EVALUATION OF ANTI-CANCER FUNCTION IN NATURAL KILLER CELLS GENERATED FROM RAT HEMATOPOIETIC STEM CELLS

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Field of Study:

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ABSTRACT

Natural killer cells (NK) cells are lymphocytes and they are the most important key constituent of mammalian immune system against cancer tumour cells. The genome of NK cells is categorically divided to inhibitory and activator genes that code ligands on the surface of NK cells. Indeed, these ligands are cancer tumour cells destroyer arms of the NK cells. On the other hand, hematopoietic stem cells (HSCs) are the origin of all blood cells, derives from BM and mature to immune cells. NK cells cannot defeat CTCs on their own. The main reason is their population (numbers are low), they cannot derive from HSCs as fast as CTCs division rate, and that is why external treatments have to be used to treat cancer. This project, designed to promote and give potency to immune system against CTCs without relying on external treatments. To obtain this goal, three specific genes of NK cells, cloned in BMHSCs and differentiated the stem cells to NK cells. The success of the colonization has been examined by western blot and flow cytometry and in term of functionality of newly generated NK cells the cytotoxicity has been examined by MTT assay and Live and Dead assay and the apoptosis ability of newly generated NK cells have been examined by RARP Cleavage and Caspase 3/7 assay. The results and data approve that the generated NK cells from BMHSCs are able to destroy cancer cells.

Keywords: Natural killer cells, Cancer Tumour Cells, Bone Marrow, Hematopoietic Stem Cells

MENILAIAN FUNGSI ANTI-KANSER OLEH SEL PEMUSHAN SEMULAJADI YANG DIJANA DARIPADA SEL STEM HEMATOPOIETIC TIKUS

ABSTRAK

Sel-sel pemusnah semulajadi (NK) adalah limfosit dan mereka adalah kunci asas sistem imun mamalia terhadap sel-sel tumour kanser. Genom sel NK terbahagi kepada gen penghalang dan pengaktif yang mengekod gen ligan di atas permukaan sel NK. Sememangnya ligan-ligan pada sel NK ini adalah senjata pemusnah CTC (sel kanser yang beredar) . Sebaliknya, sel-sel stem hematopoietik (HSC) adalah asal-usul untuk semua jenis sel-sel darah, berasal dari sum-sum tulang (BM) dan kemudiannya matang menjadi sel imun. Sel NK tidak dapat mengalahkan CTC dengan sendirinya. Alasan utama adalah populasinya (jumlahnya rendah) dan ianya tidak dapat dihasilkan dari HSC secepat CTC membahagi. Itu sebabnya teknik rawatan luaran harus digunakan untuk mengobati kanser. Projek ini, direka untuk menggalakkan dan memberikan potensi kepada sistem imun terhadap CTC tanpa bergantung kepada rawatan luaran. Untuk mencapai matlamat ini, tiga gene khusus sel NK, diklonkan kedalam BMHSCs dan membezakan sel stem tersebut menjadi sel NK. Kejayaan penaklukan telah diperiksa menggunakan 'western blot' dan 'flow cytometry' dan dari segi fungsi sel NK yang baru dijanakan, sitotoksisiti telah diperiksa dengan esei MTT, esei hidup dan mati dan kebolehan apoptosis oleh sel NK yang baru dijanakan diuji dengan esei RARP Cleavage dan Caspase 3/7. Data dan keputusan telah menunjukkan bahawa sel NK yang dijana daripada BMHSCs berkebolehan untuk memusnahkan sel kanser.

Kata kunci: Sel pemusnah semulajadi, sel tumour kanser, sumsum tulang, sel stem hematopoitik

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After all this job is done but I have to say I never wanted that this project only been as a fulfil of graduation condition only but help science one step forward and been useful to people of the world as I am just one of them.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADCC	:	Antibody-Dependent Cellular Cytotoxicity
AICL	:	Activation-induced C-type lectin
ALL	:	Acute Lymphoblastic Leukaemia
AML	:	Acute myeloid leukaemia
APC	:	Antigen Presenting Cells
BM	:	Bone marrow
BME	:	Basal Medium Eagle
BSA	:	Bovine serum albumin
CAFC	:	Cobblestone Area Forming Cell
CD 49	:	a cluster of differentiation 49
CLP	:	Common Lymphoid Progenitor
CMI	:	Cell-mediated immunity
CML	:	Chronic myeloid leukaemia
CMP	:	Common Myeloid Progenitor
CMV	:	cytomegalovirus
CTL	:	Cytotoxic T Lymphocyte
CTLD	:	C-type lectin-like domain
DECTIN	:	natural killer cell receptor-like C-type lectin
DHFR	:	Dihydrofolate reductase
DHPLC	:	Denaturing High-Performance Liquid Chromatography
DMEM	:	Dulbecco’s modified Eagle's medium
DOPE	:	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DTH	:	Delayed-type hypersensitivity
EDTA	:	Ethylene diamine tetra acetic acid

EMEM	:	Eagle's minimal essential medium
FACS	:	Fluorescence Activated Cell Sorting
FBS	:	Fetal Bovine Serum
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GFP	:	Green fluorescent protein
GvHD	:	Graft-versus-host disease
GvL	:	Graft-versus–Leukaemia
HaeIII	:	restriction enzymes (endonucleases)
HCMV	:	Human cytomegalovirus
HIV	:	Human Immunodeficiency Virus
HLA	:	Human leukocyte antigen
HPA	:	Health Protection Agency
HSC	:	Hematopoietic stem cell
HSCs	:	Hematopoietic stem cells
HSCT	:	Hematopoietic stem cell transplantation
HSV	:	Herpes Simplex Virus
IFNA	:	Interferon alpha
IFNB	:	Interferon beta
IgA	:	Immunoglobulin A
IgE	:	Immunoglobulin E
IgG	:	Immunoglobulin G
IgSF	:	Immunoglobulin superfamily
ITAM	:	Immune receptor tyrosine-based activation motifs
ITIM	:	Immune receptor tyrosine-based inhibitory motif
KIR	:	Killer cell Immunoglobulin-like Receptor

KitL	:	Stem cell factor, KIT-ligand
KLR	:	killer lectin-like receptor
LAIR	:	Leukocyte-associated Ig-like receptors
LENG	:	LRC-encoded novel gene
LILR	:	Leukocyte Ig-like receptors
LLT	:	Lectin-Like Transcript
LOU	:	myeloma cell line
LOX	:	Lectin-like oxidized receptor-1
LRC	:	Leukocyte receptor complex
LS	:	magnet separation column
LSC	:	Leukaemia stems cells
MAFA	:	Mast cell function-associated antigen
MBP	:	Mannose-binding protein
MCMV	:	murine cytomegalovirus
MHC	:	Major histocompatibility complex
MIC	:	major histocompatibility complex (MHC) class I chain-related
MICA	:	MHC Class I Polypeptide-Related Sequence A
MICB	:	MHC Class I Polypeptide-Related Sequence B
MPB	:	Mobilized peripheral blood
MSC	:	Mesenchymal stem cells
NCR	:	Natural cytotoxicity receptor
NK	:	Natural killer
NKC	:	Natural killer complex
NKCs	:	Natural Killer Cells
NKT	:	Natural killer T cells

PAMP	:	Pathogen-associated molecular patterns
PCR	:	polymerase chain reaction
PRH	:	prolactin-releasing hormone
PCR	:	Polymerase chain reaction
SCF	:	Stem cell factor
SIGLEC	:	Sialic-acid-binding immunoglobulin-like lectin
SLT	:	Secondary lymphoid tissues
SP	:	Side population
TC	:	trauma centre maturation
TCR	:	T cell receptor
TE	:	Tris, T ten E one buffer
TPO	:	gene Thyroid Peroxidase (Protein Coding)
UCB	:	Umbilical cord blood
UCBT	:	Umbilical cord blood transplants
ULBP	:	UL16 binding protein 1
WT	:	Wild-type

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Natural Killer Cells Background

Natural killer (NK) cells were discovered more than 30 years ago. NK cells are large granular lymphocytes that belong to the innate immune system because unlike T or B lymphocytes of the adaptive or antigen-specific immune system, NK cells do not rearrange T-cell receptor or immunoglobulin genes from their germline configuration. During the past 2 decades there has been a substantial gain in our understanding of origin, morphology and functions of Natural Killer cells in related to other immune cells. The most recent discoveries in NK-cell receptor biology have drove translational research that has led to extraordinary results in treating human malignancy.

The biology of the Natural Killer cell system is being investigated by many different laboratories using multiple approaches. The rationale for these investigations is the experimental evidence that NK cells play some role in inhibiting tumour growth and metastasis, convey some protective immunity and may be operative in control of differentiation from fetal life to adulthood. In preliminary experiments on cell-mediated cytotoxicity against tumour target cells, both in cancer patients and animal models, researchers consistently observed what was considered as "natural" reactivity. A certain population of cells seemed to be able to lyse tumour cells without having been previously sensitized to them. The first published study to assert that untreated lymphoid cells were able to confer a natural immunity to tumours was presented by Dr. Henry Smith at the University of Leeds, School of Medicine in 1966 (Greenberg, 1994) leading to the conclusion that the "specific cells appear to be an expression of defence mechanisms to tumour growth present in normal mice."

By 1973, 'natural killing' activity was established across a wide variety of species, and the existence of a separate lineage of cells possessing this ability was hypothesized. The discovery that a unique type of lymphocyte was responsible for “natural” or spontaneous cytotoxicity was made in the early 1970s by doctoral student Rolf Kiessling and postdoctoral fellow Hugh Pross, in the mouse (Kiessling et al., 1975) and by Hugh Pross and doctoral student Mikael Jondal in the human (Jondal & Pross, 1975). The mouse and human work were done under the supervision of professors Eva Klein and Hans Wigzell, respectively, of the Karolinska Institute, Stockholm. Kiessling’s research involved the well-characterized ability of T lymphocytes to lyse tumour cells against which they had been previously immunized. Pross and Jondal were studying cell-mediated cytotoxicity in normal human blood and the effect of the removal of various receptor-bearing cells on this cytotoxicity. Later that same year, Ronald Herberman published similar data with respect to the unique nature of the mouse effector cell (Herberman et al, 1975).

Using discontinuous density centrifugation, and later monoclonal antibodies, natural killing ability was mapped to the subset of large, granular lymphocytes known today as NK cells. The demonstration that density gradient-isolated large granular lymphocytes were responsible for human NK activity, made by Timonen and Saksela in 1980 (Timonen & Saksela, 1980) was the first time that NK cells had been visualized microscopically, and was a major breakthrough in the field.

According to recent studies about the evolution of Natural Killer cells indicates that the cytolytic effector cells that resemble Natural Killer cells have been part of the innate immune defence system long before the arrival of the seemingly more sophisticated T and B cells of the adaptive immune system approximately 500 million years ago (Cooper & Alder, 2016).

Yet today, all 3 of these lymphocyte lineages survive with NK cells outnumbering B cells in the circulation by a 3-to-1 ratio and with newly discovered functional complexity that rivals their antigen-specific memory-bearing counterparts. Clearly, NK cells must serve a very important role in host defence or they would not be here.

1.2 Hematopoietic Stem Cells Background

The discovery of hematopoietic stem cells (HSCs) provided a ground-breaking step in stem cell research. HSCs are a type of multipotent adult stem cell, characterized by their ability to self-renew and differentiate into erythrocyte (red blood cell) and leukocyte (white blood cell) cell lineages. Regarding function, these cells are responsible for the continual renewal of the erythrocytes, leukocytes, and platelets in the body through a process called haematopoiesis (Mahla, 2016).

The definition of hematopoietic stem cells has evolved since HSCs were first discovered in 1961(Till & McCulloch, 1961). The hematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors. Hematopoietic Stem Cells (HSCs) are the stem cells that give rise to other blood cells (Birbrair & Frenette, 2016b). This progress occurs in the red bone marrow, in the core of most bones.

In embryonic development, the red bone marrow is derived from the layer of the embryo called the mesoderm (Beerman et al., 2014). HSCs give rise to both the myeloid and lymphoid lineages of blood cells (Alexander et al., 2017). Myeloid and lymphoid lineages both are involved in dendritic cell formation. Myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes to platelets. Lymphoid cells include T cells, B cells, and natural killer cells.

The HSCs also play a prominent role in the formation of vital organs such as the liver and spleen during fetal development (Mahla, 2016). The early biological knowledges obtained from the studies of HSCs established the base of knowledge for understanding other stem cell systems. In addition, these cells have a vital role in furthering stem cell research for clinical applications. Regenerative medicine is a field of medicine that has applied HSCs to the treatment of blood-borne diseases such as leukaemia and lymphoma and of cancer patients undergoing chemotherapy.

Indications of blood-forming cells in humans first appeared in 1945 from studies on the citizens of Hiroshima and Nagasaki during World War II. After surviving the atomic bomb explosions, those individuals who had come across low radiation exposure died over an extended period, later research indicated that they had a compromised hematopoietic system. Their compromised systems did not allow the individuals to produce enough leukocytes to fight non-pathogenic infections or enough platelets to prevent excessive bleeding. Subsequent research with mice explored the details behind this observed phenomenon. It was discovered that when mice were given minimal lethal dosages of radiation, they all died within two weeks due to the failure of their hematopoietic systems. This mirrored what had happened to the Japanese citizens. Curiously, another experiment demonstrated that mice were able to recover from irradiation if a single bone or their spleen was protected from radiation.

1.3 Researches regarding NK Cells and HSCs application

Recently there is been many researches regarding relation of Natural Killer cells and Hematopoietic Stem Cells. Basically, since the origin of NK cells are HSCs, most of the researches focus on formation of NK cells from HSCs and involved pathways. New studies are going farther to understand the Role of Natural Killer Cells in Hematopoietic Stem Cell Transplantation (Darlington et al., 2018).

Generally, most researches regarding NK cells and HSCs relation do have three specific perspectives.

- Differentiation of NK cells from HSCs. As example, (Cappel et al., 2017), (Hara et al., 2016).
- Behaviour of NK cells in HSCs transplantation. As example, (Dezell et al., 2016), (Herrmann et al., 2016), (Cappel et al., 2017).
- Pathways of NK cells helps of Improving HSCs Engraftment, as example, (Bonnet et al., 2015; Pfeiffer et al., 2017; Eldjerou et al., 2018).

Therefore, according to most prominent researches and hardworking in this area, gives the impression that there is still a considerable gap in most researches on effectiveness and functionality applications of Natural Killer cells and Hematopoietic Stem cells together. The main hypothesis of this research was to attempt to fill up this gap by inducing Natural Killer Cell's ability of destroying cancer cells through to Hematopoietic Stem Cells.

1.4 Problem Statement

Natural killer cells use a collection of germline-encoded activating and inhibitory receptors that scan for altered protein-expression patterns. NK cells display quick and potent immunity to metastasis or haematological cancers, and major efforts are now being commenced to fully exploit NK cell anti-tumour properties in the clinic. The most important element of Immune system against cancer cells are Natural Killer Cells. NK cells in process of Haematopoiesis, derive from Hematopoietic Stem cell's lymphoid progenitors in bone marrow. Accordingly, a patient suffering from cancer won't have very much chance of survival only by relying on his/her own immune cells alone, without any external treatments such as specific drugs, chemotherapy, radiotherapy and so on. NK cells, in a time-consuming process, derive from bone marrow, they don't

divide like normal cells, consequently, their population is always very much less than cancer cells. This research has two main objectives and five sub-objectives. The objectives are, First generating NK cells from hematopoietic stem cells to obtain mass population of newly generated NK cells with dividing ability and secondly evaluating the functionality of these newly generated NK cells on killing cancer cells.

In another word, in this research, the researcher, attempted to give cancer cells killing ability of NK cells to HSCs. Regarding to this hypothesis that would be several problems ahead

- Extracting specific genes (*KLRA4*, *KLRA8*, *KLRD1*) from NK cell's entire genome for the purpose of transfecting them into the Hematopoietic stem cell.
- Methods and Materials which have to be used to activate ligand's genes after transfection procedure.
- Assays which must be used to ensure the success of the transfection process and differentiation of HSCs to NK cells.
- Assays and techniques which must be used to examine the cytotoxicity and Apoptosis ability of newly generated NK cells on targeted cancer cells.

1.5 Research Questions

This research poses the following questions

1. If the ligand's genes were successfully transfected into the stem cells genomes, could the colonized genes code NK ligands on the HSCs membrane?
2. Will the differentiated HSCs (newly generated NK cells) act as NK cell and kill cancer cells?
3. How reliable is this technique for curing cancer?

4. Will the genome changes inherit through divisions of the newly generated NK cells to achieve mass-population of newly generated NK cells (differentiated HSCs)?

1.6 Research Objectives

Based on the hypothesis of this study which is mentioned in section 1.4, the objectives of this research are:

1. To generate NK cells from hematopoietic stem cells
2. To evaluate functionality of the generated NK cells in killing cancer cells

Table 1.1: Objectives Figure Approach

OBJECTIVES	
Objective 1	Objective 2
To generate NK cells from hematopoietic stem cells.	To evaluate function of the generated NK cells in killing cancer cells.
Methodology for Objective 1	**Methodology for Objective 2 (*in vitro* – Cancer Cell Lines (control – untreated vs treated))**
1. Generation of NK cells from hematopoietic stem cells and verify the generation using western blotting to examine expression of receptors on generated NK cells	1. Cytotoxicity: MTT assay & live/dead assay
2. Flow cytometry – count the number of generated NK cells	2. Apoptosis: PARP cleavage assay & caspase 3/7 assay

In this research, Separation of Natural killer cells (NK cells) from blood, was done with LS column technique with the assistance of specific microbeads. After that, the NK cells were cultured in animal cell culture medium for growth and increase of their population. In the culture, researcher used NK cells activators such as IL 12 to activate the growth of the cells and push their transgenic factor of their regulation to help to grow more and more. For even improved growth Inomycin /PMA was used for 24 hours to provoke growth of the NK cells and their ligands (Cervera & Kamen, 2018). After purifying NK cells, genes of ly49h, ly49d and CD94 ligands (*KLRA4*, *KLRA8*, *KLRD1*)

were separated from the entire genome of NK cells and transferred to harvested hematopoietic stem cells (the stem cells were harvested from bone marrow of the rat thigh bone and been cultured in the stem cells special media (Xeno- free expansion cell culture with SCF, FLt-ligands IL3,6) to prevent maturation (Cheng et al., 2013)). With Lipofectamine as nucleotide vector the separated genes were transferred to stem cells. According to the objective of this research to examine the transfection ratio, western blotting and flow cytometry were used and to assay the functionality of newly generated NK cells regarding cytotoxicity MTT and Live/Dead assays were used and also regarding apoptosis PARP cleavage and Caspase 3/7 kit assays were used.

1.7 Research Scopes

To implicate the hypothesis of this research and fulfil the objectives mentioned in secession 1.6 there are several scopes as fallow

- To separate NK cells from the blood ficolin by the help of LS column procedure.
- To extract specific three genes of two activators and one inhibitory ligand of NK cells.
- To transfer these specific genes of NK cells ligands by the help of a vector to HSCs.
- To harvest hematopoietic stem cells from bone marrow of experimental rat.
- To culture and populating harvested stem cells with specific culture medium.
- To colonize and transform HSCs into an immune cell with NK cells characteristics.
- To provoke the transgene cells to grow cell membrane ligands with the help of Microbeads.

1.8 Research Contribution

According to American National Cancer Institute, in 2018, an estimated 1,735,350 new cases of cancer have been diagnosed in the United States and 609,640 people will die from the disease. Estimated national expenditures for cancer care in the United States in 2017 were $147.3 billion (https://www.cancer.gov/about-cancer/understanding/statistics).

According to the American Cancer Society, in the United States, about 1,620 people were expected to die of cancer each day in 2015 — this equates to nearly 590,000 people, (https://www.cancer.org/latest-news/understanding-cancer-death-rates.html).

According to the Malaysian National Cancer Registry Report (MNCR) 2007-2011, a total of 103,507 new cancer cases were diagnosed in Malaysia during the period of 2007-2011, of which 46,794 (45.2 per cent) were reported in males and 56,713 (54.8%) in females, (http://www.moh.gov.my/english.php/pages/view/402).

Cancer caused humanity suffering, this project was designed to help and strengthen the immune system to last longer against cancer that it may be a alternative treatment of cancer.

1.9 Thesis Organization

This thesis is organized in accordance with the standard structure of thesis and dissertations at the University of Malaya. As the final report of this research, the thesis is organized in a way to provide detail information on how the research is performed. This thesis consisted of seven chapters.

Table 1.2: Thesis organization based on objectives.

Chapters			
2. Literature Review	**3.** Methodology	**4.** Results	**5.** Conclusion
2.2 Overview on Immune System	**3.2** Methods for Objective 1 (Verification)	4**.2** Results of Objective 1	**5.2** Discussion on Results of Objective 1
2.3 Overview on HSCs	**3.3** Methods for Objective 2 (Functionality)	**4.3** Results of Objective 2	**5.3** Discussion on Results of Objective 2
2.4 Related Assays to Objective 1 & 2			

The first chapter of thesis brings up the background of the research. It expresses the researcher's motivation and research intention, objectives to study NK cells and hematopoietic stem cells. Thereafter the problem statement is explained, and scopes of the research and the research contributions are presented.

Chapter two is the literature review that gives a review and discussion of previous related works. In this chapter resource materials, such as journals, conference proceedings, books, seminars, thesis and online resources were used as the main references.

Chapter three is the research methodology. This chapter explains the research methodology used in this research. The methodology consists of three different stages. The first stage is performing theoretical study and literature review. The next stage is proposing the initial researchers about NK cells and stem cells. In the third stage, the applications and processes are explained.

Chapter four describes the preliminary study data collection, data analysis, and results. The preliminary study aims to verify the transfection efficiency ratio through the results and data given by the data analysis software. The collected data is being

analysed using statistical analysis. the findings from this chapter provide the basis for proposing the technique of cancer cure.

The last chapter is a general discussion about how research could cover the project objectives and answered the research questions

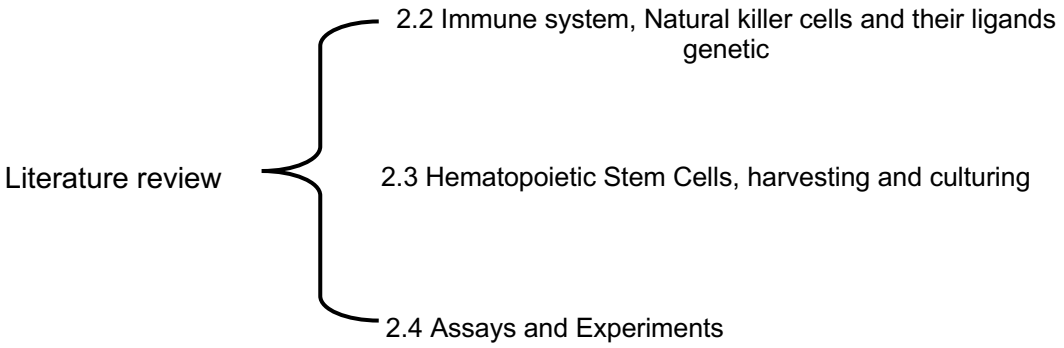
1.10 Chapter summary

This chapter involved with research backgrounds and explained the gap in previous researches and demonstrated the rationale of this research and covers the problem statements, research questions, and objectives. Research scopes are also discussed as well in this chapter. The potential theoretical and practical contributions of this research are also presented. Ultimately the summary of the thesis organization wraps up the chapter.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction:

In this chapter, complete information about the main categories of this research, is given. This chapter begins with an overview of Immune System, Natural Killer Cells and their ligands and will continue with an overview of Hematopoietic stem cells, harvesting and culturing them and at last this chapter will end with explanations about assays and experiments which have been used in this research. Therefore, this chapter contain three categories as shown below.



2.2 Overview on Immune System

2.2.1 Classification of Immune System to Innate and Acquired Immune Responses

The immunity system is responsible in responding to an invading organism or immunogens which are received by our body. This response is directed by innate and adaptive arms of the immune system. The border between adaptive and innate immune system in some aspects is not clear. In fact, these two systems are combined in many responses. The innate immune system is mediated by the cells and factors which are accountable for the fast and first defence against infection whereas the adaptive immune

response which is mainly organized by T and B cells are in charge for a long-term memory response (Johansson, 2016).

The cells intricate in innate immunity can include a different variety of myeloid and lymphoid cells such as neutrophils, basophils, macrophages, and eosinophils. The other cells like dendritic cells play a dual role in both adaptive and innate immunity (Hou et al., 2012). In current years, there are several reports about the role of NK cells in acquired immunity. Previously it was alleged that NK cells are involved in innate immunity but recent reports have indicated that NK cells have memory against pathogens and after a primary infection, they respond faster for the second time of infection. This response happens especially after viral infection (Augusto et al., 2012) .The line and situation of factors and cytokines in innate and adaptive immunity are more complicated and there are lots of statements about the role of different cytokines in both immunities (Eissens et al., 2012).

2.2.2 Innate Immune System

Microorganisms that are encountered daily in the life of a healthy individual cause disease only infrequently. Most are detected and destroyed within minutes or hours by defence mechanisms that do not rely on the clonal expansion of antigen-specific lymphocytes. These are adequate mechanisms of innate immunity(Poli et al., 2010). To recognize pathogens, both the innate and adaptive immune systems can distinguish between self and non-self, but they differ in how they do this. Innate immunity relies on a limited number of receptors and secreted proteins that are encoded in the germline and that recognize features common to many pathogens. In dissimilarity, adaptive immunity uses a process of somatic cell gene rearrangement to generate an enormous repertory of antigen receptors that are capable of fine distinctions between closely related molecules (Sattler, 2017). Nonetheless, the innate immune system discriminates very effectively

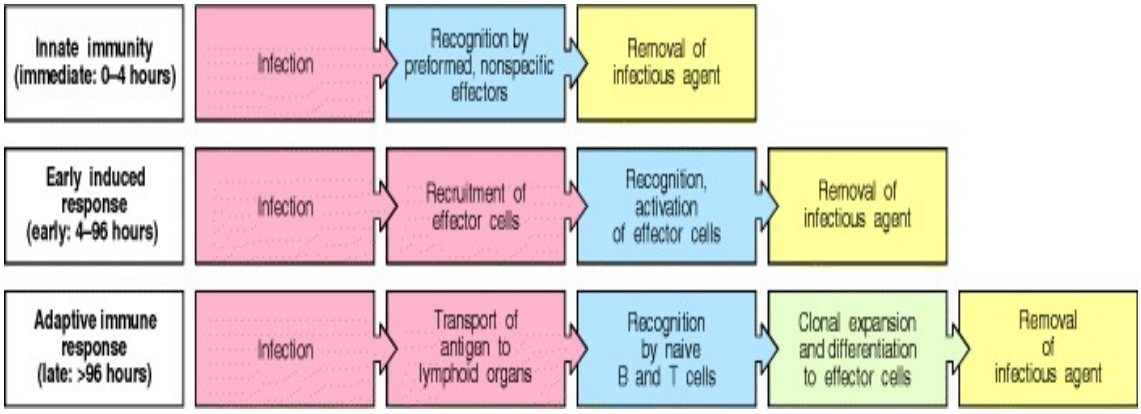
between host cells and pathogens, providing initial defences and also contributing to the provocation of adaptive immune responses (Tsuchiya et al., 2017). The importance of innate immunity is shown by the fact that defects in its components, which are very rare, can lead to increased susceptibility to infection, even in the presence of an undamaged adaptive immune system. The response to an encounter with a new pathogen occurs in three phases, when a pathogen flourishes in breaching one of the host's anatomic barriers, some innate immune mechanisms start acting immediately. The first defences include several classes of preformed soluble molecules present in blood, extracellular fluid, and epithelial secretions that can either kill the pathogen or deteriorate its effect. Antimicrobial enzymes such as lysozyme instigate to digest bacterial cell walls (antimicrobial peptides) such as the defences lyse bacterial cell membranes directly and a system of plasma proteins known as the complement system targets pathogens, both for lysis and for phagocytosis by cells of the innate immune system such as macrophages (Eissens et al., 2012).

In the second phase of the response, these innate immune cells sense the presence of a pathogen by recognizing molecules, emblematic of a microbe and not shared by host cells pathogen-associated molecular patterns (PAMPs) and become activated, setting in train several different effector mechanisms to eliminate the infection. By themselves, neither the soluble nor the cellular components of innate immunity generate long-term protective immunological memory (Maghazachi, 2010). Only if an infectious organism breaches these first two lines of defence will mechanisms be engaged to induce an adaptive immune response the third phase of the response to a pathogen. This leads to the expansion of antigen-specific lymphocytes that target the pathogen-specific call and to the formation of memory cells that provide long-lasting specific immunity.

2.2.3 The First Line of Defence

Microorganisms that cause disease in humans and animals enter the body at different sites and produce disease symptoms by a variety of mechanisms. Many different infectious agents can cause disease and damage to tissues, or pathology, and are referred to as pathogenic microorganisms or pathogens.

Table 2.1: The response to an initial infection occurs in three phases.



"The effector mechanisms that remove the infectious agent (for example, phagocytes and complement) are similar or matching in each phase, but the first two phases rely on recognition of pathogens by germline-encoded receptors of the innate immune system, whereas adaptive immunity uses variable antigen-specific receptors that are produced as a result of gene rearrangements Adaptive immunity occurs late, because the rare B and T cells specific for the invading pathogen must undergo clonal expansion before they differentiate into effector cells that can clear the infection" (Sattler, 2017. Advances in Experimental Medicine and Biology, pages 3-14).

In vertebrates, the microbial invasion is initially countered by innate defences that pre-exists in all individuals and begin to act within minutes of an encounter with the infectious agent. Only when the innate host defences have been bypassed, evaded, or overwhelmed is an adaptive immune response required (Cappel et al., 2017). Innate

immunity is sufficient to prevent the body from being routinely overwhelmed by the vast number of microorganisms that live on and in it. Pathogens are microorganisms that have evolved ways of overcoming the body's innate defences more effectively than other microorganisms (Eissens et al., 2012). Once they have gained a hold, they require the concerted efforts of both innate and adaptive immune responses to clear them from the body. Even in these cases, the innate immune system performs a valuable delaying function, keeping pathogen numbers in check while the adaptive immune system gears up for action (Bernardini et al., 2012).

2.2.4 Role of NK Cells in Innate Immune System

NK cells have an important role in innate immune responses, particularly in antiviral immunity. Recent studies have revealed a molecular basis for NK cell recognition of virus-infected cells, implicating the activating KIR and ly49 receptors and NKG2D in this process (Rahim et al., 2014). Additionally, cooperation between NK cells and dendritic cells suggests that these innate cells can shape the nature of an adaptive immune response. These findings, as well as advances in understanding NK cell development and homeostasis, indicate that NK cell biology is more sophisticated than previously appreciated (Bartel, & Steinle, 2013). NK cells have emerged as pivotal players in immune responses against pathogens and tumours. Research during the past decade has focused on the identification of the cell surface receptors and effector molecules that NK cells use in target cell recognition and destruction. Attention now turns to determining both the role of NK cells *in vivo* in innate immunity and their contribution to adaptive immunity (Bonnet et al., 2015). Although many of the NK cell activating and inhibitory receptors, their ligands and signalling pathways have been discovered , the biological relevance of these molecules in host defence, how they are regulated during development, and elucidation of the interactions between NK cells and

other hematopoietic cells are critical issues to address (P. A. Marinho, Chailangkarn, & Muotri, 2015).

The induction of IFN-g production, cytotoxicity, CD69 expression and proliferation in resting NK cells *in vitro* has been documented by using mouse DC cell lines, mouse bone marrow-derived DCs, human monocyte-derived DCs, and human cord blood-derived DCs (Jansen et al., 2013) . The mechanism by which DCs activate resting NK cells *in vitro* requires direct cell contact, but probably also involves soluble factors. These *in vitro* studies suggested that a range of cytokines produced by DCs, including IL-12, IL-18, and type I IFN, are required for the induction of the various NK effector functions, but the data are conflicting and no consensus has emerged. Interestingly, IL-2 is produced by DCs and is necessary for DC-induced IFN-g production by NK cells *in vitro* and *in vivo*. In addition, the maturation state of the DCs might influence their ability to activate NK cells (Kruse & Vivier, 2017). Several studies have shown that immature DCs require a maturation stimulus to activate NK cells, whereas others have shown that immature and mature DCs are equivalent in their ability to activate NK cells. The in vivo relevance of NK activation by DCs has been demonstrated in murine tumours and viral models, both implicating the CD8a+ DC subset. During infection of C57Bl/6 mice with mouse cytomegalovirus (MCMV), the expansion of NK cells induced by DCs was shown to specifically involve the ly49h receptor on NK cells and the cytokines IL-12 and IL-18 (Pegram et al., 2013).

2.2.5 Natural Killer Cells

Adaptive immunity, with its repositioning Immunoglobulin and T cell receptors, has caught most of the attention of up-to-date immunological research and outshined the importance of the receptors expressed by cells of the innate immune system (Middleton & Gonzelez, 2010) . NK cells have evolved two main receptor systems to carry out their

functions, both involving activating and inhibitory receptors, and include members of the Immunoglobulin-like superfamily as well as lectin-like receptors. KIR are polymorphic cell surface molecules present on NK cells which recognize classical HLA class I molecules and in doing so provide an unconventional means of modulating the immune response to infected or tumoral cells (Xing et al., 2008). Lectin–like receptors have been publicized to bind to non-classical MHC molecules such as HLA-E and the MHC Class I-related chain (MICA). These genetically defined, non-rearranging receptors have recently begun to show the magnitude of the potential inconsistency encoded within them as well as the involvement of their organization (Rajalingam, 2011) . NK cell receptors are involved in a great variety of clinical scenarios ranging from resistance/susceptibility to pathogen infections, tumour surveillance, recognition and abolition, and as important elements in solid organ and hematopoietic cell transplant outcome. The functional relationships that exist between the MHC and NK receptors provide a better perceptive of the immune responses related to pathogen incursions, malignancies, and clinical transplantation.

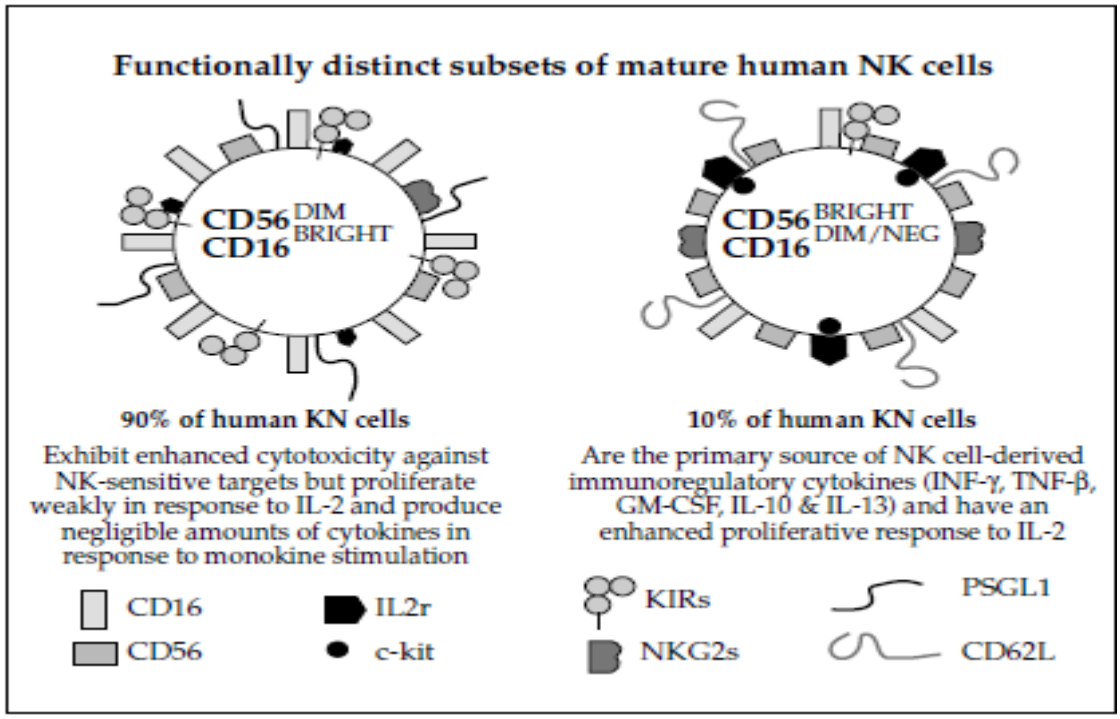


Figure 2.1: Two functionally distinct subsets of human NK are recognized. These subsets have differences in the cell surface expression density of CD56 and CD16, adhesion molecule expression and MHC receptor repertoire. These differences allow for differential trafficking, proliferative responses, and cytotoxic activity. PSGL: (P-selectin glycoprotein ligand) (Rajalingam, 2011).

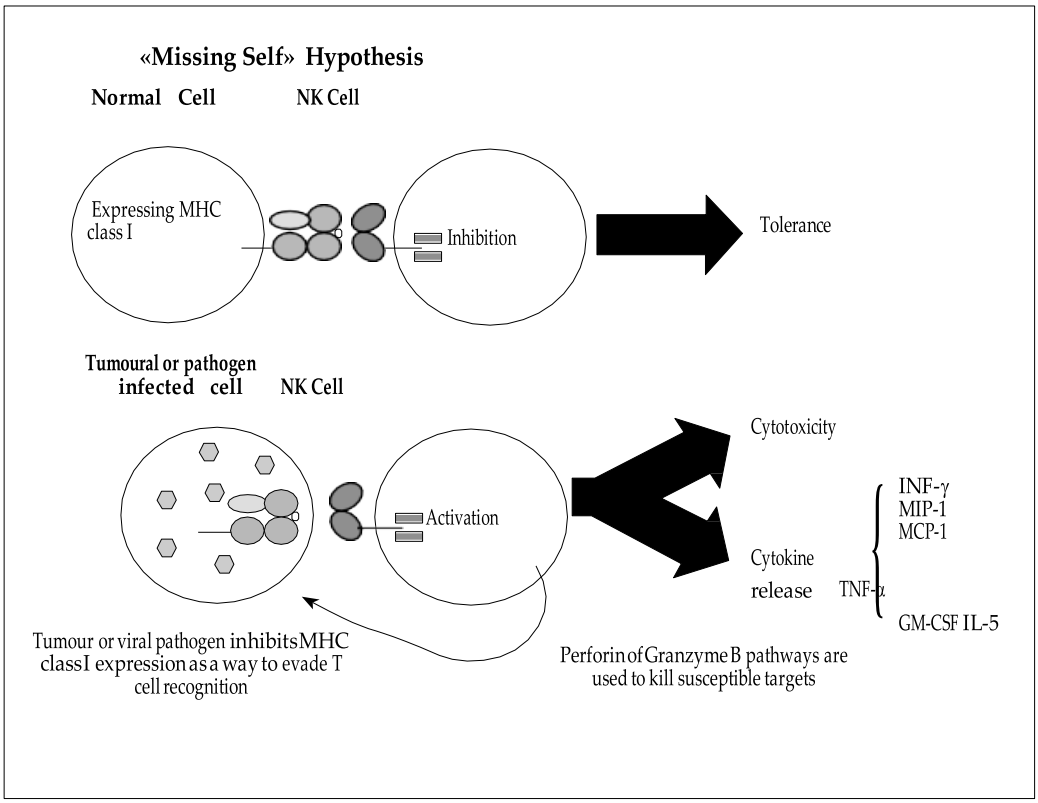


Figure 2.2: According to the «Missing Self Hypothesis» NK cells fail to recognize an appropriate MHC-ligand to inhibit the otherwise activating stimuli presented by the recognition of other «unspecific» ligands on the surface of the target cell. This stimulates the NK cell to produce cytokines, chemokines and to release lytic granules (Rajalingam, 2011).

