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Leukemia Inhibitory Factor Suppresses NKG2D mRNA Expression and Presentation on Human Natural Killer Cells

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ABSTRACT

Leukemia inhibitory factor (LIF) is a multi-functional cytokine secreted from cells such as lymphocytes and hepatocytes. This study aimed to evaluate the effect of LIF on natural killer group 2 member D (NKG2D) receptors' expression and presentation on natural killer (NK) cells.

For this purpose, peripheral blood mononuclear cells taken from 4 young male healthy blood donors were isolated and the effect of LIF (25 ng/mL) after 12, 24, and 48 hours of incubation, on NKG2D receptors expression and presentation was investigated using flow cytometry and real-time-polymerase chain reaction (PCR). All of the steps of the experiment were performed in duplicate.

After periods of 12, 24, and 48 hours, LIF reduced both the expression and presentation of the NKG2D receptor on NK cells.

The results suggest that this cytokine has a direct modulating activity on the body's immune response through suppression of NKG2D receptor expression and presentation on NK cells.

Keywords: Flow Cytometry; Leukemia inhibitory factor; Natural killer cells; NK cell lectin-like reseptor subfamily K

INTRODUCTION

Leukemia inhibitory factor (LIF) is a glycoprotein from the interleukin-6 family of cytokines that have been identified in different mammals.¹ This protein was first identified in 1984 by affecting the differentiation of

leukemia cells in mouse cell lines.² This cytokine was named so due to its ability to induce the terminal differentiation of myeloid leukemia cells and consequently prevents their continuous growth.³ This glycoprotein has shown a wide range of biological effects in cell cultures and as a growth factor on different types of cells. Depending on the cell type, it exerts its effects by controlling stem cell pluripotency, lipid and bone metabolism, differentiation of cholinergic neurons, improvement of embryo implantation, angiogenesis, and

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release of acute-phase proteins by hepatocytes.⁴ LIF regulates these complex functions by binding to its specific receptor β subunit.⁵ The receptor of this cytokine (LIFR) is expressed in different cells including hepatocytes, neurons, and lymphocytes.⁶

Natural killer (NK) cells, as an important subset of cells of the immune system, are involved in innate defense against viral infections and tumor cells.⁷ NK cell activities are regulated by establishing a balance between the signals received by inhibitory and stimulatory receptors present on this cell. The natural killer group 2 member D (NKG2D) receptor is one of the most efficient stimulatory receptors on the NK cell and plays a key role in binding to target cells.⁸ NKG2D is a type II transmembrane receptor. Furthermore, NKG2D costimulates NK cell activity by binding to a recently identified family of cell surface ligands encoded by the *MHC class I chain-related (MIC)* genes and strongly stimulates antitumor immunity, thus leading to tumor rejection.

NKG2D ligands have α - and β -like MHC domains, but they either present peptides or bind to β 2-microglobulin. These ligands include glycoproteins MicA, MicB, and UL-16 binding proteins (ULBP) in humans.⁹ The level of NKG2D ligands expression is limited in normal cells, but it increases during tumor development and is known as a marker for cellular stress. Furthermore, NKG2D serves as a primary activation receptor, which is by itself able to trigger cytotoxicity.¹⁰ Some cytokines, such as IL-2, IL-7, and IL-15 increase its expression, while β TGF- and IL-21 have a reverse effect.^{11, 12}

Despite the large number of studies on LIF and its LIFR, the mechanism (s) that orchestrate the immunomodulatory activities of LIF remain largely unknown. Furthermore, recognition of the ligands that can modulate NK cell activity can be potentially useful therapeutic targets in various immune-mediated disease conditions. Therefore, the present study aimed to examine whether the observed effects of LIF are mediated by the expression and presentation of the NKG2D receptor in NK cells.

MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

This study was performed on 4 samples of young male healthy volunteers (range of age: 28-33). Participants signed a written informed consent to

participate and this study was approved by the Medical Ethical Committee of Ahvaz Jundishapur University (IR.AJUMS.REC.1393.160). The PBMCs were separated from their heparinized blood by density gradient-centrifugation (800 \times g for 20 min) using Ficoll-Hypaque with a density of 1.077. The PBMC were washed three times with RPMI 1640 complete medium (MyBioSource, San Diego, USA) and the viability of cells was determined by trypan blue staining, and cells with more than 90% viability were used for examination. The cells were suspended at a concentration of 1×10^6 cells/mL in RPMI 1640 complete medium containing penicillin/streptomycin (160 μ g/mL), amphotericin-B (2-5 μ g/mL), glutamine (2 mM), and 10% fetal calf serum (MyBioSource, San Diego, USA). Cells with 1.25×10^6 cell concentration were cultured in 12-well plates, 25 ng/mL of LIF (R&D Systems, USA) was added and incubated at 37°C, 5% CO₂ for 12, 24, 48 hours. To overcome the interface of heparin on real-time PCR measurement, 1 U of heparinase I (Sigma-Aldrich, Germany) was added 30 min before carrying out RNA extraction.¹³

Measurement of Expression of NKG2D mRNA by Real-time PCR (qRT-PCR)

The lymphocytes were pelleted (800 \times g for 5 min) and the medium was removed. Total RNA was isolated using a high pure RNA isolation kit (Jene Biosciences, Jena, Germany) according to the manufacturer's recommendations. The concentration and quality of extracted mRNA were determined spectrophotometrically at 260 nm wavelength and 260/280 nm wavelength ratio, respectively. Two μ g of extracted RNA, diluted to a final volume of 20 μ L, was reverse transcribed into cDNA using Revert Aid™ First Strand cDNA Synthesis Kit according to the manufacturer's recommendations (Thermo Scientific, Waltham, MA, USA). Expression of NKG2D mRNA in human lymphocytes was investigated using Applied BiosystemsStepOne™ (Life Technologies, Waltham, MA, USA) real-time PCR system. Data were normalized based on GAPDH expression as the housekeeping control gene. The primers were as following:

Forward: 5'-ACTGTGGCCCATGTCCTAAA-3',

Reverse: 5'-GGTTGGGTGAGAGAATGGAG3');

Forward:

5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3',

Reverse:

5'-TAGAAGCATTTCGGGTGGACGATGGAGGG-3'

PCR reaction mixtures contained: 2 μ L of cDNA, 10 μ L SYBR Premix ExTaq™ II (2^x) (Takara, Shiga, Japan) 1 μ L from each 5 μ M for forward and reverse primers and 6 mL of DEPC treated water. After an initial denaturation step at 95°C for 10 min, temperature cycling was initiated. Each cycle consisted of 95°C for 15s followed by 60°C for 60 s. A total of 40 cycles was performed. All assessments were carried out three times and in duplicates along with appropriate negative controls. Relative Expression Software Tool (REST, 2008 v2.0.7) was used for analysis by comparative Ct method ($\Delta\Delta C_t$).¹⁴ The results are presented as ratios (number of fold increase) relative to control.

Flow Cytometry for Determination of NKG2D Receptor Presentation

To determine the level of presentation of NKG2D receptors on the isolated PBMCs flow cytometry method was employed. For this purpose, after incubation of PBMC at 37°C and 5% CO₂ with LIF, non-adherent cells were recovered and washed three times with phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin and spun at 400 \times g for 3 min.

To differentiate that the presentation of NKG2D receptors on cell types, the isolated PBMCs cells were stained with selective anti-CD56-FITC, anti-CD3-

PECY5, and anti-NKG2D-PE (e-Bio Science, USA) respectively for 30 min at 4°C in dark and followed by one washing with PBS solution. Anti-CD56-FITC was considered as a selective marker for NK cells, while anti-CD3-PECY5 and anti-NKG2D-PE were used as markers for differentiation of T cells and both NK and T-cells respectively. The cells were fixed by 1% formaldehyde and were prepared for flow cytometry (Partec Germany class I Laser Producer) technique.

Statistical Analysis

All results are expressed as mean \pm standard deviation (SD). Stemness markers expressions and proliferation assay were statistically analyzed by multiple t-tests and $p < 0.05$ was considered statistically significant.

