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Brief Communication

Niclosamide Enhances NK cell Proliferation and Anti-Tumor Activity for Cancer Immunotherapy

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NK (Natural killer) cells are innate immune cells and play important roles as the first immune cells to act when cancer occurs. In many cancer patients, NK cells can be seen to be inactivated, suggesting that NK cells are important in cancer treatment. In order to overcome the disadvantages of NK cells in cancer treatment, it is critical to develop strategies that enhance the proliferation and cytolytic function of NK cells. We applied niclosamide to measure the degree of NK cell activation, and obtained unexpected results of increased NK cell numbers and anti-tumor activity. Although further investigation is required to uncover the detailed mechanisms, our results suggest that Niclosamide is a promising candidate to increase the efficacy of cancer immunotherapy using NK cells.

Key Words: Niclosamide, NK cell, Cancer immunotherapy, Anti-tumor activity

NK cells are CD3 negative/CD56 positive innate immune cells playing a major role in the lysis of tumors and virus-infected cells, and account for 5~15% of circulating lymphocytes in humans (Laskowski et al., 2022). Activated NK cells have anti-tumor activity against tumor cells and release an effector cytokine such as interferon (IFN)-γ, and cytolytic granules containing perforin and granzyme B (Shin et al., 2020). The cytolytic activity of NK cells is regulated by an array of activating and inhibitory receptors on cell surface (Shin et al., 2020). Additionally, NK cells are involved in apoptosis by modulating death receptors such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Sordo-Bahamonde et al., 2020). The immunosuppressive and heterogeneous TME significantly hampers the function of NK cells and their infiltration into tumors (Lim et al., 2021; Shin et al., 2023). Therefore, development of a strategy to elevate cytotoxicity and expansion of NK cells would be critical for successful cancer immunotherapy.

Niclosamide is a U.S. FDA - approved and widely used anthelmintic drug for the treatment of parasitic infections (Pearson and Hewlett, 1985; Wang et al., 2022). Multiple studies have reported that Niclosamide has anti-tumor effect through inhibiting oncogenic pathways such as Stat3, Wnt/β-catenin, NF-Kb and mTOR (Li et al., 2014; Ren et al., 2022). It has been shown that Niclosamide boosts the efficacy of PD-L1 antibodies and suppresses the growth of cancer by inhibiting the binding of p-STAT3 to the PD-L1 promoter, leading to the subsequent reduction of PD-L1 expression (Luo et al., 2019; Jiang et al., 2022). In addition, Niclosamide enhances T cell-mediated lysis of cancer cells by increasing tumor infiltrating T cells and granzyme B release (Luo et al., 2019). Furthermore, Niclosamide is employed to control viral infections and metabolic diseases with its antiviral and anti-inflammatory characteristics (Chen

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et al., 2018; Al-Kuraishy et al., 2021).

The immunomodulatory and anti-tumor effects of Niclosamide led us to hypothesize that Niclosamide may activate effector immune cells such as NK cells. In this study, we investigated the effects of Niclosimide on the expansion and tumor killing activity of NK cells.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor peripheral blood using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). PBMCs were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and recombinant human interleukin-2 (rhIL-2) (500 U/mL) (rhIL-2, Proleukin; Novartis, Basel, Switzerland). Gamma-irradiated (100 Gy) K562 cells were co-cultured with PBMCs as feeder cells. Niclosamide was treated at concentrations of 1 nM, 10 nM, 100 nM and DMSO was treated as a control. Niclosamide and DMSO were treated at days of 0, 3 and 6 to the 1×10^6 total cells. Media containing Niclosamide were changed at days of 3 and 6. K562 (human leukemia cell line) and A375 (human melanoma cell line) cells were cultured in RPMI 1640, supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Niclosamide was purchased from Sigma-Aldrich (St. Louis, Missouri, United States). The study was approved by the Institutional Review Board with donors' consent (IRB# KUIRB-2021-0264-01). The absolute number of NK cells was determined by multiplying the total viable number of cells by the percentage of $CD56^+CD3^-$ cells measured by flow cytometry. Fold expansion of NK cells was calculated by dividing the number of viable NK cells present at the end of culture by the number of viable NK cells at the beginning of culture.