NK Cells are bone marrow derived peripherally circulating cytolytic lymphocytes to some extent more voluminous than B or T cells, which encompass approximately 10% of all peripheral blood lymphocytes (Johansson, 2016). Phenotypically NK cells express CD56 cell surface molecules and lack rearranging antigen receptors as well as CD3. Two individual NK cell subsets defined by the cell surface expression density of CD56 have demonstrated distinctive functional roles (Fig. 2.1 and 2.2). NK cells are derived from CD34+ hematopoietic progenitor cells and necessitate cytokines present in the bone marrow environment to mature. NKs development requires NK cell progenitors to adopt CD34+IL-2/IL-15Rβ+CD56–intermediate phenotype which then develops into a mature CD56+ NK cell in response to IL-15. Whether this is likewise true for the CD56dim population of NK cells remains unknown (Poli et al., 2009).

These two subsets show differences in the expression of IL–2r, c-kit receptor tyrosine kinase expression, Major Histocompatibility Complex (MHC)-receptor repertoire and adhesion molecule expression. Such differences allow for differential proliferative responses, cytotoxic activities and trafficking profiles (Poli et al., 2009). Most NK cells are CD56 and CD16 and represent the effector population responsible of natural cytotoxicity and Antibody-Dependent Cellular Cytotoxicity (ADCC). Unlike T cells, natural killing is not MHC restricted in the classical sense but influenced by MHC Class I expression on target cell surface according to the «missing self-hypothesis», in which NK cells eliminate MHC Class I-deficient target cells which have lost or downregulated the expression of the cognate MHC receptor ligands due to oncogenic, viral pathogenic or other cellular incursions. Although such cytotoxicity is restricted to MHC Class I-deficient hematopoietic tissues, NK cells readily kill virus-infected cells that have maintained their expression of MHC Class I molecules, possibly by recognizing pathogen-specific epitopes on the cell surface (Maltseva et al., 2011).

2.2.5.1 NK Cells Receptors

NK cell cytotoxicity is regulated by at least two families of receptors that recognize classical MHC Class I molecules on the surface of target cells and enable them to discriminate between healthy cells and pathogen-infected or tumour cells by monitoring the expression levels of MHC molecules (Hartmann et al., 2010). These two NK receptors are structurally distinguished as belonging to the Immunoglobulin (Ig) superfamily, such as KIRs or as members of the C-type lectin-like domain (CTLD) superfamily, such as CD94/NKG2s (Poli et al., 2010) . The extracellular part of lectin-like receptor resembles the carbohydrate recognition domain of a C-type lectin, whereas that the KIR receptor is made up of Immunoglobulin-like domains. Both super families include both inhibitory and activating receptor variants, which have the capacity to inhibit or activate NK cell activity (cytotoxicity and/or cytokine release) as a consequence of binding to their cognate MHC ligands. In addition to their distinctive structures, these two families complement each other's MHC-specificities. CD94/NKG2 lectin–like receptors recognize HLA-E and MICA, whereas KIR molecules recognize specific HLA-A, -B and–C allotype subsets as well as HLA-G ligands. Unlike the rearranging B and TCRs, NK cell receptors of the lectin and Immunoglobulin families, are preformed and non-rearranging, their variability being a direct consequence of the genetically defined subset of genes present for each family and later modulated during NK cell development into complex combinatorial expression patterns (Choudhary et al., 2012). It is this preformed receptor repertoire which constitutes the hallmark of innate immunity and which allows NK cells to control pathogen incursions or cellular transformation early on during the prolonged period required for the clonal expansion of antigen-specific B and T cells (Nunez et al., 2016).

NK cell MHC receptors are encoded by two large and dense immune gene complexes located on different chromosomes (Di Santo, 2016). The NKC which contains the genes

encoding the lectin-like family of receptors is located on mouse Chromosome 6 and human Chromosome 12. The LRC, which contains the KIR encoding genes, is located on human Chromosome 19. The importance of CTLDs in human innate immunity resulted from observations that NKG2D binds to the stress-induced MICA and MICB. The CTLD family of receptors include NKG2A, NKG2B, NKG2C, NKG2D, NKG2E and NKG2F (Farag et al., 2013).

In humans, NKG2A, –B, –C, –E and–F form heterodimers in conjunction with CD94 and give rise to both activating and inhibitory proteins. NKG2A, -B and -C complexes with CD94 recognize HLA-E, an MHC molecule which presents nonameric peptides derived from leader sequences of other HLA class I molecules. Such interaction confers CTLD receptors the ability to monitor the global MHC Class I repertoire. Although mice have an apparently orthologous organization of CD94 and NKG2D, KIR genes are exclusive to primates and no mouse homolog of a KIR gene has been reported (Poli et al., 2010), however, mice have evolved a CTLD molecule to fulfil the function of KIR proteins, called Ly49. Given the current knowledge regarding NK cell receptors, it seems very unlikely that a single NK receptor will be responsible for the diverse biological properties attributed to NK cells. Recent findings, however, have described three non–MHC–class–I–specific activating receptors belonging to the Ig-superfamily but not related to KIRs, termed NKp46, NKp44, and NKp30 (Human Genome Organization Gene Nomenclature Committee approved gene symbols: NCR1, NCR2, NCR3 respectively, for Natural Cytotoxicity-triggering Receptors). Unlike KIRs and CTLD receptors, NCRs are exclusively expressed on NK cells and seem to be the main receptors involved in NK cell-mediated tumour lysis. KIRs are by far the most polymorphic receptors present on NK cells (Dinescu et al., 2014).

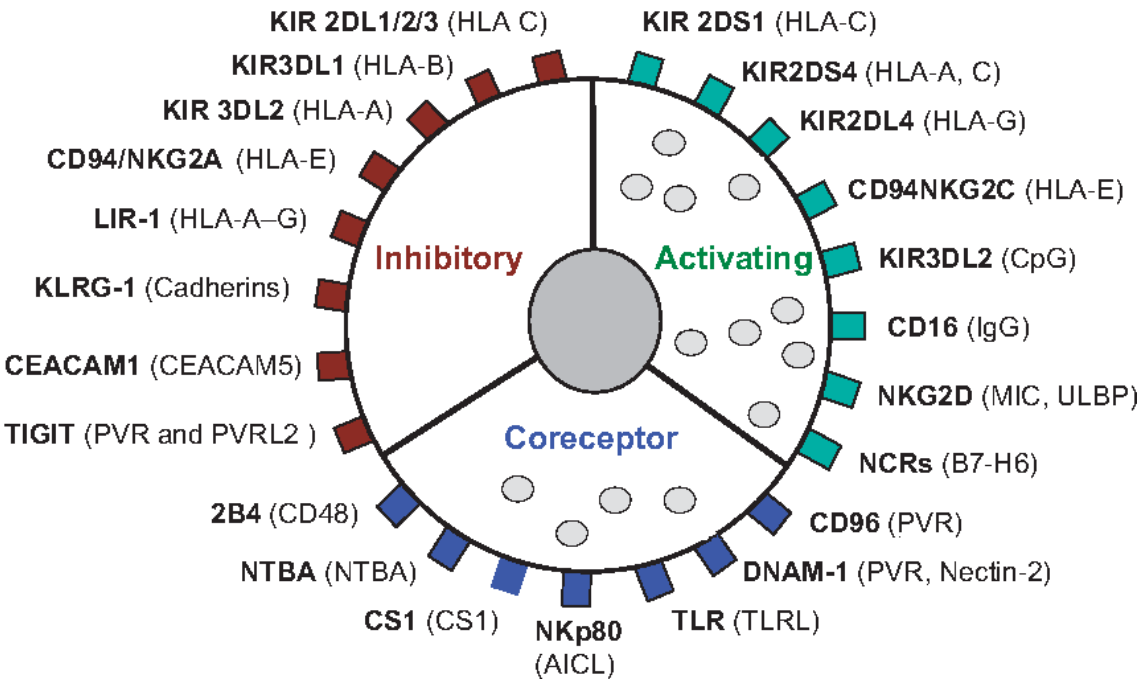


Figure 2.3: NK cell surface receptors and their ligands. Receptors are broadly classified based on their primary function (inhibitory receptors, activating receptors, and activating co-receptors). The known ligands are shown in parenthesis. Other families of receptors are not shown, including cytokine receptors (for IFN-a and IL1, -2, -12, -15, -18 and -21), chemotactic receptors (CCR-2, -5, -7; CXCR-1, -3, -4, -6; CX3CR1; and Chem23R), adhesion receptors (CD2 and b1 and b2 integrins), and inhibitory co-receptors (CD300A, LAIR-1 and Siglec7) (Darlington et al., 2018).

2.2.5.2 Killer Immunoglobulin–like Receptors (KIR)

KIRs are polymorphic cell surface molecules existing on NK cells and a small (8%) population of T cells known as Natural Killer T cells (NKT) (Kulkarni, Martin, & Carrington, 2008). They distinguish HLA class I molecules and in doing so provide an alternative means of curbing the immune response to damaged or foreign cells. KIR proteins possess characteristic Ig-like domains on their extracellular regions which are involved in classical MHC Class I ligand binding and transmembrane and cytoplasmic regions defining the type of signal which is transduced to the NK cell. KIR proteins can have two or three Ig-like domains. In the current nomenclature used to describe KIR genes, the number of Ig-like domains present are indicated by a 2D for two domain KIRs or 3D for three domain KIRs the presence of a short or long cytoplasmic tail being indicated by an S or L, respectively, at the end of the name (Augusto et al., 2012).

Two domain KIR proteins are subdivided into two large groups contingent on the origin of the membrane-distal Ig-like domains present. Type I KIR2D proteins (KIR2DL1, -2DL2, -2DL3, -2DS1, -2DS2, -2DS3, -2DS4 and -2DS5) (Hou et al., 2012) possess a membrane-distal Ig-like domain similar in origin to the KIR3D D1 Ig-like domain encoded mainly by the fourth exon of the corresponding KIR genes, and lack a D0 domain. Type II KIR2D proteins, KIR2DL4 and -2DL5, possess a membrane-distal Ig-like domain similar in origin and structure to the D0 domain present in KIR3D proteins encoded mainly by the third exon of the gene, conversely lack a D1 domain. KIRs control the response of human NK cells by delivering inhibitory or activating signals upon recognition of MHC Class I ligands on the surface of probable target cells (Maltseva et al., 2011). KIR proteins can possess short or long cytoplasmic tails. Long cytoplasmic tails usually contain Immune Tyrosine-based Inhibitory Motifs (ITIMs) which transduce inhibitory signals to the NK cell. Short cytoplasmic tail KIRs possess a positively charged amino acid residue (lysine) in their transmembrane region, which allows them to associate with a DAP12 signalling molecule capable of generating an activation signal (Campbell & Purdy, 2011). The existence of KIRs with MHC binding possessions was suggested because of observations relating to NK cell killing of HLA class I-deficient B lymphoblastic cell lines which could be reversed by transfecting these cell lines with certain HLA class I genes. Two domain KIRs recognize HLA-C allotypes while three domain KIRs recognize HLA-B allotypes. KIR2DL1 exhibits C2 specificity and recognizes HLA-C allotypes with Asn77 and Lys80 (for example HLA-Cw4, HLA-Cw2, HLA-Cw5 or HLA-Cw6) (Maltseva et al., 2011) . KIR2DL2 has a C1 specificity and distinguishes HLA-C allotypes with Ser77 and Asn80 (for example HLA-Cw3, HLA-Cw1, HLA-Cw7 or HLA-Cw8). KIR3DL1 recognizes HLA-B allotypes with a Bw4 motif on their a-helix (for example HLA-B13, HLAB38, and HLA–B51) and KIR3DL2, has been shown to identify HLA-A molecules. Although

KIRs with specificity for HLA-A, -B, -C and–G allotypes have been defined for the other KIR, the specificity currently relics unknown (Leung, 2011).

The genetic factors determining KIR repertoire diversity are related to the type of KIR genes present on any given individual. Recent studies, on the other hand, have demonstrated the role that other immune cell surface molecules, such as HLA molecules and NKG2 related receptors, have unsure the mostly expressed KIR in any given cell (Badenes et al., 2016).

2.2.5.2.1 KIR Genes

The KIR gene family comprises of fourteen genes and three pseudogenes encoded within a 150 Kb region of the LRC on Chromosome 19 (19q13.4) (Olmer et al., 2010).The LRC constitutes a large (1 Mb) dense cluster of rapidly evolving immune genes of relatively recent evolutionary origin (Hou et al., 2012). The LRC and its centromeric prolongation, the extended LRC, contain genes encoding cell surface molecules with distinctive Immunoglobulin-like extracellular domains (Carrington & Martin, 2016). KIR genes are rearranged into haplotypes, which have been defined in family segregation studies. The number of KIR genes present in any given haplotype may vary. All known KIR haplotypes are flanked at their centromeric end by KIR3DL3 and at their telomeric end by KIR3DL2, together with the centric KIR2DL4, constitute the framework genes (Fan, Wu, Ashok, Hsiung, & Tzanakakis, 2015) .

The framework genes limit two regions of variable KIR gene content where the remaining KIR genes are arranged in a head to tail fashion approximately two Kb apart from each other. KIR haplotypes show extensive haplotypic diversity pigeonholed by variability in the quantity of genes present which can range from 7 to 11 KIR genes. Most KIR genes are approximately fourteen Kb long and divided into nine exons (Kelley et al., 2015). The KIR proteins are encoded as follows exon 1 together with

exon 2 encode for the leader sequence of KIR proteins, exon 3 encodes for the membrane distal (D0) Ig-like domain (in the case of KIR3Ds and type II KIR2Ds), exon 4 encodes the middle (D1) Ig-like domain (present in type I KIR2Ds but absent in type II KIR2Ds), exon 5 encodes the membrane-proximal (D2) domain of all known KIR proteins, exon 6 encodes the stem region (classically absent in KIR3DL3), exon 7 encodes the carboxyl end of the stem region, the entire transmembrane region and the amino end of the cytoplasmic region, exon 8 encodes the first eighteen amino acids of the cytoplasmic region and finally, exon 9 with its fluctuating lengths, encodes the remaining portion of the cytoplasmic region of KIR proteins (Campbell & Purdy, 2011).

KIR genes have been shown to be polymorphic and more than 91 sequences representing alleles of the seventeen gene loci have been described (Wang et al., 2012). Dissimilar HLA class I and class II, in which most of the polymorphism of functional significance is constrained to one or two exons, KIR polymorphism is evenly distributed throughout the KIR gene. KIR nucleotide sequences are arranged into groups or KIR loci based on the number of extracellular Ig-like domains, the length of the cytoplasmic tail and sequence resemblance. Recent exclusion studies carried out in families have shown how KIR sequences previously rumored to be different genes based on cytoplasmic tail length differences may represent alleles based on the inheritance behaviour observed. This is the case of KIR3DS1 and KIR3DL1, which differ by only 6-12 amino acid residues. Interestingly, no interaction of KIR3DS1 with Bw4 motif bearing HLA-B alleles has been found to date (Uhrberg, 2015). The way in which allelic polymorphism supplementary diversifies the haplotypic variations has recently been demonstrated in high-resolution studies (Middleton & Gonzelez, 2018). The extent of such diversity makes the possibility of finding a KIR, harmonized unrelated individual very low. Whether the polymorphism of KIR genes translates into

functionally divergent proteins responsible for certain biological advantages remain unknown (Gardiner, 2018).

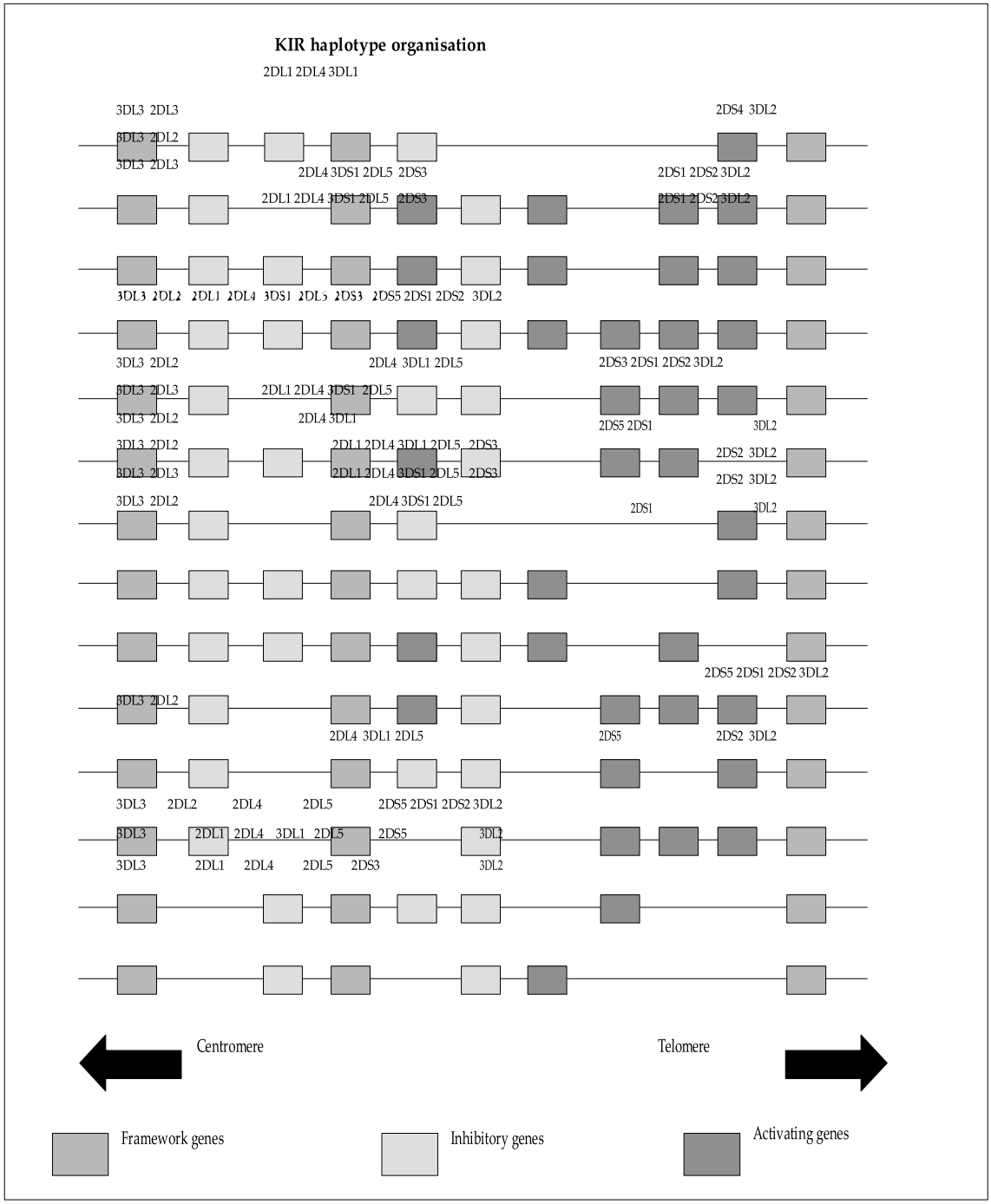


Figure 2.4: KIR genes are encoded within a 1 MB stretch of DNA in Chromosome 19 known as the LRC. All KIR haplotypes are flanked on their centromeric ends by KIR3DL3 gene and in their telomeric end by KIR3DL2. Together with KIR2DL4 located in the center of this region, these three genes represent the framework genes so called because they are present in all human individuals. Each KIR gene spans approximately fourteen Kb and is separated from neighboring genes by two Kb intergenic sequences. NOTE: the order in which KIR genes are organized within a haplotype has not been entirely determined for some KIR genes. (Maltseva et al., 2011).

2.2.6 Role of NK Cells and NK Receptors in Pathogen Incursion

NK cells have been demonstrated to be critical elements in the early immune response to a large variety of intracellular pathogens. Although limited studies have reported the importance of NK responses against cells infected with intracellular bacteria such as Listeria, Salmonella, and Legionella in humans, recent developments have shown that NK cells play an important role in the innate immune response to microbial pathogens (Chambers & Brissette-Storkus, 2012). The role of NK cell responses in such bacterial infections has been further buttressed by experimental NK-dependent lysis of bacteria infected cells and bacterial growth inhibition as well as by the description of NK selective deficiencies associated with recurrent polymicrobial infections (Ehninger & Trumpp, 2011). A role for NK cells in antibacterial responses has been demonstrated experimentally for *Toxoplasma gondii, Listeria monocytogenes* and *Leishmanial major* in murine models. NK cells have been shown to participate in anti-pathogen responses in four conducts.

The first of which is classically described as the result of the downregulation of MHC Class I molecules which intracellular pathogen infected cells endure as a consequence of a direct cytopathic effect or the presence of pathogen gene products which specifically interfere with the MHC Class I processing. *In vitro* experiments have demonstrated the downregulation of HLA-C molecules (known to serve as ligands for the inhibitory receptors KIR2DL1 or KIR2DL2/KIR2DL3) in Herpes virus infected cells and the subsequent prompting of isolated NK cell cytotoxic responses against them (Trowsdale et al., 2017). The use of this pathway by NK cells has been further corroborated by the finding of viral-TAP inhibitor ICP47 of HSV (Herpes Simplex Virus) and the MHC Class I-destroying US11 Protein of HCMV (Human Cytomegalovirus) (Kurtz et al., 2014). Bacterial downregulation of mononuclear phagocyte cell surface MHC expression has also been described in Salmonella, Yersinia

and Chlamydia pneumonia infections. As expected for rapidly evolving viruses subjected to Cytotoxic T Lymphocyte (CTL) selective pressures, Human Immunodeficiency Virus (HIV-1) has also devised a way to elude CTL responses by downregulating host cell's MHC expression. The *nef* gene product of HIV 1, is known to decrease HLA-A and–B expression levels but not those of HLA-C by accelerating the surface endocytosis rate in a highly selective manner which depends on the cytoplasmic tail region of the class I proteins involved (S M Blois et al., 2008). NK receptors specific for HLA-A and HLA-B allotypes have been defined (KIR3DL2 and KIR3DL1, respectively) and as at least one of them (KIR3DL2) is known to be a structural gene which is present in all individuals. Based on this, one should expect to find NK cell clones capable of generating potent responses against these HLA-A and–B deficient HIV-infected cells (Leung, 2011).

The second way in which NK cells can perimeter pathogen intrusions is by secreting cytokines which modulate the subsequent adaptive immune response. IFN-γ production by NK cells plays a critical role in activating macrophages and at persuading resistance to intracellular pathogen infections in other cells (Crome et al., 2013). NK cells have the potential to recognize unspecific «danger signals» expressed on the cell surface of stressed cells (Garcia et al., 2013). An example of this type of recognition involves NKG2D binding of the non-classical MICA and MICB and the GPI-linked UL16 Binding Proteins (ULBPs)belonging to the extended MHC Class I family. The expression of ULBPs or MICA/B molecules on the surface of NK-resistant target cells deliberates susceptibility to NK-dependent lysis. Such interactions result in the activation of NK cells and stimulate cytokines and chemokines production and release, proliferation, cytotoxic activity and upregulating the expression of other activating receptors on the NK cell surface. Nevertheless, some pathogens and HCMV, have evolved strategies to evade NK cell recognition and activation by producing ULBP and

MICA/B mimicking proteins such as UL16, which blocks this interaction and enables the virus-infected cell to evade NK cell lysis. Although NK cells exhibit aggressive cytotoxic activity against susceptible targets without the need of costimulatory cytokines, their exposure to IFN-α, IFN-β or IL-12 has been shown to increase such cytotoxicity 20- to 100-fold. IL–12 together with TNF-α can also stimulate NK cells to produce large amounts of IFN-γ, a cytokine known to play a crucial role at preventive some infections. The fact that NK cells constitute the main source of IFN-γ during the first days of infection and before an effective CTL response has been achieved has been demonstrated experimentally in viral, bacterial and parasitic infections (Mackensen et al., 2017).

The third way by which NK cells contribute to anti-pathogen incursions is thought to be a consequence of the direct recognition of pathogen-derived structures on the surface of the infected cell. The use of this «direct recognition» pathway by NK cells has been clearly buoyed by clinical and experimental findings (De Maria et al., 2011).The recent discovery of NK receptors (NCR1) capable of recognizing pathogen-derived structures (Influenza Virus hemagglutinin and hemagglutinin–neuraminidase of the Sendai Virus) present on the cell surface of infected cells has expanded the potential functional roles of NK cells and receptors (Maltseva et al., 2011). A similar outcome related to antibacterial responses evolved from explanations of healthy individuals who had been in close contact with Mycobacterium tuberculosis-infected patients and who had never developed a positive tuberculin skin test, portentous a possible innate immune response prior to CTL recognition of the pathogen. Subsequent studies revealed the existence of NK cell-mediated lysis of Mycobacterium tuberculosis-infected monocytes which had not downregulated their expression of MHC Class I molecules, a response which did not seem to be a consequence of heightened production of IL-18 or IFN-γ. The description of these NK cell pathogen-specificities raises the question whether certain

NK receptors are involved in the recognition of other pathogens of clinical relevance or whether the extensive polymorphism of the NK receptor families that have been pronounced so far is the result of pathogen pressures and as such confer susceptibility or protection to them (Id 2016).

The fourth way in which NK cells have the capability to eradicate pathogen infected cells is through ADCC. NK cells recognize the Fc portion of IgG antibody molecules, present on the surface of infected cells, through FcγRIII (CD16). This receptor forms part of the Ig-superfamily and is also expressed on macrophages and mast cells and as such does not represent a cytotoxic pathway exclusive of NK cells (Flemming & Stolk, 2011).

2.2.7 Role of NK Cells and NK Receptors in Tumour Surveillance

NK cells were described two decades ago as functionally capable of lysing certain tumour cells (Mocikat et al., 2013). Tumour immunity has shown to require the participation of potent lymphocyte effector responses. Both NK cells and CTLs, once activated, possess similar lytic pathways to carry out their functions, even though triggered by different antigen receptors. NK cells use at least three structurally different receptors for this purpose: KIRs, NKG2s and NCRs (Herceg & Wang, 2016). which mediate cytotoxicity via perforin and granzyme B. A unified signal cascade elicited by susceptible target cell recognition has been postulated for a common signal pathway that leads to the mobilization of lytic granules containing perforin and granzyme B toward the immune synapse (Vivier et al., 2017).

NK cells exhibit spontaneous cytotoxic activity against tumour cell lines expressing unspecific inflammatory (stress-induced) ligands (which bind to activating NK cell receptors) as well as by recognizing the absence of MHC Class I molecules on the surface of the tumour cell (Poli et al., 2010). These immune equivocal strategies

constitute an attempt to escape immune detection by CTL and comprise the downregulation of MHC Class I molecules on their cell surface, production of immune suppressive cytokines (such as TGF-β) and the increase in the levels of expression of Fas ligand (De Maria et al., 2001) .

Tumour cells that lack appropriate MHC Class I molecule expression induce NK cell insinuation, cytotoxic activation, cytokine production and induction of transcription of IFN-γ in NK cells. Of special oncological interest is the lectin-like NKG2D homodimer, which associates with the Phosphatidylinositol three kinase activator DAP10. This NK receptor is broadly expressed on NK cells, γδT cells, macrophages, and CD8+ αβT cells. This receptor can interact with a diverse family of MHC Class I correlated ligands not involved in peptide presentation, which is induced by cellular stress (such as MICA, MICB, and ULBPs). Although the expression of these NKG2D ligands is low in the normal adult tissues, the increased expression of MIC has been widely documented in many epithelial carcinomas (Campbell & Purdy, 2011). Ectopic expression of this ligand has demonstrated to provoke NK cell-mediated cytotoxicity and cytokine production. IL-2 activated NK cells are of special interest in relation to tumour Immunotherapy. These cells have been shown to infiltrate established lung and liver solid tumours and induce their regression. A further stimulation in such patients with an MHC Class I expression inhibitory such as that based on TAP inhibition by infected cell Protein (ICP)47 could possibly contribute to making this remedy more efficient (Du et al., 2006). Of similar immunotherapeutic potential is the concept of deleting inhibitory signals to optimize NK and NKT cell responses, even those activated with stimulatory cytokines, such as IL-2. This approach has demonstrated to be a powerful tool at eradicating tumours when the tumour burden is minimal as that occurs after cytoreductive therapy. This same approach could theoretically be used to elicit vigorous NK cell-mediated antiviral responses. New immunotherapeutic strategies should

consider the way in which innate immunity might be able to rheostat the development and nature of adaptive immunity by means of dendritic cell (DC)-NK cell interactions

The activation of pattern recognition receptors on DCs enables them to activate NK cells in the vicinity and consequently guide tumour recognition and lysis. The apoptotic cell bodies which result from such lysis are then taken up, transported and processed by the DCs and presented to T cells, thus affecting the outcome of the subsequent adaptive immune responses (Johansson, 2016). Enhancement of the antineoplastic cytotoxicity of NK cells and infusion of selected NK cells as alternatives to CTL seem to be very promising in the treatment of haematological patients with low tumour encumbrance (e.g., after stem cell transplantation or cytoreductive therapy) (Bartel & Steinle, 2013). The recently described Natural Cytotoxicity-triggering Receptors (NCR1-3) have also been shown to play a crucial role in antitumoural responses (Nunez et al., 2016).

2.2.8 Development of Lymphoid Lineages

Blood cells are originated from the $CD34^{+}$ pluripotent stem cells and developed from hematopoietic stem cells. They are classified into two different main groups christened lymphoid and myeloid lineages. Lymphoid lineage cells are including T and B lymphocytes as well as NK cells, while other blood cells such as erythrocytes, megakaryocytes, granulocytes, and macrophages belong to the myeloid lineage (Niemeyer et al., 2013).

A dendritic cell is not clearly categorized because it is originated from both lymphoid and myeloid progenitors (Fernandes et al., 2009). Figure 2.5 shows these two lineages.

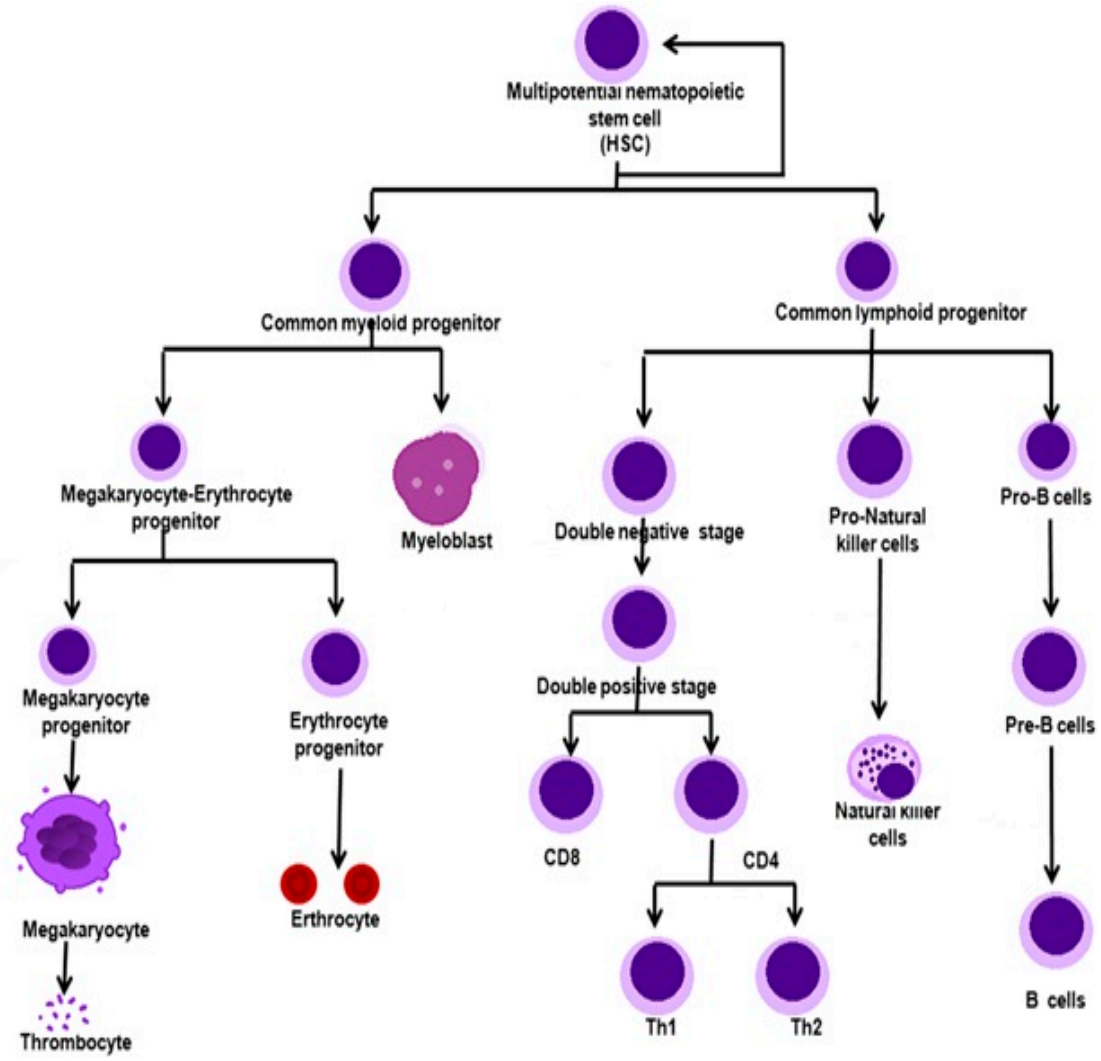


Figure 2.5: The differentiation and development of myeloid and lymphoid lineages (Fernandes et al., 2009).

2.2.9 Human NK Cells

2.2.9.1 The Phenotype of NK Cells

In humans, NK cells have been pronounced as a population of lymphocytes that do not have cell surface expression of CD3 and express the adhesion molecule CD56 (NCAM) (Dezell et al., 2012). These $CD3^{-}CD56^{+}$ lymphocytes can be divided into two major populations. The first is a $CD56^{bright}$ subset, which mostly induces chemokines and cytokines and a $CD56^{dim}$ subset which has cytotoxic activity (Shi & Gronthos, 2013).

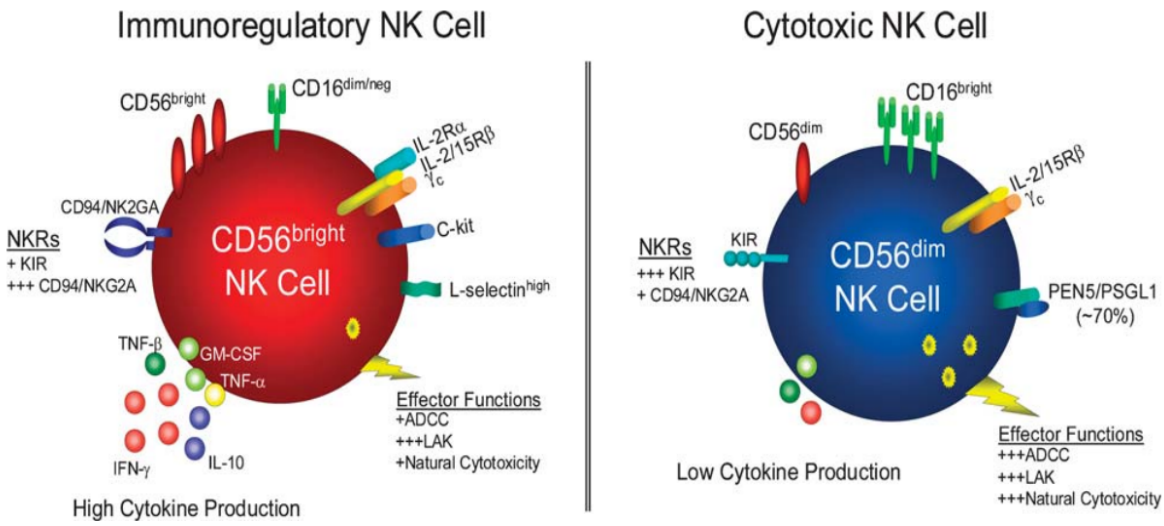


Figure 2.6: Human NK cell subsets. Following monokine stimulation, the $CD56^{bright}$ NK cells produce high levels of cytokines. This subset is an immune regulatory cell whereas $CD56^{dim}$ has cytotoxicity activity (Poli et al., 2010).

In mouse, CD56 is not articulated on the cells, so NK cells are characterized by the NKR-P1 family member NKrp1c (also known as NK1.1) (Darlington et al., 2018) or by a α2 integrin called DX5.

2.2.9.2 Structure of NK Cells Receptor Families

NK cells are defined as a group of receptors that can either activate or inhibit NK cell reactivity. Activating receptors comprises of receptors with interaction with soluble

ligands like cytokines as well as receptors that react with cell surface molecules (Fig 2.7). Some interleukin receptors such as IL-15R, IL-2R, IL-18R and IL-12Rare coupled to the common gamma chain and are involved in NK cell development and effector activity. NK cells need priming by different factors, such as IL-15 secreted by dendritic cells (DC) or macrophages, IL-12 or IL-18, to act as a functional effector (Andoniou et al., 2015).

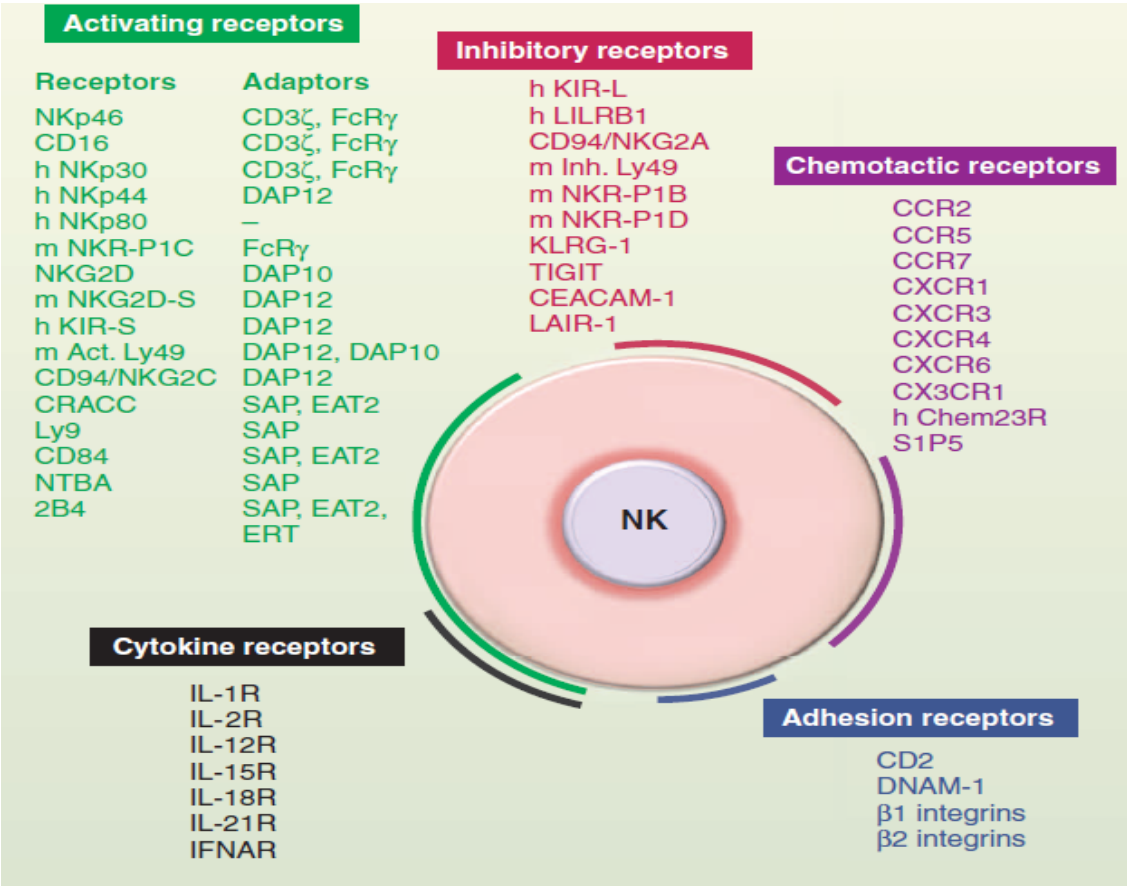


Figure 2.7: NK cells express many cell surface receptors that can be divided into different categories such as inhibitory (red), activating (green), cytokine (black), adhesion (blue), and chemotactic receptors (purple). Unless shown (h, human; m, mouse), receptors are common in both specie (Kurtz et al., 2004).

