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CTLA-4 Blockade of Natural Killer Cells Increases Cytotoxicity against Acute Lymphoid Leukaemia Cells

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Abstract

Objective: There is interest in using cytotoxic T lymphocyte antigen-4 (CTLA-4) immunotherapy to treat blood cancers. Unfortunately, patients with acute lymphoblastic leukaemia (ALL) frequently exhibit resistance to treatment and natural killer (NK) cell exhaustion. This study aims to increase the cytotoxic potency of natural killer cells by using CTLA-4 to block the Nalm-6 leukaemia cell line.

Materials and Methods: In this experimental study, NK cells were purified from the peripheral blood mononuclear cells (PBMCs) of 10 healthy people and assessed by flow cytometry for purity and viability. The purified cells were activated overnight at 37°C and 5% CO_2 with interleukin-15 (IL-15, 10 ng/ml) followed by evaluation of expressions of CTLA-4, activating and inhibitory receptors, and the release of interferon gamma (IFN-γ) and granzyme B (GZM B). CTLA-4 expression on NK cells from recurrent ALL patients was also evaluated. Finally, the cytotoxic activity of NK cells was assessed after the CTLA-4 blockade.

Results: The purity of the isolated cells was 96.58 ± 2.57%. Isolated NK cells activated with IL-15 resulted in significantly higher CTLA-4 expression (8.75%, P<0.05). Similarly, CTLA-4 expression on the surface of NK cells from patients with ALL was higher (7.46%) compared to healthy individuals (1.46%, P<0.05). IL-15 reduced NKG2A expression (P<0.01), and increased expressions of NKP30 (P<0.05) and NKP46 (P<0.01). The activated NK cells released more IFN-γ (P<0.5) and GZM B (P<0.01) compared to unactivated NK cells. Blockade of CTLA-4 enhanced the NK cell killing potential against Nalm-6 cells (56.3%, P<0.05); however, IFN-γ and GZM B levels were not statistically different between the blocked and non-blocked groups.

Conclusion: Our findings suggest that CTLA-4 blockage of Nalm-6 cells causes an increase in antitumour activity of NK cells against these cells. Our study also provides evidence for the potential of cancer immunotherapy treatment using blocking anti-CTLA-4 mAbs.

Keywords: CTLA-4, Immunotherapy, Natural Killer Cells, Nalm-6

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Introduction

Immunotherapy is increasingly being used as a successful cancer treatment. An advantage of immunotherapy is its ability to inhibit immune checkpoints, which are the sources of immune evasion for a number of malignancies. Although T cells have historically been the principal target, natural killer (NK) cells are emerging as a potential target. PD-1, TIM-3, TIGIT, LAG-3, and cytotoxic T lymphocyte antigen-4 (CTLA-4) are among the identified immune check points (1, 2). Dysfunctional NK cells have a correlation with various cancers and chronic infections (3). Through death receptors such as CTLA-4, cancer cells can also inhibit senescent or exhausted immune cells (2, 4).

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CTLA-4, known as CD152, has a superior binding affinity with CD80 and CD86 on antigen-presenting cells. It plays a negative role in the activation of immune cells. CTLA-4 expression is increased on Treg cells in some solid tumours, and this suppresses NK cell cytotoxic activity (5). CTLA-4 blockade in melanoma leads to the depletion of Treg cells and CTLA-4 therapy in melanoma is associated with an increased population of NK cells. CTLA-4 blockade may enhance the cytotoxicity effect of NK cells in both a direct and indirect manner, such as through the depletion of CTLA-4$^+$ Treg cells (6).

Immunotherapy aims to eliminate these impediments, whether molecularly or cellularly, in order to restore the immune cells' capacity to destroy tumour cells (7). In a similar manner to T cell function, NK cell function is also governed by immune checkpoint molecules (1). The impact of CTLA-4 blockade on the antitumour activity of NK lymphocytes remains unclear.

NK cells have advantages compared to T cells because they reduce the risk of Graft versus host disease (GvHD) and have greater capacity to elicit immunogenicity against recipient tumour cells from allogeneic donors (8-10).

NK cell therapy is an alternative to transplantation because of fewer side effects (11). NK cells could be an "off-the-shelf" cell source with comparable cytotoxic function and low toxicity. Numerous studies have demonstrated the efficacy of NK cell immunotherapy in the treatment of cancer; however, NK cell therapy is not effective in some patients due to exhaustion of NK cells, and strategies to overcome this problem is required (12).

Acute lymphoblastic leukaemia (ALL) is the most frequent paediatric malignancy with an incidence rate of 85% of acute leukaemia cases (13). It is characterised by the development of large numbers of immature lymphocytes. There is a direct relationship between the number of NK cells in the bone marrow and survival in patients with ALL (14). The results of a study indicate a substantial decrease in NK cell cytotoxic activity in ALL patients compared to healthy people (15). Immune-cell therapy with activated NK cells may be associated with a good prognosis in ALL patients who have acquired resistance (16, 17). On the other hand, NK cells are essential for ALL immunity, and the immune escape mechanism of lymphoblasts must be identified. Adoptive NK cell delivery that uses NK cells derived from healthy donors may improve patient prognosis (17-19).

