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Diacylglycerol Kinase ζ is a target to enhance natural killer cell function¹

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Abstract

Enhancement of natural killer (NK) cell function could be beneficial in treatment of a variety of tumors and infections. However, efforts to improve NK cell function by disrupting negative regulators that target proximal signaling pathways paradoxically results in hyporesponsive rather than hyperresponsive NK cells. In this study, we demonstrate that genetic deletion of diacylglycerol (DAG) kinase zeta (DGK ζ), a negative regulator of DAG-mediated signaling, has the desired effect of enhancing NK cell function due to its distal position in the activating receptor-mediated signaling cascade. Upon stimulation through multiple activating receptors, NK cells from mice lacking DGK ζ display increased cytokine production and degranulation in an ERK-dependent manner. In addition, they have improved cytotoxic functions against tumor cell lines. The enhancement of NK cell function by DGK ζ deficiency is NK cell-intrinsic and developmentally independent. Importantly, DGK ζ deficiency does not affect inhibitory NK cell receptor expression or function. Thus, DGK ζ KO mice display improved missing self recognition, as evidenced by enhanced rejection of a TAP-deficient tumor in vivo. We propose that enzymes that negatively regulate distal activating receptor signaling pathways such as DGK ζ represent novel targets for augmenting the therapeutic potential of NK cells.

Keywords

NK cells; natural cytotoxicity; signal transduction

Introduction

Natural killer (NK) cells are important for protection against certain viruses and tumors. They carry out their effector function through expression of a diverse set of germline-encoded activating and inhibitory receptors (1–3). Through these receptors, NK cells are able to sense their environment, allowing them to detect target cells that have upregulated activating ligands or reduced their expression of the inhibitory ligand MHC class I – events that can occur during viral infection or neoplastic transformation (1, 3).

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The ability of NK cells to target virally-infected and neoplastic cells has led to major interest in using them for therapy. Early trials have shown safety and preliminary efficacy in NK cell-based adoptive immunotherapy to treat hematological malignancies. More recently, researchers have focused on methods to improve NK function in vivo (4). These approaches can broadly be divided into three categories: 1) expansion and activation of autologous NK cells to improve their responsiveness (2, 5); 2) matching ligand expression on patient cells to killer immunoglobulin-like receptor (KIR) expression on donor NK cells (1–3, 6); and 3) genetic or molecular augmentation of NK cell signaling machinery in donor cells (1, 3, 7).

Manipulation of NK cell activating receptor-mediated signaling is complicated by the variety of signaling modules utilized by NK cell activating receptors. For example, NKG2D signals through DAP10 (co-stimulatory like), 2B4 signaling requires SAP, while NCRP1-C (NK1.1) functions through FcεRγ (ITAMs) (4, 8). Interestingly, all of these activating receptors converge upon SLP-76 and PLCγ and hence, NK cells that lack either of these signaling molecules are hyporesponsive to all three families of activating receptor stimulation (2, 5, 9, 10).

Unlike the activating receptors that function through association with adaptor molecules with activating domains, the inhibitory NK cell receptors contain ITIMs in their cytoplasmic tails. Upon interaction with their ligands, inhibitory NK cell receptors use their ITIMs to recruit phosphatases such as Src homology region 2 domain-containing phosphatase 1 (SHP-1) and SHIP, which attenuate proximal signaling pathways downstream of activating receptors. Thus, one might imagine that the inhibition of these phosphatases would enhance NK cell function and could be useful therapeutically. Paradoxically, however, SHP-1 and SHIP-deficient NK cells are hyporesponsive to activating receptor stimulation (11, 12). One potential explanation for these observations is that NK cells can continuously tune their responsiveness according to their signaling capacity (13). In fact, NK cells that are continually exposed to activating ligands or MHC class I deficient tumors become hyporesponsive to proximal signaling events (14, 15). Importantly, it has been shown that the hyporesponsive NK cell phenotype can be bypassed through PMA and ionomycin stimulation (9, 10, 12).

Given that PMA and ionomycin mimic PLCγ activation by acting as a diacylglycerol (DAG) analog and inducing Ca²⁺ flux, respectively, these data suggest that NK signal tuning occurs proximal to PLCγ activation. Thus, we hypothesized that targeting an enzyme that negatively regulates activating receptor-mediated signaling distal to PLCγ would not allow NK cells to tune to their increased signaling capacity and hence, make NK cells hyperresponsive to activating receptor stimulation. Indeed, we provide data that genetic ablation of DGKζ, a negative regulator of DAG-mediated signaling, leads to hyperresponsive NK cells in a cell-intrinsic and developmentally independent manner. Thus, enzymes that negatively regulate distal activating receptor signaling pathways such as DGKζ may represent novel targets for augmenting the therapeutic potential of NK cells.

