

Article

FcεRIγ reinforces double-negative T cell-mediated antibody-dependent cellular cytotoxicity against tumor cells

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Double-negative T (DNT) cells (TCRαβ⁺CD4⁻CD8⁻NK1.1⁻/CD56⁻) exhibit strong tumor-killing capabilities. Our single-cell transcriptome analysis has revealed high *Fcer1g* expression in DNT cells, but its role in tumor immunity remains unclear. In this study, we demonstrated that IgG1 stimulation significantly upregulated IgG Fc receptors and cytotoxic molecules in DNT cells, enhancing their cytotoxicity against MC38 tumor cells *in vitro*. FcεRIγ-deficient DNT cells failed to respond effectively to IgG1 stimulation. Inhibiting the downstream spleen tyrosine kinase (Syk) of FcεRIγ reduced cytotoxicity of DNT cells and phosphorylation levels of molecules such as AKT and NF-κB. In a subcutaneous tumor model, combined treatment with DNT cells and tumor-specific antibodies more effectively inhibited tumor growth compared to DNT cells alone, while FcεRIγ-deficient DNT cells combined with antibodies showed no significant difference in efficacy compared to DNT cells alone, suggesting that DNT cells enhance tumor cell killing via FcεRIγ-mediated antibody-dependent cellular cytotoxicity (ADCC). These results indicate that DNT cells mediate antitumor ADCC effects through high FcεRIγ expression. Binding of IgG1 to FcεRIγ activates the FcεRIγ/Syk/AKT/NF-κB pathway, consequently enhancing tumor cell killing. Thus, DNT cells may play a significant role in cancer immunity, providing a basis for novel immune cell and antibody combination therapies.

Keywords: tumor immunology, T cell therapy, double-negative T cells, antibody-dependent cellular cytotoxicity, Fc receptor γ chain (FcεRIγ)

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Introduction

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an antigen-specific cytotoxicity that contributes to the treatment of tumors (Chan and Carter, 2010). The cytotoxic activity of IgG relies on its Fc region binding to Fc receptors (FcRs) on effector immune cells (Zhu et al., 2020). Once antibodies bind to tumor cells, effector cells such as natural killer (NK) cells release perforin and granzymes, triggering apoptosis in target cells (Cigalotto and Martinvalet, 2024).

FcRs bridge antibodies with cytotoxic cells. Fc ϵ R1 γ , an FcR subunit, plays essential roles in immune responses including ADCC and allergy (Brandtsma et al., 2016). Innate T cells expressing Fc ϵ R1 γ have been reported to exhibit strong antitumor potential (Chou et al., 2022).

Upon antibody binding to target cells, the Fc portion of IgG interacts with Fc γ RIII (CD16) on effector cells. CD16 forms a signaling complex with the adaptor Fc γ R (also known as Fc ϵ R1 γ) (Lanier et al., 1991; Letourneur et al., 1991), whose immunoreceptor tyrosine-based activation motif (ITAM) domains become phosphorylated upon receptor engagement (Brandtsma et al., 2016), recruiting and activating spleen tyrosine kinase (Syk) to initiate downstream signaling cascades that mediate ADCC (Travers et al., 2019).

Double-negative T (DNT) cells, a subset of TCR $\alpha\beta$ lymphocytes lack conventional CD4, CD8, or CD56 (NK1.1 in mice), comprise 1%–3% of peripheral T cells but accumulate in local tissues, suggesting unique roles in various diseases. Recent preclinical and clinical studies have demonstrated the strong antitumor potential of DNT cells in both hematologic and solid malignancies. Allogeneic DNT cells expanded from healthy donors effectively eliminated chemotherapy-resistant acute myeloid leukemia (AML) cells *in vitro* and in xenograft models without inducing graft-versus-host disease (GvHD), highlighting their potent cytotoxicity and safety (Chen et al., 2018; Lee et al., 2018). A phase I clinical trial further confirmed the safety and efficacy of allogeneic DNT therapy for relapsed AML after allo-HSCT, showing dual anti-leukemic and GvHD-mitigating effects (Lee et al., 2025). Moreover, DNT cells display stronger cytotoxicity compared to conventional CD4⁺ or CD8⁺ T cells, underscoring their superior intrinsic antitumor activity (Merims et al., 2011). In solid tumors such as non-small cell lung cancer (NSCLC), adoptively transferred DNT cells significantly suppressed tumor growth in preclinical models and showed enhanced efficacy when combined with IL-15 or PD-1 blockade (Yao et al., 2019). Collectively, these findings establish DNT cells as a promising and safe effector population for adoptive cancer immunotherapy.

DNT cells possess potent cytotoxic capabilities against tumor cells by initiating an immune response characterized by the release of cytotoxic molecules such as perforin and granzymes, resulting in tumor cell lysis (Yao et al., 2019; Wang et al., 2022). It was reported that Fc ϵ R1 γ is highly expressed and enhances the effector functions of DNT cells

(Thomson et al., 2006). However, whether DNT cells could exert their antitumor function through ADCC is still unknown.

In this study, we found that DNT cells highly express Fc ϵ R1 γ . Following cross-linking antibody stimulation, DNT cells exhibited enhanced antitumor activities through ADCC with increased degranulation of CD107a and elevated secretion of granzyme B (GzmB) and cytokines such as IFN γ . The enhanced ADCC of DNT cells was linked to the Fc ϵ R1 γ /Syk signaling pathway. In combination with tumor-specific antibodies, both murine and human DNT cells significantly inhibited tumor growth and improved overall survival in xenograft models.

Results

DNT cells highly express Fc ϵ R1 γ

To characterize the distinct roles of DNT cells and conventional T cells, we analyzed previously published single-cell RNA sequencing (scRNA-seq) data of mouse peripheral DNT cells, CD4⁺ T cells, and CD8⁺ T cells from the GEO database (GSE129030). Gene set enrichment analysis (GSEA) revealed differential expression pathways involved in immunoglobulin-mediated immune response, response to tumor cells, and regulation of tumor necrosis factor production (Figure 1A and B), suggesting that DNT cells exhibit a stronger tumor cell-killing ability. Further analysis demonstrated that DNT cells upregulate key genes (*Fcer1g*, *Ifng*, *Gimap5*, and *Xcl1*) associated with the positive regulation of the immunoglobulin-mediated immune response pathway (Figure 1C). These genes may enhance DNT cells responses to antibodies, thereby illustrating their increased cytotoxic capacity.

Fc ϵ R1 γ is known to be expressed on NK cells and myeloid cells, mediating ADCC and antibody-dependent cellular phagocytosis (ADCP). Our results showed that *Fcer1g* is highly expressed in DNT cells compared to CD4⁺ and CD8⁺ T cells (Figure 1D). Flow cytometry analysis of freshly isolated spleen cells also confirmed significantly higher expression of Fc ϵ R1 γ on DNT cells (Figure 1E). Additionally, we analyzed the expression levels of different IgG receptors in DNT cells and indicated that, in addition to *Fcer1g*, the IgG1 receptor *Fcgr3* (gene of CD16) is also highly expressed by DNT cells (Figure 1F). These findings suggest that the high expression of Fc ϵ R1 γ and CD16 provides the molecular basis for DNT cells acting as ADCC effectors.

