Preclinical investigation of anti-tumor efficacy of allogeneic natural killer cells combined with cetuximab in head and neck squamous cell carcinoma

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ABSTRACT

Preclinical investigation of anti-tumor efficacy of allogeneic natural killer cells combined with cetuximab in head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) presents a significant therapeutic challenge because of the limited effectiveness of current treatments including immunotherapy and chemotherapy. This study investigated the potential of a novel combination therapy using allogeneic natural killer (NK) cells and cetuximab, an anti-epidermal growth factor receptor monoclonal antibody, to enhance anti-tumor efficacy in HNSCC. Allogeneic NK cells were tested against HNSCC cells *in vitro* and NOG (NOD/Shi-scid/IL-2Rγ null) xenograft mouse models for cytotoxicity. In vitro assays demonstrated enhanced cytotoxicity against HNSCC cells when NK cells were combined with cetuximab, a phenomenon attributed to antibody-dependent cellular cytotoxicity. *In vivo*, the combination therapy exhibited a significant anti-tumor effect compared to either monotherapy, with high NK cell infiltration and cytotoxic activity in the tumor microenvironment. Tumor infiltration by NK cells was confirmed using flow cytometry and immunohistochemistry, highlighting the increased presence of NK cells ($CD3^-$ $CD56^+$). These findings suggest that combination allogeneic NK cells and cetuximab could be a potential therapeutic modality for HNSCC and, provide a foundation for future clinical trials to improve patient outcomes.

Key words : Head and neck squamous cell carcinoma, Allogeneic natural killer cell, Cetuximab

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide[1]. Current systemic treatment strategies for HNSCC include cytotoxic-chemotherapy and/or immunotherapy[2]. Immunotherapy, specifically immune checkpoint inhibitors targeting the programmed death-1/programmed cell death-ligand 1 and cytotoxic T-lymphocyte-associated protein 4 pathways[3], allows immune cells to effectively recognize and attack cancer cells. Although these therapies have demonstrated promising ability to improve the outcomes of patients with recurrent or metastatic disease, they are incompletely effective, with only 20–30% of patients responding to immunotherapy[4]; thus, an unmet demand remains. Cetuximab, an antibody targeting the epidermal growth factor receptor (EGFR), is also used to treat patients with head and neck cancer[5]. Despite a targeted approach, its effectiveness as monotherapy remains limited, highlighting the need for optimized combination strategies to improve patient outcomes.

Natural killer (NK) cells have garnered attention as a promising immune cell treatment because of their selective cytotoxicity against cancer cells. They play a key role in regulating inflammation and immune responses by producing cytokines including interferon-γ and tumor necrosis factor-α and can inhibit cancer cell proliferation and metastasis. Moreover, they eliminate cancer stem cells, which are crucial for recurrence[6]. Adoptive NK cell therapies involve both autologous and allogeneic approaches. Autologous NK cells often encounter challenges, including self-recognition of human leukocyte antigens (HLA), leading to primary and/or acquired resistance[7]. Allogeneic NK cells from donors with non-matching NK cell immunoglobulin-like receptor (KIR)–HLA combinations can bypass these issues, enhancing anti-tumor activity with a minimal risk of graft-versus-host disease[8-11]. Although autologous NK cell therapy has a low immune system rejection rate, cell isolation and expansion require time, which can affect treatment feasibility. Allogeneic NK cell therapy using cells from healthy donors may offer reduced cytotoxicity and improved efficacy[12, 13].
Cetuximab induces antibody-dependent cellular cytotoxicity (ADCC)[14, 15], a mechanism primarily mediated by NK cells. In cancer patients, the detection of reduced NK cell activity[16] led to the hypothesis that the injection of allogeneic instead of autologous NK cells would enhance ADCC efficacy in cetuximab combination therapy. Here we found that allogeneic NK cells combined with

cetuximab demonstrated enhanced anti-tumor efficacy compared to monotherapy through the ADCC effect using *in vitro* and *in vivo* experiments. We also confirmed that the combination therapy of allogeneic NK cells and cetuximab is not only effective in tumor suppression in HNSCC xenograft mouse models but also helps NK cells infiltrate tumors. Our results suggest that combination therapy with allogeneic NK cells and cetuximab is promising for improving treatment outcomes in patients with HNSCC.

2. Materials and Methods

2.1. NK cell expansion

SNK02, an allogeneic NK cell product, was manufactured under Good Manufacturing Practice (GMP) conditions (NKMAX, Seongnam, Korea) for *in vivo* animal testing, following previously described methods with certain modifications[17]. Briefly, $CD3^-/CD56^+$ cells were isolated from the peripheral blood mononuclear cells (PBMCs) of the enrolled healthy donors using CliniMACS microbeads (Miltenyi Biotech GmbH, NRW, Germany) following the manufacturer's instructions. The $CD3^-/CD56^+$ cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (WELGENE Inc., Gyeongsan-si, Korea), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Tauranga, New Zealand), 20 μg/mL gentamicin (Gibco, Grand Island, NY), γ-irradiated (100 Gy) - KL-1 and LCL feeder cells, 500 IU/mL interleukin (IL)-2 (PROLEUKIN, Novartis, Basel, Switzerland), and 50 ng/mL IL-21 (NKMAX Co.). NK cells were sub-cultured every 3–4 days in fresh RPMI-1640 medium supplemented with IL-2. After 14 days of culture, the cells were harvested and either re-expanded with feeder cells in the presence of cytokines for another 14 days or cryopreserved for further expansion following the treatment schedule. On day 28 of total culture, the cells were harvested again and cultured with two types of feeder cells and cytokines for an additional 17–18 days. The expanded NK cells were collected on day 45 or 46 of the expansion culture, excluding any cryopreservation period. They were then washed three times, resuspended in a cryopreservation medium containing human serum albumin (Green Cross Corp., Yongin-si, Korea) and CryoStor® cell cryopreservation media (Sigma, St. Louis, MO), transferred to cryopreservation bags, and cryopreserved using an automatic freezing system (CRF). Once thawed, the cells were then used for further experiments.

2.2. Cell culture

FaDu/Luciferase cells (HNSCC cell line; NKMAX Co., Ltd.) were cultured in RPMI-1640 medium (Corning). The medium was supplemented with 10% heat-inactivated FBS (Gibco) and 1% penicillin-streptomycin (Gibco). The cells were cultured at 37°C and 5% carbon dioxide (CO_2). The FaDu cells were subcultured twice per week, at which time the culture medium was removed and the cells were gently washed with 5 mL of warm phosphate-buffered saline (PBS; WELGENE Inc.).

