Harbinger of Trouble; Fingerprints in the Pre-Senile Stage of NK Cell's Dysfunction

Seyhan TURK[1], Ayriana Safari BAESMAT[2], Aysegul YILMAZ[2], Umit Yavuz MALKAN[3], Can TURK[2], Ibrahim Celalettin HAZNEDAROGLU[3], Gulberk UCAR[1]

[1] Hacettepe University, Faculty of Pharmacy, Department of Biochemistry
[2] Lokman Hekim University, Faculty of Medicine, Department of Medical Microbiology
[3] Hacettepe University, Faculty of Medicine, Department of Hematology

ABSTRACT

Natural killer (NK) cells are the predominant innate lymphocyte subsets that mediate anti-tumor and anti-viral responses. The importance of NK cells in the immune system remains significant during old age. Age-associated changes in natural killer (NK) cell population, phenotype, and functions are directly attributed to the risk of several diseases and infections. This in silico study aimed to discover the potential age-related gene biomarkers in NK cells isolated from pre-elderly individuals and investigate their role in the cytotoxicity capacity of NK cells. Transcription profile data of human NK cell lines isolated from 13-62 years old individuals (GSE19067), were obtained from the gene expression omnibus database. We used a series of computational methods including GEO2R analysis, hierarchical clustering, functional pathway analysis, and Gene set enrichment analysis to identify the transcriptome alterations on age related NK cells. The impacts of nine genes (COL21A1, CYB5A, LIF, MEIS2, PTPRM, RASEF, RBMS3, TCF4, ZNF827) with substantially altered expression were assessed in relation to the age related NK cells. These nine genes have two important features, firstly, when we compare the under-40 age (group A) and over-40 age (group B) groups, they are expressed highly differently (adj-p< 0.05, log FC> 3), and secondly, the expression rates alter with age (Pearson p< 0.05, r > 0.6). All of these genes were discovered to be downregulated in over 40 years old NK samples. In this study, we investigated the transcriptional changes of NK cell lines in the pre-senile period and how these changes may affect the cytotoxicity functions of NK cells. Hope to identify early signs of alteration in NK's genome that lead to dysfunction or malfunction of these important immune cells in old age.

Keywords: Natural killer, Gene biomarkers, Aging

INTRODUCTION

Natural killer (NK) cells are a type of lymphocyte, a white blood cell that plays a critical role in the innate immune system. NK cells are responsible for detecting and destroying infected or abnormal cells, such as cancer cells or virus-infected cells, without the need for prior exposure or recognition of a specific antigen. They are called "natural" killers because they provide immediate defense against foreign cells, unlike T and B cells, which require time to recognize and respond to specific antigens.[1] NK cells recognize and attack infected or abnormal cells by using receptors that detect changes in the cells' surface proteins. NK cells also produce cytokines that can help to stimulate other cells of the immune system to mount a more targeted response. Therefore, the importance of NK cells lies in their ability to detect and destroy potentially harmful cells, which helps to prevent the spread of infection or cancer in the body.[2,3]

The importance of NK cells in the immune system remains significant during old age. However, there are changes in NK cell function and numbers that occur as a result of aging, which may impact their ability to protect against infections and cancer. One of the major changes that occur in NK cells with aging is a decline in their numbers and activity. This reduction in NK cell function may contribute to the increased susceptibility to infections and cancer that is seen in older adults.[4]

Additionally, there is evidence to suggest that chronic inflammation that occurs with aging can impair NK cell function. Despite these changes, NK cells continue to play an important role in the immune response in older adults.[5] They provide a first line of defense against infections and cancer and can help to stimulate other cells of the immune system. Therefore, it is important to maintain a healthy lifestyle, including exercise and a balanced diet, to help support NK cell function during old age. Additionally, research is ongoing to investigate ways to enhance NK cell function and improve immune responses in older adults.[6]

There are several gene biomarkers associated with NK cell aging, which can help to identify changes in NK cell function and phenotype that occur with aging. One such gene biomarker is the downregulation of NKG2D, which is a receptor on NK cells that recognizes stress-induced ligands on target cells.[7] NKG2D downregulation is associated with impaired NK cell function and increased susceptibility to infection and cancer in older adults. Another gene biomarker is the upregulation of inhibitory receptors, such as KIR, which can reduce the ability of NK cells to recognize and eliminate target cells. Additionally, aging is associated with changes in cytokine production and signaling pathways, including decreased production of IL-2, which is important for NK cell activation and proliferation. Furthermore, aging is associated with alterations in gene expression patterns, including changes in the expression of genes involved in cellular metabolism, DNA repair, and oxidative stress response, which can impact NK cell function.[8,9]

