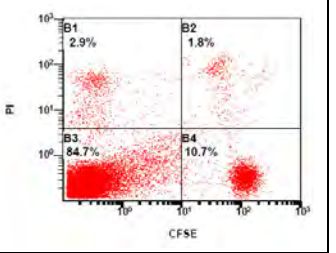
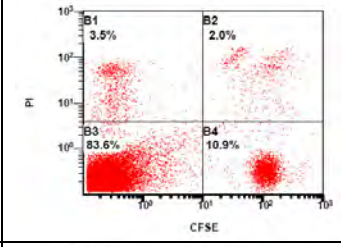
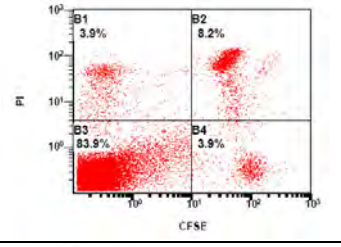
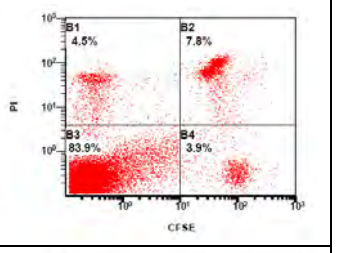
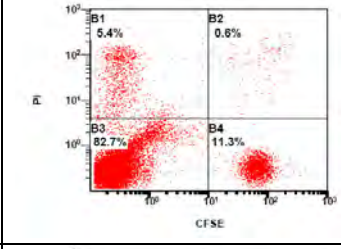
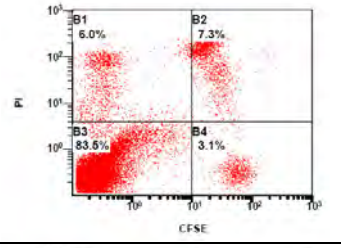
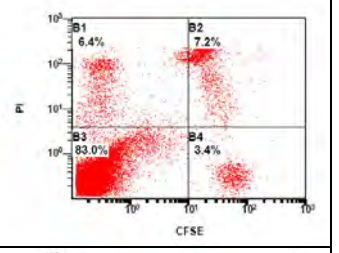
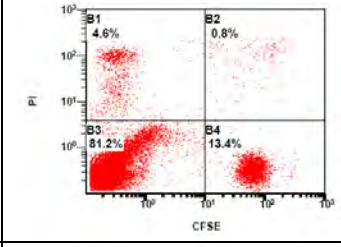
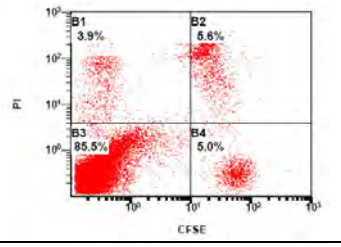
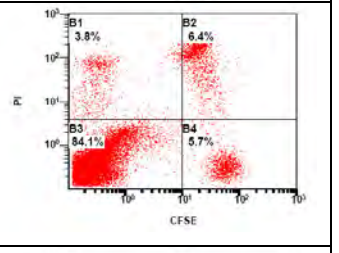
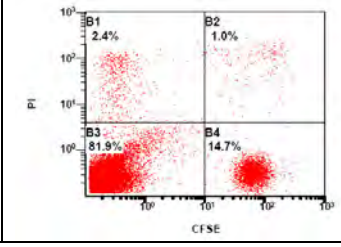
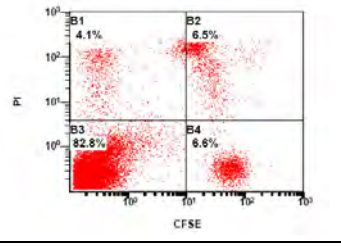
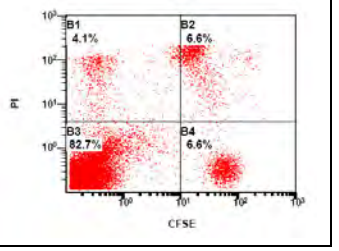
No.	Control condition	Rituximab-treated condition	
51			
52			
53			
54			
55			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