IgG1 stimulation enhances cytotoxic functions of DNT cells

To assess whether antibodies could enhance DNT cell cytotoxicity, we performed artificial ADCC activation assays using anti-OVA IgG1 and an agonistic anti-mouse kappa light chain (KLC) antibody in the absence of target cells. Cross-linking IgG1 with anti-KLC significantly boosted the cytotoxic function of DNT cells, as evidenced by increased expression of ADCC-related molecules, including the IgG1 receptors CD16 and Fc ϵ R1 γ (Figure 2A and B). In contrast, the expression of CD16 and Fc ϵ R1 γ was not increased when cells were

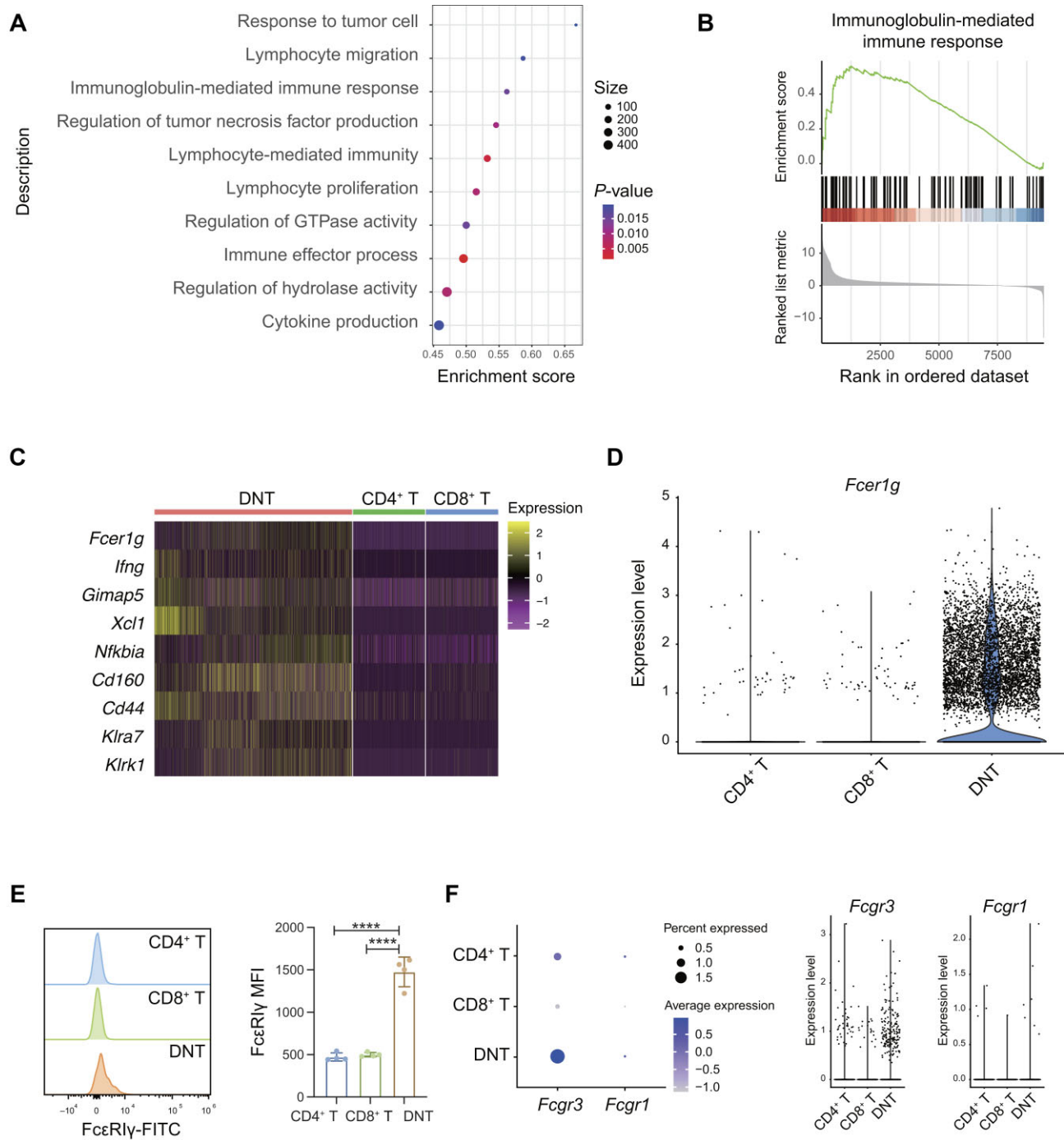


Figure 1 FcεR1 expression on DNT cells. **(A)** Single-cell transcriptome analysis of murine DNT cells. GSEA identified differential expression pathways associated with the tumor cell-killing abilities. **(B)** GSEA plot showing highly significant enrichment of positive regulation of immunoglobulin-mediated immune response in DNT cells. **(C)** A heatmap of genes related to immunoglobulin-mediated immune response in CD4⁺ T, CD8⁺ T, and DNT cells. **(D)** *Fcer1g* expression in CD4⁺ T, CD8⁺ T, and DNT cells analyzed by single-cell transcriptome sequencing. **(E)** FcεR1y expression on CD4⁺ T, CD8⁺ T, and DNT cells detected by flow cytometry. **(F)** Dot plot showing high expression of IgG1 receptors in DNT cells. $n = 3-5$ per group. One-way ANOVA was performed **(E)**. The data are represented as mean $\pm$ SD. **** $P < 0.0001$.