The cells were detached from the dish using 1 mL of 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco) and subsequently neutralized in 7–8 mL of medium. The cells were centrifuged at 1200 rpm for 3 min, pelleted, resuspended in fresh medium, and cultured in a 100mm dish.

2.3. Cytotoxicity assay

The target cancer cells (FaDu) were stained with 10 uM calcein-AM (C1430; Thermo Fisher Scientific), the stained cells (5×10^3) were seeded in 96-well clear flat bottom plates (Corning) and incubated overnight at 37°C and 5% CO_2. The target cells (FaDu) were treated with cetuximab and co-cultured with NK cells at various effector-to-target ratios (E:T ratios: 1:1, 3:1, and 10:1). After 4 h, the supernatant was transferred to a 96-well black/clear-bottom plate. The fluorescence intensities at 488 and 594 nm were measured using a microplate reader (Varioskan LUX; Thermo Fisher Scientific). NK cell–mediated killing of target cells was observed in real time using a thin imager (Leica).

2.4. *In vivo* Experiment

Six-week-old female NOG (NOD/Shi-scid/IL-2Rγnull) mice were purchased from the Central Institute for Experimental Animals (CIEA, Japan). All animal experiments were approved by the local Institutional Animal Care and Use Committee. To generate subcutaneous (s.c.) tumors, the FaDu/Luciferase cells were resuspended and 2×10^6 cells/100 μL were injected s.c. into the flank of each mouse. Tumor size was measured three times per week using electronic digital calipers. The mice were injected intraperitoneally with PBS and 2.5 mg/kg cetuximab and s.c. into the flank with IL-2 (8.4×10^4 IU/mouse). Additionally, allogeneic NK cells (NKMAX Co., Ltd.) were injected intravenously thrice per week. All *in vivo* experiments were performed at least twice.

2.5. Calculating percentage change in tumor growth inhibition

To evaluate the treatment efficacy on tumor growth inhibition, the percentage change from mean tumor size of control group was calculated using tumor measurements taken at 24 or 31 days post-tumor injection. In this formula, “0%” represents the mean tumor size of control group at the specified time point (24 or 31 days post-injection) that serves as the reference value against which

treatment responses are assessed. The formula used to calculate the percentage change in the tumor growth inhibition from the mean tumor size of control group was as follows:

$$= -(100-(\frac{\text{tumor size at day 24 or 31}}{\text{mean tumor size of the control group}})\times 100)$$

2.6. IVIS Spectrum

Bioluminescence imaging was performed weekly using the IVIS Spectrum (Perkin-Elmer, USA). The mice were intraperitoneally injected with d-luciferin (150 mg/kg). The bioluminescence imaging was performed on mice anesthetized with 2% isoflurane. Total counts were measured using Living Image software (Perkin-Elmer).

2.7. Immunohistochemistry staining

Mouse tumor tissues were fixed overnight in 4% formaldehyde. Tissue sections were stained with the anti-human antibody Ki-67 (9027S) purchased from Cell Signaling Technology. Human NKp46/NCR1(MAB1850-100) was purchased from R&D. Immunohistochemistry (IHC) images were obtained using a BX43 light microscope (Olympus, Tokyo, Japan), and Ki-67-positive cells were objectively counted.

2.8. Flow cytometry analysis

For the flow cytometry analysis, the mice were sacrificed and the peripheral blood, spleen, and tumors were harvested. The flow cytometry was performed using a CytoFLEX LX flow cytometer (Beckman Coulter). The data were analyzed using FlowJo software. The cells were washed with cold flow cytometry buffer (PBS supplemented with 2% FBS) and stained with fluorochrome-conjugated anti-human CD56 (740171), CD16 (561304), CD45 (557748), and CD3 (561807) antibodies purchased from BD Biosciences. EGFR (352928) was purchased from BioLegend, whereas the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit was purchased from Invitrogen. The antibodies were diluted 1:50 and incubated for 20 min at 4°C in the dark.

2.9. Statistics

For the survival analysis, the log-rank (Mantel–Cox) test was used to compare differences. Data are presented as mean ± standard deviation or standard error of the mean of the experimental replicates. Student's t-test was used to compare the two groups. Two-way analysis of variance (ANOVA) was used to compare three or more independent groups using GraphPad Prism v8 software (GraphPad, San Diego, CA, USA). Statistical significance was set at $p<0.05$.