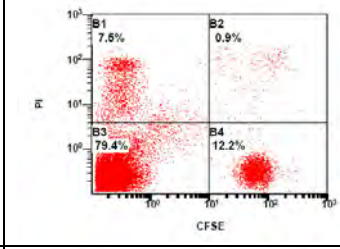
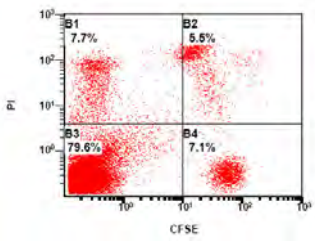
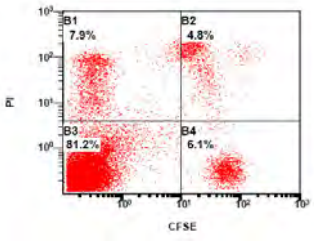
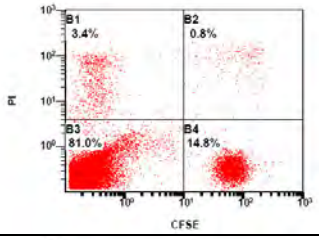
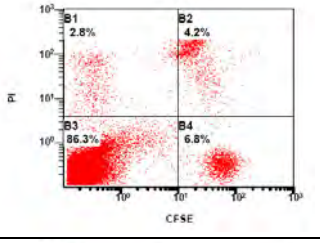
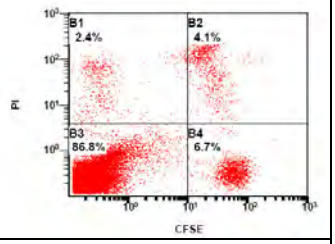
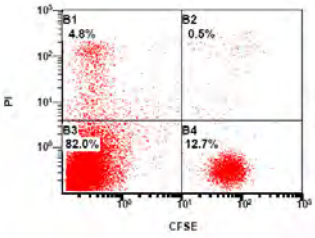
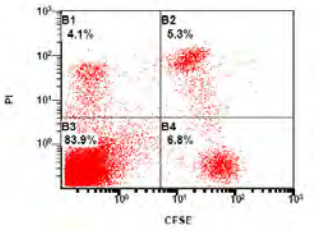
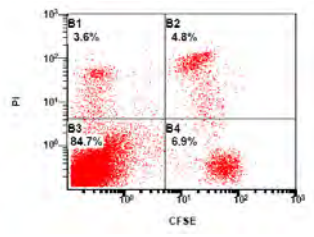
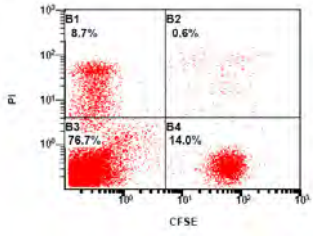
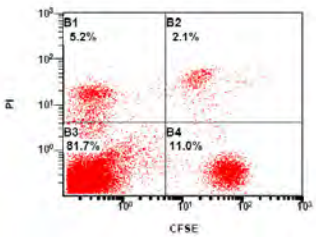
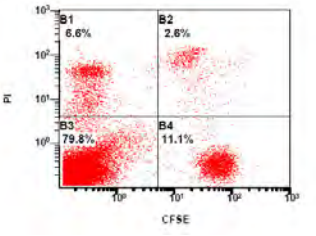
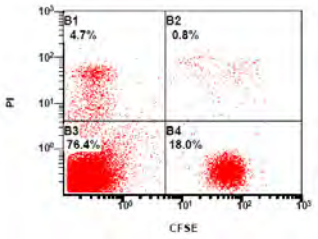
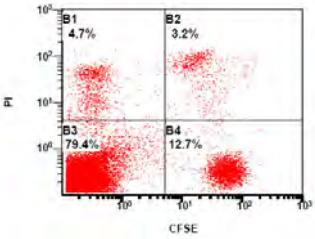
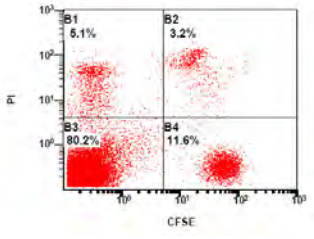
No.	Control condition	Rituximab-treated condition	
56			
57			
58			
59			
60			

Figure 13 (cont.): The dot plot histogram of rituximab-mediated cytotoxicity *in vitro*, data were presented in each individual (n=60). B1: The percentage of Peripheral mononuclear cells death ($PI^+/CFSE^-$). B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B3: The percentage of living Peripheral mononuclear cells ($PI^-/CFSE^-$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$).