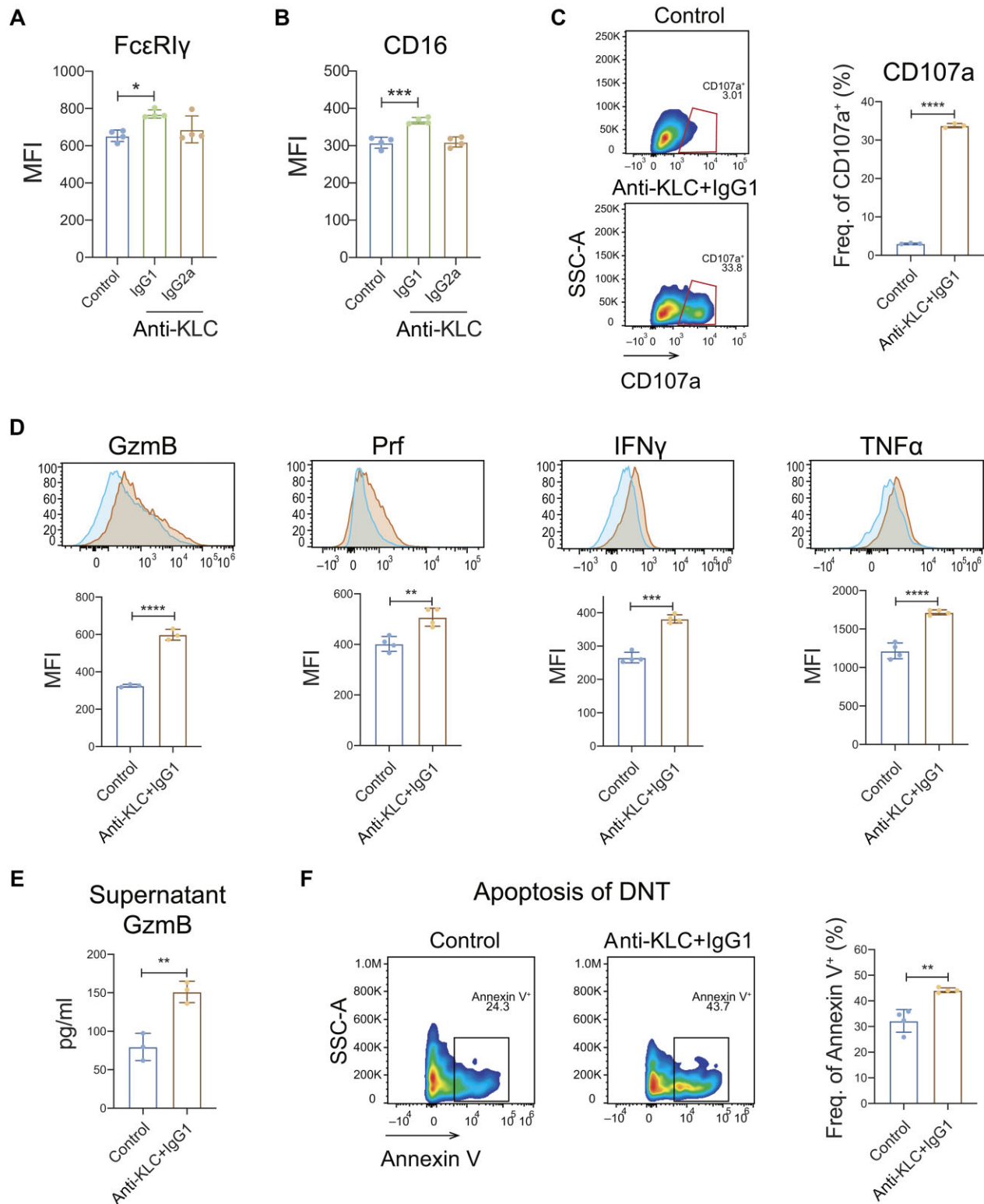


Figure 2 IgG1 stimulation enhances cytotoxic functions of DNT cells. DNT cells were stimulated with or without anti-mouse KLC and mouse IgG antibodies for 12 h *in vitro*. (A–C) Representative flow cytometry quantification and statistical analysis of FcεR1γ (A), CD16 (B), and CD107a (C) levels ($n = 4$ or 6/group) in the simulated DNT cells. (D) Cell-killing molecules expressed by the stimulated DNT cells were detected by flow cytometry. (E) Supernatant GzmB released by the stimulated DNT cells was detected by ELISA. (F) Apoptosis of the stimulated DNT cells was detected by flow cytometry. $n = 3$ –5 per group. One-way ANOVA (A and B) or Student's *t*-test (C–F) was performed. The data are represented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

stimulated with IgG2a and anti-KLC antibodies. Following cross-linking antibody stimulation, DNT cells exhibited enhanced degranulation of CD107a and elevated secretion levels of GzmB and cytokines such as IFN γ and TNF α , compared to untreated cells (Figure 2C and D). Moreover, the amount of GzmB in the culture supernatant was found to be increased (Figure 2E). Under conditions of excessive stimulation with anti-CD16 antibodies, NK cells may initiate the apoptotic program (Capuano et al., 2021; Coenon and Villalba, 2022). Similarly, we observed activation-induced apoptosis in DNT cells following treatment with cross-linking antibodies (Figure 2F). These findings indicate that IgG1 stimulation enhances both cytotoxicity and apoptosis of DNT cells via cross-linking-mediated activation, supporting their potential ADCC function.

DNT-mediated ADCC targets antibody-coated target cells

To investigate the capacity of DNT cells to exert ADCC responses, we utilized neoantigen overexpression cell lines or cancer cells as targets. Mouse colon carcinoma MC38-OVA cells were transfected with the OVA peptide (SIINFEKL) as a neoantigen, with untransfected MC38 cells serving as non-neoantigen controls (Figure 3A). Live cells were labeled with green-fluorescent Calcein, while dead cells lost the Calcein labeling. Mouse DNT cells were co-cultured with MC38-OVA cells pre-coated with or without OVA (SIINFEKL) antibody for 12 h. ADCC responses were detected by measuring the percentage of Calcein⁺ target cells. The percentage of cell death in MC38-OVA cells increased proportionally with higher effector-to-target (E:T) ratios. Specifically, the cell death of OVA antibody-coated MC38-OVA cells was increased by ~20% at an E:T ratio of 2:1 compared to uncoated MC38-OVA cells, while no such enhancement in specific cell death was observed for MC38 cells coated with the OVA antibody (Figure 3B and C). To further validate antibody-dependent cytotoxicity in human settings, human DNT cells—which express Fc ϵ R1 γ and CD16, as confirmed by flow cytometry analysis (Supplementary Figure S1)—were used against human carcinoma cell lines. The human colorectal adenocarcinoma cell line Colo205, which expresses the specific antigen HER2, was used alongside the human leukemia cell line K562, which does not express HER2, as a control (Figure 3D). The specific cell death of Colo205 cells pre-coated with the anti-HER2 antibody Herceptin increased by ~20% at an E:T ratio of 5:1, while no enhancement was observed in the control K562 cells (Figure 3E).

Additionally, immunocytochemistry (ICC) showed the increased co-localization of DNT cells with tumor cells in the presence of tumor cell-specific antibodies, indicating enhanced DNT-tumor cell interactions (Figure 3F). These findings demonstrate that DNT cells mediate specific and effective ADCC against antibody-coated tumor cells, highlighting their potential as cytotoxic effectors in immunotherapy.

Inhibition of Syk significantly suppresses IgG1-dependent DNT-mediated ADCC

Syk activation by immunoglobulin receptors triggers multiple pathways. Syk is involved in the CD16, Fc ϵ R1, PI3K-AKT, and NF- κ B pathways, according to the PathCards database (Figure 4A). CD16 and Fc ϵ R1 γ are key immune receptors in ADCC, and Syk is essential for the signal transduction during the activation of these receptors. Additionally, Syk plays a crucial role in the PI3K-AKT and NF- κ B pathways, contributing to the regulation of pathways associated with cytotoxicity, such as granule exocytosis and the production of cytotoxic cytokines by NK cells (Kwon et al., 2016). Syk is a kinase known to interact with phosphorylated Fc ϵ R1 γ molecules and mediate downstream signal transduction, a finding that was also observed in the previous study on DNT cells (Thomson et al., 2006). Using co-immunoprecipitation (co-IP) analysis, we examined the phosphorylation state of Fc ϵ R1 γ in DNT cells upon stimulation with anti-KLC and IgG1. Fc ϵ R1 γ phosphorylation peaked at 10 min post-stimulation, followed by dephosphorylation with prolonged stimulation (Figure 4B). Syk was recruited to Fc ϵ R1 γ aggregates, thereby facilitating downstream signaling pathways. Upon adding the Syk inhibitor Entospletinib during anti-KLC and IgG1 stimulation, the expression of CD16 and Fc ϵ R1 γ was suppressed, indicating a crucial role of the Syk pathway (Figure 4C). Notably, both CD107a and IFN γ levels were dramatically reduced after Syk inhibition, suggesting a significant impairment of the cytotoxic capacity of DNT cells (Figure 4D). This indicates that the inhibition of Syk impedes the downstream functional alterations of Fc ϵ R1 γ . Similarly, Fc ϵ R1 γ knockout in DNT cells, confirmed by flow cytometry analysis showing loss of Fc ϵ R1 γ expression and a concomitant reduction of surface CD16 (Fc γ RIII) (Supplementary Figure S2), downregulated CD107a on the DNT cell surface and reduced IFN γ production (Figure 4D). These findings underscore the involvement of Fc ϵ R1 γ and Syk in the IgG1-stimulated activation pathway of DNT cells.

Anti-KLC and IgG1 treatments effectively increased levels of phosphorylated ERK (pERK), phosphorylated AKT (pAKT), and p65 in DNT cells; however, when Syk transduction was inhibited by Entospletinib, these downstream signaling components were significantly obstructed (Figure 4E). These results suggest that IgG1-stimulated activation of the MAPK-ERK, PI3K-AKT, and NF- κ B signaling pathways is associated with enhanced ADCC activity of DNT cells, which can be suppressed by a Syk inhibitor.

