Experimental Gerontology 110 (2018) 73–78

Contents lists available at ScienceDirect

Experimental Gerontology

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

Deficient mitochondrial biogenesis in IL-2 activated NK cells correlates with impaired PGC1-α upregulation in elderly humans

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ARTICLE INFO

Section Editor: Richard Aspinall

Keywords:
NK cells
Mitochondria
Interleukin-2
PGC-1α
Immunosenescence

ABSTRACT

Immunosenescence has been described as age-associated changes in the immune function which are thought to be responsible for the increased morbidity with age. Human Natural Killer (NK) cells are a specialized heterogeneous subpopulation of lymphocytes involved in immune defense against tumor and microbial diseases. Interestingly, aging-related NK cell dysfunction is associated with features of aging such as tumor incidence, reduced vaccination efficacy, and short survival due to infection. It is known that NK cell effector functions are critically dependent on cytokines and metabolic activity. Our aim was to determine whether there is a difference in purified human NK cell function in response to high concentration of IL-2 between young and elder donors. Here, we report that the stimulation of human NK cells with IL-2 (2000 U/mL) enhance NK cell cytotoxic activity from both young and elderly donors. However, while NK cells from young people responded to IL-2 signaling by increasing mitochondrial mass and mitochondrial membrane potential, no increase in these mitochondrial functional parameters was seen in purified NK cells from elderly subjects. Moreover, as purified NK cells from the young exhibited an almost three-fold increase in PGC-1α expression after IL-2 (2000 U/mL) stimulation, PGC-1α expression was inhibited in purified NK cells from elders. Furthermore, this response upon PGC-1α expression after IL-2 stimulation promoted an increase in ROS production in NK cells from elderly humans, while no increase in ROS production was observed in NK cells of young donors. Our data show that IL-2 stimulates NK cell effector function through a signaling pathway which involves a PGC-1α-dependent mitochondrial function in young NK cells, however it seems that NK cells from older donors exhibit an altered IL-2 signaling which affects mitochondrial function associated with an increased production of ROS which could represent a feature of NK cell senescence.

1. Introduction

Immunosenescence is defined as age-associated changes in the immune function that contribute to the increased susceptibility to infection, cancer, and autoimmune diseases observed in old organisms, including humans (Pawelec, 1999). In the context of immunosenescence, NK cells have been concentrating attention because alterations in their number, phenotype, and functions have been shown in the elderly (Mocchegiani and Malavolta, 2004; Solana et al., 2006; Gayoso et al., 2011). It has been described that in normal elderly people the relative risk for the development of infectious diseases increase with the decrease in NK cell activity. Moreover, low NK cell activity has been associated with short survival due to infection (Ogata et al., 2001). Current evidence suggests that aging-related NK cell dysfunction can also be associated with other features of aging, such as accumulation of senescent cells or reduced vaccination efficacy (reviewed in (Hazeldine and Lord, 2013)). Besides its ability to exert cytotoxic activity against target cells, NK cells also have an immunoregulatory role as they secrete several cytokines, such as IFNγ, and chemokines following their ligand interaction with cell-surface receptors, thus acting as a link between the adaptive and innate immune response. One important cytokine that helps coordinate and potentiate NK cell function is IL-2. As a

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https://doi.org/10.1016/j.exger.2018.05.014
Received 15 October 2017; Received in revised form 4 April 2018; Accepted 15 May 2018
Available online 18 May 2018
0531-5565/

matter of fact, it has been used as an immunotherapeutic agent to promote NK cell antitumor activity (Waldmann, 2006; Ljunggren and Malmberg, 2007), and it is currently used for metastatic renal cell carcinoma and melanoma treatment. It has also been described that IFNγ produced by NK cells from vaccinated adults challenged with influenza virus is largely dependent on IL-2 secreted by specific T cells in PBMC (He et al., 2004). However, the *in vitro* stimulation of NK cells of elderly donors with IL-2 showed a reduced production of IFNγ (Krishnaraj and Bhooma, 1996). Moreover, data suggest a deficient signaling in NK cells in response to IL-2 in elderly people (Borrego et al., 1999).

In recent years there has been increased interest in understanding how metabolic pathways regulate immune cell function to generate an efficient immune response (Pearce and Pearce, 2013). Recently it was described that upon activation, T cells switch from oxidative phosphorylation to aerobic glycolysis. The authors described that the ability of activated T cells to produce IFNγ is dependent on glycolysis (Chang et al., 2013). In relation to this, we recently described that IL-2 induces an increase in mitochondrial mass as well as mitochondrial membrane potential in human NK cells, depending on the increase in the expression of the transcriptional PGC-1α cofactor. Moreover, we also observed that IFNγ secretion induced by IL-2 is partially dependent on the increase of PGC-1α mRNA expression (14). Other studies have shown that, unlike T cells, murine NK cells do not require glycolytic switch for optimal IFNγ production (Keppel et al., 2015), and in human NK cells it was reported that elevated levels of oxidative phosphorylation were required to support both cytotoxicity and IFNγ production (Keating et al., 2016). This shows that metabolic adaptation of immune cells is not only important to supply the energy for cell function, but also as a regulatory mechanism that determines the immune cell's response itself.