Materials and methods

Mice

DGK ζ KO, DGK $\zeta^{F/F}$ and DGK α KO mice were described previously (16–18). C57BL/6 (B6), B6.SJL were purchased from The Jackson Laboratory or Charles River Laboratories. CD45.1/45.2 heterozygous mice were created by breeding B6 mice to B6.SJL mice. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Flow cytometry, cell sorting, and data analysis

Antibodies for flow cytometry and functional studies were purchased from BD Pharmingen (San Diego, CA): CD3e PerCP-Cy5.5 (145-2C11), NK1.1 APC (PK136), CD45.1 AF700 (A20), CD45.2 PE-Cy7 (104), Ly49G2 APC (4D11), Ly49A PE (A1), CD244.2 Biotin (2B4), Ly49D purified or FITC (4E5), CD4 FITC (RM4-5), CD8 FITC (53-6.7), and CD27 PE (LG.3A10); Biolegend (San Diego, CA): CD49b biotin (DX5), IFN γ BV421 (XMG1.2), CD107a PE (1D4B), Ly49A Pacific Blue (YE1/48.10.6), and Streptavidin BV421; eBioscience (San Diego, CA): CD3e FITC (17A2), CD3e APC-eFluor780 (145-2C11), NKp46 PerCP-eFluor710 (29A1.4), CD49b FITC (DX5), IFN γ PE-Cy7 (XMG1.2), Ly49G2 PerCP-eFluor710 (4D11), Ly49I FITC or PE (YLI-90), NKG2D APC (CX5), Ly49H FITC, PE, APC or purified (3D10), Ly49D APC (4E5), and CD11b PE-Cy7 (M1/70); BioXcell (West Lebanon, NH): NKG2D purified (HMG2D) and NK1.1 purified (PK136); Jackson ImmunoResearch (West Grove, PA): anti-mouse-IgG3 APC, UCSF Cell Culture Facility Ly49C purified (4LO-3311) or Molecular Probes, Invitrogen (Carlsbad, CA): Live/Dead Aqua or NearIR. Flow cytometry and FACS were performed with an LSR II, FACS Canto, or a FACS Aria cell sorter (BD Biosciences). Data were analyzed with Flowjo software (Tree Star) and Prism (Graphpad), and all scatter plots have mean and SEM depicted. All flow data are pregated on live lymphocyte singlets, NK cells are CD3 $^{-}$ DX5 $^{+}$ NKp46 $^{+}$ and LAK cells are CD4 $^{-}$ CD8 $^{-}$ NK1.1 $^{+}$ DX5 $^{+}$, unless otherwise stated (Fig 1A, 1B).

NK cell functional assays

Splenocytes from B6 or DGK ζ KO mice were stained with anti-DX5-biotin antibody, enriched using anti-biotin MACs beads (Miltenyi Biotec), and expanded in hIL-2 (1000 U/ml) in tissue culture media for at least 5 days to create lymphokine activated killer (LAK) cells. After 5 days of expansion, ~80–90% of the LAK cells in culture were NK cells (CD4 $^{-}$ CD8 $^{-}$ NK1.1 $^{+}$ DX5 $^{+}$). LAK cells were used for assays after resting in fresh cytokine-free media for at least 3 hours to reduce background activation of the expanded NK cells.

For NK cell activation assays, freshly isolated splenocytes or LAK cells were cultured together with anti-CD107a antibody and Monensin for 6 hours in tissue culture plates that were pre-coated with antibodies against NK cell activating receptors (20 μ g/ml, overnight at 4°C unless otherwise specified). In experiments that examined inhibitory receptor function, anti-Ly49G2 antibody was also added (20 μ g/ml, overnight at 4°C) to the wells together with anti-NK1.1 (20 μ g/ml). For experiments that used freshly isolated splenocytes, 1000U of hIL-2 was added (except for Fig. 1G) to make the responsiveness of NK cell more robust and consistent. The addition of exogenous hIL-2 alone did not stimulate IFN γ or CD107a

upregulation in NK cells of WT or DGK ζ origin (Supplementary Fig. 1). Unstimulated controls shown in the figures did not contain exogenous hIL-2. After 6h in culture, cells were analyzed for anti-CD107a antibody staining and intracellular IFN γ by flow cytometry. In experiments involving the MEK inhibitor U0126, splenocytes were pre-incubated with the specified concentration of the inhibitor for 30 min before stimulation, and the inhibitor was maintained throughout the assay.