Substantially fewer numbers of NK cells exist in the bone marrow and peripheral blood of ALL patients (18). ALL-derived NK cells have significantly diminished cytotoxic capabilities compared to NK cells provided from healthy donors, which indicates an immunosuppressed phenotype in these cells (20). The expression of surface CTLA-4 in human NK cells has not been well studied and the advantage or effectiveness of this checkpoint inhibition for haematologic cancers such as ALL has not been evaluated.

In the present study, we aim to evaluate the effect of a CTLA-4 block on the cytotoxicity of NK cells against Nalm-6 cells, a B-lineage ALL cell line. We also intend to investigate CTLA-4 expression on human NK cells in ALL patients.

Materials and Methods

Cell lines and culture media

In this experimental study, Nalm-6 (ALL-derived cell line) and K562 (chronic myeloid leukaemia cell line) were used. The Nalm-6 cell line was kindly provided by Dr. Safa (Iran University of Medical Sciences, Tehran, Iran). The K562 cell line was purchased from Royan Institute Cell Bank (Tehran, Iran). The cell lines were cultured alone or co-cultured with NK cells in RPMI 1640 (Gibco, USA, Cat. No.: 26140-079) that contained 10% fetal bovine serum (FBS, Gibco, USA, Cat. No.: 26140-079), 3 mM L-glutamine (Gibco, USA, Cat. No.: 25030-024), 1% penicillin/streptomycin (50 µg/ml, Gibco, USA, Cat. No.: 15070-063), and 1% NEAA (Gibco, USA, Cat. No.: 11140050) in a humidified atmosphere of 5% CO_2 at 37˚C.

Patients

Ten patients with recurrent ALL (five females and five males) with a mean age of 12.35 years (range: 7 to 16 years) and 10 healthy individuals (six females and four males) with a mean age of 15.5 years (range: 10 to 25 years) enrolled in this experimental study between 2019 and 2022 at the Children's Medical Centre, Imam Khomeini Hospital (Tehran, Iran). All participation or their parent signed informed consent forms and the benefits of their participation were explained orally and in writing. The Ethics Committees of Kurdistan University of Medical Sciences, Sanandaj, Iran (IR.MUK.REC.1399.175) and Royan Institute, Tehran, Iran (IR.ACECR.RAYAN.REC.1401.064) approved all procedures in this study.

Human NK cell isolation

Peripheral blood mononuclear cells (PBMCs) from healthy donors (n=10) and patients with recurrent ALL before induction chemotherapy (n=10) were isolated using Ficoll-Paque density gradient sedimentation (Innotrain, USA, Cat. No.: 002041600). We evaluated both the frequency of the NK cells and CTLA-4 expression on NK cells that were gated as $CD3^-$ $CD56^+$ $CD16^+$ events on the mononuclear cell (MNC) flow cytometry plot in healthy donors and patients.

Then, the NK cells obtained from the MNCs of healthy donors were isolated by magnetic bead-based positive selection ($CD56^+$; Miltenyi Biotec, USA). The purity of the NK cells ($CD3^-CD56^+CD16^+$) was evaluated by flow cytometry (BD Biosciences, USA) and the results indicated a purity of 96.58 ± 2.57%. Viability of the isolated NK cells was 97.82 ± 0.94% by propidium iodide (PI) staining (Fig.1).