Overall, these gene biomarkers can help to identify changes in NK cell function and phenotype that occur with aging and may provide insights into the underlying mechanisms of immune aging and age-related diseases. However, more research is needed to fully understand the role of these gene biomarkers in NK cell aging and to develop targeted interventions to enhance NK cell function in older adults.[4,10] This in silico study aimed to discover the potential age-related gene biomarkers in NK cells isolated from pre-elderly individuals and investigate their role in the cytotoxicity capacity of NK cells.

MATERIALS and METHODS

Gene expression profiling data of NK-cell lines and determining age-based groups:

Transcription profile data of human NK cell lines (HANK, IMC1, KAI3, KHYG1, NK92, SNK1, SNK6, and SNK10) isolated from 13-62 years old individuals (GSE19067), were obtained from the gene expression omnibus database. HANK, IMC1, KHYG1, and NK92 cell lines have been isolated from patients with lymphoblastic leukemia. SNK1 and SNK6 cell lines have been isolated from patients with extranodal nasal NK/T cell lymphoma. KAI3 is a transformed NK cell line, and SNK10 has been isolated from patients with chronic active EBV infection of T- and NK-cell types, as mentioned in the manufacturer's product sheet.

Although it is important to note that human age periods are approximate and may vary depending on individual circumstances and cultural factors. Many studies categorize people over 65 as elderly. Generally, 13-19 ages belong to adolescence, 20-39 ages to early adulthood, and 40-65 to middle adulthood (pre-elderly) groups respectively.

In this study in order to determination of genes whose expression significantly alters especially in pre-elderly age (middle adulthood) we divided the NK cell lines cohort into two groups. Group A (HANK, KAI3, NKYS, SNK1, SNK10) includes NK cell lines isolated from individuals under 40 years old and group B (IMC1, KHYG1, NK92, SNK6) over 40 years old.

Processing and Normalization of Data

The raw data from the GSE19067 dataset were normalized with the Affy package in the R software (version: 4.0.5). Normalized transcription profile data consists of 22.230 different genes/54.616 probe sets. The data contains replicate gene expression values for HANK and SNK10 cell lines and a single value for the rest.

Identification of Differentially Expressed Genes Between Age-Based Study Groups

Using the limma (version 3.26.8) powers differential expression analysis, the whole normalized gene expression data of NK cells from group A

(< 40 years old; Early adulthood and adolescence; KAI3, HANK, SNK10, NKYS, and SNK1 NK cell lines) and group B (> 40years old; Pre-elderly; IMC1, KHYG1, NK92, and SNK6 NK cell lines) were compared to identify the significance and differentially expressed genes. Limma (version 3.26.8) is a R/Bioconductor (version 3.6.3) software tool for evaluating data from gene expression studies. It has a multitude of options for managing complicated experimental designs and borrowing information to overcome the issue of small sample numbers.

Linear Regression Analysis

Pearson correlation coefficient analysis was performed to identify genes that were highly correlated with age among genes that were expressed significantly differentially between the two groups. Pearson's correlation absolute P value based on the correlation coefficient was calculated using GraphPad Prism 5.0 (GraphPad Prism 5 Software, San Diego, CA, USA), and genes above 0.05 were selected. In addition, the Pearson correlation coefficient value (R-value) was calculated and genes above 0.6 were selected.

Hierarchical Clustering

Genes determined in the linear regression analysis were hierarchically clustered with mean standardized gene expression values with the Euclidean Gene Cluster 3.0 program. The data was standardized after cluster analysis, and the standardized data were viewed using Treeview. Hierarchical clustering was performed by clustering both genes and arrays using Euclidian distance as a similarity metric and complete linkage as a clustering method.

Network Analysis

To generate a network based on coexpression and genetic interactions, the GeneMANIA software was used and genes in similar pathways were identified with Cytoscape.[11] The datasets have been integrated, analyzed, and visualized to find out if they have functionally similar genes associated with each other and to identify related functions for different gene groups in the network. Thus, the network relationship of these genes was determined. The software scores each gene (node). The size of nodes directly correlated with the score.