A luciferase expressing YAC-1 cell line was co-cultured with LAK cells at varying E:T ratios for 4 hours for bioluminescent cytotoxic assays as previously described (19). Luciferase activity was detected via an IVIS Lumina II imaging system and % specific lysis was calculated by the following formula: (minimum - test well) / (minimum - maximum) \times 100%. For the cytotoxicity assay with the RMA/RMA-S cell lines, LAK cells were co-cultured with the cell line at varying E:T ratios for 6 hours, and the % of dead RMA or RMA-S cells was determined by live/dead staining and flow cytometric analysis. % specific lysis was measured by the following formula: (% dead cells in test well - % baseline death of cell line) / (100 - % baseline death of cell line). To measure cytokine production after co-culture with YAC-1 cells, LAK cells were co-cultured at a 1:1 ratio with either no targets or YAC-1 target cells for 24h in LAK media. The IFN γ content in cell-free supernatants was determined by ELISA (Biolegend).

Poly I:C injections in vivo

Poly I:C activation of NK cells *in vivo* was performed as previously described (20). In brief, WT B6 or DGK ζ KO mice were injected with 250 μ g of poly I:C intraperitoneally. 18h after injection, splenocytes were harvested from the mice for functional analysis.

Mixed BM chimeras

BM (5×10^6 cells) from control B6 or DGK ζ KO mice were mixed with CD45.1/45.2 heterozygous competitor BM (5×10^6 cells) and injected i.v. into lethally irradiated B6.SJL congenic host mice (9.5 Gy). Splenocytes were taken from the BM chimeras between 9–12 wk later for functional analysis.

Acute deletion of DGK ζ floxed alleles using ERCreT2

DGK ζ ^{F/F} Rosa26-Stop-Flox-YFP ERCreT2 or control Rosa26-Stop-Flox-YFP ERCreT2 mice were treated with Tamoxifen for 5 days as previously described (21). 1 week after the end of treatment, splenocytes were removed for functional analysis.

Western blot analysis

MACS-enriched splenic DX5⁺ NK cells (pERK, total ERK) or LAK cells were rested for 2–4 hours, and then stimulated with PK136 Ab (30 μ g/ml) for the indicated times. The cells were then lysed in 1% Ipegal in Tris-buffered saline with protease/phosphatase inhibitors (protease inhibitor cocktail solution [Roche, Sigma]), and the proteins were resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). The levels of phosphorylated ERK1/2 (Thr202/Tyr204), total ERK, phosphorylated AKT (Ser473), and total I κ B α were analyzed by Western blotting. Total PLC γ 2 or beta-actin was used as a loading control. All blots were

quantified using Fiji (ImageJ). All antibodies were from Cell Signaling (Danvers, MA), except for anti-beta-actin-HRP antibody (Sigma)

In vivo tumor challenges

In experiments involving long-term tumor burden, RMA-S cells were injected subcutaneously (1×10^6 cells) into WT or DGK ζ KO mice. 12–15 days after injection, the mice were euthanized, and tumors were harvested and weighed.

For analysis of short-term tumor rejection, RMA and RMA-S cells were labeled with CFSE and CellTrace violet, respectively and injected i.v. at a 1:3 ratio (20×10^6 cells total) into WT or DGK ζ KO mice. 18 hours after injection, spleens were harvested from these mice and the presence of tumor cells was analyzed by flow cytometry. In some experiments, NK cell depletion was performed by injecting anti-NK1.1 antibody (PK136 200 μ g i.p.) 24 hours before tumor challenge.

