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Role of CD47 In Red Cell Apoptosis (Eryptosis)

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**UNIVERSITY OF
PLYMOUTH**

Role of CD47 In Red Cell Apoptosis (Eryptosis)

by

Ibrahim Faisal Halawani

A thesis submitted to University of
Plymouth in partial fulfilment for the
degree of

DOCTOR OF PHILOSOPHY

School of Biomedical and Healthcare Sciences
(In collaboration with Taif University)

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AUTHOR'S DECLARATION

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Abstracts are shown in Appendix A

Abstract:

CD47 is a 47-52 kDa membrane spanning glycoprotein, which has 5 known isoforms and is a member of the immunoglobulin superfamily (IgSF). It possesses a large extracellular N-terminal IgV like domain. CD47 acts as a 'do not eat me' signal and consequently as a marker of self. Moreover, CD47 is reported to be upregulated in cancer cells allowing them to escape immune surveillance by binding to SIRP alpha on macrophages. The aims of this thesis were to evaluate and to confirm the presence of CD47 isoforms in erythroid and erythroleukemic cells, identify new CD47 binding proteins that may mediate the phosphatidylserine exposure pathway (eryptosis is the process of red cell death which involves the switch of phosphatidylserine from the inner leaf of the membrane to the outer leaf), investigate the functions of the red cell CD47 complexes identified, and to characterize seven different monoclonal antibodies (mAbs) against CD47 that could be used in anti-cancer therapy. To achieve these aims, Next Generation Sequencing (NGS), flow cytometry to measure phosphatidylserine exposure, western blot analysis, immunoprecipitation (IP) of CD47, and Mass Spectrometry (LC-MS/MS) were used. Results showed the presence of all CD47 five isoforms in erythroid and erythroleukemic cells, and their relative proportions have been assessed. The correct transcript sequence for CD47 isoform 5 was confirmed. CD47 isoform 2 was the most abundant isoform expressed in erythroid and erythroleukemic cells. Flow cytometry results showed three different levels of phosphatidylserine exposure by the seven different monoclonal antibodies which recognise epitopes on the N-terminus of CD47 (BRIC 32, BRIC 122, BRIC 124, BRIC 125, BRIC 126, BRIC 168 and BRIC 211). A novel finding was that a mAb to glycophorin A switched off the eryptotic effect of all anti-CD47 N-terminal monoclonal antibodies. Mass Spectrometry results showed novel protein:protein interactions in samples immunoprecipitated (IP) using N-terminal and C-terminal antibodies against CD47. S100 proteins were found to be up-regulated in CD47 IPs and five lipid raft components were found up-regulated in CD47 IPs when compared against normal erythrocyte membranes ('ghosts').

This study concludes that the CD47 eryptosis pathway involves an interaction with S100 proteins that leads to calcium influx and subsequent enhancement of protein kinase C action, which is involved in the eryptosis pathway. One CD47 monoclonal antibody, BRIC 32, could be a potential therapeutic anti-cancer agent as it shows a low level of eryptosis in erythrocytes. The novel switch-off eryptotic effect of anti-CD47 monoclonal antibodies caused by anti-glycophorin A (mAb) BRIC 256 might play a role in eryptosis pathway worthy further investigation. This pathway may well be another useful avenue to explore in cancer therapy.

Table of Contents

1	INTRODUCTION:	1
1.1	DEVELOPMENT OF ERYTHROCYTES:	2
1.2	ROLE OF ERYTHROPOIETIN IN ERYTHROPOIESIS:	3
1.3	ERYTHROCYTE STRUCTURE:	5
1.3.1	<i>Erythrocyte membrane lipids:</i>	5
1.3.1.1	Lipid Rafts:	7
1.3.2	<i>Erythrocyte membrane proteins:</i>	9
1.3.2.1	Spectrins:	10
1.3.2.2	β actin:	12
1.3.2.3	Protein 4.2:	14
1.3.2.4	Band 3:	16
1.3.2.5	Ankyrin:	18
1.3.2.6	Glycophorins:	20
1.3.2.6.1	Glycophorin A (GPA):	20
1.3.2.6.2	Glycophorin B (GPB):	21
1.3.2.6.3	Glycophorin C (GPC):	21
1.3.2.6.4	Glycophorin D (GPD):	22
1.3.2.6.5	Glycophorin E (GPE):	22
1.3.2.7	The p55 Protein (P55):	24
1.3.2.8	Protein 4.1R:	25
1.3.2.9	Rh proteins:	26
1.3.2.10	CD44:	27
1.3.2.11	Adducin:	28
1.3.2.12	CD47:	29
1.4	APOPTOSIS:	34
1.5	ERYPTOSIS:	35
1.6	AIMS:	37
2	MATERIALS & METHODS:	38
2.1	MATERIALS:	38
2.1.1	<i>Erythrocyte samples:</i>	38
2.1.1	<i>Primary Antibodies:</i>	38
2.1.2	<i>Custom Primary Antibodies:</i>	39
2.1.3	<i>Secondary antibody:</i>	40
2.1.4	<i>Pierce™ ECL Plus Western Blotting Substrate:</i>	40
2.1.5	<i>DNA and RNA Extraction kits:</i>	40
2.1.6	<i>Cell Lines RNA:</i>	41
2.1.7	<i>Next Generation Sequencing Kit:</i>	41
2.1.8	<i>The Agilent® 2100 Bioanalyzer Kit:</i>	41
2.1.9	<i>Flow Cytometry Reagents:</i>	41
2.1.10	<i>Mass Spectrometry Liquid Chromatography Mass Spectrometry (LC-MS/MS) Orbitrap Reagents:</i>	42
2.2	METHODS:	42
2.2.1	<i>Erythrocyte ghost membrane preparation (Membrane Protein Isolation):</i>	42
2.2.1.1	N-Terminal Co-immunoprecipitation:	43
2.2.1.2	C-Terminal Co-immunoprecipitation:	44
2.2.2	<i>BCA (Bicinchoninic acid) Protein quantification Assay:</i>	44
2.2.3	<i>Qubit® Protein quantitation assay:</i>	45
2.2.4	<i>Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE):</i>	45
2.2.5	<i>Western blotting:</i>	46
2.2.6	<i>Instant Blue Staining:</i>	47
2.2.7	<i>In Gel-Digestion preparation for Mass Spectrometry (MS):</i>	47
2.2.8	<i>Peptide Clean-Up Using Stage-Tips:</i>	49
2.2.9	<i>Liquid Chromatography-Mass Spectrometry (LCMS):</i>	49
2.2.10	<i>Data Analysis by MaxQuant for mass spectrometry results:</i>	51
2.2.11	<i>Annexin V-FITC binding assay:</i>	52
2.2.12	<i>Annexin V-FITC combination antibodies binding assay:</i>	52
2.2.13	<i>Statistical Analysis Software for Flow cytometry results:</i>	53
2.2.14	<i>Genomic DNA and RNA extraction from whole blood donor samples:</i>	53
2.2.14.1	DNA Extraction:	54
2.2.14.2	RNA Extraction:	55
2.2.14.3	Quality Control of extracted RNA:	57

2.2.15	<i>cDNA synthesis from Extracted RNA:</i>	58
2.2.16	<i>CD47 cDNA Primer Design:</i>	59
2.2.17	<i>PCR amplification:</i>	60
2.2.18	<i>Agarose Gel visualisation:</i>	61
2.2.19	<i>Next Generation Sequencing (NGS):</i>	61
2.2.19.1	Library preparation:	61
2.2.19.1.1	Purification:	61
2.2.19.1.2	Quantification:	62
2.2.19.1.3	cDNA ligation:	64
2.2.19.1.4	Confirming cDNA ligation by Agilent® 2100 Bioanalyzer system:	64
2.2.19.2	Template preparation:	65
2.2.19.3	Ion Torrent 316 Chip Sequencer:	66
2.2.19.4	Data analysis:	68
3	NEXT GENERATION SEQUENCING (NGS) OF CD47 GENE:	69
3.1	INTRODUCTION:	69
3.2	AIMS OF STUDY:	73
3.3	RNA-SEQ (CDNA-SEQ) RESULTS:	73
3.3.1	<i>Amplification of CD47 cDNA:</i>	74
3.3.2	<i>NGS of the CD47 gene:</i>	76
3.3.2.1	Ligation of barcoded adaptors:	76
3.3.3	<i>NGS data quality control:</i>	77
3.3.4	<i>CD47 isoforms sequence identification:</i>	81
3.3.4.1	RhD+ (R ₁ R ₁) results:	82
3.3.4.2	RhD+ (R ₂ R ₂) results:	83
3.3.4.3	RhD- (rr cde/cde) results:	84
3.3.4.4	K562 Results:	85
3.3.4.5	HL-60 Results:	86
3.3.5	<i>CD47 Isoforms Statistical Analysis:</i>	87
3.4	WESTERN BLOT ANALYSIS OF CD47 ISOFORMS IN RED BLOOD CELLS:	90
3.4.1	<i>CD47 isoform 2 monoclonal antibodies:</i>	93
3.4.2	<i>CD47 isoform 4 monoclonal antibodies:</i>	94
		94
3.4.3	<i>CD47 isoform 5 monoclonal antibodies:</i>	95
3.5	DISCUSSION:	96
3.5.1	<i>RNA-seq for CD47 in red cells and erythroleukemia cell lines:</i>	96
3.5.2	<i>Western blot analysis discussion of CD47 isoforms in red blood cells:</i>	99
4	CHARACTERIZATION OF CD47 MONOCLONAL ANTIBODIES TO BE A POSSIBLE THERAPEUTIC AGENT FOR CANCERS:	101
4.1	INTRODUCTION:	101
4.2	AIMS OF STUDY:	104
4.3	RESULTS:	105
4.3.1	<i>Western blot results:</i>	105
4.3.1.1	BRIC 32 Western blot:	105
4.3.1.2	BRIC 122 Western blot:	106
4.3.1.3	BRIC 124 Western blot:	107
4.3.1.4	BRIC 125 Western blot:	108
4.3.1.5	BRIC 126 Western blot:	109
4.3.1.6	BRIC 168 Western blot:	110
4.3.1.7	BRIC 211 Western blot:	111
4.3.2	<i>Flow Cytometry results using Annexin V FITC:</i>	112
4.3.2.1	Gating Strategy for different monoclonal antibodies PS exposure:	112
4.3.2.2	Low PS exposure group includes BRICs 32 and 122:	115
4.3.2.3	Medium PS exposure induced by monoclonal anti-CD47 BRICs 125, 126, and 168:	118
4.3.2.4	High PS exposure includes BRICs 124, 211:	121
4.3.3	<i>Trypsin Treated erythrocytes and anti-CD47 incubation followed by Annexin V FITC treatment Results:</i>	124
4.4	DISCUSSION:	126
5	ROLE OF CD47 IN RED CELL TURNOVER AND NOVEL INTERACTIONS TO ELUCIDATE CD47 FUNCTIONS:	129
5.1	INTRODUCTION:	129
5.2	AIMS OF STUDY:	132

5.3	RESULTS	133
5.3.1	<i>The binding proteins of CD47 using the N-terminal antibodies:</i>	136
5.3.1.1	BRIC 32: low PS-inducing anti-CD47	136
5.3.1.2	BRIC 256 anti-glycophorin A antibody:	139
5.3.1.3	BRIC 124: high PS-inducing anti-CD47	142
5.3.1.4	BRIC 18 anti-Kell Protein antibody:	145
5.3.1.5	BRIC 126: medium PS-inducing anti-CD47:	148
5.3.1.6	BRIC 235 anti-CD44 antibody:.....	151
5.3.2	<i>The upregulated proteins by all CD47 N-terminal antibodies:</i>	154
5.3.3	<i>Protein components identified in anti-C-terminal CD47 IPs.....</i>	157
5.3.3.1	mAbs to Isoform 2 IP components identified	157
5.3.3.2	mAbs to Isoform 4 IP components identified	160
5.3.3.3	mAbs to Isoform 5 IP components identified:.....	163
5.3.4	<i>Comparison of the protein binding partners for three of the five different CD47 isoforms:</i>	166
5.4	DISCUSSION:.....	170
5.4.1	<i>CD47 interacts with S100 Proteins</i>	170
5.4.2	<i>CD47 interacts with Lipid Rafts:</i>	172
5.4.3	<i>CD47 N-terminal antibodies interactions:</i>	175
5.4.4	<i>CD47 C-terminal antibodies interactions:</i>	176
6	GENERAL DISCUSSION AND FUTURE WORK:	178
6.1	CD47 ISOFORM 5:.....	178
6.2	CD47 ISOFORMS:.....	180
6.3	CD47 AS AN ANTI-CANCER DRUG:.....	181
6.4	ROLE OF CD47 IN RED CELL TURNOVER:	184
7	REFERENCES:	188
8	APPENDICES:	204

List of Figures:

FIGURE 1.1: THE PROCESS OF ERYTHROPOIETIC DEVELOPMENT.	4
FIGURE 1.2: THE DISTRIBUTION OF LIPIDS ON CELLULAR MEMBRANE.	6
FIGURE 1.3: DEMONSTRATION OF LIPID RAFTS IN ERYTHROCYTE MEMBRANE.....	8
FIGURE 1.4: MODELS OF HUMAN ERYTHROCYTE MEMBRANE SHOWING THREE DIFFERENT BAND 3 COMPLEXES.	9
FIGURE 1.5: STRUCTURAL HOMOLOGY OF ALPHA AND BETA SPECTRIN IN ERYTHROCYTE MEMBRANE. .	11
FIGURE 1.6: SCHEMATIC DIAGRAM OF A CROSS-SECTION THROUGH A RED BLOOD CELL SHOWING THE CORTICAL ACTIN-SPECTRIN NETWORK.	13
FIGURE 1.7: PROTEIN 4.2 INTERACTION WITH BAND 3 AND ANKYRIN COMPLEX.	15
FIGURE 1.8: THE STRUCTURE OF ERYTHROID ANKYRIN.....	19
FIGURE 1.9: GLYCOPHORIN C (GPC) TERNARY COMPLEX IN ERYTHROCYTES.	23
FIGURE 1.10: THE TOPOLOGY OF CD47 STRUCTURE.	33
FIGURE 2.1: SAMPLE PROCESS AFTER GEL ELECTROPHORESIS.	46
FIGURE 2.2: SEPARATION OF BLOOD COMPONENTS IN A 15 ML FALCON TUBE.	54
FIGURE 2.3: CONVENTIONAL PCR FOR CD8/CD4.	58
FIGURE 3.1: <i>CD47</i> GENE STRUCTURE. <i>CD47</i> HAS 12 EXONS AND 11 INTRONS.....	71
FIGURE 3.2: THE MRNA EXONS OF <i>CD47</i>	72
FIGURE 3.3: THE MRNA (cDNA) <i>CD47</i> AMPLICONS PRODUCED BY REVERSE TRANSCRIPTASE PCR..	76
FIGURE 3.4: AN ELECTROPHEROGRAM OF THE <i>CD47</i> AMPLICONS LIBRARY.	77
FIGURE 3.5: THE REPORT OF ONE SINGLE SEQUENCING RUN OF THE <i>CD47</i> LIBRARY	80
FIGURE 3.6: THE PERCENTAGE OF <i>CD47</i> ISOFORMS IN RHD+ (R ₁ R ₁ , CDE/CDE).	82
FIGURE 3.7:THE PERCENTAGE OF <i>CD47</i> ISOFORMS IN RHD+ (R ₂ R ₂ cDE/cDE).....	83
FIGURE 3.8: THE PERCENTAGE OF <i>CD47</i> ISOFORMS IN RHD- (RR).	84
FIGURE 3.9: THE PERCENTAGE OF <i>CD47</i> ISOFORMS IN K562.	85
FIGURE 3.10: THE PERCENTAGE OF <i>CD47</i> ISOFORMS IN HL-60.	86
FIGURE 3.11: THE TOPOLOGY OF <i>CD47</i>	91
FIGURE 3.12: DOTS BLOT FOR POSITIVE HYBRIDOMAS FOR <i>CD47</i> ISOFORM 2 MABS.....	93
FIGURE 3.13: THE EXPRESSION OF <i>CD47</i> ISOFORM 2 IN RED BLOOD CELLS.	93
FIGURE 3.14: THE EXPRESSION OF <i>CD47</i> ISOFORM 4 IN RED BLOOD CELLS.	94
FIGURE 3.15: DOTS BLOT FOR POSITIVE HYBIRDOMAS FOR <i>CD47</i> ISOFORM 5 MABS.....	95
FIGURE 3.16: THE EXPRESSION OF <i>CD47</i> ISOFORM 5 IN RED BLOOD CELLS.	95
FIGURE 3.17: <i>CD47</i> ISOFORM 5 SEQUENCE EXONS ARRANGEMENTS.	97
FIGURE 4.1: FOUR DIFFERENT PROPOSED MECHANISMS TO PREVENT <i>CD47</i> -SIRP ALPHA INTERACTION.	103
FIGURE 4.2: IMMUNOSTAINING OF RBC MEMBRANES FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 32.....	105
FIGURE 4.3:IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 122.	106
FIGURE 4.4: IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 124.	107
FIGURE 4.5: IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 125.	108
FIGURE 4.6: IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 126.	109
FIGURE 4.7: IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 168.	110
FIGURE 4.8: IMMUNOSTAINING OF RBC MEMBRANE FROM DIFFERENT Rh PHENOTYPES ERYTHROCYTES USING BRIC 211.	111
FIGURE 4.9:FLOW CYTOMETRY GATING STRATEGY.....	113
FIGURE 4.10:FLOW CYTOMETRY OF ERYTHROCYTES FROM DIFFERENT Rh PHENOTYPES.	114
FIGURE 4.11: FLOW CYTOMETRY FOR COMBINATION RESULTS OF BRIC 32, BRIC 122 FROM DIFFERENT Rh PHENOTYPES.	116
FIGURE 4.12: FLOW CYTOMETRY FOR COMBINATION RESULTS OF BRIC 125, BRIC 126, AND BRIC 168 FROM DIFFERENT Rh PHENOTYPES.	120
FIGURE 4.13: FLOW CYTOMETRY COMBINATION RESULTS OF BRIC 124, AND BRIC 211 FROM DIFFERENT Rh PHENOTYPES.	122
FIGURE 4.14: FLOW CYTOMETRY OF TRYPSIN TREATED ERYTHROCYTES FROM DIFFERENT Rh PHENOTYPES.....	125
FIGURE 5.1: 2D DEMONSTRATION OF BAND 3 COMPLEX AND 4.1R COMPLEX IN ERYTHROCYTES:	131

FIGURE 5.2: FLOW CHART FOR MASS SPECTROMETRY ANALYSIS OF CD47 IMMUNOPRECIPITATION..	134
FIGURE 5.3: THE PROTEIN ABUNDANCE PROFILE IN ANTI-CD47 BRIC 32 IPs.	138
FIGURE 5.4: THE PROTEIN ABUNDANCE PROFILE IN ANTI-GPA BRIC 256 IPs.....	141
FIGURE 5.5: THE PROTEIN PROFILES CONTAINED IN ANTI-CD47 BRIC 124 IPs.	144
FIGURE 5.6: THE PROTEIN ABUNDANCE PROFILE IN ANTI-KELL BRIC 18 IPs.....	147
FIGURE 5.7: THE PROTEIN PROFILES OF ANTI-CD47 BRIC 126 IPs.A- THE PIE CHARTS SHOW THE HIGHEST ABUNDANT PROTEIN DETECTED BY LS-MS/MS. THE RESULTS SHOWED THAT CD47 IS THE HIGHEST ABUNDANT PROTEIN BY BRIC 126 IP (68% MOL/MOL) WHICH IS EXPECTED AS THIS ANTIBODY IS SPECIFIC FOR CD47. B- THE PIE CHARTS SHOWING THE HIGHEST ABUNDANCE BY BRIC 126 AFTER SUBTRACTING CD47 SCORE TO ELUCIDATE DIFFERENT PROTEIN BINDING PARTNERS.	150
FIGURE 5.8: THE PROTEIN ABUNDANCE PROFILE IN ANTI-CD44 BRIC 235 IPs.	153
FIGURE 5.9: THE PROTEIN PROFILES OF ANTI-CD47 ISOFORM 2 IPs	159
FIGURE 5.10: THE PROTEIN PROFILES OF ANTI-CD47 ISOFORM 4 IPs	162
FIGURE 5.11: THE PROTEIN PROFILES OF ANTI-CD47 ISOFORM 5 IPs	165
FIGURE 6.1: THE PROPOSED RESPONSE OF GPA AFTER LIGATION WITH MONOCLONAL ANTIBODY VS THE RESPONSE OF TRYPSIN TREATED GPA AFTER LIGATION WITH MONOCLONAL ANTIBODY. .	184

List of Tables:

TABLE 1.1: THE SIMILARITIES AND DIFFERENCES BETWEEN APOPTOSIS AND ERYPTOSIS.....	36
TABLE 2.1: PURIFIED MONOCLONAL ANTIBODIES FROM INTERNATIONAL BLOOD GROUP REFERENCE LABORATORY (IBGRL).	39
TABLE 2.2: PRIMERS USED TO AMPLIFY CD47 cDNA.	59
TABLE 2.3: THERMOCYCLING CONDITIONS FOR PCR.	60
TABLE 3.1: SEROLOGY DATA SHEET PROVIDED BY NHSBT, FULTON FOR THE RH PHENOTYPE OF SAMPLES USED IN THE CD47 TRANSCRIPT NGS STUDY	74
TABLE 3.2: A SUMMARY OF THE ION PGM™ SEQUENCE RUN OUTPUT OF THE 36 CD47 SAMPLES, COLLECTIVELY PROCESSED IN 3 RUNS.....	78
TABLE 3.3: THE COMPARISON BETWEEN DIFFERENT ERYTHROCYTES PHENOTYPES AND CELL LINES BY USING T-TEST STATISTICAL ANALYSIS FOR CD47 ISOFORMS.	89
TABLE 3.4: HOMOLGY BETWEEN CD47 ISOFORM PEPTIDES:	92
TABLE 3.5: THE PEPTIDE SEQUENCES USED FOR CD47 MONOCLONAL ANTIBODY PRODUCTION:	92
TABLE 4.1: SUMMARY OF THE COMBINATIONS RESULTS:	123
TABLE 5.1: THE LFQ INTENSITY VALUES BY DIFFERENT CD47 ANTIBODIES AND THEIR CONTROL ANTIBODIES USING LC-MS/MS.....	156
TABLE 5.2: THE LFQ INTENSITY BY DIFFERENT CD47 ISOFORMS ANTIBODIES USING MS.	169

List of Abbreviations:

mAbs	Monoclonal Antibodies
BM	Bone Marrow
PS	Phosphatidylserine
GPA	Glycophorin A
EPO	Erythropoietin hormone
cAMP	cyclic adenosine monophosphate
DRMs	detergent resistance microdomains
IAP	Integrin-associated protein
ISPs	ion sphere particles
PCR	Polymerase chain reaction
NGS	Next Generation Sequencing
LFQ	label-free quantitation
CDA	congenital dyserythropoietic anaemia
NCBI	National Center for Biotechnology Information
NHSBT	National Health Service Blood and Transplant
AA	Amino Acid
EDTA	Ethylenediaminetetraacetic acid
rbm	Revolutions Per Minute
mAbs	Monoclonal Antibodies
IBGRL	International Blood Group Reference Laboratory
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
TFA	Trifluoroacetic acid
ABC	Ammonium bicarbonate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
MS	Mass Spectrometry
ACN	Acetonitrile
HRP	Horseradish peroxidase
CID	collision induced dissociation
LFQ	label-free quantitation
β -ME	β -mercaptoethanol
ISPs	Ion Sphere Particles
PVDF	Polyvinylidene fluoride
PBS-T	Phosphate Buffered Saline-Tween 20
LCMS	Liquid Chromatography-Mass Spectrometry

1 Introduction:

Blood can be described as the essential fluid of life and as well as growth and health. The main function of mature red blood cells (erythrocytes) is to maintain life by transport of oxygen and carbon dioxide between the lungs and tissues. However, blood plasma carries nourishment from digested food and hormones from glands in order to maintain growth. Moreover, blood also contains a group of cells called the white cells (leukocytes) that actively attack and provide us with resistance and memory to disease to maintain health. Blood is also responsible for carrying other disease fighting substances (e.g. Complement) and for taking waste to the kidneys for removal.

The connection between blood and disease was identified as early as 2500 BC where a tomb illustration showed a patient being bled from the foot and neck. Though the 1200s saw the first description of the pulmonary circulation, the 1660s the first recorded transfusion in animals and, in 1901, the discovery of the three main human blood groups, today, many fundamental questions remain unanswered. Scientists continue the quest for total understanding of blood, blood components and the relationships between blood and morbidity and mortality.

The first observation and description of red blood cell (erythrocyte) was in 1658 by Dutch microscopist Jan Swammerdam. However, in 1674 a more precise description was introduced by another Dutch microscopist, Antonj van Leeuwenhock, estimating their size to 25000 times smaller than a fine grain of sand. Today we know that the human adult has roughly $2-3 \times 10^{13}$ red blood cells at any given time, measuring 6-8 μm in diameter, and are turned over of 3 million per second after a controlled 120 days lifespan. The mechanism of red cell destruction remains poorly understood.

1.1 Development of Erythrocytes:

To maintain a healthy steady level of blood in the peripheral circulation, the bone marrow (BM) produces approximately 10^{11} – 10^{12} new red blood cells per a day (Lin et al., 2014).

Erythropoiesis (erythrocyte production) is a series of steps stimulated by the release of Erythropoietin hormone through a response of the kidney to the reduction of levels of oxygen in tissues (Singh et al., 2014).

Erythrocyte production and maturation takes place in the BM via a process called proliferation in which haemopoietic stem cells (HSCs) go through many events that lead to the formation of erythrocytes (figure 1.1) (Guarnerio et al., 2014, Mohandas and Gallagher, 2008). This process starts by development of a hemocytoblast which is derived from the mesoderm to become a common myeloid progenitor or lymphoid progenitors. Myeloid progenitors can be divided to give four main types, which are megakaryocytes, erythrocytes, mast cells, and myeloblasts. In the mature stage of these cells the megakaryocyte will become platelets, whilst myeloblast cells differentiate to Basophils, Neutrophils, Eosinophils and Monocytes which are the key components of the humoral immune system. Common lymphoid progenitors can mature to give either natural killer cells or small lymphocytes which can differentiate to become T and B lymphocytes (McGrath and Palis, 2008). BM is considered the factory that produces blood in adults, while the formation of the blood occurs in aggregate of blood cells in yolk sac of the developing embryos. As the fetus matures the formation of blood takes place in different location such as the spleen, liver, and lymph nodes. The liver is mainly considered as a site of haematopoiesis in the developing fetus due to lateness of the bone marrow development, the liver is enlarged during the maturation of the fetus. In certain age of fetus life (around the seventh month), the erythropoiesis takes place in the marrow of long bones. Extra-medullary haematopoiesis is a medical adult case when the erythropoiesis

occurs in the spleen, liver, or thymus causes them to enlarge (Palis, 2008).

An erythrocytes average lifespan is 120 days; platelets 8 to 12 days and granulocytes survive 3 to 9 hours. Approximately 5000 kg of blood will be formed from bone marrow during a human's lifespan (Ebdon et al., 2013).

1.2 Role of Erythropoietin in erythropoiesis:

Erythropoietin hormone (EPO) plays a key role in erythropoiesis. 90% of EPO is produced from the kidney and 10% from the liver. The human *EPO* gene is found on chromosome 7. EPO protein is highly glycosylated (39%) and consists of four amphipathic α -helical bundles which fold into a common compact globular structure. It is similar to other cytokine hormones such as growth hormone, prolactin, and interleukin 2 (Wen et al., 1994). Low EPO levels in the peripheral blood can indicate a failure either in the kidney or bone marrow. EPO works by binding and activating a high affinity, cytokine receptor (Watowich, 2011). EPO receptors (EpoR) are like other cytokine receptors, have an extracellular N-terminus, and have two extracellular immunoglobulin-like domains, and four similar spaced cysteine residues (Watowich, 2011). they are located on 'burst forming unit-erythroid' (BFU-E) and 'colony-forming unit-erythroid' (CFU-E) progenitors. Apoptosis will be lower in precursors that have high serum levels of EPO and proliferation and differentiation increases (Ebdon et al., 2013).

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Figure 1.1: The process of erythropoietic development.

In adult Bone Marrow, the progenitors starting from haematopoietic stem cells developed to erythroblasts, and as the development of a stem cell progress, there is a loss of their multipotency while increasing lineage restriction, this process takes 3-4 days before it released in the circulation as reticulocytes. In the circulation, the process of reticulocytes takes 24-48 hours to developed as mature red blood cell (erythrocyte) (Singh et al., 2014).

1.3 Erythrocyte Structure:

Red blood cells are composed of 20% H₂O, Proteins about 40% (mainly haemoglobin), 35% Lipids, and 5% are carbohydrates (Yawata, 2006, Pennell, 1964).

1.3.1 Erythrocyte membrane lipids:

Phospholipids, cholesterol and the membrane skeleton of scaffolding proteins are the main components of the lipid bilayer of the red blood cell membrane (Delaunay, 2007). Asymmetric distribution of phospholipids in the lipid bilayer of the membrane is one of the important characteristics of this membrane. The inner monolayer of the lipid bilayer consists of phosphatidylethanolamine, phosphatidylserine (PS), and phosphoinositides, while the outer monolayer contains phosphatidylcholine and sphingomyelin (Figure 1.2) (An and Mohandas, 2008). The major phospholipids in erythrocyte membrane are phosphatidylserine (13%), sphingomyelin (26%), phosphatidylethanolamine (29%) and phosphatidylcholine (31%). The role of phospholipid in the red cell membrane is significant, for example: Phosphatidylserine enhances mechanical stability of the membrane when it connects with protein 4.1R and spectrin (Ling et al., 1988, Cohen et al., 1988, Manno et al., 2002). Cholesterol is also present at the concentration of (11%) of red cell membrane (Yawata, 2006). Cholesterol play an important role in modulation of the function of membrane proteins by interacting with Band 3 and glycoporphins to maintain cellular function (Yeagle, 1991). The third components of lipids in erythrocytes membrane is lipids rafts.

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Figure 1.2: The distribution of lipids on cellular membrane.

Phosphatidylserine (PS) and phosphatidylethanolamine situated at the inner leaflets of the membrane while phosphatidylcholine and sphingomyelin founded in the outer membrane taken from (Lodish et al., 1995).

1.3.1.1 Lipid Rafts:

Within the lipid bilayer mosaic structure of the erythrocyte membrane, there are different proteins embedded and also within this lipid bilayer are microdomains representing heterogeneous distribution of various membrane components which are called lipid rafts (figure 1.3) (Salzer and Prohaska, 2001, Singer and Nicolson, 1972, Samuel et al., 2001). The most significant properties of this group are enriched in cholesterol and sphingolipids and contain specific membrane proteins. Lipid rafts proteins components in erythrocytes are glycosylphosphatidylinositol (GPI)-anchored proteins, flotillins, stomatin, aquaporin-1, and guanine nucleotide-binding protein (G α) (Salzer and Prohaska, 2001, Schnitzer and Oh, 1996, Samuel et al., 2001, Brown and Rose, 1992). Lipid rafts play an important role in cell signalling pathways in various cell types including erythrocytes (As rafts are insoluble in non-ionic detergent, they are isolated as detergent resistance microdomains (DRMs)) (Brown and Rose, 1992). Lipid rafts mediate signal transduction by binding to different membrane protein. One example for that modification is the elevation of cyclic adenosine monophosphate (cAMP) that results of phosphorylation of adducin (Kamata et al., 2008). Recently, it has been demonstrated that lipid rafts component (flotillin 2) associate with major erythrocyte membrane band 3 in stored erythrocytes (Prudent et al., 2018) and still unknown if this interaction is present *in vivo*. This will be discussed in more details in chapter 5.

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Figure 1.3: Demonstration of lipid rafts in erythrocyte membrane.

Lipid rafts are embedded in erythrocyte membrane. However, the interaction between cholesterol, glycolipid, phospholipids, and glycerosphingolipids are formed these small particles (lipids rafts). Here it shows that GPI-anchored proteins as an example of lipid rafts but also different membrane proteins which is glycosylated proteins has been described to interact with lipid rafts.

Taken from (Scolari, 2009)

1.3.2 Erythrocyte membrane proteins:

In 1963 Dodge et al. described a preparation technique, which allowed direct studies of red blood cell membrane proteins. This technique was preparing red cell membrane ghosts or ghost preparation technique (Dodge et al., 1963). After using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique, functionally there are three different groups of membrane proteins. The first group is peripheral proteins that are present on one side of lipid bilayer membrane of red cell (e.g. Spectrins). Second group are integral proteins that are embedded in the lipid bilayer itself (e.g. Band 3). Third group are anchoring proteins and their role is to give the structure good stability (e.g. Ankyrin) (Figure 1.4) (Dodge et al., 1963, Yawata, 2006). In the following sections, the most important proteins within the bilayer membrane will be discussed in detail.

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Figure 1.4: Models of human erythrocyte membrane showing three different band 3 complexes.

The first band 3 complex is showing the interaction between band 3-ankyrin. This complex includes some major membrane protein such as Rh proteins, GPA, CD47, and protein 4.2. the second band 3 complex is showing an interaction between band 3-spectrin and this complex include some key erythrocyte membrane proteins such as, protein 4.1R, P55, adducin, and dematin. The third one is showing some free band 3 membrane protein. Taken from (Kodippili et al., 2009).

1.3.2.1 Spectrins:

Spectrins are the major structural component of erythrocytes membrane; and they are rod-like protein composed of two heterodimers. There are about 200 000 copies of spectrin in each cell and the membrane will be deformable without the spectrins. Spectrin heterodimers are composed of alpha and beta subunits and associate in an antiparallel fashion to form tetramers. The alpha spectrin gene (*SPTA*) is located on chromosome 1 at position q22-23 (Huebner et al., 1985) and encodes a 2429 amino acid protein, about 280 kDa (240 kDa on SDS-PAGE) (Sahr et al., 1990, Kotula et al., 1991) while beta spectrin gene (*SPTB*) is located on chromosome 14 at position q23-24.2 and encodes a 2137 amino acid protein, with a molecular weight of 246 kDa (220 kDa on SDS-PAGE) (Prchal et al., 1987, Fukushima et al., 1990, Winkelmann et al., 1990b). Under the electron microscope the spectrins resemble slender and twisted rod structures. The spectrin molecules are almost 100 nm in length and provide stability and deformability to the erythrocyte membrane by their high degree of elasticity (Yawata, 2006).

Alpha spectrin consist of 22 subunits of which are homologous and conform to triple helical repeats and a C-terminal domain (Speicher and Marchesi, 1984, Speicher, 1984). As alpha and beta spectrins are oriented in opposite direction, the N-terminal of alpha chain consists of a self-association binding site for the C-terminal domain of beta spectrin, and also 9 identical homology segments that consists of 106 amino acids, then segment 10 is followed, which is different than the others as it has Src-homology 3 (SH3) motif. SH3 motif mediates the interaction of specific molecule to membrane proteins as it considered as a ligand (Musacchio et al., 1992). Then, repeated segments 11-22 that contain 106 amino acids and followed by EF domain which is the main binding site between alpha spectrin and calcium binding protein calmodulin. (Trave et al., 1995a,

Trave et al., 1995b). However, protein 4.2 also interacts with EF domain of alpha spectrin and this interaction is mediated by calmodulin (Korsgren et al., 2010).

Different than alpha spectrin, beta spectrins has three distinct domains; an N-terminal domain with homology to the actin binding domains of alpha-actinin and dystrophin (Winkelmann et al., 1990a), a 17 spectrin homology repeated domain and a C-terminal domains which contains phosphorylation site and ankyrin binding site. Both N and C-terminal domains lack homology to the spectrin repetitive motif. The C-terminal domain of beta spectrins and the N terminal domain of alpha spectrin are involved in the process of spectrin dimer formation (figure 1.5).

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Figure 1.5: Structural homology of alpha and beta spectrin in erythrocyte membrane.

Alpha spectrin has 22 segments that are have high homology between them except for segment 10 which has SH3 domain. However, the EF domain in alpha spectrin mediates the interaction with 4.2 protein via calmodulin.

Beta spectrin has three main domains, N and C terminal domains plus 17 repeated segments to form a worm like shape of spectrin. Segment 14 and 15 are the binding domain with ankyrin while the N-terminal domain has the binding site with actin. Taken from (Ipsaro et al., 2010)

1.3.2.2 β actin:

Each erythrocyte in humans contains around 400 000 β actin molecules. Its gene is located on chromosome 7 (7p12–p22), the β actin gene (*ACTB*) encodes a 375 amino acid protein monomer. β actin is almost 35 nm in length and arranged as double-helical F-actin protofilaments 12 to 13 monomers long. Both erythroid and nonerythroid actins have the same function and same structure. Actin polymerization is an important factor that affects the membrane flexibility. When actin polymerization is increased the membrane becomes less flexible (Vera et al., 2005). Spectrins, adducin, and protein 4.1R are known to interact with β actin to maintain erythrocyte membrane stability (Figure 1.6) (Gimm et al., 2002, Gilligan and Bennett, 1993, Picart et al., 2000). Spectrin dimers bind to the side of actin filaments at a site near the tail end of the spectrin molecule. Actually, each actin oligomer makes a complex with six spectrin ends resulting an irregular network shape which is a hexagonal shape (Byers and Branton, 1985). Protein 4.1R is stabilizing the formation of ternary complex of spectrin, and actin (Gimm et al., 2002). Moreover, the actin-spectrin binding not only mediated by protein 4.1R but also is mediated by dematin (band 4.9) which also enhance and stabilized this interaction (Koshino et al., 2012). Recently, tropomyosin (Tpm) isoforms (Tpm 1.9, and Tpm 3.1) were found also mediating the actin-spectrin interaction and also facilitate the interaction of this complex with different membrane glycoprotein such as band 3 and glycophorins (Sui et al., 2017).

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Figure 1.6: Schematic diagram of a cross-section through a red blood cell showing the cortical actin-spectrin network.

The inset is a detailed view of the network, which is comprised of the proteins actin (green), adducin (blue) and spectrin (purple) arranged in a hexagonal lattice structure: short filaments of actin capped with adducin are connected by spectrin complexes made of a mixture of α -spectrin and β -spectrin. Taken from (Stewart and Shen, 2015)

1.3.2.3 Protein 4.2:

Each erythrocyte has approximately 250 000 copies of protein 4.2. One of the major functions of protein 4.2 is to provide stability to the membrane structure, and it is a component of the membrane skeleton. Protein 4.2 has a molecular weight of 72 kDa on SDS-PAGE. Protein 4.2 interacts with the N-terminal cytoplasmic domain of Band 3 and ankyrin through critical residues 187-211 (figure 1.7) (Bruce et al., 2002, Yawata, 1994, Yawata, 2006, Bhattacharyya et al., 1999). Congenital haemolytic anaemia with micro spherocytosis will result if there is a deficiency in protein 4.2 (Yawata, 1994). Any reduction in band 3 in erythrocyte membranes will cause a reduction in protein 4.2 (Bruce et al., 2002). Protein 4.2 tightens the Band 3 membrane skeletal linkage and also binds spectrin to stabilize the spectrin-actin structure (Vera et al., 2005, Mandal et al., 2002).

The interaction between CD47 and protein 4.2 were studied in early 2000. Actually, patients who have reduction or absence of protein 4.2 has also reduction in CD47 in cell membrane (Bruce et al., 2002, Dahl et al., 2004). However, reduction of protein 4.2 in erythrocyte membrane not only affected CD47 presence but also some alteration of the membrane protein complex in general such as CD44 protein was notably increased (van den Akker et al., 2010). The major function of protein 4.2 in erythrocyte is stabilizing the membrane structure by mediating the interactions of several essential membrane proteins such as band 3, ankyrin, spectrins, and protein 4.1R (Yawata, 2006).

Protein 4.2 also binds to spectrin in erythrocyte membrane and this interaction was demonstrated by Korsgren et al. (2010). Actually, the interaction between protein 4.2 and spectrin were clearly demonstrated as protein 4.2 binds EF region of spectrin (Korsgren et al., 2010) which emphasises the major function of protein 4.2 in erythrocyte membrane as a membrane stabilizer protein.

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Figure 1.7: Protein 4.2 interaction with band 3 and Ankyrin complex.

(A) An intracellular membrane view, the erythrocyte inner structure was identified with a black line; (B) an outer view of protein 4.2 interaction with band 3, ankyrin. Protein 4.2 was displayed in pink while ankyrin was demonstrate in green and the red structure is represented band 3 protein. Protein 4.2 residues 187-211 where it binds to band 3 and ankyrin were showed in blue. Taken from (Mankelow et al., 2012)

1.3.2.4 Band 3:

Band 3- anion exchanger (AE1) is considered the major integral protein present within the erythrocyte membrane. It is a 100-102 kDa transmembrane glycoprotein and present at a concentration of 1.2 million copies per red blood cell. The responsible gene of Band 3 is *SLC4A1* which is located in chromosome 17 (17q21-q22). Band 3 is composed of 911 amino acid (Zhang et al., 2000). There are two major domains of band 3 proteins. The first domain (formed by the first 403 amino acids) is the cytoplasmic (NH₂-terminal) 43 kDa domain. The second domain has alpha helices and beta sheets to make the transmembrane (COOH-terminal) domain. Band 3 plays an important role in membrane stability by binding its COOH-terminal domain to ankyrin, protein 4.1R and protein 4.2 (Chu and Low, 2006).

There are three main band 3 complexes in erythrocyte membrane (Figure 1.4), the first one is the band-ankyrin which mainly bound to spectrin, the second is the band 3 junctional complex where it binds to the membrane skeleton through (adducin, protein 4.1R, and actin), and the last one is a free band 3 which exists as dimers or tetramers (Kodippili et al., 2009, Lux, 2016).

To start with the band 3-ankyrin macro complex, as this complex is including some major erythrocyte membrane such as the Rh protein complex, CD47, protein 4.2, and glycophorin A (GPA) (Bruce et al., 2003). The suggested function of this macro complex is to facilitate the O₂ transport through the membrane as a suggested function of Rh proteins (Soupene et al., 2001, Soupene et al., 2002). However, the main function of band 3 within this membrane is to transport chloride-bicarbonate through the cell membrane which will cause release of O₂ which is postulated to be carried out by Rh protein. Thus, the main function for this macro complex is to transport O₂/CO₂ through multi signals pathway between these proteins (Bruce et al., 2003).

The second band 3-spectrin complex in erythrocyte membranes include also some key important membrane proteins such as, protein 4.1R, actin, adducin, P55, dematin, glucose transporter 1 (GLUT1) and glycophorin C (GPC). The main function for this band 3 macro complex is to facilitate glucose transport through the erythrocyte membrane as GLUT 1 is found in this complex. However, binding band 3 to spectrin is stabilize the erythrocyte membrane as both of them are the most abundant membrane proteins (Kodippili et al., 2009, Khan et al., 2008).

Finally, the third for of erythroid band 3 is the free membrane band 3 (Che et al., 1997). However, some of these free band 3 proteins were found to bind to GPA and they are responsible for membrane trafficking (Young and Tanner, 2003). Most recently, band 3 was found also interacts with lipid raft membrane protein (flotillin 2) and the functional outcome of that interaction still unknown (Prudent et al., 2018).

1.3.2.5 Ankyrin:

Ankyrin is one of the essential membrane proteins within the red blood cell. It forms 5% of membrane protein and it has 206 kDa on SDS-PAGE (Davis et al., 1992, Bennett, 1992). It presents at a concentration of 1.2 million copies per red cell, and one of the main functions of this protein is to maintain membrane stability by binding to band 3 protein, and spectrin (Su et al., 2006, Bennett and Healy, 2008). Ankyrin is composed of three different domains that allow Ankyrin to be connected with other membrane proteins (band 3 and spectrin) (figure 1.8). The first one is the band 3 domain which has 89 kDa molecular weight at N-Terminal (Su et al., 2006). The second domain is the central domain which has 62 kDa molecular weight and it is for binding with spectrins. Moreover, the third domain is the regulatory domain at the COOH terminus and has 55 kDa molecular weight (Bennett and Baines, 2001, Yawata, 2006).

To start with the ankyrin band 3 interaction, the ankyrin binding domain with band 3 contains 24 repeated 33 amino acid units which is known as cdc10 ankyrin repeats (ANK repeats) which are subdivided into six repeat folding units. These repeats comprised of two alpha helical structures separated by an extended loop and beta hairpin divided into 6 repeats folding units (Bennett and Baines, 2001). However, these repeats can bind up to four band 3 molecules (Yawata, 2006). The main target from binding ankyrin with band 3 is to stabilize the membrane integrity.

The second domain which is the central domain is known as the spectrin binding domain as it is responsible for binding ankyrin with spectrin. The spectrin binding sites are located in the beginning and middle regions of this domain (figure 1.8). The site in the middle portion is highly conserved and appears to be the principal area of binding ankyrin with spectrin (Platt et al., 1993). However, each spectrin tetramer appears to bind only one ankyrin molecule which indicates that the binding ability of ankyrin is more strong than

the spectrin dimer (Yawata, 2006). The subdomain that is responsible for ankyrin-spectrin interaction is known as ZU5A which is located by the junction of the 14th and 15th repeats within beta spectrin (Figure 1.8) (Giorgi et al., 2001).

The third domain which is 55 kDa is located at the C-terminus is known to consist regulatory sequence that might regulate the interaction with band 3 and spectrin as the C-terminal domain of spectrin bind to this domain and also the C-terminal tails of band 3 is also bind to this domain through the N-terminus of this domain (figure 1.8). this domain is also contains different isoforms by alternative splicing (Gallagher and Forget, 1998, Gallagher et al., 1997).

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Figure 1.8: the structure of erythroid ankyrin.

The N-terminal domain consist of 24 (group functionally into 4 subdomains of 6 repeats) ankyrin repeats which has the binding sites to bind to band 3 in erythrocyte membrane. The central domain which has ZU5A region which is the binding site of ankyrin with spectrin. The C-terminal domain which is regulate the interaction between the spectrin, and band 3. Taken from (Lux, 2016)

1.3.2.6 Glycophorins:

Glycophorins are classified as integral membrane proteins, contain high sialic acid volume amount and act as target ligands for viruses and others micro-invaders (Cartron and Rahuel, 1995). There are five different subclasses for Glycophorins, which are, Glycophorin A, B, C, D and E.

1.3.2.6.1 Glycophorin A (GPA):

Glycophorin A (GPA) is considered as sialoglycoprotein in red cells. It is found at approx. concentration of one million copies per red cell. Moreover, it has a molecular weight about 36 kDa on SDS-PAGE and is composed of 131 amino acids (Fukuda, 1993). GPA was the first protein to be sequenced within red cell, and the first membrane protein ever sequenced. M and N antigens originate from GPA gene (*GYP A*) that is located in chromosome 4q28-q31 (Chasis and Mohandas, 1992, Fukuda, 1993). 60 % of GPA molecules are carbohydrates and only expressed on erythrocytes (Yawata, 2006). The exact biological function of GPA is still unknown but it is predicted to be responsible for red cell-red cell interaction or red cell-endothelial cell interaction as it contributes in the membrane net charge (Chasis and Mohandas, 1992, Yawata, 2006).