The obtained results from flow cytometry were analyzed statistically by Flomax and WIN MDI software version 2.8. A minimum of 10,000 cells was gated. Percentages of decrease in the proportion of NKG2D receptor expression were calculated by using the following equation:

% Reduction in NKG2D receptor =

$$\frac{\text{No of receptors in unexposed control} - \text{No of receptors in exposed cells}}{\text{No of receptors in exposed cells}} \times 100$$

Table 1. The percentage of decrease, relative to unexposed control, in natural killer group 2 member D (NKG2D) receptor presentation on natural killer (NK) cells following exposure to 25 ng/mL of leukemia inhibitory factor (LIF) at various time points (12, 24, and 48 hours).

Exposure Time (hr)	Unexposed Control	LIF Exposed	% of Decrease	<i>p</i>
12	22.4 \pm 1.2	11.4 \pm 2.2	49.1***	0.0005
24	18.4 \pm 1.3	7.6 \pm 1.6	58.2***	0.0007
48	18.4 \pm 1.7	9.3 \pm 1.4	49.4***	0.0005

RESULTS

Real-time PCR Results for NKG2D mRNA Expression

The mRNA expression of NKG2D on the isolated PBMCs, following exposure to 25 ng/mL of LIF, relative to unexposed controls, was highly significantly decreased ($p < 0.001$). The level of reduction of mRNA expression was reduced by 88 % after a relatively short period of 12 hours exposure, from base-line value 1 to 0.129 \pm 0.2 (95 % CI, $p < 0.001$) (Figure 1). After 24 and 48 hours of exposure the expression, relative to 12

hours of exposure, was further reduced by 50 and 67 % (to 0.063 \pm 0.2 and 0.043 \pm 0.2 respectively).

Flow Cytometry Results for Assessment of NKG2D Receptor Presentation

The antibody selective markers experiments; using selective markers for NK and T-cells, showed a highly significant ($p < 0.001$) reduction of surface NKG2D presentation on NK cells after PBMC exposure to LIF for 12, 24, 48 hours, compared with control samples (Table 1).

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The level of decrease in the percentage of NKG2D surface receptor presentation, relative to control unexposed control, at 12, 24, and 48 hours after exposure to 25 ng/mL of LIF was 49.1, 58.2, and 49.4% respectively (Figure 2, Table 1).

Gating Strategy Used to Identify NK Cells

The lymphocytes were live gated during acquisition using the side and forward scatter dot plot display. Furthermore, by using the negative gating strategy, a CD3-negative lymphocyte population was identified. The NK cell population was further identified with CD3⁻CD56⁺NKG2D⁺ (e-Bio Science, USA).

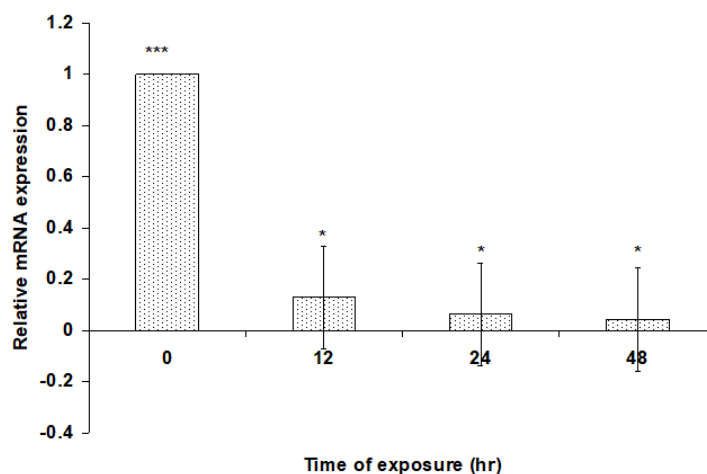
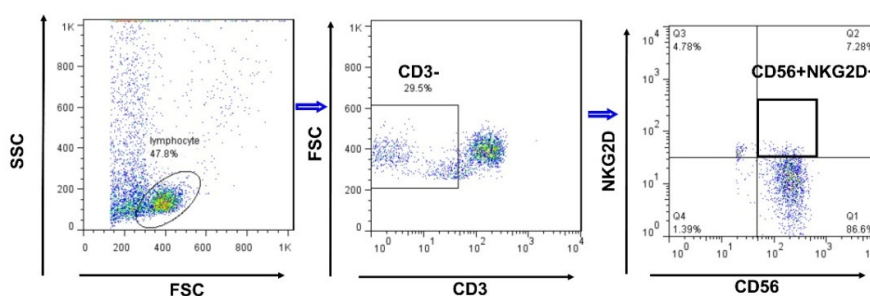
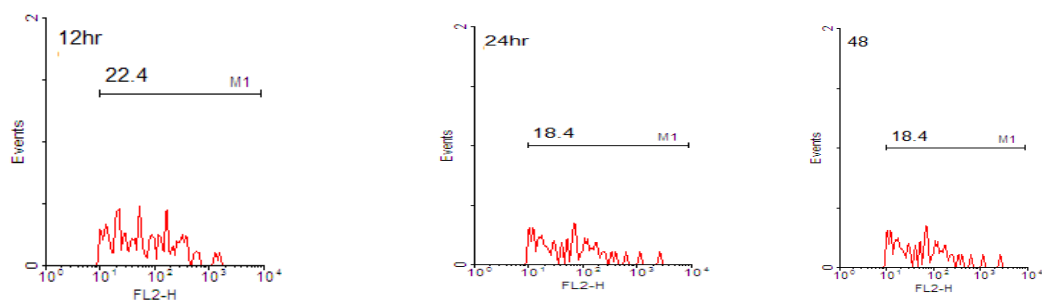


