AIX-MARSEILLE UNIVERSITÉ

FACULTÉ DE MÉDECINE DE MARSEILLE

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

T H È S E

Présentée et publiquement soutenue devant

LA FACULTÉ DE MÉDECINE DE MARSEILLE

Le 18 juin 2018

Par **Mohammed TAHA**

Né le 02 juin 1980 à RAFAH (PALESTINE)

L'UTILISATION DE CELLULES NATURAL KILLER (NK) COMME OUTIL THERAPEUTIQUE: ETUDE CLINIQUE DE PHASE I DE PERFUSION DE CELLULES NK DU DONNEUR APRES HSCT

ANNEXE: PUMILIO 2, UNE PROTEINE DE LIAISON A L'ARN SUREXPRIMEE DANS LES CELLULES NK DE PATIENTS ATTEINTS DE LAM, REPRIME LES FONCTIONS DES CELLULES NK

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Laboratoire Immunologie des Tumeurs, Centre de Recherche en Cancérologie de Marseille (UMR1068)

Membres du Jury de la Thèse :

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Dr. Martin VILLALBA	Rapporteur
Pr. Régis COSTELLO	Examinateur
Dr. Christelle RETIERE	Examinateur
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LIST OF ABBREVIATIONS

A

ADCC: Antibody-Dependent Cell-Mediated Cytotoxicity

Ago: Argonaute Protein

ALL: Acute Lymphocytic Leukemia

Allo-SCT: Allogeneic Stem Cell Transplantation

AML: Acute Myeloid Leukemia

aNKIS: Activating NK Cell Immunological Synapse

AP-1: Activator Protein 1

APA: Alternative Polyadenylation

APCs: Antigen-Presenting Cells

Auto-SCT: Autologous SCT

B

BM: Bone Marrow

BZIP: Basic Leucine Zipper

C

CEACAM: Carcinoembryonic Antigen-related Cell Adhesion Molecule 1

ChILPs: Common Helper ILC Precursors

CLP: Common Lymphoid Progenitor

CMPs: Common Myeloid Progenitors

CMV: Cytomegalovirus

CML: Chronic Myeloid Leukemia

CR: Complete Remission

CSTF: Cleavage Stimulation Factor

D

DAP10: DNAX-activation protein 10

DCs: Dendritic Cells

DD: Death Domain

DED: Death Effector Domain

DISC: Death Inducing Signaling Complex

DLI: Donor Lymphocyte Infusion

DNAM-1: DNAX accessory molecule-1

E

EAT2: Ewing's Sarcoma-Associated Transcript 2

E4BP4: E4-Binding Protein 4

eEF1A: Eukaryotic Elongation Factor 1A

eIFs: Eukaryotic Initiation Factors

eIF4E: Eukaryotic Initiation Factors 4E

Eomes: Eomesodermin

ETS-1: E26 Transformation-Specific 1

F

FADD: Fas-Associated Death Domain

FcγRs: Fragment gamma Receptors

Foxp3: Forkhead box P3

G

GITRL: Glucocorticoid-Induced TNFR-Related Protein Ligand

G-CSF: Granulocyte Colony-Stimulating Factor

GM-CSF: Granulocyte–Macrophage Colony-Stimulating Factor

GRB2: Growth Factor Receptor-Bound Protein 2

H

HA: hemagglutinin

Haplo-SCT: Haploidentical SCT

HER2: Human Epidermal Growth Factor Receptor 2

HLA: Human Leukocyte Antigen

HPCs: Hematopoietic Progenitor Cells

HSCs: Hematopoietic Stem Cells

HSCT: Hematopoietic Stem Cell Transplantation

I

iDCs: Immature DCs

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

ILCs: Innate Lymphoid Cells

iNK: immature NK cells

iNKIS: Inhibitory NK Cell Immunological Synapse

ILT-2: Immunoglobulin-like Transcript-2

ITAMs: Immunoreceptor tyrosine-based activating Motifs

ITIMs: Immunoreceptor Tyrosine-based Inhibitory

J

JAK: Janus Tyrosine-Kinase

K

KIRs: Killer Cell Immunoglobulin-like Receptors

L

LAT: Linker for the Activation of T cells

LFA-1: Lymphocyte Function-associated Antigen 1

LIR-1: Leukocyte Immunoglobulin-like Receptor-1

LPS: Lipopolysaccharide

M

mAbs: Monoclonal Antibodies

MAPK: Mitogen-Activated Protein Kinase

mDCs: mature DCs

MHC: Major Histocompatibility Complex

MICA: MHC class I-chain-related proteins A

MICB: MHC class I-chain-related proteins B

miRNAs: MicroRNAs

MLL5: Mixed-Lineage Leukemia-5

MM: Multiple Myeloma

mNK: mature NK cells

MRNPs: Messenger Ribonucleoproteins

N

NCRs: Natural Cytotoxicity Receptors

NFIL3: Nuclear Factor Interleukin-3 Regulated

NF-kB: Nuclear Factor-kappa B

NK cells: Natural Killer cells

NKG2D: Natural-Killer Group 2, member D

NKP: NK Cell Precursor

NLK: Nemo-Like Kinase

NMPD: National Marrow Donor Program

NREs: Nanos Response Elements

NTAL: Non-T cell Activation Linker

O

OS: Overall Survival

P

PABP: Poly (A) Binding Protein

PB: Peripheral Blood

PCNA: Proliferating Cell Nuclear Antigen

PD-1: Programmed Cell Death Protein-1

PDCs: Plasmacytoid DCs

PI3K: Phosphatidylinositol-3-Kinase

PLC: Phospholipase C

PRE: PUF Response Element

PUF: PUmilio-Fem-3-Binding Factor

Pum1: Pumilio 1 Protein

Pum2: Pumilio 2 Protein

Pum-HD: Pumilio Homology Domain

R

RBDs: RNA-Binding Domains

RBPs: RNA-Binding Proteins

RIC: Reduced-Intensity Conditioning

RORγt: Retinoic Acid Receptor -Related Orphan Receptor

ROS: Reactive Oxygen Species

S

S1P5: Sphingosine-1 Phosphate Receptor 5

SAP: Signaling Lymphocyte-Activation Molecule-Associated Protein

SET complex: Endoplasmic Reticulum-Associated Complex

SH2: Src Homology 2

SHP1: SH2-domain-containing Protein Tyrosine Phosphatase 1

SLAM: Signaling Lymphocyte Activation Molecule

SLTs: Secondary Lymphoid Tissues

SYK: Spleen Tyrosine Kinase

T

T-bet: T-box expressed in T-cells

TBI: Total Body Irradiation

TCRs: T Cells Receptors

TGF-β: Transforming Growth Factor- β

TH cells: T helper cells

TIGIT: T-Cell Immunoglobulin and ITIM Domain

Tim-3: T-Cell Immunoglobulin Mucin-3

TNCs: Total Nucleated Cells

TNF-α: Tumor Necrosis Factor-α

TNF-β: Tumor Necrosis Factor-β

TRADD: TNFR-Associated Death Domain

TRAIL: TNF-Related Apoptosis-Inducing Ligand

Tregs: Regulatory T cells

TSLP: Thymic Stromal Lymphopietin

U

ULBP: UL16-Binding Protein

UTRs: Untranslated Regions

Z

ZAP70: Zeta Chain-Associated Protein Kinase 70

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PROJECT I

THE USE OF NATURAL KILLER CELLS (NK) AS A THERAPEUTIC TOOL: PHASE I CLINICAL STUDY OF NK DONOR LYMPHOCYTE INFUSION AFTER HSCT

INTRODUCTION

I. NATURAL KILLER CELLS

1. Immune system

The immune system (from the Latin word immunis, meaning "free" or "untouched") is a complex system, comprised of many biological structures and processes, which protects the body against pathogens and diseases. All immune cells originate from a common hematopoietic stem cell (HSC) in a process known as hematopoiesis. Naturally, the immune system is divided into two principal structures: innate immunity and adaptive immunity (figure 1). The innate immunity is the first line of defense consisting of variety of myeloid and lymphoid cells that can exert rapid nonspecific defense mechanisms immediately or within hours of an antigen appearance in the body. In contrast, adaptive immunity refers to antigen-specific immune response controlled by two types of lymphocytes, T and B cells. The adaptive immune response is more complex than the innate because it requires recognition of specific "non-self" antigens during a process called antigen presentation. It also includes a memory that makes future responses against a specific antigen more efficient.

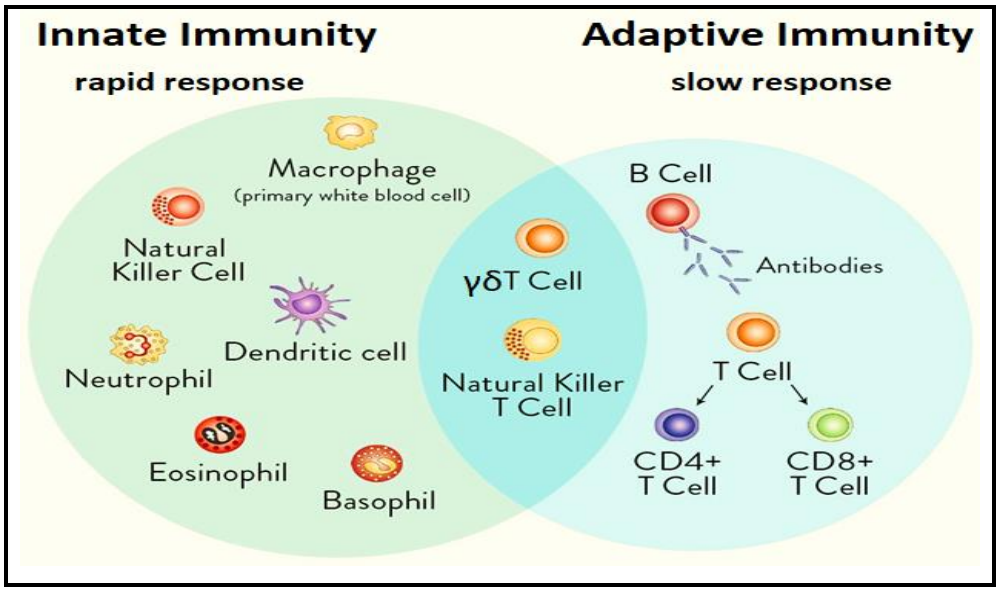

Figure 1: Cellular components of innate and adaptive immunity (adapted from Dranoff, 2004)

2. Innate lymphoid cells

Innate lymphoid cells (ILCs) are recently identified components of the innate immune system, characterized by lymphoid cell morphology with missing antigen-specific receptors (Spits and Cupedo, 2012). They are involved in innate immunity, and in tissue development and remodeling by producing several cytokines in response to their stimulation with cytokines, cellular ligands, and pathogen-associated molecular patterns. Two prototypic members of the ILCs were discovered many years ago: Natural killer (NK) cells which are involved in killing infected, stressed or transformed cells (Kiessling et al., 1975), and lymphoid tissue inducer (LTi) cells discovered in 1997 and induce the formation of lymph nodes during embryogenesis (Mebius et al., 1997). Recently, other members of the ILC family were identified and have been classified into three main groups (ILC1, ILC2, and ILC3) based on their effector cytokines, surface markers expression and to the transcription factors required for their differentiation (Juelke and Romagnani, 2016; Spits et al., 2013). ILC1s are characterized by the need of T-box expressed in T-cells (T-bet) transcription factor for their development and production of interferon-γ (IFN-γ), a potent stimulator of phagocytes for antimicrobial activity effectively controlling intracellular infections. They include NK cells and other IFN-γ-secreting ILCs cells distinct from NK cells. In addition to T-bet and IFN-γ production, NK cells are characterized by cytotoxic activity, and expression of perforin and Eomesodermin (Eomes).

ILC2s require GATA3 for their development and produce type 2 cytokines, especially interleukin-5 (IL-5), IL-9 and IL-13 in respond to IL-25, IL-33, and thymic stromal lymphopietin (TSLP) stimulation. They are involved against helminthic infections as well as to the pathogenesis of allergic inflammation (Juelke and Romagnani, 2016; Spits et al., 2013).

The third group of ILCs (ILC3s) is characterized by the production of cytokines typically produced by T_H17/T_H22 cells, IL-17 and IL-22, and depends on retinoic acid receptor-related orphan receptor (RORγt) for its development and/or survival (Juelke and Romagnani, 2016; Spits et al., 2013). Moreover, ILC3s are subdivided into $NKp44^+$ and $NKp44^-$ ILC3s in human subjects, where $NKp44^+$ ILC3s highly produce IL-22, while $NKp44^-$ ILC3s produce mostly IL-17A (Hoorweg et al., 2012).

The ILC populations develop from common lymphoid progenitor (CLP) that lead to T cell and B cell precursors, NK cell precursor (NKP), and the recently identified common helper ILC precursor

(ChILP) that express Id2 and variable levels of PLZF transcription factors (figure 2), (Klose et al., 2014; Spits and Cupedo, 2012). ChILPs generate all ILCs groups except NK cells, whereas $PLZF^{+}$ ILC precursors generate all ILC groups but not NK cells or LTi cells (Constantinides et al., 2014; Klose et al., 2014). For ILCs development from CLP (via NKP or ChILP), B and T cell development pathways are restricted through expression of specific transcription factors that activate or repress target genes required for subset-specific lymphocyte differentiation. In this context, several transcription factors are critical at the ILC precursor stage, including Id2, nuclear factor interleukin-3 regulated (NFIL3) and GATA3 (Eberl et al., 2015). ID2 is a transcriptional repressor acting to reduce the activity of E-box transcription factors which are critical in early B and T cell development (Yokota et al., 1999). Consequently, NKP and ChILP express variable levels of Id2, whereas CLP do not express Id2 (Klose et al., 2014; Male et al., 2014). Similarly, GATA3 represses B cell development by blocking EBF1 and thereby supports T and ILC differentiation from CLPs (Yagi et al., 2014).

3. Natural killer (NK) cells

NK cells are large, granular, bone marrow-derived lymphocytes arising from the lymphoid lineage which were identified in 1975 as effector lymphocytes because of their ability to lyse certain tumor cells (Herberman et al., 1975; Kiessling et al., 1975; Sendo et al., 1975). Since then, copious knowledge has been acquired with respect to their origin, differentiation and receptors as well as effector functions. NK cells have been classified as lymphocytes depending on their morphology, their expression of some lymphoid markers, and their origin from CLP in the bone marrow, however, they are considered to be crucial components of the innate immune defense due to the direct kill of their target cells in the absence of specific immunization and lack of antigen-specific cell surface receptors. Currently, NK cells have been reclassified as a subset of cytotoxic innate lymphoid cells (ILCs) (Spits et al., 2013; Vivier et al., 2011).

As NK cells do not fully conform to the definition of adaptive immunity, they also differ from members of the innate immune system. For instance, NK cells do not mediate phagocytosis and lack bactericidal enzymatic systems. Rather, they express intracellular proteins associated with effector functions, including granzymes and perforin. Generally, resting NK cells circulate in the blood and have the ability to infiltrate into most tissues that contain pathogen-infected or malignant cells following activation by cytokines (Biron, 1997; Fogler et al., 1996; Glas et al., 2000). Subsequently,

the activated NK cells are capable to kill their target cells by direct cytotoxicity through the release of granules containing cytotoxic enzymes (perforin and granzymes), and/or by releasing cytokines as IFN-γ and tumor necrosis factor-α (TNF-α) (Lieberman, 2003; Trinchieri, 1989; Vivier et al., 2011).

4. NK cell phenotype and subsets

In humans, NK cells are considered as the third largest population of lymphocytes following T and B cells comprising approximately 10 – 15 % of all peripheral blood lymphocytes (Cooper et al., 2001a; Timonen et al., 1982). Phenotypically, they are defined as $CD3^{-}CD56^{+}$ lymphocytes upon their expression of CD56 (NCAM1, Neural Cell Adhesion Molecule, 140 kDa isoform) and lack of expression of the T cell marker CD3 (Lanier et al., 1986). Moreover, NK cells can be subdivided into various subsets with diverse functions based on the surface expression of CD56 and CD16 or inhibitory and activating receptors (Bryceson et al., 2006a; Jonges et al., 2001). Broadly, two circulating major populations of human NK cells were initially described based on the expression of CD56 and CD16: $CD56^{bright}$ $CD16^{-}$ and $CD56^{dim}$ $CD16^{+}$ (Cooper et al., 2001a; Ferlazzo and Munz, 2004)(Jacobs et al., 2001). $CD56^{bright}$ $CD16^{-}$ NK cells are immature, cytokine-producing NK cells (5 – 10% of NK cells), being dominant in the lymph nodes and probably function as immunoregulatory cells by secreting cytokines such as IFN-γ, TNF-α, IL-10, IL-13 and granulocyte–macrophage colony-stimulating factor (GM-CSF). Phenotypically, $CD56^{bright}$ $CD16^{-}$ cells highly express NKG2A inhibitory receptor and have low to absent expression of killer cell immunoglobulin-like receptors (KIRs) (Cooper et al., 2001a; Ferlazzo and Munz, 2004; Jacobs et al., 2001). In contrast, around 90 - 95% of peripheral blood NK cells are $CD56^{dim}$ $CD16^{+}$ cells which are cytotoxic cells with weak proliferation activity and low levels of cytokines production at resting state (Cooper et al., 2001b; Fehniger et al., 2003; Vivier, 2006). However, $CD56^{dim}$ NK cells largely secrete proinflammatory cytokines and chemokines more than $CD56^{bright}$ cells after target cell stimulation (Fauriat et al., 2010a).

Additional NK cell subpopulations have been discovered under specific pathological conditions, such as $CD56^{-}$ $CD16^{+}$ NK cells in HIV-infected patients which have a poor cytotoxic function due to the extreme expression of inhibitory NK receptors and the low expression level of natural cytotoxicity receptors (NCRs) in the $CD56^{-}$ NK subset (Alter et al., 2005; Mavilio et al., 2005). Further, two extra NK cell subsets, characterized by immature and noncytotoxic properties, are

found in the peripheral blood of patients with advanced breast cancer referred as $CD56^{bright}CD16^{+}$ and $CD56^{dim}CD16^{-}$ NK cells (Mamessier et al., 2013).

5. NK cell development

Hematopoietic stem cells (HSCs) are multipotent, self-renewing progenitor cells which give rise to CLPs (the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells) during the process of hematopoiesis (figure 2), (Reya et al., 2001). In general, NK cells develop during fetal life as well as after birth from $CD34^{+}$ hematopoietic stem cells which differentiate into CLPs that can give rise to NKP. It has been commonly reported that the bone marrow (BM) is considered to be the main site of NK cell development similar to B cells (Colucci et al., 2003; Galy et al., 1995; Haller et al., 1977). However, NK development might not occur exclusively in BM because $CD56^{bright}$ NK cells are relatively dominant in secondary lymphoid tissues (SLTs) as lymph nodes compared with $CD56^{dim}$ NK cells which are abundantly clear in BM (Ferlazzo et al., 2004a). Activation of SLTs $CD56^{bright}$ NK cells increases the expression of NK receptors that are characteristic of $CD56^{dim}$ NK subset. In addition, some studies have notified that NK cell development and maturation can take place in other sites such as the thymus (Vosshenrich et al., 2006) and lymph nodes (Freud et al., 2005).

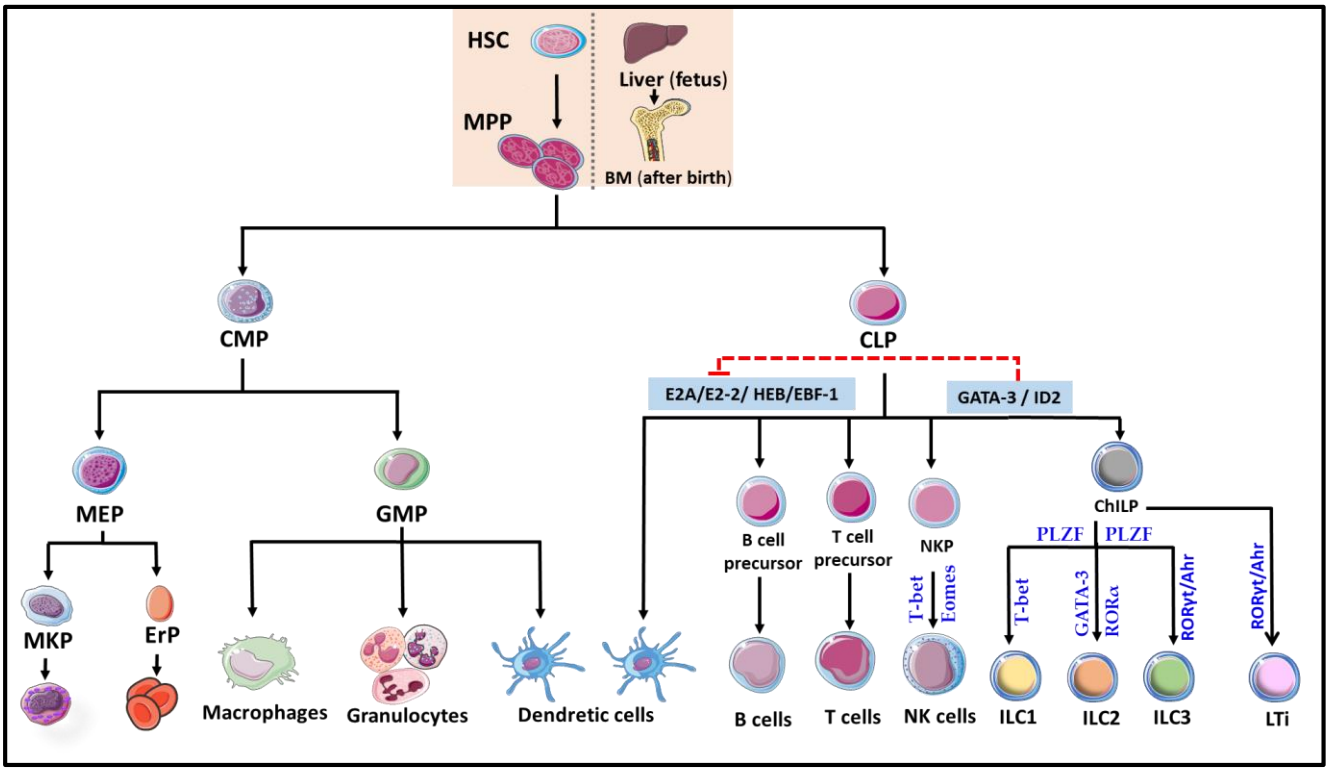

Figure 2: Development of hematopoietic stem cells (adapted from Fuchs, 2016; Reya et al., 2001)

6. NK cell differentiation and maturation

The differentiation pathway of human NK cell is complex, characterized by losing the expression of some surface antigens as CD34 and increasing the expression of other surface antigens such as CD56, NKG2A, CD16, KIRs and CD57 which results in a heterogeneous $CD3^{-}CD56^{+}$ NK cell population. Moreover, a variety of activating and inhibitory receptors are acquired during maturation of immature NK cells into functional NK cells. In general, two major subpopulations of blood $CD3^{-}CD56^{+}$ NK cells can be discriminated based on CD56 expression, $CD56^{bright}CD16^{-/+}$ and $CD56^{dim}CD16^{+}$. Since their discovery, it has been assumed that blood $CD56^{bright}$ and $CD56^{dim}$ NK cells may represent two sequential stages of NK cell differentiation based on the following explanations: (i) only blood $CD56^{bright}$ NK cells express CD117 (c-kit), which is typically expressed by immature hematopoietic progenitor cells (HPCs) (Matos et al., 1993); (ii) culture of $CD34^{+}$ cells in IL-2 or IL-15 results in development of $CD56^{bright}CD16^{-}KIR^{-}$ NK cells *in vitro* (Jaleco et al., 1997; Mingari et al., 1997; Mrózek et al., 1996); and (iii) at $CD34^{+}$ stem cell transplantation, donor-derived $CD56^{bright}$ NK cells appear in the recipient's blood immediately after engraftment, whereas $CD56^{dim}$ NK cells appear later,

concomitant with a decrease of $CD56^{bright}$ NK cells (Shilling et al., 2003; Vitale et al., 2004). (iv) a reduced frequency of $CD3^{-}C56^{dim}$ cells and accumulation of $CD3^{-}CD56^{bright}$ cells in PB due to a maturation defect, which exhibited an altered activation in response to IL-2 and IL-15 (Domaica et al., 2012). There are different stages of human NK cell differentiation have been recognized based on phenotypic analysis of several surface markers including CD34, CD45RA, CD117, CD94/NKG2A, CD56, NKRs, KIR CD16, CD57) (Montaldo et al., 2013; Scoville et al., 2017). Figure 3 shows differentiation stages of NK cells from the precursors.

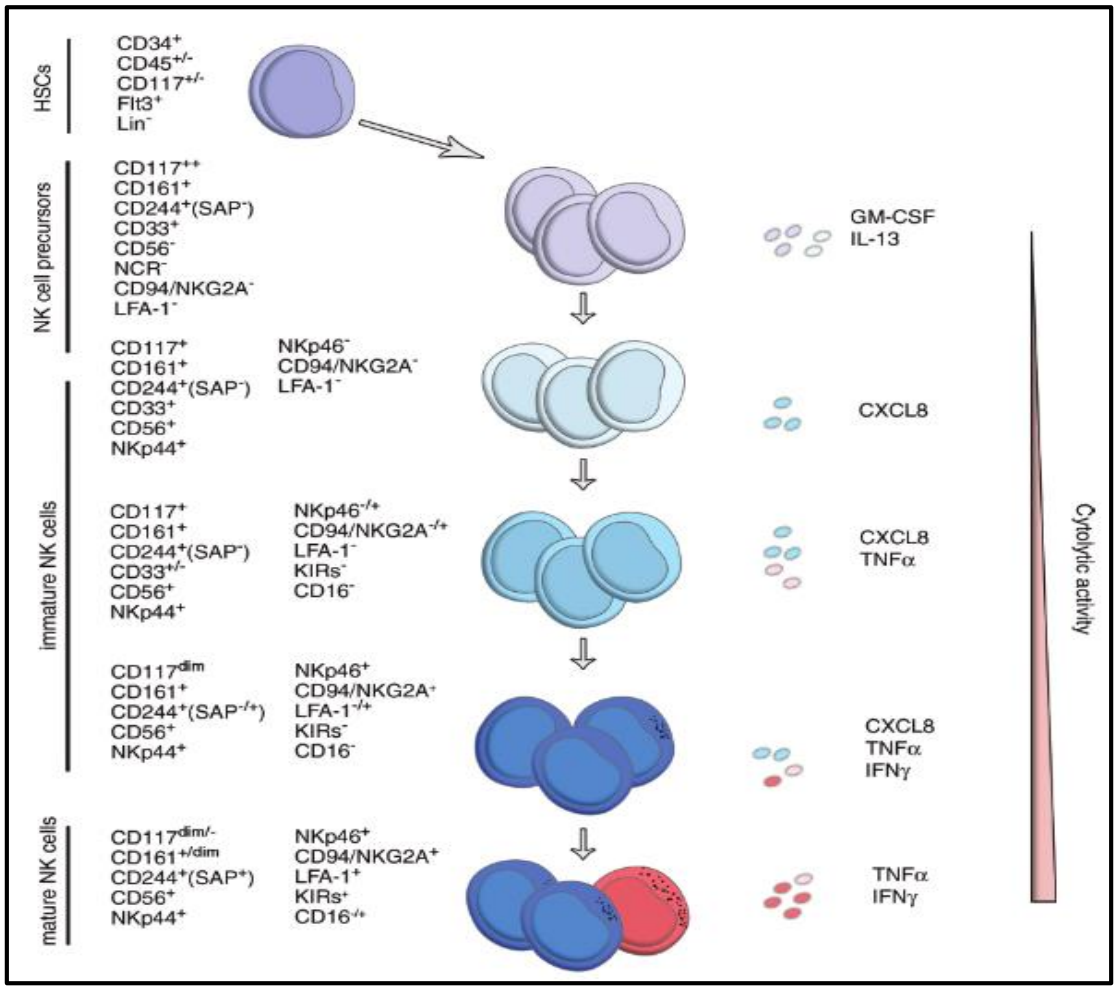

Figure 3: Stages of NK cells differentiation and maturation (Montaldo et al., 2013)

7. Factors regulating NK cell differentiation

NK cell differentiation process seems to be a coordinated mechanism that involves many factors, including transcription factors and cytokines that are required at different stages in NK cell development (Reviewed by Boos et al., 2008). Additionally, several studies have shown the importance of microRNAs (miRNAs) in the differentiation and maturation of immune cells including NK cell (reviewed by Kumar Kingsley and Vishnu Bhat, 2017).

i- Transcription factors

Transcription factors have a prominent effect on immune cell development and differentiation by regulating gene transcription of specific molecules controlling these processes. There are numerous transcription factors involved in regulation of NK cell differentiation and maturation. One of these factors is Id2, which belongs to inhibitor of DNA binding (ID) family and contains a helix-loop-helix (HLH) domain (Benezra et al., 1990). By its HLH domain, Id2 antagonizes the activity of E-box binding (E) protein transcription factors (E2A, E12 and E47) and prevent their binding with DNA (Sun et al., 1991). Boos *et al.* demonstrated that Id2 has a role in NK cell differentiation but at later stage because they found that Id2 deficient mice have low number of mature (mNK) cells in the periphery while having normal number of iNK cells and NKPs in the BM (Boos et al., 2007). Another example is E4-binding protein 4 (E4BP4, also called NFIL3), being a basic leucine zipper (bZIP) transcription factor that has a potential regulatory role in the immune system (Reviewed by Male et al., 2012). E4BP4-deficient mice showed a sharp reduction in both iNK and mNK numbers, suggesting that E4BP4 is required for the progression from NK cell precursors to iNK cells and then from iNK cells to mNK cells (Gascoyne et al., 2009; Kamizono et al., 2009). Recently, Male *et al.* showed that E4BP4 is necessary for NKP development from CLP, and for NK cell development at early stage by controlling the expression of Eomes and Id2 (Male et al., 2014). In addition, T-box transcription factors family including T-bet (also known as Tbx21) and Eomes are defined as basic drivers of immune cell development and cytolytic function (Knox et al., 2014). In humans, Knox et al. have found that Eomes was expressed in both $CD56^{bright}$ and $CD56^{dim}$ NK cells, but it was significantly higher in $CD56^{bright}$ suggesting that Eomes is likely essential for $CD56^{bright}$ maturation (Knox et al., 2014). With respect to T-bet, T-$bet^{-/-}$ mice exhibited a reduced number of NK cells in the spleen, liver and peripheral blood, while an increase of NK cells in LNs and BM because T-bet regulates the expression of sphingosine-1 phosphate receptor 5 (S1P5) that plays an important role in NK cell

recirculation (Jenne et al., 2009; Townsend et al., 2004). T-bet$^{-/-}$ NK cells also showed an impaired cytotoxicity and IFN-γ production (Townsend et al., 2004). ETS-1 also has an important role in differentiation of NK cell lineage which belongs to Ets (E26 transformation-specific) family. ETS-1-deficient mice have a reduced number of NK cells especially in the spleen and BM (Barton et al., 1998). Ramirez *et al.* found that ETS-1 acts at early stages of NK cell development to boost the expression of fundamental transcriptional regulators including T-bet and Id2. Regarding of GATA-3 which binds DNA at "GATA" sequence, normal NK-cell number was observed in the BM and spleen of GATA-3-deficient mice but having immature phenotype with normal cytotoxic activity and poor production of IFN-γ (Samson et al., 2003).

ii. Cytokines

Commonly, cytokines play an important regulatory role in variety events of the immune system, including development, proliferation, homeostasis, and activation status. In the context of NK cells, IL-2, IL-7, IL-15 and IL-21 are the major cytokines that regulate NK cell development, differentiation, survival, and function acting via receptors using the common cytokine receptor γ-chain (γc). Their receptors are signaling through three main transduction pathways: the Janus tyrosine kinases (JAK)-STAT pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, and the mitogen activated protein kinase (MAPK) pathway (figure 4), (reviewed by Marçais et al., 2013; Romee et al., 2014; Vosshenrich et al., 2005). In contrast to other cytokines, IL-18 receptor (IL-18R) principally transduces signals through the adapters MyD88 and TRAF6 leading to MAPK and nuclear factor-kappa B (NF-kB) activation (reviewed by Dinarello et al., 2013). Table 1 summarizes the key interleukins regulating NK cell differentiation and other biological functions (reviewed by Dinarello et al., 2013; Marçais et al., 2013; Romee et al., 2014; Vosshenrich et al., 2005).

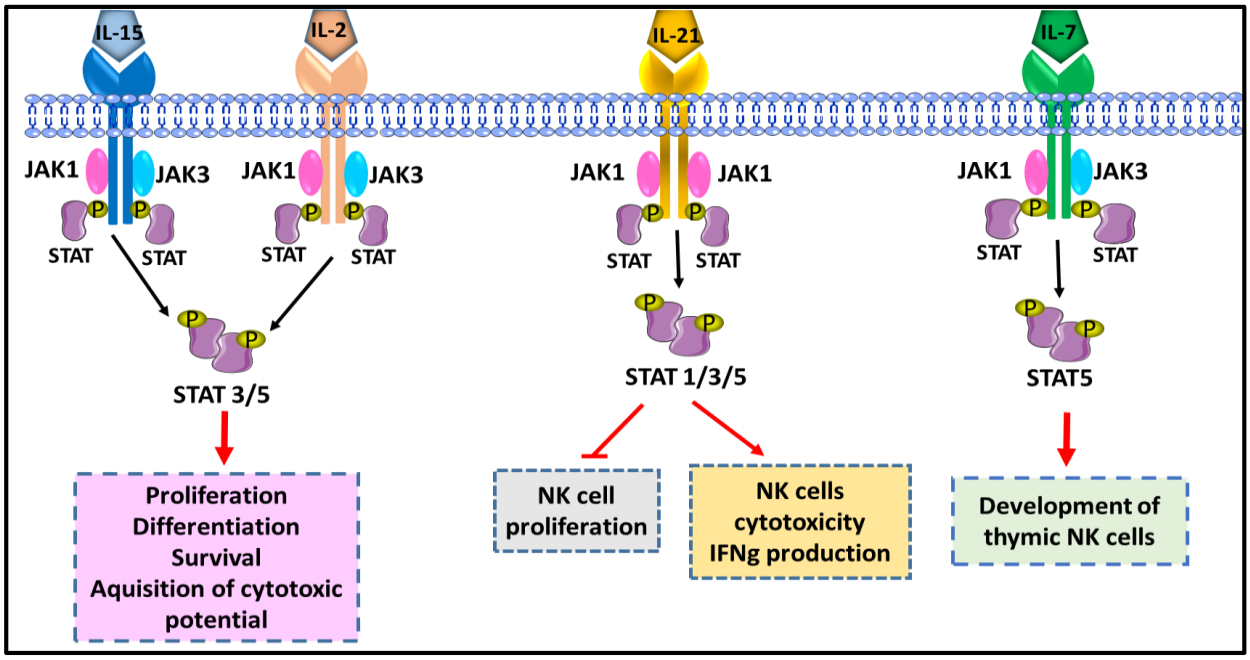

Figure 4: γc Cytokines regulating NK cell biology (adapted from Marçais et al., 2013)

Table 1: Major interleukins regulating NK cell differentiation and other biological functions

Cytokine	Source	Signaling	Function
IL-2	T cells	JAK1/3 STAT3/5 MAPK	Cytokine production Proliferation Survival Enhanced cytotoxicity
IL-7	Stromal cells	JAK1/3 STAT5	Development of thymic NK cells Proliferation
IL-12	DCs Macrophages	JAK2/ TYK2 STAT3/4/5	Differentiation Proliferation Cytokine production Enhanced cytotoxicity
IL-15	DCs Macrophages Stromal cells	JAK1/3 STAT3/5 MAPK	Cytokine production Proliferation Survival Maturation Enhanced cytotoxicity
IL-18	DCs Macrophages	MyD88 MAPK NF-kB	Cytokine production Proliferation Cytokine production Proliferation
IL-21	T cells	JAK1/3 STAT1/3/5	Differentiation Enhanced cytotoxicity Cytokine production Limits proliferation

vii. MicroRNAs

MicroRNAs (miRNAs) are a large family of short non-coding (~22 nucleotides) RNA molecules that regulate gene expression post-transcriptionally by targeting the 3' untranslated regions (UTRs) of mRNAs, resulting in translational inhibition and/or mRNA degradation (Bartel, 2004). The primary indication of immune response regulation by miRNAs was established based on the expression of some miRNAs as miR-181 and miR-223 in immune cells (Chen et al., 2004). There are several miRNAs have shown affecting the development and maturation of NK cells. For example, a consistent increase in the expression levels of miR-181a/b is associated within different stages of NK cell development, suggesting that miR-181 has an impact on human NK cells development from $CD34^{+}$ hematopoietic progenitors (Cichocki et al., 2011). MiR-181 establishes its effect on NK cells development through downregulation of nemo-like kinase (NLK), which is a target of miR-181 in NK cells. NLK is a protein kinase that negatively regulates Notch-dependent transcriptional activation pathway, where previously has been shown that Notch signaling pathway is important in NK cell development (Bachanova et al., 2009).

Another example is miRs-15/16 which are highly expressed in NK cells and are involved in regulation of NK cell development by controlling the level of *Myb* (also known as *c-Myb*) (Sullivan et al., 2015). *Myb* is a transcriptional activator factor related to *Myb* (myeloblastosis) family that is highly expressed in iNK cells ($CD56^{bright}$ NK cells), however, its expression is decreased during NK cell maturation ($CD56^{dim}$ NK cells). It has been found that iNK cells are accumulated, while mNK cells are reduced in the absence of miR-15/16 (Sullivan et al., 2015).

Further, it was noted by Mundy-Bosse and his colleagues that iNK cells were extremely reduced in acute myeloid leukemia (AML) patients due to a disruption in NK cell differentiation (Mundy-Bosse et al., 2016a). They also found low levels of T-bet and Eomes, transcription factors having a vital role in NK cell differentiation, as a result of elevation of miR-29b level among leukemic NK cells (miR-29b is a regulator of T-bet and Eomes). As a consequence of miR-29b deletion in NK cells, the iNK subset is returned to appear again (Mundy-Bosse et al., 2016a).

8. NK cell receptors

It is clear that maturation of iNK cells into totally functional NK cells comprises acquisition of a series of cell surface receptors that also regulate NK cell effector functions. Although MHC class I molecules are essential for protection from NK cells lysis, inhibition effect by MHC class I is not always sufficient to avoid NK cytotoxicity. For instance, NK cells are able to kill some virus-infected cells that keep expression of MHC class I at their cell surface (Malnati et al., 1993; Routes, 1992). Also, some NK cell-resistant targets have been lysed by IL-2 activated NK cells, suggesting that IL-2 activated NK cells have increased lytic activity compared with circulating NK cells (Caligiuri et al., 1990). Accordingly to these observations, there are additional surface molecules involved in regulating NK cell effector functions. Generally, these molecules can be classified into different groups: inhibitory receptors, activating receptors, adhesion molecules, cytokine and chemotactic receptors (figure 5).

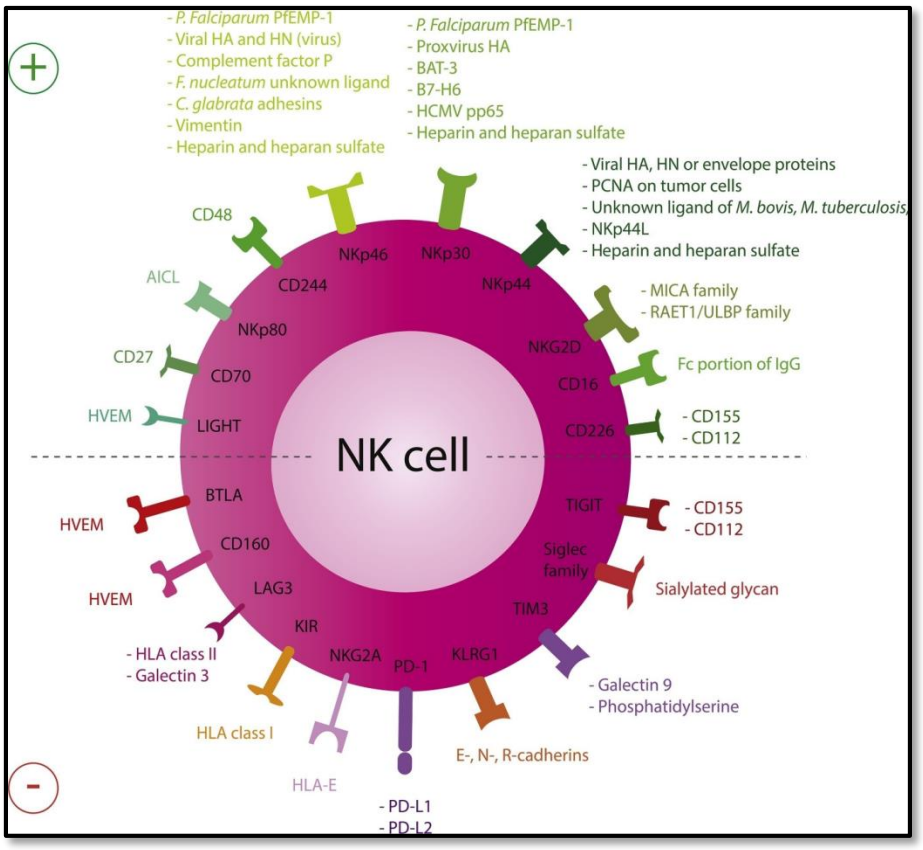

Figure 5: Human NK cell receptors (Chiossone et al., 2017)

i. Inhibitory receptors

NK cell functions are negatively controlled by a set of surface expressed inhibitory receptors via recognition and engagement of specific ligands expressed on the surface of their targets. Normally, human NK cells express many inhibitory receptors including CD94/NKG2A heterodimers, inhibitory KIRs, immunoglobulin-like transcript-2 (ILT-2), LAIR-1, and CEACAM1. Among these receptors, NKG2A and inhibitory KIRs are the major inhibitory receptors where their corresponding ligands are related to human leukocyte antigens (Reviewed by Lanier, 2003; Pegram et al., 2011).

Inhibitory receptors transmit their inhibitory signals through intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), located in the cytoplasmic tail of these receptors. Following binding the ligand with its receptor, the tyrosine residues in ITIMs are phosphorylated, and thus recruit and activate Src homology 2 (SH2) domains of the intracytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2. Then, these phosphatases inhibit the activation of NK cells by dephosphorylation of specific intracellular signaling molecules at multiple stages of signaling cascade (reviewed by Long et al., 2013; Ravetch and Lanier, 2000).

a. CD94/NKG2A

NKG2A is a transmembrane protein structurally characterized by C-type lectin extracellular domains, covalently coupled to CD94 subunit (Brooks et al., 1997; Carretero et al., 1997). CD94/NKG2A receptor is found as a heterodimer that recognizes non-classical HLA class I molecule, HLA-E (Braud et al., 1998). It is naturally expressed on about half of all NK cells, and its expression is not stable and can be affected by cytokines present in the surrounding environment. CD94 has a short cytoplasmic domain lacking signaling function, whereas NKG2A contains ITIM domain in its intracellular structure, which upon tyrosine phosphorylation can recruit SHP-1 or SHP-2 providing inhibitory signal transduction (Carretero et al., 1998).

b. KIRs

KIRs (CD158) are transmembrane glycoproteins, encoded on chromosome 19q13.4, and belong to immunoglobulin (Ig) superfamily. KIRs family have been named and classified into 14 groups (*2DL1* to *2DL5*, *3DL1* to *3DL3*, *2DS1* to *2DS5*, and *3DS1*) according to number of extracellular Ig-like domains, and cytoplasmic tail length (Marsh et al., 2003; Vilches and Parham, 2002). They are monomeric receptors, structurally characterized by either 2 extracellular Ig-like domains

(designated D1 and D2 in KIR2D) or 3 extracellular Ig-like domains (designated D0, D1 and D2 in KIR3D). KIRs recognize and engage MHC class I ligands, including alleles of HLA-A (Pende et al., 1996), HLA-B (Litwin et al., 1994; Wagtmann et al., 1995), and HLA-C (Wagtmann et al., 1995). This group of receptors is a very polymorphic receptor family, but all KIRs members have an identical extracellular domain and consequently, have the same ligands (HLA-A, B and C). In contrast, these receptors have differences in their cytoplasmic domains and are subdivided into KIRs with long (L) cytoplasmic tails (KIR2DL and KIR3DL) and others with short (S) cytoplasmic tails (KIR2DS and KIR3DS). Based on these variances, KIRs are classified to two functionally distinct sets: inhibitory KIRs (KIR2DL and KIR3DL, where the long tails generate an inhibitory signal) and activating KIRs (KIR2DS and KIR3DS, wherever short tail KIRs generate an activation signal), (figure 6). A current listing of known KIRs and their known ligands can be found in table 2 (Rajalingam, 2011).