GPA are considered as members of the band 3 macro complex as naturally occurring red cell band 3 knock outs shows an absence of GPA (Southgate et al., 1996). However, the membrane shape and integrity is not affected by absence of GPA but it shows an increase glycosylation of band 3 and that is because of increase of sialic acids that must be in GPA molecules (Tanner and Anstee, 1976). Interestingly, deficient GPA red cells are more resistant to malaria invasion as it is considered as receptors for *plasmodium* via erythrocyte binding antigen 17 (Wassmer and Carlton, 2016, Chishti et al., 1996). GPA and band 3 express the Wright (Wr) blood group antigen in erythrocyte membranes by an interaction

of the specific amino acid Glu658 in band 3 with Arg61 in GPA interaction (Bruce et al., 1995). This interaction was specifically studied recently and they found that this interaction organizing Wr blood group antigen and also emphasizes how GPA affects the transport role of band 3 in erythrocytes by a direct interaction of band 3 with GPA outside the ankyrin membrane complex (Kalli and Reithmeier, 2018).

1.3.2.6.2 Glycophorin B (GPB):

Although 95% sequence similarity between GPA and GPB, the difference is in the exoplasmic domain and cytoplasmic tails. Moreover, GPB is composed of 72 Amino Acids and molecular weight of 20 kDa in SDS and the concentration of GPB per red cell is much less than GPA (Palacajornsuk, 2006). GPB is present at the concentration of 150 000 per red cell. There are five exons of its gene *GYPB*. The exact function of GPB is still needs to be discovered and the only thing related to GPB is that the antigen Ss is expressed by GPB (Yawata, 2006, Chasis and Mohandas, 1992). Moreover, GPB was also found to be used during *plasmodium* invasion via erythrocyte binding ligand 1 (EPL-1) (Mayer et al., 2009).

1.3.2.6.3 Glycophorin C (GPC):

Glycophorin C (GPC) has molecular weight on the SDS-PAGE 32 kDa but when calculated it is 14 kDa because it is highly glycosylated protein, and it is composed of 128 amino acids (Schawalder et al., 2004). There are almost 143 000 copies of GPC per red cell which is derived GPC gene (*GYPC*) that located on chromosome 2q14_q21. Molecular *GYPC* composed of four exons and the glycosylation site located on the extracellular domain (Cartron et al., 1993). GPC has an important role in membrane shape integrity by interaction major membrane proteins and resulting more stability of the

membrane of red cell and also carries the Gerbich blood group antigen (Colin et al., 1989).

Red cell membrane that lack glycophorin A or B will still have normal shape but red blood cells that are GPC deficient will show elliptocytic morphology and less membrane stability than normal cell as GPC interacts with major membrane protein such as P55, and 4.1R to generate the membrane stability (figure 1.9) (Reid et al., 1987). However, GPC is a membrane receptor for *plasmodium falciparum* via the erythrocyte binding antigen 140 (EBA-140) (Maier et al., 2003).

The invasion of erythrocytes by malarial parasite will cause eryptosis by switching the phosphatidylserine (PS) from the inner membrane to the outer membrane thorough releasing of EPA-140 which binds to GPC (Lobo et al., 2003, Mayer et al., 2006). Previous work within Avent group has also shown that ligation of GPC with anti-GPC antibody will cause eryptosis and used as a control for similar work in this thesis but this is abolished in Gerbich-variant red cells that are resistant to Malarial invasion (chapter 5) (Head et al., 2005a).

1.3.2.6.4 Glycophorin D (GPD):

GPD is similar to GPC but 9 kDa smaller (23 kDa) and both of them are encoded by the same *gene GYPC* that located on 2q14-q21 (Cartron et al., 1993). GPD is present at the concentration of 83 000 copies per red cell. The difference between GPD and GPC is in the first 21 amino acid sequence that missed in GPD and for that GPD is considered as truncated version of GPC. Moreover, GPD is related to Gerbich blood group (Schawalder et al., 2004).

1.3.2.6.5 Glycophorin E (GPE):

Molecular cloning has identified GPE. *GYPE* is composed of four functional exons. GPE is consist of 59 Amino Acids and has molecular weight of 20 kDa on SDS. Moreover,

GPE is raised from *GYPE* gene on chromosome 4q28.2-q31.1. (Chasis and Mohandas, 1992).

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Figure 1.9: Glycophorin C (GPC) ternary complex in erythrocytes.

GPC complex in erythrocytes shows the interaction of GPC with protein 4.1R and p55 proteins in erythrocyte cytoskeleton. The YFI region is specific for p55 interaction while the RHK binding site is specific for 4.1R interaction. This complex is also interact with spectrin to support the membrane stability taken from (Jaskiewicz et al., 2018).

1.3.2.7 The p55 Protein (P55):

P55 protein is composed of 466 Amino Acids and has a molecular weight of 55 kDa on SDS and encoded by the P55 gene (*MPP1*) that is located on chromosome X (Xq28) (Seo et al., 2009). P55 protein is a member of membrane-associated guanylate kinase homologues (MAGUKs) family which is characterized by the presence of PDZ domain, SH3 domain, and a catalytically inactive guanylate kinase-like (GUK) domain (Dimitratos et al., 1999).

P55 is a palmitoylated phosphoprotein in erythrocytes are present at the concentration of 80×10^3 copies per erythrocytes (Chishti, 1998, Marfatia et al., 1997). P55 protein interacts with GPC (YFI tripeptide) via PDZ domain as shown in (figure 1.9). However, it also interacts with protein 4.1R via SH3 domain (Seo et al., 2009). This ternary complex with 4.1R and GPC is believed important for the stabilization of erythrocyte membrane as it connected to spectrins (Hemming et al., 1995). The third domain is composed of (GUK) domain and is a tyrosine phosphorylation domain (Seo et al., 2009, Marfatia et al., 1997).

The interaction of p55 with 4.1R via SH3 domain and the specific motive that binds to 4.1R is known as D5 domain which is has high lysine conserved lysine residues (KKKKYKDK) that are always constant within MAGUKs family (Hough et al., 1997, Seo et al., 2009). Most recently, p55 proteins was found to interact directly with lipid rafts (flotillin 1, flotillin 2) in erythrocytes membrane. During palmitoylation the ability of p55 protein molecules to bind 4.1R and GPC in ternary complex are decreased and p55 turned to bind flotillin 1, flotillin 2 tetramer directly which is facilitating the functional domain formation (protein-lipid) domain which is reflected the importance of p55 protein in erythrocyte membrane (Biernatowska et al., 2017).

1.3.2.8 Protein 4.1R:

Protein 4.1R is present at the concentration of approx. 200 000 copies per red cell and composed of 588 amino acids that form four functional domains (Scott et al., 2001, Leto and Marchesi, 1984). Protein 4.1R is an important phosphoprotein within cell membrane as it has a molecular weight of 66 kDa but on SDS it migrates to 80 kDa (Baklouti et al., 1997, Huang et al., 1993). Protein 4.1R gene (*EL1 or EPB41*) is located on chromosome 1p36.1 (Kim et al., 1998).

The main four functional are the N-terminal domain (and known as FERM domain which is 30 kDa), the second is 16 kDa domain, the third is 10 kDa domain, and the fourth is the C-terminal domain (24 kDa) (Conboy et al., 1986, Takakuwa, 2000).

To start with the FERM domain (F refer to 4.1R protein, E refer to ezrin, R refer to radixin, and M refer to moesin) (Ward et al., 2001). This domain has 30 kDa and responsible for binding membrane protein and lipids. This domain is formed from three lobes (A, B, and C). Lobe A is represented by amino acids (1-90) of FERM domain and includes the binding site for band 3 protein while lobe B is started from amino acids (91-190) and contain the binding site for GPC, XK, and Duffy receptors. The third lobe which is lobe C (amino acids191-280) is known as the C-terminal lobe and contains the binding site for p55 protein (Baines, 2006, Han et al., 2000, Pearson et al., 2000). Moreover, lobe B of 4.1R protein is also binds to Kell blood group protein via XK interaction as XK indeed is covalently linked to the Kell glycoprotein by a single disulphide bond (Azouzi et al., 2015, Russo et al., 1998). These interactions with different membrane protein via this domain functionally regulate the signal pathways of multi-cellular process such as the binding with GPC is essential for stability of the membrane as it is connected to spectrins (Nunomura et al., 2000).

The second functional domain is the 16 kDa domain which has the binding site for Protein kinase A (PKA) and Protein kinase C (PKC) (Baines, 2006). Phosphorylation of 4.1R protein by PKA and PKC regulates the mechanical function of erythrocyte membrane (Manno et al., 2005).

The third functional domain is the 10 kDa domain which binds to myosin, actin, and spectrins. The binding to spectrins has high importance as it maintains the membrane stability (Baines, 2006).

The fourth domain which is the C-terminal domain is highly acidic, and the phosphorylation binding sites are located close to this domain (Yawata, 2006).

1.3.2.9 Rh proteins:

In 1940, Landsteiner and Wiener were identified Rhesus (Rh) blood group antigens using antisera of immunised rabbits and guinea pigs with red cells taken from *Macacus rhesus* monkeys (Avent and Reid, 2000). The Rh family includes three erythroid gene members: *RHD*, *RHCE*, and *RHAG*. The erythroid genes are involved in forming the essential complex of Rh antigen expression and red blood cell membrane integrity. *RHD* and *RHCE* genes are closely linked and share high homology about almost 92%, and are located at the *RH* locus on chromosome 1p34-1p36, whilst the *RHAG* gene is present on chromosome 6p12- 6p21 (Matassi et al., 1999, Cherif-Zahar et al., 1990, Huang, 1997). If RhD antigen is present this is indicated by the term RhD positive and RhD negative if absent. In the Rh blood group system Cc and Ee antigens are also present. However, 85% of Caucasians are RhD positive and 99.5% of Japanese are also RhD positive (Avent and Reid, 2000). The D protein is composed of 416 amino acids and 32 kDa, encoded by the *RHD* gene. The Cc and Ee is also composed of 416 amino acids and 33 kDa, encoded by

RHCE gene. Cytoplasmic N and C termini are present in *RHD* and *RHCE* proteins and both have 12 membrane-spanning domains. RhAG (Rh associated glycoprotein) composed of 409 amino acids and shares only 36% homology with RhD and RhCE polypeptides (Avent et al., 1992).

Rh proteins (RhD, RhCE) are erythroid specific but RhAG and its homologs RhBG and RhCG is widely distributed in many cells and shares many homologues in both the plant and animal kingdoms (Suyama et al., 2000). A prospective role has been identified for RhAG as a carbon dioxide channel in the human red cell membrane (Burton and Anstee, 2008, Endeward et al., 2008).

Previously, the structure of the Rh complex was thought to be a tetrameric configuration contained of two Rh and two RhAG units (Eyers et al., 1994, Ridgwell et al., 1994). The Rh molecular complex is now known to be a trimeric configuration. RhCE and RhD associate as an Rh core complex that involves of two Rh-associated glycoproteins (RhAG) and one RhD/CE protein (Avent et al., 2006). Actually, Rh protein superfamily members share similar homology. The function of Rh proteins are ammonium transporters in erythrocytes (Callebaut et al., 2006, Avent et al., 2006).

The Rh complex interacts with CD47, LW and GPB. These proteins are linked the Rh complex with the membrane and the erythroid skeleton by noncovalent linkages (Mouro-Chanteloup et al., 2003). Moreover, protein 4.2 deficient human red cells have shown lack CD47 which indicates an interaction between the Rh complex and Band 3 complex, via the relation between protein 4.2 and CD47 (Bruce et al., 2002, Oldenborg et al., 2001).

1.3.2.10 CD44:

CD44 is a transmembrane glycoprotein implicated in different cellular functions such as,

T cell activation, the maturation of haemopoietic cells, and extracellular matrix adhesion (Gallatin et al., 1989, Parsons et al., 1994, Bollyky et al., 2009). CD44 structures are constant in different cell types which includes a single polypeptide chain that crosses the membrane once with its amino terminus in the extracellular domain (Parsons et al., 1994). The extracellular domain composed of a proximal O-glycan rich domain and a distal disulphide bond domain (Jackson et al., 1992, Parsons et al., 1994). CD44 induces tethering and rolling on a synthetic substrate in shear conditions by acting as an erythrocyte ligand on binding to hyaluronan (Kerfoot et al., 2008). CD44 binds to the 30 kDa C-terminal domain of protein 4.1R via its C-terminal domain as it has the internal sequences SRRRC and QKKKL, and this interaction reduced by Ca^{2+} and calmodulin (Nunomura et al., 1997). CD44 expresses the Indian antigens (In^a and In^b). There has been reported to cause a novel form of congenital dyserythropoietic anaemia (CDA) if there is a reduce or deficiency of CD44 (Parsons et al., 1994).

1.3.2.11 Adducin:

Adducin is present in erythrocyte membrane at the concentration of 60 000 copies per erythrocyte. There are two heterodimers of adducin in erythrocytes alpha and beta (Joshi et al., 1991). They are both structurally similar, but they encoded by different genes. The alpha adducin gene is *ADDA* and located on chromosome 4q16.3 and composed of 737 amino acids while the beta adducin gene is *ADDB* and located on chromosome 2q13-p14 and composed of 726 amino acids. The molecular weight of alpha adducin is 81 kDa but on SDS-PAGE is 103 kDa due to glycosylation while beta adducin is 80 kDa and also on SDS-PAGE it is 97 kDa (Katagiri et al., 1996, Joshi et al., 1991, Hughes and Bennett, 1995). There are three main domains on adducin, the first is the N-terminal domain and has 39 kDa, the second is a 9 kDa domain of the connecting neck, the last one is the C-terminal domain which is a protease-sensitive domain (Hughes and Bennett, 1995, Joshi

et al., 1991). The C-terminal domain is mainly hydrophilic and has 22 amino acid segments that regulates Ca^{2+} / calmodulin capping and bundle of actin filaments (Li et al., 1998). Actually, the function of adducin in erythrocyte is a membrane stabilizer protein as it connects the spectrin to actin to increase the membrane stability. However, the adducin does not bind to spectrin directly without the presence of actin as the tails of adducin bind to the actin binding domain at the N-terminal of spectrin (Li and Bennett, 1996). Most recently, adducin has been reported to bind band 3 protein in erythrocyte. Adducin binds to residues 246-264 of the C-terminal domain of band 3 protein and this interaction prevent ankyrin, which is a part of band 3 complex, from binding to band 3 protein. the outcome of this interaction suggested that this interaction affects the membrane integrity (Franco et al., 2016).

1.3.2.12 CD47:

CD47 is a transmembrane receptor with 5 membrane-spanning segments and also is known as Integrin-associated protein (IAP) (figure 1.10). The history behind the discovery of CD47 is interesting, as CD47 was discovered as an erythroid membrane protein during testing investigation of the Rh molecules, when found CD47 were reduced in Rh_{null} cells (Avent et al., 1988). Five years later, they found that the complete cDNA sequence of IAP was reported, a five membrane domain spanning molecule that copurified with the integrin $\alpha\text{v}\beta 3$ from leucocytes and placenta and with $\beta 3$ integrin from platelets. CD47 was shown to be identical to ovarian tumour marker (OA3). It was elucidated that it has the same structure and sequence of CD47 (Lindberg et al., 1993, Brown et al., 1990, Lindberg et al., 1994, Mawby et al., 1994, Campbell et al., 1992).

CD47 is 47-52 kDa on SDS-PAGE and it is a member of the immunoglobulin (Ig) superfamily of membrane proteins. Moreover, it has glycosylated extracellular

immunoglobulin variable domain (IgV) at its N-terminal domain, and multi spanning domain that cross the membrane five times and a C-terminal domain (Brown and Frazier, 2001, Reinhold et al., 1997, Reinhold et al., 1999).

CD47 gene is located on chromosome 3q13.1-q13.2 (Lindberg et al., 1994). Alternatively spliced mRNA isoforms were found to encode different C-terminal domains found in erythrocytes. Historically, four different isoforms were discovered that have different amino acids sequences on the C-terminal domain of CD47. These differences on the amino acids sequence leads to produce four different CD47 protein isoforms. Each longer tail came from the addition of a short peptide sequence encoded by a short exon, each joining a common 3' exon (Reinhold et al., 1995).

However, in 2002 a fifth isoform was discovered on skeletal muscle and named isoform 5. This isoform has an extra exon named as exon EN and it contains 47 base pair (bp) between exon 7 and exon F2 and the cDNA of this isoform was shown a differ from the already known isoform 2 due to the addition of EN exon (Schickel et al., 2002). Furthermore, the corrected sequence of isoform 5 sequence was updated by Avent's group in 2007 (Plummer, 2007) (more details in chapter 3).

CD47 is expressed widely in all human tissues as it described as ubiquitous glycoprotein. Starting with haemopoietic cells in peripheral blood, megakaryocytes in fetal liver and sinusoidal cells, fatal hepatocytes, hepatocytes and portal tracts in adult liver (Avent et al., 1988). Moreover, CD47 is also distributed on mesenchyme and epithelia at multiple sites on various human tissues which includes placenta, tongue, bile duct, epidermis, gall bladder, pancreas, brain cortex, stomach, kidney, liver, spleen, skeletal muscle, and renal cortex (Mawby et al., 1994, Schickel et al., 2002).

In erythrocytes, the immune globulin superfamily (IgSF) domain within CD47 drives its interactions with its receptors and ligands. There are different ligands and receptors which interact with CD47 such as: $\alpha v \beta 3$, $\alpha 2 \beta 1$ integrins, thrombospondin (TSP-1) and signal-

regulatory protein alpha (SIRP α) (Brown, 2001).

As previously mentioned, the life of a red blood cell starts after it is released from the bone marrow (BM) and ends when it is engulfed by splenic macrophages (120 days). Factors that lead to red cell engulfment mechanism are still poorly understood. Oldenborg et al. in 2000 hypothesized that CD47 interacts with SIRP α in the macrophage and induces an inhibitory signal “do not eat me signal” that leads to recognition that the cell is a self-cell (Oldenborg et al., 2000). Thus, CD47 regulates cell-cell interaction by binding to SIRP α on the macrophage. CD47 reduced cells were found to be removed from the circulation (Oldenborg et al., 2000). This research showed that in mice, CD47 antigens on erythrocytes are recognized by the SIRP alpha receptors thus inhibiting a phosphorylation cascade and subsequently inhibiting macrophage activation. Erythrocytes from CD47 null mice were found to be rapidly cleared from the bloodstream of wild type mice whilst the presence of CD47 on normal murine erythrocytes, when transfused into null recipients, prevented their elimination. Hence, it is suggested by Oldenborg et al that macrophages use the presence of the CD47 antigen to determine whether an erythrocyte is self or foreign.

Recently, the elimination process of foreign cells that lack CD47 are also include the involvement of dendritic cell (DC) by activation of CD11c, which is known as integrin alpha x. For instant, if foreign cells that lack CD47 fail to interact with SIRP alpha could induce rapid capture by splenic DCs (Wu et al., 2018). These finding were found in Vevo by identifying the elimination process which include the activation of CD11c and its regulator Talin 1 in splenic DC (Wu et al., 2018).

CD47 has a peptide (KRFYVVMWKK) residue which acts as a receptor for thrombospondin 1 (TSP-1) and other thrombospondin members (Brown and Frazier, 2001). The C-terminal binding domain of TSP-1 interacts with CD47 VVM residues. This interaction leads to different cellular pathways activation such as platelet activation, adhesion, migration, and phagocytosis. CD47 acts as TSP-1 receptor on platelets (Chung et al., 1997).

Recently, CD47 was found to be over-expressed in erythroid cancer cells (Uchendu et al., 2018, Jaiswal et al., 2009). The observation of increased CD47 levels in cancer cells were first studied in 2009 by Jaiswal et al. The results were achieved when they investigated CD47 expression first on normal mice against leukemic mice and they found CD47 was increased, so they extended these experiments on normal and dysplastic human hematopoietic cells by flow cytometry. The conclusions of this research is that CD47 is upregulated in human cancer cells which might be a future solution to eliminate cancer cells (Jaiswal et al., 2009). However, anti-CD47 was used in a combination with anti-cancer treatment to enhance the elimination of cancer cells (Chao et al., 2010). The anti-CD47 were used in that experiments (BRIC126 and B612.2) and the anti-cancer drug was rituximab. This combination was tested *in vitro* by using non-Hodgkin lymphoma cell lines. They found that there was a synergistic effect when using these two drugs together. Furthermore, they tested that combination again in mice and they have not found the same synergistic pattern as *in-vitro* which is due to increased toxicity level from the elimination target of this combination as anti-CD47 enhance the macrophage while the chemotherapy enhance natural killers (NK) (Chao et al., 2010). Five years later, the first preclinical development of the combination were achieved when Liu et al, humanised anti-CD47 (5F9) (Liu et al., 2015). The humanized version of this antibody (5F9) called Hu5F9-G4 were tested on human (AML) HL-60 cell lines. They performed toxicokinetic studies in non-human primates (Monkeys) which conclude that Hu5F9-G4 could be safely

administrate intravenously at doses able to achieve therapeutic levels (Liu et al., 2015). Most recently, the clinical trials on humans have shown that using anti-CD47 (Hu5F9-G4) as a therapeutic agent for cancers have an adverse effect by causing Anaemia (Advani et al., 2018, Chen et al., 2018). Hence, the core of this thesis is to studied different mAbs against CD47 to reduce the adverse effect of causing Anaemia (Chapter 4)

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Figure 1.10: The topology of CD47 Structure.

CD47 *gene* located at chromosome 3 q13.1-q13.2. After the discovery of alternative splicing, CD47 has 5 different isoforms at C-terminal. CD47 is a member of the immunoglobulin (Ig) superfamily of membrane proteins. (Plummer, 2007; Brown & Frazier, 2001).

1.4 Apoptosis:

Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). This process is important in early the development of tissues, during morphogenesis and in mature organisms to remove senescent cells to allow regular turnover.

Apoptotic cells undergo plasma membrane changes that help trigger the macrophage response such as translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Cells exposing PS at the cell surface are recognised by macrophages which are equipped with specific PS receptors (Lang et al., 2006a, Lang et al., 2005a). The translocation of PS is a calcium dependant process and is carried out by an phospholipid scramblase (Delaunay, 2007). This phosphatidylserine exposure can be exploited and can be measured using flow cytometry methods with the use of a fluorescent marker (Annexin V-FITC) which is used in this thesis (Chapter 4). Annexin V-FITC can be used to label the phosphatidylserine and therefore the amount of apoptosis can be measured in response to a specific death signal.

There are two ways to mediate apoptosis which is by intrinsic or extrinsic pathways. Intrinsic pathways often include pathways activated by environmental damage, whereas extrinsic pathways are mediated through specific death receptors (CD95) (Lang et al., 2006b, Lang et al., 2006a).

1.5 Eryptosis:

A term eryptosis is a description of erythrocytes cascade process that will lead to death of red cell and removal of this cell from the circulation. As erythrocytes are don't have a mitochondria and nuclei, this process is slightly different than apoptosis which happens in other cell types. The term eryptosis was first coined, by Florian Lang in University of Zurich and it is defined as red blood cell death as apoptosis in other biological human cells (Lang et al., 2005b).

Eryptosis shares some properties with apoptosis, which takes place in all other cell types, like phosphatidylserine exposure, membrane blebbing and cell shrinkage. There are many factors which can induce eryptosis such as: hyperthermia, oxidative stress, and increase of cytosolic Ca^{+2} activity. On other hand, other factors like, adenosine, amitriptyline, blebbistatin, and caffeine could inhibit eryptosis by stimulate erythropoietin release which leads to Ca^{+2} efflux (Lang et al., 2006a, Lang et al., 2012).

Within Avent's group, there were two studies described the eryptosis in erythrocytes. The first was found that (BRIC 10) which is a specific antibody against glycophorin C induces eryptosis in erythrocytes. The second one was shown that ligation of CD47 with anti-CD47 antibodies (BRIC 126) will also lead to PS switching from the inner leaflet of the membrane to the outer leaflet (Head et al., 2005b, Head et al., 2005a). Moreover, the finding of these studies suggested the contribution of CD47 in erythrocyte signal pathway that results in PS exposure in erythrocytes. A further study within the same group showed that ligation of CD47 with anti-CD47 antibody with or without presence of 4.1R protein in rare blood samples will lead to different results (Jeremy et al., 2009). The PS exposure level was shown higher level in 4.1R deficient cells compared to normal. These finding suggested CD47 is interacts with protein 4.1R in signal pathway. Finally, the red cell eryptosis protocol were used in this thesis to differentiate between seven different CD47 monoclonal antibodies (chapter 4).

Table 1.1: The similarities and differences between Apoptosis and Eryptosis.

Apoptosis	Eryptosis
PS exposure observed	PS exposure observed
Membrane Blebbing (Zeiosis)	Membrane Blebbing (Zeiosis)
Calpains involved	Unknown if calpains involved
Caspase independent and dependant pathways	Caspase independent and dependant pathways
Extrinsic (death receptor) and Intrinsic (mitochondrial mediated) pathways exist.	No mitochondria
DNA degradation and DNA repair enzyme degradation (esp. PARP) key feature of apoptosis pathway	No Nucleus
	Still poorly understood

Table 1.1: The similarities and differences between Apoptosis and Eryptosis. Taken from (Jeremy, 2012).

1.6 Aims:

1. Evaluate the expression of CD47 isoforms using Next Generation Sequencing:
 - 1.1. Confirm the presence of CD47 isoform 5 in erythrocytes
 - 1.2. Compare the presence of CD47 isoforms in different erythrocyte phenotypes.
2. Characterization of different CD47 monoclonal antibodies that could be used as an anti-cancer drug:
 - 2.1. Determine whether all CD47 mAbs bind to to the same epitope.
 - 2.2. What are the outcomes of combine two CD47 mAbs in erythrocytes?
3. Elucidate the role of CD47 in erythrocytes by studying protein-protein interactions using Mass Spectrometry:
 - 3.1. Comparing between different CD47 N-terminal mAbs IP.
 - 3.2. Comparing between different CD47 C-terminal mAbs IP.

2 Materials & Methods:

2.1 Materials:

2.1.1 Erythrocyte samples:

Ethylenediaminetetraacetate acid (EDTA) tubes containing at least 5 ml of whole blood, or blood donor bags containing 450 ml of blood for IP experiments were obtained from random blood donors. All samples were with complete awareness of ethical donor consent form from National Health Service Blood and Transplant (NHSBT), Filton Bristol, UK. For each sample, serology information was provided. The blood was spun at 1500 rpm for 10 minutes, and the plasma was removed, and packed red blood cells were added to 1.5 mL Eppendorf tubes except for DNA and RNA extractions, the blood was kept in EDTA tube after centrifugation to extract DNA/RNA from the buffy coat.

2.1.1 Primary Antibodies:

Purified Anti-CD47 monoclonal antibodies (mAbs) (BRIC 32, BRIC 122, BRIC 124, BRIC 125, BRIC 126, BRIC 168, and BRIC 211), anti-GPC mAbs (BRIC-10 and IBGRL-100), anti-CD44 mAb (BRIC 235), anti-Band 3 mAbs (BRIC 170 and BRIC 6), anti-Kell mAb (BRIC 18) and anti-GPA mAb (BRIC 256) were obtained from the International Blood Group Reference Laboratory (IBGRL), National Blood Service, Bristol, U.K. (Table 2.1).

2.1.2 Custom Primary Antibodies:

Purified and tissue culture supernatant anti-isoforms 2, 4, and 5 of CD47 antibody was customised and ordered from Dundee Cell Product, Dundee, UK. For full details see chapter 3 section (3.4)

Table 2.1: Purified Monoclonal Antibodies from International Blood Group Reference Laboratory (IBGRL).

Monoclonal Antibody	IgG subclass	Target Protein
BRIC 32	IgG1	CD47
BRIC 122	IgG1	CD47
BRIC 124	IgG2a	CD47
BRIC 125	IgG2b	CD47
BRIC 126	IgG2b	CD47
BRIC 168	IgG2a	CD47
BRIC 211	IgG1	CD47
BRIC 10	IgG1	GPC
IBGRL 100	IgG1	GPC
BRIC 6	IgG3	Band 3
BRIC 170	IgG1	Band 3
BRIC 235	IgG2b	CD44
BRIC 256	IgG1	GPA
BRIC 18	IgG2a	Kell

There are three different IgG subclasses among the seven CD47 monoclonal antibodies used in this study (Table 2.1). For monoclonal antibodies (BRIC 32, BRIC 122, and BRIC 211), BRIC 256 which is specific for Glycophorin A (GPA) were used. For monoclonal antibodies (BRIC 124, and BRIC 168), BRIC 18 which is specific for Kell blood group glycoprotein. For monoclonal antibodies (BRIC 125, BRIC126), BRIC 235 which is specific for CD44 protein. BRIC 10 which is anti- Glycophorin C protein were used as a positive controls.

2.1.3 Secondary antibody:

Horseradish peroxidase-conjugated rabbit anti-mouse IgG-HRP secondary antibody (1:3000) was purchased from DAKO, Cheshire, U.K.

2.1.4 Pierce™ ECL Plus Western Blotting Substrate:

An enhanced chemiluminescent substrate for detection of horseradish peroxidase (HRP) activity from antibodies and other Western blot probes was purchased from Thermo Fisher, Paisley, U.K.

2.1.5 DNA and RNA Extraction kits:

DNA (QIAamp DNA blood mini kit) and RNA (QIAamp RNA blood mini kit) extraction kits were purchased from Qiagen, Manchester, UK. The protocol suggested by the manufacturer was done with details in the method sections (2.2.13.1 and 2.2.13.2).

2.1.6 Cell Lines RNA:

K562 Cell line RNA and H1-60 cell line RNA were purchased from the American Type Culture Collection (ATCC), Guernsey, UK.

2.1.7 Next Generation Sequencing Kit:

Next-generation sequencing kit included: End Repair Buffer, End Repair Enzyme, Ligase Buffer, DNA Ligase, Adapters, Platinum® PCR SuperMix High Fidelity, Library Amplification Primer Mix, Nick Repair Polymerase, dNTP Mix, Low TE and was purchased from Thermo, Paisley, UK.

2.1.8 The Agilent® 2100 Bioanalyzer Kit:

The kit was purchased from life technology, Paisley, UK. The protocol was followed according to the manufacturer's recommendations with details in the method section.

2.1.9 Flow Cytometry Reagents:

Flow Cytometry reagents include Hepes buffer from Sigma, UK, Annexing V FITC and binding buffer (10 x) from NEB, UK, Alexa Flour 488 from life technology, Paisley, UK.

2.1.10 Mass Spectrometry Liquid Chromatography Mass Spectrometry (LC-MS/MS) Orbitrap Reagents:

Most of mass spectrometry reagents are made in house. Buffer A which contains (0.5% Acetic Acid (v/v), 1% Trifluoroacetic acid (TFA) (v/v)), Buffer B which contains (80% Acetonitrile (v/v), 0.5% Acetic Acid (v/v), 1% TFA (v/v)), 50 mM Ammonium bicarbonate (ABC), 10 mM dithiothreitol (reduction buffer), 50 mM 2-chloroacetamide in 50 mM ABC, 2% (v/v) TFA, 12.5 ng/μl trypsin in 50 mM ABC.

2.2 Methods:

2.2.1 Erythrocyte ghost membrane preparation (Membrane Protein Isolation):

Red blood samples were centrifuged at 1500g for 10 minutes to get rid of plasma and the buffy coat. After that, the samples washed three times at least with 0.15 M NaCl solution. Monobasic (NaH_2PO_4) and dibasic solutions (Na_2HPO_4) were mixed to have the stock solution of Phosphate buffer (0.1M Na_2PO_4 pH8.0). The samples must be washed at least three times with the stock solution or until the supernatant is clear. Moreover, the pellet must be incubated at 4°C for at least 10 minutes after the samples have been washed three times with the stock solution to prevent the membranes from resealing again without complete washing. Samples were transferred to centrifuge tubes. Samples were mixed thoroughly and incubated for 5 minutes at 4°C with 5M Na_2PO_4 . Incubated samples were centrifuged at 16000g at 4°C for 5 minutes, and the supernatant was removed. This procedure was repeated until the membranes were creamy pink. Membranes were transferred to 1ml Eppendorf tubes. 1ml of 5mM phosphate buffer pH 8.0 was added, and

membranes were centrifuged at 21000g for 10 minutes. Supernatant should be clear and removed while the ghosts are still in the tube, and 100µl 5mM phosphate was added along with 10µl 2mM phenylmethanesulphonyl fluoride (Sigma, Dorset, UK) to prevent proteolysis of samples during long-term storage. Samples were labelled and stored at -80°C until required.

2.2.1.1 N-Terminal Co-immunoprecipitation:

Red blood samples were centrifuged at 1500g for 10 minutes to get rid of plasma and the buffy coat. After that, the samples were washed three times with 0.15 M NaCl solution. Then, cells were washed once with 1x PBS solution. After that, cells were incubated with 100 µl of Purified 1mg/ml N-terminal binding antibody such as (BRIC 32, BRIC 122, BRIC 124, BRIC 125, BRIC 126, BRIC 168, BRIC 211) for an hour at 37°C. After that, cells were washed again with 1x PBS to remove any excess unbound antibody. Similar to section 2.2.1 in washes till creamy ghost founded. Ghost then washed once with PBS containing 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0 at 18000 g for 15 minutes to solubilise the membrane protein. Then, the supernatant containing the proteins was transferred to 15 ml Falcon tubes containing 100 µl Protein G sepharose (from Abcam, West Sussex, UK). Cells were agitated for 30 minutes at room temperature. After that, the supernatant was centrifuged for 10 minutes at 2000g. Remove supernatant and washed again with 10 ml PBS contains 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0. Finally, remove the supernatant and resuspend the pellet in 100 µl PBS contains 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0. Samples were labelled and stored at -80°C until required.

2.2.1.2 C-Terminal Co-immunoprecipitation:

Started steps similar to the steps in section 2.2.1 for making ghosts until the step of the creamy pink ghostformation. Then, ghost membranes are incubated with 10 mg/ml tissue culture supernatant C-terminal antibody such as (CD47 isoform 2, isoform 4, and isoform 5) overnight at 4°C in cold room. Then, membrane proteins were washed three times with lysis buffer (5M Na₂PO₄ pH 8.0) to remove any excess unbound antibody. After that, ghosts were then washed once with PBS contains 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0 at 18000 g for 15 minutes to solubilise the membrane protein. Then, the supernatant was transferred to 15 ml Falcon tubes and 100 µl Protein G sepharose (Abcam, UK) was already added in the tubes. Cells were agitated for 30 minutes at room temperature. After that, the supernatant was centrifuged for 10 minutes at 2000g. Supernatant was removed and the sample was washed again at least twice with 10 ml PBS contains 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0. Finally, the supernatant was removed and resuspend the pellet in 100 µl PBS contains 2% (w/v) Triton X-100, 200mM PMSF, and 0.1 M EDTA pH 8.0. Samples were labelled and stored at -80°C until required.

2.2.2 BCA (Bicinchoninic acid) Protein quantification Assay:

BCA (Bicinchoninic acid) Protein Assay Reagent kit was purchased from Thermo Fisher, Paisley, UK. According to the manufacturer's instructions, Bovine Albumin Standard Ampule, was diluted into nine different dilutions by phosphate buffer to make a set of protein standards for a standard calibration curve. BCA reagent A and BCA reagent B were mixed at a ratio of 50:1 in 15ml falcon tubes to create a working reagent. Working reagent was added to samples to determine protein concentration (8:1). 25µl of a sample and 200µl of working reagent was mixed and incubated at 60°C for 30 minutes in a heat

block, along with the BSA standards. After cooling to room temperature, samples and standards were analysed at 562nm in a spectrophotometer from Becton Dickinson (BD), (Plymouth, UK). All samples were analysed within 10 minutes as the BCA reaction has no actual end point. Moreover, the results from BCA assay have been confirmed by using Qubit® for protein quantification.

2.2.3 Qubit® Protein quantitation assay:

Qubit® protein assay kit for protein quantification was purchased from Thermo Fisher Scientific, Paisley, UK. First, the Qubit reagent and Qubit working solution were mixed a ratio of 1:200. According to manufacturer's instructions, the recommended ratio for each standard and each sample is 1:200. After that, the standard and the samples were incubated at room temperature for 15 minutes before being measured by using Qubit® Fluorometer 2.0.

2.2.4 Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE):

For each 375µl of erythrocyte ghost membranes, 125µl of 4x loading sample buffer from BioRAD, Hertfordshire, U.K. was added. In experiments where samples need to be run under reducing conditions, 5µl of reducing agent was added BioRAD, Hertfordshire, U.K. Before loading samples onto gels, samples were heated in a hot block at 80°C for 10 minutes. BlueEye Marker from GeneFlow, Staffordshire, UK and Precision Plus Protein (Western C) from Bio Rad, Hertfordshire, UK were used to determine the molecular protein weight of any target proteins during further analysis.

1x of 3-N-morpholino propanesulphonic acid (MOPS) running buffer was prepared from concentrate. Pre-cast gel (XT Bis-Tris 4-12%, 12+2 well, 45µl) were purchased from BioRad, Hertfordshire, UK. Gels were loaded with 15µg of protein. Criterion Cell from BioRad, Hertfordshire, UK. was used. Gels were run at 120V max for 2 hours or until the dye went to the lowest point of the gels. Gels were either stained (2.2.6) or immunoblotted (Western blotted) (2.2.5) (Figure 2.1).

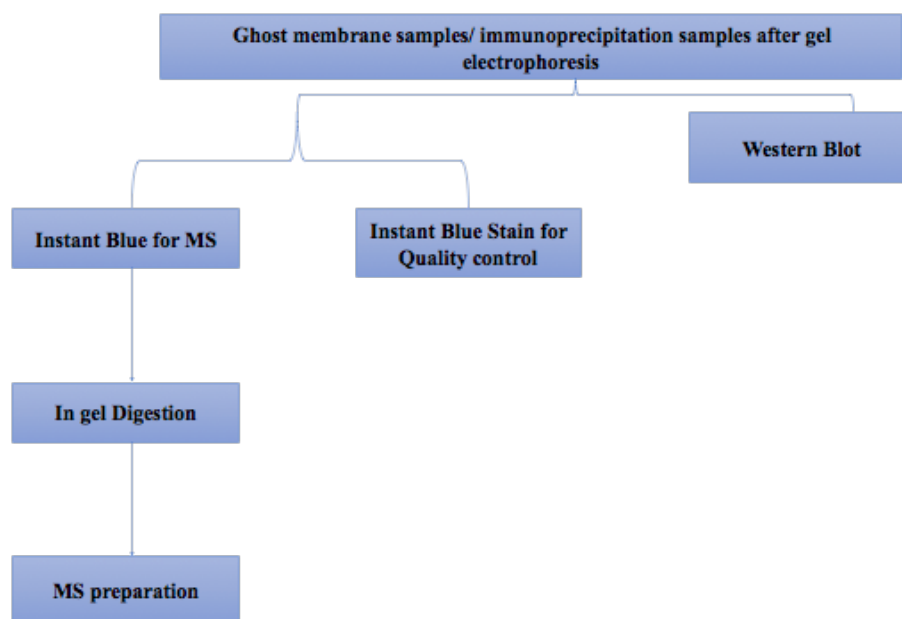


Figure 2.1: Sample process after gel electrophoresis.

2.2.5 Western blotting:

Samples were transferred onto an active PVDF membrane after electrophoresis. PVDF was activated by immersing the membrane in Methanol for 20 secs followed by 2 min in dH₂O then in cold transfer solution (190 mM Glycine, 25mM Tris base, and 20% (v/v) Methanol). After activating the membrane, wet transfer blotting was used in Bio-Rad system for less than an hour with a current of 100 mA. Moreover, after transfer proteins to PVDF membrane, blocking solution (PBS-T, plus 5% w/v bovine milk (dried, and

skimmed)) was added to the membrane for an hour rocking at room temperature. Then, PVDF membrane was incubated in cold room overnight with (1:2500) for purified antibodies, and (1:10) for tissue culture supernatant Primary antibody in blocking buffer. Followed by three washes for 10 minutes each at room temperature with PBS-T and then, the secondary antibody was added to the membrane from Bio-Rad (1:3000) in PBS-T and incubated for an hour at room temperature. Finally, PVDF membrane washed with PBS-T and then one wash with ECL pulse (Western blotting substrate) from Thermo, Paisley, UK. to enhance the membrane for chemiluminescent visualization.

Briefly, the ECL kit contains two reagents called reagent 1 and reagent 2. The first step was to mix both reagents at the concentration ration (4:1) between both reagents. Thus 1 ml of reagent 2 was added to 4 ml of reagent 1.5 ml of ECL was added to PVDF membrane and rocked for 5 min before imaging the PVDF membrane.

2.2.6 Instant Blue Staining:

To check the quality control of the gel electrophoresis, the gels were immersed overnight at room temperature in Instant Blue stain from Expedeon, Cambridgeshire, UK. Then, the gel was rinsed with PBS before imaging (photograph) the gel or directly scanned using an office scanner.

2.2.7 In Gel-Digestion preparation for Mass Spectrometry (MS):

After staining the gel with instant blue stain, using a blade, the desired gel area which is needed to run on MS had extracted and transferred to 1.5 ml Eppendorf. Equilibration and destaining by adding 300µl of 50 mM ABC and incubated for five mins at room temperature, then all liquid was discarded. After that, 300µl of 25% ACN and 75% ABC (v/v) were added and incubated for 5 mins; then all liquid was discarded. To finish

equilibration and destaining 300µl of 100% ACN were added and incubated for 5 min at room temperature. Meanwhile, all the previous steps were repeated two times to make sure equilibration and destaining reach completion. Gel pieces turn slightly white and this indicates that they were ready for the next step, which was the reduction step. This was done by adding 300µl of reduction buffer and samples were incubated for 20 mins at 56°C followed by the gel shrinking step with addition of 300µl of ACN, then alkylation step with addition of 300µl of alkylation buffer and incubated for 20 min in the dark at room temperature. Then, Equilibration was needed to be done now to get rid of extra alkylation buffer. 300µl of 100% ACN was added and incubated for 5 min at room temperature; then all liquid was discarded. After that 300µl of 50 mM of ABC was added and samples were incubated for 5 min at room temperature, then all liquid was discarded. Moreover, 300µl of 100% ACN was added and samples were incubated for 5 min at room temperature. To make sure the equilibration was done correctly all the steps were repeated a second time. After the equilibration step, the gel pieces were then ready for the trypsin digestion step, which is done by adding 150µl of trypsin (12.5ng/ml) and the samples are incubated for 20 min at room temperature, followed by the addition of 300µl of ABC and an overnight incubation at 37°C. The next day, extraction of the peptides took place by adding 200µl of TFA and incubating the samples for 20 min at room temperature on the shaker at 800 g (Fisher, Paisley, UK). After that, all supernatant transferred to a new Eppendorf. Then, repeat the previous step but using buffer B instead of TFA. Meanwhile, all supernatant was placed on evaporator (Thermo, Paisley, UK) for 3 hours at 30°C. Finally, now the samples were ready for peptide clean up procedure using Stage-tips and were stored at 4°C.

2.2.8 Peptide Clean-Up Using Stage-Tips:

The purpose of peptide clean-up is to purify the peptide solutions before MS analysis.

This procedure is performed using home-made stage tips (Stop and go extraction tips).

The procedure starts by adding 50µl of TFA to the samples. After that, high-performance extraction disk (C18) should be prepared. First, label the tips with sample number or label then pick a piece of the disk (C18) using a picking tool and transfer the piece of the disk (C18) to the pipette tip and push it until fixed. Meanwhile, the tips with (C18) disk should be applied to 2 ml Eppendorf, and 50µl of methanol was added to the stage tips and centrifuged for 10 min at 1500g, then 50µl of buffer B is added to the stage tip and centrifuged for 10 min at 1500g, all liquid passed the filter should be disposed. After that, 50µl of buffer A was added to the stage-tip and centrifuged for 10 min at 1500g, then repeat the last step which is 50µl of buffer A is added and centrifuged and discard all liquid. Now the stage tip is ready to hold the purified peptide. However, sample now is added to the stage tip and centrifuged at 1500 g for 10 min. After that, take just the tip and place it to a new 1.5 Eppendorf. Elute the peptide by adding 25µl buffer B and centrifuged for ten mins at 1500 g then, repeat the previous step. Meanwhile, place the Eppendorf in the evaporator for 30 mins at 30°C. Finally, 35µl of buffer A to the Eppendorf and transfer it into glass vials to run them on MS.

2.2.9 Liquid Chromatography-Mass Spectrometry (LCMS):

The LCMS was conducted by Dr Vikram Sharma who runs the Proteomics Facility of the Systems Biology Centre at University of Plymouth. Briefly, on a Dionex Ultimate

3000 RSLC nanoflow system (Dionex, Camberley UK), peptides were divided. Then, using bypassing the analytical column concept and with a flow rate 5 $\mu\text{l}/\text{min}$, a 3 μl of sample was added in 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile (2% acetonitrile in 0.1% TFA) onto an Acclaim Pep Map100 $\mu\text{m} \times 2 \text{ cm}$, 3 μm C18 nano trap column. Next, with a linear gradient of 96% buffer A and 4% buffer B to 60% buffer A and 40% buffer B, (Buffer A: 0.5% Acetic Acid, Buffer B: 80% acetonitrile in 0.5% acetic acid) at a constant flow rate of 300nl/min over 120 minutes, elution of bound peptides was performed with the trap column in line with an Acclaim PepMap C18 nanocolumn 75 $\mu\text{m} \times 25 \text{ cm}$, 3 μm , 100 \AA (Analytical Column). After that, using a Proxeon nanospray ESI source (Thermo Fisher Hemel UK) and analysed in an Orbitrap Velos Pro FTMS (Thermo Finnigan, Bremen, Germany), the sample was ionised in positive ion mode. Then, the Orbitrap Velos Pro instrument underXcalibur2.1 software was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition and MS spectra of intact peptides (m/z 350-1600) with an automated gain control accumulation target value of 1000000 ions were gained with a resolution of 60000. Moreover, in the linear ion trap by collision-induced dissociation (CID) at a target value of 10,000 or maximum ion time of 200 ms, the ten most intense ions were sequentially isolated and fragmented. A dynamic exclusion of ions previously sequenced within 45 angle degree was applied. However, all the singly charged and unassigned charge state ions were omitted from sequencing. Finally, the gold standard mass spectrometric conditions were spray voltage, 2.3 kV; heated capillary temperature, no sheath and auxiliary gas flow; 275°C; normalised CID collision energy 30% for MS2 in LTQ. The ion selection threshold was 10000 counts for MS2 and an activation time of 30 ms and an activation $q = 0.25$ were used.

2.2.10 Data Analysis by MaxQuant for mass spectrometry results:

Raw data were obtained from LCMS, then by using Andromeda via automated database searching of all tandem mass spectra against a curated target/decoy database (forward and reversed version of the Human protein sequence database (<http://www.uniprot.org/>, UniProt. Release May 2017) containing all Human protein entries from Swiss-Prot and TrEMBL, Peptides and proteins were recognized and identified. Spectra were initially searched with a mass tolerance of 6 ppm in MS mode and 0.5 Da in MS/MS mode and strict trypsin specificity and allowing up to 2 missed cleavage sites. N-acetyl protein deamidated NQ, and oxidized methionine was investigated as variable modification, whereas Cysteine carbamidomethylation was investigated as a fixed modification. Moreover, inbuilt MaxQuant software modules for further processing, label-free quantitation (LFQ) and a maximum false discovery rate of 1% was fixed for the result output files were used to analyse the results which are on Andromeda peak list-output files. To carry on data analysis, data from all samples were transferred to excel sheet. In excel software, the mean LFQ value for each protein in each sample was calculated. Then, the negative control sample for the mass spectrometry work was the sample only contain G-spharose beads, and the protein hits for these samples of the negative control were subtracted from the protein hits for all the other samples of interests. Then, we have a true reflection of the protein hits that relevant for each protein in each IP. The final list of proteins per sample was showed in pie charts as the results show in chapter 5. For the ratio calculation, I have divided the hits of the proteins of the interests in the IP samples against the control (ghost sample). If the results were more than two that means this protein is upregulated in my IP sample and if the results were less than 0.5 that means this protein is down regulated in my IP sample.