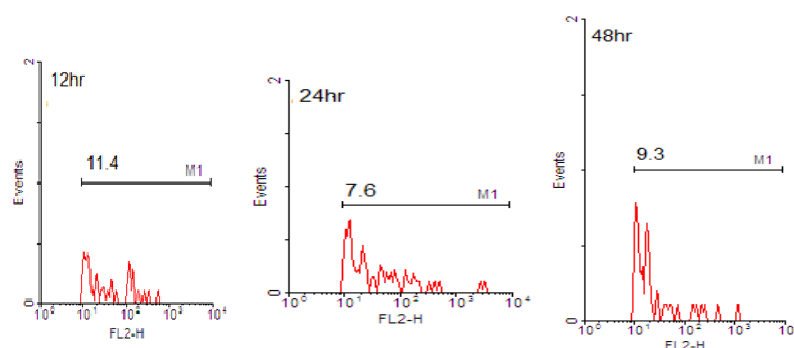
Figure 1. Relative expression of natural killer group 2 member D (NKG2D) mRNA following exposure to leukemia inhibitory factor (LIF) after 12, 24, and 48 hours of exposure. The result means \pm SEM of duplicates of three independent experiments. *** $p < 0.001$ between unexposed control and other treatment groups. * $p < 0.05$ between 12 and 24 and 48 hours exposures to 25 ng/mL of LIF.



A. Unexposed



B. Exposed



C. Exposed

Figure 2. (A) The results related to select the NK cell expressing CD3, CD56, and NKG2D using flow cytometry. (B) This part shows the expression of NKG2D on natural killer (NK) cells after various times of incubation (12, 24, and 48 hours) with peripheral blood mononuclear cell (PBMCs) was taken from healthy donors (in unexposed control). (C) Flow cytometry analysis of natural killer group 2 member D (NKG2D) receptor suppression effect of leukemia inhibitory factor (LIF) on the expression of NKG2D on natural killer (NK) cell after various times of incubation (12, 24, and 48 hours) with peripheral blood mononuclear cell (PBMCs) was taken from healthy donors. Samples were analyzed by 3-color flow cytometry. Anti-CD56-FITC was considered as a selective marker for NK cells, while anti-CD3-PECY5 and anti-NKG2D-PE were used as markers for differentiation of T cells and both NK and T-cells respectively.

DISCUSSION

The present study attempted to assess the capacity of LIF in decreasing the expression and presentation of NKG2D-receptor in healthy human isolated CD3-CD56⁺ NK cells; using real-time PCR (qRT-PCR) and flow cytometry techniques. The results showed that LIF, following 12, 24, and 48 hours incubation, produced potent inhibitory activity upon both

expression and presentation of NKG2D receptors in these cells.

