CORRELATING THE TRANSCRIPTIONAL REGULATION OF uNK AND pNK CELLS WITH FUNCTIONAL MODALITIES THROUGH META-ANALYSIS APPROACH

A DISSERTATION

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I, Priyanka Rawat, Roll No. 2K20/MSCBIO/23 of MSc. Biotechnology, hereby declare that the project Dissertation titled "***CORRELATING THE TRANSCRIPTIONAL REGULATION OF uNK AND pNK CELLS WITH FUNCTIONAL MODALITIES THROUGH META-ANALYSIS APPROACH***" which is submitted by me to the Department of Biotechnology, Delhi Technological University, New Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any degree, Diploma Associateship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Dissertation titled "***CORRELATING THE TRANSCRIPTIONAL REGULATION OF uNK AND pNK CELLS WITH FUNCTIONAL MODALITIES THROUGH META-ANALYSIS APPROACH***" which is submitted by Priyanka Rawat, 2K20/MSCBIO/23, Department of Biotechnology, Delhi Technological University, New Delhi is in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record of the work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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ABSTRACT

Natural killer cells form the first line of defense against the viral infections and tumor cells in the body. NK cells are present abundantly in the body in various organs covering primary and secondary lymphoid to non-lymphoid organs as well. Here, the two extremes, pNK and uNK have been discussed in detail. The differences in NK cells can be correlated to the expression of different transcriptional factors by them. Transcriptional factors play a key role in the regulation of NK cells functionality and it becomes important to understand the expression of different transcriptional factors by NK cells. Besides regulating NK cell development, transcription factors such as GATA-3 is involved in preventing infections such from *Listeria monocytogenes*. Studying the transcriptional factors would help in designing novel immunotherapeutics by modulating the expression of transcription factors in NK cells. The work focuses on correlating the transcriptional regulation of peripheral and uterine NK cells to functionality and then comparing the biological pathways involved in these NK cells in tumor microenvironment. Here, hepatocellular carcinoma (HCC) pNK cells would be used to carry out gene enrichment analysis with respect to the healthy pNK cells. Tumor microenvironment such as of HCC provides an amalgamation of multiple pathways ultimately covering all of them and providing insights about the pathways that are downregulated or upregulated in that environment. NK cells in HCC have been found to have a downregulation of cytotoxic pathways such as interferon-gamma/alpha compared to the healthy NK cells. This downregulation of the cytotoxic pathways results in progression of the cancer. Therefore, the gene-enrichment analysis for understanding the functionality of NK cells in different environment is of importance.

Keywords: *Natural killer cells, Peripheral NK cells, Uterine NK cells, Transcriptional factors, GATA-3, Hepatocellular carcinoma, Gene enrichment analysis*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

NK	Natural killer
CLP	Common lymphoid progenitor
CD	Cluster of differentiation
PNK	Peripheral NK
UNK	Uterine NK
MHC	Major histocompatibility complex
TR-NK	Tissue resident NK
IFN	Interferon
GATA-3	GATA binding protein 3
DBA	Dolichos biflorus lectin
NFIL-3	Nuclear factor interleukin 3 regulated
ID2	Inhibitor of DNA binding 2
TF	Transcriptional factors
STAT	Signal transducer and activator of transcription
IRF	Interferon regulatory factor
EOMES	Eomesodermin
RUNX	Runt related transcription factors
MEF	Myocyte enhancement factor
ZEB2	Zinc finger E homeobox-binding transcription factor
IL	Interleukin
AR	Activating receptors
IR	Inhibitory receptors
NCR	Natural cytotoxicity receptor
TNF	Tumor necrosis factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
TGF	Transforming growth factor
KIR	Killer cell immunoglobulin receptors
eNK	Endometrial NK
dNK	Decidual NK
ILC	Innate lymphoid cell
cNK	Circulating NK

VEGF-C	Vascular endothelial growth factor-C
PLGF	Placental growth factor
TCF-1	T-cell factor 1
KLF	Kruppel like factor
GEO	Gene expression omnibus
HCC	Hepatocellular carcinoma
NES	Normalized enrichment scores
FDR	False discovery rate

CHAPTER 1: INTRODUCTION

NK cells are predominantly found in innate lymphocyte subsets with cytolytic activity against tumor and viral infections. [1] NK cell development process includes originating from CLP that develops into mature NK cells with cytotoxicity. [2] They are usually characterized in two principal NK cell subsets with $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ phenotype in peripheral blood. $CD56^{dim}$ subset is abundantly present in the blood, while the $CD56^{bright}$ subset is more present in abundance in peripheral and lymphoid tissues. [3] These two NK cells subsets in peripheral blood are collectively called Peripheral Natural Killer (pNK) cells. [4] After comparing the Uterine Natural Killer (uNK) cells found in uterus to either of the pNK cell subsets; they were characterized as a different subset. However, uNK cells have more similarities with the $CD56^{bright}$ subset. [5] uNK cells share many similarities with pNK cell $CD56^{bright}$ subset but are different in more ways. [6] Besides the differences between pNK and uNK on the basis of cytokine production or general appearance, the thesis focuses majorly on the differences between the two based on the differentially expressed transcriptional factors (TF).

1.1 NK cells

NK cells are granulated in appearance with cytolytic activity and are a crucial component of innate immunity. Their ability to lyse viral and tumor-infected cells makes them popular in immunotherapeutics. Unlike T-lymphocytes, they do not require MHC molecules to carry out killing and no prior sensitization is required. NK cells kill the MHC-I defective cells, helping in the identification of healthy “self” cells which constitutively express MHC-I. [7]

1.2 NK cells in different organs

All the experiments that have been carried out for decades involved pNK cells but now other organs of the body have also been explored to find tr-NK cells. Besides the primary and secondary lymphoid organs, several non-lymphoid organs such as the lungs, liver, and uterus also have NK cells. [8]

1.2.1 Lymph nodes

It is commonly populated with B or T cells but not NK cells. Recruitment gives rise to the majority of the NK cells found in lymph nodes. IFN-gamma receptor is required for the recruitment process. Mice deficient in the receptor did not experience any NK cell recruitment. [8]

1.2.2 Thymus

It has NK cells with phenotype in mice as $CD127^{+}$ $CD69^{high}$ $Ly49^{low}$ $CD11b^{low}$. GATA-3 is one crucial transcriptional factor involved in development stages of thymic NK cells. GATA-3 deficient mice reported an absence of thymus tr-NK cells. The development of these NK cells requires a functional thymus besides GATA-3. [8]

1.2.3 Spleen

The phenotype for splenic NK cells in mice is $CD127^{-}$ $CD69^{-}$ $Ly49^{+}$ $CD11b^{high}$. [8] In humans, the splenic NK cells express $CD56^{dim}$ $CD16^{+}$.

1.2.4 Liver

Not so long ago, it has been found in mice that mutual expression of CD49a and DX5 results in the formation of two subsets in liver. One with the phenotype: $CD49a^{-}$ $DX5^{+}$ and the other: $CD49a^{+}$ $DX5^{-}$. The former subset resembles the spleen NK cells phenotypically and circulate in the blood while the $CD49a^{+}$ $DX5^{-}$ subset subsides in the liver as liver-resident NK cells. [9]

In humans the surface markers for liver tr-NK cells have the phenotype: $CD56^{bright}$ $CD16^{-}$, $CXCR6^{+}$, $CD69^{+}$, $CD57^{-}$. [9]

1.2.5 Uterine NK cells

uNKs are found in uterus with a $CD56^{superbright}$ phenotype. uNKs go through a development phase starting from early pregnancy to the end of pregnancy. They are also normally found in the human endometrium in non-pregnant females. uNKs expand and becomes more granulated in the first trimester as a result of progesterone release after ovulation. This is the time when uNKs are at their peak in uterus. After the first trimester progressing towards the end of pregnancy, they become less granulated and diminish. Only a small numbers of uNKs are present at the end of pregnancy. [10]

uNKs are similar to CD56bright in terms of being poorly cytotoxic and cytokine effector cells. The heavily granulated uNKs produce granzymes, granulysin, and perforin but have poor

cytotoxicity. The inhibitory receptors such as KIR and NKG2A are expressed highly in uNKs than the other NK subsets found. [10]

On the basis of the reactivity of uNKs in mice to DBA, they are divided into two subsets, which is acquired after the increase in their granularity. In mice, uNKs are not heavily granulated until the blastocyst implantation. The inhibitory receptors such as Ly49 which is involved in recognizing the MHC-I is highly expressed in uNKs compared to the pNKs. Killer-cell lectin like receptor is reportedly overexpressed by murine uNKs than the murine pNKs. [10]

Table1.1 Different subsets of uNK in mice

Mice uNK subsets	Function	References
DBA^+	Express transcripts for antigenic factors	[10]
DBA^-	Express transcripts for interferon	

Despite the physiological and anatomical differences between the human and murine uNKs, they are comparable based on their function and regulation. From a broader view, the differences are acceptable making mice, a decent and useful animal model to carry out experiments to understand reproductive immunology. [10]

1.3 Transcriptional factors in NK cells

Transcriptional factors are responsible for regulating the expansion and function of NK cells. Developmental stages of NK cells right from the very early stages to the maturation are under the regulation of TFs. Some common TFs that help in NK cell expansion are NFIL3, and ID2 in the early developmental stages of NK cell.

To summarize, some of the many TFs involved in expansion, function, and maturation of NK cells have been described below briefly.

1.3.1 GATA-3

GATA-3 is important as it regulates functioning of other TFs like T-bet. It also regulates the expression of interferon-gamma in mice. In human, GATA-3 regulates the expression of CD94-NKG2A which is a NK cell inhibitory receptor. Also, thymic NK cell deficient in GATA-3 reported that it is essentially required for their development. [11]

1.3.2 STAT-5

It is an IL-15 downstream component. IL-15 has a key role in NK cell expansion and is responsible for activating STAT-5. STAT-5 has two components, STAT-5a and STAT-5b and are very similar in human and mice. Mice deficient in STAT-5b component is observed to have severe impairment in development and function of NK cells. [11]

1.3.3 IRF

Interferon regulatory factor (IRF) regulates hematopoiesis and defense against pathogens. IRF1 and IRF2 deficient mice have been observed to be at a severe loss of pNK cells. IRF1 is also important to the uNK cells with their involvement in development and localization of uNK cells. Deficiency of IRF2 results in the loss of cytotoxicity in NK cell. One reason for the losing the cytotoxicity could be decrease in numbers of NK cell. [11]

1.3.4 Nfil3 (E4BP4)

Nfil3 is an important TF in the early stages of NK cell expansion. The idea behind the involvement of Nfil3 in early developmental stages comes from the fact that a loss of Nfil3 in the later stages of development does not affect the NK cell numbers. It is involved in the lineage commitment and directly regulates Id2 and Eomes expression. It has also been observed that overexpressing Id2, compensates the deficiency of Nfil3. [11]

1.3.5 Id2

Inhibitor of differentiation (Id)-2 regulates NK cell expansion. Mice deficient in Id2 results in a severe loss of pNK cells. [11]

1.3.6 T-bet and Eomes

They are from the T-box family of transcriptional factors and are involved in NK cell expansion and maturation. T-bet specifically in NK regulates the terminal maturation with regulation of S1P5 expression. Mice deficient in T-bet reported an increase of NK cells in bone marrow and lymph node unlike peripheral NK. This is speculated to be due to the downregulation of S1P5 which results in difficulty to exit the bone marrow and lymph nodes.

Between T-bet and Eomes, a compensatory mechanism has been observed as mice deficient in T-bet seem to express high level of Eomes. Tumor microenvironment leads to a decrease in interferon gamma production and a decreased expression of T-bet and Eomes. [11]

1.3.7 Runx1 and Runx3

IL-15 has a key role in NK cell expansion and Runx3 regulates the proliferation of genes downstream to the IL-15 pathway. This makes Runx3 an important TF in the NK cell expansion and function. [12]

For Runx3, inflammation works to drive its upregulated expression whereas for Runx1, antigen receptor signaling is also required besides inflammation. [12]

1.3.8 Ets-1

Ets-1 is a crucial TF with Ets-1 deficient mice reporting severely impaired expansion of NK cells and function. Ets-1 is involved in very early developmental stages of NK cell and regulates Id2 and T-bet expression. [13]

In mice, Ets-1 regulates the expression of Ly49H and Ly49D (some important NK cell receptors). [13]

1.3.9 Mef

It has its role to portray in regulating the NK cell maturation. Mef deficient mice show decreased NK cell numbers with defects in their functionality. Mef deficient splenic NK cells lose their lytic ability against tumor cells with decreased production of perforin and IFN-gamma. [14]

1.3.10 Zeb2

Zeb2 is the most highly expressed TF in NK cell maturation stages. It is also involved in the epithelial-mesenchymal transition. Zeb2 deletion has been observed to result in impaired maturation and preventing their exit from bone marrow. [15]

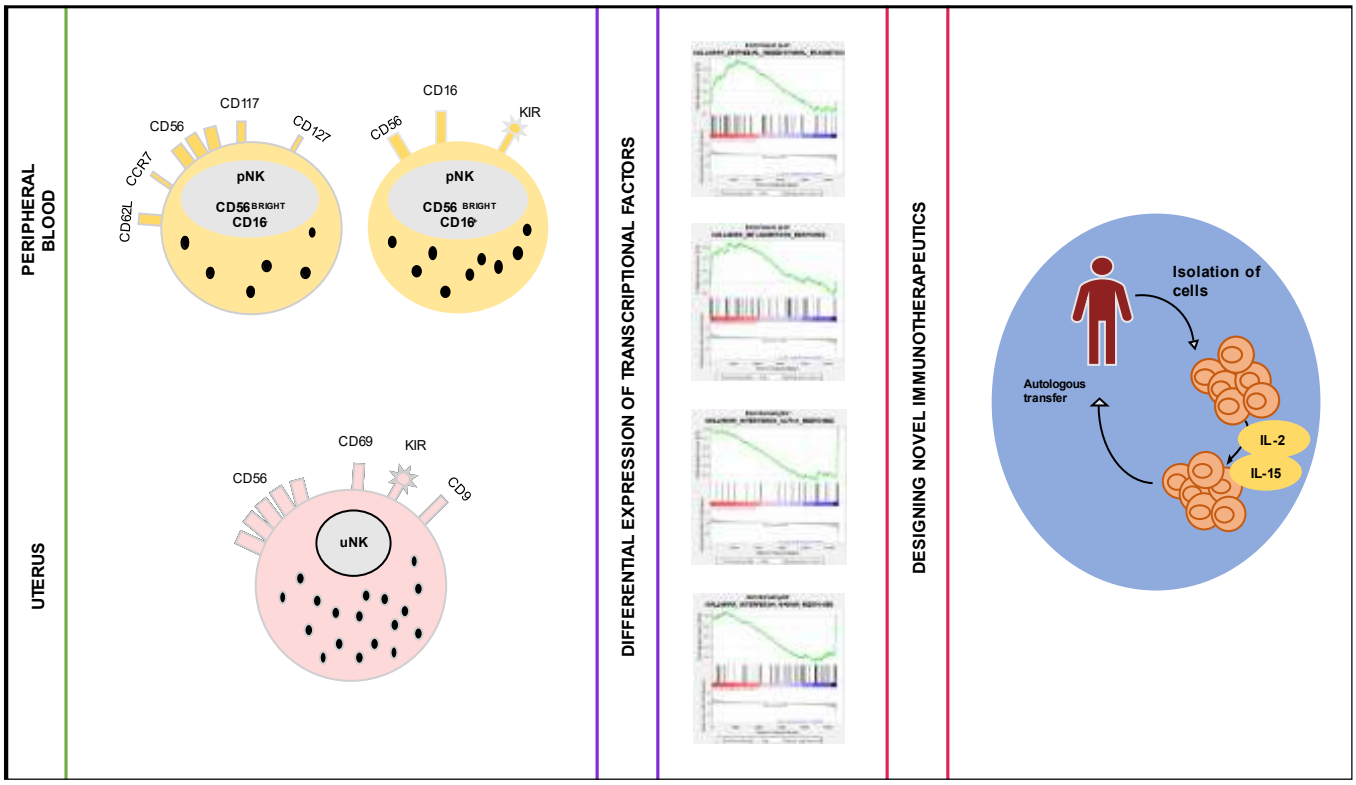


Figure 1.1 Graphical introduction

CHAPTER 2: LITERATURE REVIEW

2.1 Natural Killer cells: An Overview

A crucial component of innate immune system with granular appearance because of the presence of typical cytoplasmic azurophilic granules. [16] NK cells fight diseases and malignancies being the first in the line of defense against pathogens. [17] Commonly, we define NK cells considering their cytotoxic activity against infections and tumors. [18] NK cells are considered as main producers of IFN- gamma. [19] They are much more diverse in the terms of functionality and phenotype, due to the expression of different receptors. [18]

Table2.1 Different phenotypic markers of NK cells in humans and mice.

NK cells	Phenotypic marker	References
Humans	CD56 in absence of CD3	[17], [20], [21]
Mice	CD11b and CD27 CD49b and/or NK1.1	[22] [23]

The cytotoxic attack of the NK cells is executed via wide range of NK cell receptors. Cytotoxic attack of the NK cells via their receptors does not require any antigen priming prior to the infection and is immediate. [17], [18] The activating receptors (AR) are involved in ligand recognition, present on the cells in distress. NK cell inhibitory receptors (IR) however, prevent the lysis of healthy 'self' cells. IRs recognize the MHC-I molecules which are constitutively expressed by the healthy cells in the body and is lost by the cells in conditions of stress. [22]

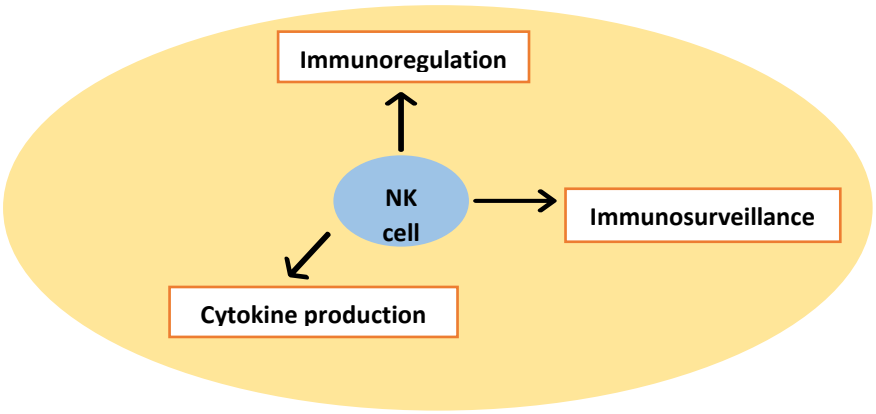


Figure 2.1 Overview of the NK cell functions

2.1.1 NK cell receptors

They are capable of differentiating between the healthy-self-cells from non-self-cells or infected self-cells. This is achieved by maintaining a balance between the AR and IR of NK cells. Since, NK cells are involved in crucial functions of innate immune system and release of various cytokines, it becomes important to learn about the cell-surface receptors of NK cells. These receptors are generally divided into AR and IR but we can also segregate natural cytotoxicity receptors which comes under the umbrella of AR.

- Activating receptors

  Activating receptors work on the cells with defected or no MHC-I molecules. The inhibitory signal on such cells is weakened and a chain of NK cell activating receptors take over to further activate the NK cells resulting in the lysis of such cells.

  Natural cytotoxicity receptors

  NCR are type 1- membrane glycoprotein receptors that are responsible for cytokine production and NK cell cytotoxic activity. NKp44 and NKp46 recognizes viral proteins (hemagglutinin of influenza). [24]

- Inhibitory receptors

NK cell inhibitory receptors are responsible for maintaining the autoimmune tolerance by inhibiting the function of NK cells by recognizing the MHC-I molecules. MHC-I is expressed by healthy cells of the body except the ones that are in some stress, helping inhibitory receptors to work on the cells where no MHC-I molecules have been presented. [25]

Table2.2 Different receptors present on NK cell.

NK cell		References
AR	CD94/NKG2C, NKG2D, KIR-2DS, KIR-3DS, 2B4, CD226	[17], [20], [24]–[28]
NCR	NKp30, NKp44 (only expressed under activation), NKp46, DNAM1	
IR	KIR, CD94-NKG2A, TIM-3, KIR-2DL, KIR-3DL, CD94/NKG2A, and TIGIT	

2.1.2 Cytokines and NK cells

NK cells lyse tumor or virally infected cells but what actually helps them in carrying out the lysis of such cells are the cytokines. Cytokines regulate the functioning and expansion of NK cells. Some of the important cytokines have been described below briefly.

- Interleukin 2 and 15

  These are two of the very important cytokines of NK cells with a positive impact on their development and functioning. Reportedly, the functional maturation of $CD56^{bright}$ NK cell subset has also been regulated by IL-2. IL-2 activates the NK cells for an anti-tumor response and is widely used for designing immunotherapeutics against the tumor. The only drawback with using the IL-2 for activating an enhanced anti-tumor response by NK cells is its toxic cytotoxicity.

  Perforin and granzymeB production are regulated together by IL-2 and 15 further mediating the NK cell cytotoxicity. IL-2 and 15 with IL-12 combined results in the upregulated expression of IFN-gamma, TNF-alpha, and GM-CSF. This upregulated expression would not have been possible just by IL-2 and 15 alone, as they are considered poor inducers of cytokine production.

  Although, IL-15 has been reported to be less toxic than IL-2 when used in designing immunotherapeutics, its post-therapy effects has limited its use in therapeutics. [29]

- Interleukin 21

IL-21 can have positive and negative effects on NK cells and shows upregulated NK cell proliferation and cytotoxicity when combined with other interleukins such as IL-2 and 15. [29]

- Interferon-alpha

IFN-alpha is involved in anti-viral and anti-tumor functions. It helps in the proliferation and cytotoxicity of NK cells. [29]

- Interferon-gamma

It is responsible for the cytotoxic response, anti-tumor activity, and regulating NK cell mediated adaptive immune response. It is a Type II interferon with it production being stimulated by the activation of NK cells either by IL-12 and 18 or mitogens. [29]

- TGF-beta

TGF-beta1 supports the recruitment of $CD56^{bright}$ NK cells while restricting or not favoring the other $CD56^{dim}$ NK cell subset. It limits the NK cell cytotoxicity and downregulates the expression of T-bet with their involvement in the SMAD-dependent pathway. [29]

- Interleukin 6

It has been designated as the cytokine that mediates inflammation, linking inflammation to tumor development. IL-6 downregulates the cytotoxicity and blocking of IL-6 has shown good results in clinical trials of cancer. [29]

Table2.3 List of cytokines and chemokines produced by NK cells.

	Cytokines	**Chemokines**	**Growth factors**	**References**
NK cell	IFN- gamma, TNF-alpha, IL-10, IL-2, IL-15	CCL2, CCL3, CCL4, CCL5, XCL1, IL-8	GM-CSF, G-CSF, IL-3	[19], [29]

2.2 NK cell v. B and T cells

Our immune system has two arms consisting of innate and adaptive immunity. While the NK cells are a crucial part of innate immunity, adaptive immunity stands on the shoulders of B and T lymphocytes. [30] For the longest time, immunotherapeutics has been developed using T-

lymphocytes but currently the much ignored NK cells have come in focus. [30] NK cells can differentiate between abnormal and normal cells leading to more specific anti-tumor cytotoxic activity. [30]

2.3 NK cells in different organs

They are widespread in the lymphoid as well as non-lymphoid tissues in the body. [22] Besides the NK cell present in peripheral blood/circulating blood, other numerous subsets of NK cell are present in different organs in the body. There are referred as tissue-resident NK cells (tr-NK). [18]

Table2.4 Comparison between NK cells found in different organs

Properties	Uterine NK cells	Lymph node NK cells	Thymus	Liver NK cells	References
Phenotype	$CD56^{superbright}CD16^{-}$ (Main phenotype)	$CD56^{bright}$ $CD16^{-}$	$CD56^{dim}CD16^{-}$ /$CD16^{+}$	$CD56^{bright}$ $CD16^{-}$	[10], [31]–[40]
NKG2A/CD94	+	↓	Can't say	↑	
KIR	↑	-	Can't say	↓	
CD69	+	↓	+	+	
CD11b	+	↓	-/+	↑	
Cytotoxicity relative to pNK	Reduced cytotoxicity	Reduced cytotoxicity	Reduced cytotoxicity	Debatable Burt – Low Chuang - high	

2.4 Peripheral NK cells

pNK cells appear granular and constitutes 10-15% of circulating lymphocytes. [41] They have the two phenotypes $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ in peripheral blood. [42] $CD56^{dim}$ subset is more cytotoxic than $CD56^{bright}$.

2.4.1 pNK cells during menstrual cycle

No notable changes have been observed in the pNK cells between the follicular and luteal phase of menstrual cycle. [42]

Table2.5 A comparative account of two principal NK cell subsets.

General Properties	**$CD56^{dim}CD16^{+}$**	**$CD56^{bright}CD16^{-}$**	**References**
Abundance	95% pNK cells	~5% of the total pNK cells	[20], [22], [26], [43], [44]
Cytolytic granules	High	Low	
Cytotoxicity	High	Low	
General function	Cytotoxic effector cells	Cytokine producers	
Found majorly in	Peripheral blood and lung tissues	Lymph nodes and tonsils	
Perforin expression	+	-	
Receptors			
KIR	+	-	
CD94/NKG2A	↓	↑	
CD16	++	-	
CD57	+	-	
CD127	-	+	
CD117	-	+	

2.5 Uterine NK cells

2.5.1 Origin

uNK cell origin has always been a question and many hypotheses have been proposed contemplating their origin. The absence of CD16 in uNKs similar to pNKs led to the hypothesis that uNK originated from pNK through migration an differentiation. [45] The other hypothesis proposes the idea that uNK originated from hematopoietic progenitor cells present in the tissues of uterus or by migration via blood. [46] Keskin et al experimentally observed that $CD16^{+}$ pNK cells converts into $CD16^{-}$ with similarities to the uNK cells. [43]

2.5.2 Phenotype

uNK cells are heavily granulated and have the $CD56^{superbright}$ $CD16^{-}$ phenotype with some similarity to both the pNK subsets to an extent. [47], [48]

2.5.3 Functionality

Although, uNK cells have low cytotoxic activity they express cytokines and chemokines with no stimulation required. The major cytokine for the differentiation of uNK cells is IL-15. [47] uNK cells are capable of killing the CMV-infected cells in decidua and carry out effective responses against *Chlamydia trachomatis*. [49]

2.5.4 Receptors

Activating receptors of uNK cells include CD244, NKG2D, NKp30, NKp44, and NKp46. The surface expression of inhibitory receptors such as KIR, CD94/NKG2A, and ILT-2 by uNK cells prevent them from killing the trophoblast cells. [47] A pregnant women highly expresses HLA-C binding killer immunoglobulin receptors (KIR2DL1/S1^{+} and KIR2DL2/3/S2^{+}). [50]

2.5.5 Subsets

uNK cells comprises of endometrial NK cells (eNK) and decidual NK cells (dNK) and the two differs in functional aspects. dNK cells are abundantly present (~70%) in the uterus during pregnancy while only ~30% eNK cells are found. [27]

Decidual NK cells

dNK cells are localized to the decidua basalis. [48] dNK cells show a difference in expression of some cytokines and chemokines in first trimester and terminal pregnancy.

- First trimester dNK: dNK cells show high expression of chemokine receptors including CCR1, 3, CXCR2, and 3 contrary to the terminal dNK. [48]

Endometrial NK cells

eNK cells have CD56^{bright} CD16^{-} CD57^{-} CD49a^{+} CD69^{+} CD9^{+} phenotype and are found in the proliferative phase. However, they proliferate vigorously in the secretory phase after ovulation. The proliferation of eNK cells is due to the release of IL-15 in response to progesterone by stromal cells in. The KIR inhibitory receptor expressed by eNK cells is different from the one expressed by pNK or dNK cells. [49]

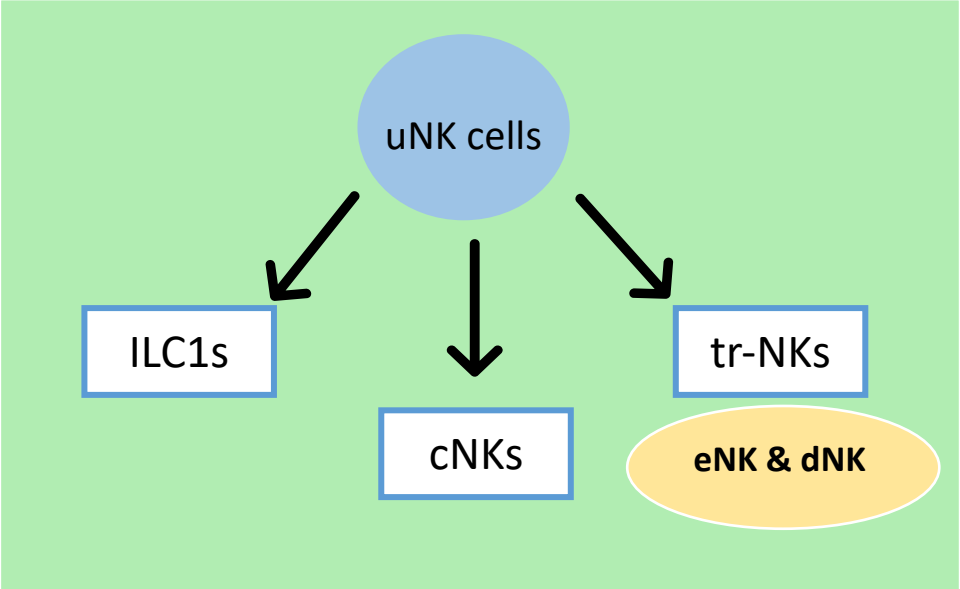


Figure 2.2 NK cells in uterus

Table2.6 Differences between two types of uNK cells (eNK v. dNK).

	eNK	dNK	References
CD49a	+++	+	[27]
TIM-3	-	+	[27]
Primary function	Involved in embryo implantation	• Regulation of maternal-fetal tolerance. • Vascularization	[27]
Abundance	30% in uterus	70% in uterus	[27]

2.6 pNK and uNK cells

NK cells are found abundantly in the body of both human and mice, in both various lymphoid as well as non-lymphoid organs. The thesis however, focuses on two extremes of NK cell found in the body that is pNK and uNK. Both the pNK and uNK cells differ on the basis of their functionality and phenotypical differences.