During aging, mitochondria are the most damaged organelles, and their dysfunction has been closely related to the aging process. Recent work has related mitochondrial dysfunction with other cellular pathways of aging and with accelerated aging phenotypes in different tissues (Payne and Chinnery, 2015; Sahin et al., 2011; Ziegler et al., 2015; Gonzalez-Freire et al., 2015). This mitochondrial decline function may result from multiple mechanisms, including reduced biogenesis (Lopez-Otin et al., 2013; Wenz, 2011; Lopez-Lluch et al., 2008). Several transcription factors and cofactors have been implicated in the activation and regulation of mitochondrial biogenesis. However, all the signaling pathways involved in this process seem to share the common PGC-1α key component, which is a co-transcription factor that seems to be critical to activate mitochondrial biogenesis and respiration (Lopez-Lluch et al., 2008). The regulatory network formed by this coactivator is associated with its capacity to coactivate multiple transcription factors, coordinating diverse processes necessary for mitochondrial biogenesis (Lin, 2009). Furthermore, PGC-1α gene expression is rapidly increased in response to different external stimuli that increase energy demand in different tissues (Kupr and Handschin, 2015; Liu and Lin, 2011; Villena, 2015).

Since mitochondrial dysfunction is considered a hallmark of aging, and because mitochondrial biogenesis is critical for optimal NK cell activation, the aim of the present work was to analyze the relation between PGC-1α expression and mitochondrial behavior in response to IL-2 in NK cells from healthy older human adults. In this study, we present data that contribute to understand some of the mechanisms involved in preserving NK cell function, which could be useful to promote healthy aging.

2. Materials and methods

2.1. Donors

For this study, we recruited 27 healthy elderly volunteers of both sexes living in Metropolitan Santiago. The age at inclusion ranged from 63 to 95 years, with a mean age of 75 years. Blood samples of 13 healthy young adults were obtained from the blood bank of the Hospital Clínico of the Universidad de Chile. The age at inclusion ranged from 19 to 50 years, with a mean age of 29 years. We excluded elderly participants with chronic diseases such as diabetes, cancer, heart failure, kidney or liver failure, people taking medications that could affect their immune status or history of severe allergies, evidence of respiratory infection or any symptoms that could have pointed to an underlying illness. An overnight fasting blood sample was obtained to measure routine laboratory parameters (glucose, total cholesterol, HDL cholesterol, triglycerides, and creatinine), performed using routine automated laboratory methods.

This study was approved by the Ethics Committee of the Universidad de Santiago de Chile, and the volunteers gave their written informed consent.

2.2. NK cell purification and cell culture

Human peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation of blood samples over lymphocyte separation medium (Cellgro, Mediatech). Monocytes were depleted by plating PBMC on Petri dishes for 1 h at 37 °C. The lymphocytes were then harvested, washed with pH 7.4 phosphate-buffered saline (PBS), and suspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (HyClone, Thermo Scientific), and 20 μg/mL gentamycin (Invitrogen). NK cells were further enriched using the NK cell negative isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. This technique regularly produced highly purified NK cells with > 90% $CD3^-$ $CD16/CD56^+$ cells confirmed by flow cytometry (Gate strategy for NK cell purification analysis in Supplementary Fig. S1).

K562 cells used as target cells were grown at 37 °C in supplemented RPMI with 5% CO_2. Experiments were carried out with cells in the logarithmic growth phase.

2.3. NK cell activation

Purified human NK cells were incubated for 48 and 72 h with 2000 U/mL of IL-2 in supplemented RPMI at 37 °C and 5% CO_2 for activation. For control conditions, purified NK cells were incubated for the same duration with 50 U/mL of IL-2 for maintenance.

2.4. Cytotoxicity assay

A ^{51}Cr-release assay was used to quantify NK cytotoxic activity. 1×10^6 K562 cells were labeled by incubation in supplemented medium containing 100 μCi $Na_2{}^{51}CrO_4$ (PerkinElmer) for 1 h at 37 °C. We used 5000 ^{51}Cr-labeled cells/well as target cells, and human NK cells as effector cells, employing different E:T ratios. When indicated, effector cells were previously incubated with 5 μM oligomycin for 30 min and washed twice with RPMI. All the conditions were tested in triplicate. After 4 h of incubation at 37 °C and 5% CO_2, 100 μL of supernatant were recovered from each well and tested for radioactivity release. The cytotoxicity percentage was calculated as follows: 100 x [(experimental release – spontaneous release)/(maximum release – spontaneous release)]. Maximum release was obtained from target cells lysed with 2% Triton X-100 (Sigma, St Louis, MO, USA). Spontaneous release was always below 10% of maximum release.

2.5. PGC-1α, ATP5β, and MCAD expression quantification by RT-qPCR

Total RNA was isolated with E.Z.N.A. Total RNA Kit II (Omega, Bio-Tek, Inc.), and cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific). Quantitative RT-PCR for PGC-1α was performed using Maxima SYBR Green qPCR