Table 11: The summarize data of RTX-mediated cytotoxicity *in vitro* from 60 healthy volunteer

Sample No.	Control (%)			RTX1 (%)			RTX2 (duplicated) (%)			% Average cytotoxicity (RTX)	% RTX mediated cytotoxicity
	B2	B4	cytotoxicity	B2	B4	cytotoxicity	B2	B4	cytotoxicity		
1	0.50	14.10	3.42	2.30	7.80	22.77	2.00	7.90	20.20	21.49	18.06
2	0.50	12.00	4.00	3.40	5.80	36.96	3.70	6.00	38.14	37.55	33.55
3	0.40	12.80	3.03	3.80	6.40	37.25	3.70	6.90	34.91	36.08	33.05
4	0.40	13.20	2.94	4.70	5.40	46.53	4.30	5.80	42.57	44.55	41.61
5	0.40	9.00	4.26	6.30	3.80	62.38	7.00	4.10	63.06	62.72	58.46
6	1.30	7.30	15.12	3.80	5.10	42.70	3.70	5.00	42.53	42.61	27.50
7	0.50	10.40	4.59	1.50	7.40	16.85	1.50	7.40	16.85	16.85	12.27
8	0.30	13.40	2.19	0.90	9.30	8.82	0.70	8.40	7.69	8.26	6.07
9	0.50	6.00	7.69	3.10	4.20	42.47	3.10	4.00	43.66	43.06	35.37
10	1.10	11.00	9.09	5.60	7.40	43.08	5.70	7.50	43.18	43.13	34.04
11	0.80	11.40	6.56	3.60	7.60	32.14	3.60	7.70	31.86	32.00	25.44
12	0.80	10.00	7.41	3.40	6.90	33.01	3.70	6.70	35.58	34.29	26.89
13	0.70	13.60	4.90	1.60	9.90	13.91	1.70	10.70	13.71	13.81	8.92

*B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$). RTX: Rituximab.

Table 11 (cont.): The summarize data of RTX-mediated cytotoxicity *in vitro* from 60 healthy volunteers

Sample No.	Control (%)			RTX1 (%)			RTX2 (duplicated) (%)			% Average cytotoxicity (RTX)	% RTX mediated cytotoxicity
	B2	B4	cytotoxicity	B2	B4	cytotoxicity	B2	B4	cytotoxicity		
14	1.20	9.10	11.65	6.50	3.70	63.73	6.00	3.80	61.22	62.47	50.82
15	1.10	10.20	9.73	2.30	6.10	27.38	2.20	6.70	24.72	26.05	16.32
16	0.90	9.70	8.49	2.00	8.10	19.80	2.40	8.10	22.86	21.33	12.84
17	1.70	7.60	18.28	7.10	2.50	73.96	7.00	2.90	70.71	72.33	54.05
18	0.80	14.40	5.26	4.70	9.20	33.81	5.10	8.60	37.23	35.52	30.26
19	0.70	12.90	5.15	2.60	9.50	21.49	3.00	9.00	25.00	23.24	18.10
20	0.50	12.50	3.85	1.80	8.30	17.82	1.60	8.30	16.16	16.99	13.15
21	2.20	9.60	18.64	5.30	5.80	47.75	5.40	6.10	46.96	47.35	28.71
22	1.00	9.30	9.71	3.30	5.00	39.76	3.00	4.80	38.46	39.11	29.40
23	1.20	12.10	9.02	2.50	7.60	24.75	2.60	7.30	26.26	25.51	16.48
24	0.70	8.40	7.69	6.60	3.10	68.04	6.10	3.40	64.21	66.13	58.43
25	0.70	8.30	7.78	2.90	5.00	36.71	3.40	5.00	40.48	38.59	30.81
26	0.50	8.90	5.32	4.40	4.40	50.00	4.90	4.30	53.26	51.63	46.31

*B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$). RTX: Rituximab.