The origin of uNK cells from a pNK cell subset has also been proposed but there is no hard evidence supporting the hypothesis.

Table2.7 Differences between peripheral and uterine NK cells

Properties	pNK	uNK	References
Phenotype	Two subsets: $CD56^{dim}CD16^{+}$ $CD56^{bright}CD16^{-}$	$CD56^{superbright}$ $CD16^{-}$	[31], [51]–[58]
Granularity	Less	High	
Cytotoxicity	$CD56^{dim}CD16^{+}$: High $CD56^{bright}CD16^{-}$: Low	Low	
VEGF-C	-	+	
PLGF	-	+	

2.7 Transcriptional factors

Transcriptional regulation of NK cells is involved in the expansion, function, and various effector functions of NK cells. Learning about these TFs in detail to understand their involvement is crucial to further understand the NK cells and use them in therapeutics.

2.7.1 NFIL-3

NK cell development and functionality is under the regulation of a crucial TF; NFIL3/ E4BP4. Mice deficient in Nfil3 lacked mature NK cells suggesting the contribution of Nfil3 in various NK cell development stages (NKP → iNK → mNK). Besides the lack of mature NK cells, Nfil-3 deficient mice were also observed to have low cytotoxicity and decreased production of IFN-gamma. Nfil-3 is involved in lineage specification and are present downstream of IL-15 pathway while being upstream to the Id2. [13] Nfil3 has been found to be involved in regulating anti-inflammatory response and circadian rhythm in mammals. [59]

2.7.2 T-bet

T-bet regulates the terminal stages of NK cell expansion. Wang et al. found that majority of the dNK cells are deficient in T-bet unlike pNK cells that are $T\text{-}bet^{+}$. [60]

2.7.3 Id2

ID2 regulates early development stages of NK cell. Using western blot analysis, Wang et al. reported the expression of ID2 by dNK cells exclusively. [60]

2.7.4 PBX-1

PBX-1 deficient mice were reported to lack NK cells completely. It is very homologous between human and mice and is important because of its involvement in NK cell

differentiation. [60] Zhou et al. reported the expression of PBX-1 in dNK cells and not pNK cells. PBX-1 regulates the expression of two important growth factors (PTN and OGN) produced by the dNK cells. [61]

2.7.5 Hoxa

Hoxa-10 and Hoxa-11 are two crucial transcription factors expressed by uNK cells and affect the decidualization process in the uterus. The deletion of Hoxa-10 has shown dysregulation of uNK functional maturation while not affecting the numbers. [47]

2.7.6 Eomes

It is upregulated in pNK cells and controls careful regulation of early developmental stages of NK cell. The dependency of several other factors on Eomes makes it an important TF as its downregulation leads to downregulation of other important factors too. [62]

2.7.7 GATA-3

Ali et al. experimentally concluded and explained the involvement of GATA-3 in maturation and exit of NK cells from bone marrow. Ali et al. also explained that GATA-3 is essentially required by liver for the development of $CD49a^{+}$ tr-liver NK cells. [63]

2.7.8 TCF-1

It is encoded by tcf7 and regulates NK cell expansion. Liu et al observed the downregulation of several other important TFs in Tcf1 deficient mouse, resulting in NK cell impairment. [64]

2.7.9 Zeb2

It is the major TF required during maturation of NK cell and is overexpressed during the maturation stages. It is also involved in the epithelial to mesenchymal transition. Zeb2 expression is dependent on T-bet leading to a similar phenotype in either of their deficiency. [15]

2.7.10 KLF

KLF-2 is an important TF for the pNK cell maturation and development. Rabacal et al. experimentally reported a decrease in pNK cells with KLF-2 deficiency. However, KLF-2 deficiency does not affect the numbers of the tr-liver NK cells. [65]

On the other hand, there has been no reports regarding the effect of KLF-2 on uNK cells but KLF-5 seem to be involved in angiogenesis and vascular remodeling and is expressed by uNK cells. [65]

2.7.11 STAT

JAK-STAT pathway regulates crucial functions of NK cells, making it difficult for them to survive without STAT-5. However, it has been reported that STAT-5 deficiency can be overcome by upregulating the expression of Bcl-2. STAT-5 is found downstream to IL-7, 2, and 15, making STAT-5 an important TF for the NK cell expansion and survival. Gotthardt et al. reported tumor-promoting effects in cells deficient in STAT-5. [66]

2.7.12 IRF

IRF-1 is induced by the interferon-gamma and is involved in the regulation of inflammation. Blocking IRF-1 results in nullifying of the IFN-gamma effect on tumor cells. IRF-1 itself was not found to affect the tumor cells but has a key role in activation of NK cells and attracting them towards the tumor cells. Tumor cells expressing IRF-1 are easy to target by NK cells than in the absence of IRF-1. [67]

2.8 NK cells and their functional impact on diseases

NK cells are involved in preventing infections and diseases. Several components of NK cells such as receptors, cytokines produced, and TFs are involved in regulating an immune response. Some conditions, infections, and diseases where some defect or deficiency in NK cells led to the condition are described below briefly.

Recurrent miscarriages

For a successful pregnancy, the inhibitory receptors presented by the uNK cells play an important role. Zhou et al. examined the uNK cells of recurrent spontaneous abortion females to find out that a deficiency or downregulation of PBX-1 is present in such patients. This indicates the role of PBX-1 TF in a successful pregnancy. [61]

Inflammatory disorders

A decrease has been observed in the numbers of NK cell and an impairment of their function in disorders (Type-1 diabetes, multiple sclerosis, and systemic lupus erythematosus) resulting in high flares. This directs towards the conclusion that NK cells help in controlling inflammation in such disorders. [68]

Infectious diseases

It has been reported that dNK cells limit the HIV-infection in pregnancy. This is not only achieved by killing of infectious cells but also through production of several cytokines. [69]

Cancer

Gill et al. reported that forced expression of Eomes leads to a reduction in tumor burden. The Eomes was expressed in adoptively transferred NK cells and resulted in survival of the NK cells in mice. [70] Going into specificalities, hepatocellular carcinoma is one the most malignant and is the third leading carcinoma. Deeper analysis revealed the dysfunctioning of NK cells in HCC patients. This involves lower cytotoxic responses and decreased cytokine production in the patients when compared to their healthy counterparts. [71]

2.9 Designing novel immunotherapeutics

NK cells can be used to design novel immunotherapeutics against infections, cancer, or preventing miscarriages by modulating the NK cell receptors or transcriptional factors regulating important cytotoxic or other relevant pathways.

Some of the strategies that can be adopted for designing immunotherapeutics against diseases are:

1. Adoptive transfer of autologous NK cells

   Using autologous NK cells for adoptive transfer after modulating the NK cells with the desired TF is one strategy that can be used to design immunotherapeutics against infections, cancer, and recurrent spontaneous miscarriages.

   For instance, being aware of the PBX-1 requirement for carrying out smooth successful pregnancy we can forcefully express PBX-1 into the NK cells in patients with low PBX-1 expression. [61]

2. Transfecting NK cell using a nanoparticle delivery system

   Using nano-particle delivery system for transfecting NK cells instead of using a viral vector would help eliminate the risks that come along with viral vectors. [72]

   For instance, a negative regulator of the desired TF can be silenced using transfection method and result in smooth operation of the function of the TF.

3. Si-RNA mediated gene silencing

   This could help in designing immunotherapeutics by targeting the negative regulators of the TF and preventing the production of such regulators helping in the unrestricted expression of the TF. [73]

CHAPTER 3: METHODOLOGY

3.1 Data retrieval from GEO

Using the Gene Expression Omnibus (GEO) [74] NCBI platform, a GEO dataset with the accession number GSE79939 was retrieved consisting of pNK (3 - $CD56^{dim}$ and 3 - $CD56^{bright}$) and dNK (4) samples. GSE79939 is based on the platform GPL97 (HG-UI33B Affymetrix Human genome UI33B array).

The other dataset retrieved from GEO [74] is with the accession number GSE120123 consisting of 2 Hepatocellular carcinoma (HCC) pNK cells and 2 healthy pNK samples. GSE120123 is based on the platform GPL6480 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Probe Name version).

3.2 GEO2R analysis

The GEO datasets were analyzed with GEO2R to retrieve the differentially expressed genes (DEGs). Firstly, the dNK (test) samples were selected followed by the selection of $CD56^{dim}$/$CD56^{bright}$ (control) pNK samples. Similarly, HCC pNK samples (test) were selected followed by the healthy pNK cells (control) before carrying out the GEO2R analysis.

The adjusted P-values in GEO2R analysis were calculated using Benjamini & Hochberg (False discovery rate).

Master table consisting of all the differentially expressed genes with their p-values and more, after the GEO2R analysis was downloaded to carry out further analysis from it.

3.3 Normalization of data graphs with R

GEO2R analysis also provides a R script for all the normalization graphs produced during the analysis. This R script was used to create the graphs in R studio 4.1. These graphs consist of box-plots and density plot.

Besides the normalization graphs, volcano plots and histograms of all the p-values distributed in the sample was also generated with R studio 4.1. (Appendix A.1,2,3)

3.4 Gene enrichment analysis with FunRich

FunRich software [75] was used to generate a gene enrichment report. Before loading the data in FunRich, the master table downloaded after the GEO2R analysis was used to create two separate data sheets, one with log Fc > 1 and the other with log Fc < -1. These two data sheets were imported into FunRich for creating a venn diagram of overlapped significant genes in the datasets and gene enrichment analysis report.

The gene enrichment analysis report was used for correlating different transcriptional factors to the biological pathways found to be enriched for the sample.

3.5 Gene set enrichment analysis with GSEA

Gene set enrichment analysis software [76], [77] was used to generate results showing the enrichment of the genes in different biological pathways. The snapshots of all the biological pathways both up and down regulated for the samples was generated using the GSEA software. (Appendix B.1,2,3)

Using GSEA software to generate data with positively and negatively enriched pathway in our data involves some basic steps:

Firstly, the data was loaded with an extension .rnk into the software, then a pre-ranked dataset was selected from the software itself. For this thesis, hallmark genes dataset provided by the software was selected to run against the retrieved GEO datasets. Then, the data is run to generate the results.

3.6 Graphs with excel

The data for gene enrichment analysis was exported to excel to create bar graphs showing the fold enrichment of different transcriptional factors in dNK compared to the $CD56^{dim}/CD56^{bright}$ and HCC pNK compared to the healthy pNK. The transcriptional factors were correlated to the biological pathways.

The hypergeometric test p-values were used for biological pathways, transcriptional factors analysis data visualization.

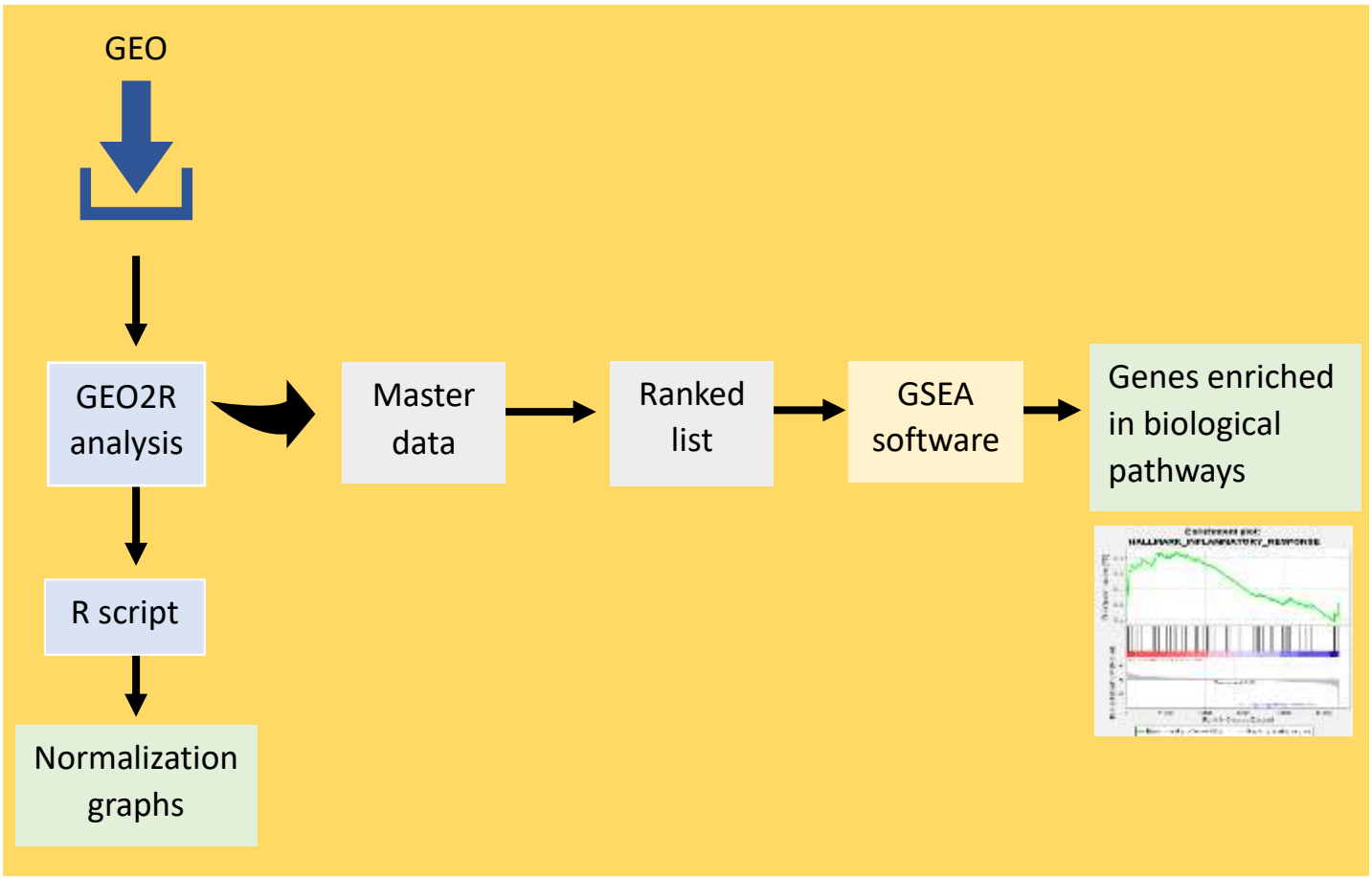


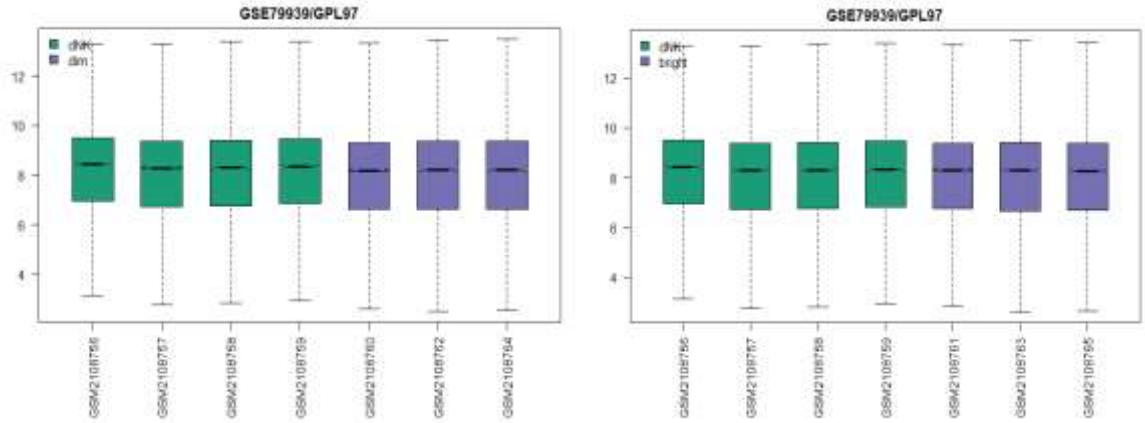
Figure 3.1 Basic steps for the methodology

CHAPTER 4: RESULTS

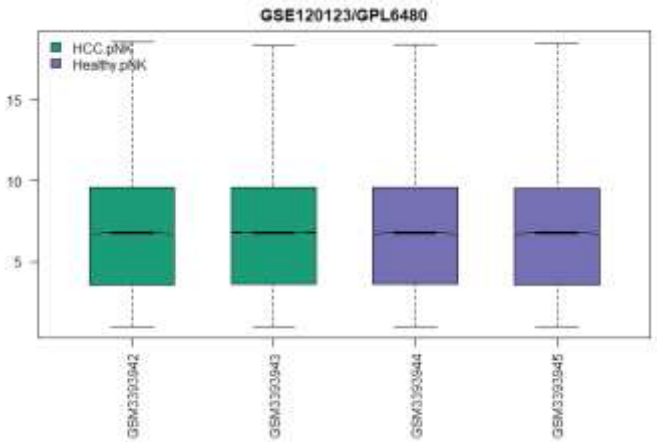
4.1 Data normalization with R

The data was normalized before beginning the differential expression analysis. This helps in confirming that the data is okay to compare and carry out further analysis with it.

For the box-plots, generally, when the mean centered values are obtained it indicates that the data is normalized and is cross comparable.



A)



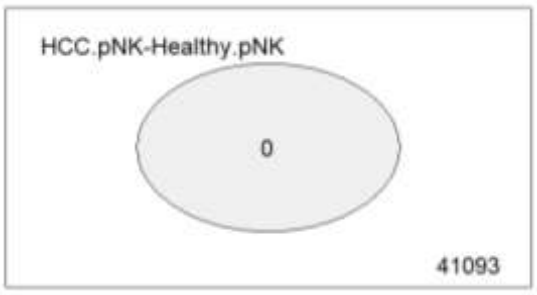
B)

Figure 4.1 Box-plots with the normalization of GEO datasets for **A)** dNK and $CD56^{dim}/CD56^{bright}$ pNK **B)** HCC pNK and healthy pNK samples retrieved using R.

4.2 Differentially expressed genes (DEGs) with venn diagrams

The DEGs for GSE79939 and GSE120123 were retrieved after the GEO2R analysis. The R script generated from the analysis was used to generate venn diagrams showing the overlap of DEGs in different samples of the two GEO datasets.

A



B

Figure 4.2 Venn diagrams showing the overlapping DEGs in **A)** dNK and CD56dim/CD56bright NK cells and **B)** HCC pNK and healthy pNK cells.

4.3 Gene set enrichment analysis

4.3.1 dNK v. CD56^{dim} NK

1. Interferon gamma response is negatively correlated with the dNK cells. The negative enrichment scores indicate the downregulation of the response in dNK cells. The genes that are enriched in CD56^{dim} for the interferon gamma response are: LYSMD2, SP110, TRAFD1, GBP4, STAT1, PFKP, SPPL2A, PLSCR1, PTPN2, HELZ2, NLRC5, APOL6, RNF213, PSMA3, IFIT3, B2M, ZBP1, LATS2, IFIT2, SLAMF7, and PELI1.

   The transcriptional factors associated with the pathway are: IRF1 and STAT1. The TFs are highly enriched in CD56^{dim} NK cells. The lower enrichment values in IFN-gamma response for the TFs in dNK cells correlates to the lower enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between IFIT2 and IFIT3 but not with the other genes enriched in the response.

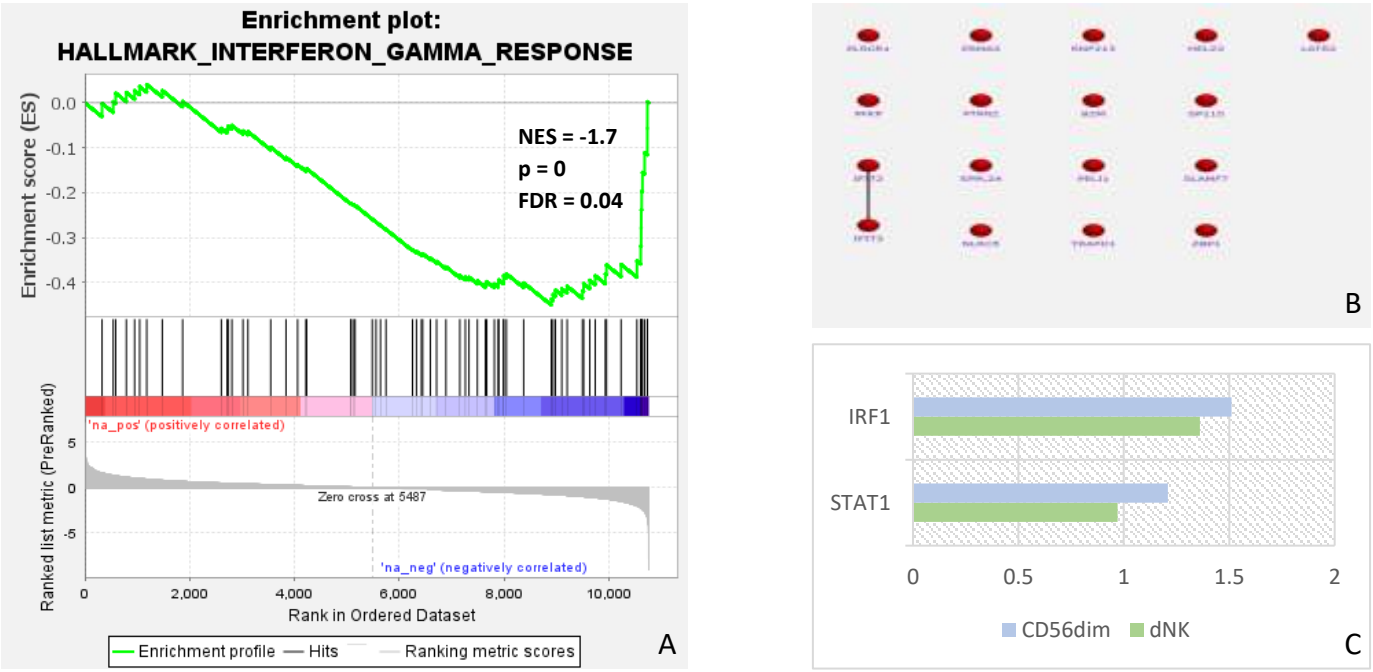


Figure 4.3 **A)** Gene set enrichment analysis showing negative correlation of dNK cells with the Interferon gamma pathway. **B)** Interaction analysis of the genes enriched for the IFN-gamma response. **C)** The fold enrichment of transcriptional factors involved in the interferon-gamma response in dNK cells.

2. Interferon alpha response is negatively correlated with the dNK cells. The negative enrichment scores indicate the downregulation of the response in dNK cells. The genes that are enriched in CD56^{dim} for the interferon alpha response are: PARP9, SP110, TRAFD1, NUB1, GBP4, PLSCR1, HELZ2, PSMA3, IFIT3, B2M, NCOA7, GBP2, IFIT2, and MOV10.

   The transcriptional factor associated with the pathway is: IRF1. The TF is relatively enriched in CD56^{dim} NK cells. The lower enrichment values in IFN-alpha response for the TFs in dNK cells correlates to the lower enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between IFIT2, IFIT3, and NUB1 but not with the other genes enriched in the response.

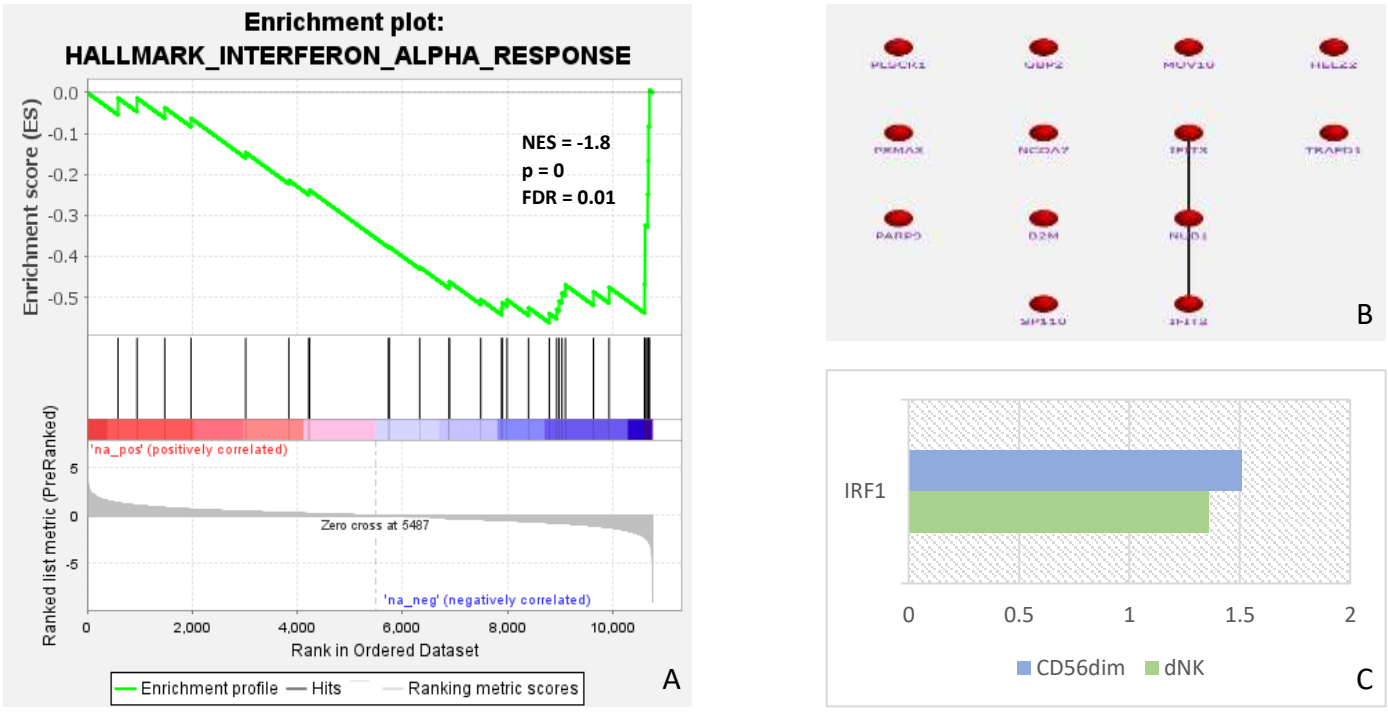


Figure 4.4 **A)** Gene set enrichment analysis showing negative correlation of dNK cells with the Interferon alpha pathway. **B)** Interaction analysis of the genes enriched for the IFN-alpha response. **C)** The fold enrichment of transcriptional factor involved in the interferon-alpha response in dNK.

3. TGF-beta signaling

TGF-beta signaling is negatively correlated with the dNK cells. The negative enrichment scores indicate the downregulation of the response in dNK cells. The genes that are enriched in CD56^{dim} for the TGF-beta signaling are: SKI, SMAD1, SKIL, CDK9, THBS1, CTNNB1, PMEPA1, LTBP2, and NOG.

The transcriptional factor associated with the pathway is: SMAD1. The TF is enriched in CD56^{dim} NK cells. The lower enrichment values in TGF-beta response for the TFs in dNK cells correlates to the lower enrichment score of the response in dNK cells.

The interaction analysis shows an interaction between SKI, SKIL, and SMAD1 but not with the other genes enriched in the response.