2.2.11 Annexin V-FITC binding assay:

Briefly, fresh packed red blood cells were washed at least three times with Hanks buffer (Sigma, Gillingham, U.K.). Then, cells were counted using a haemocytometer. Then, an appropriate number of red cell 6×10^6 per 200 μ l was transferred to 96 well plates. Cells were incubated with 10 μ g/ml of each mAb, or control mAb, for an hour at 37°C (in case of competitive binding assay, please see section 4.3.2). After the incubation, cells were washed three times with 500 μ l Hanks buffer. Then cells were washed once with 500 μ l of 1x binding buffer (BD, Plymouth, U.K.). Supernatants were removed, and the cells re-suspended in 100 μ l binding buffer. 3 μ l of Annexin V-FITC (BD, Plymouth, U.K) was added to the cells. Samples were incubated for 15 minutes in the dark at room temperature. After incubation, samples were transferred into FACS tubes and analysis completed by FACS ARIA II from (BD, Plymouth, U.K). The events recorded for each sample was 10 000 events.

2.2.12 Annexin V-FITC combination antibodies binding assay:

Erythrocytes were ligated with CD47 monoclonal antibodies for (1-3 hours) and control isotype as shown in table 4.2. BRIC 10 which is specific for Glycophorin C, used as a positive control for the experiment because it is ligated to GPC resulting high PS exposure (Head et al., 2005).

However, the combination experiments were carried out as 5 μ l of purified 1 mg/ml of the first purified monoclonal antibody (X) were added first to the red cells then after 30 minutes of incubation at 37 °C another 5 μ l of purified 1 mg/ml of the purified monoclonal antibody (X) again added to the reaction to used it as a control for the combination experiment. The concentration used for these antibodies was 1mg/ml which is a purified

antibody to make sure we have targeted every CD47 present in the samples for each erythrocyte. The concentration of CD47 in normal erythrocytes around 45 000 per cell and in every samples the concentration of erythrocytes are $1 \times 10^6/200\mu\text{l}$. In the other samples; $5\mu\text{l}$ of the purified 1 mg/ml of the first purified monoclonal antibody (X) were added first to the blood donor samples then after 30 minutes of incubation at 37°C another $5\mu\text{l}$ of purified 1 mg/ml of the purified monoclonal antibody (Y) added to the reaction. For example, starting with BRIC 32 as an X antibody added to the blood samples and after 30 minutes incubation, then in the first tube antibody X were added again to be used it as a control (to make sure we completely saturated all epitopes) or the other monoclonal antibodies Y were added on the following tubes. For instance, Y antibodies in this case includes BRICs (122,124,125,126,168, and 211) and also the controls BRICs (18, 235, and 256). Finally, the results were shown that there is no change in the results if the orders of the antibodies were changed.

2.2.13 Statistical Analysis Software for Flow cytometry results:

A website GraphPad Software calculated statistic (<http://www.graphpad.com>) were used to calculate *p*-Value for flow cytometry results.

2.2.14 Genomic DNA and RNA extraction from whole blood donor samples:

DNA and RNA extraction kits from whole blood were purchased from Qiagen, Manchester, U.K. The RNA extraction has been done according to manufacturer's instruction, which is depending on column separation method.

The whole sample of blood needs to centrifuge at 800 g for ten mins to have three different layers as shown in (figure 2.2). After that, RNA was taken from the

reticulocytes (nucleated red blood cells), so from the first ml under the packed red cells while DNA was taken from buffy coat (containing white blood cells).

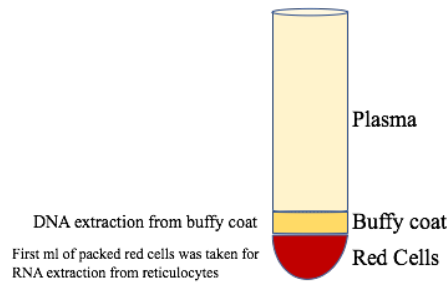


Figure 2.2: Separation of blood components in a 15 ml falcon tube.

2.2.14.1 DNA Extraction:

DNA was extracted using a QIAamp DNA Blood Mini Kit (QIAGEN, Manchester, UK), which is based on a spin column procedure extracting and purifying DNA from the buffy coat. The kit contained the following: buffers (lysis buffer AL and washing buffers AW1 and AW2 with 96-100% ethanol added) and lyophilised QIAGEN Protease, dissolved in 1.2ml protease solvent (preservative). The buffy coat (containing concentrated nucleated leukocytes) was transferred in 1.5ml tubes and centrifuged at 6000 g to purify the buffy coat from the extra serum and red cells, following which 200µl of the buffy coat was transferred into a new 1.5ml tube. The DNA extraction procedure followed: 20µl QIAGEN Protease was mixed with 200µl buffy coat and equivalent volume amount (200µl) of buffer AL by vortexing for 15 seconds until a homogeneous solution was observed. Then, tubes were briefly centrifuged and then incubated at 56°C for 10 minutes which is suggested to give the best DNA yield, followed by a pulse spin to collect a residual sample from the inside of the tube lids. 200 µl ethanol (96-100%) was added to the samples and they were vortexed for 15 seconds. This mixture was then carefully transferred to the QIAamp mini spin column in the provided 2ml collection tube and centrifuged at full speed to avoid clotting of buffy coat

for 1 minute, before transferring the column to new 2ml tubes and discarding the collection tube that contains the rest. After that, 500µl Buffer AW1 was added to the mixture and spun for 1 minute at 3500 g, and the above column centrifugation process was repeated once. Following this, 500µl Buffer AW2 was added to the QIAamp mini spin column and centrifuged at 9000 g for three mins. Then, the collection tubes were changed with new 2ml Eppendorf tubes to perform another centrifugation at 9000 g for 1 minute, which is recommended by the manufacturer, to remove any residual Buffer AW2. The QIAamp mini spin column was then placed into new sterile 1.5ml Eppendorf tubes and liquid discarded. Following this, 200µl Buffer AE was added for DNA elution to the column and incubated for 5 minutes to increase DNA yield at room temperature. Samples were then centrifuged at 3500 g for 1 minute. DNA was divided into two separate sterile aliquots and stored at -20°C. DNA was ready for quantifying by using several approaches, including NanoDrop™ (Thermo Fischer Scientific Inc, Paisley, UK), and Qubit® 2.0 Fluorometer with Qubit® dsDNA Broad range (BR) Assay Kit (Invitrogen™, Paisley, UK).

2.2.14.2 RNA Extraction:

Samples planned for RNA extraction were processed immediately upon received by centrifugation at 800 g for 10 minutes at room temperature in order to separate the whole blood components, plasma, buffy coat, and red cells (Figure 2.2).

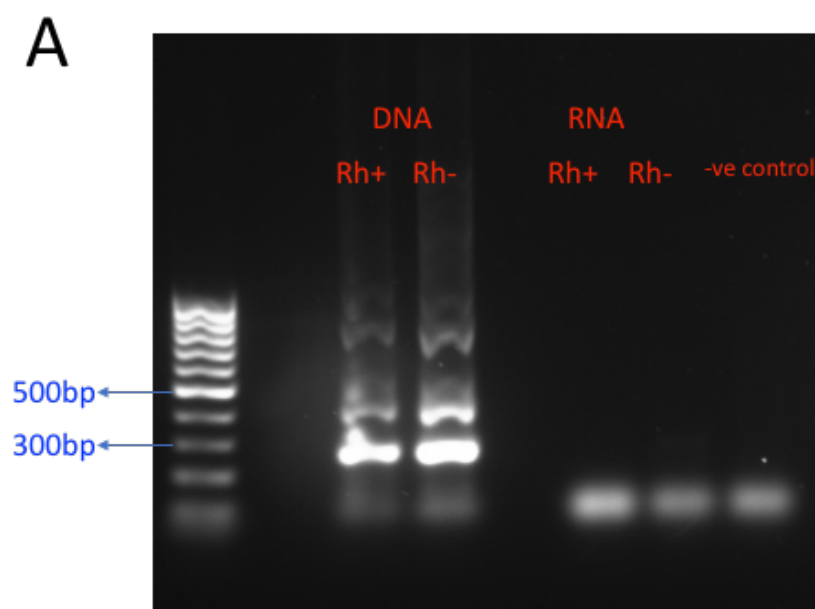
RNA extraction and purification were achieved using QIAamp RNA Blood Mini Kit (QIAGEN, Manchester, UK), with an addition of RNase-Free DNase Set (QIAGEN, Manchester, UK). The first ml of RBC layer where the reticulocytes are located was transferred into low bind DNA 1.5 ml Eppendorf tubes and mixed with 5x the sample volume (5 ml) EL buffer, incubated for 15 minutes on ice and briefly vortexed twice to

get a transparent solution which indicated lysis of erythrocytes. Then, samples were spun at 400 g for 10 minutes at 4°C before removing the supernatant carefully, leaving a small amount of the supernatant 100µl which is advised for RNA extraction from reticulocytes instead of leucocytes, for which the manufacturers intended the kit. Next, 2 ml Buffer EL was added to the samples, and cells were re-suspended by vortexing briefly, followed by centrifugation at 400 g for 10 minutes at 4°C. Then, the supernatant was discarded, leaving a small amount 50µl which was mixed with 600µl RLT buffer containing 143 mM β -mercaptoethanol (β -ME) (Sigma-Aldrich Company Ltd, UK) after that, vortexed and transferred directly onto a QIAshredder spin column, placed in 2 ml collection tubes. Samples were then spun for 2 minutes at maximum speed to obtain homogenized lysates, while columns were discarded. 600 µl 70% ethanol was added to the homogenized lysates and mixed by pipetting 5 times, then carefully transferred into new QIAamp spin column tubes without touching the borders. The columns were then centrifuged at 1500 g for 15 seconds, then the filtrate was discarded, and collection tubes were changed with new collection tubes. Then, DNase digestion was obtained by washing the spin column membrane, via the addition of 350µl of Buffer RW1 to the column and centrifuged at 1500 g for 15 seconds. Then, the filtrate was discarded, and collection tubes were changed with new collection tubes. After that, 80µl incubation mix was prepared by adding 10µl DNase I stock solution to 70µl Buffer RDD and mixed gently due to the sensitivity of DNase to physical denaturation by inverting the tube. After that, a brief spinning of the solution and its direct addition to the QIAamp spin column membrane to ensure a full DNase digestion for incubation at room temperature for 15 minutes. Then, 350µl Buffer RW1 was added to the column; the sample was centrifuged for 15 seconds at 1500 g and the filtrate discarded. The columns were then transferred into 2ml collection tubes, followed by the addition of a 500µl working solution of Buffer RPE mixed with 4 volumes of (96-100%) ethanol,

and then spun again for 15 seconds at 1500 g discarding the collection tubes containing the filtrate. This step was repeated once; however, samples were then centrifuged at 8000 g for 3 minutes. Then, columns were transferred into new 2 ml Eppendorf tubes and re-spun at 8000 g for 1 minute in order to prevent Buffer RPE carryover. For RNA elution, the QIAamp spin columns were transferred into 1.5 ml microcentrifuge tubes and 40µl RNase-free water was directly added to the column membrane, followed by centrifugation at 1500 g for 2 minutes. 2µl RNA was used for quantification by NanoDrop 2000™ (Thermo Scientific, Paisley, UK).

2.2.14.3 Quality Control of extracted RNA:

To check the quality control of the extracted RNA and make sure that there was no contamination from DNA. CD4 and CD8 checks were done (Figure2.3). CD4/CD8 are only present in the DNA and they are missed from RNA.



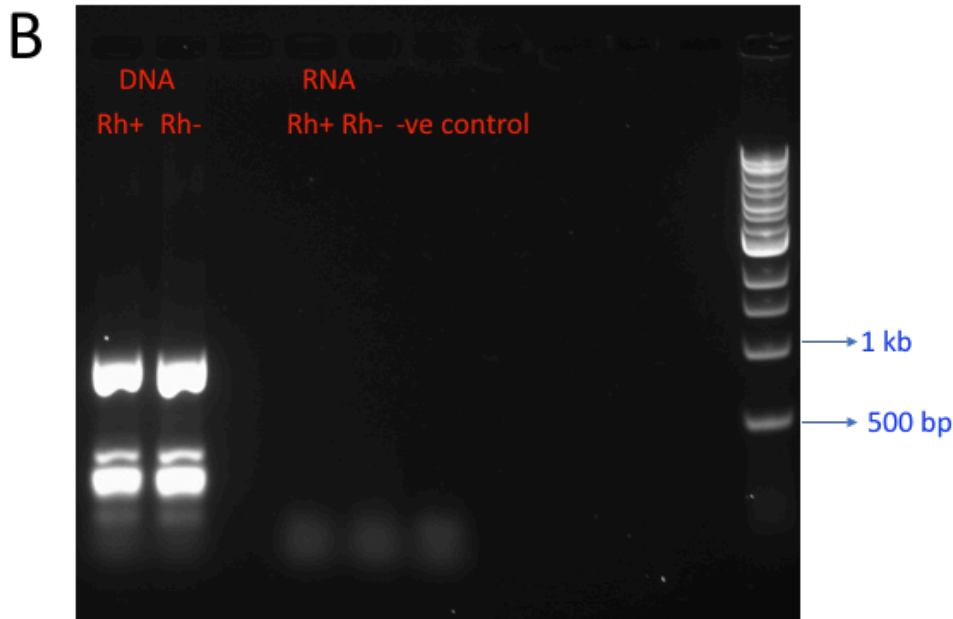


Figure 2.3: Conventional PCR for CD8/CD4.

A- show CD4 band in DNA samples at (~300bp) while it was missing in RNA samples.

B- show CD8 band in DNA samples at (~900bp) while it was missing in RNA samples.

2.2.15 cDNA synthesis from Extracted RNA:

SuperScript III First-Strand Synthesis System for RT-PCR from Life Technologies, Paisley, UK. was purchased to make cDNA from RNA. The kit is designed to convert 1pg-5µg of RNA into first strand DNA. Briefly, 3µl of RNA (total concentration 5µg/µl) in a 0.2ml sterile PCR tube was added to the primer mixture from the kit which contains; 50µM of oligo(dT) primers (1µl), 10mM dNTP mix (1µl), DEPC-treated water (5µl) to make a final volume of 10µl and incubated for 5 mins at 65°C then placed in ice for 1 minute at least. The cDNA synthesis Mix was prepared which includes; 10x RT buffer (2µl), 25mM Mg Cl₂ (4µl), 0.1 M DTT (2µl) RNaseOUT (40U/µL) (1µl), and Superscript III RT (200U/µl) (1µl). Subsequently, the cDNA synthesis mix was

added to the primer mixture and mixed gently by pipetting. Then, incubated for 50 mins at 50°C, then the reaction was terminated by increasing the temperature to 85°C for 5 mins and the sample was chilled on ice. Finally, 1µl of RNase H was added to the 0.2 mL PCR tube and incubated for 20 mins at 37°C. cDNA now is ready and can be used immediately or stored at -20°C till needed. However, the cDNA for the cell lines used for this experiment (HL-60, K562) were purchased directly from the European collection of authenticated cell culture (ECACC), Salisbury, UK.

2.2.16 CD47 cDNA Primer Design:

The *CD47* gene is located on chromosome 3. A primer pair was designed to cover the *CD47* gene from exon five towards exon 12 as the difference between the isoforms are located in this area. Primer3 website was used to design the primer (<http://primer3.ut.ee/>). Primers specific to cDNA of CD47 (Table 2.2):

Table 2.2: Primers used to amplify CD47 cDNA.

Amplicon Location		Primers (5'-3') (Primers base pair length)	Amplicon size (bp)
Exon 5 CD47	F	AAGAATGCTACTGGC	384
		CTTGG (20)	
Exon 12 CD47	R	TCGGAGTCCATCACTT	384
		CACTT (21)	

2.2.17 PCR amplification:

In this step, Hot Start *Taq* 2x Master Mix (New England Biolabs, Hitchin, UK.) was used. Briefly, to make 25 μ l PCR reaction, 12.5 μ l of Hot Start *Taq* master mix was mixed with 0.5 μ M of forward and 0.5 μ M of reverse primers (Table 2.2). The final volume was 25 μ l by adding 2 μ l of cDNA. After that, PCR reaction was done by using Veriti Thermal cycler (Thermo Fisher Scientific, Paisley, U.K.) according to the conditions in Table 2.3.

Table 2.3: Thermocycling Conditions for PCR.

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C 56°C 72°C	10 seconds 30 seconds 30 seconds
Final Extension	72°C	2 minutes
Hold	4°C	

2.2.18 Agarose Gel visualisation:

After PCR amplification, visualising the amplicon is the next step. To do this step, 1% w/v Hi-Res Standard Agarose (from Geneflow, Staffordshire, UK) was prepared by dissolving 1g of agarose powder in 100 ml 1xTAE (40mM Tris-acetate-1mM EDTA) and stained by 1X GelRed (Cambridge Bioscience, Cambridge, UK). 10µl of the Generuler 1kb Plus DNA ladder (Thermo Fisher, Paisley, U.K.) was loaded as a marker for the gel electrophoresis run to see the size of the amplicon. Gels were run at 120V max for 1 hour.

2.2.19 Next Generation Sequencing (NGS):

After amplifying PCR product, NGS protocol was started. The NGS protocol was used here is prepare amplicon libraries without fragmentation using the Ion plus fragment library kit.

2.2.19.1 Library preparation:

After running and visualising the PCR, library preparation, which include purification, quantitation, and ligation for the amplicons are the next steps.

2.2.19.1.1 Purification:

In order to purify the PCR products, AMPure® XP beads Reagent (Beckman Coulter, High Wycombe, UK.) was used. The remaining volume of PCR reaction after having used some to run a gel, was purified using magnetic beads purification, which confirms removal of primer dimers and free nucleotides that may interfere with downstream processing. Beads were re-suspended at room temperature for 30 minutes

before starting the purification. 70% molecular grade-ethanol (Fisher Scientific, Paisley, UK) diluted in nuclease-free water (Ambion[®], Applied Biosystems, Thermo Fisher Scientific, USA) was used for washing steps.

In a nuclease-free 1.5ml Eppendorf (Thermo Scientific, Paisley, UK), 72µl bead was mixed with solution 40µl PCR product by vortexing and incubated at room temperature for 5 minutes. This particular bead-sample ratio is suggested to attract a PCR product of >100bp and eliminate contaminants. The Eppendorf containing PCR product was then placed on a magnetic rack (DynaMag[™]-2 Magnet, Thermo Fisher, Paisley UK) for 3 minutes, which enhances the attachment of amplicon-carrying beads to the side of the Eppendorf, leaving a clear supernatant on the side, which was then carefully discarded. Then, without removing the tube from the magnet, two washing steps were done: 30µl of 70% ethanol was added to the tube and incubated for 30 seconds at room temperature before the supernatant was carefully discarded without disturbing the pellet. After that, without removing the tube from the magnet, residual ethanol was removed by pipetting with smaller size pipette tips (20µl; to avoid disturbing the pellet) and the beads air dried for 3 minutes at room temperature. Then, purified amplicons were eluted by removing the tube from the magnet plate and re-suspending the pellet by adding 15µl of nuclease-free water (Ambion[®], Applied Biosystems, Thermo Fisher Scientific, USA). The solution, containing purified DNA (amplicons), was then transferred to 0.2 ml sterilised nuclease-free tube strips (BIOplastics, Landgraaf, the Netherlands), with 1µl used for amplicon quantification. The Qubit[®] 2.0 Fluorometer with broad range (BR) assay kit was used to quantify the PCR amplicons.

2.2.19.1.2 Quantification:

The determination of DNA concentration was measured using the Qubit[®] dsDNA BR assay kit (Life Technologies, Paisley, UK). This method is highly selective for double-

stranded DNA (dsDNA) and therefore has a higher tolerance to contaminants, such as salts, free nucleotides, solvents, detergents or even protein. According to manufacturer's recommendation, the total volume (of Qubit[®] working solution and Qubit[®] standard/ sample) is 200 µl. So, ten µL of the standard is added to 190 µl of working solution, but the amount of sample added can be anywhere between 1-20 µl. For all experiments, one µl of sample was added to 199 µl of working solution. After two mins incubation at room temperature, the standards and samples were vortexed and run on the on the Qubit[®] 2.0 Fluorometer (Life Technologies, Paisley, UK). The Qubit[®] Fluorometer generates concentrations in ng/ ml, which corresponds to the concentration of the sample after dilution. To calculate the starting concentration of the sample, the following calculation was used:

$$\text{Concentration of sample (ng/ } \mu\text{L)} = \text{QF value} = \left(\frac{200}{x} \right) / 1000$$

Where QF value = the value given by the Qubit[®] 2.0 Fluorometer and x = the number of microliters of sample added to the assay tube (1µl). The total concentration for each sample was adjusted to 100ng to carry on with cDNA ligation.

2.2.19.1.3 cDNA ligation:

cDNA Ligation: After cDNA quantitation step, ligation of cDNA to adapters (P1 and Ion Xpress™ Barcode X adapter) from the Ion Xpress™ Barcode Adapters Kit (Life Technologies, Paisley, U.K.) took place. P1 in the universal adapter that is compatible and recognised by the Ion PGM™ sequencer and X adapter holds a distinct barcode (about 13bp) to distinguish the samples when pooled prior sequencing. The size of the adapters together is about 70bp.

2.2.19.1.4 Confirming cDNA ligation by Agilent® 2100 Bioanalyzer system:

The confirming of cDNA ligation was done by Dr Michele Kiernan (Research Fellow, Plymouth University Systems Biology Centre) by using Agilent® 2100 Bioanalyzer system. The Bioanalyzer™ (Agilent Technologies, California, USA) is an on-chip electrophoresis system that provides digital data and information regarding the size distribution, quantitation, and quality control not only for DNA but also for RNA, protein, and cells, which are displayed as electropherograms (peaks) and gel-like images (bands). To start this experiment, a gel-dye mix is needed. A volume of 15 µl of High Sensitivity DNA dye concentrate (blue dye) was mixed with a High Sensitivity DNA gel matrix with a red vial. The mixture was then vortexed and moved to the spin filter. Then, the tube was spun at 2500g for 15 minutes. Then, the solution was kept in the dark at 4°C after the filter was discarded.

Before starting the experiment, the gel-dye mix needed to be settled at room temperature for 30 minutes. A new High Sensitivity DNA chip was placed in the chip priming station.

A volume of 9 µl of sample was added to the first indicated well. The plunger was positioned at 1000 µl in the chip priming station and was then closed. After that, the

plunger was held and pressed by the chip for 1 minute. Then, the plunger was slowly pulled back to the one mL position. The chip priming station was opened, and a volume of 9 µl of the gel-dye mixture was added to the three indicated wells. Then, a volume of 5 µl of the marker was added to the twelve indicated wells. After that, a volume of 1 µl of High Sensitivity DNA ladder was added to the indicated well. Then, the samples were loaded in each well separately with one µl. Finally, the chip was placed horizontally onto the adapter of the IKA vortex mixer (Model MS3, supplied together with the Bioanalyzer[®] instrument) and vortexed at 1500 g for 1 minute. Within the next 5 minutes, the chip was placed on the 2100 Bioanalyzer[®] instrument, and the readout was carried out, which took 45 minutes. The Agilent 2100 Expert software was used to display the results.

2.2.19.2 Template preparation:

The template preparations were done by Dr Michele Kiernan (Research Fellow, Plymouth University Systems Biology Centre) by using Ion OneTouch[™] System (Life Technologies, Paisley, UK). For template preparation, the Ion OneTouch[™] 2 System (Life Technologies, Paisley, UK) was used to prepare enriched, clonally amplified cDNA (400bp average insert libraries) with template-positive Ion PGM[™] Template OT2 200 Ion Sphere[™] Particles, by the Ion PGM Template OT2 200 Kit and used on the Ion Personal Genome Machine[®] (Ion PGM[™]) System. The Ion OneTouch[™] 2 System contains the Ion OneTouch[™] 2 Instrument, and the Ion OneTouch[™] ES Instrument (Life Technologies, Paisley, UK). Template-positive immobilised to ion sphere particles (ISPs), containing clonally amplified DNA, were prepared using the Ion PGM Template OT2 200 Kit (Life Technologies, Paisley, UK), for 400 base-read libraries, with the Ion OneTouch[™] 2 Instrument (which is based on emulsion PCR (emPCR)). First, this was

according to the following: protocol Ion PGM™ Template OT2 200 Kit for use with the Ion OneTouch™ 2 (Thermo Fisher Scientific, Paisley, UK). The sequencing libraries were diluted with nuclease-free water to give a total volume of 25 µl. The samples were then mixed vigorously for 5 seconds, spun down for 2 seconds and placed on ice. Then, mixing a volume of 25 µl of the diluted sequencing libraries with the following reagent: 500 µl of Ion PGM™ Template OT2 200 Reagent Mix, 300 µl of Ion PGM™ Template OT2 200 PCR Reagent B, 50 µl of Ion PGM™ Template OT2 200 Enzyme Mix and 25 µl of nuclease-free water to prepare an amplification solution. The mixture was mixed vigorously for 10 seconds and spun down for 3 seconds. 100 µl of ISP was added to the mixture and then mixed vigorously. The amplification mixture was mixed vigorously for 5 seconds. The prepared amplification solution was loaded to the sample port of the Ion PGM™ OneTouch Plus Reaction Filter Assembly. Then, 1ml of Ion OneTouch™ Reaction Oil was loaded through the sample port. Then, a further 0.5 ml was added to the sample port. Then the filled Ion PGM™ OneTouch Plus Reaction Filter Assembly was inverted and installed into the three holes on the top stage of the Ion OneTouch™ 2 Instrument. Finally, the clonal amplification was performed by running the emulsion mixture on the Ion OneTouch™ 2 Instrument.

2.2.19.3 Ion Torrent 316 Chip Sequencer:

After template preparation, the samples were loaded to 316 chip sequencers (Thermo Fisher, UK). The runtime was 2.5 hours, and it is a semiconductor-based sequencer. The chip loading and sequencing steps were conducted by Dr Michele Kiernan who runs the Genomics Facility of the Systems Biology Centre at the University of Plymouth. Before processing the samples started, the Ion PGM™ should be clean and ready for sequencing which was recommended by the manufacturer. Templates were

loaded onto the 316™ chip after processing with the Ion PGM™ 200 Sequencing Kit v2 (Life Technologies, Paisley, UK). Briefly, 5µl of control Ion Sphere particles were added to the enriched template-positive ISPs to prepare the enriched template-positive ISPs mixture in 200 µl PCR tube. The solution was vortexed and was spun at 16000 g for 3 minutes. Without disturbing the pellet, the supernatant was removed, but 10 to 15 µl was left in the tube. Then, to obtain a total volume of 27 µl, a total volume of 12-17 µl of sequencing primer was added and mixed carefully by pipetting. Next, the reaction was transferred and run on a thermocycler using the following programme: 95°C for 2 minutes followed by 37°C for 2 minutes with utilising the heated lid option to anneal the sequencing primer to the ISPs. Then, to obtain a total volume of 30 µl, three µl of Ion PGM™ Sequencing 200 v2 Polymerase was added to the ISPs. The sequencing reaction was then vortexed well and incubated at room temperature for 5 minutes. Next, the liquid inside the chip required to be removed to be able to load the sequencing reaction to the chip. Then, the pipette tip inserted firmly into the loading port while the chip was tilted to 45 degrees and the liquid was removed from the lower port on the chip. After that, in the centrifuge adapter bucket and the bucket was transferred to the MiniFuge with the chip tab pointing in, the chip was then placed in an upside-down position. Then, the chip was spun for 10 seconds to empty the chip entirely. After that, on a flat surface, the chip was positioned in the bucket, and the loading was done slowly and carefully into the loading port with a rate of 1µl/second. The chip and bucket were spun in the MiniFuge for 45 seconds. After that, the pipette was adjusted to 20 µl to mix the sequencing reaction while tilting the chip to 45 degrees three to five times. Next, in the MiniFuge, the chip was spun again for 45 seconds, and the sample was remixed thoroughly by pipetting the solution three to five times. Finally, the chip was placed in the Ion PGM™ sequencer. According to the manufacturer's instructions, run the sequencing programme to start the sequencing reaction. The sequencing on the Ion

PGM™ is constructed for sequencing by synthesis in which a polymerase incorporates a nucleotide. Thus, a hydrogen ion is released, and the base is called, and this is in what way the sequencing data was generated on this platform.

2.2.19.4 Data analysis:

After each run on Ion PGM™, the raw sequence data was generated and was transferred to the Ion Torrent server, after that the Ion Torrent Suite™ converted data into FASTQ file formats. Then, software was used to analyse the data such as Integrative Genomics Viewer (IGV, <https://www.broadinstitute.org/igv>) to visualise the sample reads within the human genome 19 (hg19). CLC genomic workbench 8.0 was used to align the sequence reads to human CD47 mRNA isoform 1 (XM_005247909.2), isoform 2 (NM_198793.2), isoform 3 (XR_924220.2), isoform 4 (NM_001777.3), and isoform 5 which is taken from (Plummer, 2007). All CD47 isoforms sequences are attached in appendix B. Briefly, all reads from each sample were mapped against CD47 isoforms transcripts sequences at the same time as CLC has the ability to align the sample against different references. Then, the results were shown the percentage of the isoforms expression within the samples. Furthermore, each category of Rh phenotype or erythroid cell lines results were averaged, then the results of each Rh phenotype or erythroid cell lines shown in chapter 3.

3 Next Generation Sequencing (NGS) of CD47 Gene:

3.1 Introduction:

Alternative splicing (AS) is the process whereby the mRNA precursor (heterogenous nuclear RNA) is processed utilising different splice sites to produce more than one transcript from a gene (Lapuk et al., 2010, Black, 2003). At least five different erythrocyte membrane protein isoforms are being encoded by single gene such as CD47, although only varying at the cytoplasmic C-terminal domain.

CD47 is also known as Integrin-associated protein (IAP) and its gene *CD47* is located at chromosome 3 q13.1-q13.2. When CD47 cDNA was cloned and expressed, antibodies specific to CD47 also recognised IAP, hence they were identified as being the same molecule (Mawby et al., 1994). CD47 was first identified as an erythroid component during investigations into the Rh molecule. When multiple monoclonal antibodies specific to CD47 agglutinated with Rh_{null} cells (which lack all components of the Rh and LW protein complexes), it was observed that reactivity was reduced compared with normal cells (Avent et al., 1988, Anstee and Tanner, 1993). CD47 is a 47-52 kDa transmembrane receptor with 5 membrane spanning segments. It is a member of the immunoglobulin (Ig) superfamily and has a highly glycosylated extracellular immunoglobulin variable domain (IgV) at its N terminal domain. The Ig domain is necessary for interaction with its associated integrins $\alpha v \beta 3$ and $\alpha 2 \beta 1$, and for binding to SIRP α and TSP-1 (Brown and Frazier, 2001).

Initially, studies of *CD47* alternative splicing led to the discovery of four different isoforms of CD47 (isoforms 1-4) (Reinhold et al., 1995). Then in 2002 a fifth isoform was discovered (Schickel et al., 2002) (Figure 3.1). The exons that can be alternatively spliced to create the different CD47 isoforms are shown in Figure 3.2.

K562 cells are myeloid cells of the erythroid lineage, harvested from a 53-year-old female with blast crisis chorionic myeloid leukaemia (CML) (Klein et al., 1976). K562 cells express on their membrane many erythroid proteins such as GPC, CD47, GPA, CD99, Kell, and CD44 (Gane et al., 2001). Moreover, HL-60 cells are also myeloid cells of the erythroid lineage, harvested from a 36-year-old female with an acute myeloid leukaemia (AML) (Gallagher et al., 1979). The use of these cell lines is to evaluate CD47 isoforms in these cell lines and compare them with erythrocytes.

Next Generation Sequencing (NGS) is the new technology of research and discovery not only because its high-throughput sequencing approach but also due to its low costs and time saving.

Moreover, with the significant coverage depth of Next Generation Sequencing (NGS), it will allow evaluation of the expression levels of the different isoforms of CD47, in terms of monitoring their abundance in reticulocytes, and by inference red cells. Next Generation Sequencing (NGS) platforms can rapidly sequence entire genomes in the form of millions of short cDNA fragments in a cost-effective and high-throughput manner (Zhang et al., 2016). Furthermore, using NGS to evaluate CD47 isoform expression abundance in erythroid cells has not yet been described in the literature.

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Figure 3.1: *CD47* Gene Structure. *CD47* has 12 exons and 11 introns.

5 isoforms are formed at the region of the mRNA encoding the C-terminal domain of *CD47*. The *CD47* gene is located at chromosome 3 q13.1-q13.2 and consists of 47995 base pairs. E1-E7: show the common exons for *CD47* gene. UTR: Untranslated region, which is the section of the mRNA that immediately follows the translation codons. Adapted from (Plummer, 2007, Schickel et al., 2002)

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Figure 3.2: The mRNA exons of CD47.

In silico mRNA of CD47 in red blood cells. A) The forward primer starts from exon 5 while the reverse primer starts from exon 12 (3'UTR). B) Alternative splicing results in expression of five different isoforms, isoform 4 is the longest isoform while isoform 1 is the shortest.

3.2 Aims of Study:

Although there is a large body of research on CD47 in the literature, little work has been done to date looking at the expression levels of the CD47 isoforms. The aim of this study was to evaluate the expression of CD47 isoforms in erythroid cells and also to confirm the presence of CD47 isoform 5 and its isoform specific sequence by using the power of NGS. In addition, isoform-specific (2, 4, and 5) monoclonal antibodies designed specifically for this project were used to confirm the presence of these isoforms on the protein level in red blood cell membranes. Moreover, NGS was used to evaluate the expression of CD47 isoforms in different blood cell lines such as K562 and HL60, in addition to the analysis of peripheral blood derived reticulocytes.

3.3 RNA-seq (cDNA-seq) Results:

Thirty blood donor samples were used in this study with ethical consent. Moreover, K562 and HL60 cells were also used in this experiment to evaluate the expression of

CD47 isoforms in these cells. However, different blood phenotypes were used (Table 3.1). Subsequently, as well as assessing the presence of the different CD47 isoforms in red blood cells, we also looked to evaluate the expression of CD47 with respect to whether the RhD protein was expressed or not. Thus, using NGS is a novel approach for comparing the expression of CD47 in erythroid and erythroleukemic cell lines.

3.3.1 Amplification of CD47 cDNA:

The first step after designing the primers for mRNA (cDNA) is performing a conventional PCR experiment, to confirm the presence of CD47 isoforms in red blood cells. The PCR amplicon amplifies the region from exon 5 to the untranslated region (UTR), which is exon 12, of the *CD47* gene, and the longest product was (384 bp) which represents isoform 4 as shown in figure 3.2. The forward and the reverse primers were mixed with Hot Start Taq 2x Master Mix (New England Biolabs, Hitchin, UK.) as described in materials and methods. According to the manufacturer's the fidelity of this master mix is two-fold greater than that of *Taq* DNA polymerase alone. cDNA PCR amplicons were loaded to a 1% (w/v) agarose gel and electrophoresed at 90 V for an hour. Amplicons were detected as multiple ~400bp band as expected (Figure 3.3).

Table 3.1: Serology data sheet provided by NHSBT, Filton for the Rh phenotype of samples used in the CD47 transcript NGS study

#	Sample ID	D status	Rh	D	C	E	c	e
1	918 K	D+	R ₁ R ₁	+	+	–	–	+
2	533 M	D+	R ₁ R ₁	+	+	–	–	+
3	541 M	D+	R ₁ R ₁	+	+	–	–	+

4	552 G	D+	R ₁ R ₁	+	+	-	-	+
5	386 X	D+	R ₁ R ₁	+	+	-	-	+
6	130 X	D+	R ₁ R ₁	+	+	-	-	+
7	900 K	D+	R ₁ R ₁	+	+	-	-	+
8	910 G	D+	R ₁ R ₁	+	+	-	-	+
9	920 C	D+	R ₁ R ₁	+	+	-	-	+
10	921 A	D+	R ₁ R ₁	+	+	-	-	+
11	887 O	D+	R ₂ R ₂	+	-	+	+	-
12	519 I	D+	R ₂ R ₂	+	-	+	+	-
13	169 3	D+	R ₂ R ₂	+	-	+	+	-
14	187	D+	R ₂ R ₂	+	-	+	+	-
15	908 Y	D+	R ₂ R ₂	+	-	+	+	-
16	920 5	D+	R ₂ R ₂	+	-	+	+	-
17	050 J	D+	R ₂ R ₂	+	-	+	+	-
18	083 1	D+	R ₂ R ₂	+	-	+	+	-
19	266 I	D+	R ₂ R ₂	+	-	+	+	-
20	295 8	D+	R ₂ R ₂	+	-	+	+	-
21	687 8	D-	rr	-	-	-	+	+
22	693 C	D-	rr	-	-	-	+	+
23	840 X	D-	rr	-	-	-	+	+
24	841 V	D-	rr	-	-	-	+	+
25	849 F	D-	rr	-	-	-	+	+
26	581 R	D-	rr	-	-	-	+	+
27	855 J	D-	rr	-	-	-	+	+
28	906 8	D-	rr	-	-	-	+	+
29	907 6	D-	rr	-	-	-	+	+
30	957 C	D-	rr	-	-	-	+	+

After
cDNA
synthesis
from
mRNA
derived
from

reticulocytes or cell lines, CD47 specific forward and reverse primers were mixed together with Hot Start Taq 2x Master Mix. Then, amplicons were loaded to 1% (w/v) agarose gel. As expected there is more than one band in each sample which represents 30 samples were used in this study to sequence the most abundant isoforms of CD47 in red blood cells. The first 10 samples are of phenotype of D⁺ (R₁R₁ CDe/CDe) samples, while from samples 11 to 20 represent D⁺ (R₂R₂ cDe/cDe) sample; and from sample 21 to 30 are represent D⁻ (rr cde/cde) samples. The rationale behind choosing different Rh phenotypes (R₁R₁, R₂R₂, and rr) to evaluate CD47 isoforms presence in different erythrocyte phenotypes.

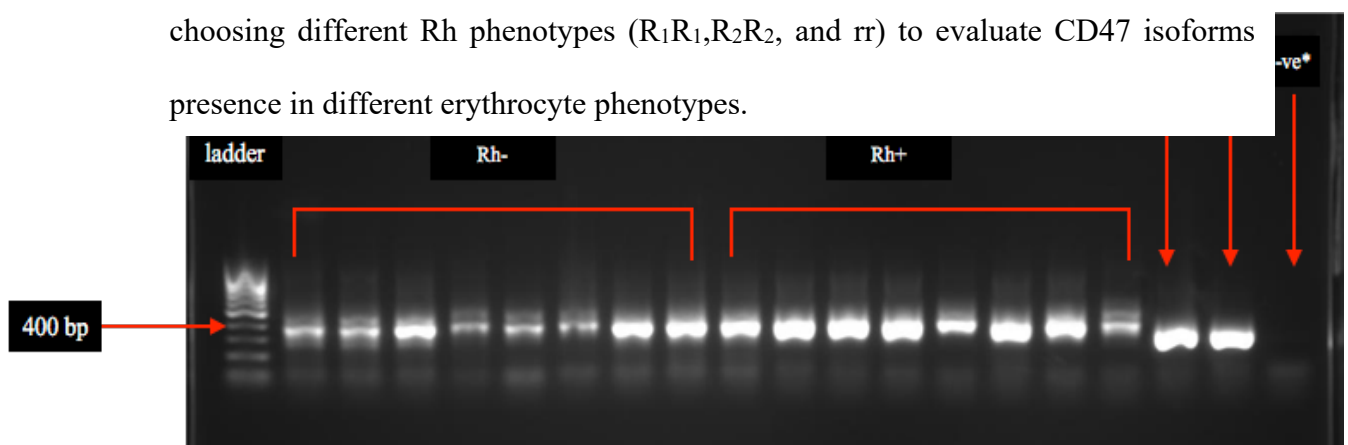


Figure 3.3: The mRNA (cDNA) CD47 amplicons produced by reverse transcriptase PCR.

In this example, the amplicons of 8 D+ (R₁R₁) and 8 D- (rr) samples are shown. * The last column (-ve) represents a negative control, which is a master mix without DNA added (with RNA free water). The GeneRuler™ 1Kb Plus DNA ladder (Thermo Fisher Scientific) was used as a marker of DNA size.

3.3.2 NGS of the CD47 gene:

3.3.2.1 Ligation of barcoded adaptors:

The samples were ligated to barcoded adapters, in which P1 and the Ion Xpress™ Barcode X adapter (provided by the Ion Xpress™ Barcode adapters Kit) were used along with DNA ligase. The ligated libraries were then purified and analysed using Agilent® 2100 Bioanalyzer with Agilent High Sensitivity DNA Kit. (Figure 3.4) shows the size distribution of the sequencing libraries after ligation.

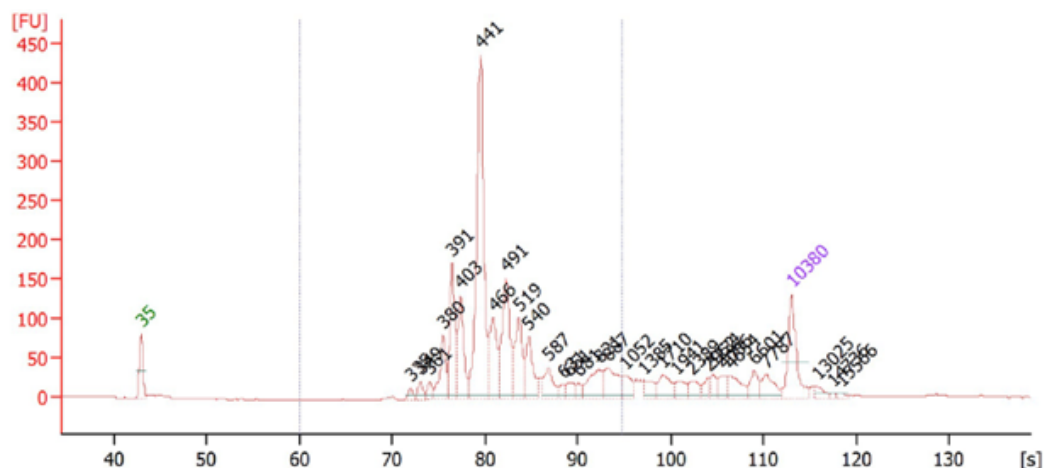


Figure 3.4: An electropherogram of the CD47 amplicons library.

The library was purified with some peaks of around 400-450bp. A purified library showing some peaks to the right (caused by adapter ligation (size about 70 bp). The green labelled peak (35bp) is the lower marker and the purple labelled peak (10380bp) is the upper marker. Results shown were obtained using the Bioanalyzer® instrument.

3.3.3 NGS data quality control:

The 30 peripheral blood samples used in this experiment and three replicates of each sample of K562 and HL60 cell lines, (total of 36 samples), were sequenced using Ion PGM™ in three separate runs. The data was then processed, and a summary was produced by the Torrent Suite™ Software Version 4.4 and CLC software version 9.9. The average of the total reads produced from each of the 36 CD47 samples was 2,828,657. In this report, the Ion Sphere Particle (ISP) loading density was 65%, while 35% of the 316™ chip wells were empty. A summary of the CD47 sequencing report from the 3 separate runs is shown in Table 3.2. A brief sequencing report of a single representative CD47 sequencing run is displayed in figure 3.5. For downstream analysis, there were around 3 million usable reads in total, which is 70% of the total reads containing the library ISPs usable. These reads were thoroughly processed by well classification and read filtering to ensure their quality. Clonal ISPs (in which single template fragments were amplified) was made up 77 % of reads, while 23 % were polyclonal ISPs. After filtering out 1 % of test

fragments and 9 % of low-quality reads, the final percentage of the final library was 70% with a mean read length of 267 bp (Figure 3.5).

	No. of samples	ISP Loading %	Total usable reads	Usable reads%	Mean read length	Chip Used
1st run report	4	40	270,674	57	190 bp	314
2nd run report	16	63	2,961,213	77	236 bp	316
3rd run report	16	65	2,828,657	70	267 bp	316

Table 3.2: A summary of the Ion PGM™ sequence run output of the 36 CD47 samples, collectively processed in 3 runs.

The chip used for the first run was 314 which is capable to give up to 500,000 reads per run. The used reads were 57% (270,674) which is considered acceptable as the sample numbers were only four samples only. Usable reads mean that these reads are mapped

to CD47 and the non-usable reads are indicated these reads not match to CD47 and couldn't be used for further analysis.

However, in the following runs a larger chip (316). 316 chips are capable of giving 3-4 millions reads per run. Actually, the usable numbers in the second run were 77% (2,961,213) which is great as the number of samples were 16 samples. Furthermore, in the third run the usable number were dropped to 70% (2,828,657) while the same numbers of samples were used. Usable reads mean that these reads are mapped to CD47 and the non-usable reads are indicated these reads not match to CD47 and couldn't be used for further analysis.