Anticancer effects of DNT cells are enhanced in combination with tumor-specific antibody in vivo

To investigate the antitumor effects of DNT cells in combination with anti-OVA antibody on targeting solid tumors *in vivo*, we established an allograft mouse colon cancer model (MC38-OVA) in NSG mice (Figure 5A). When the tumor volumes reached an average of ~45–55 mm³, the mice were randomly injected with anti-OVA or IgG1 antibody 24 h before

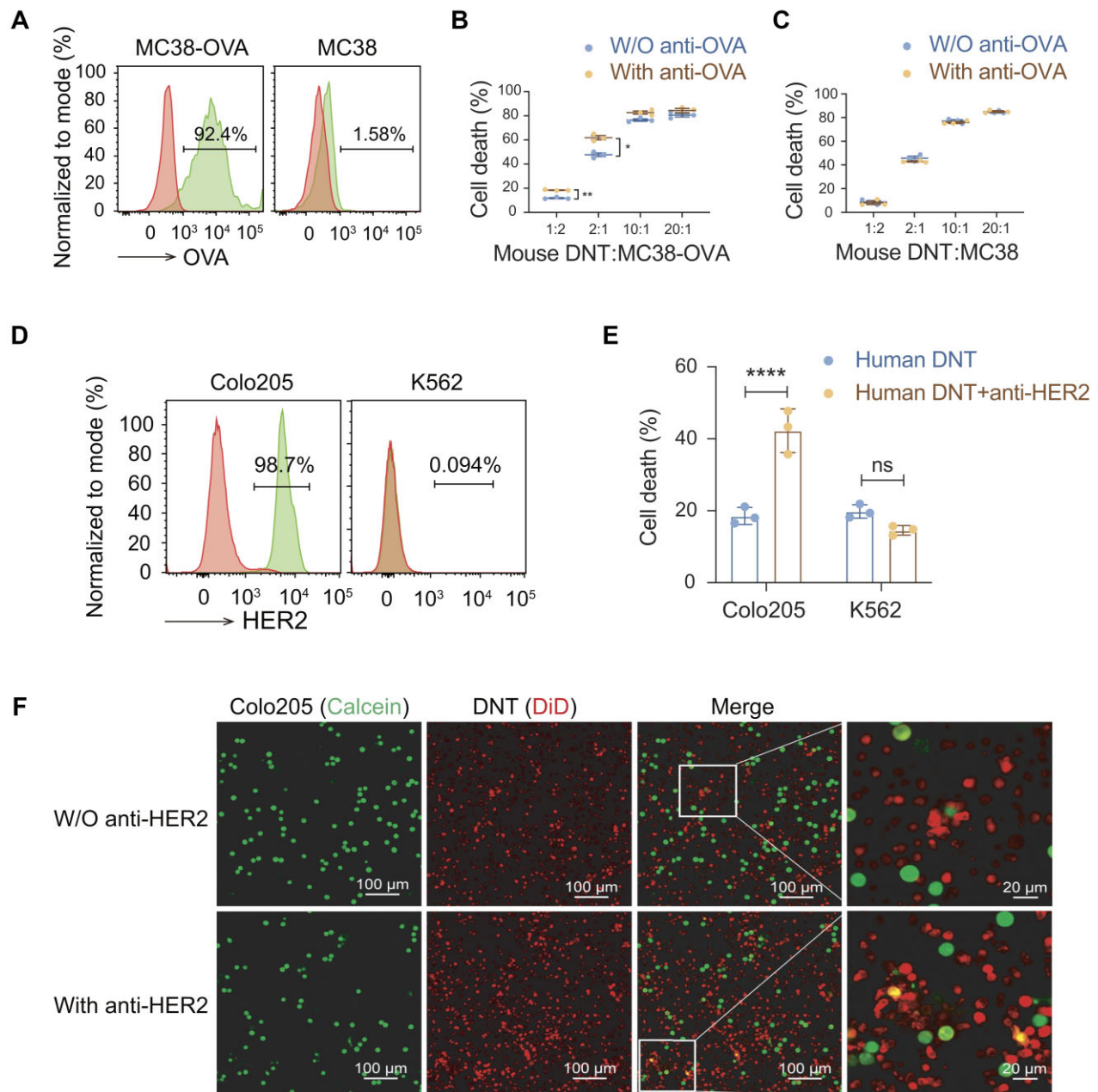


Figure 3 DNT-mediated ADCC targets antibody-coated target cells. (A) OVA (SIINFEKL) expression on target cancer cells (MC38-OVA and MC38) was analyzed by flow cytometry. (B and C) DNT-mediated ADCC response against anti-OVA antibody-coated MC38-OVA (B) or MC38 (C) cells was detected by flow cytometry. (D) HER2 expression levels on target cancer cells (Colo205 and K562) were analyzed by flow cytometry. (E) DNT-mediated ADCC response against anti-HER2-coated Colo205 and K562 cells was detected by flow cytometry. (F) Co-localization of DNT cells and anti-HER2-coated Colo205 was shown by ICC. $n = 3-5$ per group. Student's t -test (B and C) or two-way ANOVA (E) was performed. The data are represented as mean $\pm$ SD. ns, not significant; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

intravenous adoptive transfer of wild-type (WT) or $Fc\epsilon R1\gamma^{-/-}$ DNT cells. All mice received intraperitoneal IL-2 injection twice weekly.

Representative photographs of the excised tumors are shown in [Supplementary Figure S3A](#). On Day 7 post-treatment, mice receiving the combination therapy of DNT cells and anti-OVA antibody exhibited significantly reduced

tumor growth by 50.9% ($P = 0.0153$), 33.8% ($P = 0.0232$), and 36.1% ($P = 0.0368$) compared to those receiving the saline control, the combination of DNT cells and IgG1, and the combination of $Fc\epsilon R1\gamma^{-/-}$ DNT cells and anti-OVA, respectively (Figure 5B).

Mice treated with the WT DNT+anti-OVA combination exhibited significantly higher percentages of DNT cells