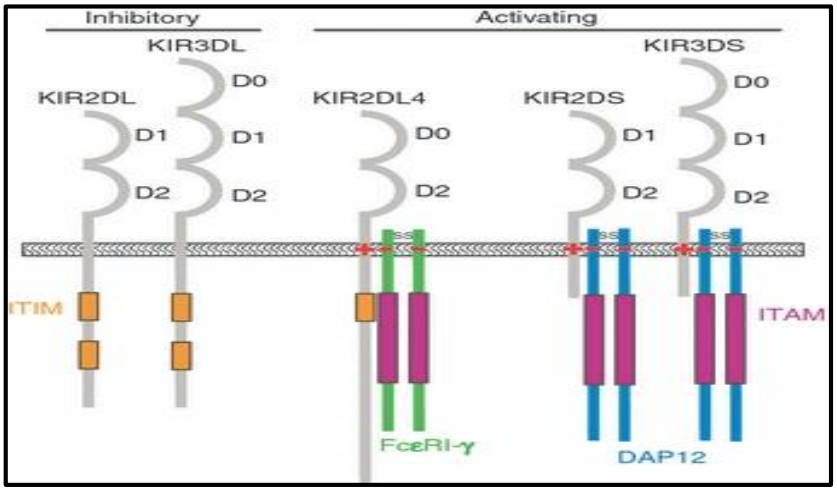

Figure 6: Structural differences between inhibitory and activating KIRs (Campbell and Purdy, 2011)

- ***Inhibitory KIRs***

Inhibitory KIRs signal through their ITIMs in the cytoplasmic domain with long tail. Following engagement with their ligands, the tyrosine residues of ITIMs are phosphorylated by Src family kinases, and then recruit protein tyrosine phosphatases, including SHP-1, which are critical for mediating inhibitory function. The known ligands for inhibitory KIRs are HLA-class I (HLA-A, B and C) molecules. For example, HLA-A is recognized by KIR3DL2. HLA-C is the dominant HLA class I locus that provides ligands for many KIR receptors. All HLA-C allotypes carry valine at position 76, while

position 80 displays a dimorphism of either asparagine (C1 epitope, binding with KIR2DL2 and 2DL3) or lysine (C2 epitope, binding with KIR2DL1), (Wagtmann et al., 1995; Winter and Long, 1997). Likewise, all HLA-B allotypes have either the Bw4 or Bw6 epitope, but only the Bw4 epitope is a ligand for KIRs (KIR3DL1) (Gumperz et al., 1995; Wagtmann et al., 1995).

Another long cytoplasmic tail KIR, KIR2DL4, has the ability to bind with HLA-G, non-classical MHC class I allele HLA-G (Rajagopalan and Long, 1999). In contrast to other KIRs, KIR2DL4 is thought to be expressed by all NK cells including NK $CD56^{bright}$ cells and resides primarily in endosomes (Rajagopalan, 2010). This receptor was initially classified as an inhibitory receptor because of the presence of ITIM in its cytoplasmic domain, and also it has an arginine residue capable of recruiting the FcεRIγ protein possessing ITAMs to perform activation activity. Although it has a long cytoplasmic tail that is typical of inhibitory KIR, engagement of KIR2DL4 results in the activation of NK cells, not for cytotoxicity, but for IFN-γ production (Rajagopalan et al., 2001).

Table 2: Activating and inhibitory KIRs and their ligands

KIR		Ligand
Inhibitory KIRs	2DL1	HLA-C group 2
	2DL2/3	HLA-C group 1, B46, B73 and some HLA-C group 2
	3DL1	HLA-Bw4
	3DL2	HLA-A3, A11
	3DL4	HLA-G
	3DL5	Not known
	3DL3	Not known
Activating KIRs	2DS1	HLA-C group 2
	2DS2	Not known
	2DS3	Not known
	2DS4	HLA-A11 and subsets of HLA-C
	2DS5	Not known
	3DS1	Not known

ii. Activating receptors

NK cells express a wide group of activating receptors, which upon ligation transmit activating intracellular signals to initiate the effector functions of NK cells (figure 5). The major activating receptors include NKG2D (Natural-Killer Group 2, member D), NCRs, DNAX accessory molecule (DNAM)-1, CD16 (Fcγ RIII) and activating KIRs (reviewed by Lanier, 2003; Long et al., 2013). In addition, several co-receptors act in concert with the major activating receptors for NK cell activation including NKp80, CD2, CD160 and the SLAM family members 2B4 and NTB-A (reviewed by Lanier, 2003; Long et al., 2013). Commonly, NK activating receptors signal through two prominent signaling pathways: immunoreceptor tyrosine-based activating motifs (ITAMs) pathway, and DNAX-activation protein 10 (DAP10) pathway.

a. NKG2D

NKG2D is one of the most important activating receptors expressed by NK cells, having the ability to recognize induced-self ligands. These ligands are not expressed or are expressed at low levels on most normal cells, but are upregulated on unhealthy cells due to the activation of pathways associated with malignancy, infection or stress (Raulet, 2003). It is a C-type lectin surface receptor which was identified in 1991 and related to the NKG2 family (Houchins et al., 1991). $CD8^+$ T cells and γδT cells also have NKG2D receptor on their surface, and it plays a central role in their immunity (Bauer et al., 1999). NKG2D binds to stressed-ligands that are overexpressed on the abnormal cells, including MHC class I-chain-related proteins A and B (MICA and MICB), and up to six different proteins called ULBPs (UL16-binding proteins) (Cosman et al., 2001; Raulet et al., 2013; Steinle et al., 2001). Recognition of target cells by NK cells via NKG2D is considered a major mode of natural killing of tumor cells (Bauer et al., 1999; Jamieson et al., 2002).

The intracellular domain of NKG2D does not have ITAM signaling motif, therefore, it signals through its association with adaptor protein called DAP10 (Wu et al., 1999). When the receptor is engaged, DAP10 is phosphorylated and binds with p85 subunit of PI3K or growth factor receptor-bound protein 2 (GRB2), initiating downstream signaling pathways that promote NK cell degranulation and production of cytokines and chemokines such as IFN-γ, TNF-α, GM-CSF, CCL4 and CCL1 (Upshaw et al., 2006; Wu et al., 1999).

b. Natural cytotoxicity receptors (NCRs)

Natural cytotoxicity receptors (NCRs) are non-MHC class-I activating receptors and belong to Ig superfamily, which are expressed almost exclusively by NK cells. In humans, three NCRs (NKp46, NKp44, and NKp30) have been recognized, where NKp46 (NCR1 or CD335) and NKp30 (NCR3 or CD337) are constitutively expressed by all peripheral blood NK cells (Pende et al., 1999; Pessino et al., 1998; Sivori et al., 1997). On other hand, NKp44 (NCR2 or CD336) is expressed on NK cells mainly after IL-2 stimulation (Vitale et al., 1998).

NCRs include an extracellular ligand-binding domain and a transmembrane domain, while they lack functional cytoplasmic tails. So, each receptor is coupled with single dimeric ITAM-containing adaptor proteins (CD3ζ, FcRIγ and DAP12) to generate their activating signals. Among NCRs, NKp30 and NKp46 associates with FcRγ and/or CD3ζ, whereas DAP12 is connected with NKp44 (Joyce and Sun, 2011; Koch et al., 2013). Engagement of NCRs by their ligands results in phosphorylation of tyrosine residue in ITAMs of the adaptor molecules by Src family kinases. The phosphorylated ITAMs recruit and activate both SYK and ZAP70 tyrosine kinases. Consequently, these kinases phosphorylate other adaptor molecules and signaling molecules, including PI3K, phospholipase C (PLC), VAV2 and VAV3 proteins (Cella et al., 2004; Spaggiari et al., 2001; Tassi et al., 2005). By these signaling proteins, calcium flux is induced which finally leads to cellular cytotoxicity and secretion of cytokines such as IFN-γ and TNFα (Koch et al., 2013).

Although realizing the cellular ligands of NCRs represents a big challenge, many ligands have been identified. For example, all of the NCRs bind to cellular heparin or heparan sulfate proteoglycans upregulated on tumor cells, where this binding lead to NK-mediated killing (Hecht et al., 2009). Concerning NKp30, it initiates NK cytotoxic activity via binding to the tumor antigens B7-H6 (Brandt et al., 2009; Kaifu et al., 2011) and BCL-2-associated athanogene 6 (BAG6, also known as BAT3: B-associated transcript 3), (Pogge von Strandmann et al., 2007). Regarding NKp46, few cellular and pathogen-associated ligands have been shown. It recognizes hemagglutinin (HA) expressed by virus infected cells such as Influenza, Sendai and Poxivirus viruses (Mandelboim et al., 2001; Mendelson et al., 2010). Recently, a new ligand for NKp46 was discovered called complement factor P, a soluble plasma glycoprotein involved in the alternative complement pathway (Narni-Mancinelli et al., 2017). For NKp44, several molecules have been described as ligands that stimulate NK cell

cytotoxicity. These ligands include sialylated and sulfated cellular proteoglycans (Ito et al., 2012), influenza virus HA and other viral HA–neuraminidase proteins (Arnon et al., 2001; Jarahian et al., 2009). It also binds with MLL5 (Mixed-Lineage Leukemia-5) expressed mainly on AML blasts (Baychelier et al., 2013). On the other hand, proliferating cell nuclear antigen (PCNA), which is overexpressed by cancer cells, has been described as an inhibitory ligand of NKp44 (Rosental et al., 2011).

Commonly, NCRs are one of the main mechanisms by which NK cells kill their targets (Alvarez-Breckenridge et al., 2012; Pende et al., 1999; Pessino et al., 1998). As a consequence, tumors and viruses alter NCRs and their ligands to escape NK cell immunosurveillance (Costello et al., 2002; De Maria et al., 2003; Fauriat et al., 2007; Venton et al., 2016).

c. Activating KIRs

In contrast to inhibitory KIRs, KIRs with short cytoplasmic tail are activating KIRs. These receptors are associated with ITAM-bearing DAP12 adaptor protein. When activating KIRs are ligated, src family kinases phosphorylate the ITAM-containing adaptor molecule DAP12. Consequently, DAP12 activates zeta chain-associated protein kinase 70 (ZAP70) and spleen tyrosine kinase (SYK) which generate the downstream activation cascade (Vilches and Parham, 2002). There are several activating KIRs such as KIR2DS1 which has similar Ig-like domains to inhibitory KIR2DL1 and also binds HLA-C2 (Stewart et al., 2005). KIR2DS4 is the oldest and most predominant activating KIR receptor, having the ability to bind with C1 and C2 epitopes of HLA-C allotypes and HLA-A11 (Graef et al., 2009).

d. CD16A (FcγRIIIA)

In humans, several constant fragment gamma receptors (FcγRs) are found on the surface of NK cells, DCs, neutrophils, monocytes, macrophages, B cells and some $CD8^+$ T cells including activating receptors such as (FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB) and a single inhibitory receptor, FcγRIIB, which plays a key role in inhibition of IgG-mediated inflammation (Reviewed by Nimmerjahn and Ravetch, 2007). These receptors bind to Fc portion of IgG antibodies to initiate intracellular signaling pathways.

CD16 is a low-affinity IgG Fc receptor (FcγRIII) that is expressed on the surface of NK cells, neutrophils, DCs, monocytes and macrophages (Lanier et al., 1988; Nimmerjahn and Ravetch, 2007). These are two isoforms of CD16, FcγRIIIA (CD16A) which is expressed mainly on majority of $CD56^{dim}$ NK cells, and FcγRIIIB (CD16B) which is found on neutrophils. It has ITAM linked with CD3ζ and Fc-εRIγ. Once CD16 binds to the Fc region of IgG, the tyrosine residues of ITAM are phosphorylated via Src-kinases resulting in activation of PI3K and PLC-γ, which activate PKC and calcium flux. CD16 is responsible on a mechanism of immune defense called antibody dependent cell cytotoxicity (ADCC). Upon ligation of CD16A, NK cells release cytotoxic granules containing perforin and granzymes, causing lysis of target cells such as tumors and virus-infected cells.

e. DNAM-1

DNAM-1 (CD226) is an activating receptor of the immunoglobulin superfamily expressed on NK cells, $CD8^+$ T cells, and other immune cells (de Andrade et al., 2014; Kojima et al., 2003; Shibuya et al., 1996). DNAM-1 recognizes two ligands related to nectin family: poliovirus receptor (PVR; CD155, also known as nectin-like molecule 5) and Nectin-2 (CD112) which are expressed by various healthy tissues as well as tumor cells (Bottino et al., 2003; Tahara-Hanaoka et al., 2004). The interaction between DNAM-1 and its ligands boosts cytokines production and cell-mediated cytotoxicity against DCs, tumor cells and virus infected cells (El-Sherbiny et al., 2007; Iguchi-Manaka et al., 2008; Magri et al., 2011; Matusali et al., 2012; Pende, 2005, 2006).

f. 2B4

2B4 (CD244) is a cell surface glycoprotein of the Ig-superfamily structurally related to CD2 subset called signaling lymphocyte activation molecule (SLAM) subfamily, expressed on all human NK cells, $CD8^+$ T cells, monocytes and mast cells (Garni-Wagner et al., 1993; Kubota, 2002; Mathew et al., 1993). Its cytoplasmic domain contains two or more cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSMs), associated directly with both signaling lymphocyte-activation molecule-associated protein (SAP) adaptor molecule and LAT (Bottino et al., 2000; Latour et al., 2001). CD48 is identified as the high-affinity ligand of 2B4, which is a glycophosphatidylinositol-linked molecule expressed on all nucleated hematopoietic cells, including NK cells themselves (Brown et al., 1998). 2B4-mediated activation is initiated by its binding to CD48 which leads to phosphorylation of ITSMs by Src family kinases Fyn and Lck. Subsequently, both SAP and LAT, which

are directly associated with 2B4, are activated. SAP then mediates its activating signals by recruiting other signaling molecules, resulting in enhancement of NK cell cytotoxicity, and IFN-γ production (Latour et al., 2001).

9. Acquisition of NK cell functions

NK cells play a key role in immuno-surveillance and host defense against certain infected or transformed cells mediated by direct cytolysis via perforin/granzyme, death receptors (Fas, TRAIL) and/or ADCC pathways (Reviewed by Smyth et al., 2005). Alongside, NK cells secrete cytokines and chemokines which influence the host's immune response by regulating other immune cells (Assarsson et al., 2005; Kalinski et al., 2005; Zingoni et al., 2005).

To acquire the ability for recognition of target cells, NK cells have to be educated by detection of host MHC class I molecules, and adapted to their environment by cytokines through priming process. Moreover, the integration of inhibitory and activating pathways following interaction of activating and inhibitory receptors with ligands available on the neighboring cells to regulate NK cell activation (figure 7).

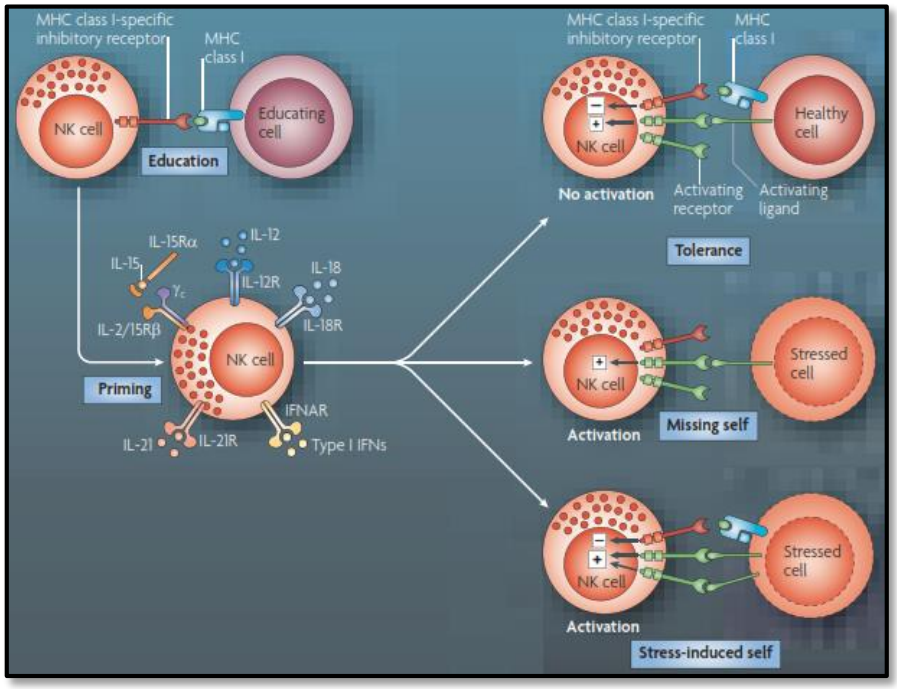

Figure 7: Acquisition of NK cell functions (Vivier and Ugolini, 2010)

i. NK cell education

NK cells express many inhibitory receptors that recognize diverse self-molecules to prevent self-reactivity against healthy cells, however, self-MHC class I molecules recognized by NK inhibitory receptors are essential for NK cell education (Anfossi et al., 2006; Kim et al., 2005; Yokoyama and Kim, 2006). NK cell education, also known as licensing or arming, is a process developed during NK cell maturation to acquire the effector functions that are adapted to the host in which they develop (reviewed by Brodin and Höglund, 2008; Orr and Lanier, 2010). It has become clear by several reports that binding of inhibitory receptors on NK cells by self-MHC class I molecules is important to determine whether an NK cell will be functionally capable of mediating missing-self recognition, or it will be hyporesponsive following stimulation. To illustrate, NK cells developing in the absence of MHC class I are unable to kill MHC class I-deficient tumor cell lines *in vitro* and fail to reject MHC class I-deficient bone marrow *in vivo* (Bix et al., 1991; Liao et al., 1991).

Interestingly, Fauriat and his colleagues demonstrated that NK cell education can be also mediated by activating KIRs (Fauriat et al., 2010b). Their results showed that the expression of KIR2DS1 (an activating KIR) and its ligand (HLA-C2) reduces the responsiveness of NK cells against their targets in both presence and absence of NKG2A (Fauriat et al., 2010b). They found also that hyporesponsiveness is limited to target cell recognition because KIR2DS1^{+} NK cells are stimulated by exogenous cytokines. Several models have been described the various aspects of NK cell education by MHC class I molecules.

a. Arming model

In the arming model, self-MHC class-I specific inhibitory receptors induce the functional activity of NK cells precursors where absence of self-MHC class-I specific inhibitory receptors leads to hypo-responsive immature NK cells (figure 8) (Höglund and Brodin, 2010; Raulet and Vance, 2006).

b. Disarming model

In the disarming model, the NK cell is activated by default; however, the lack of inhibitory receptors for self-MHC class I molecules induces hyporesponsive NK cell by chronic stimulatory encounters with self-cells (figure 8) (Höglund and Brodin, 2010; Raulet and Vance, 2006).

c. Rheostat model

The rheostat model represents a quantitative interpretation of NK cell education. Based on the strength of the inhibitory signal during NK cell education, NK cell responsiveness can either be increased (arming-like) or decreased (disarming-like) (figure 8) (Höglund and Brodin, 2010; Raulet and Vance, 2006).

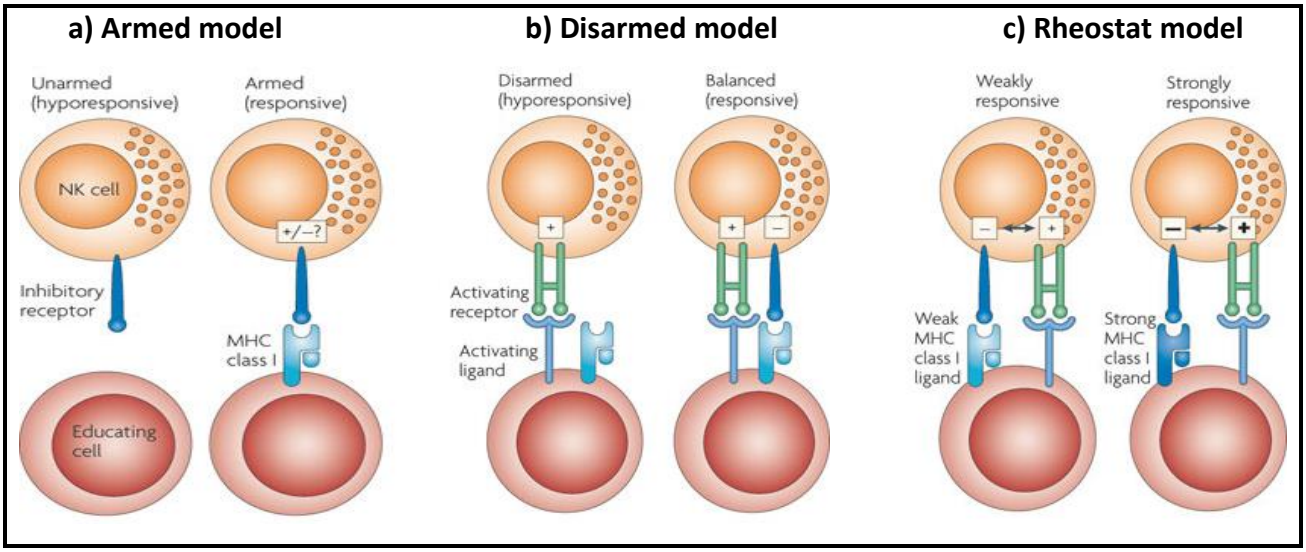

Figure 8: Models for NK cell education (Höglund and Brodin, 2010)

ii. NK cell priming

Resting human NK cells show a little cytotoxic functions when incubated *in vitro* with tumor target cells, suggesting that resting NK cells require additional signals for their complete activation (Bryceson et al., 2006b). To achieve their effector functions, NK cells should be activated in a process called priming. Several *in vitro* studies have demonstrated the possible role of myeloid cells in activation of resting NK cells (Andoniou et al., 2005; Baratin et al., 2005; Ferlazzo et al., 2004b). In this context, DCs play a critical role in priming resting NK cells (Fernandez et al., 1999; Gerosa et al., 2002; Granucci et al., 2004; Yu et al., 2001). These studies proposed that acquiring of NK effector functions is related to cytokines produced by DCs. In 2007, Lucas and his colleagues found that DCs and the consequent production and trans-presentation of IL-15 by DCs are required for priming resting NK cells to respond against viral and bacterial pathogens (Lucas et al., 2007). Moreover, other cytokines as IL-12 (Guia et al., 2008), IL-18 (Chaix et al., 2008) and IL-27 (Ziblat et al., 2015) also play a key role in priming and maturation of NK cells (figure 9). For instance, IL-27, a cytokine of the IL-

12 family produced by DCs, improves NK cell effector functions by upregulating CD25, CD69 and NKp46 expression and primes NK cells for IL-18 responsiveness (Ziblat et al., 2015).

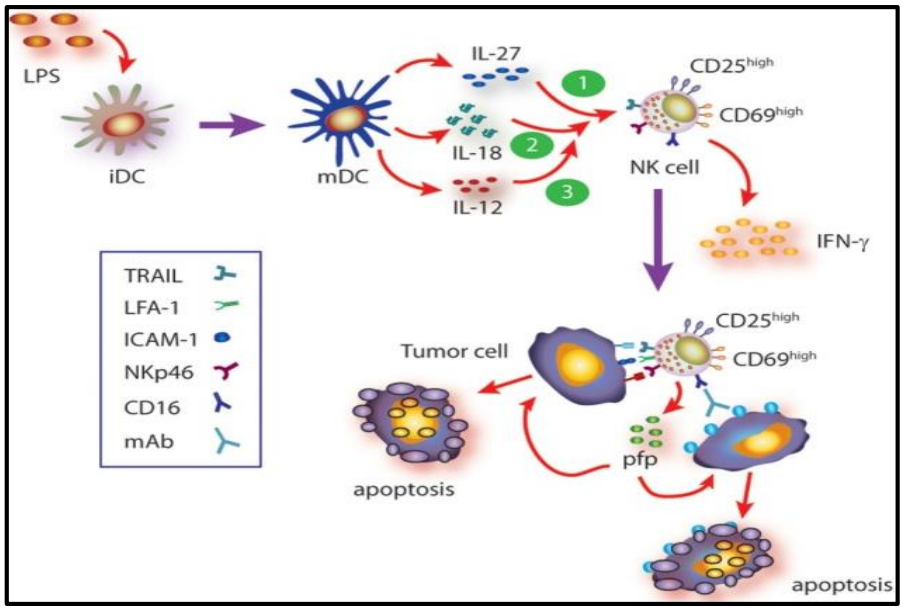

Figure 9: Involvement of DCs and their secreted cytokines in NK cell stimulation (Zwirner and Ziblat, 2017)

iii. Inhibitory/activating signals balance

NK cells have a varied group of inhibitory and activating receptors expressed on their cell surface that control their activation, proliferation and effector functions. Under normal circumstances, NK cells can discriminate between normal healthy cells and abnormal cells (infected or transformed) through MHC class I molecules. Healthy cells constitutively express MHC class I molecules which bind to NK cell inhibitory receptor (CD94/NKG2A) to avoid NK cell-mediated lysis (figure 10-a), (Chiesa et al., 2005; Moretta and Moretta, 2004). On contrary, tumors and virus-infected cells down-regulate the expression of MHC class I ligands to escape the cytotoxic T cells (figure 10-b), (Horst et al., 2011). Interestingly, these cells have displayed an upregulation of some ligands for activating NK receptors (figure 10-c). Consequently, NK cells are able to attack and kill their target cells due to the imbalance between inhibitory and activating signals.

- **Missing self-hypothesis**

MHC class I molecules play a critical role in controlling the effector functions of both cytotoxic T-cells and NK cells. By which, APCs display viral or tumor antigens to specific immune cells as cytotoxic T cells. Moreover, these molecules are considered as ligands for a large group of activating and inhibitory NK cells. Unlike T-cells, which recognize antigen as peptide fragments bound to MHC molecules, NK cells become functionally active in the absence or altered their expression on target cells. Down-regulation of MHC I molecules by abnormal cells is a phenomenon called missing-self hypothesis which allows NK cells to target and kill these abnormal cells (Ljunggren and Kärre, 1990).

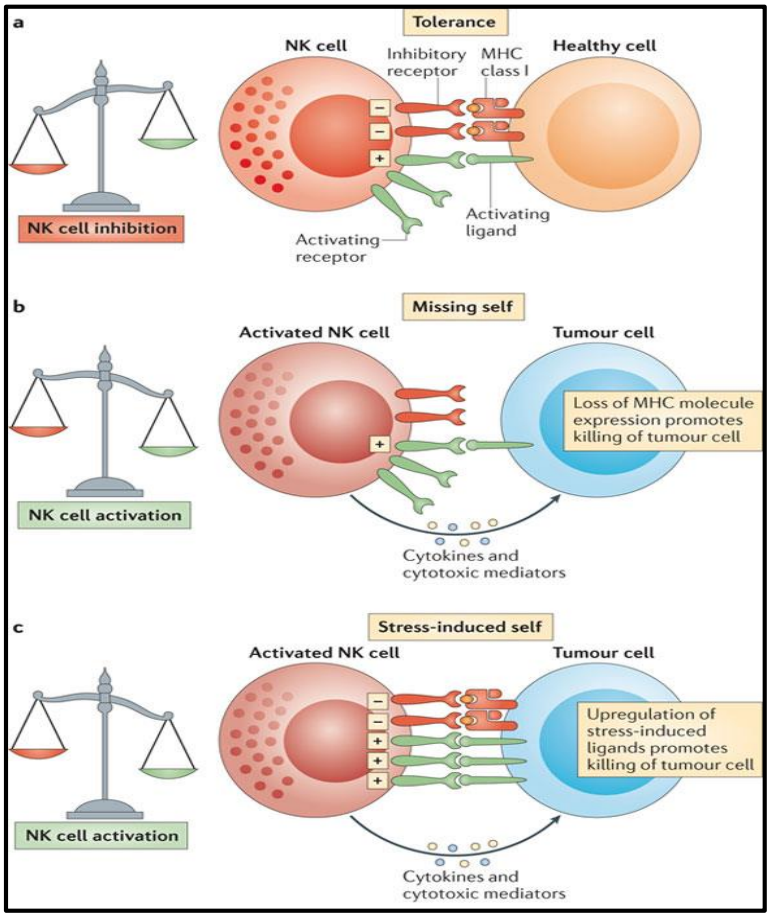

Figure 10: Outcome of inhibitory and stimulatory signals balance (Vivier et al., 2012)

10. NK cell effector functions

NK cells are key players in the effector arm of the immune system that maintain the homeostasis via recognition and killing abnormal and pathogen-infected cells. Stressed cells undergo surface marker changes including losing the expression of HLA class I molecules and/or upregulating damage-associated proteins. Therefore, NK cells can recognize tumor cells via two models: “missing-self” recognition, and “stress-induced recognition”. Several damage-associated proteins have been found in tumor cells such as MICA, MICB, ULBPs binding with NKG2D, ligands of NKp30 as B7-H6 and HLA- BAT3 (reviewed by Fang et al., 2017). Further, NKp44 can recognize an isoform of a mixed-lineage leukemia protein, and CD155 and CD112 which interact with DNAM-1 (reviewed by Fang et al., 2017).

Beside its activation by tumor cells and pathogens, NK cell can be directly or indirectly receive regulated signals from other immune cells during the immune response (reviewed by Fang et al., 2017). For instance, DCs can regulate NK cell proliferation and function by secreting IL-12, type I IFN, and IL-15 (reviewed by Fang et al., 2017). Moreover, macrophage play a role in NK cell activation and cytotoxicity by secreting cytokines such as IL-12, IL-18, IL-1β and IFN-β (reviewed by Fang et al., 2017). Monocytes control NK proliferation and function by secreting IL-2, IL-12, IL-18 and IL-21 (reviewed by Fang et al., 2017). In addition, $CD4^+$ T cells can secrete IL-2, which is critical for NK cell survival and proliferation (reviewed by Fang et al., 2017). Otherwise, Treg cells can suppress NK cell proliferation and activity by secreting transforming growth factor- β (TGF-β) and IL-10 (reviewed by Fang et al., 2017).

Activated NK cells are able to kill their target cells in a process called apoptosis (a process of programmed cell death) though a variety of mechanisms, including cytolytic granule-dependent exocytosis pathway, signaling through the TNF death receptor family members such as FAS (CD95) and TRAIL (TNF-related apoptosis-inducing ligand), the release of cytokines IFN-γ and TNF-α, and ADCC via CD16 (Chavez-Galan et al., 2009). Besides the cytotoxicity mediated by NK cells, immune response can be regulated by NK cells through the recruitment of other immune cells (figure 11).

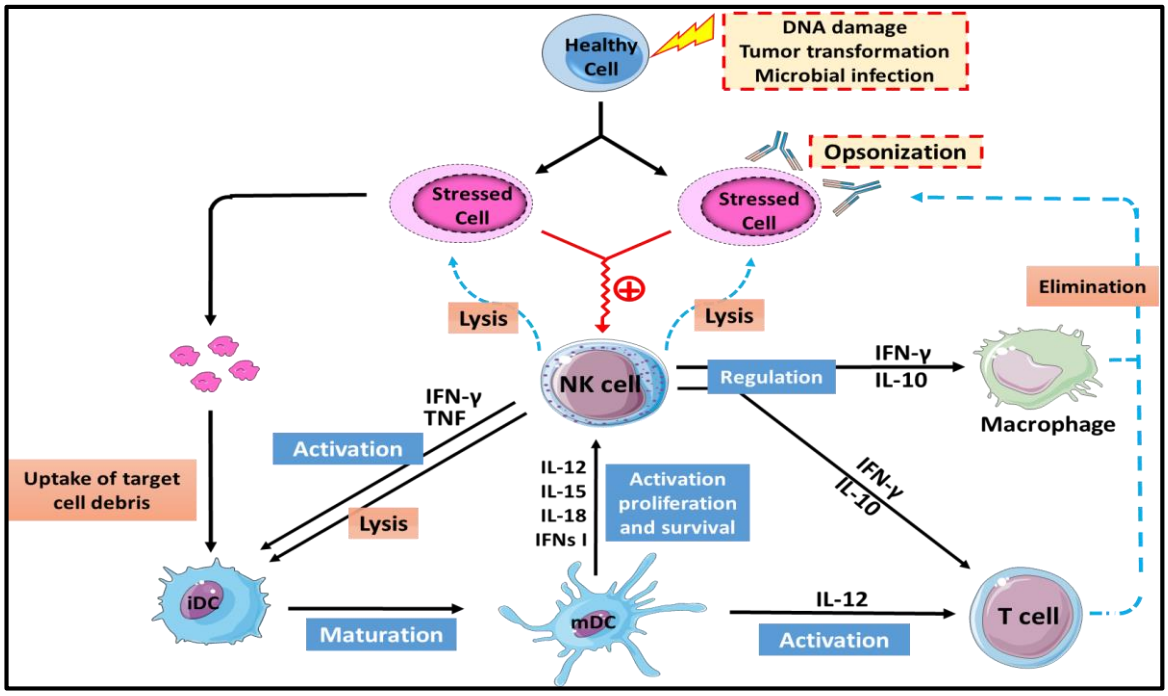

Figure 11: NK cell functions and its interaction with other immune cells (Adapted from Vivier and Ugolini, 2010)

Normally, the activation and inhibition signals are generated when NK cell interacts with its target cell at specific site known as immunological synapse. In the case of NK cell, the immunological synapse is defined as an intercellular contact between NK cell and its target that causes proteins segregation at the cell-cell interface into micrometer-scale three-dimensional domains (Davis, 2002; Davis and Dustin, 2004; Orange, 2008). NK cells are capable to form different types of immunological synapses such as activating NK cell immunological synapse (aNKIS), and inhibitory NK cell immunological synapse (iNKIS), based on both their targets and receptor ligands. To clarify, when NK cell interacts with MHC-I positive cell as healthy cell, an iNKIS is generated to prevent NK cell activation and killing the normal cell (Krzewski and Strominger, 2008).

On the other hand, the interaction between NK cell and a target lacking MHC-I molecules as tumors or virus-infected cells leads to generation of an aNKIS (Krzewski and Strominger, 2008). Formation of the aNKIS is a sequential and tightly controlled process involving several steps: i) contact and adhesion based on LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) adhesion molecules, ii) receptor binding and signaling, iii) rearrangements of actin cytoskeleton, iv) further receptor clustering and sustained signaling (signal amplification), v) translocation of the microtubule

organizing center (MTOC) with associated lytic granules toward the immunological synapse (MTOC polarization), and vi) granule degranulation (Krzewski and Strominger, 2008; Orange, 2008). Together, these processes allow the lytic granules to transport to the synapse followed by their close association with the NK-cell membrane to which they can fuse and release their contents onto the target cell to destroy it.

i. NK cell cytotoxicity

a. Cytolytic granule-dependent exocytosis

The cytotoxic granules of NK cells are specific secretory lysosomes with storage and secretory functions (Burkhardt et al., 1990). They contain a mixture of cytotoxic mediators that cause death of the target cells. These mediators are represented by perforin, granzymes, Fas ligand (FasL; CD178), TRAIL (CD253), and granulysin. Moreover, the membrane of these granules comprise highly glycosylated membrane proteins called lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and LAMP-2 or CD107b (Winchester, 2001). CD107a expression on NK cell surface has been described as a marker of NK cell degranulation and is upregulated on the cell surface following NK cell stimulation (Alter et al., 2004).

- **Perforin**

Perforin is a pore-forming protein (67 kDa), where its gene is constitutively transcribed in NK cells and is regulated by receptor activation signals and cytokines (IL-2 and IL-15) (Salcedo et al., 1993). It is required for the function of other granule components mainly granzymes family that are released with perforin following formation of the immunological synapse. The exact molecular mechanism induced by perforin remains indefinable, but there are two suggested hypotheses describe the function of perforin (Reviewed by Voskoboinik et al., 2006). The first one proposes that perforin makes pores in the membrane of target cell, so this allows granzymes to diffuse into the target cell and also allows an ionic exchange, which causes an osmotic unbalance. The second suggests that perforin binds to the target-cell membrane (through electrostatic or possibly receptor-mediated interactions) and is internalized into its endosomes (containing granzymes) to disrupt them, thereby granzymes are released into the cytosol (Voskoboinik et al., 2006).

- **Granzymes**

Granzymes are proteases related to serine proteases family. In humans, there are five granzymes (A, B, H, K and M) have been defined where granzymes A and B are the most abundant granzymes (Grossman et al., 2003; Smyth and Trapani, 1995). Granzymes are synthesized as pro-enzymes and are activated by cathepsin C and H. Although the most accepted suggestion is that the perforin pores can serve as passive conductors of granzymes through the target cell membrane, Motyka *et al.* have shown that in perforin absence, granzyme B is introduced into the target cell because it forms a complex with the mannose-6-phosphate receptor, and then the complex is internalized by endocytosis (Motyka et al., 2000). Compared with other granzymes, granzyme B has the strongest pro-apoptotic activity, and can cleave its targets after aspartate residues. It induces targets death based on caspase-dependent apoptosis rapidly through two pathways. In the first pathway, granzyme B directly activates caspase 3, which enhances DNA destruction (Metkar et al., 2003). The second is characterized by promotion of permeability of the mitochondrial outer membrane and cleaving of a molecule from the Bcl-2 family called BID (BH3-interacting-domain death agonist) (Pinkoski et al., 2001). In turn, BID induces cytochrome-C release from mitochondria which decreases the mitochondrial function, and triggers the activation of caspase 9 (which enhances caspase 3 activation).

- **Granulysin**

Granulysin is a cationic protein related to the saposin-like protein family expressed as a 15 kDa protein which is converted to its active form (9 kDa) by proteolytic cleavage from 3 to 5 days after cell activation (Hanson et al., 1999; Peña et al., 1997). It exists in cytolytic granules of cytotoxic T lymphocytes and NK cells, and is secreted by exocytosis to perform its cytolytic activity against tumors and microbial targets (Krensky and Clayberger, 2009; Stenger et al., 1998). It makes pores in the mitochondrial membrane of target cell, disrupting the transmembrane potential ($\Delta\psi$) in mitochondria (Kaspar et al., 2001). Another mechanism by which granulysin induces cell death is through activation of caspase-3 (Kaspar et al., 2001).

b. Death receptor pathway

Death receptors are cell surface receptors related to TNF super family that transmit apoptotic signals initiated by specific ligands such as Fas ligand, TNF-α and TRAIL. They play an important role in apoptosis via activation of caspase cascade. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors contain an intracellular death domain (DD), which upon ligand binding associates directly with an adaptor protein called Fas-associated death domain (FADD) or indirectly via TNFR-associated death domain (TRADD). FADD also contains death effector domain (DED) which interacts with pro-caspase-8 to form a complex at the receptor called the death inducing signaling complex (DISC). The final step in this process is mobilization of caspase 8 to the DISC causing its activation and initiation of apoptosis (Thorburn, 2004).

- **Fas (CD95)**

Fas and its ligand (FasL) play an important role in killing of targets such as virus-infected cells or cancer cells by NK cells. NK cells express FasL on their surface to suppress tumor growth (Bradley et al., 1998). In some cases tumor cells do not express Fas, but NK cells have the ability to induce Fas expression on these cells via IFN-γ secretion (Screpanti et al., 2001). Binding of Fas with FasL promotes receptor clustering, DISC formation and activation of caspase 8 by auto-proteolysis process (Carrington et al., 2006). Accordingly, caspase 8 converts procaspase 3 to its active form (caspase 3) which initiates apoptosis process via DNA cleavage (Carrington et al., 2006). Further, caspase 8 can also hydrolyze BID, which destroys the mitochondrial membrane and triggers cytochrome c release.

- **TRAIL**

TRAIL, also known as Apo2 ligand, is a member of TNF superfamily which is capable to induce NK cell-mediated apoptosis for many tumors and transformed cells. Five receptors have been recognized for TRAIL in humans where two of them, TRAIL-R1 (death receptor DR4) and TRAIL-R2 (DR5), are able to initiate an apoptotic signal (Wang and El-Deiry, 2003). Ligation of TRAIL to its receptor (DR4 and DR5) results in engagement of FADD proteins in their cytoplasmic domain, and then formation of DISC. As a result, caspase-8 is activated which is able to trigger the apoptosis process as mentioned above in Fas.

c. Antibody-dependent cell-mediated cytotoxicity (ADCC)

Antibody-dependent cell-mediated cytotoxicity (ADCC), also called antibody-dependent cellular cytotoxicity, is an immune mechanism whereby the effector cells bearing Fc receptors can recognize and lyse antibody-coated target cells (opsonized cells). Activating low affinity FcγRIIIA receptor (CD16A) is highly expressed on the cytotoxic $CD56^{dim}$ $CD16^{+}$ NK cell subset, and mediates ADCC by binding to the Fc portion of IgG antibody (Lanier et al., 1988). After recognition of their targets, $CD56^{dim}$ $CD16^{+}$ NK cells interact with the Fc region of IgG antibody coating the target cell which is followed by phosphorylation of ITAMs via cellular src kinases to activate the signaling pathways in NK cells. As a result, cytotoxic granules containing perforin, granzymes and others are secreted, causing lysis of target cells (Gómez Román et al., 2014).

ii. NK cell cytokines secretion

Beside their cytotoxicity, NK cells also have important effector and regulatory effects mediated by cytokines and chemokines secretion following either cytokine- or activating receptor stimulation (Fauriat et al., 2010a; Vivier et al., 2008). It is thought that NK cells participate in a complex interaction network with other lymphocytes, DCs, and macrophages to effectively control immune responses (Newman and Riley, 2007). For instance, NK cells are considered as a major producer of IFN-γ in response to their stimulation, which has immune regulatory activity as well as direct effector activity (Martín-Fontecha et al., 2004; Wang et al., 2012). Activated NK cells also secrete TNF-α which has both direct cytotoxic activity by triggering caspase-8-mediated apoptosis and immune regulatory activity by inducing DCs maturation (Gerosa et al., 2002; Wang et al., 2008). Additionally, NK cells have the ability to secrete several other factors, including GM-CSF growth factor, immunoregulatory cytokines such as IL-5, IL-10, IL-13, and the chemokines MIP-1α, MIP-1β, IL-8, and RANTES allowing NK cells to co-localize with other immune cells such as at inflammation sites.

11. NK cells and cancer

NK cells play a major role in the immunosurveillance of malignancy (hematological or solid tumors) by direct killing of malignant cells and/or releasing a number of cytokines that regulate both innate and adaptive immune responses. This concept is demonstrated by several *in vitro* as well as *in vivo* studies. The cytotoxic activity of NK cell against tumors have been firstly confirmed in animal models, where transplanted hematopoietic tumors or chemically-induced tumors were rejected in various mice models due to NK activity. In contrast, eradicating NK cells in such models often led to an aggressive tumor growth and metastasis (Waldhauer and Steinle, 2008).

In humans, several studies have shown a relationship between low levels of NK cell activity and an increase in the risk of cancer. For instance, a prospective epidemiological follow-up study has performed in Japan for 11 years and included 3625 individuals showed that a low NK cell cytotoxicity is associated with an increased risk of cancer occurrence (Imai et al., 2000). In addition, the infiltration of NK cells into tumors is associated with good prognosis in patients with colorectal carcinoma, gastric carcinoma and lung cancer (reviewed by Waldhauer and Steinle, 2008). Many clinical trials have been used NK cells as adoptive immunotherapy based on the alloreactivity of donor's NK cells to treat the hematological malignancies and solid tumors (Geller et al., 2011; Miller, 2005; Ruggeri et al., 2007a). More recently, a variety of *ex vivo* expansion and activation methods have been used to increase both number and function of the infused NK cells to improve their antitumor activity (Denman et al., 2012; Fujisaki et al., 2009; Sutlu et al., 2010). However, tumor cells can develop various strategies to escape immunosurveillance of NK cell and other effector cells, which can be explained by general mechanisms such as saturation of the immune system by rapid growth of the tumor as well as alterations of NK cells and other immune cells leading to dysfunction of the immune system (Costello et al., 1999; Farnault et al., 2012). These alterations well be explained in details later.

12. NK cells and acute myeloid leukemia

i. Acute myeloid leukemia (AML)

Leukemias are cancers starting in cells that would normally develop into different types of blood cells. AML, one type of leukemias, is a heterogeneous clonal disorder of HSC characterized by accumulation of immature, non-functional myeloid precursors (blasts) in the bone marrow and blood without the ability to differentiate normally and to respond to normal regulators of proliferation (Estey and Döhner, 2006). According to the national cancer institute, the prevalence of AML is 4.2 cases per 100,000 (21,380 new cases of AML in 2017) rising to 17.9 cases per 100,000 adults aged 65 years and older (https://seer.cancer.gov).

Conventional treatment of AML is multiple cycles of intensive chemotherapy regimens, divided into two phases: induction phase, and consolidation phase. Induction phase is an aggressive chemotherapy aiming to produce complete remission (CR) by clear peripheral blood from leukemia cells and reducing blasts number in bone marrow to less than 5%. Usually it is known as "7+3" because it consists of a combination of an anthracycline (daunorubicin or idarubicin, given IV for 7 days) and cytarabine, administrated IV for 3 days (Fernandez et al., 2009; Ravandi et al., 2010). Consolidation phase is used to maintain CR and prevent relapse by eliminating the non-detectable leukemia cells. It usually consists of high or intermediate dose of cytarabine, and hematopoietic stem cell transplantation (HSCT) for patients having high risk of relapse. In general, patients younger than 60 years with newly diagnosed AML, cytotoxic chemotherapy results in CR in approximately 60 to 80% (Tallman et al., 2005). However, most of them relapse and about 40 to 45% of patients achieving CR remain alive at 5 years. In elderly patients (more than 60 years), the CR rate is approximately 40-50% and around 10-15% remain alive at 5 years (Tallman et al., 2005). In order to minimize relapse and to improve survival rate of in elderly patients, immunotherapy is used in combination with standard therapy (Barrett and Le Blanc, 2010; Lichtenegger et al., 2015).