Table 11 (cont.): The summarize data of RTX-mediated cytotoxicity *in vitro* from 60 healthy volunteers

Sample No.	Control (%)			RTX1 (%)			RTX2 (duplicated) (%)			% Average cytotoxicity (RTX)	% RTX mediated cytotoxicity
	B2	B4	cytotoxicity	B2	B4	cytotoxicity	B2	B4	cytotoxicity		
27	1.40	8.20	14.58	4.90	5.20	48.51	4.90	5.00	49.49	49.00	34.42
28	0.90	8.50	9.57	4.30	6.70	39.09	3.50	4.80	42.17	40.63	31.06
29	1.60	12.40	11.43	3.70	7.50	33.04	4.20	7.70	35.29	34.16	22.74
30	1.80	11.50	13.53	5.30	5.60	48.62	4.80	5.40	47.06	47.84	34.31
31	1.40	14.00	9.09	2.00	10.50	16.00	2.10	10.80	16.28	16.14	7.05
32	0.60	13.40	4.29	2.50	8.00	23.81	2.40	8.70	21.62	22.72	18.43
33	0.50	14.90	3.25	1.30	10.80	10.74	1.50	11.40	11.63	11.19	7.94
34	0.80	7.70	9.41	4.10	5.10	44.57	3.40	4.30	44.16	44.36	34.95
35	1.70	7.10	19.32	4.30	5.60	43.43	4.60	5.30	46.46	44.95	25.63
36	0.90	9.10	9.00	2.00	9.90	16.81	1.90	9.70	16.38	16.59	7.59
37	0.70	18.80	3.59	6.10	18.80	24.50	5.60	11.30	33.14	28.82	25.23
38	0.90	17.10	5.00	8.50	7.90	51.83	8.50	7.70	52.47	52.15	47.15
39	0.80	17.20	4.44	3.10	11.70	20.95	3.40	11.50	22.82	21.88	17.44

*B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$). RTX: Rituximab.

Table 11 (cont.): The summarize data of RTX-mediated cytotoxicity *in vitro* from 60 healthy volunteers

Sample No.	Control (%)			RTX1 (%)			RTX2 (duplicated) (%)			% Average cytotoxicity (RTX)	% RTX mediated cytotoxicity
	B2	B4	cytotoxicity	B2	B4	cytotoxicity	B2	B4	cytotoxicity		
40	0.80	16.70	4.57	2.70	12.10	18.24	3.20	10.60	23.19	20.72	16.14
41	0.70	17.90	3.76	2.70	11.40	19.15	3.30	12.00	21.57	20.36	16.60
42	0.70	19.50	3.47	4.30	11.40	27.39	5.10	11.00	31.68	29.53	26.07
43	0.30	13.50	2.17	1.20	9.70	11.01	1.20	10.50	10.26	10.63	8.46
44	0.30	13.50	2.17	3.40	8.50	28.57	3.50	8.70	28.69	28.63	26.46
45	0.40	11.70	3.31	3.70	7.10	34.26	3.60	6.30	36.36	35.31	32.01
46	0.40	10.50	3.67	2.30	7.70	23.00	2.40	7.70	23.76	23.38	19.71
47	0.30	12.00	2.44	1.80	10.00	15.25	1.80	8.40	17.65	16.45	14.01
48	0.30	10.60	2.75	4.40	5.00	46.81	4.30	4.70	47.78	47.29	44.54
49	1.80	18.70	8.78	1.90	14.90	11.31	2.20	14.30	13.33	12.32	3.54
50	1.60	16.40	8.89	7.30	7.90	48.03	7.20	8.10	47.06	47.54	38.65
51	1.10	12.20	8.27	1.80	10.70	14.40	1.70	10.70	13.71	14.05	5.78

*B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$). RTX: Rituximab.

Table 11 (cont.): The summarize data of RTX-mediated cytotoxicity *in vitro* from 60 healthy volunteers

Sample No.	Control (%)			RTX1 (%)			RTX2 (duplicated) (%)			% Average cytotoxicity (RTX)	% RTX mediated cytotoxicity
	B2	B4	cytotoxicity	B2	B4	cytotoxicity	B2	B4	cytotoxicity		
52	2.00	10.90	15.50	8.20	3.90	67.77	7.80	3.90	66.67	67.22	51.71
53	0.60	11.30	5.04	7.30	3.10	70.19	7.20	3.40	67.92	69.06	64.02
54	0.80	13.40	5.63	5.60	5.00	52.83	6.40	5.70	52.89	52.86	47.23
55	1.00	14.70	6.37	6.50	6.60	49.62	6.60	6.60	50.00	49.81	43.44
56	0.90	12.20	6.87	5.50	7.10	43.65	4.80	6.10	44.04	43.84	36.97
57	0.80	14.80	5.13	4.20	6.80	38.18	4.10	6.70	37.96	38.07	32.94
58	0.50	12.70	3.79	5.30	6.80	43.80	4.80	6.90	41.03	42.41	38.63
59	0.60	14.00	4.11	2.10	11.00	16.03	2.60	11.10	18.98	17.50	13.39
60	0.80	18.00	4.26	3.20	12.70	20.13	3.20	11.60	21.62	20.87	16.62

*B2: The percentage of Ramos cells death ($PI^+/CFSE^+$). B4: The percentage of living Ramos cells ($PI^-/CFSE^+$). RTX: Rituximab.