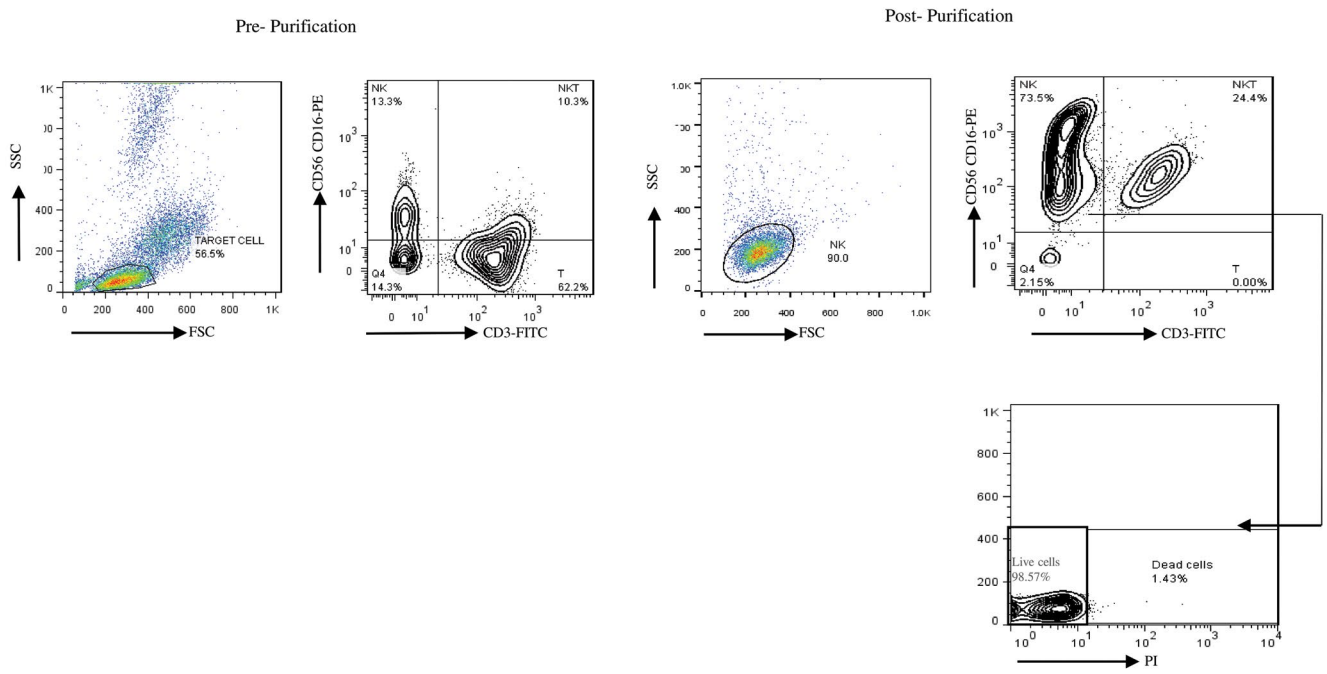

Fig.1: Isolation and viability of NK cells ($CD3^-$ $CD56^+$ $CD16^+$). The $CD3^-$ $CD56^+$ $CD16^+$ cells before purification was about 9.87%. After purification with MACS, purity was 96.58 ±2.57% with 97.82 ± 0.94% cell viability. NK; Natural killer, and MACS; Magnetic activated cell sorting.

Human NK cell activation by IL-15

A total of 1×10^6 isolated NK cells from the MNCs of healthy donors per 1 ml of RPMI were placed in a 24-well plate (Corning, Cat. No.: 3524) and allowed to incubate overnight with IL-15 (10 ng/mL; Miltenyi Biotech, USA, Cat. No.: 130-095-760) at 37°C. These cells were washed twice with PBS, and 1×10^5 NK cells were incubated with special antibodies for 30 minutes at 4°C. After washing the NK cells with PBS, we used flow cytometry to measure the expressions of NKG2D, NKP30 and NKP46, as activating receptors, and NKG2A and CTLA-4, as inhibitory receptors. The expressions of NKG2D, NKP30, NKP46, NKG2A, and CTLA-4 on the NK cells before IL-15 treatment was compared to after treatment. The condition media from the NK cells after overnight incubation with IL-15 was harvested, and the level of interferon gamma (IFN-γ) and granzyme B (GZM B) secretion of the NK cells was evaluated by ELISA. The analytical data after treatment with IL-15 was compared to before treatment. In addition, we evaluated the expression of CTLA-4 on NK cells obtained from the patients by incubating 1×10^5 isolated MNCs with an anti-CTLA-4 antibody. The NK cells were gated on flow cytometry plots, and the percent of $CTLA\text{-}4^+$ NK cells from the patients were compared to the IL-15-treated $CTLA\text{-}4^+$ NK cells from healthy donors.

Immunophenotyping of cell populations

Surface molecule expression was measured by flow cytometry. The NK cells were quantitatively evaluated by anti-human CD3-FITC/CD16 CD56-PE (2 µg/ml, BioLegend, USA, Cat. No.: 342403) and FITC anti-human CD335 (NKP46, 4 µg/ml, Biolegend, USA, Cat. No.: 331922), PE anti-human CD159a (NKG2A, 4 µg/ml, BiolLegend, USA, Cat. No.: 142803), PE anti-human CD314 (NKG2D, 4 µg/ml, BioLegend, USA, Cat. No.: 320806), PE anti-human CD337 (NKP30, 2 µg/ml, BioLegend, USA, Cat. No.: 325207), and PerCP-eFluor 710 anti-human CD152 (CTLA-4, 0.06 µg/ml, eBioscience, USA, Cat. No.: 46-1529-42).

Evaluation of NK cell-mediated cytotoxicity

The K562 cell line is the gold standard cell line that lacks HLA-I and HLA-II and is sensitive to NK cell-mediated cytotoxicity. K562 and Nalm-6 cells were inactivated with mitomycin C (20 µg/ml; Sigma, USA, Cat. No.: M0503) for 45 minutes at 37°C in 5% CO_2. The cells were washed with phosphate buffered saline (PBS) and resuspended in RPMI that contained 10% FBS. Subsequently, 1×10^5 cells were added per well to 24-well plates. K562 cells were used to optimise the effector:target (E:T) ratio. The effector NK cells were co-cultured with K562 target cells at different times (6, 12, and 24 hours) at 37°C and 5% CO_2 and different effector ratios (1:1, 5:1, and 10:1). The capacity of NK cell cytotoxicity was evaluated using PI staining. After optimising the E:T ratio, NK cells that were treated with IL-15 (10 ng/ml) and CTLA-4 blocker [purified anti-human CD152 (CTLA-4) antibody; 5 µg/ml, BioLegend, USA, Cat. No.: 349902] were added at a 10:1 effector to the Nalm-6 ratio (1×10^6 NK cells: 1×10^5 Nalm-6 cells) in 24-well plates. The cells were co-cultured at 1×10^6 cells/well for 12 hours in the presence of 1 mL complete media. NK cells without CTLA-4 blocker

served as the control. The capacity of these NK cells to kill Nalm-6 cells was quantified by flow cytometry and PI staining, and the cell lysis frequency was determined.