Results

DGK ζ KO but not DGK α KO NK cells are hyperresponsive to activating receptor stimulation

NK cells from WT and DGK ζ KO mice were stimulated through multiple cell surface activating receptors. Although slight decreases in the proportion of NK cells expressing Ly49A, Ly49C, and 2B4 was seen in DGK ζ KO compared to WT NK cells, the development of NK cells was largely similar between WT and DGK ζ KO mice with regards to inhibitory receptor expression, activating receptor expression, and maturity (Fig. 1 A–C, Supplementary Table I). Upon activation through three distinct activating receptor families (ITAM-dependent: NK1.1, Ly49D; costimulatory-like: NKG2D; SAP-dependent: 2B4), an increased fraction of DGK ζ KO NK cells degranulated and produced IFN γ compared to WT NK cells (Fig. 1D, 1E). Importantly, IFN γ production downstream of cytokine activation (IL-12 + IL-18) or by PMA/ionomycin was similar between DGK ζ KO and WT NK cells (Fig. 1F). Since exogenous IL-2 was added to the NK cell stimulation assays to make the stimulations more robust and consistent, we additionally tested whether DGK ζ deficiency augmented the activity of NK cells in the absence of exogenously added IL-2. Although the response of NK cells was more variable, an increased fraction of DGK ζ KO NK cells degranulated and produced IFN γ compared to WT NK cells stimulated with anti-NK1.1 antibody in the absence of IL-2 (Fig. 1G), suggesting that DGK ζ deficiency augmented activating receptor-mediated stimulation. Increased NK cell function was also observed in NK cells isolated from DGK ζ KO mice treated with Poly I:C, which mimics a viral infection and primes NK cell responses through type I IFN (Fig. 1H). Thus, DGK ζ deficiency enhances NK cell function even when isolated from an inflammatory environment.

Next, to directly examine cytotoxicity by NK cells, WT and DGK ζ KO NK cells were expanded in IL-2 to create lymphokine-activated killer (LAK) cells. Similar to freshly isolated NK cells, an increased proportion of DGK ζ KO LAK cells degranulated and produced IFN γ upon activating receptor stimulation (Fig. 2A). Moreover, this correlated with an increased ability of DGK ζ KO LAK cells to kill the NK cell-sensitive tumor cell

line, YAC-1, and against the MHC class I-deficient tumor cell line, RMA-S (Fig. 2B). Importantly, no differences in cytotoxicity were seen between the ability of WT and DGK ζ KO LAK cells to kill MHC class I-sufficient RMA cells (Fig. 2B). Increased IFN γ release was also detected by ELISA when DGK ζ KO LAK cells were cocultured with the YAC-1 cell line (Fig 2C). Although we consistently observed an increase in cytotoxicity and IFN γ production by DGK ζ KO LAK cells in all experiments, the results were not statistically significant (Supplementary Fig. 2).

Previous studies have highlighted the importance of other isoforms of DGK such as DGK α in T cell activation (18, 22). Thus, to test if DGK α was also important in NK cell function, we stimulated NK cells from WT and DGK α KO mice using platebound anti-NK1.1 antibody. We found that there was no significant difference between DGK α KO mice and WT controls in both degranulation and cytokine production, suggesting that DGK α does not contribute to limiting NK cell function (Fig 3A, 3B).

DGK ζ KO NK cells are hyperresponsive in an NK cell-intrinsic and developmentally independent manner

Since DGK ζ KO mice display increased activation in multiple hematopoietic lineages, it was conceivable that the hyperresponsive phenotype of NK cells was not NK cell-intrinsic. To test this possibility, bone marrow (BM) chimeric mice were created by BM transplantation of WT competitor BM mixed with WT control or DGK ζ KO BM. Upon stimulation of NK cells from these mice, NK cells in the WT competitor/WT control BM chimeric mice were similarly responsive, while NK cells of DGK ζ KO origin in the WT competitor/DGK ζ KO BM chimeras were significantly more responsive than the WT competitor controls (Fig. 4A, B). Next, to test whether the hyperresponsive phenotype of DGK ζ KO NK cells was independent of altered NK cell development, we acutely deleted DGK ζ from NK cells using a Tamoxifen-inducible Cre (Cre-ERT2) system. NK cells from Tamoxifen-treated mice bearing floxed alleles of DGK ζ were more responsive to activating receptor stimulation than Cre⁺ WT controls (Fig. 4C, 4D). Together, these data suggest that DGK ζ deficiency enhances NK cell responsiveness to activating receptor stimuli in an NK cell-intrinsic and developmentally independent manner.