HSCT is the oldest immunotherapeutic treatment for AML post chemotherapy and/or total body irradiation (TBI), where the level of leukemia cells at the time of transplant is an important predictor of HSCT outcomes. Its effectiveness is related to the graft-versus-leukemia (GvL) effect

mediated by the immune cells in the graft. By GvL effect, cytotoxic immune cells are able to eradicate the residual leukemia cells, thus minimizing the risk for disease relapse. However, its major complications are infections, graft-versus-host disease (GVHD) and high rate of TRM (Copelan, 2006). Because the majority of elderly patients are not eligible for HSCT and to minimize the relapse rate, new immunotherapy strategies for AML are developed to use as adjuvant therapy or alternative immunotherapy, including i) monoclonal antibodies; ii) adoptive transfer of NK and T cells; iii) systemic administration of cytokines; and iv) therapeutic vaccinations with peptides, modified leukemic cells and monocyte-derived DCs (Geiger and Rubnitz, 2015; Lichtenegger et al., 2015, 2017).

ii. Mechanisms of AML escape from NK cell immunity

Although the cytotoxic activity of NK cells against leukemia cells and their beneficial role in immunotherapy, many tumors including AML can evade the immunosurveillance of NK cell by destroying the precise balance between inhibitory and activating signals (Costello et al., 2002; De Maria et al., 2003; Fauriat et al., 2007; Stringaris et al., 2014; Venton et al., 2016). Commonly, AML cells are able to escape NK cell immunosurveillance through various mechanisms: i) alteration of NK cells; ii) immunosuppressive properties of AML cells; and iii) interactions with other immune cells (reviewed by Lion et al., 2012), (figure 12).

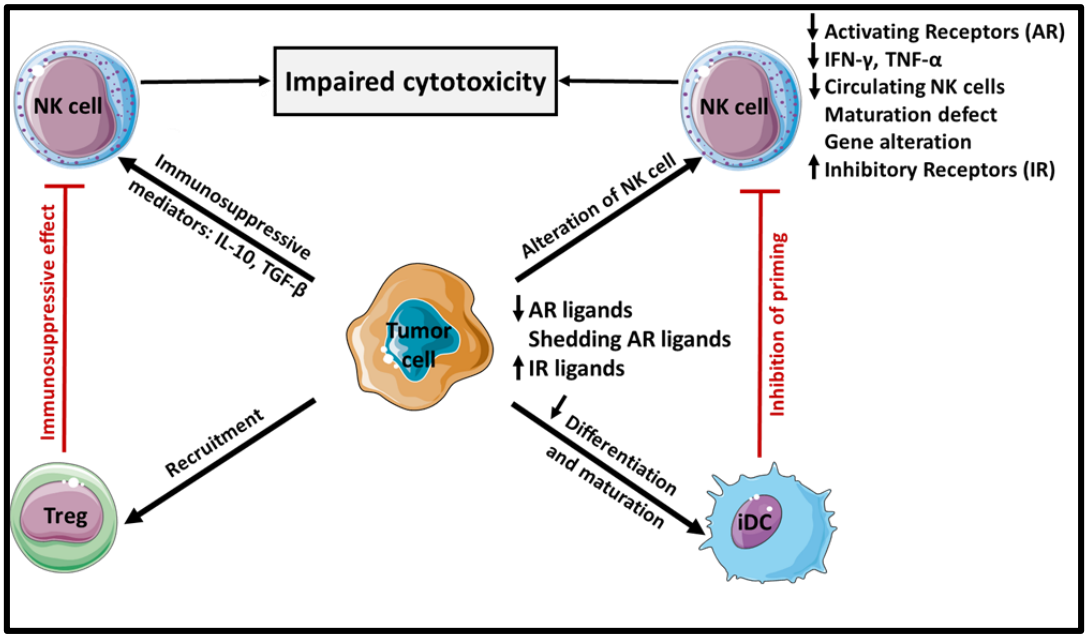

Figure 12: NK cell immune escape mechanisms in AML.

a. Alterations of NK cell by AML

AML cells are capable to alter expression of NK receptors and their ligands, resulting in a significant impairment of NK cell functions, however, the molecular mechanisms responsible for these alterations are still unknown.

- **Alterations of NK cell receptors expression**

Several reports have shown a clear decrease in the expression of NK activating receptors on circulating NK cells of AML patients such as NCRs (NKp30, NKp44 and NKp46), NKG2D and DNAM-1 (Costello et al., 2002; Fauriat et al., 2007; Khaznadar et al., 2015a; Sanchez-Correa et al., 2011; Stringaris et al., 2014; Szczepanski et al., 2010). These alterations are associated with impaired anti-leukemic activity of NK cells, a decreased IFN-γ and TNF-α production, and a higher risk of relapse. Notably, our team showed that NCRs downregulation on NK cells was associated with poor prognosis for AML patients, significantly lower 5-year survival rates than their NCR^{bright} counterparts (Fauriat et al., 2007). Interestingly, the phenotypic and functional abnormalities of NK cells are partially or totally restored in patients achieving remission suggesting that the presence of AML cells is responsible for NK cells abnormalities. Moreover, our team showed a strong correlation between NKp46 expression on NK cells of AML patients at diagnosis and the clinical outcomes after allogeneic stem cell transplantation (allo-SCT). We found that patients with high expression of NKp46 at diagnosis had better progression-free survival and overall survival (OS) than patients with low expression of NKp46 (Chretien et al., 2017a). Regarding the expression of inhibitory receptors, it is clear that failure to achieve remission in AML patients is strongly associated with NKG2A and iKIRs overexpression (Khaznadar et al., 2015a; Stringaris et al., 2014).

In parallel to phenotypic and functional alterations of NK cells, it was found that the percentage of circulating NK cells is lower in AML patients at diagnosis compared to the NK cells of healthy volunteers. Also, $CD56^{bright}CD16^{-}$ NK cells in AML patients is significantly higher, while frequency of $CD56^{dim}CD16^{+}$ NK cells is significantly lower (Szczepanski et al., 2010).

- **Alterations of NK cell receptors ligands expression**

Another strategy by which AML can escape from NK cell immunosurveillance is decreasing the expression and shedding of surface ligands for various NK cell activating receptors on AML cells

themselves (Diermayr et al., 2008; Nowbakht et al., 2005; Pende et al., 2005; Salih et al., 2003; Sanchez-Correa et al., 2011). For example, leukemic blasts are characterized by a sharp decrease in the expression of MICA/B and ULBPs (ligands of NKG2D), CD48 (a ligand for 2B4), NCR-specific ligands, and DNAM-1 ligands (CD112/CD155) (Hilpert et al., 2012; Kearney et al., 2016; Nowbakht et al., 2005; Pende et al., 2005; Sanchez-Correa et al., 2011).

On other hand, DNAM-1 ligands (CD112 and CD155) are highly expressed on AML blasts of patients younger than 65 years. However, NK recognition and killing of leukemic blasts is reduced due to downregulation of DNAM-1 on NK cells of AML patients, hypothesizing a converse relationship between DNAM-1 ligands expression on leukemic blasts and DNAM-1 expression on NK cells (Sanchez-Correa et al., 2012). This hypothesis was supported by *in vitro* culturing NK cells of healthy volunteers with leukemic blasts expressing DNAM-1 ligands, where the results showed downregulation of DNAM-1 expression on healthy donors' NK cells (Sanchez-Correa et al., 2012).

Besides the classical alterations of the expression of NK receptors and their ligands in AML, our team reported three different groups of AML patients based on NK maturation profile: hypomaturation, intermediate maturation, and hypermaturation) (Chretien et al., 2015). Recently, we have evaluated the clinical outcomes of AML patients based on NK maturation profile. Our findings revealed that patients with hypomaturation profile have decreased overall survival and relapse-free survival compared to patients with intermediate and hypermaturation (Chretien et al., 2017b).

- **Alterations of NK cell at genetic level**

Some attempts were performed at gene level to try to identify the molecular mechanisms of NK functions defect in hematological malignancies. In this context, Costello team aimed to realize the mechanisms underlying NCRs down-regulation in NK cells from AML patients (Venton et al., 2016). They found that AML-NK cells showed a specific transcriptomic signature compared to NK cells from healthy volunteers, disappeared by NK cells expansion. Although, the gene expression of E26 transformation-specific 1 (ETS-1) transcription factor (a potential regulatory element of NCRs expression) was decreased in presence of AML blasts, the expression of ETS-1 and NCRs was restored following AML-NK cells expansion. This proposes that ETS-1 may regulate NCRs expression (Venton et al., 2016).

In addition, miRNAs, which play an important role in fundamental NK cell processes (Leong et al., 2014), can be well accepted to participate in many aspects of AML, including proliferation, differentiation, survival, apoptosis and invasion by targeting oncogenes or tumor suppressors (Marcucci et al., 2011; Wallace and O'Connell, 2017). Recently, a study was performed to clarify the molecular mechanisms by which AML evades NK cell surveillance using a mouse model of de novo AML (Mundy-Bosse et al., 2016). The findings showed a selective loss of immature NK cells subset and a clear reduction in the cytolytic granules containing perforin and granzyme B among NK cells in leukemic mice. Moreover, NK cells in leukemic mice showed lower levels of T-bet and Eomes, critical transcription factors for terminal NK cell differentiation. They demonstrated that these results are related to miR-29b overproduction, a negative regulator of T-bet and Eomes, in NK cells of leukemic mice because deletion of miR-29b in NK cells reversed these alterations (Mundy-Bosse et al., 2016). More future studies are required to understand how miRNAs are involved in AML escape mechanisms of NK cell immunity.

b. Immunosuppressive properties of AML blasts

Besides phenotypic and functional alterations of NK cells, AML blasts are able to diminish NK cell immunosurveillance by other immunosuppressive factors. For example, Baessler and co-workers found an overexpression of CD137 ligand (CD137L) and glucocorticoid-induced TNFR-related protein ligand (GITRL) on AML blasts, where these molecules are ligands for receptors belonging to tumor necrosis factor receptor (TNFR) family (Baessler et al., 2009, 2010). Engagement of CD137L and GITRL with their receptors on human NK cells are directly associated with impairing NK cell-mediated killing and IFN-γ secretion or indirectly via secretion of IL-10 by AML cells. Oppositely, they showed that both CD137 and GITR mediate a stimulatory signal in mouse NK cells after their binding with CD137L and GITRL, respectively (Baessler et al., 2009, 2010). Cytotoxic functions of NK cells are also impaired by TGF-β, which is secreted by AML blast as well as by regulatory T cells, myeloid derived suppressor cells (MDSCs) and other stromal cells in the tumor microenvironment (Baginska et al., 2013; Otegbeye et al., 2018).

c. Alterations of interaction between NK cells and other immune cells

Cellular interactions between NK cells and other immune cells are also altered in AML patients resulting in more possibilities of immune escape. NK cells play a vital role in regulation of DCs by killing iDCs to limit inflammation and inappropriate T cell tolerization. Fauriat and his colleagues have noticed the inability of NK cells from AML patients to kill iDCs which might result in an abnormal interaction between T cells and iDCs and induction of tolerogenic T cells (Fauriat, 2005). Further, several studies have reported an increase in Treg numbers among AML patients compared to healthy donors, while their numbers are reduced in patients with CR (Shenghui et al., 2011). Generally, Treg cells are potent immune suppressor cells mediated their inhibitory action toward effector immune cells via cell-to-cell contact as well as secretion of suppressor soluble factors such as IL-10 and TGF-β (Ghiringhelli et al., 2005; Shenghui et al., 2011). In murine AML model, it was observed that Treg cells depletion resulted in more NK cell activation (Hallett et al., 2008).

13. NK cell-based cancer immunotherapy

Based on their ability to sense the altered expression of MHC molecules and stress markers through various receptors, tumor cells become reachable targets for NK cell lysis due to down-regulation of MHC expression as well as enhancement of stress ligands expression. As NK cells are considered as key players against malignant cells, mainly AML, many attempts and clinical trials have been previously and still performed to utilize NK cells as immunotherapy to treat solid and hematopoietic tumors (https://clinicaltrials.gov). Now, different approaches are being used to enhance effectiveness of anti-tumor function of NK cells such as: i) cytokines, ii) monoclonal antibodies as checkpoint inhibitors and immunomodulators, and iii) adoptive transfer of NK cells (Chiossone et al., 2017; Fang et al., 2017; Guillerey et al., 2016a; Mehta et al., 2018).

i. Cytokines

Several cytokines have been confirmed to enhance NK cell proliferation and/or cytotoxicity against several types of tumors. Cytokines are used for this purpose either by direct infusion of cytokines *in vivo* to boost the autologous NK cell numbers and functions or by *in vitro* incubation of allogeneic NK cells with cytokines before adoptive NK cell immunotherapy.

a. IL-2

IL-2 is the first cytokine approved for use in patients to improve NK cells activity, however, infusion of IL-2 into patients was accompanied by limited clinical outcomes because IL-2 activates not only NK cells but also Treg cells, which express CD25 (high affinity receptor for IL-2) that can compete with NK cells for IL-2, and subsequently suppress their effector function (Rosenberg et al., 1985). Additionally, high doses of IL-2 can cause severe side effects, including capillary leakage, neuropathy and renal failure. Therefore, a modified form of IL-2 called "superkine or super-2" has been evolved which has a high affinity to IL-2Rβ and can function independently of IL-2Rα (CD25) (Levin et al., 2012). IL-2 is also used to expand and activate PBMC-derived NK cells to be used as adoptive immunotherapy (Miller, 2005).

b. IL-15

IL-15 has been reported to control development, homeostasis and cytotoxicity of NK cells, which can be presented to NK cells *in vivo* through several cell types, including monocytes, macrophages and DCs (reviewed by Floros and Tarhini, 2015). Infusion of IL-15 into patients resulted in 4 to 8-fold increase in cell numbers of circulating NK cells and CD8 effector memory T cells (Conlon et al., 2015). Several fusion proteins have been developed to improve the efficiency and half-life of IL-15. For example, dsNKG2D-IL-15 fusion protein has an enhanced NK cell-targeting ability and exhibits a greater tumor growth-suppressing capacity against human gastric cancer (Chen et al., 2017). Furthermore, super-agonist ALT-803 (a modified form of IL-15 by replacing asparagine to aspartic acid at amino acid 72) can prolong the half-life of the heterodimeric IL-15 to nearly 25 h and improves NK cell cytotoxicity. Recently, ALT-803 is used as an immunomodulatory agent to improve NK cell cytotoxicity against B cell lymphomas (Rosario et al., 2016). The results show that ALT-803 stimulation amplifies NK cells cytotoxicity by increasing the expression of granzyme B and perforin, degranulation and IFN-γ production. Moreover, IL-15 is also used alone or in combination with feeder cells to expand and activate PBMC-derived NK cells to be used as adoptive immunotherapy (Fujisaki et al., 2009).

c. Other cytokines

Other cytokines, such as IL-12, IL-18 and IL-21, have been shown to improve NK cell functions (reviewed by Floros and Tarhini, 2015). For example, IL-21 infusion suppresses tumor growth

through NK and $CD8^+$ T cell-mediated cytotoxicity in a mouse tumor-bearing model. In adoptive NK cell immunotherapy, IL-21 is used to increase both the number and activity of purified allogeneic NK cells (Denman et al., 2012; Li et al., 2015; Sutlu et al., 2010).

ii. Monoclonal antibodies

Over the past decade, the efficiency of antibodies in treatment of cancer has been increasingly approved. There are two groups of monoclonal antibodies (mAbs) treatment based on NK cells (Chiossone et al., 2017; Fang et al., 2017; Guillerey et al., 2016b). First, mAbs which target tumor-associated antigens to induce NK cell via interaction with CD16. Second, mAbs which target and block immune checkpoint proteins to enhance NK cell cytotoxicity.

a. Tumor antigen-specific antibodies

Several therapeutic antibodies have been developed and approved for treatment of various types of tumors (Chiossone et al., 2017; Fang et al., 2017; Guillerey et al., 2016b; Weiner et al., 2010). These antibodies directly target tumor-associated antigens and also bind to FcγRIIIA (CD16a) receptor expressed by NK cells to induce NK-mediated ADCC (figure 13). In addition to tumor-specific antibodies, tumor microenvironment can be also targeted by antibodies to slow tumor growth (Weiner et al., 2010).

Many examples of tumor-targeting mAbs have been successfully used in treatment of various malignancies such as Obinutuzumab (type 2 anti-CD20 mAb) and Rituximab (type 1 anti-CD20 mAb) for treatment of B cell lymphoma, trastuzumab (mAb to human epidermal growth factor receptor 2, HER2, for treatment of HER2 overexpressing invasive breast cancer), cetuximab (mAb to epidermal growth factor receptor, EGFR, for treatment of colorectal cancer), and daratumumab (anti-CD38 for treatment of multiple myeloma), (Capuano et al., 2017; Guillerey et al., 2016b; Raedler, 2016; Weiner et al., 2010). Moreover, there are some modifications on tumor-targeting mAbs to increase the affinity of FcγRIIIA receptor to the Fc region of IgG to boost NK cell cytotoxicity. Recently, an Fc-optimized CD133 antibody shows a greater affinity to NK cells and more cytotoxic activity for NK cells without relevant toxicity to hematopoietic progenitors in a human AML xenograft model (Koerner et al., 2017).

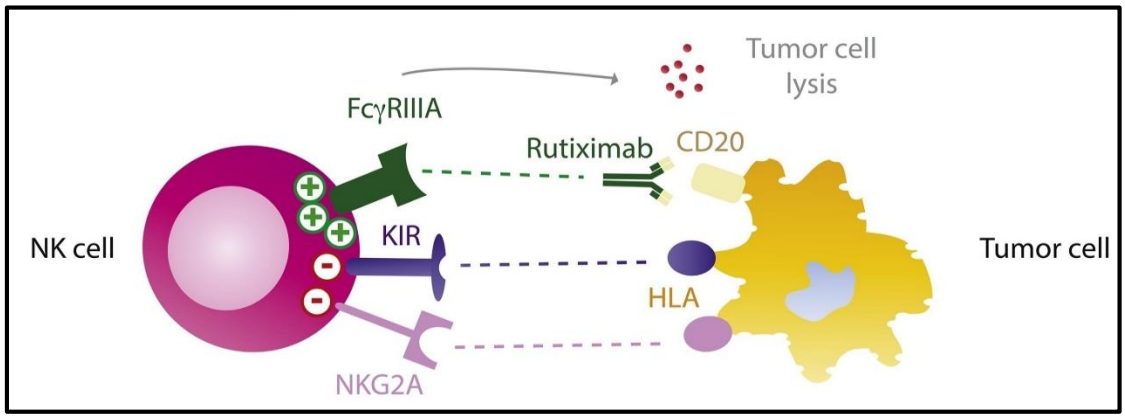

Figure 13: Tumor antigen-specific antibodies (Chiossone et al., 2017)

b. Immune checkpoints inhibitors

One mechanism used by tumor cells to escape NK cell immunosurveillance is expression of ligands for NK cell inhibitory receptors. Some studies have tested the ability of antibodies to block the interaction of certain NK cell inhibitory receptors and checkpoints with their ligands to increase the antitumor activity of NK cells (reviewed by Chiossone et al., 2017; Fang et al., 2017; Guillerey et al., 2016; Weiner et al., 2010).

- **Inhibitory KIRs**

The inhibitory receptors KIR2DL1, KIR2DL2 and KIR2DL3 are an important group of NK cell inhibitory receptors that bind to HLA-C expressed on tumor cells to reduce the cytotoxicity of NK cells. The effect of KIRs on NK cell cytotoxic activity against cancer has been confirmed in patients with AML undergoing haplo-SCT, where KIR/HLA-mismatched has significantly reduced relapse rate without GVHD (Ruggeri et al., 2007a). 1–7F9 (IPH2101) and Lirilumab (IPH2102) are mAbs blocking the three inhibitory KIR receptors for HLA-C to increase NK cell cytotoxicity in HLA-C-expressing tumor cells, and they have been confirmed to promote NK cell-mediated lysis *in vitro* and in a xenograft mouse model of AML (Romagné et al., 2009). 1–7F9 (IPH2101) was the first approved mAb to be used in clinical trials, where it enhances the activity of NK cells against multiple myeloma without side effects (Benson et al., 2010a). Moreover, lirilumab is still being evaluated in many phase I/II clinical trials against different solid and hematological cancers as monotherapy or in combination

(see https://clinicaltrials.gov: NCT02399917, NCT02599649, NCT02252263, NCT02481297, NCT01687387, NCT01714739, NCT01592370, and NCT01750580).

- **NKG2A**

NKG2A is an inhibitory receptor expressed in NK and $CD8^+$ T cells, and binds with HLA-E ligand. The expression of HLA-E is often upregulated in cancer cells to escape from NK cell cytotoxicity. In addition, NKG2A expression on tumor-infiltrating NK and T cells has increased in breast and cervical cancer, which is related to TGF-β secretion by tumor cells (Mamessier et al., 2011; Sheu et al., 2005). So, blocking NKG2A by a novel humanized antibody called monalizumab (PH2201) improves the cytotoxic activity of NK cells against leukemia Epstein-Barr virus cell lines in a mouse model (Ruggeri et al., 2016). Currently, several clinical trials are designed to evaluate safety and effectiveness of monalizumab as a single agent or in combination with other drugs for treatment of cancer (see https://clinicaltrials.gov). For example, our team in 2016 designed a phase 1 clinical trial (NCT02921685) in collaboration with Institut Paoli-Calmettes (IPC-Marseille, France) to determine the maximal tolerated dose of monalizumab and its effect on the anti-tumor activity of NK cells.

- **Programmed cell death protein 1 (PD-1)**

PD-1 is an immunoinhibitory receptor of the CD28 family classically expressed on T, B and myeloid cells (Riley, 2009), however, recently its expression has been also found in a subset of healthy mature $CD56^{dim}$ NK cells with $NKG2A^-KIR^+$ phenotype where $PD\text{-}1^+$ NK cells have poor anti-tumor activity and cytokine-induced proliferation (Pesce et al., 2017). Additionally, circulating $PD\text{-}1^+$ NK cells have been increasingly found in peripheral blood of cancer patients, including multiple myeloma, Kaposi sarcoma as well as ovarian carcinoma (Beldi-Ferchiou et al., 2016; Benson et al., 2010b). PD-1 has two ligands: PD-L1 which is constitutively expressed in most hematopoietic cells, vascular endothelial cells and various tumor cells, such as melanoma, breast cancer, ovarian and hematopoietic tumor cells, and PD-L2 which is only expressed in macrophages and DCs.

A number of PD-1 antibodies such as pidilizumab, lambrolizumab and nivolumab, preventing PD-1/PD-L1 interaction, have been developed. Although these antibodies are able to kill tumor cells mainly by targeting T cells, their effect to enhance endogenous NK cell cytotoxic function remains attractive (reviewed by Romero, 2016). In 2015, Huang and co-workers have found that intracranially implanted of IL-2-activated NK cells pre-treated with a PD-1-blocking antibody into

mice resulted in improved survival in a mouse glioma model (Huang et al., 2015). Moreover, the combination of PD-1 antibody (pidilizumab) with rituximab in phase II clinical trial was active and well-tolerated in patients with relapsed follicular lymphoma where the CR rate was significantly increased comparing with CR rate in patients retreated with rituximab monotherapy (Westin et al., 2014). These findings propose that blocking the inhibitory pathway of PD-1 and its ligand PD-L1 on NK cells may improve their effector function, and encourage to design clinical trials to assess the efficiency of using PD-1 antibodies as NK cell based immunotherapy.

- **T-cell immunoglobulin mucin 3 (Tim-3)**

Tim-3 is a type I glycoprotein expressed by innate and adaptive immune cells such as macrophages, DCs, $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells. Upon activation by binding with galectin-9, it functions as an inhibitory receptor on T cells which diminishes cytotoxicity and cytokine production (Sakuishi et al., 2010). In 2014, Gallois et al. found that circulating NK cells in metastatic melanoma patients are exhausted and characterized by high expression of Tim-3 (Gallois et al., 2014). In patients with lung adenocarcinoma, upregulated expression of Tim-3 on NK cells has been detected, and using anti-Tim-3 antibody increases the cytotoxicity and IFN-γ production of peripheral NK cells (Xu et al., 2015). Thus, Tim-3 expression in NK cells can be used as a predictive biomarker in human cancers and further studies are needed to fully understand the role of Tim-3 in the cell biology of NK cells.

- **TIGIT and CD96**

TIGIT (T cell immunoglobulin and ITIM domain) and CD96 are co-inhibitory receptors expressed on NK and T cells which recognize CD155 and CD112 ligands (DNAM-1 receptor binds to the same ligands, but with lower affinity) expressed on APCs, T cells, and various types of tumors (Dougall et al., 2017). Blocking TIGIT improves NK cell functions (Bi et al., 2014). Interestingly, using anti-CD96 antibody alone or in combination with anti-PD-1 in $TIGIT^{-/-}$ mice significantly reduces lung metastases and boosts IFN-γ production by NK cells (Blake et al., 2016). Overall, these data suggest that clear understanding of the regulation role of TIGIT and CD96 in NK cell can be a key to design new immunotherapies alone or in combination with other existing immune checkpoint inhibitors.

Currently, a human anti-TIGIT mAb (MTIG7192A, RG6058) is being tested in a phase I clinical trial in combination with anti-PD-1 therapy in various solid tumors.

iii. NK cell as adoptive immunotherapy

This title well be addressed in details in NK cell-donor lymphocyte infusion section.

Cellular immunotherapy is a promising alternative to severe chemotherapy for cancer mainly in the context of HSCT for treatment of a hematopoietic malignancy. However, HSCT remains a procedure associated with high mortality rates of up to 50% depending on the transplant type (related or unrelated, matched or mismatched) and conditioning regimen used. NK cells appear to be the best players in adoptive immunotherapy for cancer, where several clinical trials have proved their safety and efficacy to improve GvT effect without serious toxic effects as GVHD. Among the various sources of adoptive transfer NK cells, allogeneic haploidentical NK cells, which have been expanded and activated *ex vivo*, have demonstrated effective anti-tumor effects with a low rate of graft rejection and side effects.

Additionally, CAR-NK cells, genetically modified NK cells, show enhanced NK cell cytotoxicity, specificity and targeting. However, this approach has been successful with NK cell lines such as NK-92 because achieving stable transgene expression in primary NK cells has been challenging (Miah and Campbell, 2010). Moreover, more studies are essential to establish the safety and feasibility of treatment with genetically modified NK cells in the clinical setting.

II. HEMATOPOIETIC STEM CELL TRANSPLANTATION

1. Hematopoietic stem cell transplantation (HSCT)

Hematopoietic stem cells (HSCs) are multipotent cells that have the ability to self-renew and produce all mature blood lineages in a process called hematopoiesis. In 1959, Thomas and his colleagues found that a patient with end-stage leukemia who was treated with TBI, followed by infusion of bone marrow from her identical twin, had a three-month remission (Thomas et al., 1959). Since that time, numerous trials had been performed and different methods had been evolved based on the scientific and technical development to improve HSCT method. Generally, this method is favored to be used in many conditions such as: i) the body cannot make the blood cells that it needs, ii) the bone marrow or blood cells have become diseased and need to be replaced, or iii) a disease treated with high doses of chemotherapy and/or radiation, which destroy both diseased cells and normal stem cells at the same time.

HSCT, which is considered as the established therapy for numerous life-threatening malignant and nonmalignant disorders, is infusion of stem cells from either bone marrow (BM), peripheral blood (PB) or umbilical cord blood (UCB) into patients to establish functions of the bone marrow and the immune system. Every year, more than 25,000 HSCT process are performed for treatment of lymphoma, leukemia, immunodeficiency diseases, congenital metabolic defects, hemoglobinopathies, and myelodysplastic and myeloproliferative syndromes (reviewed by Hatzimichael and Tuthill, 2010).

2. Types of HSCT

Based on who gives the stem cells, HSCT is categorized into autologous SCT (auto-SCT) and allogeneic SCT (allo-SCT).

i. Autologous SCT

In this type of transplantation the patient receives stem cells from his own blood, where the patient's own stem cells are collected (apheresis) and frozen before treatment with chemotherapy and/or irradiation (conditioning regimen). Following conditioning treatment, the patient’s stem cells

are infused again into the body to produce healthy blood cells. One advantage of this procedure is that the body recognizes the cells and therefore does not reject or attack them. In addition, it avoids the difficulty of finding a suitable donor. However, auto-SCT has disadvantages including tumor relapse due to possibility of the presence of tumor cells within the injected stem cells. Another disadvantage is that the immune system is the same as before transplantation, meaning that tumor cells have the ability to escape from the immune system again.

ii. Allogeneic SCT

Allo-SCT is a potentially curative therapy for a variety of hematologic malignancies in which a recipient (patient) receives stem cells collected from a healthy donor. Primarily, the donor should be human leukocyte antigens (HLA)-matched (identical twin or sibling) to minimize transplantation complications. However, lack of the suitable donor is considered one of the major restrictions of allo-SCT, because only around third of patients have HLA-matched siblings. So, alternative sources of stem cells are involved as matched unrelated donors, umbilical cord blood and HLA haploidentical donors. Moreover, GVHD is a lethal obstacle that limits the application of allo-SCT, whereby the donor's immune cells attack the patient's tissues due to HLA mismatch. HLA is a type of protein found on the outer surfaces of cells that regulates the immune system and can identify which cells belong to the body and which do not. Undoubtedly, the closer of HLA matching between a donor and patient, the lesser risk of GVHD, and the greater chance of transplant success. Depending on the donor, allo-SCT can be classified into different subtypes.

a. Match related allo-SCT

In this subtype, the stem cells are isolated from HLA genoidentical matched sibling donors where these donors are the standard source of stem cells for allo-SCT. However, about 70% of patients do not have an available HLA-identical sibling donors (reviewed by Hatzimichael and Tuthill, 2010).

b. Match unrelated allo-SCT

This method is used for patients who have not a family donor, where stem cells are isolated from phenotypically HLA-matched unrelated donors (Anasetti et al., 2001). Unrelated donors can be found through a registry of bone marrow donors such as the National Marrow Donor Program (NMPD) and Bone Marrow Donor Worldwide (BMDW) database. In 2016 for instance, over 27

million donors are registered in the international database (*www.bmdw.org*) where in most European populations a 10/10 HLA-matched donor can be found for at least 50% of patients and 20-30% of patients may have a 9/10 HLA-matched donor (Tiercy, 2016).

c. Syngeneic allo-SCT

In this form of transplantation, bone marrow or peripheral blood stem cells are obtained from a donor who is a genoidentical twin to the patient. This method is essentially the same as auto- SCT, without tumor contamination within the graft.

iii. Haploidentical SCT

Although allo-SCT has been successfully used to treat many hematologic malignancies, its best results have been achieved in patients receiving an allograft from a HLA matched related donor (reviewed by Koh et al., 2007). The major obstacle of allo-SCT is the difficulty to find the suitable HLA genoidentical sibling donor. Moreover, application of allo-SCT using HLA matched unrelated donors remains limited by some difficulties including: 1) the possibility of finding a HLA matched unrelated donor ranges from less than 10% in ethnic minorities to 60-70% in Caucasians (Beatty et al., 1995); 2) the process of searching a suitable unrelated donor is complex and takes about 4 months, where some patients might relapse or even die during this waiting period; and 3) allo-SCT using HLA matched unrelated donors is associated with a high TRM (Davies et al., 2000; Sierra et al., 2000). To overcome these limitations, researchers have developed a modified method of HSCT known as a haplo-SCT or HLA-mismatched SCT.

Haplo-SCT is an alternative transplantation method which provides the chance for approximately 70% of patients, who do not have an HLA-identical related donor, to benefit from HSCT by using half-matched related donors, where the haploidentical related donor is a 50% HLA match to the patient and usually be the patient's parent, sibling or child (Beatty et al., 1985; Mehta et al., 2004; Powles et al., 1983). The first haplo-SCT has been carried out by Powles and colleagues from more than 30 years (Powles et al., 1983). The use of haplo-SCT offers several advantages, 1) practically all patients have at least one immediately available HLA-partially matched donor; 2) it is easy to repeat donation of donor's stem cells if donor-derived cellular therapy is needed for the treatment and/or prophylaxis of relapse, or for second transplantation due to graft failure; and 3) a

potentially stronger GvL effect (reviewed by Huang, 2008). Due to HLA-mismatch, the major obstacles of haplo-SCT are T cell mediated alloreactions in two directions: the host-versus-graft direction which causes graft rejection, and in the graft-versus-host direction causing GVHD, and severe infections resulting from delayed or incomplete immune reconstitution.

3. Complications of HSCT

As HSCT has been the major curative therapy for several disorders, its efficacy and perceptible benefits are frustrated by serious complications including tumor relapse, infections, and GVHD which are associated with a higher incidence of TRM (Copelan, 2006). Both type and risk of HSCT complications depends on the conditioning regimen received prior to transplant, patient's age and disparity of HLA that affect integrity of the immune system. Conditioning regimen is the treatment used to prepare the patient for HSCT by providing a sufficient immunosuppression to avoid graft rejection (Vriesendorp, 2003). The conventional regimen is known as myeloablative conditioning regimen, where chemotherapy and/or TBI are given immediately prior to transplant at high doses. Although myeloablative regimens produce a rapid engraftment of donor's cells, they are associated with high risk of toxicities as infections, GVHD and TRM mainly at elderly patients.

In order to reduce TRM associated with myeloablative regimens and to make transplantation available for the older patients, a newer treatment approach, known as non-myeloablative conditioning regimen or reduced-intensity conditioning (RIC), has been developed using low doses of chemotherapy and radiation to prevent eradicate all the bone marrow cells of the recipient (Alyea et al., 2006; Diaconescu et al., 2004). Non-myeloablative regimens are characterized by lower risks of serious infections, acute GVHD and TRM; however, chronic GVHD remains a significant cause of mortality (Mielcarek et al., 2003).

i. Relapse of malignancy

As tumor relapse is a fatal complication of HSCT, its occurrence rate varies by transplantation method, tumor type, type of conditioning regimen and the presence of GVHD. In most malignancies, relapse rates after auto-SCT are higher than after allo-SCT due to contamination of the autograft with tumor cells, while the graft in allo-SCT is free from contaminating tumor cells and also contains donor-derived immune-competent cells which can produce GvT. About 40-45% of recipients of HLA-

identical grafts and 35% of recipients of unrelated donor transplants may be relapsed with their original malignancy (Barrett and Battiwalla, 2010). Commonly, tumor relapse can occur after transplant due to many causes: i) the conditioning regimen is insufficient to destroy most of tumor cells, ii) the immune system is unable to establish an effective GvL effect, or iii) the tumor undergoes immune escape. Unfortunately, management of patients suffering from tumor relapse is a major challenge due to the absence of the standard treatment.

ii. Graft-versus-host disease (GVHD)

GVHD, the major cause of mortality following allo-SCT, is a serious immunological complication post HSCT, observed by Barnes and Loutit in a murine model in 1956 (Barnes and Loutit, 1957). GVHD can be considered as an undesirable and exaggerated aspect of normal inflammation process, in which donor T-lymphocytes attack recipient's tissues. In general, GVHD is classified as acute GVHD (occurs within 100 days after transplantation) or chronic GVHD (occurs beyond 100 days) according to presentation time and the involved organ. However, distinguish between them is not always clear as acute GVHD can occur after 100 days and also it can progresses into chronic GVHD. In general, acute GVHD can develop during three phases where both innate and adaptive immune systems are involved: (a) damage of the recipient's tissue by the conditioning regimens; (b) donor T-cell activation, differentiation and migration (afferent phase); and (c) damage of the target organs by a group of cellular and inflammatory factors (efferent phase) (Reviewed by Ghimire et al., 2017).

a. Conditioning-mediated tissue damage

In this phase, patient's tissues have been extremely damaged by the underlying disease and the conditioning regimens prior to transplantation process. Consequently, proinflammatory cytokines as IL-1, IL-6 and TNF-α are released, and then activate the patient's APCs by increasing the expression of their MHC antigens, costimulatory molecules and cell surface adhesion molecules which are critical for activation of allogenic T cells and initiation of GVHD (Ferrara et al., 2009). Moreover, gut damage from conditioning regimens plays an essential role because it allows systemic translocation of microbial products as lipopolysaccharide (LPS), enhancing activation of host APCs (Ferrara et al., 2009). This clarifies why using of non-myeloablative regimens is associated with less risk and severity of GVHD.

b. Afferent Phase

It is the critical phase in GVHD pathogenesis in which donor T cells proliferate and differentiate by recipient APCs (figure 14). T-cell responses and GVHD frequency are directly related to the degree of mismatch between HLA proteins of donors and recipients; however, about 40% of HLA-identical grafts recipients have GVHD due to mismatch in minor histocompatibility HLAs (minor HLAs). These antigens are associated with both GVHD and GvL effects based on their tissue distribution. For example, HY and HA-3 antigens expressed on all tissues are required for both GVHD and GvL, whereas HA-1 and HA-2 antigens may enhance GvL effect because their expression is restricted on haemopoietic cells (including leukemic cells) (reviewed by Ghimire et al., 2017).

After activation of T cells by host APCs, they proliferate and differentiate into T subsets as: $CD4^+$ cells which can develop GVHD to MHC class II differences, and $CD8^+$ cells which are able to produce GVHD to MHC class I mismatch. In contrast, regulatory T cells ($CD4^+CD25^+$ cells) have the ability to suppress T-cell alloreactivity without decreasing the GvL effect (Edinger et al., 2003). Interestingly, Cohen and Boyer found that addition of regulatory T cells to donor graft can reduce conventional T cells proliferation and prevent GVHD in animal models (Cohen and Boyer, 2006). Moreover, T-helper 1 cytokines (TNF-α, IL-2 and IFN-γ) produced by activated T cells play an effective role in progression of GVHD. For instance, IL-2 augments the alloreactivity of allogeneic T-cells as well as stimulates macrophages to release TNF-α and enhances inflammation damage of skin and gut (Ferrara et al., 2009). IFN-γ is another cytokine playing a significant role in development of GVHD through: increasing the expression of chemokine receptors, MHC proteins and adhesion molecules; promoting the susceptibility of monocytes and macrophages to stimuli as LPS; direct damage of epithelium in gut and skin (Ferrara et al., 2009).

c. Efferent Phase

This phase is characterized by apoptosis of target cells, mediated by the activity of both innate and adaptive immune cells which work together to exacerbate the T cell-induced inflammation. Targets cells are mainly destroyed via Fas/FasL pathway and perforin/granzyme pathway, which are regulated by cytotoxic T cells and NK cells (reviewed by Ghimire et al., 2017). Additionally, LPS can leak through the damaged intestinal mucosa and skin and stimulate monocytes and macrophages

to secret inflammatory cytokines, which result in more damage of epithelial cells mostly in gut (figure 14) (Reviewed by Ghimire et al., 2017).

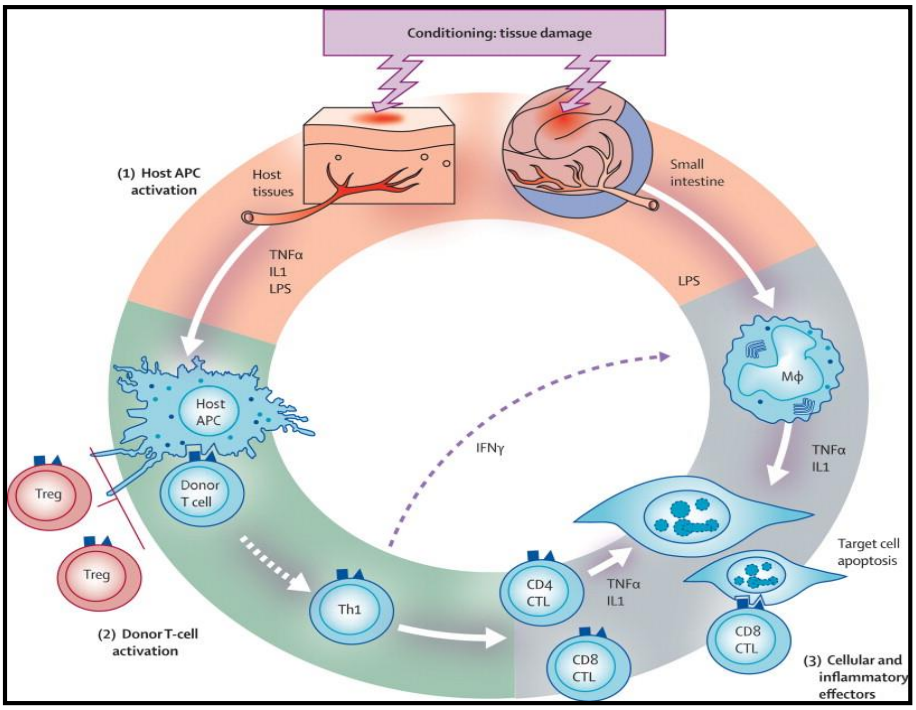

Figure 14: Pathophysiology of acute GVHD *(Ferrara et al., 2009)*

With respect to chronic GVHD, its pathophysiology remains unclear. There are different theories can explain how it develops (Min, 2011). i) Disruption of immune tolerance to self-antigens due to thymic epithelial damage. ii) Regulatory T cell deficiency, where Tregs may suppress chronic GVHD secretion of TGF-β and IL-10. iii) Auto-antibody production by abnormal B cells.

4. Reconstitution of immune cells after HSCT

It is clear that the conditioning regimens before HSCT are used to eradicate patient's HSCs to facilitate the engraftment and prevent graft rejection. Accordingly, donor graft from bone marrow or peripheral blood is used to recover patient's immunity. Reconstitution of host's immune cells after HSCT can improve the outcomes of HSCT and the delay in this process is associated with transplantation related complications such as infections and relapse. After transplantation, the functions of immune system are regulated by both mature immunocompetent cells within the graft and by immune cells arising from transplanted stem cells. Recovery of fully functional immune cells is slow and occurs at different time points where the innate immune cells are reconstituted firstly

within few weeks after transplantation, followed by the adaptive immune cells that may take nearly 2 years for complete recovery (reviewed by Storek et al., 2008).

i. Reconstitution of innate immune cells

a. Reconstitution of monocytes and DCs

Monocytes are recovered in 1 month post-transplant. Although G-CSF mobilized PB grafts contain a large number of monocytes, most of them become undetectable by day 7 after transplantation either due to their death or their development to tissue macrophages (reviewed by Storek et al., 2008).

DCs are APCs which play a key role in regulation of the adaptive immune responses. They are a rare immune population where their precursors in blood constitute less than 1% of mononuclear cells. DCs can be divided into many subsets according to their location: (a) epithelial DCs that migrate to the lymph nodes extrafollicular areas upon encounter of antigen, (b) DCs located in the extrafollicular areas of lymph nodes and spleen, where their main function is to present antigens to T cells and provide costimulatory signals, (c) blood DCs, which may represent the precursors of DCs in the epithelium and the extrafollicular areas of lymph nodes, (d) thymic DCs that play a role in deletion of autoreactive T cells, and (e) follicular DCs in lymph nodes germinal centers that play a role in maturation of B cell (reviewed by Storek et al., 2008). Post transplantation, blood DCs are detectable within 2-3 weeks and stay low in quantity in the first 3 months. Consequently, counts of myeloid DCs (mDCs, the major stimulator of T cells) can be normalized while plasmacytoid DCs (pDCs, the principle producers of interferon-α) are still low even at 1 year post transplantation (Chklovskaia et al., 2004a). Follicular DCs recover slowly, 1 year after transplantation that could clarify the late reconstitution of memory B cells.

b. Reconstitution of NK cells

NK cells are the first lymphocytes to reconstitute following HSCT and their rapid recovery is associated with better outcomes in patients due to NK cell activity against tumor cells and pathogens (Chklovskaia et al., 2004b; Dunbar et al., 2008; Ruggeri et al., 2002; Storek et al., 2008). While numbers of NK cell are commonly reconstituted within approximately 1 month, the acquisition of maturation markers and full functional activity can occur in 6 months to one year after transplant

depending on donor source, underlying disease, GVHD and functional efficiency of the NK cells in the graft itself and NK cells developing from the graft, which is difficult to distinguish (reviewed by Ullah et al., 2016).