Evaluation of IFN-γ production and GZM B release from NK cells

IFN-γ and GZM B ELISA Kits (R&D Systems, Inc., Cat. No.: DIF50C and FineTest Biotech, Inc., Cat. No.: EH0157) were used to test GZM B release and IFN-γ secretion by NK cells. Briefly, for IFN-γ, duplicate aliquots of 100 µl per well of the diluting solution or samples were added to all wells of a 96-well plate, and the plate was incubated for two hours at room temperature. After disposing of the contents of the plate, 200 µl of IFN-γ antibodies were added per well. After three washes with PBS (Gibco, USA, Cat. No.: 21600-051), 200 µl of an ABC working solution was added to each well. Then, absorbance was read at 450 nm in a microplate reader after adding a 50 µl/well stop solution. For GZM B measurement, 100 l per well of the diluting solution or samples were added to all wells of a 96-well plate and the plate was incubated for two hours at room temperature. Without disposing of the contents of the plate, 100 µl of GZM B antibodies were added to each well. After three washes with PBS, 100 µl of a SABC working solution was added to each well. Finally, absorbance was read at 450 nm in a microplate reader after adding a 50 µl/well stop solution.

Statistical analysis

All results are reported as mean ± standard deviation (SD), and statistical analysis was conducted using GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA, USA). The unpaired t test was utilised to determine the statistical significance of the two groups. The two-way ANOVA test was used to determine the statistical significance of more than two groups. $P<0.05$ indicated statistical significance.

Results

IL-15 upregulated CTLA-4 expression on the NK cells

NKG2D, NKP30, NKP46, NKG2A, and CTLA-4 expressions were measured to evaluate the effect of IL-15 on NK cells. NKG2D expression was not statistically different (P=0.401, Fig.2A). Both NKP30 (20.54 ± 7.02%) and NKP46 (26 ± 5.89%) increased their expressions after IL-15 compared to before treatment (Fig.2B, C). There was a decrease in NKG2A (1.99 ± 6.94%) expression after IL-15 administration (Fig.2D). Notably, IL-15 increased the number of CTLA-4 positive NK cells (8.75 ± 2%, P=0.025; Fig.2E). Both IFN-γ (90.33 ± 8.1 pg/ml, P=0.012) and GZM B (12.45 ± 5.8 pg/ml, P=0.003) upregulated after IL-15 treatment of the NK cells (Fig.2F). Statistical analyses were performed using the unpaired t test.