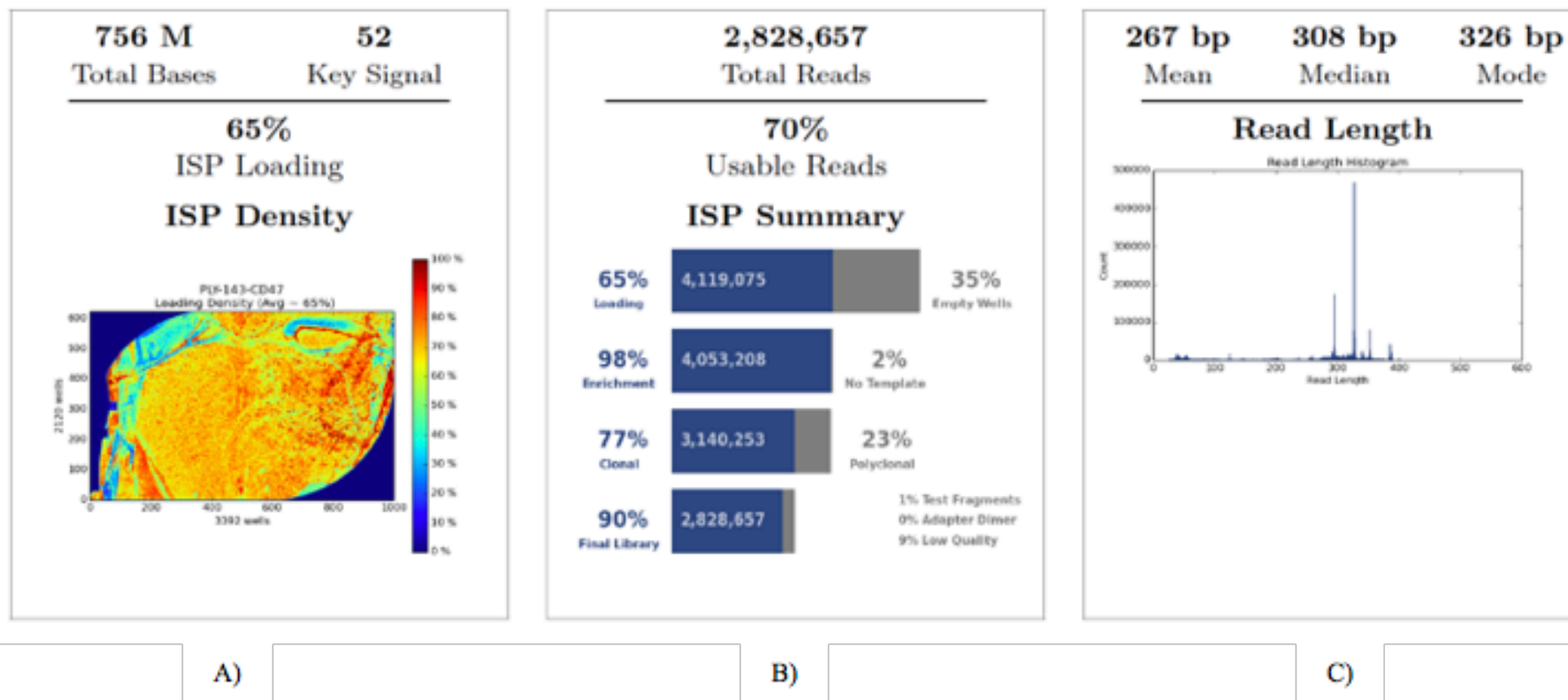


Figure 3.5: The report of one single sequencing run of the CD47 library

- A) The percentage of ISP loading density of the ISP shown by the 316TM chip wells was 65 %. Different colours represent the loading percentage of ISP through the physical 316TM chip plate surface (red is highest; blue is lowest).
- B) The total number of usable reads is 2,828,657, after trimming and filtration from empty wells, non-templated and polyclonal reads. The percentage 70% is obtained by dividing these reads by the number of reads containing the library ISPs (4, 119, 075). The live/enrichment percentage is 100%, which indicates that ISPs contain a strong sequence signal from test fragment and library (templated).
- C) Histogram shows a mean reading length of 267bp. The read count is displayed in the y-axis, while the read length, in bp, is shown on the x-axis.

3.3.4 CD47 isoforms sequence identification:

NGS work confirmed the presence of the mRNA species coding for the different CD47 protein isoforms. CD47 isoforms encode different C-terminal domains of the CD47 protein, starting with isoform 1 which is formed from common exons (figure 3.2b). Isoform 1 is composed of 1245bp and 8 exons while isoform 2 is composed of 1276bp and 9 exons. Isoform 3 is composed of 1301bp and 10 exons while isoform 4 is the longest at 1334bp and 11 exons. Last but not least isoform 5 which is composed of 1292bp and 9 exons.

The current study detected the presence of different isoforms by using NGS (RNA-seq, (cDNA-seq)) in erythroid lineage blood cells.

As previously mention above, the results were grouped depending on RhD phenotypes as following:

3.3.4.1 RhD+ (R₁R₁) results:

After mapping the results of 10 blood samples against CD47 isoform sequence references (see 2.2.18.4), the read coverage for all the ten samples were averaged which indicates the most abundant isoform by the highest coverage. Subsequently, the least coverage indicated the least abundant isoform. The results show isoform 2 to be the most abundant isoform with 35% of reads in all RhD+ (R₁R₁) samples. The second most abundant isoform is isoform 4 with 27% of reads (Figure 3.6).

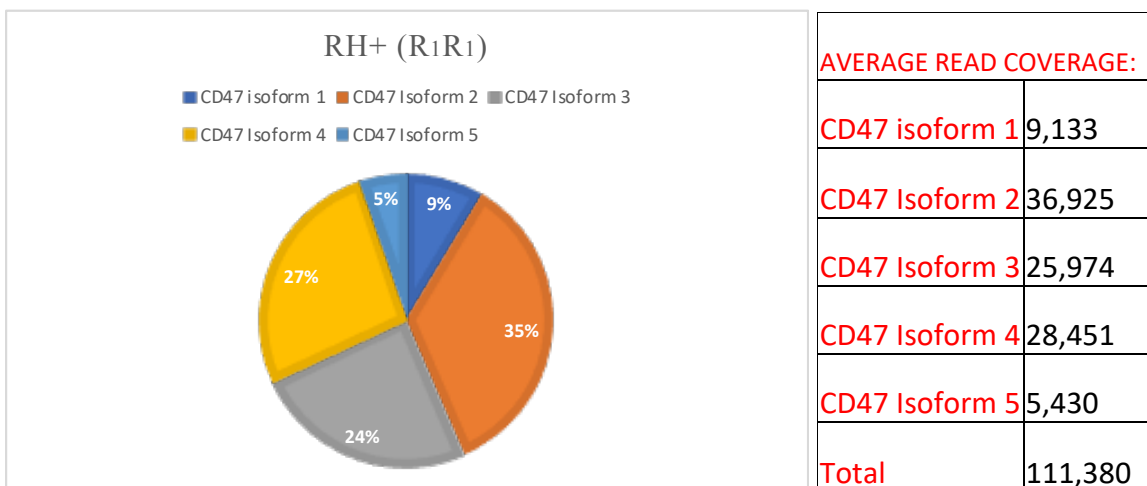


Figure 3.6: The percentage of CD47 isoforms in RhD+ (R₁R₁, CDe/CDe).

The average value of the reads coverage from 10 samples of red blood was calculated to evaluate the expression of CD47 isoforms. The most abundant isoform is isoform 2. However, the presence of isoform 5 and its sequence reference were confirmed. CLC version 9.9 was used to map the reads to CD47 isoforms references.

3.3.4.2 RhD+ (R₂R₂) results:

Different phenotype of RhD+ (R₂R₂ cDE/cDE) were used here to elucidate the presence of CD47 in different erythroid cells of this phenotype. RhD+ (R₂R₂) expresses the D, E, and c antigen in red cell membrane. CD47 isoform 2 is the most abundant with 32% of reads while CD47 isoform 5 is the least expressed isoform with only 5% of the reads.

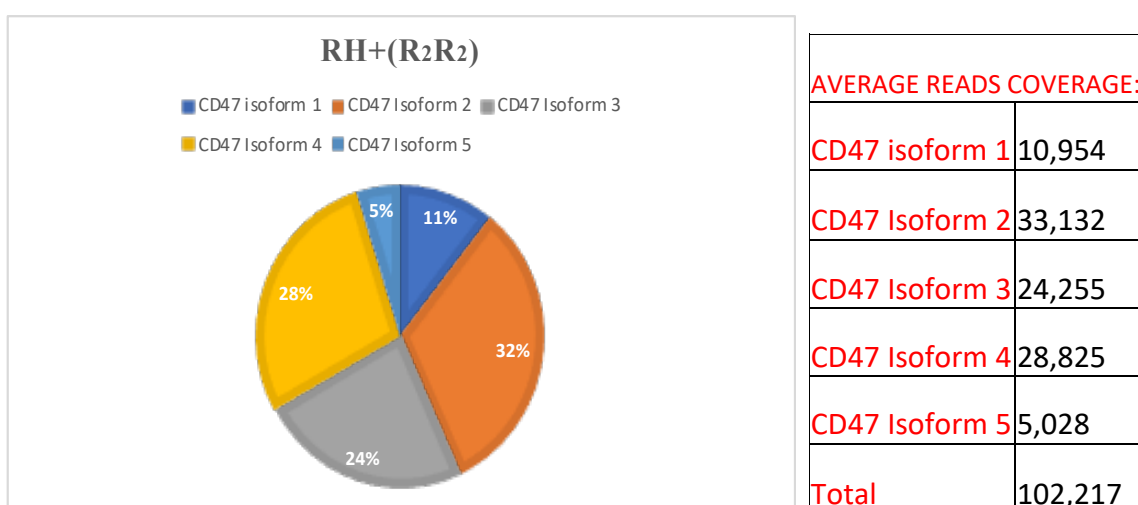


Figure 3.7: The percentage of CD47 isoforms in RhD+ (R₂R₂ cDE/cDE).

The average value of the reads coverage from 10 samples of red blood cells was calculated to evaluate the expression of CD47 isoforms. The most abundant isoform is isoform 2. However, the presence of isoform 5 and its sequence reference were confirmed. CLC version 9.9 was used to map the reads to CD47 isoforms reference.

Although there were a few differences between R₁R₁ and R₂R₂ results, the *t*-test were performed on R₁R₁ against R₂R₂ and it was statically not significant see (table 3.3).

3.3.4.3 RhD- (rr cde/cde) results:

Here we have used RhD- (rr cde/cde) phenotype which is characterized by the absence of D antigen in red blood cell membrane while still express c, and e antigens on red cell membranes. These results show there is a reduction in reads coverage of CD47 in RhD- phenotype (Figure 3.8). This may suggest that the abundance of CD47 transcripts in rr (cde/cde) peripheral blood is lower than in D-positive peripheral blood. CD47 isoform 2 is the most abundant isoform with 28% while CD47 isoform 4 is expressed almost as much as CD47 isoform 2 with 27% of reads.

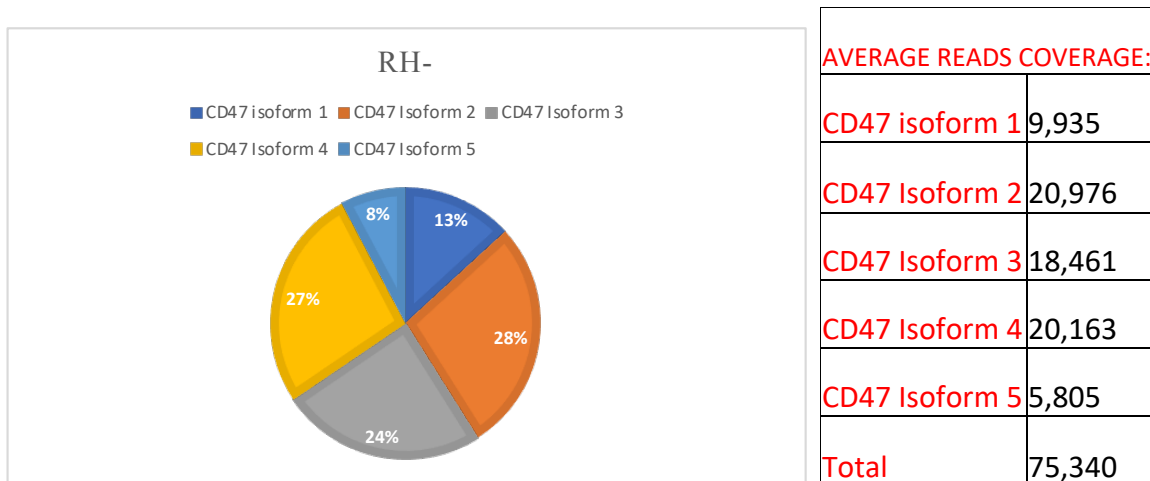


Figure 3.8: The percentage of CD47 isoforms in RhD- (rr).

The average value of the reads coverage from 10 samples of red blood was calculated to evaluate the expression of CD47 isoforms. The most abundant isoform is isoform 2. However, the presence of isoform 5 and its sequence reference was confirmed. CLC version 9.9 was used to map the reads to CD47 isoforms reference.

The statistical analysis for Rh- against Rh+ (R_1R_1 , R_2R_2) were not significant see (table

3.3)

3.3.4.4 K562 Results:

K562 represents a malignant cell line derived from an individual with chronic myeloid leukaemia (CML). Ready-made cDNA from the European Collection of Authenticated Cell Cultures (ECACC) was purchased to minimize cell culture limitation such as cell contamination and high cell passages. NGS was completed with three replicate samples from the K562 cell line and then averaged the number of reads from the three samples. The most abundant isoform expressed in K562 cell lines is isoform 2 with 72% of reads (Figure 3.9)

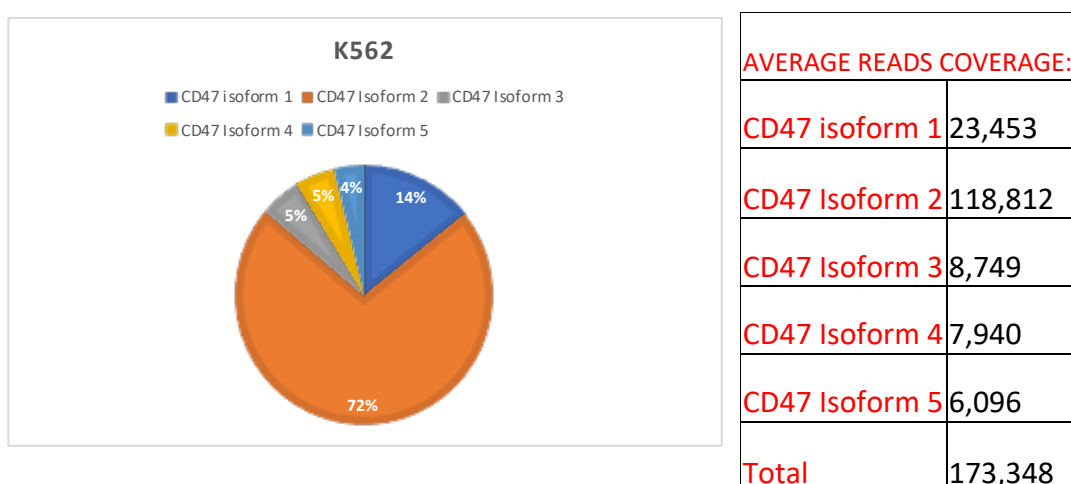


Figure 3.9: The percentage of CD47 isoforms in K562.

The average value of the reads coverage from 3 samples of K562 was calculated to evaluate the expression of CD47 isoforms. The most abundant isoform is isoform 2 (72%). However, the presence of isoform 5 and its sequence reference was confirmed. CLC version 9.9 was used to map the reads to CD47 isoforms reference.

The statistical analysis for this results comparing to CD47 in erythrocytes (Rh+,Rh-) were statically significant for all isoforms except isoform 5 as the P-value <0.01. For Isoform 5 the statistical analysis shows that the difference is not statically significant see (table 3.3).

3.3.4.5 HL-60 Results:

HL-60 represents a malignant cell line which is derived from an individual with acute myeloid leukaemia (AML). To minimize cell culture limitation such as cell contamination and high cell passages, we have purchased ready-made cDNA from the European Collection of Authenticated Cell Cultures (ECACC). Triplicate samples of this cell line were done and average the reads coverage. The most abundant CD47 isoform expressed in HL-60 is isoform 2 with 64% of reads (Figure 3.10).

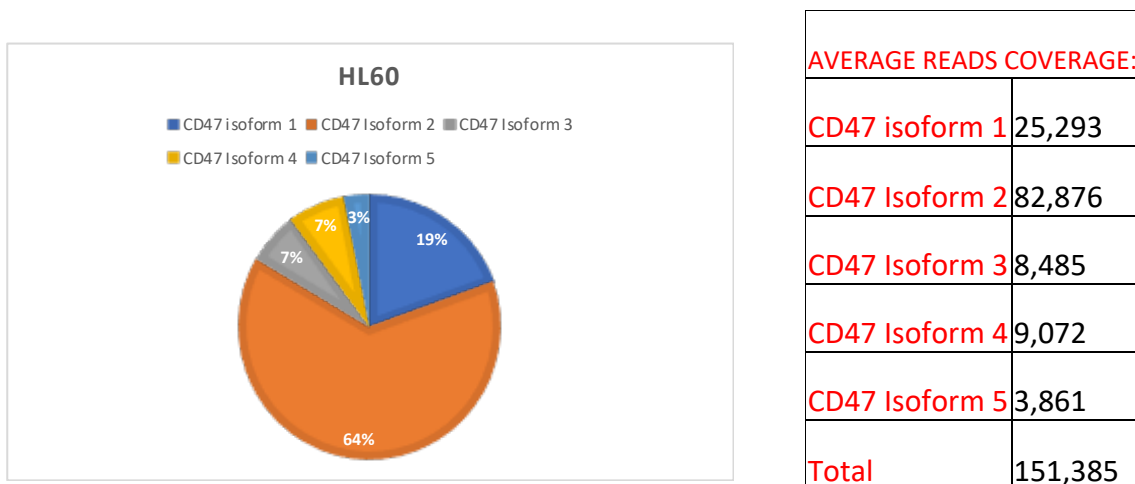


Figure 3.10: The percentage of CD47 isoforms in HL-60.

The average value of the reads coverage from 3 samples of HL-60 was calculated to evaluate the expression of CD47 isoforms. The most abundant isoform is isoform 2 (64%). However, the presence of isoform 5 and its sequence reference was confirmed. CLC version 9.9 was used to map the reads to CD47 isoforms reference.

The statistical analysis for this results comparing to CD47 in erythrocytes (Rh+,Rh-) were statically significant for all isoforms except isoform 5 as the P-value <0.01. For Isoform 5 the statistical analysis shows that the difference is not statically significant see (table 3.3).

3.3.5 CD47 Isoforms Statistical Analysis:

To compare CD47 isoforms between different erythrocyte phenotypes and cell lines, *t*-tests were performed to calculate p-values and determined how statistically significant the difference between these erythrocytes and cell lines as well. All tests were performed for each isoform of CD47 by using GraphPad software (Table 3.3).

p-value	R ₁ R ₁ vs R ₂ R ₂	R ₁ R ₁ VS rr	R ₁ R ₁ vs K562	R ₁ R ₁ vs Hl- 60	R ₂ R ₂ vs rr	R ₂ R ₂ vs K562	R ₂ R ₂ vs Hl- 60	rr vs K562	rr vs Hl-60	K562 vs HL- 60
Isoform 1	0.2 (ns)	0.5 (ns)	<0.01 (s)	<0.01 (s)	0.3 (ns)	<0.01 (s)	<0.01 (s)	<0.01 (s)	0.6 (ns)	0.8 (ns)
Isoform 2	0.3 (ns)	0.4 (ns)	<0.01 (s)	<0.01 (s)	0.7 (ns)	<0.01 (s)	<0.01 (s)	<0.01 (s)	0.5 (ns)	0.2 (ns)
Isoform 3	0.6 (ns)	0.7 (ns)	<0.01 (s)	<0.01 (s)	0.8 (ns)	<0.01 (s)	<0.01 (s)	<0.01 (s)	0.7 (ns)	0.5 (ns)
Isoform 4	0.8 (ns)	0.9 (ns)	<0.01 (s)	<0.01 (s)	0.9 (ns)	<0.01 (s)	<0.01 (s)	<0.01 (s)	0.5 (ns)	0.4 (ns)

Isoform	0.1	0.5 (ns)	0.3	0.4	0.2	0.6	0.8	0.2	0.5	0.7
5	(ns)		(ns)	(ns)	(ns)	(ns)	(ns)	(ns)	(ns)	(ns)

Table 3.3: the comparison between different erythrocytes phenotypes and cell lines by using *t*-test statistical analysis for CD47 isoforms.

ns: indicate that the p-value is more than 0.05 and the result are not statistically significant.

s: indicate that p-value is less than 0.01 and the result are statistically significant

3.4 Western blot analysis of CD47 isoforms in red blood cells:

Isoform-specific monoclonal antibodies (mAb) specific against CD47 isoforms 2, 4, and 5 amino acid sequences were raised. The C-terminal CD47 amino acids sequence of each isoform were sent to Dundee Cell. The mAb production was performed by Dundee Cell Products, Dundee, UK. The high homology shared between the amino acid sequences of the CD47 isoforms presented some technical challenges in the design of peptides for immunisation to produce mAbs (table 3.4). Isoform 1 as previously mentioned has 8 common exons (Figure 3.2) and shares five common amino acids in the sequence at C-terminal domain with isoform 5 (YMKFV) and only 3 amino acids with isoform 2 (KFV). Moreover, isoform 2 has 9 exons and shares 7 amino acids with isoform 3 (KTIQPPR) at its C-terminal domain. Furthermore, isoform 3 has 10 exons and shares 4 amino acids with isoform 4 (EPLN) in the C-terminus while isoform 4 has 11 exons and the longest unique amino acids sequence at C-terminal domain. Additionally, isoform 5 also has 9 unique amino acids in the C-terminal domain. Isoforms 2, 4, and 5 were chosen (Figure 3.11) (Table 3.5) to start raise monoclonal antibodies against, because these may represent the least challenging to obtain isoform specific mAbs.

Secondly, the time scale and the cost to finish these antibodies projects were really critical. Briefly, the project to be accomplished by Dundee cell biosciences should process within 2 phases. Phase 1 which includes the peptide synthesis and coupling to keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA). Moreover, peptide sequence is very critical in this phase as the high homology between isoforms. Then, Phase 2 was started with immunisation; 4 mice were immunised with 50 µg of antigen (4 injections per mouse). Next, cell fusion step which include fusion of splenocytes and myeloma cells. Then, screening of hybridomas for production of non-specific Ig and recognition of specific antigen and the propagation of hybridomas and production of cell

lines. Moreover, recloning of all positive hybridomas cell colonies and Ig isotype characterization. Then, production of 25ml of supernatant from each clone and preparation of a cell bank for each cloned hybridoma. Thus, each project for each isoform took almost 10 months to be accomplished. However, the cost for each isoform specific antibody was £5000 and thus a considerable expense to this project.

Figure has been removed due to Copyright restrictions.

Figure 3.11: The topology of CD47.

CD47 has five spanning segments crossing the membrane. It is a 47-52 kDa and it is highly glycosylated at the N-terminal domain (IgV). 5 different isoforms are formed at C-terminal domain. Isoform 4 has the longest C-terminal domain. Isoform 2 is the most abundant isoform in red cell membrane. (adapted from (Plummer, 2007, Brown and Frazier, 2001))

Table 3.4: Homology between CD47 isoform peptides:

CD47 Isoform:	Amino Acid Sequence
CD47 Isoform 1	NH ₂ - QLLGLVYM KFVE-COOH
CD47 Isoform 2	NH ₂ - QLLGLVYM KF VASNQK TIQPPRNN-COOH
CD47 Isoform 3	NH ₂ - QLLGLVYM KF VASNQK TIQPPR KAVEEPLN -COOH
CD47 Isoform 4	NH ₂ - QLLGLVYM KF VASNQK TIQPPR KAVEEPLN AFKESKGMMNDE- COOH
CD47 Isoform 5	NH ₂ - QLLGLVYM KFVGFRGTPTVS-COOH

Table 3.5: The peptide sequences used for CD47 monoclonal antibody production:

CD47 Isoform:	Amino Acid Sequence
CD47 Isoform 2	NH ₂ - KFV ASNQ KTIQPPR NN-COOH
CD47 Isoform 4	NH ₂ - EPLN AFKESKGMMNDE-COOH
CD47 Isoform 5	NH ₂ - YMKFV GFRGTPTVS-COOH

3.4.1 CD47 isoform 2 monoclonal antibodies:

Tissue culture supernatant from 6 positive hybridoma clones was sent for testing (Figure 3.12). Testing was completed using ghost membrane preparations from red blood cells and western blotting. Figure 3.13 shows the best example of these antibodies (5A12).

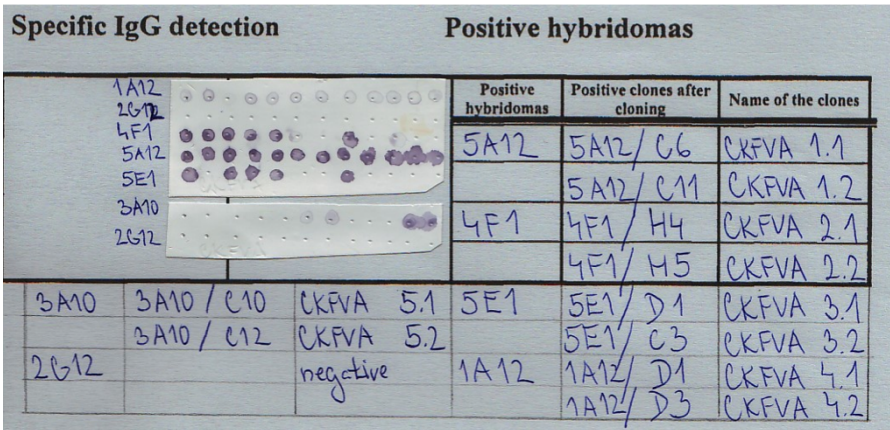


Figure 3.12: Dots blot for positive hybridomas for CD47 isoform 2 mAbs.

Six positive dots blot for CD47 isoform 2 mAbs were shown as (1A12, 4F1, 5A12, 5E1, 3A10, and 2G12)

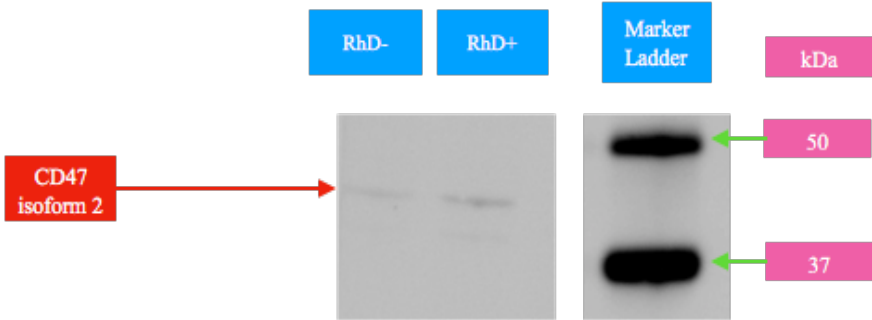


Figure 3.13: The expression of CD47 isoform 2 in red blood cells.

Red blood cell membrane ghost was blotted with isoform 2 specific CD47 monoclonal antibody. RhD positive and negative phenotypes were used. The western blot was used under non-reducing condition. The concentration of primary antibody was 1:10 (v/v) in blocking buffer. The acrylamide gel percentage was 4-12%. The secondary antibody was rabbit-anti mouse. There are no other bands were visible on these Western blots.

3.4.2 CD47 isoform 4 monoclonal antibodies:

Dundee cell product provided tissue culture supernatant antibodies from 12 positive clones to test them and choose the best hybridoma that produce antibody against CD47 isoform 4. To test these antibodies, we have chosen western blot technique using erythrocyte membrane ghosts. Figure 3.14 shows the best example of these antibodies.



Figure 3.14: The expression of CD47 isoform 4 in red blood cells.

Red blood cell membrane ghost was blotted with isoform 4 specific CD47 monoclonal antibody. RhD positive and negative phenotypes were used. The western blot was used under non-reducing condition. The concentration of primary antibody was 1:10 (v/v) in blocking buffer. The acrylamide gel percentage was 4-12%. The secondary antibody was rabbit-anti mouse. There are no other bands were visible on these Western blots.

3.4.3 CD47 isoform 5 monoclonal antibodies:

Dundee cell product has sent us tissue culture supernatant antibodies from 5 positive clones to test them and choose the best hybridoma that produce antibody against CD47 isoform 5 (Figure 3.15). To test these antibodies, western blot technique was chosen using erythrocyte membrane ghost. Figure 3.16 shows the best example of these antibodies (1B5).

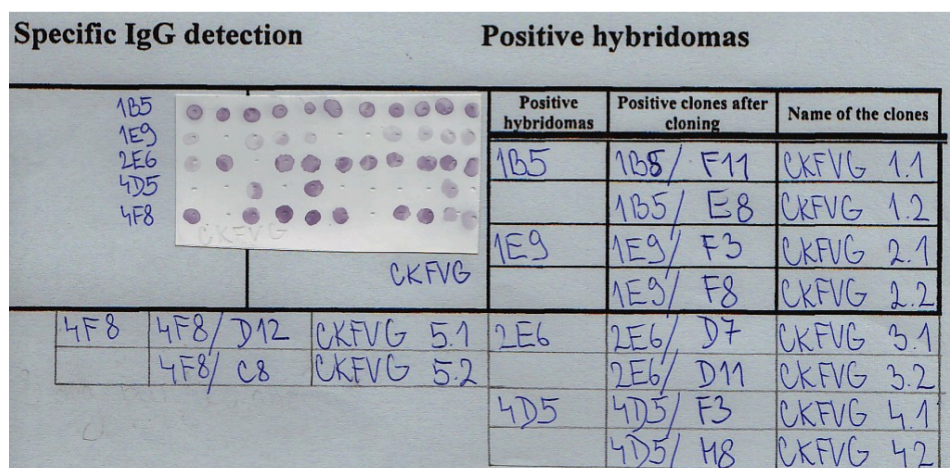


Figure 3.15: Dots blot for positive hybridomas for CD47 isoform 5 mAbs.

Five positive dots blot for CD47 isoform 5 mAbs were shown as (1B5, 1E9, 2E6, 4D5, and 4F8)

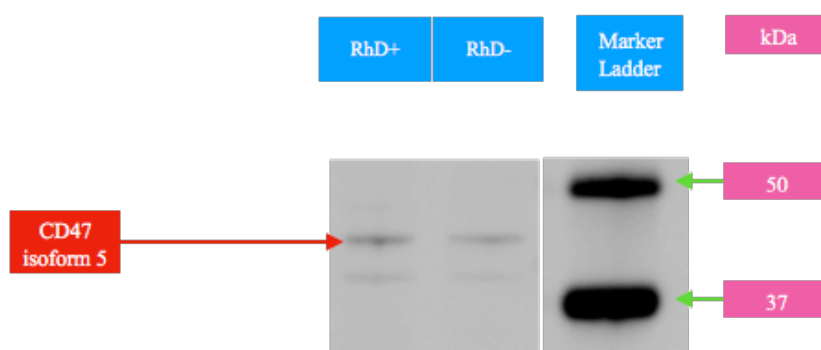


Figure 3.16: The expression of CD47 isoform 5 in red blood cells.

Red blood cell membrane ghost was blotted with isoform 5 specific CD47 monoclonal antibody. RhD positive and negative phenotypes were used. The western blot was used under non-reducing condition. The concentration of primary antibody was 1:10 (v/v) in blocking buffer. The acrylamide gel percentage was 4-12%. The secondary antibody was rabbit-anti mouse. There are no other bands were visible on these Western blots.

3.5 Discussion:

3.5.1 RNA-seq for CD47 in red cells and erythroleukemia cell lines:

This chapter describes using NGS (RNA-seq) for CD47 isoform expression in erythroid cells and erythroleukemia cell lines which is novel in transfusion science to explore which isoform of CD47 has the most abundant expression. NGS (RNA-seq) is now considered the gold standard technique to assess gene expression over other techniques such as microarray and real time polymerase chain reaction (RT-PCR) due to its high throughput data, low cost, and time saving (Sirbu et al., 2012, McGettigan, 2013).

CD47 isoforms were detected in many human tissues previously. Reinhold *et al.* in 1995 published the detection of all CD47 isoforms in keratinocytes (outermost layer of skin) while they were only able to detect CD47 isoform 2 in HL-60 cell lines (Reinhold et al., 1995). Moreover, Mordue *et al.* in 2017 were able only to detect CD47 isoform 2 in red blood cells which conflicted with Plummer (2007) who was able to detect all CD47 isoforms in red blood cells (Mordue et al., 2017, Plummer, 2007). Furthermore, the need for a gold standard technique to resolve the debate of CD47 isoforms expression in human tissue is essential, to elucidate the fundamental role of CD47 not only in red cell turnover but also in different biochemical signalling pathways. Thus, NGS (RNA-seq) is the most advanced and sophisticated technique to assess the expression of CD47 isoforms in human cells and tissues.

Here the results of RNA-seq which is quantitative of CD47 expression in erythroid cells and erythroleukemia cell lines (K562, and HL-60) were shown depending on depth and high coverage of CD47 isoforms. The sequencing results were mapped against all CD47 5 isoforms reference sequences using CLC genomics suites version 9.9 which gave the most abundant CD47 isoforms in red blood cell and erythroleukemia cell lines. As a result, isoform 2 was confirmed as the most abundant isoform. However, isoform 5

sequence which was discovered by Schickel *et al.* in 2002 and corrected by Plummer (2007) was confirmed by NGS (RNA-seq) in this chapter.

Moreover, CD47 isoform 5 sequence as published by Schickel *et al.* in 2002 is proposed to contain the F2 exon which is incorrect, and Plummer (2007) had shown the correct sequence of CD47 isoform 5. Here the right sequence of CD47 isoform 5 which is suggested by Plummer (2007) was confirmed by using NGS (cDNA-seq)(Figure 3.17) because 98% of the reads for CD47 isoform 5 mapped to the sequence suggested by Plummer (2007) as opposed to the sequence suggested by Schickel et al. (2002) or might the sequence achieved by Schickel is a different sequence could be isoform 6, although this was not detected in any of the experiments described in this thesis.

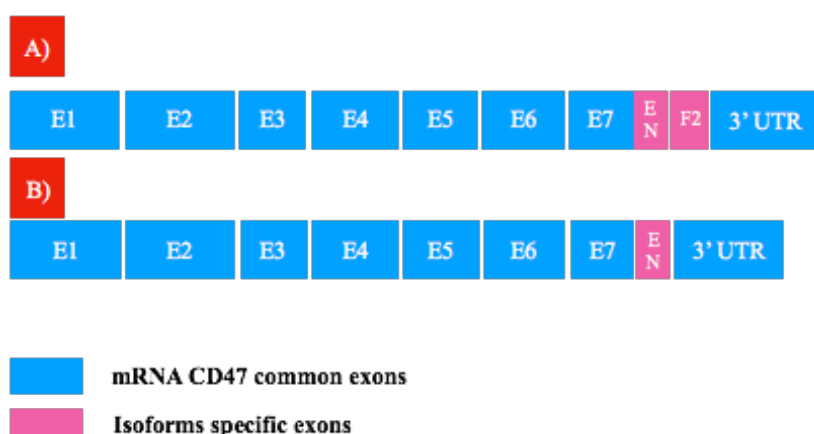


Figure 3.17: CD47 isoform 5 sequence exons arrangements.

A) Shows CD47 isoform 5 sequence exon arrangement suggested by Schickel *et al.* (2002) which has the F2 exon present in the sequence for isoform 5.
 B) CD47 isoform 5 sequence exon arrangements suggested by Plummer (2007) without the F2 exon which is confirmed by NGS (RNA-seq) (Plummer, 2007, Schickel et al., 2002)

Actually, according to National Center for Biotechnology Information (NCBI), which is updated in 28-02-2018, CD47 has 11 exons and 10 introns. On the other hand, Schickel et al, has discovered isoform 5 in 2002 as mentioned above but the exact sequence of

isoform 5 has been confirmed by Plummer et al, in 2007 (Schickel et al., 2002, Plummer, 2007). Since 2007, NCBI has not been updating the isoform 5 sequence for unknown reasons. NGS (RNA-seq) data confirm that CD47 isoform 5 is present in positive and negative Rh phenotype (Figures 3.6-3.8). To date, EN exon (Figure 3.2) specific for isoform 5 is present in the NCBI database as an intron. Moreover, NGS data confirmed that CD47 has 12 exons and 11 introns, thus we feel that the NCBI database entry is incorrect.

The results also showing a reduction in CD47 coverage reads numbers on all RhD- samples compared to the coverage reads number on RhD+ samples (Figures 3.6-3.8) which suggests CD47 transcripts are reduced in RhD- phenotype. Moreover, Avent et al. in 1988 showed that CD47 is reduced in Rh_{null} phenotype (Avent et al., 1988). It therefore is conceivable that the absence of the RhD protein in D-negative individuals may in some unknown way contribute to the reduction in levels of *CD47* transcripts. Indeed, another approach to confirm these results was done by using immunoprecipitation with monoclonal antibodies against CD47 (BRIC 32, 124, and 126) to confirm CD47 protein:protein interactions by Mass Spectrometry in chapter 5.

CD47 isoform transcripts were also detected in K562 and HL-60 in this chapter. The presence of all CD47 isoforms in these cell lines was confirmed (Figures 3.9 and 3.10). Interestingly, they showed that the most abundant isoform is isoform 2 with 72% and 64% respectively for K562 and HL60 cells. However, Reinhold *et al.* in 1995 screened different tissues and cells in humans and they confirmed the presence of isoform 2 in (keratinocytes, monocytes, macrophages, and nasal carcinoma cells); and in 2000 Oldenberg *et al* elucidated the function of CD47 as ‘a self-recognition’ antigen by binding to SIRP α in macrophages (Oldenberg et al., 2000).

Thus, the data suggested the isoform that responsible for this function is isoform 2 as it is the most abundant isoform not only in red blood cells but also the major isoform in erythroleukemia cell lines (K562, and HL-60) but to confirm this practically is hard at the moment as these isoforms in C-terminal domain which is an intracellular domain and there is no practical test could do that at least at the moment . Moreover, the most targeted aim for erythroleukemia cells is to keep growing and mobilising in the circulation without being tackled by the immune system (macrophages) (Jaiswal et al., 2009). Thus, CD47 isoform 2 is the most abundant isoform in K562 and HL-60 cells. Furthermore, the increased number of reads for CD47 in these cells were higher than normal red blood cells (Figures 3.6-3.10), which confirmed that CD47 is increased in erythroleukemia cells.

3.5.2 Western blot analysis discussion of CD47 isoforms in red blood cells:

After confirming the presence of all CD47 isoforms in erythroid cells on molecular base level using NGS (RNA-seq) and different RhD phenotypes, monoclonal antibodies against CD47 isoforms (2, 4, and 5) were designed to confirm the presence of these isoforms on protein base level in mature red cell membranes. As mentioned previously, although there is a great homology between CD47 isoforms (Table 3.4) we were still able to produce CD47 isoform specific monoclonal antibodies. However, Mordue *et al.* in 2017 published that CD47 isoform 2 is the only isoform that is present in erythroid cells and they used polyclonal antibodies to prove that point. Moreover, they used also the homology between isoform 3 and 4 in their work to conclude any results positive for isoform 4 is also positive for isoform 3 while they only shared 8 amino acids (KAVEEPLN) between each other. Furthermore, they also showed CD47 isoforms 2, 3, and 4 were present in the K562 cell line but completely missed CD47 isoform 5.

CD47 isoform 2 is the most abundant isoform in erythroid cells in a different RhD phenotype. Moreover, figure 3.12 shows that clearly CD47 isoform 2 is present in erythroid cells and this result was detected by using the western blot technique. Furthermore, isoform 4 is also present in RhD⁺ and RhD⁻ phenotypes; that was confirmed by using a specific CD47 isoform 4 monoclonal antibody (Figure 3.13). Although CD47 isoform 4 is not the most abundant isoform in erythroid cells but make a close percentage to CD47 isoform 2 in RhD⁻ phenotype (figure 3.9) on molecular basis; and we have confirmed the presence of CD47 isoform 4 by western blot. Moreover, CD47 isoform 5 is the least abundant CD47 isoform that is detected in erythroid cells, and we have confirmed its presence by western blot using CD47 isoform 5 specific isoform (Figure 3.14).

To summarise, CD47 isoforms (1-5) could be present ubiquitously in all cells that have CD47 expressed on its membrane, but it is varying between one type of cell and another. For instance, CD47 isoform 2 is the major CD47 isoform in erythroleukemia cells keeping them growing and proliferating to avoid phagocytosis. On other hand, CD47 isoform specific monoclonal antibodies will have the ability elucidate the role of these isoforms in red blood cells as we show the difference between CD47 isoform antibodies by using the immunoprecipitation technique to determine what proteins are pulled down by using these isoform specific antibodies in chapter 5.

4 Characterization of CD47 monoclonal antibodies to be a possible therapeutic agent for cancers:

4.1 Introduction:

CD47 was first recognized to be involved in red cell turnover in 2000 (Oldenborg et al), but shortly afterwards, it was recognized also as a regulator of red cell death (eryptosis) (Head et al., 2005). CD47 plays an important role in recognizing self-red blood cells (Oldenborg et al., 2000). However, to recognize self-cells in normal cells, CD47 interacts with macrophage SIRP-alpha. The interaction between CD47 and SIRP alpha in macrophages induces a phosphorylation pathway activation through the Src-homology 2 domain containing phosphatase 1 (SHP1), and this induces a “do not eat me signal” (Oldenborg et al., 2001). However, in cancer cells CD47 is upregulated (Jaiswal et al., 2009, Chao et al., 2012) which indicates that cancer cells may escape immune surveillance by upregulating CD47 to bind to SIRP-alpha in the macrophages. To block this mechanism, researchers have developed four different mechanisms (Majeti et al., 2009). The first was to directly blocking CD47 with anti-CD47 antibody to the cancer cells with blocking of SIRP alpha by anti SIRP alpha antibody or using SIRP alpha recombinant protein. This mechanism is dependent on phagocytes as the macrophage recognized these cells are lack of CD47 and remove it (Figure 4.1 A) (Chao et al., 2012, Majeti et al., 2009). The second mechanism is eradicating tumour cells through Fc-dependent mechanisms including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) by anti-CD47 antibodies (Chao et al., 2012, Kim et al., 2008). The third mechanism is that CD47 antibodies eradicate cancer cells by apoptosis (eryptosis in case of erythroid malignant cells) and it is dependent on the type of antibody used and where it binds to CD47 (will discussed more in this chapter)(Chao et al., 2012, Manna and Frazier, 2004). Finally, the fourth mechanism is the CD47 antibody will eradicate cancer cells by activation of dendritic cells (Figure 4.1). All these

mechanisms have been explored *in vitro* in mice and they showed considerable effective rate of successes (Chao et al., 2012, Latour et al., 2001, Matozaki et al., 2009).

Programmed cell death (apoptosis or eryptosis) is one of the mechanisms by which that eradicate cancer cells by monoclonal antibodies against CD47. The mechanism of induced programmed cell death (apoptosis) in cancer cells involves caspase pathway activation which will lead to a death cascade, membrane blebbing, and Phosphatidylserine (PS) flipping from the inner leaflet to the outer leaflet of the membrane (Shalini et al., 2015). Eryptosis which is the programmed cell death in erythroid cells, is shown to have similar properties of apoptosis except DNA degradation (Lang et al., 2012, Lang et al., 2006a). PS switching from the inner leaflet to the outer leaflet is detectable by annexin V FITC flow cytometry techniques.

Different murine CD47 monoclonal antibodies have been produced against CD47 by the International Blood Group Reference Laboratory (IBGRL), which are BRIC (32,122,124,125,126,168, and 211) (Table 4.1), and investigated whether they may induce different levels of PS exposure and they have the ability to ligate to CD47 in cancer cells. BRIC 126 is the most described in the literature, but all the other six monoclonal antibodies are poorly described. Currently, clinical trials have started using anti-CD47 as an anti-cancer therapy with an antibody called Hu5F9-G4. This antibody is produced by Forty-Seven Inc, USA, and the biggest challenge now is to prevent the side effect of causing anaemia as CD47 expressed in all cell types (Weiskopf, 2017).

Furthermore, different CD47 eryptosis level leads to differentiate between these antibodies by using Annexin V FITC assay to differentiate between these antibodies to determine whether using these mAbs are suitable as therapeutic agents for cancer therapy. IBGRL have produced seven different antibodies as mentioned earlier but none have been tested as a therapeutic anti-cancer agent.

Figure has been removed due to Copyright restrictions.

Figure 4.1: Four different proposed mechanisms to prevent CD47-SIRP alpha interaction.

A- Monoclonal antibodies bind to CD47 and induce apoptosis (eryptosis) and inhibit CD47 SIRP alpha pathway.

B- CD47 antibody will bind to CD47 and eradicate cancer cells by binding to CD47 and lead to activation of nature killer cells which will eradicate cancer cells.

C- Whether CD47 antibody or recombinant SIRP alpha will do this strategy, which is bind to CD47/SIRP alpha in the cancer cell and activate phagocytosis by macrophage.

D- CD47 antibodies will remove cancer cells by binding to CD47 and this will activate dendritic cell which will activate CD4 and CD8 T cells.

Adapted from (Chao et al., 2012).

4.2 Aims of study:

Confirm IBGRL monoclonal antibodies BRIC's (32,122,124,125,126,168, and 211) bind to CD47 in erythrocyte by using western blot. Moreover, characterize and differentiate between seven different monoclonal antibodies against CD47 using Annexin V FITC to measure the PS exposure levels in erythrocytes. Moreover, competitive binding assay were used to determine and characterize where these antibodies are binding to within the N-terminal domain of CD47 and whether they make an additive effect by combining two monoclonal antibodies together or they compete with each other for the same binding epitopes on CD47 N-terminal domain.

4.3 Results:

4.3.1 Western blot results:

All seven BRICs (32,122,124,125,126,168, and 211) CD47 murine monoclonal antibodies were tested against Rh⁺, Rh⁻ phenotypes ghost membrane samples to confirm that these antibodies bind to CD47 by western blot. All western blot results were done using a control antibody (BRIC10) which is anti-GPC to confirm the protein concentration used in different Rh phenotype samples (data not shown).

4.3.1.1 BRIC 32 Western blot:

BRIC 32 is a monoclonal antibody specific for CD47. However, it was used previously as an anti-CD47 antibody as well (Bruce et al., 2002). BRIC 32 blots with Rh⁺(R₁R₁), Rh⁻ samples showed a clear smear around 47 kDa with no other band were showed (figure 4.2).



Figure 4.2: Immunostaining of RBC membranes from different Rh phenotypes erythrocytes using BRIC 32.

10 µg of proteins were used in both samples under non-reducing conditions, Rh⁺ samples shows higher density of CD47. Also, CD47 was observed at 47-52 kDa as a smear because it is heavily glycosylated glycoprotein.

4.3.1.2 BRIC 122 Western blot:

10 µg of protein from ghost membranes with different Rh phenotypes were run using 1D gel electrophoresis prior to transfer onto PVDF membranes. Purified BRIC 122 was used at the concentration of 1:2500 as a primary antibody followed by rabbit anti-mouse horseradish peroxidase as a secondary antibody (figure 4.3). BRIC 122 with Rh- phenotype samples were shown less glycosylation effect compared to Rh+(R₁R₁). BRIC 122 results showed less glycosylated band around 47 kDa comparing to the one caused by BRIC 32.

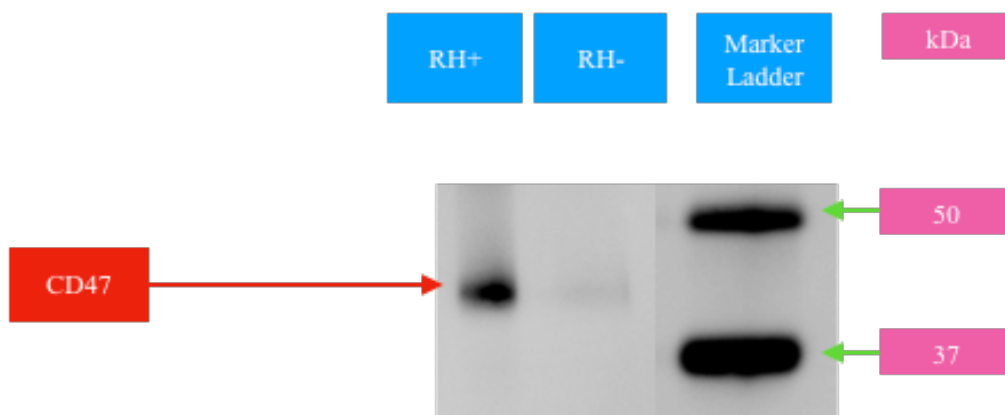


Figure 4.3: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 122.
Rh+ samples show higher density of CD47. Also, CD47 was observed at 47-52 kDa as a smear because it is heavily glycosylated glycoprotein.