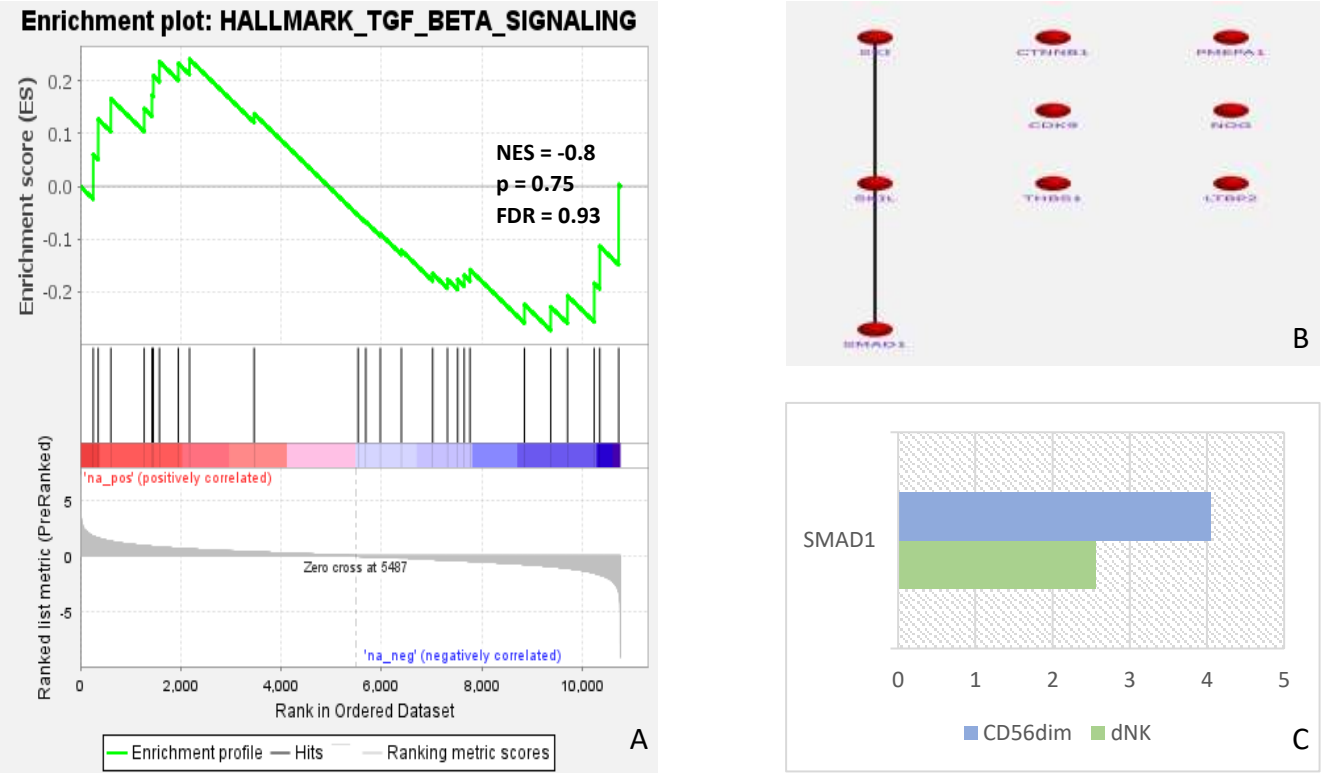


Figure 4.5 **A)** Gene set enrichment analysis showing negative correlation of dNK cells with the TGF-beta signaling. **B)** Interaction analysis of the genes enriched for the TGF-beta signaling. **C)** The fold enrichment of transcriptional factor involved in the TGF-beta signaling in dNK cells.

4. TNF-A signaling via NFK-B is positively correlated with the dNK cells. The positive enrichment scores indicate the upregulation of the response in dNK cells. The genes that are enriched in dNK cells for the TNF-A signaling via NFK-B are: DUSP4, EGR1, PHLDA1, RHOB, TNFAIP8, MCL1, MARCKS, PDLIM5, PHLDA2, ETS2, SPSB1, TNIP2, SOCS3, ACKR3, ICOSLG, TRAF1, PMEPA1, FOSL2, TNFRSF9, and REL.

   The transcriptional factors associated with the pathway are: EGR1, NR4A2, PLAU, and TGIF1. The TFs are highly enriched in dNK cells. The higher enrichment values in TNF-A signaling via NFK-B for the TFs in dNK cells correlates to the high enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between TRAF1, TNFRSF9 and TNIP2, REL but not with the other genes enriched in the response.

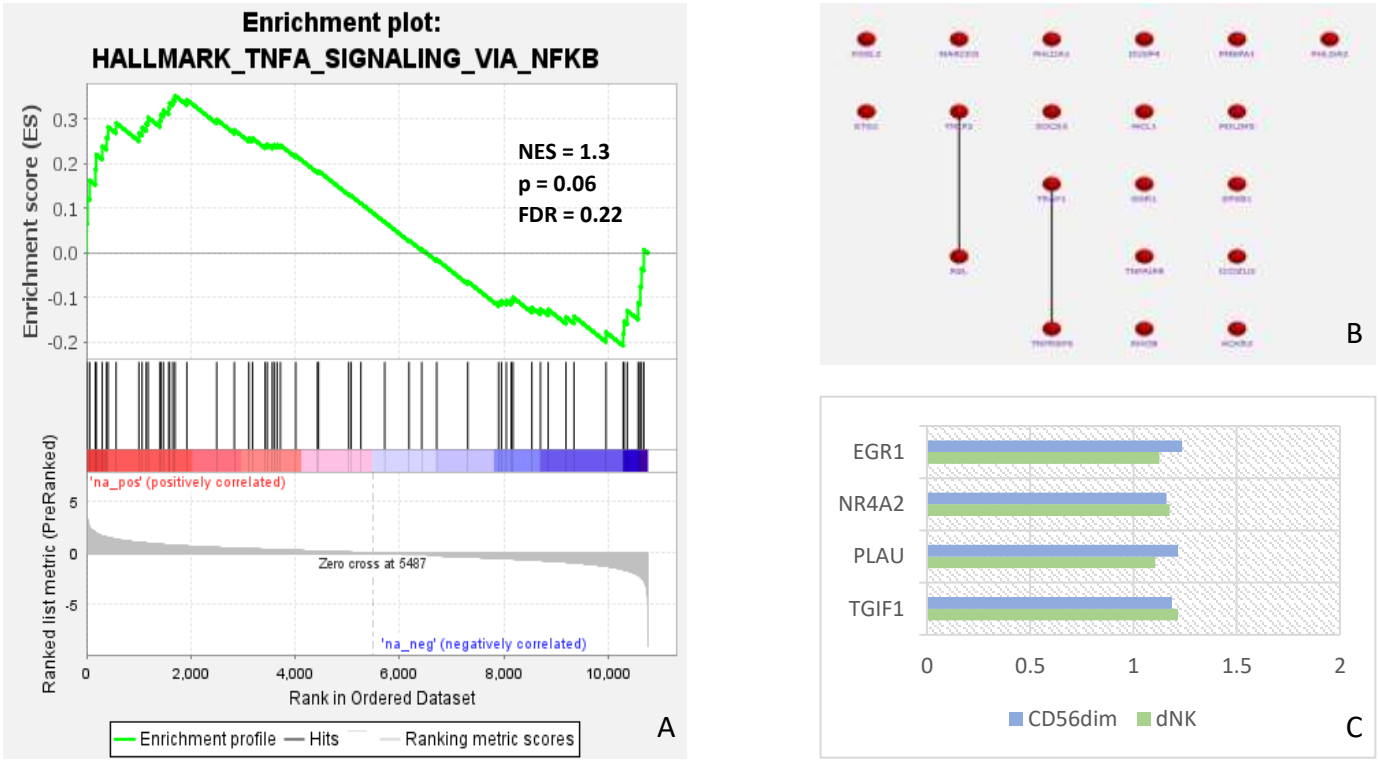


Figure 4.6 **A)** Gene set enrichment analysis showing positive correlation of dNK cells with the TNF-A via NFK-B signaling. **B)** Interaction analysis of the genes enriched for the TNF-A via NFK-B signaling. **C)** The fold enrichment of transcriptional factors involved in the TNF-A via NFK-B signaling in dNK NK cells.

5. Epithelial mesenchymal transition is positively correlated with the dNK cells. The positive enrichment scores indicate the upregulation of the transition in dNK cells. The genes that are enriched in dNK cells for the epithelial-mesenchymal transition are: SERPINE2, PVR, RHOB, CD59, MGP, ABI3BP, ITGA2, PRRX1, TPM4, LUM, NNMT, SFRP1, QSOX1, SGCB, THBS1, SGCD, PMEPA1, COL11A1, LOXL2, CADM1, TAGLN, FSTL1, CDH11, and CDH6.

   The transcriptional factors associated with the pathway are: MSX1 and PRRX1. The TFs are highly enriched in dNK cells. The higher enrichment values in epithelial mesenchymal transition for the TFs in dNK cells correlates to the high enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between SGCB and SGCD but not with the other genes enriched in the response.

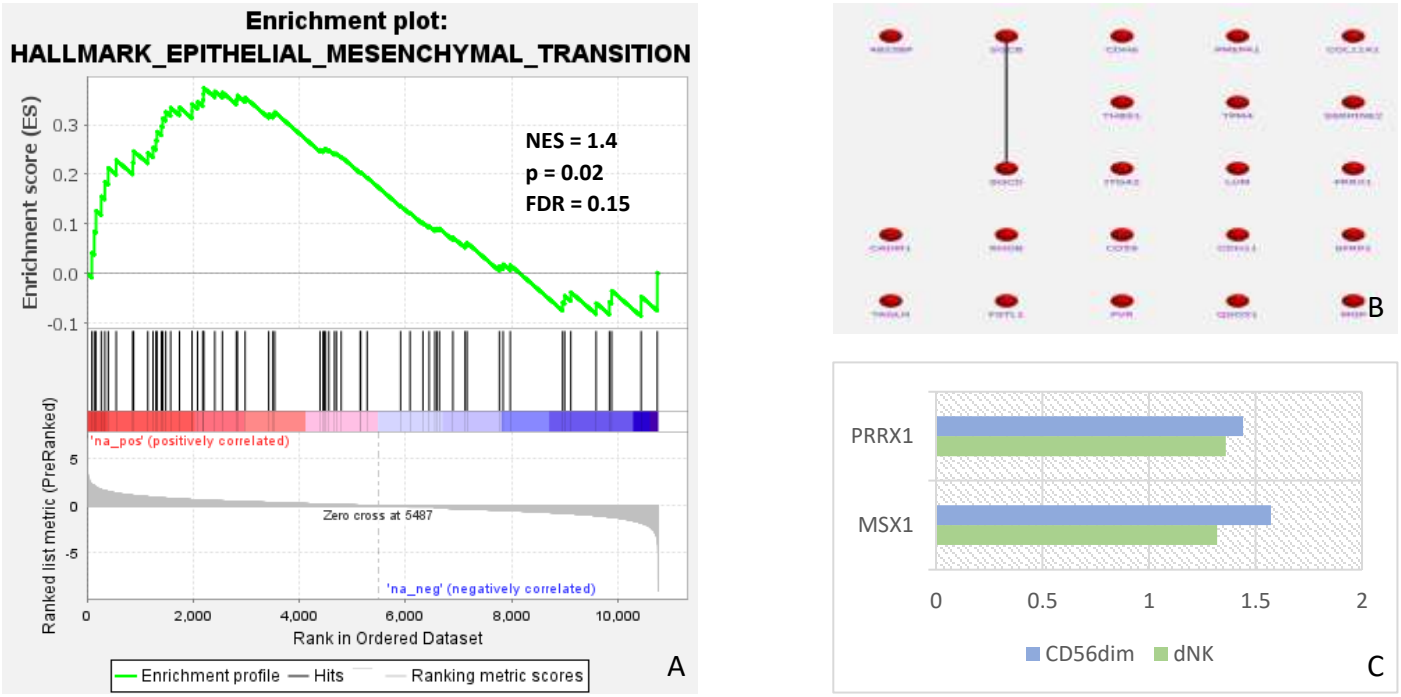


Figure 4.7 **A)** Gene set enrichment analysis showing positive correlation of dNK cells with the epithelial-mesenchymal transition. **B)** Interaction analysis of the genes enriched for the epithelial-mesenchymal transition. **C)** The fold enrichment of transcriptional factors involved in the epithelial-mesenchymal transition in dNK cells.

6. Inflammatory response is positively correlated with the dNK cells. The positive enrichment scores indicate the upregulation of the transition in dNK cells. The genes that are enriched in dNK cells for inflammatory response are: CD82, GPR132, OSMR, PVR, ROS1, KCNMB2, TACR1, CHST2, ITGB8, SLC7A2, ICOSLG, ATL3, IRAK2, TNFRSF9, SLAMF1, OSM, TPBG, TNFSF15, HAS2, and HBEGF.

   The transcriptional factors associated with the pathway are: IRF1 and MYC. The TFs are highly enriched in dNK cells. The higher enrichment values in inflammatory response for the TFs in dNK cells correlates to the high enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between HBEGF, CD82 and OSM, OSMR but not with the other genes enriched in the response.

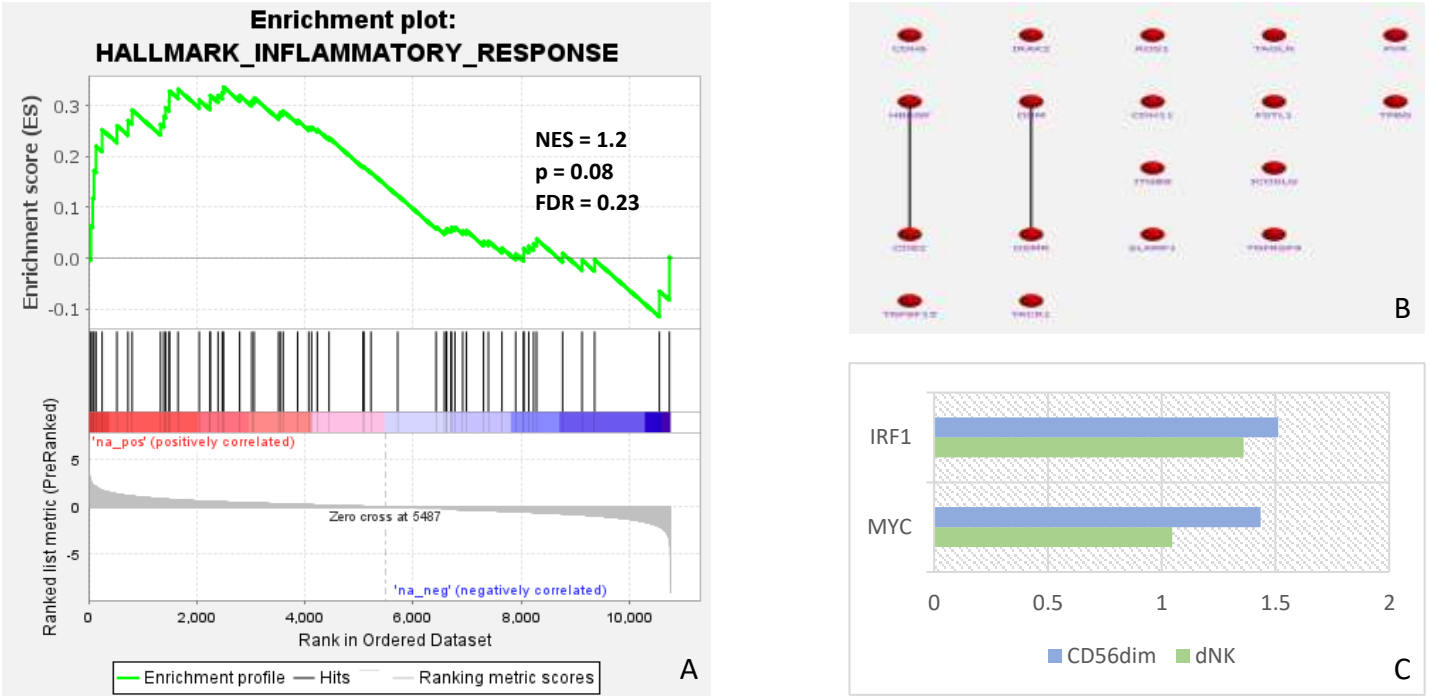


Figure 4.8 **A)** Gene set enrichment analysis showing positive correlation of dNK cells with the inflammatory response. **B)** Interaction analysis of the genes enriched for the inflammatory response. **C)** The fold enrichment of transcriptional factors involved in the inflammatory response in dNK.

4.3.2 dNK v. CD56^{bright} NK

1. Interferon gamma response is negatively correlated with the dNK cells. The negative enrichment scores indicate the downregulation of the response in dNK cells c. The genes that are enriched in CD56^{bright} for the interferon gamma response are: CMPK2, NFKBIA, LYSMD2, AUTS2, SPPL2A, PTPN2, OAS3, RSAD2, CMKLR1, GBP4, IFIT3, ZBP1, PLSCR1, RNF213, B2M, NLRC5, SLAMF7, PSMA3, LATS2, IFIT2, and PELI1.

   The transcriptional factors associated with the pathway are: IRF1 and STAT1. The TFs are highly enriched in CD56^{bright} NK cells compared to the dNK cells. The lower enrichment values in IFN-gamma response for the TFs in dNK cells correlates to the lower enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between IFIT2, IFIT3, and SPPL2A but not with the other genes enriched in the response.

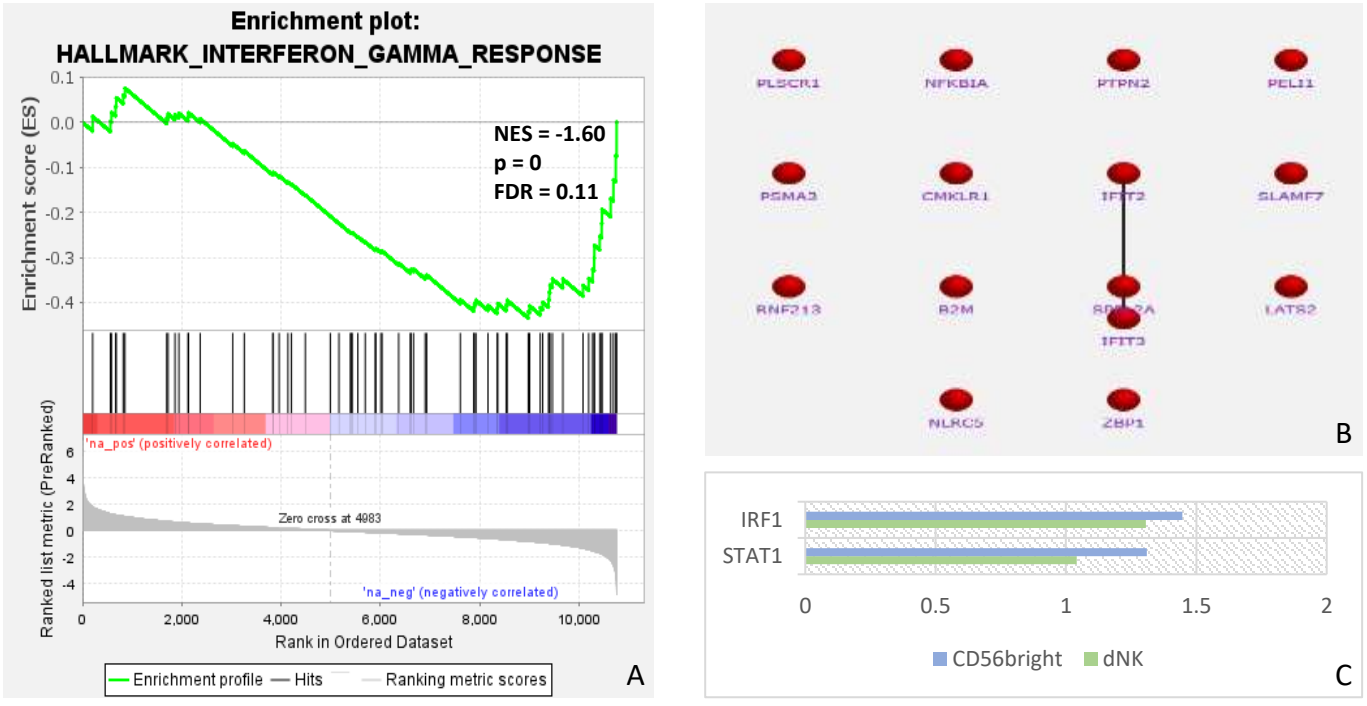


Figure 4.9 **A)** Gene set enrichment analysis showing negative correlation of dNK cells with the Interferon gamma pathway. **B)** Interaction analysis of the genes enriched for the IFN-gamma response. **C)** The fold enrichment of transcriptional factors involved in the interferon-gamma response in dNK compared to the CD56^{bright} NK cells.

2. Interferon alpha response is negatively correlated with the dNK cells. The negative enrichment scores indicate the downregulation of the response in dNK cells. The genes that are enriched in CD56^{bright} for the interferon alpha response are: GBP2, RSAD2, CD47, NUB1, GBP4, IFIT3, PLSCR1, B2M, NCOA7, MOV10, PSMA3, and IFIT2.

   The transcriptional factor associated with the pathway is: IRF1. The TF is enriched in CD56^{bright} NK cells. The lower enrichment values in IFN-alpha response for the TFs in dNK cells correlates to the lower enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between IFIT2, IFIT3, and NUB1 but not with the other genes enriched in the response.

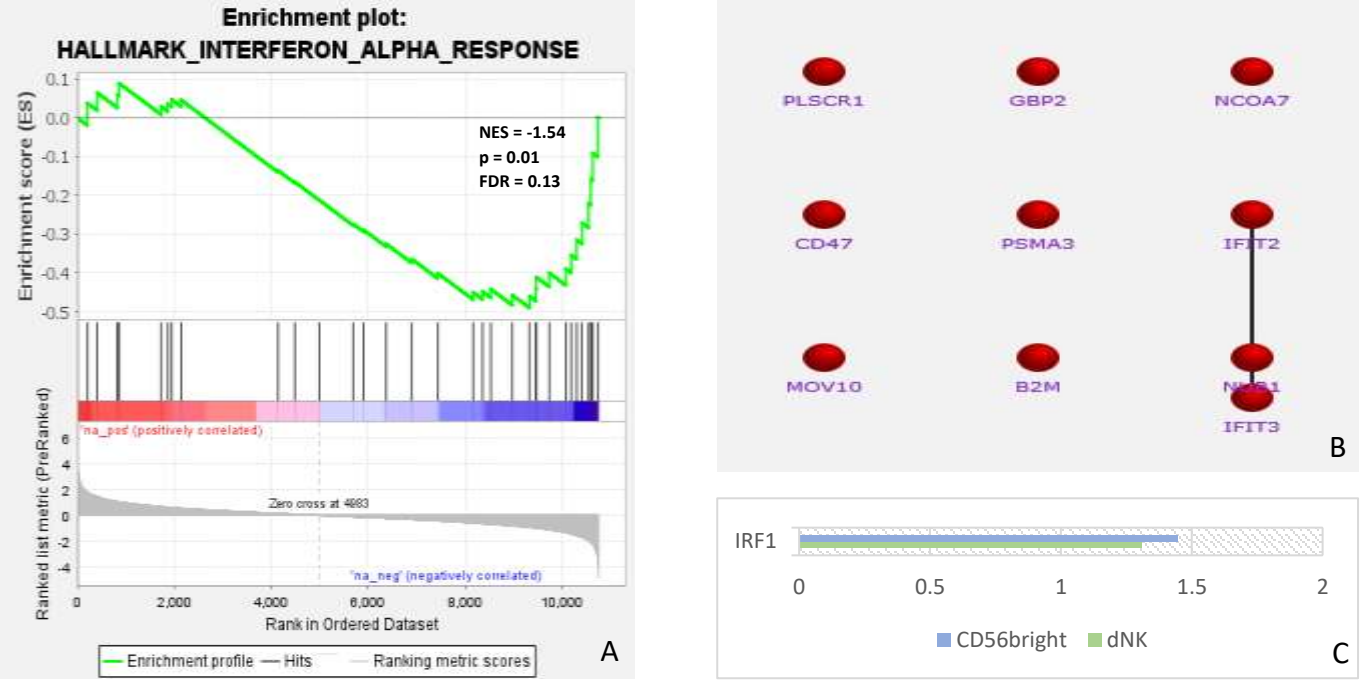


Figure 4.10 **A)** Gene set enrichment analysis showing negative correlation of dNK cells with the Interferon alpha pathway. **B)** Interaction analysis of the genes enriched for the IFN-alpha response. **C)** The fold enrichment of transcriptional factor involved in the interferon-alpha response in dNK cells.

3. TNF-A signaling via NFK-B is positively correlated with the dNK cells. The positive enrichment scores indicate the upregulation of the response in dNK cells. The genes that are enriched in dNK cells for the TNF-A signaling via NFK-B are: PHLDA1, EGR1, MCL1, PDLIM5, DUSP4, TNIP2, FOSL2, TNC, RHOB, SPSB1, DDX58, JAG1, REL, TRAF1, and PHLDA2.

   The transcriptional factors associated with the pathway are: CEBPD, IRF1, ATF3, PLAU, MYC, and TGIF1. The TFs are highly enriched in dNK cells. The higher enrichment values in TNF-A signaling via NFK-B for the TFs in dNK cells correlates to the high enrichment score of the response in dNK cells.

   The interaction analysis shows an interaction between TNIP2 and REL but not with the other genes enriched in the response.

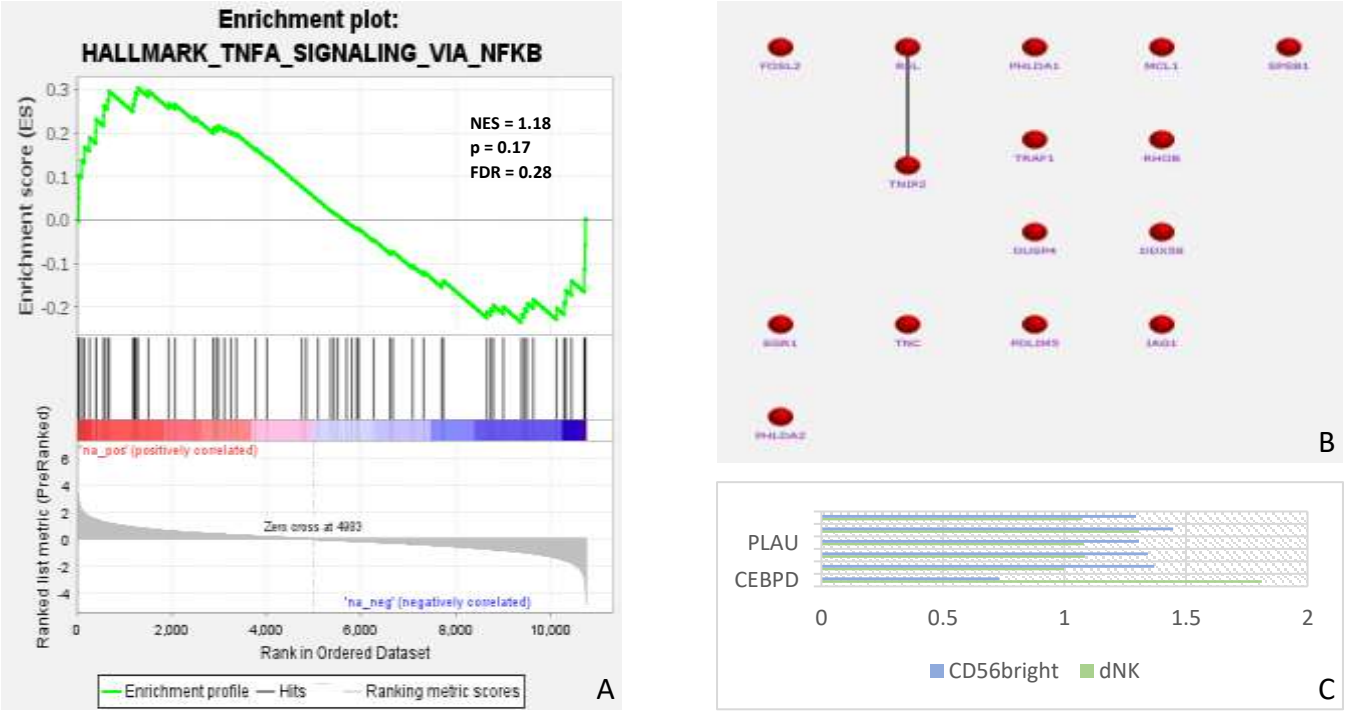


Figure 4.11 **A)** Gene set enrichment analysis showing positive correlation of dNK cells with the TNF-A via NFK-B signaling. **B)** Interaction analysis of the genes enriched for the TNF-A via NFK-B signaling. **C)** The fold enrichment of transcriptional factors involved in the TNF-A via NFK-B signaling in dNK cells.

4. Epithelial-mesenchymal transition is positively correlated with the dNK cells. The positive enrichment scores indicate the upregulation of the transition in dNK cells. The genes that are enriched in dNK cells for the epithelial-mesenchymal transition are: ABI3BP, TPM4, PVR, NT5E, TNC, RHOB, QSOX1, CD59, and LUM.