In summary, the early reconstituting NK cells are characterized by a high percentage of $CD56^{bright}$ immature NK cells (40-60% of the NK cells in the first months post HSCT), higher NKG2A expression (around 90%), lower KIRs, variant NCRs expression, and lower than normal cytotoxic function (Dulphy et al., 2008; Foley et al., 2014; Nguyen et al., 2005; Pical-Izard et al., 2015). After the first 6 months of HSCT, the normal receptor repertoires and functions are gradually restored by differentiation of $CD56^{bright}$ NK cells into $CD56^{dim}$ mature NK cells which are characterized by a significant decrease in NKG2A expression and an increase in the expression of NCRs, CD16, KIRs and CD57 (Foley et al., 2014; Ghasemzadeh et al., 2016; Pical-Izard et al., 2015). As a consequence of improving the effector functions of reconstituting NK cells, the GvL effect is enhanced and the residual tumor cells are killed, while GVHD can be minimized due to lysis of both APCs and cytotoxic T cells (Palmer et al., 2013). We are going to mention in details about their phenotypic characteristics, their functional activity, and their role in GvL and GVHD effects in NK cells-donor lymphocyte infusion section.

ii. Reconstitution of adaptive immune cells

In contrast to the fast recovery of the innate immune cells after HSCT, reconstitution of lymphoid cells of the adaptive immune system is slow and may take years for complete recovery.

a. Reconstitution of B cells

B cells are lymphocytes formed in BM from CLPs and represent the humoral immunity by secreting antibodies. The earliest B cells formed in BM are immature cells which migrate to secondary lymphoid organs and differentiate into mature B cells (naïve B cells). Then, mature B cells are activated by binding with antigens of $CD4^+$ T cells or DCs via their B cells receptors (BCRs). As a result, they differentiate into plasma cells that secrete antibodies or memory B cells (reviewed by Kurosaki et al., 2015). B cells are the slowest immune cells to reconstitute following HSCT. In the first few months, very few circulating B cells have been observed but their full reconstitution can be achieved within 2 years psot HSCT. Even though the total B cells counts may reach levels of adult

controls, most of the reconstituting B cells in the first year are immature and naïve B subsets, whereas memory B cells recovery occurs later (reviewed by Ogonek et al., 2016). Additionally, the regenerating B cells have impaired humoral functions in the first 2 years following HSCT due to: low levels of circulating B cell subsets; and the insufficient $CD4^+$ T cell recovery because signaling of $CD4^+$ T cell is required for activation of naïve B cells and isotype switching for memory B cells (reviewed by Ogonek et al., 2016; Williams and Gress, 2008).

b. Reconstitution of T cells

T cells, or T-lymphocytes, are a subset of lymphocytes mainly characterized by the expression of CD3 protein complex on their cell surface. They are the main constituent of adaptive immune system having a central role in cell-mediated immunity. Normally, progenitors of T-lymphocytes arise in BM from CLPs; and then these progenitors leave the BM to the thymus to complete their maturation and differentiation into functional T-lymphocytes subpopulations. In the thymus, progenitor cells lack most of the surface molecules characterized mature T cells mainly CD4 and CD8 co-receptors. Therefore, these cells are called double-negative ($CD4^-CD8^-$) thymocytes which have rearrangement for their TCR genes (αβ TCR genes and γδ TCR genes) to produce the main two T-cell lineages: γδ T cells (the minor population of T-lymphocytes which lack CD4 or CD8 even after maturation), and the major population of T-lymphocytes (αβ T cells) which have the ability to express both CD4 and CD8 at the same time forming $CD4^+CD8^+$ double-positive thymocytes (figure 15). About 98% of $CD4^+CD8^+$ double-positive thymocytes die during either positive selection or negative selection processes, while the other 2% survive, lose expression of either CD4 or CD8 and leave the thymus to peripheral blood and tissues as a mature single-positive $CD4^+$ or $CD8^+$ T cells (reviewed by Janeway et al., 2001).

$CD4^+$ cells, also known as T helper cells (T_H cells), are activated via binding to antigens presented by MHC class II which are expressed on the surface of APCs like macrophages and DCs. Once activated, they divide and differentiate into several subtypes such as T_H1, T_H2, T_H17 or regulatory cells, which secrete different cytokines to regulate or assist different immune responses, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages (reviewed by Zhu and Paul, 2008).

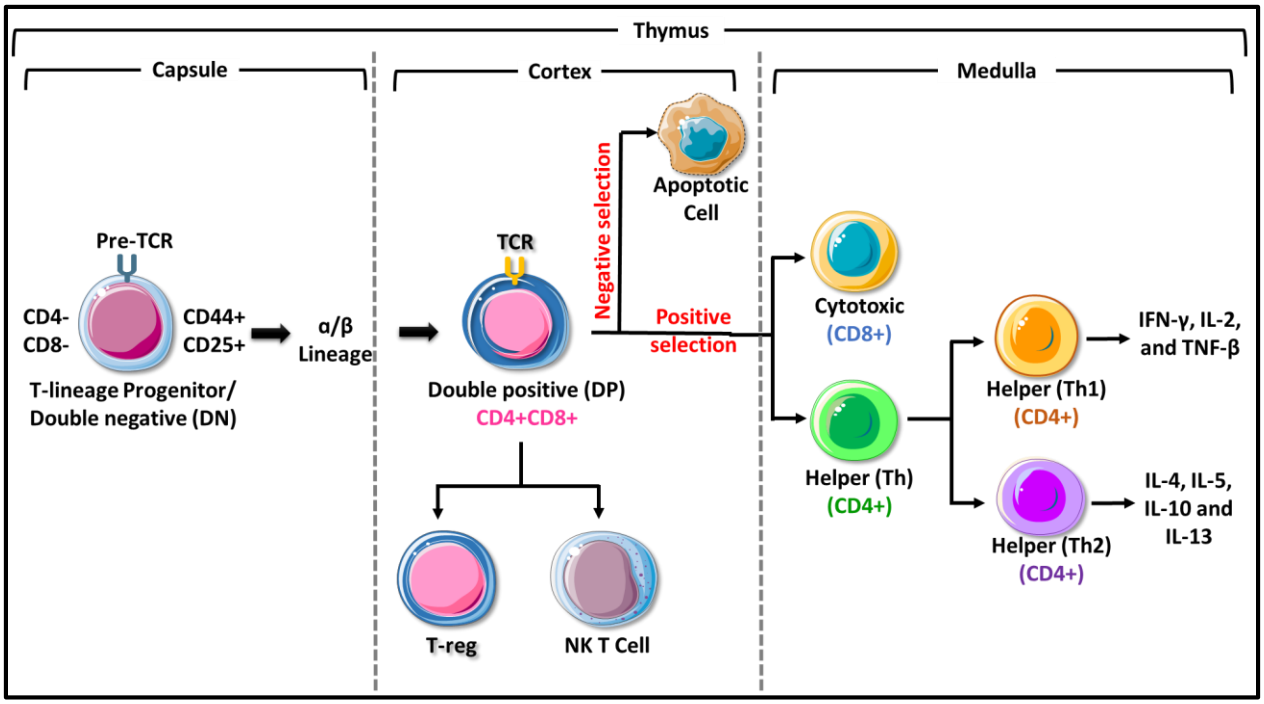

Figure 15: Development and differentiation of T cell (adapted from Janeway et al., 2001)

$CD8^+$ cells, also called cytotoxic T cells, are very important for immune defense against intracellular pathogens, including viruses and bacteria, and tumor cells. In HSCT, $CD8^+$ cells are considered the main effector cells against malignant cells via GvT effect, however, they have a basic role in development of HSCT complications as GVHD and graft rejection. These cells become activated through binding to their targets antigens linked with MHC class I molecules, which are expressed on all nucleated cells. Activated $CD8^+$ cells have three major mechanisms to kill infected or malignant cells, including secretion of cytokines, primarily TNF-α and IFN-γ; production and release of cytotoxic granules which contain perforin and granzymes; and Fas/FasL interactions. Activated $CD8^+$ T cells express FasL on their cell surface that binds to its Fas receptor on surface of its target cell resulting in apoptosis of the target cell via activation of caspase cascade (reviewed by Zhang and Bevan, 2011).

The delay in T lymphocytes recovery and the deficiency of their immune functions after HSCT are related to the destruction of the patient's T cells and the inability of the thymus to produce appropriate numbers of naïve T cells, which are caused by the damage effect of the conditioning regimens and the alloreactivity of donor T cells within the graft. After HSCT, $CD8^+$ T cells reconstitute faster than $CD4^+$ T cells, which occurs around 3 months or later and is indicated by the inversion of

the ratio of $CD4^+/CD8^+$ T cell early after HSCT. However, $CD4^+$ T cells need 1 or 2 years after HSCT to achieve their complete reconstitution (reviewed by Mehta and Rezvani, 2016).

Commonly, T lymphocytes reconstitute within the first year through two pathways: thymus-independent and thymus-dependent pathways (figure 16). Indeed, the initial recovery of T-cells during the first months after HSCT depends mainly on thymus-independent pathway in which the peripheral mature T cells (either infused with the graft or patient's T cells that have survived after the conditioning regimen) are expanded to generate $CD8^+$ T cells more than $CD4^+$ T cells (reviewed by Mehta and Rezvani, 2016). When donor's graft is engrafted in patient's BM, T lymphocytes progenitors migrate to the thymus, the primary site for T cell development and differentiation, to perform T lymphocytes recovery based on thymus-dependent pathway. This pathway is characterized by producing naïve $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and $CD4^+$ $CD25^+$ regulatory T cells (reviewed by Mehta and Rezvani, 2016).

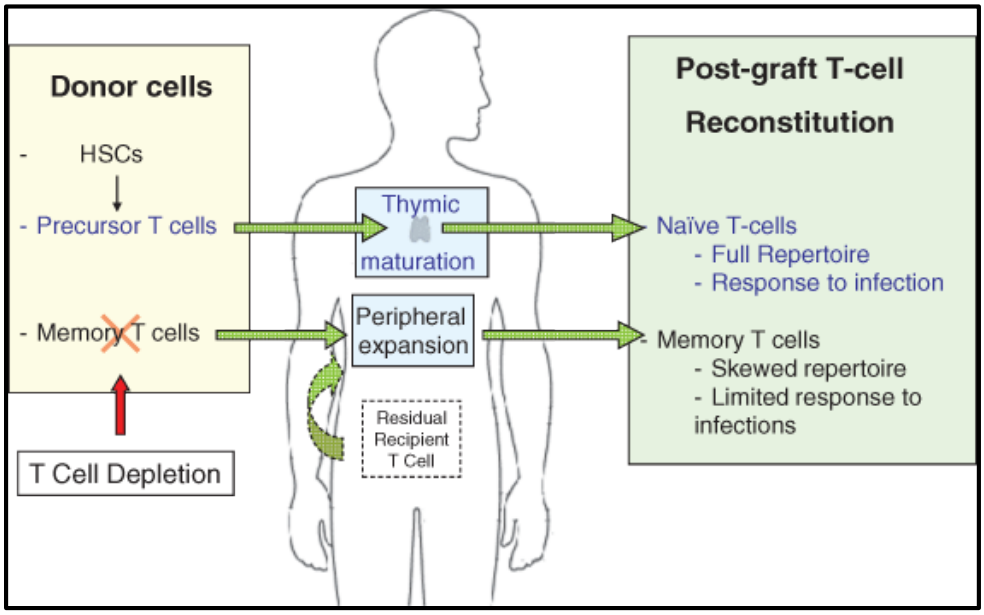

Figure 16: Reconstitution of T-lymphocytes after HSCT (Toubert et al., 2012)

5. Regulatory T cells and HSCT

Regulatory T cells (Tregs) play an essential role in suppressing excessive immune responses and maintaining immunological unresponsiveness to self-antigens by suppression of the activation and effector functions of many immune cells such as $CD4^+$ and $CD8^+$ T cells, NK cells, B cells and APCs (reviewed by Sakaguchi et al., 2008). Most of reports have focused on Tregs arising from $CD4^+$ T cells which were discovered in 1995 by Sakaguchi and his colleagues (Sakaguchi et al., 1995), however, new population ($CD8^+Foxp3^+$) has been described developing from $CD8^+$ T cells and has the ability to suppress immune responses (Xystrakis et al., 2004).

Tregs arising from $CD4^+$ T cells are phenotypically characterized by the expression of transcription factor forkhead box P3 ($Foxp3^+$) and high expression of CD25 ($CD4^+CD25^+Foxp3^+$). There are two groups of $CD4^+CD25^+Foxp3^+$ Tregs based on their origin: natural Tregs (nTregs) which develop in the thymus during maturation and differentiation of T progenitors, and induced Tregs (iTregs) that are generated in peripheral lymphoid organs from $CD4^+CD45RA^+$ naive T cells in the presence of TGF-β (reviewed by Sakaguchi et al., 2008).

Tregs can suppress excessive immune responses by different mechanisms (figure 17) including: (a) production of inhibitory cytokines such as IL10 and TGF-β, where IL-10 inhibits the activity of APCs (DCs and macrophages) by inhibiting the expression of co-stimulators and MHC II molecules on those cells and suppressing the production of IL-12 by activated DCs and macrophages. Moreover, TGF-β, inhibits the proliferation, differentiation and effector functions of T cells and the activation of macrophages (reviewed by Sakaguchi et al., 2008); (b) competition of effector cells for interaction with APCs by binding co-stimulatory receptors on the surface of APCs; (c) apoptosis of effector T cells or DCs by cell-to-cell contact through the production of perforin/granzyme B; and (d) metabolic disruption by enhancing APCs to produce enzymes that consume essential amino acids required to proliferation of effector cells (reviewed by Sakaguchi et al., 2008).

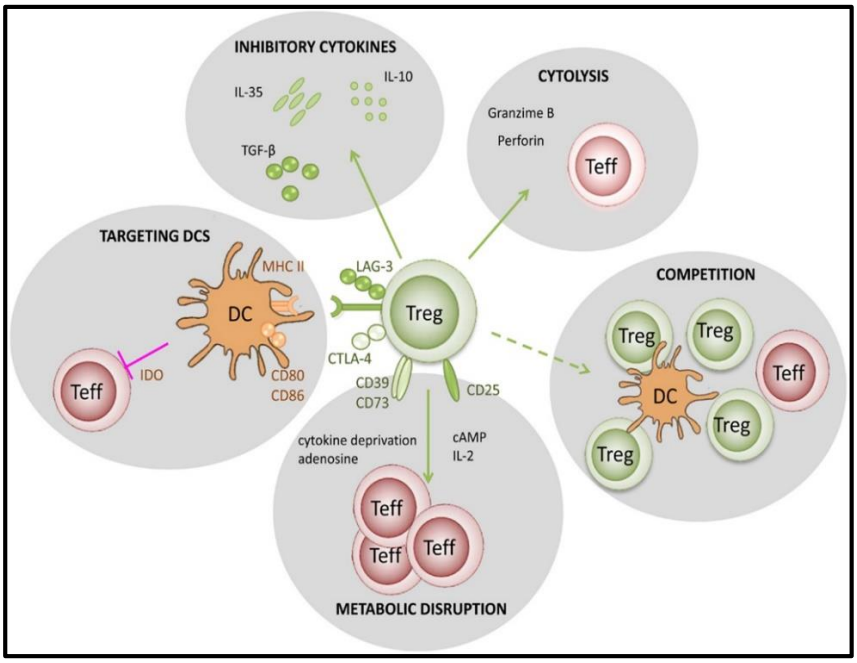

Figure 17: Mechanisms used by regulatory T cell suppress immune system (Caridade et al., 2013)

Tregs play an indispensable role in allograft tolerance after HSCT, however, their scarce numbers in PB (0.5-1%) is the main challenge to evaluate their immunity post-transplant. There are many animal models used to investigate the transplantation tolerance mediated by Tregs, mainly $CD4^+CD25^+Foxp3^+$ Tregs (reviewed by Ogonek et al., 2016). For example, Edinger and co-workers have found in a mouse model that $CD4^+CD25^+Foxp3^+$ Tregs suppress GVHD after BM transplantation without annulling the GvL effect (Edinger et al., 2003b). Furthermore, Tregs numbers after HSCT has been evaluated by many researchers where they have noted that $CD4^+CD25^+Foxp3^+$ Tregs frequency was significantly decreased in patients with severe GVHD (reviewed by Ogonek et al., 2016).

In term of $CD8^+Foxp3^+$ Tregs role in transplantation tolerance, Robb and colleagues have reported that GVHD and its related mortality after BM transplantation in a mouse model were suppressed by $CD8^+Foxp3^+$ Tregs; and interestingly they have found that $CD8^+Foxp3^+$ Tregs have more suppression efficiency than $CD4^+CD25^+Foxp3^+$ Tregs (Robb et al., 2012).

III. NK CELL-DONOR LYMPHOCYTE INFUSION (NK-DLI)

1. Donor lymphocyte infusion

Even though efforts to improve the efficacy of conditioning regimens and GvL effect for patients undergoing allo-SCT, tumor relapse remains the major cause of HSCT failure. While, different techniques have been used to treat tumor relapse including immunosuppression cessation, re-induction of chemotherapy or transplant repeat, the majority of relapsed patients die due to the insufficiency of these methods (Porter et al., 2010). Alternatively, donor lymphocyte infusion has been frequently used to treat and prevent tumor relapse after allo-SCT based on GvL effect of donor derived T cells (Bar et al., 2013; Bethge et al., 2004; Lewalle et al., 2003; Michallet et al., 2005; Schmid et al., 2007; Takami et al., 2014).

Donor lymphocyte infusion (DLI), also known as donor leukocyte infusion, is adoptive immunotherapy used to induce GvL effect by intravenous infusion of a donor T lymphocytes into a patient previously received HSCs from the same donor. This approach has been used by Kolb and his colleagues in 1990 to treat patients with relapsed chronic myeloid leukemia (CML) after allo-SCT, and the efficacy of DLI was confirmed by several studies which showed an CR in 70-80% of patients with relapsed CML (Reviewed by Chang and Huang, 2013; Kolb et al., 1990). Although DLI was initially used to treat patients with relapsed CML, its effectiveness to produce CR in patients with other hematological malignancies relapse as AML, acute lymphocytic leukemia (ALL) and multiple myeloma (MM) is limited (reviewed by Luznik and Fuchs, 2002). Beside its valuable GvL effect, regrettably DLI is accompanied by a high incidence of severe complications such as GVHD (40-60%) and aplasia (20-40%) due to using large number of $CD3^+$ T cells (Marks, 2002).

It is clear that GvL effect of DLI and the incidence of GVHD are highly related due to the activity of $CD3^+$ T cells. Consequently, it is very important to separate GvL effect from GVHD to avoid DLI limitations. Different mechanisms are used to enhance the GvL effect or to minimize GVHD, including: selection of the ideal donors; dose-escalation regimen (Dazzi et al., 2000); *ex vivo* activation of T lymphocytes prior infusion (Porter et al., 2006); selection and depletion of allo-reactive T lymphocytes subsets using CD8-depleted lymphocytes or administration of purified $CD4^+$

lymphocytes because $CD8^+$ T cells play a critical role in pathogenesis of GVHD (Alyea et al., 1998; Wagner et al., 2012); and combination of DLI with pharmacological agents to improve the effectiveness of GvL effect (Schroeder et al., 2015). Unfortunately, some of these modifications are associated with an insufficient GvL effect resulting in disease relapse, graft rejection and infectious complications. Moreover, infusion of T cells is not applicable to all patients and not idealistic in some conditions as haplo-SCT due to increase the incidence of GVHD (reviewed by Koh and Chao, 2008). For these reasons, there is a necessary need for novel adoptive cellular immunotherapy to enhance GvL effect without or with minimal GVHD development, and to reduce the risk of relapse posttransplant.

2. NK cell based adoptive immunotherapy

Since the past decade, clinical trials focused on NK cells to use as adoptive immunotherapy due to their strong GvL effect without worsening GVHD effect (Miller, 2005; Palmer et al., 2013; Ruggeri et al., 2002, 2007b; Sutlu and Alici, 2009).

i. NK cell alloreactivity

Normally, NK cell function is regulated by a balance between activating and inhibitory receptors. KIRs, clonally distributed receptors recognizing HLA class I allele ligands, play a critical role in determination of NK cell function. KIRs distribution on NK cells is diverse due to the random expression of KIR genes, however, all individuals have NK cells expressing KIR2DL2/3 receptors which are specific for HLA-C group 1 alleles, KIR2DL1 receptor (specific for HLA-C group 2 alleles) is found in 97%, and around 90% express KIR3DL1 receptor which is specific for HLA-Bw4 (Uhrberg et al., 1997). Interestingly, only NK cells which express inhibitory receptors for self-HLA ligands as KIRs and NKG2A become functional cells (education theory). Missing specific HLA class-I allele by allogeneic target cells allows KIRs to sense the absence of self HLA class-I ligand (‘missing self’ recognition), resulting in target lysis. However, NK cell alloreactivity is not initiated among CD94-NKG2A receptor because HLA-E, NKG2A ligand, is expressed on cells from all individuals. This suggests that KIRs and their ligands are the main players in NK cell alloreactivity via KIRs and the incompatibility or missing of their ligands hypothesis (reviewed by Nguyen et al., 2011).

Several clinical reports, mainly in haplo-SCT, have shown that donor-versus-recipient NK cell alloreactivity is a key therapeutic element in the success of transplant due to its activity against remaining malignant cells (GvL effect) without development of GVHD as well as controlling infections (Ruggeri et al., 2007b; Suck et al., 2016).

ii. NK cell and GVHD

Many studies have demonstrated that alloreactive NK cells act as potential modulators of GVHD following transplant. For example, Ruggeri *et al*. found that infusion of murine alloreactive NK cells into haploidentical mouse recipient did not cause GVHD. Moreover, they also found in human that NK cell alloreactivity eliminate leukemia relapse and graft rejection and protect patients against GVHD (Ruggeri et al., 2002). In general, effect of NK alloreactivity on the incidence of acute GVHD is varying between reducing the incidence of acute GVHD and no significant difference (reviewed by Ruggeri et al., 2007). NK cells can minimize GVHD via different mechanisms (figure 18) including: i) the ability of NK cells to lyse active $CD4^+$ T cells through interaction between DNAM-1 receptor and its ligands (CD155 and CD161) on T cells; ii) the binding between NKG2D receptor and T cell marker H60 which also results in damage of activated T cells; iii) competition of IL-15 by NK cells which is necessary for $CD8^+$ T cells proliferation; and iv) lysis of DCs which are involved in GVHD development (reviewed by Palmer et al., 2013, Simonetta et al., 2017). In addition, non-hematopoietic healthy tissues of recipient are safe from NK alloreactivity because they lack the expression of ligands for NK activating receptors.

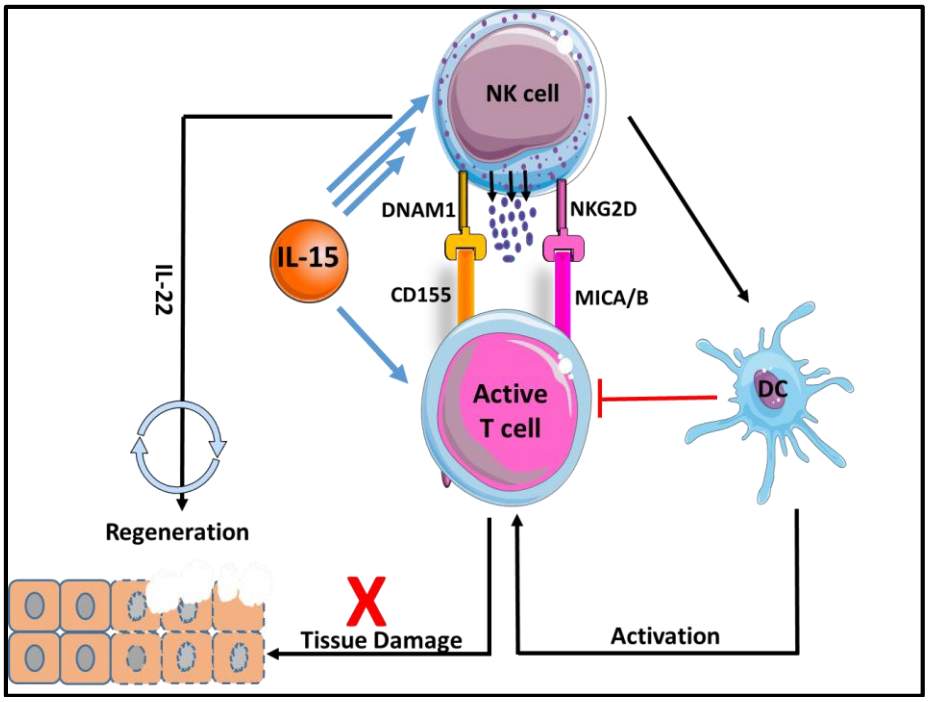

Figure 18: NK cells as potential modulators of GVHD (Adapted from Simonetta et al., 2017)

iii. NK Donor lymphocyte infusion (NK-DLI)

Unfortunately, the acquirement of full functional activity for the reconstituting NK cells requires long time due to several conditions other than graft type and reconditioning regimens such as: i) it is assumed that the reconstituting NK cells are primarily resulting from differentiation and maturation of progenitor cells after engraftment rather than expansion of mature NK cells within the graft (Foley et al., 2014; Nguyen et al., 2005). As the early reconstituting NK cells have an immature $CD56^{bright}$ phenotype, they are associated with an impaired cytotoxicity due to a high expression level of NKG2A inhibitory receptor and decreased levels of KIRs expression. With the time, NK cells differentiate and mature to $CD56^{dim}$ subset characterized with a gradual decrease of NKG2A expression and high levels of NCRs which abolish the inhibitory effect of NKG2A receptor and recover the impaired NK cell effector function (Ghasemzadeh et al., 2016; Pical-Izard et al., 2015), ii) development of GVDH where the recovery of NK cell numbers and its effector function was delayed in patients suffering from GVHD (Huenecke et al., 2017), and iii) low levels of Eomes and T-bet, two T-box transcription factors regulating NK cell maturation and function, which are associated

with reduced production of both IFN-γ and perforin by NK cells post HSCT (Simonetta et al., 2015). iv) hyporesponsiveness of developing NK cells expressing inhibitory KIRs for non-self HLA class I ligands (uneducated NK cells) (Björklund et al., 2010). However, Hsu et al. have reported that the developing NK cells in patients who lacked HLA class I ligands for donor inhibitory KIR are alloreactive (Hsu et al., 2005).

The first trials in humans to improve the anti-tumor activity of NK cells focused on activation of autologous NK cells by IL-2 where IL-2 was administrated directly to patients or autologous NK cells were activated *ex vivo* via IL-2 (Benyunes et al., 1995; Burns et al., 2003). However, IL-2 stimulated NK cells resulted in a limited efficacy in the recovery of NK cell cytotoxicity *in vivo* due to: i) competition between the recipient's lymphocytes for IL-2, ii) inhibition of autologous NK cells by self-MHC molecules, iii) chronic impaired host's immunity induced by the tumor, and iv) the possibility to stimulate Tregs by low doses of IL-2 (reviewed by Foley et al., 2014).

Interestingly, several studies have demonstrated that the higher absolute count of NK cells posttransplant and their alloreactivity were associated with lower tumor relapse and improved survival (Chklovskaia et al., 2004b; Dunbar et al., 2008; Ruggeri et al., 2002; Storek et al., 2008). Consequently, clinicians have proposed to infuse mature and fully functional alloreactivity NK cells into patients as a novel immunotherapy protocol (NK donor lymphocyte infusion, NK-DLI) to improve HSCT outcomes. NK-DLI is an alternative technique to DLI in which NK cells are sorted from the donor involved in all-SCT, then the sorted NK cells are activated ex-vivo and infused in the patient. In 2004, NK-DLI was firstly established to be a safe technique for patients following haplo-SCT. In this study, infusion of purified NK cells from PB of donors into AML patients was well tolerated without GVHD development (Passweg et al., 2004). From that time until now, several trials have been performed using allogeneic NK cells as adoptive immunotherapy. Due to differences in the strategies used for separation, expansion and activation of NK cells, effect of the injected NK cell alloreactivity on transplant outcomes are variable. For instance, Stefan and co-workers accomplished a phase 1 dose-escalation study of membrane-bound IL-21 expanded donor NK cells which were infused before and after haplo-SCT for high-risk myeloid malignancies. They found that infusion of high doses of *ex vivo*-expanded NK cells ($1x10^8$/kg per dose) is safe without adverse effects and associated with decreased GVHD, lower TRM, significantly improved NK cell function,

and low relapse rate posttransplant (Ciurea et al., 2017). In 2016, *ex vivo*-expanded and activated NK cells derived from unrelated healthy donors were infused into patients with malignant lymphoma or solid tumors. The findings showed that infusion of activated NK cells improved the effector function of patients' immune system via increasing the expression of NKG2D on $CD8^+$ T cells, reducing both regulatory T cells and myeloid-derived suppressor cells, and repressing TGF-β production (Yang et al., 2016). Another study was carried out by Curti et al., in 2011, where infusion of alloreactive NK cells into elderly patients with high-risk AML was associated with a decrease in AML relapse and enhancing survival of patients without development of GVHD (Curti et al., 2011). In addition, a prospective phase II study was performed in two centers (Basel-Switzerland, and Frankfurt-Germany), where a median dose of $1{,}21 \times 10^7$/kg of purified NK cells was administered to 16 high-risk leukemia patients treated with haplo-SCT. After a median follow-up of 5.8 years, 4 patients were still alive and 12 patients had died due to relapse (5 patients), GVHD (3 patients), graft failure (3 patients), and transplant related neurotoxicity in one patient (Stern et al., 2013).

Regarding solid tumors, the hopeful evidence of adoptive immunotherapy treatment with NK cell remains scarce. Low effectiveness of NK cell adoptive therapy for solid tumors is related to insufficient *in vivo* expansion of NK cells, homing to tumor sites, or suppression of the tumor microenvironment (reviewed by Fang et al., 2017). In 2010, a phase I clinical trial was designed to evaluate safety and possible clinical efficacy of infusion of allogeneic activated NK cells accompanied by immunodepletion chemotherapy into patients with advanced non-small cell lung cancer (NSCLC). They found that only 2 patients showed a partial response from sixteen patients who received allogeneic activated NK cells (Iliopoulou et al., 2010). Moreover, Geller and co-workers assessed the tumor response and *in vivo* expansion of allogeneic NK cells in recurrent ovarian and breast cancer. Twenty patients (14 ovarian cancer and 6 breast cancer), who received lymphodepleting preparative regimen, were injected with *ex vivo* activated NK cells from haploidentical related donors. Although the adoptive NK cells could be detected in the blood after 1 week of infusion, they did not show better expansion *in vivo* and the outcome was poor which may be related to the high increase in Tregs (Geller et al., 2011).

iv. Sources of NK cells for adoptive immunotherapy

a. Peripheral blood-derived NK cells

Peripheral blood is a convenient source to obtain NK cells due to the ease of collection as well as they can be derived from either autologous or allogeneic sources. Furthermore, NK cells derived from PBMCs can be obtained by separating or non-separating protocol from ex vivo expanded cells, and their activation is induced by cytokine or feeder cells. Separation methods of NK cells from PBMCs can be performed via mixing immunomagnetic beads by depleting $CD3^+$ cells, followed by enriching the $CD56^+$ cells. As large numbers of NK cells are usually required for clinical use, apheresis technology and the CliniMACS device are used, where more than 10^8 NK cells can be obtained in each batch. The enrichment of NK cells by depleting $CD3^+$ cells only from whole blood can lead to a mean purity of 20% among cells. Otherwise, selecting $CD56^+$ cells from peripheral blood can provide a purity of 95%. Usually to separate NK cells from PBMCs, both $CD3^+$ cell depletion and $CD56^+$ cell enrichment are used together to enhance the purity for 99% (reviewed by Knorr et al., 2014). Obtaining sufficient numbers of NK cells from PBMCs to achieve a therapeutic effect has been a major limitation. So, *ex vivo* expansion and activation strategies for NK cells remain an essential issue.

b. Stem cell-derived NK cells

Stem cells are an essential source for NK cells production. Umbilical cord blood (UCB) is characterized by a higher concentration of HSCs and progenitor cells than is normally found in PB, making it as another potentially rich source of NK cells for adoptive immune therapy (reviewed by Mehta et al., 2016). NK-cell progenitors in UCB can differentiate into NK cells after *ex vivo* stimulation with cytokines, including IL-2, IL-15, and/or FLT-3 ligand. NK cells from UCB are slightly different from PBMC-derived NK cells. The main differences, which are limited the use of UCB-derived NK cells in tumor therapy, are fewer expression of inhibitory KIRs, higher expression of NKG2A and almost completely lack of CD57 expression on UCB-derived NK cells (reviewed by Mehta et al., 2016). As a result, resting CB $CD56^{dim}$ NK cells have poor cytotoxicity compared with PB NK cells. To improve the activity of UCB-derived NK cells, CB $CD34^+$ cells should be expanded in the presence of stimulators as IL-2 alone or in combination with IL-15, or stem cell factor and FLT3-

ligand, or artificial APCs such as K562 cells expressing membrane-bound IL-21 (reviewed by Mehta et al., 2016).

v. Ex vivo NK cell expansion methods

Because NK cells comprise only 10-15% of PB lymphocytes, obtaining sufficient numbers of NK cells from donor's PBMC is the main limitation of using alloreactive NK cells as adoptive immunotherapy. Initially, Miller and colleagues sought to expand the infused NK cells *in vivo* by injecting the patients with IL-2 after infusion of alloreactive NK cells. They have found an increase in circulating NK cell numbers and their cytotoxicity against leukemia cells (Miller, 2005). However, induction of NK cell apoptosis upon contact with vascular endothelium and not enough expansion of NK cells resulted in poor outcomes when cytokines are used for this purpose (Rodella et al., 2001). As a result of poor expansion with limited life spans of NK cells *in vivo*, other investigators have developed methods to expand and activate NK cells *ex-vivo* by using cytokines and/or feeder cells to accomplish the requirements for clinical use (Denman et al., 2012; Fujisaki et al., 2009; Li et al., 2015; Miller, 2005; Sutlu et al., 2010).

Many types of original cells or genetically modified cells are used as feeder cells such as PBMCs, Epstein-Barr virus-transformed lymphoblastoid cell lines, and genetically modified K562 (reviewed by Koepsell et al., 2013). The combination of feeder cells and cytokines can largely induce NK cell expansion and activation, ranging from 100 - to 40,000-fold in about 2-3 weeks (Denman et al., 2012; Fujisaki et al., 2009; Li et al., 2015; Sutlu et al., 2010). For example, K562 cell line expressing membrane-bound IL-15 and 41BB ligand (K562-mb15-41BBL) was used by Fujisaki and collaborators to generate human NK cells with enhanced cytotoxicity. They found that after seven days of co-culture with irradiated K562-mb15-41BBL, the median of $CD56^{+}CD3^{-}$ cell recovery was 22-fold which increased to 152-fold and 277-fold after 14 and 21 days of culture, respectively (Fujisaki et al., 2009). Regarding the cytotoxicity of the expanded NK cells, the findings showed that the cells could be noticed for >1 month when infused into immunodeficient mice and could eradicate leukemia in AML murine models (Fujisaki et al., 2009).

Because *ex vivo* expansion of NK cells using genetically-modified artificial APCs expressing membrane-bound IL-15 has been limited by telomere shortening, Denman *et al.* developed a method to expand NK cells *ex vivo* using K562 feeder cells expressing membrane-bound IL-21

(mbIL21) (Denman et al., 2012). In contrast to mbIL15, mbIL21-expressing K562 cells promoted more NK cell expansion without evidence of senescence for up to 6 weeks of culture. By day 21, NK cells expanded up to 32,000-fold and showed a highly cytotoxic activity against tumor cell lines due to an increase in the expression of NCRs, CD16, and NKG2D (Denman et al., 2012).

Another group has used different feeder cells to expand NK cells for clinical use, where NK cells were co-cultured with irradiated Epstein–Barr virus-transformed lymphoblastoid cells (Berg et al., 2009). They found that NK cells have expanded up to 500-fold over 21 days of co-culture. Further, the expanded NK cells have higher cytotoxicity against targets due to the increase in TRAIL, FasL and NKG2D expression, and cytokines (IFN-γ, GM-CSF, TNF-α, MIP-1α and MIP-1β) secretion (Berg et al., 2009).

OBJECTIVES

Many attempts have been performed to improve the immune system efficacy post-transplant in order to minimize tumor relapse and other complications. Interestingly, studies have shown that higher absolute NK cell count post-transplant is associated with lower relapse and improved survival. However, it is evidenced that NK cell count normally reconstitute around 1 month, but the reconstituting NK cells are characterized by immature phenotypic properties and impaired cytotoxicity. Consequently, clinicians have proposed infusion of mature, fully functional NK cells into patients as a novel immunotherapy protocol to improve transplant outcomes.

In this context, the transplantation department as well as the cell therapy unit of the IPC (Marseille, France) have designed a clinical trial, which is infusion of IL-2 activated purified $CD3^-$ $CD56^+$ NK cells at three different concentrations into hematological malignancies patients after HLA-identical allo-SCT. In addition, we have designed an ancillary study aiming at monitoring the immune systems, especially NK cells during the course of the trial.

The goal of our study is to assess the biological effects of infusing activated purified NK cells into hematological malignancies patients early after allo-SCT. My thesis project aims to meet the following objectives:

i. To evaluate the impact of NK-DLI on reconstitution of immune populations and subpopulations as T cells, B cells, Monocytes, DCs, and NK cells.

ii. To evaluate the impact of NK-DLI on the reconstituting NK cell biology by assessing:
    - Expression of activating and inhibitory NK receptors.
    - Expression of activation and maturation markers.
    - Effector functions

RESULTS

ARTICLE IN PREPARATION

Infusion of high doses of *ex-vivo* activated NK cells is associated with improved phenotype and functions of reconstituting NK cells post allo-SCT.

Mohammed Taha[1], Raynier Devillier[2], Bechara Mfarrej[3], Daniel Olive[1], Didier Blaise[2], Claude Lemarié[3], Boris Calmels[3], Christian Chabannon[4], Cyril Fauriat[1]

Author Affiliations

1. Inserm, U1068, Centre Recherche en Cancérologie de Marseille, Institut Paoli-Calmettes, CNRS, UMR7258, Aix-Marseille University, Marseille, France.

2. Department of Hematology, Institut Paoli-Calmettes, Inserm U1068, CNRS UMR 7258, Centre de Recherche en Cancérologie de Marseille, Aix-Marseille University, UM 105, Marseille, France.

3. Cell Therapy Facility, Institut Paoli-Calmettes, CIC Biothérapies: Inserm CBT-1409, Marseille, France.

4. Inserm, U1068, Centre Recherche en Cancérologie de Marseille, Institut Paoli-Calmettes, CNRS, UMR7258, Aix-Marseille University; Centre de Thérapie Cellulaire, Département de Biologie du Cancer, Institut Paoli-Calmettes; Inserm CIC-1049, Centre d'Investigations Cliniques en Biothérapie, Marseille, France.

* **Corresponding author:** Cyril FAURIAT, email: cyril.fauriat@inserm.fr

Abstract

Allogeneic stem cell transplantation (allo-SCT) has become the treatment of choice for hematological malignancies, but disease relapse remains the main cause of treatment failure. Cellular immunotherapy with *ex-vivo* activated NK cells after allo-SCT is used to enhance graft-versus-leukemia (GvL) effect. The current study aims to evaluate the effect of activated NK cells infused post allo-SCT on the phenotype and functional activity of developing NK cells as well as the effect on patient's immune system recovery. Based on the injected dose of activated NK cells post allo-SCT, sixteen patients with hematological malignancies were divided into three groups: dose 1 group (10^6 NK cell/Kg, n=3), dose 2 group ($5x10^6$ NK cell/Kg, n=7) and dose 3 group (>$5x10^6$- <$5x10^7$ NK cell/Kg, n=6), and followed up to six months. Blood samples were taken directly before NK cells infusion (baseline) and at different time points post NK cell treatment. Reconstituting NK cells were phenotypically and functionally assessed compared with a similar group of patients not receiving NK cells. Our findings showed higher frequency of NK cells in the periphery of patients treated with larger doses of activated NK cells. Although the notable immature phenotype shortly after treatment, the circulating NK cells in patients receiving larger doses of activated NK cells displayed more activation status with improved maturation profile after 6 months of treatment. We also found that the expression of activating NK receptors (NKG2D, NKp30, and NKp46) augmented on circulating $CD56^{dim}$ NK cells of patients receiving larger doses of activated NK cells. Moreover, these cells showed a significant increase in degranulation capacity as well as cytokine secretion (IFN-Y and TNF-α) throughout study period. No significant changes in frequencies of other immune cells ($CD3^+$, γδ T, $CD8^+$, $CD4^+$, $CD4^+$Tregs, monocytes and DCs) were observed during study period. In conclusion, we suppose that infusion of high doses of *ex-vivo* activated NK cells might be associated with improving the phenotype and functions of reconstituting NK cells post allo-SCT.

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a promising alternative to chemotherapy and radiation therapy for hematological malignancies treatment. Unfortunately, it is associated with serious complications such as tumor relapse, infections as well as graft-*versus*-host disease (GVHD) (Copelan, 2006; Ferrara et al., 2009; Hatzimichael and Tuthill, 2010). As immune system play a critical role in tumors surveillance, performance of patient's immune system post-transplant is a basic determinant in allo-SCT outcomes. Due to the slow reconstitution of fully functional lymphoid cells post allo-SCT, using cellular adoptive immunotherapy is sufficient to improve the efficacy of immune system to achieve complete remission (CR) as well as to reduce transplantation-related complications (Bouchlaka et al., 2010; Cirillo et al., 2018).

In this context, donor T lymphocyte infusion (DLI) protocol is used following transplant to eradicate residual malignant cells and to minimize tumor relapse by enhancing graft-*versus*-leukemia (GvL) effect (Bar et al., 2013; Bethge et al., 2004; Lewalle et al., 2003; Michallet et al., 2005; Schmid et al., 2007; Takami et al., 2014). Unfortunately, DLI is associated with high incidence of severe GVHD and is not suitable to produce CR in patients with acute myeloid leukemia (AML) (Koh and Chao, 2008; Marks, 2002). Consequently, a novel adoptive cellular immunotherapy technique is required to enhance GvT effect without or with minimal GVHD development.

Natural killer (NK) cells play a key role in mediating GvL effect against hematological malignancies, particularly AML (Miller et al., 2007; Ruggeri et al., 2002). NK cells are lymphocytes of the innate immune system which have the ability to recognize tumors and virus-infected cells without prior specific sensitization (Caligiuri, 2008; Moretta et al., 2005; Vivier et al., 2008). NK cell function is regulated by the expression of numerous inhibitory and activating receptors which bind to ligands on healthy or transformed cells (Caligiuri, 2008; Moretta et al., 2005; Vivier et al., 2008). The antitumor activity of NK cells is mediated via direct cytotoxic function as well as regulation of other immune cells by cytokine-secreting function. It was noticed that NK cells are the first reconstituting lymphocytes following transplant and their rapid recovery is accompanying with lower relapse and improved survival (Chklovskaia et al., 2004b; Dunbar et al., 2008; Ruggeri et al., 2002; Storek et al., 2008). However, the early reconstituting NK cells are characterized with an immature phenotype and impaired killing and cytokine-secreting functions. Moreover, normal

receptor repertoires and functions are gradually acquired within one year after transplant (Foley et al., 2014; Ghasemzadeh et al., 2016; Pical-Izard et al., 2015). To enhance the effector function of reconstituting NK cells, first trials have used IL-2 to stimulate autologous NK cells post-transplant; however, the recovery of NK cell cytotoxicity *in vivo* was limited (Benyunes et al., 1995; Burns et al., 2003).

Subsequently, infusion of mature, fully functional allogeneic NK cells into patients following transplant (NK-donor lymphocyte infusion, NK-DLI) was applied as a novel immunotherapy protocol to improve transplant outcomes (Miller, 2005; Sutlu and Alici, 2009). Some trials have shown that using allogeneic NK cells as adoptive immunotherapy post-transplant is associated with strong GvL effect and without or with minimal GVHD effect (Palmer et al., 2013; Ruggeri et al., 2002, 2007b). Due to diverse strategies used for separation, expansion and stimulation of NK cells, there are variations in the effect of infused NK cells on transplantation outcomes. Further, most published studies about NK-DLI post transplantation did not clearly address the phenotype and function profiles of reconstituting NK cells following NK cell adoptive immunotherapy.

In the current study, three different doses of *ex vivo* IL-2 stimulated CD56 NK cells were infused into patients 2 months after allo-SCT for hematological malignancies. Patients were monitored before and at different time points following treatment to evaluate the effect of activated purified NK cell infusion on the reconstitution of immune populations. Moreover, we performed a detailed analysis to assess the phenotype and functional activity of reconstituting NK cells post NK-DLI.

Methods

Patients

Between 2013 and 2016, sixteen patients from Institut Paoli-Calmettes (IPC, Marseille, France) with an intermediate, high or very high risk index of hematological malignancies according to the disease risk index developed by the Dana Farber Cancer Institute were enrolled in our study. Patients' characteristics are described in Table 1. The median age was 59 years (range: 39-68 years) and all participants gave written informed consent in accordance with the Declaration of Helsinki. All patients received a RIC regimen comprising (Fludarabine, Busulfan and Thymoglobuline) and they have undergone HLA identical allo-SCT with granulocyte colony stimulating factor (G-CSF) mobilized peripheral blood stem cells between 2013 and 2016. All patients were in CR before DLI-NK at day 0. After 60 to 90 days of transplantation, the patients were injected with activated purified NK cells based on a protocol approved by the ethical review boards from the IPC and registered at www.clinicaltrials.gov (NCT01853358). Exclusion criteria included: active grade ≥ 2 aGVHD or corticosteroid therapy ≥ 0.5 mg/kg/day at time of NK cell infusion, active infections, pregnancy, or breast-feeding.