4.3.1.3 BRIC 124 Western blot:

BRIC 124 results showed heavier immunostaining with Rh+(R₁R₁) samples compared to Rh- samples. However, 10 µg of protein from ghost membranes with different Rh phenotypes (figure 4.4).

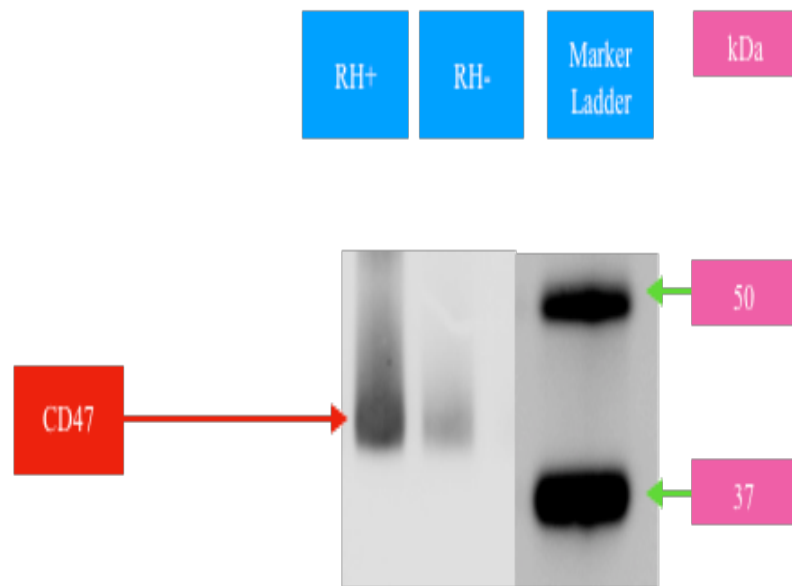


Figure 4.4: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 124.

CD47 was observed at 47-52 kDa as a smear because it is a heavily glycosylated glycoprotein.

4.3.1.4 BRIC 125 Western blot:

BRIC 125 results showed a very heavy smear with Rh⁺(R₁R₁) samples compared to the Rh⁻ samples (figure 4.5).

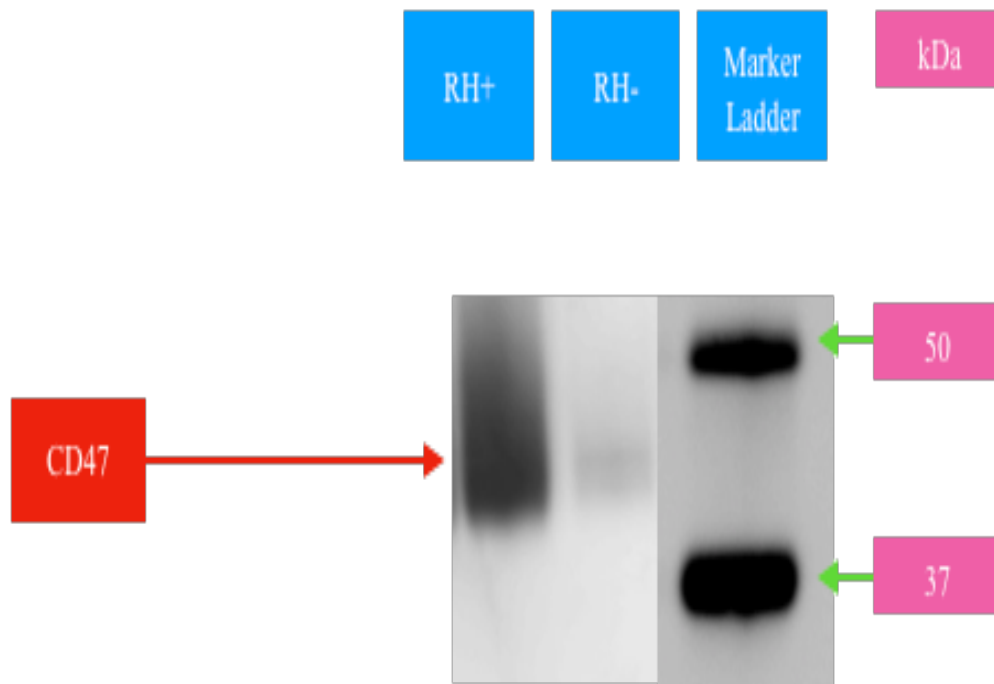


Figure 4.5: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 125.

CD47 was observed at 47-52 kDa as a smear because it is a heavily glycosylated glycoprotein.

4.3.1.5 BRIC 126 Western blot:

BRIC 126 is the most frequent monoclonal antibody used for CD47 in the literature. Moreover, the results of BRIC 126 with Rh⁺(R₁R₁) shows heavier immunostaining than Rh⁻ samples (figure 4.6).

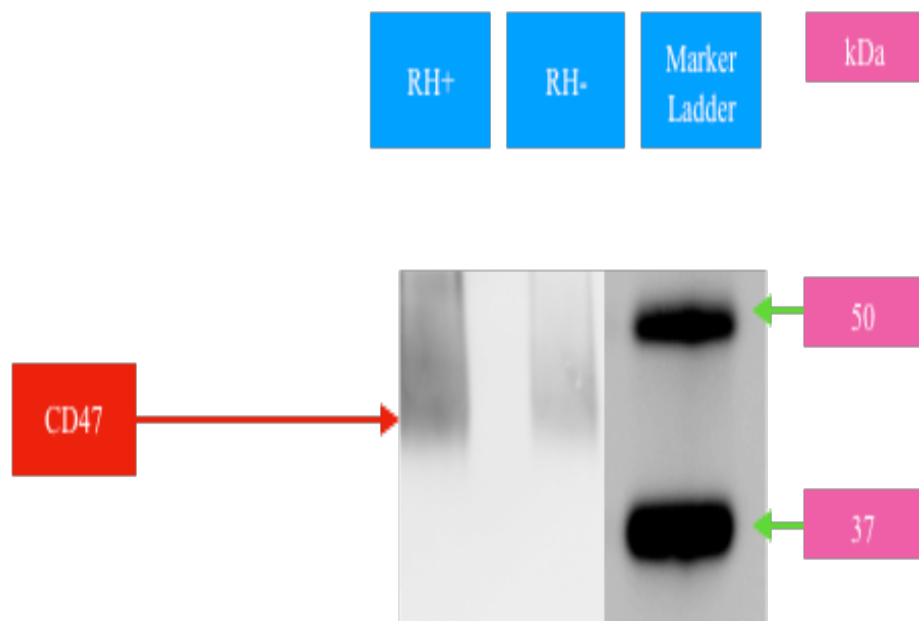


Figure 4.6: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 126.
CD47 was observed at 47-52 kDa as a smear because it is a heavily glycosylated glycoprotein.

4.3.1.6 BRIC 168 Western blot:

BRIC 168 results showed a heavier immunostaining band with Rh+(R₁R₁) membranes than the results with Rh- membranes. (figure 4.7).

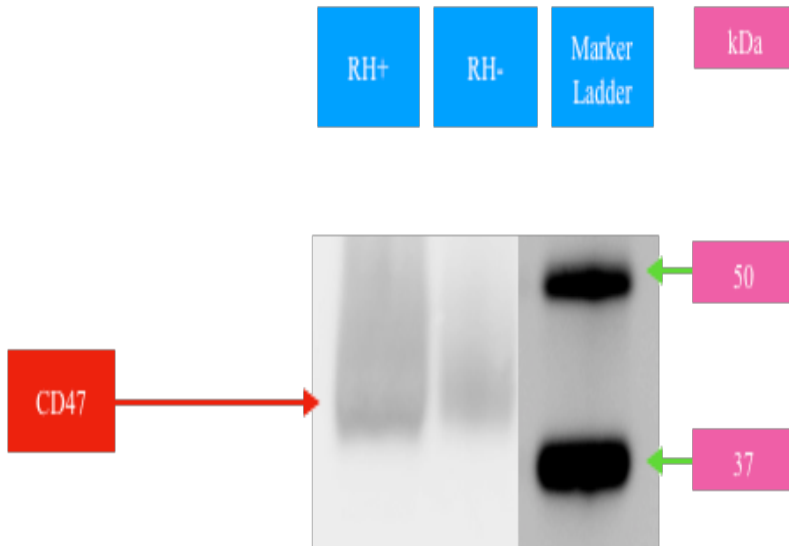


Figure 4.7: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 168.

CD47 was observed at 47-52 kDa as a smear because it is a heavily glycosylated glycoprotein.

4.3.1.7 BRIC 211 Western blot:

BRIC 211 is the only CD47 monoclonal antibody that shows almost the same degree of immunostaining with both Rh⁺(R₁R₁) and Rh⁻ membrane samples (figure 4.8).

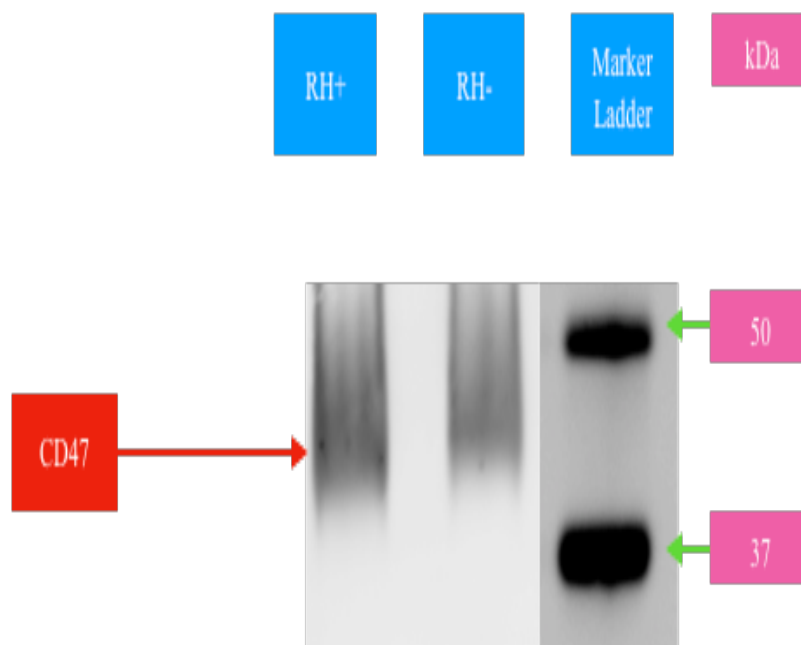


Figure 4.8: Immunostaining of RBC membrane from different Rh phenotypes erythrocytes using BRIC 211.

CD47 was observed at 47-52 kDa as a smear because it is a heavily glycosylated glycoprotein.

4.3.2 Flow Cytometry results using Annexin V FITC:

To determine PS exposure levels in red blood cells that were induced by CD47 monoclonal antibodies BRICs 32, 122, 124, 125, 126, 168, and 211 an annexin V fluorescein isothiocyanate (FITC) assay was used. Twelve biological samples were tested and each of these samples were tested in triplicate with a total number of 36 samples. The twelve biological replicates were; 4 Rh⁺ (R₁R₁), 4 Rh⁺ (R₂R₂), and 4 Rh⁻ (rr) phenotypes.

The results of antibodies incubated with erythrocytes cells showed different PS exposure levels (figure 4.10). the control used with no antibody at all. BRIC 32, and BRIC 122 showed the lowest eryptosis level with less than 10% of eryptotic erythrocytes. However, BRIC's (125, 126, and 168) showed a medium level of eryptosis with almost 20% eryptotic level of erythrocytes. BRIC 124, and BRIC 211 showed the highest eryptosis level in erythrocytes with almost 40% of eryptotic level.

4.3.2.1 Gating Strategy for different monoclonal antibodies PS exposure:

The assessment of PS exposure was carried out by using FACS-ARIA II. For example, negative control was set to have the minimal PS exposure. Then the assessment of other monoclonal antibodies was fixed as the negative control as shown in figure 4.9.

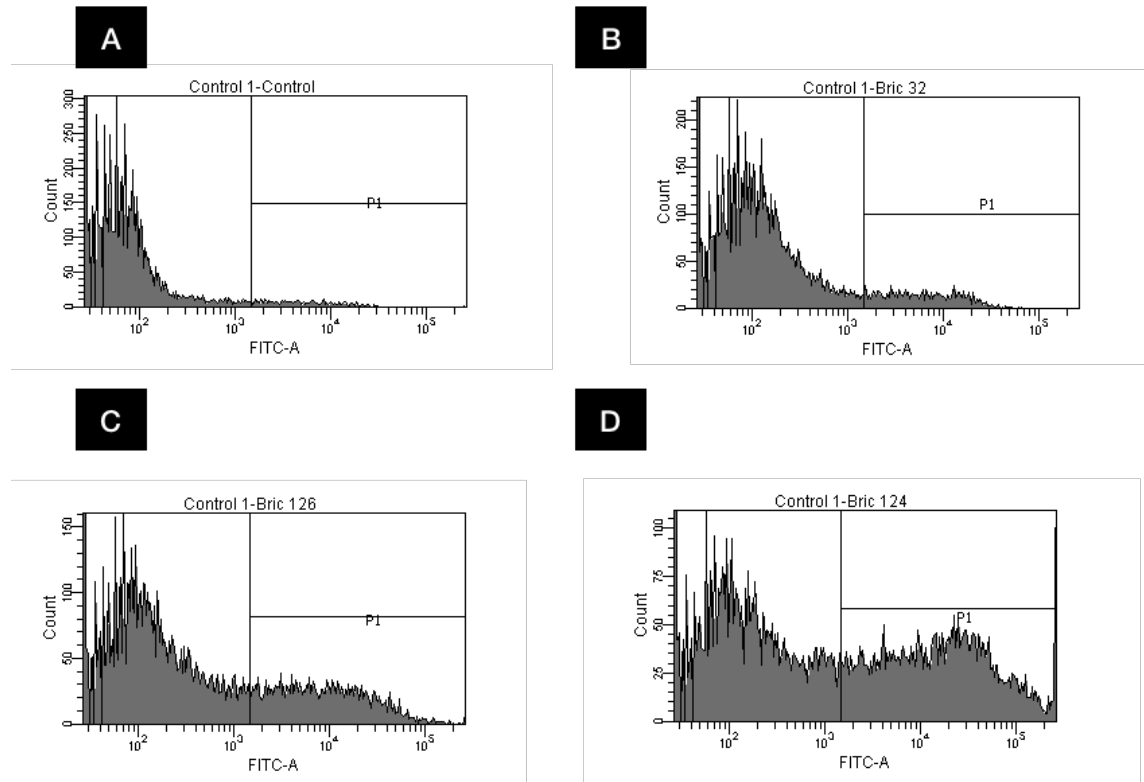


Figure 4.9:Flow Cytometry gating strategy.

- A- Shows the negative control example of PS exposure < 2% of control cells as there are no mAbs were added.
 - B- Shows an example of low PS exposure <10% of erythrocytes show PS exposure.
 - C- Shows an example of medium PS exposure <20% of erythrocytes show PS exposure.
 - D- Shows an example of high PS exposure <40% of erythrocytes show PS exposure
- In all images P1 indicates the population of cells were in count for PS exposure.

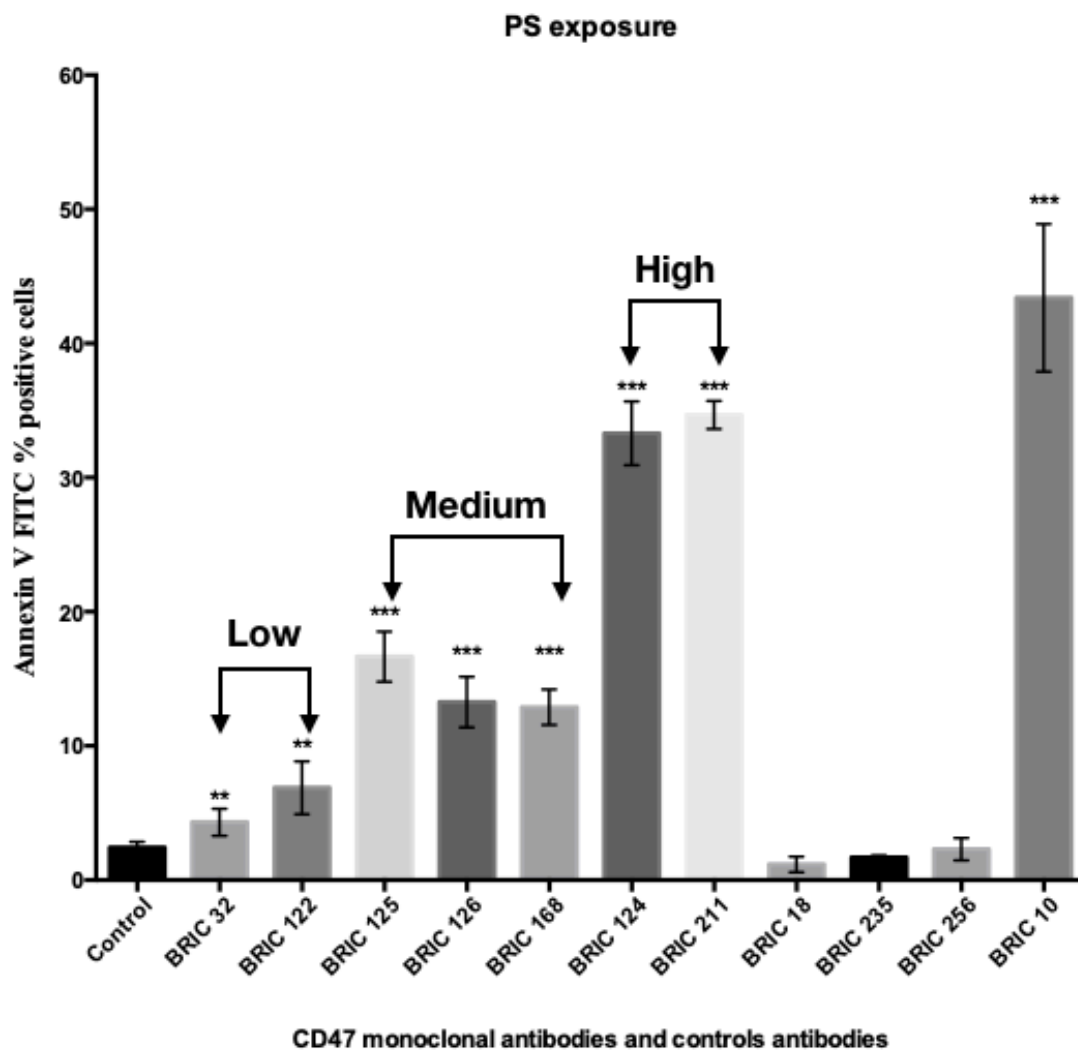


Figure 4.10:Flow Cytometry of erythrocytes from different Rh phenotypes. There are three different levels of PS exposure, BRIC 32,122 are induced the lowest resulting PS exposure in erythrocytes while BRIC 124, 211 are the highest. Each experiment was repeated three times, and statistical analysis was performed using paired student's t test by graph pad Prism. The statistical significance is indicated on the figure as follows (*) P<0.05, (**) P<0.01, (***) P<0.0001. Using the percentage of PS exposure is reflecting the number of cells were releasing PS that can be measured by flow cytometer (FACSARIA II).

4.3.2.2 Low PS exposure group includes BRICs 32 and 122:

BRIC 32 and BRIC 122 were classified as low eryptotic level to induce eryptosis as they showed in (figure 4.10). The competitive binding assay was established to characterize the binding site for CD47 monoclonal antibodies. As mentioned previously these antibodies bind to the N-terminal domain of CD47 but unknown if they compete with each other for the same CD47 epitope(s) figure 4.11.

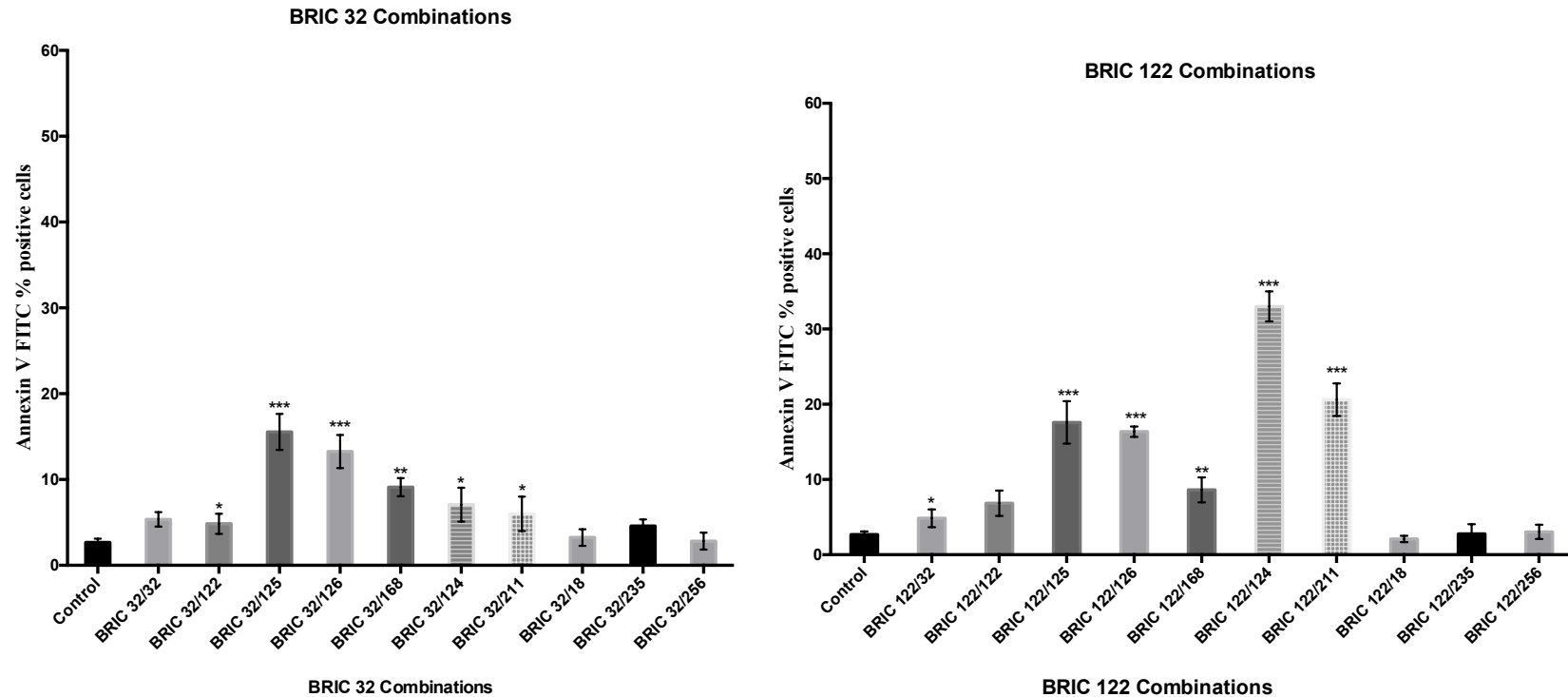


Figure 4.11: Flow Cytometry for combination results of BRIC 32, BRIC 122 from different Rh phenotypes.

Ligation of erythrocytes with different CD47 monoclonal antibodies and controls resulting PS exposure, which is detecting by annexin V-fluorescein isothiocyanate (FITC). Erythrocytes were incubated with two CD47 monoclonal antibodies or CD47 monoclonal antibody and isotype controls. Each experiment was repeated three times, and statistical analysis was performed using paired student's t test by graph pad Prism. The statistical significance is indicated on the figure as follows (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.0001$.

For BRIC 32 the most interesting results was how BRIC 32 blocks the eryptotic effect of BRIC 124 and BRIC 211 which are known to be among the high eryptotic antibodies while with BRIC 122 this effect is absent.

Firstly, BRIC 32 results showed how this antibody could be suitable for cancer therapy to alleviate the side effect of haemolytic anaemia. The results of BRIC 32/BRIC 32, which is the control, caused 4% of eryptosis in erythrocytes. The BRIC32/BRIC 122 combination results showed a reduced eryptotic effect of BRIC 122 even though the effect of BRIC 122 is not significantly different from BRIC 32 (higher by 2 % (6.8%)). Interestingly BRIC 32 reduces the eryptotic effect of high eryptotic antibodies (BRIC's 124, 211). On the other hand, the medium eryptotic antibodies (BRIC 125, 126 and 168) were unaffected by BRIC 32 as they shown higher eryptotic level than the control which is BRIC 32 with itself. So, BRIC 32 is not blocking the eryptotic level of medium eryptotic antibodies (figure 4.11).

BRIC 122 results suggest that this antibody could be a good candidate for cancer therapy, or in combination with other anti-CD47. Starting with the control which is BRIC122 with an extra amount of BRIC 122 after 30 minutes of incubation; the results showed 6.8% eryptotic level of erythrocytes.

Then, BRIC 122 with medium eryptotic antibodies (BRIC's 125, 126, 168) does not show any effect against this category as these antibodies diminished the eryptotic effect of BRIC 122. In fact, BRIC122/BRIC 168 result showed the eryptotic effect were reduced of BRIC 168 which indicates they were competing with each other for the same epitope but still BRIC 168 can induce its medium non-significant eryptotic effect (~10%).

The most noticeable difference between BRIC 32 and BRIC 122 is that BRIC 122 along with high eryptotic antibodies (BRIC's 124, 211), does not reduce this eryptotic effect which indicates this antibody possibly binds a different epitope to BRIC 32. However, the effect of BRIC 122 (X-antibody) with BRIC 32 (Y-antibody) as mentioned earlier indicates that BRIC 32 reduces the effect of BRIC 122 (figure 4.10).

4.3.2.3 Medium PS exposure induced by monoclonal anti-CD47 BRICs 125, 126, and 168:

In this category there are three CD47 monoclonal antibodies which are BRIC's (125, 126, and 168).

BRIC 126 is the most widely described antibody within the three antibodies and is commonly used in CD47 studies while BRIC 125, BRIC 168 are rarely used. In this study, all of these antibodies appear to show similar levels of eryptosis effect in erythrocytes (figure 4.10).

Firstly, BRIC 125 previous incubation results showed that this antibody is capable of eliminating the BRIC 32 effect (as BRIC 32 on its own causes 7% eryptosis) but BRIC 125 with BRIC 32 cause (~17%) similar to BRIC 125 on itself effect. BRIC 125 has also an additive effect with BRIC 122 while interestingly it reduces the BRIC 124 induced levels of eryptosis (~20%) while normally BRIC 124 causes (~35%). BRIC125/BRIC 126 results showed that they possibly compete for the same epitopes. BRIC 125 increases the BRIC 168 eryptotic effect and noting that the eryptotic level is significantly increased to reach almost 20%. Finally, BRIC 125 reduces the high eryptotic level of BRIC 211 (~16%) (figure 4.12).

Secondly, BRIC 126 results showed that it diminished the eryptotic level of BRIC 32 as BRIC 32 on its own causes 7% eryptosis while with BRIC 126 causes a similar effect of BRIC 126 on its own (17%). BRIC 126 with BRIC 122 there is a slightly increased eryptosis level (additive effect ~20%) than the results shown by BRIC 126 individually (~17%). Furthermore, with BRIC 124 reduces the high eryptotic effect of this antibody

and with BRIC 125 as mentioned earlier, they may compete with each other for the same epitopes. BRIC 126 with BRIC 168 the results showed that there is an additive effect (~25%). Finally, BRIC 126 showed a reduction of BRIC 211 eryptotic level as the result was only 20% of eryptosis whilst BRIC 211 normally causes around 40%.

Thirdly, BRIC 168 results showed that BRIC 32 used as antibody Y reduced the eryptotic effect of BRIC 168 which leads to reducing the eryptosis level to less than 10%. Also, BRIC 122 used as antibody Y showed the same effect with BRIC 168 as the eryptotic level decreased to less than (10%). Furthermore, BRIC 168 reduces the eryptotic level of BRIC 124 statistically and reduces the eryptotic level to 10%. BRIC 125 has increased the eryptotic effect of BRIC 168 level to ~20%. Furthermore, BRIC 168 and BRIC 126 showed an additive effect on erythrocytes by inducing (~25%) eryptotic level. Finally, BRIC 168 reduces BRIC 211 eryptotic effect but also shows a less eryptosis level than a BRIC 168 control.

Similar to low eryptotic monoclonal antibodies, the combinations experiments were applied to all the medium PS exposure eryptotic monoclonal antibodies.

BRIC 125 combination results are showing blockage of high eryptotic monoclonal antibodies BRIC's (124, and 211) while with BRIC 126 the effect of BRIC 125 is abolished which indicates that BRIC 126 is blocking the binding site of BRIC 125. On other hand, BRIC 125 is also blocking the effect of low eryptotic monoclonal antibodies BRIC's (32, and 122) (figure 4.12).

Moreover, BRIC 126 combination results show a dominant effect on all other CD47 antibodies except BRIC 125.

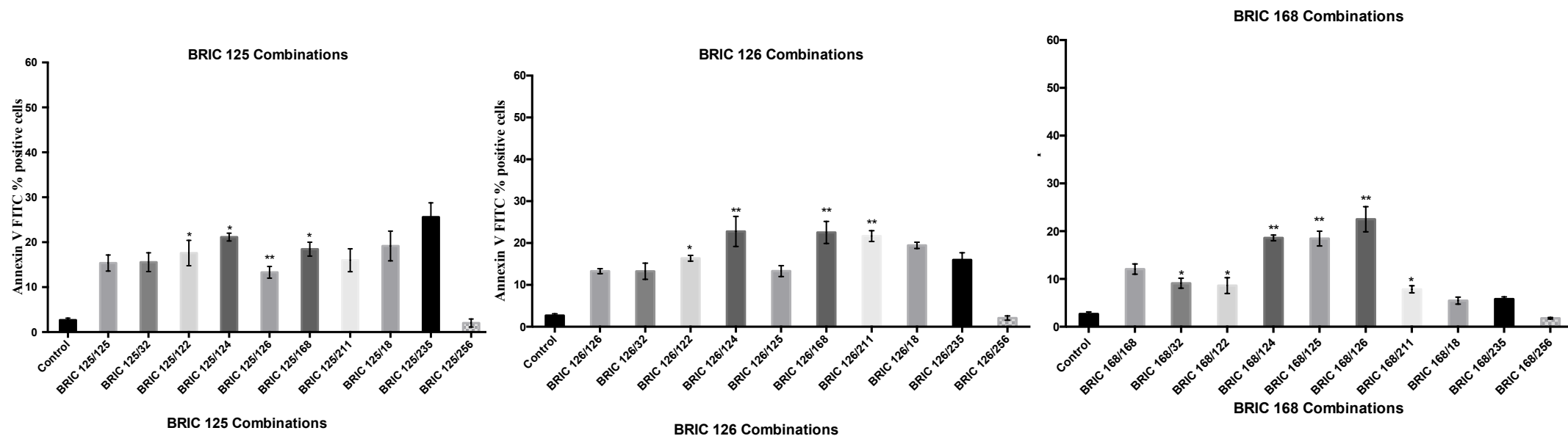


Figure 4.12: Flow Cytometry for combination results of BRIC 125, BRIC 126, and BRIC 168 from different Rh phenotypes.

Ligation of erythrocytes with different CD47 monoclonal antibodies and controls resulting PS exposure, which is detecting by annexin V-fluorescein isothiocyanate (FITC). Erythrocytes were incubated with two CD47 monoclonal antibodies or CD47 monoclonal antibody and isotype controls. Each experiment was repeated three times, and statistical analysis was performed using paired student's t test by graph pad Prism. The statistical significance is indicated on the figure as follows (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.0001$.

4.3.2.4 High PS exposure includes BRICs 124, 211:

BRIC 124 and BRIC 211 show high level of eryptosis in erythrocytes (~30%). However, these results showed that these antibodies do not induce high PS exposure in the presence of other antibodies such as BRIC 32.

To start with BRIC 124 results, the data showed that BRIC 32 reduces BRIC 124 eryptotic effect (~8%); but interestingly with BRIC 122 the effect of BRIC 124 is still effective (~30). However, not only BRIC 32 is reducing BRIC 124 eryptotic effect but also medium eryptotic antibodies (BRIC's 125, 126, and 168). BRIC 211 is also reducing the eryptotic effect of BRIC 124 although the eryptosis level is still highly induced (~27%). Finally, BRIC 256, which was considered as an isotype control, is switching off the eryptosis effect of BRIC 124 to levels of controls. This startling effect is present in all anti-CD47 combination experiments, the use of the anti-GPA BRIC 256 appears to be a “master switch” to eliminate all anti-CD47 induced PS exposure

BRIC 211 results showed interesting data. BRIC 211 produced almost 30% of eryptosis in erythrocytes. However, BRIC 32 is reducing this effect by dropping the eryptosis level to less than 10%. Furthermore, BRIC 122 reduces the eryptotic level of BRIC 211, but not as much as BRIC 32 (~20%). BRIC 211 reduces the eryptotic level of BRIC 124 and switching off its effect. However, BRIC 125 is switching BRIC 211 eryptotic effect off and also BRIC 126 (~16%, ~20%) respectively. Finally, the results of BRIC 168 with BRIC 211 showed that they compete with each other for the same epitope but neither of them able to dominate its eryptotic effect on erythrocytes.

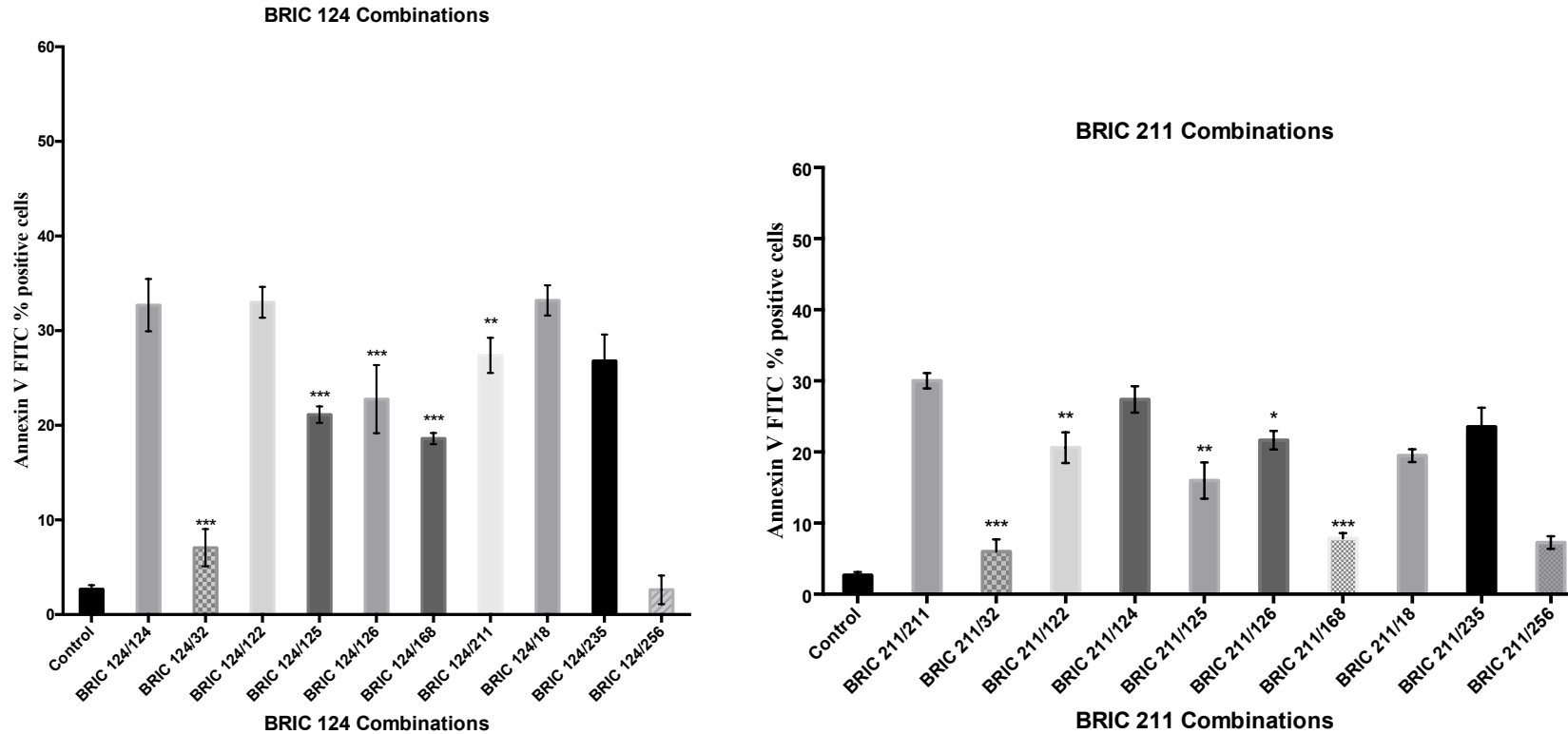


Figure 4.13: Flow Cytometry combination results of BRIC 124, and BRIC 211 from different Rh phenotypes.

Ligation of erythrocytes with different CD47 monoclonal antibodies and controls resulting PS exposure, which is detecting by annexin V-fluorescein isothiocyanate (FITC). Erythrocytes were incubated with two CD47 monoclonal antibodies or CD47 monoclonal antibody and isotype controls. Each experiment was repeated three times, and statistical analysis was performed using paired student's t test by graph pad Prism. The statistical significance is indicated on the figure as follows (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.0001$.

Table 4.1: Summary of the combinations Results:

	BRIC 32 (Low)	BRIC 122 (Low)	BRIC 124 (High)	BRIC 125 (Medium)	BRIC 126 (Medium)	BRIC 168 (Medium)	BRIC 211 (High)	BRIC 256 (Off)
BRIC 32		Low	Low	Medium	Medium	Medium	Low	Off
BRIC 122	Low		High	Medium	Medium	Compete each other (ND)	High	Off
BRIC 124	Low	High		Medium	Medium	Medium	High	Off
BRIC 125	Medium	Medium	Medium		Compete each other (ND)	Medium	Medium	Off
BRIC 126	Medium	Medium	Medium	Compete each other (ND)		Additive effect	Medium	Off
BRIC 168	Medium	Compete each other (ND)	Medium	Medium	Additive effect		Compete each other (ND)	Off
BRIC 211	Low	High	High	Medium	Medium	Compete each other (ND)		Off
BRIC 256	Off	Off	Off	Off	Off	Off	Off	

*Black squares indicate controls.

*ND: Not Determined

Off means that the cryptotoc effect is switched off.

4.3.3 Trypsin Treated erythrocytes and anti-CD47 incubation followed by Annexin V FITC treatment Results:

To characterize the seven different monoclonal antibodies against CD47 BRIC's (32, 122, 124, 125, 126, 168, and 211) prior trypsin treatment of the erythrocytes was used as within our group we were able to characterize anti-GPC monoclonal antibody (BRIC 10) by using trypsin (Head et al., 2005a) as the binding epitope of BRIC 10 was cleaved when trypsin was added to erythrocytes. As a result, BRIC 10 binding site were discovered as there was only one known region for trypsin in the glycophorin C (N-terminal domain).

Triplicates of three different blood donor samples were used (Rh⁺ (R₁R₁), Rh⁺ (R₂R₂), and Rh⁻ (rr)) at the concentration of $1 \times 10^6/200\mu\text{l}$ erythrocytes; they were incubated with 10 μl of 100 mg/ml trypsin for 3 hours at 37 °C. Then, 10 μl of 1 mg/ml purified monoclonal antibody or corresponding control were added (Table 2.1). However, as a positive control for this experiment BRIC 10 (GPC) monoclonal antibody was used.

The results of trypsin treated erythrocytes followed by incubation with CD47 monoclonal antibodies showed there are no effect of trypsin on PS exposure with CD47 monoclonal antibodies except with BRIC 168 and BRIC 211 (figure 4.14). BRIC 168 caused (~13%) of PS exposure and after the trypsin treated the percentage dropped to (~7%) while BRIC 211 the PS exposure level was (~35%) and dropped to (~20.5%) with trypsin treated erythrocytes.

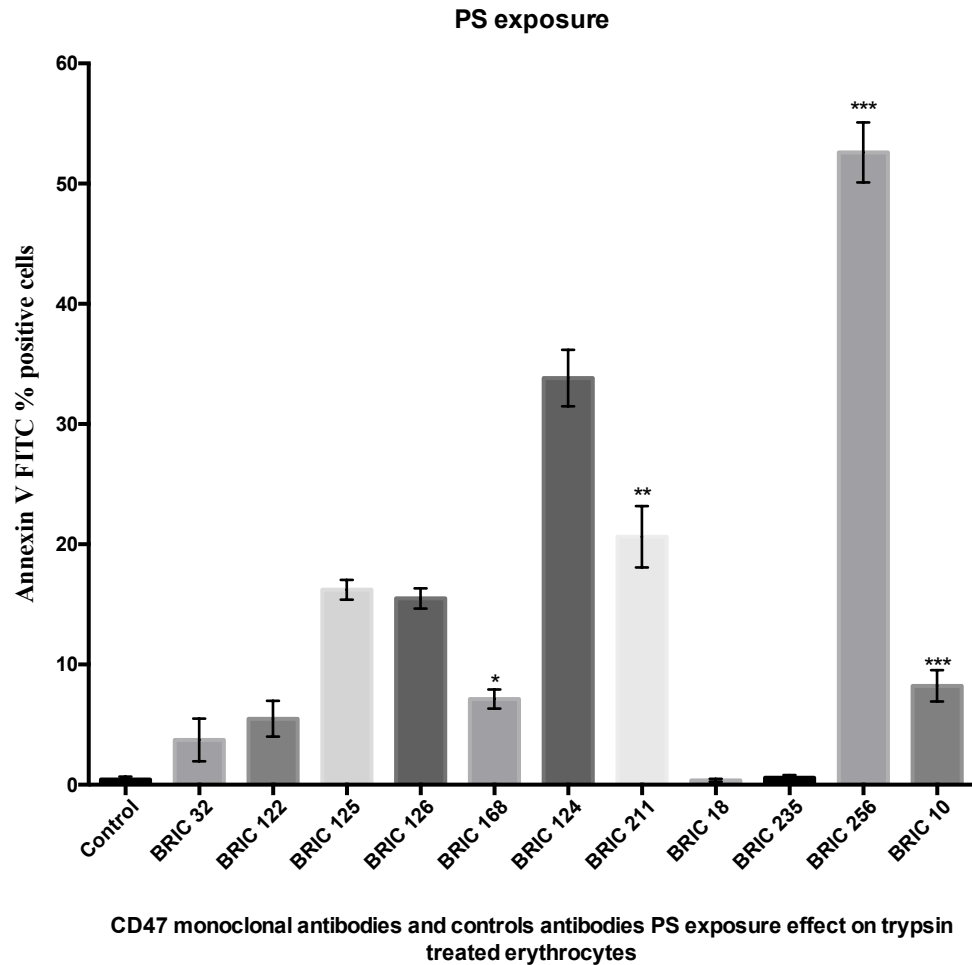


Figure 4.14: Flow Cytometry of trypsin treated erythrocytes from different Rh phenotypes.

Ligation of erythrocytes with different CD47 monoclonal antibodies and controls resulting PS exposure, which is detected by annexin V-fluorescein isothiocyanate (FITC). Erythrocytes were incubated with trypsin for 1-3 hours before CD47 monoclonal antibodies and isotype controls were added. However, BRIC 10 which is specific to GPC were used as positive control. BRIC 256 with trypsin results showed an increase the apoptotic level to ~60%. There The statistical significance is indicated on the figure as follows (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.0001$ and it was done against the antibody itself without trypsin treated erythrocytes (figure 4.9).

4.4 Discussion:

CD47 is a transmembrane receptor with 5 membrane-spanning segments and has a molecular weight of 47-52 kDa on SDS-PAGE. Eryptosis is known as programmed erythrocyte cell death (Lang et al., 2005b). PS exposure is one of the markers of eryptosis when PS flips from the inner layer of red cell membrane to the outer layer. In 2005, Head et al, suggested that CD47 plays an important role in the PS signalling pathway of red blood cells (Head et al., 2005b). We used this approach for characterization of monoclonal anti-CD47 antibodies and found that they induced a significant erythrocyte cell death compared to control samples.

In 1991, Gardner et al, have characterized these antibodies with two different assays (named as quantitative binding assay and competitive binding assay) (Gardner et al., 1991). They concluded that all these antibodies bind to the IgV in N-terminal domain of CD47. However, it was the only previous study has the all seven monoclonal antibodies in one experiment.

Moreover, the different of PS exposure level reflects the different binding affinities and epitopes specification of these monoclonal antibodies in erythrocytes but these differences confirm that the PS signals is mediated through CD47.

Actually, the combination results were showed an unexpected result with BRIC 256 which is binding to Glycophorin A (GPA). The data showed that GPA is a “master off switch” of PS exposure caused by CD47 monoclonal antibody ligation regardless whether it was (low, medium, or high) PS exposure levels.

Moreover, although trypsin was very sufficient when characterizing BRIC 10 for glycophorin C (GPC) as it cleaves the Arginine-Glycine peptide bond in the N-terminal domain of GPC (Head et al., 2005a); but with CD47 as there is a cleavage site for trypsin (figure 4.13) unfortunately all the seven monoclonal antibodies against CD47 still can

bind to the N-terminal domain and induce its PS exposure effect, possibly suggesting that trypsin is unable to cleave the N-terminal domain of CD47. However, BRIC 168 and BRIC 211 showed a significant reduction of PS exposure, but it is not completely ineffective which may indicate these two antibodies have epitopes that are sensitive to trypsin. However, trypsin treated erythrocytes with BRIC 256 (anti-GPA) results showed a significant increase in the eryptosis level. This result was novel as it indicates the master switch off epitopes were destroyed by trypsin (Figure 4.13).

Furthermore, since 2009; studies have focussed on CD47 as a therapeutic agent against most cancer cells as the cancer cell upregulates CD47 to avoid innate immunity (phagocytosis) by binding to SIRP alpha (Jaiswal et al., 2009, Majeti et al., 2009). However, there are some studies suggesting using CD47 monoclonal antibodies in combinations with anti-cancer drugs to stimulate the innate immunity for phagocytosis and eradicate cancer cells (Chao et al., 2010); the problem with this approach or the other suggestion which is also include CD47 monoclonal antibodies as a therapeutic agent against cancer cells are that CD47 monoclonal antibodies will cause Anaemia when it used as therapeutic agent in clinical trials (Advani et al., 2018) . The only used antibody for this objective was BRIC 126 which have identified here as a medium PS exposure antibody. Thus, it would be strongly suggested that BRIC 32 is the best monoclonal antibody to be used as a therapeutic agent against CD47 as it is binding to CD47 and it is not enhancing the PS exposure level. It remains to be determined if this antibody blocks the CD47-SIRP-alpha interaction.

Recently, the use of CD47 as a therapeutic agent is now under clinical trials (Liu et al., 2015); in which CD47 antibody with anti-cancer therapeutic agents but as mentioned early there are some obstacles are prohibiting using this strategy such as Anaemia. However, their antibody that they used for that purpose was called (Hu5f9-G4) which is a humanized monoclonal antibody against CD47 but unfortunately

when they have used it *in vivo* in mice with combination of other anti-cancer drugs, they could not switch off the side effect of using CD47 monoclonal antibody negative impact on normal cells which is leading to Anaemia. Hu5f9-G4 were identified as anti-CD47 monoclonal antibody that bind in the N-terminal domain and has similar effect of BRIC 126. Then, we strongly suggest that using humanized BRIC 32 instead for that purpose. Perhaps more valuable data from our combination results (figures 4.10, 4.11, and 4.12) that showed BRIC 256 (anti-GPA) is a “master switch off” of the CD47 eryptotic effect; this antibody could therefore be used in combination with one or more anti-CD47 with anti-cancer drugs to eliminate the negative side effect of CD47 monoclonal antibody that induce eryptosis for normal cells.