3. Results

3.1. Allogeneic NK-high cells effectively managed HNSCC in NOG xenograft mouse model

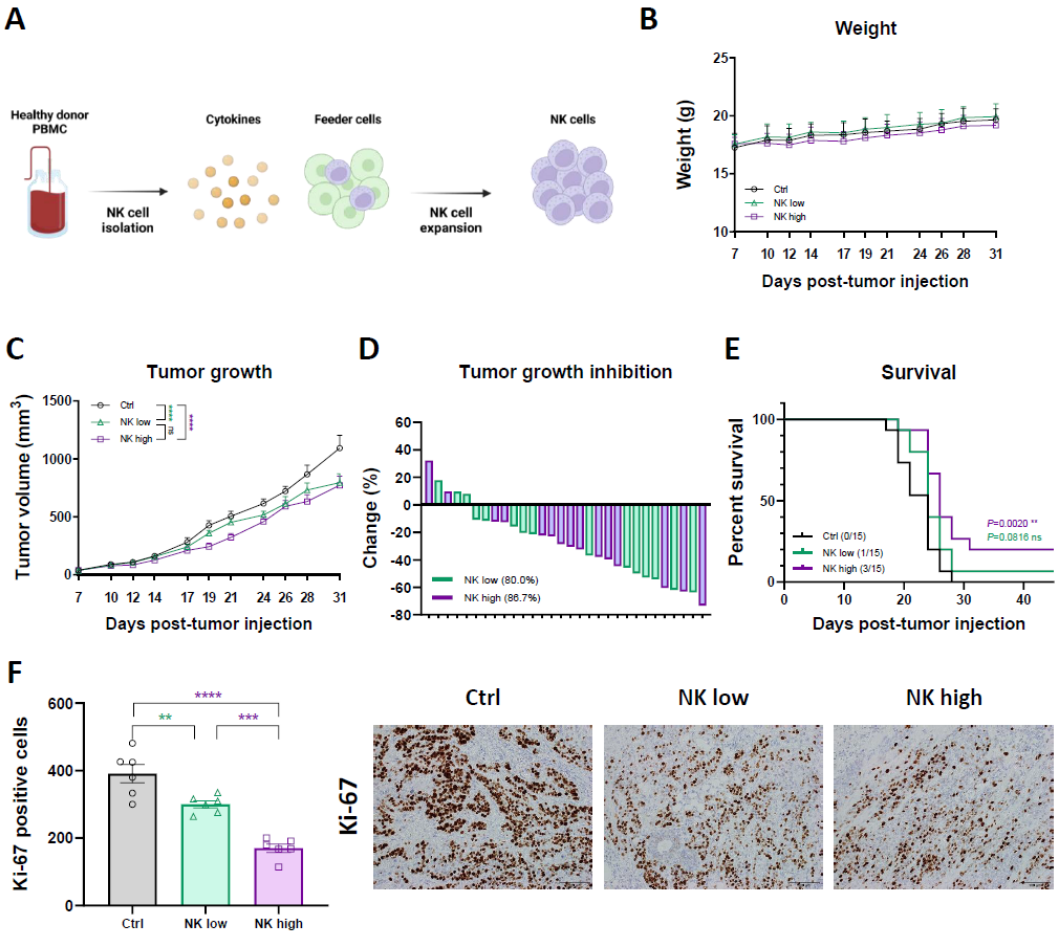


Figure 1. High-dose allogeneic natural killer (NK) cells effectively controlled head and neck tumors in a NOG (NOD/Shi-scid/IL-2Rγ null) xenograft mouse model. (A) Scheme of NK isolation and expansion. (B) Graphs of mouse weights. (C) Graphs of mouse tumor growth. Statistical significance was determined by two-way analysis of variance (ANOVA). (D) Graphs of tumor growth inhibition. (E) Graphs of survival rate. N = 15 mice per group. Statistical significance was determined by two-way ANOVA or a paired t-test. (F) Immunohistochemistry staining with Ki-67 antibody of the mice tumor and summary graph (×20).

In the current study, we first assessed the feasibility of allogeneic NK cell transplantation. NK cells were isolated from PBMCs from healthy donors and expanded using cytokines and feeder cells. This expansion process significantly increased the number of NK cells (3.16±0.67×1010 folds), ensuring sufficient quantities for subsequent experiments **(Figure 1A**).

Next, we evaluated the ability of various allogeneic NK cell doses to control HNSCC in a NOG xenograft mouse model. The study included control (Ctrl), low-dose NK (NK-low; 1×10^6 cells/mouse), and high-dose NK (NK-high; 1×10^7 cells/mouse) groups. The body weights of the mice were evaluated throughout the experiment to monitor systemic toxicity. All groups maintained stable body weights without significant differences among them (**Figure 1B**), indicating that NK cell treatment did not cause notable adverse effects, even at high doses. Tumor growth was significantly reduced in the NK-low and NK-high groups versus the Ctrl group. Tumor volume was monitored over time, and a statistical analysis using two-way ANOVA indicated a significant difference in tumor growth suppression between the NK-low and NK-high groups (**Figures 1C**, **S1A** $p<0.05$).

Analysis of the tumor growth inhibition revealed a notable difference. The NK-high group cell group exhibited a higher tumor growth inhibition (86.7%) than that noted in the NK-low group (80.0%), highlighting the enhanced efficacy of the elevated dose NK cell treatment (**Figure 1D**). Although the NK-low group exhibited no significant improvement in survival versus the Ctrl group, the NK-high group had a clear survival advantage (**Figure 1E**), highlighting the efficacy of the increased dose of NK cells.

We also evaluated the effect of NK cells on tumor cell proliferation. Ki-67 staining revealed the highest inhibitory effect on tumor cell proliferation in the NK-high group, with fewer Ki-67-positive cells than in the NK-low group (**Figure 1F**). Based on these findings, an increased dose of NK cells was selected for subsequent studies owing to its superior ability to improve survival outcomes and inhibit tumor cell proliferation despite no marked differences weight between the NK-low and NK-high groups.

3.2. NK-high and IL-2 combination therapy did not enhance anti-tumor response

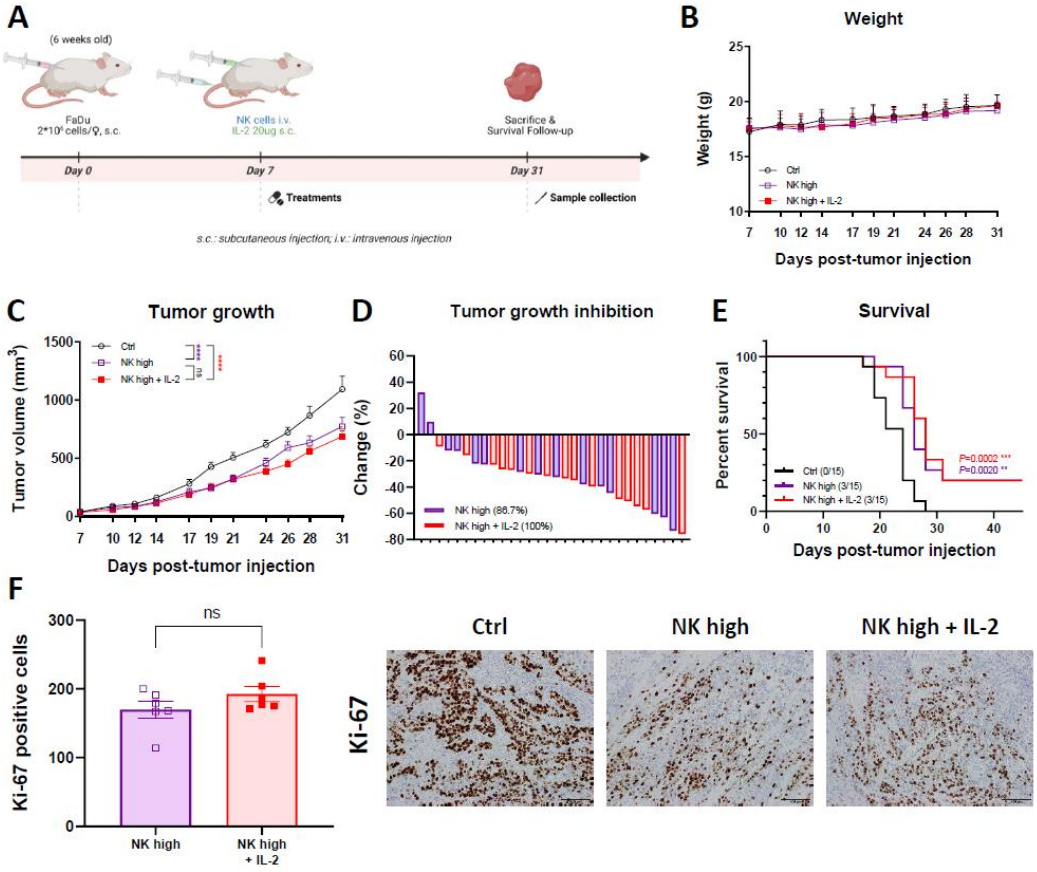


Figure 2. Combination therapy consisting of high-dose natural killer (NK) cells and interleukin (IL)-2 did not enhance the anti-tumor response. (A) Study design for combination therapy of allogeneic NK cells and IL-2. (B) Graphs of mouse weight. (C) Graphs of mouse tumor growth. Statistical significance was determined by two-way analysis of variance (ANOVA). (D) Graphs of tumor growth inhibition. (E) Graphs of survival rate. N = 15 mice per group. Statistical significance was determined by two-way ANOVA or a paired t-test. (F) Immunohistochemistry staining with Ki-67 antibody of the mice tumor and summary graph (×20).