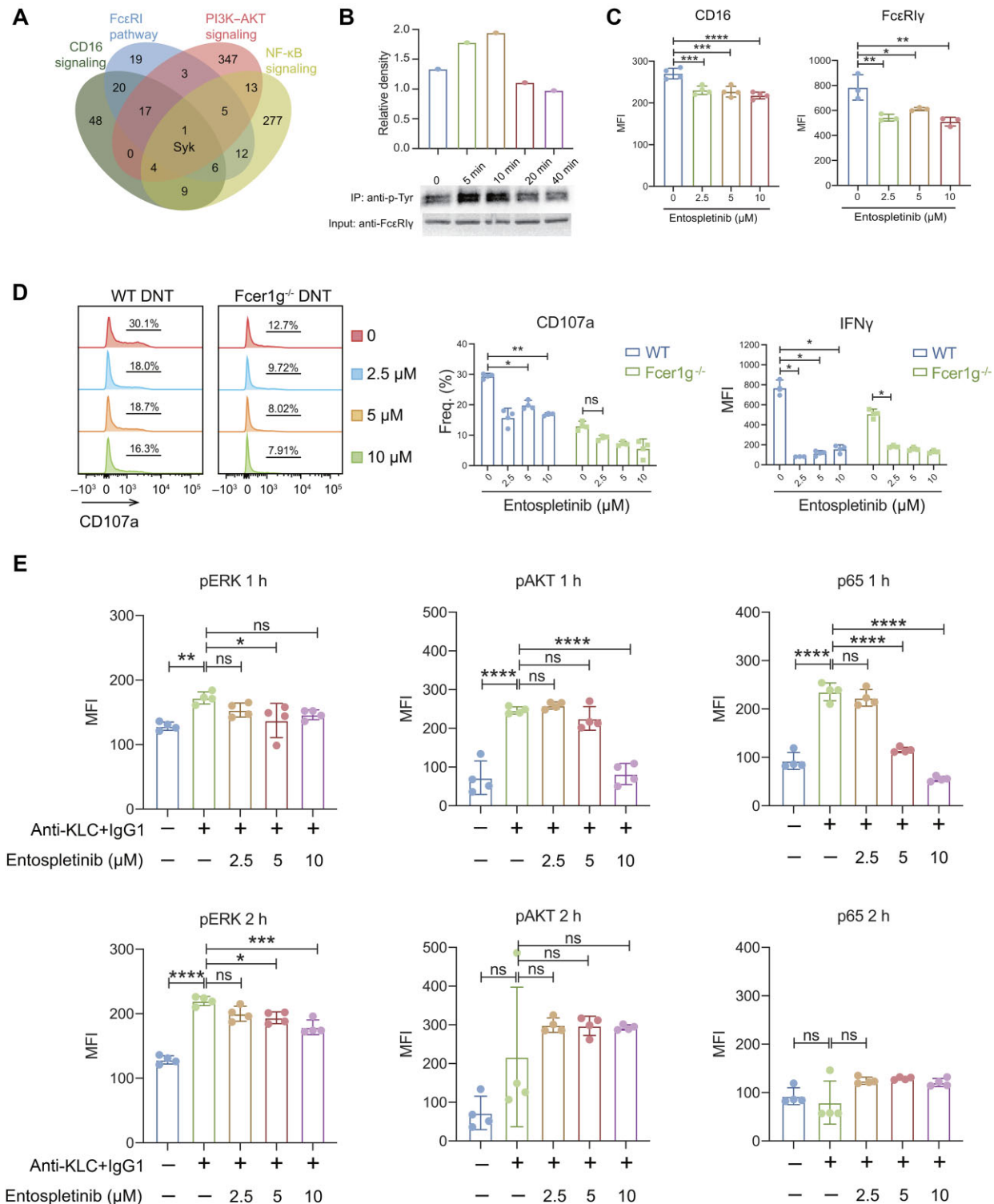


Figure 4 Inhibition of Syk significantly suppresses IgG1-dependent cell-killing ability of DNT cells. **(A)** Venn diagram depicting the overlapping molecules among the CD16, FcεRI, PI3K-AKT, and NF-κB signaling pathways, derived from data in the PathCards database. **(B)** Co-IP analysis showing the phosphorylation state of FcεRIγ in DNT cells upon stimulation with anti-KLC and IgG1. **(C and D)** The levels of CD16 and FcεRIγ **(C)** and CD107a and IFNγ **(D)** in DNT cells stimulated with anti-KLC and IgG1 antibodies and treated with or without the Syk inhibitor Entospletinib were detected by flow cytometry. **(E)** After 1-h or 2-h stimulation as indicated, pERK, pAKT, and p65 levels were detected by flow cytometry. $n = 3-5$ per group. One-way ANOVA **(C and E)** or two-way ANOVA **(D)** was performed. The data are represented as mean $\pm$ SD. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

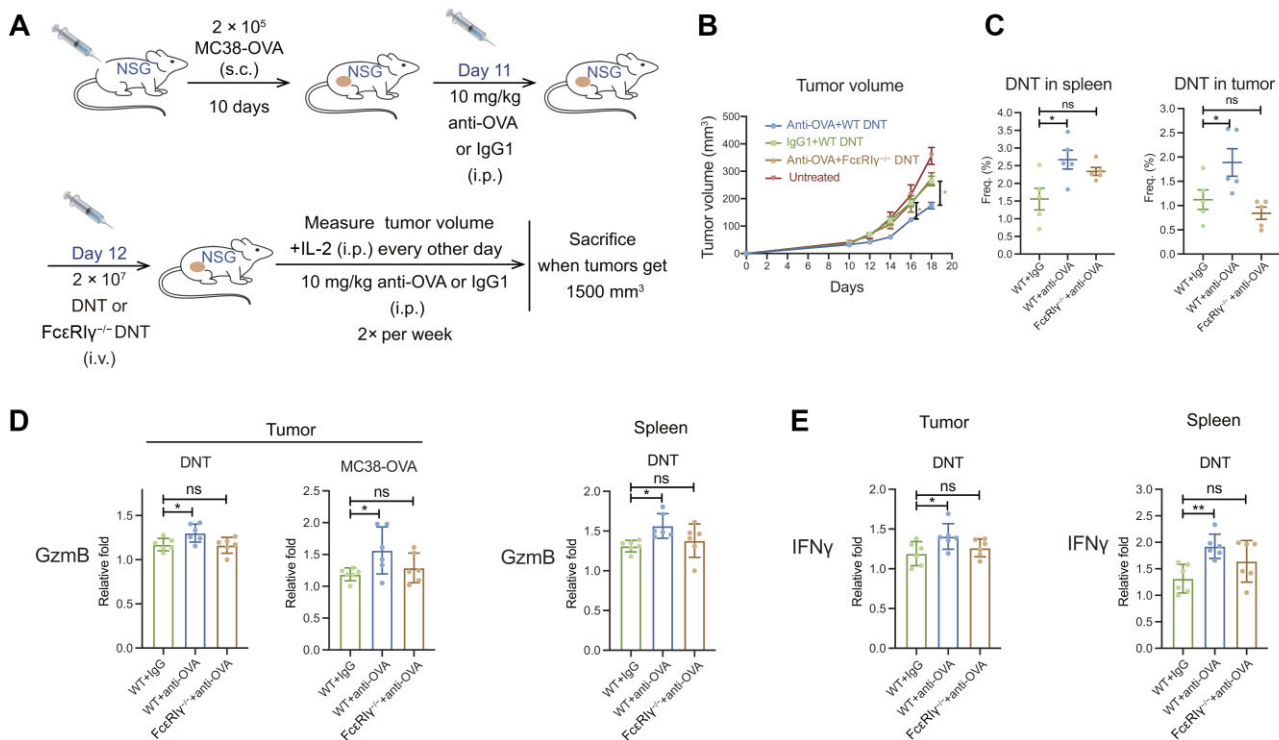


Figure 5 Combination with anti-OVA antibody enhances the antitumor activity of DNT cells in the MC38-OVA tumor model. (A) Schematic of the subcutaneous MC38-OVA allograft model and adoptive transfer of WT or FcεRIγ^{-/-} DNT cells with anti-OVA antibody or IgG1 treatment. (B) Statistical analysis of tumor volumes in mice from the four groups (anti-OVA+WT DNT, IgG1+WT DNT, anti-OVA+FcεRIγ^{-/-} DNT, and untreated). (C) Statistical analysis of the percentages of DNT cells in the spleens and tumors of mice from the three treated groups (IgG1+WT DNT, anti-OVA+WT DNT, and anti-OVA+FcεRIγ^{-/-} DNT). (D and E) Statistical analysis of GzmB levels in infiltrating DNT cells, MC38-OVA tumor cells, and spleen DNT cells (D) and IFNγ expression levels in infiltrating DNT cells and spleen DNT cells (E) from the three treated groups. $n = 6$ per group. One-way ANOVA (B–E) was performed. The data are represented as mean $\pm$ SD. ns, not significant; * $P < 0.05$, ** $P < 0.01$.

in spleens and tumors compared to those treated with WT DNT+IgG1; this effect was not observed in FcεRIγ^{-/-} DNT+anti-OVA-treated mice (Figure 5C). To determine whether the increased infiltration of DNT cells exhibited stronger antitumor functions, GzmB and IFNγ expression levels in spleens and tumors were assessed by flow cytometry. As shown in Figure 5D and E, higher levels of GzmB and IFNγ were detected in the tumors and spleens of mice treated with DNT+anti-OVA compared to those treated with DNT+IgG1 or FcεRIγ^{-/-} DNT+anti-OVA. A significantly higher level of GzmB was also detected in MC38-OVA cells in the tumor tissues of mice treated with WT DNA+anti-OVA (Figure 5D). These results demonstrate that the combination of DNT cells and a tumor-specific antibody enhances the quality and function of DNT cells in targeting tumors.

To further evaluate the antitumor activity of human DNT cells in combination with tumor-specific antibodies *in vivo*, a cell line-derived xenograft model was established using Colo205 cells in NSG mice (Figure 6A). NSG mice were subcutaneously injected with Colo205 cells (2×10^5 cells). After 8 days, when tumor volumes reached ~ 40 – 45 mm³, the mice were randomly assigned to five groups: untreated,

anti-HER2 (Pertuzumab) monotherapy, human DNT+IgG, human DNT+anti-HER2, and human DNT (Fc-blocked)+anti-HER2. For FcR blockade, DNT cells were pre-incubated with Human TruStain FcX™ reagent before peritumoral injection. All mice received intraperitoneal injections of human recombinant IL-2 twice weekly for 10 days.