Furthermore, using CD47 monoclonal antibodies is not only effective in erythroid cancers, but also in solid tumours as well (Willingham et al., 2012). This group has used BRIC 126 in their study to eradicate solid tumour cells. Moreover, they have also found that using CD47 as a therapeutic agent will also prevent tumour metastasis, but the toxicity level was unacceptable due the temporary Anaemia that caused by using CD47 monoclonal antibodies.

Finally, Characterization of CD47 antibodies important not only to transfusion science by understanding the mechanism of red cell eryptosis and the important role of CD47 by interaction with SIRP α in normal haemostasis and in senescent red blood cells, but also in immune-oncology science as our understanding of its function has evolved from an integral associated protein to a regulator of programmed cell death whether eryptosis or apoptosis.

5 Role of CD47 in Red Cell turnover and Novel Interactions to Elucidate CD47 Functions:

5.1 Introduction:

The erythrocyte membrane contains more than 100 different membrane proteins to facilitate membrane function and to provide structure through interactions with membrane skeletal components. These proteins are essential to manage different tasks such as gas transportation, signal pathway transduction, and glycolysis, membrane transport of solutes, complement control, cell-cell interactions etc; due to these different tasks and also the circulation between different capillary sizes, the 8µm erythrocyte size membrane is dynamic and configurable which may lead to different proteins doing multiple functions depending on the location of the erythrocyte in the circulation.(Tse, 2001, Yawata, 2006). One of these proteins is CD47 which has been reported to interact with the Rh membrane complex (Avent and Reid, 2000), Band 3 complex, and protein 4.2 (Mouro-Chanteloup et al., 2003, Bruce et al., 2002) (Figure 5.1).

The molecular mechanism for the arrangement of protein complexes of erythroid CD47 is poorly understood. The direct interactions between membrane proteins, membrane skeletal proteins and cytosolic proteins with CD47 have been poorly characterized in the erythrocyte. CD47 is known to interact with the Rh complex and protein 4.2; actually, CD47 is notably reduced in Rh_{null} and protein 4.2 deficient erythrocytes (Avent et al., 1988, Bruce et al., 2002) (Figure 5.1).

CD47 has five known isoforms, each with a different C-terminal tail and therefore each CD47 isoform can potentially bind different proteins. CD47 isoform 1 seems to be the most abundant in keratinocytes, whilst isoform 2 is the most abundant in erythrocytes (chapter 3). Furthermore, isoform 3 appears the most abundant isoform in macrophages whilst isoform 4 is the most abundant in neurocytes, intestine, and testis (Brown and Frazier, 2001, Reinhold et al., 1995). However, till now there has been no further research

on isoform 5 expression, despite the fact that it has a different C-terminal peptide sequence compared to the others. Thus, CD47 isoforms 2, 4, and 5 are the candidates for this analysis as these isoforms bind PLICs (protein linking IAP to cytoskeleton). PLIC 1 which is mainly represented by ubiquitin 1 interacts with CD47 in ovarian cells to anchor vimentin filaments to the plasma membrane (Wu et al., 1999); but it is still unknown if this interaction is isoform specific or not.

There are three different categories of CD47 antibodies that probably bind to different CD47 epitopes within the extracellular erythroid IgSF N-terminal domain causing different phosphatidylserine (PS) exposure as described in chapter 4. The purpose to explore whether these three distinct groups of antibodies showed differential binding to other red cell components. This is hypothesized based on the novel finding of differential PS-exposure with these monoclonal antibodies (mAbs). The aim is to further characterize these interactions, with possible clues of downstream pathways they illicit in the mature circulating erythrocyte. Furthermore, this thesis is the first to describe the production and characterization of isoform specific anti-CD47 monoclonal antibodies by mass spectrometry. For this analysis, BRIC 32 were choose as it induced low PS exposure, BRIC 126 as it induced medium PS exposure, and BRIC 124 as it induced high PS exposure.

To elucidate the protein-protein interactions in this study, Liquid Chromatography-Mass Spectrometry (LC/MS) was used. Protein samples are first run on SDS-PAGE then excised and treated with trypsin before loaded to MS. These tryptic peptides are identified by sequencing, and then mapped to the human protein sequence database. This technique has been developed in the recent years and used as a gold standard technique to identify the peptides in a biological sample (Bereman et al., 2008, Yates et al., 2006). The principal of the orbitrap mass analyser is based on the orbital trapping of ions. Furthermore, ions particles in the biological samples that are injected into the orbitrap,

which consists of an inner (central) and an outer electrode, are processed in a quadrupole logarithmic electrostatic potential. The injected ions cycle around the central electrode and at the same time oscillate along the horizontal axis (Hu et al., 2005). Thus, Orbitrap is able to detect the different tryptic peptides by complicated logarithmic process. The results produced by Orbitrap were then aligned to *Homo sapiens* protein database for downstream analysis.

This approach was used to identify the proteins that bind to CD47 immunoprecipitated (IP) samples from solubilized red cell membranes with anti-N-terminal CD47 antibodies, or C-terminal antibodies (isoform-specific antibodies). The anti-C terminal IPs were thus used on pre-prepared red cell membrane ghosts in order to facilitate the access of the Mab to the cytoplasmic-localised C-terminus.

Figure has been removed due to Copyright restrictions.

Figure 5.1: 2D demonstration of Band 3 complex and 4.1R complex in erythrocytes:

This diagram shows the Band 3 and 4.1R complexes in erythrocytes. The Band 3 complex includes interaction with Ankyrin, Glycophorin A, LW, the Rh complex, protein 4.2, and CD47. The interaction with CD47 is thought to be mediated by protein 4.2. However, the 4.1R complex also shows an interaction between band 3, 4.1R protein, Glycophorin C, Dematin, p55, and Kell. The erythrocyte membrane is dynamic, so these interactions could change according to the situation of erythrocytes. Taken from (Salomao et al., 2008)

5.2 Aims of Study:

The aim of this study was to use Mass Spectrometry (MS) to analyse CD47 binding protein partners and to identify protein: protein interactions. However, many CD47 protein binding partners have been recognized in the last decade, but the data presented in this chapter includes a novel approach as previously mentioned based on the different classes of CD47 mAb reactivity.

5.3 Results

Two units of red cell concentrate of phenotype were Rh⁺ (R₁R₁) and Rh⁺ (R₂R₂) were obtained from the NHSBT (Filton, Bristol) with ethical approval. The first step of preparation of the samples was to aliquot them in 5 ml Falcon tubes and incubate them with the appropriate antibody prior to extraction of the proteins. For the N-terminal CD47 study, BRIC 32, BRIC 124 and BRIC 126 (described earlier in this thesis Chapter 4), as different levels of PS expression induced by binding of the antibodies to their epitopes. BRIC 32 induces low PS exposure in erythrocyte whilst BRIC 124 induces high PS exposure, BRIC 126 induces medium level of PS exposure in erythrocyte. Moreover, for CD47 C-terminal domain, isoform 2,4, and 5 antibodies were used which have been reported earlier in this thesis (Chapter 3) for CD47 C-terminal antibody analysis.

For quality control of our results we have also selected controls for each of these N-terminal antibodies as described in table 2.1 according to the IgG subclass.

The C-terminal antibodies, as described in chapter 3, have not had their IgG subtype classified yet. So, for that reason a negative control was chosen which only contained protein G-sepharose incubated with the donor's samples.

After the incubation of the samples with the appropriate antibody, immunoprecipitation method was used (see Materials & Methods section 2.2.1.1 and 2.2.1.2). Then, 1D SDS-PAGE was performed for all samples and for each biological replicate every antibody was done in triplicate. Soon after the 1D gel separation the whole lane of the gel was excised by a sharp blade. These samples were then prepared for Mass Spectrometry analysis by using an in gel tryptic digestion method (Figure 5.2).

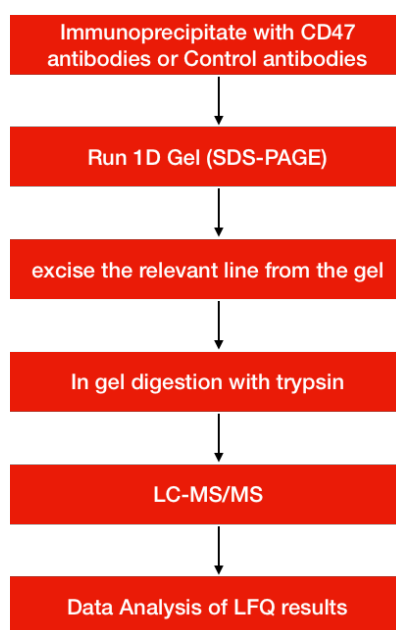


Figure 5.2: Flow chart for mass spectrometry analysis of CD47 immunoprecipitation

Finally, after analysis of the digested samples by LC-MS/MS, the data were aligned to *Homo sapiens* protein database and exported into Excel for further analysis.

The first step in the analysis of the data in Excel was to take the averages of the Label Free Quantification (LFQ) intensities for each protein across the replicates for each antibody. LFQ intensities are normalised values whereas the peptide counts are dependent on the molecular mass of the protein (Cox et al., 2014, Goeminne et al., 2016). After the averages were calculated, the next step was to divide the LFQ intensities for the highest abundant protein in the sample of interest, for example the CD47 protein in immunoprecipitated (IP) sample with BRIC 126 antibody, the LFQ intensity of this protein in BRIC 126 sample divided by LFQ intensity of CD47 protein in the control antibody sample (BRIC 235). Finally, if this ratio of the highest abundance was 2-fold or more in the BRIC 126 IP sample that indicate the chosen protein was upregulated in the BRIC 126 IP compared to the control IP (BRIC 235) (personal communication with DR. Vikram Sharma).

For the C-terminal CD47 antibodies, as they were novel specificities, we have compared the results of each isoform to the other isoforms to have a clear indication about which proteins bind which isoforms. To date, CD47 isoform 4 has the largest C-terminal tail and may be expected to bind more protein species.

5.3.1 The binding proteins of CD47 using the N-terminal antibodies:

The results from Mass Spectrometry were given as label free quantification (LFQ) values, which depend on the molecular abundance of the peptides by Mass Spectrometry. However, to validate these results, triplicates of each biological replicate samples were processed. For instance, the three used antibodies (BRIC 32, BRIC 124, and BRIC 126) were shown different PS exposure due to binding to different epitopes of CD47. Moreover, the results with using IP from these antibodies (BRICs 32, 124, and 126) , were shown slightly different protein profiles within the IPs indicating that CD47 binding in erythrocytes may vary in response to antibody binding used as the erythrocyte membrane is dynamic.

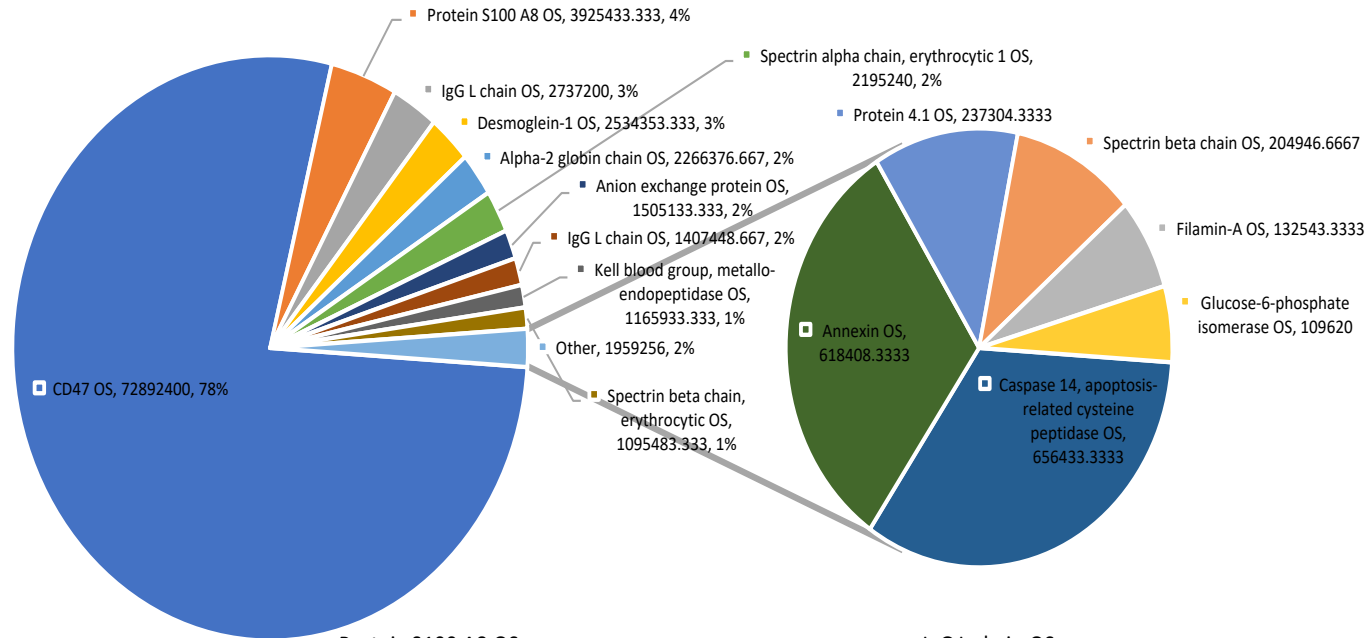
5.3.1.1 BRIC 32: low PS-inducing anti-CD47

The similar protein results from the BRIC 32 IP samples were averaged on both biological replicates and were done in triplicate. There was minimal variation between the samples (statically insignificant). The summary of the full list of proteins that binds to CD47 by using BRIC 32 antibody in IP sample is shown in figure 5.3.

Furthermore, the results showed as expected that the most abundant protein is CD47 (78%) and can give a measure of internal control to assess the molar amounts of other binding partners in the IPs Figure 5.3 (it is important to make sure mass spectrometry results confirm that this antibody is specific for CD47 protein). BRIC 32 IP sample showed Protein S100 (A8), (which is has a role in Ca₂ transport, see later), was expressed at 19% mol/mol and Desmoglein 1 was expressed at 12% mol/mol. Also, alpha and beta spectrin were expressed at 11% mol/mol. Band 3 protein is also present (7% mol/mol). Finally, Protein 4.1R is also highly expressed in CD47 BRIC 32 IP (1% mol/mol) only found low levels (1% mol/mol) of protein 4.2 in these IPs.

A

CD47 (BRIC 32) IP Proteins Profile



- CD47 OS
- Desmoglein-1 OS
- Anion exchange protein OS
- Spectrin beta chain, erythrocytic OS
- Protein 4.1 OS
- Glucose-6-phosphate isomerase OS
- Protein S100 A8 OS
- Alpha-2 globin chain OS
- IgG L chain OS
- Caspase 14, apoptosis-related cysteine peptidase OS
- Spectrin beta chain OS
- IgG L chain OS
- Spectrin alpha chain, erythrocytic 1 OS
- Kell blood group, metallo-endopeptidase OS
- Annexin OS
- Filamin-A OS

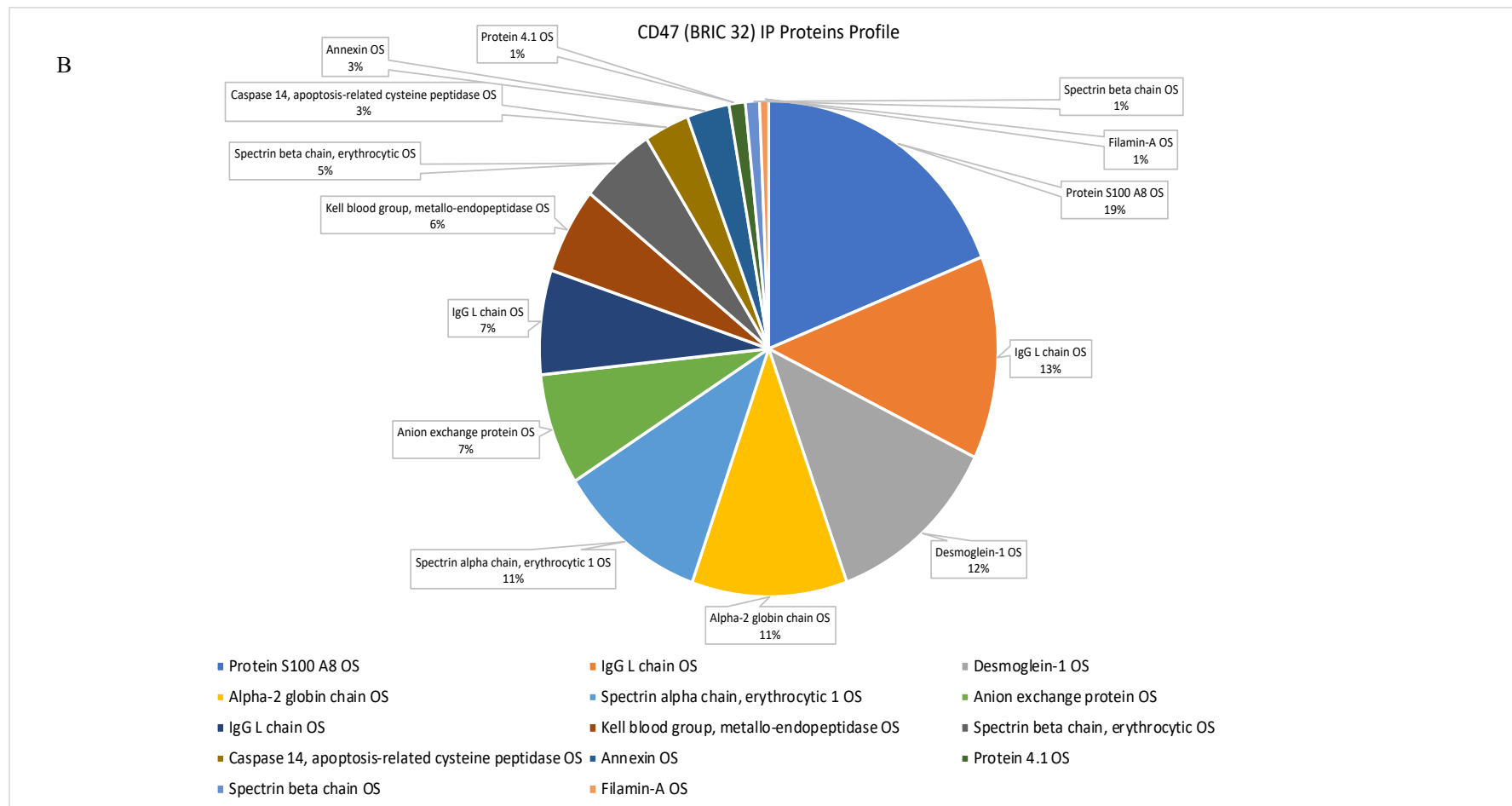


Figure 5.3: The protein abundance profile in anti-CD47 BRIC 32 IPs.

A- the pie charts show the highest abundant protein detected by LS-MS/MS. The results showed that CD47 is the highest hit by BRIC 32 (78%) which is expected as this antibody is specific for CD47.

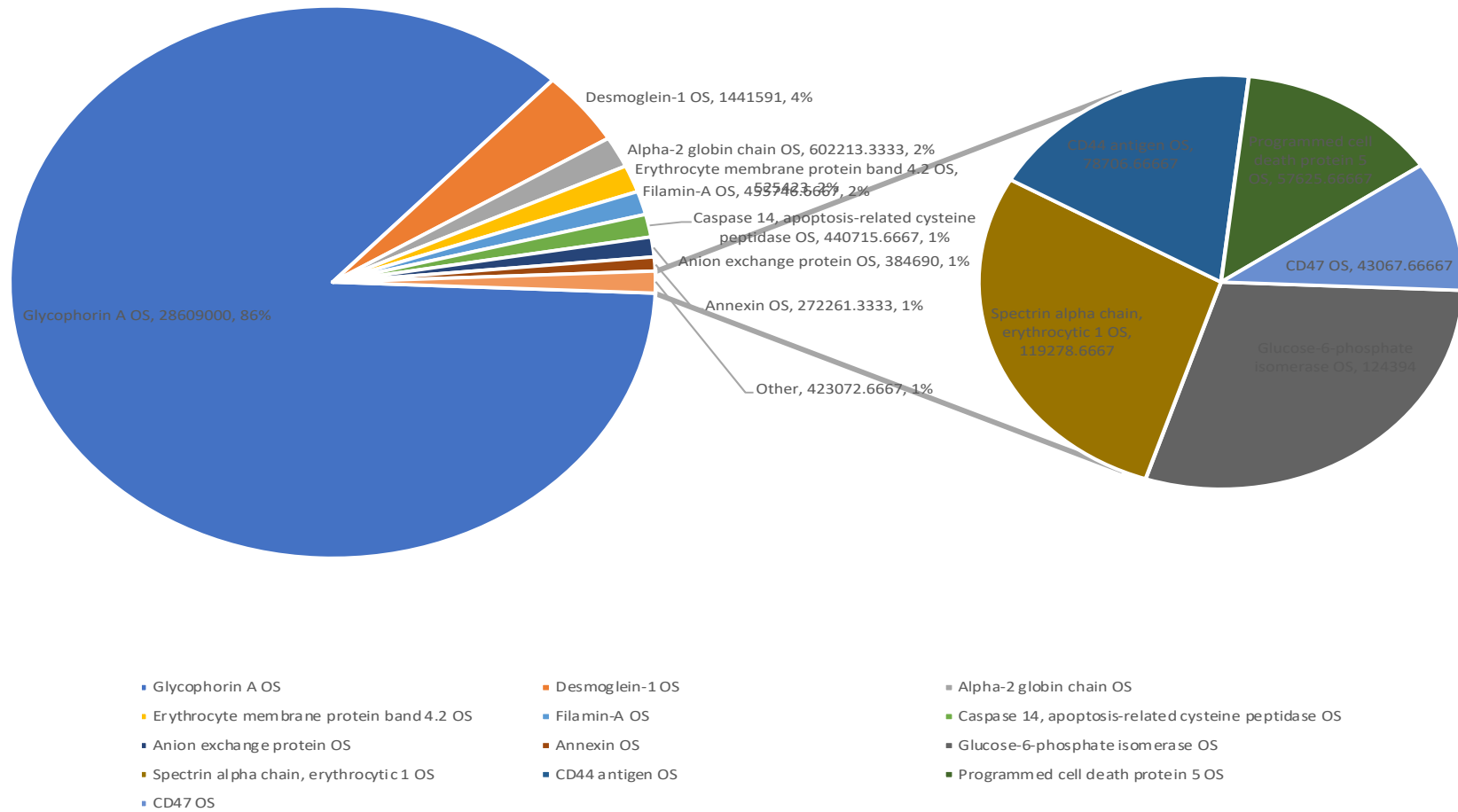
B- the pie charts show the highest abundance protein by BRIC 32 after removing CD47 score from the results to elucidate what is the role of CD47 in erythrocytes.

5.3.1.2 BRIC 256 anti-glycophorin A antibody:

BRIC 256 which is an antibody against Glycophorin A (GPA) were used. This was a subclass control for CD47 BRIC 32. GPA results are shown in Figure 5.4. GPA has a high sialic acid content and is a major provider to the net charge of erythrocyte membrane (Chasis and Mohandas, 1992). The IP results of anti-GPA (BRIC 256) show that the most abundant protein is GPA (86% mol/mol), which is not surprising as BRIC 256 is specific for this protein but it is important to make sure mass spectrometry results confirm that this antibody is specific for GPA protein. After subtracting the GPA results, the remainder will be the most abundant proteins that are pulled down by anti-GPA IP.

After the GPA itself, the most abundant protein pulled down by the IP with BRIC 256 is desmoglein 1 protein (32% mol/mol) which might be due to contamination, followed by alpha 2 globin with (13% mol/mol). Protein 4.2 is the third most abundant protein pulled down by BRIC 256 with (11% mol/mol). This result showing that band 3 is one of the most abundant protein pulled down by GPA with (8% mol/mol each). GPA IP results is showing CD44 is one of the abundant proteins as well with (2% mol/mol) and CD47 is with (1% mol/mol) (figure 5.4).

Glycophorin A (BRIC 256) IP Proteins profile



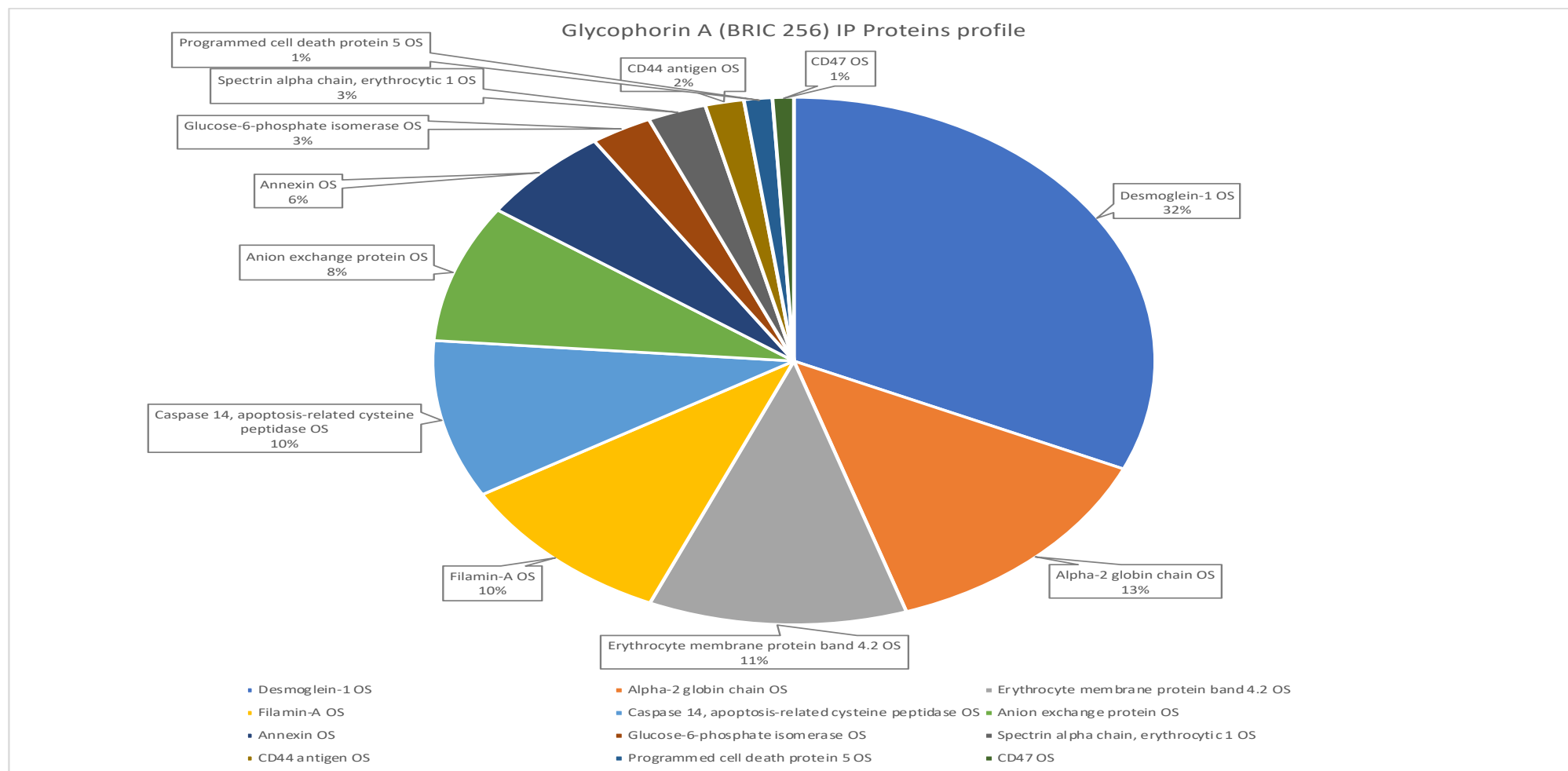


Figure 5.4: The protein abundance profile in anti-GPA BRIC 256 IPs.

A- the pie charts show the highest abundant proteins detected by LS-MS/MS. The results showed that GPA is the highest hit by BRIC 256 (86%) which is expected as this antibody is specific for GPA. B- the pie charts showing the highest abundance by BRIC 256 after removing GPA score.

5.3.1.3 BRIC 124: high PS-inducing anti-CD47

CD47 BRIC 124 as described earlier in this thesis, causes high PS exposure and thus induces high level of eryptosis in erythrocytes. Here in this study was selected to represent this category. We assume that antibodies of the BRIC124 class mimic unknown ligands that induce PS erythroid exposure.

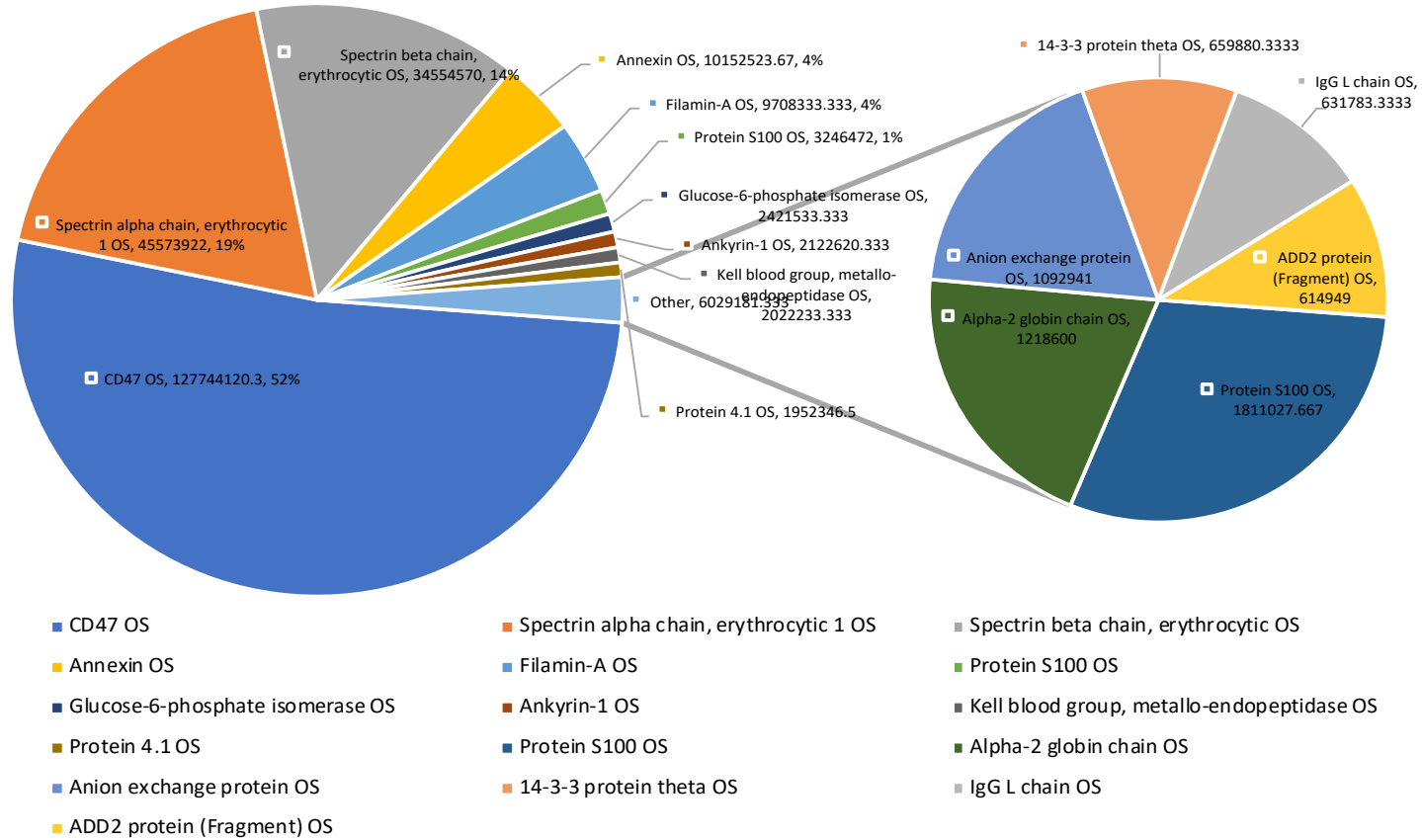
Proteins list from BRIC 124 IP samples results were consistent between two different biological replicates which were repeated in triplicate. BRIC 124 was incubated with erythrocytes prior to immunoprecipitation (100 μ l/5ml of erythrocyte) the concentration of the antibody is (1mg/1ml).

The summary of the highest protein abundance found in BRIC 124 IPs is shown in (Figure 5.5).

The protein profiles of the IPs of BRIC 124 by LC-MS/MS shows a different protein profile from that of BRIC 32, which indicates that binding different epitopes of CD47 pull down different proteins and indicate a possible different function of this protein, and may we speculate, result in a conformational change in the CD47 protein recruiting binding to red cell membrane, membrane skeletal and cytoplasmic components In BRIC 124 IPs the most abundant protein after the expected most abundant CD47 (52% mol/mol) are alpha and beta spectrin (39%, and 30% mol/mol) respectively. Annexin were pulled down by CD47 BRIC 124 (9% mol/mol) and Filamin A, which is an actin binding protein, were present in IPs also by (8% mol/mol). Protein S100 A8 was present being 3% (mol/mol) of the proteins in CD47 BRIC 124 IPs.

A

CD47 (BRIC 124) IP Proteins Profile



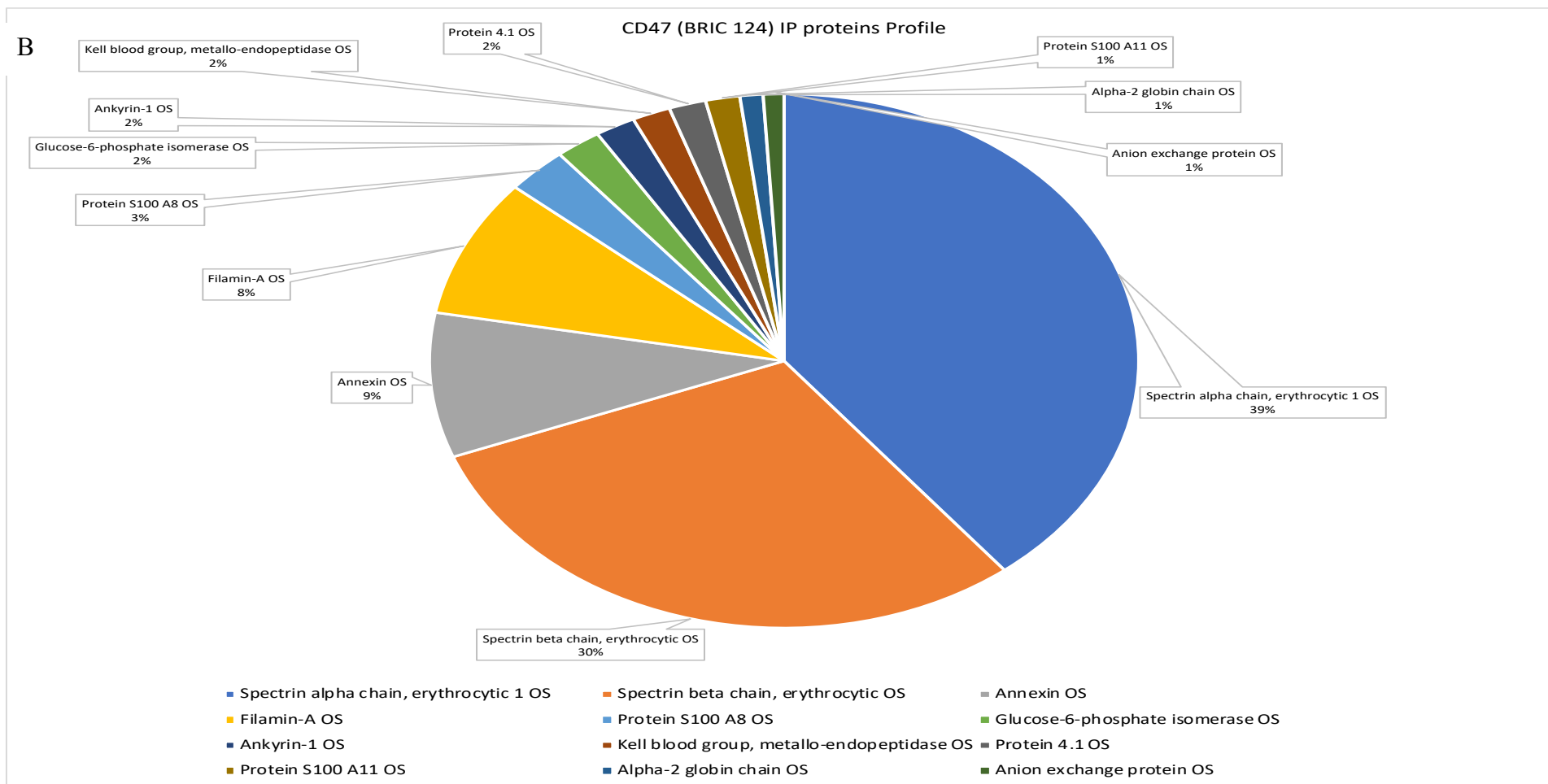


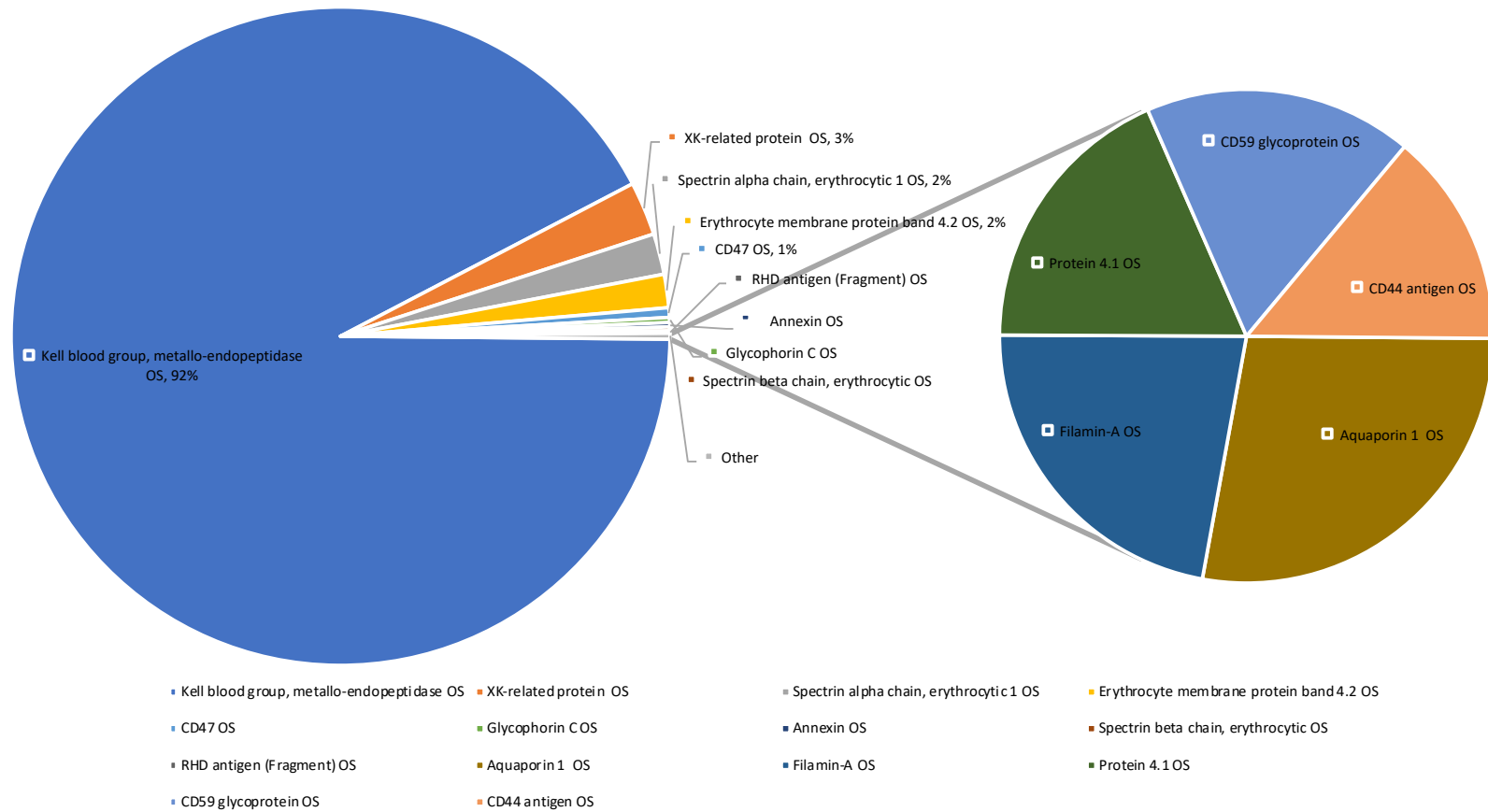
Figure 5.5: The protein profiles contained in anti-CD47 BRIC 124 IPs.

A-the pie charts show the highest abundant detected by LS-MS/MS. The results showed that CD47 is the highest abundance (52% mol/mol) which is expected. B- the pie charts showing the highest abundance by BRIC 124 after CD47 to elucidate what is the role of CD47 in erythrocyte on binding BRIC124 class ligands.

5.3.1.4 BRIC 18 anti-Kell Protein antibody:

BRIC 18, which is Kell protein antibody as a subclass control for CD47 BRIC 124. The Kell glycoprotein is a type II single-pass membrane with a size of 93 kDa. It is expressed on the surface of erythrocytes at a level of 3500–17000 copies per red cell (Masouredis et al., 1980). The Kell glycoprotein is covalently attached to another protein in the red cell membrane, known as the XK protein via a disulphide bond (Khamlichi et al., 1995). In this study, anti-Kell IP results are shown in Figure 5.6. the most abundant protein is Kell protein (92% mol/mol) as BRIC 18 is specific for this Kell protein (it is important to make sure mass spectrometry results confirm that this antibody is specific for Kell protein). After removing the Kell protein from the results, it is possible to tell the most abundant proteins pulled down by the BRIC 18 IPs. Anti-Kell IP showed XK protein as the most abundant protein (34% mol/mol), followed by alpha spectrin with (26% mol/mol). However, protein 4.2 is the third most abundant protein with (21% mol/mol). Also, this result showing that CD47 also pulled down by Kell with (6% mol/mol each). Furthermore, anti-Kell IP results is showing Glycophorin C is abundant as well with (3% mol/mol) and RhD with (1% mol/mol) (figure 5.6).

Kell protein (BRIC 18) IP protein profile



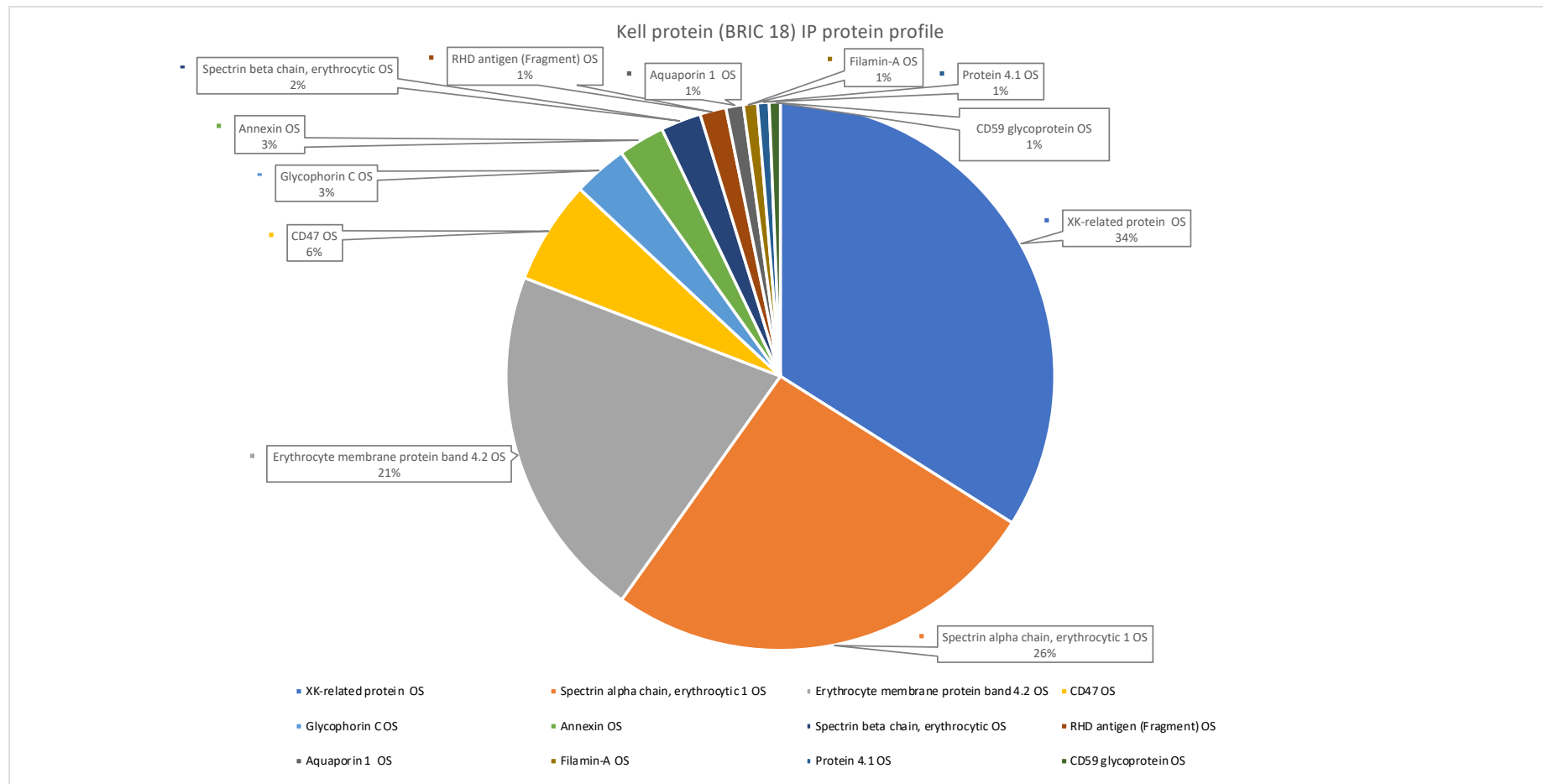


Figure 5.6: The protein abundance profile in anti-Kell BRIC 18 IPs.