Table 12: Data of primary clinical response from 17 Non-Hodgkin's lymphoma patients

Subject No	Drug regimens	Clinical outcome	FcγRIIIa genotype	Type of disease	Notation
1	R-CHOP	CR	VV	DLBCL	
2	R-CHOP	CR	VF	DLBCL	
3	R-CVP	No assessment	VF	FL	Drug complication
4	R-ESHAP	PR	FF	DLBCL	
5	R-EPOCH	CR	VV	DLBCL	
6	R-CVP	CR	VV	FL	
7	R-CHOP	CR	VF	DLBCL	
8	R-EPOCH	CR	VV	DLBCL	
9	R-PCM	CR	VV	FL	
10	R-CHOP	CR	VV	DLBCL	
11	R-CHOP	No assessment	FF	DLBCL	Drug complication
12	R-CHOP	CR	VV	DLBCL	
13	R-CHOP	No assessment	FF	BL	The treatment was changed to triple therapy
14	R-CVP	PR	FF	MALT-NHL	
15	R-CHOP	CR	FF	DLBCL	
16	R-CHOP	PR	FF	MCL	
17	R-CHOP	CRu	VV	DLBCL	

* CR: Complete response, CRu: Uncertain complete response, PR: Partial response

DLBCL: Diffuse Large B-cell Lymphoma, FL: Follicular Lymphoma, BL: Burkitt's Lymphoma,

MALT-NHL: Mucosa-Associated Lymphoid Tissue Lymphoma, MCL: Mantle cell Lymphoma

R-CHOP: Rituximab+cyclophosphamide/doxorubicin/vincristine/prednisone

R-CVP: Rituximab+Cyclophosphamide/etoposide/cisplatin

R-ESHAP: Rituximab+Etoposide/methylprednisolone/cytarabine/cisplatin

R-EPOCH: Rituximab+etoposide/vincristine/doxorubicin/cyclophosphamide/prednisolone