Pathway Enrichment Analysis

To understand the biological linkage behind these genes, "Database for Annotation, Visualization and Integrated Discovery" (DAVID) software was used. The pathways associated with our genes were identified.

Gene Set Enrichment Analysis (GSEA)

The gene set enrichment analysis (GSEA) was carried out in concordance with GSEA guideline procedure.[12] E-MEXP-3496 data was used in order to perform the analysis. Analysis was performed between group B versus group A. The main purpose of this analysis is to determine which gene is significantly enriched in which gene set belongs to the GSEA as well as to understand which gene set is enriched in which groups.

GSEA calculates the enrichment score (ES), normalized enrichment score (NES), nominal P value (NOM P value), false discovery rate q value (FDR q value), and familywise error rate P value (FWER). The ES value indicates gene's maximum deviation in gene sets; in other words, this score helps to find most upregulated genes. NES value represents the connection or difference between gene sets and gene expression. The higher NES value shows the elevation of permutations. Hence, higher NES value increase significance of gene sets. In addition to ES and NES values, NOM P value evaluates the importance of ES calculation. Therefore, NOM P value is directly correlated with ES as well as NES value. Increase of NOM P value shows critical role of ES. On the other hand, FWER P value indicates false positives probability of NES and so, lower FWER P value directly and significantly correlated with correctness of NES calculation. Moreover, the FDR q value is the most vital value of this analysis. This value needs to be lower than 0.25 and even, when this value becomes smaller, the enrichment of gene sets is more meaningful.

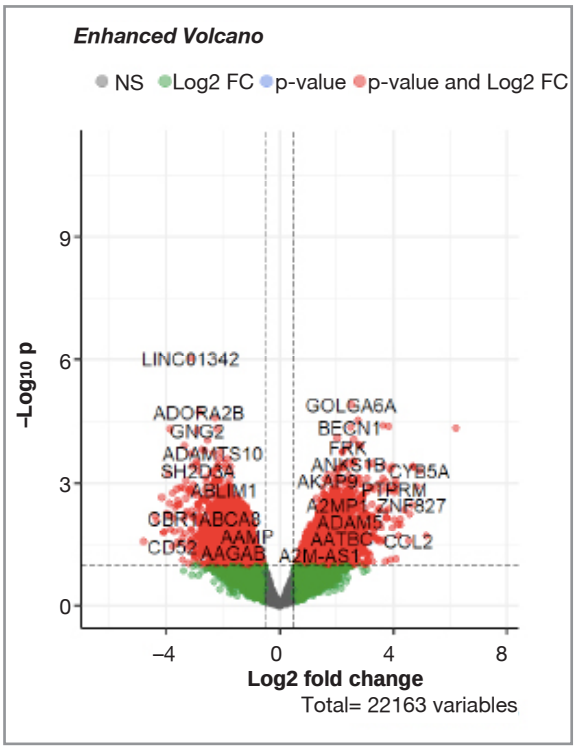

Figure 1. Comparison of whole genome expression between group A (< 40 years old; Early adulthood and adolescence) and group B (> 40 years old; Pre-elderly). Over 500 genes are significantly differentially

Table 1. Linear Regression analysis of nine genes whose expression values show a high and significant correlation with age.

Gene	p-value	r-value
RBMS3	0.00083	0.0098
PTPRM	0.0014	0.013
ZNF827	0.0001	0.03
COL21A1	0.0103	0.03
TCF4	0.0004	0.035
RASEF	0.0001	0.03
LIF	0.0003	0.042
MEIS2	0.001	0.031
CYB5A	0.0003	0.04

Statistical Analysis

All statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA).

A value of p and q < 0.05 was statistically significant.

Ethical Considerations

Ethical permission was not required for this study as it solely utilized publicly available or commercially obtained cell lines and their transcriptomic data and did not involve human subjects or animal experimentation.

RESULTS

In order to determine genes whose expression alters age-wise in NK cells and investigate their effect on cytotoxicity function we divided our sample cohort into two groups. Group A consists of five NK cell lines (HANK, KAI3, NKYS, SNK1, and SNK10), and all of them were isolated from individuals under 40 years old (mean age: 17.21) while group B includes four samples (IMC1, KHYG1, NK92, SNK6) isolated from over 40 years old individuals (mean age: 49.20).