2.2.9.3 Regulation of NK Cell Response by Activating and Inhibitory Receptors

In mammals, NK cell receptors based on their effects on cytotoxic activity are grouped into two major groups, inhibitory and stimulating receptors (Pegram et al., 2013). All inhibitory NK cell receptors consist of one or more copies of ITIM in their

cytoplasmic domains. In contrast, several activating NK receptors employ adapter proteins including an immune receptor tyrosine-based activation motif to pass on their signals. Structurally, NK cell receptors are also classified into two main groups, one group called NK C-type lectin receptor entailing of a dimeric type II transmembrane domain, a domain very similar to C-type lectin, and the other group named NK Immunoglobulin (Ig) like receptors (KIR) having a type I transmembrane domain and Ig-like domains (Kurtz et al., 2014).

Figure 2.7 depicts two major types of ligands for NK cell receptors. Members of the CLEC2 subfamily as ligands for NKRP1 subfamily resemble C-type lectin-like receptors (left). Ligands of CD94/NKG2 receptors and Ly49 receptors (mouse only) are MHC Class I complexes consisting of a heavy chain, β2 macroglobulin, and a peptide (right). Symbols "+" and "-" show activating and inhibitory functions, respectively. For NKrp1a, NKrp1f, and NKrp1g, functional activity remains unknown (Du et al., 2015) .

Many of the activating or co-activating NK cell receptors expressed by mouse and human NK cells recognize self-antigens (Table 2.2 and 2.3). For example, the activating NKG2D receptor recognizes plentiful self-ligands in the host. Several of the activating KIR, ly49, and CD94-NKG2C receptors are capable of recognizing self-MHC Class I proteins, and members of the "natural cytotoxicity receptors" group (such as NKp30, NKp44, and NKp46) appear able to bind as yet undefined self-ligands in the host (Lanier, 2008 ; Moretta et al., 2017). In addition, several "co-activating" receptors have been identified on NK cells, such as CD2, LFA-1, CD244 (2B4), and CD226 (DNAM-1), which also recognize self-ligands that are approximately distributed in many tissues in the host (Krampera, 2012). Thus, NK cells express many activating receptors for self that could potentially drive autoreactivity.

Table 2.2: Activating and Co-activating NK Cell Receptors.

Common Name	Ligand	Gene	Species
Ly49D	H-2D^{d} Hamster MHC Class I	*KLRA4*	Mouse
Ly49H	MCMV-m157	*KLRA8*	Mouse
Ly49P	MCMV	Klra16	Mouse
NK1.1, NKR-P1C	Unknown	Klrb1c	Mouse
NKR-P1F	Clrg	Klrb1f	Mouse
PILRβ	O-glycosylation CD99	Pilrb1	Mouse
2B4	CD48	CD244	Human
LFA-1, CD11a	ICAM-1, 2, 3	ITGAL	Mouse, human
CD94-NKG2C, E	Mouse Qa-1^{b} Human HLA-E	*KLRD1*-KLRC2, 3	Mouse, human

Table 2.3: NK Cell Inhibitory Receptors.

Common Name	Ligand	Gene	Species
Ly49A, C, G2, I	Various H-2 class I	Klra1, 3, 7, 9	Mouse
NKR-P1B, D	Ocil (Clr-b)	Klrb1b, d	Mouse
gp49b1	$a_v\beta_3$	Lilrb4	Mouse
PILRα	O-glycosylated CD99	Pilra	Mouse
2B4	CD48	CD244	Mouse
MAFA	E-, N-, R– cadherins	KLRG1	Mouse, human
CD94-NKG2A	Mouse Qa-1^{b}, Human HLA-E	*KLRD1*-KLRC1	Mouse, human
NKR-P1A, CD161	LLT1 (CLEC2D)	KLRB1	Human
LAIR-1, CD305	Collagen XVII	LAIR1	Human, mouse
ILT2, LIR1, CD85j	HLA class I	LILRB1	Human

It has been recommended that NKp46, a member of the highly conserved natural cytotoxicity receptor family (NCR) (Eissens et al., 2012), is competent to define NK cells cross-species . Indeed, this receptor has been described to be specific for NK cells

in humans (De Maria et al., 2001), mice, monkeys, rats and chattel and may be useful in comparative NK cell analyses between species.

2.2.10 Model of NK Cell Development

The NK cell is originated from $CD34^+$ hematopoietic stem cells as a part of the hematopoietic system. Human B and T cells develop in the bone marrow and thymus, in that order. Nevertheless, the development of NK cell pathway from $CD34^+$ HPCs is not well demarcated.

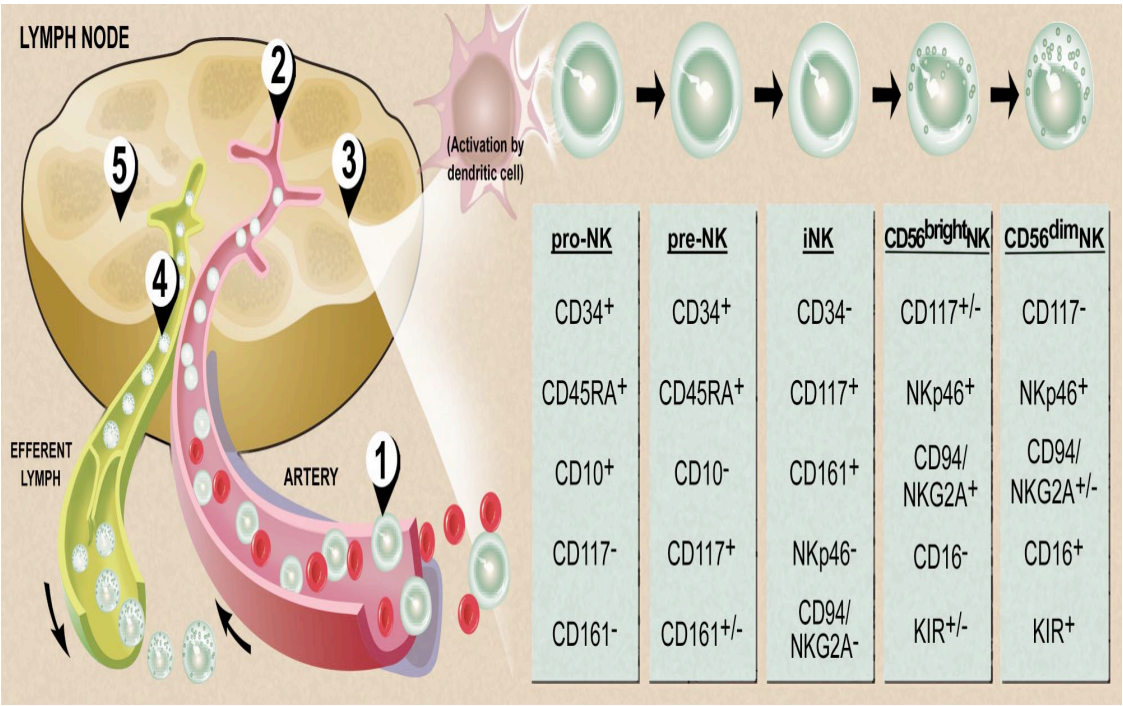


Figure 2.8: The model of human NK cell development.

It has been suggested that NK cell development may not completely happen in the bone marrow. This impression came from the expression of $CD56^{bright}$ (Human NK cells can be subdivided into different populations based on the relative countenance of the surface markers CD16 and CD56. The two major subsets are CD56 bright CD16dim/− and CD56dim CD16+, These cells are arithmetically in the minority in peripheral blood but establish most NK cells in secondary lymphoid tissues. They are copious cytokine

producers but are only weakly cytotoxic before activation. Recent data recommend that under certain conditions, they have immune regulatory possessions and that they are probably immediate precursors of CD56dim NK cells. CD56 bright NK cell percentages are prolonged or abridged in a certain number of diseases, but the significance of these variations is not yet clear) (Robertson & Ritz, 2017) and its isolation from lymph nodes and tonsils as secondary lymphoid tissues (SLT). $CD56^{bright}$ in these secondary lymphoid organs is less mature than $CD56^{dim}$ found in bone marrow, blood and spleen. A specific population of pre-NK cells with $CD34^{+,\ CD}45RA^{+}$ markers have been found with a high frequency in SLT whereas they are at a low frequency in bone marrow and spleen. It approves that they are the first $CD34^{+}$ cells in these tissues and are being developed there.

2.2.11 the Interaction Between Human NK Cells and Other Immune Cells During Response to Antigens

The response of NK cells is regulated by a balance of signals from activating and inhibitory receptors. Figure (2.9a). When an interaction between activating receptor and ligand is inattentive, the lysis is blocked. In fact, the inhibitory signalling is activated inside the NK cell. Figure (2.9b) exemplifies when activating receptors are attached to their ligands on target cells, lysis is carried out. It is happening in the lack of inhibitory receptor/ligand interactions. Figure (2.9c) states when the activation receptors and/or ligands are upregulated, the pure activating signal is going over the inhibitory signal. This is because the activating receptor/ligand interactions are stronger than inhibitory receptor/ligand signals and finally, Figure (2.9d) shows which the main interaction between the inhibitory receptor and ligand results in a negative signal that stops NK cell lysis. It is happening when the activating receptor/ ligand are weaker than inhibitory receptor/ ligand signals (Gardiner, 2008).

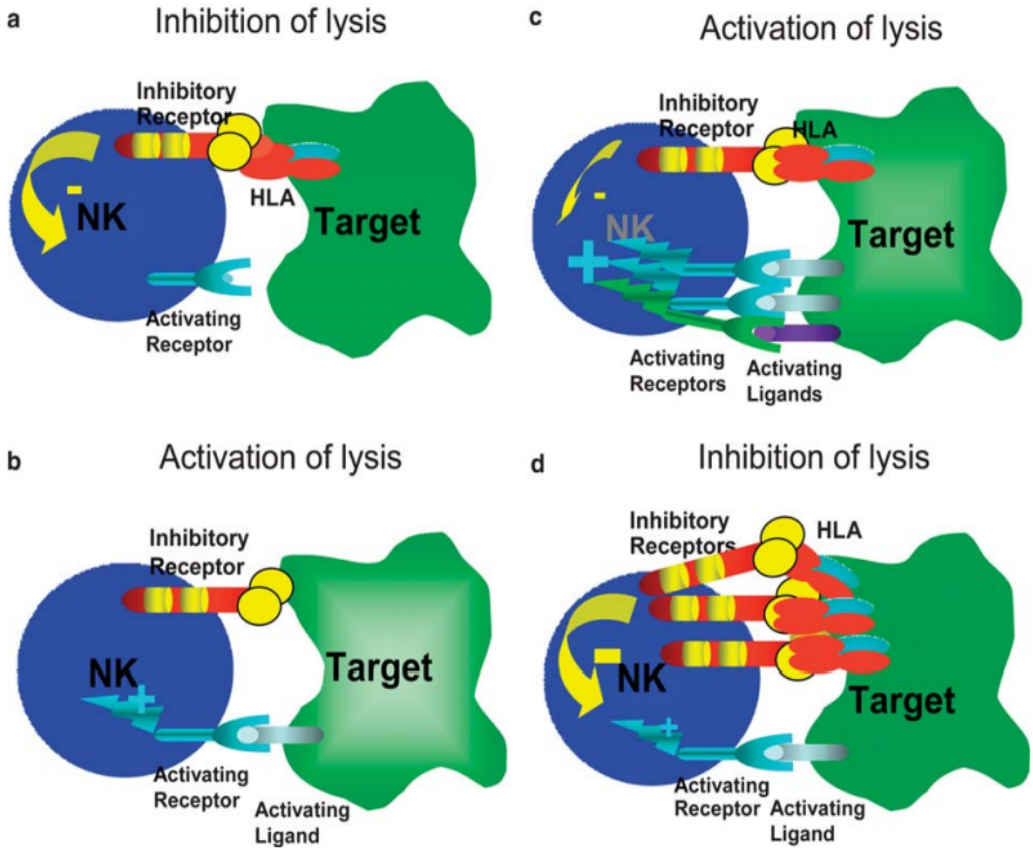


Figure 2.9: The activating and inhibitory receptors of NK cells and their role in the regulation of NK cells.

2.2.12 NK Cells Activating and Inhibitory Receptors Gene Complex

2.2.12.1 Immune Receptor Tyrosine–based Inhibitory

NK cells were initially described on the basis of their 'natural' capability to kill certain tumour cells *in vitro* , a process well-known as 'natural killing' (Wang et al., 2011). This function does not necessitate host sensitization to the tumour cell and its discovery led to studies of NK cell activities in tumour surveillance. Nevertheless, NK cells are also crucial for innate host justification against pathogens, against viral infections. Prominently, NK cells use the same mechanisms to resist tumours and infections. Many membrane proteins, such as CD2, 2B4, CD11a–CD18 and CD69, can induce or modulate NK cell cytotoxicity, and cytokines can enhance NK cell killing (Bott, 2014). Orchestrated interactions between these molecules subsidize to the natural killing of target cells. Despite these complexities, it is useful to categorize NK cell

receptors into two broad categories of inhibitory and activating receptors. The susceptibility of tumour targets to natural killing is inversely related to target cell expression of MHC Class I molecules, as determined by crucial studies carried out by Kärre and colleagues. This observation formed the basis for the 'missing self' hypothesis, which proposes that NK cells survey tissues for expression of MHC Class I molecules. In the absence of otherwise ubiquitously expressed MHC Class I molecules ('missing self') (Shimazaki & Okano, 2017), NK cells are released from the negative influence of MHC Class I molecules and kill the target. 'Missing self' is now explained by the expression of NK cell inhibitory receptors specific for MHC Class I molecules. These receptors include the human KIRs, the rodent Ly49 receptors and the human and rodent CD94–NKG2 molecules. In general, the effect of the MHC Class I-specific inhibitory receptors dominates over NK cell activating receptors. Inhibitory receptors mediate their effect through a MOTIF (ITIM) in their cytoplasmic domains (Chambers & Storkus, 2012).

After ligand binding, the ITIM turn out to be tyrosine phosphorylated by an SRC-family tyrosine kinase, which then recruits and activates SH2-domain-containing Protein tyrosine phosphatase 1 (SHP1) and potentially other phosphatases, such as SHP2. Recent studies also implicate a role for SH2-domain-containing inositol polyphosphate 5′ phosphatase (SHIP1), which can also bind to ITIMs (Eric Vivier et al., 2014). Even though the specific targets of the activated phosphatases are yet unclear, they presumably inhibit NK cell functions by interrupting the early phosphorylation pathways that are responsible for NK cell activation that is, the inhibitory receptors block stimulation by NK cell activating receptors. Many NK cells activating receptors have extracellular domains that are like those of the inhibitory receptors but lack the intracellular consensus ITIM. Instead, they generally have charged transmembrane residues that facilitate association with signalling chains that are often required for

optimal surface expression. On NK cells, these signalling chains include (ITAM) containing molecules, such as DNAX-activating Protein of 12 kDa (DAP12**),** also known as Killer activating receptor associated Protein (KARAP) , FcεRIγ and CD3ζ (Jansen et al., 2013).

Receptor crosslinking leads to tyrosine phosphorylation of the ITAM on the signalling chain and downstream events that culminate in cytotoxicity and cytokine production. The details of this NK cell activating and inhibitory pathways are the topics of active investigation (Figure 2.10).

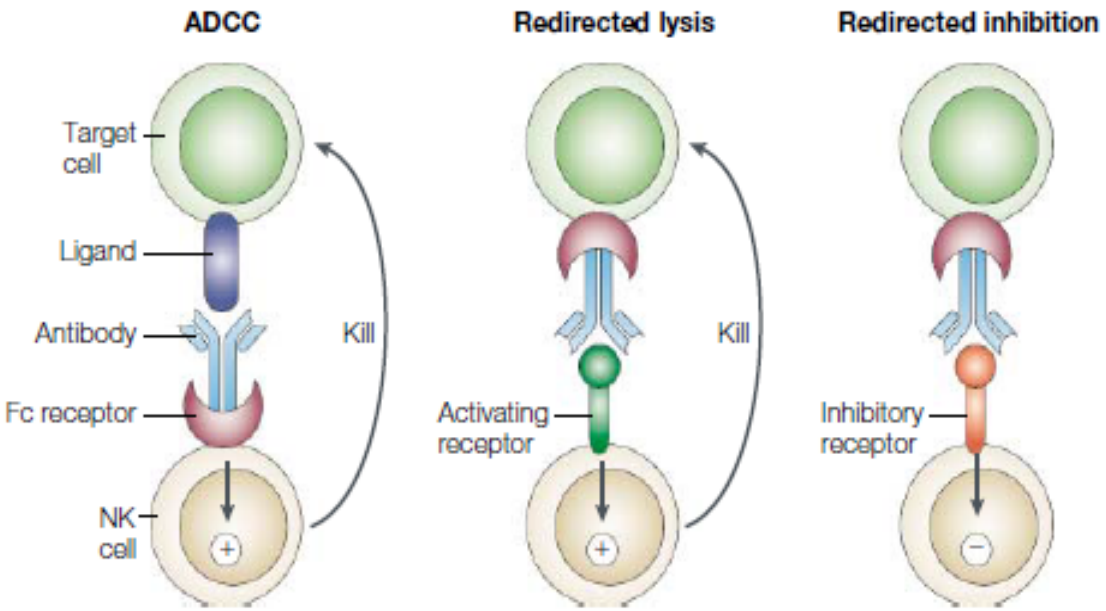


Figure 2.10: NK cells activator and inhibitory ligands kill.

2.2.12.2 Activating and Inhibitory NK Cell Receptors Genomic Regions

Most activating and inhibitory NK cell receptors are encoded by genes in two genomic regions, the NK gene complex (NKC) and the LRC. Whereas molecules encoded in the LRC belong to the Immunoglobulin superfamily, are expressed more approximately on a wide variety of hematopoietic cells and have been the topic of recent reviews, the NKC-encoded molecules have marked differences from LRC-

encoded receptors and form. As will become clear, the NKC encodes many molecules with related structures and functions that are important for NK cell activities (Bernardini et al., 2012).

The cell surface molecules NK cell receptor Protein 1 **(**NKrp1**)** and ly49 were among the first surface molecules to be recognized as being constitutively and selectively articulated by rodent NK cells, functionally active in NK cell-mediated lysis and encoded by genes located on distal mouse Chromosome 6 (Sandra M Blois et al., 2008). Both molecules have a type II transmembrane Protein orientation, share sequence homology with C-type lectins and are expressed as bisulfide-linked dimers. Subsequent studies resulted in the identification of related human molecules, such as the CD94–NKG2 heterodimers that are encoded in a locus on the syntenic (In classical genetics, synteny designates the physical co-localization of genetic loci on the same Chromosome within a discrete or species. Today, conversely, biologists usually refer to synteny as the preservation of blocks of order within two sets of chromosomes that are being compared with each other. This concept can also be referred to as shared synteny of human Chromosome 12p13 (Current & Id, 2016). It is now acknowledged that most of these genes are conserved across mouse, rat and human genomes.

These genetic regions are known as the NKC. Whereas the NKC encodes a few molecules that do not seem to be important to NK cells, most molecules encoded by the NKC are receptors that are highly germane to NK cell function and have features that were first recognized for NKrp1 and ly49 molecules (Wang et al., 2013).

The main NK receptors for MHC Class I molecules in humans have its place to the IgSF and are encoded in the LRC on Chromosome 19q13.4 (Dinescu et al., 2014) of the 45 genes in the LRC, the 30 IgSF receptors can be congregated into several related gene families based on gene organization, phylogeny, and structure. These families consist of

the Killer Cell Immunoglobulin–like receptors (KIRs), leukocyte Ig-like receptors (LILRs; also, called LIRs and ILTs), and the leukocyte-associated Ig-like receptors (LAIRs). The furthermost centromeric end of the human LRC contains genes for the platelet glycoprotein VI (GP6), the natural cytotoxicity triggering receptor 1 (NCR1; also, called NKp46), and the receptor of the IgA Fc fragment (Fcar; also, called CD89). These proteins are structurally analogous to KIR genes but differ from other loci in the LRC by interacting with ligands other than MHC Class I molecules (Kelley et al., 2015). The organization of the human LRC is shown in the comparative maps of Figure 2.11.

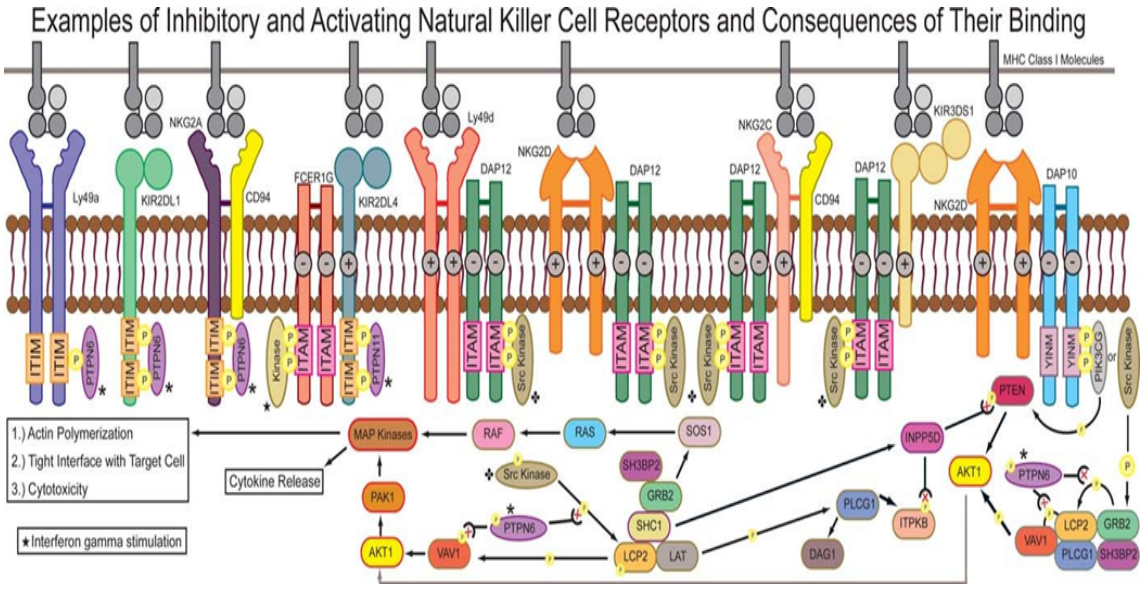


Figure 2.11: Examples of Inhibitory and Activating NK Cell Receptors and the Consequences of Their Binding This Figure shows the association of NK receptors with MHC Class I molecules and portions of the resulting signaling pathway, as described in the text. ‘‘Src Kinase’’ represents various kinases capable of participating in this interaction, such as Syk and ZAP70. SHP2 (PTPN11) is used in place of SHP1 (PTPN6) in some circumstances, such as correlating with KIR2DL4. Symbols show the continuation of the pathway where arrows are not drawn.negatively charged residue; þ, positively charged residue; AKT1, v-akt murine thymoma viral oncogene homolog 1 (also called RAC); DAG1, dystroglycan 1; FceRI-c, receptor for Fc fragment of IgE, high affinity I, gamma polypeptide; GRB2, growth factor receptor-bound Protein 2; INPP5D, inositol polyphosphate-5-phosphatase, 145kDa (also called SHIP); ITPKB, inositol 1,4,5-trisphosphate 3-kinase B (also called IP3KB); LAT, liNKer for activation of T cells; LCP2, lymphocyte cytosolic Protein 2 (also called SLP76); P, phosphate group; PAK1, p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast); PIK3CG, phosphoinositide-3-kinase, catalytic, gamma polypeptide (also called phosphatidylinositol 3-kinase); PLCG1, phospholipase C, gamma 1; PTEN, phosphatase and tensin homolog; PTPN6, Protein tyrosine phosphatase, nonreceptor type 6 (also called SHP1); RAF, raf Protein; RAS, rat sarcoma viral oncogene homolog; SH3BP2, SH3 domain–binding Protein 2; SHC1, SHC (SH2 domain containing) transforming Protein 1; SOS1, son of sevenless homolog 1; VAV1, vav one oncogene; YINM, tyrosine-containing motif (YINM) (Gerosa et al., 2002a).

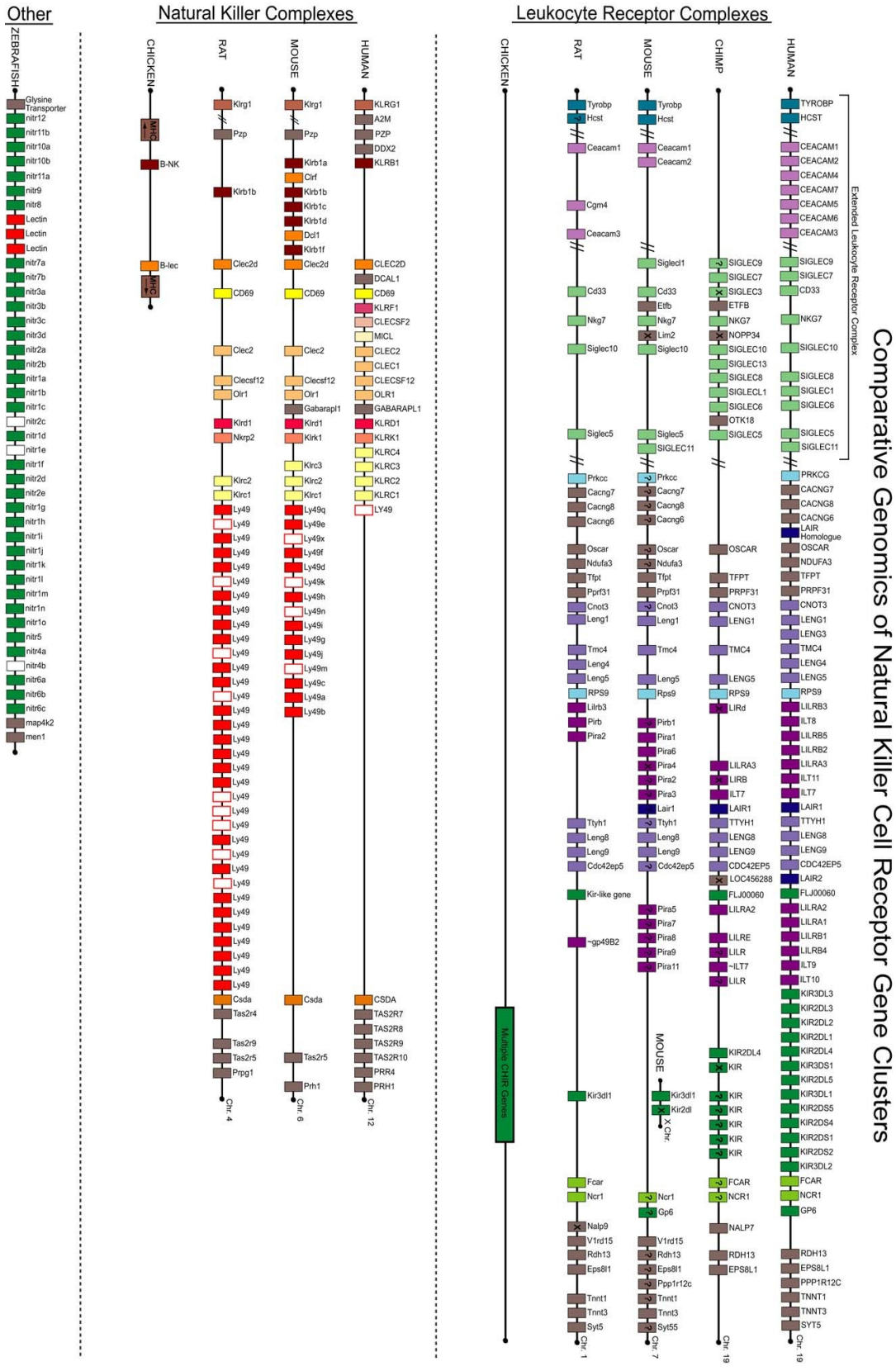


Figure 2.12: Comparative Genomics of Natural Killer Cell Receptor Complexes.

This Figure, screening LRCs and NKCs in various species, is not drawn to scale; however, the linear arrangement of genes is precise and is aligned vertically by homology within each region, where possible. Colours point toward genes related by gene organization, structure, and phylogeny. Gray indicates genes that are not considered NK receptors. White boxes mark pseudogenes. Slash marks represent large expanses in the genomic sequence. Some non-NK receptor genes that are located within this region may not be represented in this Figure. A question mark indicates that the gene has been mapped to the corresponding Chromosome, but the specific chromosomal position is not acknowledged. An ‘‘X’’ indicates that the gene is not homologous with genes sharing its vertical alignment. The NKC contains a variety of C-type lectin genes, some of which are expressed specifically on NK cells. There is only one member of the ly49 gene family, KLRA1 (also called Ly49L), in humans, as contrasting to the multiple homologous genes encoding MHC Class I ligands in rodents (Kulkarni et al., 2018). While KLRA1 is transcribed, a point mutation causes the production of a non-functional molecule.

2.2.13 This Research Genes Cluster

In this research the target ligands are ly49h, ly49d and CD95

1. **Ly-49H:** Lacks the characteristic ITIM (immunoreceptor tyrosine-based inhibitory motif) and contains an arginine in the transmembrane domain implying these molecules act as activation receptors. Ly-49H is expressed in a subset of NK cells but not NKT (NK1.1CD3+) cells. Natural killer cells can express several Ly-49 proteins. It has been shown that there is preferential staining of Ly-49H with Ly-49D (cat 12-5783). Expression has been confirmed on C57BL/6 and NWNA but not Balb/c or DBA/2Ly49H is a member of the C-type lectin Ly-49 multigene family of receptors found on natural killer cells. Ly49H, like Ly49D, lacks the characteristic ITAM (immunoreceptor tyrosine-based inhibitory motif) and contains an arginine in the transmembrane domain implying these molecules act as activation receptors. Ly49H is expressed in a subset of NK cells but not NKT (NK1.1/CD3+) cells. Natural killer cells can express several Ly49 proteins (Kruse & Vivier, 2017).

2. **Ly-49D:** An activating member of the Ly-49 family of NK cell receptors. This family of receptors form homodimers that recognize MHC Class I molecules. The activating Ly-49 receptors (Ly-49D and Ly-49H) do not contain immune receptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains, distinguishing them from the inhibitory Ly-49 family members. Instead, activating Ly-49 molecules have been shown to associate with DAP12 which contains an immunoreceptor tyrosine-based activation motif (ITAM) that is required for positive signalling. Expression of different Ly-49 family members is not linked; therefore, several Ly-49 proteins can be expressed on the same cells. Expression of Ly-49D on subsets of NK cells is strain-dependent and is found in

C57BL/6, CB6F1 and SJL mice, while BALB/c, DBA/2, AKR and CBA/J mice are negative for Ly-49D. In 129/J mice, eBio4E5 also recognizes Ly-49O, Ly-49V and Ly-49R (Current & Id, 2016).

3. **Natural killer cells, like CD94:** are a distinct lineage of lymphocytes that mediate cytotoxic activity and secrete cytokines upon immune stimulation. Several genes of the C-type lectin superfamily, including members of the NKG2 family, are expressed by NK cells and may be involved in the regulation of NK cell function. *KLRD1* (CD94) is an antigen preferentially expressed on NK cells and is classified as a type II membrane protein because it has an external C terminus. Three transcript variants encoding two different isoforms have been found for this gene (Id, 2016).

2.2.13.1 Ly49 (Lectin-Like Receptor) Family

ly49 receptors or killer cell lectin-like receptor subfamily A (KLRA), are a class of natural killer cell receptor(Augusto et al., 2012). ly49 proteins are a diverse set of C-type lectins that are expressed on NK cells in some mammals, together with rodents but not humans. Their primary function is to bind host MHC class I as a mechanism of self/health recognition. Upon binding ligands, most ly49 receptors will deliver an inhibitory signal, averting killing of the target cell. In the case of cancer or virally infected cells, MHC-I will often be downregulated in order to limit cytotoxic T cell mediated killing of the cell, whereby NK cells will lack ly49 inhibitory signal and be able to kill infected or cancerous target cells (Beers et al., 2012). The homologous human KLRAP1 gene has been classified as a transcribed pseudogene because all associated transcripts are candidates for nonsense-mediated decay. ly49 receptors are lectin-like type II transmembrane bisulfide-bonded homodimers expressed on natural killer (NK) cells and some T-cell subsets. Cell-mediated cytotoxicity and release of

cytokines/chemokines are functions regulated by ly49 recognition of class I major histocompatibility complex proteins (MHC-I) or virus-encoded MHC-like product(s) (Biassoni, 2008).

MHC appreciation is central to both innate and adaptive immune recognition (Johansson, 2016). The occupation of both the innate immune effectors, such as NK cells and the adaptive immune effectors, such as T cells, hinge on the recognition of MHC-I molecules expressed on aberrant cells; nevertheless, the mode of recognition varies between the two lymphocyte subsets. T cell receptors (TCR) have specificity to the antigenic peptide bound to MHC-I and form contacts to the peptide as well as to the MHC molecule. NK cell receptors, such as the ly49 in mice, bind MHC-I molecules in a peptide–dependent but not peptide-specific manner. An exclusion is the ly49c, a member of the ly49 receptor family, whose binding to H-2Kb haplotype appears to be peptide-specific (Rahim et al., 2014). The binding interface between ly49 and MHC-I, revealed from crystal constructions of ly49a and H-2Db molecules, is distinct and away from the peptide binding groove on MHC-I. While signals downstream of TCR engagement specifically activate T cell functions, the ly49 receptors can be activating or inhibitory in nature, and their expression is not limited to NK cells. In addition to NK cells, other leukocytes have also been shown to express inhibitory ly49 receptors, such as the CD8+ T cells, CD3+ cells, intestinal epithelial lymphocytes (IELs), NKT cells, uterine NK cells (uNK) cells, and cells of the myeloid lineage. At this point, table 2.5 reviews the expression of ly49 receptors on different cells of the innate and adaptive immune system and their contribution to immunity (Id et al., 2016).

Table 2.4: Mouse Ly49 and human KIR receptors for MHC-I (Rahim et al., 2014).

Common Name	Ligand	Gene	Species
ly49d	H-2D^{d} Hamster,MHC class I	*KLRA4*	Mouse
ly49h	MCMV-m157	*KLRA8*	Mouse
ly49p	MCMV	*Klra16*	Mouse
NK1.1, NKR-P1C	Unknown	*Klrb1c*	Mouse
NKR-P1F	Clrg	*Klrb1f*	Mouse
PILRβ	Oglycosylation CD99	*Pilrb1*	Mouse
CD94-NKG2C, E	Mouse, Qa-1 Human HLA-E	*KLRD1-KLRC2, 3*	Mouse, human

2.2.13.2 ly49 Receptors

The innate MHC-I receptors include the human KIR, NKG2/CD94, and mouse ly49 families of receptors(Current & Id, 2016). The genes that encode the mouse MHC–I receptors are clustered together in the NKC on mouse Chromosome 6. The ly49 receptors are homodimeric type II glycoproteins of the C-type lectin-like superfamily. They are structurally distinct but functionally similar to human KIR in terms of MHC-I recognition, and therefore provide a use full model system to study the role of this class of receptors in immune regulation (Rahim et al., 2014). A list of activating and inhibitory ly49 receptors in different mouse strains and KIR in humans is publicized in Figure 2.13. MHC-I receptors generally inhibit NK cell function when they are engaged by self MHC ligands. Henceforth, inhibitory ly49 receptors are generally agreed to be important for the prevention of autoimmunity by suppressing NK cell activation. The acquisition of inhibitory ly49 for self MHC-I is also a key step in the "licensing" of developing NK cells to avoid a hyporesponsive state .In dissimilarity, the activating ly49 receptors identify ligands that are expressed on abnormal or infected cells and activate cytokine production and cellular cytotoxicity by NK cells (Johansson, 2016). The integration of signals from the activating and inhibitory ly49 receptors ultimately determines the functionality of NK cells. The inhibitory and activating members of ly49 and KIR families are

characterized by the presence or absence of ITIM domains in their cytoplasmic tail. NK cell stimulation results in discrepancy phosphorylation of ly49 receptors. Mason and co-workers first reported that tyrosine phosphorylation was restricted to the inhibitory ly49 molecules such as ly49 A, C/I, and G2, while the activating ly49d was not phosphorylated (Long & Wagtmann,2013).

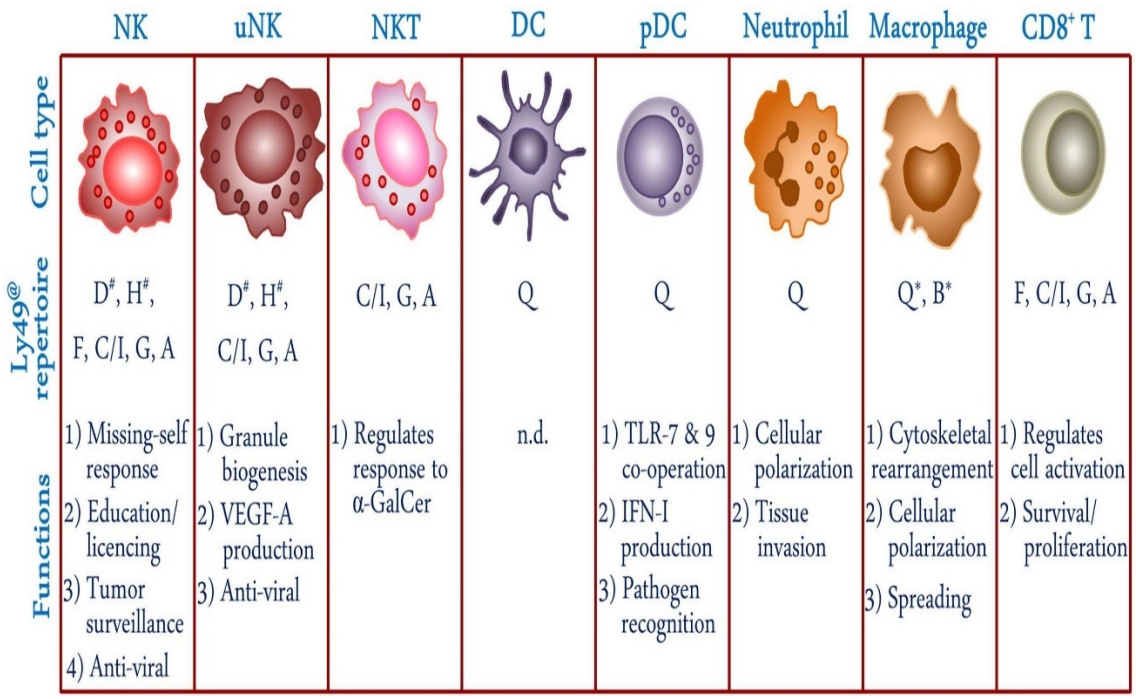


Figure 2.13: Schematic representation of cell types expressing ly49 receptors. Receptor repertoire and proposed functions of ly49 in different cellular subsets are shown based on literature reports in C57BL/6 mice. NK, Natural killer; uNK, uterine Natural killer; NKT, Natural killer T; DC, dendritic cells; pDC, plasmacytoid dendritic cell; α-GalCer, α-galactosyl ceramide; VEGF-A, vascular endothelial growth factor A; TLR, Toll-like receptor; IFN-I, type I interferon; n.d., not determined (Rahim et al., 2014).

2.2.13.3 ly49 Expression and Function on NK Cells

ly49 receptors are best known for their role in the regulation of NK cell functions. Both the activating and inhibitory ly49 receptors are expressed by NK cells (Figure 2.14). Murine NK cells express up to six members of the ly49 receptor family in an overlapping fashion, such that the average NK cell expresses two to three ly49 receptors. ly49 expression begins early during NK cell development in the bone marrow (BM)(Rahim et al., 2014). An *in vivo* differentiation study demonstrated

that there are five stages of NK cell development in murine BM, as assessed by surface expression of NK cell markers. In the first three stages of development, NK cells sequentially accomplish expression of CD122 (stage I; $CD122^{+}$ $NK1.1^{-}$ $DX5^{-}$ $Ly49^{-}$), then NK1.1, DX5, and NKG2A (stage II; $CD122^{+}$ $NK1.1^{+}$ $DX5^{lo}CD94/NKG2A^{+}$ $Ly49^{-}$)(Rahim et al., 2014), and finally ly49 at stage III. Afterward, immature NK cells undergo extensive cell division and expansion in stage IV followed by functional maturation at stage V of their development. ly49e is the only member of the ly49 family that is expressed on fetal NK cells and its expression is lost early after birth. The countenance of other ly49 family members is detected on developing NK cells during the first 2–3 weeks after birth and reach optimal levels within 6–8 weeks after birth (Johansson, 2016).