R-PCM: Rituximab+mitoxanthrone/fludarabine/cyclophosphamide

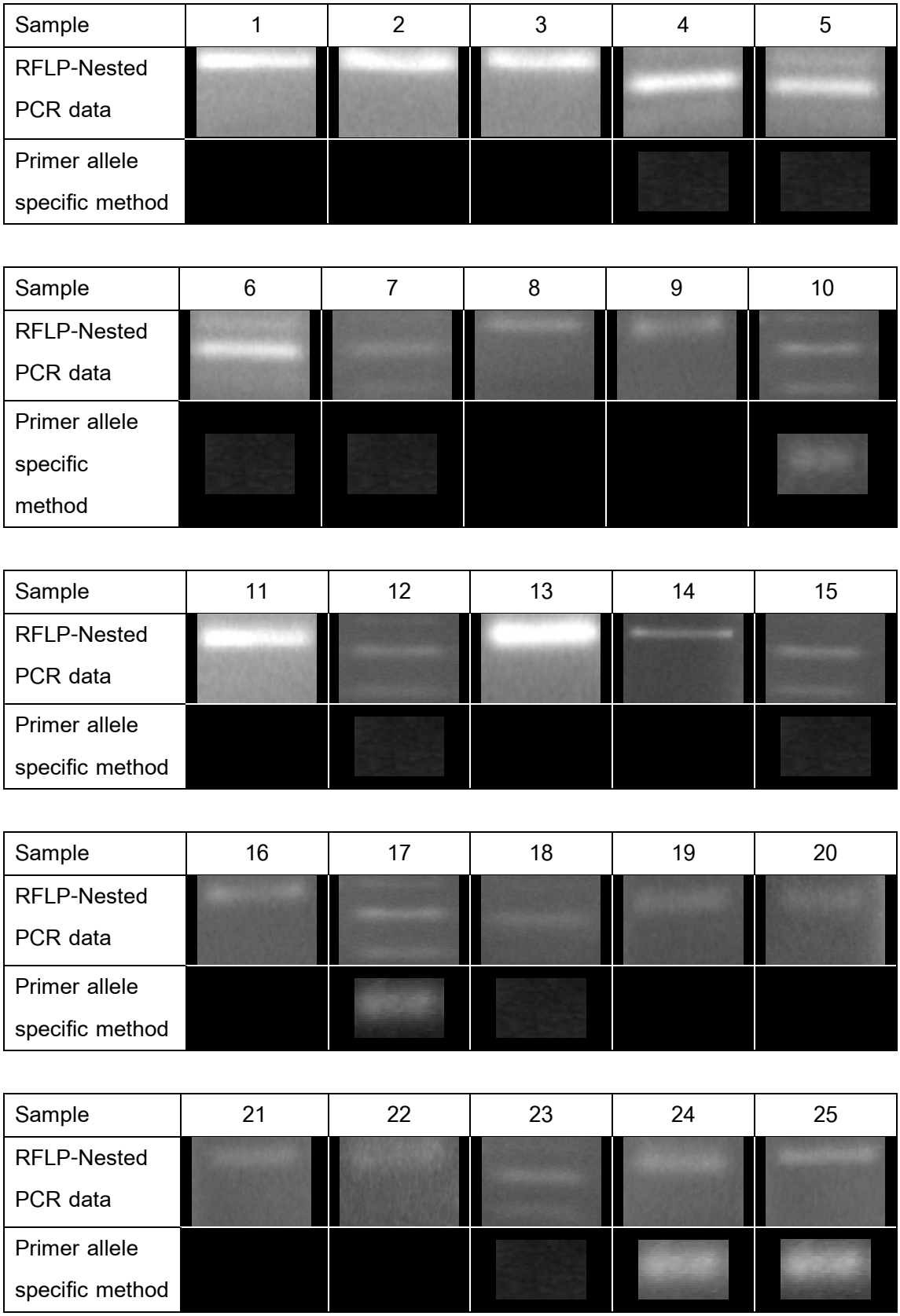


Figure 14: The interpretation of FcγRIIIa polymorphism by the RFLP-nested PCR method and Primer allele specific method from 60 healthy volunteers.

Sample	26	27	28	29	30
RFLP-Nested PCR data					
Primer allele specific method					

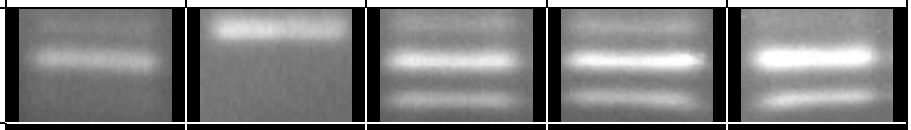
Sample	31	32	33	34	35
RFLP-Nested PCR data					
Primer allele specific method					

Sample	36	37	38	39	40
RFLP-Nested PCR data					
Primer allele specific method					

Sample	41	42	43	44	45
RFLP-Nested PCR data					
Primer allele specific method					

Sample	46	47	48	49	50
RFLP-Nested PCR data					
Primer allele specific method					

Figure 14 (cont.): The interpretation of FcγRIIIa polymorphism by the RFLP-nested PCR method and Primer allele specific method from 60 healthy volunteers.

Sample	51	52	53	54	55
RFLP-Nested PCR data					
Primer allele specific method					

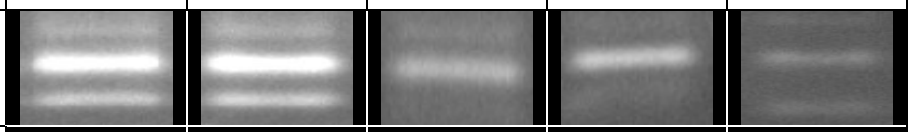
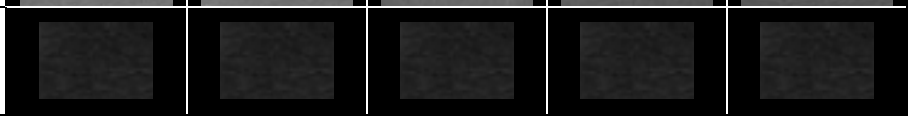
Sample	56	57	58	59	60
RFLP-Nested PCR data					
Primer allele specific method					

Figure 14 (cont.): The interpretation of FcγRIIIa polymorphism by the RFLP-nested PCR method and Primer allele specific method from 60 healthy volunteers.