Over 500 genes were found to be significantly differentially expressed as a result of comparing the group A with the group B (Figure 1). Among these large number of genes, 21 genes were determined to have p values < 0.01 and absolute Log fold change (FC) values above 4.

Then we asked whether these differentially expressed genes are related to age or not. Pearson correlation coefficient analysis shows that the expression of nine (RBMS3, PTPRM, ZNF827, COL21A1, TCF4, RASEF, LIF, MEIS2, CYB5A) out of 21 genes were significantly (p value < 0.05) downregulated (r value > 0.6) as the age increases (Table 1). Figure 2 shows the two genes (RBMS3 and ZNF827) with the lowest p and FC values of these nine genes that have the highest correlation with age.

COL21A1, CYB5A, LIF, MEIS2, PTPRM, RASEF, RBMS3, TCF4, ZNF827 have two important features, firstly, when we compare the under-40 and over-40 age groups, they are expressed highly differently (Data not shown). All of these genes were discovered to be downregulated in over 40 years old NK samples.

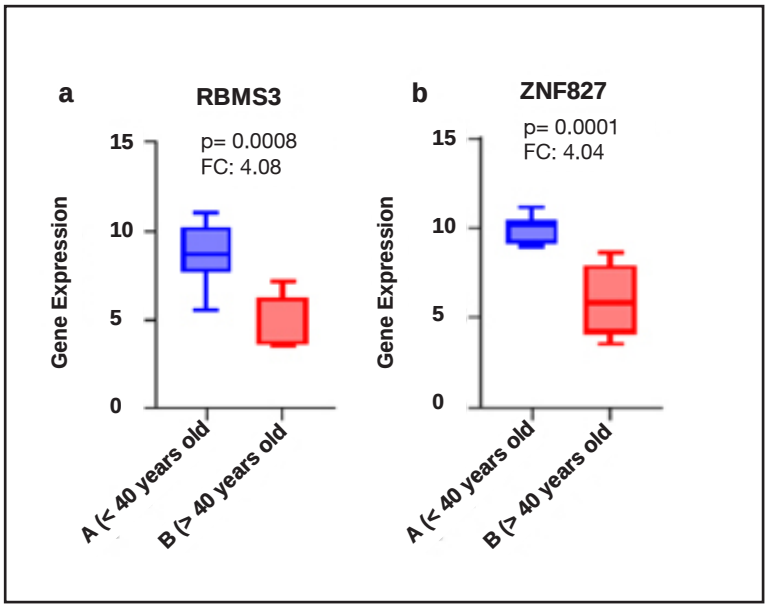

Figure 2. The p and FC values of RBMS3 and ZNF827 genes, which are among the genes with the highest correlation with age.

In addition, hierarchical cluster analysis showed that these genes can distinguish perfectly between Nine differentially expressedelated and age-related genes could well distinguish between group A and group B (Figure 3).

Clustering analysis of functional annotations for these genes revealed two distinct groups. According to their enrichment scores, the first cluster (ES: 0.78) contains the majority of genes involved in transcription regulation and DNA binding, and the second cluster (ES: 0.21) contains genes associated with signaling significantys.

To better demonstrate the biological linkage of these nine genes whose expression are downregulated in NK cell lines isolated from over 40 years old individuals, network analysis was performed. There is a strong genetic interaction network link between eight of these genes (COL21A1, CYB5A, LIF, MEIS2, PTPRM, RASEF, RBMS3, TCF4).

We used GSEA to discover which gene sets were enriched in the NK cell lines isolated from over 40 years old individuals (group B). 616 out of 3922 genesets were found to be significantly enriched at FDR < 25%. Table 2 shows the top significant 20 genesets enriched in group B. Among these top 20 genesets, it was remarkable that six of them were directly related to mitochondrial functions.