The CD107a and IFN-γ were measured according to the following procedures. NK cells treated with Dimethyl sulfoxide (DMSO) or Niclosamide were co-cultured with K562 and A375 cells with Effector:Target (E:T) ratio of 1:1 in a round-bottomed 96-well plate with anti-CD107a-FITC monoclonal antibody (mAb) and Golgistop (BD Pharmingen) for 6 h. The samples were fixed and permeabilized using Cytofix/Cytoperm intracellular staining kits (BD Pharmingen), and IFN-γ-PE mAb was added to measure intracellular IFN-γ of NK cells. Flow cytometry was performed with Fluorescence-activated cell sorting (FACS) CantoII

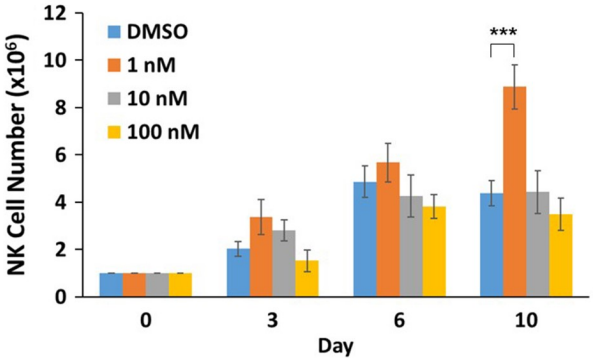

Fig. 1. Effects of Niclosamide on NK cell proliferation. Average cell counting results from four different donors showed that 1 nM Niclosamide increased NK cell proliferation. Data obtained from four different individual donors are presented as the mean ± SEM. ***, $P < 0.001$.

(BD Bioscience), and CD3-PerCP negative and CD56-APC positive NK cells were gated for analyzing with the FlowJo (Tree Star) software. Tumor killing activity of NK cells treated with 1 nM Niclosamide for 10 days was measured using lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Diagnostics). Niclosamide treated NK cells were co-cultured with K562 or A375 target tumor cells, and LDH activity released from the damaged tumor cells was measured using spectrophotometer. The percentage of specific lysis was calculated as follows: [((absorbance (abs) 492 nm effector/target mix − abs 492 nm effector only) − abs 492 nm spontaneous)/(abs 492 nm maximum − abs 492 nm spontaneous)] × 100 (Lim et al., 2021).

NK cells treated with 1 nM Niclosamide reached up to average 8.875±0.943 fold expansion by day 10, and those cultured in DMSO proliferated only up to average 4.380± 0.543 fold expansion by day 10 from four different donors (Fig. 1, ***, $P < 0.001$). We then examined the effect of Niclosamide on tumor cytotoxicity and cytokine secretion in NK cells. After Niclosamide treatment, we measured CD107a degranulation and IFN-γ production by NK cells against two different tumor targets—a human leukemia cell line K562 and a human melanoma cell line A375. The percentage of CD107a+ NK cells increased in a Niclosamide treated NK cells against K562 and A375 target tumor cells (Fig. 2A; for K562; 2.3 fold higher in Niclosamide relative to DMSO, and for A375; 1.7 fold higher in Niclosamide