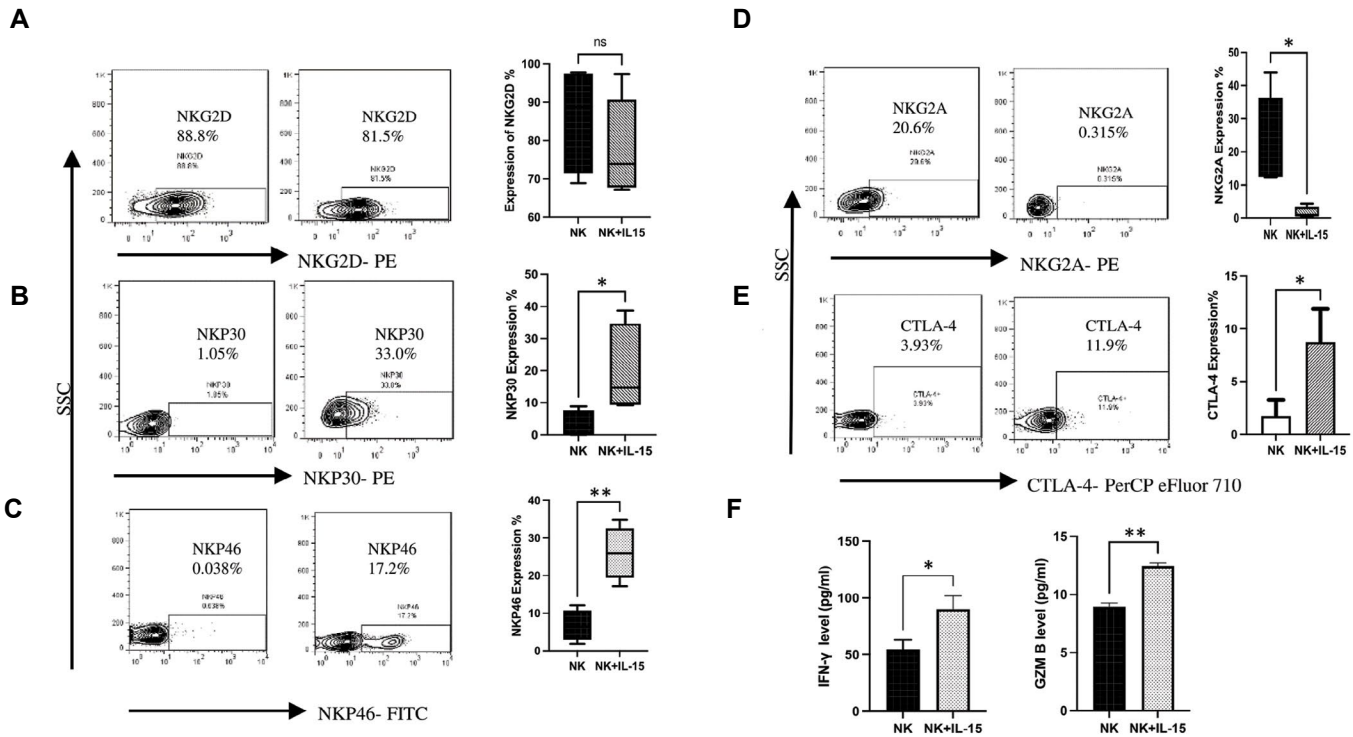

Fig.2: Expressions of activation markers on NK cells before and after exposure to IL-15. Purified NK cells were activated in the presence of IL-15 (10 ng/ml) after an overnight incubation. **A.** NKG2D expression did not statistically differ (P=0.401). **B.** NKP30 expression and **C.** NKP46 expression, as activating receptors, increased after exposure to IL-15**. D.** NKG2A expression decreased after IL-15-exposure. **E.** CTLA-4 expression increased after IL-15 treatment. (P=0.025). **F.** IFN-γ (P=0.012) and GZM B (P=0.003) levels significantly increased after treatment with IL-15 compared to the untreated NK cells. Statistical analysis was performed using the unpaired t test. *; P<0.05, **; P<0.01, NS; Not significant, NK; Natural killer, CTLA-4; Cytotoxic T lymphocyte antigen-4, IL; Interleukin, IFN-γ; Interferon gamma, and GZM B; Granzyme B.

We evaluated CTLA-4 expression on NK cells that were gated in an MNC flow cytometry plot from whole blood samples of ten ALL patients (Fig.3A). The findings indicated that 5.3% of total lymphocytes were $CD3^{-}$ $CD56^{+}$ $CD16^{+}$ (NK cells), of which 7.46 ± 3.48% were CTLA-4 positive. There were fewer NK cells in patients compared to healthy individuals (P0.002=, Fig.3B). However, we observed a significant increase in CTLA-4 expression on NK cells that were gated in MNC flow cytometry plot from patients' whole blood samples compared with healthy people (P=0.0002, Fig.3C). This result was similar to the result obtained from increased CTLA-4 expression after IL-15 activation.

CTLA-4 blockade increased NK cell-mediated cytotoxicity

The gold standard for NK cell cytotoxicity assays is the assessment of the cytotoxic effects of NK cells against K562 cells. NK cells were co-cultured with K562 at E:T ratios of 1:1, 5:1, and 10:1. After 6, 12 and 24 hours, we assessed cytotoxic function of the NK cells (Fig.4). Our results showed the highest NK cell cytotoxicity at 12 hours after the co-culture. There was greater cell lysis at the 10:1 ratio after 12 hours co-culture with K562 compared to the 5:1 and 1:1 ratios (Fig.4A). Statistical analyses were performed using two-way ANOVA.

As mentioned earlier, IL-15 upregulated CTLA-4 expression on the NK cells. We used purified anti-human CD152 (CTLA-4) antibody (5 g/ml) as the CTLA-4 blocker. According to our results, this concentration significantly reduced CTLA-4 expression (Fig.4B). Next, we sought to determine if the CTLA-4 blockade could affect the cytotoxic potential of NK cells. Our finding demonstrated that the rate of IFN-γ and GZM B release was not significantly different in the $IL\text{-}15^{+}$ CTLA-4 blocker-treated group compared to the IL-15 group (P=0.230 and P=0.050, respectively, Fig.4C). Assessment of the cytotoxicity of NK cells on the target Nalm-6 cells showed significant enhancement in the IL-15+CTLA-4 blocker group (56.3%, P=0.014, Fig.4D). Statistical analyses were performed using the unpaired t-test. Figure 4E shows the flow cytometry results and morphological illustration of the effect of CTLA-4 inhibition on NK cell cytotoxic activity toward the Nalm-6 cells.