Study design and samples

The following study started in 2013, where patients diagnosed with hematological malignancies have been included and were treated with chemotherapy to achieve CR (Figure 1). Then, the selected patients have received allo-SCT from HLA-matched related or unrelated (10/10) donors. Around day 60 post transplantation, PBMCs were collected from the original and consenting donors, and purified $CD3^-CD56^+$ NK cells were obtained after double selection using $CD3^+$ depletion followed by magnetic $CD56^+$ selection (Miltenyi Biotec). Purified $CD3^-CD56^+$ NK cells were then *ex-vivo* activated for 7 days by culturing in RPMI 1640 medium supplemented with 10% of heat-inactivated fetal calf serum (FCS) and IL-2 (1000U/ml) at 37°C. Phenotypic and functional characteristics of donors NK cells were analyzed before their activation and after 7 days of IL-2 activation (before infusion into patients), (data not shown). Between day 60 to day 90 post graft, activated NK cells were infused into patients based on dose escalation regimen where patients were divided into 3 groups as follows: dose 1 ($1x10^6$ NK cells/kg, 3 patients), dose 2 ($5x10^6$ NK cells/kg, 3 patients), and

dose 3 group (>5x10^6 - <5x10^7 NK cells/kg, 10 patients). PB samples were collected from both healthy donors and patients at intervals over the course of 6 months, and were cryopreserved until use. For healthy donors, the samples were collected before NK cell sorting, after NK cell sorting (before activation with IL-2), and after 7 days of IL-2 activation. While for patients, the samples were taken directly pre NK-DLI (Day 0, baseline) to month 6 after infusion of activated NK cells as the following time frame: at Day 1, Day 9, Day 16, Month 1, Month 3, and Month 6. HLA-genoidentical transplanted patients (Pical-Izard et al., 2015) were used as controls in which PB samples corresponding to patients' time frame samples were used.

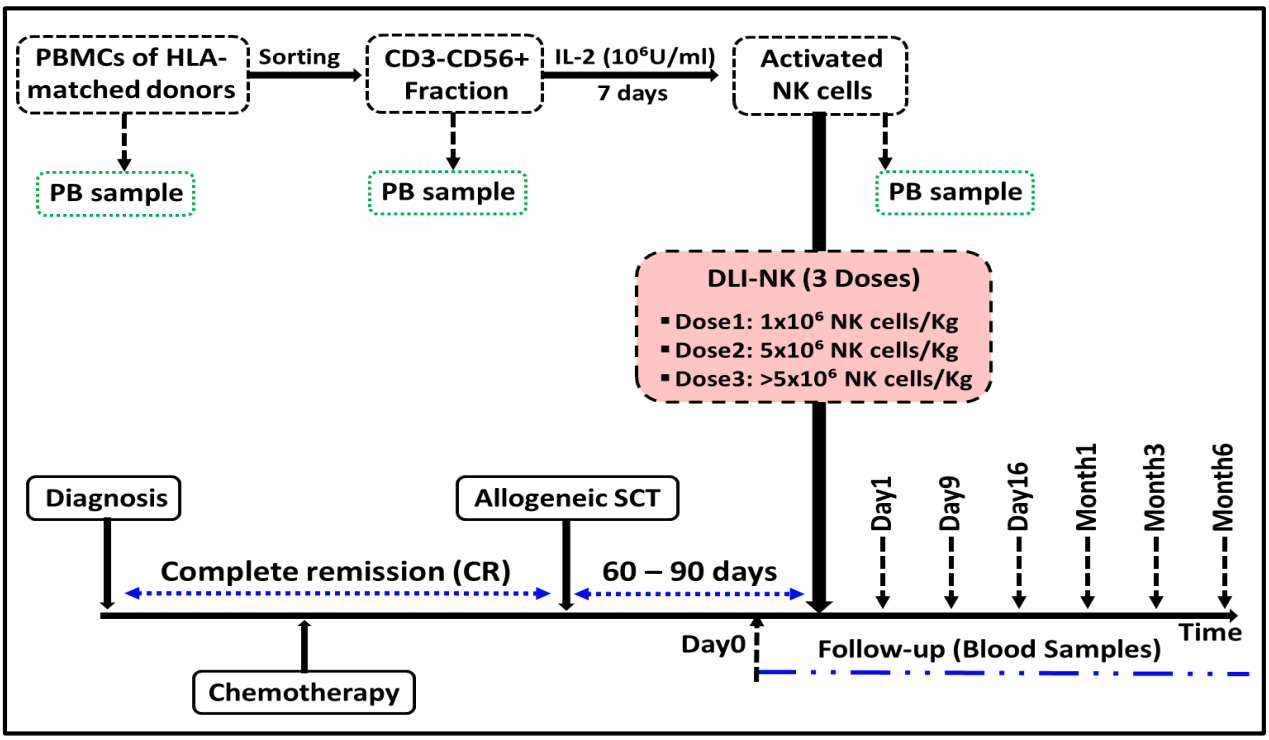

Figure 1: Time frame of the study. Patients suffering from hematological malignancies have received allo-SCT after CR achieved by chemotherapy. *Ex-vivo* IL-2 stimulated purified allogeneic NK cells were infused into patients within escalation dose regimen (3 doses). PB samples were taken from patients and their respective donors at different time points.

Flow cytometry

After thawing, the cells were immunostained with fluorochrome-labeled antibodies of phenotypic panels listed in Supplementary Table 1. Acquisition was performed on a FACS LSR II (BD Biosciences) and analysis was done using FlowJo v10 software (LLC, Ashland, Oregon). The gating strategy based on elimination of the doublets using FSC-A/FSC-H parameters, followed by the

removal of dead cells using a cell viability marker. NK cell population was defined as $CD3^{-}CD56^{+}$ cells within the lymphocyte gate. The phenotypic data are represented as percentage of cells positive for a given marker.

NK cell functions

PBMCs at all time points were thawed up and rested overnight at 37°C within RPMI 1640 medium supplemented with 10% FCS (complete medium) without interleukins (IL-2 or IL-15). Cytokines production and degranulation capacity were determined by stimulating NK cells with the erythroleukemia cell line K562 (Bryceson et al., 2010). Briefly, $1x10^6$ PBMCs were incubated with K562 cells (ratio 10:1) at 37°C and 5% CO_2 for 4 hours in the presence of GolgiPlug® (BD Biosciences, #555029) and FITC-conjugated anti-human CD107a (BD Biosciences, clone H4A3). After 4 hours of incubation, the cells were collected, washed and immunostained for surface markers with fluorochrome-labeled antibodies listed in Supplementary Table 1. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm® kit (BD Biosciences) according to the manufacturer's instructions, and immunostained with fluorochrome-conjugated anti-cytokine antibodies listed in Supplementary Table 1. The cells were finally prepared for flow cytometry analysis using a FACS LSR II (BD Biosciences) and FlowJo v10 software (LLC, Ashland, Oregon).

Statistical analysis

Graphics were generated with Graphpad Prism software (San Diego, CA, USA), Multiple Experiment Viewer (MeV) software and Microsoft Excel 2013-Radar chart. For radar chart, data were normalized to baseline values (D0) and expressed as the frequency of change. Statistical analyses were carried out using Graphpad Prism software. Multiple comparisons were performed among NK-DLI treated patients, healthy donors (donors for HSCs and injected NK cells) and genoidentical patients (non-treated patients, controls) using the Kruskal-Wallis nonparametric test. Comparisons between two different groups were performed using the Mann-Whitney test; while comparisons between the same individual were performed using the Wilcoxon matched pairs T test. For all tests, P values ≤ 0.05 were considered significant.

Results

Because no effective changes in the results were observed at the 9th and 16th day post NK-DLI infusion, figures did not include the analyses both of them. Moreover, it was difficult to find separated controls (a cohort of HLA-genoidentical transplanted patients in a previous study performed by our team (Pical-Izard et al., 2015)) before treatment (day 0) and at day 1 of NK-DLI due to the variations in infusion time of activated NK cells. So, findings of control group at day 1 presented in the figures represent data of day 0 and day 1. Obtaining the sufficient number of purified NK cells following expansion step is a critical limitation for NK-DLI technique. So, new classification for patients was performed based on the available numbers of activated NK cells post expansion (Table 1).

Reconstitution of NK cells after DLI-NK

To evaluate the effect of activated NK cells infusion post allo-SCT on NK cell recovery, we analyzed PB samples of patients (n=16) before and after indicated time points of NK-DLI. In parallel, NK cell reconstitution was also estimated among genoidentical patients (GI, control group) at the same time points of treated patients. NK cells were identified within PBMC as $CD3^{-}CD56^{+}$ among viable lymphocytes (Figure 1A). Our findings showed that frequencies of NK cell were significantly increased at day 1 after infusion of activated NK cells compared to day 0 (before NK-DLI), but mainly in the dose 3 group (median, day 1: 32.7% versus day 0: 13.1%, p= 0.003) (Figure 1B). NK cell frequency of dose 3-infused patients remained significantly higher than that of day 0 until the end of the study. Interestingly, the median frequency of NK cells in dose 3 recipients was higher than that of control subjects during the study period at the corresponding time points (day 1: 32.7% versus 11.5%, p= 0.002; month 1: 20.7% versus 8.8%, p= 0.03; month 3: 18.9% versus 10%, p= 0.48; month 6: 17.2% versus 13.5%, p= 0.59) (Figure 1C). For patients related to dose 2, frequencies of NK cell decreased after day 1 of NK-DLI, and reached donors and controls levels by month 6; dose 2-patients: 12.8%, donors: 13.5% and controls: 13.4%. In contrast, dose 1 recipients NK-DLI did not show any significant raise in NK cell frequencies during study period. Together, these results suggest that infusion of high doses of activated NK cells into patients post allo-SCT may enhance NK cell recovery among those patients.

Activation profile of the reconstituting NK cells after NK-DLI

We next analyzed the expression of NKp44, CD69 and HLA-DR markers on $CD56^{dim}$ NK cells to assess the activation state of the reconstituting NK cells in patients after NK-DLI. Percent change in term of activation markers expression was calculated at all-time points relative to the baseline values (day 0). When we compared the expression of NK-activation markers in dose 3 treated group with donors after 7 days of IL-2 stimulation, we strikingly found a sharp decrease in their expression at day 1 of NK-DLI therapy (Data not shown). Over the study period, no statistical significant variations were observed in the expression of activation markers in dose 1-treated patients as well as controls. Contrary, we found a significant increase in mean percent change of CD69 expression over 6 months following NK-DLI in dose 2-treated patients (mean ± SD, day 1: 42.3% ± 13, p= 0.03, month 1: 35.7% ± 18, p= 0.007, month 6: 62.9% ± 29.6, p= 0.004) and dose 3-treated patients (day 1: 49% ± 20, p= 0.03, month 1: 44% ± 41.8, p= 0.04, month 6: 74.1% ± 26, p= 0.002) (Figure 2B). Concerning NKp44 expression, figure 2C displayed a significant increase in the mean percent change among dose 3-treated patients at day 1, month 1 and month 6 post treatment compared to baseline value (p= 0.03, 0.01 and 0.003, respectively). In dose 2-treated group, the mean percent change from baseline significantly increased at the 1^{st} day and the 6^{th} month (p=0.03 in both) post NK-DLI (Figure 2C). With respect to HLA-DR expression, we found a significant rise in the mean percent change among dose 2- and dose 3-treated patients shortly after infusion of activated NK cells (day 1: p= 0.03 in both; month 1: p= 0.2 and p= 0.01, respectively) (Figure 2D). However, no significant changes were observed in HLA-DR expression among those patients from the 3^{rd} month unto study end (Figure 2D). Comparing with controls, patients receiving dose 3 showed significantly higher expression of NKp44 and CD69 (insignificant at month 1) markers during study period, while no change in HLA-DR expression (Supplementary Figure 1A-C). These findings suggest that the circulating NK cells in PB of patients having received high doses of activated NK cells following allo-SCT display a more active status.

Reconstituting NK cells show a gradual increase in NK maturation behavior post DLI-NK

In order to investigate the maturation status of NK cells developing post DLI-NK, PBMCs were stained with specific mAbs and analyzed by cytometry. Firstly, surface density of CD56 was assessed on total NK cells of all enrolled patients, donors and controls at the indicated time points (Figure 3A). As predicted, frequencies of immature NK cells ($CD56^{bright}$) significantly increased directly after infusion of dose 2 as well as dose 3 activated NK cells (p= 0.03 in both compared to day 0) (Figure 4B). At late time points, frequencies of $CD56^{bright}$ NK cells were restored to baselines but still lower than that of controls mainly in dose 3 recipients. In contrast, $CD56^{dim}$ NK cells frequencies were greatly reduced in dose 2 and dose 3 patients directly after DLI-NK compared with day 0 (p= 0.03 in both), but returned gradually with time to become comparable to day 0 values (Figure 3C). Additionally, we analyzed the expression of NKG2A and CD57 on $CD56^{dim}$ NK cells because NK cells maturation is also associated with a loss of NKG2A expression and acquisition of CD57 (Bjorkstrom et al., 2010). We found that $NKG2A^{+}CD56^{dim}$ NK cell frequencies were significantly high at day 1 of NK-DLI in patients receiving dose 2 and dose 3 compared to day 0 (p= 0.03 in both groups), and began to decrease to reach levels of day 0 at the first month of NK-DLI (Figure 3D-left). Interestingly, the decrease in $NKG2A^{+}CD56^{dim}$ NK cell frequency continued by the time for lower levels than that of day 0 in dose 3-treated patients (month 6, median frequency: 35% versus 50% of day 0, p= 0.03). Although the frequency of $NKG2A^{+}CD56^{dim}$ NK cells was significantly higher than that of controls until the 1^{st} month post NK-DLI, dose 3 recipients revealed a similar profile of NKG2A expression as controls at the end of the study (Figure 3D-right). With respect to CD57 and KIRs expression, a significant downregulation was observed in patients of dose 3 at day 1 of therapy compared with baseline (p= 0.03 in both) (Figure 3E, F-left). Comparing with controls, patients of dose 3 also showed a significant decrease in KIRs expression at day 1 (p= 0.04) (Figure 3F-right). After the 1^{st} month of treatment, there was an increase in their expression in patients receiving dose 2 and dose 3 compared to day 0, but the increase was greater in dose 3 recipients. Moreover, the frequencies of $CD57^{+}$ and $KIRs^{+}CD56^{dim}$ NK cells were higher in dose 3 recipients compared with that of controls at month 6 (Figure 3E, F-right). In conclusion, we observed that reconstituting NK cells display immature properties shortly after infusion of activated NK cells and change inversely to mature status faster than non-treated patients.

Reconstituting NK cells display an increase in activating NK receptors expression after NK-DLI

We further analyzed whether NK cell receptors expression was affected following infusion of activated NK cells by measuring the frequencies of activating receptors expression including DNAM-1, NKp30, NKp46, NKG2D and NKG2C on $CD56^{dim}$ NK of patients, donors and controls at the indicated time points. The radar chart in figure 4A presenting percent of changes of activating NK receptors expression in patients at different time points relative to day 0 showed a clear upregulation in receptors expression on $CD56^{dim}$ NK cells of patients receiving dose 3 NK-DLI compared with other groups (Figure 4A). Our findings revealed that no significant change was observed in the expression activating receptors on $CD56^{dim}$ NK cells among dose 1- treated patients and controls compared to day 0. Interestingly, the frequency of $NKp30^{+}$ $CD56^{dim}$ as well as $NKp46^{+}$ $CD56^{dim}$ NK cells significantly increased in patients who received dose 3 during the study period compared to baseline as well as control subjects at the corresponding sampling points, whereas dose 2- treated patients showed a significant upregulation in NKp30 and NKp46 expression at the 1^{st} day post NK-DLI (Figure 4B, C). With respect to NKG2D, patients of dose 2 as well as dose 3 showed a remarkable increase in its expression which remained significantly higher than that of day 0 until the end of the study (Figure 4D). Moreover, a significant increase in $NKG2D^{+}$ $CD56^{dim}$ NK cell frequency was noticed in dose 3-treated patients at all-time points compared with controls, while the expression was significantly higher in patients of dose 2 compared with controls only at the beginning of the study (day 1, p= 0.02) (Figure 4D). Regarding DNAM-1, a notable increase in DNAM-1^{+} $CD56^{dim}$ NK cell frequency was shown among dose 2-, and dose 3-related patients directly after infusion of activated NK cells compared to day 0 (P= 0.03 in both), which decreased by time to become close to controls values at late time points (Figure 4E). On the other hand, expression of NKG2C did not change significantly during the study period in all patients and controls when was compared to day 0 except for dose 3-received patients who showed a significant increase in the expression at month 6, p= 0.03 (Supplementary Figure 2). Overall, based on these findings, we may expect more efficient NK cells after infusion of activated NK cells post allo-SCT due to upregulation of activating receptors.

Reconstituting NK cells display an increased cytolytic activity and cytokine secretion post NK-DLI

We next studied the functional properties of reconstituting $CD56^{dim}$ NK cells by monitoring their capacity to produce cytokines (IFN-Y and TNF-α) and MIP-1β chemokine, and their degranulation level by measuring CD107a expression in response to stimulation with K562 target cells (Bjorkstrom et al., 2010; Bryceson et al., 2010). It was observed that $CD56^{dim}$ NK cells of dose 2- and dose 3-related patients obviously produced higher cytokines, MIP-1β chemokine and CD107a at all-time points post NK-DLI compared with patients who received dose 1 (Figure 5A). To illustrate, our findings revealed a low degranulation levels at day 0 for all patients and controls which were significantly increased after infusion of dose 2 and dose 3 activated NK cells (CD107a frequency was higher in dose 3 than dose 2) compared to day 0 (Figure 5B). Interestingly, we found that degranulation capacity of reconstituting $CD56^{dim}$ NK cells in patients receiving dose 3 activated NK cells was higher than controls at all-time points (Figure 5B). Regarding cytokines production, we also showed a low level of both IFN-Y and TNF-α in response to K562 stimulation among controls and patients before NK-DLI. However, a significant increase was obtained directly following dose 2 and dose 3 infusion (more cytokines levels were shown in dose 3-treated patients) and continued to the end of the study compared to day 0 (Figure 5-C, D left). Compared with control subjects, frequencies of IFN-Y^{+} $CD56^{dim}$ and TNF-α^{+} $CD56^{dim}$ NK cells were visibly greater in dose 2- and dose 3-treated patients at the 1^{st} day post NK-DLI (IFN-Y: p= 0.002, 0.01; TNF-α: p= 0.03, 0.01 respectively), however, cytokine secretion profile became similar at late time points (Figure 5-C, D right). With respect to MIP-1β, patients showed significantly higher frequency of MIP-1β^{+} $CD56^{dim}$ NK cells following infusion of dose 2 as well as dose 3 activated NK cells, which reduced by time but still higher than that of controls and dose 1-treated patients (Supplementary Figure 3). Comparing with healthy donors, we found a low degranulation level and cytokine production in all patients before NK-DLI therapy. Interestingly, the percentage of $CD56^{dim}$ NK cells in dose 3 treated patients expressing CD107a was significantly greater compared with donors after therapy (Supplementary Figure 4A). Regarding cytokine production, no difference was observed between donors and dose 3 treated group during study period (Supplementary Figure 4B, C). Together, these data displayed that more degranulation and proinflammatory cytokines were produced by reconstituting $CD56^{dim}$ NK cells following NK-DLI, suggesting that infusion of activated NK cells post allo-SCT may improve the effector functions of NK cell.

Reconstitution of other immune cells after NK-DLI

To study immune cells reconstitution after allo-SCT followed by NK-DLI, FACS analysis was used to measure the frequencies of immune cells in treated patients and controls at different sampling points. Concerning B cells, no change was shown in patients shortly after infusion of activated NK cells compared to baseline values (Figure 6A). Unlike the late recovery of B cells post HSCT, dose 2- and 3-treated patients displayed more increase in B-cell frequency at the 6^{th} month after treatment compared controls. No significant changes in frequencies of $CD3^{+}$, $TC\gamma\delta^{+}$, $CD8^{+}$, $CD4^{+}$, monocytes-$CD16^{-/+}$, and DCs were observed during study period among NK-DLI treated patients and control group compared with baseline values (Figure 6B-G). However, the frequencies of $CD3^{+}$, $CD8^{+}$ subset and monocytes-$CD16^{-/+}$ were clearly lower in dose 2 and dose 3 recipients month 6 after NK-DLI compared with controls (Figure 6B, D, F). Regarding $CD4^{+}$Tregs, there was a significant increase in the median percentage among controls at the end of the study compared to baseline ($p= 0.03$) and also was higher than that of dose 3-treated patients ($p= 0.007$) (Figure 6H). Though no significant differences in DCs frequencies were shown throughout study period among patients and controls, NK-DLI treated patients tended to have lower median frequency of pDC and mDC subsets at the latest time point compared to controls (Supplementary Figure 5A, B).

Discussion

Considering NK cells play a vital role in the first line of defense against malignantly transformed cells, clinical trials have indicated that NK cell-based immunotherapy represents a promising antitumor immunotherapeutic approach by mediating a strong GvL effect against hematological malignancies, particularly in AML (Boyiadzis et al., 2017; Ciurea et al., 2017; Miller, 2005; Palmer et al., 2013; Passweg et al., 2004; Ruggeri et al., 2002, 2007b). However, the data regarding the developing NK cells after NK cell adoptive immunotherapy are few.

In the current study, we have evaluated the recovery of NK cells and the acquisition of their phenotypic profile as well as their effector functions in patients following infusion of activated NK cells post allo-SCT. Our findings showed that infusion of high doses of activated NK cells is associated with an improved recovery of NK cells. Interestingly, the developing NK cells are characterized by an enhancement of their phenotypic properties in term of maturation markers, activating receptors and activation markers. Because were not able to distinguish between the infused NK cells and NK cells developing from donor's graft mainly at early time points, we thought that most of these cells may be the infused cells. So, we compared the expression of activation markers at day 1 with donor's NK cells after 7 days of activation. Surprisingly, we found a sharp downregulation of activation markers directly after infusion of the cells (day 1). These findings suggest that maybe the cells were lost after infusion, the cells lost their activation markers sharply after infusion or the cells were diluted at the periphery. Moreover, we thought that maybe we have a possibility to distinguish between the cells phenotypically based on KIRs expression and when we assess all of markers in KIR^+ NK cells. Because NK-DLI therapy was used after 3 months of allo-SCT, this time was enough for the developing cells to acquire some of KIRs. So, when we compared the expression of some KIRs on NK cells of patients at day 0, day 1 and month 6 with donor's NK cells, we found that the cells have a similar KIRs expression profile.

At functional level, more degranulation capacity and cytokine secretion by the developing NK cells were observed in response to target cell stimulation. Moreover, we found that no significant change in the recovery of other immune cells was shown between NK-DLI treated and non-treated

patients, except $CD4^+$Treg cells which were lower in patients receiving higher doses of activated NK cells. These findings suggest that using high doses of activated NK cells as adoptive immunotherapy post-transplant are safe and improve both phenotypic profile and functional activity of developing NK cells.

Here, we were able to detect an increase of NK cell frequencies after NK-DLI, however, it was not possible to clearly differentiate by phenotype between the adoptively infused allogeneic NK cells and those that derived from the donor graft. Although the frequency of circulating NK cells gradually reduced after NK-DLI therapy, we found more NK cells throughout study period in patients receiving higher doses of activated NK cells. This gradual decrease in circulating NK cells may be related to up-regulation of homing molecules on NK cells such as L-selectin (CD62L), P-selectin and α4β7 (Olson et al., 2009). Few studies have addressed the trafficking of transferred NK cells *in vivo*. During the first 24 hours post infusion, NK cells were distributed mainly to the lungs and then to liver, spleen, lymph nodes, bone marrow and tumor site (Brehm et al., 2011; Meller et al., 2004; Olson et al., 2009; Pegram et al., 2010). In agreement with our results, myeloid malignancies patients treated with three doses of mbIL-21 *ex vivo*-expanded allogeneic NK cells before (-2 days) and after (+7 and +28 days) haplo-SCT, showed higher NK-cell numbers after treatment compared with the control group (Ciurea et al., 2017).

After HSCT, it was previously demonstrated that the early reconstituting NK cells are characterized by an immature phenotype (high frequency of $CD56^{bright}$ NK cells) although their numbers are restored within approximately 1 month. Moreover, this immature picture is associated with impaired cytotoxic activity as well as cytokine production (Dulphy et al., 2008; Ghasemzadeh et al., 2016; Nguyen et al., 2005; Pical-Izard et al., 2015). In our study, we found a similar picture following NK-DLI therapy where circulating NK cells showed a significant increase in frequency of $CD56^{bright}$ NK cells shortly after treatment with higher doses of activated NK cells. After month 3, frequencies of $CD56^{bright}$ NK cells were restored to baselines but lower than that of controls mainly in dose 3 recipients. As NK cell maturation is also characterized by losing NKG2A expression and acquiring of CD57 and KIRs (Bjorkstrom et al., 2010), a significant decrease in $NKG2A^+CD56^{dim}$ NK cell frequency was noticed at the end of study in dose 3 treated patients. Although CD57 and KIRs expression significantly decreased after injection of dose 3 activated NK cells, frequencies of $CD57^+$

and KIRs^{+}CD56^{dim} NK cells started to highly increase after month 3 of therapy, and were higher than that of controls at month 6.

It was demonstrated by our team and others that downregulation of NK cell activating receptors expression is a mechanism used by tumor cells to escape NK cell immunosurveillance (Costello et al., 2002; Fauriat et al., 2007; Stringaris et al., 2014). Strikingly, we found that the frequency of NKp30^{+}, NKp46^{+} and NKG2D^{+} CD56^{dim} NK cells remarkably increased in patients receiving dose 3 NK-DLI during the study period compared to baseline as well as controls at the corresponding sampling points. It has been shown previously that stimulation with IL-2 or IL-15 leads to highly upregulation of activating NK receptors including NKp30, NKp46 and NKG2D (Hromadnikova et al., 2013; Huenecke et al., 2010). Contrary, a recent study pointed to no significant differences in percentage of activating receptors expression (DNAM-1, NKp30, NKp46, NKp80, 2B4, CD16, NKG2D and NKG2C) on total NK cells between treated patients with IL-21 expanded NK cells and non-treated patients (Ciurea et al., 2017). However, a significant difference in the expression of CD16, 2B4 and NKG2C receptors on mature KIR^{+} NK cells was observed between those treated or non-treated at day 28 of treatment. This gives us a hint to check the phenotype of NK KIR^{+} subset after NK-DLI therapy. Together the findings of improved NK cell maturity and acquisition of activating receptors suggest that infusion of activated NK cells may alter the patient's cytokines required for NK cell differentiation as IL-2 and IL-15.

Based on the improvement of phenotypic characteristic mentioned above, we asked whether these modifications might have an impact on functional activity of NK cells. On the other hand, it was shown that the early developing NK cells post-transplant are characterized by an impaired cytotoxic function as well as cytokine production (Foley et al., 2014; Ghasemzadeh et al., 2016; Pical-Izard et al., 2015). Noteworthy, we observed that circulating CD56^{dim} NK cells in patients treated with higher doses of NK-DLI showed a higher degranulation level, and more secretion of proinflammatory cytokines (IFN-Y and TNF-α) and MIP-1β chemokine than did CD56^{dim} cells either in dose 1-treated patients and controls. Different from our findings, circulating NK cells of patients treated with mbIL-21 *ex vivo*-expanded allogeneic NK cells pre/post haplo-SCT revealed a pronounced increase in production of proinflammatory cytokines (IFN-γ and TNF-α) in response to target cell stimulation compared with non-treated patients (Ciurea et al., 2017). In the context of

degranulation capacity, they found no variation in CD107a expression between treated and non-treated patients. In a previous study, we evaluated the functional properties of NK cells post allo-SCT (Pical-Izard et al., 2015). Similar to healthy volunteers, the findings pointed to a faster recovery of degranulation level and MIP-1β chemokine production by reconstituting NK cells compared to IFN-γ and TNF-α cytokines in response to K562 stimulation (Pical-Izard et al., 2015). These results were compatible with Foley *et al.* results who found that target cell-induced IFN-γ production is decreased after allo-SCT, while there was no difference in CD107a expression between healthy donors and recipients (Foley et al., 2011). Remarkably, our data showed a complete restoration of cytokine production in dose 3 NK-DLI patients comparable to healthy donors as represented by a better IFN-γ /TNF-α production in response to target cell stimulation in treated patients compared to controls (Genoidentical patients from Pical-Izard et al. study). With respect to degranulation, DLI-NK cells displayed higher responsiveness to target cell stimulation compared to healthy donors, themselves higher to genoidentical controls. These data suggest that injection of high dose of highly activated NK cells improve NK cell anti-tumor activity and warrants further demonstration.

We consider that functional activity improvement of developing $CD56^{dim}$ NK cells post larger doses of NK-adoptive immunotherapy might be related to different justifications including: i) the improvement of NK-cell reconstitution; ii) the increase in activating receptors expression on circulating $CD56^{dim}$ NK cells, where stimulation of these receptors is associated with higher ability to kill and to produce IFN-γ (Anfossi et al., 2006; Fauriat et al., 2010a; Fernandez, 2005; Jamieson et al., 2002); iii) the activation profile characterizing $CD56^{dim}$ NK cells in periphery post NK-DLI (Benlahrech et al., 2009; BORREGO et al., 1999; Clausen et al., 2003; Esin et al., 2008; Forte et al., 2005); iv) the rapid recovery of NK cell maturity following NK-DLI as it was demonstrated by previous studies that mature $CD56^{dim}$ NK cells are the major source of proinflammatory cytokines and chemokines after target cell recognition beside their cytotoxic activity (Bryceson et al., 2010; Cooper et al., 2001c; Fauriat et al., 2010a; Juelke et al., 2010; Lopez-Verges et al., 2010).

With respect to immune cell reconstitution, the performance of patient's immune system is insufficient post-transplant due to the slow reconstitution of fully functional immune cells. In general, innate immune cells usually recover earlier (within few weeks) than that of adaptive immunity which requires 2 years to reach normal level (Storek et al., 2008). We expected that

infusion of activated NK cells post allo-SCT could improve immune cell reconstitution due to the changes in the patient's cytokine environment and killing the remaining leukemic cells by the GvL effect of NK cells. Our results found that no significant change in the percentage of innate immune cells (monocytes $CD16^{-/+}$ and DCs) among treated and non-treated patients throughout study period. Our findings, in agreement with a previous study (Ciurea et al., 2017), revealed that no significant change was shown in frequencies of the lymphoid cells during study period compared to baseline, but a significant decrease in $CD3^{+}$ and $CD8^{+}$ frequencies was observed in patients receiving higher doses of activated NK cells compared with controls at month 6. As $CD4^{+}$Tregs compete NK cells on IL-2 which is critical for their development, we did not notice a change in frequency of $CD4^{+}$Tregs in NK-DLI treated patients, which may be related to the increase in circulating NK cells in those patients. Interestingly, these findings were different from those of non-treated patients who showed a significant increase in $CD4^{+}$Tregs frequency at month 6.

In conclusion, this phase I clinical study showed that infusion of IL-2 activated NK cells into hematological malignancies patients post allo-SCT is safe according to immunological parameters. The findings showed more recovery of circulating NK cells after infusion activated NK cells mainly at doses superior to 5 million cells per kilo. These cells were characterized by improved phenotypic characteristics and functional activity, suggesting a positive effect of injecting activated NK cells. These findings justify further evaluation with phase II clinical study and a larger cohort to assess the potential benefits from infusion of high doses activated NK cells on allo-SCT outcomes.

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Figures

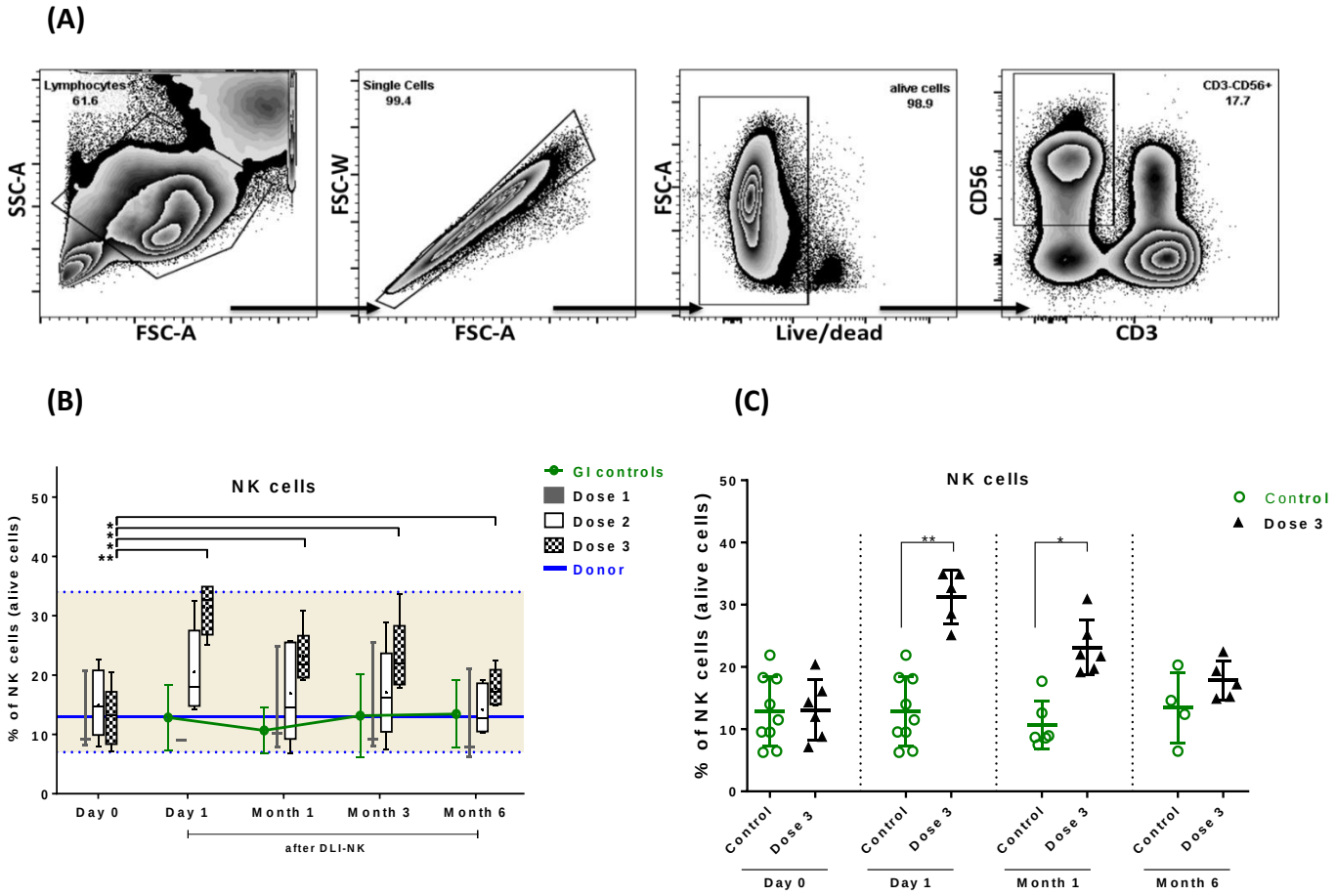
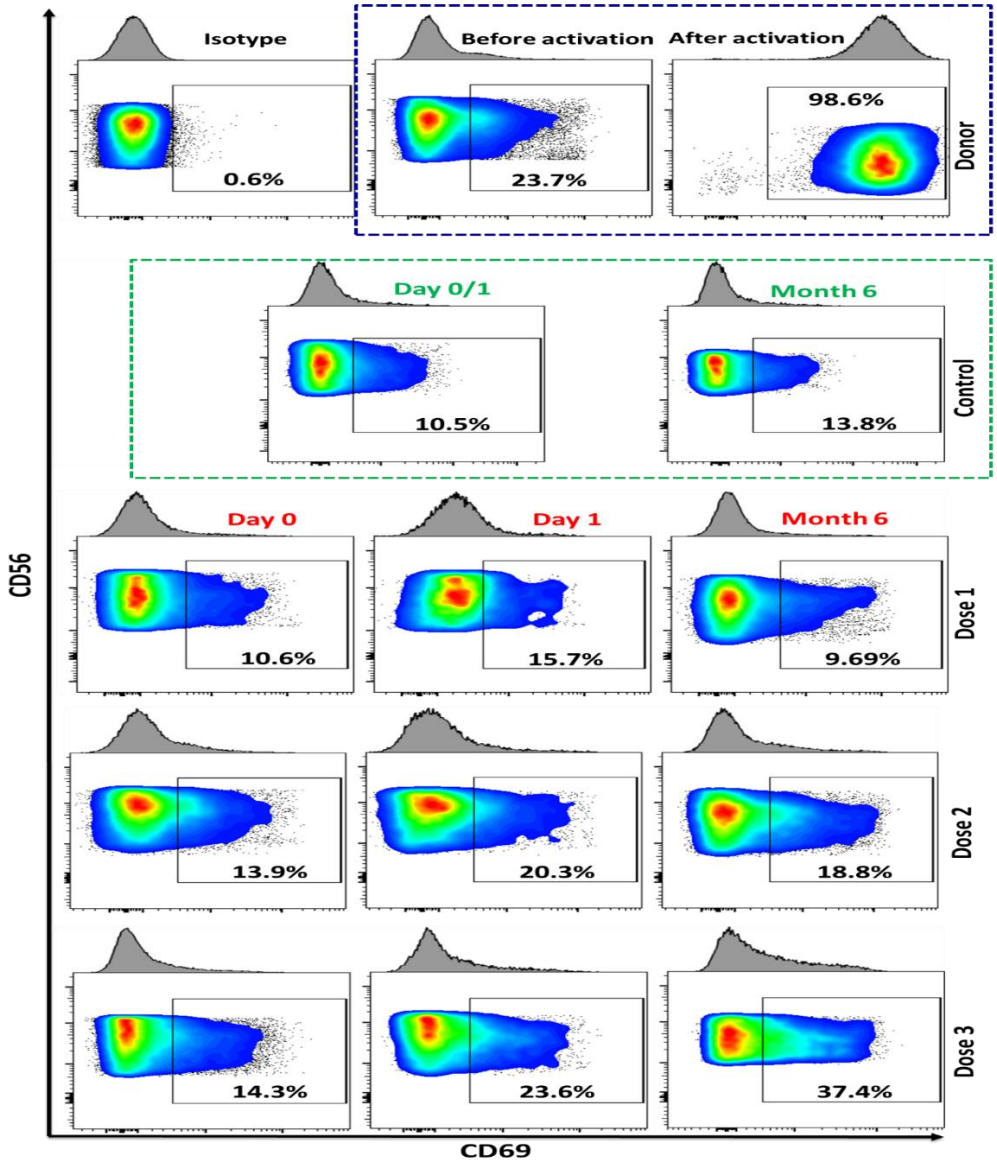
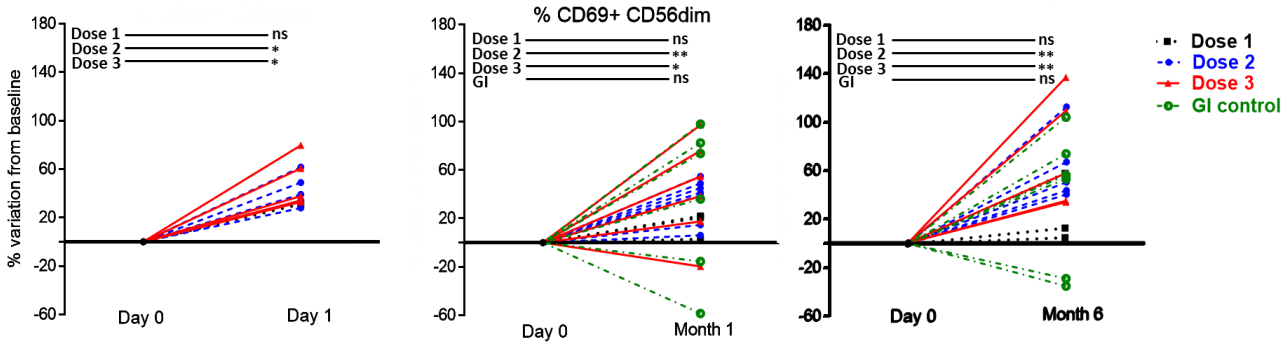


Figure 1: NK-cell reconstitution post allo-SCT in patients treated with *ex-vivo* IL-2 activated NK cells (NK-DLI). Monitoring of NK cell was performed based on CD56 expression in patients before and after NK-DLI, and their respective donors, as well as in non-treated patients (controls) at specific time points. (A) A representative fluorescence-activated cell sorting (FACS) profile showing gating strategy to identify NK cells, where $CD3^{-}CD56^{+}$ NK cells were identified within PBMC after gating singlets and viable lymphocytes. (B) Box and whisker plots displaying NK cell frequencies (median, range) in PB of patients treated with dose 1 (n=3, gray lines), dose 2 (n=7, white boxes), and dose 3 (n= 6, black boxes) as well as controls (green line) before and after different time points of NK-DLI. (C) Scatter dot plots showing a comparison between NK cell frequencies (mean ± SD) of dose 3-treated patients (black triangle) and controls (green circle) pre NK-DLI (day 0), and at day 1, month 1 and 6 post NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$). Blue lines represent NK cell frequency (mean, min-max) of donors (n=16)

(A)


Isotype
Before activation
After activation
0.6%
23.7%
98.6%
Donor
Day 0/1
Month 6
10.5%
13.8%
Control
Day 0
Day 1
Month 6
10.6%
15.7%
9.69%
Dose 1
13.9%
20.3%
18.8%
Dose 2
14.3%
23.6%
37.4%
Dose 3
CD56
CD69

(B)


% CD69+ CD56dim
% variation from baseline
Dose 1 ns
Dose 2 *
Dose 3 *
Day 0
Day 1
Dose 1 ns
Dose 2 **
Dose 3 *
GI ns
Day 0
Month 1
Dose 1 ns
Dose 2 **
Dose 3 **
GI ns
Day 0
Month 6
Dose 1
Dose 2
Dose 3
GI control

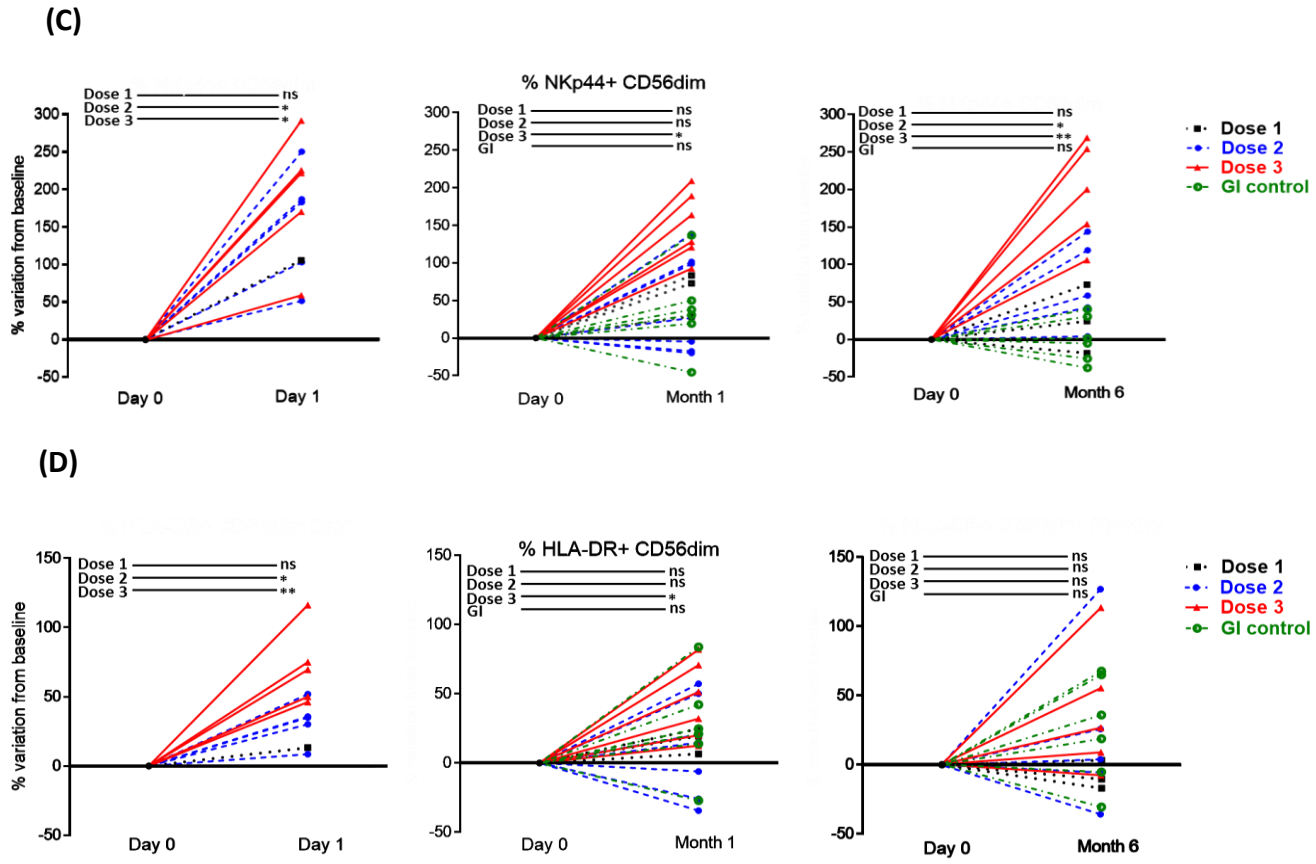
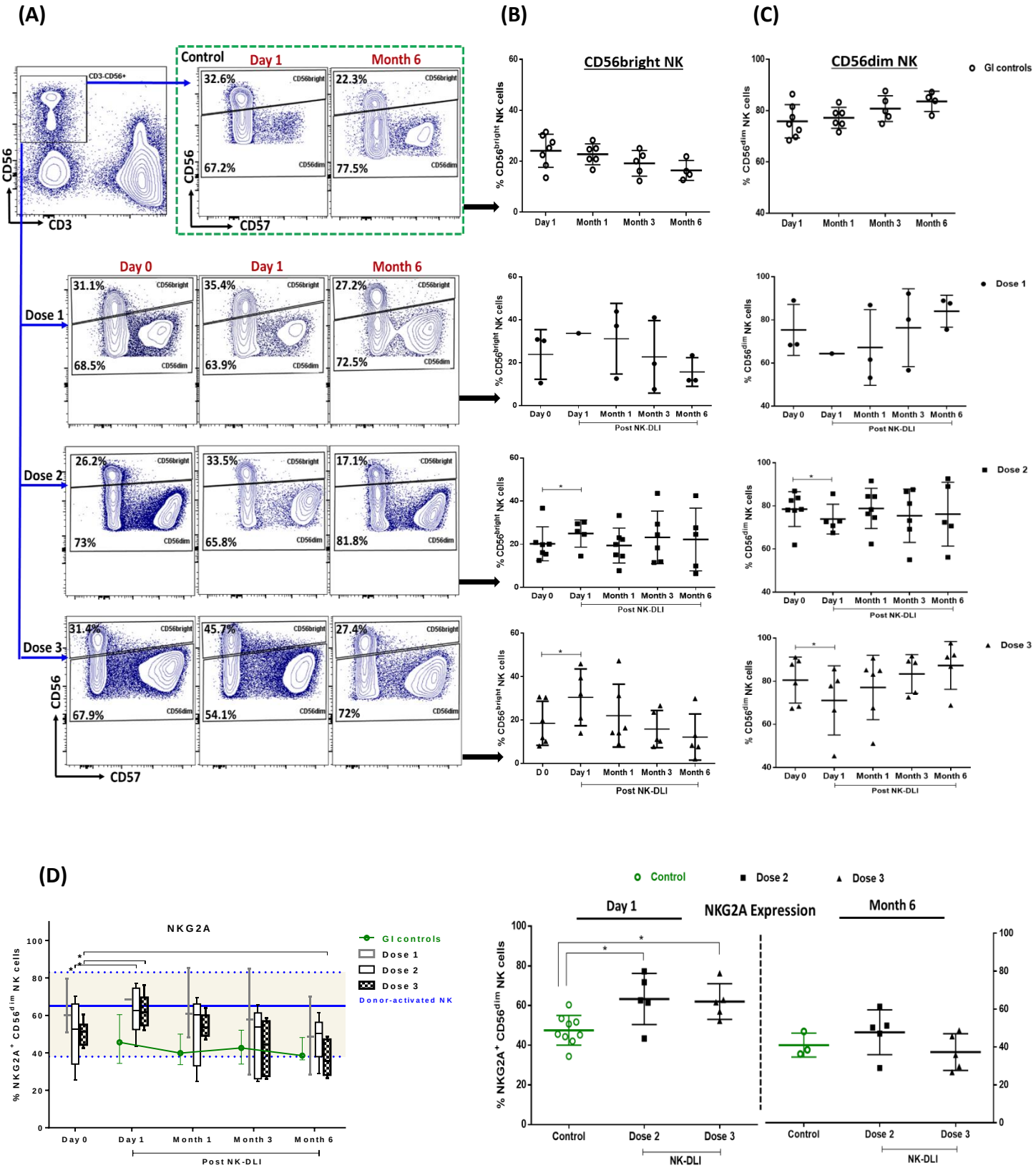


Figure 2: Assessment of activation profile of circulating NK cells in NK-DLI treated and non-treated patients. (A) Representative flow cytometry plots showing expression of CD69 on $CD56^{dim}$ NK cell subset for one patient from each treated group as well as one control at baseline (day 0), day 1 and month 6 post NK-DLI. Plots also show the expression of CD69 on $CD56^{dim}$ NK cell subset of a donor following before and after 7 days of IL-2 stimulation. Values indicate the percentage of $CD69^{+}$ $CD56^{dim}$ NK cells. (B-D) Percent of change in expression of CD69, NKp44 and HLA-DR, respectively, on $CD56^{dim}$ NK cells of dose 1-recepients (dotted black lines), dose 2-recepients (dashed blue lines), dose 3-recepients (plain red lines), and controls (dashed green lines) at day 1, month 1 and month 6 post NK-DLI compared to baseline (day 0). Statistical analyses were performed using Wilcoxon non parametric t. test (* $p \leq 0.05$, ** $p < 0.01$).