The hyperresponsive phenotype of DGK ζ KO NK cells is associated with a trend towards enhanced ERK activation

DGK ζ deficiency has been shown to enhance at least 3 distinct downstream signaling pathways: ERK, AKT, and NF κ B. To test which of these pathways are altered in DGK ζ KO NK cells, we stimulated WT or DGK ζ KO NK cells with anti-NK1.1 antibody, and analyzed the levels of pERK, pAKT and I κ B degradation by Western blot analysis. Consistent with previous results, DGK ζ KO NK cells consistently displayed increased levels of pERK upon stimulation (although not statistically significant due to variability in the fold-increase), while showing no difference in the expression of total ERK (Fig. 5A–C), suggesting that the fraction of pERK was increased in DGK ζ KO NK cells. However, to our surprise, we found that stimulation of DGK ζ KO NK cells resulted in no difference in pAKT or I κ B degradation compared to WT controls (Fig. 5D–F).

To test whether the enhancement in pERK was responsible for the increased function of DGK ζ KO NK cells, we treated anti-NK1.1 antibody-activated NK cells with the MEK inhibitor U0126. The degranulation of WT NK cells was inhibited by U0126 in a dose-dependent manner, suggesting that pERK was important for NK cell degranulation. Although DGK ζ KO NK cells were also inhibited by U0126 in a dose-dependent manner, they required a higher concentration of U0126 to completely attenuate their ability to degranulate (Fig. 6A). Moreover, the % of functional inhibition (% of baseline) observed at each concentration of MEK inhibitor was similar between WT and DGK ζ KO NK cells (Fig. 6B). Given that U0126 is a non-competitive MEK inhibitor (23), these data suggest that the pERK level observed in anti-NK1.1-stimulated DGK ζ KO NK cells is not at a functionally saturating level. Together with increased pERK activation, these data suggest that increased pERK contributes to the enhanced responsiveness of DGK ζ KO NK cells.

DGK ζ KO NK cells are licensed and exhibit intact inhibitory receptor function

One unique function of NK cells is their ability to recognize reduced MHC class I expression (missing self) on target cells through their inhibitory receptors. To test whether DGK ζ deficiency affected the function of inhibitory receptors, we examined whether DGK ζ KO NK cells displayed intact licensing (24), a process by which an NK cell that expresses an inhibitory receptor for self-MHC I (e.g., Ly49C in B6 mice) is more likely to respond than one that does not. Both WT and DGK ζ KO NK cells that expressed Ly49C⁺ were more likely to respond to activating receptor stimulation than their Ly49C⁻ counterparts, suggesting that licensing and therefore inhibitory receptor signaling was intact in these mice. Importantly, both Ly49C⁺ and Ly49C⁻ DGK ζ KO NK cells were hyperresponsive compared to their WT subset counterparts, suggesting that licensing was not the major cause of NK cell hyperresponsiveness in DGK ζ KO mice (Fig. 7A, 7B).

To further test if the loss of DGK ζ in NK cells affected inhibitory receptor function, we tested whether co-ligation of an inhibitory Ly49 receptor would attenuate NK cell function in DGK ζ KO NK cells. NK cells from WT or DGK ζ KO mice were stimulated with anti-NK1.1 antibody with or without an antibody against the inhibitory receptor Ly49G2. Upon co-ligation of Ly49G2, the degranulation of Ly49G2⁺ NK cells from WT and DGK ζ KO was similarly reduced by ~25% (Fig. 7C). Importantly, no inhibition of degranulation was seen in Ly49G2⁻ NK cells from either WT or DGK ζ KO mice. These data suggest that DGK ζ deficiency did not alter the function of inhibitory receptors.

DGK ζ KO NK cells exhibit increased clearance of TAP-deficient tumor cells *in vivo*

To test whether DGK ζ deficiency leads to improved anti-tumor responses *in vivo*, we subcutaneously injected WT and DGK ζ KO mice with RMA-S tumor cells. 12–15 days post injection, rapid tumor growth was observed in WT mice injected with RMA-S cells. Although tumors also grew in DGK ζ KO mice, there was a significant reduction in tumor burden compared to WT controls. These data suggest that DGK ζ deficiency enhances protection against tumors by delaying tumor growth *in vivo* (Fig. 8A).

To further test if this protection was NK cell specific, we performed an additional experiment where both RMA (TAP-sufficient) and RMA-S (TAP-deficient) tumor cells were

differentially labeled with dyes and injected at a 1:3 ratio into WT or DGK ζ KO hosts. 18 hours after injection into hosts, we found that the RMA-S:RMA ratio was significantly reduced in DGK ζ KO compared to WT mice, suggesting that DGK ζ KO NK cells were better at clearing MHC-Class I deficient tumor cells than WT NK cells. This difference was lost when mice were depleted of NK cells prior to the tumor challenge, suggesting that DGK ζ KO NK cells were responsible for the significant increase in the rejection of the RMA-S cells (Fig 8B).