Representative photographs of excised tumors are shown in Figure 6B, and the corresponding tumor growth curves are graphed in Figure 6C. Mice treated with human DNT cells combined with Pertuzumab exhibited markedly slower tumor growth compared with those treated with DNT+IgG or Pertuzumab monotherapy. Blocking FcRs markedly diminished the antitumor effect of the DNT+anti-HER2 treatment, indicating the dependence on FcγR-mediated ADCC.

Flow cytometry analysis of tumor-infiltrating lymphocytes showed the significantly higher proportion of human DNT cells in the DNT+anti-HER2 group than in the DNT+IgG group, whereas FcR blockade reduced the intratumoral DNT cell frequency (Figure 6D). Moreover, tumor-infiltrating DNT cells from the DNT+anti-HER2 group expressed higher levels of GzmB and perforin compared to those from the DNT+IgG group, and this upregulation was attenuated by FcR blockade

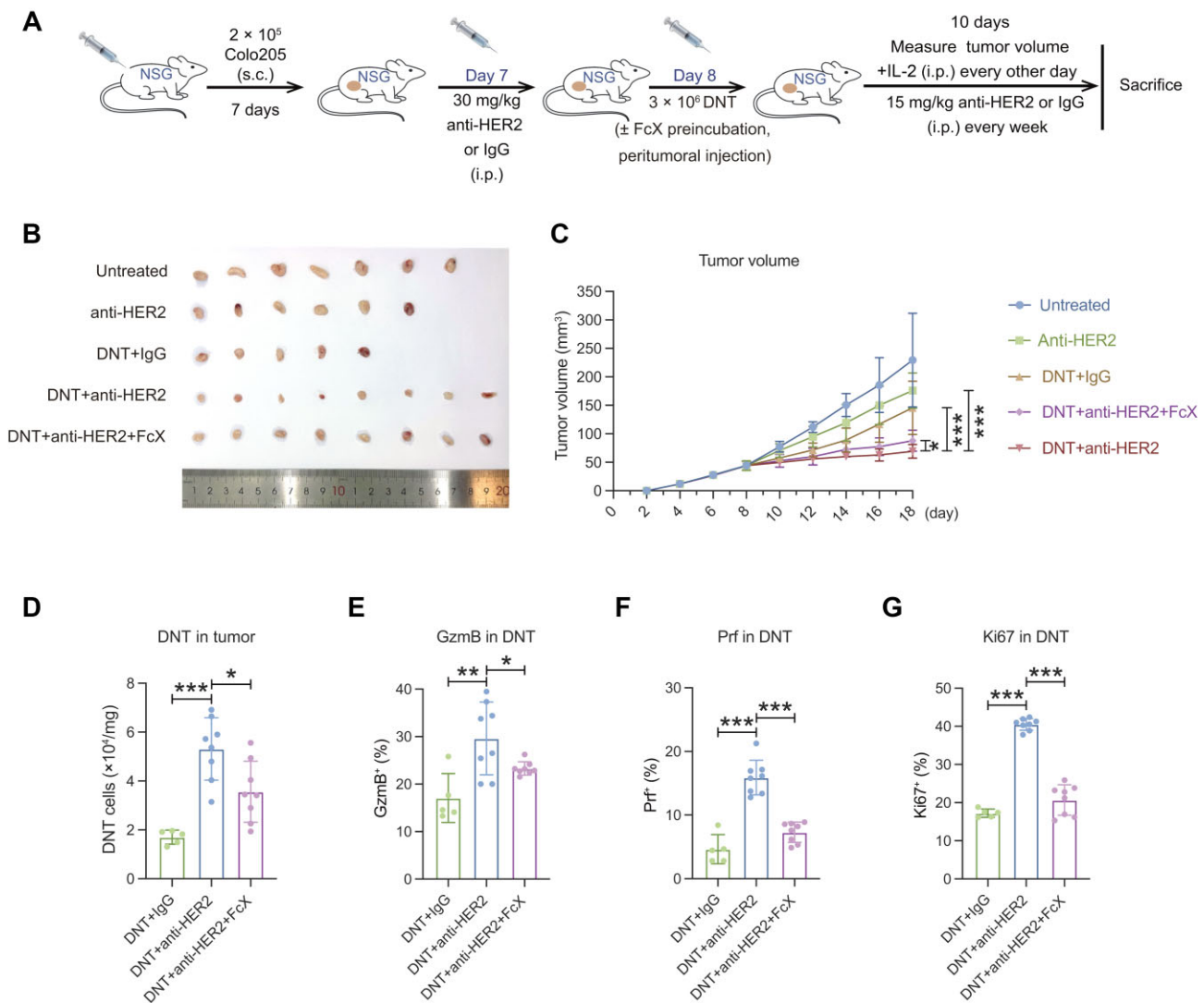


Figure 6 Pertuzumab enhances the antitumor activity of human DNT cells in the Colo205 xenograft model. **(A)** Schematic diagram of the Colo205 xenograft model and adoptive transfer of human DNT cells in combination with anti-HER2 antibody, human IgG, or FcR blockade. **(B)** Images of excised tumors from mice in the five groups: untreated, anti-HER2, DNT+IgG, DNT+anti-HER2, and DNT+anti-HER2+FcX. **(C)** Statistical analysis of tumor volumes in mice from the five groups. **(D)** Quantification of tumor-infiltrating human DNT cells in the three DNT-treated groups (DNT+IgG, DNT+anti-HER2, and DNT+anti-HER2+FcX). **(E–G)** Flow cytometry analysis of intratumoral DNT cells showing expression levels of GzmB **(E)**, perforin **(F)**, and Ki67 **(G)** in the three DNT-treated groups. $n = 5–8$ per group. One-way ANOVA **(C–G)** was performed. The data are represented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Figure 6E and F). In addition, the Ki67 expression was significantly higher in intratumoral DNT cells from the DNT+anti-HER2 group, which was reduced by FcR blockade (Figure 6G). Together, these results indicate that the enhanced antitumor activity of DNT cells in combination with anti-HER2 antibody is mediated through Fc γ R dependent ADCC.

To evaluate potential systemic effects, serum cytokine levels were measured at the experimental endpoint (Supplementary Figure S4A and B). Levels of murine cytokines (IL-6, IL-1 β , and IFN γ) were similar across the untreated, DNT+IgG, and DNT+anti-HER2 groups and remained substantially lower than those associated with

cytokine release syndrome, indicating the absence of a systemic inflammatory response. Levels of human cytokines related to DNT cell functions (IFN γ , TNF α , GzmB, and perforin) were below the detection limit, suggesting minimal systemic dissemination of effector molecules derived from DNT cells. Furthermore, histopathological examination of major organs (liver and lung) from representative groups revealed no evidence of tissue damage or inflammatory infiltration, and the tissue architecture was well preserved (Supplementary Figure S4C). These findings confirm that the combined administration of DNT cells and anti-HER2 antibody does not induce detectable systemic toxicity.

Discussion

DNT cells play a crucial role in antitumor immunity (Young et al., 2001, 2003). Upon recognizing tumor antigens via TCR, they mediate rapid tumor cell lysis through the perforin/GzmB pathway (Fang et al., 2023). Certain intrinsic signaling pathways in DNT cells are crucial for regulating their survival and effector functions. For example, OX40 promotes DNT cell maintenance and effector function (Liu et al., 2018). Lag-3 contributes to MHC-II antigen binding, thereby modulating the antigen recognition of DNT cells (Tian et al., 2019). The expression of NKG2D on DNT cells enhances the release of GzmB, thereby boosting their immunoregulatory functions (Hu et al., 2021). Our findings reveal high Fc ϵ RI γ expression on DNT cells, indicating its novel role in mediating ADCC during antitumor responses.