   The transcriptional factors associated with the pathway are: MSX1 and PRRX1. The TFs are enriched in dNK cells. The higher enrichment values in epithelial mesenchymal transition for the TFs in dNK cells correlates to the high enrichment score of the response in dNK cells.

   The interaction analysis shows no interaction between the genes enriched in the response.

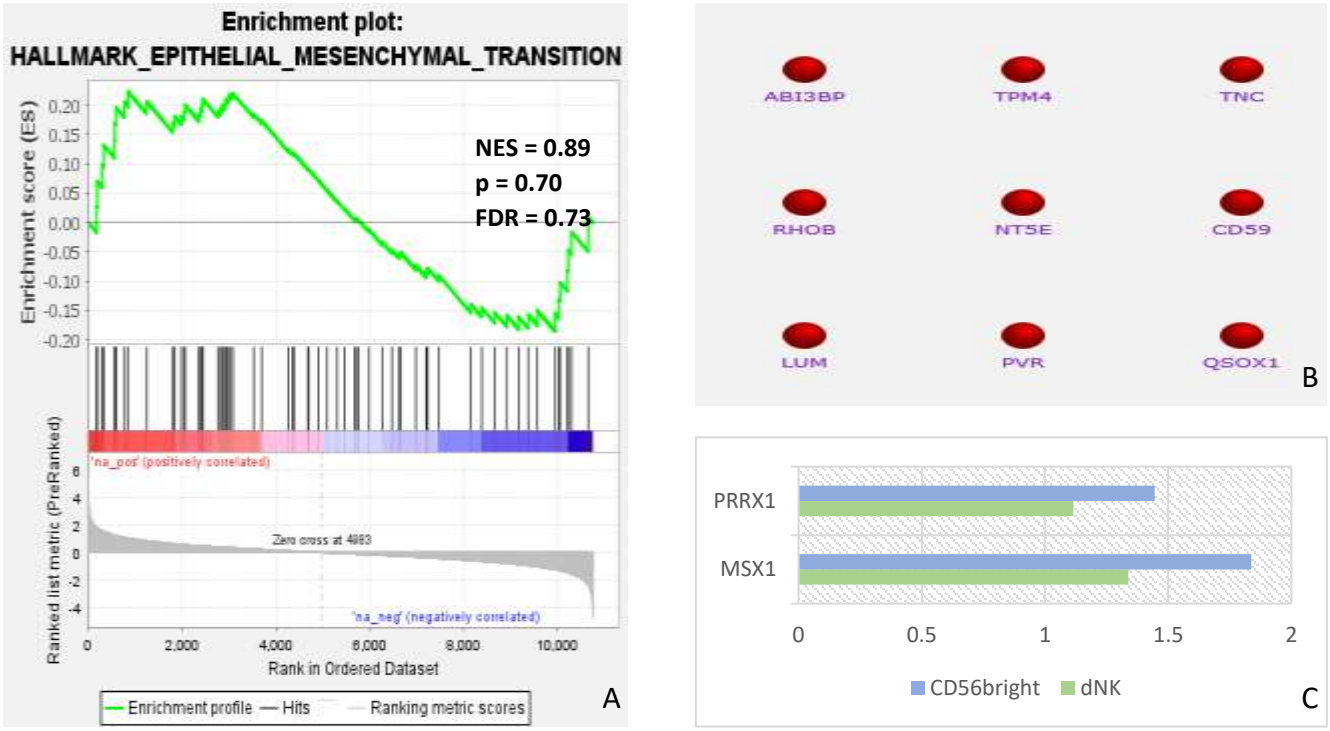


Figure 4.12 **A)** Gene set enrichment analysis showing positive correlation of dNK cells with the epithelial-mesenchymal transition. **B)** Interaction analysis of the genes enriched for the epithelial-mesenchymal transition. **C)** The fold enrichment of transcriptional factors involved in the epithelial-mesenchymal transition in dNK cells.

4.3.3 HCC pNK v. Healthy pNK

1. Interferon alpha response positively correlated with the HCC pNK cells. The positive enrichment scores indicate the upregulation of the response in HCC pNK cells. The genes that are enriched in HCC pNK cells for the interferon alpha response are: TXNIP, IL7, ISG20, BATF2, ELF1, IL15, CASP1, GMPR, IFI27, CD47, RTP4, IFI44, DHX58, IFI44L, SP110, C1S, UBA7, PARP9, IRF9, and DDX60.

   The interaction analysis does not show any interaction between the genes enriched for the interferon alpha response.

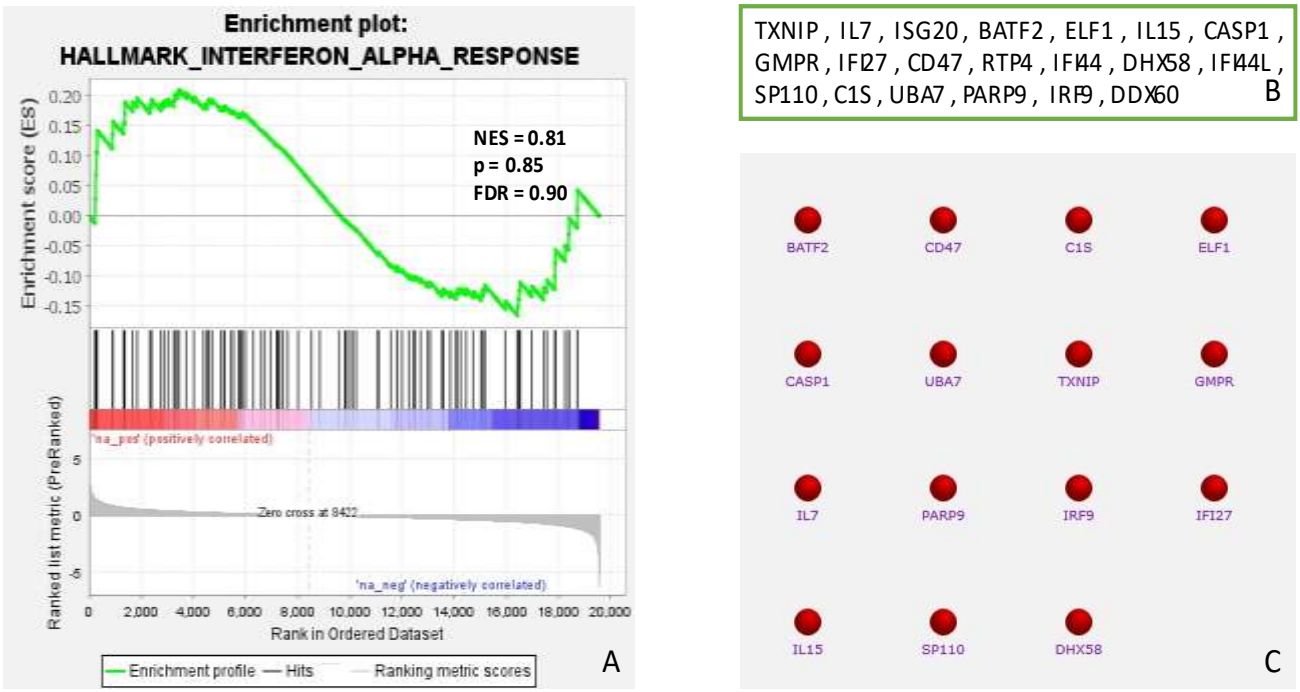
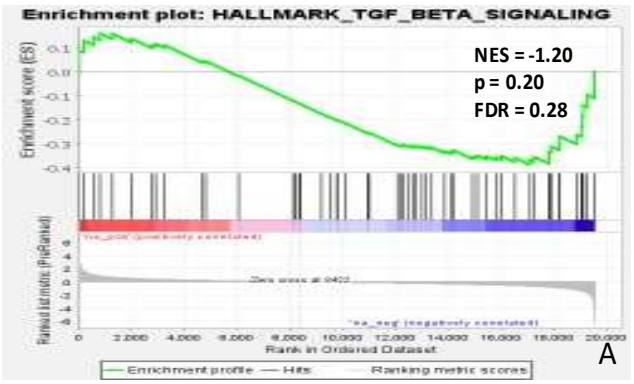


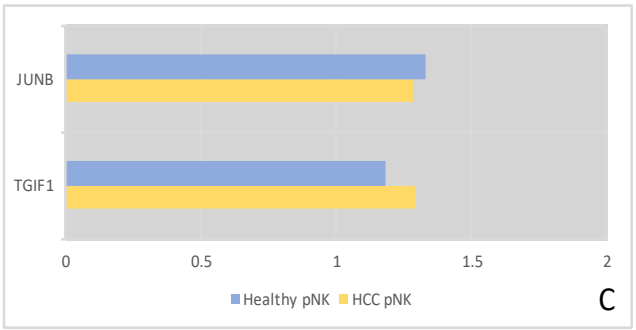
Figure 4.13 **A)** Gene set enrichment analysis showing positive correlation of HCC pNK cells with the Interferon alpha response. **B)** Genes enriched in HCC pNK cells for interferon alpha response **C)** Interaction analysis of the genes enriched for the inflammatory response.

2. TGF-beta signaling is negatively correlated with the HCC pNK cells. The negative enrichment scores indicate the downregulation of the response in HCC pNK cells compared to healthy pNK cells. The genes that are enriched in healthy pNK cells for the TGF-beta signaling are: TGFBR1, TGFB1, TJP1, PPP1R15A, CDKN1C, SKI, SPTBN1, SMAD7, KLF10, PMEPA1, BMP2, JUNB, and ID1.

   The transcriptional factors associated with the pathway are: TGIF1 and JUNB. The TFs are highly enriched in healthy pNK cells compared to the HCC pNK cells. The lower enrichment values in TGF-beta signaling response for the TFs in HCC pNK cells correlates to the lower enrichment score of the response in HCC pNK cells.

   The interaction analysis shows an interaction between SMAD7, TGFBR1, TGFB1, and BMP2 but not with the other genes enriched in the response.





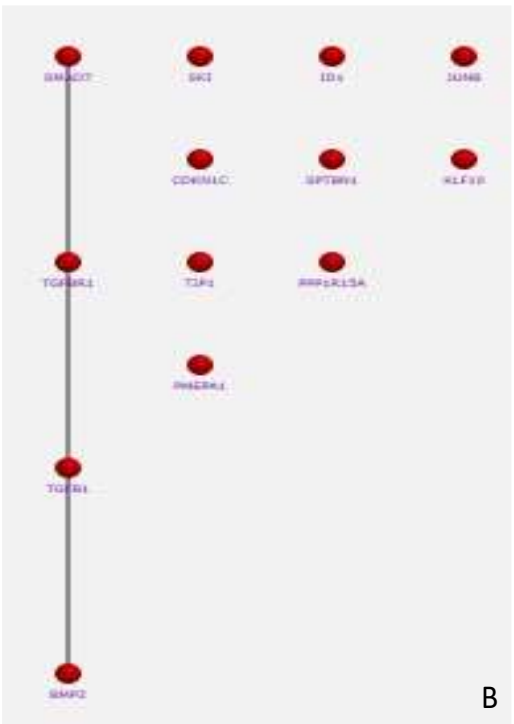


Figure 4.14 **A)** Gene set enrichment analysis showing negative correlation of HCC pNK cells with the TGF-beta signaling. **B)** Interaction analysis of the genes enriched for the TGF-beta signaling. **C)** The fold enrichment of transcriptional factors involved in the TGF-beta signaling in HCC pNK cells compared to the healthy pNK cells.

3. TNF-A signaling via NFK-B is negatively correlated with the HCC pNK cells. The negative enrichment scores indicate the downregulation of the response in HCC pNK cells compared to healthy pNK cells. The genes that are enriched in healthy pNK cells for the TNF-A signaling via NFK-B are: JAG1, CLCF1, ZFP36, TNFSF9, IL1A, CFLAR, BCL3, DUSP4, NINJ1, PTX3, LIF, RNF19B, SGK1, KYNU, LDLR, NFKB2, PPP1R15A, CCRL2, CSF1, CD44, SPSB1, GEM, NFKBIA, BHLHE40, DRAM1, PLAUR, NFKBIE, PLAU, RELA, CDKN1A, PTGS2, IRF1, DENND5A, RELB, FOSL1, EFNA1, IL6, IRS2, GPR183, IL23A, CD69, IFIT2, KLF4, GADD45A, NR4A2, MAFF, FOS, TNFAIP2, FOSL2, ZBTB10, MARCKS, CXCL6, PDE4B, KDM6B, EGR3, MSC, RHOB, KLF10, ZC3H12A, PMEPA1, BMP2, MYC, IER2, CCND1, ICAM1, IER5, PDLIM5, SIK1, SOCS3, JUNB, GADD45B, PLK2, FOSB, EGR2, OLR1, BTG2, PHLDA1, JUN, PHLDA2, CCL20, G0S2, CSF2, NR4A3, TNFRSF9, CXCL2, CD83, CXCL3, TNF, IL1B, NR4A1, IER3, EGR1, and HES1.

   The transcriptional factors associated with the pathway are: EGR1, PLAU, NR4A2, JUNB, JUN, FOSB, FOS, and TGIF1. The TFs are highly enriched in healthy pNK cells compared to the HCC pNK cells. The lower enrichment values in TNF-A signaling via NFK-B for the TFs in HCC pNK cells correlates to the lower enrichment score of the response in HCC pNK cells.

   The interaction analysis shows an interaction between IRS2 and SOCS3, PLAUR and PLAU, CSF1 and TNF, SGK1 and MARCKS, and IL1A, NFKBIE, FOSB, FOSL1, JUNB, FOS, BCL3, NFKB2, RELB, NFKBIA, PPP1R15A, MYC, RELA, JUN, FOSL2, CCND1, GADD45B, GADD45A, CDKN1A, EGR1, and IRF1 but not with the other genes enriched in the response.

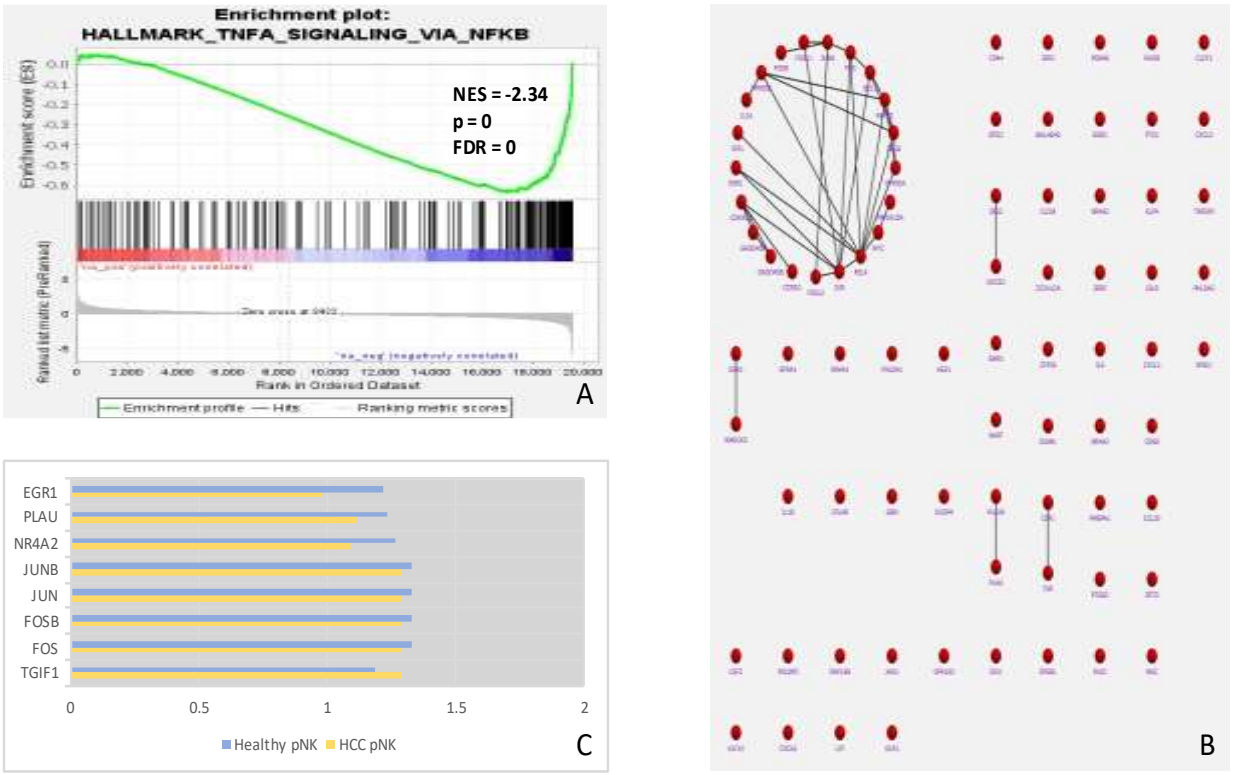


Figure 4.15 **A)** Gene set enrichment analysis showing negative correlation of HCC pNK cells with the TNF-A signaling via NFK-B. **B)** Interaction analysis of the genes enriched for the TNF-A signaling via NFK-B. **C)** The fold enrichment of transcriptional factors involved in the TNF-A signaling via NFK-B in HCC pNK cells compared to the healthy pNK cells.

4. Epithelial-mesenchymal transition is negatively correlated with the HCC pNK cells. The negative enrichment scores indicate the downregulation of the response in HCC pNK cells compared to healthy pNK cells. The genes that are enriched in healthy pNK cells for the epithelial-mesenchymal transition are: QSOX1, TNFAIP3, FLNA, APLP1, COMP, MMP14, BGN, SERPINH1, FMOD, WIPF1, TFPI2, SDC1, SLC6A8, TPM4, COL7A1, TGFBR3, FOXC2, IL32, LAMC2, CTHRC1, SNAI2, PDLIM4, LRRC15, PLOD3, COL1A1, FGF2, NT5E, ELN, CXCL12, COL6A3, TNFRSF12A, IGFBP4, ANPEP, GPC1, COL12A1, LOXL1, LAMA1, TGFB1, CAPG, PTX3, EMP3, LAMC1, TGFBI, CD44, GEM, THBS2, IGFBP3, PLAUR, COL8A2, CDH6, MATN2, DST, SNTB1, CALD1, MSX1, IL6, CDH11, GADD45A, PVR, CXCL6, SLIT2, FBLN1, HTRA1, RHOB, FAP, PMEPA1, SERPINE2, GADD45B, ADAM12, CXCL8 , SCG2, JUN, and PRRX1.

The transcriptional factor associated with the pathway is: JUN. The TF is highly enriched in healthy pNK cells compared to the HCC pNK cells. The lower enrichment values in epithelial-mesenchymal transition for the TF in HCC pNK cells correlates to the lower enrichment score of the response in HCC pNK cells.

The interaction analysis shows an interaction between GADD45A, GADD45B and FAP, PLAUR and HTRA1, NT5E, LAMA1, LAMC2, COL7A1, MMP14, CXCL8, SDC1, FGF2, CD44, MATN2, COL1A1, IGFBP3, ADAM12, FBLN1, LOXL1, ELN, FMOD, TGFER3, TGFB1, BGN but not with the other genes enriched in the response.

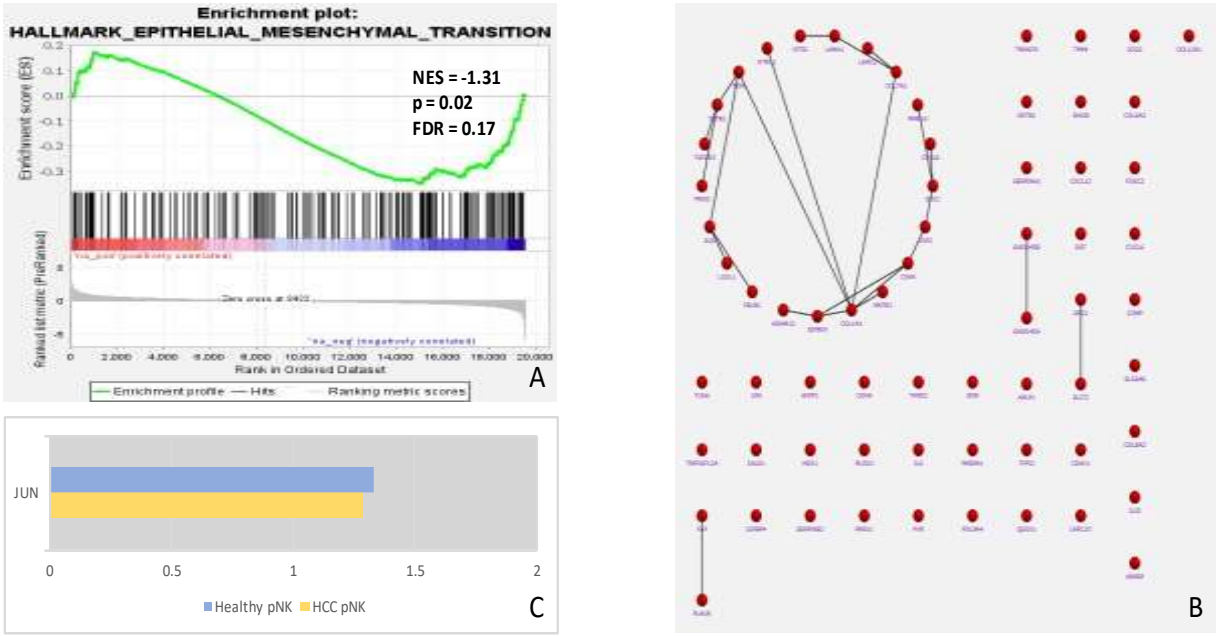


Figure 4.16 **A)** Gene set enrichment analysis showing negative correlation of HCC pNK cells with the epithelial-mesenchymal transition. **B)** Interaction analysis of the genes enriched for the epithelial-mesenchymal transition. **C)** The fold enrichment of transcriptional factors involved in the epithelial-mesenchymal transition in HCC pNK cells compared to the healthy pNK cells.

5. Inflammatory response is negatively correlated with the HCC pNK cells. The negative enrichment scores indicate the downregulation of the response in HCC pNK cells compared to healthy pNK cells. The genes that are enriched in healthy pNK cells for the inflammatory response are: SLC28A2, NAMPT, HBEGF, GNA15, CD14, CSF3, SLAMF1, ACVR1B, MET, CCR7, CCL22, IL2RB, FZD5, F3, MEFV, TNFSF10, NDP, OSM, AHR, TNFSF9, IL1A, LIF, EMP3, OPRK1, VIP, LAMP3, AXL, BDKRB1, LDLR, CCRL2, ICAM4, CSF1, TAPBP, NFKBIA, IRAK2, SLC4A4, STAB1, CX3CL1, PLAUR, FFAR2, NLRP3, RELA, CDKN1A, CMKLR1, RASGRP1, SELL, IRF1, RGS1, IL6, LTA, GPR183, CD69, PVR, CXCL6, PDE4B, MARCO, MYC, EREG, ICAM1, TNFSF15, OLR1, CXCL8, BTG2, CCL20, SLC1A2, ITGB8, CXCR6, TNFRSF9, IL1B, and RGS16.

   The interaction analysis shows an interaction between RELA, MYC, AHR, IRF1, NFKBIA and GNA15, OPRK1 but not with the other genes enriched in the response.

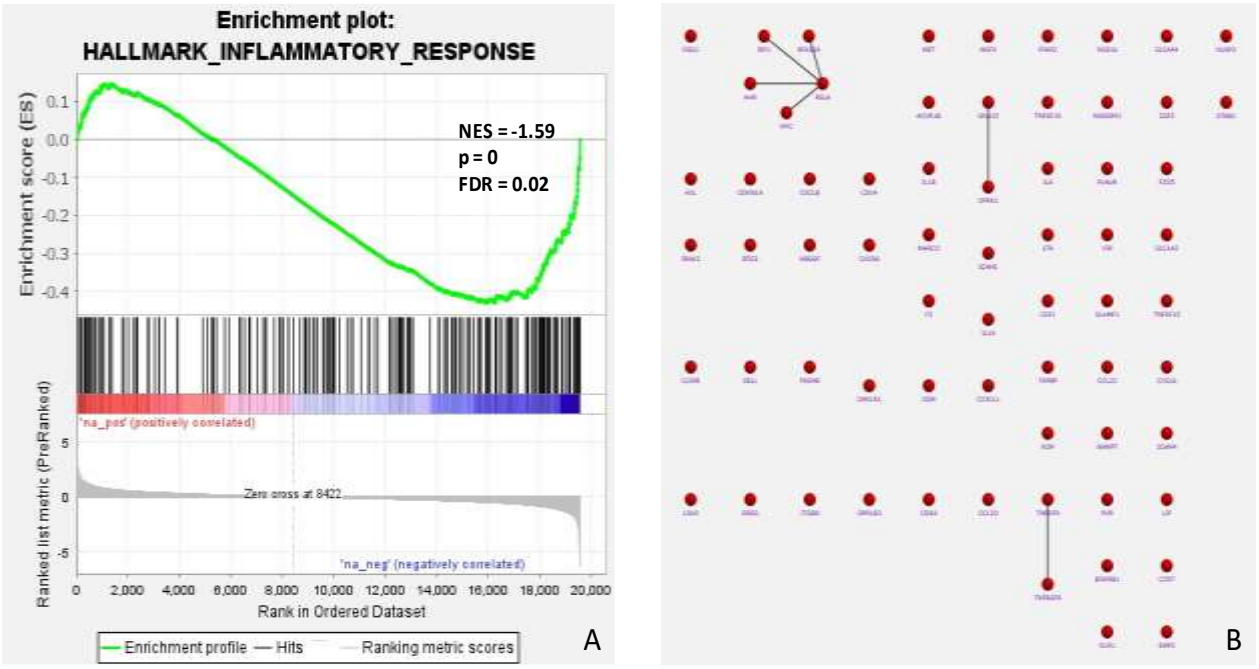


Figure 4.17 **A)** Gene set enrichment analysis showing negative correlation of HCC pNK cells with the inflammatory response. **B)** Interaction analysis of the genes enriched for the inflammatory response.

CHAPTER 5: DISCUSSION & CONCLUSION

Different populations of NK cells are found in the body (Human and mice), but the thesis focuses on the two extremes; pNK and uNK cells. Besides, differing in granular appearance and abundance, pNK and uNK cells differ vastly in regulation of same and different transcriptional factors. [31] This difference in expression of TFs by pNK and uNK can be correlated to the biological pathways they are involved in and the reason behind the differences in cytotoxicity between the two.

TFs have been reportedly involved in preventing diseases and causing severe conditions with lack of their expression. For instance, PBX-1 has been found to be repressed in recurrent spontaneous abortion patients indicating their role in helping the uNK cells carry out a smooth pregnancy. [61] Several other TFs such as GATA-3 has been observed to prevent infections by *Listeria monocytogenes* and a repression of GATA-3 would cause the infection. [78]

Therefore, studying the expression of different transcriptional factors in the two populations of NK cells becomes important to further design immunotherapeutics modulating these TFs.

In the gene set enrichment analysis, the two pNK subsets ($CD56^{dim}$ and $Cd56^{bright}$) has been compared with dNK cells to look out for biological pathways enriched in the population.

The enrichment stats from GSEA indicated the enrichment of TNF-A signaling via NFK-B and Epithelial to mesenchymal transition in dNK cells while the cytotoxic responses such as interferon-gamma response, interferon-alpha response, and TGF-beta responses have negative enrichment scores. The results indicate the enrichment of these cytotoxic responses in $CD56^{dim}$/$CD56^{bright}$ NK cells rather than dNK cells. Similar results were obtained when dNK was compared with CD56dim or with Cd56bright except the absence of TGF-beta signaling when the genes for dNK and $CD56^{bright}$ NK cells were enriched.

To look out for biological pathways and genes enriched in NK cells in diseased state, HCC pNK cells were compared to the healthy pNK cells. HCC provides a tumor microenvironment that is an amalgamation of multiple pathways, ultimately covering all the pathways, making an ideal environment to study differences.

HCC pNK cells were enriched in interferon-gamma response and with negative enrichment scores for TGF-beta signaling, TNF-A signaling via NFK-B, epithelial-mesenchymal

transition, and inflammatory responses. The response with negative enrichment scores for HCC pNK cells is positively enriched in healthy pNK cells. Sung et al also mentioned how the NK cells in HCC patients have low cytotoxic potential and decreased production of cytokines. [71] The results here clearly indicate the downregulation of cytotoxic pathways in HCC pNK cells (Fig. 4.13 & 4.14) confirming the fact that low cytotoxic potential of HCC pNK results from the negative enrichment of cytotoxic pathways in HCC pNK cells.

Important transcription factors enriched in the results

STAT-1 has been reported to be involved in regulating the effector functions of pNK cells. [11] The results show STAT-1 enriched in interferon-gamma response for both $CD56^{dim}$ (Fig. 4.3) and $CD56^{bright}$ (Fig. 4.9) pNK cells which is true to the expected regulation as per the literature.