Following the selection of an increased dose of NK cells based on our earlier findings, we investigated IL-2 as a partner drug to enhance NK cell activity *in vivo* (**Figure 2A**). As IL-2 is widely utilized to expand allogeneic NK cells, we hypothesized that combining it with NK-high infusions would enhance the anti-tumor response. The weights of the mice were monitored to evaluate their overall health and the potential treatment-related side effects (**Figure 2B**). However, a tumor growth analysis revealed no significant differences between the NK-high + IL-2 and NK-high groups (**Figures 2C, S1A**).

Similar trends were observed in the tumor growth inhibition and survival data in that no meaningful reduction in tumor growth inhibition or improvement in survival was observed in the combination therapy versus NK-high group (**Figures 2D, 2E**).

Furthermore, IHC staining using the Ki-67 antibody to assess tumor cell proliferation demonstrated no significant differences between the combination therapy and NK-high groups (**Figure 2F**). Similar experiments were conducted using reduced doses of NK cells combined with IL-2, and the results mirrored those of the NK-high group. No significant differences were observed in tumor growth, burden, or survival between the NK-low + IL-2 and NK-low groups, further confirming that IL-2 did not enhance NK cell anti-tumor activity at either dose (**Figures S2A–E**).

These results collectively indicate that IL-2, when combined with NK-high or NK-low, did not augment the anti-tumor response *in vivo*. The lack of an improved anti-tumor response through the IL-2 combination *in vivo* prompted us to investigate other combination treatment partners.

3.3. Cetuximab enhanced cytotoxic effect of allogeneic NK cells *in vitro*

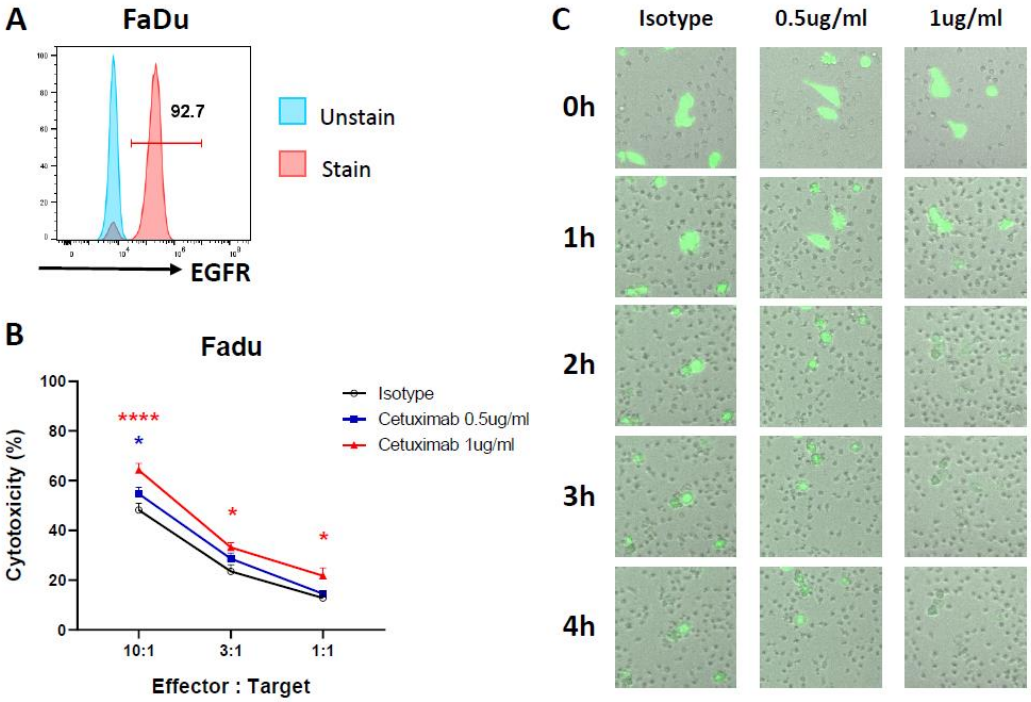


Figure 3. Cetuximab enhanced cytotoxic effect of allogeneic NK cells in vitro. (A) Expression of epidermal growth factor receptor on FaDu cells (head and neck squamous cell carcinoma cell line). (B) Antibody-dependent cellular cytotoxicity assay using calcein-AM for 4 h at 10:1, 3:1, and 1:1 effector-to-target ratios of allogeneic NK cells:FaDu cells. (C) Images of FaDu cells (stained green by calcein-AM) being co-cultured with NK cells.

Cetuximab, an EGFR-targeting antibody, was selected for subsequent experiments due to its potential to enhance NK cell-mediated ADCC. In preparation for the subsequent experiments with cetuximab, we first confirmed the EGFR expression in FaDu cells The expression of EGFR in FaDu cells (**Figure 3A**) provided a suitable rationale for using cetuximab as the drug-induced ADCC when combined with NK cells. This EGFR expression served as a critical indicator that cetuximab could be a promising drug for enhancing NK cell-mediated anti-tumor activity. *In vitro* experiments confirmed the ADCC effect, as confirmed by the results of the calcein-AM assay, where NK cells demonstrated effective cytotoxicity against FaDu cells at effector-to-target (E:T) ratios (10:1, 3:1, and 1:1) (**Figure 3B**). Images of FaDu cells co-cultured with NK cells further illustrated this effect (**Figure 3C**).

Collectively, while IL-2 showed limitations in enhancing NK cell activity *in vivo*, the ADCC effect of cetuximab was enhanced by NK cell combination treatment in HNSCC.