ADCC is a key mechanism of antibody-mediated tumor clearance, involving various FcR-expressing cells such as NK cells, macrophages, and neutrophils, which kill opsonized tumor cells through ADCC or ADCP (Clemenceau et al., 2015; Behrens et al., 2023). Our study demonstrated that IgG1 stimulation enhances the cytotoxic and antitumor functions of DNT cells *in vivo* and *in vitro*. Although antibody stimulation alone can upregulate the expression of cytotoxic molecules in DNT cells and partially enhance their cytotoxic activity, our results indicate that ADCC also contributes significantly to the target cell killing. DNT cells combined with anti-HER2 antibodies exhibited stronger killing of HER2⁺ Colo205 cells compared to HER2⁻ K562 cells (Figure 3E), confirming that the enhanced cytotoxicity is driven by both antigen-specific antibody-mediated ADCC and antibody-induced molecular upregulation in DNT cells. Therefore, both mechanisms collaboratively enhance DNT cell cytotoxicity. The high expression of Fc ϵ RI γ serves as the molecular basis for DNT cells acting as ADCC effector cells. Furthermore, ADCC has been shown to play an essential role in and provide therapeutic benefits for autoimmune diseases (Michel et al., 2024) and is also involved in the elimination of virus-infected cells (Bruel et al., 2016). Thus, the ADCC effect mediated by DNT cells might offer a promising strategy for treating autoimmune and infectious diseases.

Fc ϵ RI γ , an ITAM-containing adaptor, recruits Syk upon IgG binding and phosphorylation. Activated Syk initiates downstream cytotoxic signaling. Compared to other T cells, DNT cells show enriched Fc ϵ RI γ expression, which is further upregulated by IgG1. This activation induces Fc ϵ RI γ phosphorylation and Syk recruitment. In our study, Syk inhibition or *Fcer1g* knockout reduced CD107a and IFN γ expression, impairing DNT cell cytotoxicity. IgG1 also activated the ERK, AKT, and p65 pathways, which was suppressed by Syk inhibition, highlighting the importance of Fc ϵ RI γ /Syk in ADCC by DNT cells.

Combining DNT cells with other immunotherapy approaches is a promising strategy. Fang et al. (2019) found that combining DNT cells with Nivolumab (an anti-PD-1 antibody) significantly inhibited the growth of advanced

lung cancer xenografts. Chen et al. (2018) showed that chemotherapy-resistant AML cells become more sensitive to DNT-mediated cytotoxicity after pretreatment with daunorubicin. Yao et al. (2019) found that stimulating DNT cells with the immunomodulator IL-15 increased the expression of effector molecules, such as TRAIL, and cytotoxicity against NSCLC both *in vitro* and *in vivo*. Our study adds that DNT cells cooperate with tumor-specific antibodies to induce ADCC, further boosting their antitumor potential and providing new therapeutic insights.

To further validate that this antitumor effect depends on FcR engagement, we utilized Human TruStain FcX reagent in our xenograft model. This reagent is formulated to block the ligand-binding sites of CD16 and CD32. We observed that blocking these receptors significantly attenuated the therapeutic efficacy of the combination treatment. Consistent with our finding that DNT cells highly express Fcgr3 (CD16) (Figure 1F) and given that Pertuzumab is a human IgG1 antibody known to trigger ADCC through the engagement with Fc γ Rs (primarily CD16) (Scheuer et al., 2009), these results collectively suggest that the enhanced tumor inhibition is mediated through the engagement of Fc γ Rs, particularly CD16, on DNT cells. Although the use of *Fcer1g*^{-/-} recipient mice would provide a more definitive model to exclude host FcR contributions, our data from multiple approaches strongly support the intrinsic ADCC capacity of DNT cells. First, *in vitro* co-culture assays demonstrated their ability to directly lyse antibody-coated tumor cells in the complete absence of accessory cells (Figure 3A–E). More importantly, in our *in vivo* model, the enhanced antitumor response was primarily DNT cell-dependent: WT DNT cells combined with anti-OVA exhibited markedly stronger tumor inhibition compared to Fc ϵ RI γ ^{-/-} DNT cells under identical NSG conditions. This indicates that Fc ϵ RI γ signaling within DNT cells drives the enhanced antitumor response, despite the potential presence of residual host myeloid cells.

Moreover, Fc ϵ RI γ /Syk/AKT/NF- κ B signaling supports DNT cell survival and proliferation *in vivo*, consistent with the higher Ki67 levels in WT DNT cells than in Fc ϵ RI γ ^{-/-} DNT cells (Supplementary Figure S3B) and in the human DNT+anti-HER2 model (Figure 6G). These findings explain the increased accumulation of WT DNT cells *in vivo* and are not contradictory to the short-term activation-induced cell death observed under acute stimulation *in vitro*.

In summary, DNT cells exert enhanced antitumor activity when combined with monoclonal antibodies via Fc ϵ RI γ /Syk signaling, which drives ADCC and promotes CD107a and GzmB expression. These results reveal the cytotoxic mechanisms of DNT cells and highlight their potential in antibody-based cancer immunotherapy.

Materials and methods

Mice

Six- to eight-week-old male WT C57BL/6J, NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{em15moc}), and *Fcer1g*^{-/-} C57BL/6J mice were

purchased from Shanghai Model Organisms. The animals were housed and bred under specific pathogen-free conditions, maintained at 20°C with 60% relative humidity and under 12-h light/dark cycles at Beijing Friendship Hospital. Mice were randomized divided into subgroups (5–10 mice/group). All animal experiments were reviewed and approved by the Animal Ethics Committee of Beijing Friendship Hospital, Capital Medical University (approval number: 24-2026) and performed in accordance with the ARRIVE guidelines and institutional animal welfare regulations.

Conversion of DNT cells in vitro and adoptive transfer

The conversion of DNT cells *in vitro* was performed as previously described (Zhang et al., 2011). Briefly, mature dendritic cells (mDCs) were harvested from lipopolysaccharide-stimulated bone marrow cells derived from C57BL/6J mice and separated according to CD86-positive selection. CD4⁺ cells of WT or *Fcer1g*^{-/-} C57BL/6J mice were incubated with mDCs, 50 ng/ml recombinant mouse IL-2 (PeproTech), and 1 µg/ml 2-mercaptoethanol (Sigma-Aldrich) for 7 days. CD3⁺CD4⁻CD8⁻NK1.1⁻ DNT cells were sorted using a FACSaria II sorter (BD Biosciences). The information of antibodies used in this study is listed in [Supplementary Table S1](#).

Human samples

Human peripheral blood samples were collected from healthy adult volunteers, with written informed consent obtained from all participants. The research followed a protocol approved by the Ethics Committee of Beijing Friendship Hospital (approval number: 2020-P2-174/DR20200174). Peripheral blood lymphocytes were isolated using density gradient centrifugation with Human Lymphocyte Separation Medium (Biosci, 7111012).

Ex vivo expansion of human DNT cells

Human DNT cells were isolated from peripheral blood mononuclear cells using the Double-negative T Cell Isolation Kit (Miltenyi Biotec). The isolated human DNT cells were cultured in complete X-VIVO™ 15 medium (Lonza), supplemented with recombinant human IL-2 (50 ng/ml), on cell culture plates pre-coated with anti-human CD3 antibody (3 µg/ml) and soluble anti-human CD28 antibody (1 µg/ml) (BioLegend). For experiments, human DNT cells from Days 12–20 of culture were used. Before conducting the experiments, additional purification was performed using a FACSaria II sorter (BD Biosciences).