IRF1 is an important transcription factor for pNK cell development. Mice deficient in IRF1 has been observed to lack pNK cells entirely. [11], [79], [80] The results obtained indicate the enrichment of IRF1 in interferon gamma (Fig. 4.3 & 4.9), interferon alpha (4.4 & 4.10), inflammatory responses (Fig. 4.8), and TNF-A signaling via NFK-B (Fig. 4.11) for $CD56^{dim}$, $CD56^{bright}$ pNK cells. IRF1 has also been observed to be enriched in dNK cells in the TNF-A signaling via NFK-B.

CEBPD is involved in NK cell function but the deficiency of CEBP does not affect the numbers of pNK cells.[11] The results indicate enrichment of CEBPD in TNF-A signaling via NFK-B (Fig. 4.11) for dNK cells indicating their role in the pathway in dNK cells.

The meta-analysis becomes important here to understand the overall impact of transcriptional regulation and ultimately helping in designing immunotherapeutics.

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APPENDICES

APPENDIX A Statistical analysis using R studio and the R script retrieved from GEO2R analysis.

A.1 dNK v. CD56dim NK cells

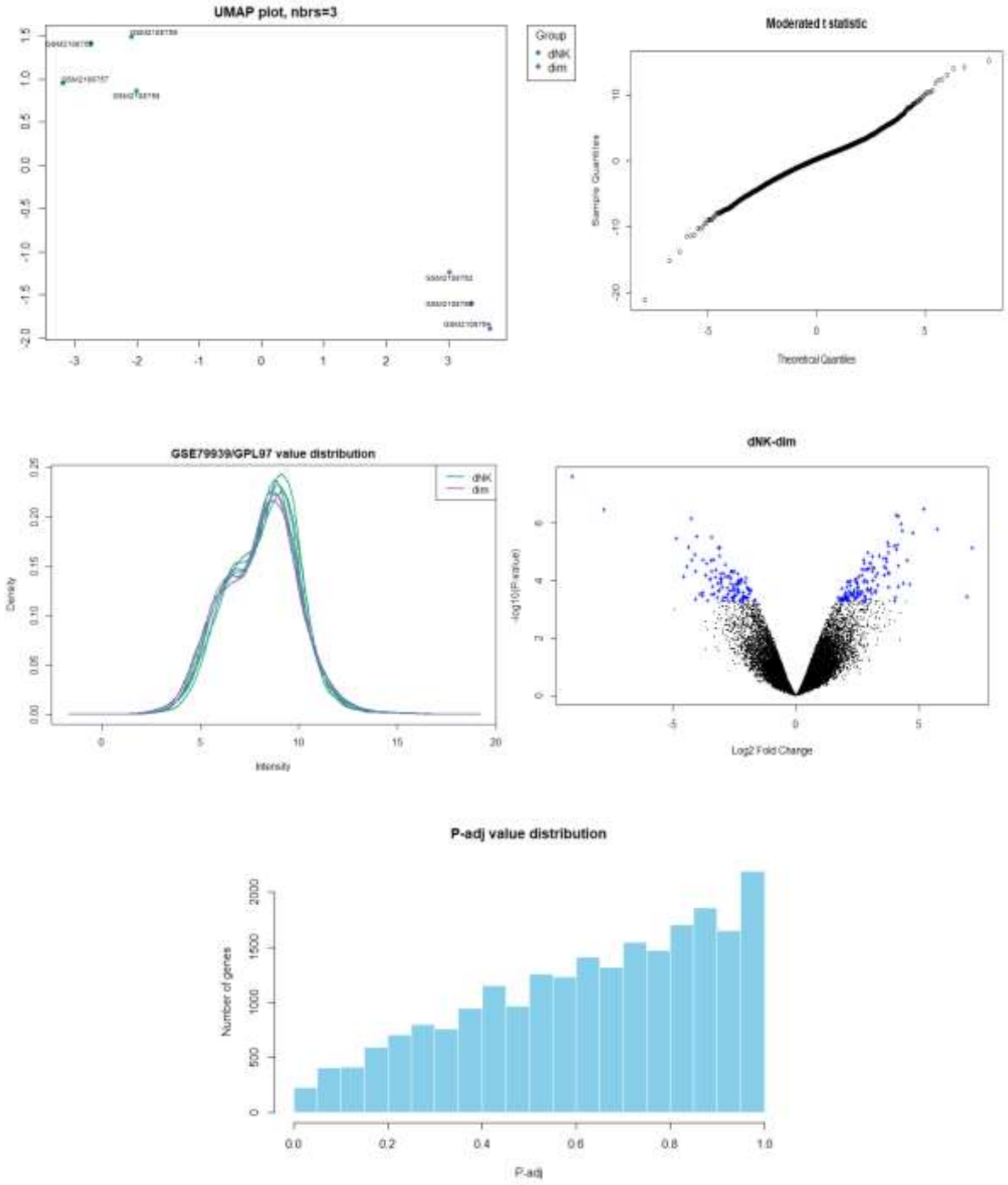


Figure A.1.1 A) UMAP plot B) t-moderated statistic C) Density plot D) Volcano plot E) Histogram showing adjusted p-value distribution in the sample.

A.2 dNK v. CD56bright NK cells

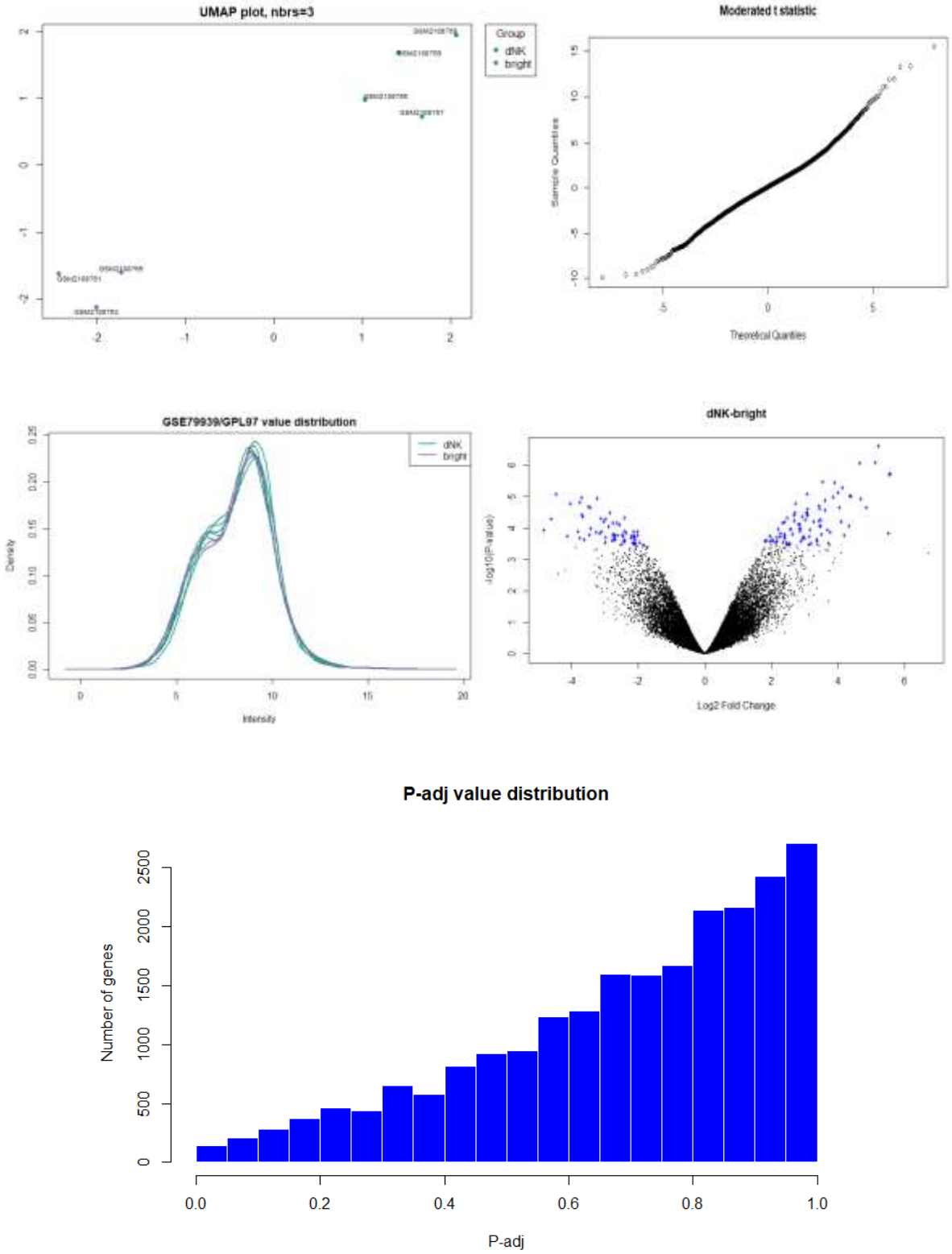


Figure A.2.1 A) UMAP plot B) t-moderated statistic C) Density plot D) Volcano plot E) Histogram showing adjusted p-value distribution in the sample.

A.3 HCC pNK cells v. Healthy pNK cells

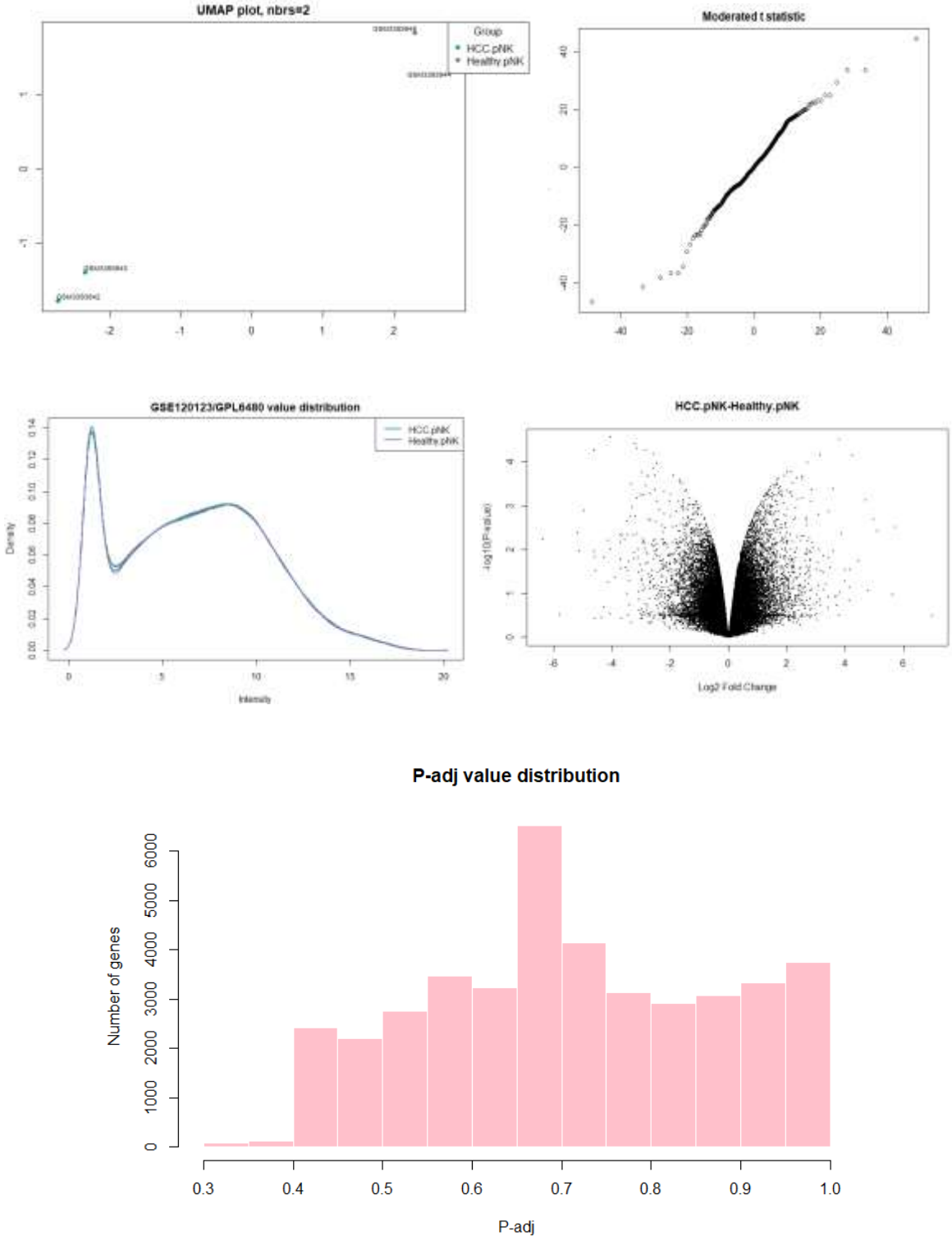
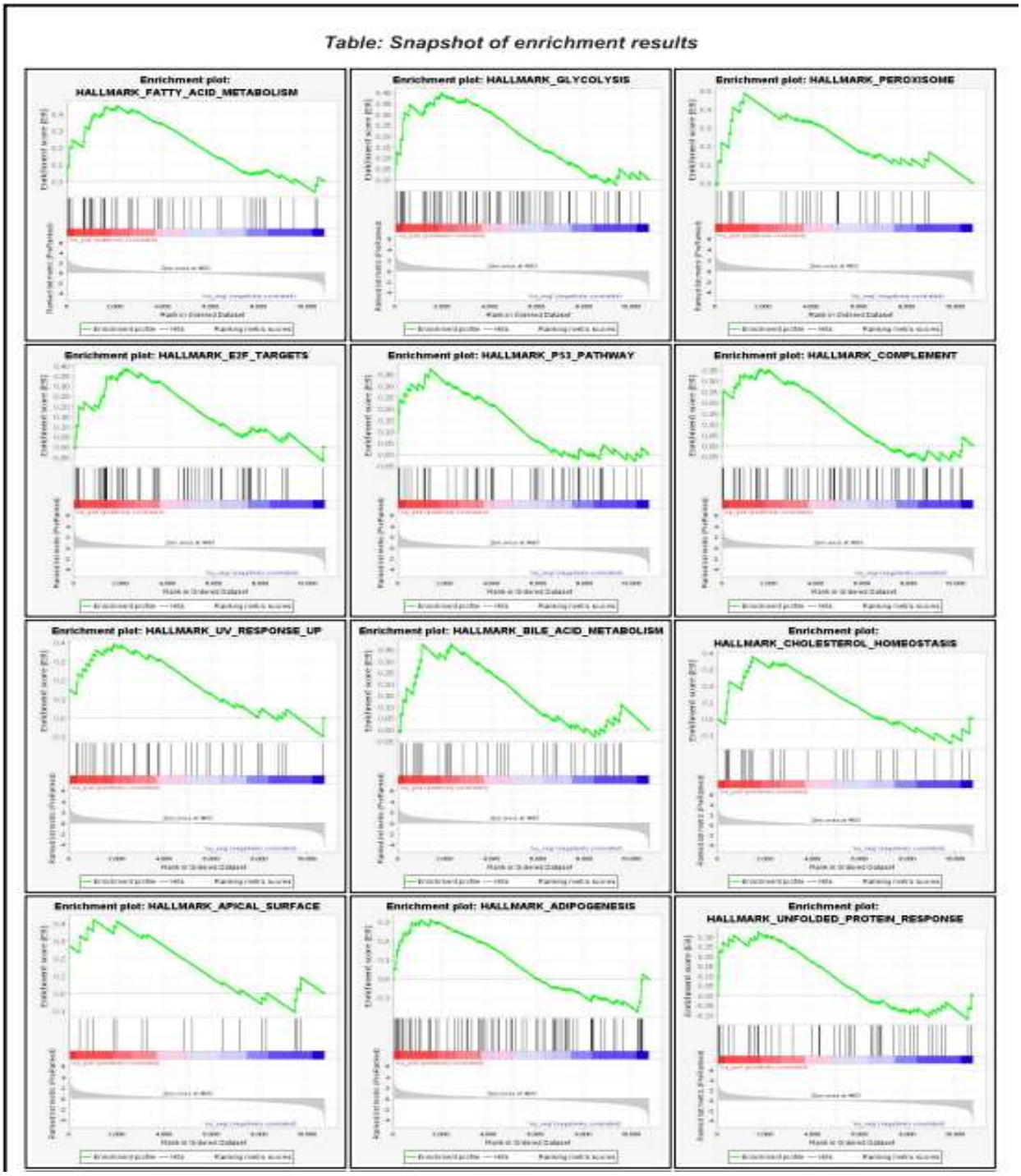


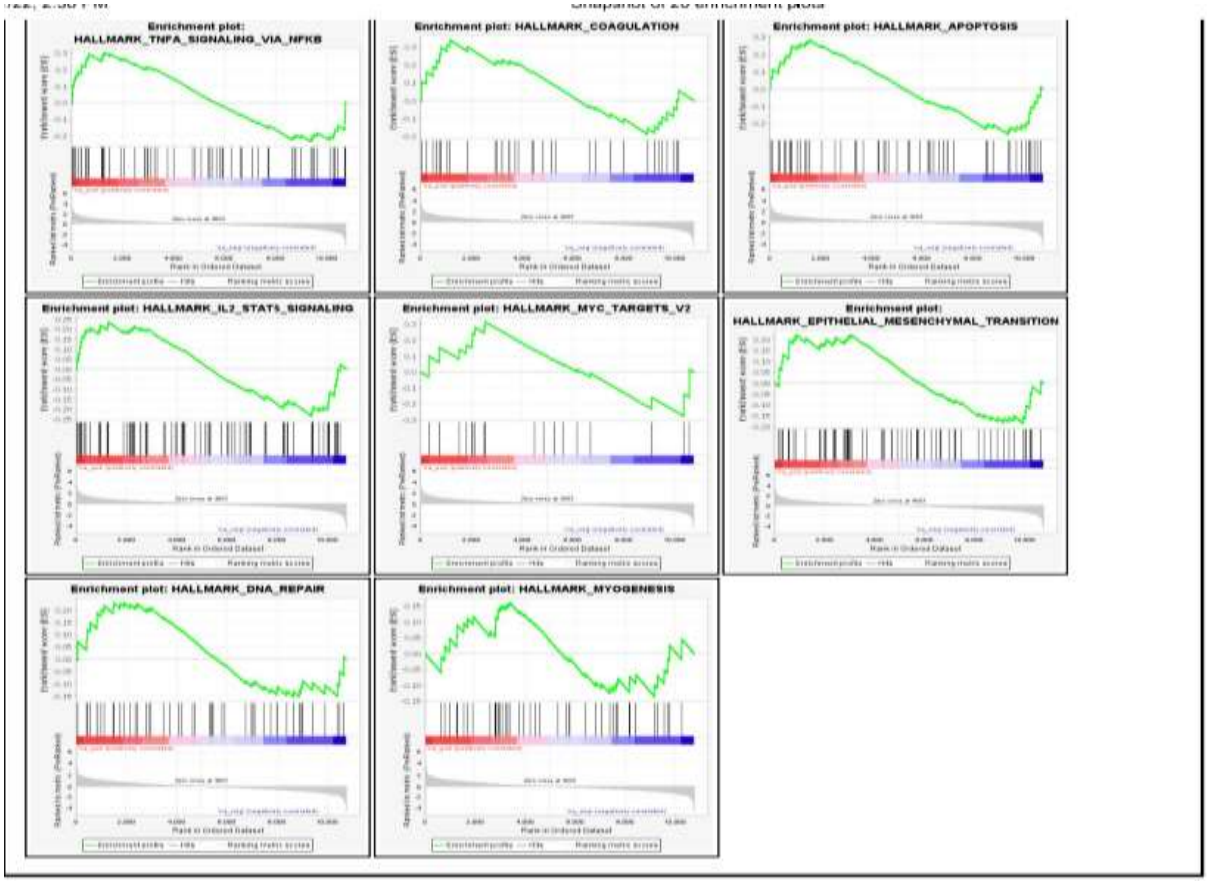
Figure A.3.1 A) UMAP plot B) t-moderated statistic C) Density plot D) Volcano plot E) Histogram showing adjusted p-value distribution in the sample.

APPENDIX B Gene enrichment results from GSEA software analysis

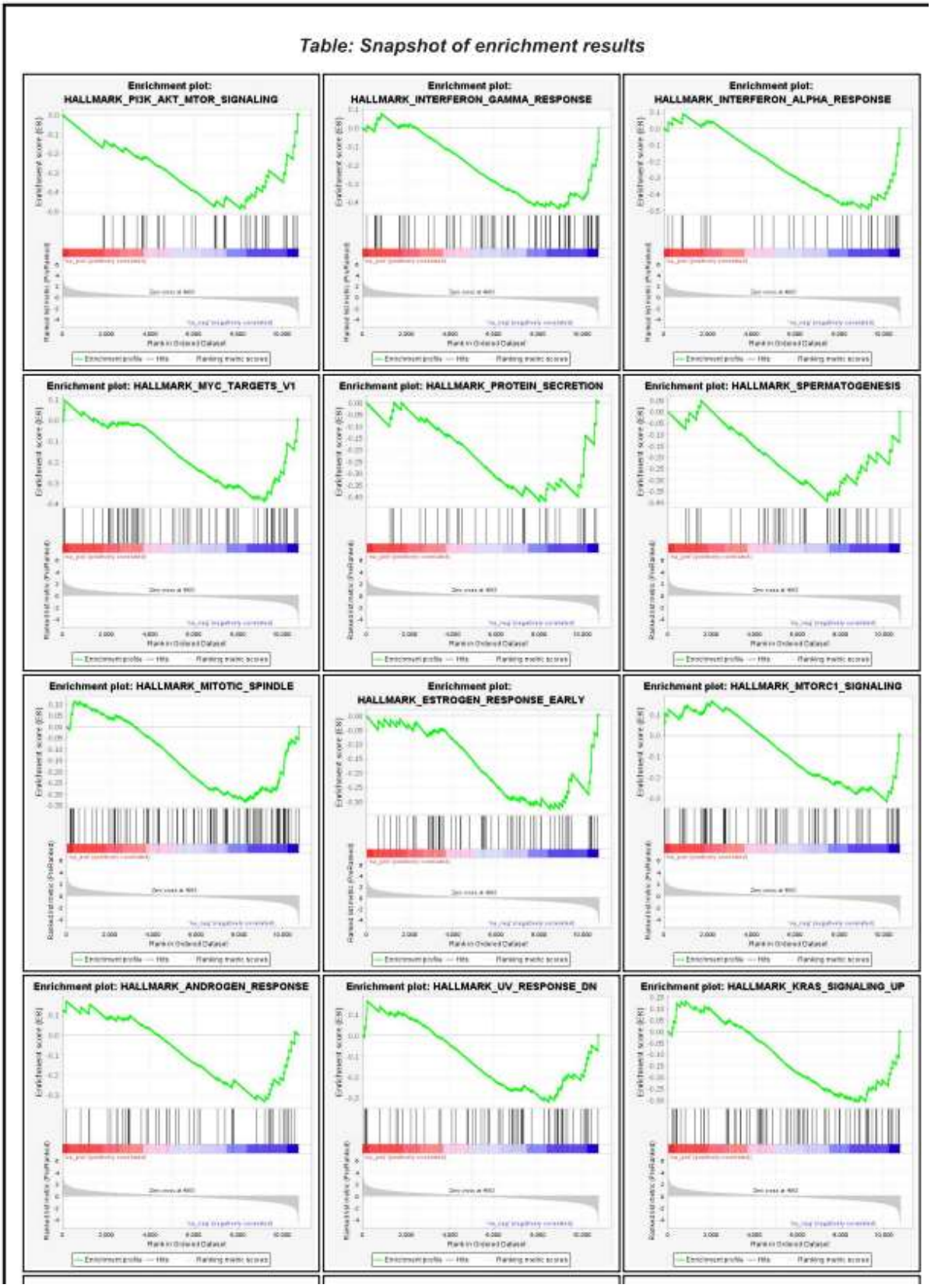
B.1 dNK v. CD56 dim NK cells

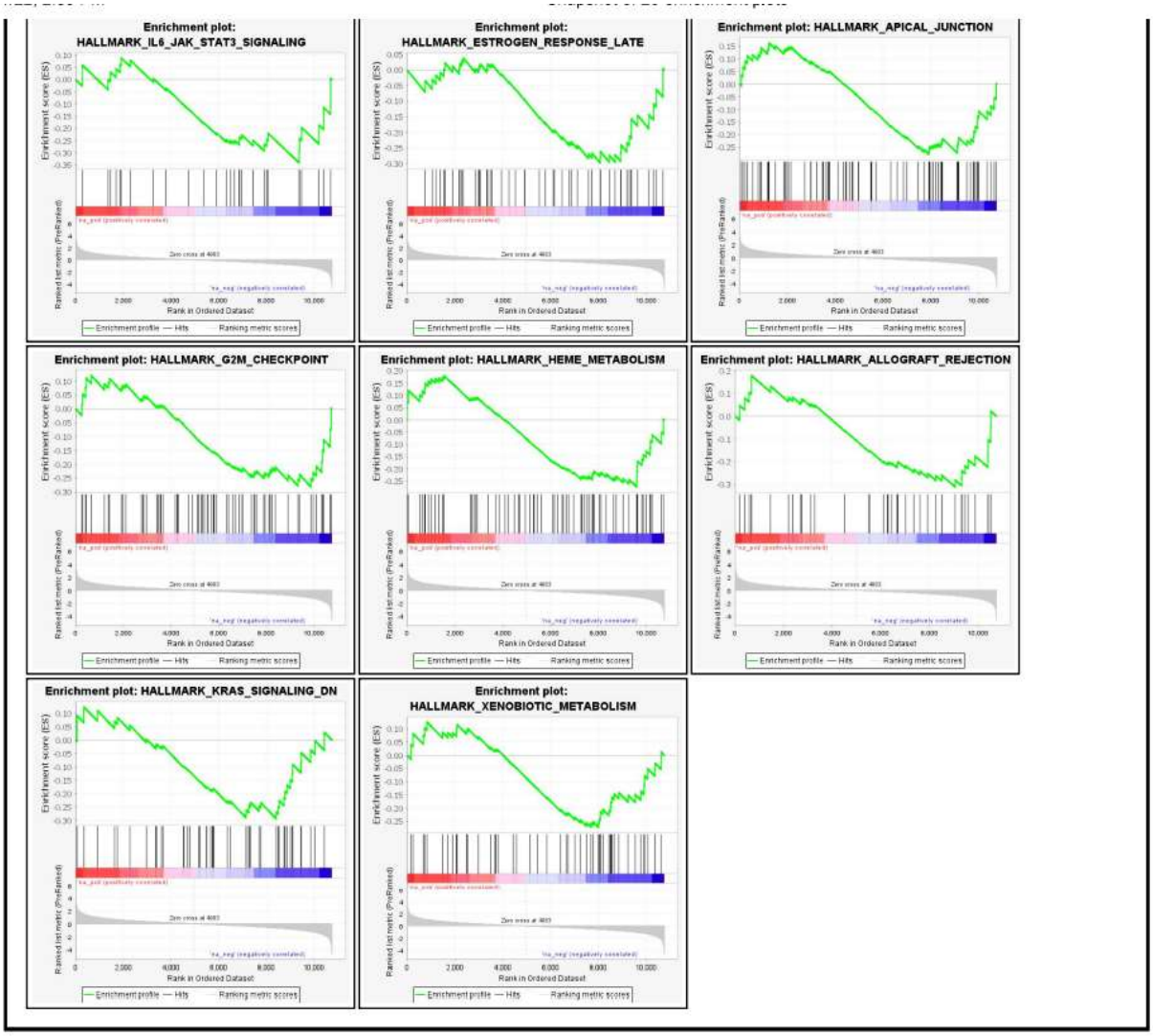
Biological pathways with positive enrichment scores for dNK cells




Enrichment plot:
HALLMARK_TNFA_SIGNALING_VIA_NFKB
Enrichment plot: HALLMARK_COAGULATION
Enrichment plot: HALLMARK_APOPTOSIS
Enrichment plot: HALLMARK_IL2_STAT5_SIGNALING
Enrichment plot: HALLMARK_MYC_TARGETS_V2
Enrichment plot:
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
Enrichment plot: HALLMARK_DNA_REPAIR
Enrichment plot: HALLMARK_MYOGENESIS