3.4. Synergistic effect of allogeneic NK cells and cetuximab on head and neck tumor suppression in NOG xenograft mouse model

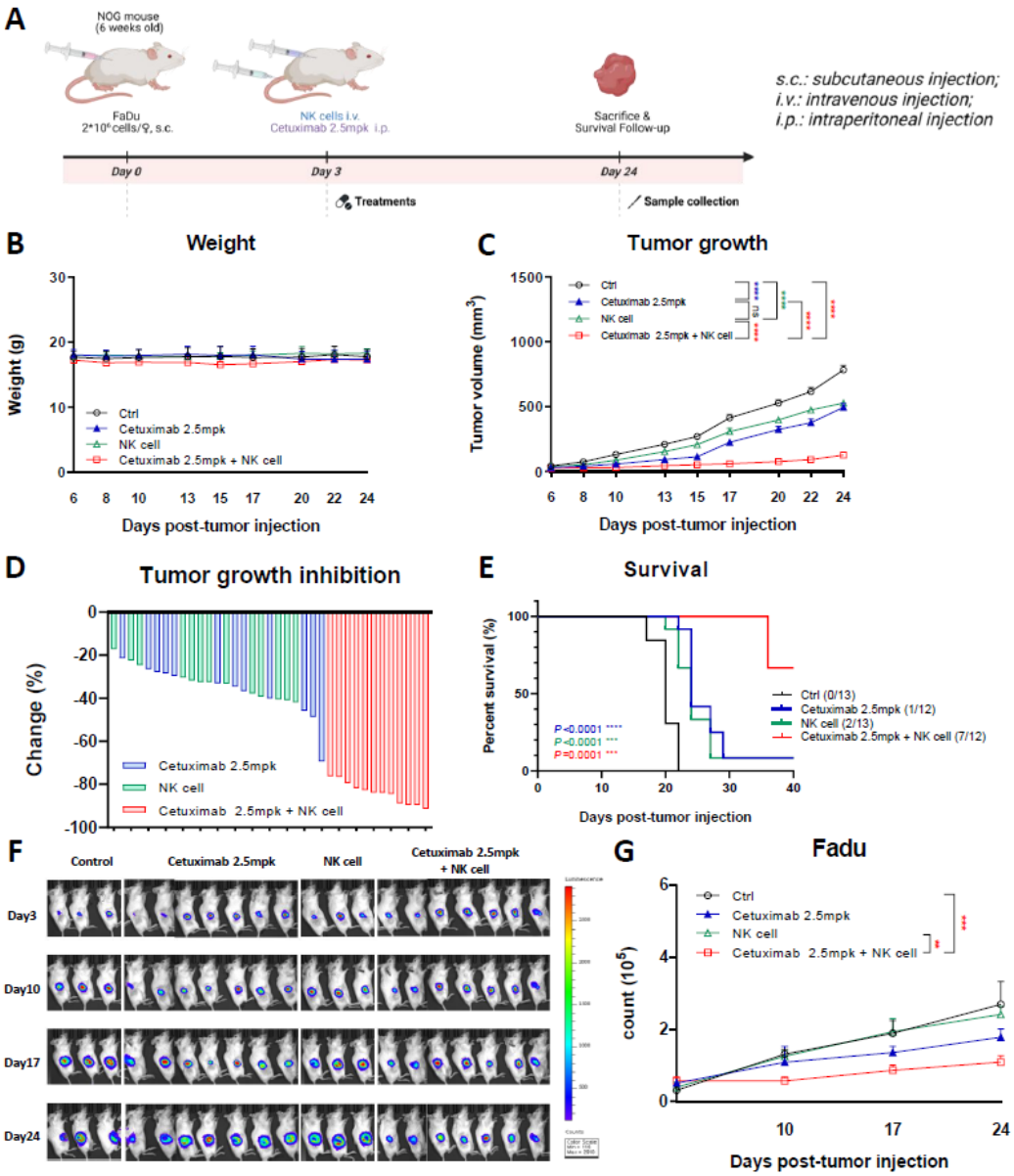


Figure 4. Combination therapy consisting of allogeneic natural killer (NK) cells and cetuximab effectively managed head and neck tumors in the NOG (NOD/Shi-scid/IL-2Rγ null) xenograft mouse model. (A) Study design for combination therapy of allogeneic NK cells and cetuximab. (B) Graphs of mouse weight. (C) Graphs of mouse tumor growth. Statistical significance was determined by two-way analysis of variance (ANOVA). (D) Graphs of tumor growth inhibition. (E) Graphs of survival rate. N = 12–13 mice per group. Statistical significance was determined by two-way ANOVA or a paired t-test. (F) IVIS Spectrum images. (G) Graphs of tumor burden (count). N = 12–13 mice per group.

Building on previous experiments in which NK-high cells (1×10^7 cells/mouse) were selected, we investigated the combination of allogeneic NK cells and cetuximab in the NOG xenograft mouse models to evaluate the ADCC effect and overall therapeutic efficacy of the combination treatment (**Figure 4A**). The study included four groups: ctrl, cetuximab 2.5mg/kg (mpk), NK cells (1×10^7 cells/mouse), and cetuximab 2.5mpk + NK cell.

The weights of the mice were monitored to evaluate their overall health and potential treatment-related side effects (**Figure 4B**). No significant weight loss was observed in any groups, suggesting that the treatment was well tolerated. Additionally, the cetuximab + NK cell group exhibited significantly reduced tumor growth compared with that in the control and single-agent groups (cetuximab and NK cells alone) (**Figures 4C**, **S3A**). The tumor growth inhibition was significantly lower in the cetuximab + NK cell group than in the other groups (**Figure 4D**), and survival analysis revealed improved survival in the cetuximab + NK cell group than in the control and monotherapy groups (**Figure 4E**). This demonstrated that the therapeutic benefit was statistically significant and highlighted the superior effect of combination therapy. The reduced tumor burden was visually identified in the cetuximab + NK cell group (**Figure 4F**), and the results were further confirmed by quantitative analysis using IVIS Spectrum imaging (**Figure 4G**). Collectively, the combination of allogeneic NK cells and cetuximab effectively managed HNSCC in NOG xenograft mouse model, showing significant reductions in tumor growth and burden and improved survival rates. These results highlight the potential of this combination therapy to enhance anti-tumor efficacy.