DISCUSSION

Aging of natural killer cells is associated with an increased susceptibility to specific diseases. Changes in the function and activity of aged NK cells contribute to disease susceptibility and progression. Aging leads to significant impairments in the two main mechanisms by which NK cells provide host protection: direct cytotoxicity and the secretion of immunoregulatory molecules. Viral infections become more severe in older individuals due to the reduced cytotoxicity and impaired immune response of aged NK cells. Additionally, impaired NK cell function allows cancer cells to evade immune surveillance, increasing the risk of cancer development and progression. Aged NK cells may also disrupt immune tolerance, contributing to the initiation of autoimmune diseases. The chronic low-grade inflammation associated with aging, driven by alterations in NK cell activity, can exacerbate chronic inflammatory diseases. Moreover, changes in NK cell activity in the retina are associated with age-related macular degeneration, a leading cause of vision loss in older individuals. In this study, we investigated the transcriptional changes of NK cell lines in the pre-senile period and how these changes may affect the cytotoxicity functions of NK cells. Hope to identify early signs of alteration in NK's genome that lead to dysfunction or malfunction of these important immune cells in old age.

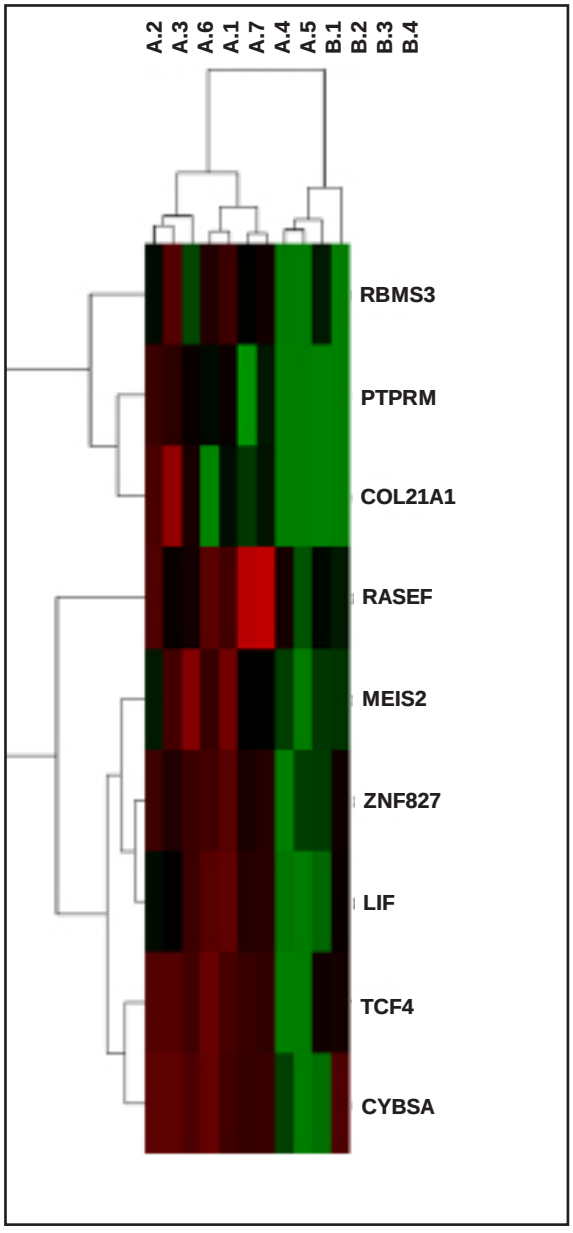

Figure 3. Nine differentially expressedelated and age-related genes could well distinguish between group A (< 40 years old; Early adulthood and adolescence) and group B (> 40years old; Pre-elderly).

We found nine genes have been downregulated in NK cell lines isolated from pre-elderly individuals. COL21A1 is a type of collagen protein that has been found to play a role in various biological processes, including cell adhesion, tissue development, and wound healing.[13] While there is limited research on the specific effects of COL21A1 on NK cell cytotoxicity, some studies suggest that it may indirectly influence NK cell function. One study found that COL21A1 expression was upregulated in the liver during viral infection and that this upregulation was associated with increased NK cell activation and cytotoxicity. Specifically, the researchers found that COL21A1 expression was induced by type I interferons, which are signaling molecules that are produced in response to viral infections and are known to enhance NK cell activity.[14] Another study found that COL21A1 may play a role in regulating the activity of immune cells in the tumor microenvironment. The researchers found that COL21A1 was highly expressed in tumors and that its expression was associated with the recruitment of immune cells, including NK cells, to the tumor site.[15] However, the exact mechanism by which COL21A1 affects NK cell activity in this context is still unclear. Overall, while there is some evidence to suggest that COL21A1 may play a role in modulating NK cell function, further research is needed to fully understand the mechanisms involved.