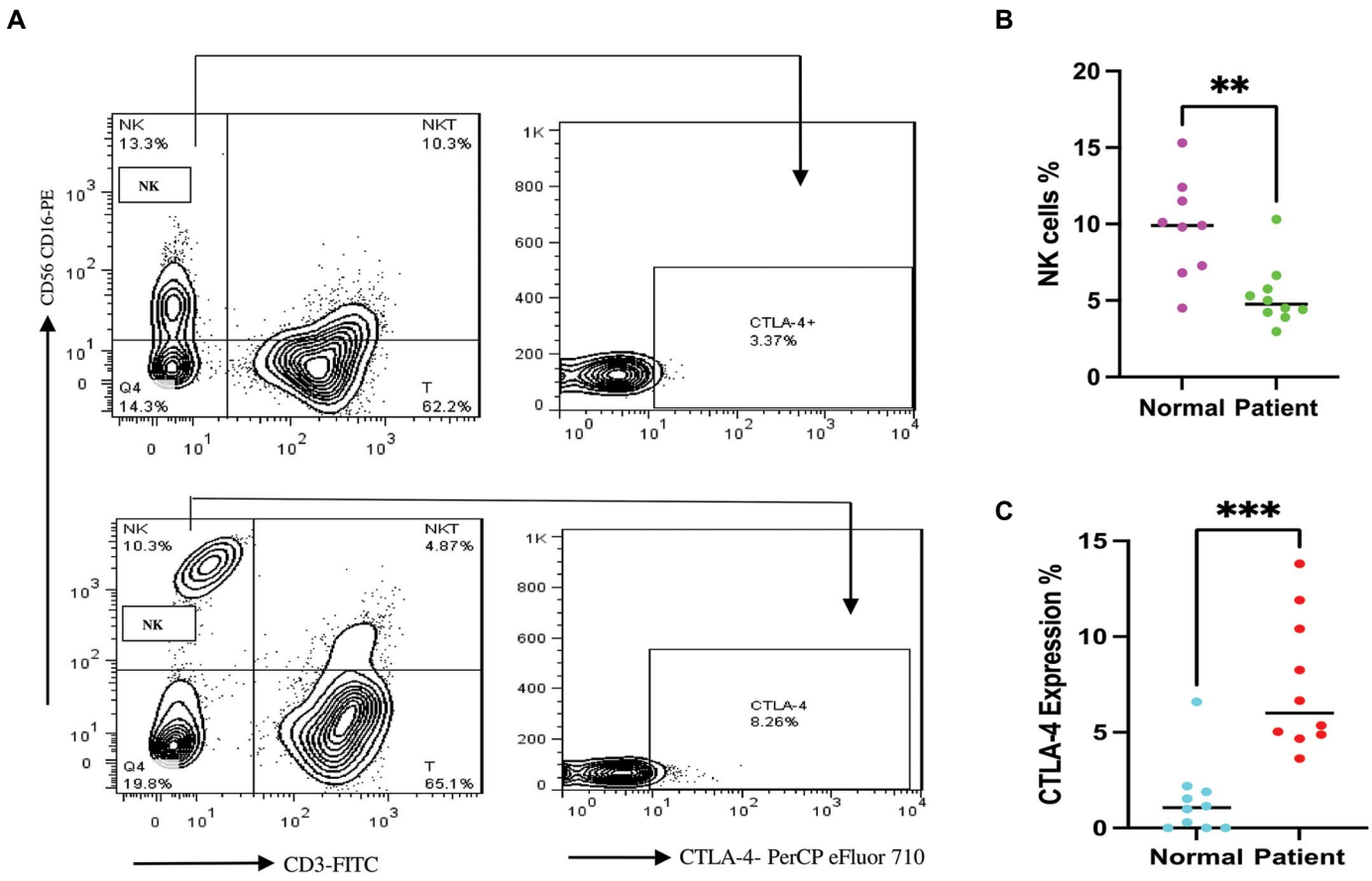

Fig.3: CTLA-4 expression on NK cells gated from MNCs of healthy individuals and recurrent ALL patients. **A.** Schematic presentation of flow cytometry data from healthy individuals and recurrent ALL patients. **B.** Comparison of the percent of NK cells in patients (5.3%) and healthy donors (10.7%). The results show that 5.3% of total lymphocytes were $CD3^{-}$ $CD56^{+}$ $CD16^{+}$ cells (NK cells), and the proportion of NK cells was reduced in patients compared to healthy individuals (P0.002=). **C.** Comparison of the percent of $CTLA\text{-}4^{+}$ NK cells in normal individuals and ALL patients. CTLA-4 expression on NK cells was 7.46% in patients and 1.46% in normal donors. CTLA-4 expression on NK cells gated in the MNC flow cytometry plot from patients' whole blood samples was significantly higher than in healthy people (P=0.0002). Statistical analysis was conducted using the unpaired t test (n=10). **; P<0.010, ***; P<0.001, NK; Natural killer, CD; Cluster of differentiation, MNCs; Mononuclear cells, CTLA-4; Cytotoxic T lymphocyte antigen-4, and ALL; Acute lymphoblastic leukaemia.