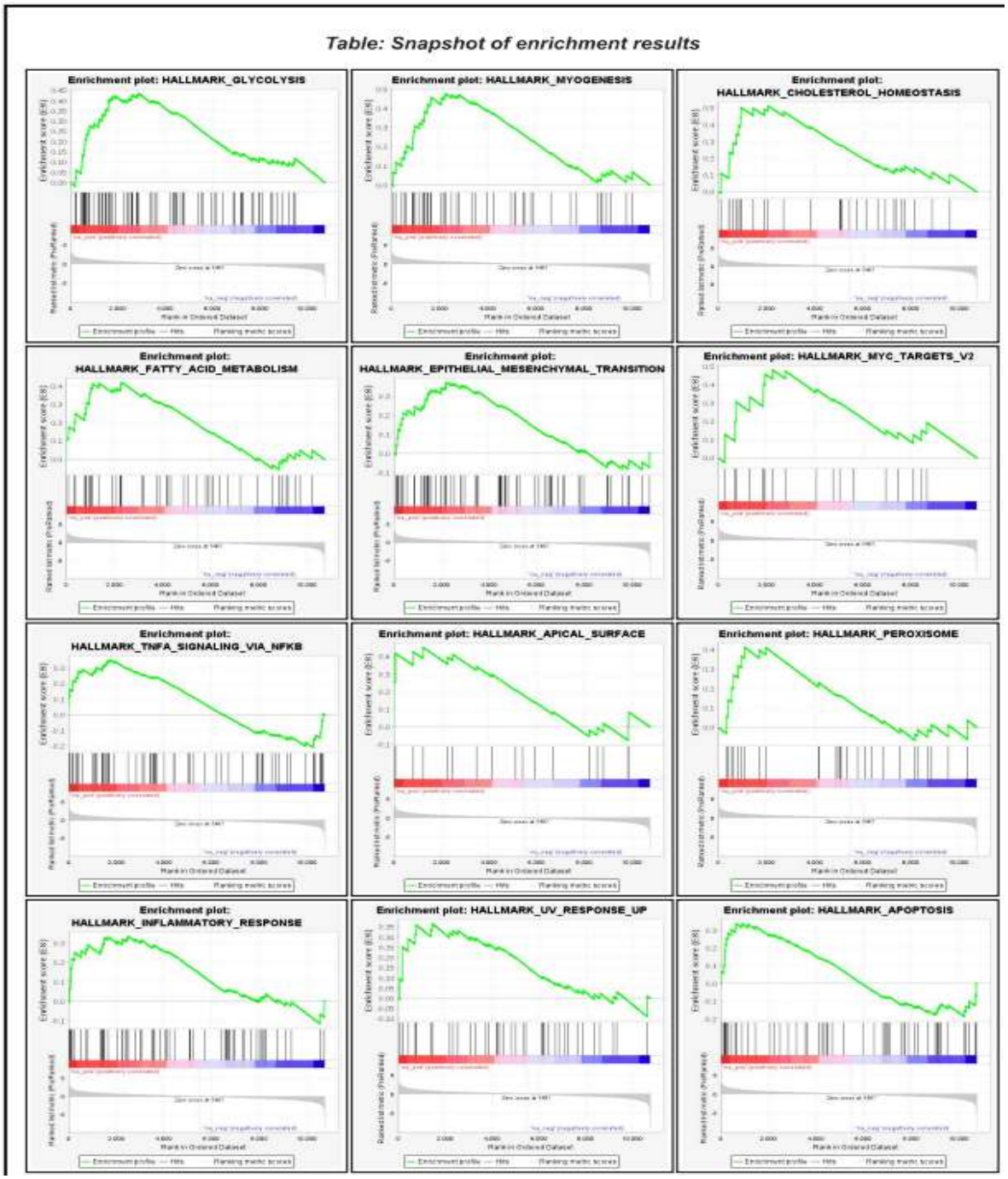
Biological pathways with negative enrichment scores for dNK cells

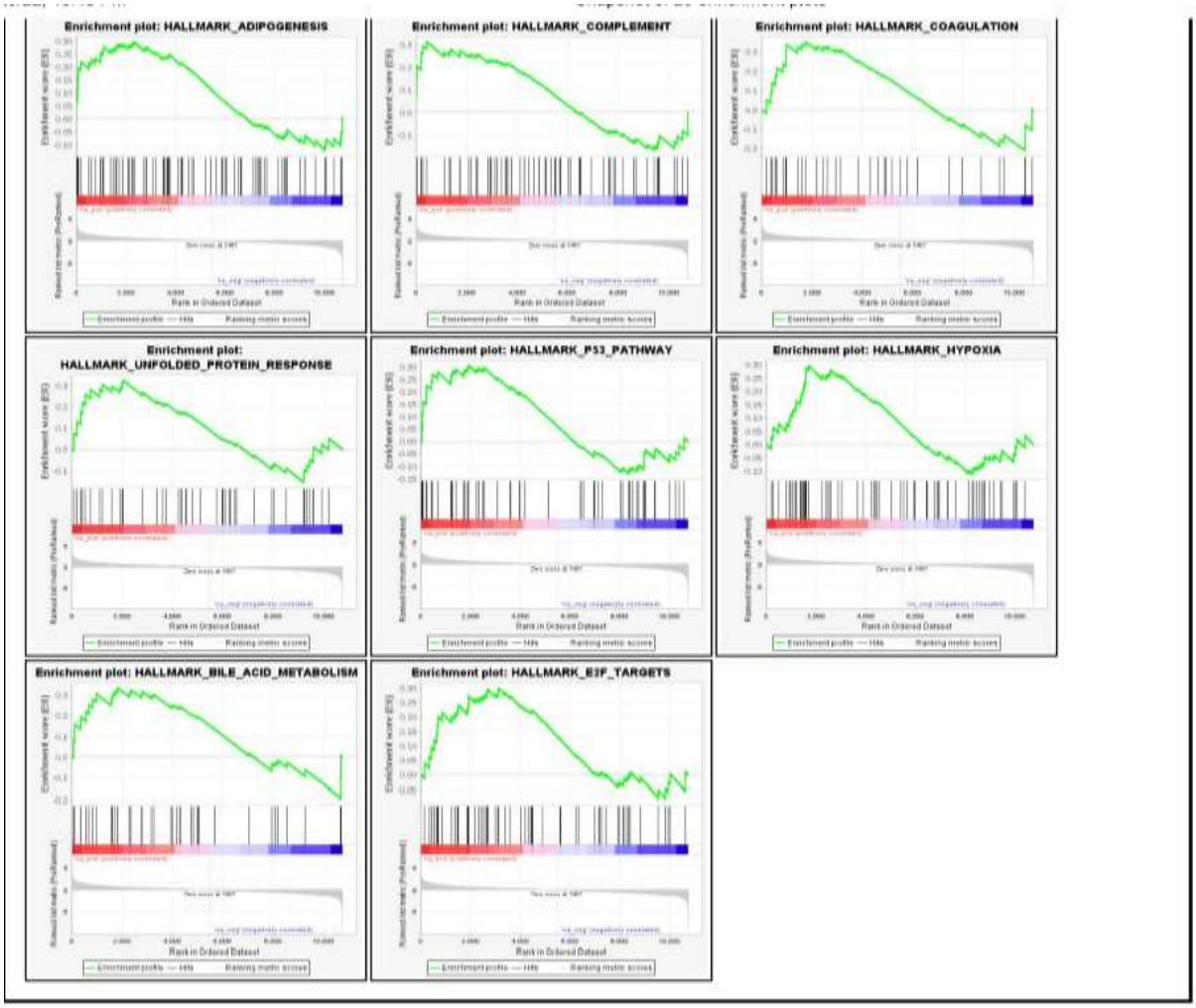



Enrichment plot: HALLMARK_IL6_JAK_STAT3_SIGNALING
Enrichment plot: HALLMARK_ESTROGEN_RESPONSE_LATE
Enrichment plot: HALLMARK_APICAL_JUNCTION
Enrichment plot: HALLMARK_G2M_CHECKPOINT
Enrichment plot: HALLMARK_HEME_METABOLISM
Enrichment plot: HALLMARK_ALLOGRAFT_REJECTION
Enrichment plot: HALLMARK_KRAS_SIGNALING_DN
Enrichment plot: HALLMARK_XENOBIOTIC_METABOLISM
Enrichment score (ES)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

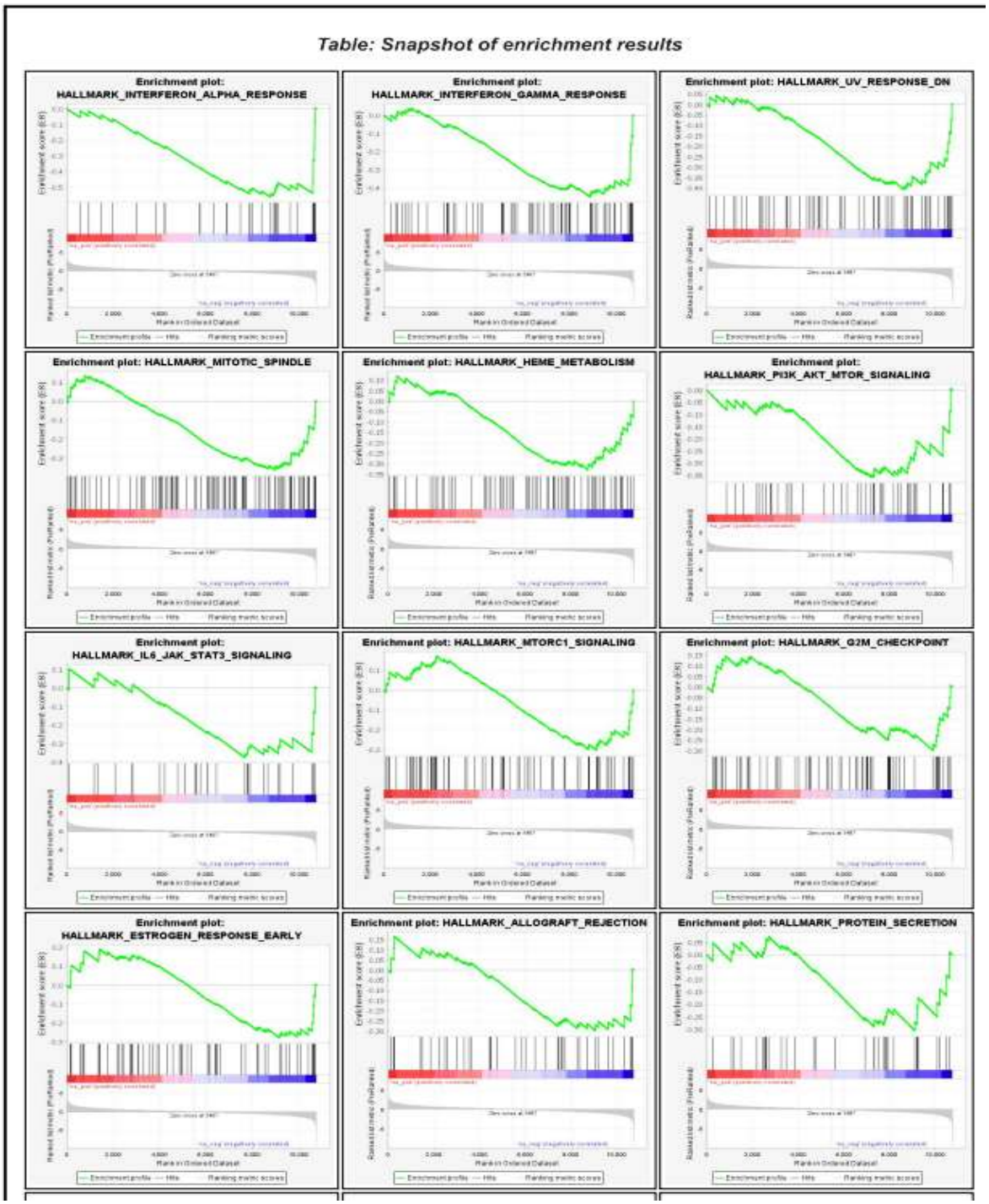
B.2 dNK v. CD56 bright NK cells

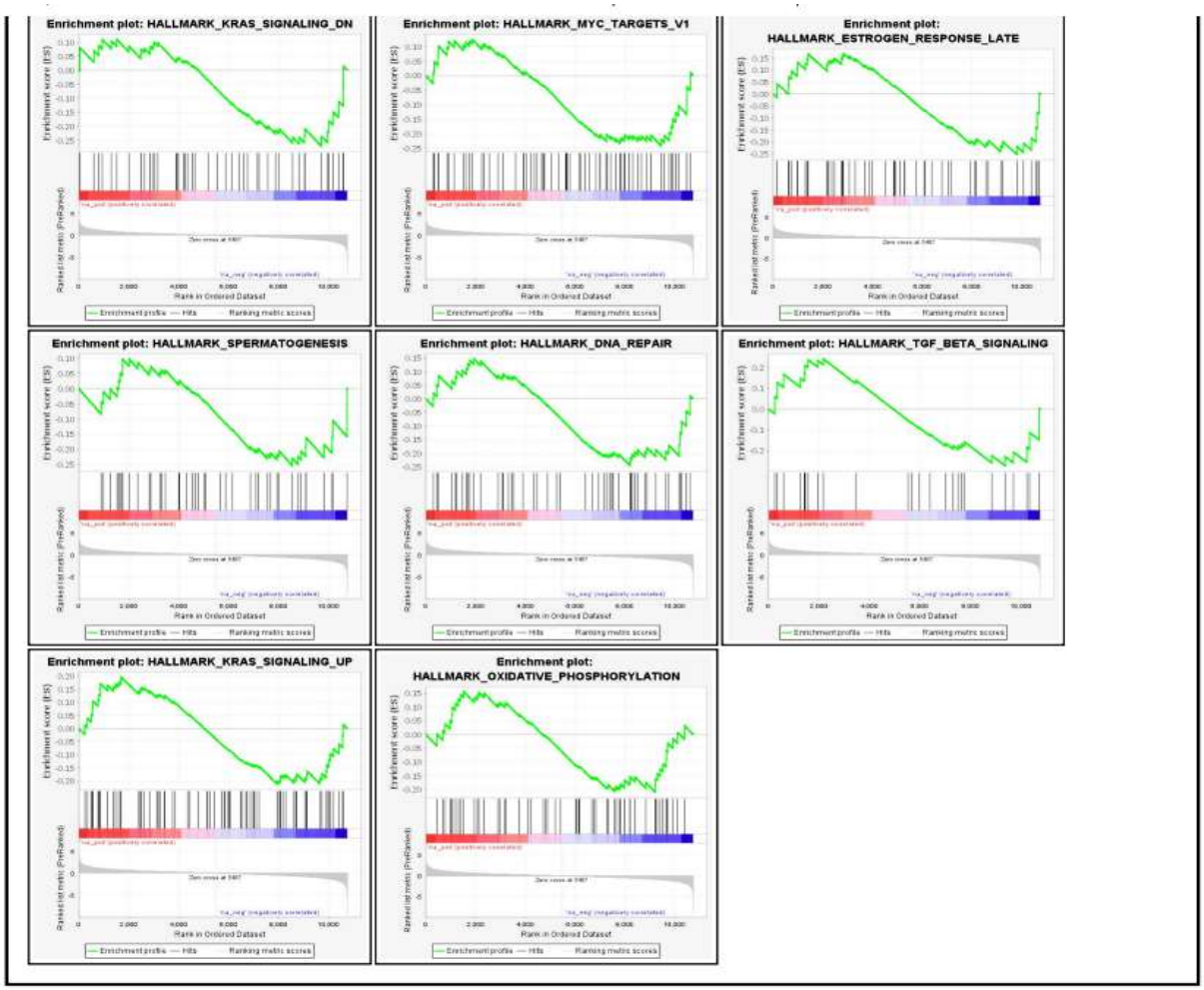
Biological pathways with positive enrichment scores for dNK cells




Enrichment plot: HALLMARK_ADIPOGENESIS
Enrichment plot: HALLMARK_COMPLEMENT
Enrichment plot: HALLMARK_COAGULATION
Enrichment plot: HALLMARK_UNFOLDED_PROTEIN_RESPONSE
Enrichment plot: HALLMARK_P53_PATHWAY
Enrichment plot: HALLMARK_HYPOXIA
Enrichment plot: HALLMARK_BILE_ACID_METABOLISM
Enrichment plot: HALLMARK_E2F_TARGETS

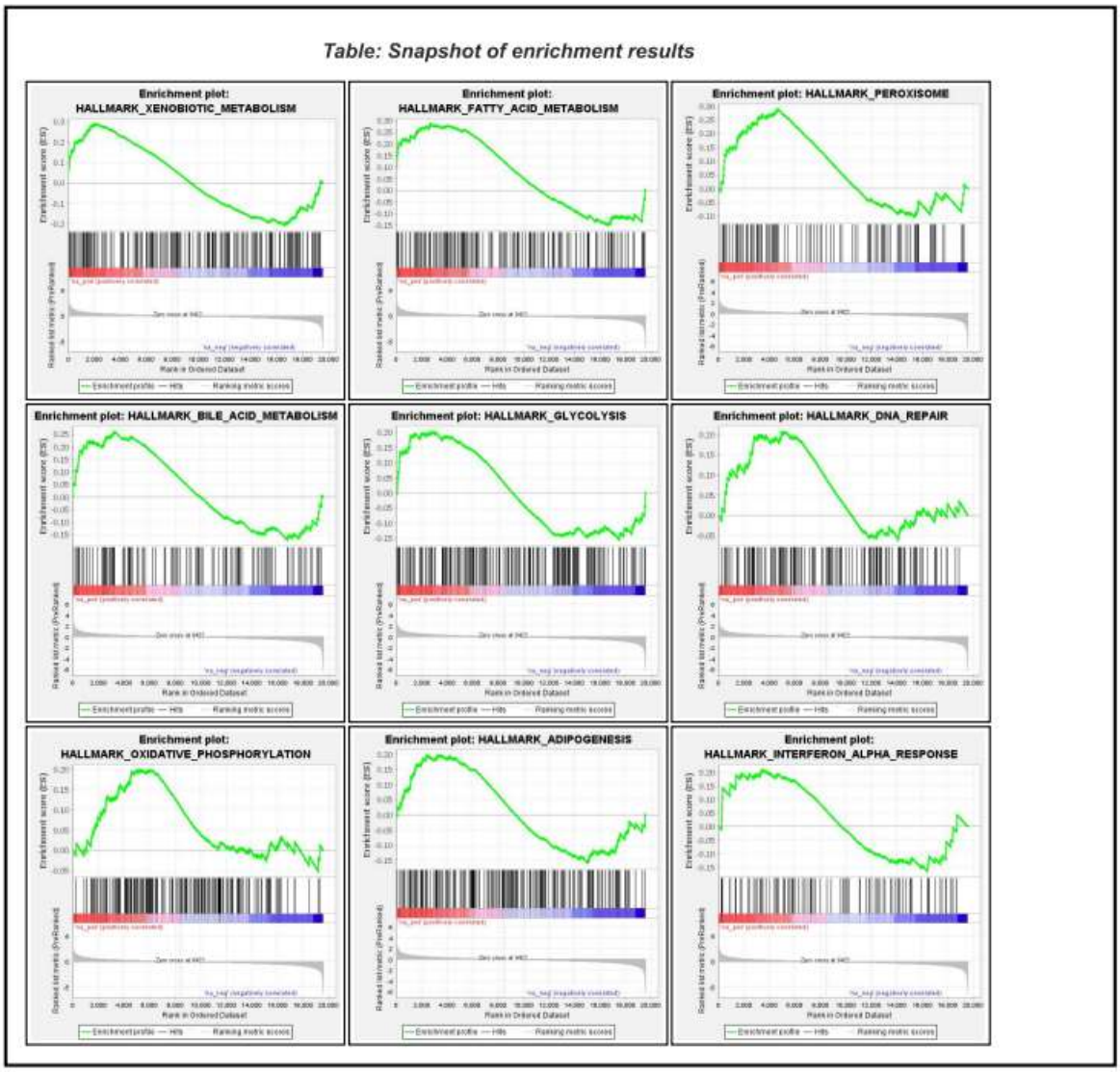
Biological pathways with negative enrichment scores for dNK cells



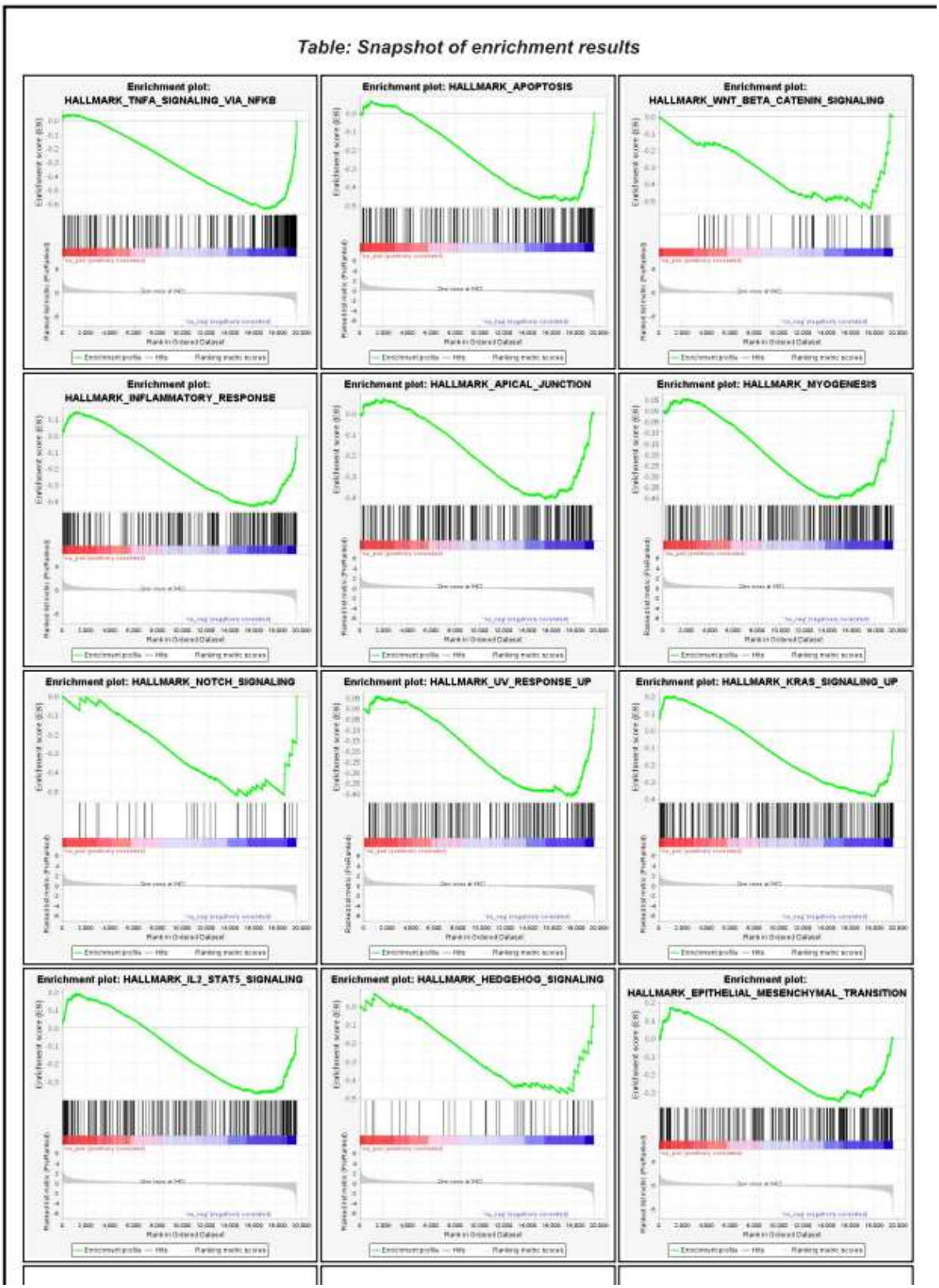

Enrichment plot: HALLMARK_KRAS_SIGNALING_DN
Enrichment plot: HALLMARK_MYC_TARGETS_V1
Enrichment plot: HALLMARK_ESTROGEN_RESPONSE_LATE
Enrichment plot: HALLMARK_SPERMATOGENESIS
Enrichment plot: HALLMARK_DNA_REPAIR
Enrichment plot: HALLMARK_TGF_BETA_SIGNALING
Enrichment plot: HALLMARK_KRAS_SIGNALING_UP
Enrichment plot: HALLMARK_OXIDATIVE_PHOSPHORYLATION

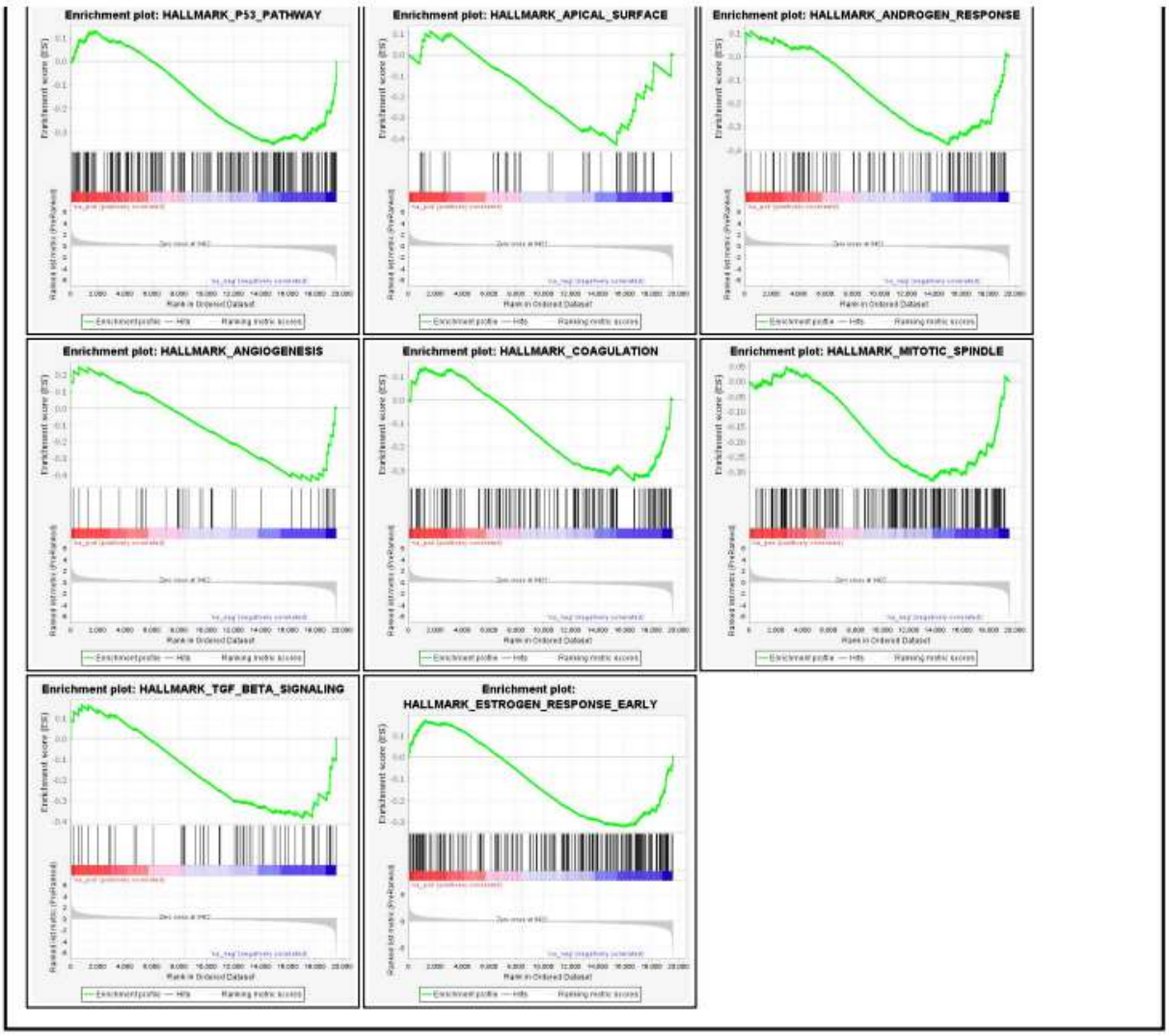
B.3 HCC pNK cells v. healthy pNK cells

Biological pathways with positive enrichment scores for HCC pNK cells



Biological pathways with negative enrichment scores for HCC pNK cells





APPENDIX C List of Publications

- **Priyanka Rawat**, Asmita Das*, Differential expression of disparate transcription factor regime holds the key for NK cell development and function modulation, Life Sciences, 2022, 120471, ISSN 0024-3205, https://doi.org/10.1016/j.lfs.2022.120471. **(IF – 5.037)**
- **Rawat P**., Dhingra M., Kosta K., and Das A., "Microflora impacts immune system and its anti-tumour function," in *Microbial Crosstalk with Immune system*. To be published by Elsevier.

PROOF OF PUBLICATION

The pdf document for the paper titled, “Differential expression of disparate transcription factor regime holds the key for NK cell development and function modulation,” published by Life sciences Elsevier is attached.