Discussion

In the present study, we tested the role of DGK ζ in NK cell function. Using genetic ablation, we showed that the absence of DGK ζ in NK cells lead to increased function of NK cells upon stimulation through multiple activating receptors in vitro. Moreover, this enhanced function correlated with an increased ability of DGK ζ KO NK cells to exhibit increased rejection and limit growth of an MHC class I-deficient target cell in vivo. Thus, DGK ζ serves to limit NK cell activation and could represent a novel target for the enhancement of NK cell function.

As DGK ζ is a negative regulator of DAG signaling, one might predict that all immune cells that lack DGK ζ would be hyperresponsive to activating stimuli. Indeed, DGK ζ KO T cells show enhanced activation and resist anergy induction upon TCR stimulation (25, 26). However, the absence of DGK ζ does not always lead to an enhancement in all cellular functions. For example, DGK ζ -deficient mast cells produce more cytokines but display reduced degranulation upon IgE receptor engagement (27). Thus, depending on the cell type or cellular function, the responses that are regulated by DGK ζ could be different. Thus, it was important to test the role of DGK ζ in regulating NK cell function. Interestingly, we found that both degranulation (cytotoxicity) and cytokine production were increased in DGK ζ KO NK cells compared to WT NK cells, suggesting that degranulation in mast cells and NK cells are potentially regulated by different signaling pathways.

To the best of our knowledge, this study is the first to demonstrate that a negative regulator of activating signals can be targeted in NK cells to improve their function. Previous attempts to enhance the function of NK cells have been hampered by the ability of NK cells to quickly tune their activation threshold to the environment. NK cells that lack enzymes that negatively regulate proximal signaling pathways (SHP-1 and SHIP) tune their responsiveness, which results in NK cells with diminished function (11, 12). Similarly, NK cells subjected to an MHC I-deficient environment also leads to hyporesponsiveness (15), as do NK cells that experience constant stimulation through their activation receptors (14). However, in each of these situations, NK cells are normally responsive to PMA and ionomycin, suggesting that the tuning process occurs upstream of PLC γ activation. Thus, in this study, we hypothesized that the removal of DGK ζ , which is a negative downstream of PLC γ function, would enhance the function of NK cells downstream of the three major pathways of activating signals (8). Indeed, we found that DGK ζ KO NK cells showed an increased propensity to degranulate and produce cytokines after activation through receptors that associate with either SAP or with adaptor molecules containing ITAM or costimulatory-like domains.

In addition to bypassing tuning, targeting a negative regulator of distal signaling pathways could be beneficial for the retention of inhibitory signaling pathways that are required for missing-self recognition by NK cells. The removal of negative regulators that are directly associated with MHC I-binding inhibitory receptors such as SHP-1 and SHIP abrogates the ability of NK cells to conduct missing self recognition (12, 28). In contrast, our data demonstrate that the loss of DGK ζ does not affect the ability of inhibitory receptors to attenuate activation and license NK cells. Thus, missing-self recognition by DGK ζ KO NK cells remains intact as evidenced by increased killing of the RMA-S cell line.

One potential caveat with our studies is that our NK cell preparation could be contaminated with T cells, which may confound our interpretations. In experiments using LAK cells, the NK cell purity was typically in the 80–90% range. Although we used T cell markers (CD4 and CD8) to avoid measuring T cell activation in our flow cytometry-based assays, T cells could potentially contribute to cytotoxicity in assays involving co-culture with tumor cell targets or in vivo tumor rejection. However, we believe that the contribution of T cells to these assays is minimal, given that RMA-S cells express very low levels of MHC class I and are prime targets for missing self recognition by NK cells. Furthermore, no enhancement in killing was observed against MHC class I-sufficient RMA cells by the LAK cells derived from DGK ζ KO mice. In our in vivo RMA-S rejection assay, NK cell depletion abrogated the ability of NK cells to skew the RMA-S:RMA ratio in WT and DGK ζ KO mice, suggesting that the enhancement in RMA-S killing in vivo was NK cell-dependent. Hence, we conclude that it is likely that the enhanced activity we see in our data is derived from NK cells, and not from T cell contaminants.