CYB5A, or cytochrome b5 type A, is a protein that plays a role in electron transfer reactions in cells. It has been shown that CYB5A is upregulated in response to the activation of immune cells, including NK cells. The researchers found that CYB5A is involved in the regulation of cellular respiration and that its upregulation is associated with increased cellular metabolism and oxidative phosphorylation, which can enhance the function of NK cells.[16] Another study found that CYB5A is overexpressed in some types of cancer and that its expression is associated with decreased NK cell activity. The researchers found that CYB5A expression in cancer cells leads to the production of reactive oxygen species (ROS), which can impair the function of NK cells and other immune cells.[17,18]

LIF, or leukemia inhibitory factor, can enhance the cytotoxic activity of NK cells. The researchers found that treatment with LIF resulted in increased expression of NK cell activation markers and enhanced cytotoxicity against target cells. The mechanism by which LIF enhances NK cell activity is not fully understood, but it may involve the activation of signaling pathways that promote NK cell function.[19] Another study found that LIF can enhance the survival and proliferation of NK cells. The researchers found that treatment with LIF resulted in increased expression of anti-apoptotic

Table 2. Top significant 20 genesets enriched in group B (> 40 years old; Pre-elderly).

GS	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK at MAX
GOCC_MITOCHONDRIAL_PROTEIN_CONTAINING_COMPLEX	244	-0,5	-2,55	0	0	0	6335
GOBP_RRNA_METABOLIC_PROCESS	236	-0,5	-2,53	0	0	0	6079
HP_INCREASED_CSF_LACTATE	79	-0,58	-2,45	0	0	0	7060
GOBP_MITOCHONDRIAL_GENE_EXPRESSION	154	-0,51	-2,45	0	0	0	6614
GOBP_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS	413	-0,46	-2,45	0	0	0	6097
GOBP_NADH_DEHYDROGENASE_COMPLEX_ASSEMBLY	46	-0,63	-2,44	0	0	0	5669
GOCC_ORGANELLAR_RIBOSOME	87	-0,55	-2,41	0	0	0	4876
GOBP_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY	84	-0,56	-2,4	0	0	0	5669
GOBP_RIBOSOME_BIOGENESIS	278	-0,47	-2,36	0	0	0,002	6079
GOBP_MITOCHONDRIAL_TRANSLATION	125	-0,51	-2,34	0	0	0,003	6436
HP_ABNORMAL_CSF_METABOLITE_CONCENTRATION	105	-0,52	-2,32	0	0	0,003	7112
GOBP_NCRNA_PROCESSING	382	-0,43	-2,31	0	0,001	0,006	6079
HP_ABNORMAL_ACTIVITY_OF_MITOCHONDRIAL_RESPIRATORY_CHAIN	86	-0,53	-2,29	0	0,001	0,006	7488
HP_INCREASED_SERUM_LACTATE	161	-0,47	-2,27	0	0,001	0,011	5705
GOCC_MITOCHONDRIAL_LARGE_RIBOSOMAL_SUBUNIT	55	-0,56	-2,26	0	0,001	0,011	4876
GOCC_NADH_DEHYDROGENASE_COMPLEX	38	-0,61	-2,26	0	0,001	0,011	6895
GOBP_RNA_MODIFICATION	159	-0,47	-2,23	0	0,002	0,025	6030
GOCC_ORGANELLE_INNER_MEMBRANE	488	-0,41	-2,23	0	0,002	0,025	5285
GOCC_SNO_S_RNA_CONTAINING_RIBONUCLEOPROTEIN_COMPLEX	23	-0,7	-2,22	0	0,002	0,028	3821
GOCC_MITOCHONDRIAL_MATRIX	447	-0,41	-2,2	0	0,003	0,039	6465

Abbreviations: GS, Gene Sets; ES, Enrichment Score; NES, Normalized Enrichment Score; NOM, Nominal; FDR, False Discovery Rate; FWER, Familywise Error Rate.