(A)
(B)
(C)
Control
Day 1
Month 6
CD3-CD56+
CD56
CD3
CD57
32.6%
67.2%
22.3%
77.5%
CD56bright
CD56dim
CD56bright NK
CD56dim NK
GI controls
% CD56bright NK cells
% CD56dim NK cells
Day 1
Month 1
Month 3
Month 6
Day 0
Post NK-DLI
Dose 1
31.1%
68.5%
35.4%
63.9%
27.2%
72.5%
Dose 2
26.2%
73%
33.5%
65.8%
17.1%
81.8%
Dose 3
31.4%
67.9%
45.7%
54.1%
27.4%
72%
D 0
(D)
NKG2A
GI controls
Dose 1
Dose 2
Dose 3
Donor-activated NK
% NKG2A+ CD56dim NK cells
NKG2A Expression
Control
NK-DLI

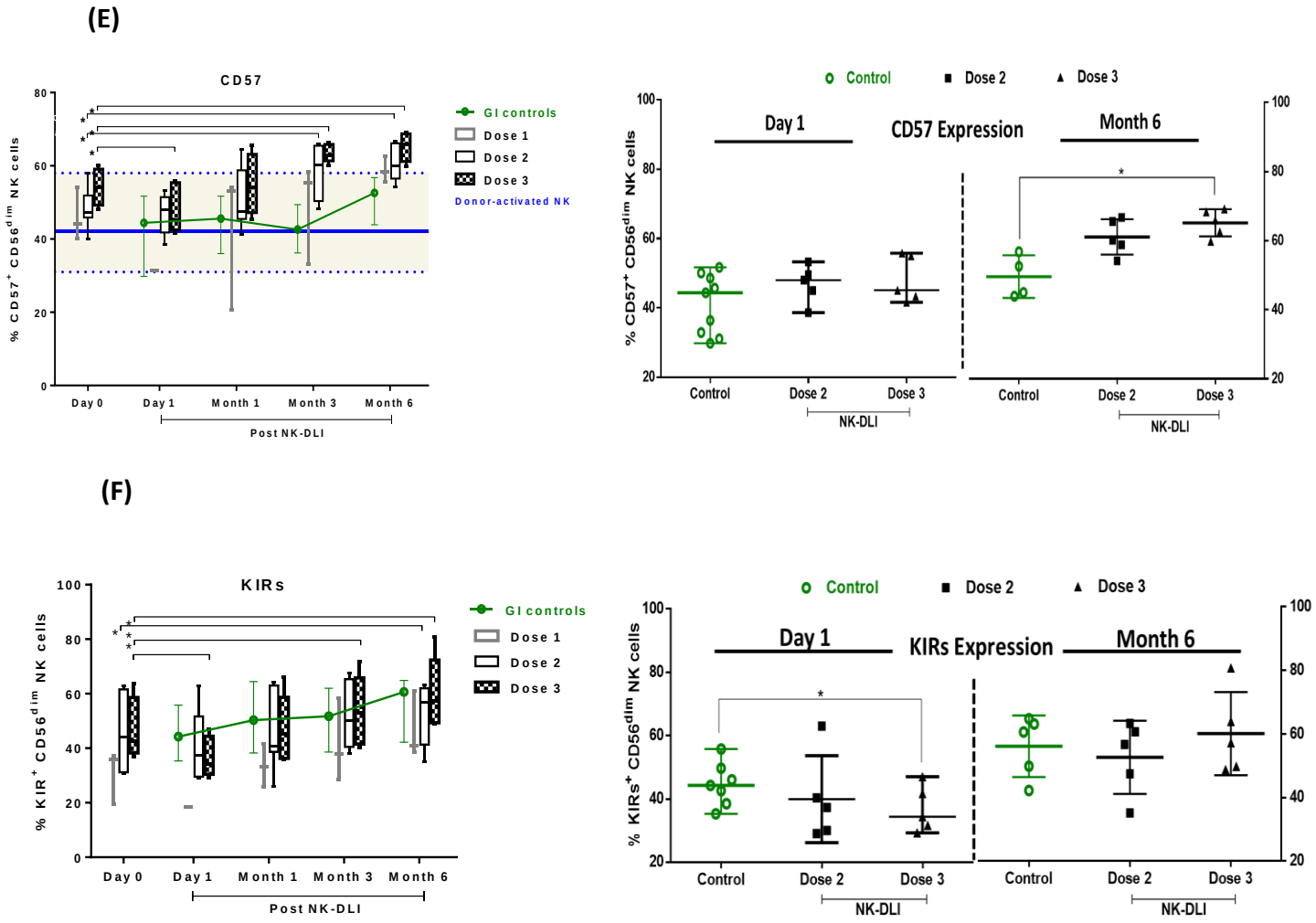
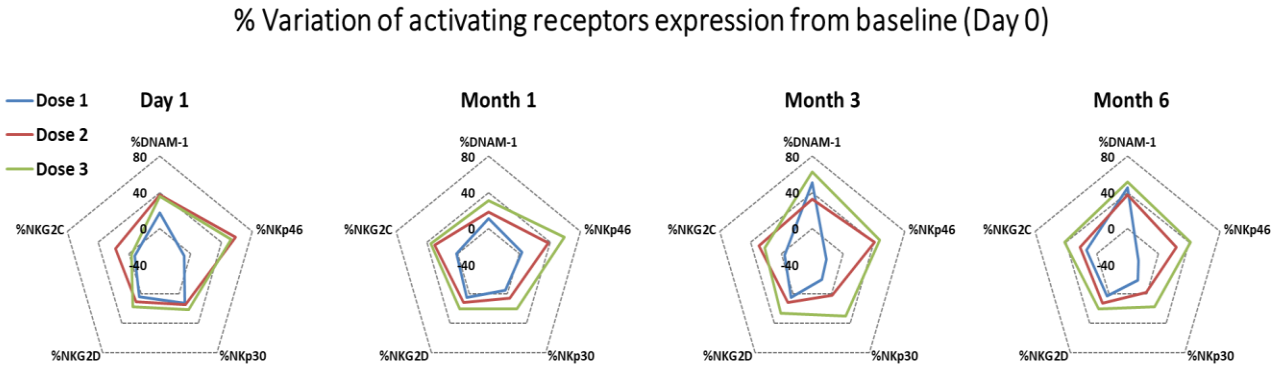
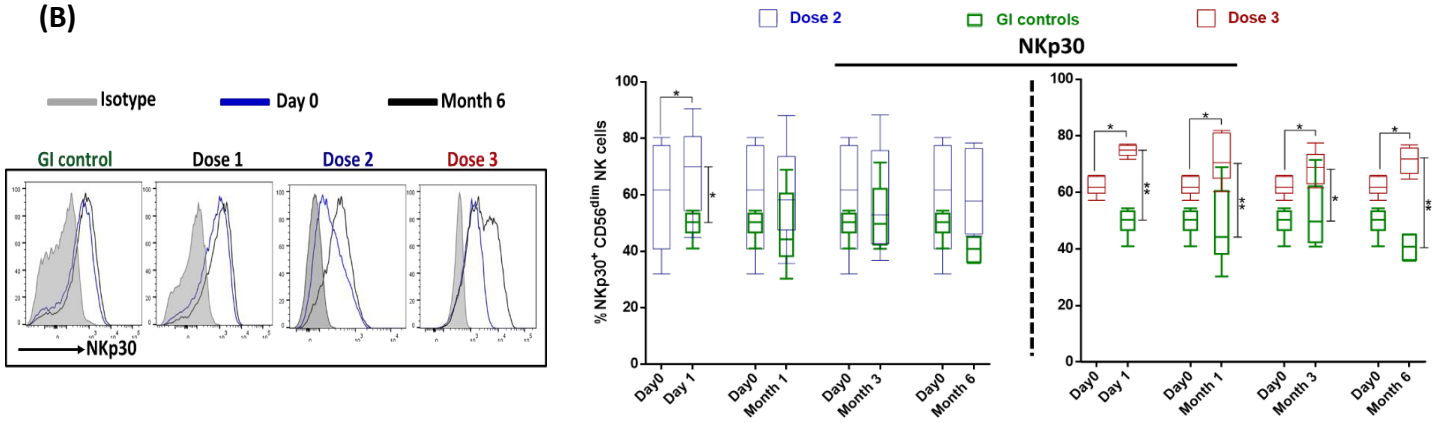


Figure 3: Assessment of maturation behavior of circulating NK cells in NK-DLI treated and non-treated patients. (A) Representative flow cytometry plots showing frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK subsets within $CD3^{-}CD56^{+}$ NK cells for one patient from each treated group as well as one control at baseline (day 0), and day 1, month 6 post NK-DLI. (B, C) Scatter dot plots showing frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK subsets (mean ± SD) within circulating $CD3^{-}CD56^{+}$ NK cells of controls (open circle), dose 1-treated patients (black filled circle), dose 2-treated patients (square) and dose 3-treated patients (triangle) before NK-DLI (day 0) and at day 1, month 1, 3, 6 post NK-DLI. (D-F) Expression profile of NKG2A, CD57 and KIRs on $CD56^{dim}$ NK subset of treated patients and controls before and at different times after NK-DLI. Data are represented as box and whisker (Left: min to max; horizontal lines represent median values) graphs and scatter dot plots (Right: mean ± SD). The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$). Blue lines represent NK cell frequency (mean, min-max) of donors (n=16).

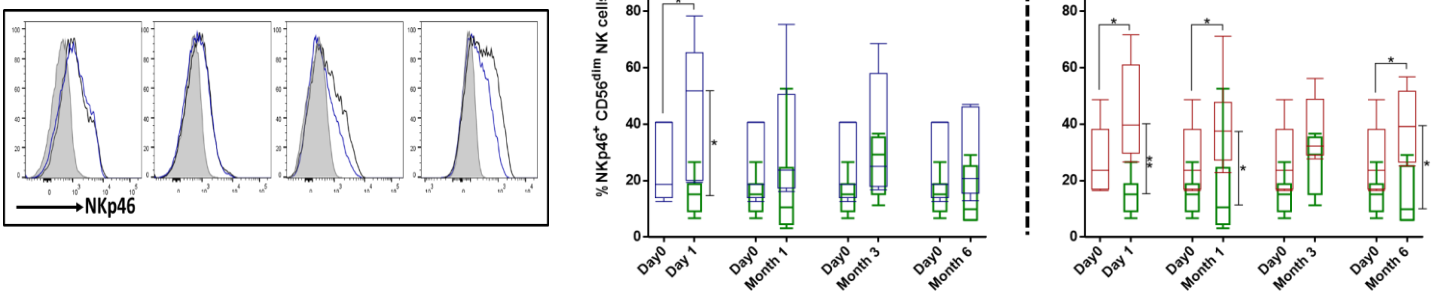
(A)


% Variation of activating receptors expression from baseline (Day 0)
Dose 1
Dose 2
Dose 3
Day 1
Month 1
Month 3
Month 6
%DNAM-1
%NKp46
%NKp30
%NKG2D
%NKG2C
80
40
0
-40

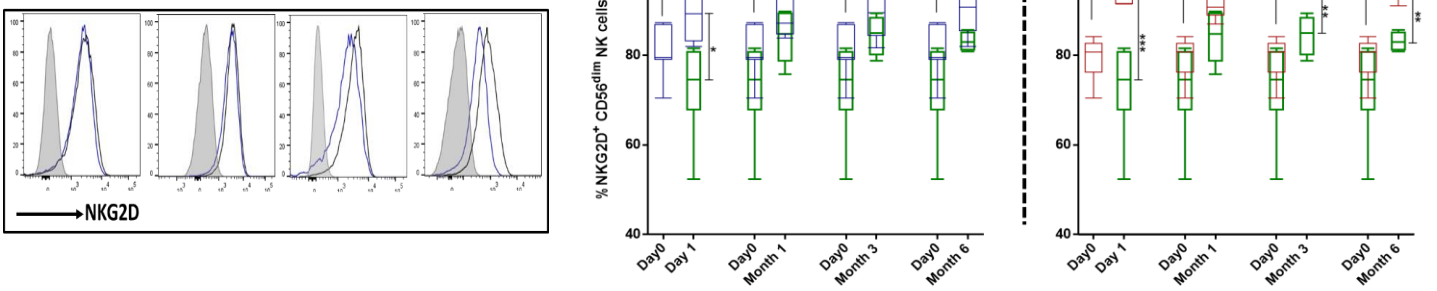
(B)


Isotype
Day 0
Month 6
GI control
Dose 1
Dose 2
Dose 3
NKp30
Dose 2
GI controls
Dose 3
NKp30
% NKp30+ CD56dim NK cells
Day0
Day 1
Month 1
Month 3
Month 6

(C)


NKp46
NKp46
% NKp46+ CD56dim NK cells
Day0
Day 1
Month 1
Month 3
Month 6

(D)


NKG2D
NKG2D
% NKG2D+ CD56dim NK cells
Day0
Day 1
Month 1
Month 3
Month 6

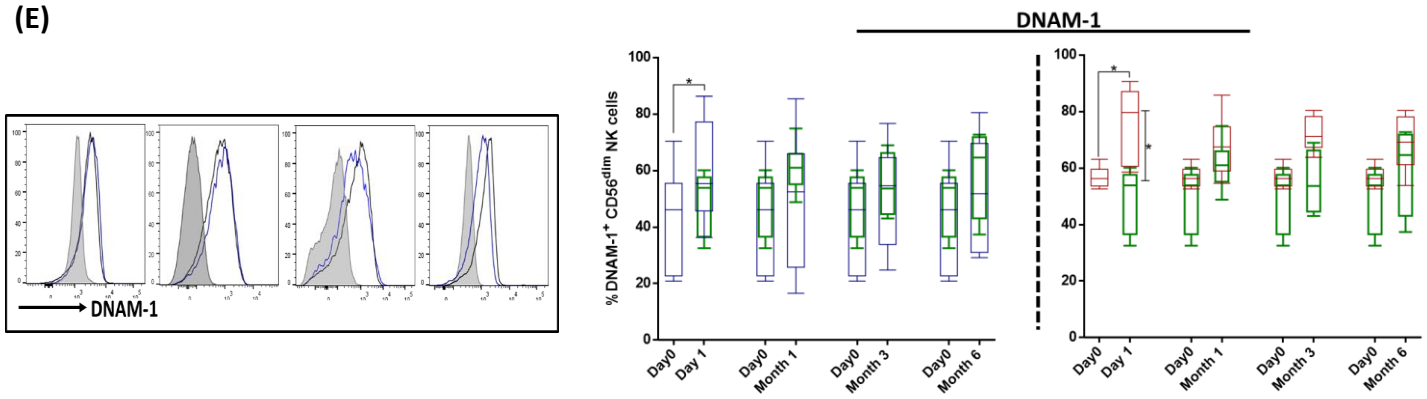
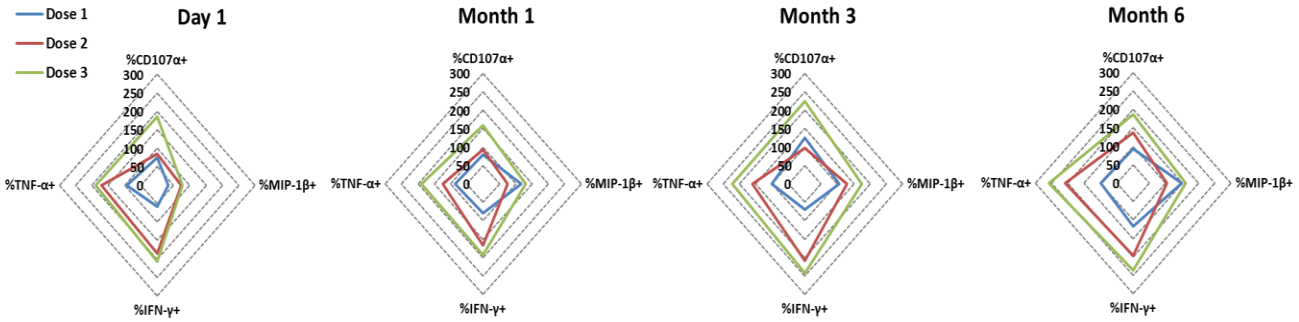
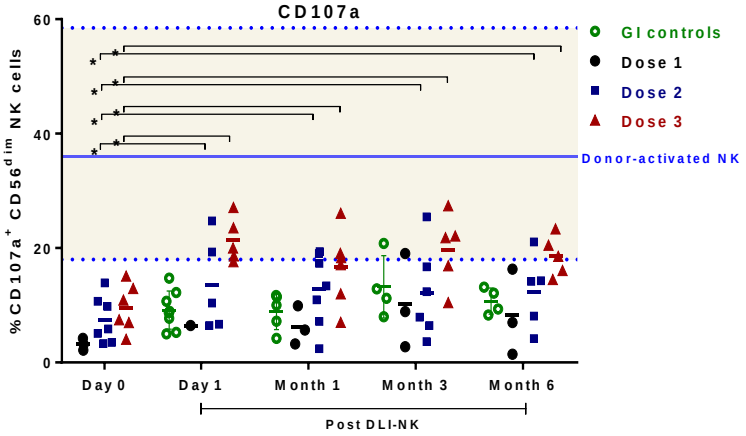


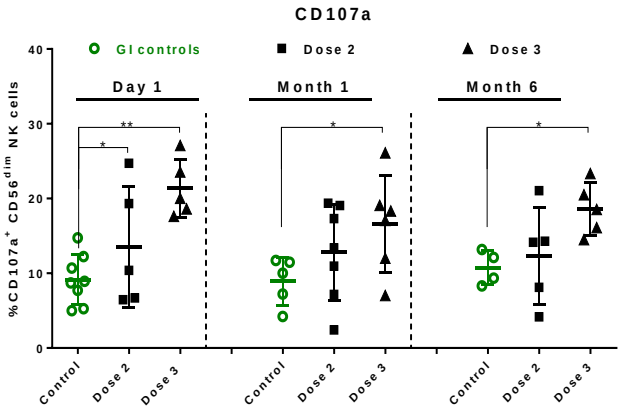
Figure 4: Expression profile of activating NK cell receptors on CD56^{dim} NK cells among NK-DLI treated and non-treated patients. (A) Representative radar chart displaying percent of change in the expression of DNAM-1, NKp30, NKp46, NKG2D and NKG2C receptors on CD56^{dim} NK cells of dose 1-recepients (blue), dose 2-recepients (red) and dose 3-recepients (green) at different time points post NK-DLI in comparison to baseline value (day 0). (B-E left) Representative histogram profiles for each receptor expressed on PB CD56^{dim} NK cells from a control and a treated patient with dose 1, dose 2 and dose 3, respectively at day 0 (blue line) at month 6 (black line). Isotype-matched negative controls are shown as gray filled histograms. (B-E right) Box and whisker graphs displaying frequency of positive CD56^{dim} NK cell (min to max; horizontal lines represent median values) for NKp30, NKp46, NKG2D, DNAM-1 receptors in PB of patients treated with dose 2 (blue boxes), and dose 3 (red boxes) as well as controls (green boxes) before and after different time points of NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$).

(A)

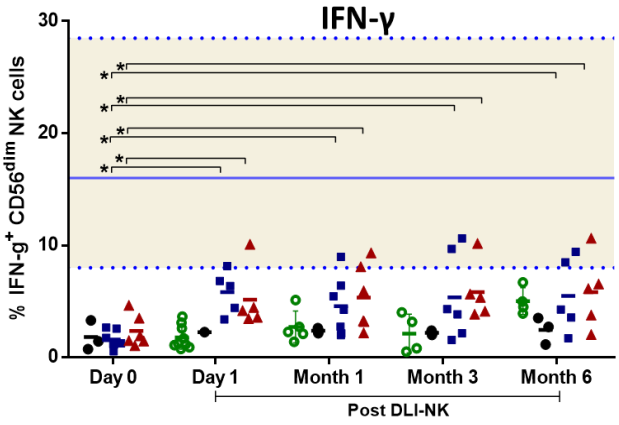

% Variation of degranulation, cytokines and chemokine production from baseline (Day 0)
Dose 1
Dose 2
Dose 3
Day 1
Month 1
Month 3
Month 6
%CD107α+
%MIP-1β+
%IFN-γ+
%TNF-α+
300
250
200
150
100
50
0

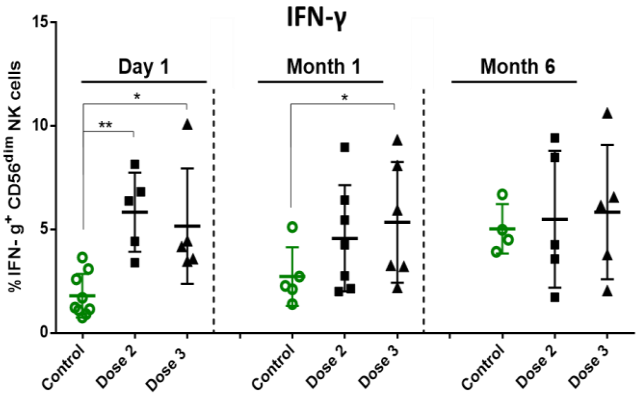
(B)


CD107a
GI controls
Dose 1
Dose 2
Dose 3
Donor-activated NK
% CD107a+ CD56dim NK cells
Day 0
Day 1
Month 1
Month 3
Month 6
Post DLI-NK


CD107a
GI controls
Dose 2
Dose 3
Day 1
Month 1
Month 6
% CD107a+ CD56dim NK cells
Control
Dose 2
Dose 3

(C)


IFN-γ
% IFN-g+ CD56dim NK cells
Day 0
Day 1
Month 1
Month 3
Month 6
Post DLI-NK


IFN-γ
Day 1
Month 1
Month 6
% IFN- g+ CD56dim NK cells
Control
Dose 2
Dose 3

(D)

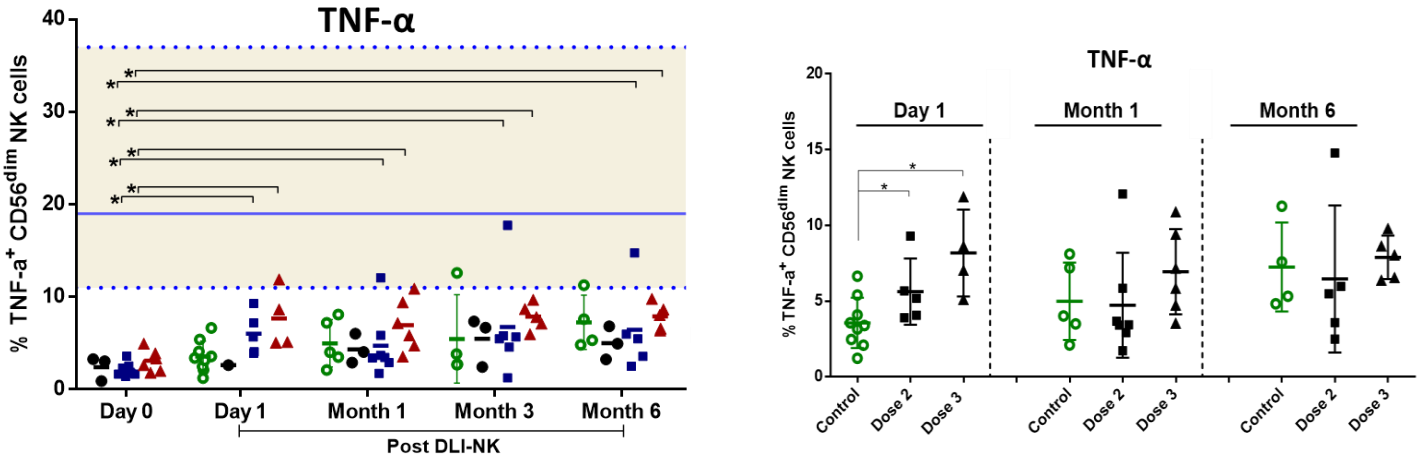
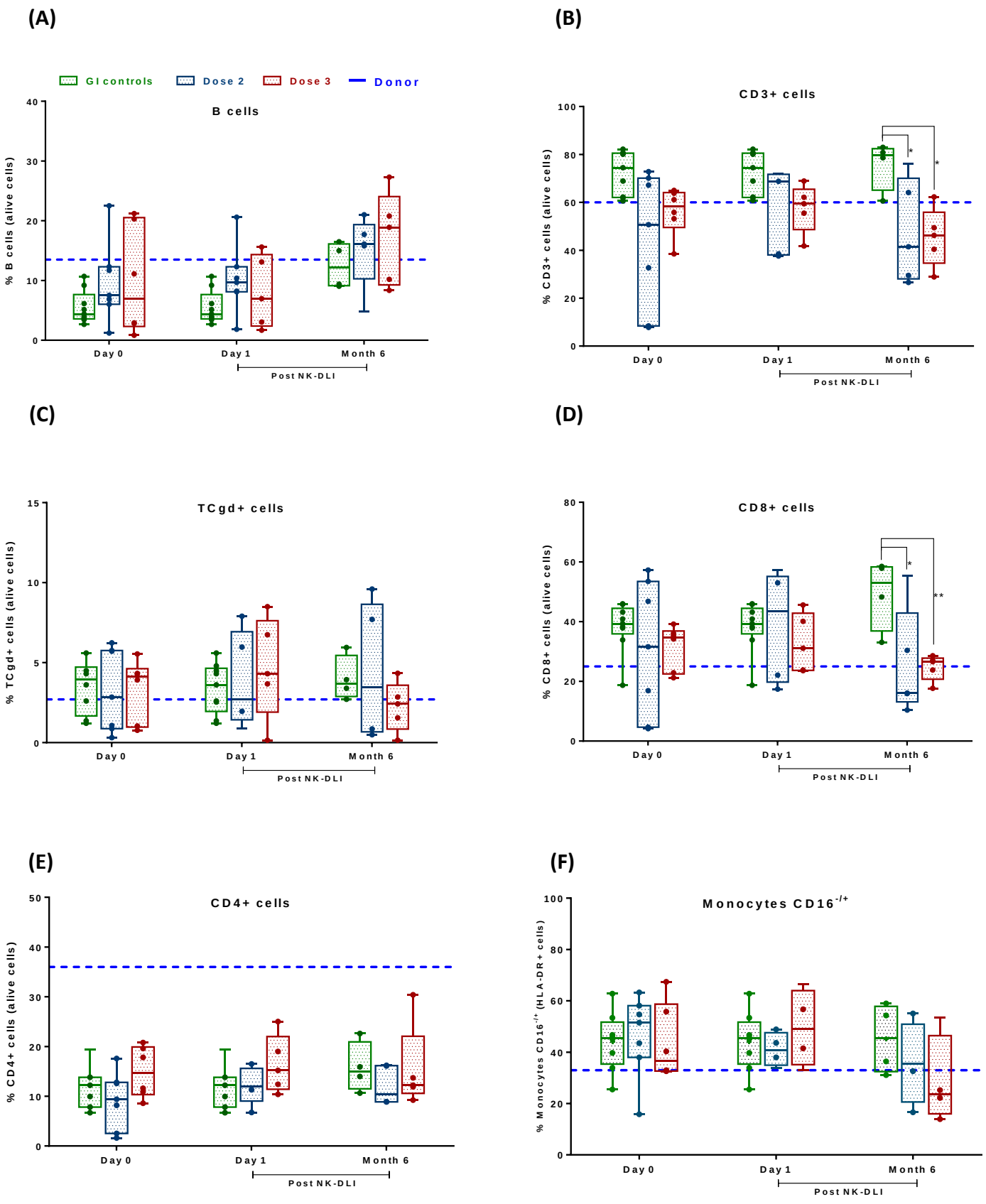


Figure 5: Functional activity of reconstituting NK cells in NK-DLI treated and non-treated patients. (A) Representative radar chart depict MIP-1β chemokine and cytokines production as well as degranulation capacity of $CD56^{dim}$ NK cells in dose 1-recepients (blue), dose 2-recepients (red) and dose 3-recepients (green) at different time points post NK-DLI in comparison to baseline value (day 0). (B-D left) Scatter dot plots showing frequencies (mean ± SD) of $CD107a^{+}$ $CD56^{dim}$ NK cells (B); IFN-Y^{+} $CD56^{dim}$ cells (C); TNF-α^{+} $CD56^{dim}$ NK cells (D) in controls (green circle), dose 1-treated patients (black circle), dose 2-treated patients (blue square) and dose 3-treated patients (red triangle) before NK-DLI (day 0) and at different time point post NK-DLI. (B-D right) Scatter dot plots showing a comparison of frequencies (mean ± SD) of $CD107a^{+}$ $CD56^{dim}$ NK cells (B); IFN-Y^{+} $CD56^{dim}$ cells (C); TNF-α^{+} $CD56^{dim}$ NK cells (D) between controls (green circle), dose 2-treated patients (black square), and dose 3-treated patients (black triangle) at day 1, month 1 and month 6 post NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$). Blue lines represent NK cell frequency (mean, min-max) of donors (n=16).


(A)
GI controls
Dose 2
Dose 3
Donor
B cells
% B cells (alive cells)
Day 0
Day 1
Month 6
Post NK-DLI
(B)
CD3+ cells
% CD3+ cells (alive cells)
(C)
TCgd+ cells
% TCgd+ cells (alive cells)
(D)
CD8+ cells
% CD8+ cells (alive cells)
(E)
CD4+ cells
% CD4+ cells (alive cells)
(F)
Monocytes CD16-/+
% Monocytes CD16-/+ (HLA-DR+ cells)

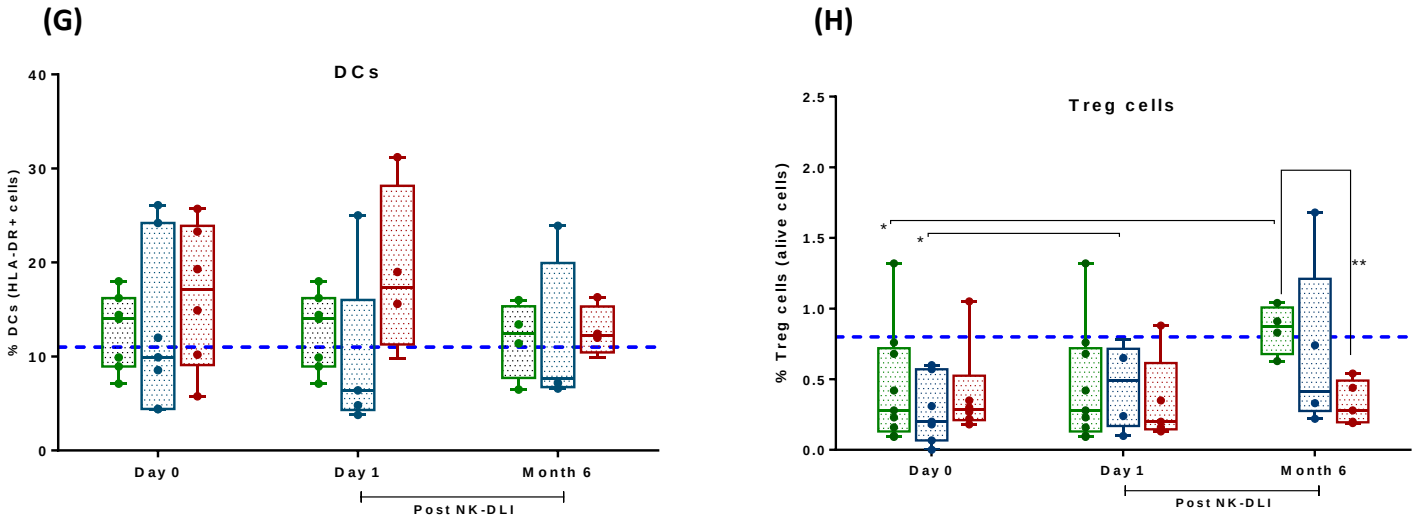
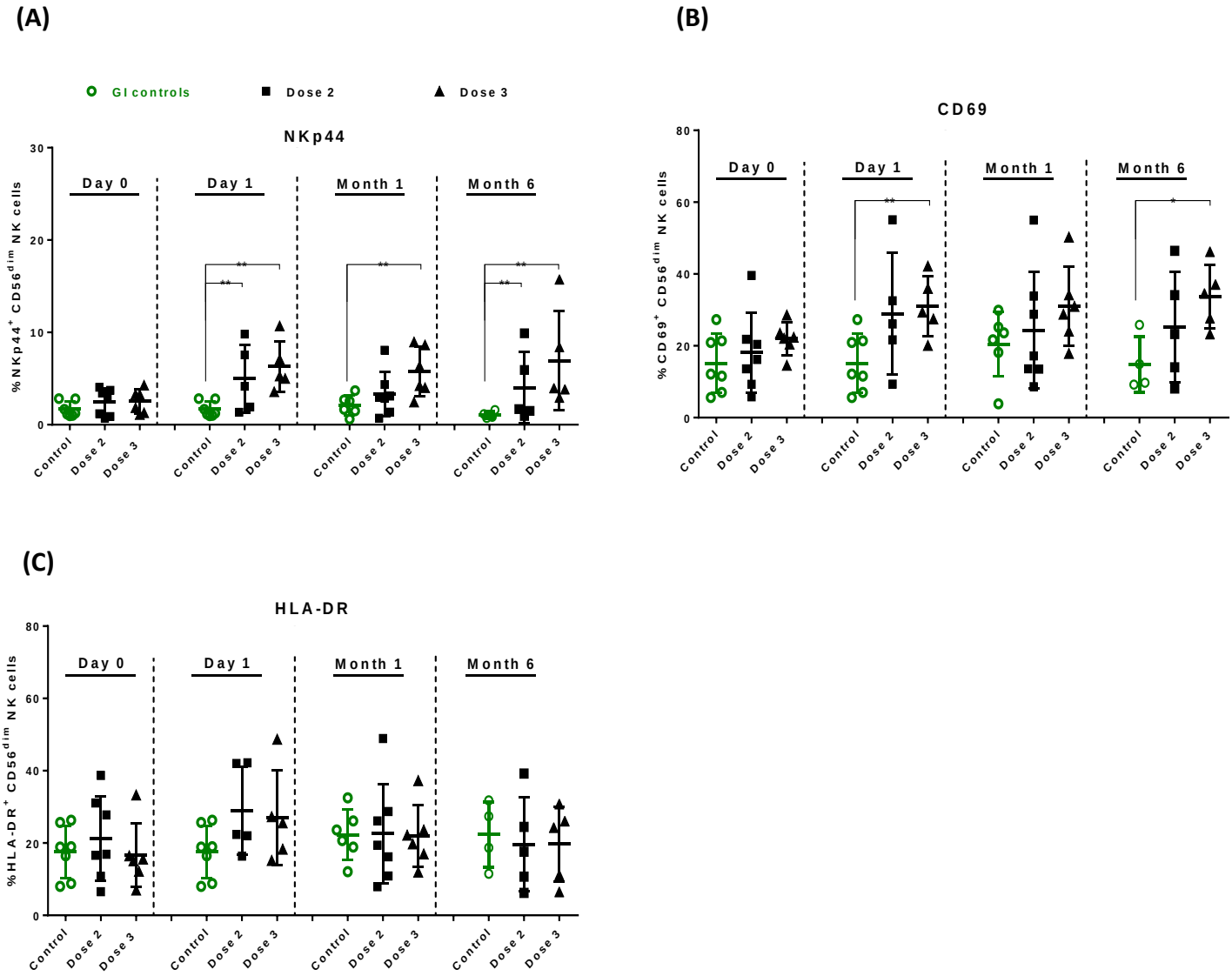
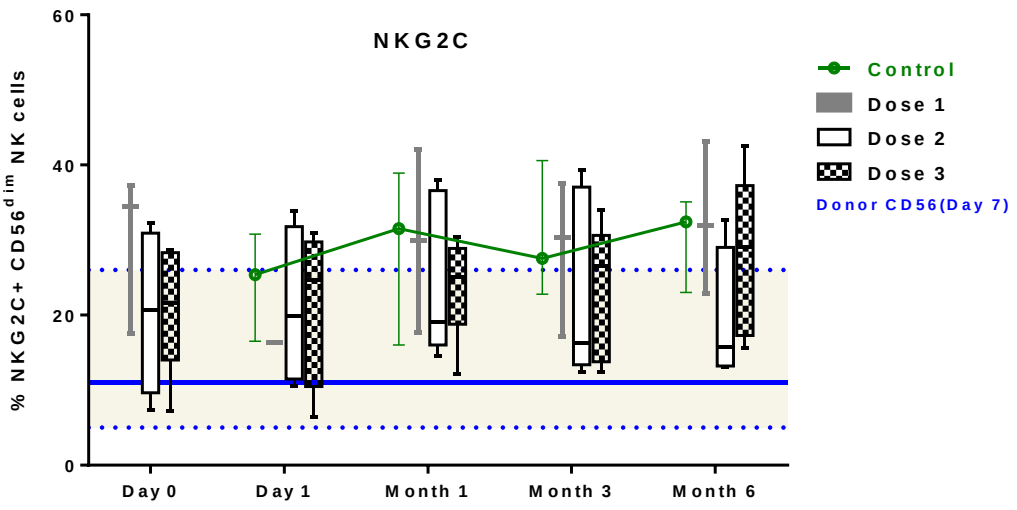


Figure 6: Evaluation of immune cells reconstitution post allo-SCT in patients treated with *ex-vivo* IL-2 activated NK cells (NK-DLI). (A-H) Box and whisker graphs displaying frequencies (median, range) of immune cells: (A) B cells, (B) $CD3^+$ cells, (C) $TC\gamma\delta^+$, (D) $CD8^+$ cells, (E) $CD4^+$ cells, (F) monocytes $CD16^{-/+}$, (G) DCs, (H) $CD4^+$Treg in PB of patients treated with dose 2 (blue boxes), and dose 3 (red boxes) as well as controls (green boxes) before NK-DLI (day 0) and at day 1, month 6 of NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$). Dashed blue line represents mean frequency of donors (n=16).

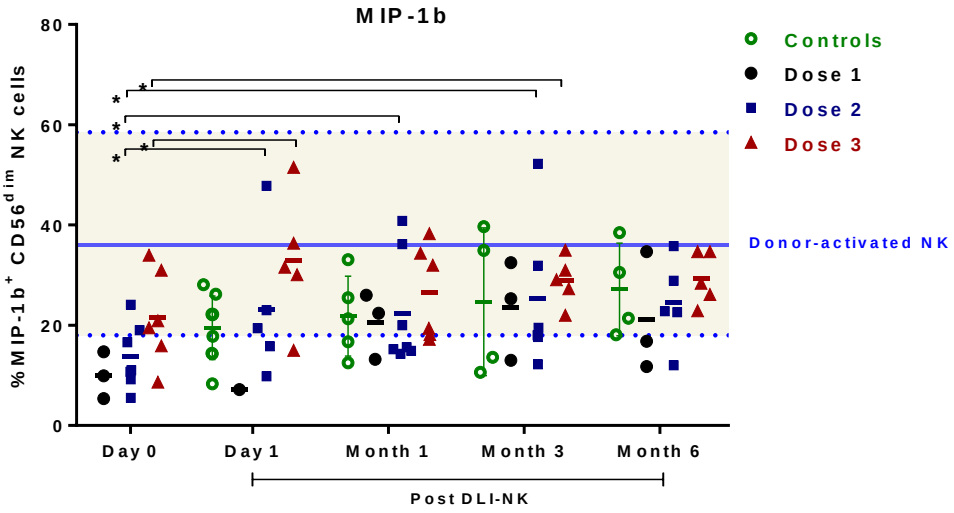
Supplementary Figures



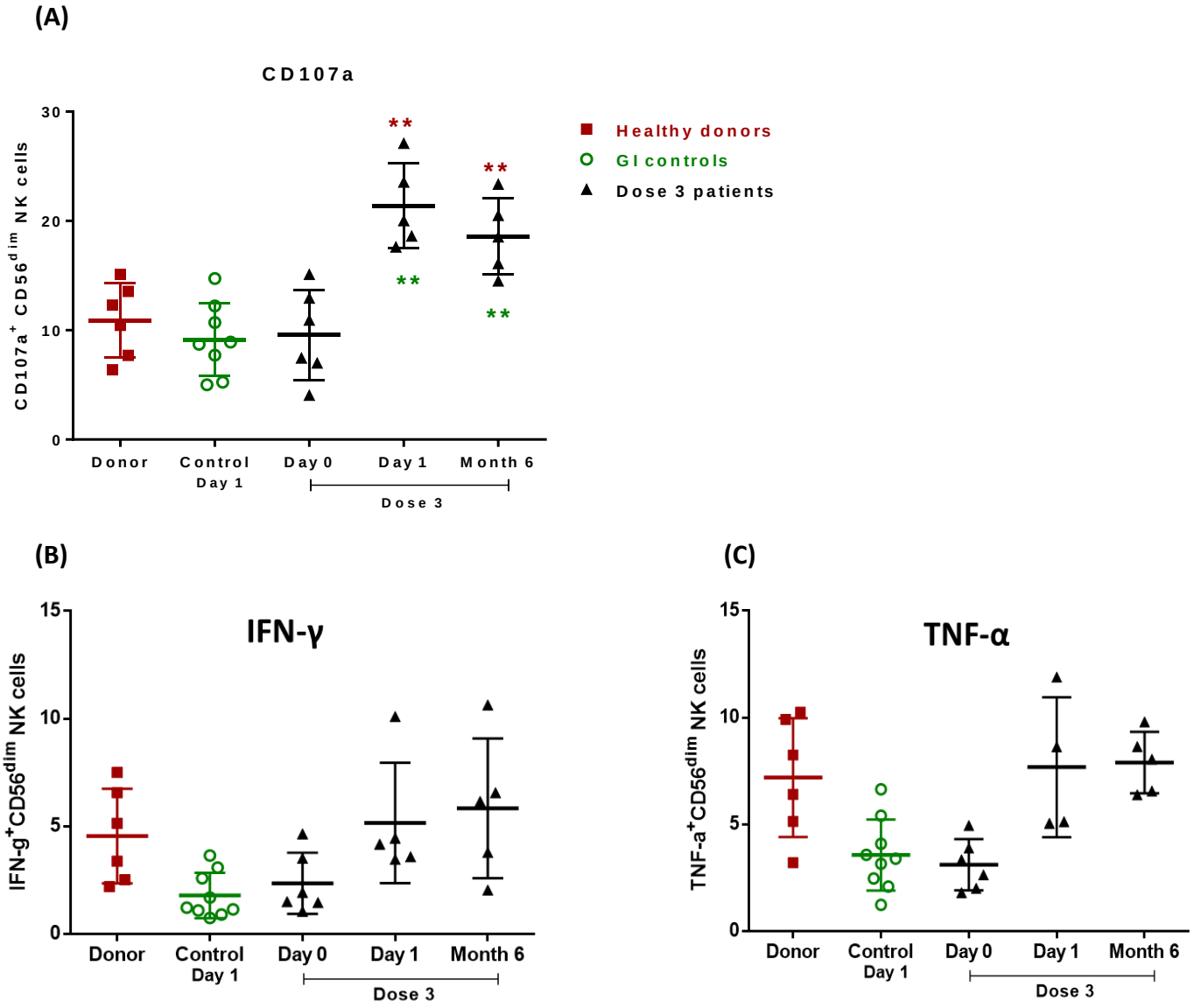
Supplementary Figure 1: Injection of high dose of activated NK cells shows an increase in NK cell activation markers expression. (A-C) Scatter dot plots showing a comparison of frequencies (mean ± SD) of NKp44^{+} CD56^{dim} NK cells (A); CD69^{+} CD56^{dim} cells (B); HLA-DR^{+} CD56^{dim} NK cells (C) between controls (green circle), dose 2-treated patients (black square), and dose 3-treated patients (black triangle) before (day 0) and at day 1, month 1 and month 6 post NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$).