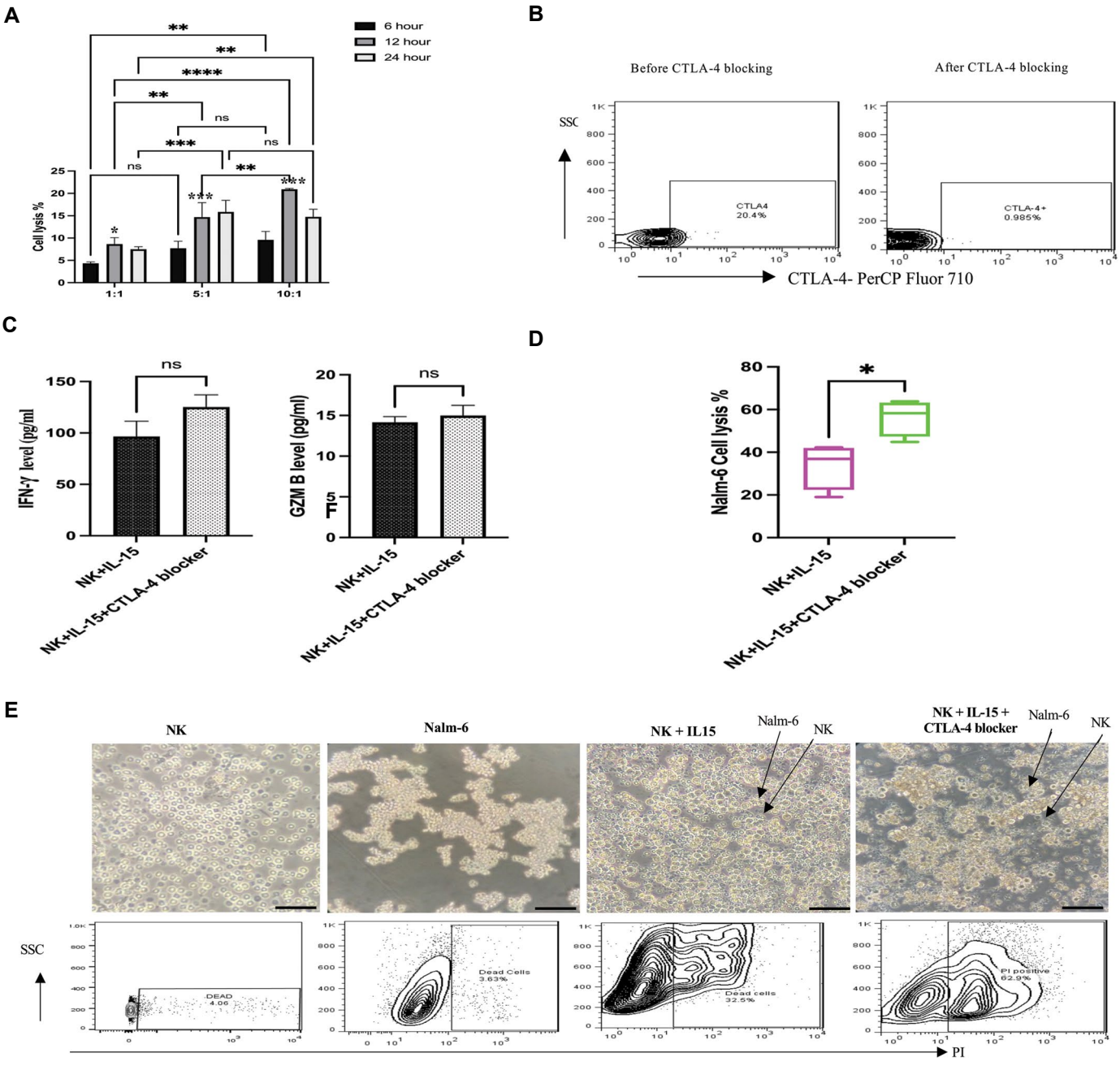

Fig.4: The effect of CTLA-4 blocker on NK cell cytotoxicity. **A.** Cell lysis of NK cells against K562 cells at E:T ratios of 1:1, 5:1, and 10:1 at 6, 12, and 24 hours of incubation (n=4). Statistical analysis was performed using two-way ANOVA. **B.** Flow cytometry results of CTLA-4 reduction after the CTLA-4 blocker (5 µg/ml). **C.** Secretion levels of IFN-γ and GZM B after CTLA-4 blockade as measured by ELISA. The results did not statistically differ between the blocked and non-blocked group (P=0.230 and P=0.050, respectively). **D.** Cytotoxic activity of NK cells increased in the blocked group compared to the unblocked IL-15 group (n=4, P=0.014). **E.** Flow cytometry analysis and morphological illustration of the effect of CTLA-4 inhibition on NK cell cytotoxic activity toward Nalm-6 cells (20x magnification, scale bar: 100 µm for NK, Nalm-6 cells, and co-cultured Nalm-6 cells with NK cells). Statistical analysis was performed using the unpaired t test. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001, NS; Not significant, NK; Natural killer, E:T; Effector: target ratio, IFN-γ; Interferon gamma, GZM B; Granzyme B, CTLA-4; Cytotoxic T lymphocyte antigen-4, and IL-15; Interleukin-15.

Discussion

The principal aim of this investigation was to assess the impact of CTLA-4 blockade on the activation and cytotoxic activity of NK cells derived from healthy individuals against an ALL cell line. The findings of this study revealed that IL-15 upregulated CTLA-4 expression on the NK cells, and the CTLA-4 blockade increased the antitumour activity of NK cells against Nalm-6 cells.

Sanseviero et al. (6) reported that ipilimumab (an anti-CTLA-4 blocker) in combination with IL-15 in a mouse model of melanoma increased NK cell activation and depletion of intratumoural Treg cells.