proteins and enhanced proliferation of NK cells. This suggests that LIF may play a role in the maintenance of NK cell populations and function. The available evidence suggests that LIF may enhance NK cell cytotoxicity and promote the survival and proliferation of NK cells.[20] In this regard downregulation of this gene may be lead to the dysfunction of NK cells in the elderly ages. Myeloid ecotropic viral integration site 2 (MEIS2), is a transcription factor that plays a role in various biological processes, including embryonic development, cell differentiation, and immune responses. MEIS2 may play a role in modulating NK cell function through its effects on gene expression and the production of immunosuppressive factors. However, more research is needed to fully understand the mechanisms involved and to determine the specific effects of MEIS2 on NK cell cytotoxicity.[21]

It has been shown that PTPRM (protein tyrosine phosphatase receptor type M) is expressed on NK cells and that its expression is associated with enhanced cytotoxic activity. The researchers found that PTPRM promotes the activation of signaling pathways that enhance NK cell function and that its expression is correlated with increased expression of NK cell activation markers.[22]

RASEF may play a role in modulating NK cell function through its effects on signaling pathways and the production of immunosuppressive factors. It has been shown that its expression is correlated with increased expression of NK cell activation markers.[23]

RNA-binding motif, single-stranded interacting protein 3 (RBMS3), is involved in the regulation of the IFN-gamma response in NK cells. The researchers found that RBMS3 promotes the translation of IFN-gamma mRNA and that its expression is correlated with increased IFN-gamma production in response to NK cell activation.[24,25] Another

study showed that RBMS3 is downregulated in NK cells from patients with chronic lymphocytic leukemia (CLL) and that its downregulation is associated with decreased cytotoxic activity. The researchers found that RBMS3 expression in NK cells is necessary for their proper development and function and that its downregulation in CLL can lead to impaired cytotoxic activity.[26,27]

TCF4 is a transcription factor that plays a role in various biological processes, including the development and function of the immune system. TCF4 is involved mainly in the regulation of NK cell development and function. TCF4 is expressed in developing NK cells and its expression is necessary for their proper maturation and cytotoxic activity. It also regulates the expression of several genes involved in NK cell function, including genes involved in cytokine production and cell signaling.[28] While we were unable to find any clear insights into the specific effects of ZNF827 on NK cell cytotoxicity in the literature, it is possible that it may play a role in regulating NK cell function through its effects on gene expression. Further studies are needed to investigate the potential role of ZNF827 in the immune system and its effects on NK cell cytotoxicity.[29,30]

Interestingly our results demonstrated that mitochondria-associated gensets were enriched in the over-40 age group. This raises the question, whether dysregulation in mitochondria-related genes leads to cytotoxic loss of function of NK cells in old age. Mitochondria are organelles in cells that are responsible for energy production through the process of oxidative phosphorylation. In NK cells, mitochondrial activity can affect the production of reactive oxygen species (ROS) and adenosine triphosphate (ATP), which are important for NK cell function.[31,32] Studies have shown that increased mitochondrial activity in NK cells can lead to enhanced cytotoxicity against target cells. This is because higher mitochondrial activity leads to higher production of ATP and ROS, which can activate NK cells and promote their ability to recognize and kill target cells.[33,34] On the other hand, reduced mitochondrial activity can impair NK cell function and decrease their cytotoxicity. For example, NK cells from individuals with mitochondrial dysfunction have been found to have reduced cytotoxicity against target cells. Overall, the effect of mitochondrial activity on NK cell cytotoxicity is complex and depends on multiple factors, including the level and duration of mitochondrial activity, as well as the specific mechanisms through which mitochondrial activity affects NK cell function.[35]

Conclusion

Our results suggest novel nine genes as early biomarkers for NK cell dysfunction during old age. In addition based on our results, dysregulation in mitochondria-associated gensets might be responsible for the dysfunction of NK cells in the elderly.

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Correspondence:
Seyhan TURK, Ph.D.
Hacettepe Universitesi, Eczacilik Fakultesi
Biyokimya Anabilim Dali
Sihhiye, ANKARA / TURKIYE

Tel: (+90-544) 4501832
e-mail: seyhan.turk@hacettepe.edu.tr

ORCIDs:

Seyhan Turk	0000-0003-3843-4173
Ayriana Safari Baesmat	0000-0002-2969-8655
Aysegul Yilmaz	0000-0002-9541-9853
Can Turk	0000-0003-1514-7294
Umit Yavuz Malkan	0000-0001-5444-4895
Ibrahim Celalettin Haznedaroglu	0000-0001-8028-9462
Gulberk Ucar	0000-0002-8647-1545