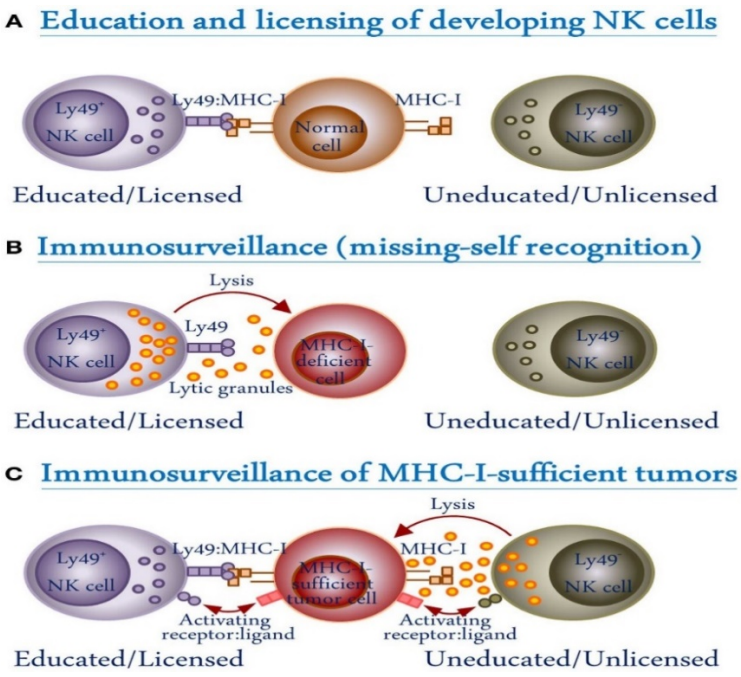


Figure 2.14: Schematic representation of the role of ly49 receptors in NK cell development and function. (A) During NK cell development, interactions between the inhibitory ly49 receptors and their self MHC-I ligands on normal cells result in NK cell functional maturation (education/licensing). (B) Licensed ly49+ but not unlicensed ly49− NK cells recognize MHC-I-deficient cells and kill them through the release of lytic granules (missing self-recognition). (C) Tumour cells express ligands which are recognized by activating receptors on NK cells (Kelley et al., 2005).

ly49 receptors play a significant role in NK cell education and acknowledgment of target cells. The mechanism behind NK cell recognition of target cells was discovered by Kärre and colleagues and accordingly named the "missing self-hypothesis" (Figure 2.15). According to the hypothesis, NK cells survey MHC-I expression on cells with which they come into contact. Abnormal or infected cells often a down-regulate expression of MHC-I on their surface in order to avoid detection and killing by cytotoxic T cells. This "missing self" hypothesis was further supported by the finding that cells isolated from mice lacking expression of β2m, the light chain component of MHC-I that is necessary for its surface expression, were keenly recognized and killed by NK cells, while re-introduction of a β2m transgene restored the resistance to NK cell killing. In addition to the increased susceptibility of cells isolated from MHC-I-deficient mice to NK cell killing, it's been also renowned that NK cells from $\beta 2m^{-/-}$ mice exhibited a diminished ability to kill traditional NK cell targets when compared to wild-type (WT) mice. This led to the proposal that MHC-I interactions during NK cell development are needed for induction of NK cytolytic activity (Bartel et al., 2013). The NK cells from ly49-deficient mice are unlicensed and show reduced missing self-response against MHC-I-deficient target cells. This education requirement has also been found in human NK cells with the interaction of MHC–I and KIR. The licensed NK cells are functionally active while at the same time self-tolerant due to the recognition of MHC-I through their inhibitory Ly49 receptors (Di Santo et al., 2016). In contrast, the unlicensed NK cells which lack self-MHC-specific inhibitory receptors are tolerant due to their hypo receptive nature (Trowsdale et al., 2012).

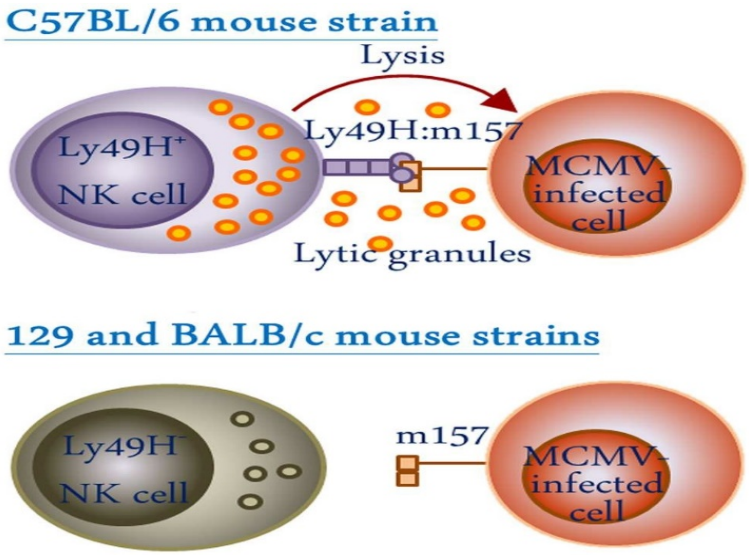


Figure 2.15: Schematic representation of the role of the activating Ly49H receptors in recognition of MCMV-infected cells. NK cells from C57BL/6 but not 129 or BALB/c mouse strains express Ly49H and are capable of recognizing m157 viral Protein on the surface of MCMV-infected cells, thereby, conferring resistance to MCMV infection (Gardiner, 2008).

2.2.13.4 CD94 Receptors

Natural killer (NK) cells, like CD94, are a distinct lineage of lymphocytes that mediate cytotoxic activity and secrete cytokines upon immune stimulation. Several genes of the C-type lectin superfamily, including members of the NKG2 family, are expressed by NK cells and may be involved in the regulation of NK cell function. *KLRD1* (CD94) is an antigen preferentially expressed on NK cells and is classified as a type II membrane protein because it has an external C terminus. Three transcript variants encoding two different isoforms have been found for this gene. inhibitory receptors in the human, and Ly-49 homodimers in rodents. CD94, NKG2 and Ly-49 belong to the C-type lectin super-family. As yet, CD94 and NKG2 molecules have not been detected in rodents or Ly-49 in humans. It has therefore been proposed that the two receptors represent functional equivalents in these species. The present study describes the cDNA cloning of a novel rat gene encoding a protein of 179 amino acids, 54.2 % identical to human CD94. The single-copy Cd94 gene is localized to the rat NK gene complex (NKC), within 50 kb from

Nkrp2, between the Nkrp1 and Ly49 gene clusters. By Northern blot analysis, the researcher showed that rat CD94 is selectively expressed by NK cells and a small subset of T cells, similar to the human orthologue. This expression is strain dependent, with high expression in DA NK cells and low in PVG NK cells. Evidence is presented that this difference is not due to receptor repertoire shaping by MHC-encoded ligands, but is controlled by genetic elements residing within the NKC. The identification of a rat CD94 orthologue suggests that NK cell populations utilize two different C-type lectin receptors for MHC class I molecules in parallel.

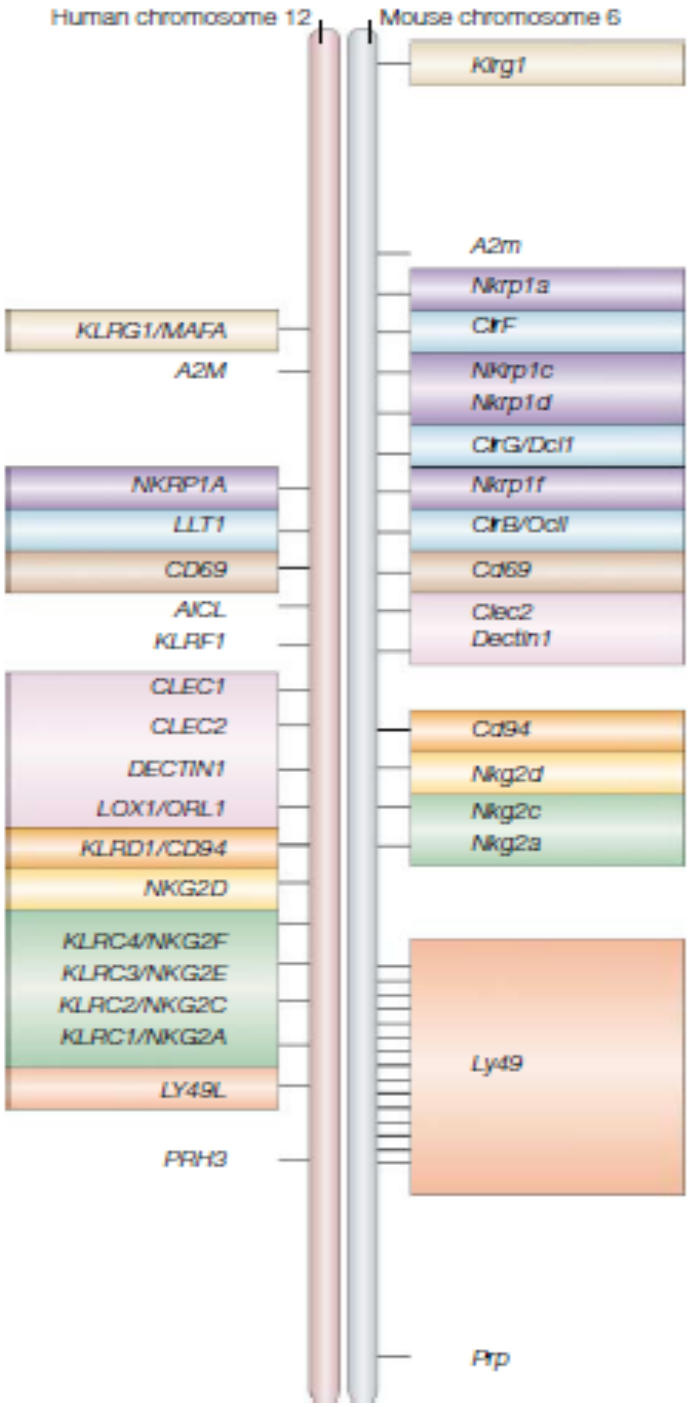


Figure 2.16: Lectin-like molecules encoded in the NKC. The genes for lectin-like receptors are present on human Chromosome 12p13.1 and mouse distal Chromosome 6. Colored boxes indicate families of genes that are present in both species. This Figure is not drawn to scale. AICL, activation-induced C-type lectin; A2M, α-2 macroglobulin; CLEC, C-type lectin-like receptor; Clr, C-lectin related; NK cell receptor-like C-type lectin (DECTIN), dendritic cell-associated C-type lectin; KLR, Killer cell lectin-like receptor; Lectin-Like Transcript (LLT), lectin-like transcript; Lectin-like oxidized receptor-1 (LOX), oxidized low-density lipoprotein; MAFA, mast cell function-associated receptor; NKG2, NK group 2; NKRP, NK cell receptor Protein; Ocil, osteoclast inhibitory lectin;), proline-rich restriction enzymes (endonucleases) HaeIII; Prp, proline- (Placeholder1)rich Protein (Gerosa et al., 2012).

2.2.14 Rodent NK Receptor Gene Complexes

The mouse LRC is located on Chromosome 7 (Hou et al., 2012) , even though it contains none of the KIR loci that form a cornerstone of MHC Class I recognition in humans. However, two murine KIR-like sequences have been detected outside the LRC on the X Chromosome (Hou et al., 2012) . There are conflicting reports on the function of one of these murine KIR-like sequences, One study found that Kir3dl1 (Kirl1) lacks an ITIM and any residue proficient of binding to an activating adaptor molecule, while another reported the presence of two ITIMs in Kir3dl1 (Contreras et al., 2007). Surprisingly, the other KIR-like sequence, Kirl2, is selectively expressed in defined areas of the mouse brain. The murine LRC contains orthologs of human GP6, NCR1, RPS9, and LAIR1. The Pir gene family members (including Pira1 to Pira11 and Pirb), which share sequence individuality with the human LILRs, are found between Ncr1 and Rps9 (Kulkarni et al., 2018).

In addition to sequence similarity, the Pir genes are arranged into two clusters similar to the LILRs and encode products that may interact with certain MHC Class I molecules. Genes of the murine LRC approximately display syntenic homology with the human arrangement apart from the absence of KIR loci.

In the rat, the LRC is located on Chromosome 1 and comprises an ortholog of murine Pirb, Ncr1 (Rahim et al., 2014) , and one KIR sequence (Kir3dl1) with a potential ITIM (Kulkarni et al., 2018) . The rat also has Fcar, which, while also present in humans, was lost in the mouse lineage. Orthologs of human NKC genes are reported in a syntenic region of murine Chromosome 6. The gene families are arranged in a similar order to the human NKC, except for expansions and interchanges of the C-type lectin-related (Clr) (ortholog of human Ocil) and NKrp1 (Klrb1) gene families. Interestingly, the

products of these two genetically entwined families in mouse interact, suggesting potential functional advantages for their adjacent gene locations.

There are several Klrb1 (NKrp1) genes in mouse, with Klrb1a, Klrb1c, and Klrb1f taking activating forms and Klrb1b and Klrb1d being inhibitory (Lu & Clark, 2012) . Members of the NKC-encoded C-type lectin-related gene family have been found as ligands for Klrb1d and Klrb1f. In the rat, the NKC is located on Chromosome 4. In fact, the first NKC gene discovered in any species was rat NKrp1. Human, mouse, and rat all-share orthologs of CD69 and *KLRD1* (CD94) however, there are still many differences between human and rodent in the gene content of the NKC (Kruse et al., 2017).

In contrast to the single human ly49 locus, the polymorphic ly49 gene family in mice contains at least sixteen genes and pseudogenes, named ly49a to ly49q, although the gene content can differ significantly in different mouse strains (Gust et al., 2017). While framework ly49 genes are present in all murine haplotypes, the ly49 gene content between these framework genes varies at some loci, in a similar manner to what is observed in the human KIR family.

The ly49 molecules have a variety of functions and expression patterns. Ly49a, ly49c, ly49g, and ly49i contain ITIMs and act as NK inhibitory receptors when recognizing MHC class I molecules. Conversely, ly49d and ly49h exhibit activating properties. ly49e functions in fetal NK cell development, when other Ly49 molecules are not present (Cappel et al., 2017). ly49q is found on plasmacytoid dendritic cells and not NK cells (Xu et al., 2017) . In the rat, this family has expanded even further. The most recent report lists 19 functional rat ly49 genes and fifteen pseudogenes. The rapid expansion of rat ly49 genes appears to result from repeated tandem and block gene duplications, which occurred after species divergence from the mouse, and might be

related to the higher number of MHC Class I genes in the rat genome than in the mouse genome.

Nonetheless, the Ly49 family in mouse parallels the human KIR both functionally and genetically (Current & Id, 2016) . Insight into the evolutionary drive behind gain or loss of Ly49 loci was provided by studying the relationship between Ly49 genes and murine cytomegalovirus (mCMV). When the ly49h receptor in mice is not present, is blocked by a monoclonal antibody, or has its pathway interrupted through mutation of the DAP12 molecule, uncontrolled viral replication of mCMV befalls, demonstrating that the function of this activating receptor is essential for mCMV resistance. Ly49h interrelates specifically with mCMV Protein m157 to counteract inhibition imposed by the viral Protein engaging Ly49i an example of the biological arms race (Birbrair & Frenette, 2016).

The KLR gene family in mouse contains numerous members, including Klrc1, Klrc2, Klrc3, *KLRD1*, Klri1, and Klri2. Similar to the recognition of HLA-E in humans, members of this family recognize the mouse HLA-E functional homolog H_2-T23. NKg2d binds minor histocompatibility molecule H60, the retinoic acid early transcript 1 (Rae-1) family, and mouse UL-16 binding Protein–like transcript 1 (Mult1) (Hou et al., 2012).

Fascinatingly, the Mill gene family, the murine functional equivalent of human MIC genes, which are encoded in the human MHC Class I region and bind NKG2D, are found near the murine LRC (Dezell et al., 2012). In mice, NKg2d is proposed to have activating properties through association with Dap12 and Dap10 (Pfeiffer et al., 2012). Human NKG2D cannot associate with DAP12 toward producing an activating signal. A direct ortholog of KLRF1 is not present in the mouse. The murine NKC also contains Klrg1 and Cd69, and genes other than those encoding NK receptors genes such as

alpha-2-macroglobulin, Clecsf12, and Clec2 (Caligiuri et al., 2011). Genes encoding three Klrc molecules, NKg2d, CD94, Klrb1, and Klrh1 (an inhibitory receptor) have been testified in rat.

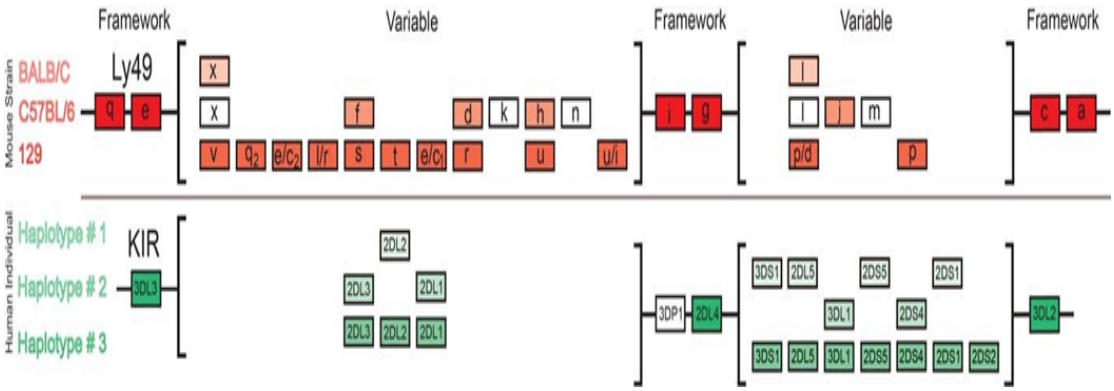


Figure 2.17: Differences in Organization of KIR Genes in Human Haplotypes and ly49 Genes in Various Mouse Strains (Kulkarni et al., 2008).

This Figure is not drawn to scale, but it exemplifies the presence of framework genes and the variable gene content in ly49 genes in different mouse strains and KIR sequences in human haplotypes. The vertical arrangement of genes within a family shows allelic relationships. White boxes indicate known pseudogenes. Please note that while many KIR haplotypes are possible, only a small selection has been shown here.

2.2.15 Natural Killer Cells and Cancer

Natural killer (NK) cells are classified as a member of the innate lymphoid cells (ILCs) group 1(Current & Id, 2016). ILCs have been recently recognized and grouped on the basis of their phenotypical and functional physiognomies. They are effectors of innate immunity and are involved in secondary lymphoid organ generation and tissue remodelling. NK cells are powerful cytotoxic lymphocytes capable to recognize and eliminate tumour- and virus-infected cells by limiting their spread and tissue damage. The recognition of tumour cells is mediated by both activating and inhibitory receptors (Johansson, 2016). While in haematological malignancies the role played by NK cells is

extensively known, their role in recognizing solid tumours remains unclear. Of late, tumour cell populations have been divided into two compartments: cancer-initiating cells (CICs) or cancer stem cells (CSCs) and senescent tumour cells (Tilgner et al., 2013). CSCs are a small subset of malignant cells with stem-like properties that are involved in tumour maintenance and recurrence due to their ability to survive to traditional therapies; they are, moreover, poorly recognized by T lymphocytes (Hara et al., 2016). Recent data showed that NK cells recognize *in vitro* cancer-initiating cells derived from colon cancer, glioblastoma, and melanoma. Nevertheless, more *in vivo* studies are urgently required to fully understand whether these new antitumour NK cells with cytotoxic competence may be considered in the design of new immunotherapeutic interventions.

The large granular innate lymphoid cells (ILCs) are named as natural killer (NK) cells for their protective and cytotoxic role in infectious and tumour diseases(Nakada et al., 2015). They play an active role in transplant rejection and in pathological conditions. The main effectors mechanisms of NK cells are lymphocytotoxicity and proinflammatory cytokine production. Recently, they were classified in Group 1 of the ILCs.

In human, NK cells are phenotypically characterized by the surface expression of molecules like CD56 and CD16 (FcγRIII) and by the absence of CD3 (Darlington et al., 2018), while in mice, they are defined as cells expressing NK1.1, DX5 and not expressing CD3 (Rahim et al., 2014). NK cells rely on a set of germ-line encoded activating and inhibitory receptors. The activating receptors include CD16, NKG2D, DNAM-1, and NKp46, all common to both human and mouse, while the other NK receptors (NCRs: NKp44 and NKp30) are expressed solely by human NK cells (Bonnet et al., 2015). Apart from the CD16 receptor recognizing Fc domain antibodies, the other receptors interact with ligands on infected and transformed cells. Molecules associated

with a “stress signature,” such as, MICA, MICB, ULBPs, and Hsp90 in human and Rae-1, MULT-1, and H60 in mouse, have been identified as major activating ligands. Other activating NK ligands are PVR and NECTIN-2 bound by DNAM-1, expressed both in human and mice. NCR ligands are still unknown, except for NKp30, whose ligands are B7-H6, BAG-6, and Gal-3.

The inhibitory receptors of NK cells interact with major histocompatibility complex (MHC-I) molecules. In human, most of these receptors belong to the family of killer cell immunoglobulin like receptors (KIRs) that directly bind to certain HLA-A, -B, and -C alleles. KIRs are highly polymorphic receptors that interact with MHC-I molecules and elicit inhibitory or activating signals (Tuttolomondo et al., 2017). Their name is correlated with the number of Ig-like domains in their extracellular regions and with the presence of a long (inhibitory) or short (activating) cytoplasmic tail (Johansson, 2016). Other types of inhibitory and activating NK cell receptors are the lectin-like heterodimers of NKG2 family. The best-known members are the inhibitory heterodimer CD94/NKG2A and its activating counterpart CD94/NKG2C, both recognizing the non-classical MHC class I molecule, HLA-E. In mice, the inhibitory receptors belong to the superfamily of lectin-like type II integral membrane proteins, known as Ly49 and CD94/NKG2A receptors.

The lack or reduced expression of MHC-I on the target cells’ surface leads to “missing self” recognition. There are clinical observations and ongoing clinical trials based on the idea that NK cells transferred from one individual to a non-MHC-I-matched patient can exert antileukemic effects based on “missing self” recognition.

In both species, the fully matured highly cytotoxic NK phenotype is acquired after a, still poorly understood, MHC class I molecule-dependent oncogenic process. The NK cells ontogeny process has been termed “education – licensing – arming/disarming”. Recently, Kärre and colleagues have proposed to explain the NK cells’ education in the

"Rheostat Model". The "Rheostat Model" postulates that education of NK cells represents a tuning mechanism that goes on continuously and reversibly accordingly to the MHC environment, even after NK cell maturation. This model has implications for immunotherapy based on exploiting "missing self" reactivity when NK cells are transferred to a cancer patient from a healthy, non-MHC-matched individual. If the NK cells are "re-tuned" in the new environment, as proposed in the rheostat model, this may affect their efficacy to react against the recipient's leukaemia cells and can influence the reactivity of NK cells against tumour cells in different situations. It has been shown that NK cells can exert antitumour effects in the new host environment. Lately, Wagner and colleagues (Wang et al., 2013) provided further support to the rheostat model demonstrating that NK cells infused in a recipient become tolerant to healthy cells representing "missing self" in the new environment, while the retuned NK cells will retain activity against tumour cells representing "missing self," due to a higher expression of ligands for activating NK receptors on tumour cells.

Another new concept that has contributed to change our knowledge about the NK cells biology derives from the observations made during human and mice CMV infection, where NK cell subsets recognizing CMV-infected cells expanded (Pegram et al., 2013). The expanded CMV-specific population has been proposed to be a relevant NK cell memory subset able to provide immunity in the second infection round (Palmer et al.,2013). Therefore, the information that are now available on the NK cells biology are pushing the field toward their use in clinical settings.

The history of NK cells has evolved closely related with the history of cancer immunotherapy since they were discovered in the 1970s for their capability to target and kill cancer cells. NK cells preventing leukaemia relapse in mismatched hematopoietic stem cell transplant (HSCT) patients stirred a renewed interest in the use of NK cells in the fight against cancer. They are the most efficient effectors against

tumours and are considered suitable candidates for adoptive immunotherapy of both haematological and non-haematological malignancies. Nearly 50 years after their discovery, we are now starting to realize the potential of these NK cells in tumour immune therapy trials.

2.3. Hematopoietic Stem Cells

Stem cells are generally divided to two main categories

- Pluripotent Stem Cells
- Multipotent stem cells

2.3.1 Pluripotent Stem Cells

Two generally studied types of pluripotent stem cells (PSCs) are embryonic stem cells (ESCs) and induced PSCs (iPSCs) (Kashin et al., 2017). Human ESCs are isolated from the inner cell mass of the blastocyst stage of a developing embryo. iPSCs are similar or equivalent to ESCs but are generated via ectopic expression of reprogramming genes such as OCT4, KLF4, SOX2, and c-MYC in adult somatic cells (Guo et al., 2016). iPSCs have developed stem cell research by simplifying the derivation of certain stem cells that can then be used to model diseases *in vitro*. These research models can be used in defining the mechanisms of disease pathology and can therefore play a vital role in the possible identification of therapeutic targets and drug discovery. According to multiple researches, the unlimited supply of iPSCs that can be directed to become functionally mature cells also holds great promise as a source material for cell therapies that discourse a variety of diseases such as diabetes, liver diseases, and Parkinson's and Alzheimer's diseases (Avior & Benvenisty, 2016).

To maintain the undifferentiated stem cell state, both iPSCs and ESCs require the expression of key transcription factors such as Nanog and OCT4 that regulate genes

important for cell division, differentiation, and development (Pasca et al., 2015). In addition to these transcription factors, a specific set of cell-surface proteins such as TRA-1-60, TRA-1-81, SSEA3, and SSEA4 are characteristically expressed by the PSCs (Kashin et al., 2017). Recognition of the presence of these markers is one of the first steps in characterizing newly derived iPSC and ESC lines.

2.3.2 Multipotent stem cells

Multipotent stem cells are unspecialized cells that have the ability to self-renew for long periods of time and differentiate into specialized cells with specific functions (Mirzaei et al., 2017). For example, multipotent adult stem cells in bone marrow, or hematopoietic stem cells, can give rise to all blood cell types, and neural stem cells in the brain can give rise to glial and neuronal cells (Vicinanza et al., 2017). Though, mesenchymal stem cells can mature to several cell types found in bone, muscle, cartilage, fat and other tissues (Nunez et al., 2016).

Hematopoietic stem cells (HSCs) are multipotent stem cells that can give rise to all cell types in the blood, including B and T lymphocytes, natural killer cells, dendritic cells, monocytes, platelets, and erythrocytes (Smith et al., 2017). HSCs are very small and are non-adherent cells, which make them very difficult to purify or visualize by microscopy. In their inactive state, HSCs can be found in the red bone marrow. Typically, HSCs are identified by flow cytometry using 10-to-14 antibody panels targeting various cell-surface antigens (Vicinanza et al., 2017). CD34 is a highly glycosylated monomeric with a molecular weight range of 111-115 kDa surface protein that is present on many stem cell populations (Nunez et al., 2016).

Hematopoietic stem cells (HSCs) are able clonally and persistently to produce all blood cells while maintaining the HSC pool size, permitting sustained haematopoiesis

throughout life. HSCs have served as an exceptional model in stem cell biology, and HSC transplantation has served as a prototype for stem cell therapy in regenerative medicine. The exploration field of stem cell biology and regenerative medicine has been conceptually and essentially established through the study of HSCs. After many years of work, we have finally begun to understand self-renewal and multilineage differentiation in HSCs at the molecular level (Marinho et al., 2015).

Haematopoiesis is the tightly regulated process of blood cell formation. Short-lived mature blood cells are replenished on a daily basis by hematopoietic stem cells through large numbers of HSC progeny such as multipotent progenitors and lineage-committed progenitors (Miranda et al., 2015). HSCs are the first identified and the best-characterized stem cells, providing a first model for the stem cell system pertinent to many other stem cells. Figure 2.15 shows the hematopoietic system, which is organized hierarchically.

Many basic concepts in stem cell biology have been created based on the study of HSCs. An asymmetric division model, a stochastic model (Till & McCulloch, 2016), a clonal succession model (Kay ,2010), and a stem cell niche model are good examples of such work. Although much progress has recently been made in this field, our understanding of the molecular mechanisms underlying the regulation of HSCs has just begun. This literature review presents the current understanding of the regulation of HSCs at the molecular level and highlights molecules controlling self-renewal.

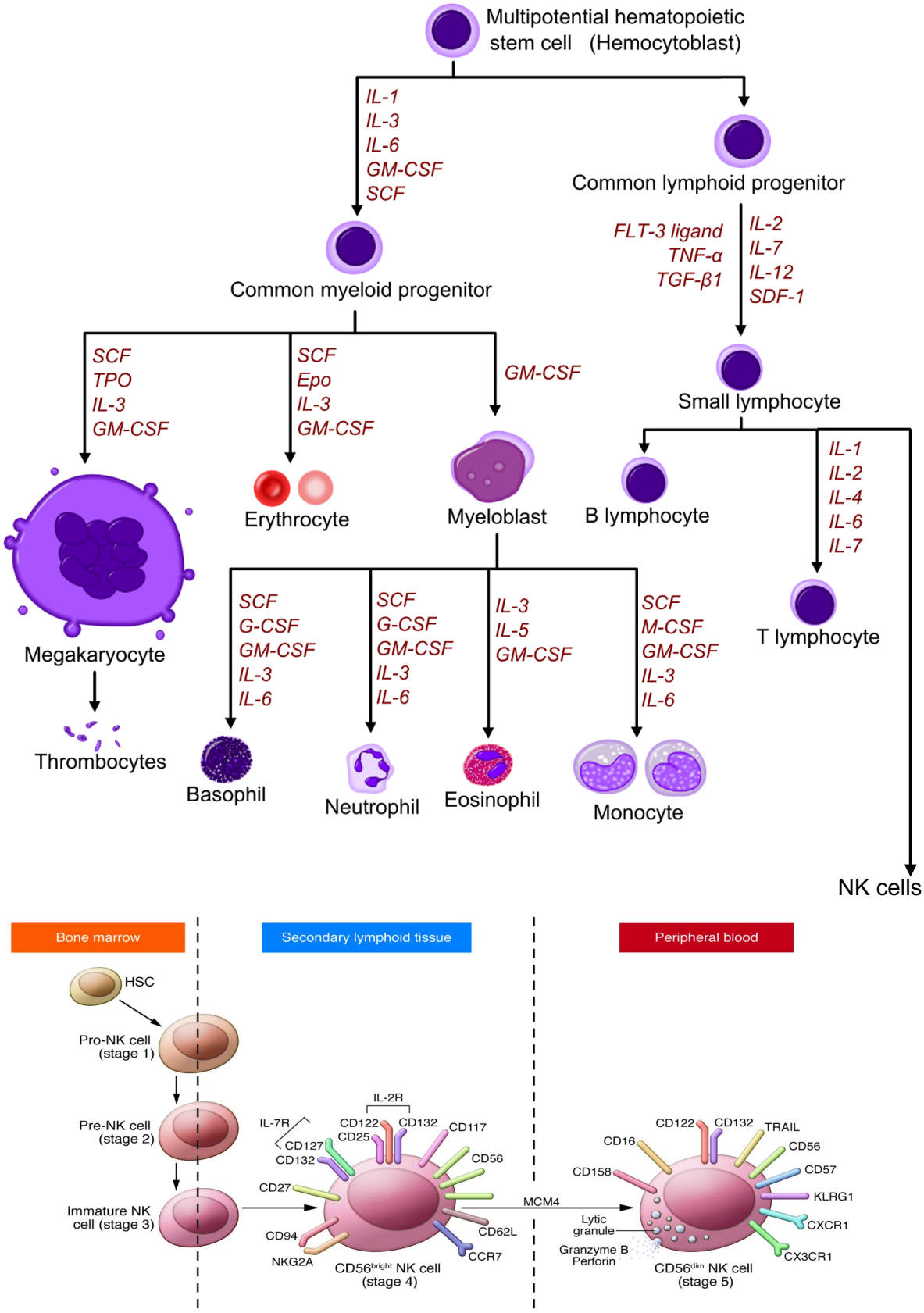


Figure 2.18: The haematopoiesis system. The hematopoietic hierarchy is illustrated. In concept, one stem cell asymmetrically gives rise to a stem cell to maintain its line and to a daughter cell destined for multilineage differentiation. Multipotent progenitor cells rather rapidly undergo symmetric self-renewal a limited number of times, leading to their amplification in a short interval. The entire hematopoietic system is established and maintained by a hematopoietic stem cell (Ritter et al., 2013).

2.3.4 The Definition and Entity of HSCs

Stem cells are demarcated as cells with both self-renewal and differentiation potentials. This definition was primarily framed by J. E. Till and E. A. McCulloch, based on their discovery and characterization of colony forming units in the spleen (CFU-S) (Smith et al., 2017). When mouse BM or spleen cells are injected into lethally irradiated mice, visible colonies develop in the spleen of the receiver mice during a relatively narrow window of time after transplantation (e.g., 8–12 days). These colonies consist mainly of myeloid cells such as neutrophils, macrophages, erythroblasts, and megakaryocytes. The clonal nature of CFU-S was proven using chromosomal markers (Schulz et al., 2012). The magnitudes and cell compositions of colonies change with time after transplantation, indicating that CFU-S are heterogeneous. It is now acknowledged that CFU-S belong to a certain class of progenitors distinct from HSCs. The multipotent progenitor population is highly supplemented in day 12 CFU-S (Patrikoski et al., 2013), and the erythroid/ megakaryocytic progenitor population is highly enriched in day 8 CFU-S (Batlle & Clevers, 2017).

Nevertheless, most of the concepts established, based on comprehensive analyses of CFU-S, have been proven to apply to stem cells, an instance of how concepts or models can remain important throughout the history of research in a particular field.

To ‘self-renew’ is a process of producing one or two daughter cells identical to their parental cell via cell division. More accurately, it copies the complete genome, including epigenetic modifications. In repetition, an act of self-renewal is a cell division that produces one or two daughter cells functionally equivalent to the parental cell. Both self-renewal potential and the full range of differentiation potentials (“multipotency”) are maintained throughout the self-renewing division. Differentiation is the process by which cells become more specialized, functional cells. Stem cells least likely

differentiate without cell division. Incidentally, to differentiate is a process of epigenetic change via cell division. In practice, an act of differentiation is a cell division that produces one or two daughter cells in which self-renewal potential or some part of multipotency is lost.

HSCs give rise to daughter cells that increasingly restrict their differentiation potentials during their differentiation. Lineage obligation is the event that allows cells to differentiate further along one or more particular lineage, but not along other lineages. It can occur in cells at all differentiation stages, from HSC daughter cells to bipotent progenitor cells. In general, cells cannot revoke lineage commitment or reverse differentiation sequences. It is hoped that these events self-renewal, differentiation, and commitment will be more clearly and simply defined in molecular terms in the near future (Wilson et al., 2008).

2.3.5 Historical Overview

The search for stem cells began in the repercussion of the bombings in Hiroshima and Nagasaki in 1945. Those who died over a prolonged period from lower doses of radiation had compromised hematopoietic systems that could not stimulate either sufficient white blood cells to protect against otherwise non-pathogenic infections or enough platelets to clot their blood. Higher doses of radiation also killed the stem cells of the duodenal tract, resulting in more rapid death. Later, it was demonstrated that mice that were given doses of whole-body X-irradiation developed the same radiation syndromes; at the minimal lethal dose, the mice died from hematopoietic failure approximately two weeks after radiation exposure. Significantly, however, shielding a single bone or the spleen from radiation prevented this irradiation syndrome. Soon thereafter, using inbred strains of mice, scientists showed that whole-body-irradiated mice could be rescued from otherwise fatal hematopoietic failure by injection of

suspensions of cells from blood-forming organs such as the bone marrow (Ashley Ng & Warren, 2017). In 1956, three laboratories demonstrated that the injected BM cells directly regenerated the blood-forming system, rather than releasing factors that instigated the recipients' cells to repair irradiation damage. Thus far, the only known treatment for hematopoietic failure following entire body irradiation is transplantation of BM cells or HSCs to regenerate the blood-forming system in the host organisms.

2.3.6 HSC Assays

Assays have been developed to characterize hematopoietic stem and progenitor cells in vitro and in vivo. In vivo assays that are used to study HSCs include till and McCulloch's classical spleen colony forming (CFU-S) assay, which measures the ability of HSC (as well as blood-forming progenitor cells) to form large colonies in the spleens of lethally irradiated mice. Its' main advantage (and limitation) is the short-term nature of the assay (now typically 12 days). Conversely, the assays that truly define HSCs are reconstitution assays. Mice that have been "preconditioned" by lethal irradiation to accept new HSCs are injected with purified HSCs or mixed populations containing HSCs, which will repopulate the hematopoietic systems of the host mice for the life of the animal. These assays typically use different types of markers to distinguish host and donor-derived cells (Srikanth et al., 2015).

For instance, allelic assays distinguish different versions of a particular gene, either by direct analysis of DNA or of the proteins expressed by these alleles (Hunt, Gates, Rancourt, Kallos, & Meng, 2013). These proteins may be cell surface proteins that are recognized by specific monoclonal antibodies that can distinguish between the variants CD45 or cellular proteins that may be recognized through methods such as gel-based analysis (Fan et al., 2015). Other assays take advantage of the fact that male cells can be detected in a female host by detecting the male cell specific Y-Chromosome by

molecular assays (e.g., polymerase chain reaction, or PCR). Small numbers of HSCs (as few as one cell in mouse experiments) can be assayed using competitive reconstitutions, in which a small amount of host-type BM cells (enough to radio protect the host and thus ensure survival) is mixed in with the donor-HSC population (Birbrair & Frenette, 2016).

To inaugurate long-term reconstitutions in mouse models, the mice are followed for at least 4 months after receiving the HSCs. Serial reconstitution, in which the BM from a beforehand irradiated and reconstituted mouse becomes the HSC source for second irradiated mouse, extends the potential of this assay to test lifespan and expansion limits of HSCs. Inappropriately, the serial transfer assay measures both the lifespan and the transplant ability of the stem cells. The transplant ability may be altered under various conditions, so this assay is not the sine qua none of HSC function (Marinho et al., 2013). Testing the *in vivo* activity of human cells is obviously more problematic.

These assays are clearly more stringent, and thus more informative, but also more difficult than the in vitro HSC assays discussed below. Nevertheless, they can only assay a fraction of the lifespan under which the cells would usually have to function. Statistics on the long-term functioning of cells can only be derived from clinical HSC transplantations (Perrotti et al., 2010). A number of assays have been developed to recognize HSCs in vitro (e.g., in tissue culture). These are especially important when assaying human cells. Since transplantation assays for human cells are limited, cell culture assays often represent the only viable option. In vitro assays for HSCs include Long-Term Culture-Initializing Cell (LTC-IC) assays and Cobblestone Area Forming Cell (CAFC) assays. LTC-IC assays are based on the ability of HSCs, but not more mature progenitor cells, to maintain progenitor cells with clonogenic potential over at least a five-week culture period. CAFC assays to measure the ability of HSCs to

maintain a specific and easily recognizable way of growing under stromal cells for five to seven weeks after the initial plating. Progenitor cells can only grow in culture in this manner for shorter periods of time (Herencia et al., 2011).