Sequencing analyses of Fcer1g expression

The scRNA-seq data from GEO (GSE129030) were analyzed by GSEA to identify pathways involving *Fcer1g* upregulation in DNT cells compared to CD4⁺ T cells. The dataset was preprocessed to retain high-quality cells and genes, followed by normalization, scaling, principal component analysis, uniform manifold approximation and projection, and clustering to identify CD4⁺ T cells and DNT cells. Differential expression

analysis ranked genes between these cell types by log-fold change. GSEA was performed using predefined gene sets, with enrichment scores computed by a weighted Kolmogorov-Smirnov-like statistic. The significance of enrichment score was assessed via permutation testing, and nominal *P*-values were adjusted using the Benjamini-Hochberg method. Gene sets with false discovery rate-adjusted *P*-values <0.05 were considered significantly enriched, indicating pathways where *Fcer1g* is upregulated in DNT cells.

RNA-seq compared CD4⁺ T cells and DNT cells for relative *Fcer1g* expression using FPKM (fragments per kilobase of transcript per million mapped reads) normalization. High-quality cells and genes were selected, normalized, and scaled. Expression levels were visualized with bar charts, and statistical significance was determined through differential expression analysis (*P* < 0.05).

In vitro Syk inhibitor treatment and IgG1 antibody stimulation

For signaling analysis, cells were plated at a density of ~2 × 10⁵ cells/well in a 96-well tissue culture-treated plate pre-coated with anti-CD3 (2 µg/ml). Cells were treated with anti-OVA (as an IgG1 isotype antibody, 3 µg/ml; SIINFEKL) pre-incubated with anti-KLC antibody (to enhance the stimulating effect of IgG antibodies) and/or Entospletinib (0, 2.5, 5, 10 µM; MedChemExpress) according to the manufacturer's protocol for 12 h. The release of GzmB into the supernatant was detected using a mouse GzmB ELISA Kit (Elabscience), as per the manufacturer's instructions. Before the detection of CD107a, monensin (Merck) was added to the culture medium at a concentration of 10 µM for 4 h to inhibit vesicular transport within the endoplasmic reticulum and Golgi apparatus, enhancing surface expression of CD107a. Dimethyl sulfoxide (Sigma-Aldrich) was used as a vehicle control.

Flow cytometry analysis

DNT cells were harvested and subjected to flow cytometry analysis to assess the expression levels of a range of cell surface and intracellular markers, including GzmB, perforin, IFNγ, TNFα, pERK, pAKT, and p65. Anti-CD11b (M1/70), anti-CD45 (30-F11), anti-TCRβ (H57-597), anti-NK1.1 (PK136), anti-TNFα (MP6-XT22), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD3 (17A2), anti-CD86 (PO3), anti-CD3 (17A2), anti-CD107a (1D4B), anti-Granzyme B (QA16A02), anti-Perforin (S16009A), and anti-IFNγ (XMG1.2) were purchased from BioLegend. The antibody information is listed in [Supplementary Table S1](#). Flow cytometry analysis was conducted using a FACSaria II system (BD Biosciences), and the resulting data were processed and analyzed with FlowJo software (Tree Star).

Flow cytometry-based ADCC assay

Flow cytometry was performed on a 48-well plate per treatment. Target cells (K562, Colo205, MC38, or MC38-OVA) were harvested and labeled with 1 µM Calcein-AM (Invitrogen) for

40 min at 4°C, followed by washing to remove excess dye with phosphate-buffered saline (PBS). The labeled target cells were then plated at a concentration of 1×10^4 cells per well and incubated with 60 ng/ml anti-HER2, anti-OVA (SIINFEKL), or IgG1 isotype and 5×10^4 DNT cells (effector cells) at an E:T ratio of 5:1 for 12 h. After the incubation, samples were analyzed by flow cytometry. The percentage of specific target cell death was calculated using the following formula:

Cell death MC38-OVA (%)

$$= (100 - \text{number of Calcein}^+ \text{ live cells in MC38-OVA} / \text{number of Calcein}^+ \text{ cells in MC38}) \times 100.$$

Three control conditions were used for each experiment: (i) unstained control, (ii) Calcein-AM only control, in addition to multiple experimental test conditions as described above, and (iii) Calcein-AM-stained target cells with/without antibody and with/without DNT cells.

For ICC, Colo205 cells were first labeled with Calcein-AM to assess cell viability, followed by incubation with anti-HER2 antibody to target the specific antigen. Concurrently, DNT cells were labeled with the fluorescent dye DiD (Invitrogen) to enable their detection. The labeled Colo205 cells and DiD-labeled DNT cells were then co-cultured to allow interaction and potential cytotoxic activity. The killing effect of DNT cells on Colo205 cells was observed and quantified using ICC methods, enabling the visualization of DNT-mediated cytotoxicity.

In vivo treatment

Male NSG mice (6–8 weeks old) were subcutaneously inoculated with 2×10^5 MC38-OVA or Colo205 cells into the right flank.

For the MC38-OVA tumor model, on Day 11 after tumor inoculation, when tumors reached $\sim 50 \text{ mm}^3$, mice were randomized into four groups: anti-OVA+WT DNT, IgG1+WT DNT, anti-OVA+Fc ϵ RI $\gamma^{-/-}$ DNT, and untreated. The mice received intraperitoneal injections of anti-mouse OVA antibody, mouse IgG1 antibody, or PBS. On the following day, the mice were intravenously injected with 2×10^7 mouse DNT cells or PBS. Recombinant mouse IL-2 (10^4 IU per mouse) was administered intraperitoneally at the time of DNT infusion and twice weekly thereafter for a week. The mice were sacrificed 7 days after the treatment, and tumor and spleen tissues were collected to assess DNT cell infiltration using Zombie viability dye and specific antibodies.

For the Colo205 xenograft model, on Day 8 after tumor inoculation, when tumors reached 40–45 mm^3 , mice were randomized into five groups: untreated, anti-HER2 (Pertuzumab) monotherapy, human DNT+human IgG, human DNT+anti-HER2, and FcR-blocked human DNT+anti-HER2. The mice received intraperitoneal injections of anti-human HER2, human IgG, or PBS. For FcR blockade, human DNT

cells were pre-incubated with Human TruStain FcXTM reagent before peritumoral injection. On the following day, mice were peritumorally injected with 3×10^6 human DNT cells (either unblocked or Fc-blocked) or PBS. Recombinant human IL-2 (10^4 IU per mouse) was administered intraperitoneally at the time of DNT infusion and twice weekly thereafter for 10 days. The mice were sacrificed 10 days after the treatment, and tumor, liver, lung, and serum samples were collected. Tumor-infiltrating cells were analyzed for DNT cell frequency, cytotoxic molecules (GzmB and perforin), and the proliferation marker Ki67, while serum cytokine levels were determined by ELISA.

For both tumor models, tumor volumes were measured three times per week using digital calipers and calculated using the formula: $1/2 \times (\text{length} \times \text{width}^2)$. Long-term survival was monitored throughout the experiment. The maximum allowable tumor volume was 1000 mm^3 , and no mice reached this threshold at the time of sacrifice. No body-weight loss, adverse outcomes, or severe signs of illness were observed in any mice. All experiments were performed by independent research teams responsible for animal handling and data analysis.

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8 (GraphPad Software). Data were expressed as the mean and standard deviation (SD). Two-tailed unpaired or paired Student's *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA with Bonferroni multiple comparison posttest was performed (ns, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

Supplementary material

[Supplementary material](#) is available at *Journal of Molecular Cell Biology* online.

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Conflict of interest: D.Z. is an inventor on a patent for the *ex vivo* generation of DNT cells (China). Other authors do not have a conflict of interest.

Author contribution: S.S. and X.H. participated in performing the research, analyzing the data, and initiating the original draft of the article. T.Z., L.Y., M.L., Z.Z., X.D., and H.J. participated

in performing the research. Y.Y., Z.Z., D.Z., D.T., and G.S established the hypotheses, supervised the studies, analyzed the data, and co-wrote the manuscript. S.W. reviewed and edited the manuscript. All listed authors participated meaningfully in the study, who have read and approved the submission of this manuscript.

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