NK cells are of significant importance in the surveillance and eradication of tumour cells. They could potentially play a role in the treatment of leukaemia (21). The exhaustion and reduced number of NK cells in ALL patient are important considerations for treatment of this disease (22, 23). We have reported a decrease in NK cells in recurrent

ALL patients that may be due to previous chemotherapy treatments. Recent research on ALL patients in Mexico has identified impaired cytotoxicity and a reduction in the proportion of NK cells in patients with ALL (24).

In this study, we showed that CTLA-4 expression increased on MNC-gated NK from ALL patients. According to other studies, a high level of PD-1 expression on the surface of T cells in paediatric B-cell ALL patients was associated with a poor prognosis (25). Our study is the first report on the CTLA-4 expression in human NK cells obtained from ALL patients. The increased expression of CTLA-4 may be due to the exhaustion of NK cells after exposure to lymphoblasts.

IL-15 supports NK cell proliferation, survival and cytolytic activity. NK activity is controlled by the relative balance of signals received from cell surface receptors (17). We observed that IL-15 activated NK cells by reducing the NKG2A inhibitory receptor, and increasing the NKP30 and NKP46 activating receptors. Other studies reported decreased expression of NKG2A and a nonsignificant difference in NKG2D expression after IL-15 treatment (26). IL-15 can activate NK cells by increasing the expressions of the activating receptors NKP30 and NKP46, and the inhibitory receptor, NKG2A. This changes the equilibrium between activating and inhibitory signals, and leads to NK cell activation (27, 28). The antitumour activity of NK cells ultimately results from one of the pathways initiated by IFN-γ derived from NK cells (29). Our results indicate an increase in IFN- and GZM B secretion of NK cells after IL-15 treatment. Other studies have also shown that activated NK cells express IFN- and GZM B (26, 30, 31).

We observed that IL-15 activated NK cells and also upregulated CTLA-4$^+$ NK cells. Simone et al. (32) reported an elevated circulating soluble form of CTLA-4 in most B-ALL patients and another study reported increased IL-15 levels in blood samples from ALL patients (33). These findings suggest IL-15 can activate NK and also upregulate CTLA-4 expression. Therefore, IL-15 could act as a double-edged sword. The underlying mechanism is still unclear.

We further noted that the release of IFN- and GZM B from NK cells after CTLA-4 blockade was not significantly different than before the CTLA-4 blockade. However, both NK cells and IFN- were necessary for tumour control and IFN- expression by NK cells was enhanced by therapeutic mAbs (34). Gotthardt et al. (35) reported that IFN- production from NK cells is detectable after four hours, but and its nondetectable after eight hours. Since we removed the condition media after 12 hours. Therefore, maybe our results are because we missed the golden time to measure interferon secretion.

It has been shown that blockade of immune checkpoints can enhance the efficiency of the antitumour function of NK cells (36, 37). It appears that recent developments in combination therapy that target immune checkpoint molecules are beneficial for NK cell therapy (36-38). Here, we found that the CTLA-4 blockade increased NK cell-mediated cytotoxicity against the Nalm-6 cells. Another study showed that neutralising anti-CTLA-4 antibodies enhanced the antitumour immunity against transplanted and established colon carcinoma and fibrosarcoma in mice (39). The results of a clinical trial showed that inhibition of PD-1 could efficiently treat human B-cell malignancies (25). However, the role of the CTLA-4 blockade remains unclear. The current study is the first to use the CTLA-4 as a blocker to increase the cytotoxicity function of NK cells toward a B-lineage ALL cell line. Further studies with *in vivo* models are needed to confirm our findings. Additionally, optimisation of the activation pathways is critical. Anti-CTLA-4 antibodies used to treat ALL could enhance the efficacy of NK cell monotherapy.

Conclusion

Our results demonstrate that IL-15 upregulates CTLA-4 expression on NK cells. Therefore, we suggest that IL-15 may function as a double-edged sword to enhance the cytotoxic function of these cells. We suggest that inhibition of CTLA-4 combined with IL-15 can increase NK cell-mediated cytotoxicity against a B-lineage ALL cell line (Nalm-6 cells). We observed that treatment with anti-CTLA-4 improved the cytotoxic properties of NK cells. However, the limitations of this study include a low number of samples, use of only one cell line to evaluate the cytotoxic activity of NK cells, and the lack of lymphoblast samples from patients. Large-scale studies that use patient-derived lymphoblasts and in vivo models are necessary to confirm these findings. We suggest that double blockade of PD-1 and CTLA-4 may more efficiently increase NK cell-mediated cytotoxicity against tumour cells. Our study also provides evidence for the potential of cancer immunotherapy treatment using blocking anti–CTLA-4 mAbs.

Acknowledgments

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Authors' Contributions

N.P.; Conception, Study design, Data collection, All experimental work, Statistical analysis, and Writing the manuscript. M.E.A., A.A.H.; Medical consultation, Assisted with sample collection, and Revised the manuscript. F.F., A.A.A.; Contributed to the revised the manuscript and Advisored the study. M.E., Z.V.; Conceptualisation of the study, Revised the manuscript, and Supervised the study. All authors performed manuscript editing, participated in the

finalisation of the manuscript, and approved the final draft for submission.

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