To test the contribution of signaling pathways downstream of DAG that were responsible for the enhanced NK cell function, we examined the activation of ERK, AKT, and NF κ B signaling pathways. In contrast to what has been reported in T cells (17, 18), DGK ζ KO NK cells exhibited increased pERK, but not pAKT or enhanced I κ B α degradation, suggesting that increased pERK could be a major contributor to the enhanced NK cell function seen in DGK ζ KO NK cells. Consistent with previous findings (29), we found that the addition of a MEK inhibitor suppressed NK cell degranulation in a dose-dependent manner. Importantly, the NK cell response in WT and DGK ζ KO NK cells was reduced by the same magnitude even at the lowest doses of the MEK inhibitor. These data suggest that the elevation of pERK seen in DGK ζ KO NK cells is functionally meaningful (not saturated) and supports the notion that elevated pERK contributes to heightened NK cell function in the absence of DGK ζ .

Aside from DGK ζ , DGK α is another negative regulator of DAG signaling in lymphocytes. DGK α seems to possess both positive and negative roles in T cell function. Although the phenotype is weaker than seen with DGK ζ KO T cells, DGK α KO T cells display increased cytokine production and phosphorylation of ERK (18, 25), suggesting that DGK α negatively regulates TCR signaling. DGK α is involved in the stabilization of the immune synapse IS in T cells (22), suggesting that DGK α also plays a positive role in T cell function. However, in NK cells, we found that the loss of DGK α did not influence NK cell effector function. Thus, our data suggest DGK α does not negatively regulate signaling downstream of NK cell

activating receptors. However, a more in-depth study into the role that DGK α plays in IS formation in NK cells could potentially demonstrate a role for DGK α in NK cell function.

Overall, our data support a model where NK cell tuning acts proximal to the generation of DAG, suggesting that therapeutic targeting of distal negative regulators of NK cell activation may represent a strategy for improving the clinical efficacy of NK cells. In particular, our data regarding the acute deletion of DGK ζ in NK cells, as well as its key position in T cell function, highlights its potential as a specific target for clinical purposes in anti-tumor responses (30).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

NK	natural killer
DGKζ	diacylglycerol kinase zeta
NK1.1	NKRP1-C
SHP-1	Src homology region 2 domain-containing phosphatase 1
DAG	diacylglycerol
DGKζ KO	DGK ζ deficient
DGKα KO	DGK α deficient
DGKζ^{F/F}	DGK ζ floxed mice

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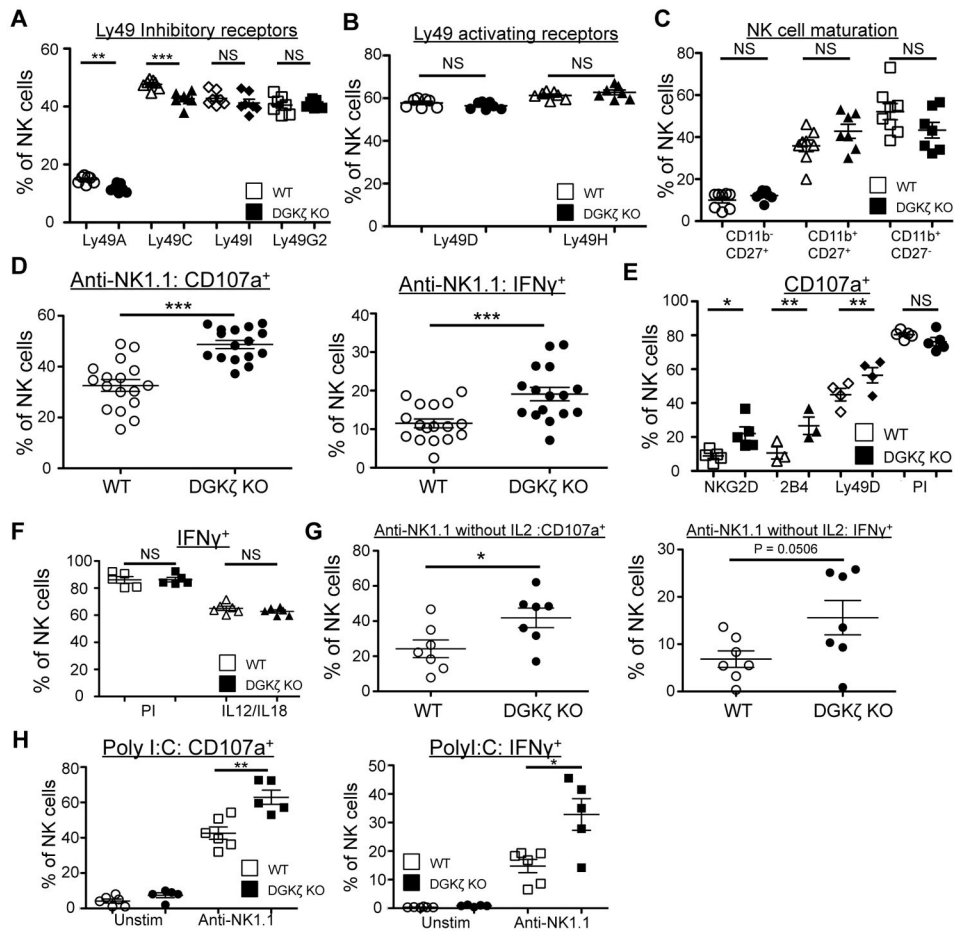