3.5. Enhanced NK cell infiltration and ADCC in HNSCC NOG xenograft mouse model therapy treated with allogeneic NK cells and cetuximab

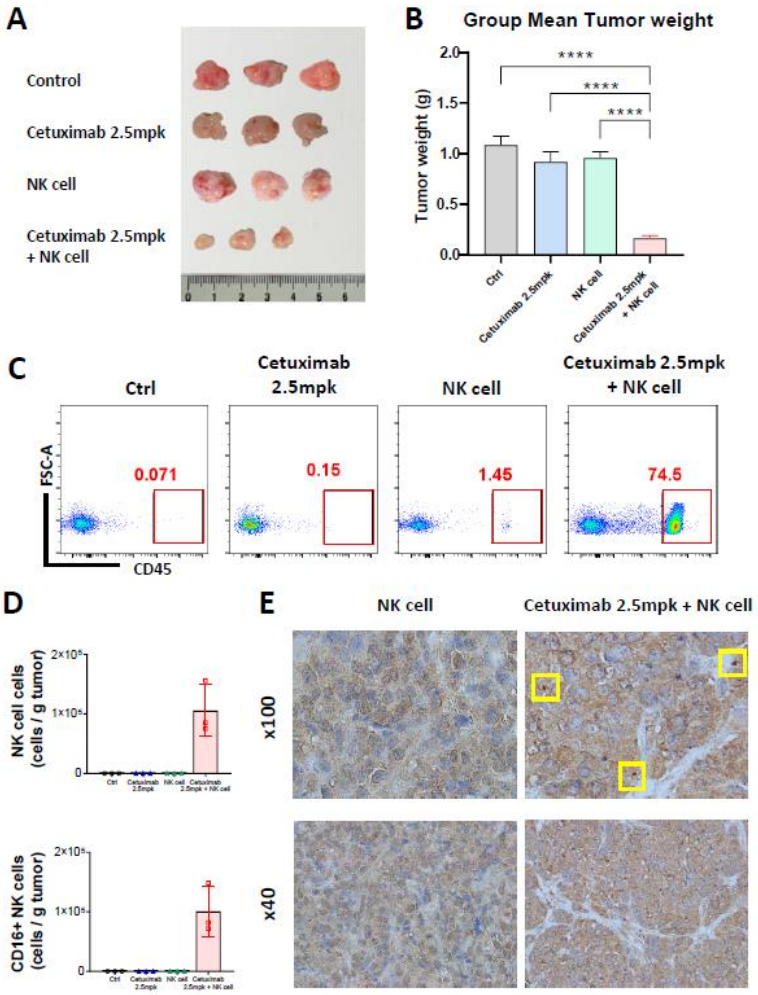
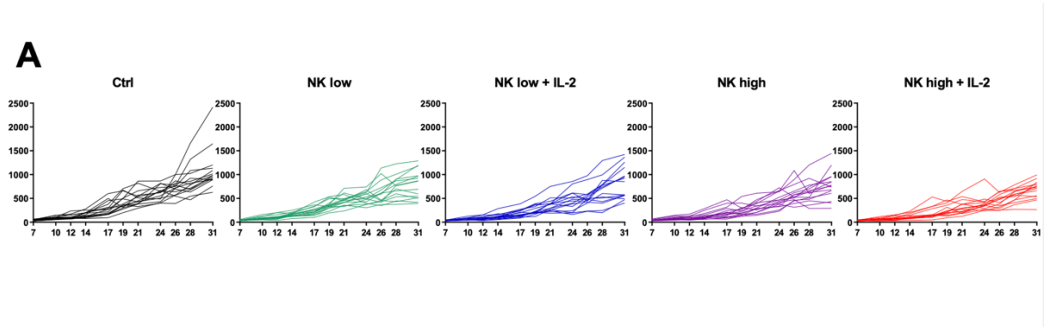


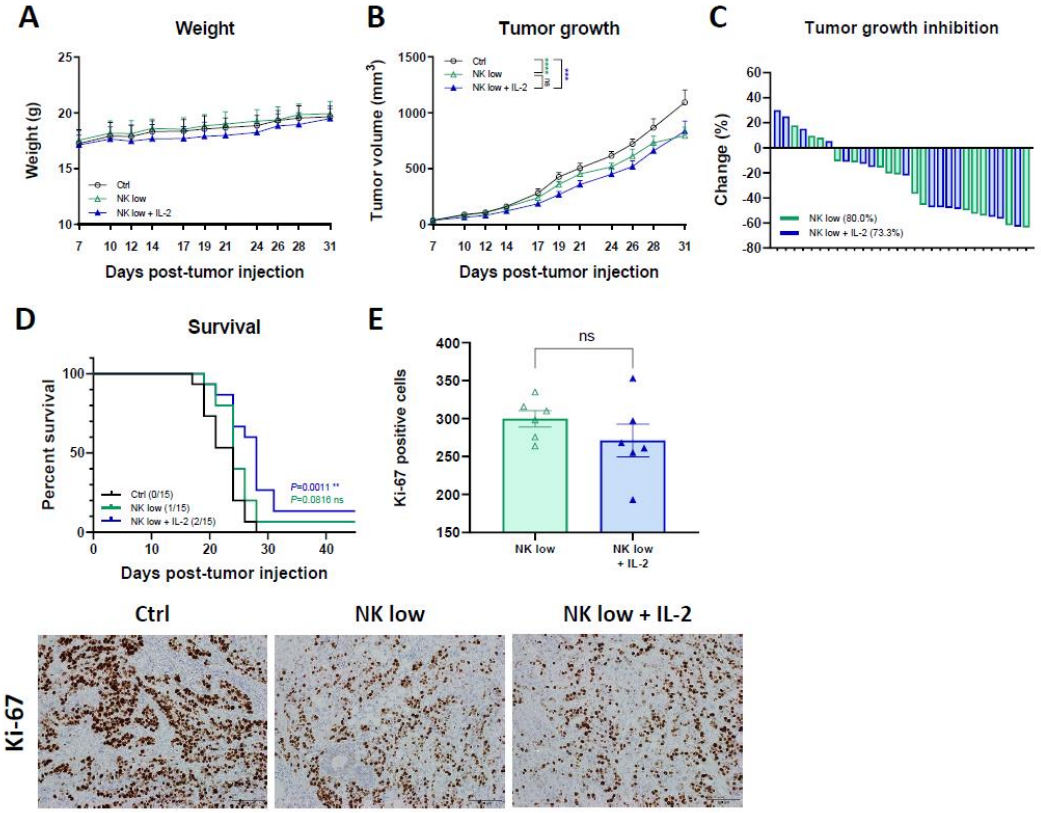
Figure 5. Allogeneic natural killer (NK) cells infiltrated tumors via antibody-dependent cellular cytotoxicity. (A) Tumors in each group of mice. (B) Tumor weights. (C) Flow cytometry analysis revealed the proportion of $CD45^+$ cells in the tumors. (D) Graphs of flow cytometry results displaying the proportion of NK ($CD3^-$ $CD56^+$) and cytotoxic NK ($CD16^+$) cells within the tumors. (E) Immunohistochemistry staining with NKp46 antibody of the mice tumor (×40, ×100).