Life Sciences 297 (2022) 120471

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journal homepage: www.elsevier.com/locate/lifescie

Differential expression of disparate transcription factor regime holds the key for NK cell development and function modulation

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Keywords:
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Peripheral natural killer cells
Uterine natural killer cells
Transcriptional factors
Eomes
T-bet

ABSTRACT

Natural killer (NK) cells are involved in providing immunity against autoimmune diseases and perpetuation of a successful pregnancy in addition to protecting us from viral infections and providing tumor immunity. NK cells are present in various organs such as spleen, blood, lymph nodes, skin, liver and uterus and differential expression of transcription factors in divergent subsets of NK cells lead to differences in their cytotoxicity and cytokine profile. Tissue-specific expression and regulation of the TFs involved, has a profound effect on the cytokine profile and surface markers on NK cells, thus impacting NK cell function. Nfil-3, Id-2, Ets-1, GATA-3, and Eomes are TFs varying in abundance in peripheral NK (pNK) and uterine NK cells (uNK), which further highlights the functional variations in the two subsets of NK cells. GATA-3 mediated regulation of IFN-γ production, NK cell maturation, protection against pathogens, and regulation of expression of inhibitory NK cell receptor (NKG2A), exemplifies a potential mechanism for immune-modulation, involving NK cells. This review highlights the differences in the regulation of TFs in pNK and uNK cells, which can be crucial for development of novel immune-therapeutics.

1. Introduction

Natural killer (NK) cells are cytotoxic lymphocytes [1] that modulate immune responses by secreting immunoregulatory cytokines and chemokines. They are abundantly present in the human body, enriched in lymphoid and non-lymphoid tissues. Between the two subsets, $CD56^{bright}$and $CD56^{dim}$NK cells, the former accumulates in lymph nodes, tonsil, stomach, gut, liver, uterus, adrenal gland, and visceral adipose tissue, and the latter in lung, kidney, mammillary tissue, bone marrow, and spleen [2]. Lymphoid tissue, liver, and uterus possess some unique subsets of tissue-resident $CD56^{bright}$ NK cells [2].

Liver NK cells majorly comprise of tissue-resident (tr) $CD56^{bright}$ NK cells with CD69 and CXCR6 expression. Their low cytotoxic function is explained by the low IFN-γ, perforin, and granzyme production [2]. The thymus comprises a unique NK cell population of GATA-3^{+}cells with a low expression of Ly49 receptors. Thymic NK cells are very similar to liver trNKs in cytotoxicity but differ from others for requiring both IL-7 and IL-15 signaling during their development [3].

Lymphocytes in the peripheral blood comprise 5–15% NK cells, originating in the bone marrow with possible development in lymphoid (majorly secondary) tissues and thymus. These are immune cells innate to the host and can kill tumor cells and cells infected by viruses. Peripheral blood has two subpopulations of NK cells; one with $CD56^{dim}$ $CD16^{+}$ surface markers and the other with CD56 ^{bright} $CD16^{-}$ [4]. The former subpopulation is more abundant and more cytotoxic than the latter. The ability of $CD56^{bright}$ $CD16^{-}$ expressing NK cells to express CCR7, CXCR3, CXCR4, and L-selectin also contribute to their potential to migrate to secondary lymphoid organs and non-lymphoid organs.

Most of the theories surrounding the origin of Uterine NK (uNK) cells ascertain that the migration of $CD56^{bright}$ $CD16^{-}$ subset to the uterus, accompanied by high expression of CD56, a gain of KIR receptor, and loss of CD127 by pNK cells, results in the origin of uNK cells and differential functionality of the uNK cells [5]. The uterus is a non-lymphoid organ that accommodates CD56bright CD16- markers on the NK cell population, known as uNK cells [6]. Longer telomeres are observed in $CD56^{bright}$ NK compared to the $CD56^{dim}$ NK cells [2].

Several factors differentiate pNK cells from the tissue-resident (tr) uterine NK cells comprising of surface markers, transcription factors,

Abbreviations: NK, Natural Killer; TF, Transcriptional factor; pNK, Peripheral NK; uNK, Uterine NK; tr-NK, Tissue-resident NK; HSC, Hematopoietic stem cells; LMPP, Lymphoid-primed multipotent progenitors; CLP, Common lymphoid progenitors; NKP, Natural killer cell progenitor; iNK, Immature natural killer cell; mNK, Mature natural killer cells.

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Table 1
Comparative account of characteristics, cytokines/angiogenic factors produced, and surface markers present on pNK and uNK cells.

Properties	pNK cells	uNK cells	References
Abundance	2–3% (Spleen and blood)	Present in uterus	[13,15]
Appearance	Less granular	Highly granular	[12,13,64–68]
Cytotoxicity	High	Low	[6,9,18,19,28,64,65,67,69]
Surface markers			
$CD56^{Dim}$ and $CD56^{Bright}$	$CD56^{Dim}$ and $CD56^{Bright}$	$CD56^{Bright}$ (CD56 marker's expression is high)	[2,6,9,12,18,22,28,70–75]
Integrin	DX5	CD49a	[2,14,15]
CD69	Absent	Present	[2,28]
CCR5	Present	Absent	[2]
CCR7	Present Expressed by $CD56^{bright}$	Absent Expressed by only $CD56^{bright}$	[2,6]
CD9	Absent	Present	[6]
CD62L	Present	Absent	[2,28]
KIR	• Absent • Present on $CD56^{dim}$	• Present (%) • Absent	[2,6,19,28,67,73,74,76]
CD127	Expressed (%)	Absent	[2]
CD117	Expressed (%) on $CD56^{bright}$	• Absent • Highest on $CD56^{bright}$	[2,6]
CD16	+	–	[65]
CYTOKINES & ANGIOGENIC MOLECULES			
VEGF-C (Humans) (Vascular endothelial growth factor)	Does not secrete	Secretes +++	[18,27,47,73,77]
VEGF-A (Mice)	- (Hypoxia + TGF-β1 leads to secretion of VEGF-A)	Secretes +++	[22,27,47,73,78]
CSF-1 (Colony stimulating factors)	+	+++ (When stimulated by IL-15 & 12)	[6,28,68,73]
TNF-α	+	+++ (When stimulated by IL-15 & 12)	[6,68,73]
INF-γ	+	+ Not produced in abundance +++ (When stimulated by IL-15 & 12)	[6,9,64,68,73,79]
IL-10	+	+++ (When stimulated by IL-15 & 12)	[73,80]
IL-2	+	+++	[14]
PLGF (Placental growth factor)	–	+	[54,73,81]
Ang2 (Angiopoietin)	–	+	[27,73,81]
Granzyme A	Expressed in both	Expressed in uNK cells; 3-fold of $CD56^{Dim}$ and 10-fold of $CD56^{Bright}$	[28,82]

and properties such as abundance and appearance. NFIL-3, T-bet, GATA-3, and Eomes are some of the many crucial transcription factors that differ in expression in pNK and uNK. NFIL-3 is an essential factor in the development of pNK cells but has no effect whatsoever on tr-NK cells in organs, including the liver, salivary gland, and uterus. Apart from Nfil-3, T-bet and Eomes are also essential in the development of NK cells which is proved by the absence of NK cells in all organs in T-bet and Eomes deficient cells. However, uNK cells have shown no such dependency on T-bet. GATA-3 helps sustain the NFIL-3 expression and is also reportedly involved in regulating inhibitory receptors of NK cells; NKG2A expression is involved in eliciting inflammatory responses [7]. GATA-3 deficiency has been reported to result in a low IFN-γ production by NK cells [8]. Studies have reported the involvement of Eomes in increasing the IFN-γ production and reducing the TNF production compared to Eomes deficient cells, thus exemplifying the role of Eomes. However, uNK cells produced both TNF and IFN-γ. Therefore, we will discuss the transcription factors produced and their effects on uNK and pNK cells [9].

Based on the functionality and cytotoxicity profile, pNK and uNK cells represent the two contrasting populations and hence have been focused on in this paper. Our extensive comparative evaluation of the disparate transcription factors involved in these two diverse populations of NK cells is crucial in understanding the role of NK function modulation by transcription factor expressions and hence may also give valuable insights into the development of novel immunotherapeutics.

2. Comparative account of pNK cells with uNK cells

The pNK cells are similar to uNK in some aspects but differ more. Both the pNK and uNK cells require IL-15 signaling in their early development, as confirmed by the reports showing IL-15Rα deficient mice with no signs of pNK and uNK cells. pNK cells consist of $CD56^{Dim}$ $CD16^{+}$ and $CD56^{Bright}$ $CD16^{-}$cells (co-expressing L-selectin). uNK cells express $CD56^{Bright}$ $CD16^{-}$ cells and are lacking in the expression of L-selectin. uNK cells are tissue residents, restricting them to the tissue they are in while pNK cells can circulate throughout the body. Though the expression of IL-2 receptor is more in uNK cells than in pNK, it does not lead to higher cytotoxicity. Sharma and Das [10] showed an increase in the NK cell population with IL-2 stimulation with no change in hyperactivity. Furthermore, it has been experimentally proven by Das, Gupta and Saxena [11] that IL-2 when induced in NK cells in the presence of circulating immune complexes, results in a significant increase in its cytotoxicity. Though, uNK cells have low cytotoxicity compared to the pNK cell and produce cytokines (IFN-γ, CSF) and angiogenic molecules (PLGF, VEGF, Ang2). A detailed account of differences in pNK and uNK cells based on the surface expression of markers and cytokines produced has been provided in the table below (Table 1)

3. Uterus harboring NK cells

The lineage-specific development and differentiation of pNK and uNK concerning the differential expression of a multitude of transcription factors are summarized in Fig. 1. The differential expression of transcription factors results in differential functionality of peripheral and uterine NK, and there are multiple theories towards their diverse ontology. The ontology of uNK cell has been speculated for a long time and can be listed as three possible theories [12–14,5]:

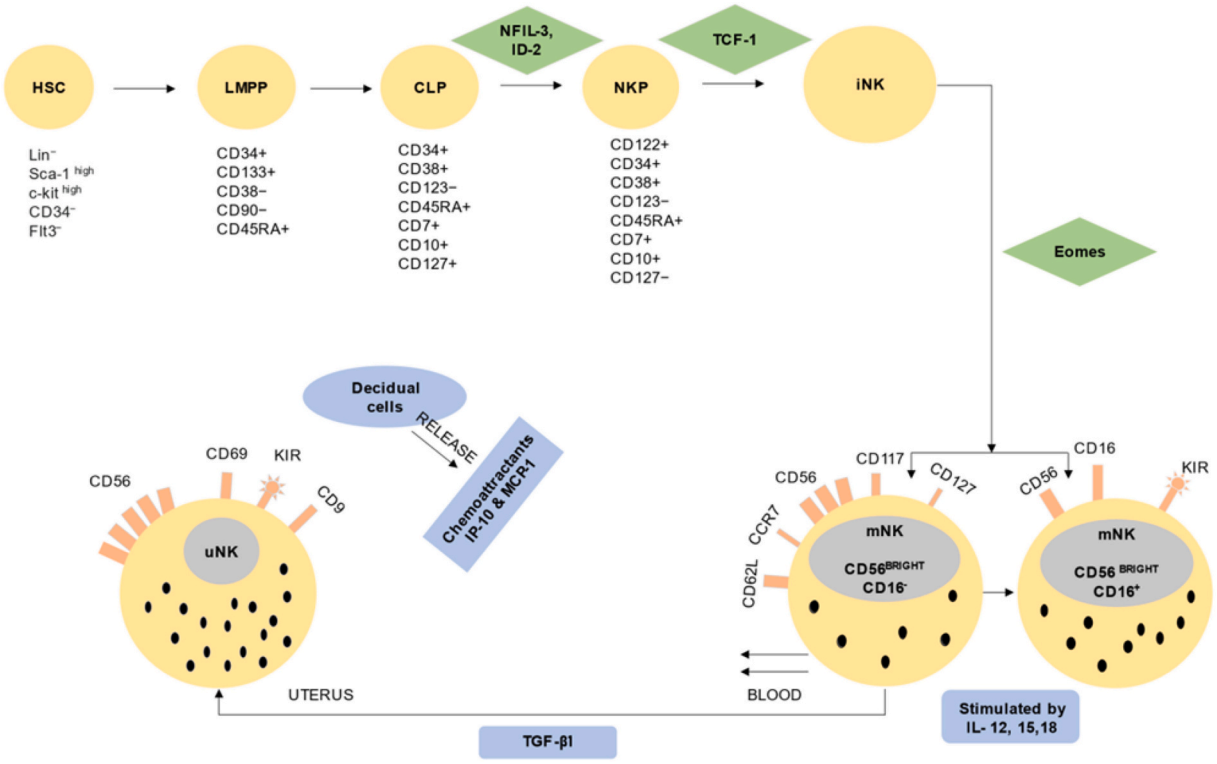

Fig. 1. Schematic representation showing the different stages of development observed in pNK cells and uNK cells: HSC differentiates into LMPP to CLP. CLP further divides into NKP giving rise to iNK and finally to mNKs (CD56 + CD16- and CD56 + CD16+) subset.
(HSC- Hematopoietic stem cells, LMPP- Lymphoid-primed multipotent progenitors, CLP- Common lymphoid progenitors, NKP- Natural killer cell progenitor, iNK- Immature natural killer cell, mNK- Mature natural killer cells).

1. Differentiation of pNK cells into uNK cells, supported by the secretion of NK cell chemo-attractants (IP-10 and MCP-1) by decidual cells and trophoblasts.
2. Development from endometrial NK cells, supported by pregnancy-associated factors and hormones.

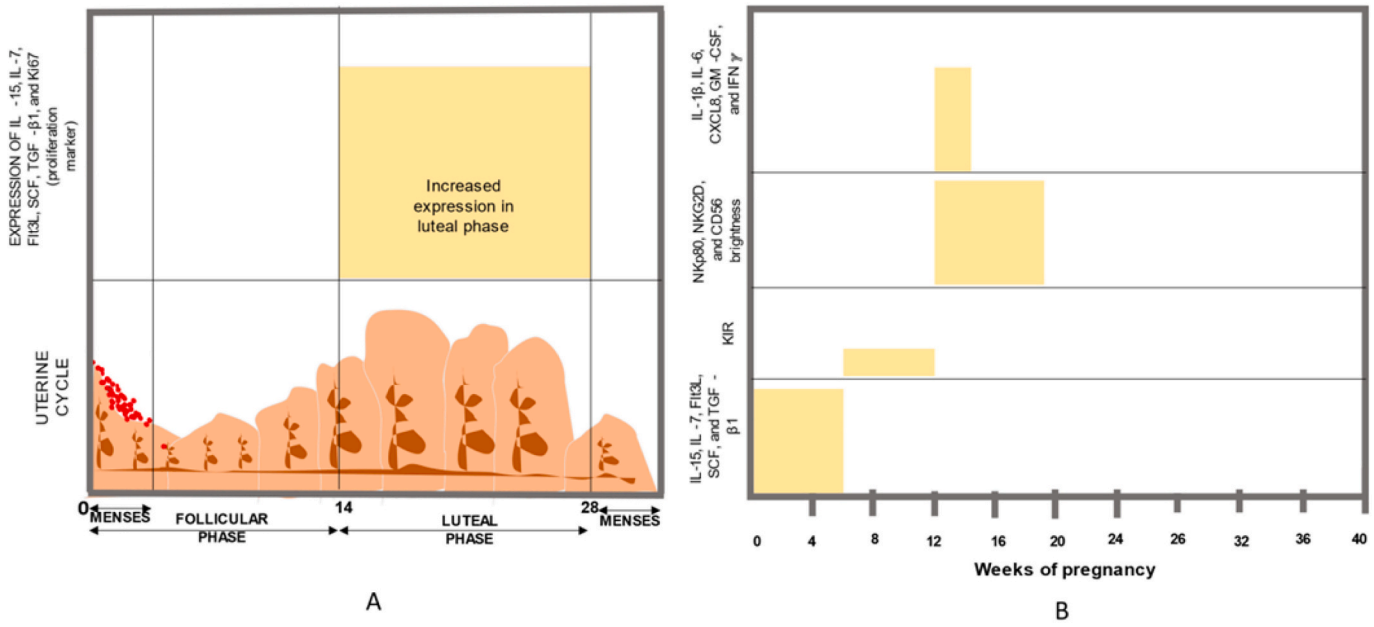

Fig. 2. A) Changes in the uterus during different phases of the menstrual cycle. The figure represents the expression levels of IL-15, IL-7, FIt3L, SCF, TGF-β1, and Ki67 observed in different phases of the menstrual cycle. (Data obtained from [13,73]).
B) Changes in the uterus throughout the different stages of pregnancy. The figure represents the expression levels of interleukins and cytokines in different stages of pregnancy. IL-15, IL-7, FIt3L, SCF, and TGF-β1 are highly expressed in early weeks of pregnancy; KIR is highly expressed in 6–12 weeks of pregnancy; NKp80, NKG2D, and CD56 brightness are highly expressed in 12–10 weeks of pregnancy; IL-1β, IL-6, CXCL8, GM-CSF, and IFN- γ are highly expressed in 12–14 weeks of pregnancy. (Data obtained from [13,73]).

3. Differentiation from hematopoietic precursors in the decidua, supported by the release of decidual stromal factors.

Besides being cyclically exposed to sex hormones, Uterine tissue harbors tr-NK cells and a few conventional NK cells, constituting uNK [15]. uNK cells in humans are restricted to the mucosa, decidua, and endometrium. The expression levels of uNK cells present in the uterine tissue change concerning the uterus's stage. uNK cells in a pregnant mouse have been found in greater abundance than when a mouse is not pregnant. This raises the question of the role of uNK cells in supporting pregnancy. Some reports have suggested the pivotal role of uNK cells in maintaining a healthy pregnancy. In contrast, some reports indicate a chance of abnormal pregnancy or miscarriage due to the abundance of uNK cells in the uterus of a pregnant mouse [16]. Robson et al. [17] confirmed the involvement of uNK cells in the remodeling of spiral arteries in the uterus, which is crucial to avoid pregnancy complications. Differentiation of uNK cells occurs in every menstrual cycle, three to 5 days after the LH (Luteinizing hormone) surge [16].

3.1. Effect of Uterine NK cells on pregnancy

During pregnancy, of the total lymphocytes present in the uterus, 70% of them are NK cells. Among these NK cells, the majority are of the subset, $CD56^{bright}$ $CD16^{-}$. The CD56 ^{bright} level in these NK is higher than the intensity of $CD56^{bright}$ present in pNK cells [2,15,18–20]. Suggestively, uNK cells that have a distinctive cytokine profile as compared to the pNK exert a positive effect on pregnancy. Confirmed by the experiments of Yadi et al. [21], no changes are observed in the receptor profile of pNK cells during pregnancy (mid-gestation), unlike uNK cells that observe changes in cytokines and other factors during pregnancy are presented in Fig. 2.

The trophoblast cell invasion by uNK cells in pregnancy is prevented by the secretion of factors including TGFβ, and a demethylating agent in hypoxic conditions, leading to the production of VEGF-A, which is responsible for reduced cytotoxicity [22]. A difference in the expression of various receptors and their levels of expression by uNK cells has been observed. Yokota et al. [23] gave experimental evidence for the involvement of NKp46 in facilitating normal pregnancy. Expression levels of NKp46 on uNK cells have been reported to upregulate the production of cytokines involved in a successful normal pregnancy [23]. Manaster et al. [24] performed experiments confirming the role of IL-15 in increasing the expression of activating receptors (NKp30/44) in the uterus. uNK cell abundance is significantly increased in the first trimester of pregnancy and decreases by the end of the second trimester. The hemochorial placenta of humans and mice is responsible for fluctuations in the abundance of uNK cells throughout the pregnancy. This type of placenta is invasive, allowing interaction between fetal tissue and the mother's immune system. The fetus's trophoblast infiltration triggers the mother's body, leading to an increased abundance of immune cells, including uNK in the endometrium. uNK during early pregnancy exhibits substantially reduced cytotoxic potential compared to pNK cells. There is a higher expression of NKG2A levels on these NK cells and due to the engagement of these inhibitory receptors on NK cells by the overexpressed non-classical MHC molecules on trophoblast cells, thus resulting in diminished cytotoxicity in these uNK populations [12].

NK cell receptors are critically involved in regulating cytotoxicity in NK cells which is dependent on polarized granulation. Das and Long [25] examined the effect of inhibitory receptors on the NK cell cytolytic granule polarization and degranulation and conclusively demonstrated that polarization of granule was the preferred site for inhibition of NK cytotoxicity. NKp46 found on both resting and activated NK cells is one of the primary NK cell natural cytotoxicity receptors involved in inducing NK cell cytotoxicity [26]. uNK cells differ from pNK due to a different receptor profile which includes increased expression of NKG2A/E/C, KIR2DL4, CD31, and CXCR3/4. uNK cells have a profound cytokine secreting profile rather than cytotoxicity; the increased expression of inhibitory receptors could be the reason for this [27]. Two primary NK cell inhibitory receptors are NKG2A and members of KIR. The ligand for NKG2A is HLA-E which is expressed on the trophoblast. The increased expression of NKG2A inhibitory receptor on uNK cells results in blocking of the perforin polarization and target cell killing by the engagement of the NKp46 receptor, which is successfully carried out in pNK cells [27]. 2B4; SLAM (Signaling lymphocyte activation molecule) related receptor is responsible for stimulating NK cell cytotoxicity [28]. The interaction of 2B4 with inhibitory receptors expressed by uNK cells like NKG2A and KIR results in blockage of the raft recruitment and functionality of 2B4 [29].

The poor cytotoxicity of uNK cells is associated with increased expression of inhibitory receptors specific for HLA-E, HLA-G, and HLA-C expressed by trophoblast. HLA-C is the only detectable classical MHC-I expressed by trophoblast [28]. The uNK receptors interact with trophoblast MHC (Major histocompatibility complex) molecules, which may affect the cytotoxicity of uNK cells. Some NK cell receptors decrease NK cell activity by binding to MHC-I present on the target cells [30–32]. Ly49 is an inhibitory receptor in mice similar to KIR found in humans. Das and R.K. Saxena [33] conducted experiments showing higher expression of Ly49 by spleen cells (containing NK) induced with IL-12 in the presence of fixed tumor cells. With the inverse relation between NK cell lysis and MHC-I expression, Ly49 in mice is responsible for interacting with MHC-I and preventing lysis, helping normal cells survive. However, in the absence of Ly49, normal cells of the body would be lysed. Das and Saxena [34] tested NK cells, blocking the interaction of Ly49 and MHC-I using specific antibodies, resulting in inhibition of its cytotoxic activity. The NKG2A/CD94 receptor's binding with the MHC-I molecule, HLA-E, expressed by trophoblast, is responsible for overriding the cytotoxicity signals secreted by NKp46 receptor, expressed by uNK cells [27]. KIR receptor binding to fatal HLA expressed by trophoblast is linked to the increased risk of pre-eclampsia caused by defects in spiral arteries formation [28].

4. Transcriptional factors

4.1. NFIL3

Nuclear factor interleukin-3 (NFIL3) is a widely expressed transcription factor in natural killer cells. It is a basic leucine zipper TF, also known as E4-binding protein 4 (E4BP4) [35,36]. Experiments conducted on mice deficient in Nfil3 showed early defects in the development of natural killer cells, which resulted in the near-complete loss of cNKs in the periphery. The requirement of Nfil-3 in the early natural killer cell developmental stage has been reported [3]. Sequencing of the transcriptome by Seillet, Huntington et al. [37] of NKP, iNK, and mNK cells to compare them with all the transcriptomes published revealed the expression of Nfil-3 at all lymphoid progenitors stage which was slightly reduced during lymphoid development. It can be interpreted from the data that Nfil-3 is expressed in all lymphoid progenitors, continuing to express throughout the natural killer cell development. Nfil3 is expressed before other TFs such as ID2, T-bet, and Eomesodermin which defines NK lineage [1]. The regulation of circadian rhythm, development of immune cells, and regulation of ovulation have been reported as some crucial functions of Nfil-3 [38] in mice lacking Nfil-3. Experiments conducted on E4bp4 $^{-}/^{-}$ mice by Gascoyne et al. [39] revealed the absence of a natural killer cell population in the periphery. However, the populations of natural killer cells present in salivary glands were found to be independent of Nfil-3, as shown by Cortez et al. [40] comparing Nfil-3 $^{-}/^{-}$ from wild-type controls. This result implicated the presence of few populations of NK cells present, which would develop independently of Nfil-3. It is also reported that Nfil-3 deficiency has no impact on tissue-resident NK cells of the skin, liver, and uterus [14,38]. Sojka et al. [14] revealed the presence of tr-NK cells in the liver, uterus, and skin of Nfil-3 deficient mice. Extensive studies indicate the existence of a distinct lineage of tr-NK cells from cNK cells. Apart from involving in the

direct development of pNK cells, Nfil3 is also involved in the regulation of other TFs. One of these transcription factors is ID2 (Inhibitor of DNA binding 2), involved in natural killer cell differentiation and maturation [3]. However, Seillet, Huntington et al. [37] showed no such dependence of ID2 on Nfil-3. E4bp4 deficient NK cells in mice were reported to restore development by overexpression of ID2, Eomes, or T-bet. This led to the belief that Nfil-3 acts upstream of them [1].

4.2. Eomes

Eomesodermin is a T-box TF constituting a T-box DNA-binding domain and a shared homology with T-bet. Eomesodermin is involved in vertebrate embryogenesis and is expressed at high levels in pNK cells. In NK cell development, Eomes is expressed downstream of Tbet [1]. Death reports of Eomes$^{-/-}$ mice directed towards the importance of Eomes at an early embryonic stage. This led to the studies on Eomes +/− Tbx21−/− mice, which reported a significant impairment of natural killer cells compared to the mice deficient in Tbet only. As a consequence of the involvement of Eomes with CD122, and IL-2R & IL-15R (β chains), a severe downregulation of them was observed with one loss of allele of Eomes [41]. Eomes deficiency severely affected the development of natural killer cells because of the downregulation of certain other factors (also required for the development of NK cells) with its deficiency. Though a significant reduction of natural killer cells has been reported by blood and spleen deficient in Eomes, only a slight decrease of natural killer cells was seen in the liver, LN (lymph node), and BM (bone marrow) [41]. BM and pNK cells are severely reduced with deficiency of Eomes. Uterine NK cells reported concomitant expression of Eomes and are largely responsible for uNK being different from liver and skin tr-NK cells in being Tbet independent [14,42].

4.3. T-bet

T box expressed in T cells is a phosphorylated protein and tyrosine T-box TF with a T-box DNA-binding domain. Cells of hematopoietic origin express this TF, encoded by the Tbx21 gene [41]. Originally seen and identified in T-cells, it is involved in the production of interferon-γ (IFN-γ), which commits CD4 to Th-1 lineage. T-bet is reported to be involved in NK cell maturation and not in the early stages of development in NK cells [1]. T-bet supports the proliferation of NK cells and the survival of mature cNKs. Experiments on T-bet deficient mice reported a slightly higher number of natural killer cells in BM and reduction in the spleen, liver, and peripheral blood. Peripheral cNK cells were moderately impacted by the T-bet deficiency, while the bone marrow observed minimal impact in cNK numbers. The requirement of T-bet is reported for the development of tr-NK cells in the liver and skin. However, mice deficient in T-bet showed had no decrease in the number of uNK cells [14,42]. T-bet and Eomes deficient mice showed a severe reduction of NK cells in all the organs [1].

4.4. GATA-3

GATA binding protein regulates the natural killer cell inhibitory receptor, NKG2A. NKG2A regulates cytotoxic and inflammatory responses of natural killer cells. GATA3 regulates the NKG2A gene as reported by the experiments by inducing mutation in GATA binding site II resulting in lowered promoter activity. GATA-3 sustains the expression of other TFs such as Id2, T-bet, and NFIL3. GATA3 deficient cNKs in mice showed impaired maturation and BM egress. Thymic NK cells require GATA-3 since their development does not rely upon T-bet or ID2 but on IL-7 [1]. No change in tr-NK cells in the liver was found in GATA3 deficient mice. However, severe reduction of splenic and BM NK cells is observed with genetic deletion of GATA-3 at the iNK stage. GATA-3 deficiency did not affect the cell cytotoxicity for tumor cells, but an immature phenotype was observed with high CD27 expression, low T-bet, and lower production of IFN-γ in vitro [1]. GATA-3 deficient NK cells failed to provide protection against infection by *Listeria monocytogenes* and *Citrobacter rodentium,* demonstrating the crucial role of GATA-3 in NK cell mediated protection [8,43].

4.5. TCF-1

T cell factor 1 consists of an HMG box domain [44] and regulates the early natural killer cell development. It is encoded by Tcf7. Tcf7 deficient mice studied for all the natural killer cell development stages showed severe reduction in NK cell (pNK) abundance. Immature NK cells have a very high expression of TCF-1 [1] Nfil-3, and TCF-1 expression has been reported at or before the NKP-ILCP development stage, implicating their involvement in proper pNK lineage differentiation [3]. Low expression of inhibitory NK receptor Ly49A and high expression of Cd11b and KLRG1 in Tcf7 deficient mice is reported [1]. This phenotype is hypermature but pro-apoptotic, associated with terminal maturity [3]. TCF1 deficient natural killer cells in mice showed elevated expression levels of granzymes. This involvement of TCF-1 in regulating the production of granzyme prevents the NK cells from apoptosis [1]. TCF-1 deficient mice reported fewer pre-rNKPs, pre-NKPs, and mNKs in BM. TCF-1 regulates the development of pNK cells by modulating the time of maturation and expression of the effector gene [3].