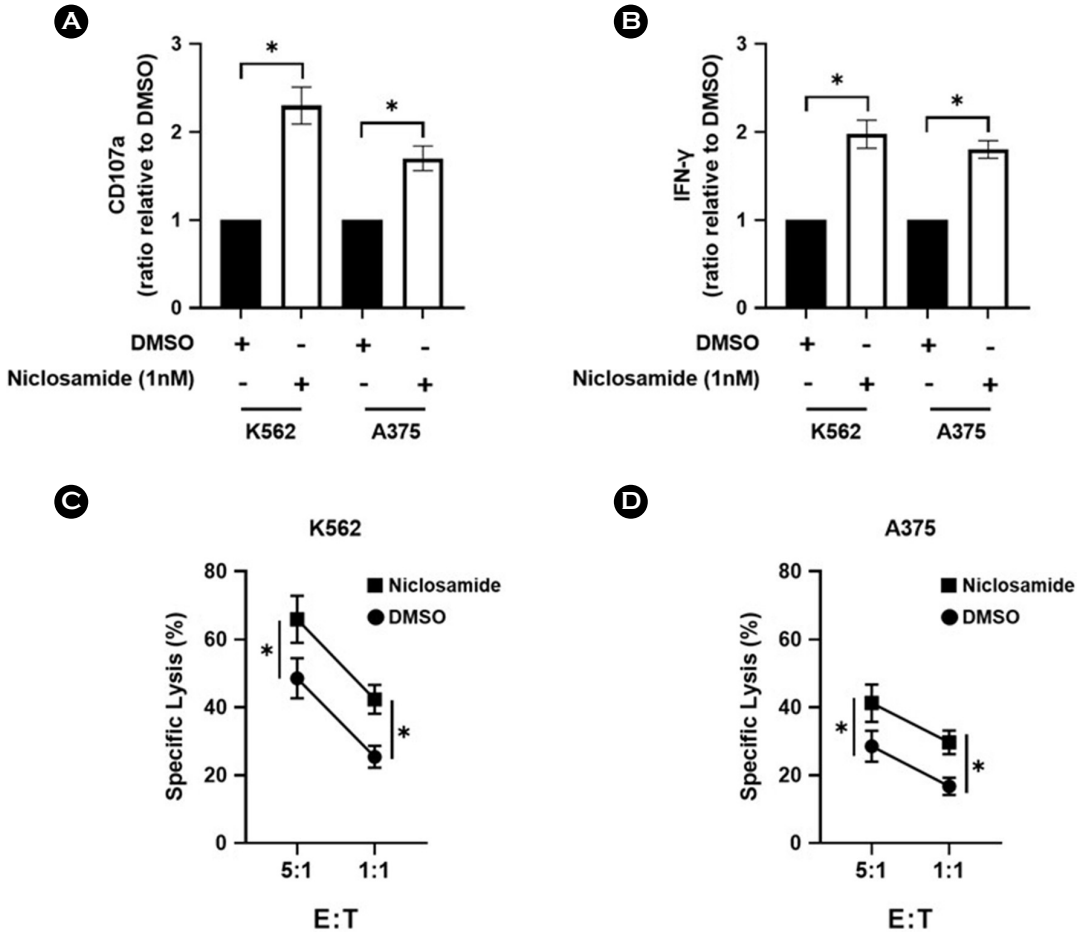

Fig. 2. Effects of Niclosamide on anti-tumor effects of NK cells. (A, B) The level of degranulation and cytokine release of NK cells against K562 and A375 tumor targets was analyzed by surface CD107a and intracellular IFN-γ-staining. (C, D) NK cell tumor cytotoxicity was measured by LDH activity. Data obtained from three independent experiments, each using PBMCs from different individual donors, are presented as the mean ± SEM. *, $P < 0.05$.

relative to DMSO, *, $P < 0.05$). The percentage of IFN-γ+ NK cells also increased in a Niclosamide treated group against K562 and A375 target tumor cells (Fig. 2B; for K562; 1.97 fold higher in Niclosamide relative to DMSO, and for A375; 1.80 fold higher in Niclosamide relative to DMSO, *, $P < 0.05$). NK cell tumor cytotoxicity measured by LDH cytotoxicity kit was also increased in Niclosamide treated group, indicating that Niclosamide enhanced anti-tumor activity of NK cells against K562 and A375 tumor cell lines (Fig. 2C, D; *, $P < 0.05$).

Our results uncover a previously unidentified role of Niclosamide that can enhance expansion and effector phenotypes of NK cells for adoptive cellular therapy for cancer treatment. These results show that Niclosamide represents a promising candidate for repurposing to potentiate the anticancer activity by enhancing the function of surrounding immune cells. Further studies are necessary to investigate the detailed cellular and molecular mechanisms regarding the role of Niclosamide in activating NK cells.

Abbreviations

Absorbance (abs)
Dimethyl sulfoxide (DMSO)
Effector:Target (E:T)
Fas ligand (FasL)
Fetal Bovine Serum (FBS)
Fluorescence-activated cell sorting (FACS)
Interferon (IFN)

Lactate dehydrogenase (LDH)
Monoclonal antibody (mAb)
Natural killer (NK)
Peripheral blood mononuclear cells (PBMCs)
Recombinant human interleukin-2 (rhIL-2)
TNF-related apoptosis-inducing ligand (TRAIL)
Tumor microenvironment (TME)

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None.

CONFLICT OF INTEREST

The researcher claims no conflicts of interest.

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