LIF is a member of IL-6 cytokines with pleiotropic functions on various contrasting metabolic activities. However, the clinical significance and the underlying mechanism(s) remain unknown. Among the conditions in which LIF has been shown that have an important anti-inflammatory protective role in various physiological conditions such as in embryo implantation and development,¹⁵ materials-fetal

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conflicts during pregnancy,¹⁶ promotion of sensory neuron survival and formation,¹⁷ as well as in transplant tolerance.¹⁸ Among the proposed mechanisms by which LIF produces these effects is related to its direct inhibitory action on effectors T cells and NK cells.¹⁹ In addition, it may indirectly polarize tumor-associated-myeloid cells (TAMC) inducing the expression of CCL22, one of the best characterized and effective T-regs chemotactic factors, leading to cancer development and progression,²⁰ and autoimmune diseases.²¹ In support of this hypothesis, recent experimental evidence showed a decrease in T-regs after anti-LIF anti-body treatment was accompanied by an increase in NK and T-cells resulting in CT26 tumor cell death.²²

Selection of the concentrations of the LIF employed in these series of experiments was based on previous findings where the mean concentration in ovarian tumor ascites was reported to be 25 ng/mL and with a concentration range between 0.05 ng/mL-125 ng/mL.²³ Although, prudently, other concentrations of LIF could be tested, the main objective of this study was to assess the effect of a LIF present at a concentration that was proved to be a present pathological condition such as cancer. Furthermore, at normal physiological conditions of serum level is 0-5 pg/mL which is highly increased under pathological conditions such as cancer and can increase dramatically, up to 10000 fold, in inflammatory pathophysiological conditions such as cancer.²⁴ On the other hand, the concentration of LIF reported by many researchers vary widely ranging from <5 pg/mL in normal subjects, >15 pg/mL in the serum of patients with metastatic nasopharyngeal cancer,²⁵ and with a median concentration of 3.9 ng/mg in the amniotic fluid in pregnant women with intra-amniotic infection,²⁶ at a concentration range of 1 to 43 ng/mL in the in synovial fluid of 23% of patients with rheumatoid arthritis (RA) or other inflammatory or infectious arthritides,²⁷ to more than 125 ng/mL in ascites fluid.²³ Overall, these data suggest that LIF has a wide target-cell range, and fulfills different functions in different tissues which may be partly determined by its concentration and the prevailing physiological and pathological condition.^{23,28}

In these series experiment real-time PCR and flow cytometry were used for evaluation of changes in mRNA expression and presentation of NKG2D receptor on human PBMC respectively. The results showed that LIF, at a low concentration equal to its

endogenously released level produced a time-dependent and significant reduction of NKG2D receptor mRNA expression. This finding is the first to present evidence on the inhibitory effects of LIF on NK cells. Previous studies have demonstrated that LIF, extracted from ovarian tumor ascites fluid, was involved in the generation of tumor-associated macrophages, facilitating tumor escape from immune surveillance.²⁴

Although the clinical utilization of LIF in the clinical setting is hurdled by many limitations, in experimental autoimmune encephalomyelitis²⁹ and CT26²² tumor animal models, administration of LIF was found useful in ameliorating the symptoms by preventing demyelination and resulted in regression of tumor size. Furthermore, LIF was found to increase in multiple sclerosis patients,³⁰ which suggests its possible role in autoimmune diseases by increasing T-reg and limiting Th1 development in these patients by supporting the expression of FOXP3.³¹ In the present *in vitro* study the level of expression and presentation of NKG2D receptor on isolated NK cells were selectively assessed in NK cells by employing specific antibody. These results reveal that the mechanism by which LIF produces its effect is by direct inhibitory activity on normal human NK cells. The implications of these effects need to be assessed in future studies. Prudently, to gain more understanding of the role of LIF on other PBMCs, further similar studies are needed.

In conclusion, the results of this study showed that there were relatively rapid significant decreases in expression and presentation of the NKG2D receptor at all the incubation time points following exposure to LIF. The trend of decrease in the expression of NKG2D receptor mRNA on NK cells was more rapid, reaching 88% after 12 hours, and was highly reduced by 96% after 48 hours. In contrast, the level of presentation was found to be significantly reduced by 49.1% after 12 hours of exposure and remained low after 24 and 48 hrs without any significant difference from that measured after 12 hours. Based on the results from the present study, it is difficult to explain this discrepancy. However, one possible postulation is that the process of expression of NKG2D receptor by NK cells seems to be very efficient, therefore a longer time was needed to fully inhibit. On the other hand, the translocation of this receptor to the cell membrane seems to be a slow process and is readily subjected to inhibition at a much earlier time point.²³

CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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