Following the initial experiments demonstrating the therapeutic efficacy of allogeneic NK cells combined with cetuximab, we conducted further investigations to assess NK cell infiltration and ADCC within tumors (**Figure 5A**). Differences in tumor size between the control, cetuximab, NK cell, and cetuximab + NK cell groups were visually identified, and the cetuximab + NK cell group had a significantly lower mean tumor weight than the other groups (**Figure 5B**). This tumor weight reduction is consistent with previous findings and enhances the anti-tumor activity of combination therapy. Flow cytometry analyzed the composition of immune cells within the tumor. The proportion of $CD45^+$ (marker for human immune cell) cells was assessed, and immune cell penetration was disproportionally augmented in the cetuximab + NK cell group compared to that in the other treatment groups (**Figure 5C**). Further analysis focused on NK cell infiltration, and the proportions of NK ($CD3^-$ $CD56^+$) and cytotoxic NK ($CD3^-$ $CD56^+$ $CD16^+$) cells were significantly increased in the cetuximab group with NK cells (**Figures 5D**, **S4A, S4B**). This increase in the number of NK cells within the tumor microenvironment supports the hypothesis that combined treatment enhances NK cell-mediated cytotoxicity via ADCC.

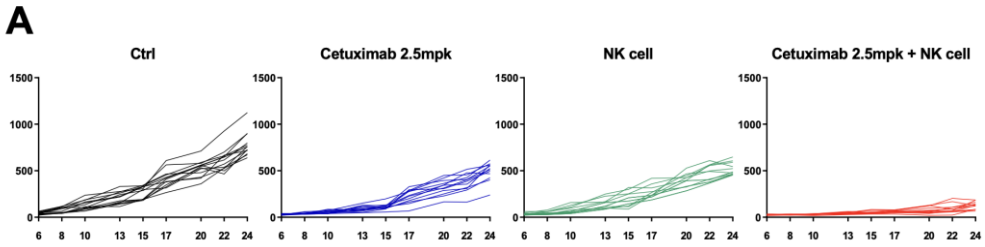
The IHC analysis of the tumor sections confirmed the presence of NK cells within the tumor by staining with NKp46 antibody. Compared to the NK cell monotherapy group, the addition of cetuximab enhanced NK cell infiltration within the tumor (**Figure 5E**). These observations were consistent with the flow cytometric data. In summary, the combination of allogeneic NK cells and cetuximab reduced tumor size and prolonged survival and enhanced NK cell infiltration and ADCC within the tumor microenvironment, highlighting the potential of this combination therapy to treat HNSCC.



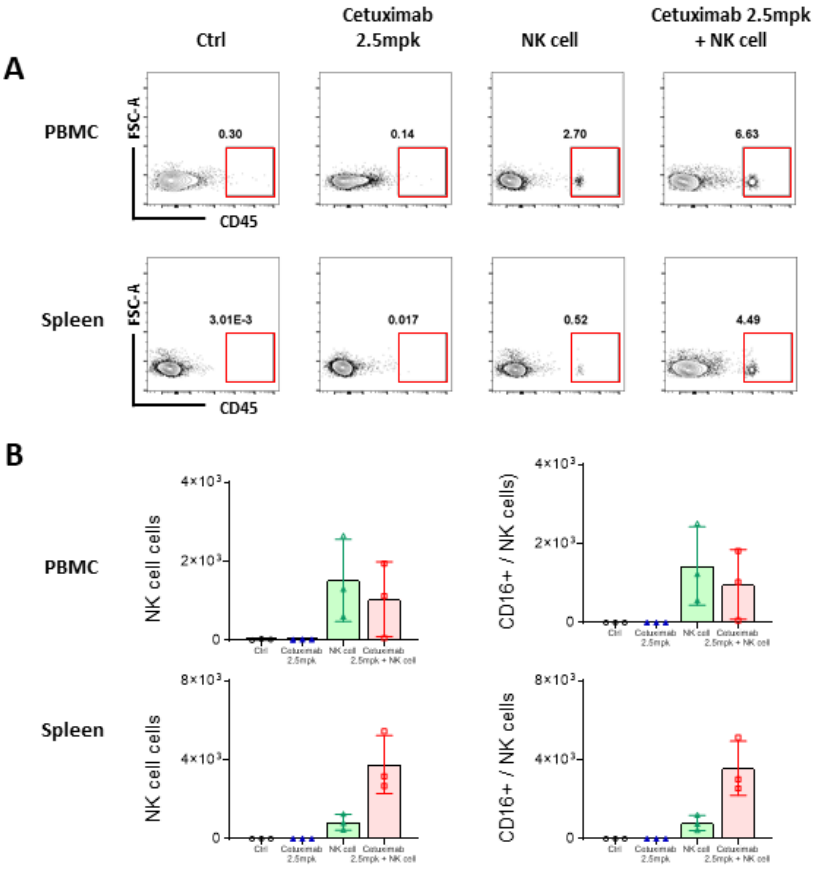
Supplementary Figure 1. Individual tumor growth curves. (A) Individual tumor growth curves from the xenograft mouse model experiment determining natural killer (NK) cell dose and combination therapy experiment with interleukin (IL)-2. X-axis: tumor volume (mm^3); Y-axis: days post-tumor injection.



Supplementary Figure 2. Combination therapy of low-dose natural killer (NK) cells and interleukin-2 did not enhance the anti-tumor response. (A) Graphs of mouse weight. (B) Graphs of mouse tumor growth. Statistical significance was determined by two-way analysis of variance (ANOVA). (C) Graphs of tumor growth inhibition. (D) Graphs of survival rate. N = 15 mice per group. Statistical significance was determined by two-way ANOVA or a paired t-test. (E) Immunohistochemistry staining with Ki-67 antibody of the mouse tumors and summary graph (×20).



Supplementary Figure 3. Individual tumor growth curves. (A) Individual tumor growth curves from xenograft mouse model experiment with NK cells and cetuximab combination. X-axis: tumor volume (mm^3); Y-axis: days post-tumor injection.



Supplementary Figure 4. Flow cytometry analysis results demonstrated the proportion of natural killer (NK) cells in the peripheral blood mononuclear cells (PBMCs) and spleen. (A) Flow cytometry analysis displaying the proportion of $CD45^+$ cells in the PBMCs and spleen. (B) Graphs of flow cytometry results demonstrating the proportion of NK ($CD3^-$ $CD56^+$) and cytotoxic NK ($CD16^+$) cells in the PBMCs and spleen.