2.3.7 Transplantation Assays

2.3.7.1 Rat Cells

The presence of hematopoietic stem cells in non-cultured or cultured cell preparations can only be tested experimentally by transplanting the cells into lethally or sub lethally irradiated mice and measuring the ability of these cells to reconstitute haematopoiesis. Haematopoiesis derived from donor cells can be measured by employing genetic differences between donor and recipient mouse strains, such as the expression of different CD45 isoforms (CD45.1 and CD45.2) (Herencia et al., 2011), which can be detected by flow cytometry after staining blood, spleen or BM samples with appropriate antibodies 18 Co-staining with lineage-specific antibodies is used to measure the contribution of donor cells to different blood cell lineages.

By examining serial peripheral blood samples of the transplanted mice over weeks and months it is possible to get information on the ability of the transplanted cells to perform short-term engraftment and long-term multilineage haematopoiesis. Some cultured cells may be able to reconstitute donor haematopoiesis during a short period (e.g. several weeks) and disappear later, and/or only reconstitute a single lineage (e.g. only granulocytes). A cell population can only be considered to contain true pluripotent stem cells if donor cells can be detected for prolonged periods of time, usually at least five months after transplantation and in different blood cell lineages, specifically granulocytes and/or monocytes and lymphocytes.

The donor cells should also contribute to the erythroid and megakaryocytic lineages, but RBC and platelet chimerism cannot be easily measured in the CD45.1/CD45.2 transplantation model as mature RBCs and platelets lack CD45 expression. If measuring the contribution of transplanted cells to RBC and/or platelet reconstitution is important, other transplantation models could be used. These include transplantation with cells from transgenic EGFP-expressing donor mice, which can be detected based on their fluorescence. RBC derived from normal stem cells can also be measured. If mutant strains carrying the W or alpha-thalassemia mutations are used as recipients of HSCs from normal mice RBC chimerism can be measured based on size differences between donor-derived normal RBCs and mutant recipient RBCs using the forward scatter parameter of a standard flow cytometer (Storm et al., 2010).

2.3.8 Cell Markers Can Identify HSCs

While initial experiments studied HSC activity in mixed populations, much progress has been made in specifically describing the cells that have HSC activity. A variety of markers has been discovered to help recognize and isolate HSCs. Initial marker efforts focused on cell size, density, and recognition by lectins (carbohydrate-binding proteins derived largely from plants), but more recent efforts have focused mainly on cell surface Protein markers, as defined by monoclonal antibodies (Lang et al., 2006).

For mouse HSCs, these markers include panels of 8 to 14 different monoclonal antibodies that recognize cell surface proteins present on differentiated hematopoietic lineages (Jansen et al., 2013), such as the red blood cell and macrophage lineages (thus, these markers are collectively referred to as “Lin”), as well as the proteins Sca-1, CD27, CD34, CD38, CD43, CD90.1, CD117(c-Kit), AA4.1,and MHC Class I, and CD150 (Lam et al., 2014).

2.3.8.1 Cell Surface Marker Combinations That Define Hematopoietic Stem Cells

HSC assays, when combined with the ability to purify HSCs, have provided increasingly detailed insight into the cells and the early steps involved in the differentiation process. Several marker combinations have been developed that describe murine HSCs, including [CD117high, CD90.1low, Linneg/low, Sca-1pos], [CD90.1low, Linneg, Sca-1pos Rhodamine123low], [CD34neg/low, CD117pos, Sca-1pos, Linneg], [CD150 pos, CD48neg, CD244neg], (Shimazaki & Okano, 2008) and "side population" cells using Hoechst-dye. Each of these combinations' countenances purification of HSCs to near-homogeneity. Similar strategies have been developed to purify human HSCs, employing markers such as CD34, CD38, Lin, CD90, CD133 and fluorescent substrates for the enzyme, aldehyde dehydrogenase (Poon et al., 2014a). The use of highly purified human HSCs has been mainly experimental, and clinical use typically employs enrichment for one marker, usually CD34. CD34 enrichment yields a population of cells enriched for HSC and blood progenitor cells but still contains many other cell types.

Nevertheless, limited trials in which highly FACS-purified CD34pos CD90pos HSCs were used as a source of reconstituting cells have demonstrated that rapid reconstitution of the blood system can reliably be obtained using only HSCs. The purification strategies described above recognize a rare subset of cells. Exact numbers depend on the assay used as well as on the genetic background studied. In mouse BM, 1 in 10,000 cells is a hematopoietic stem cell with the ability to support long-term haematopoiesis following transplantation into a suitable host (Pidala et al., 2018). When short-term stem cells, which have a limited self-renewal capacity, are included in the estimation, the frequency of stem cells in BM increases to 1 in 1,000 to 1 in 2,000 cells in humans and mice (Mirzaei et al., 2017). The numbers present in normal blood are at least ten-fold lower than in marrow.

None of the HSC markers currently used is directly linked to an essential HSC function, and subsequently, even within a species, markers can differ depending on genetic alleles, mouse strains, progressive stages, and cell activation stages. Regardless of this, there is a clear correlation in HSC markers between divergent species such as humans and mice. However, unless the ongoing attempts at defining the complete HSC gene expression patterns will yield usable markers that are linked to essential functions for maintaining the "stemness" of the cells, functional assays will remain necessary to identify HSCs unequivocally (Miranda et al., 2015).

2.3.9 Clinical use of HSCs

The clinical use of stem cells holds great promise, although the application of most classes of adult stem cells is either currently untested or is in the earliest phases of clinical testing. The only exception is HSCs, which have been used clinically since 1959 and are used increasingly routinely for transplantations, albeit almost exclusively in a non-pure form. By 1995, more than 40,000 transplants were performed annually worldwide. Currently, the main suggestions for BM transplantation are either hematopoietic cancer (leukaemia and lymphomas) or the use of high-dose chemotherapy for non-hematopoietic malignancies (cancers in other organs) (Bresagen, 2014). Other indications include diseases that involve genetic or acquired BM failure, such as aplastic anaemia, thalassemia sickle cell anaemia, and increasingly, autoimmune diseases.

2.3.9.1 Stem Cell Therapy

In a directive to translate stem cell biology into stem cell therapy, we must know how to control the destiny of stem cells. If not, the possibility of unsolicited outcomes is ostensible, with harm due on the one hand to self-renewal capacity and on the other to pluripotency or multipotency. Malignant tumours originate from cells with uncontrolled

self-renewal and shortage of variance. Overproduction of only, a particular lineage may result in a functional failure of the organ. ESCs have become an extremely attractive source for adult stem cells since human ESCs and persuaded pluripotent stem cells were established (Gust et al., 2017). In the field of haematology, efficient generation of HSCs from ESCs is the most important key to successful stem cell therapy. Ex vivo expansion of HSCs has been difficult. This problem should be overcome by use of ESCs. Nonetheless, it remains difficult to generate HSCs from ESCs. ESCs allegedly give rise to HSCs through multiple steps (Hara et al., 2016). In mice, compulsory expression of HOXB4 supports the generation of HSCs from ESCs (Rodriguez Sawicki et al., 2017). Certain developmental stages of HSCs seem skipped by HOXB4. The consequential ESC-derived repopulating cells remain significantly mediocre to adult HSCs from BM, conversely, particularly in the context of repopulating activity or self-renewal potential. In humans, repopulating cells have not been successfully generated from ESCs by any means including obligatory HOXB4 expression. Therefore, in this study, the researcher has not gone to the area of achieving practical use of human ESCs and induced pluripotent stem cells as a source of HSCs because our target experimental animal is rat.

2.4 Transfection Efficiency and Functionality Assays

In this project related the transfection method, Lipofectamine was used to transfer target genes to HSCs and regarding objective one of this project which is generating transgenic Natural Killer Cells, the experiments used to evaluate the transfection, were

- Western Blotting
- Flow Cytometry

and for the second objective of this project which is examine the functionality of newly generated NK cells, the researcher examined cytotoxicity and apoptosis ability of

newly generated NK cells. In case of cytotoxicity evaluation used and regarding apoptosis.

- MTT assay
- Live/Dead assay
- PARP cleavage
- Caspase 3/7 kit assays

were used. Full explanations about these techniques are given in their related sections in chapter methodology.

CHAPTER 3: METHODOLOGY

3.1 Introduction

In this chapter the methods, material and applications which was used in this research will be explained. According to the objectives of this project as they have been mentioned in section 1.6, the methods and applications were used to fulfil the scopes of this project.

According to the objectives which have been mentioned in section 1.6, the Methodology of this research has two main pillars

1. Generation of NK cells from HSCs and verification of transfection success by western blotting and flow cytometry.
2. Examine the functionality of newly generated NK cells on cancer cells in two aspects of cytotoxicity and apoptosis. To examine the cytotoxicity of newly generated NK cells, MTT assay and Live & Dead assay was used and secondly to evaluate the apoptosis ability of newly generated NK cells, PARP cleavage and Caspase 3/7 assays was used.

Figure 3.1 shows the methods, materials and applications which were used to implement the objectives.

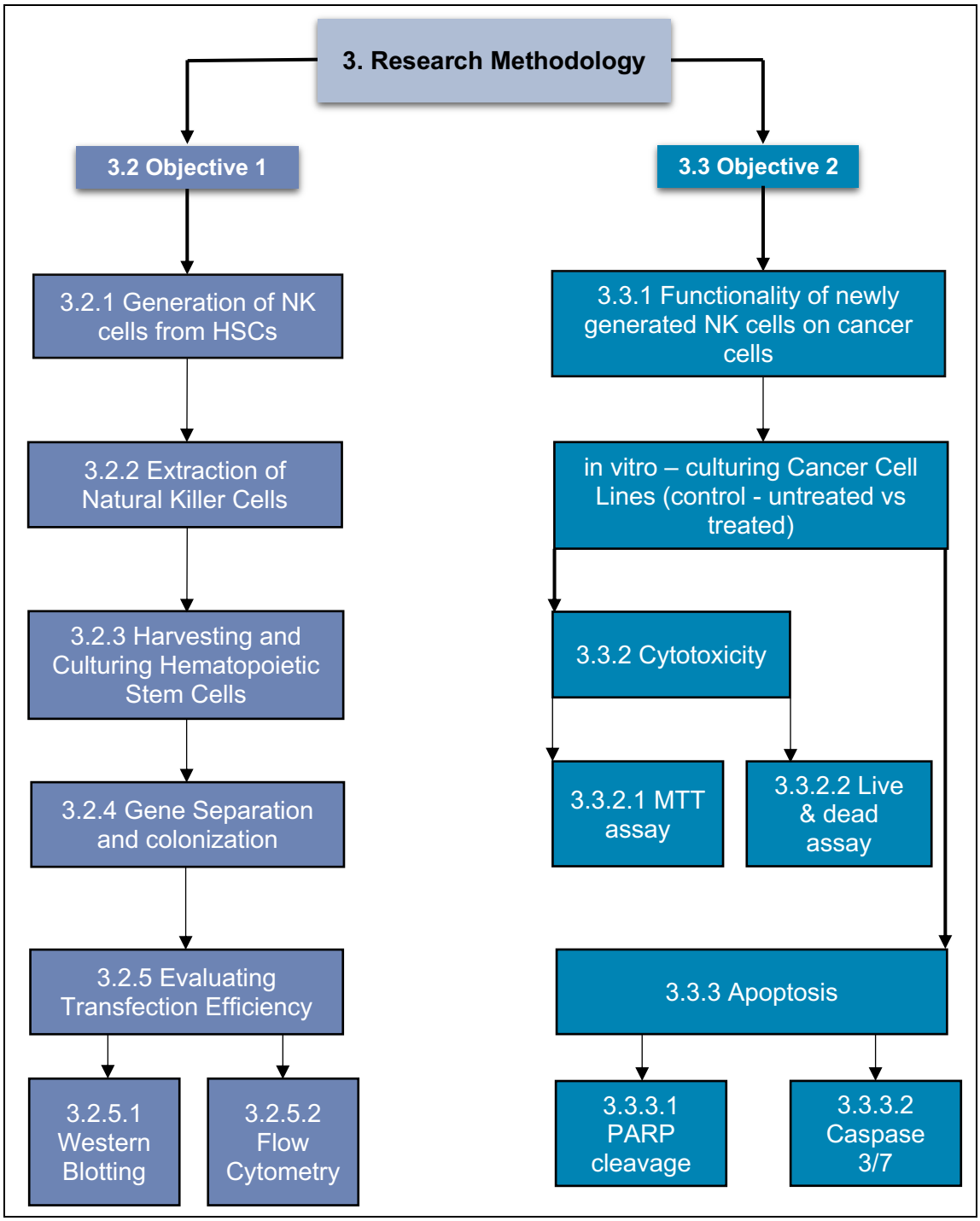


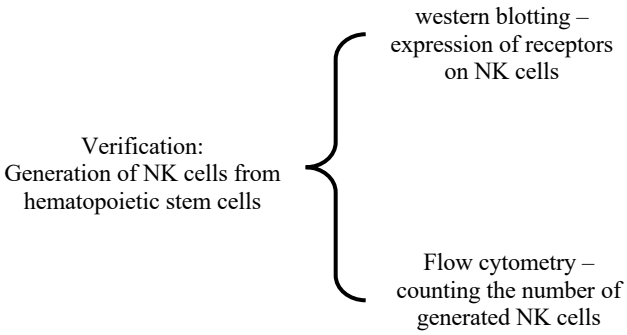
Figure 3.1: The figure approach of Research Methodology.

3.2 Research Methodology for objective 1 (verification of transfection success)

3.2.1 Generation of NK cells from Hematopoietic Stem Cells

To fulfil the objective 1, three main process were done

- Natural Killer Cells were extracted from experimental animal's peripheral blood.
- Hematopoietic stem cells were harvested from the thigh bone marrow of the experimental animal (three Sprague Dawley rat).
- Three specific genes were separated from the DNA of separated NK cells and colonized in Hematopoietic stem cells.



3.2.2 Extraction of Natural Killer Cells

For this section, 5 processes were done. table 3.1.

Table 3.1: The process of Natural Killer Cells separation from blood.

Extraction of Natural Killer Cells	
1	10 ml of the rat's peripheral blood was extracted & transferred to an EDTA tube
2	Separation of lymphoid
3	Separation of NK cells by Ls column magnet with the help of microbeads
4	Culturing the separated NK cells in DMEM
5	Treating the culture with IL2,12 and Inomysine 10ng/ml for 24 hours

3.2.2.1 Magnet Separation

Regarding separation of Natural Killer cells, an albino breed Sprague Dawley rat was purchased and according to University of Malaya Institute of Animal Care protocol, the experimental animal was kept and treated in laboratory for one week. The experimental animal was male and 280 gram in weight. For the purpose of blood sample collection, the direct venepuncture of rat lateral tail vein procedure was done. The rat's tail was washed and warmed in 39°C for 10 minutes. With 21G needle, 2ml of blood was obtained. During 24-hour period, 5 samples, each 2ml of blood was collected from the tail vein. The blood was collected in Purple cap blood collecting tubes (specialized for haematology tests and full blood count (FBC)) with EDTA (ethylene diaminete traacetic acid) to prevent of blood clotting. Each tube with blood was refrigerated in 4°C immediately after collection.

The Magnet Technology or the Column Technology is founded on the use of Microbeads. Magnetic-activated cell sorting (MACS) is a method for separation of various cell populations depending on their surface antigens (CD molecules). The MACS method allows cells to be separated by using magnetic nanoparticles coated with antibodies against a surface antigen. This causes the cells expressing this antigen to attach to the magnetic nanoparticles.

LS Columns have been developed for the tender isolation of Microbeads labelled cells. As Microbeads are extremely small, superparamagnetic particles, a high-gradient magnetic field is required to recollect the labelled cells. LS Columns contain an augmented matrix to generate this strong magnetic field when placed in an enduring magnet such as the Midi Separator, Quadro Separator, Vario Separator, Super MACS II Separator, or Multi MACS Cell Separator Plus. LS Columns have been industrialized for positive selection of human and animal cells, particularly rare cells, out of a

heterogeneous cell suspension in amalgamation with a Separator. LS Columns can also be used for exhaustion of cells which strongly express the magnetically labelled surface antigen. They can also be used to distinct other biological material such as plant cells, bacteria, viruses, protozoa, cell organelles etc. LS Columns are not suitable for particles larger than 30 µm.

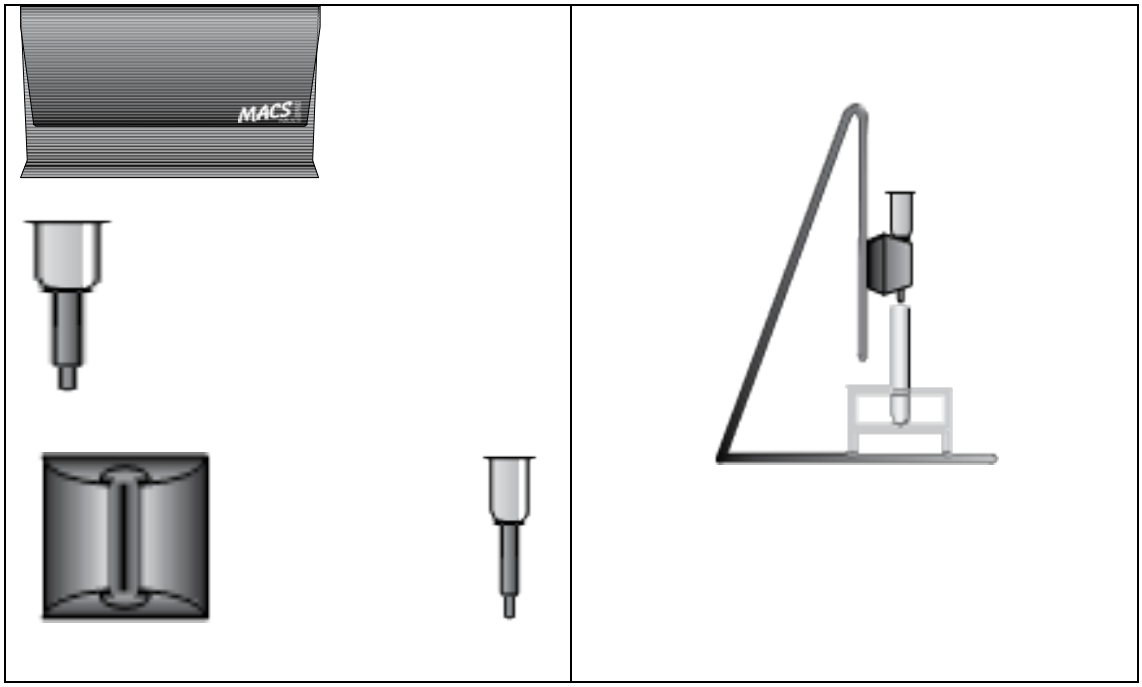


Figure 3.2: Assembly of LS Column.

Rat CD49b (DX5) Microbeads were developed for the isolation of Rat NK cells from single-cell suspensions of lymphoid and nonlymphoid tissue, as well as from peripheral blood or body fluids. The Microbeads were formerly called Anti-NK Cell (DX5) Microbeads. The CD49b is expressed on NK cells and a small population of T cells (CD4+CD3+TCRαβ+). These cells can be depleted prior to NK cell enrichment by using CD90 (Thy1.2) Microbeads or CD5 Microbeads. CD49b is less rat strain-specific than other NK cell markers and is expressed by the most common inbred Rat strains.

In this step of the experiment the blood samples were Passed through 30 µm nylon mesh (Merck company, Pre-Separation Filters) to remove cell clumps which may clog

the column. The samples were centrifuged for 300×g at 4-8° for 10 minutes. After that the supernatant was pipped off completely. 10 µL of CD49b (DX5) Microbeads was added to each sample and was mixed well and incubated for 15 minutes at 4−8 °C. after that again the cells were washed by adding 2 mL of buffer and again was centrifuged at 300×g for 10 minutes. The supernatant was Pipetted off completely. After that 500 µL of buffer was added and now the samples are prepared for loading in to the columns.

After that 3 columns were put in the magnetic field Separator. The columns were prepared by rinsing with 3 mL of buffer (PBS: phosphate-buffered saline). The cell suspension was applied onto the columns. After that the unlabelled cells which was passing through was collected and the columns was washed with 3×3 mL of buffer and collected. Washing steps was repeated for three times by adding buffer. 5 mL of buffer was flushed onto the column to wash out fraction with the magnetically labelled cells. Subsequently the separation is done, after that the NK cells suspension was cultured in DMEM (Dulbecco's Modified Eagle's medium)[1]. The media was treated with IL12[2] (invivoGen company, Recombinant human interleukin-12) and Inomysine 10ng/ml for 24 hours to aggravate NK cells for improved growth.

3.2.3 Harvesting and Culturing Hematopoietic Stem Cells (HSCs)

Several research groups have combined cDNA cloning and sequencing with array-based analysis to begin to define the full transcriptional profile of HSCs from different species and developmental stages and compare these to other stem cells (Michel et al., 2017). While transcriptional profiling is clearly a work in progress, comparisons among

[1] Among defined media for cell and tissue culture, the Dulbecco's modification of Eagle medium (DMEM) is the most broadly suitable medium for many adherent cell phenotypes. The Dulbecco's modification is an enhanced supplementary formulation that boosts select amino acid and vitamin content of the original Eagle's medium by up to fourfold. Our selection includes a range of glucose concentrations, as well as formulations with and without L-glutamine (Yao & Asayama, 2017).

[2] Interleukin 12 (IL-12) is a pleiotropic cytokine, the actions of which create an interconnection between the innate and adaptive immunity. IL-12 was first described as a factor secreted from PMA-induced EBV-transformed B-cell lines. Based on its actions, IL-12 was initially designated as "cytotoxic lymphocyte maturation factor" and "natural killer cell stimulatory factor"(Stern et al., 2017).

various types of stem cells may eventually identify sets of genes that are involved in defining the general "stemness" of a cell, as well as sets of genes that define their exit from the stem cell pool (e.g., the beginning of their path toward becoming mature differentiated cells, also referred to as commitment) (Srikanth et al., 2015). Furthermore, these datasets will reveal sets of genes that are associated with specific stem cell populations, such as HSCs and MSCs, and thus define their unique properties. Assembly of these datasets into pathways will greatly help to understand and to predict the responses of HSCs (and other stem cells) to various incentives.

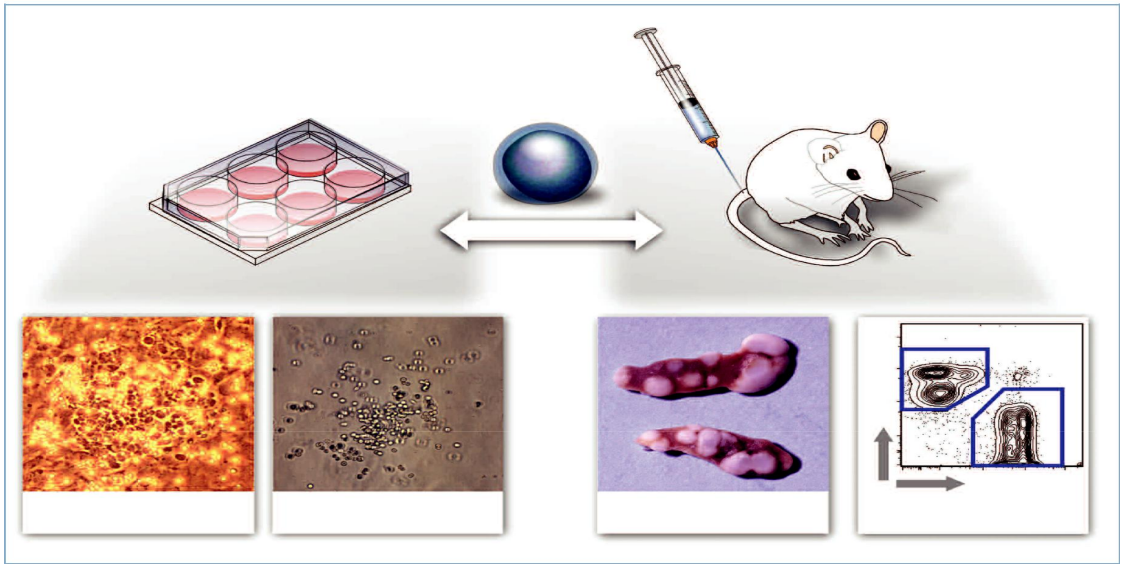


Figure 3.3: Assays used to detect hematopoietic stem cells. The tissue culture assays, which are used frequently to test human cells, include the ability of the cells to be tested to grow as "cobblestones" (the dark cells in the picture) for 5 to 7 weeks in culture. The Long-Term Culture-Initiating Cell assay measures whether hematopoietic progenitor cells (capable of forming colonies in secondary assays, as shown in the picture) are still present after 5 to 7 weeks of culture. *In vivo* assays in mice include the CFU-S assay, the original stem cell assay discussed in the introduction. The most stringent hematopoietic stem cell assay involves looking for the long-term presence of donor-derived cells in a reconstituted host. The example shows host-donor recognition by antibodies that recognize two different mouse alleles of CD45, a marker presents on nearly all blood cells. CD45 is also a good marker for distinguishing human blood cells from mouse blood cells when testing human cells in immunocompromised mice such as NOD/SCID. Other methods such as PCR-markers, chromosomal markers, and enzyme markers can also be used to distinguish host and donor cells (Peister, 2014).

The identification of cytokines and other stimuli which support stem cell proliferation and differentiation has led to the development of cell culture methods for ex vivo generation of hematopoietic cells for a variety of applications, such as:

- Generation of large numbers of mature blood cells or lineage-specific cells, for example, erythroid progenitors, mature red blood cells, granulocytes, monocyte/macrophages, dendritic cells and megakaryocytes.
- Expansion of stem cells capable of reconstituting haematopoiesis after transplantation.
- Identification of novel regulators of haematopoiesis.
- Genetic modification, for example, retroviral or lentiviral gene transfer of stem cells and progenitor cells.
- Characterization of leukemic stem cells and regulation of leukemogenesis.

Large numbers of progenitors and mature blood cells of individual lineages can be generated with culturing HSC and hematopoietic progenitor cell (HPC) preparations in appropriate culture media, cytokines and other stimuli. However, culture conditions that promote HSC and HPC proliferation and differentiation usually lead to irreversible loss of pluripotent HSCs (Naithani, Dayal, & Dixit, 2017). Normal HSCs cannot be cultured for more than a few days without losing their stem cell properties (i.e. self-renewal and ability to reconstitute haematopoiesis after transplantation) and entering into the pathway of lineage commitment and terminal differentiation (Mével et al., 2016). In this respect, hematopoietic stem cells differ from other types of stem cells, such as embryonic stem cells, which can undergo multiple cell divisions in culture while still retaining their stem cell properties (Palmer & Malarkannan, 2013).

3.2.3.1 Hematopoietic Expansion Cultures

3.2.3.1.1 Serum-Free Culture Media

Media used to culture HSCs and HPCs have historically contained serum. However, serum may contain inhibitors of proliferation and/or differentiation, for example, transforming growth factor γ (TGF-γ) (Nunez et al., 2016). In addition, serum

constituents vary in type and concentration between different batches, which can cause considerable variability between experiments. In order to minimize these variables, the use of serum-free media is essential.

In this state of experiment, it was very important to keep in mind that the starting cultures with unfractionated cells or partially processed cells is possible but not recommended as the large numbers of mature and non-hematopoietic cells present in cultures of unfractionated cells can potentially stimulate HSC and HPC through paracrine mechanisms or inhibit growth by secretion of inhibitory cytokines, competition for nutrients and accumulation of toxic waste products. These effects are variable and unpredictable and can make data interpretation difficult. For these reasons the use of purified CD34+, lineage-depleted cells Lin or more highly purified cells obtained by cell sorting is preferable (Heng et al., 2012). Purified cells can also be plated at higher cell densities and this may provide a stronger and more reproducible proliferative response, provided other culture conditions have been optimized. The optimal number of cells plated is dependent on the types and purity of the progenitors, the types and concentrations of cytokines and other stimuli, the culture conditions and the purpose of the experiment. In our experiment the minimum plating densities for CD34+ or Lin-cells are in the order of 2000 – 3000 cells per ml.

Based on our research in University of Malaya, data diverge significantly among individual single cell transplantation experiments. This might result from several explanations, but is perhaps due to variations among individual donor rat, the number of HSCs included in contestant cells, and conditions of competitor cells and receiver rat. To control, these conditions appear difficult. Nonetheless, the criteria for long-term multilineage repopulating cells must be unified. The source and number of competitor cells, with the definition of long-term and multilineage reconstruction, are the most

important circumstances. From both logical and practical points of view, this research proposes that 8- to 12-week-old male or female C57BL/6 rat be used as HSC donors and recipients, where normal and unmanipulated BM cells be used as competitor cells, that long-term be defined as at least 4 months, and that 1% or more peripheral blood chimerism with detectable test donor cell-derived myeloid, B-lymphoid, and T-lymphoid cells be defined as multilineage reconstitution. This percentage is obtained by ([% test donor cells] × 100)/ (% test donors-derived cells + % competitor-derived cells). In some laboratories, 0.1–0.3% chimerism is adopted as defining multilineage reconstitution, which is useful for certain purposes. We, however, believe that if one wishes to compare purification levels between different protocols, the above criteria should be agreed on and shared.

For this section four process was done. Table 3.2. Mature hematopoietic cells of both the lymphoid and myeloid lineages are continuously generated from a small self-renewing pool of pluripotent hematopoietic stem cells (Hara et al., 2016). HSCs can be found primarily in the bone marrow (BM) of healthy adults, in umbilical cord blood (CB) and in adult blood after mobilization from the bone marrow with cytokines, such as G-CSF, or other agents. HSCs can differentiate into all mature blood cells. During differentiation to mature blood cells, the progeny of HSCs will go through intermediate stages prior to reaching maturity, including multi-potential progenitors and lineage-committed progenitors (Mahla, 2016). Several stimulatory and inhibitory factors present in the hematopoietic stem cells maturation.

Table 3.2: The procedure of harvesting and culturing the HSCs.

Harvesting and Culturing Hematopoietic Stem Cells	
1	Removing the rat's thigh bone
2	Breaking the bone and washing the BM with Saline
3	Transferring the liquid to Xeno- free expansion cell culture with SCF and IL3,6
4	Incubating the culture

After preparation of the medium, the experimental animal was sacrificed and after that, the thigh bone was discarded. The bone was cracked and the bone marrow was removed and washed with normal saline. By this method, very few stem cells were gathered. After that, they were transferred to the liquid xeno-free expansion cell culture with SCF-FLt, ligands and IL 3,6 (company: thermofisher scientific). This medium is particularly designed for culturing of stem cells .IL 3,6 helps the growth of the stem cells but the medium prevents the stem cells from differentiation. The medium and HSCs were incubated for 14 days to raise the population of hematopoietic cells to the adequate measurement.

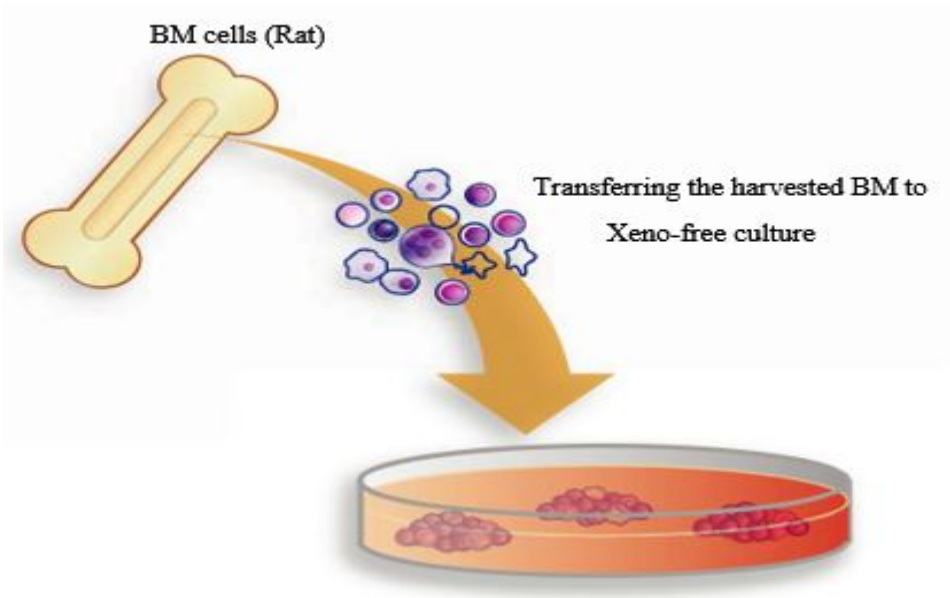


Figure 3.4: In vitro colony assays. Cells from hematopoietic tissues are plated in semisolid xeno free medium with a combination of cytokines, such as stem cell factor (SCF), thrombopoietin (TPO), interleukin-3 (IL-3), and erythropoietin (EPO) at a minimum.

3.2.4 Gene Separation and Colonization

After harvesting the Hematopoietic Stem Cells and extracting the Natural Killer Cells it was time to separate our three targeted genes from the genome of extracted NK cells and colonize them into the HSCs.

NK cells express cell surface receptors that recognize class I major histocompatibility complex (MHC-I) molecules to distinguish between healthy and unhealthy cells. The multigenic and polymorphic nature of the MHC-I genes has influenced the convergent evolution of likewise polymorphic and diversified NK cell receptor families: The C-type lectin-like ly49 receptors in mice, and the KIRs in humans. Although structurally distinct, both receptor families have similar functions in terms of MHC-I recognition and downstream signal transaction, and they regulate multiple aspects of NK cell biology during development and after maturation as fully differentiated and functionally competent cells. The ly49 gene locus has undergone rapid, lineage-specific expansions and contractions resulting in multiple distinct haplotypes of variable gene number, allelic diversity, and MHC-I ligand specificity. Genes package from ly49 family are ly49d receptor with gene name *KLRA4* and ly49h with gene name *KLRA8* (Dezell et al., 2012) and for the inhibitory ligand CD94 with the gene name *KLRD1*("Bos taurus CD94 (CD94) mRNA, CD94¬04 allele, complete CDs," 2016) in case of rats. Must be mentioned that the ly49d subfamily code a ligand called H-2D and ly49h subfamily code a ligand called MCMV-M157 (Cooper et al., 2013).

As it has been mentioned in the research literature review and appendix A, B and C, these specific genes are (ly49d (*KLRA4*), ly49h (*KLRA8*) and CD94 (*KLRD1*)). The reason for choosing these three specific genes is because they code an activator and inhibitory ligand. As it has been mentioned in literature review, Natural Killer cells to act against cancer cells need to have two activator and one inhibitory ligand activated.

Therefore, for HSCs to have cancer destroying ability, need to have both inhibitory and activator ligands on their cell membrane.

Table 3.3: Activating and Co-activating NK Cell Receptors (Caligiuri et al., 2011).

Common Name	Ligand	Gene	Species
ly49d	H-2D^{d} Hamster MHC class I	*KLRA4*	Mouse
ly49h	MCMV-m157	*KLRA8*	Mouse
ly49p	MCMV	Klra16	Mouse
NK1.1, NKR-P1C	Unknown	Klrb1c	Mouse
NKR-P1F	Clrg	Klrb1f	Mouse
PILRβ	Oglycosylation CD99	Pilrb1	Mouse
CD94	Mouse, Qa-1^{b}. Human HLA-E	*KLRD1*-KLRC2, 3	Mouse, human

3.2.4.1 DNA Extraction and Transfection of DNA & RNA

The progression of introducing nucleic acids into eukaryotic cells by non-viral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a prevailing tool to study gene function and Protein expression in the context of a cell. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology, together with the availability of transfection reagents, provides the foundation to study mammalian promoter and garnish sequences, trans-acting proteins such as transcription factors, mRNA processing, Protein-Protein interactions, translation and recombination events (Cardarelli et al., 2016).

Transfection is a method that neutralizes or precludes the issue of introducing negatively charged molecules (e.g., phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Chemicals like calcium phosphate and DEAE-

dextran or cationic lipid-based reagents coat the DNA, neutralizing or even creating an overall positive charge to the molecule (Ranganathan et al., 2015).

This makes it easier for the DNA-transfection reagent complex to cross the membrane, particularly for lipids that have a “fusogenic” component, which enhances fusion with the lipid bilayer. Physical methods like microinjection or electroporation simply thump through the membrane and introduce DNA directly into the cytoplasm. Each of these transfection technologies is discussed in the following sections (Trivedi et al., 2015).

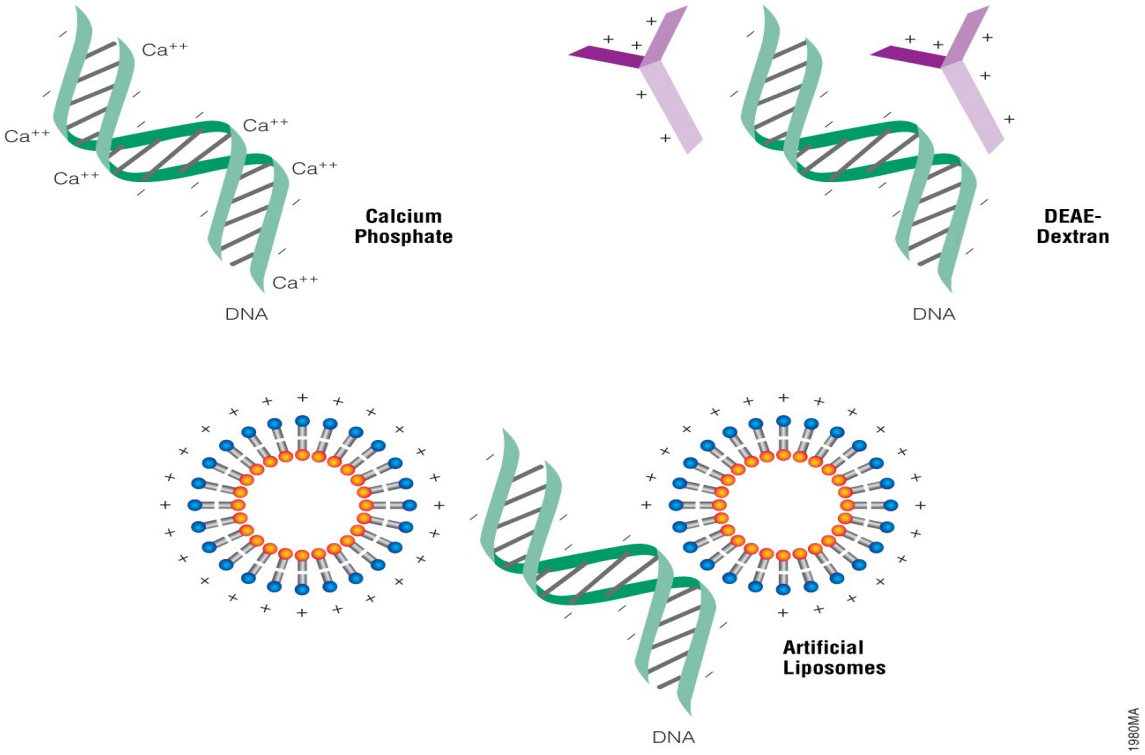


Figure 3.5: Schematic representation of various transfection technologies and how they neutralize the negatively charged DNA. Note that lipid-based reagents also can coat DNA in addition to forming micelles and associating with DNA by attraction as depicted.

3.2.4.1.1 Chemical Reagents

One of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells was DEAE-dextran (Mével et al., 2016). DEAE-dextran is a cationic polymer that tightly acquaintances with negatively charged nucleic acids (Trivedi et al.,

2015). An excess of positive charge, contributed by the polymer in the DNA polymer complex, allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. This method successfully delivers nucleic acids into cells for transient expression; that is, for short-term countenance studies of a few days in duration. Nonetheless, this technique is not generally useful for stable or long-term transfection studies that rely upon the integration of transferred DNA into the Chromosome. Other synthetic cationic polymers have been used to transfer DNA into cells, including polybrene, polyethylene mine and dendrimers (Ranganathan et al., 2015).