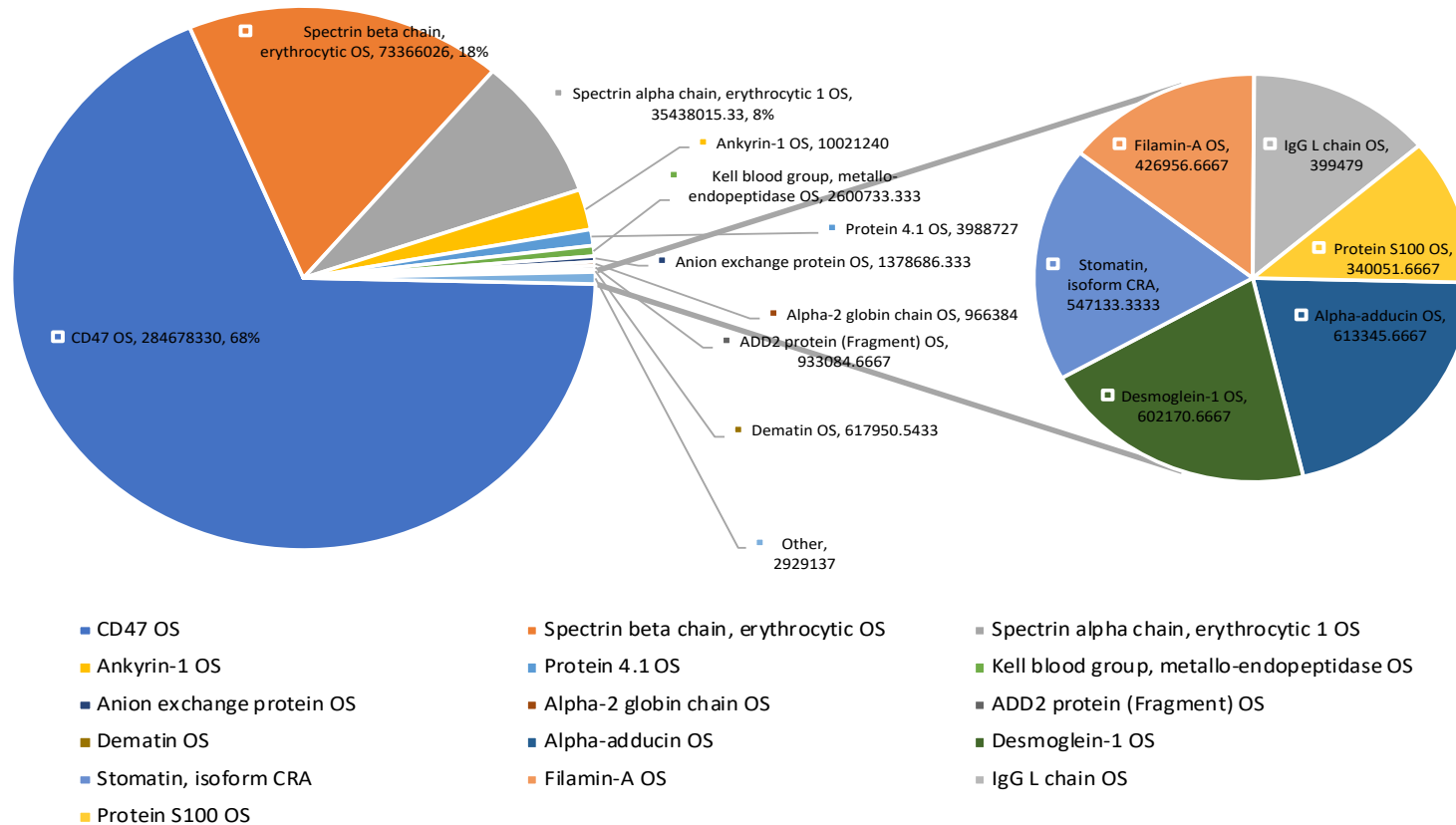
A- the pie charts show the highest abundant detected by LC-MS/MS. The results showed that Kell is the highest hit by BRIC 18 (92%) which is expected as this antibody is specific for Kell. B- the pie charts showing the highest abundance by BRIC 18 after removing Kell score.

5.3.1.5 BRIC 126: medium PS-inducing anti-CD47:

CD47 BRIC 126 is widely described for the analysis of CD47. as described earlier in chapter 4 this antibody causes “medium” PS exposure, but here results describe a different protein profile based on LFQ analysis in BRIC126 IPs which may reflect different natural ligand actions. Mordue et al (2017) , used MS results for BRIC 126 IPs, but they used unique peptide identifications only for their analysis, which does not account for the molecular weight of the protein while the LFQ is a normalized value with a logarithmic equation (Mordue et al., 2017). Thus, in our CD47 BRIC 126 IP results shows a different protein profile compared to BRIC 32 IPs but they are similar to the BRIC 124 IPs. For instance, alpha and beta spectrin were the most abundant (27% (mol/mol), and 57% (mol/mol respectively)) after subtraction of CD47 (68% mol/mol) from the analysis, which is different from BRIC 124 IPs. In all IPs containing alpha and beta spectrin, one may expect equimolar ratios of both of them as they are tightly complexed. Our observations may thus be experimental artefact or reflect dissociation of the spectrin tetramer following anti-CD47 binding However in contrast, Annexin was present in BRIC 126 IPs (8% mol/mol) followed by protein 4.1R (3% mol/mol) and Kell protein (2% mol/mol) see figure 5.7. Thus, CD47 BRIC 126 mAb may reveal a different site of ligand binding on CD47 which results in the recruitment of different red cell components, or the configuration of a subpopulation of CD47 is different due to pre-existing interactions.

A

CD47 (BRIC 126) IP Proteins Profile



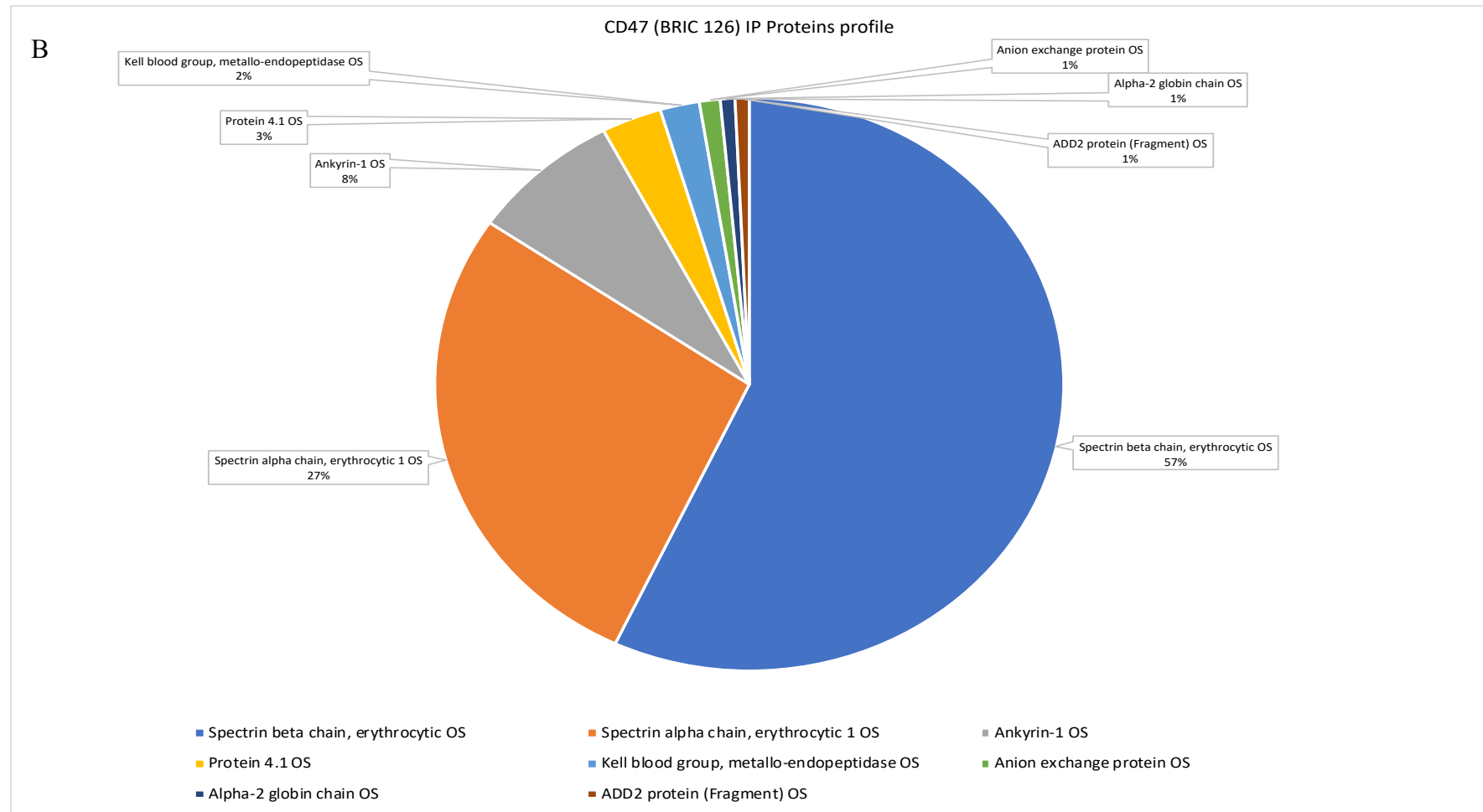
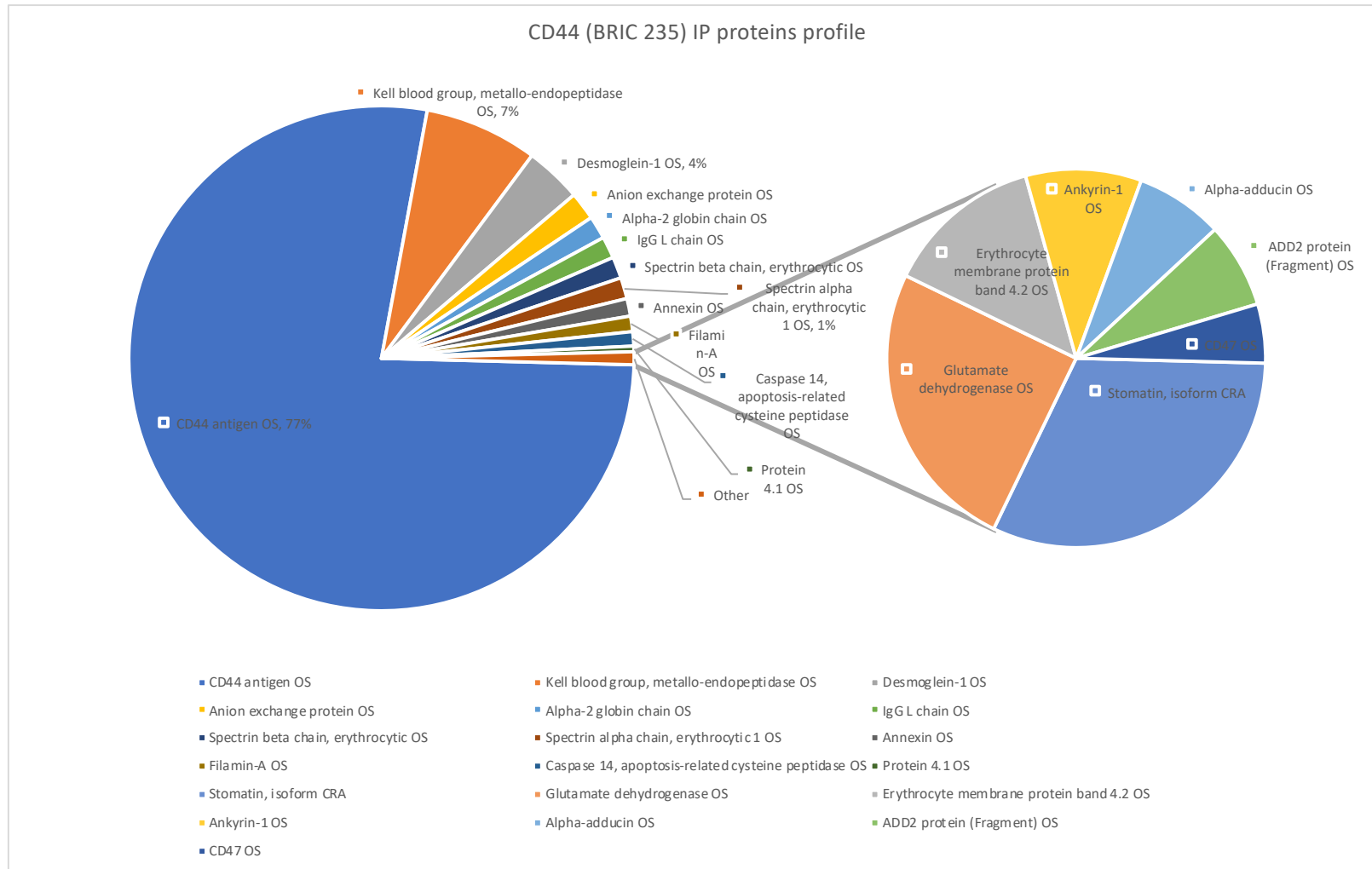


Figure 5.7: The protein profiles of anti-CD47 BRIC 126 IPs.A- the pie charts show the highest abundant protein detected by LS-MS/MS. The results showed that CD47 is the highest abundant protein by BRIC 126 IP (68% mol/mol) which is expected as this antibody is specific for CD47. B- the pie charts showing the highest abundance by BRIC 126 after subtracting CD47 score to elucidate different protein binding partners.

5.3.1.6 BRIC 235 anti-CD44 antibody:

BRIC 235, which is an antibody against CD44, as a subclass control for CD47 BRIC 126. CD44 is a transmembrane glycoprotein which has a major role in extracellular matrix adhesion, T cell activation and the maturation of haemopoietic cells (Bollyky et al., 2009). The BRIC 235 IP results are shown in Figure 5.8. The most abundant protein is CD44 (77% mol/mol) as BRIC 235 is specific for this protein (it is important to make sure mass spectrometry results confirm that this antibody is specific for CD44 protein). However, when subtracted CD44 results it will show that the most abundant proteins that upregulated by CD44.

Kell protein is the most abundant protein (33% mol/mol), followed by Desmoglein 1 with (16% mol/mol). However, band 3 is the third most abundant protein pulled down by CD44 with (8% mol/mol). Also, this result showing that spectrins (alpha & beta) are also abundant in anti-CD44 IP with (6% mol/mol each). Furthermore, CD44 IP results show Protein 4.1R is abundant as well with (2% mol/mol) and protein 4.2 with (1% mol/mol) (figure 5.8).



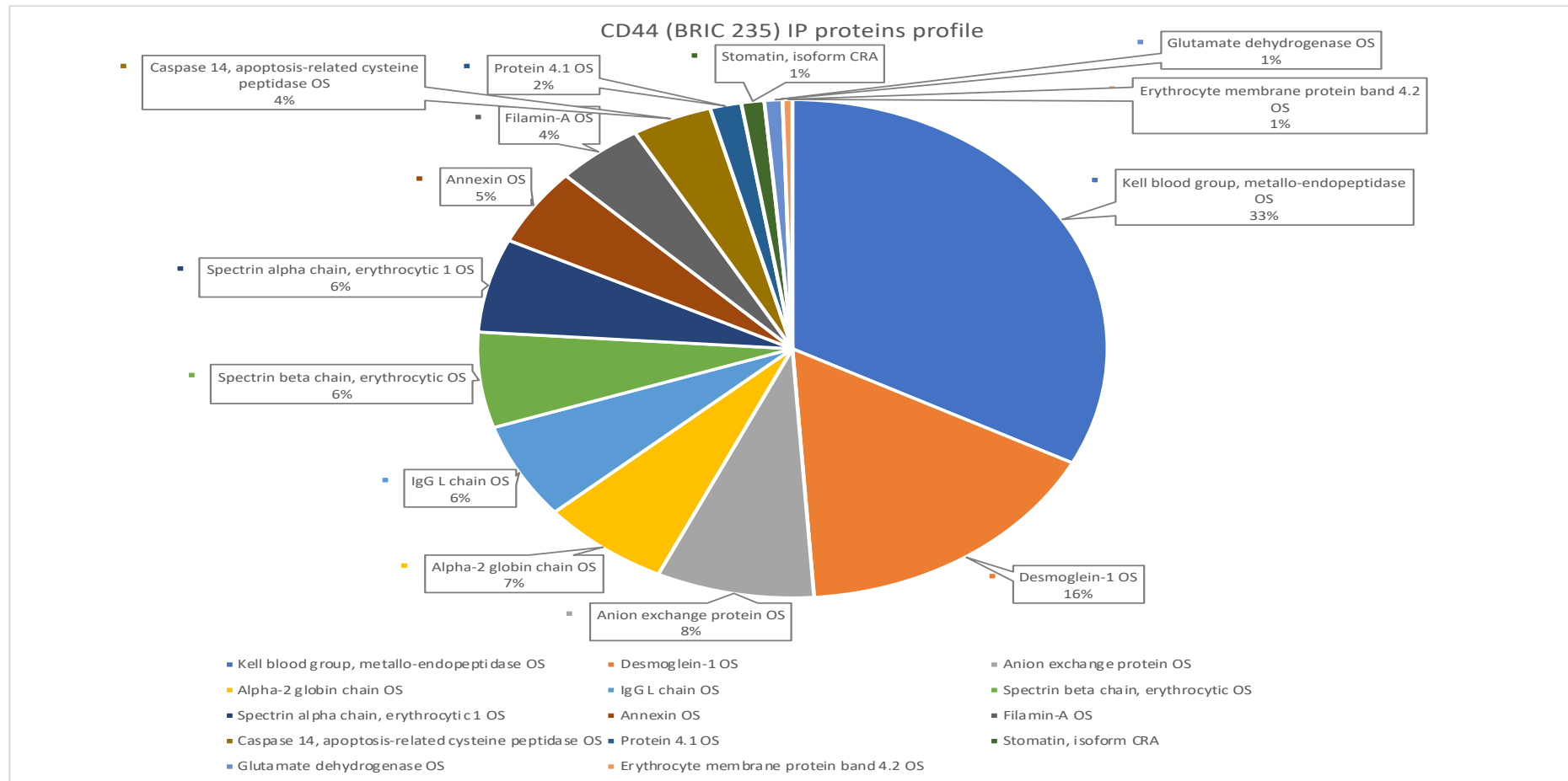


Figure 5.8: The protein abundance profile in anti-CD44 BRIC 235 IPs.

A- the pie charts showing the highest abundant by LS-MS/MS. The results showed that CD44 is the highest abundant by BRIC 235 (77%) which is expected as this antibody is specific for Kell.

B- the pie charts showing the highest abundance by BRIC 235 after removing CD44 score.

5.3.2 The upregulated proteins by all CD47 N-terminal antibodies:

In this section, the constant upregulated proteins by all the three antibodies used earlier were collected to elucidate the function of CD47 in erythrocytes. To validate the results shown by the three antibodies, the comparison against a specific antibody from the same IgG subclass (Table 5.1) were done.

The results showed a constant interaction of some proteins with CD47 whether using any of CD47 antibodies. However, there are differences between different CD47 as the pie charts (figures 5.3, &5.5, & 5.7) showed due to presumably different binding sites mimicking natural ligands of CD47.

Furthermore, here in this part the analysis of the results were different than previous part, it was shown the highest proteins hits by using different CD47 antibodies earlier but here in this part, the results showing the constant upregulated proteins that pulled down by the different categories of PS exposure CD47 antibodies. First, the LFQ value of a certain protein were compared in the CD47 antibody divided by the same protein LFQ value by the control and if the results was 2-fold or more that protein upregulated as showing in (table 5.1) (Personal communication with Dr. Vikram Sharma). Moreover, results in table 5.1 showed below indicates Protein G and flotillin 1, which are an important parts of lipid raft, were upregulated on ligation of CD47 antibodies and also confirming the interaction between CD47 and protein 4.1R as well. Furthermore, on ligation of CD47, Ankyrin and dematin were upregulated.

Accession Number	Protein Description	BRIC 126 (LFQ)			BRIC 124 (LFQ)			BRIC 32 (LFQ)		
		Total LFQ in BRIC 126	IgG control (BRIC 235) anti-CD44	Ratio	Total LFQ in BRIC 124	IgG control (BRIC 18) Anti-Kell	Ratio	Total LFQ in BRIC 32	IgG control (BRIC 256)	Ratio
O95837	Guanine nucleotide-binding protein	29471	*	29471	3377	120.7	28	78226.7	*	78226.7
Q8WZ56	Serine/threonine-protein phosphatase	7266.7	*	7266.7	69836.7	*	69836.7	24572	3190	7.7
Q08495	Dematin	617950.5	2407.3	256.6	37437.3	9500	3.9	10963.3	2664.7	4.1
P16157	Ankyrin-1	10021240	39340.0	254.7	2122620.3	191853.3	11.0	51292	4714.7	10.8
P11277	Spectrin beta chain, erythrocytic	73366026	665556.7	110.22	34554570	1907033.3	18.1	1095483.3	144799	7.5
P02549	Spectrin alpha chain, erythrocytic 1 OS	35438015.3	654996.7	54.1	45573922	20701666.6	2.2	2195240	119278.6	18.4
Q05DK5	ADD2 protein	933084.7	28903	32.2	614949	88300	6.9	20249	2027.7	9.9
P11171	Protein 4.1	3988727	172940	23.0	1952346.5	546393.3	3.5	237304.3	16306.3	14.5
Q5ST80	Flotillin 1	17629.1	1459.5	12.0	4015.3	813.3	4.9	5501	1837.47	2.9

A0A024R882	Stomatin	547133.3	126214.3	4.3	68057.7	9346.7	7.2	17552.7	742.7	23.6
E5RGX5	Stathmin	13524	8214.6	1.6	460731.7	*	460731.7	10919.3	661.3	16.5
G4V2I9	Anion exchange protein	1378686.3	869746.7	1.5	1092941	15146.7	72.1	1505133.3	384690	3.9
P05109	Protein S100 A8	340051.7	2551733.3	0.13	3246472	214340	15.1	3925433.3	1759884	2.2
P27348	14-3-3 protein theta	*	8206	*	659880.3	*	659880.3	13938.7	*	13938.7

*indicates that protein is not detected in this sample.

Table 5.1: The LFQ intensity values by different CD47 antibodies and their control antibodies using LC-MS/MS.

The ratio was calculated by dividing the LFQ intensity score of a certain protein in the CD47 antibody IP sample dividing by the same LFQ intensity of the same protein in the control sample. If the score was 2-folds or more of the ratios, that indicates the protein is enriched or over-represented in the IP sample by using CD47 antibody. However, in some cases as Guanine nucleotide-binding protein in BRIC 126 sample is not detectable in the control sample which is indicating that CD47 BRIC 126 is highly upregulated that protein. Moreover, for some proteins for example 14-3-3 protein theta is only present in the control of CD47 BRIC 126 but is not detectable by CD47 BRIC 126 antibody while it is detectable by CD47 BRIC 124 and BRIC 32.

5.3.3 Protein components identified in anti-C-terminal CD47 IPs

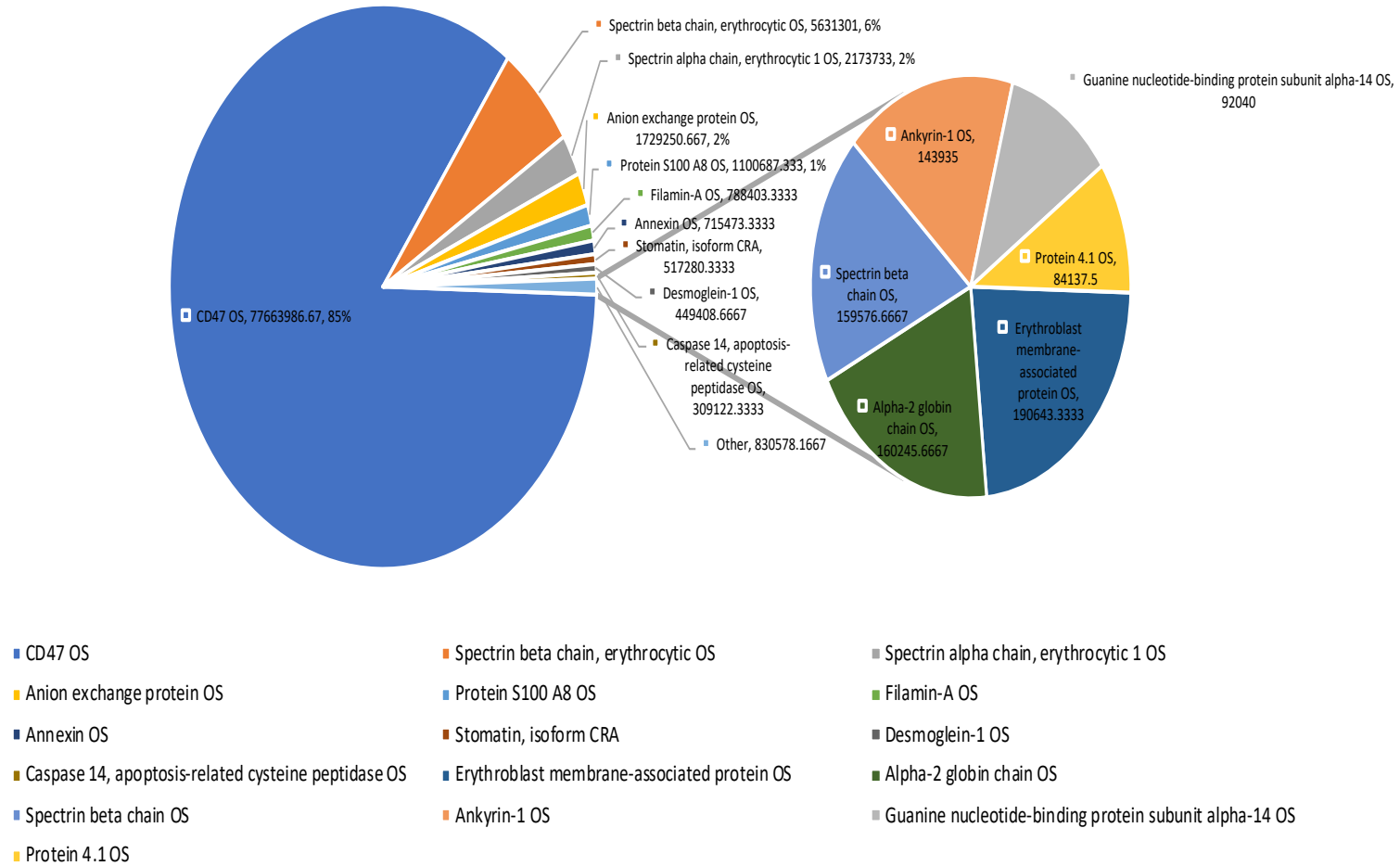
Different proteins profile that pulled down by a C-terminal IP antibody for CD47 will be investigated. Antibodies against isoform 2, 4, and 5 were used. These antibodies were customized for this project as described in chapter 3. These three isoforms of CD47 have different peptide sequences at the C-terminal domain of CD47. Isoforms 2 and 4 have highly homologous peptide sequences whilst isoform 5 has a unique peptide sequence, compared to the others (table 3.4). The protein profiles contained in the anti-CD47 C-terminal IPs were investigated, membrane ghosts as starting material to make their epitopes accessible.

5.3.3.1 mAbs to Isoform 2 IP components identified

Isoform 2 is the most abundant isoform of CD47 in erythrocytes, as shown by the NGS work in this thesis (Chapter 3). Isoform 2 IP results showed CD47 was the most abundant protein with (85% mol/mol) of the abundance by MS and after removing it from the analysis, the proteins which were highly represented in anti-isoform 2 antibody IPs (figure 5.9). Alpha and beta spectrin were the most abundant proteins in isoform 2 antibody IPs (15%, and 39% mol/mol respectively), whilst Band 3 and S100 A8 also showed high abundance (12%, and 8% mol/mol respectively). Filamin is also present with (6% mol/mol) while annexin with (5% mol/mol). Stomatins are also present with (4% mol/mol), and desmoglein 1 (3% mol/mol). Finally, it is worthy of note that the following proteins are present caspase 14, ankyrin, and protein 4.1R with (1% mol/mol) each (figure 5.9).

A

CD47 Isoform 2 IP Proteins Profile



B

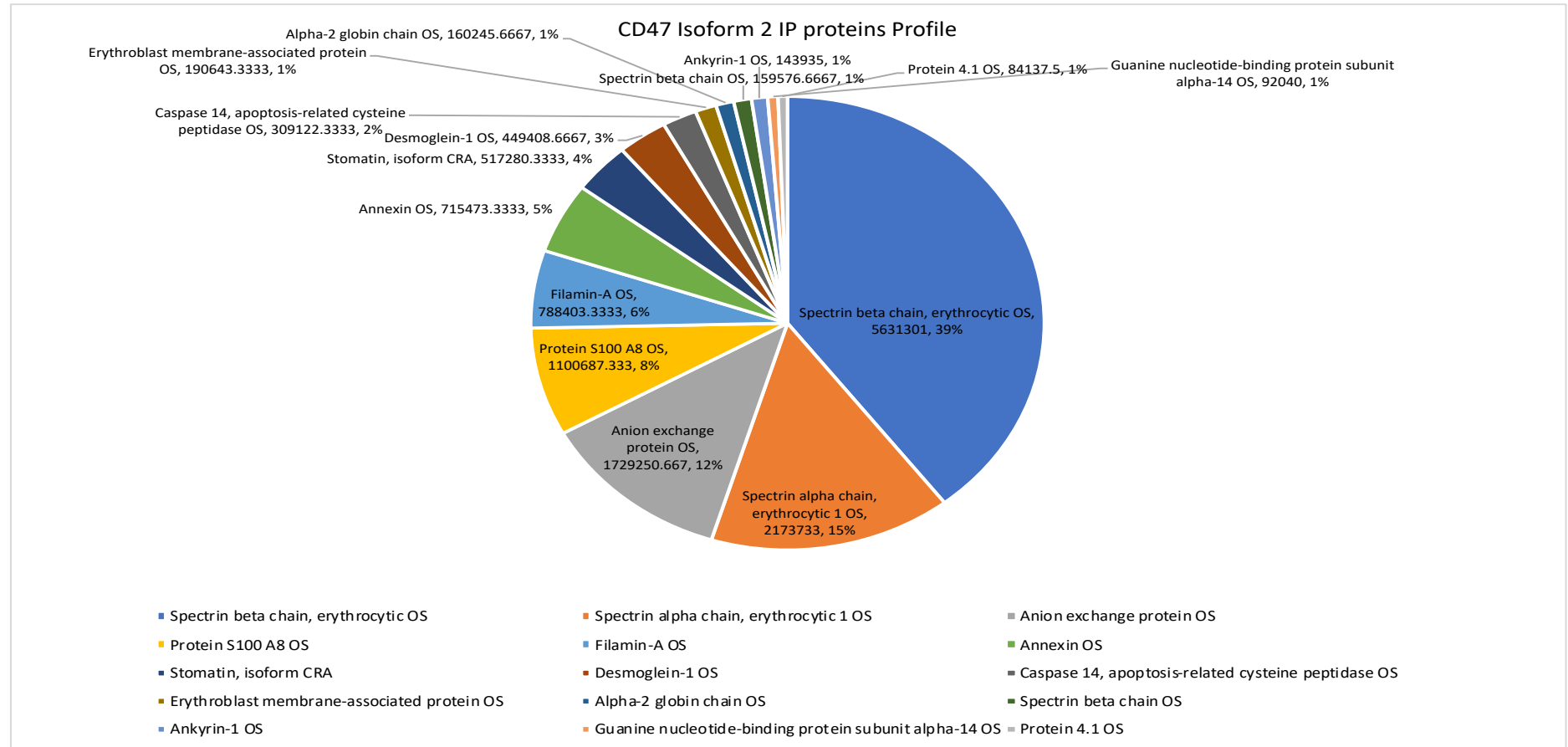


Figure 5.9: The protein profiles of anti-CD47 isoform 2 IPs

A- the pie charts showing the highest abundant by LS-MS/MS. The results showed that CD47 is the highest abundance by isoform 2 (85%) which is expected as this antibody is specific for CD47.

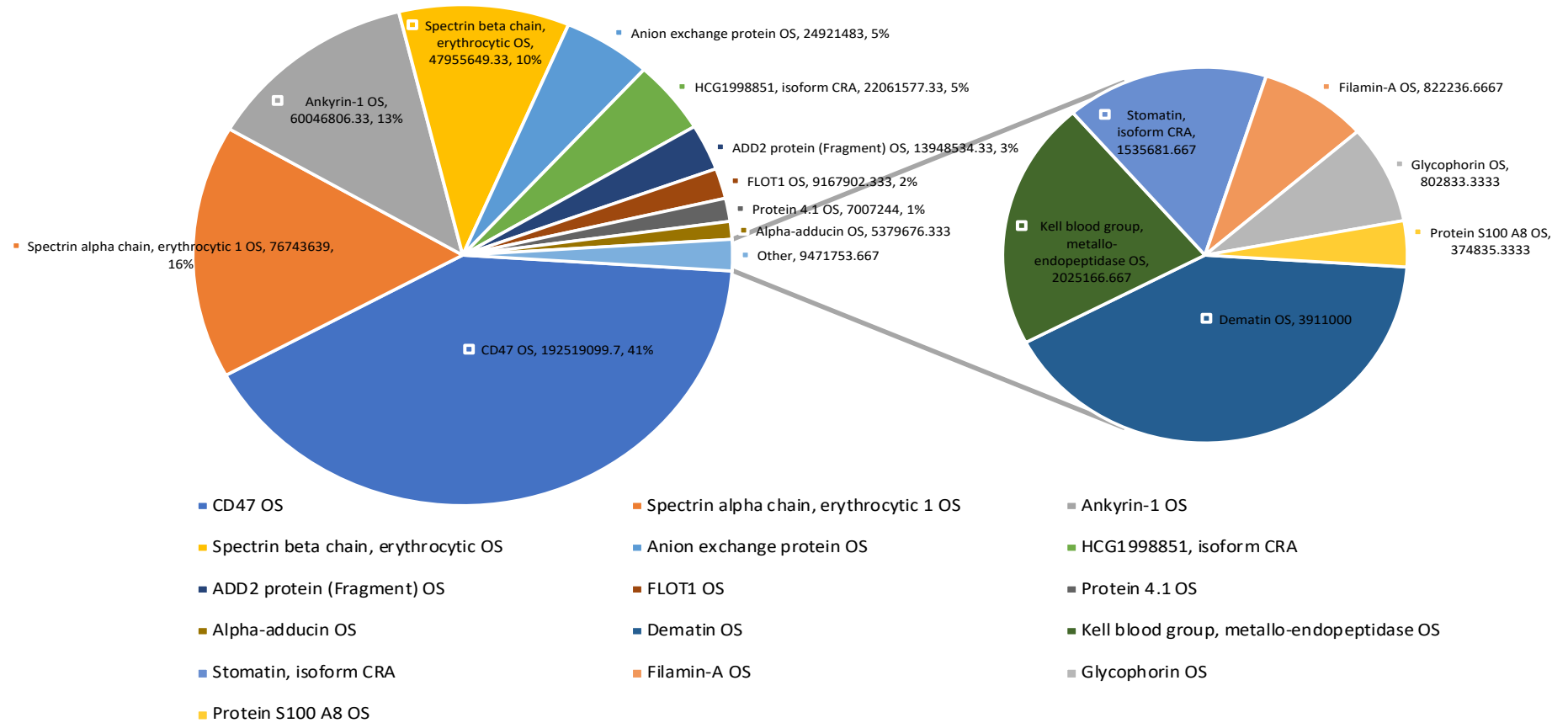
B- the pie charts showing the highest abundance by isoform 2 after removing CD47 score to elucidate specific binding partners for CD47 isoform 2.

5.3.3.2 mAbs to Isoform 4 IP components identified

Although CD47 isoform 2 and isoform 4 have high homology isoform 4 is longer than isoform 2 and isoform 4 demonstrated a different protein profile than that of isoform 2. First, CD47 was the most abundant protein in anti- CD47 isoform 4 IPs (41% mol/mol) (which indicated that the antibodies were customised are working against CD47 isoform 4) and when we subtracted CD47 from these results, it was found that alpha spectrin is present with (28% mol/mol) (different than isoform 2) ankyrin is the second-most abundant protein (22% mol/mol) while beta spectrin is (17% mol/mol) (Figure 5.10). Band 3 is present with (9% mol/mol) while HCG1998851 (flotillin 2) is present with (8% mol/mol). Beta adducin is presented with (5% mol/mol) while Flotillin 1 and protein 4.1R are present at (3% mol/mol) each. Alpha adducin is present with (2% mol/mol) while dematin, stomatin, and kell protein are present with (1% mol/mol) each.

A

CD47 Isoform 4 IP Proteins Profile



B

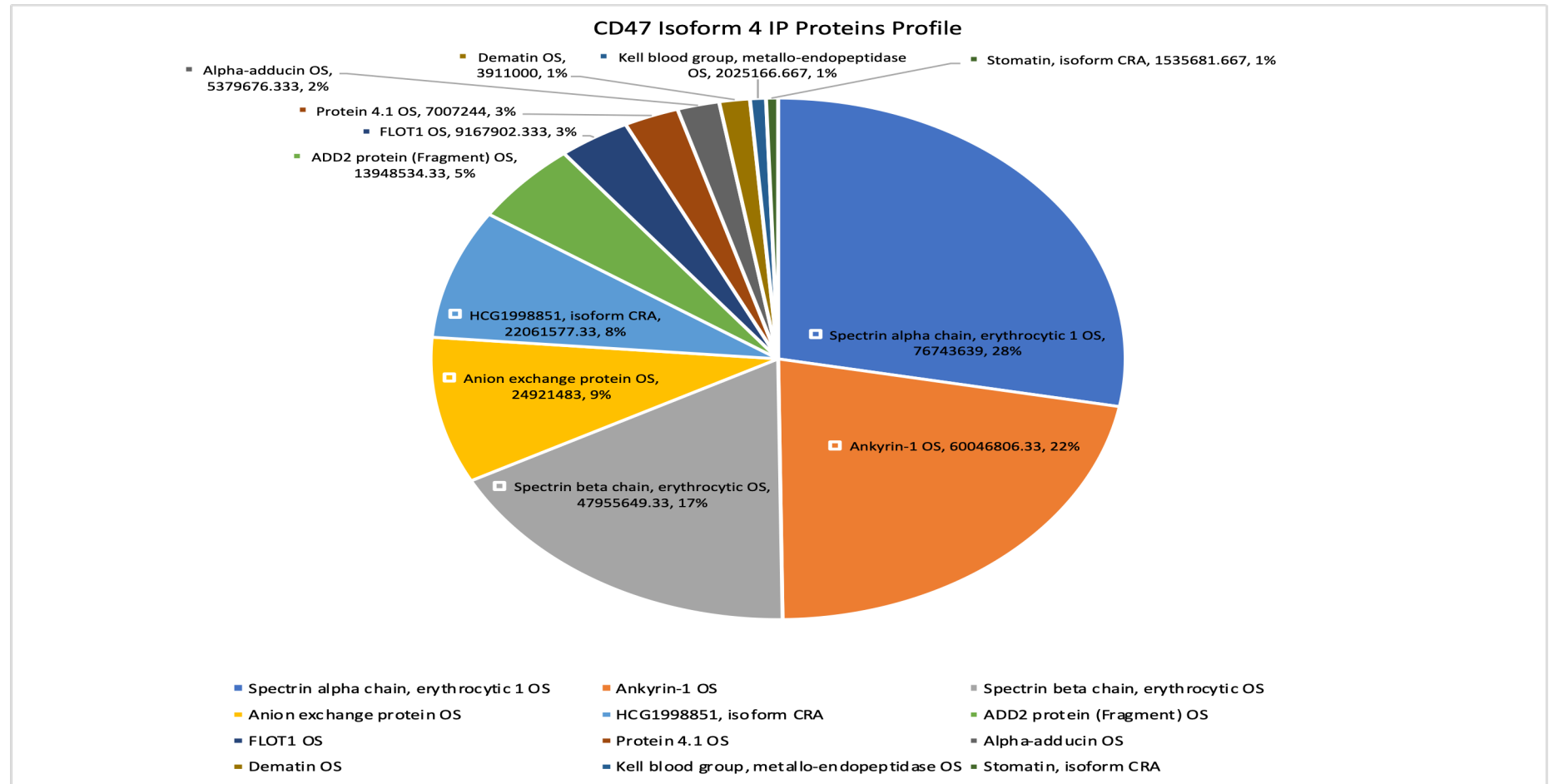


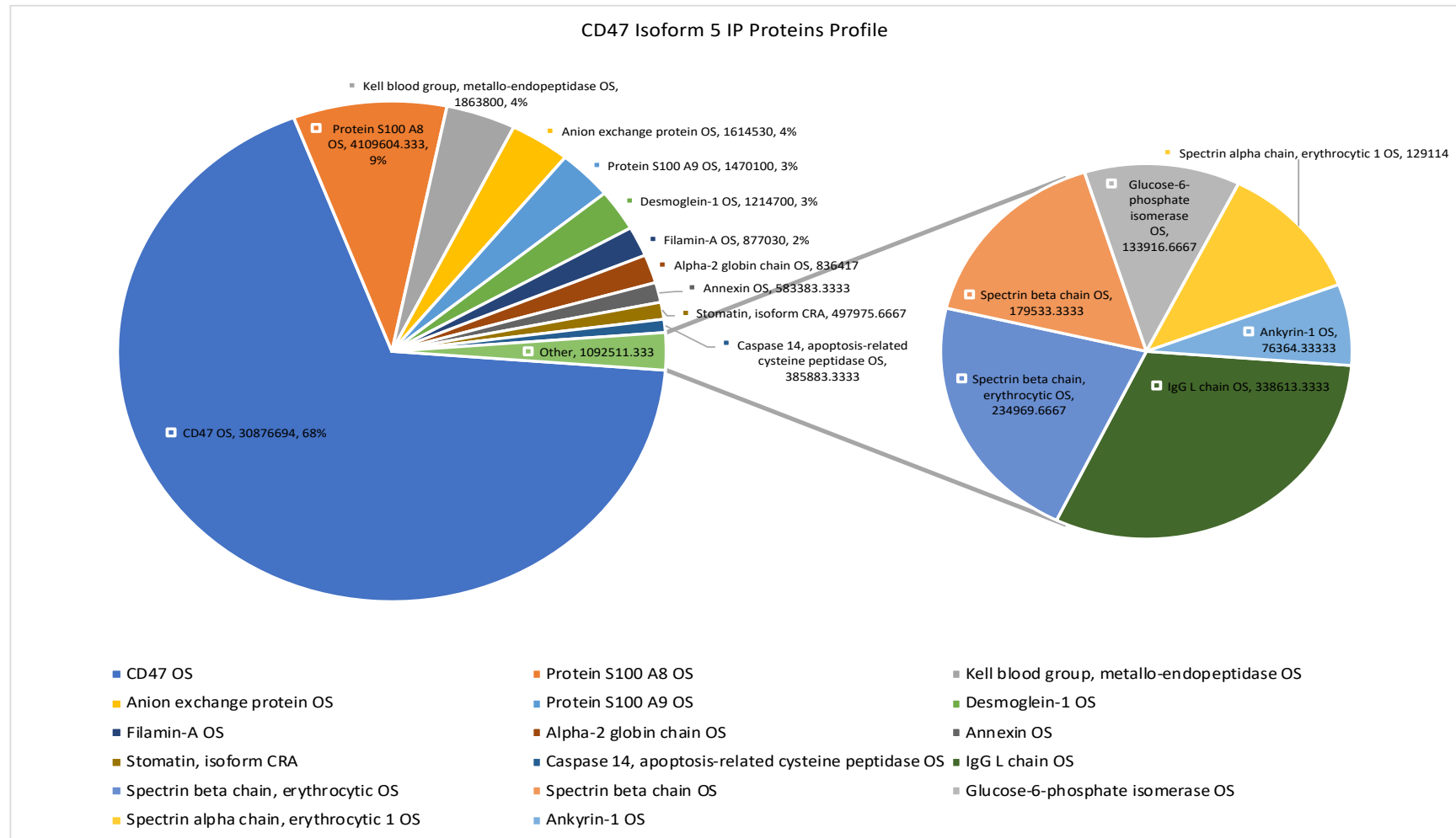
Figure 5.10: The protein profiles of anti-CD47 isoform 4 IPs

A- the pie charts show the highest abundant protein detected by LS-MS/MS. The results show that CD47 is the highest abundance by isoform 4 (41%) which is expected as this antibody is specific for CD47. B- the pie charts showing the highest abundance by isoform 4 after removing CD47 score to elucidate CD47 isoform 4 binding partners in IPs.

5.3.3.3. mAbs to Isoform 5 IP components identified:

CD47 isoform 5 was the latest isoform discovered (Schickel et al., 2002). Its presence in erythroid cells by NGS and by immunoblot using this antibody was confirmed in chapter 3. CD47 isoform 5 has a different peptide sequence than the other 4 isoforms. I was the first to raise antibodies to the peptide sequences that were sent to Dundee cells to make a specific antibody for this isoform since its discovery in 2002. Furthermore, the LC-MS/MS data shows a different protein profile in the IPs of CD47 isoform 5 antibodies compared to other isoforms analysed in this study. CD47 itself, is present at 68% mol/mol of the highest LFQ intensity abundance and when CD47 is subtracted from the results, it was found that protein S100A8 has the highest LFQ abundance (28% mol/mol) while Kell glycoprotein (13% mol/mol). Band 3 is (11% mol/mol) of the highest LFQ abundance and S100 A9 (10% mol/mol) whilst desmoglein is (8% mol/mol). Also, filamin 1 is (6% mol/mol) and alpha globin is (6% mol/mol). Moreover, annexin is (4% mol/mol) whilst stomatin and caspase 14 are (3% mol/mol) each from the highest LFQ hits. Finally, alpha and beta spectrin, ankyrin, Guanine nucleotide-binding protein are (1%) each from the highest LFQ intensity abundance (Figure 5.11).