4. Discussion

This study used a NOG xenograft mouse model to demonstrate the enhanced anti-tumor efficacy of combined allogeneic NK cell and cetuximab treatment for HNSCC. During the selection process for allogeneic NK cells and their partner drugs, IL-2 (commonly used for NK cell expansion) was selected as the partner drug and tested in the xenograft mouse model. However, it did not display a synergistic effect, suggesting that IL-2 alone is insufficient to significantly enhance NK cell anti-tumor activity and highlighting the need for alternative combination strategies. Therefore, we explored the effects of cetuximab combined with NK cells. The combination therapy consisting of cetuximab and allogeneic NK cells resulted in significant tumor suppression. Moreover, cetuximab facilitated NK cell infiltration into tumors via ADCC.

Several approaches have been explored in the context of NK cell therapy for cancer treatment. Traditionally, autologous NK cells have been used; however, such approaches have significant limitations, particularly regarding cellular persistence and activity. Therapies aimed at enhancing NK cell activity, such as those involving the use of monalizumab, an immune checkpoint inhibitor targeting NKG2A[18], were recently introduced and subjected to clinical trials. Despite efforts to block NK cell inhibition signals, clinical trials of combination cetuximab treatment have not demonstrated appreciable efficacy compared to cetuximab monotherapy[19]. Given the limitations of autologous NK cells and therapies focusing solely on inhibitory checkpoint molecules, here we explored the preclinical activity of allogeneic NK cells. Here cetuximab was incorporated to enhance the ability of infused NK cells to mediate ADCC.

Our findings highlight the advantages of using high-dose allogeneic NK cells combined with cetuximab. Allogeneic NK cells offer key benefits over autologous NK cells, which often exhibit reduced cytotoxicity due to self-HLA recognition. In contrast, allogeneic NK cells obtained from donors with mismatched KIR-HLA combinations can bypass these self-recognition mechanisms and exhibit enhanced anti-tumor activity[8-11]. Additionally, allogeneic NK cells are readily available for treatment, whereas autologous therapies require time-consuming isolation and expansion, making the former more practical, particularly in urgent treatment scenarios. The current *in vitro* experiments demonstrated that the combination of cetuximab and NK cells significantly enhanced

the cytotoxic effects against FaDu cells, a human HNSCC cell line. This synergy is likely attributable to the ability of cetuximab to bind to the EGFR on tumor cells, flagging them for destruction by NK cells.

In this study, we used NOG (NOD/Shi-scid/IL-2Rγnull) mice, which lack mature T, B, and NK cells, making them ideal models for human cell transplantation and cancer research. The absence of an adaptive immune response in the NOG mice enabled us to effectively evaluate human immune cell therapies, such as allogeneic natural NK cell treatment, without interference from the host's immune system. Therefore, we conducted *in vivo* experiments that considered these advantages. First, these *in vivo* experiments revealed that high doses of allogeneic NK cells were more effective at tumor suppression than lower doses. Moreover, the infusion of high-dose allogeneic NK cells did not affect safety, including weight loss, and its anti-tumor efficacy was further increased. Therefore, high-dose allogeneic NK cells were used in subsequent experiments. Next, we investigated the effect of cetuximab and allogeneic NK-high cells on the tumor burden of NOG xenograft mice. Tumor growth was significantly suppressed in the combination versus Ctrl and monotherapy groups, providing additional evidence of the enhanced anti-tumor efficacy of this therapeutic strategy. These findings support our hypothesis that cetuximab enhances NK cell–mediated cytotoxicity via ADCC, resulting in significant tumor suppression.

NK cell populations generally have characteristics such as $CD56^+$ and $CD16^+$, which are markers of cytotoxic potential; NKp46 is also being emphasized as a key marker of natural killer cytotoxicity[20, 21]. Flow cytometry revealed a substantial increase in the infiltration of NK cells into the tumors of mice treated with combined cetuximab and high-dose NK cells. The proportions of $CD45^+$ immune cells, NK cells ($CD3^-CD56^+$), and cytotoxic NK cells ($CD16^+$) were significantly higher in the combination versus other treatment groups. These findings indicate that the combined therapy not only enhances NK cell infiltration but also promotes cytotoxic activity within the tumor microenvironment. The IHC analysis provided additional evidence of enhanced NK cell activity and tumor suppression. The increased NK cell infiltration and cytotoxic markers in the cetuximab + NK-high group corroborated the flow cytometry results and validated the occurrence of ADCC with this treatment.

In conclusion, this study provides compelling evidence that the combination of high-dose allogeneic NK cells and cetuximab significantly improves tumor suppression in patients with HNSCC. This observed synergy suggests that the combination therapy enhances NK cell–mediated cytotoxicity via ADCC, leading to improved treatment outcomes. Allogeneic NK cells, with their practical advantages over autologous cells, offer a promising therapeutic strategy for meeting the unmet needs of patients with HNSCC, especially those demonstrating a limited response to current treatments.

Keywords:
Head and neck squamous cell carcinoma, Allogeneic natural killer cell, cetuximab, Antibody-dependent cellular cytotoxicity

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Abstract in Korean

두경부 편평세포암에서 세툭시맙과 병용된 동종 유래 자연살해(NK) 세포의 항종양 효능에 대한 전임상 연구

두경부 편평세포암은 면역 요법과 화학 요법을 포함한 현재 치료법의 효과가 제한적이기 때문에 중요한 치료 과제를 제시합니다. 이 연구는 동종 자연 살해 세포와 항-EGFR 단일 클론 항체인 세툭시맙을 사용한 새로운 병용 요법이 두경부 편평세포암에서 항종양 효능을 향상시킬 수 있는 잠재력을 조사합니다. 동종 자연 살해 세포는 시험관 내 및 NOG 이종 이식 마우스 모델에서 두경부 편평세포암에 대한 세포 독성을 테스트했습니다. 시험관 내 분석 결과, 항체 의존성 세포매개 세포 독성에 기인한 동종 자연 살해 세포와 세툭시맙을 병용했을 때 두경부암 세포에 대한 세포 독성이 향상된 것으로 나타났습니다. 생체 내에서 병용 요법은 각 단독 요법에 비해 유의미한 항종양 효과를 보였으며, 종양 미세 환경에서 더 높은 자연 살해 세포 침투 및 세포 독성 활성을 보였습니다. 자연 살해 세포에 의한 종양 침투는 유세포 분석 및 면역조직화학을 통해 확인되어 자연 살해 세포($CD3^-$ $CD56^+$)의 존재 증가를 강조했습니다. 이러한 발견은 동종 자연 살해 세포와 세툭시맙의 병용이 두경부 편평세포암에 대한 잠재적 치료 전략을 제공할 수 있음을 시사하며, 향후 환자 결과를 개선하기 위한 임상 시험의 기반을 제공할 수 있음을 시사합니다.

핵심되는 말 : 두경부 편평세포암, 동종 자연 살해 세포, 세툭시맙