Calcium phosphate co-precipitation turns out to be a popular transfection technique following the systematic examination of this method in the early 1970s (Jondal & Pross, 1975). The authors scrutinized the performance of various actions and effects of cation concentration, phosphate concentration, and pH on transfection. Calcium phosphate co-precipitation is widely used because the components are easily available and inexpensive, the protocol is easy-to-use, and it is effective with many different types of cultured cells. The protocol encompasses mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. The controlled addition generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by cells via endocytosis or phagocytosis (Hadaya et al., 2008). Calcium phosphate transfection is routinely used for both transient and stable transfection of a variety of cell types. In addition, calcium phosphate appears to provide protection against intracellular and serum nucleases (Pistone & Hiorth, 2017).

Conversely, calcium phosphate co-precipitation is prone to variability and is not suited for *in vivo* gene transfer to whole animals (Weber, & Goycoolea, 2016). In

addition, small pH changes (± 0.1) can compromise the effectiveness of calcium phosphate transfection (Cardarelli et al., 2016).

After separation of three specific genes (ly49d (*KLRA4*), ly49h (*KLRA8*) and CD94 (*KLRD1*)), Lipofectamine 2000 was used to transfer the genes to the harvested HSCs. Lipofectamine is a cationic liposome founded reagent that provides high transfection efficiency and in elevation levels of transgene expression in a range of mammalian cell types *in vitro* using an unpretentious protocol (Federal & Year, 2014). Optimum transfection efficiency and subsequent cell viability depend on several experimental variables such as cell density, liposome and DNA concentrations, liposome–DNA complexing time, and the presence or absence of media components such as antibiotics and serum. The importance of these factors in Lipofectamine 2000 mediated transfection will discourse together with some specific applications transfection of primary neurons, high throughput transfection, and delivery of small interfering RNAs.

As it has been mentioned, Lipofectamine reagent contains lipid subunits that can form liposomes in an aqueous environment, which entrap the transfection payload, i.e. DNA plasmids (Trivedi et al., 2015). Lipofectamine is a cationic liposome formulation, which complexes with negatively charged nucleic acid molecules to allow them to overcome the electrostatic repulsion of the cell membrane. Lipofectamine's cationic lipid molecules are formulated with a neutral co-lipid (helper lipid) (Cardarelli et al., 2016). The DNA-containing liposomes (with positive charge on their surfaces) can fuse with the negatively charged plasma membrane of living cells, due to the neutral co-lipid mediating fusion of the liposome with the cell membrane, allowing nucleic acid to cross into the cytoplasm and contents to be available to the cell for replication or expression. Lipofectamine is a mixture of (N-(1-(2,3-dioleyloxy)propyl)-N-(2

(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate (“DOSPA”) and (“DOPE”)(Cardarelli et al., 2016).

For the purpose of DNA extraction from the separated NK cells the sample was taken out from the 4°C storage and left for 15 minutes in room temperature. After that the specimen was mixed well by inversion. Then 4mL of the specimen was pour into a 10 mL tube containing 6 mL of RBC Lysis Solution (Promega DNA kit). The tube was caped and mixed well by inversion (>5 times). Incubated for 2 minutes at 4°C and mixed once more by inversion (>5 times). Then the tube was centrifuged at 2000 g for 10 minutes at room temperature. The supernatant was carefully poured out into an appropriate biohazard container, leaving the NK cells and a small amount of residual liquid pallet at the bottom of the tube. The tube was vortexed to resuspend the NK cells in the residual fluid. After that, 10 mL RBC Lysis Solution was added, tube caped, mixed by inversion, and centrifuged again at 2000g for 10 minutes. Again, the supernatant was poured out into an appropriate biohazard container, leaving the NK cells in the bottom of the tube. 1.0 mL of NaCl Solution, (1M) was added. Tube vortexed vigorously to resuspend and disperse the NK cells. 6.0 mL of Cell Lysis Solution was added to the tube. Then 50 µL RNase Solution was added to the tube to destroy RNA. Tube caped and vortexed gently to mix. Incubated at 37 degrees C, for 60 minutes to allow complete lysis of the NK cells and degradation of RNA. After one-hour centrifuging, 1.0 mL Sodium Acetate Solution, (3M) was added to sample tube. Vortexed briefly to mix. lysate was transferred to a 15ml Phase lock light tube then added 1.0 mL of Phenol chloroform isoamyl alcohol 25:24:1 to the sample. Tube caped tightly and vortexed vigorously for 10 seconds. After that the tube was again centrifuged at 1500 g for 5 minutes at room temperature for phases separation. The supernatant was poured into a 10 mL centrifuge tube containing 5.0 mL of Isopropanol, 100%. The 10 mL sample tube was tightly caped and inverted for 25 times slowly to

facilitate precipitation of the DNA. After that the DNA was visible. The sample was Centrifuged at 2000 g for 5 minutes at room temperature. The supernatant was gently discarded, leaving the DNA pellet at the bottom of the tube. Then 10 mL of Ethanol, 70% was added to the sample and centrifuged at 2000g for 3 minutes at room temperature. Ethanol was poured out from the tube to keep the DNA pellet at the bottom of the tube. 500 μL of TE Buffer was added to the tube. Then the tube was caped and vortexed gently for 5-10 seconds. At the end the sample was left overnight at 37°C to dissolve the DNA pellet completely. The day after the sample was taken from storage and vortexed gently to mix. Now the sample was ready for quantitation, storage, or testing.

3.2.4.2 Transfection

After extraction the NK cell's DNA, the three specific genes had to be separated from the entire genome for cloning into the HSCs. The main purpose of this stage is because this research intended to solve the problem of NK cells division. Any change in HSCs genome had to be able to pass through generations to have mass product of newly generated NK cells.

To separate our three specific genes (ly49d (*KLRA4*), ly49h (*KLRA8*) and CD94 (*KLRD1*)), from the DNA of NK cells, the process of PCR was required. For that matter, based on our genes FASTA from NCBI, the proper Forward and Reverse primer was designed. Table 3.4, shows the designed primers for the PCR. The primers length for each gene, is 70 base pairs.

Table 3.4: Designed Forward and Reverse primers for PCR.

KLRA4									
Primer pair 1	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self-complementarity	Self3' complementarity
Forward primer	ATTTGCATAGCAAGGCTGTAGATG	Plus	24	1	24	59.72	41.67	6	1
Reverse primer	AAGGATAGAATAGGGAGGGCACA	Minus	23	70	48	60.38	47.83	2	0
Product length	70								
KLRA8									
Primer pair 1	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self-complementarity	Self3' complementarity
Forward primer	TTAGCTAGCACAAGGCTGTAG	Plus	21	1	21	57.47	47.62	6	2
Reverse primer	AAGGATAGAATAGGGAGGGCACA	Minus	23	70	48	60.38	47.83	2	0
Product length	70								
KLRD1									
Primer pair 1	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self-complementarity	Self3' complementarity
Forward primer	ATCGAATGCACAAGGCTGTAGA	Plus	22	1	22	60.09	45.45	4	2
Reverse primer	AAGGATAGAATAGGGAGGGCAC	Minus	22	70	49	58.75	50	2	2
Product length	70								

To prepare the PCR cocktail 50 µL of distilled water was added to PCR tube then buffer 1x, after that 0.05 units/µL of Taq polymerase was added. After that 200 µM of dNTP mix was added to the tube. Then 0.5 mM of $MgCl_2$, and 0.5 µM of each Forward primer and 0.5 µM of Reverse primer was added to the tube. At last, 200 pg/µL of DNA template was added to the tube and set for PCR and amplification of genes.

Afterward Invitrogen Lipofectamine 2000 Transfection Reagent was used for the purpose of transfection. Lipofectamine is a Transfection Reagent suitable for transfection of nucleic acids (DNA and RNA) into eukaryotic cells. In this part of the experiment, the extracted and separated genes had to be transfer to the Hematopoietic Stem Cells. To perform One day before doing transfection procedure, transgene HSCs were cultured in 4- 4-8 $x10^5$ cells plates with 500 µl of growth medium without antibiotics. After that, Diluted DNA in 50 µl of Opti-MEM I Serum Medium without

serum and mixed. Mixed Lipofectamine 2000 before use. After 5 minutes incubation, added the diluted DNA with 100 µl diluted Lipofectamine 2000, then mixed gently and incubated for 20 minutes at room temperature, solution turned to cloudy appearance. Afterward, added 100 µl of complexes to each well containing cells and medium, and mixed gently by rocking the plate back and forth. After that Incubated cells at 37°C in a CO_2 incubator for 48 hours. prior to testing for transgene expression. Hematopoietic stem cells are quite sensitive, and lipofectamine could be toxic to them. To solve this matter titrated the amount of Lipofectamine used (keeping the ratio Lipo/DNA constant), and changed the medium every 6 hours. It also makes a difference whether cells are attached to plastic or a cover glass during transfection, plastic being preferred. Finally avoid splitting cells right after transfection and let them recover o/n. now the samples were ready for the transfection efficiency assessment. For this part of the experiment two techniques were used.

- Western Blotting, to check the expression of receptors on NK cells.
- Flow Cytometry, to count the number of generated NK cells.

3.2.5 Evaluating Transfection Efficiency

After isolation of three specific gene and transfection procedure, to ensure that the transfection is successful or on the other hand, the transgenic Hematopoietic Stem Cells are generated or not, western blotting used to detect the expression of ligands proteins and second used flow cytometry for generated cells count.

3.2.5.1 Western Blotting

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. A western blot experiment, or western blotting (also called immunoblotting, because an antibody is

used to specifically detect its antigen) was introduced by Towbin in 1979 and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semi-quantitative data about the protein of interest (Mahmood & Yang, 2012).

Western blotting technique is mostly used for identification of particular protein from the mixture of protein. In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test.

Western blotting is a very crucial and useful technique which use in cell and molecular biology. Basically western blot, Ables researchers to identify specific proteins from a complex mixture of proteins extracted from cells (Irnidayanti & Sutiono, 2018).

This technique uses three fundamental categories to accomplish this task:

- separation by size
- transfer to a solid support
- marking target protein using a proper primary and secondary antibody to visualize.

As previously mentioned, western blotting refers to use specific antibodies to identify proteins which have been separated based on size by gel electrophoresis (Table 3.5). This assay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride) (Mahmood & Yang, 2012). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

Table 3.5: The project's target genes and their proteins molecular information.

Ly49d, *KLRA4*: Killer cell lectin-like receptor, subfamily A, member 4				
Genomic Location	**Size/bases**	**Protein Symbol**	**Size/ aa**	**Molecular mass/D**
F3; 4 63.44 6	48.039	Q5QGZ9	265	30582
Ly49h, *KLRA8*: Killer cell lectin-like receptor, subfamily A, member 8				
Genomic Location	**Size/bases**	**Protein Symbol**	**Size/ aa**	**Molecular mass/D**
F3;4 6 63.44 6	45.198	Q9UBE3	266	31104
CD94, killer cell lectin-like receptor, subfamily D, member 1				
Genomic Location	**Size/bases**	**Protein Symbol**	**Size/ aa**	**Molecular mass/D**
F3;4 63.42 6	39.819	Q13241	179	17109

Along with the lack of known ligands for many Ly49 genes, there is very limited characterization of cells expressing Ly49. Only 11 of the Ly49 receptors have monoclonal antibodies that recognize them. Further complicating the issue, many of the antibodies are cross-reactive for more than one Ly49 gene. The protein's entities are given in table 3.5.

The Procedure is as follows

1. Extraction of protein.
2. Gel electrophoresis: SDS PAGE.
3. Blotting: electrical or capillary blotting.
4. Blocking: BSA.
5. Treatment with primary antibody.
6. Treatment with secondary antibody (enzyme labelled anti Ab).
7. Treatment with specific substrate; if enzyme is alkaline phosphatase, substrate is p-nitro phenyl phosphate which give colour.

Several genes of the C-type lectin superfamily and ly49 superfamilies' including members of the NKG2 family, are expressed by NK cells and are involved in the regulation of NK cell function *KLRD1* (CD94) is an antigen preferentially expressed on NK cells and is classified as a type II membrane protein because it has an external C terminus. Three transcript variants encoding two different isoforms have been found for this gene. Regarding performing the Western Blotting, the related antibodies were prepared and the other materials were ready to run Western Blot and Flow Cytometry. Table 3.5 shows the information about the *KLRD1*, gene/ protein and its Monoclonal antibody, used Western Blotting and Flow Cytometry.

Table 3.6: Information about the used Antibodies in Western Blotting.

GENE	PROTEIN		ANTIBODIES SPECIFICATIONS						
Gene Name	**Name and Reference Sequence**		**Species reactivity**	**Host/ Isotype**	**Class**	**Type**	**Immunogen**	**Form**	**WB**
KLRA4	Killer cell lectin-like receptor 4 isoform 2 [Mus musculus]	NP_001239506.1	Rat	Mouse IgG	Monoclonal	Antibody	Purified recombinant fragment of *KLRA4* expressed in E. Coli	Liquid	1/500
KLRA8	Killer cell lectin-like receptor 8 isoform X2 [Mus musculus]	XP_012499956.1	Rat	Mouse IgG	Monoclonal	Antibody	Purified recombinant fragment of *KLRA8* expressed in E. Coli	Liquid	1/500
KLRD1	NP_001107868.1, 179 amino acids	NP_001092139.1	Rat	Mouse IgG	Monoclonal	Antibody	Purified recombinant fragment of KlLRD1 expressed in E. Coli	Liquid	1/500

Regarding running western blotting for each ligand, three samples for each ligand were prepared. First to examine expression of ly49d, *KLRA4* ladder (30KD), extracted NK cells and treated HSCs were prepared as sample 1. Second to examine expression of ly49h, *KLRA8* ladder (31KD), extracted NK cells and treated HSCs were prepared as sample 2. Third to examine expression of CD94, *KLRD1* ladder (17KD), extracted NK cells and treated HSCs were prepared.

Sample 1,2 and 3 was combined with loading buffer, reducing agent and distilled water, after that the samples were transferred to tube and treated for 10 minutes in a preheated heating block set to 80 degrees Celsius so that only the primary structure remains, meanwhile prepared 800 ml of the running buffer and took 200 ml from this and add 500 microliters of acting antioxidant. This ensures that the protein maintains a reduced state. After that removed the jar from as packaging and rinsed it with distilled water and took the plastic comb to create the wells. Took the samples from the heating block and centrifuged. Transferred 15 ml of the protein sample into separate wells of the gel. In the first well loaded 5 microliters of protein ladder which will separate from visible bands of determined molecular weights. These bands will enable us to determine the molecular weight of the protein detected in the sample (sample 1, *KLRA8*,31KD. Sample 2, *KLRA4*,30KD. Sample 3, *KLRD1*,17KD) each on separated gel.

Transferred the lid on the tank and connected the wires to the voltage sockets (60mv according to the supplier instruction). The separated proteins on gel must now be transferred to a membrane in which this can be done through the semi-dry transfer method. Prepared the transfer buffer (Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.3 with 20% methanol and 0.1 %SDS)). The nitrocellulose membrane was cut and put in methanol then rinsed in distilled water before soaking it in transfer buffer. Two thick filter papers for each gel were cut to size and immersed in transfer buffer. After that in the semi dry transfer machine prepared the gel sandwich by placing one piece of pre wetted filter paper from the bottom followed by the membrane. Then removed the won gel from the tank used a chisel to separate the top panel of the case from the gel and cut away the well section and placed the gel on top of the membrane and then the second piece of pre wetted filter paper. Used roller to remove any air bubbles secured the lid and connected the wires to their correct sockets using the voltage for 30 minutes. The electric current will draw the positively charged proteins towards the cathode and onto

the membrane. After that the blocking reagent was applied to the surface of the membrane to prevent the antibody binding to unspecific proteins. After the semi-dry transfer is complete separated the gel sandwich and kept the membrane mark which side of the membrane the protein has been transferred onto by cutting the top right-hand corner. Immersed the membrane in blocking reagent made up of 5% non-fat dried milk and 5% fetal bovine serum. After that incubated the membrane at 37 degrees Celsius for 60 minutes in order to detect the specific protein. The primary antibody was applied to each gel. Antibodies are critical to the success of the western blot technique. They allow for the selective detection of the protein of interest. Typically, a primary antibody is used to specifically bind the protein of interest and a labelled secondary antibody is used for detection. (The antibodies information and brands are given in appendix).

- Sample/gel,1: ly49d: *KLRA4*: ly49d primary: ZO-41 Monoclonal Antibody (ZO41-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 18.
- Sample/ gel,2: ly49h: *KLRA8*: ly49h primary: ZO81- Monoclonal Antibody (ZO81-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 11.
- Sample/gel,3: CD94: *KLRD1*: MA5-15818 Monoclonal Antibody, NKG2/CD94/ secondary: Mouse anti Rat CD94 Antibody.

The blocking reagent was used to delete the primary antibody. Removed the membranes from the blocking reagent and placed them inside a small bag made from plastic film. Added the primary antibody solution and incubated the membrane at four degrees Celsius overnight on a shaker. The next day any unbound antibody needed to be washed away with tris buffer saline +0.1% tween 20, to do this removed the membrane from the bag and immersed it in a container with TBST.

Placed each gel on shaker for 30 minutes (repeating three times). The secondary antibody applied. This binds to the primary antibody enabling to be visualized. The secondary antibody is typically conjugated to horseradish peroxidase an enzyme which when bound to a specific substrate creates a detectable signal. Diluted the secondary antibody to 1500 µl using the blocking buffer. Removed TBST from the membrane and then added the secondary antibody solution to each gel. Left on a shaker at room temperature for one hour. After that any unbound secondary antibody was removed by washing the membrane with TBST on shaker for 30 minutes (repeating three times). After that by aligning the protein with the ladder of molecular weight, expression of the target proteins was detected.

3.2.5.2 Flow Cytometry

In this research, Flow Cytometry was used to detect the protein translation of Natural Killer cells which been transfected into the Hematopoietic Stem cells. Three KLRA4, KLRA8 and KLRD1 genes were transferred to HSCs and to examine the transfection success flow cytometry was used to detect the expression of these genes in newly generated NK cells. The flow cytometry results for expressed proteins of these three specific genes with specific monoclonal antibodies full information are given in appendixes A6, B6 and C6.

Flow cytometry is a wide used laser-based technology to analyse the characteristics of cells or particles (Picot & Boulanger, 2012). This technique is used for analysing the characterizing and defining different cell types expression of cell surface and intracellular molecules in a heterogeneous cell population, assessing the purity of isolated subpopulations and analysing cell size and volume (Nilsson & Pronk, 2013). Flow cytometry generally is a cell analysis technique that was first used in the 1950s to

measure the volume of cells in a rapidly flowing fluid stream as they passed in front of a viewing gap. Since that time, innovations from many engineers and researchers have concluded in the modern flow cytometer, which is able to make measurements of cells in solution as they pass by the instrument's laser at rates of 10,000 cells per second (or more) (Picot & Boulanger, 2012). Because of its speed and ability to inspect at the single-cell level, this technique allows cell biologist the statistical power to rapidly analyse and characterize millions of cells, although at low cost.

Flow cytometry allows for identification of cellular subsets based on cell essential properties, most often by the use of fluorochrome-conjugated antibodies recognizing distinct cell-surface epitopes that define the cells of interest (Hara et al., 2016). Advances in technical instrumentation and the availability of an ever-increasing number of fluorophores, today allows identification of multicolour defined cellular populations to a formerly unreachable resolution. However, these possibilities put an increasing call on preparation, acquisition, and subsequent analysis of the investigated samples. Identification of very rare cellular subsets, such as the bone marrow-residing Hematopoietic Stem Cells (HSCs), causes further complexity to such analysis (Rundberg Nilsson et al., 2013).

It is principally used to measure fluorescence intensity produced by fluorescent-labelled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules such as propidium iodide binding to DNA (Herrmann et al., 2016). The staining procedure involves making a single-cell suspension from cell culture or tissue samples. The cells are then incubated in tubes or microtiter plates with unlabelled or fluorochrome-labelled antibodies and analysed on the flow cytometer.

The possible applications of analysis by flow cytometry are frequent, including the detection and measurement of (Cervera & Kamen, 2018):

- Protein expression—throughout the entire cell, even the nucleus.
- Protein post translational modifications, includes cleaved and phosphorylated proteins.
- RNA—including miRNA, and mRNA transcripts
- Cell health status—from viability to late-stage apoptosis or programmed cell death.
- Cell cycle status—providing a powerful tool to assess cells in G0/G1 phase versus S phase, G2, or polyploidy, including analysis of cell proliferation and activation.
- Identification and characterization of distinct subsets of cells within a heterogeneous sample—including distinguishing central effector memory cells from exhausted T cells or even regulatory T cells.

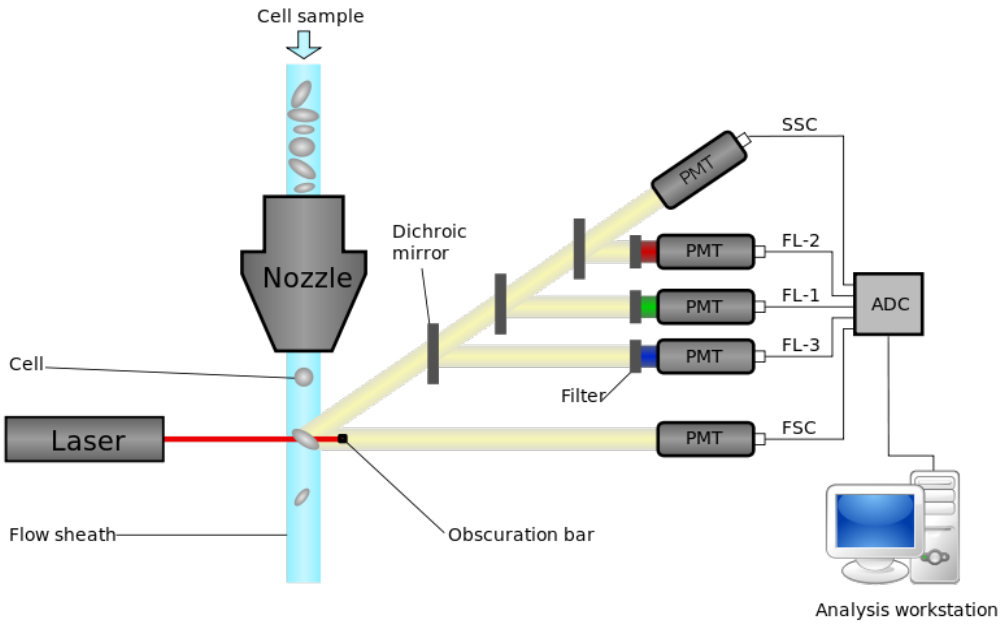


Figure 3.6: Overview of the flow cytometer. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam one cell at a time. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells (Cervera & Kamen, 2018).

Cells or particles passing through the beam scatter light, which is detected as FS and SS. FS relates with cell size and SS is related to the granularity of the cells (A. K. Chen et al., 2012). In this means, cell populations can often be illustrious based on differences in their size and granularity alone. A useful example of this is when running blood samples on the flow cytometer (Rundberg Nilsson, Bryder, & Pronk, 2013b).

- Larger and more granular granulocyte cells produce a large population with high SS and FS.
- Monocytes are large cells, but not so granular, so these produce a separate population with high FS but lower SS.
- Smaller lymphocytes and lymphoblasts produce a separate population with less FS. They are not granular cells, so also have low SS.
- Therefore, these cells can be separated into different populations based on their FS and SS alone.

As well as separating cells based on FS and SS, cells can also be separated by whether they express a particular protein (Rundberg Nilsson et al., 2013). In this case, a fluorochrome is often used to stain the protein of interest. Fluorochromes used for the detection of target proteins emit light when excited by a laser with the corresponding excitation wavelength. These fluorescent stained cells or particles can be detected individually. Forward and side scattered light and fluorescence from stained cells are split into defined wavelengths and channelled by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered so that each sensor will detect fluorescence only at a specified wavelength. These sensors are called photo multiplying tubes (PMTs) (Eissens et al., 2012).

The pulse area is calculated by adding the height values for each time slice of the pulse, determined by the speed of the analog to digital converter (ADC), which is 10

MHz (i.e. 10 million per second or 10 per microsecond) (Nakada et al., 2015). These events are assigned channels based on pulse intensity (pulse area). This signal can be amplified by turning up the voltage going through the PMT. Each event is given a channel number depending on its measured intensity; the more intense the fluorescence, the higher the channel number the event is assigned (Lock & Tzanakakis, 2018).

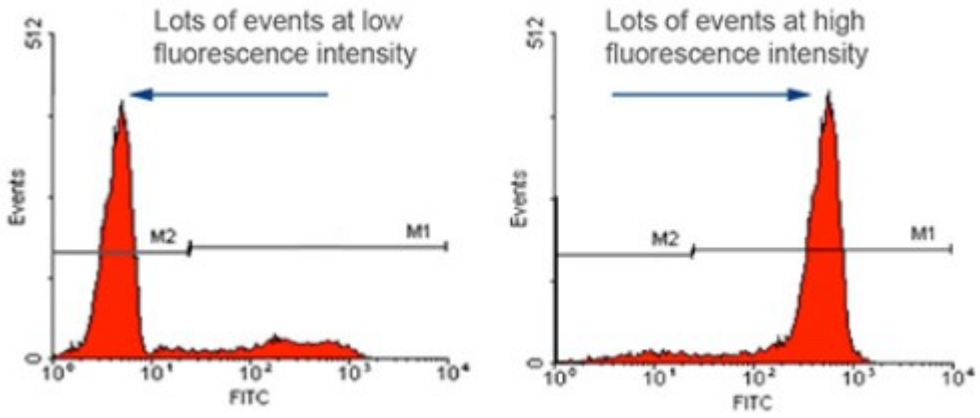


Figure 3.7: Fluorescence intensity measurements for a negative and positive result. The negative result shown on the left has no staining and many events at low fluorescence intensity. A positive result is shown on the right, this has a large number of events at high fluorescence intensity (Nakada et al., 2015).

In this research the Western Blotting technique was used to detect the expressed proteins of transfected genes of Natural Killer Cells into the Hematopoietic Stem Cells, after this stage the Flow Cytometry also used to measure the population of NK cells were Generated from HSCs. Both these techniques were used for the purpose of examine the transfection efficiency ratio. Although NK cells are typically present within very heterogeneous immune cell populations, they can be identified by characteristic cell surface markers.

Regarding antibody staining there are three type of staining have been used (Picot et al., 2012)

1. **Direct staining:** In direct immunofluorescence staining, cells are incubated with an antibody directly conjugated to a fluorochrome (e.g., FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody. This approach is particularly useful for intracellular staining, where large antibody-fluorochrome complexes including secondary antibodies can become trapped causing non-specific binding, or fail to enter the cell preventing primary antibody detection.

2. **Indirect staining:** In indirect staining, the primary antibody is not fluorochrome-labelled, but is detected by a fluorochrome-labelled secondary antibody. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used, whereby an antibody is conjugated to biotin and detected with fluorochrome-labelled avidin. With the wide range of conjugated antibodies now available, this method means that unconjugated primary antibodies raised against many different targets can be used in conjunction with a labelled secondary antibody for FACS analysis. This widens the choice of target proteins for the researcher.

3. **Intracellular staining:** Staining of intracellular antigens for flow cytometry protocols depends on various fixation and permeabilization methods to allow access of antibodies to internal cellular proteins. A successful staining procedure in all cases is dependent on optimization of experimental conditions through tittering of antibodies, use of appropriate controls to set up the flow cytometer correctly and optimized fixation and permeabilization procedures.

In this research, our target genes are *KLRA4* to express ly49 deactivating ligand, *KLRA8* to express ly49h activating ligand and *KLRD1* to express CD94 inhibitory ligand. To evaluate the expression of these transfected genes Flow Cytometry was used but for each ligand. As it has been shown in Table 3.6, specific antibodies were used. (The antibodies entire information and brands are given in appendix).

Table 3.7: Specific Antibodies for each gene in Flow cytometry assay

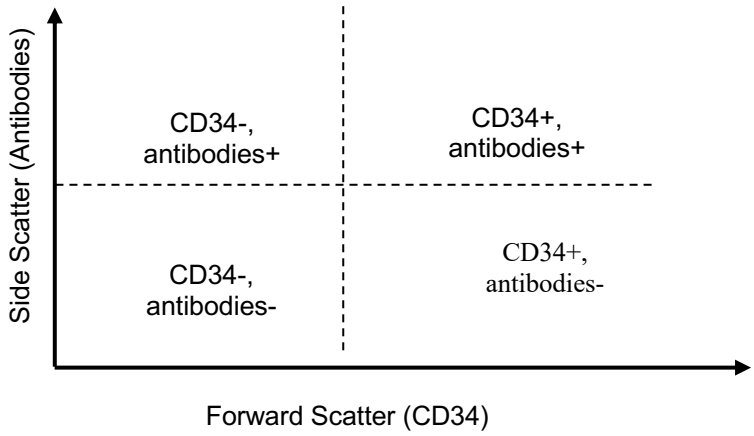
Proteins Monoclonal Antibodies for Flow Cytometry		
ly49d (KLRA4)	ly49h (KLRA8)	CD94 (KLRD1)
4E5,CD34	**3D10PE, CD34**	**18d3PE, CD34**

1. The monoclonal antibody eBio4E5 (4E5) reacts with Ly-49D, an activating member of the Ly-49 family of NK cell receptors. This family of receptors form homodimers that recognize MHC Class I molecules. The activating Ly-49 receptors (Ly-49D and Ly-49H) do not contain immune receptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains, distinguishing them from the inhibitory Ly-49 family members. Instead, activating Ly-49 molecules have been shown to associate with DAP12 which contains an immunoreceptor tyrosine-based activation motif (ITAM) that is required for positive signalling. Expression of different Ly-49 family members is not linked; therefore, several Ly-49 proteins can be expressed on the same cells.
2. The monoclonal antibody 3D10 recognizes Ly-49H, a member of the C-type lectin Ly-49 multigene family of receptors found on natural killer cells. Ly-49H, like Ly-49D, lacks the characteristic ITIM (immunoreceptor tyrosine-based inhibitory motif) and contains an arginine in the transmembrane domain implying these molecules act as activation receptors. Ly-49H is expressed in a subset of NK cells but not NKT (NK1.1CD3+) cells. Natural killer cells can express several Ly-

49 proteins. It has been shown that there is preferential staining of Ly-49H with Ly-49D (cat. 12-5783). Expression has been confirmed on C57BL/6 and NWNA but not Balb/c or DBA/2 mice. Other mice strains have not been tested. No cross reactivity to Ly-49A, C, D or G2 has been observed. Addition of monoclonal antibody 3D10 to cell cultures induces lysis of target cells.

3. The 18d3 monoclonal antibody reacts with rat CD94, a 70 kDa type II transmembrane glycoprotein. CD94 belongs to the C-type lectin superfamily and is present as a heterodimer with NKG2 on the surface. CD94 is expressed by NK cells, a subset of T cells, and NKT cells and plays an important role in adhesion and activation of NK cell lineage. DBA/2J mice are naturally CD94-deficient and do not express cell-surface CD94/NKG2A receptors, even on neonatal NK cells.
4. CD34 is a stem cell marker although its expression on human hematopoietic stem cells is reversible. CD34 may serve as a surface receptor that undergoes receptor-mediated endocytosis and regulates adhesion, differentiation and proliferation of hematopoietic stem cells and other progenitors. CD34 expression is likely to represent a specific state of hematopoietic development that may have altered adhering properties with expanding and differentiating capabilities in both in vitro and in vivo conditions. CD34 is possibly an adhesion molecule with a putative role for mediating the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells. Further, CD34 could act as a scaffold for the attachment of lineage specific glycans, allowing stem cells to bind to lectins expressed by stromal cells or other marrow components. CD34 is thought to have a role in presenting carbohydrate ligands to selectins. The intracellular chain of the CD34 antigen is a site of phosphorylation by activated protein kinase C suggesting a putative role in signal transduction. Diseases associated with CD34 dysfunction include dermatofibrosarcoma and neurofibroma.

Table 3.8: Interpretation of Flow Cytometry results.



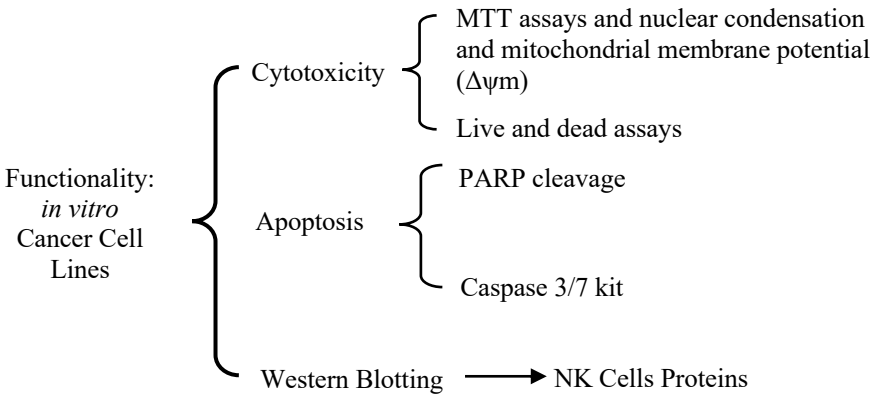
Regarding interpretation of flow cytometry results, in this research 4 different antibodies used to detect transgene Hematopoietic Stem Cells with specific ligands. The flow cytometry charts divide to four quarters and express or not express of antibodies, tells researches the true results. In flow cytometry charts, the forward scatter (FS) shows the size of cells which is in case of Hematopoietic Stem cells 0.2- 6 μM in diameter and the side scatter (SS) shows the complexity of cells which is to detect the expression of ligand or granularity of cells. The samples of treated HSCs are divided to three groups.

- Sample 1: 3D10PE monoclonal antibody to detect expression of ly49h ligand and CD34 as a stem cell marker to detect hematopoietic stem cells.
- Sample 2: eBio4E5 (4E5) monoclonal antibody to detect expression of ly49d ligand and CD34 as a stem cell marker to detect hematopoietic stem cells.
- Sample 3: 18d3 monoclonal antibody to detect CD94, and CD34 as a stem cell marker to detect hematopoietic stem cells.

3.3 Methodology for objective 2

3.3.1 Functionality of Newly Generated NK Cells on Cancer Cells (In vitro culturing cancer cell line)

To fulfil the objective 1 of this project, this research used Western Blotting and Flow cytometry to verify the differentiation of Hematopoietic stem cells from Natural Killer Cells which harvested from experimental animals. After that to fulfil the second objective of this research, the functionality of newly generated NK cells, *in vitro* cancer cell lines, was examined by two categories of cytotoxicity and apoptosis.



3.3.2 Cytotoxicity

cell cytotoxicity refers to the ability of certain chemicals or mediator cells to abolish living cells by using a cytotoxic compound (Riss & Moravec, 2014). Healthy living cells can either be induced to undergo necrosis (accidental cell death) or apoptosis (programmed cell death). The ability to precisely measure cytotoxicity can prove to be a very valuable tool in identifying compounds that might pose certain health risks in humans. Cell cytotoxicity, also proves to be quite essential in the process of developing therapeutic anti-cancer drugs. By decisive the cytotoxicity ratio of cancer cells, anti-cancer medications can delay the proliferation of target cells either by messing with their genetic material or by blocking the nutrients that the cells need to survive (Stern et

al., 2015). Cells exposed to a cytotoxic compound can respond in a number of ways. If the treat is lethal, the cells may experience necrosis, during which they lose membrane integrity and die quickly, or the cells may follow another pathway of cell death, such as apoptosis or autophagy (Riss & Moravec, 2014). Cells exposed to a sublethal compound may stop growing and dividing. Any of these responses can be measured individually or with multiplex assays to monitor whole cells or subcellular components or organelles.

Natural Killer Cells are innate effector lymphocytes necessary for resistance against malignant cells, microbe-infected, or stressed. NK cells kill target cells by either of two major mechanisms that require direct contact between NK cells and target cells. In the first pathway, cytoplasmic granule toxins, principally a membrane-disrupting protein known as perforin, and a family of structurally related serine proteases (granzymes) with various substrate specificities, are secreted by exocytosis and together induce apoptosis of the target cell. The granule-exocytosis pathway effectively activates cell-death mechanisms that operate through the activation of apoptotic cysteine proteases (caspases), but can also cause cell death in the absence of activated caspases. The second pathway involves the engagement of death receptors on target cells by their related ligands on NK cells, resulting in classical caspase-dependent apoptosis (Riss & Moravec, 2014). In this research to determine the destruction ability and examine the cytotoxicity of our newly generated NK cells, two assays were used

- MTT Assay
- Live and Dead Assay

3.3.2.1 MTT Assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NADPH-dependent cellular oxidoreductase enzymes may, under well-defined conditions, reflect the number of viable cells present (Mosmann, 2016). These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour (Rundberg Nilsson et al., 2013). This enzyme has a tendency to convert tetrazolium dye into a purple coloured compound or formazan which could be visualize with our eye (Bryceson et al., 2009). Once the cell suspension is added to the dye, if the colour of the solution changes to purple, we can tell that the cell suspension is viable and also the density of the colour gives the assessment of how many cells are viable, it's the kind of qualitative and quantitative test (Picot et al., 2012).

The reason for using MTT assay is to find out the cytotoxicity of the cell or in other hand to find out the viability of the cell, whether the cell is living or not, whether the cell is dividing or not. If the cell is living and everything is going on fine inside the cell and the mitochondria also working properly, there are multiple enzymes and metabolic pathways are present in mitochondria. The NADPH dependent oxide reductase enzyme is also produced by mitochondria and when the cell is living only when the cell is viable then the NADPH dependent oxidoreductase enzymes could be found in cells medium.

For most cell populations the number of viable cells is related to total mitochondrial activity. This assay is mostly used to measure the in vitro cytotoxic effects of drugs or treatments on primary patient cells or cell lines (Riss & Moravec, 2014). The general purpose of the MTT assay is to measure alive cells in relatively high throughput (96-well plates) without the need for intricate cell counting. The principle of the MTT assay is that for most viable cells activity of mitochondrial is continuous, so an increase or

decrease in the number of viable cells is linearly related to mitochondrial activity. The activity of the mitochondrial is reflected by the adaptation of the tetrazolium into formazan crystals, which can be solubilized for homogenous measurement. Consequently, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 and 720 nm (Stockert et al., 2018). The MTT assay also is applicable for the measurement of drug sensitivity in cell lines as well as primary cells. For dividing cells (usually cell lines) the reduction of cell number reflects cell growth inhibition and the drug sensitivity are then usually specified as the attentiveness of the drug that is required to achieve 50% growth inhibition as compared to the growth of the untreated control (50% inhibitory concentration, IC50) (Eldjerou et al., 2010). For primary (nondividing) cells, drug sensitivity is measured as improved cell kill of treated cells as compared to the loss of cells already commonly seen in untreated cells (50% lethal concentration, LC50)(Eldjerou et al., 2010).

ZR-75-l human breast cancer cells obtained from the source and were routinely cultured in phenol red-free RPM 1640. The cells were supplemented with 2 mM-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, streptomycin, and 10% (v/v) fetal bovine serum and incubated under a humidified atmosphere of 95% air/5% CO2 at 37°C. Cells were passaged weekly by treatment with 0.05% trypsin/0.02% EDTA (w/v). The ZR-75-l cells used in the present study were at their 93rd passage at the time of inoculation. Regarding the MTT assay, our target cells were seeded into control, treated and positive control (untreated), divided in 96 well plate, and after that incubated at 37°C for 24 hours. Medium was removed and cells were washed with PBS to remove dead cells. After that added 20 µl of 5mg/ml MTT to control and treated. Then incubated at 37°C for 4 hours. Then the medium was removed and 200µl of DMSO

(Dimethyl sulfoxide) was added to control and treated tube. After that the well plate was put for absorbance read at 595 nm.

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

Table 3.9: To normalize the data, the wells are divided to (1) zero control which has only newly generated NK cells, (2) negative control which contains cancer cells lines and medium and (3) positive control which to normalizing data started from zero control to negative control.