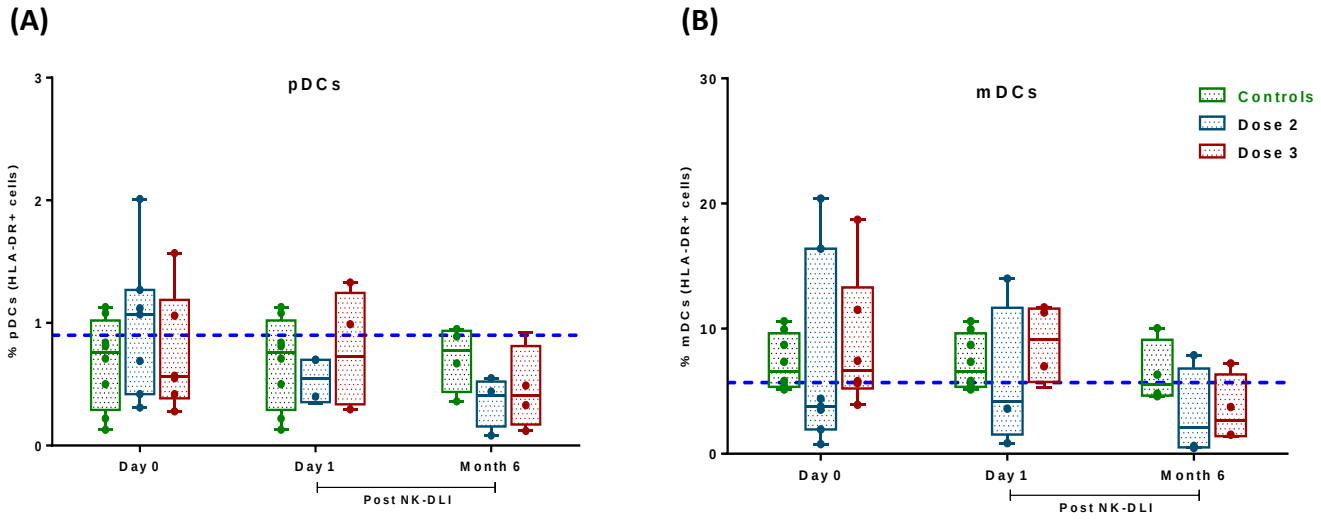
Supplementary Figure 2: NKG2C expression on CD56^{dim} NK cells in NK-DLI treated and non-treated patients. Expression profile of NKG2C on CD56^{dim} NK subset of treated patients and controls before and at different times after NK-DLI. Data are represented as box and whisker (min to max; horizontal lines represent median values) graphs. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test. Blue lines represent NK cell frequency (mean, min-max) of donors (n=16).



Supplementary Figure 3: More MIP-1b chemokine secretion by CD56^{dim} NK cells of patients after treatment with high dose of activated NK cells. Scatter dot plots showing frequency (mean ± SD) of MIP-1b^{+} CD56^{dim} NK cells in controls (green circle), dose 1-treated patients (black circle), dose 2-treated patients (blue square) and dose 3-treated patients (red triangle) before NK-DLI (day 0) and at different time point post NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (* $p \leq 0.05$, ** $p < 0.01$). Blue lines represent NK cell frequency (mean, min-max) of donors (n=16).



Supplementary Figure 4: Restoration of degranulation capacity and cytokine production of NK cells in response to target cell stimulation after NK-DLI therapy. (A-C) Scatter dot plots showing a comparison of frequencies (mean ± SD) of CD107a^{+} CD56^{dim} NK cells (A); IFN-γ^{+} CD56^{dim} cells (B); TNF-α^{+} CD56^{dim} NK cells (C) between healthy donors (red square), controls (green circle), and dose 3-treated patients (black triangle) at different time points post NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test (** $p < 0.01$).



Supplementary Figure 5: Reconstitution of pDCs and mDCs post allo-SCT in patients treated with *ex-vivo* IL-2 activated NK cells (NK-DLI). Box and whisker graphs displaying frequencies (median, range) of pDCs (A), and mDCs (B) in PB of patients treated with dose 2 (blue boxes), and dose 3 (red boxes) as well as controls (green boxes) before NK-DLI (day 0) and at day 1, month 6 of NK-DLI. The comparisons between each time point post NK-DLI and day 0 were performed using Wilcoxon non parametric t. test. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between each two groups were performed using non-parametric Mann-Whitney U test. Dashed blue line represents mean frequency of donors (n=16).

Table 1: Patients Characteristics

N (%)	16
Age	
Mean (SD)	57.3 (9.6)
Median (Min-Max)	59 (39 - 68)
Sex	
Male	8 (50)
Female	8 (50)
Diagnosis	
AML	5 (31.25)
CML	1 (6.25)
ALL	1 (6.25)
NHL	4 (25)
HL	1 (6.25)
MM	2 (12.5)
Others	2 (12.5)
Injected Dose	
Dose 1: 1x10^6 NK/Kg	
N (%)	3 (18.75)
Median (Min-Max)	1 (1 - 1)
Dose 2: >1x10^6 - ≤5x10^6 NK/Kg	
N (%)	7 (43.75)
Median (Min-Max)	4 (1.3 - 5)
Dose 3: >5x10^6 - < 5x10^{7}NK/Kg	
N (%)	6 (37.5)
Median (Min-Max)	6.6 (5.1 - 10)
Median time to NK-DLI (days)	91 (61 - 106)
cGVHD	
Faible	
Before NK-DLI (N %)	1 (6.25)
Day1-Day30 after NK-DLI (N %)	1 (6.25)
After day30 (N %)	-
Moderate	
Before NK-DLI (N %)	-
Day1-Day30 after NK-DLI (N %)	1 (6.25)
After day30 (N %)	3 (18.75)
Severe	
Before NK-DLI (N %)	-
Day1-Day30 after NK-DLI (N %)	1 (6.25)
After day30 (N %)	1 (6.25)

OS at month6 (%, number of events)	88% (2)
PFS at month6 (%, number of events)	88% (2)

AML indicates acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoid leukemia; NHL, non-Hodgkin lymphoma; HL, Hodgkin lymphoma; MM, multiple myeloma; cGVHD, chronic graft *versus* host disease; OS, overall survival; PFS, progression-free survival.

Supplementary Table 1: Antibodies used for flow cytometry in the study.

Antibody	Clone	Supplier	Panel
CD45RA-V450	HI100	BD Biosciences	TCs Panel
CD3-BV605	SK7	BD Biosciences	
CD8-PE-CF594	RPA-T8	BD Biosciences	
CD4-AF700	RPA-T4	BD Biosciences	
TcRγ/δ-FITC	REA591	Miltenyi Biotec	
CD27-APC-H7	M-T271	BD Biosciences	
CD25-APC	M-A251	BD Biosciences	
FoxP3-PE	236A/E7	eBioscience	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	
HLA-DR-BV605	G46-6	BD Biosciences	MoDCs Panel
CD141 (BDCA3)-FITC	AD5-14H12	Miltenyi Biotec	
CD303 (BDCA2)-FITC	AC144	Miltenyi Biotec	
CD1c (BDCA1)-BV421	L161	BioLegend	
CD3-APC-H7	SK7	BD Biosciences	
CD56-BV510	NCAM16.2	BD Biosciences	
CD19-BV510	SJ25C1	BD Biosciences	
CD16-AF700	3G8	BD Biosciences	
CD14-PE-CF594	MϕP9	BD Biosciences	
CD33-PE-Cy7	WM-53	eBioscience	
HLA-DR-V450	L243	BD Biosciences	NK Activation Panel
CD69-PE	IM1943U	Beckman Coulter	
CD336 (NKp44)-APC	2.29	Miltenyi Biotec	
CD25-FITC	M-A251	BD Biosciences	
CD3-BV605	SK7	BD Biosciences	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	

CD19-PE-CF594	HIB19	BD Biosciences	
CD14-AF700	M5E2	BD Biosciences	
CD16-APC-Vio770	VEP13	Miltenyi Biotec	
NKG2A-Pacific Blue	Z199	Beckman Coulter	**NK Maturation Panel**
CD57-FITC	TB01	eBioscience	
NKG2C-PE	FAB138P	RD Systems	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	
CD3-PE-CF594	UCHT1	BD Biosciences	
CD158b1/b2,j (KIR2DL3/L2/S2)-PE-Cy5.5	A66900	Beckman Coulter	
CD158a,h (KIR2DL1/S1)-APC	A22332	Beckman Coulter	
CD158e1 (KIR3DL1)-AF700	DX9	BioLegend	
CD16-APC-Vio770	VEP13	Miltenyi Biotec	
CD335 (NKp46)-ef450	9E2	eBioscience	**NKRs Panel**
CD226 (DNAM-1)-FITC	DX11	BD Biosciences	
CD337 (NKp30)-PE	AF29-4D12	Miltenyi Biotec	
CD314 (NKG2D)- PerCP-efluor710	1D11	eBioscience	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	
CD3-BV605	SK7	BD Biosciences	
CD19-PE-CF594	HIB19	BD Biosciences	
CD14-AF700	M5E2	BD Biosciences	
CD16-APC-Vio770	VEP13	Miltenyi Biotec	
CD158a (KIR2DL1)-FITC	FAB1844F	RD Systems	**KIRs Panel**
KIR2DL3-PE	FAB2014P	RD Systems	
CD158b1/b2,j (KIR2DL3/L2/S2)-PE-Cy5.5	A66900	Beckman Coulter	
CD158a,h (KIR2DL1/S1)-APC	A22332	Beckman Coulter	
CD158e1 (KIR3DL1)-AF700	DX9	BioLegend	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	

CD3-PE-CF594	UCHT1	BD Biosciences	
NKG2A-Pacific Blue	Z199	Beckman Coulter	
CD16-APC-Vio770	VEP13	Miltenyi Biotec	
CD107a-FITC	H4A3	BD Biosciences	**NK Functional Panel (1)**
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	
CD3-PE-CF594	UCHT1	BD Biosciences	
CD16-APC-Vio770	VEP13	Miltenyi Biotec	
TNF-α-ef450	MAb11	eBioscience	
MIP-1β-PE	D21-1351	BD Biosciences	
IFN-γ-AF700	B27	BD Biosciences	
NKG2A-Pacific Blue	Z199	Beckman Coulter	**NK Functional Panel (2)**
CD57-PE	HCD57	BioLegend	
pan-KIR-APC	NKVFS1	Miltenyi Biotec	
CD56-PE-Vio770	AF12-7H3	Miltenyi Biotec	
CD3-PE-CF594	UCHT1	BD Biosciences	
CD107a-FITC	H4A3	BD Biosciences	
IFN-γ-AF700	B27	BD Biosciences	
Live/dead Discriminator	Fixable Aqua Dead cell stain kit	Life Technologies	

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PROJECT II

PUMILIO 2, A RNA BINDING PROTEIN UPREGULATED IN NK CELLS FROM AML PATIENTS, REPRESSES FUNCTIONS OF NK CELLS.

INTRODUCTION

Gene expression refers to the conversion of genetic information from genes via mRNA (transcription process) to proteins (translation process by ribosomes). So, mRNA is the key molecule that links genes to proteins. In humans and other eukaryotes, mRNAs are firstly synthesized in the nucleus as pre-mRNAs. Then, these molecules are undergone through some processing steps called post-transcriptional modifications to become mature mRNAs which are able to leave the nucleus to cytoplasm in order to translate into proteins by ribosomes. Post-transcriptional gene regulation concerns processes involved in maturation, transport, stability and translation of coding and non-coding RNAs. These modifications include 5'-end capping, splicing, 3'-end cleavage, and polyadenylation tail. Throughout these processes, the mRNA is packaged with proteins, together called messenger ribonucleoprotein particles (mRNPs), which protect the mRNA from destruction by nuclear enzymes until reach to the cytoplasm for translation into proteins. In general, hundreds of RNA-binding proteins (RBPs) and miRNAs are involved in post-transcriptional modifications via their binding to specific sequences at 3'-UTRs of mRNA (Filipowicz et al., 2008; Gebauer and Hentze, 2004).

RBPs play key roles in post-transcriptional control of RNAs to regulate gene expression by controlling processing, localization, translation and degradation of cellular RNAs. They have one or more RNA-binding domains (RBDs) to bind directly to RNA or to protein complexes that bind to RNA (Lee, 2006). The work presented here highlights one of these RBPs called Pumilio 2 (Pum2) protein to investigate its role on NK cell biology at genetic level.

1. PUF family proteins

PUmilio-Fem-3-binding factor (PUF) proteins are a conserved family of RNA-binding proteins, which are important regulators of mRNAs translation and stability. The family name is related to the first proteins discovered in this group, **Pu**milio in *Drosophila melanogaster* and **F**BF in *Caenorhabditis elegans*. The mechanisms by which these proteins regulate mRNAs remain unclear, however, PUF proteins suppress the target mRNA expression by stimulation of mRNA decay and/or inhibition of translation (Miller and Olivas, 2011; Quenault et al., 2011; Wickens et al., 2002). In general, they are

involved in various physiological processes such as stem cell proliferation and differentiation, fertility, neuron function and memory formation.

PUF proteins can perform their regulatory functions via binding to specific sequences in the 3' UTR of mRNAs called PUF response element (PRE), a cis element consisting of 8-12 nucleotide consensus sequence that directs regulation of mRNAs (Quenault et al., 2011; Wickens et al., 2002). Moreover, all PUF proteins are characterized by the presence of an RBD, also known as Pumilio homology domain (Pum-HD), that contains 8 α-helical repeats of a motif of ≈36 amino acids and folds into an arc-like shape (Wickens et al., 2002; Zamore et al., 1997). Subsequently, PUF proteins bind to specific RNA element located in PRE that comprises a core 'UGUR' tetranucleotide.

Hundreds of PUF proteins have been identified in different eukaryotic organisms. Some organisms such as *Drosophila* express only one PUF protein, while others such as *C. elegans* and *Trypanosoma brucei* express six or more PUF proteins. In humans, it has been thought only two PUF proteins are expressed: Pum1 and Pum2. However, two novel PUF proteins: Puf-A and C14orf21 have been discovered (Kuo et al., 2009).

2. Human pumilio proteins: Pum1 and Pum2

As mentioned above, both human Pum1 and Pum2 belong to PUF family of RBPs that post-transcriptionally regulate protein expression by inhibiting target mRNAs translation and/or enhancing mRNAs decay (Quenault et al., 2011; Spassov and Jurecic, 2002; Wickens et al., 2002). Normally, Pum1 and Pum2 are often co-expressed in diverse tissues such as fetal tissues, adult central and peripheral nervous system, cardiovascular, gastrointestinal, urogenital, hematopoietic and endocrine systems (Spassov and Jurecic, 2002).

3. Structure of Pum1 and Pum2

From biochemical point of view, human Pum1 (127 kDa) and Pum2 (114 kDa) consist of 1186 and 1064 amino acids, respectively (Spassov and Jurecic, 2002). Also they have three different domains: glutamine/alanine rich domain, serine rich domain and PUM-HD domain. In contrast to PUM-HD domain, the function of the glutamine/alanine rich and serine rich domain is still unclear. Regarding Pum1 gene, it consists of 22 exons while Pum2 gene has 20 exons, and in both genes PUM-HD spans eight exons encoded by exons 15-22 in Pum1 gene and exons 13-20 in Pum2 gene

(Spassov and Jurecic, 2002). Commonly, Pum1 and Pum2 proteins share 83% overall similarity and 75% identity, while their PUM-HD share 91% identity and 97% similarity (Spassov and Jurecic, 2003, 2002).

PUM-HD, the typical feature of PUF family proteins, is the RNA-binding domain located in the C-terminal part of Pum proteins (Spassov and Jurecic, 2002; Zamore et al., 1997). It contains a canonical 361-amino acid long sequence, including eight tandem repeats of 36 amino acids where each repeat is composed of three α helices (H1, H2 and H3), (figure 19).

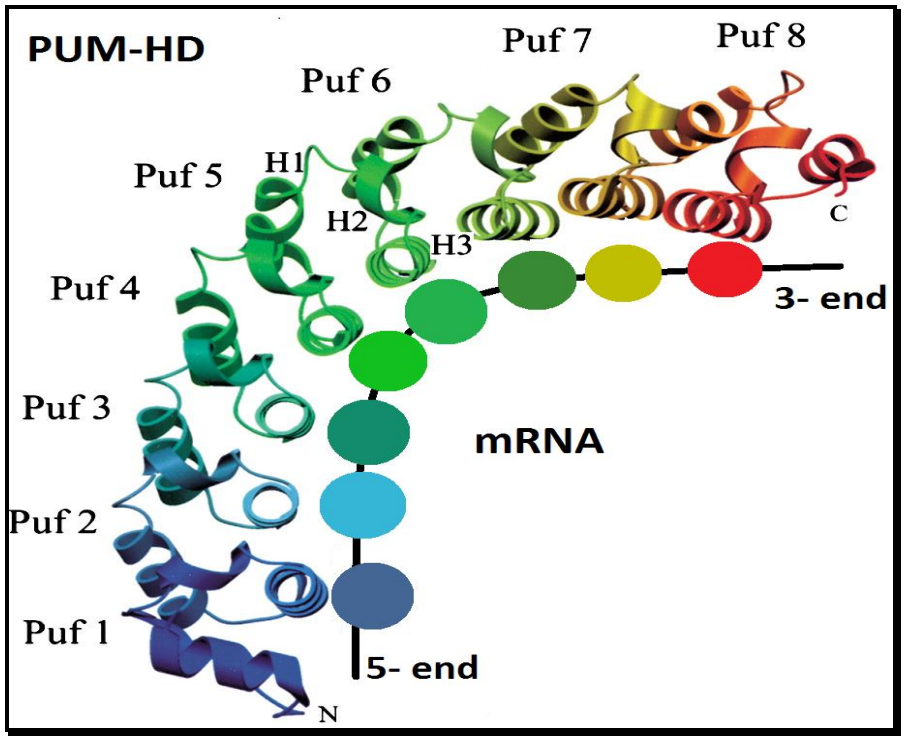


Figure 19: Structure of PUM-HD (Adapted from Edwards et al., 2001)

In each repeat, three amino acids can recognize a ribonucleotide base of the target mRNA where the amino acids at position 12 and 16 of the PUF repeat bind each RNA base via hydrogen bonding or van der Waals contacts, whereas the amino acid at position 13 makes a stacking interaction (Wang et al., 2002). Previously, characteristic sequence motifs have been found in the 3'-UTRs of the mRNA targets of different PUF-family members containing a core 'UGUR' tetranucleotide and known as Nanos Response Elements (NREs) or PBE (Wang et al., 2002; Wickens et al., 2002; Zamore et al., 1997). For human Pum1 and Pum2, Galgano and his colleagues have

identified a 8-nt consensus motif (5'-UGUANAUA, where N is any nucleotide) highly associated with human Pum1 and Pum2 (Galgano et al., 2008). Further, the 8-nt consensus sequence is highly related to the *Drosophila* Pum mRNA binding site (figure 20). They also analyzed the distribution of this motif among whole mRNA targets (3'-UTR, coding sequences (CDSs) and 5'-UTR), and the findings showed that approximately 85% of human Pum1 and Pum2 mRNAs targets contain the motif exclusively in the 3'-UTRs, 3-5% only in the CDSs, ~17% bear the motif in both the CDSs and 3'-UTRs, and it is almost absent in 5'-UTRs (Galgano et al., 2008).

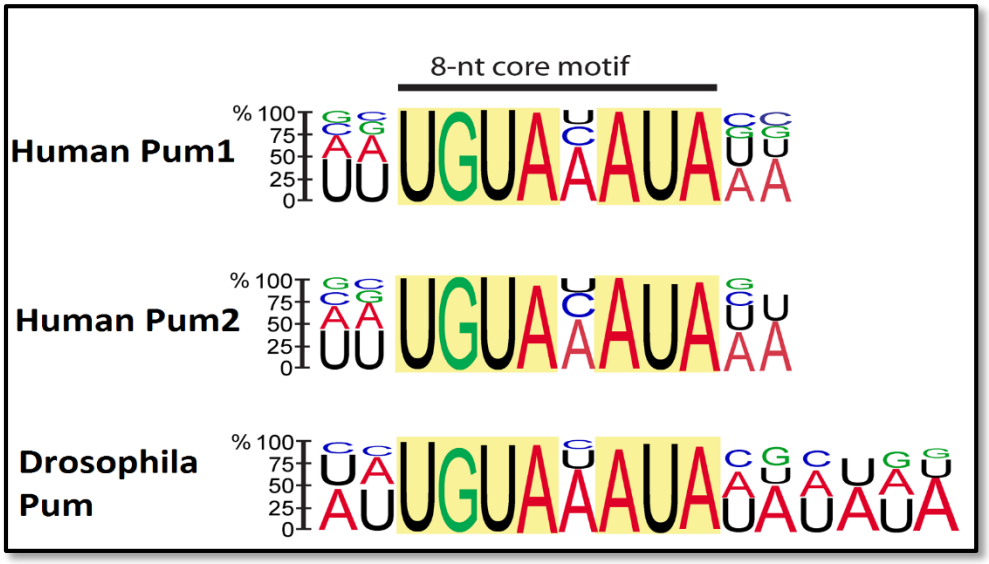


Figure 20: Human Pum1 and Pum2 motifs in 3'-UTR sequences (Adapted from Galgano et al., 2008)

4. Function of PUF proteins

The official role of PUF proteins, as posttranscriptional regulators of gene expression, is applied by binding to specific PBEs that are generally located in the 3'-UTRs of their mRNA targets. Indeed, PUFs are involved in mRNA translational repression via different pathways. Moreover, studies have indicated the role of PUF proteins in translational activation and stability of mRNAs (Quenault et al., 2011; Suh et al., 2009).

i. Translational repression

As the efficacy of mRNA translation is promoted by the 5' 7-methyl guanosine cap and the 3' poly (A) tail, enzymatic removal of the poly (A) tail (deadenylation) and/or 5' cap (de-capping) can suppress translation and initiate mRNA degradation. Although the complete understanding of

mRNA regulation by human Pums remains to be clarified, PUF proteins generally control gene expression by binding to PRE at the 3'-UTRs of specific mRNAs and triggering mRNA decay or translational repression in different pathways:

a. Deadenylase induction

PUF proteins action is associated with removal of the poly (A) tail of target mRNAs. So, many attempts were performed to recognize how these proteins enhance deadenylation and mRNA decay. The first mechanism of mRNA repression by PUF proteins was described by the Wickens laboratory, where yeast PUF proteins bind specifically to the Pop2 subunit of the Ccr4-Pop2-NOT mRNA deadenylase complex (Goldstrohm et al., 2006). By binding Pop2p, the PUF proteins simultaneously recruit the major cytoplasmic deadenylase (Ccr4p deadenylase) to the target mRNAs resulting in poly (A) tail deadenylation and shortening at 3' UTR that lead to translational inhibition and mRNA degradation (Quenault 2010) (figure 21).

In human, Ccr4-NOT (CNOT) complex contains CNOT7 and CNOT8 proteins which are related to yeast Pop2p, whereas CNOT6 and CNOT6L are orthologous to yeast Ccr4p deadenylase (Goldstrohm and Wickens, 2008). It has evidenced that both human Pum1 and Pum2 repress mRNA translation and enhance mRNA decay based on their direct binding with CNOT deadenylase complex subunits (CNOT7 and CNOT8), which are associated with deadenylase enzymes, CNOT6 and CNOT6L (Van Etten et al., 2012) (figure 21).

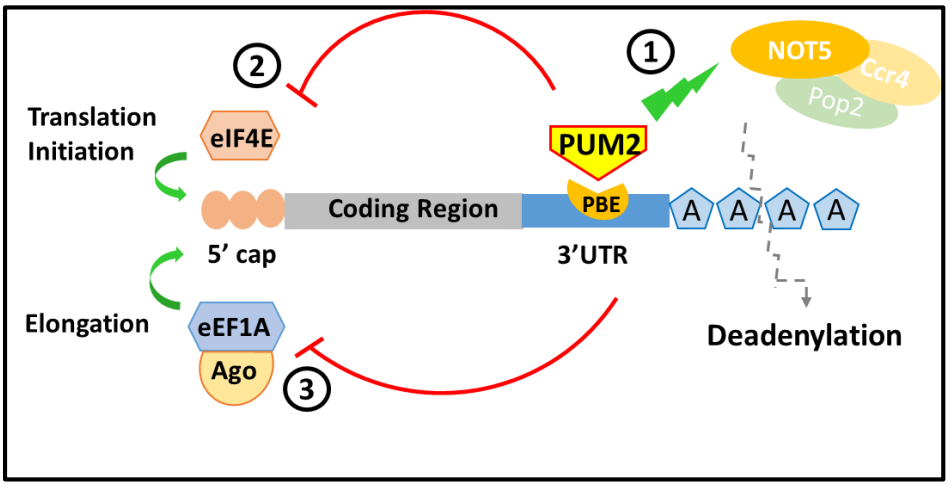


Figure 21: Mechanisms of action of Pum2 protein

b. Inhibition of translation initiation

Translation initiation process is a critical step in protein synthesis which is regulated with several factors such as eukaryotic initiation factors (eIFs). One of these factors is eukaryotic initiation factor 4E (eIF4E) playing a central role in translation initiation through its binding with 5' cap of mRNA (Jackson et al., 2010). PUF proteins have the ability to interrupt translation initiation process by different ways. For example, binding of *Drosophila* Pum at 3' UTR can recruit d4EHP (translation inhibitor) to mRNA, which inhibits translation by competing with binding of eIF4E to the 5' cap (Cho et al., 2005). In yeast, Ccr4-Pop2- NOT complex interacts with decapping activator and translational repressor cofactors, Dhh1 and Dcp1, which are recruited to mRNAs by the Pop2–Puf5 interaction causing decapping by hydrolysis of the 5' cap (Goldstrohm et al., 2006) (figure 21).

In human, poly (A) tail plays a fundamental role in mRNA translation through the action of poly (A) binding protein (PABP, is a RNA-binding protein which binds specifically to the poly (A) tail of mRNA), where the major form in humans is known as PABPC1 (Kühn and Wahle, 2004). PABPC1 interacts with eIF4G, eukaryotic initiation factor plays a central role in translation initiation through its interaction with the cap-binding protein eIF4E. EIF4E-eIF4G interaction is required for the recruitment of the 40S ribosomal subunit to the 5' end of mRNA to initiate translation process. By binding PRE at the 3'-UTR, human Pums inhibit translation initiation process by antagonizing the ability of PABPC1 to bind with eIF4G (Weidmann et al., 2014).

c. Inhibition of translation elongation

After initiation of translation process, translation elongation factor 1A (eEF1A) is required for translation elongation based on its GTPase activity. It was noticed that PUF proteins (*C. elegans* FBF and human Pum2) form a ternary complex with an Argonaute protein (Ago) and eEF1A (Friend et al., 2012). Argonaute is protein family plays a central role in microRNA-mediated mRNA repression by binding with small non-coding RNAs. By binding PRE at the 3'-UTR, *C. elegans* FBF and human Pum2 interact with Ago then form a complex with eEF1A which blocks its GTPase activity and attenuate translation elongation (Friend et al., 2012) (Figure 21). Surprisingly, Goldstrohm team have found that mutations in the Ago and eEF1A binding motifs do not alter *Drosophila* and human PUFs PUF-mediated repression of protein expression *in vivo* (Weidmann et al., 2014). This is suggesting that Ago is not necessary for repression elicited by *Drosophila* and human PUFs.

d. Cooperation with miRNAs

miRNAs are a large family of small non-coding RNAs binding to their target mRNAs by recognizing specific short sequences usually located in 3'UTRs (Bartel, 2009). miRNAs and RBPs play a fundamental role in post-transcriptional gene regulation as both of them have definite target sequences at 3'UTRs and function in mRNA decay and translation repression independently. Although RBPs and miRNAs have generally been considered as two distinct regulatory mechanisms for mRNA expression, growing studies hypothesize that these two mechanisms act coordinately (reviewed by Ciafrè and Galardi, 2013).

In HeLa S3 cancer cells, human Pums motifs are enriched around predicted miRNA binding sites and that high-confidence miRNA binding sites are significantly enriched in the 3'-UTRs of experimentally determined pum1 and Pum2 targets (Galgano et al., 2008). This suggest an interplay between PUF proteins and miRNA post-transcriptional regulatory systems. For instance, it was investigated by Kedde and coworkers that binding of Pum1 and Pum2 to 3'UTR of *p27* mRNA induce a conformational change that uncovers a miR-221 and miR-222-binding site. As a consequence, miR-221 and miR-222 can easily interact with *p27* target site at 3' UTR and mediate repression of the *p27* tumor suppressor (Kedde et al., 2010).

ii. Translational Activation

Some findings have suggested that PUFs can activate mRNA translation process to increase protein production, however, the mechanisms of PUF-dependent activation are poorly defined (reviewed by Quenault et al., 2011). Enhancement of polyadenylation process, which is considered a key step for translational activation, is a possible mechanism. For instance, *C. elegans* FBF interacts with GLD-1 mRNA, where GLD-1 is a protein required for regulation of mitosis and meiosis division during germline development (Suh et al., 2009). As a result, the cytoplasmic poly(A) polymerase GLD-2 and GLD-3 are recruited to FBF/GLD-1 complex leading to poly(A) tail elongation and translational activation (Suh et al., 2009), (figure 22).

Recently, it was shown that FOXP1 levels are higher in human HSCs exhibited high Pum1 and Pum2 levels, and its expression intensely decreased by knockout of Pum1 and Pum2 (Naudin et al.,

2017). The increase in FOXP1 level in HSPCs is related to positively regulation of FOXP1 mRNA expression by Pum1 and Pum2 via two PBEs located in 3'UTR of FOXP1 transcript.

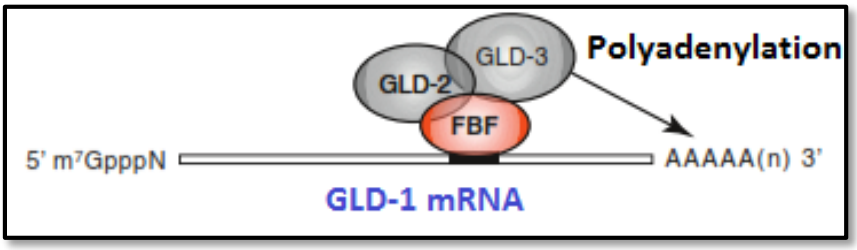


Figure 22: mRNA activation by PUF proteins (Quenault et al., 2011)

5. Human Pums and physiological functions

As posttranscriptional mRNA expression regulators, PUF proteins play a critical role in diverse functions, including differentiation, germline maintenance, embryogenesis, cell proliferation, organelle biogenesis, neurological processes and memory formation (reviewed by Wickens et al., 2002). In human, much less is known about the biological roles and regulatory functions of Pums due to the scarcity of information about the genes functionally regulated by human Pums.

For instance, human pum2 is expressed in human embryonic stem cells and germ cells, and has a role in germ cell development through formation a stable complex with deleted in azoospermia (DAZ) and DAZ-like (DAZL) proteins, RBPs required for germ cell development (Moore et al., 2003). It was found that Pum2 downregulates the expression of MAPK1 (or ERK2) and MAPK14 in human embryonic stem cells. MAPK1 and MAPK14 are kinases acting in the MAPK/ERK pathway that represses stem cell self-renewal and regulates programmed cell death. So, the negative regulation of MAPK/ERK pathway by Pum2 may propose a mechanism for tumor progression (Lee et al., 2007).

Further, it was reported that human Pum2 mRNA is expressed in both cell body and dendritic compartment of neurons during neuronal development, and it may contribute to neuronal morphology and synaptic responses through formation of stress granules (Vessey et al., 2006).

6. Human Pums and HSCs

Indeed, several strides have been made to understand the regulatory mechanisms responsible for HSCs and progenitor cells development, however, the role of RBPs and posttranscriptional regulation of gene expression in regulation of HSCs and their progenitor cells remains poorly studied. Commonly, homeobox (HOX) transcription factors are considered as important regulators of human HSCs proliferation and differentiation, where homeobox B4 (HOXB4) homeoprotein appears to be the major positive regulator in this family because its overexpression obtains a significant *in vivo* and *ex vivo* expansion of HSCs without functional impairment (Antonchuk et al., 2001, 2002). Moreover, it was reported that the HOXB4 upregulates expression of Pum1/2 in human and murine HSCs suggesting that human Pums are involved in HSCs expansion (Auvray et al., 2012).

Recently, it was demonstrated that FOXP1 play a vital role in hematopoiesis process by downregulation of two cell cycle inhibitors, p21 and p27, where FOXP1 knockout in HSPCs is associated with inhibition of $CD34^{+}$ cell expansion followed by enhanced cell death (Naudin et al., 2017). PFOXP1 is a transcription factor related to FOXP subfamily, which regulate a large number of genes involved in cell proliferation, differentiation and development. Interestingly, it was shown that FOXP1 levels are higher in human HSCs exhibited high Pum1 and Pum2 levels, and its expression strongly reduced by Pum1 and Pum2 knockout (Naudin et al., 2017). The increase in FOXP1 level in HSPCs is related to positively regulation of FOXP1 mRNA expression by Pum1 and Pum2. In order to absolutely decide the biological relationship between FOXP1 and Pum1/2, FOXP1 was constitutively expressed in Pum1/2 knockdown HSPCs, where exogenous FOXP1 overturned growth defects and apoptosis of Pum1 or Pum2 KD HSPCs. Together, these findings demonstrate that human Pums are essential for HSPCs proliferation and survival (Naudin et al., 2017).

7. Human Pums and cancer

Human Pums can negatively regulate the translation of hundreds mRNAs including oncogenes and tumor suppressor genes (Galgano et al., 2008). However, some tumors have the capability to bypass the undesirably mRNA posttranscriptional control of these proteins to enhance their growth and survival. For example, Bladder and prostate cancers are characterized by overexpression of E2F3 gene, a target suppressed by human Pum1 and Pum2, and this overexpression of E2F3 is associated with increased tumor proliferation and aggressiveness (Olsson et al., 2007). Interestingly, Dyson

laboratory revealed that the inhibitory regulatory effect of human Pums on E2F3 is circumvented in bladder cancer cells through downregulation of miRNAs (503 and 125b) that cooperate with Pum1 and Pum2 to negatively target E2F3, and by shortening the 3' UTR of E2F3 mRNA which consequently removes PRE (Miles et al., 2012).

Additionally, they also verified in 2016 that the posttranscriptional suppressive regulation of human Pum1 and Pum2 is evaded in triple negative breast cancer by alternative polyadenylation process for mRNAs of their targets NRas and c-Jun (Miles et al., 2016). Alternative polyadenylation (APA) is a process that alters mRNA stability and translation potential by addition or deletion regulatory elements at 3' UTR. c-Jun is a protein that homodimerizes with c-Fos protein to form activator protein 1 (AP-1) which mediates genes expression contributing with cell proliferation, differentiation, and transformation (Shaulian and Karin, 2001). NRas is a protein with intrinsic GTPase activity that is involved in transmitting signals from outside the cell to the nucleus to enhance transcription of genes involved in cell growth and division, and to inhibit apoptosis-mediated genes. They found that mRNAs of NRas and c-Jun are targets of PUM-mediated repression, where knockdown of Pum1 or Pum2 in triple-negative breast cancer cells increases NRas and c-Jun levels (Miles et al., 2016). Furthermore, the results show an overexpression of polyadenylation factor called cleavage stimulation factor (CSTF) which enhances APA process, causing shortening of 3' UTR of NRas and c-Jun mRNAs and losing their PREs. As a consequence of bypassing the repression effect mediated by Pum1 and Pum2, the functional activity of NRas and c-Jun proteins is abnormally enhanced to change the expression of their downstream targets genes.

On the other hand, our team has compared the gene expression of NK cells from AML patients with NK cells from healthy individuals. Among the selected genes, Pum2 gene was highly upregulated in NK cells from AML patients.

Newly, the expression of Pum1 and Pum2 is significantly increased in primary human AML cells and AML cell lines compared with healthy $CD34^{+}$ cells (Naudin et al., 2017). Impressively, reducing the expression of Pum1 and Pum2 in human AML cells and AML cell lines is linked with repression of cell proliferation and induction of apoptosis later (Naudin et al., 2017). Moreover, lower FOXP1 level is confirmed in AML cell lines with Pum1 or Pum2 knockdown and the level is conversely higher in human AML cells showed high Pum1 and Pum2 levels. Altogether, these results indicate that

FOXP1 expression is correlated with human Pum1 and Pum2 levels in AML cells. Collectively, these findings demonstrate that both human Pum1 and Pum2 are important regulators of myeloid leukemia cell growth and survival (Naudin et al., 2017).

OBJECTIVES

As mentioned in the introduction part, Pums can negatively regulate the translation of hundreds mRNAs including oncogenes and tumor suppressor genes (Galgano et al., 2008). However, some tumors have the capability to bypass the undesirably mRNA posttranscriptional control of these proteins to enhance their growth and survival. Moreover, cancerous cells can develop immunosuppressive mechanisms to escape immune surveillance.

My team and others have found that NK cells from AML patients have impaired anti-leukemic activity (Costello et al., 2002; De Maria et al., 2003; Fauriat et al., 2007; Stringaris et al., 2014; Venton et al., 2016). Until now, the molecular mechanisms involved in the alterations of anti-leukemic activity of NK cells from AML patients are not defined. So, there is a persistent need to understand how AML escapes the natural defenses of immune system. Our team is mainly interested in NK cell biology. Many projects are developed to study NK cells in AML at diagnosis and during clinical evolution. In this project, we found that Pum2 gene was highly up-regulated in NK cells from AML patients.

My work on this project aims to study the role of Pum2 in NK cell biology. In this context, we will:

- Identify the genes regulated by Pum2 in NK cells.
- Assess impact of Pum2 on the phenotype and functional activity of NK cells.
- Evaluate effect of Pum2 on the anti-tumor activity of NK cells *in vivo.*

MATERIALS AND METHODS

Cell lines and cell culture

NK-92 and KHYG-1 are human IL-2 dependent NK cell lines derived from a patient with non-Hodgkin's lymphoma and aggressive NK leukemia, respectively (Gong et al., 1994; Yagita et al., 2000). The cells were maintained in RPMI 1640 medium (Life technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 100 µg/ml penicillin, 100 µg/ml streptomycin (all supplements from Life Technologies), and IL-2 (100 U/mL for NK-92, 450 U/ml for KHYG-1) at 37°C in a humidified atmosphere containing 5% CO_2. Erythroleukemia cell line K562, K562 HLA-E and Raji cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. HEK293 (human embryonic kidney 293) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% heat-inactivated FCS.

Immunoprecipitation (IP)

The ribonucleoprotein-immunoprecipitation was performed depending on a published protocol. NK92 and KHYG1 cells were grown in complete culture medium with IL-2 addition, washed in PBS and collected by centrifugation at 1500 rpm for 5 min. The cells ($10x10^7$ cells) were resuspended in an equal volume of polysome lysis buffer (PLB). Protein concentration was determined by the Bradford method. Protein A sepharose beads (Amersham) were equilibrated in NT2-coupling buffer and then 50 mg of rabbit anti-Pum2 antibody was coupled to the beads, which were further incubated overnight at 4°C. Afterward, the beads were washed and resuspended in NT2-RIP buffer. The total protein extract was added to the antibody-coupled beads, and mixed for 6 hours at 4°C. Finally, the beads were thoroughly washed in ice-cold NT2 buffer and Pum2-mRNA complexes were eluted with SDS-EDTA for 10 min at 65°C.

Plasmids and lentivirus production

Because we expected that PUM2 protein may have a long half-life in cells, we excluded a transient knock-down of PUM genes. In addition, the mechanisms of action of PUM proteins, acting by repression of mRNA translation in a microRNA manner, we also excluded a si/shRNA approach.

Therefore, we used LentiCRISPR/Cas9 technique to increase the efficacy and maximize our results. In brief, three sgRNA sequences were purchased from Thermo Scientific and used against human Pum1: sgPum1 #1 (forward primer: 5'-CACCGGCTGCTCATCAGCCGCACAT-3' and reverse primer: 5'-AAACATGTGCGGCTGATGAGCAGCC-3'); sgPum1 #2 (forward primer: 5'-CACCGCGCGCCTGCATTCACTACAA-3' and reverse primer: 5'- AAACTTGTAGTGAATGCAGGCGCGC-3'); and sgPum1 #3 (forward primer: 5'- CACCGTCTTCCTCTCGTTTGCGATA-3' and reverse primer: 5'-AAACTATCGCAAACGAGAGGAAGAC-3'). Also, three sgRNA sequences were used against human Pum2: sgPum2 #1 (forward primer: 5'-CACCGGCTCAGCAGCCACATATAGC-3' and reverse primer: 5'-AAACGCTATATGTGGCTGCTGAGCC-3'); sgPum2 #2 (forward primer: 5'-CACCGCATGGAACGCCGTAATACTG-3' and reverse primer: 5'- AAACCAGTATTACGGCGTTCCATGC-3'); and sgPum2 #3 (forward primer: 5'- CACCGGCGTCCTCTTACTCCCAATC-3' and reverse primer: 5'-AAACGATTGGGAGTAAGAGGACGCC-3'). All the selected sgRNA sequences were cloned into BsmBI digested LentiCRISPRv2 vector (Addgene plasmid #52961) as previously described (Sanjana et al., 2014). For the negative control plasmid, no sgRNA sequence was inserted into the construct. The positive clones were validated with sequencing using LentiCRISPRv2-sgRNA-sequencing primer: 5'-GTACAAAATACGTGACG-3'. Lentiviruses were produced in collaboration with International Institute of Molecular and Cell Biology, Laboratory of Cell Biology (Warsaw-Poland) by using HEK293 cell line which were transfected with lentiCRISPRv2 clones, psPAX2 (packaging plasmid, Addgene #12260) and pMD2.G (envelope expressing plasmid, Addgene plasmid #12259) according to manufacturer's instructions.

Cell transduction

For transduction of NK-92 cell lines, 3×10^5 cells per well were seeded into 24-well plates in 500µl of culture medium supplemented with IL-2 (final concentration: 200U/ml), BX-795 (final concentration: 6µM BX-795) and protamine sulfate (final concentration: 15µg/ml). The cells were transduced with sgPum1 and sgPum2 lentivirus at MOI: 10, where the total volume was adjusted to 1 ml. The plates were centrifuged at 750 rpm for 1 hour at RT followed by incubation at 37°C, 5% CO_2 overnight. Day 2: the virus containing supernatant was removed and the cells were resuspended in 500µl fresh culture medium supplemented with IL-2, BX-795 and protamine sulfate at the same concentrations. Then, the lentiviruses were added as described at day 1. Day 3, the virus containing supernatant was removed, and 1ml fresh growth medium supplemented with IL-2 (final

concentration: 200U/ml) was added, followed by incubation at 37°C, 5% CO_2 overnight. Day 4: the transduced cells were sorted by puromycin selection (final concentration: 3µg/ml) for 4 days, followed by single-cell cloning.