NHL patients sample	1	2	3	4	5
RFLP-Nested PCR data					
F allele specific method					

NHL patients sample	6	7	8	9	10
RFLP-Nested PCR data					
F allele specific method					

NHL patients sample	11	12	13	14	15
RFLP-Nested PCR data					
F allele specific method					

NHL patients sample	16	17
RFLP-Nested PCR data		
F allele specific method		

Figure 15: The interpretation of FcγRIIIa polymorphism by the RFLP-nested PCR method and Primer allele specific method from 17 Non-Hodgkin's lymphomas.

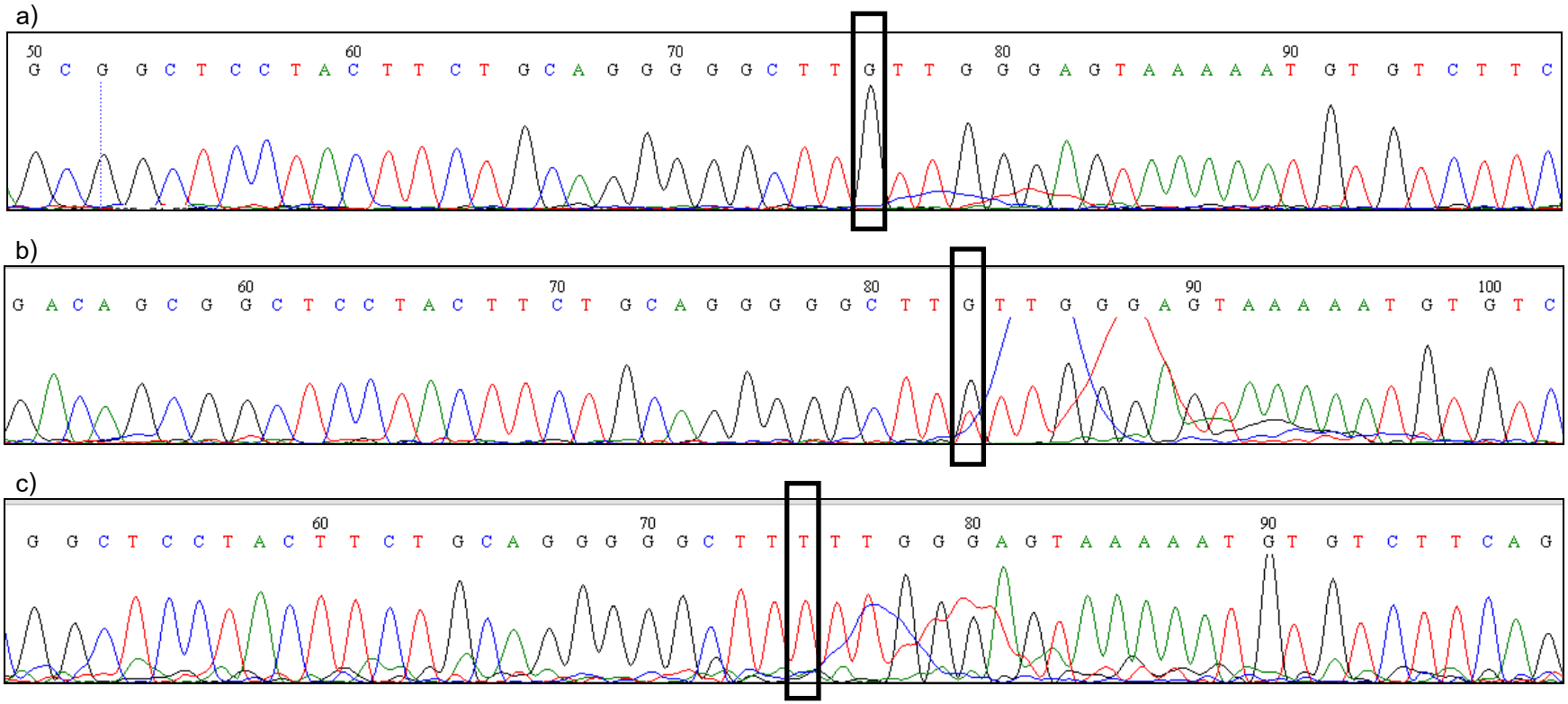


Figure 16: The three of the sixty genomic DNA sequencing data. a) The homozygous V/V genotype (n32). b) The heterozygous V/F genotype (n34). c) The homozygous F/F genotype (n1).

BIOGRAPHY

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