Assays Design by Control/Treated/Untreated							
1	Zero Control	NK cells only/100%, 5µL					
2	Negative Control (Untreated)	Cancer Cells only + Medium/100%, 5µL					
3	Positive Control (Treated)	0µL cancer cells+5µL,NK cells	1µL cancer cells+4µL,NK cells	2µL cancer cells+3µL,NK cells	3µL cancer cells+2µL,NK cells	4µL cancer cells+1µL,NK cells	5µL cancer cells+0µL,NK cells

3.3.2.2. Live Dead Assay

Regarding experimenting the cytotoxicity effect of our newly generated Natural Killer Cells on this research cell lines, after the MTT assay, the live/dead assay also used for comparative results purposes. Live Dead Assay, accurately and differentially labels live and dead cells with fluorescent dyes. It is used for the quick quantitation of cell viability using flow cytometry or fluorescent microscopy. The Live Dead assay staining solution is a mixture of two fluorescent dyes that differentially label live and dead cells (Pfeffer & Fliesler, 2017).

Live cells are distinguished by the presence of omnipresent intracellular esterase activity, determined by the enzymatic change of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein (Jiang et al., 2014). The polyanionic dye calcein is well retained within live cells, producing an intense uniform

green fluorescence in live cells (EX/EM ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (EX/EM ~495 nm/~635 nm) (Pfeffer & Fliesler, 2017). The purpose of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are integrally low with this assay technique, because the dyes are virtually nonfluorescent before interacting with cells.

The Live/Dead Viability/Cytotoxicity Assay provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability intracellular esterase activity and plasma membrane integrity (Paten et al., 2017). Molecular Probes has found that calcein AM and ethidium homodimer (EthD-1) are optimal dyes for this application. The assay is designed for use with fluorescence microscopes or fluorescence multi well plate readers and easily usable for usage with flow cytometers and other fluorescence detection systems. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells and certain tissues. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, 51Cr release and similar methods for determining cell viability and cytotoxicity(Stockert et al., 2018). This technique is generally faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods.

The seal of viable cells is an intact plasma membrane and intracellular enzymatic activity. These two features form the basis of Live and Dead Cell Assay. Live cells are identified on the basis of intracellular esterase activity (generating green fluorescence)

and exclusion of the red dye. Dead cells are identified by the lack of esterase activity and non-intact plasma membrane which allows red dye staining (Paten et al., 2017).

The Live cell dye labels intact, viable cells green. It is membrane permeant and non-fluorescent until ubiquitous intracellular esterase remove ester groups and render the molecule fluorescent. The Excitation and Emission are 494nm and 515nm (Jiang et al., 2014).

The Dead cell dye labels cells with compromised plasma membranes red. It is membrane-impermeant and binds to DNA with high affinity. Once bound to DNA, the fluorescence increases >30-fold. The Excitation and Emission are 528nm and 617nm (Jiang et al., 2014). The Live Dead assay protocol also uses a one-step staining procedure that is simple and fast. It can be used directly in cell culture media.

For running the assay, a 32 well plate was used and added suspension cell lines, in zero control, negative control and positive control. 2µL of cells were stained with 5x diluted concentration of live and dead dye with 1mL PBS. After that incubated for 10 min at room temperature in the dark. Alternatively set for imaging as Green = live cells and Red = dead cells.

3.3.3 Apoptosis

Cell death can occur by a spectrum of morphologically and biochemically distinct pathways, including apoptosis, necrosis and autophagy. The term “apoptosis”, coined by Kerr et al., describes cell death characterized by defined morphological changes, including cell shrinkage, chromatin condensation, loss of nuclear membrane integrity, plasma membrane blebbing and eventually apoptotic body formation. Although apoptosis refers to a purely morphological change, biochemical events accompany these morphological changes. These events include initiator and effector caspase activation,

release of cytochrome c from mitochondria, externalization of phosphatidylserine on the plasma membrane, poly (ADPribose) polymerase (PARP) cleavage and internucleosomal DNA fragmentation.

Biochemical occasions lead to cell morphology changes and demise (Poon et al., 2014a). These progressions incorporate blebbing, cell shrinkage, atomic discontinuity, chromatin build-up, chromosomal DNA fracture, and mRNA rot (Campuzano & Cerquera, 2011a). The normal grown-up human loses somewhere in the range of 50 and 70 billion cells every day because of apoptosis. Since apoptosis can't stop once it has started, it is a profoundly managed process. Apoptosis can be started through one of two pathways. In in the extrinsic pathway the cell kills itself because of signals from other cells, while in the intrinsic pathway the cell kills itself because it senses cell stress (Campuzano & Cerquera, 2015). Powerless outer signs may likewise enact the characteristic pathway of apoptosis. Both pathways actuate cell demise by initiating caspases, which are proteases, or catalysts that corrupt proteins (Poon et al., 2014a). The both of two pathways, activate initiator caspases, which then activate executioner caspases, which then kill the cell by degrading proteins indiscriminately (Srikanth et al., 2015).

Research on apoptosis has expanded generously since the mid-1990s. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. A few factors like Fas receptors and caspases advance apoptosis, while a few individuals from the Bcl-2 group of proteins restrain apoptosis (Campuzano & Cerquera, 2015).

The inception of apoptosis is firmly controlled by initiation components, in light of the fact that once apoptosis has started, it definitely prompts the demise of the cell (Poon et al., 2014). The two best-comprehended actuation instruments are the

characteristic pathway (additionally called the mitochondrial pathway) and the extrinsic pathway. The intrinsic pathway is enacted by intracellular signs created when cells are pushed and relies upon the arrival of proteins from the intermembrane space of mitochondria (Mattson & Chan, 2013). The extrinsic pathway is initiated by extracellular ligands official to cell-surface passing receptors, which prompts the arrangement of the passing instigating flagging complex (Mattson & Chan, 2013).

A cell initiates intracellular apoptotic signalling, which may realize cell suicide. The official of atomic receptors by glucocorticoids, heat, radiation, supplement deprivation, viral infection, hypoxia, expanded intracellular centralization of free greasy acids and expanded intracellular calcium concentration, for instance, by harm to the layer, would all be able to trigger the arrival of intracellular apoptotic motions by a harmed cell (Lee, Lu, & Madhukar, 2010). Various cell segments, for example, poly ADP ribose polymerase, may likewise help control apoptosis. Single cell vacillations have been seen in exploratory investigations of stress actuated apoptosis (Poon et al., 2014).

Before the real procedure of cell demise is accelerated by catalysts, apoptotic signals must reason administrative proteins to start the apoptosis pathway. This progression enables those signs to cause cell passing, or the procedure to be halted, should the cell never again need to die. A few proteins are included, yet two principle techniques for regulation have been distinguished: the targeting of mitochondria functionality, or directly transducing the signal via adaptor proteins to the apoptotic mechanisms (Böhm & Schild, 2003, pages 2-14). An extrinsic pathway for inception distinguished in a few toxin studies is an expansion in calcium fixation inside a cell brought about by medication action, which likewise can cause apoptosis by means of a calcium restricting protease calpain (Lee et al., 2010).

Not all of these biochemical events are specific to apoptosis, and not all events occur in all apoptotic cells, in all stages of apoptosis or in response to all apoptosis-inducing stimuli. The time course of these biochemical events often differs, depending upon a wide variety of factors, such as the cell line or tissue, apoptosis-inducing agent, drug concentration or stimulus intensity, and exposure time. Therefore, it is critical to choose wisely the biochemical events that will be measured to show that cell death occurs by apoptosis and to understand the kinetics of cell death.

Apoptosis is a naturally occurring programmed and targeted cause of cellular death (Redza Dutordoir & Averill Bates, 2016) on the other hand, Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma which result in the unregulated digestion of cell components (Su, & Yu, 2015). Regarding this study, the MTT Assay and Live/Dead Assay used to examine the cytotoxicity ability of the newly generated Natural Killer Cells or transgenic Hematopoietic Stem Cells on ZR-75-l breast carcinoma cells. Nonetheless for comparative purposes this study examines the apoptosis capability of newly generated NK cells by two applications

- RARP Cleavage
- Caspase 3/7 Kit

3.3.3.1 PARP Cleavage

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in a number of cellular processes such as programmed cell death, DNA repair and genomic stability (Fisher et al., 2017). The PARP family contains 17 members such as PARP1, PARP2, VPARP (PARP4), Tankyrase-1 and 2 (Audebert et al., 2014). They have different structures and functions in the cell. The main duty of PARP is to detect and begin an immediate cellular response to radiation-induced single-strand DNA breaks (SSB), by

signalling the enzymatic machinery involved in the SSB repair (Chaitanya et al., 2017). Once PARP detects the SSB, it binds to the DNA, undergoes a structural change, and begins the synthesis of a polymeric adenosine diphosphate ribose (poly (ADP-ribose) or PAR) chain, which acts as a signal for the other DNA-repairing enzymes. Target enzymes include DNA ligase III (LigIII), DNA polymerase beta (polβ), and scaffolding proteins such as X-ray cross-complementing gene 1 (XRCC1). After repairing, the PAR chains are degraded via Poly (ADP-ribose) glycohydrolase (PARG) (Herceg & Wang, 2016). The PARP is deactivated by caspase-3 cleavage during programmed cell death.

PARP enzymes are essential in a number of cellular functions, including expression of inflammatory genes. PARP1 is required for the induction of ICAM-1 gene expression by smooth muscle cells, in response to TNF (Lee et al., 2010). Lymphoblastoid cell lines recognized from blood samples of humans who were lived (100 years old or older) have meaningfully higher PARP activity than cell lines from younger (20 to 70 years old) individuals, again indicating a linkage between longevity and repair capability. These findings suggest that PARP-mediated DNA repair capability contributes to mammalian longevity. Thus these findings lend support to the DNA damage theory of aging which assumes that un-repaired DNA damage is the underlying cause of aging and that DNA repair ability contributes to permanency (Audebert et al., 2014).

PARP can be activated in cells experiencing stress and/or DNA damage. Activated PARP can deplete the ATP of a cell in an attempt to repair the damaged DNA. ATP depletion in a cell leads to lysis and cell death (Herceg & Wang, 2016). PARP also has the capability to induce programmed cell death, via the production of PAR, which stimulates mitochondria to release AIF (Dantzer et al., 2015). This mechanism appears to be caspase-independent. Cleavage of PARP, by enzymes such as caspases or cathepsins, typically inactivates PARP. The size of the cleavage fragments can give

understanding into which enzyme was responsible for the cleavage, and can be useful in determining which cell death pathway has been activated.

This ELISA based assay detects poly (ADP-ribose) deposited by PARP-1 onto immobilized histones in a 96-well format. An anti-PAR monoclonal detecting antibody followed by addition of a goat anti-rabbit IgG-HRP secondary and a colorimetric HRP substrate yields relative absorbance that correlates with PARP-1 activity (Yuan et al., 2017). During apoptosis PARP-1 is cleaved with a resulting drop in activity (Tomita, 2016). Etoposide is included as a control apoptosis inducer (Sun et al., 2016).

Poly (ADP-ribose) polymerase (PARP) is a highly abundant nuclear enzyme involved in DNA repair that synthesizes poly ADP ribose from NAD+ in response to damaged DNA (Zhu et al., 2015). PARP also known as ADPRT and PPOL, is a 118-kDa enzyme that uses NAD as a substrate to catalyse the covalent transfer of ADP-ribose to a variety of nuclear protein acceptors. ADP ribosyl-transferase is required for cellular repair, and PARP expression is induced by single-strand breaks in DNA. PARP is proteolytically cleaved by Caspase-3 into two fragments of 89 and 24-kDa in one of the hallmark events of apoptosis (Liu, & Sun, 2015). When cells are exposed to a critical level of damaging agents, cellular apoptosis is induced through an intracellular signalling cascade. Activation of the caspase family of apoptosis-specific proteases results in the cleavage of PARP by caspase-3. Cleavage of PARP produces the N-terminal 24 kDa DNA binding domain fragment, and the C-terminal 89 kDa catalytic domain fragment, rendering it inactive (Yogo, & Urano, 2017). The 24 kDa fragment has been shown to retain its DNA binding affinity for strand breaks, thereby inhibiting further DNA repair, ADP-ribose polymer formation, and transcription. Caspase-7 is also involved in PARP cleavage during apoptosis. High levels of PARP activity have been implicated in a caspase-independent cell death pathway through the depletion of cellular energy stores

(NAD+) and the downstream effector AIF (Apoptosis-Inducing Factor) (Sun et al., 2016).

Regarding PARP/Apoptosis assay, it should be mentioned that, during apoptosis, PARP-1 which catalyses the NAD+-dependent addition of poly (ADP-ribose) (PAR) onto various cytoplasmic and nuclear proteins, is cleaved from about 116 kDa to 85 kDa. Moreover, this enzyme is a therapeutic target for BRCA1 and BRCA2 associated cancers. The HT PARP/Apoptosis Assay is ideal for measuring the activity of PARP in cell extracts before and during apoptosis. This ELISA semi-quantitatively detects PAR deposited onto immobilized histone proteins in a 96-well format. An anti-PAR monoclonal antibody, goat anti-mouse IgG-HRP conjugate, and HRP substrate are used to generate a signal (Johansson, 2016). Thus, signals correlate with PARP activity. Etoposide is a topoisomerase II inhibitor that stabilizes this enzyme after it cleaves DNA. It is included as a control apoptosis inducer.

To monitoring PARP Activity Before and During Apoptosis, Trevigen's HT PARP/Apoptosis kit was used. The following is the protocol which this research followed to reach the results.

- On Day 0, the ZR-75-l actively-growing human breast cancer cells were seeded in 200 µl fresh medium in a 96 well V-bottom plate. And on the other hand, a triplicate well containing healthy cells was set for controls.
- On Day 1, 1µl of 10 mM Etoposide, was added to triplicate wells, and incubated at 37°C/5% CO2.
- Continued as above (add 1 µl of 10 mM Etoposide, and/or other agents to triplicate wells) to set up the wells for the 4 hr, 2 hr and remaining time points.

- Centrifuged the v-bottom plate at 1,000 x g for 5 minutes at 4°C, and carefully aspirate off the supernatants. Washed the cell pellets twice with 200 µl/well ice cold 1X PBS. Added 100-200 µl Cell Extraction Buffer which was prepared already, and incubated lysates on ice with periodic mixing for 30 minutes. Using a multi-channel pipettor, transferred 25 µl of each lysate to corresponding wells in the histone-coated plate, and proceeded with the ribosylation reaction.
- Removed the strip wells from the Ziploc bag and added 50 µl/well of 1X I-PAR Assay Buffer to rehydrate the histones. Incubated at room temperature (25°C) for 30 minutes.
- Removed the 1X I-PAR Assay Buffer from the wells by tapping the strip wells on paper towels. Added 25 µl, in triplicate, to histone-coated wells in a 96 well plate after that, added 25 µl, in triplicate, of the serial dilutions of PARP standard. Then again added 25 µl, in triplicate, of the cell lysates directly from the tissue culture plate.
- The negative Control or untreated cancer cells, without Newly generated NK cells, include wells without PARP or cell extract to provide the background absorbance that will be subtracted from the experimental sample values.
- After that, distributed 25 µl of the PARP Substrate Cocktail (20X I-PAR Assay Buffer,125 µl, 10X Activated DNA, 250 µl and 20 mM NAD 250 µl plus dH2O 18.75 µl) into each well using a multichannel pipettor. And Incubated the strip wells at room temperature for 30 minutes.

Continued for detection:

- Washed strip wells 2 times with 1X PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS.

- Added 50 µl per well of diluted anti-PAR monoclonal antibody (prepared already according to brochure), and Incubated at room temperature (25°C) for 30 minutes.
- Washed the strip wells 2 times with 1X PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.
- Added 50 µl per well of diluted goat anti-mouse IgG-HRP conjugate and Incubated at room temperature (25°C) for 30 minutes.
- Washed strip wells 2 times with 1X PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. After each wash, the researcher ensured that all the liquid is removed following each wash by tapping strip wells onto paper towels.
- Added 50 µl per well of pre-warmed TACS-Sapphire™ colorimetric substrate and incubated, in the dark, for 15 minutes at room temperature (25°C). Stoped the reactions by adding 50 µl per well of 0.2M HCl or 5% Phosphoric Acid and began to set the negative and positive controls for wester blotting.

3.3.3.2 Caspase-3/7 Green Detection assay

For this part of the experiment, the Caspase-3/7 Green Detection Reagent, was used. Caspase-3/7 Green Detection Reagent is a novel fluorogenic substrate for activated caspases 3 and 7 (Velatooru, & Janapala, 2016). Members of the caspase family of cysteine proteases play central roles in coordinating the stereotypical events that occur during apoptosis. Because the major executioner caspases, caspase-3 and caspase-7, exhibit almost indistinguishable activity toward certain synthetic peptide substrates, this has led to the widespread view that these proteases occupy functionally redundant roles within the cell death machinery. These distinct phenotypes could be related to differences in the relative expression levels of caspase-3 and caspase-7 in vivo, or due

to more fundamental differences between these proteases in terms of their ability to cleave natural substrates.

The Caspase-3/7 Assay is designed for use with multi well-plate formats, making it ideal for automated high throughput screening of caspase activity or apoptosis. The assay has been automated in 96-, 384- and 1536-well formats. Cell washing, removing medium and multiple pipetting steps are not required. The caspase and luciferase enzyme activities reach steady state so that the luminescent signal peaks in approximately one hour and is maintained for several hours with a minimal loss of signal. This results in a rapid, sensitive and flexible caspase-3/7 activity assay. The assay system may be used with purified enzyme preparations and has been tested on a variety of cell model systems including monolayer cultures of adherent or suspension cells, 3D microtissue cultures and zebrafish embryos. This assay also can be multiplexed with other homogeneous assays to measure more than one parameter from a single well.

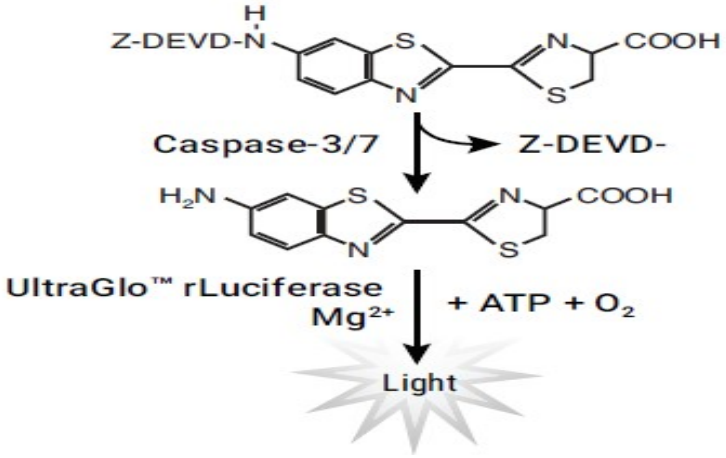


Figure 3.8: Schematic Diagram of the Caspase-Glo® 3/7 Assay Technology. Following caspase cleavage of the proluciferin DEVD substrate, a substrate for luciferase (amino luciferin) is released and, in the presence of luciferase and ATP, results in the luciferase reaction and the production of light.

The Caspase-3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. The reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye (Toleung et al., 2016). This cell-permeant substrate is intrinsically non-fluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 or caspase-7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with an absorption/emission maximum of ~502/530 nm (Pyrshev, & Klymchenko, 2017).

Adding a single Caspase 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present. The Caspase 3/7 Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to generate a stable "glow-type" luminescent signal and improve performance across a wide range of assay conditions.

There are several benefits of the Caspase-3/7 Green Detection Reagent compared to other methods of detecting activated caspase 3/7. One important advantage of this assay is that it does not require wash steps, which helps preserve the fragile apoptotic cells typically lost during wash steps (Nguyen et al., 2017). To use the Caspase-3/7 Green Detection Reagent, add the substrate to your cells in complete growth medium or buffer, incubate for 30 minutes, and image. Apoptotic cells with activated caspase-3/7 show bright green nuclei, while cells without activated caspase 3/7 exhibit minimal fluorescence signal.

This robust assay is highly specific for caspase-3/7 activation, and can be used to monitor caspase-3 or -7 activation with live-cell fluorescence imaging. Because the cleaved reagent labels nuclei of caspase 3/7–positive cells, this stain can also provide information on nuclear morphology, including condensed nuclei typical of late-stage apoptosis. Additionally, the fluorescent signal from Caspase-3/7 Detection Reagent survives formaldehyde fixation and detergent permeabilization. This provides flexibility in assay workflow and extends the multi flexibility of this probe for the detection other proteins of interest using immunocytochemistry (Mahajan et al., 2016).

In addition to traditional fluorescence microscopy, the Caspase-3/7 Green Detection Reagent has been validated for high-content imaging and analysis. The drastic change in fluorescence between normal and apoptotic cells within a population provides an excellent assay window, and the z-factor value indicates the reagent is robust enough for use in high-content imaging assays (Hanson & Finkelstein, 2019).

Here the researcher used the caspase-3 and caspase-7 exhibit differential, to examine the apoptosis inhibitory ability of the newly generated NK cells on our target cells. To proceed for this experiment, the untreated cancer cells and treated cells with newly generated NK cells were plated onto a 96-well plate as positive control.

- Zero control: Caspase 3/7 Reagent, vehicle and cell culture medium without cells.
- Negative control: Caspase 3/7 Reagent and vehicle-treated cells in medium.
- Positive control: Caspase 3/7 Reagent and treated cancer cells with newly generated NK cells in medium.

The zero control is used to measure background luminescence associated with the cell culture system and Caspase 3/7 Reagent. Subtract the value for the blank reaction from experimental values. The negative control reactions are important for determining the basal caspase activity of the cell culture system.

The protocol for this assay which been used in a 96-well plate, is as follows

- First equilibrate the Caspase 3/7 Buffer and lyophilized Caspase 3/7 Substrate to room temperature and allow the reagent to be equilibrated.
- The 96-well plates containing treated cells was removed from the incubator and allowed the plates to equilibrate to room temperature.
- Added 100µl of Caspase 3/7 Reagent to each well of a white-walled 96-well plate containing 100µl of zero, negative and positive control cells which was treated cells in culture medium. And after that covered the plate with a plate sealer.
- After the mixing process, gently mixed contents of wells using a plate shaker at 300–500 rpm for 30 seconds. Incubated at room temperature for 2 hours.
- After the incubation. The plate and samples were ready to be measured by the luminescence of each sample in a plate-reading luminometer.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Introduction

In this chapter the results and discussions about them are given. Regarding objectives of this project the results also follow two main categories. The first objective of this project is generating transgene Hematopoietic Stem Cells from Natural Killer Cells with same ability. As it has been discussed in methodology chapter, three specific activating and inhibitory genes of NK cells been cloned in HSCs. To evaluate the success of this transfection, western blot was used to examine expression of transfected gens and flow cytometry as well was used for cell count and specific detection of newly generated NK cells on breast cancer cells. After that regarding objective two as examining the functionality of newly generated NK cells, MTT and Live and Dead Assay were used to evaluate the cytotoxicity of generated NK cells and RARP Cleavage and Caspase 3/7 Kit assays were used to evaluate the apoptosis of generated NK cells. This chapter contains the results and interpretations of these results.

4.2 Results of objective one; Transfection Efficiency

The progression of transfection technologies has empowered scientists to investigate Protein function and gene regulation in a variety of cell types, tissues, and organisms. Transfection is generally attained by three different methods, chemical, physical, and biological. The choice of the method will be contingent on the application, the transfected molecule, and the cell type because various cells may respond differently to a particular method. Once cells have been transfected, various methods can be used for analysis post-transfection and for assessing transfection efficiency.

There are many factors that can influence transfection efficiency, a number of which are definite to the target cell. Cell-related factors affecting transfection include cell density, cell size, replication state, passage number, the health of cells, biomolecule type, and concentration. Some factors are method specific, for example, electroporation parameters such as voltage, capacitance, and resistance strongly affect transfection efficiency. Therefore, to obtain high efficiencies, all relevant factors should be considered when planning transfection experiments.

Recurrently, after any transfection experiment, it is imperative to assess the efficiency of transfection and the impact of transfection on the cells. Analysis of transfection efficiency can be as simple as confirming the expression of the gene of interest. However, in many cases assessment of transfection involves determining the total expression level of the gene of interest, determining the number of positive cells within a transfected population (percentage of positive cells), and/or visual confirmation of the Protein of interest (Jordan et al., 2017).

The methods for measuring Protein expression level of the transfected cells will determine the total expression from a population of transfected cells. Measurement of total gene expression can be done through western blotting and fluorometry. Determining the number of positive cells within a transfected cell population can be done through flow cytometry.

4.2.1 Western Blotting

Western blotting uses antibodies to spot individual proteins among a cell or tissue lysate. Antibodies bind to extremely specific sequences of amino acids, called epitopes (Vicinanza et al., 2017). Because amino acid sequences vary from protein to protein, western blotting analysis can be used to identify and quantify a single protein in a lysate that contains thousands of different proteins (Pfeffer & Fliesler, 2017). First, proteins are separated from each other based on their size by SDS-PAGE gel electrophoresis or

Nitrocellulose gel. Next, the proteins are transferred from the gel to a membrane by application of an electrical current. The membrane can then be processed with primary antibodies specific for target proteins of interest. Next, secondary antibodies bound to enzymes are applied and finally a substrate that reacts with the secondary antibody-bound enzyme is added for detection of the antibody/protein complex.

To detect the expression of target proteins, western blot used. These bands will enable us to determine the molecular weight of the protein detected in the sample (sample 1, *KLRA4*,3kD. Sample 2, *KLRA8*,31kD. Sample 3, *KLRD1*,17KD) each on separated gel. Three specific primary and secondary antibodies added to each specific sample.

- Sample/gel,1: ly49d: *KLRA4*: ly49d primary: ZO-41 Monoclonal Antibody (ZO41-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 18.
- Sample/ gel,2: ly49h: *KLRA8*: ly49h primary: ZO81- Monoclonal Antibody (ZO81-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 11.
- Sample/gel,3: CD94: *KLRD1*: MA5-15818 Monoclonal Antibody, NKG2/CD94/ secondary: Mouse anti Rat CD94 Antibody.

Each sample loaded in separated gel as shown in figure 4.1 and table 4.1 and set for running the western blot.

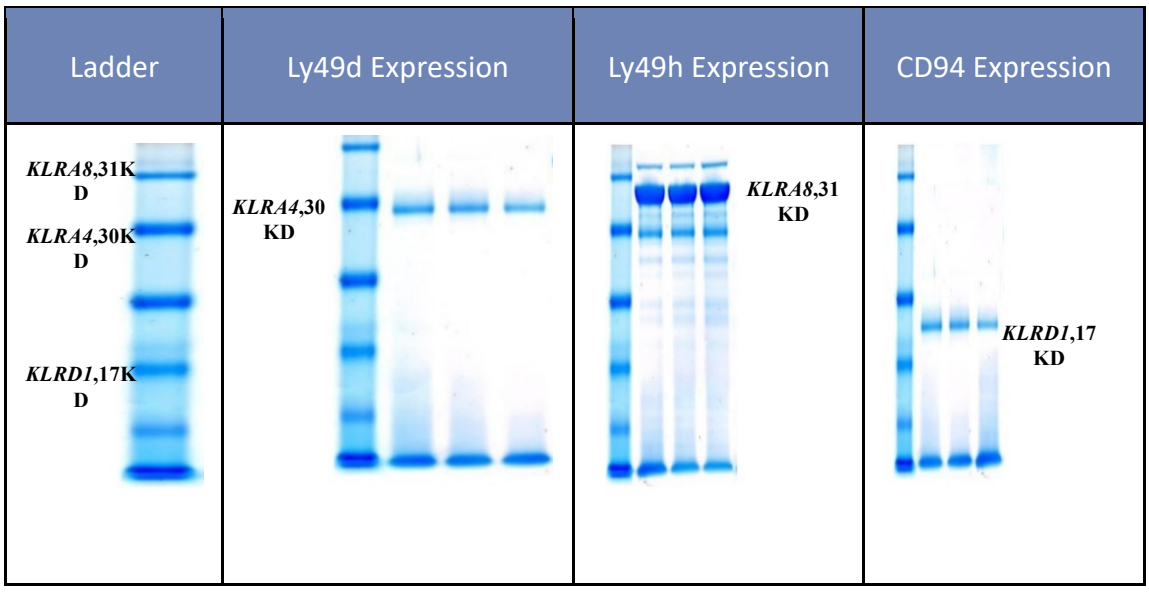
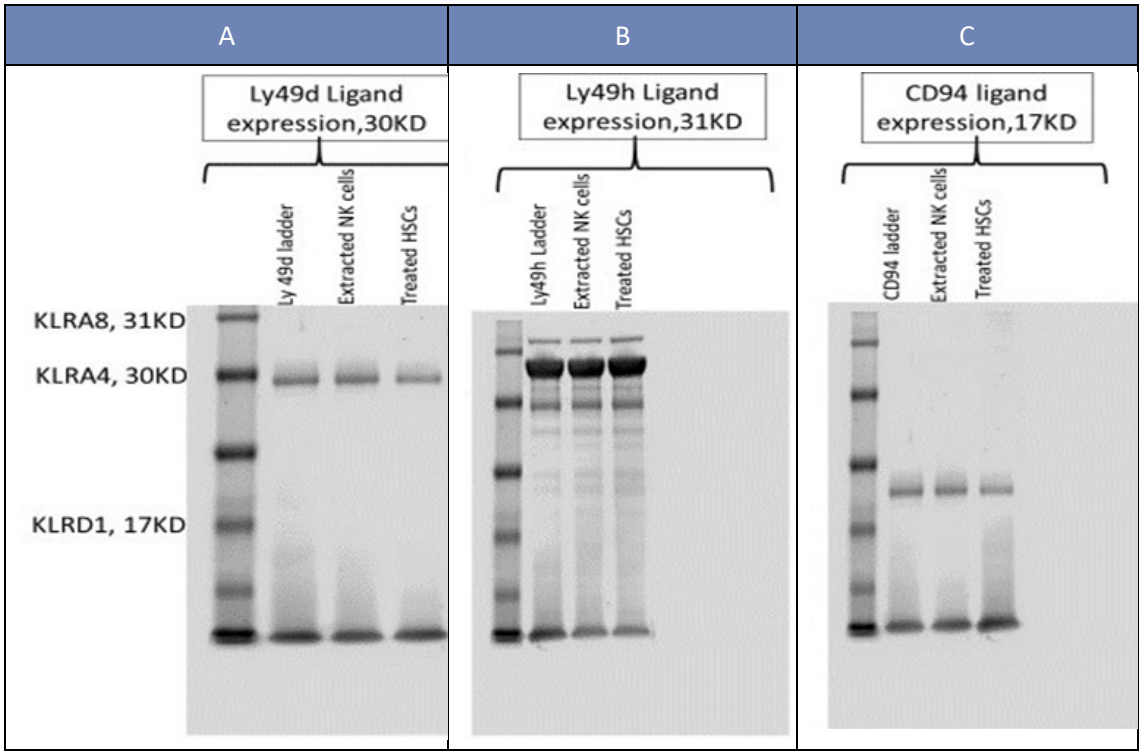


Figure 4.1: Shows the sample 1,2 and 3, western blotting gels and expression of targeted proteins in untreated and treated samples.

Table 4.1: Western Blotting results.



4.2.1.1 Western Blotting Data analysis

According to the western blotting results, showing in Table 4.1,

- A: Contains the results of sample/gel,1: ly49d: *KLRA4*: ly49d primary: ZO-41 Monoclonal Antibody (ZO41-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 18. The firs column is the ligands ladder shows the molecular weight of 30 kD for *KLRA4* gens. The second column shows the ly49d ladder, the third column shows the expression of ly49d protein (30kD) in untreated, extracted NK cells as control and the fourth column shows the expression of ly49d protein in treated HSCs with *KLRA4* gens.
- B: Contains the results of sample/gel,2: ly49h: *KLRA8*: ly49h primary: ZO81-Monoclonal Antibody (ZO81-1A12)/ secondary: Mouse anti-Rat IgG (Heavy chain) HRP 11. The first column shows the ly49h ladder, the second column shows the expression of ly49h protein (31kD) in untreated, extracted NK cells as control and the third column shows the expression of ly49h protein as well in treated HSCs with *KLRA8* gens.
- C: Contains the results of sample/gel,3: CD94: *KLRD1*: primary: MA5-15818 Monoclonal Antibody, NKG2/CD94/ secondary: Mouse anti Rat CD94 Antibody. The first column shows the *KLRD1* ladder, the second column shows the expression of CD94 protein (17kD) in untreated, extracted NK cells as control and the third column shows the expression of CD94 protein as well in treated HSCs with *KLRA8* gens.

It has been proofed that Hematopoietic stem cells are not possess any KLRA4, KLRA8 and KLRD1, genes in their genome. Consequently, there should be no expression of the ly49d, ly49h and CD94 receptor's proteins. Nonetheless the results as shown in each forth column of the table 4.1, shows the expression of ly49 h, d and

CD94 in treated HSCs with newly generated NK cells. These results suggest that the transfection of these three genes were successful and the treated hematopoietic stem cells differentiated to NK cells.

4.2.2 Flow Cytometry

Flow cytometer can determine the number of positive cells within a transfected cell population (percentage of positive cells) (Naithani et al., 2017). In addition, a flow cytometer with sorting aptitudes can enrich for positive cell populations. Nevertheless, flow cytometry requires that the cells either express a fluorescent Protein, such as GFP, or the Protein of interest must be labelled with a fluorescent molecule (Hara et al., 2016). Flow cytometry is for the identification of the cellular subsets based on cell essential properties, most often by the use of fluorochrome-conjugated antibodies, recognizing distinct cell-surface epitopes that define the cells of interest. In case of the specific receptors of this project,

- The monoclonal antibody eBio4E5 (4E5) reacts with Ly-49D, an activating member of the Ly-49 family of NK cell receptors.
- The monoclonal antibody 3D10 recognizes Ly-49H, a member of the C-type lectin Ly-49 multigene family of receptors found on natural killer cells. Ly-49H, like Ly-49D, lacks the characteristic ITIM (immunoreceptor tyrosine-based inhibitory motif) and contains an arginine in the transmembrane domain implying these molecules act as activation receptors.
- The 18d3 monoclonal antibody reacts with rat CD94, a 70 kDa type II transmembrane glycoprotein. CD94 belongs to the C-type lectin superfamily and is present as a heterodimer with NKG2 on the surface.
- CD34: Is a stem cell marker, although its expression on human hematopoietic stem cells is reversible. CD34, serves as a surface receptor that undergoes receptor-mediated

endocytosis and regulates adhesion, differentiation, and proliferation of hematopoietic stem cells and other progenitors. In flow cytometry charts, the forward scatter (FS) shows the size of cells which is in the case of Hematopoietic Stem cells, is 0.2- 6 µM in diameter and the side scatter (SS) shows the complexity of cells which is to detect the expression of ligand or granularity of cells.

Table 4.2 indicates the separation of samples based of the receptors and their specific antibody.

Table 4.2: Separation of samples regarding specific antibodies.

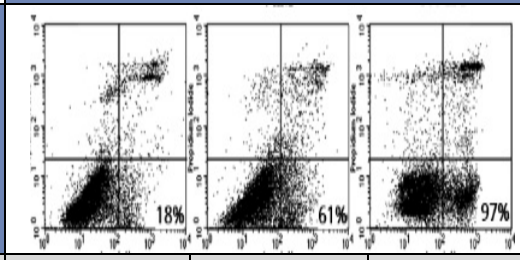
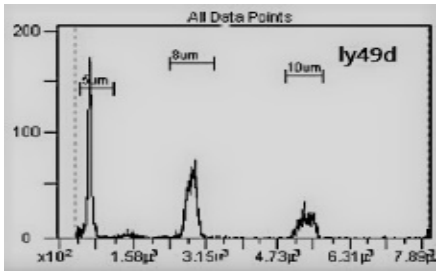
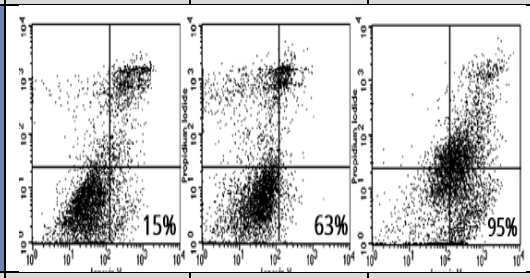
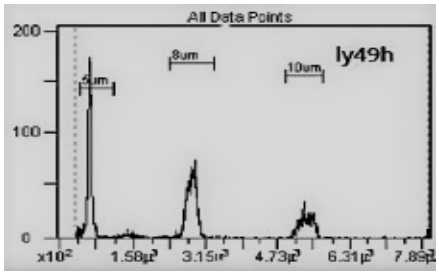
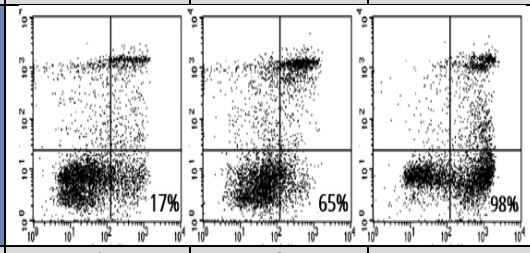
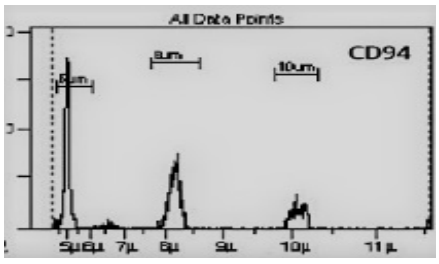
KLRA8 Expression		*KLRA4* Expression		*KLRD1* Expression	
3D10PE-,CD34+	3D10PE+, CD34+	4E5-, CD34+	4E5+, CD34+	18d3PE-,CD34+	18d3PE+, CD34+

After transfection, totally three samples with each own specific antibody were set for Flow Cytometry for three times. The first time, day after transfection and second time 2 days after transfection and the last time, seven days after transfection. Each time three samples were run in flow for detection of transfection ratio.

- Sample 1: 3D10PE monoclonal antibody to detect the expression of ly49h ligand and CD34 as a stem cell marker to detect hematopoietic stem cells.
- Sample 2: eBio4E5 (4E5) monoclonal antibody to detect the expression of ly49d ligand and CD34 as a stem cell marker to detect hematopoietic stem cells.
- Sample 3: 18d3 monoclonal antibody to detect CD94, and CD34 as a stem cell marker to detect hematopoietic stem cells.

The table 4.3 shows the percentage of differentiated and non-differentiated hematopoietic stem cells to NK cells based on passing time.

Table 4.3: Flow cytometry results.

	A			B
Ly 49d				
Day	1	2	7	
Ly 49h				
Day	1	2	7	
CD 94				
Day	1	2	7	

According to the Flow Cytometry results, showing in table 4.3, (A): ly49d: increase in population of transfected HSCs with expressed of 3D10PE+, CD34+, (A): ly49h: increase in population of transfected HSCs with expressed of 4E5+, CD34+. (A): CD94: increase in population of transfected HSCs with expressed of 18d3PE+, CD34+. (B): ly49d Dot plots shows significantly increasing in size of treated HSCs form 5µ to 10µm by incubation with 3D10PE+. (B): ly49h Dot plots shows significantly increasing in size of treated HSCs form 5µ to 10µm by incubation with 4E5. (B): CD94 Dot plots shows significantly increasing in size of treated HSCs form 5µ to 10µm by incubation with 18d3PE.

4.2.2.1 Flow cytometry statistical data analysis

Regarding the expression of target genes in samples (Table 4.4)

- 24 hours after the transfection;
    - Sample 1: 18% expression of ly49d, 82%, no expression of ly49d ligand.
    - Sample 2: 15% expression of ly49h, 85% no expression of ly49h ligand.
    - Sample 3: 17% expression of CD94, 83% no expression of CD94 ligand.
- 48 hours after the transfection;
    - Sample 1: 61% expression of ly49d, 39%, no expression of ly49d ligand.
    - Sample 2: 63% expression of ly49h, 37% no expression of ly49h ligand.
    - Sample 3: 65% expression of CD94, 35% no expression of CD94 ligand.
- 7 days after the transfection;
    - Sample 1: 97% expression of ly49d, 3%, no expression of ly49d ligand.
    - Sample 2: 95% expression of ly49h, 5% no expression of ly49h ligand.
    - Sample 3: 98% expression of CD94, 2% no expression of CD94 ligand.