Western blot analysis

NK92 cells were harvested, washed with PBS and lysed in 1% NP-40 lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1mM Na3VO4, 0.1% SDS, and protease inhibitor cocktail [Roche# 11836170001]) at 4°C. After 10 min of incubation with lysis buffer, the lysate was centrifuged at 13000 rpm for 15 min at 4°C. Samples of total protein lysate (50µg) were prepared in 5x loading dye, denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to PVDF membranes (Immobilon-P, Millipore), where nonspecific binding was blocked by incubating the membranes in PBS containing 5% bovine serum albumin (BSA) for 30min at RT. After that, the membranes containing the resolved proteins were incubated with the primary antibody of interest in 5% BSA in TBS-T 0.1% overnight at 4C°. After washing, the membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. The bands were detected by enhanced western blot chemiluminescence detection reagent (West Pico, Thermo Scientific), and the signals were quantified using the ImageJ software. The following primary antibodies were used: anti-STAT5 antibody (Santa Cruz Biotechnology, SC-835). Anti-Pum1 (A300-201A), anti-Pum2 (A300-202A) antibodies were purchased from Bethyl Laboratories, Inc. Anti-Erk1/2 (#9102), anti-phospho-Erk1/2 (S473, #4377), anti-phospho-STAT5 (Y694, #9351), anti-Akt (#9272), and anti-phospho-Akt (#9271) antibodies were purchased from Cell Signaling Technology (CST). Anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG secondary antibodies were purchased from Jackson ImmunoResearch.

Antibodies and flow cytometry

NK cell activating and inhibitory receptors expression was analyzed on NK92 cells by flow cytometry. Cells were processed and immunostained with fluorochrome-labeled antibodies and viability dye (LIVE/DEAD Fixable Dead Cell Stain Kit, Life Technologies) at 4°C for 20 min. The following conjugated anti-human antibodies were used for flow cytometry analysis: anti-CD56 (PE-Vio770, clone: AF12-7H3) and anti-NKp30 (PE, clone: AF29-4D12) antibodies were purchased from Miltenyi Biotec. Anti-NKp44 (PE, clone: Z231), anti-NKp46 (PE, clone: BAB281), anti-NKG2D (PE,

clone: ON72), anti-CD69 (PE, clone: TP1.55.3) and anti-NKG2A (Pacific blue, clone: Z199) antibodies were purchased from Beckman Coulter. Anti-DNAM-1 (PE, clone: DX11) antibody was purchased from BD Biosciences. FACS analysis was performed in the IPC immunomonitoring platform using a FACS LSR Fortessa (BD Biosciences) and FlowJo v10 software (LLC, Ashland, Oregon).

Effector functions

Effector functions of NK92 cell were evaluated by flow cytometry. Cytokines production and degranulation capacity (CD107a expression) were determined by stimulating NK cells with the K562 and K562 HLA-E cells as previously described in a published protocol (Bryceson et al., 2010). Briefly, NK92 cells ($2.5x10^5$) were incubated with K562 cells (ratio 1:1) at 37°C and 5% CO_2 for 4 hours in the presence of GolgiPlug® (BD Biosciences, #555029) and FITC-conjugated anti-CD107a (BD Biosciences, clone H4A3). After 4 hours of incubation, the cells were collected, washed and immunostained for surface markers with fluorochrome-labeled antibodies (anti-CD56 PE-Vio770, and anti-CD107a FITC). For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm® kit (BD Biosciences) according to the manufacturer's instructions, and immunostained with fluorochrome-conjugated anti-cytokine antibodies: anti-IFN-γ AF700 (BD Biosciences, clone: B27) and anti-TNF-α ef450 (eBioscience, clone: MAb11). The cells were finally prepared for flow cytometry analysis using a FACS LSR Fortessa (BD Biosciences) and FlowJo v10 software (LLC, Ashland, Oregon).

Cell stimulation

NK-92 cells were rested overnight in complete culture medium without IL-2 addition. For NKp30 stimulation, rested NK-92 cells were incubated for 15 min at 4°C with biotinylated anti-Nkp30 (Biolegend, #325206). Cells were washed and stimulated at 37°C for the indicated time by adding streptavidin (20µg/ml final concentration). For IL-2 stimulation, rested NK-92 cells were stimulated with 1000 U/ml of IL-2 at 37°C for the indicated time. Then, stimulated cells were lysed at 4°C for 10min in 1% NP-40 lysis buffer and the lysates were prepared for western blot analysis. Detailed protocol and the corresponding primary antibody were mentioned in western blot analysis section.

Statistical analysis

Graphics were generated with Graphpad Prism software (San Diego, CA, USA), and Microsoft Excel 2013. Data were presented as mean ± SEM. Statistical analyses were performed using unpaired Student's *t* test (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

The results referred to in this section concern NK92 cells because we did not success to make knockout (KO) of Pum1 or/and Pum2 in KHYG1 cells. Moreover, we succeed to obtain 2 clones of NK92-Pum1KO and 4 clones of NK92-Pum2KO. The data presented below concern one clone for pum1 and pum2 and are representative of all clones obtained.

Insufficient quantity and quality of mRNA bound with Pum2 following immunoprecipitation

Pum2 protein was detected in primary NK cells and human NK-cell lines (NK92, KHYK1) by immunoblotting. After that, we sought to isolate mRNA bound with Pum2 to identify the target genes regulated by Pum2. Pum2-mRNA complexes were immunoprecipitated based on a published protocol (Galgano and Gerber, 2011). By western blot, we found that most of Pum2-mRNA complexes were precipitated from the total protein extract of NK92 and KHYG1 cells (Figure 1A). Next, we isolated mRNA from the complexes for microarray analysis. We found that both quality (260/230 ratio < 0.5) and quantity (10-40ng) of isolated mRNA were insufficient for microarray analysis (Figure 1B). To improve the quality and quantity of mRNA, we used 3 different kits: mirVana™ PARIS kit (Ambion®), µMACS™ One-Step cDNA Kit (Miltenyi Biotec), and Trizol®. Unfortunately, no improvement was achieved either in quality or quantity of the isolated mRNA.

Pum 2 gene expression is induced upon NK cell stimulation and in NK cell from AML patients

We sought to recognize the role of Pum2 protein in NK cell biology by assessing its expression and the consequences of its deletion. Firstly, we evaluated the expression of Pum2 gene in NK cells from AML patients. We found that Pum2 gene was highly expressed in NK cells from AML patients (Figure 2A). Moreover, we tested the expression of Pum2 gene in NK cells of healthy volunteers at different conditions of stimulation. We observed an up-regulation of Pum2 gene after stimulation of primary NK cells from healthy donors (Figure 2B). These findings suggest that the overexpression of Pum2 in NK cells is linked to their activation. We believed that these preliminary findings provided a good rationale for the study of PUM2 in NK cells as potential and partial mechanism of AML-NK defects. We used Lenti-CRISPR/Cas9 technique to generate Pum2KO cells in NK92 cell line to explore the role of Pum2 expression in NK cells (Figure 2C).

Effect of Pum2 on the expression of activating and inhibitory receptors of NK cells

After obtaining several clones of NK92-Pum1KO and NK92-Pum2KO, we evaluated the expression of activating receptors (CD69, NKG2D, DNAM-1, NKp30, NKp44, and NKp46) and NKG2A inhibitory receptor on NK92WT, NK92-transduced with empty vector (NK92-EV), NK92-Pum1KO and NK92-Pum2KO cells using flow cytometric analysis (Figure 3A-D). Regarding the expression of NCR (NKp30, NKp44, NKp46), we found a significant increase in the MFI expression of NKp30 and NKp44 on NK92-Pum1KO and NK92-Pum2KO cells compared with NK92WT and NK92-EV cells, while no difference was observed in the expression of NKp46 receptor (Figure 3B). For NKG2D and CD69 receptors, both Pum1KO and Pum2KO cells showed a significant boost in the MFI of their expression compared with other NK92 cells, whereas no change in DNAM-1 expression was noticed between the different NK92 cells (Figure 3C). Concerning the inhibitory receptor NKG2A, we found a decrease in its expression within NK92-Pum1KO ($p = > 0.05$) and NK92-Pum2KO ($p = \leq 0.05, <.001$ respectively) cells compared with NK92WT and NK92EV cells (Figure 3D). Together, these findings suggest that Pum2 protein might decrease the expression of some activating receptors and increase NKG2A inhibitory receptor expression in NK cells.

Pum2 knockout increases degranulation level as well as cytokine secretion

NK cell functions are regulated by the expression of numerous inhibitory and activating receptors which bind to ligands on healthy or transformed cells (Caligiuri, 2008; Moretta et al., 2005; Vivier et al., 2008). As a consequence of overexpression of some activating receptors in NK92 Pum1&2KO cells, we hypothesized that these cells would have a better effector functions against their target cells (K562). Moreover, we expected an improvement in the effector functions of NK92 Pum1&2KO cells against K562HLA-E target cells (target cells which express HLA-E, the ligand of NKG2A receptor) because our results revealed a decrease in NKG2A inhibitory receptor expression. We tested the ability of NK92 cells (WT, Pum1KO and Pum2KO) to produce IFN-Y and TNF-α as well as their degranulation level by measuring CD107a expression in response to stimulation with target cells (K562 and K562HLA-E) (Bjorkstrom et al., 2010; Bryceson et al., 2010). Our findings showed a significant increase in CD107a expression among NK92-Pum2KO cells in response to stimulation with K562 ($p=<0.05$ in both) as well as K562HLA-E cells ($p=<0.001$ in both) compared with NK92WT and NK92EV cells (Figure 4B). Although the increase in the frequency of $CD107a^{+}$ NK92-Pum1KO cells, the increase was significant only in response to K562HLA-E cells compared with NK92WT and

NK92EV cells (p= <0.05 in both) (Figure 4B). With respect to cytokine secretion, we showed a significant increase in the frequency of INF-γ^+ NK92-Pum2KO cells following stimulation with K562 (p=<0.05 in both) as well as K562HLA-E cells (p=<0.01 in both) compared with NK92WT and NK92EV cells, whereas the frequency of INF-γ^+ NK92-Pum1KO cells was significantly higher in response to K562HLA-E cells (p=<0.05 in both) (Figure 4C). Concerning TNF-α, we observed an increase in its secretion by NK92-Pum1KO and NK92-Pum2KO cells as a result of K562 stimulation, but the increase was significant (p=<0.01 in both) only with NK92-Pum2KO cells compared with NK92WT and NK92EV cells (Figure 4D). In the case of K562HLA-E stimulation, the frequencies of TNF-α^+ NK92-Pum1KO and NK92-Pum2KO were insignificantly higher than that of NK92WT and NK92EV cells (Figure 4D). From these findings, it appears that Pum2 absence is associated with improving cytokine secretion as well as degranulation capacity of NK cells in response to target cell stimulation.

Pum2 negatively regulates the NK-cell signaling pathway triggered by IL-2 and NKp30

We next sought to evaluate the effect of Pum2 knockout on signaling pathways of NK cells: IL-2 and NKp30 activation pathways. NK92 cells (WT, Pum1KO and Pum2KO) were stimulated for 2 and 5 minutes with IL-2 (50IU/ml) and NKp30 mAb (10µg/ml), separately. After that, lysates from NK92 cell subsets were blotted to check the expression of p-Stat5, p-Erk1/2 and p-Akt by western blot. We found a high level of p-Stat5 among NK92-Pum2KO cells after 5 min of IL-2 stimulation compared to WT and Pum1KO cells (Figure 5A). Also, the level of Erk1/2 and Akt phosphorylation was increased in NK92-Pum2KO cells after 2 min of NKp30 mAb stimulation (Figure 5B, C), respectively. These results suggest that Pum2 expression can diminish these signaling pathways of NK cells. These data require further investigation to verify whether the phosphorylation of Erk1/2 is due to the increase of NKp30 expression and if the intrinsic signaling machinery is partly controlled by Pum2 associated protein, especially since with regard to IL-2 signaling, we did not notice changes in IL-2 receptor expression.

Figures

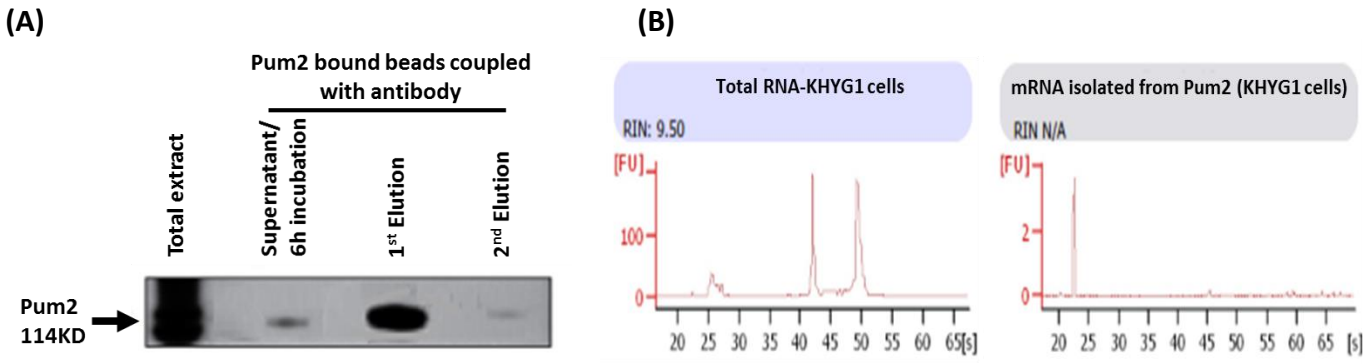


Figure 1: Extraction of mRNA bound with Pum2 protein after immunoprecipitation. Total protein extract was incubated with beads coupled with Pum2 specific antibody for 6h. Then Pum2-mRNA complexes were separated from the beads. mRNA was isolated from Pum2 and analyzed by Agilent. A) Western blot showing Pum2 after immunoprecipitation and following elution from the beads. B) RNA electropherogram showing RNA integrity number (RIN) of total RNA and mRNA isolated from Pum2 protein.

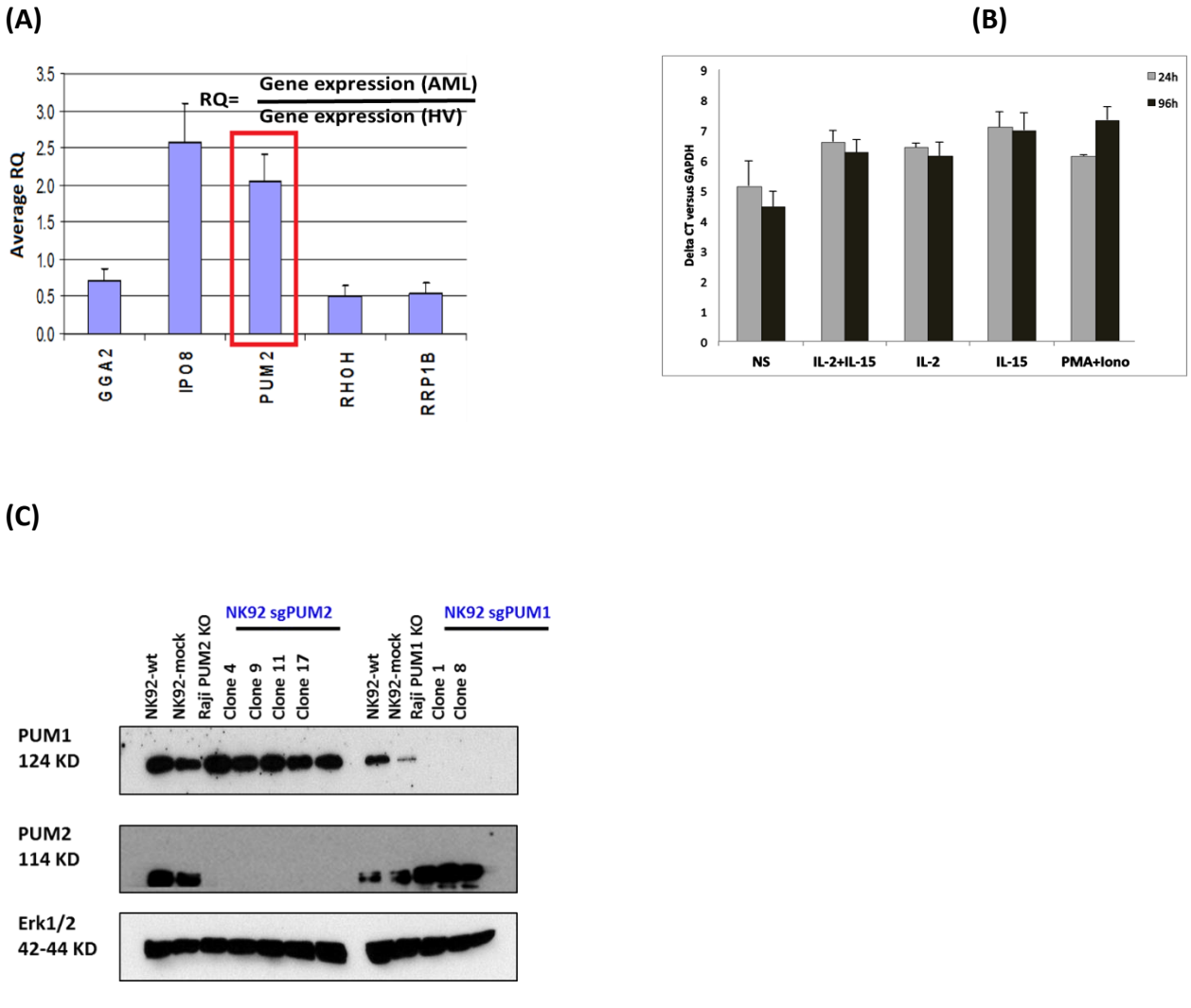
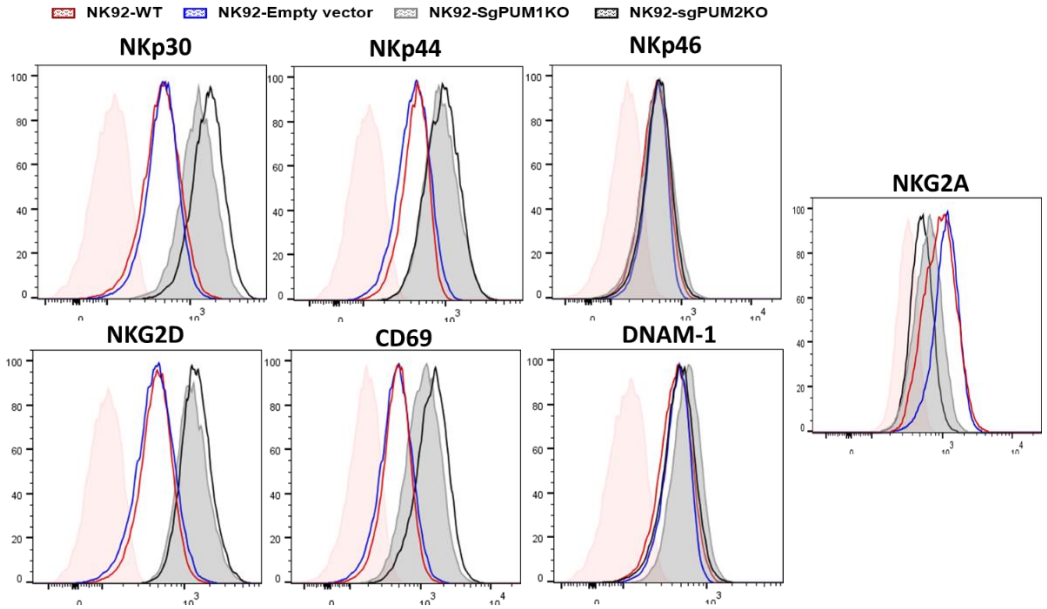


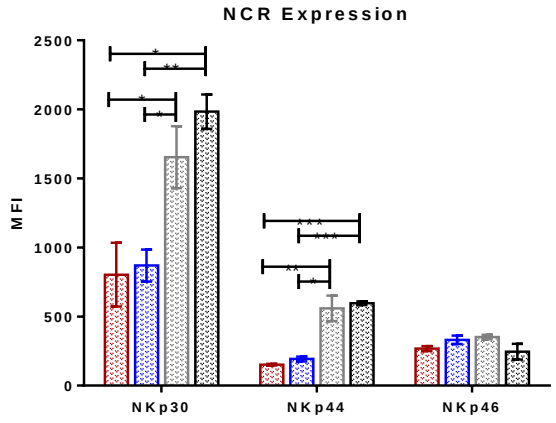
Figure 2: NK cell activation was associated with overexpression of Pum2. (A) Real-time PCR was used to evaluate the expression of Pum2 gene in NK cells from AML patients and healthy donors. (B) Expression of Pum2 gene in primary NK cells from healthy donors after 24 and 96 h of activation with different conditions (1000IU/ml IL-2, 15ng/ml IL-15, 1µg/ml PMA, 1µg/ml Ionomycine). (C) Western blot showing the expression of Pum1 (124KD) and Pum2 (114KD) proteins in several clones of sgPum1 and sgPum2 NK92 cells after sorting with puromycin (3µg/ml), where NK92 WT is the positive control while sgPum1 and sgPum2 Raji cells are the negative control (KO) for Pum1 and Pum2, respectively.

(A)

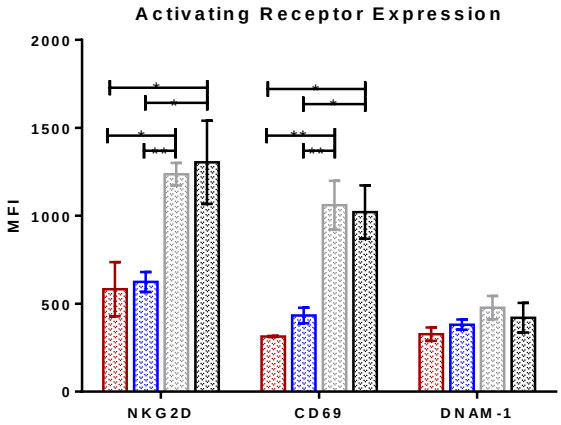

NK92-WT
NK92-Empty vector
NK92-SgPUM1KO
NK92-sgPUM2KO
NKp30
NKp44
NKp46
NKG2A
NKG2D
CD69
DNAM-1


NK92-WT
NK92-Empty vector
NK92-SgPUM1KO
NK92-sgPUM2KO

(B)


NCR Expression
MFI
2500
2000
1500
1000
500
0
NKp30
NKp44
NKp46

(C)


Activating Receptor Expression
MFI
2000
1500
1000
500
0
NKG2D
CD69
DNAM-1

(D)

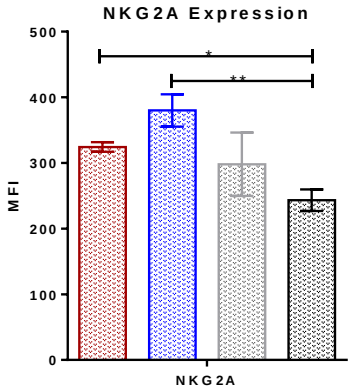


Figure 3: Pum2 deletion enhances the expression of NK activating receptors and reduces the expression of NKG2A inhibitory receptor. NK92 cells were stained with specific mAbs for activating and inhibitory receptors and analyzed by flow cytometry. (A) Representative histogram profiles for each receptor expressed on NK92WT (red line), NK92-EV (blue line), NK92-Pum1KO (gray filled histogram), and NK92-Pum2KO (black line). Isotype-matched negative controls are shown as red filled histograms. (B-D) MFI of NCRs (B), MFI of NKG2D, CD69 and DNAM-1 (C), and MFI of NKG2A (D). Values are shown as mean ± SEM. *n*= 3; three independent experiments. Statistical analyses were performed using unpaired Student's *t* test (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$).

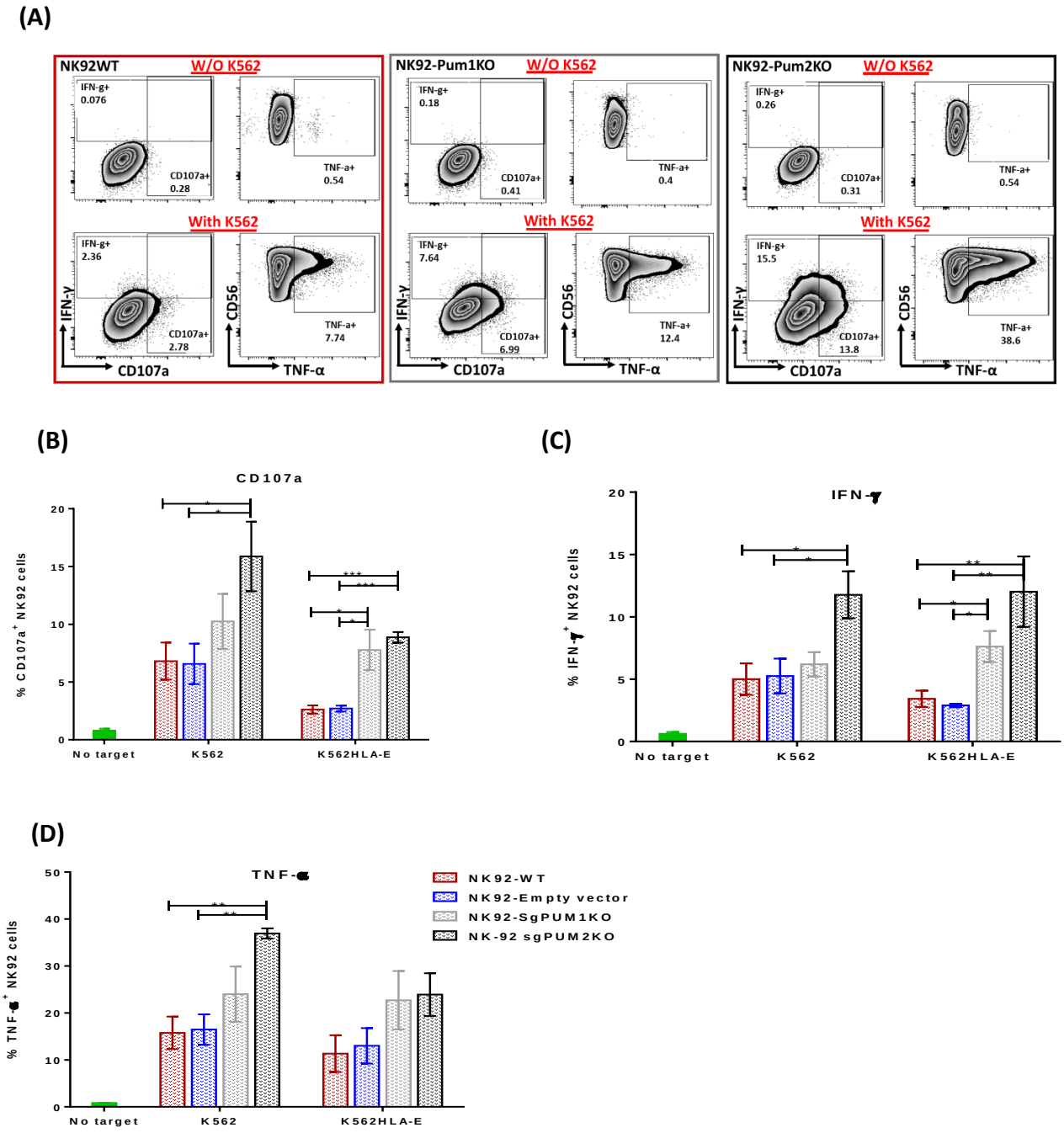
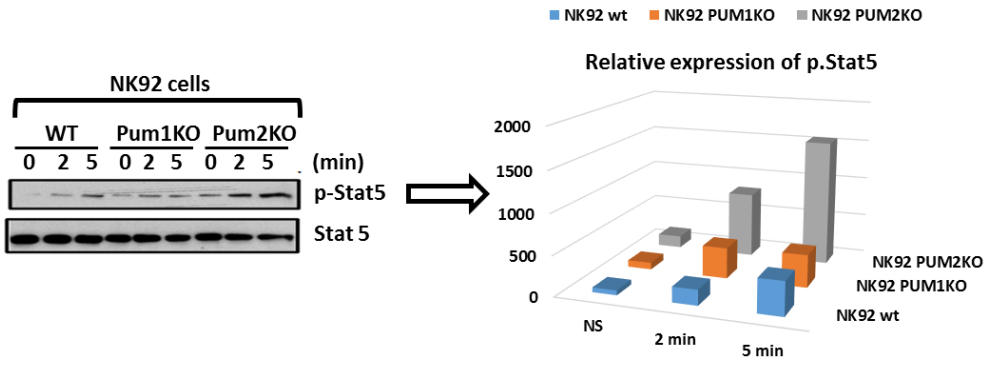
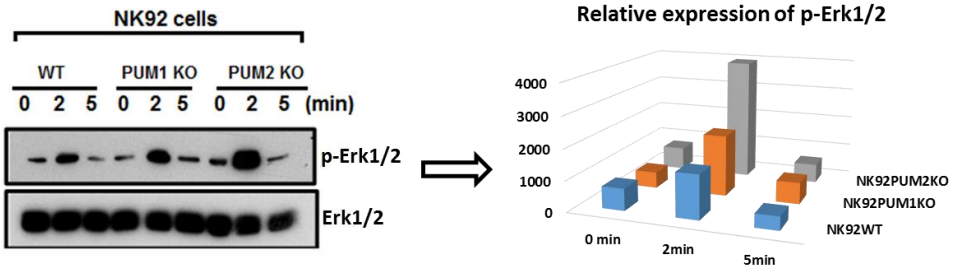


Figure 4: Pum2 deletion enhances degranulation level as well as cytokine secretion in response to target cell stimulation. NK92 cells (WT, EV, Pum1KO and Pum2KO) were co-cultured with K562 and K562 HLA-E (E/T ratio was 1:1) for 4 hours. After stimulation, the cells were stained for surface markers, intracellular cytokines (IFN-Y and TNF-α), and analyzed by flow cytometry. (A) Representative flow cytometry plots showing frequencies of $CD107a^+$, $INF\text{-}\gamma^+$ and $TNF\text{-}\alpha^+$ cells in NK92WT cell (red box), NK92-Pum1KO cells (gray box) and NK92-Pum2 KO cells (black box). (B-D) Frequencies (mean ± SEM) of $CD107a^+$ cells (B), $INF\text{-}\gamma^+$ cells (C) and $TNF\text{-}\alpha^+$ cells (D) among different NK92 subsets. *n*= 3; three independent experiments. Statistical analyses were performed using unpaired Student's *t* test (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(A)



(B)



(C)

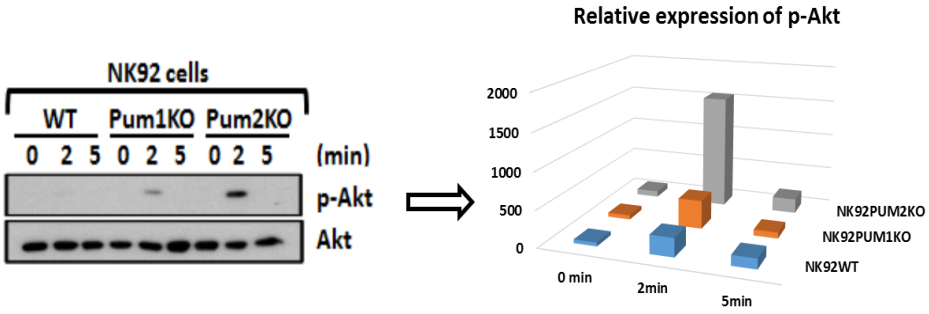


Figure 5: Pum2 inhibits IL-2 and NKp30 signaling pathways of NK cell. NK92 cells (WT, Pum1KO and Pum2KO) were stimulated with IL2 (50IU/ml) and NKp30 mAb (10µg/ml) for 2 and 5 minutes. A, B and C *(left)*: Western blot analysis of p-Stat5, Stat-5, p-Erk1/2, Erk1/2, p-Akt and Akt expression after stimulation of NK92 subsets at the indicated times. A, B and C *(right)*: Band intensity of western blot results was quantified using imageJ software. The relative expression of p-Stat5, p-Erk1/2 and p-Akt was calculated based on the expression of Stat5, Erk1/2 and Akt (loading controls), respectively.

DISCUSSION AND PERSPECTIVES

Although NK cells play a major role in immunosurveillance against AML cells, it was demonstrated by our team and others that NK cells in AML patients have impaired anti-leukemic activity (Costello et al., 2002; De Maria et al., 2003; Fauriat et al., 2007; Stringaris et al., 2014; Venton et al., 2016). However, specific molecular mechanisms involved in these alterations are still not well defined. Performing a microarray analysis, we have found a high expression of Pum2 gene in NK cells from AML patients. Since little was known about the function of Pum2, especially in NK cells, we hypothesized that its RNA translation repressor function may affect the biology of NK cells notably when overexpressed in AML patients. We first verified that Pum2 protein was expressed by human NK cells from healthy volunteers and found it expressed and even upregulated after activation. We first aim to identify RNA targets of Pum2 by immunoprecipitation and Pum2-bound RNA isolation, however, due to technical issues, we did not able to progress in this approach. Therefore, we decided to generate human NK-cell lines (NK92, KHYG1) deficient for Pum2 in order to study the impact of the deletion on NK cell phenotype, function and gene expression. Because these cell lines are hard to transfect, it took us almost 2 years to get NK92Pum2KO clones. Although still preliminary, our data showed an increase in degranulation level, cytokine production and signaling pathways in Pum2 deficient cells. This suggests that Pum2 protein negatively affects NK cells in the context of phenotype and functional activity.

Hence, we found that knocking out Pum2 protein resulted in upregulation of NK cell activating receptors as well as a decreased NKG2A inhibitory receptor expression. The impact of Pum2 deletion on NK cell functions was also tested and we showed an increase in degranulation capacity and cytokine production among Pum2KO cells in response to activation with target cells. We don't know if this improvement of functional activity of Pum2KO cells was related to upregulation of activating receptors as well as downregulation of NKG2A inhibitory receptor or signaling pathways from these receptors are controlled by Pum2 protein. In this context, we evaluated the impact of knocking out Pum2 on NK cell activation induced by NKp30 signaling pathway. Interestingly, the level of NKp30-induced Erk1/2 and Akt phosphorylation was highly increased in Pum2KO cells. In parallel, we assessed NK-cell activation induced by IL-2 in WT and Pum2KO cells. We found that the level of Stat5

phosphorylation was increased in the absence of Pum2 protein. In light of these observations, it would be interesting to investigate the potential negative role of Pum2 in NK-cell signaling pathways below NKRs (NKp30) and cytokine receptors (IL-2). Based on Galgano et al. findings (2008), Pum2 targets different signaling molecules in HeLa S3 cells (Cervical cell line) including Mitogen-activated protein kinase (MAPK), A-RAF (Serine/threonine-protein kinase A-Raf) and PIK3 (phosphatidylinositide 3-kinase) (Galgano et al., 2008). Together, these findings propose that Pum2 protein might target one or more molecule(s) involved in signaling pathways of NK cells. So, more experiments are required to study others signaling molecules involved in NK signaling pathways.

In general, this potential negative feedback is common in lymphocytes. For instance, it has been shown that overexpression of Dok1/2 (downstream of tyrosine kinase) proteins in mice and human NK cells reduces cell activation induced by NK-cell-activating receptors (Celis-Gutierrez et al., 2014). In T lymphocytes, a SOCS family protein CISH (cytokine inducible SH2 containing protein) has been recently described as a repressor of T cell activation (Guittard et al., 2018). In both cases, the absence of Dok1/2 or CISH resulted in an enhanced effector functions and anti-tumor activity of lymphocytes.

Based on the findings of Galgano *et al.* showing that the mRNA targets of Pum2 in HeLa S3 cells are pleiotropic and are involved in multiple cellular processes such as cell proliferation and differentiation, metabolism, cytokine response, intracellular protein traffic, and intracellular signaling cascade (Galgano et al., 2008), it might be possible that the activity of Pum2 protein in NK cells affects several physiological processes. Therefore the alteration Pum2 in AML-NK might affect broad processes that in turn reduced effector functions. In this context, we tried to identify Pum2 targets in NK cells by microarray analysis for mRNA bound with Pum2. Unfortunately, we were not able to identify the genes regulated by Pum2 protein because the quantity of mRNA isolated from Pum2 by immunoprecipitation method was low with insufficient quality. So, to recognize the genes regulated by Pum2 in NK cell, a comparative gene expression between mock-transfected NK92 (wt) and Pum2KO cells may be an alternative.

Another justification supporting our hypothesis of the repressive role of Pum2 in NK cells is the findings of Mundy-bosse and co-workers indicating that AML evades NK cell immunity through microRNA-mediated dysregulation of NK cell development (Mundy-Bosse et al., 2016a). They

recently identified the microRNA miR-29b, a negative regulator of T-bet and EOMES, was elevated in NK cells of AML patients and this alteration was associated with maturation defects in AML-NK cells. Because the binding sites of Pum2 protein and microRNAs are significantly enriched in the 3'-UTRs, we can assume that alteration of RNA binding proteins (as Pum2), similar to microRNAs, in AML patients' NK cells may be a common mechanism of NK cell alteration. One explanation that supports this hypothesis comes from a recent study by Khaznadar *et al.* showing that NK cells in AML have broad gene sets alterations, and most of them concern immune processes (Khaznadar et al., 2015b). More experiments and hypotheses are required to prove repressive role of Pum2 in NK cell biology.

We found similarities in the consequences of Pum1 and Pum2 KO among NK92 cells. Moreover, it was identified in HeLa S3 cell line that 88% of the Pum2 target genes are also defined as Pum1 targets beside the similarity in their RNA-binding domains (PUM-HD, 91% identity) (Galgano et al., 2008). In order to identify whether Pum1 compensates for Pum2-KO, and inversely, NK92 cells with double KO for Pum1 and Pum2 should be generated. Moreover, despite having several clones of Pum1 or Pum2 KO NK92 cells, our observations should be confirmed in another NK cell model, such as primary NK cells or the KHYG-1 cell line. Thus, it is important to find a suitable vector and methodology to transduce KHYG1 cells. After that, we will confirm if the improvement of phenotypic characteristics and functional activity of NK92 cells is related to Pum2KO and/or Pum1KO by re-inducing the expression of Pum1 and Pum2 separately in the double KO cells. Ultimately, having a robust transfection technique to modify primary NK cells and reduce Pum2 protein level in these cells would be an important progress.

As our data showed an enhanced responsiveness of Pum2KO cells toward HLA negative targets, we should verify that this is also true with AML cell lines (KG-1, HL-60, and Kasumi) not *in vitro* but also *in vivo*. These cells can be injected into NSG mice and expanded. Once the leukemia is installed in the mice, effector NK92 variants will be injected intravenously. Anti-tumor activity will be analyzed by flow cytometry through measuring the frequency of leukemic cells in mice or bioluminescence (we have the leukemic cell lines transfected with luciferase, collaboration TrGET, CRCM) (Gertner-Dardenne et al., 2012).

Altogether, our data suggest that Pum1&2 proteins are repressors of NK cell effector functions and potentially anti-tumor activity. Several experiments are required to confirm and strengthen these data and to explain how Pum proteins affects these functions in human NK cells.

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Summary

Natural killer (NK) cells are effector lymphocytes of the innate immune system that have the ability to kill transformed cells without prior stimulation. They play a critical role in hematological malignancies surveillance, however, tumor cells develop immunosuppressive mechanisms to escape NK cell-mediated killing. So, maintaining or improving NK cell performance is considered a major challenge. In this work we focused on NK cells in the context of acute myeloid leukemia (AML) as well as post allogeneic hematopoietic stem cell transplantation (allo-SCT).

During my PhD study, I have been involved into 2 projects. The first project concerned the monitoring of NK cells after a phase I clinical trial consisting of infusing allogeneic NK cells 2 months after allo-SCT. The second project concerned the role of pumilio 2, a RNA binding protein involved in the repression of gene expression, in NK cell functions. Unfortunately I faced several technical issues while conducting this fundamental project; therefore the data remain preliminary.

In the first project, three different doses (dose 1: 10^6 NK cell/Kg, n=3; dose 2: $5x10^6$ NK cell/Kg, n=7; dose 3: $>5x10^6$ NK cell/Kg, n=6) of *ex-vivo* IL-2 activated NK cells were infused into patients with hematological malignancies after all-SCT. The goals of this part are to evaluate the impact of activated NK cells infusion on the recovery and biology of circulating NK cells post allo-SCT and the effect on the reconstitution of other immune populations. Our results showed higher frequency of NK cells in the periphery of patients treated with larger doses of activated NK cells. Although the notable immature phenotype shortly after treatment, the circulating NK cells in patients receiving larger doses of activated NK cells displayed more activation status with improved maturation profile after 6 months of treatment. We also found that the expression of activating NK receptors (NKG2D, NKp30, and NKp46) augmented on circulating $CD56^{dim}$ NK cells of patients receiving larger doses of activated NK cells. Moreover, these cells showed a significant increase in degranulation capacity as well as cytokine secretion (IFN-Y and TNF-α) throughout study period. No significant changes in frequencies of other immune cells ($CD3^+$, γδ T, $CD8^+$, $CD4^+$, $CD4^+$Tregs, monocytes and DCs) were observed during study period.

In the second project, we sought to recognize the role of Pum2 protein in NK cell biology by assessing its expression and the consequences of its deletion. We observed an up-regulation of Pum2 gene in NK cells from AML patients as well as in primary NK cells from healthy volunteers after stimulation. After that we used Lenti-CRICPER/Cas9 technique to generate Pum2KO cells in NK92 cell lines to explore the role of Pum2 in NK cells. Our results showed a significant upregulation in the expression of some activating receptors (NKp30, NKp44, NKG2D and CD69) in NK92-Pum2KO cells, while the expression of NKG2A inhibitory receptor was significantly decreased compared with NK92WT cells. Concerning NK cell functions, we showed an enhancement of cytotoxic function as well as cytokine secretion (INF-γ and TNF-α) by NK92-Pum2KO cells in response to stimulation with K562 as well as K562HLA-E target cells. In addition, we studied the effect of Pum2KO on IL-2 and NKp30 activation pathways in NK cells. We found more phosphorylation of Stat5 as well as Erk1/2 and Akt among NK92-Pum2KO cells in response to IL-2 stimulation and NKp30 mAb stimulation, respectively.

Résumé

Les cellules Natural Killer (NK) sont jouent un rôle essentiel dans la surveillance des hémopathies malignes. Cependant, les cellules tumorales développent des mécanismes immunosuppresseurs pour échapper à la reconnaissance et la lyse par les cellules NK. Ainsi, le maintien ou l'amélioration des performances des cellules NK sont considérés comme des défis majeurs. Dans ce travail, nous nous sommes concentrés sur les cellules NK dans le contexte de la leucémie myéloïde aiguë (AML) ainsi que la greffe de cellules souches hématopoïétiques post-allogéniques (allo-SCT).
Au cours de mon doctorat, j'ai participé à 2 projets. Le premier concernait la surveillance des cellules NK après un essai clinique de phase I consistant en l'infusion de cellules NK allogéniques 2 mois après allo-SCT. Le second projet concernait l'étude du rôle de pumilio 2, une protéine de liaison à l'ARN impliquée dans la répression de l'expression génique, dans les fonctions des cellules NK. J'ai fait face à plusieurs problèmes techniques lors de la réalisation de ce projet fondamental; par conséquent, les données restent préliminaires.

Dans le premier projet, des doses croissantes de cellules NK activées par IL-2 ex-vivo ont été perfusées chez des patients atteints de tumeurs malignes hématologiques 2 mois après allo-SCT. Les objectifs de cette partie étaient d'évaluer l'impact de la perfusion de cellules NK activées sur la récupération et la biologie des cellules NK circulantes après l'allo-SCT et l'effet sur la reconstitution d'autres populations immunisées.
Nos résultats ont montré une fréquence plus élevée des cellules NK dans la périphérie des patients traités. Bien que le phénotype immature soit remarquable peu après le traitement, les cellules NK circulantes, présentaient un état d'activation avec un profil de maturation amélioré après 6 mois de traitement. Nous avons également constaté que l'expression des récepteurs NK activateurs (NKG2D, NKp30 et NKp46) augmentait sur les cellules NK CD56dim circulantes des patients. De plus, ces cellules ont montré une augmentation significative de la capacité de dégranulation ainsi que de la sécrétion de cytokines (IFN-Y et TNF-α) tout au long de l'étude. Ces différences ont notamment été observées chez les patients ayant reçu des doses plus importantes de cellules NK activées. En conclusion, nous supposons que la perfusion de fortes doses de cellules NK activées ex-vivo pourrait être associée à l'amélioration du phénotype et des fonctions des cellules NK au cours de la reconstitution immunitaire après allo-SCT.

Quant au second projet, nous avons étudié le rôle de la protéine Pum2 dans la biologie des cellules NK en évaluant son expression et les conséquences de son invalidation. Nous avons observé surexpression du gène Pum2 dans les cellules NK de patients atteints de LAM. Afin d'explorer le rôle de Pum2 dans les cellules NK, nous avons généré des cellules Pum2KO dans les lignées cellulaires NK92. Nos résultats ont montré une augmentation significative de l'expression de certains récepteurs activateurs dans les cellules NK92-Pum2KO, et une diminution du récepteur inhibiteur NKG2A. Nous avons également observé une amélioration de la fonction cytotoxique ainsi que de la sécrétion de cytokines (INF-γ et TNF-α) par les cellules NK92-Pum2KO en réponse à la stimulation des cellules cibles. Enfin, nous avons observé une augmentation de phosphorylation de Stat5 ainsi que Erk1/2 et Akt parmi les cellules NK92-Pum2KO en réponse à la stimulation par IL-2 (pSTAT5) et la stimulation par NKp30.