4.6. Inhibitor of DNA binding 2

ID2, encoded by the Id2 gene, is involved in developing natural killer cells and helper ILC lineages. It is reported to be expressed throughout the early stages of natural killer cell development with a peak in expression in mNK cells [1]. Deficiency of Id2 results in a severe reduction of pNK cells showing its important role in cNK maturation and IL-15 signaling sensitivity [3]. Id2 deficient mice were observed with a normal abundance of NKPs and iNK cells but a reduction in mNK cells. Studies revealed that administration of high doses of IL-15 or deletion of the negative regulator of IL-15 (SOCS3) overcame ID2 requirement in the development of NK cells [1].

4.7. Zinc-finger e homeobox binding 2

Zeb2 is a TF that is strongly induced during NK cells' maturation and helps control the epithelial to mesenchymal transition [45]. Reportedly, Tbet plays a critical role in regulating the expression of Zeb2. Studies on natural killer cells deficient in Tbet, Zeb2, or both led to similar phenotypes, which direct towards the working of Zeb2 and T-bet together [1,45]. Zeb2 reportedly controls the maturation of NK cells downstream of T-bet. Zeb2 mRNA expression in mice being administered with graded doses of T-bet showed a decrease in expression of Zeb2 in Tbx21+/− and even more Tbx21−/− compared to the ones with Tbx21+/+. This concluded the requirement of T-bet by Zeb2 [45]. The dependence of Zeb2 on T-bet implicates the absence of Zeb2 in uNK cells because they develop normally in Tbx21 deficient mice too. Zeb2 deficient mice showed high numbers of immature cNKs in BM while very few mature cNKs were in the periphery. Targeted deletion of Zeb2 in mice by van Helden et al. [45] resulted in impairment of natural killer cell maturation, survival, and exit from BM. Breeding experiments on Zeb2$^{fl/fl}$ mice (Higashi et al., 2002) by van Helden et al. [45] led to the observation that Zeb2 deficient natural killer cells accumulate in BM with a significant reduction in blood and peripheral organs including LN, lungs, liver, salivary glands, and spleen. However, overexpression of Zeb2 led to a decrease in natural killer cell abundance in BM with no impact on the number of splenic natural killer cells. To determine the TFs that show increased mRNA expression in mature CD28 natural killer cells compared to immature natural killer cells, van Helden et al. [45] screened microarray data and found Zeb2 to be one such TF. Maturation stages of natural killer cell development show high expression of Zeb 2 with its expression linked to T-bet in human NK cells, reportedly [45].

4.8. Kruppel like factor 2

KLF-2 is a TF regulating the differentiation and maturation of pNK cells. Maturing cNK cells depend on KLF-2 for support in survival and restriction of abnormal proliferation in them. mRNA and protein analysis carried out by Rabacal et al. [46] showed expression of KLF-2 in early NK cell development, increasing with maturation. KLF-2 deficiency has resulted in reduced numbers of mature cNKs in the periphery. Excision of Klf2 in gene-targeted mouse models by Rabacal et al. [46] has been observed to help in promoting the proliferation of iNK cells in peripheral tissues. Tr-NK cells in the liver are not affected by the loss of KLF-2. Low levels of KLF-2 limit the proliferation of natural killer cells (antigen-independent) in all the tissues; this is supported by the proliferative burst observed in NK cells with the excision of the gene responsible for KLF-2 [46]. Although there have been no reports until now about the effect of KLF-2 on uNK cells, it is known that KLF-5 is important, and its expression has been observed in uNK cells. KLF-5 is involved in the uterus for helping in the regulation of vascular remodeling and angiogenesis with high expression during implantation [47].

4.9. Runx3

Runt-related TF is a transcription factor from the Runx family, critically involved in hematopoiesis. It consists of a Runt domain and is expressed in natural killer cells in the development stage. The expression increases as maturation progress [1] Runx3 is involved in promoting later stages of cNK maturation and is suggestively able to do so with T-box and Ets family TFs. Deficiency of Runx3 or its co-regulator (CBF-β) in mice leads to a severe reduction in pNK cell numbers. Impaired development of iNK and mNK cells is observed in Runx3 deficient cells. Runx3 targets CD122 in NK cells and reduce CD122 expression in mice deficient in Runx3. The lower levels of CD122 expression lead to low responsiveness to IL-15, which the pNK and uNK cell uses for signaling. Lower IL-15 signaling in Runx3 deficient NK cells leads to alteration of expression of genes involved in proliferation, survival, migration, and NK cell function. Suggestively these genes consist of Ets-binding motifs, directing towards the co-regulation by Runx and Ets-1 in them [1]. Western blot analysis shows the mouse and human NK cells being high in the expression of Runx3 [48]. IFN-γ production was not affected by the Runx3 deficiency, but natural killer cells were reduced in BM and spleen. Upregulation of Runx3 in uNK cells is observed with an absence of uNK cells in Runx3 deficiency [48].

4.10. Ets protooncogene 1

Ets protooncogene 1 of the Ets family has a winged-helix turn helix. Ets1 deficient mice showed a severe reduction in NK cell compartment, with few rNKPs, iNK cells, and mNK cells in BM. It can be concluded from the observation that Ets1 regulates the early stages of natural killer cell development [1]. Reduced natural killer cell population in Ets1 deficient mice, directing towards the involvement of ETS-1 in NK cell differentiation [49]. With the reduction in natural killer cells, Ets-1 deficiency also leads to a lowered activating natural killer cell receptor's expression and an elevated expression in inhibitory receptors of NK .cells, failures in degranulation upon stimulation, and functional defects are observed [1]. Ets-1 is also believed to modulate the regular expression of ID2 and T-bet, which was confirmed by the fact that Ets-1 binds directly to the Id2 gene locus. Ets-1 deficient NK cells showed an increase in proliferation, size, granularity, and production of granzyme. Ets-1 can also limit the responsiveness of IL-15 [1], which is required by both pNK and uNK cells for signaling. Taveirne et al. [49] revealed the involvement of human ETS-1 in regulating the expression of TFs such as E4BP4, GATA-3, and T-bet.

4.11. STAT-5

The signal transducer and activator of transcription-5 is a TF encoded by Stat5a and Stat5b. It acts downstream of the IL-15 receptor. Stat5 regulates homeostasis and natural killer cells development. STAT-5 deficient mice showed a reduction in the number of NK cells in which stat5b had a significant impact [1]. The involvement of STAT-5 in the early stages of natural killer cell development was proved by Villarino et al. [50] using $Stat5a^{+}/^{-}$ $Stat5b^{+}/^{-}$ mice. STAT-5 was found to be involved in generating NKPs and ILC progenitors. STAT5 regulates T-bet expression to some extent. STAT-5 and T-bet regulate the expression of overlapping sites present in genes of NK cells. This overlapping showed the cooperative relationship between STAT-5 and T-bet, two important NK lineage-defining transcription factors [1]. Reportedly, STAT5 regulates adaptive immune responses [51].

4.12. .Mysm-1

Mysm-1 expanded as Myb like swirm and mpn domains, is a histone H2A deubiquitinase functioning as a transcriptional regulator to promote NK cell development. Mysm-1 maintains an open configuration at the Id2 transcription factor gene locus and facilitates the transfer of Nfil3 to the Id2 promoter [1]. Mysm-1 is involved in the maturation of natural killer cells and not in the early stages [52]. Hematopoiesis examined by Mysm-1 deficient mice by Nandakumar et al. [52] led to the observation of a reduced number in various lymphoid tissues of $NK1.1^{+}CD3^{-}$ natural killer cells. Real-time PCR analysis on Mysm-1 deficient NK cells in mice by Nandakumar et al. [52] revealed a reduction in natural killer cells in the bone marrow to threefold while pNK cells were lowered by three to nine folds in the spleen, blood, LN, liver, and lung. Drastic reduction in the number of mNK cells was observed in Mysm-1 deficient mice by assessing the surface expression by NK cells. ID2 transcription is suggestively linked to Mysm-1, as its absence leads to defective ID2 transcription leading to faulty maturation. ChIP assays carried out by Nandakumar et al. [52] revealed the association of NFIL-3 promoter region with Id2 locus and requirement of MYSM-1 for the successful association of NFIL-3 at Id2 locus.

4.13. Rroid

RNA demarcated regulatory region of ID2 is a nuclear long non-coding RNA that is highly expressed in ILC1 [53]. It is found upstream of Id2, promoting chromatin accessibility and recruitment of STAT-5 to Id2 locus leading to enhancement of expression of ID2. The later stages of natural killer cell development essentially require Rroid, whereas other TFs can replace their function for lineage specification stages [1]. Rroid maintains homeostasis, repression of E-protein target genes, ILC1 lineage identity, and ID2 activity promotion [51].

4.14. Pbx homeobox 1

PBX1 is a TALE (3-amino acid loop extension) family TF involved in B-cell development. Y. Zhou et. al [54] studied the role and expression of PBX1 in NK cells. The experiments conducted by [54] involving transcriptome-wide screening confirmed the presence of PBX1 in uNK cells and not pNK. The transcriptome-wide screening also revealed the production of Growth promoting factors (GPF) by uNK. The regulation of expression of GPFs (PTN and OGN) by PBX1 in uNK cells is confirmed by [54]. Analysis of uNK cells of patients with RSA (Recurrent spontaneous abortion) and URSA (Unexplained recurrent spontaneous abortion) by Zhou et al. [54] revealed the reduced expression of PBX1 in these patients as compared to the controls. This suggests the involvement of PBX1 in abortion and fetal growth restriction was also observed in the PBX1 deficient uNK cells [54].

The significance of differentially expressed TFs in pNK and uNK cells has been summarized in Table 3.The differences in the regulation of

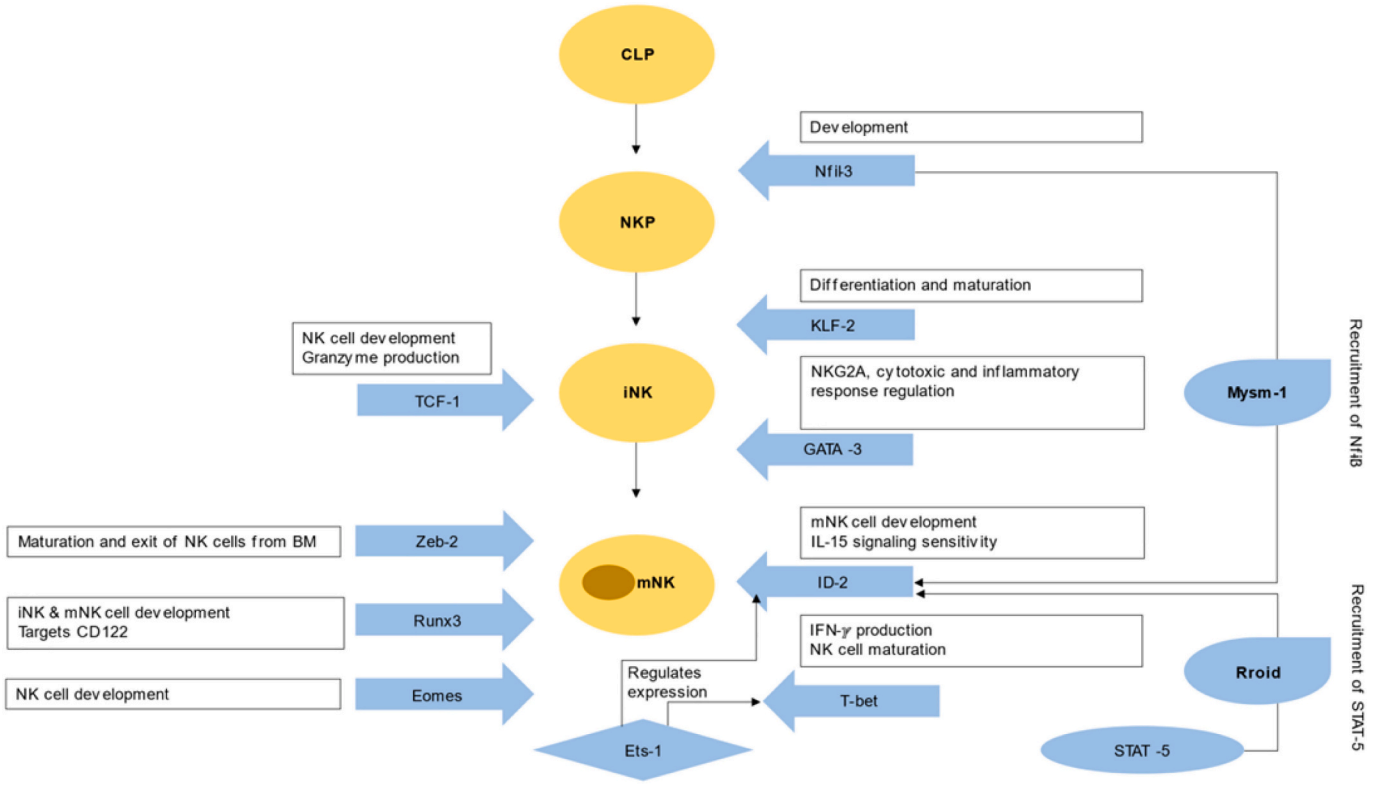

Fig. 3. The figure represents the Transcription Factors involved in the different stages of peripheral natural killer cell development.

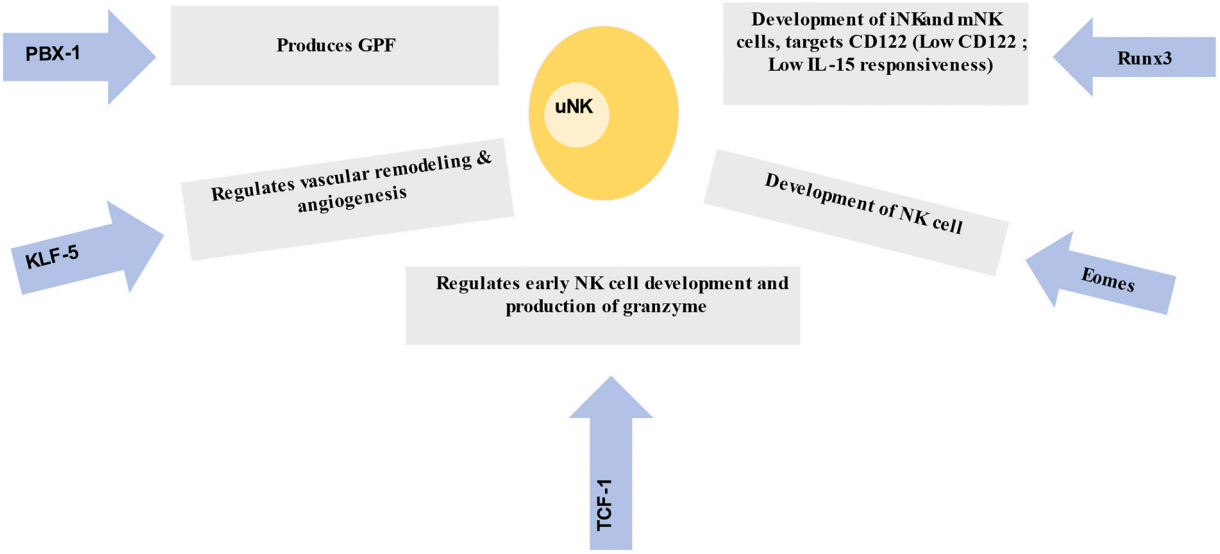

Fig. 4. The figure is a schematic representation of the Transcription factors, Runx3, Eomes, TCF-1, KLF-5, and PBX-1 involved in uterine NK cell development and their impact on NK cell function. *Production of PBX-1 and KLF-5 exclusively by uNK cells in addition to common TFs. (GPF-Growth promoting factors).

transcriptional factors in pNK and uNK cells have been summarized and contextualized in Figs. 3 and 4 and Table 2.

4.15. Strategies to manipulate TF expression to regulate NK cell function

The methodologies used to manipulate TF expression in order to regulate NK cell function involve the autologous transfer of the desired TF into the NK cells in-vitro (Fig. 5a) or using a nanoparticle delivery system to transfect an NK cell and silence the negative regulator of the desired TF (Fig. 5b).

Adoptive transfer of autologous NK cells by Parkhurst et al. on patients with metastatic melanoma showed the technique is efficient for treating patients with cancer [55]. Hence, it can serve as a prospective strategy to manipulate the TF expression to regulate NK cell function in conjunction with other immunomodulation therapies involving T cell modulation.

For NK cell transfection, the first vectors used were retroviral vectors. Their efficiency was too low. Therefore, there has been a progression in developing transfection strategies to improve the NK cell transfection [56]. The nanoparticle delivery method is currently the

Table 2
Comparison between the regulation of transcriptions factors in pNK and uNK cells.

Transcriptional factors	pNK	uNK	References
NFIL-3[a]	Unaltered	Absent	[3,14,15,36,38–40,83–89]
Id-2	Unaltered	Absent (Because it is regulated by NFIL-3 which is not required by uNK)	[3,8,83,90]
TCF-1	Unaltered By $CD56^{bright}$ cells	Unaltered	[44]
KLF-2[b]	Unaltered	(KLF-5 present)	[3,46,47]
Ets-1	Unaltered	Absent (ETS-1 deficiency leads to upregulation of Eomes, required by uNK in abundance.)	[3,49]
Rroid	Unaltered	Absent	[3]
GATA-3	Unaltered	Absent	[3,8]
T-bet	Upregulated	Absent	[3,14,41,42]
Zeb-2	Unaltered	Absent	[3,45]
STAT-5	Unaltered	Absent	[3,50,51]
Mysm-1	Unaltered	Absent	[3,52]
Eomesodermin	Unaltered	Upregulated	[9,14,41,42,58]
Runx3	Unaltered	Upregulated	[3,48]
PBX1	Absent	Unaltered	[54]

[a] Nfil-3: Expressed in early stages of natural killer cell development with constitutive expression throughout the development [37].
[b] KLF-2: Expressed in early stages of natural killer cell development with increasing levels with maturation of natural killer cells [46].

most widely used technique. Using nanoparticles for transfection instead of viral vectors prevents random insertional mutagenesis concerns. Electroporation, trogocytosis-mediated methods, and charge-altering releasable transporters (CARTs) are other non-viral transfection strategies that would help achieve the same [56].

5. Conclusion

Uterine and Peripheral natural killer cells differ in expression levels of various surface markers, cytokines, and transcriptional factors. We can account for this difference as the cause of uNK cells being less cytotoxic than pNK and its involvement in pregnancy complications. $CD56^{bright}$ pNK cells and uNK cells are similar in various aspects involving the increased expression of CD56 markers. However, NFIL-3, a crucial transcriptional factor that regulates the early stages of natural killer cell development, is absent in uNK cells. Since NFIL-3 regulates the expression of ID-2, we can conclude that ID-2 is also probably absent in uNK cells. uNK cells show a highly increased expression of Eomesodermin, Runx3, and PBX1, which is not observed in pNK cells. PBX1 expression by uNK cells is involved in the production of growth-promoting factors, and a decrease in the expression of PBX1 leads to fetal growth restriction and recurrent spontaneous abortions. Pre-eclampsia and recurrent spontaneous abortion have been reportedly caused due to the loss of uNK cells [27]. This is probably due to the reduced expression of factors produced by uNK, and the uNK deficient uterus leads to these complications. A sudden migration of immature NK cells has also been observed in pregnancy, which leads to their maturation by interacting with the components of the uterus. This also leads to changes in their phenotype [27]. The absence or reduced number of uNK cells in cases of pre-eclampsia and recurrent spontaneous abortion have also been confirmed by Fraser et al. [20]. Also, Kofod et al. [57] observed infertile women with a decrease in the number of uNK cells

Table 3
Significance of differential expression of transcriptional factors (TF).

TF	Biological pathways involved	Function	References
NFIL-3	• NF-kappaB signaling • IL-4 signaling pathway • Circadian rhythm related genes • Development & heterogeneity of the ILC family	• Required in early NK cell development • Regulation of circadian rhythm • Development of immune cells and regulation of ovulation	[3,38,91]
EOMES	• NF-kappaB signaling • Development & heterogeneity of the ILC family • IL-12 mediated signaling events • Endoderm differentiation • Mesodermal commitment pathway	• Involved in vertebrate embryogenesis	[1,92]
T-BET	• NF-kappaB signaling • IL-12 mediated signaling events • Innate lymphoid cells differentiation • Calcineurin-regulated NFAT-dependent transcription in lymphocytes • Development & heterogeneity of the ILC family	• Involved in NK cell maturation • Supports proliferation of NK cells • Production of IFN-γ	[1,93]
GATA-3	• Cytokine signaling in immune system • G-AlphaQ Signaling • Interleukin-4 and 13 signaling	• Regulates NK cell inhibitory receptor, NKG2A • Provides protection against infection by *Listeria monocytogenes* and *Citrobacter rodentium*	[1,8,43,94]
TCF-1	• Human embryonic stem cell pluripotency • Wnt signaling pathway • Regulation of Wnt-mediated beta catenin signaling and target gene transcription	• Regulates production of granzyme • Regulates the development of pNK cells	[1,3,95]
ID2	• Innate lymphoid cells differentiation • Wnt/Hedgehog/Notch pathway • Human early embryo development	• Development of NK cells and helper ILC lineages	[1,96]
ZEB2	• TGF-beta signaling pathway • TGF-beta receptor signaling	• Controls maturation of NK cells	[45,97]
KLF-2	• Embryonic and Induced Pluripotent Stem Cells and Lineage-specific Markers • EV release from cardiac cells and their functional effects	• The proliferation of NK cells	[46,98]
RUNX3	• TGF-beta signaling pathways • Notch signaling pathways	• Hematopoiesis • NK cell development	[99,1]
ETS-1	• Innate lymphoid cells differentiation	• Regulates early stages of NK cell development	[1,100]
STAT-5	• Immune response IL-23 signaling pathways • IL-2 pathway • IL-21,7,9,15 signaling pathways	• Homeostasis and NK cell development • Regulates adaptive immune responses	[1,51,101]
MYSM-1	• Metabolism of proteins • Deubiquitination	• Promote NK cell development	[1,102]

(*continued on next page*)

Table 3 (*continued*)

TF	Biological pathways involved	Function	References
		• Facilitates transfer of Nfil3 to Id2	
Rroid	• Targets Id2 gene, hence the biological pathways related to it.	• Later stages of NK cell development • Promotes ID2 activity	[1,51]
PBX1	• NF-kappaB signaling • Transcriptional regulation of pluripotent stem cells • Mesodermal commitment pathway	• Regulates the expression of growth-promoting factors	[54,103]

(CD56^{bright}) compared to fertile women. This leads to a connection between uNK cells, expression levels of transcriptional factors produced by them, and pregnancy complications.

The differential expression of transcription factors is exploited in treating diseases like cancer and arthritis by devising therapeutics targeting TF [58]. Inhibiting the activity of the transcription factor involved in the progression of a disease or using gene therapy to introduce the corrected genes (using CRISPR) are some possible therapeutic strategies. Knowing the effects of PBX-1 deficiency in uNK, an adoptive transfer strategy can be used for PBX-1 present in Uterine NK cells by inducing their expression in-vitro in patients with recurrent spontaneous abortion [54]. The involvement of transcription factors in development, differentiation, and function has led to their use in devising therapeutics. Acquiring a better understanding of TFs, their downstream targets, and their effects on disease progression would help prevent pathogenesis and help in therapeutics as represented by Fig. 5. TFs can be modulated using several strategies like CRISPR for targeted genome editing of TFs in NK cells, leading to repression or increased expression of that TF [59]. Using the adoptive transfer of NK cells (Fig. 5) autologous NK cells can be modulated for expression of specific TF and transferred in vivo. Alternatively, in vivo TF modulation in combinatorial therapy especially in the context of T cell modulation in tumor microenvironment may be a more effective therapy in cancer immunotherapy [60]. Peptide inhibitors could also be used to design therapeutics aiming to interfere with the interaction of TFs, preventing their expression [61]. Using siRNA-mediated gene silencing (Fig. 5), the negative regulators of TFs can be silenced, resulting in the prevention of protein production and repression of TF [62]. Combinatorial drug development for concomitant immunoregulation has immense potential, and transcription factor-mediated regulation of key immune sentinels like NK cells and T cells are essential in the context of therapeutics in cancer and viral infections [63]. Differential expression of TFs during development is also crucial in preventing autoimmunity and rejection of fetus and thus perpetuating pregnancy. Our understanding of this differential TF expressions may help modulate the NK activity in tumor microenvironment and thus affect cancer prognosis.

CRediT authorship contribution statement

The first author Priyanka Rawat has contributed in primary data compilation and writing original draft of the review.

The corresponding author Dr. Asmita Das has contributed in conceiving the concept of the review, supervision of data curation, reviewing and editing the manuscript.

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Consent statement/ethical approval

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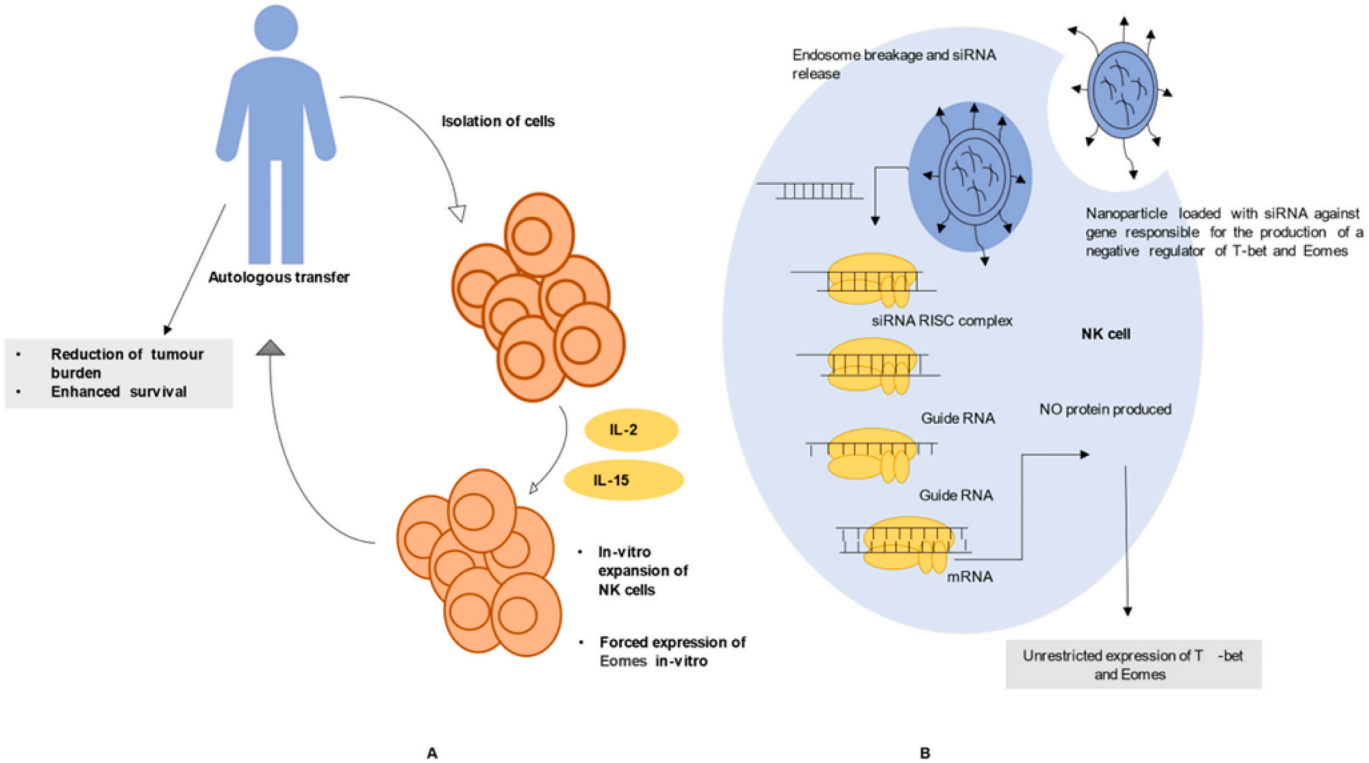

Fig. 5. Modulation of TFs using Adoptive transfer of NK cells and siRNA mediated gene silencing. A) Autologous adoptive transfer of NK cells with forced expression of Eomes reducing tumor burden. B) siRNA-mediated gene silencing to prevent the production of a negative regulator of T-bet and Eomes.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The present study does not contain any human participants or animals and has been carried out in the Department of Biotechnology, Delhi Technological University, following all ethical principles of the university. The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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I hereby certify that the Dissertation titled "***CORRELATING THE TRANSCRIPTIONAL REGULATION OF uNK AND pNK CELLS WITH FUNCTIONAL MODALITIES THROUGH META-ANALYSIS APPROACH***" which is submitted by Priyanka Rawat, 2K20/MSCBIO/23, Department of Biotechnology, Delhi Technological University, New Delhi is in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record of the work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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