Table 4.4: Flow Cytometry Statistical Data

TIME/DAY	KLRA8 EXPRESSION		KLRA4 Expression		KLRD1 Expression	
	3D10PE-, CD34+	3D10PE+, CD34+	4E5-, CD34+	4E5+, CD34+	18d3PE- ,CD34+	18d3PE+, CD34+
1	82	18	85	15	83	17
2	39	61	37	63	35	65
7	3	97	5	95	2	98
Expression mean/percent		58.6		57.6		60
Negative Antibody	3D10PE- ,CD34+	4E5-, CD34+	18d3PE-, CD34+			
	82	85	83			
	39	37	35			
	3	5	2			
Correlation (R value)	0.9979					
Significance F (P value)	0.00016001					
Positive antibody	3D10PE+, CD34+	4E5+, CD34+	18d3PE+, CD34+			
	18	15	17			
	61	63	65			
	97	95	98			
correlation (R value)	0.9984					
Significance F (P value)	0.00016127					

Table 4.4 shows Separation of samples regarding specific antibodies for flow cytometry. Correlation coefficients (r) were calculated between three antibodies used for flow cytometry assay. The relation between reducing undifferentiated stem cells in samples medium shown in negative antibodies raw. The percentage of undifferentiated stem cells reduces due to rising the percentage of differentiated stem cells or transgene NK cells. The R-value for samples with no antibodies or negative antibodies is 0.9979 against very small P-value. On the other hand, the growth in the number of transgene NK cells in samples contained positive antibodies has a correlation of 0.9984 against the very small P-value.

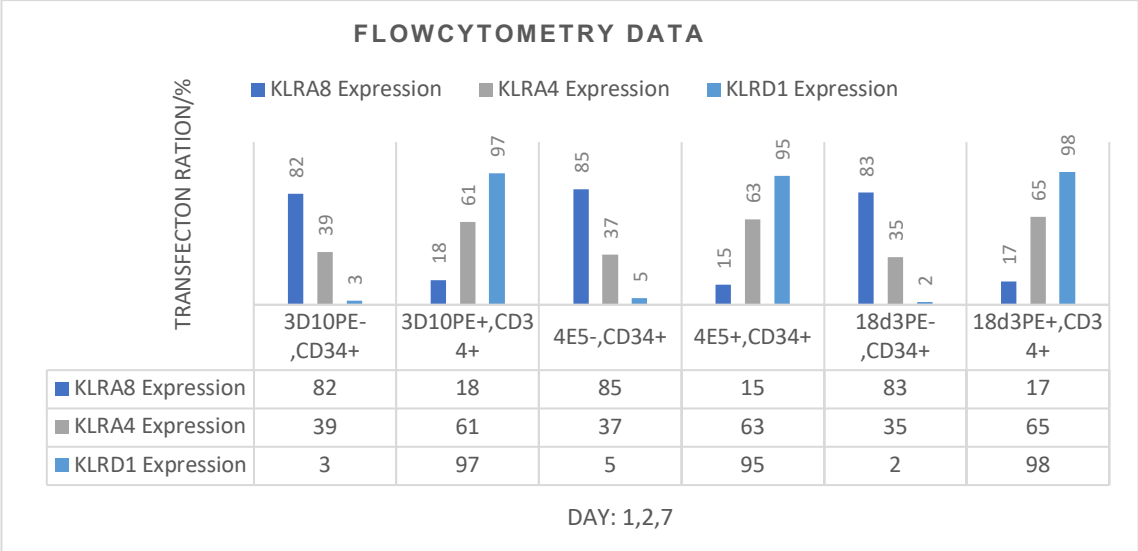
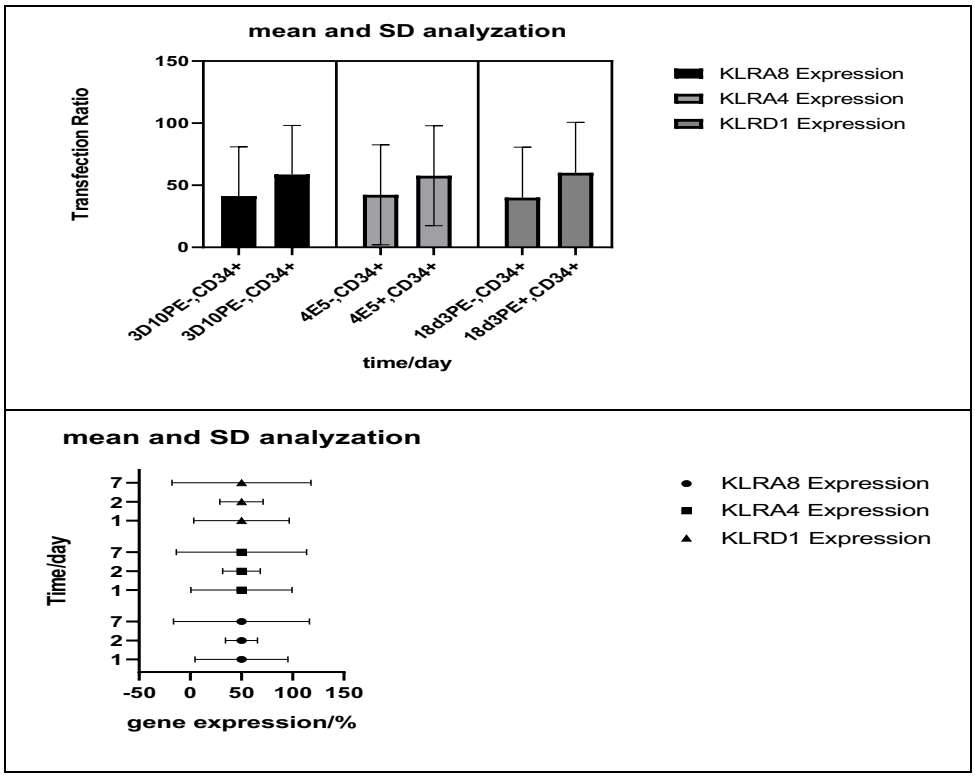


Figure 4.2: Transfection Ratio Based on Time.

The Table 4.5 show the average and standard deviation of each sample per each day of running experiment.

Table 4.5: Flow Cytometry Statistical Measurements.



Regarding the expression of target genes in samples, the diagram shows the statistical measurements of the transfection efficiency. There were three samples each contains specific antibodies. The Table 4.5 show the average and standard deviation of each sample per each day of running experiment. According to statistics for sample 1- *KLRA8* expression, after 7 days, totally 58.6% of all transfected HSCs have ly49d ligand on their cell membrane. The data shows 0.39 standard deviation. Second according to statistics for sample 2- *KLRA4* expression, after 7 days, totally 57.6% of all transfected HSCs have ly49h ligand on their cell membrane. The data shows 0.40 standard deviation. According to statistics for sample 3- *KLRD1* expression, after 7 days, totally 40% of all transfected HSCs have CD94 ligand on their cell membrane. The data shows 0.40 standard deviation as well. By assessing the data, 58.9% of all treated HSCs with target genes, express target ligands on their cell membrane.

4.3 Results of The Objective Two

The objective 2 of this research, is to examine the functionality of the newly generated NK cells on cancer cell lines by the aspect of cytotoxicity and apoptosis. For this portion of the research, the researcher used four different assays for each regard. As examining the cytotoxicity, the MTT Assay and Live and Dead Assay, were used and for the purpose of examining the apoptosis RARP Cleavage and Caspase 3/7 Kit, were used. In this part of the thesis, the results of these four assays are given.

For this research ZR-75-l human breast cancer cell line (properties of the cell line explained in section 3.2.2.1) was chosen and provided to examine the cytotoxicity and apoptosis preventing ability of the Newly Generated NK cells on real cancer cell line.

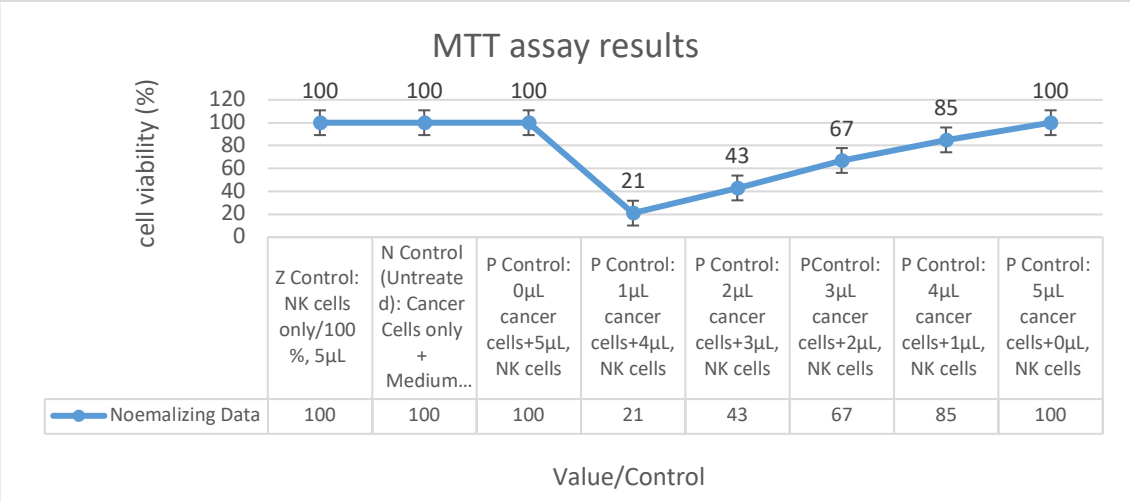
4.3.1 MTT Assay Results (Cytotoxicity)

The ZR-75-l carcinoma cells were seeded at a density of 1×10^3 per well in 96 well plate and incubated for 24 hours. Cells were treated with Newly Generated NK cells at a con-centration ranging from 0 to 5 (μM) for 24 hours according to Table 3.7. Viability assay was carried out with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma, MO, USA) as described previously in literature review chapter.

Regarding MTT assay, bilaterally the researcher assessed the nuclear condensation and mitochondrial membrane potential ($\Delta\psi m$) of Treated and untreated cells by staining them with 0.5 mg/mL of nuclear stain Hoechst 33342 (Sigma, MO, USA) for 10 minutes. After the staining and after 2 hours incubation, the images were captured with DS-Qi2 camera using UV-filter of Nikon Ti-U Inverted fluorescent microscope and documented with NIS element software. Cells with apoptotic condensed nuclei were scored as percentage per sample and for each assay three random fields were scored. For

analysing Δψm, the medium was gently aspirated and 100 µL of serum-free Opti-MEM (Invitrogen, CA, USA) containing 50 nM TMRM dye (Molecular Probes, OR, USA) was added. After 10 min incubation at 37 °C, images were captured using TRITC filter of fluorescent micro-scope. The results of the MTT assay and the nuclear condensation and mitochondrial membrane potential (Δψm), is given in Figure 4.3 and Table 4.6.

Table 4.6: Cell Viability by MTT assay is shown at different concentrations (0–5 µl) of newly generated NK cells treatment on cancer cells for 24 hours.



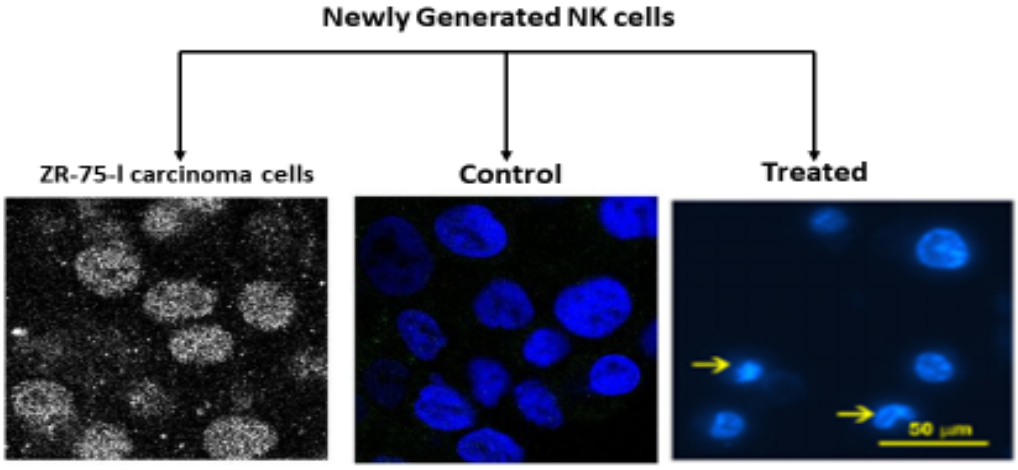


Figure 4.3: The exposure of newly generated NK cells (15 µl for 48 h) induced nuclear condensation as illustrated in Hoechst-stained images (scale bar 50 µm). Arrows indicate condensed nuclei.

In this part of the research the cytotoxic effect of Newly generated NK on ZR-75-1 carcinoma cells at various concentrations (0–5 μM) of NK cells was investigated. Table 4.5, shows Cell Viability (value/control) by MTT assay at different concentrations (0–5 μl) of newly generated NK cells treatment on cancer cells for 24 hours. In MTT assay, NADPH-dependent cellular oxidoreductase enzymes, reflect the number of viable cells presented. These enzymes are capable of reducing the tetrazolium dye to its insoluble form which is called formazan. Formazan has purple colour. In this assay by the help of Spectrophotometry at Higher absorbance of light at 595 nm, is possible to measure the colour change. The results which are given in Table 4.5, indicate that by adding 4 μl of the newly generated NK cells to the medium containing cancer cells, the viability of cancer cells reduce drastically and by reducing the amount of the Newly generated NK cells in the medium, the viability of cancer cells, gradually increasing again.

On the other hand, the Microscopic images of stained treated and untreated cancer cells with newly generated NK cells (Figure 4.3), shows that adding 15 μM of newly generated NK cells in the cancer cells medium, in an experiment with 48-hour time, causes nuclear condensation and consequently destruction for cancer cells.

4.3.2 Live and Dead Assay Results (Cytotoxicity)

Cell viability is an important component of any *in-vitro* cell-based assay. Culture conditions and experimental treatments can affect cell viability by directly or indirectly inducing cytotoxicity, apoptosis and/or necrosis (Pfeffer & Fliesler, 2017). A hallmark of viable cells is an intact plasma membrane and intracellular enzymatic activity (Cho, & Jang, 2018). These two features form the basis of this Live and Dead Cell Assay. Live cells are identified on the basis of intracellular esterase activity (generating green fluorescence) and exclusion of the red dye. Dead cells are identified by the lack of

esterase activity and non-intact plasma membrane which allows red dye staining (X. Liu, & Andreescu, 2016).

The Live and Dead assay stain solution is a mixture of two highly fluorescent dyes that differentially label live and dead cells:

- The Live cell dye labels intact, viable cells green. Its membrane is permeant and non-fluorescent until intracellular esterase remove ester groups and render the molecule fluorescent. The Excitation (max) and Emission (max) are 494 nm and 515 nm. (X. Chen et al., 2019).

- The Dead cell dye labels cells with compromised plasma membranes red. Its membrane is impermeant and binds to DNA with high affinity. Once bound to DNA, the fluorescence increases >30-fold. The Excitation (max) and Emission (max) are 528 nm and 617 nm. (Oni Biton, & Segal, 2016).

For running the assay, a 32 well plate was used and added suspension of the ZR-75-l carcinoma cells cell lines, in zero control without any treatment, and the mixture of the newly generated NK cells added as negative control and for positive control, 2µL of cancer cell lines was treated with newly generated NK cells. All treated and non-treated samples were stained with 5x diluted concentration of live and dead dye with 1mL PBS. After that, samples were incubated for 4 hours at room temperature in the dark. Alternatively set for imaging as Green = live cells and Red = dead cells. After the treatment, the culture was stored for 4 hours and then the results were analysed by flow cytometry and fluorescent microscopy. LIVE: Emission (max): 495 nm, Excitation (max): 515 nm and DEAD: Emission (max): 528 nm, Excitation (max): 617 nm.

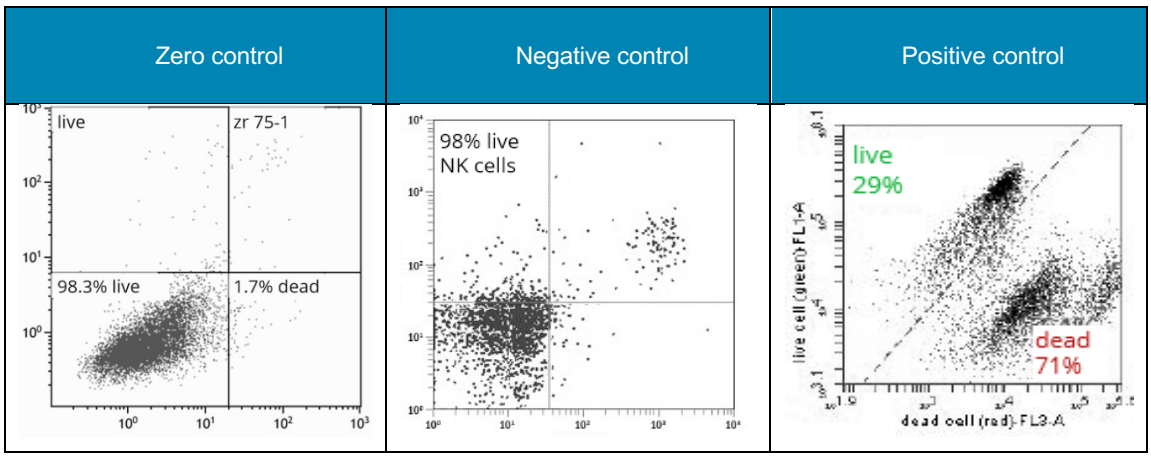


Figure 4.4: The Flow Cytometry Results of Live and Dead Assay.

The flow cytometry results of live and dead assay which is shown in Figure 4.4, shows, the A. zero control, which is untreated ZR-75-l carcinoma cells cell lines, 98.3 percent viability. The zero control, contains only cancer cells without treating them with newly generated NK cells. In the negative control which is shown in the results table as B, contains only newly generated NK cells, without mixing them with cancer cells. And the viability is 98 percent live cells and almost 2 percent of them is already dead. At the last sample, which is C. treated control, the newly generated NK cells, are added to ZR-75-l carcinoma cells, and after 8 hours, the positive control, set for flow cytometry. The flow result of the positive control, shows that the newly generated NK cells are likely killed 71 percent of the cancer cells, after 8 hours. By analysing these results, it's been proved that, the Newly Generated NK cells, are capable of destroying cancer cells.

The next step of this assay, was to observe the samples by fluorescent microscope, to see the formation and shape of the dead cells and comparing them with the flow cytometry results. Regarding the fluorescent microscopy, the alive cells are glowing green and the dead cells are glowing red. The results of the fluorescent microscopy on zero control, negative control and the positive control, are given in Figure 4.5.

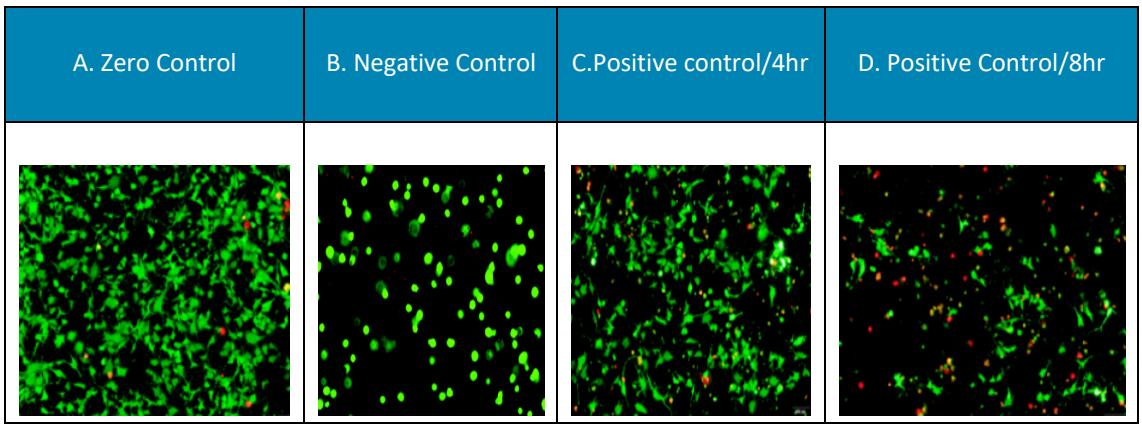


Figure 4.5: Fluorescent Microscopy Results.

In Figure 4.5, the fluorescent microscopy results are given. The A. zero control, shows the untreated ZR-75-l carcinoma cells with intracellular esterase activity which are generating green fluorescence and the dead cells, which are identified by the lack of esterase activity and non-intact plasma membrane which allows red dye staining. In the second part, the B. negative control, shows the newly generated NK cells. The bone marrow NK cell's size is normally 12–15 µm, which here the Newly generated NK cells, showing 5 µm, which is similar to small size of Hematopoietic stem cells as 4 and 5 µm diameter. At the third part of the results, the C. positive control, or treated sample, shows the destruction ability of newly generated NK cells on the ZR-75-l carcinoma cells, after 4 hours. And the last part of the results, D. positive control, shows that the newly generated NK cells, have the cytotoxicity capability on cancer cells and killed most of the cancer cells.

4.3.3 PARP Cleavage Assay Results (Apoptosis)

The control of apoptosis is the most intensely studied form of programmed cell death which has been a long sought-after goal for the treatment of cardiovascular, neurological, autoimmune and malignant diseases. Poly (ADP-ribose) polymerase (PARP-1) becomes a mediator of cell death by triggering the translocation of apoptosis inducing factor from the mitochondria to the nucleus. In experimental models, PARP-1

inhibition can prevent unwanted tissue damage following myocardial and neuronal ischemia, diabetes, septic shock, and vascular stroke (Su et al., 2015). Apoptosis involves many changes in cell component structure including exposure of phosphatidylserine in the outer plasma membrane, caspase activation, cytochrome C release from the mitochondria, chromatin condensation in the nucleus, and DNA ladder formation (Redza Dutordoir & Averill Bates, 2016). During apoptosis, PARP-1 which catalyses the NAD-dependent addition of poly (ADP-ribose) (PAR) onto various cytoplasmic and nuclear proteins, is cleaved from about 113 kDa to 85 kDa.

In this part of the experiment, the Trevigen's HT PARP/Apoptosis Assay was used which is ideal for measuring PARP activity in cell extracts prepared before and during apoptosis. The HT PARP/Apoptosis Assay is an ELISA which semi-quantitatively detects PAR deposited onto immobilized histone proteins in a 96-well format. An anti-PAR monoclonal antibody, goat anti-mouse IgG-HRP conjugate, and HRP substrate are used to generate a colorimetric signal. Thus, absorbance correlates with PARP activity. Etoposide is a topoisomerase II inhibitor that stabilizes this enzyme after it cleaves DNA.

After following the protocol, which is given in methodology chapter, the results are achieved as they have been shown below.

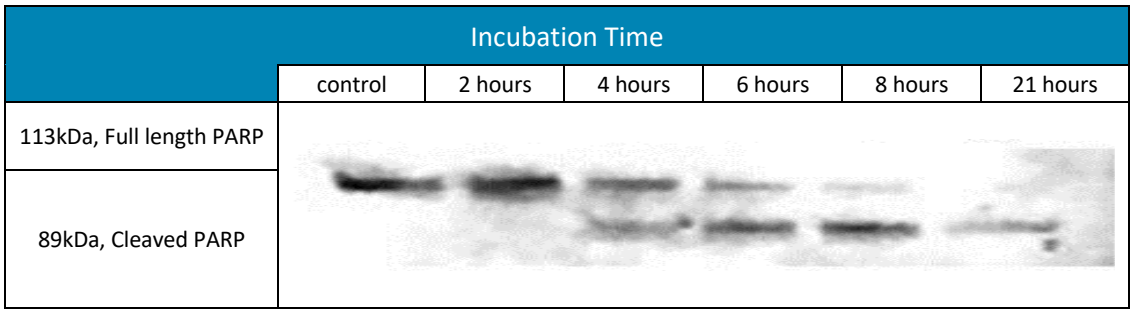


Figure 4.6: Western blot of a time course of ZR-75-l carcinoma cells, treated with 50 µM etoposide for the indicated time periods. The amount of extract theoretically derived from 100,000 cells were resolved, per lane, on an 8%-16% SDS-PAGE gel and analysed by immunoblotting for PARP-1.

The results of the Effects of the newly generated NK cells on the expression of cleaved PARP in the ZR-75-l carcinoma cells. Western blot analysis was used to measure the expression of cleaved PARP. Two independent experiments were performed in duplicate. Data are expressed as the mean ± SD; the results are expressed as a ratio of the normal control group. $P<0.05$, vs. the treated sample. The results showing that the newly generated NK cells have effect on the apoptosis of the sample cancer cells.

4.3.4 Caspase 3/7 Kit Results (Apoptosis)

Apoptosis is one of the most important intracellular events in living cell, which is a programmed cell death interrelated with caspase enzyme activity for maintaining homeostasis in multicellular organisms (Yogo et al., 2017). Therefore, direct apoptosis imaging of living cells can provide enormous advantages for diagnosis, drug discovery, and therapeutic monitoring in various diseases. However, a method of direct apoptosis imaging has not been fully validated, especially for live cells in in vitro and in vivo (Liu et al., 2019).

Following treatments cells were subjected to Caspase 3/7, activities measurement with Caspase-Glo assay kit (Promega, Madison USA). The plates containing cells were removed from the incubator and allowed to equilibrate to room temperature for 30 minutes. 100 µl of Caspase-Glo reagent was added to each well, the content of well was gently mixed with a plate shaker at 300–500 rpm for 30 seconds. The plate was then incubated at room temperature for 2 hours. Caspase-dependent pathway can be further divided into extrinsic or intrinsic pathway, as determined by involvement of caspase-8 or caspase-9, respectively. Both intrinsic and extrinsic pathway involved activation of caspase-3/7 which is important for inducing downstream DNA cleavage molecules. To examine the molecular mechanism underlying apoptosis process, the researcher, stained

cells with amino luciferin-labelled substrate of caspase and determined the caspase-3/7, activities by measuring the luminescence intensities every three hours. As shown in Figure 4.7, and Table 4.7, it's been observed a slowly increase of caspase-3/7 activity, which peaked at 18 hours in ZR-75-l carcinoma cell lines treated with newly generated NK cells. The activity of caspase-3/7 increased significantly from 6 to 12 hours, but remained high even after 30 hours of treatment, indicating a more latent effect of Newly Generated NK cells on ZR-75-l carcinoma cell lines.

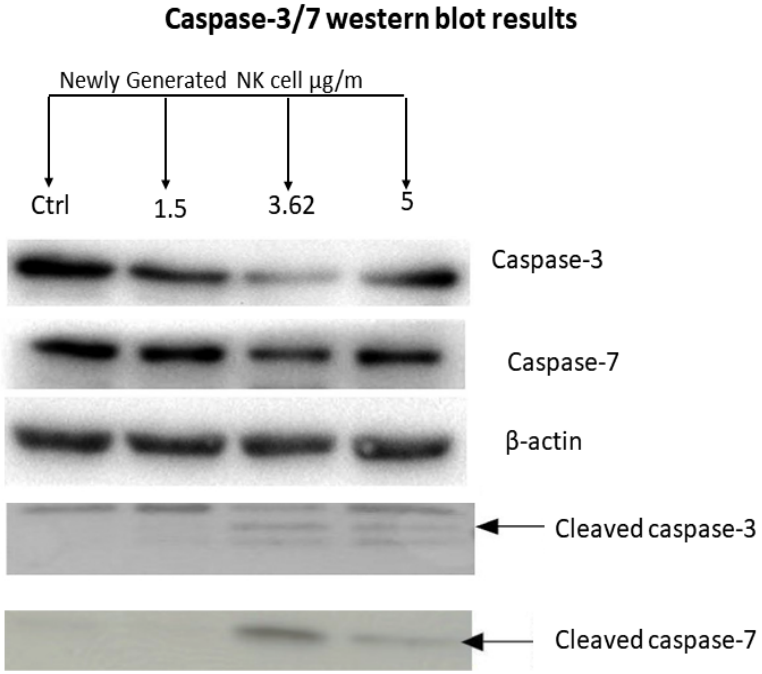
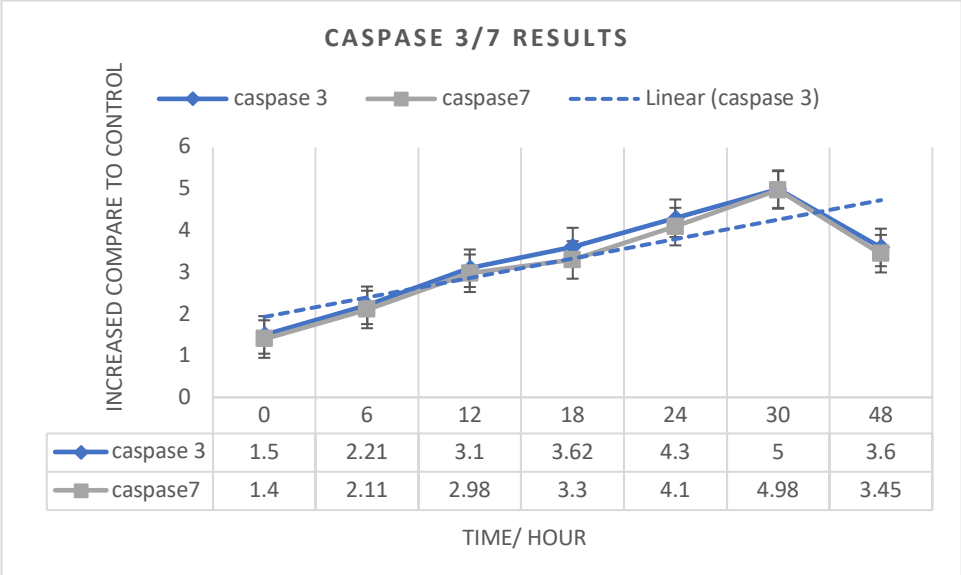


Figure 4.7: Western blot results showing the expression levels of cleaved caspase 3 and 7 in ZR-75-l carcinoma cell lines treated with various concentration (1.5, 3.62 and 5 µg/ml) of newly generated NK cells. β-actin served as a loading control.

Table 4.7: Caspase-3/7, activities in presence of newly generated NK cells on treated cancer samples determined as increase in luminescence and shows gradual increment activity of caspase-3/7 after treatment.



	0	6	12	18	24	30	48
caspase 3	1.5	2.21	3.1	3.62	4.3	5	3.6
caspase7	1.4	2.11	2.98	3.3	4.1	4.98	3.45

Incubation of the cancer cell lines with newly generated NK cells causes a time-dependent activation of caspase-3/7 activity. These results suggest that newly generated NK cells induced apoptosis via mitochondrial-dependent intrinsic pathway. Of note, caspase-3/7 cleaves several target proteins, one of which is DNA repair enzyme, PARP. Interestingly, DNA fragmentation was detected in NK cells-treated cancer cells.

4.5 Objective Two, Data Analysis and Mining

Accordingly, regarding to objective two of this project, a set of experiments were designed to support the hypothesis of this research. The objective one of this research was to examine whether the cloned specific genes of the natural killer cells in hematopoietic stem cells, are expressing in their host and are they giving the stem cells the ability of NK cells or no? that's why in this portion of this research, the western blot and flow cytometry was used.

Nonetheless for the second objective of this project, the goal was to examine the functionality of the newly generated NK cells. And for this portion of the research, the

set of experiments were used to examine whether the newly generated NK cells, have the ability of cytotoxicity and causing apoptosis on cancer cells or not?

In this regard, the MTT assay and Live and Dead assay was used to examine the cytotoxicity effect of the newly generated NK cells on the chosen cancer cell line, which is ZR-75-l carcinoma cell in this research. According to the MTT assay results, the expose of the newly generated NK cells, reduce the cell viability up to 80 percent. And by increasing the quantity of the newly generated NK cells, the percentage of cytotoxicity effects of NK cells on cancer cells, increases. Bilaterally by MTT and live & dead assays, the apoptosis effect of the newly generated NK cells with causing malformation, also examined. The results indicate that the newly generated NK cells cause nucleus malformation of the ZR-75-l carcinoma cell lines.

To examine the cell viability the live and assay also was used on ZR-75-l carcinoma cell lines. According to the results of this assay which is given in tables 4.7 and 4.8, the presence of the newly generated NK cells, cause cancer cells dead up to 71 percent.

After examining the cytotoxicity effect of the newly generated NK cells on ZR-75-l carcinoma cell lines, the research continued to examining the apoptosis effect of the newly generated NK cells. For this regard the PARP Cleavage Assay and Caspase 3/7 assay was used.

According to the PARP results which are given in figure 4.4, the expression of the cleaved PARP which is appeared in western blot experiment, shows that the newly generated NK cells has apoptosis effect on the ZR-75-l carcinoma cell.

At last, to examine the apoptosis effect of the newly generated NK cells on the ZR-75-l carcinoma cells, and for the matter of comparison, the Caspase 3/7 assay also was

used. According to the results of this assay which id given in figure 4.4 and table 4.9, the exposure of the newly generated NK cells on ZR-75-l carcinoma cells, cause cancer cells apoptosis. The western blot results showed the expression of the cleaved caspase 3 and caspase 7, which indicates that the newly generated NK cells cause apoptosis on ZR-75-l carcinoma cells.

4.6 Discussion

This project was designed to differentiate hematopoietic stem cells, collected from the bone marrow of experimental animals to natural killer cells, to advance the immune system of the experiment creature against cancer tumour cells. According to data examination, it's been cleared that above 95 percent of all gathered HSCs which were complexed with DNA in addition to vector, hereditarily changed to NK cells, which means stem cells separated ordinarily developed as an immune component. With the assistance of these newly generated NK cells and their ability to divide as typical cells with regular division speed (that would be less time consuming than deriving from BM), it could be a chance for the destruction of tumour cells.

No research is without limitation; ours is not an exception. The results showed that the ultimate goals had been reached but this research was done *in vitro,* The results have not been obtained from animal models because of many barriers such as facilities, permissions and allowances required. Also, this research has a weakness or gap. A very exclusive question. The NK cells are basically aggressive cells. By producing a mass amount of NK cells in a live model, that would be a chance for the NK cells, attack the body’s organs. This research has no answer for this question but to find the answer, is the future work of this research.

4.7 Conclusion

To conclude, the results have given in this chapter indicates that the Newly Generated NK cells, which were harvested from the Hematopoietic stem cells, have both cytotoxicity and apoptosis effect on cancer cell line which is the breast cancer. The results approve that the hypothesis of this research reached and objectives made.

4.8 Chapter Summary

This chapter started with results of the objective one, which was examine the transfection efficiency by western blotting and flow cytometry. And it continued with discussion of the objective one results. After that the results of the objective two has been given. First cytotoxicity ability of newly generated NK cells on cancer cells and second point of view was apoptosis ability of newly generated NK cells on cancer cell line. And at the end the discussion regarding the results and data achieved has been given in this chapter.

CHAPTER 5: CONCLUSION AND FUTURE WORKS

5.1 Conclusion and Future Works

5.1.1 Introduction

Concluding remarks on the overall research are presented in this chapter. Limitations of the research are also declared and potential future works are suggested.

5.2 Discussion

There are several published researches with the same hypothesis of this research. For example, there is a research which recently has been published with the title of (NK cell therapy after hematopoietic stem cell transplantation: can we improve anti-tumour effect?), (Van Elssen & Ciurea, 2018), which has quite similar ultimate goal with this research but different approach. All of these researches also trying to solve the problem of low population of NK cells against cancer cells. But most of these researches have different approach. In previous researches, the researches tried to produce large number of NK cells by inducing HSCs with immune cells markers such as CD64, CD86. In these researches the results support the success of this attempt but there are two significant differences with this research and most of the others. First is, by inducing immune cells or specifically NK cells markers to HSCs, the mass population of NK cells from HSCs happens, but the problem is, these changes on HSCs will not pass to next generations of newly generated NK cells from HSCs. Which this research filled this gap by transfecting three NK cells genes to the HSCs. The transfection causes genome changes in HSCs and the changes with pass through generations.

The second significant differences of this research with previous related researches is, most of the other researchers used immune cells markers to transform HSCs to NK cells, to produce mass population of NK cells, but the limitation of these researches are that the newly generated NK cells, generation one, will divide as fast as other peripheral cells but after they die, the population will again reduce drastically against cancer cells. But in this research, by transfecting the HSCs, the daughter cells are also dividing as fast as peripheral cells though the population of newly generated NK cells, will maintain against cancer cells. These are two most significant gaps which this research has filled and have the supporting results.

5.3 Practical Implications and Limitations

First, in order to open a new chapter of cancer genetics and stem cells genetics in the orientation of immunology.

Another implication is to consider the meaning of cure in the world of cancer. As mentioned already, most or perhaps all methods of curing cancer are from outside the body but this method would be the hope of curing cancer from the inside of the body using its own immune system and obviously would be less dangerous and with less side effects.

Last but not least, the development of this method would be another option and alternative for curing cancer. Added on focusing on improving of the existing methods, this method would be the new chance for curing cancer.

No research is without limitation; ours is not an exception. Some noteworthy limitations of this study are as follows:

For claiming the implication that this method works the researcher had to have results. For this purpose, after the transfection and cloning, three different laboratory techniques were used to gather data that proved the transfection had built ligands on the cell membrane of stem cells and cloning has changed the genome of the stem cells. The results showed that the ultimate goals had been reached but it had to be checked on human. The results have been explained but the main limitation was to check the results on first on animal models before human and that is not done because of many barriers existing such as permissions and allowanced required for

5.4 Directions for Future Works

Knowledge can be assessed from different perspectives. In this study, the focus was on assessing completeness, correctness, and understandability of this new stem cells technology. Apart from this, the ability of this technique would be beyond doubt if can be tested on human patient with the clinical trials. That would be the main future work of this study.

5.5 Summary

This chapter brings an end to the study with anticipation regarding the prospective contributions as proposed especially to the field of cancer genetics and cancer cure.

It has also hoped that the contributions extend to the existing literature of genetics and immunology. The hypothesis of this research was to find a new method for curing cancer which is causing humanity so much so many. The results are supporting the achievements of the ultimate goal. The only hope that is left is to have chance to examine this technique *in-vivo.*

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LIST OF PUBLICATIONS AND PAPER PRESENTED

PUBLICATIONS

1. **Alireza, P**., Shaharudin,Ab, R,. Shamsul Azlin B Ahmad Shamsuddin, (2020). Evaluation of Anti-Cancer Function of Transgenic Hematopoietic Natural Killer Cells. *Journal of cancer, 19*(7), 214–218.

2. **Alireza, P**., Shaharudin,Ab, R,. Shamsul Azlin B Ahmad Shamsuddin, (2018). Hematopoietic Natural Killer cells against cancer tumor cells by comparative results of Flow Cytometry and FACs. *Heliyon, 31*(13), 178–186.

3. **Alireza, P**., Shaharudin,Ab, R,. Shamsul Azlin B Ahmad Shamsuddin, (2017). Cloning and characterization of ly49h, ly49d and CD94 Genes in hematopoietic stem cells. *Iranian Journal of Allergy, Asthma and Immunology. 11*(8), 358-365.

PAPERS PRESENTED

1. Alireza, P., Shaharudin,Ab, R, (2018). *Hematopoietic natural killer cells against cancer by comparative results of flow cytometry and FACs*, paper presented at the 3rd International Congress & Expo on Biotechnology and Bioengineering: Recent Innovations and applications in Biotechnology and Bioengineering, 25-26th June 2018. Kuala Lumpur, Malaysia.

2. Alireza, P., Shaharudin,Ab, R, (2016). *Transgenic hematopoietic stem cells*, paper presented at the 5th International Conference on advances in Applied Science and Environmental Engineering (ASEE), 12-13th March 2016. Kuala Lumpur. Malaysia.

3. Alireza, P., Shaharudin,Ab, R, (2016). *Cloning and characterization of ly49h, ly49d and CD94 genes in hematopoietic stem cells*, paper presented at the 5th International Postgraduate Research Awards Seminar (InPRAS2016), 7– 8th March 2016. Kuala Lumpur, Malaysia.