A



B

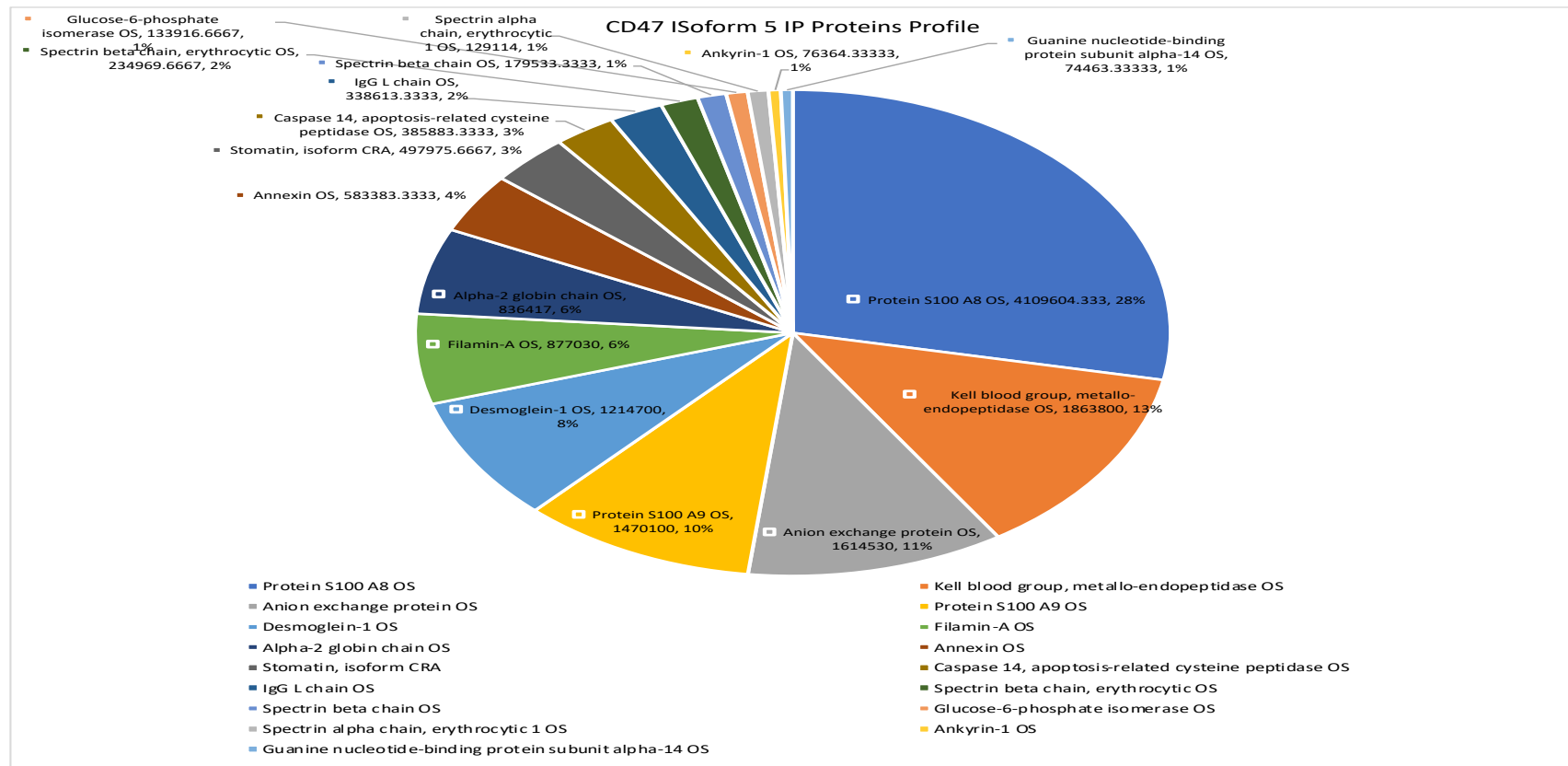


Figure 5.11: The protein profiles of anti-CD47 isoform 5 IPs

A- the pie charts showing the most abundant protein detected by LS-MS/MS. The results showed that CD47 is the most abundant by anti-CD47 isoform 5 IP (68% mol/mol) which is expected as this antibody is specific for CD47. B- the pie charts showing the highest abundance by isoform 5 after removing CD47 score to elucidate are the binding proteins of CD47 isoform 5 either before or after ligation

5.3.4 Comparison of the protein binding partners for three of the five different CD47 isoforms:

To differentiate between the three different C-terminal antibodies against CD47 isoforms, the LFQ of a certain protein that is present in by one isoform IP was divided. Some of these proteins were found to be unique to a specific isoform, whilst others were shared between the three, that were investigated. Due to financial constraints and the difficulty in raising mAbs to identical sequences, it was not possible to investigate isoforms 1 and 3 which have identical sequences (but of different lengths) to isoforms 2 and 4.

As these antibodies are designed against CD47, one would expect it to be the most abundant component in anti-C terminal IPs, which was indeed the case. Anti-Isoform 2 has CD47 four times more than isoform 4 and twenty-five-fold more than isoform 5, which is confirming that CD47 isoform 2 is the most abundant isoform in erythrocytes (Table 5.2). Band 3 (Anion Exchanger) was pulled down by all CD47 C-terminal isoform antibodies. The data shows that CD47 isoform 4 has abundance of Band 3 fourteen-fold, fifteen-fold more than isoform 2, and isoform 5 respectively. CD47 isoform 4 is the longest isoform and this possibly indicates it has the capacity to bind more proteins. Protein 4.1R is also bind to CD47. Protein 4.1R is upregulated isoform 4 in eighty-two-fold more than isoform 2 and two hundred eighteen folds more than isoform 5 while protein 4.1 is upregulated in isoform 2 two-fold more than isoform 5.

Protein 4.2 has been previously proposed to directly interact with CD47 and it is shown here to be pulled down by the antibodies against the CD47 isoforms antibodies. The data shows that CD47 isoform 4 is upregulated protein 4.2 five-fold more than isoform 2 and seven-fold more than isoform 5. On the other hand, CD47 isoform 5 is has the presence of stathmin while the other isoforms (1&4) it was not found. Finally, the summary of the

detailed comparisons between the isoforms are shown in table 5.2.

Accession Number	Protein name	Isoform 2 (LFQ)	Isoform 4 (LFQ)	Isoform 5 (LFQ)	ISO2/ISO 4 Ratio	ISO2/ISO5 Ratio	ISO4/ISO 2 Ratio	ISO4/ISO5 Ratio	ISO5/ISO 2 Ratio	ISO5/ISO 4 Ratio	The summary
A0A0A1TSG4	CD47	776639866.7	192519099.7	30876694	4	25.1	0.2	6.2	0.03	0.16	Iso2>Iso4>Iso5
G4V2I9	Anion exchange protein (Band 3)	1729250.7	24921483	1614530	0.06	1.07	14.4	15.43	0.9	0.06	Iso4>Iso2~Iso5
P11171	Protein 4.1	84137.5	7007244	32039.5	0.012	2.6	83.2	218.70	0.38	0.004	Iso4>Iso2>Iso5
P16157	Ankyrin-1	143935	60046806.3	76364.3	0.002	1.8	417.18	786.3	0.53	0.001	Iso4>Iso2~Iso5
P16452	Erythrocyte membrane protein band 4.2	48230.17	244789.4	31443.03	0.19	1.5	5.07	7.7	0.6	0.1	Iso4>Iso2~Iso5
Q5ST80	Flotillin 1	18970	9167902.3	12889.1	0.002	1.4	483.2	711.2	0.6	0.00	Iso4>Iso2~Iso5
U4PCV3	Kell blood group, metallo-endopeptidase	*	2025166.7	1863800	*	*	*	1.08	*	0.9	Iso2~Iso4~Iso5
Q6JSD7	Aquaporin 1	*	85506.6	*	*	*	85506.6	85506.6	*	*	Iso4>Iso2~Iso5
P27348	14-3-3 protein theta	15141.3	55526.7	24350.3	0.27	0.6	3.6	2.2	1.6	0.43	Iso4>Iso2~Iso5
P02549	Spectrin alpha chain,	5631301	47955649.3	234969.7	0.11	23.9	8.5	204.09	0.04	0.004	Iso4>Iso2>Iso5

	erythrocytic 1										
K9JIQ8	Glycophori n A	31417.3	802833.3	30333.7	0.03	1.0	25.5	26.4	0.9	0.03	Iso4>Iso2~Iso5
H0YD13	CD44 antigen	37393.3	48974	38143.3	0.7	0.9	1.3	1.2	1.02	0.7	Iso2~Iso4~Iso5
Q08495	Dematin	16014.6	3911000	3349.1	0.004	4.78	244.2	1167.7	0.20	0.0008	Iso4>Iso2>Iso5
E5RGX5	Stathmin	*	*	11242.7	*	*	*	*	11242.7	11242.7	Iso5>Iso2~Iso4
E7EV99	Adducin	17779	5379676.3	8568.7	0.003	2.07	302.5	627.8	0.48	0.001	Iso4>Iso2>Iso5
Q14254	FLOT 2	284147	22061577.3	21933.8	0.001	1.29	776.4	1005.8	0.77	0.0009	Iso4>Iso2~Iso5
O95837	Guanine nucleotide- binding protein	92040	4360	74463.3	21.11	1.2	0.047	0.05	0.8	17.0	Iso2>Iso4<Iso5 Iso2~Iso5
A0A024R882	Stomatin	517280.3	1535681.7	497975.7	0.33	1.03	2.9	3.08	0.9	0.32	Iso4>Iso2~Iso5

Table 5.2: The LFQ intensity by different CD47 isoforms antibodies using MS.

The ratio was calculated by dividing the LFQ intensity score of a certain protein in the CD47 isoform sample dividing by the same LFQ intensity of the same protein in the other isoforms sample. If the score was 2-folds or more of the ratios, that indicates the protein is upregulated by using CD47 antibody. The red numbers indicate the downregulation of the proteins while the green numbers indicate the upregulation of the proteins.

5.4 Discussion:

To elucidate the pathway that CD47 participates in erythrocytes, protein: protein interactions were studied using a variety of different monoclonal antibodies directed against both external and internal localized regions of the CD47 molecule.

CD47 has been reported to be a part of several biological pathways in erythrocytes such as eryptosis (Head et al., 2005b), glycosylation (Mawby et al., 1994), Calcium influx (Ca^{2+})(Schwartz et al., 1993), and recognition of self-cells “do not eat me signal” by binding to SIRP alpha in the macrophages (Oldenborg et al., 2000); but it is still unknown if binding to different epitopes of CD47 will affect these pathways and furthermore is this pathways are an isoform dependent.

Here novel interactions are described in erythrocytes for CD47 which have not been described before in the literature.

5.4.1 CD47 interacts with S100 Proteins

The S100 proteins were first discovered in the nervous system by Moore in 1965 (Moore, 1965). S100 proteins are a protein family comprising 25 different members. S100 proteins participate in different extra or intracellular activities such as energy metabolism, inflammation, cell apoptosis, migration, phosphorylation, proliferation and differentiation by regulating calcium balance (Xia et al., 2018).

S100 proteins, a family of calcium-binding cytosolic proteins, are not only present in nervous system but also in many different human cells and tissues such as, keratinocytes, macrophages, intestine, liver, colon, skeletal muscles (Zimmer et al., 1995). S100 proteins have been used as a marker for different injuries and diseases such as, active brain damage (haemorrhage), Alzheimer's, Down syndrome, melanoma and cancers (Heizmann, 2002,

Gazzolo and Michetti, 2010). The S100 protein family are distributed around different human cells and tissues, the abundance of them are different among cells and tissues such as S100A1 is present in nervous system while S100A8 and S100A9 are present in macrophages, and S100A11 is present in bone marrow (Zimmer et al., 1995).

S100 proteins were identified in the erythrocyte proteome (Goodman et al., 2007) but the functions of these proteins are unknown. S100 proteins that were detected in our study were S100A8, S100A9, and S100A11. Calcium dependent channels are formed by S100A8/S100A9 homo and heterodimers that mediate calcium influx in the membrane of myeloid cell differentiation (Teigelkamp et al., 1991). Interestingly, CD47 may be bound to these complexes in erythrocytes during an eryptosis pathway. Accordingly, upregulating Ca^{2+} influx will trigger scramblase which will lead to flip the PS from the inner leaflet of the membrane to the outer leaflet of the membrane which is a hallmark of eryptosis (Lang et al., 2005b). According to our results (Figure 5.11), Protein S100A8/S100A9 upregulation is related to CD47 isoform 5 more than other isoforms (figure 5.9-5.10).

Protein S100A11 is characterized in keratinocytes (Ruse et al., 2001); but it has yet to be described in erythrocytes. This study was the first to identify this protein in erythrocytes by proteome analysis. However, CD47 BRIC 124 is the only antibody that upregulates S100A11 protein (figure 5.5) which indicates this protein is upregulated with “high PS” exposure antibody. Protein S100A11 is related to phosphorylation process of the membrane by binding to annexin protein (Dorovkov and Ryazanov, 2004); which is also upregulated in CD47 BRIC 124 results (figure 5.5). these results might indicate the phosphorylation of CD47 is related to the interaction with S100A11 protein in the membrane.

5.4.2 CD47 interacts with Lipid Rafts:

Lipids Rafts are one of the most important components of the cell membrane, as the membrane is composed of a lipid bilayer in which different proteins are embedded. Recent evidence suggests the presence of lipid rafts in erythrocytes membranes act as microdomains which represent heterogeneous distribution of various membrane components. Lipid rafts contain of different membrane proteins such as, GPI-anchored proteins, aquaporin-1, flotillin1, flotillin 2, stomatin, and Guanine nucleotide-binding protein (G α); also, lipid rafts are enriched in cholesterol and sphingolipids (Salzer and Prohaska, 2001, Samuel et al., 2001, Schnitzer and Oh, 1996). Lipids rafts regulate different functions in the erythrocyte membrane such as, vesicular trafficking and signal transduction (Simons and Ikonen, 1997).

This study found different lipid raft proteins upregulated within IPs using different CD47 N-terminal antibodies. Furthermore, the results demonstrated that some lipid raft proteins are upregulated within IPs using specific CD47 isoform antibodies.

Firstly, flotillin 1, which is the first protein discovered as a lipid raft member, is a 45 kDa protein (Bickel et al., 1997). Flotillin 1 acts as separate scaffolding component at the cytoplasmic face of erythrocyte lipid rafts. For instance, the membrane proteins have been found interacts with flotillin 1 were actin, spectrin, protein 4.1R, and protein 4.2 (Salzer and Prohaska, 2001).

The results indicate that also CD47 IPs binds to flotillin 1 in erythrocytes. Actually, flotillin 1 is highly upregulated in the CD47 isoform 4 IPs (483-fold more than isoform 2, and 711-fold more than isoform 5) (table 5.3). These data suggest that the upregulation of flotillin 1 by CD47 is isoform dependent, possibly due to ligand binding that is

mimicked anti-CD47 isoform 4.

Secondly, flotillin 2, which is highly similar to flotillin 1 as they share ~50% amino acids identity (Stuermer et al., 2001). Moreover, flotillin 1 and flotillin 2 forms part of a complex that has a prohibitin homology (PHB) domain in various tissues (Browman et al., 2007). Until now, this complex has not reported in erythrocytes, but it is known that flotillin 2 is upregulated in protein 4.1R deficient erythrocytes (Jeremy, 2012) which may suggest lipid rafts increase the fluidity of the membrane to compensate the absence of protein 4.1R.

The results here show that flotillin 2 is upregulated by CD47 isoform 4 almost 777-fold more than isoform 2 and also 1005-fold more than isoform 5 (Table 5.3). This interaction with isoform 4 is might increase the fluidity of the membrane and also facilitate signal transduction inside the membrane.

Thirdly, the next protein that is a part of lipid rafts and found in erythrocytes is stomatin (band 7). Stomatin is ~31 kDa and named after the human haemolytic anaemia hereditary stomatocytosis (Lande et al., 1982). Stomatin is an oligomeric integral membrane protein which is palmitoylated (Snyers et al., 1998). However, different than other lipid rafts component like flotillins, stomatin is a calcium dependent protein (Salzer et al., 2002). Thus, in the results show that, anti-CD47 (N-terminal and C-terminal) IPs contain stomatin as which may result in Ca^{2+} influx during eryptosis. Furthermore, stomatin is highly upregulated by in CD47 isoform 4 IPs two-fold more than isoform 2 IPs and three-fold more than isoform 5 IPs (Table 5.3).

Fourthly, Aquaporin 1 is also a lipid raft membrane protein that is present in erythrocytes (Murphy et al., 2004, Schnitzer and Oh, 1996). Aquaporin 1 has a molecular weight ~28 kDa and it is present at the concentration about 200,000 copies per erythrocytes (King et

al., 2000). Aquaporin 1 plays an important role in CO₂ transport through the membrane as interacts with Band 3 (Hsu et al., 2017, Endeward et al., 2006). However, aquaporin 1 main function is to transport water (water channel) through erythrocyte membranes and also interact with Band 3 to transport CO₂ through the membrane (Sugie et al., 2018, Murata et al., 2000). CD47 is a part of the band 3 complex and our data suggest that aquaporin is contained by only CD47 isoform 4 IPs and it is not present in the other isoforms IPS based on proteome results (Table 5.3). Moreover, band 3 is also upregulated by CD47 isoform 4 IPs and this upregulation with isoform 4 is 14, and 15-fold more than isoform 2, and 5 IPs respectively (Table 5.3).

Finally, the last but not least protein that has been detected by the proteome data and is belonging to lipid rafts is Guanine nucleotide-binding protein (G α) (Lauer et al., 2000). The function of this protein in erythrocytes is believed to be as a signal transducer by enhancing Cyclic Adenosine Monophosphate (cAMP) through phosphorylation of adducin (Kamata et al., 2008). Actually, Adducin is a part of band 3 complex which is include Rh proteins, p55, protein 4.1R, ankyrin, protein 4.2 and CD47 (Anong et al., 2009). According to the data, Guanine nucleotide-binding protein is upregulated in CD47 isoform 2 IPs and isoform 5 IPs as well (table 3.5). This upregulation is not specific to only one isoform of CD47 but could possible happened with either isoform 2 IPs or isoform 5 IPs; as the results show isoform 2 IPs is upregulated in Guanine nucleotide-binding protein 21 folds more than isoform 4 IPs but it is just about 1.8 folds more than isoform 5 which is not significant. On the other hand, CD47 isoform 5 IPs is upregulated in Guanine nucleotide-binding protein 17 folds more than CD47 isoform 4 IPs but it is less than 0.8-folds in compare to isoform 2 IPs (table 3.5). So, depending on the situation of the erythrocyte, CD47 isoform 2 is probably the main driver for this interaction but in

certain circumstances CD47 isoform 5 might be able to interact with Guanine nucleotide-binding protein.

5.4.3 CD47 N-terminal antibodies interactions:

Binding of CD47 with different N-terminal antibodies revealed different scenarios that CD47 may play in the mature erythrocyte. Here three different murine monoclonal antibodies were used that give rise to three different PS exposure levels in erythrocytes. BRIC 32 represents low PS exposure, BRIC 124 represents high PS exposure, and BRIC 126 represents medium PS exposure. The number of proteins that are pulled down by CD47 BRIC 124 (high PS exposure) is higher than the others.

Band 3 is a major component of the erythrocyte's membrane. It was found that CD47 IPs consistently pulled down band 3. However, the quantity of this upregulation differs between the three categories. CD47 BRIC32 band 3 ~4 fold more than its GPA control (table 5.2), while CD47 BRIC 124 ~72 fold more than its Kell control. On the other hand, CD47 when ligated with antibody BRIC 126 does not cause CD47 to bind Band 3 significantly, it is only 1.5-fold more than the CD44 control (table 5.2). The minimum fold to considerate as significant upregulation is 2-fold.

Another interesting interaction that is found when the antibody binds to the CD47 on the external surface of the membrane, the C-terminal end of CD47 binds to protein 4.1 R. Protein 4.1R is one of the most important erythrocyte membrane components as people with protein 4.1R deficiency have hereditary elliptocytosis (Dalla Venezia et al., 1992, Delaunay et al., 1996). Both CD47 and protein 4.1R is a part of Band 3 complex in erythrocytes (Bruce et al., 2003). The proteome data presented here show that different upregulation levels of protein 4.1R in IPs with CD47 N-terminal antibodies. To start with

CD47 BRIC 32 IPs shows upregulation of protein 4.1R with 14-fold than its control (GPA) while CD47 BRIC 126 IPs shows upregulation of protein 4.1R 23-fold more than its control, and CD47 BRIC IPs 124 is also show this upregulation with 3-fold more than its control (table 5.2).

Finally, Kell protein is also showing a constant upregulation with anti-CD47 IPs results. Kell is a major blood group antigen and it is always present in the erythrocyte membrane with XK protein; with the proteins being linked by a single disulfide bond (Lee et al., 2000). In the anti-Kell IP results, shown that XK is present and it is the second most abundant protein after Kell protein (figure 5.6); but with anti-CD47 IPs results the only protein was pulled down was Kell while XK is absent (data not shown) and also the score of Kell protein was not shown in the table 5.2 as BRIC 18 which is anti-Kell antibody is used as a control for BRIC 124 and it will not be upregulated comparing to CD47 (BRIC 124) IP results. This result might indicate that there is a monomer of Kell protein that binds to CD47 and is not bound to XK protein.

5.4.4 CD47 C-terminal antibodies interactions:

This part different CD47 isoforms antibodies were used to elucidate the differences between CD47 isoforms in erythrocytes using three different antibodies that are specific to CD47 isoform C-terminal peptide sequences, CD47 isoform 2, isoform 4, and isoform 5. CD47 C-terminal domain is intracellular but information about the functional significance of the different isoforms is limited. This study is the first to elucidate differences between these isoforms. However, to identify functional differences between them, the proteome results from different IPs using these antibodies were examined. These results have suggested novel interaction of CD47 with S100 protein and with lipids

rafts. Furthermore, there are also other differences with already known interactions of cytoskeletal proteins with CD47. For instance, CD47 isoform 4 is the isoform that is responsible for interaction with band 3 as the results suggest. Band 3 is upregulated 14, 15 folds with isoform 4 IPs more than CD47 isoform 2 IPs, and isoform 5 IPs respectively (table 5.3). Actually, most of CD47 intercellular interaction is mediated by CD47 isoform 4 which may be due to longer peptide sequence (table 5.3). However, there is also an importance of the other isoforms such as CD47 isoform 5 is the only isoform that upregulates Stathmin which is a protein that regulate different intracellular signal transduction (Sobel, 1991) (table5.3).

6 General discussion and Future work:

The aims of this project were to elucidate the functions of CD47 in erythrocytes, characterize different CD47 monoclonal antibodies, evaluate the expression of CD47 isoforms in erythrocytes, and study protein-protein interactions when CD47 is immunoprecipitated by CD47 monoclonal antibodies. To achieve these aims, the advantages of Next Generation Sequencing (NGS), Mass Spectrometry, and flow cytometry were utilized.

6.1 CD47 Isoform 5:

Until now, the majority of research CD47 research has suggested CD47 has four isoforms, but actually CD47 has 5 isoforms and this study has proved this important point about this gene transcripts and protein. NGS was used in chapter 4 to evaluate the expression of CD47 isoforms in reticulocytes and to confirm the presence of CD47 isoform 5 in mature erythrocytes. Previously, Plummer, 2007 in her PhD thesis, confirmed the presence of CD47 isoform 5 in erythrocytes by using RT-PCR and western blot, but Mordue et al., in 2017 published that CD47 isoform 3 and isoform 4 are diminished during erythropoiesis although they did not look for CD47 isoform 5 expression at all (Mordue et al., 2017, Plummer, 2007).

CD47 Isoform 5 transcript analysis was used to prove the presence of CD47 isoform 5 in reticulocytes (as it has an RNA (which is converted to cDNA for NGS)), NGS started by amplifying the relevant region of cDNA in all the samples by designing the forward primer to amplify the region that shared by all isoforms in exon 5 and the reverse primer was started from the common 3' exon that also shared by all isoforms (figure 3.2) in order

to be able to capture all the relevant CD47 transcripts. The non-fragmented PCR amplicons protocol was used to ensure that full read was obtained and hence be able to distinguish between different multiple transcripts that give rise to different isoforms.

To achieve the aim of studying CD47 isoform expression in erythroid cells, different obstacles had to be overcome. The first was in the NCBI database, which is updated regularly, only the sequence for four isoforms of CD47 are present, with no sequence for the transcript for isoform 5. However, Plummer (2007) detected the presence of isoform 5 in mature erythrocytes. She also corrected the right isoform sequence that originally published by Schickel et al., (2002). The sequence of the transcript that give rise to CD47 isoform 5 as shown by NGS in this study matches that described by with Plummer (2007) rather than Schickel (2002).

Monoclonal antibodies for CD47 isoforms 2, 4, and 5 were raised and ordered to confirm the presence of these isoforms in mature erythrocytes, by the synthesis of synthetic peptide immunogens corresponding to each isoform which has a unique C-terminal domain. Thus, the presence of CD47 isoform 5 in reticulocytes on transcriptomics level as well as detecting an isoform 5 specific product in immunoblots from red cell membranes was confirmed (figure 3.14). Not only detected CD47 isoform 5 in reticulocytes hence imply that is in erythrocytes, but it also presents erythroid cell lines K562, and HL-60.

Isoform 5 isoform binding partners were studied in chapter 5 using isoform 5 IP experiments. CD47 isoform 5 was found to be the only isoform that interacts with erythrocyte protein stathmin in erythrocytes which is a protein that responsible for signal transduction in erythrocyte membranes (Machado-Neto et al., 2014).

Confirming the presence of CD47 isoform 5 in erythrocyte is important as CD47 isoform 5 upregulates stathmin which is found overexpressed in myeloid leukaemia cell line

(K562) (Rubin et al., 2003). The information about CD47 in cancer are limited, at the moment the only thing that known about CD47 in cancer is that it is overexpressed but is this overexpression just for isoform 2 as detected in K562, and HL-60 or are all the isoform are overexpressed? Knowing CD47 isoform 5 interacts with stathmin which is a phosphorylated protein in HL-60 cell line (Feuerstein and Cooper, 1983), suggests that CD47 isoform 5 is involved in phosphorylation pathway.

6.2 CD47 isoforms:

After confirming the presence of CD47 isoform 5 in mature erythrocytes, the expression of transcripts giving rise to the five known isoforms were studied. 30 random samples were tested that have different Rh status (ten Rh⁺ (R₁R₁) samples, ten Rh⁺ (R₂R₂), and ten Rh⁻ (rr) samples) as CD47 has only around 45 000 copies per erythrocyte in positive Rh phenotype and around 20 000 copies per erythrocyte in negative Rh phenotype (Mouro-Chanteloup et al., 2003). The most abundant isoform of CD47 in erythrocytes is isoform 2 in all samples (Rh⁺ and Rh⁻) which is in agreement with previous research (Brown and Frazier, 2001).

Erythroid cell lines also express CD47 isoforms. Evaluation the expression of CD47 in erythroid cell lines K562, and HL-60 was achieved and found that CD47 isoform 2 is the most abundant isoform.

Using genomics aspect to evaluate the expression of transcripts that rise the isoforms has been used before in Plummer (2007) to confirm the presence of CD47 in different tissues and cells. She used specific primers for each isoform, and she managed to confirm the

presence of all CD47 isoforms in erythroid cells. However, this investigation was continued as the aim was not only to confirm the presence of CD47 isoform but also using the advantage of NGS to study which one of these isoforms have the highest abundance in erythrocyte (Plummer, 2007).

For future studies, this NGS study is important for these type of similar studies as it showed the CD47 isoform 2 is the most abundant isoform in normal erythrocytes but CD47 isoform 4 is only less than CD47 isoform 2 by (8%) in Rh+(R₁R₁), (5%) in Rh+(R₂R₂), and only (1%) in Rh-(rr) but in erythroid cancer cell lines K562 CD47 isoform 4 was expressed lower than CD47 isoform 2 (67%) and in HL-60 CD47 isoform 4 was lower than CD47 isoform 2 by (57%). These findings indicate that CD47 isoform 2 is overexpressed in cancer cells but the question here is this the case with all cancer cell lines, and if this is the case it may suggest CD47 isoform 2 is responsible for the interaction between CD47 and SIRP alpha to escape immune surveillance. Thus, we would like to evaluate CD47 isoform expression in different tissues and cells such as intestine, testis, liver, kidney, and central nervous system (CNS) and tumours derived from these tissues.

6.3 CD47 as an anti-cancer drug:

The second approach in this thesis was characterization of different CD47 monoclonal antibodies. These antibodies bind to different external regions of CD47 and induce different levels of PS exposure indicating eryptosis in erythrocytes. Seven different monoclonal antibodies that bind to CD47 all of which produce eryptosis in erythrocytes were characterized. Three different level of eryptosis were seen, suggesting three

different epitopes are recognised by the mAbs. Flow cytometry was used for characterization of PS exposure levels with an Annexin V FITC binding assay was following ligation with CD47 antibodies. The different degrees of PS exposure following ligation of CD47 using different monoclonal antibodies are a novel observation. This finding was broadened to investigate whether there was competition for epitope binding amongst the different Mabs utilised. Those different CD47 monoclonal antibodies fell into three different categories. One group induced low PS exposure, one group medium PS exposure, and one group high PS exposure. Low PS exposure antibodies were induced a low PS exposure even when they are used in combination with high PS exposure antibodies. For example, BRIC 32 induced a low level of eryptosis, but also blocked the high PS exposure level induced by BRIC 124. Thus, according to these results, it can be hypothesized that there are some CD47 epitopes that are dominant and if we CD47 is ligated with the antibodies against these, it will result in the “switching off or downregulating” at the eryptosis of the other high PS exposure inducing CD47 monoclonal antibodies.

During the study of different CD47 monoclonal antibodies, an isotype control for the IgG1 CD47 monoclonal antibodies (BRIC 256) were used. BRIC 256 is a monoclonal antibody against GPA which is an important protein in erythrocytes as it found at the concentration of 1 million copies per erythrocytes while CD47 as mentioned earlier has only around 45 000 copies per erythrocyte in positive RhD phenotype and around 20 000 copies per erythrocyte in negative RhD phenotype (Mouro-Chanteloup et al., 2003). BRIC 256 constantly switched off the entire eryptotic effect whether from the same IgG subclass or different IgG subclass. This finding was surprising as GPA is not known to be involved in the eryptosis pathway.

Furthermore, this study showed that BRIC 256 ligation affects were abolished using trypsin treated red cells, which removes the extracellular domain of GPA. Thus, a BRIC256 anti-GPA interaction must be responsible for the “master off switch” response seen by anti-GPA ligation. Based on the experiments, whether starting ligating erythrocytes with BRIC 256 followed by anti-CD47 monoclonal antibodies or starting with anti-CD47 monoclonal antibodies then added BRIC 256 the results always showed the eryptotic effect of CD47 is not achieved, hence this finding led to the suggestion that using anti-GPA (BRIC256) when trying to use anti-CD47 monoclonal antibodies as an anti-cancer therapy to achieve the results of blocking CD47 from binding to SIRP alpha. This may lead to better therapies to abolish the anaemia seen in some trials using anti-CD47.

For future work, it would be helpful to investigate the effect of GPA (BRIC 256) on GPC (BRIC 10) as previous results within our group have shown that BRIC 10 is involved in eryptosis pathway (Head et al., 2005a). BRIC 10 were used in this experiment as a positive control, but the question is the switching off effect of GPA is still working with anti-GPC or is only with anti-CD47 monoclonal antibodies. Moreover, we would like to characterize the binding site of BRIC 256 and where it binds to GPA. The preliminary data using trypsin treated erythrocytes has shown a remarkable increase in eryptosis (figure 4.13). Trypsin was characterised according to (Reid et al., 2012) to cleave GPA at positions 50 and 58 aa residues in the N terminal domain. This cleavage was led to switch on the eryptosis pathway. In GPC scenario, the switch off was noticed after the trypsin cleavage as GPC was involved in eryptosis pathway. The interpretation of “switch on” the eryptotic effect might cause trypsin treated GPA is involved in eryptosis pathway or

it might be that the monoclonal antibody (BRIC 256) recognizes an eryptotic epitopes of GPA that were not been exposed on non-trypsin treated erythrocytes (figure 6.1).

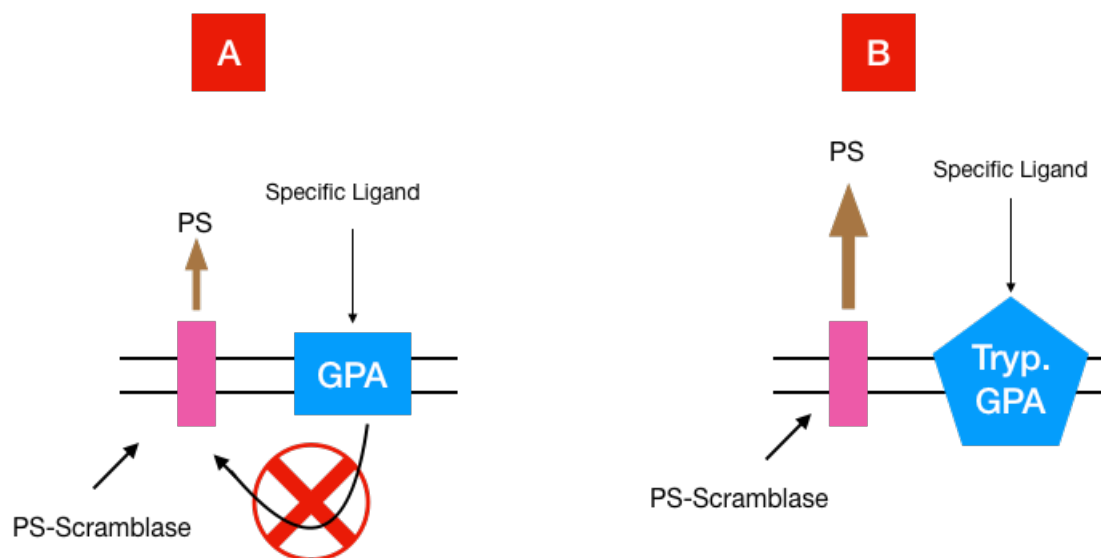


Figure 6.1: The proposed response of GPA after ligation with monoclonal antibody vs the response of trypsin treated GPA after ligation with monoclonal antibody.

Ligation of GPA with specific ligand such as BRIC 256 in normal conditions will not trigger the eryptosis pathway as shown (A). However, trypsin treated GPA when ligated with a specific ligand such as BRIC 256 will trigger the eryptosis pathway (B).

6.4 Role of CD47 in red cell turnover:

Mass Spectrometry was used to analyse the protein-protein interactions in erythrocytes using IP samples that were incubated with anti CD47 monoclonal antibodies. Some of the findings were novel as there is an interaction of CD47 with S-100 protein for the first time

in erythrocytes and this interaction was constant with high PS exposure CD47 monoclonal antibody (BRIC 124), and also with C-terminal monoclonal antibodies (isoform 2, isoform 4, and isoform 5).

S-100 protein is found in keratinocytes and was reported in erythrocytes by Goodman et al., (2007) following proteomics assessment but the function of this protein in erythrocytes remain unknown (Goodman et al., 2007, Wilson et al., 2016). S100 is analogous to calmodulin as a Ca^{2+} binding protein, thus may implicate CD47 is a Calcium signalling pathway. CD47 induces eryptosis in erythrocytes but there are different explanations for this phenomenon. The one given by Jeremy (2012) was that CD47 binds to protein 4.1R in erythrocytes and induces eryptosis by a different pathway which is include inhibition of Protein Kinase A (PKA) that led to increase eryptosis while the inhibition of Protein Kinase C (PKC) led to decrease the eryptosis pathway. Furthermore, he suggested that PKA play a role to prevent eryptosis while PKC is involved in eryptosis process. P4.1R is also known to bind calmodulin (Gauthier et al., 2011, Jeremy, 2012).

On the other hand, S-100 proteins are interacting with PKC, S-100 proteins mediate the non-classical apoptosis pathway as previously described (Viemann et al., 2007, Donato et al., 2013, Słomnicki et al., 2009). The interpretation of the interaction of CD47 with S-100 protein is that these proteins regulate Ca^{2+} influx through the membrane which is an initial step in eryptosis which also involved the PKC pathway.

Moreover, the MS results also showed an interaction of C-terminal CD47 IPs with lipid raft proteins in erythrocytes (flotillin 1, flotillin 2, stomatin, and Guanine nucleotide-binding protein ($\text{G}\alpha$)). The interaction of CD47 with lipid rafts was prominent with CD47 isoform 4. CD47 isoform 4 interacts with more intracellular proteins and that might

be the reason behind this extra peptide length that it has. The interaction of lipid rafts with CD47 is suggested to facilitate the transduction and signal pathway. The importance of discovering that CD47 are involved in lipid rafts is that CD47 can play an important role in erythrocyte membranes not only act as a self-recognition protein by binding to SIRP alpha but also participate in the transduction process by interaction with other lipid rafts membrane proteins.

For future work, we would like to confirm the interaction of CD47 with S-100 protein and lipid rafts by different technique such as western blotting. Expanding the MS results to do all the CD47 monoclonal antibodies and compare all proteins profile that were pulled down against each other as currently we have taken three N-terminal monoclonal antibodies (BRIC 32, BRIC 124, and BRIC 126) that represent each category that caused different eryptosis level. We would like to investigate the remaining N-terminal monoclonal antibodies (BRIC 122, BRIC 125, BRIC 168, and BRIC 211) and also the remaining C-terminal monoclonal antibodies (isoform 1, isoform 3).

Finally, the finding within this thesis is contributing in understanding the importance of CD47 not only in transfusion field but also in the cancer field as clinical trials are ongoing at the moment for optimization of the use of CD47 as an anti-cancer drug. The side effect of using anti-CD47 as an anti-cancer therapy is the causing of anaemia as CD47 present in all cell types. Thus, low eryptosis inducing anti-CD47 is required as the aim is to block CD47 in cancer cells without causing eryptosis. The best candidate for this option is BRIC 32. To investigate more about anti-CD47 monoclonal antibodies, it is hypothesized that BRIC 32 may only be binding to specific isoform of CD47 and that is why it is causing

lower eryptosis levels than the other BRIC antibodies against CD47. The best method to test this idea practically is to do western blot using CD47 isoform IP samples with BRIC 32 as the primary antibody. The results from knowing whether BRIC 32 has a CD47 isoform specific epitope will lead to targeting that CD47 isoform as it may not involve in the eryptosis pathway. As previously mentioned, anti-GPA could possibly be used in combination with anti-CD47 to ameliorate the anaemia seen with anti-CD47 therapy. The current study, did not test the isoforms directly by Annexin V FITC assay as they are intracellular isoforms. Also, there are only successfully raised CD47 isoforms 2, 4, and 5 monoclonal antibodies. In the future, the aim is to have all CD47 antibodies against all 5 isoforms of CD47 to fully study the role of all the CD47 isoforms in the erythrocyte.

To sum up, in this project the goal of knowing the most abundant CD47 isoform in erythrocytes and confirming the presence of the new isoform of CD47 in erythrocytes by using NGS were achieved. Moreover, the suggestion of an antibody as a candidate for using CD47 as a therapeutic agent in cancer was introduced. Finally, a novel protein: protein interaction was founded in erythrocyte membrane that involve CD47.

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8 Appendices:

Appendix A:

- 1) Annual Research Event, University of Plymouth. (April 2016).

Participation type: Poster.

Participation title: Role of CD47 In Red Cell Eryptosis.

A red blood cell starts its journey when it is produced from the bone marrow and ends its life in the liver or spleen when it is engulfed by Kupffer cells or splenic macrophages. In normal haemostasis, red blood cell lifespan is ~100-120 days. Eryptosis is programmed red cell death. The term eryptosis was first coined by Florian Lang in University Of Zurich (Lang et al., 2005). Eryptosis shares some properties with apoptosis, which takes place in all other cell types, like phosphatidylserine exposure, membrane blebbing and cell shrinkage.

CD47 is a transmembrane receptor with 5 membrane-spanning segments and is also known as Integrin-associated protein (IAP). CD47 is 47-52 kDa on SDS-PAGE and it is a member of the immunoglobulin (Ig) superfamily of membrane proteins. Moreover, it has glycosylated extracellular immunoglobulin variable domain (IgV) at its N terminal domain. The IgSF domain within CD47 drives its interactions with its receptors and ligand. There are different ligands and receptors that interact with CD47 such as: $\alpha\text{v}\beta 3$, $\alpha 2\beta 1$ integrins, thrombospondin (TSP) and signal-regulatory protein alpha (SIRP α) (Brown and Frazier, 2001).

Oldenborg et al. in 2000 hypothesized that CD47 interacts with SIRP α in the macrophage and induces an inhibitory signal “do not eat me signal” that leads to it recognizing that cell as a self-cell (Oldenborg et al., 2000). Moreover, Burger et al. in 2012 have an update about that theory which is CD47 not only acts as an inhibitory signal, but also acts as an activator signal when it bound to thrombospondin-1 (TSP-1) derived peptide which is causing engulfment of that cell. Burger et al. in 2012 mimicked a TSP-1 derived peptide by using the 4N1K peptide (with amino acid sequence: KRVYVVMWKK) that binds to CD47 because it has the VVM residue on its C-terminal binding domain and they used a control peptide 4NGG (with amino acid sequence: KRVYGGMWKK) that binds to CD47 in the same site but this peptide does not cause engulfment.

Here in this study we used Flow Cytometry (FACS ARIA II) and annexin V FITC assay protocol to measure the eryptosis rate within our red blood cell samples, which are randomly selected from Filton Blood Center, Bristol. We suggest that BRIC 126, which is Monoclonal antibody that binds specifically to CD47, might produce a synergistic effect when it is used in combination with the 4N1K peptide. Our results confirm that BRIC 126 and 4N1K bind to different sites on CD47 and suggest that the synergistic effects will increase the eryptotic rate of red blood cells. We conclude that BRIC 126 is binding to CD47 at the variable domain N-terminal while 4N1K is binding to CD47 at the C-terminal as previously mentioned.

- 2) International Society of Blood Transfusion (ISBT), Copenhagen, Denmark.
(June 2017)

Participation type: Talk.

Participation title: Anti-CD47 induces different degrees of phosphatidylserine exposure.

CD47 is a transmembrane receptor with 5 membrane-spanning segments and also is known as Integrin-associated protein (IAP). CD47 is 47-52 kDa on SDS-PAGE and it is a member of the immunoglobulin (Ig) superfamily of membrane proteins. It has a glycosylated N-terminal extracellular immunoglobulin variable domain (IgV). The IgSF domain within CD47 drives its interactions with its receptors and ligands, which include $\alpha v\beta 3$, $\alpha 2\beta 1$ integrins, thrombospondin-1 (TSP-1) and signal-regulatory protein alpha (SIRP α) which behaves as an inhibitory “do not eat me signal” that leads to recognition of self. Eryptosis is known as the mechanism of RBC death. It shares some properties with apoptosis, which takes place in all other cell types, like phosphatidylserine exposure, membrane blebbing and cell shrinkage. CD47 monoclonal antibodies (MAbs) are thought to bind to the external N-terminal of CD47 and have been shown to activate eryptotic pathway by switching phosphatidylserine (PS) from the inner leaflet of the membrane to the outer leaflet of the membrane. There are seven different CD47 MAbs from International Blood Group Reference Laboratory (IBGRL) BRIC antibodies (32,122,124,125,126,168 and 211) and have been tested on RBCs from normal blood donors to evaluate them on blocking CD47 and induce eryptosis in RBCs. Evaluation of different CD47 antibodies blocks CD47 and not induce eryptosis in RBCs and to investigate their epitope mapping by competitive binding. Red cells from ten random blood donors were obtained from NHSBT, Filton, UK. A flow cytometry assay was used to characterize the CD47 mAbs. Briefly, fresh packed red blood cells were washed at least 3 times with HEPES buffer (Sigma, U.K.). Cells were counted by using a haemocytometer. 1×10^6 cells were incubated with 10 μ g/ml of each mAb, for an hour at 37°C. Cells were then washed three times with 500 μ l Hanks buffer. Supernatants were removed and the cells re-suspended in 100 μ l binding buffer. 3 μ l of Annexin V-FITC (BD, U.K) was added to the cells. Samples were transferred into FACS tubes and analysis by FACS ARIA II (BD, U.K). Also, FACSARIA II software was used to analysis flow cytometry results while Graph pad software was used to calculate *t*-test. Data shows that BRIC 32, and 122 bind to red cell CD47 with minimal PS exposure, while BRIC 125,126,168, and 211 induce eryptosis via PS exposure. Moreover, different MAbs combinations were tested to find out if synergistic effects are found or competitive binding is demonstrated. Our data suggest that BRIC 32/122 bind to different CD47 epitopes compared to BRIC 124/125/126/168 and 211.

We have shown that PS exposure induced by red cell binding with various anti-CD MAbs is variable with BRIC 32 and 122 inducing only low levels of PS exposure compared to BRIC 124-126,168 and 211. Studies are underway to investigate the domains on which CD47 bind. We have also shown by RNA-seq experiments that there are five different red cell CD47 isoforms, and experiments are underway to investigate which isoforms are bound by all CD47 MAbs under investigation.

- 3) British Blood Transfusion Society (BBTS), Glasgow, UK. (September 2017).

Participation type: Poster.

Participation title: Molecular Studies of Erythroid CD47.

CD47 (also known as Integrin-associated protein) is a 47-52KDa transmembrane receptor with 5 membrane-spanning segments and is an immunoglobulin superfamily (IgSF) member. It has a glycosylated N-terminal extracellular immunoglobulin variable domain. The IgSF domain within CD47 has interactions with its receptors and ligands, which include $\alpha v \beta 3$, $\alpha 2 \beta 1$ integrins, thrombospondin-1 and macrophage signal-regulatory protein alpha, which behaves as an inhibitory “do not eat me signal” leading to recognition of self. We have previously shown that CD47 ligation in red cells induces phosphatidylserine (PS) exposure, a key event in eryptosis, indicating the key role for CD47 in red cell turnover.

CD47 has five differently spliced isoforms that give rise to different C-terminal domains. We have used next generation sequencing (NGS) to evaluate the expression of CD47 in reticulocytes and have confirmed not only the presence of isoform 5 but also its sequence. Using NGS on erythroid and erythroleukaemic transcripts we have defined the relative proportions of each CD47 isoform in these cells. We have raised monoclonal antibodies (mAbs) to CD47 isoform 4 and are in the process of producing mAbs to other CD47 isoforms to investigate the specific CD47 interactions with red cell membrane components. Immunoprecipitation with anti-CD47 mAbs (BRIC's 32, 122, 124, 125, 126, 168 and 211) has shown distinct reproducible interactions with the band 3 complex/protein 4.2, and with components of erythroid lipid rafts (stomatin, flotillin A and B spectrin and actin) which form signalling units within membranes.

Moreover, we have shown variability of PS exposure following mAbs ligation extending on previous studies. We have shown by competitive binding that there are at least three distinct epitopes of CD47, which show varying levels of PS exposure, with BRIC 32 showing the least and BRIC 124 showing the most exposure. These may indicate that CD47 ligation induces different conformations in the molecule leading to differing signalling pathways, including PS exposure, a hallmark of eryptosis. This may also inform the choice of anti-CD47 mAb used as an anti-tumour drug in that those that express low levels of PS exposure on red cells may not cause, or produce less, anaemia in cancer patients.

- 4) Annual Research Event, University of Plymouth. (March 2018).

Participation type: Poster.

Participation title: Evaluation of CD47 isoforms expression in red blood cells.

CD47 is a transmembrane receptor with 5 membrane-spanning segments and also is known as Integrin-associated protein (IAP). CD47 is 47-52 kDa on SDS-PAGE and it is a member of the immunoglobulin (Ig) superfamily of membrane proteins. Moreover, it has glycosylated extracellular immunoglobulin variable domain (IgV) at its N terminal domain.

The IgSF domain within CD47 drives its interactions with its receptors and ligand. There are different ligands and receptors which interact with CD47 such as: $\alpha v\beta 3$, $\alpha 2\beta 1$ integrins, thrombospondin (TSP) and signal-regulatory protein alpha (SIRP α).

In 1988, Avent et al. introduced CD47 as a red cell membrane protein by multiple monoclonal antibodies reactivity against Rh_{null} phenotype erythrocyte. A few years later and after knowledge of alternative splicing, four different isoforms of CD47 were discovered and named isoforms 1-4. However, in 2002 a new isoform was discovered and named isoform 5. Thus, CD47 mapping has 12 exons, 11 introns, and 5 isoforms. Twenty random blood samples were taken from NHSBT, Filton, UK. Next generation sequencing was applied to them. Briefly, RNA was extracted from reticulocytes in 20 samples (5 R1R1, 5 R2R2, and 10 rr). cDNA synthesis were made by using synthetic III kit from Life technology, UK. PCR primers were design to extracted CD47 gene from cDNA samples. Next generation sequencing preparation include: Library construction, Templates preparation, Run sequence, and data analysis. Our results confirm the presence of 5 CD47 isoforms in red blood cells. Moreover, the data shows isoform 2 is the most abundant isoform in red cells at average presence 28% of CD47 while in cancer cell lines such as HL60 and K562 isoform 2 is the dominant isoform of CD47 at presence average 72%.