Figure 1. DGK ζ -deficient NK cells exhibit enhanced function downstream of activating receptors
 The proportion of NK (CD3⁻ NK1.1⁺) cells expressing A) inhibitory and B) activating Ly49 receptors, and C) CD27/CD11b in WT and DGK ζ KO cells is shown. N=7. D) Splenocytes from WT and DGK ζ KO mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a (left) and intracellular anti-IFN γ antibody (right) is shown. N=17. E) Splenocytes from WT and DGK ζ KO mice were stimulated with plate-bound antibodies against the indicated activating receptors or with PMA/Ionomycin (PI). The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a antibody is shown. N=4–5 per condition. F) Splenocytes from WT and DGK ζ KO mice were stimulated with PMA/ionomycin or with IL-12 and IL-18. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) expressing intracellular anti-IFN γ is shown. N=5–6 per condition G) Splenocytes from WT and DGK ζ KO mice were stimulated with plate-bound anti-NK1.1 antibody without IL-2. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a (left) and intracellular anti-IFN γ antibody (right) is shown. N=7. H) Splenocytes from polyI:C-treated WT and DGK ζ KO mice were stimulated with plate-bound anti-NK1.1 antibodies. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a (left) and intracellular anti-IFN γ antibody (right) is shown. N=5. *, ** and *** represent statistical significance of p<0.05, p<0.01, and p<0.001 by Student's t-test, respectively. NS = not significant. Data shown are compiled

from 2 separate experiments are shown in A–C and E, and from at least 3 separate experiments for figures D, F and G.

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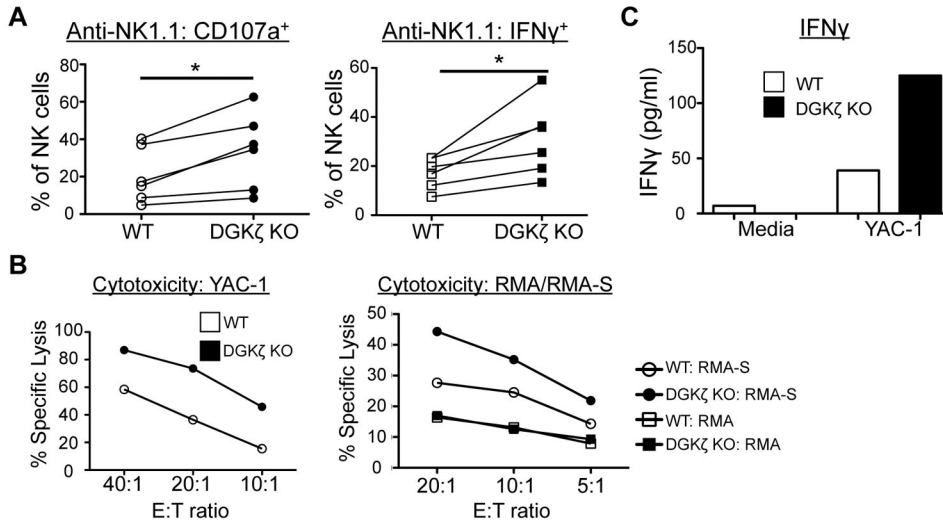


Figure 2. DGK ζ KO LAK cells display increased cytotoxicity and cytokine production upon interaction with tumor cells

A) WT or DGK ζ KO LAK cells were stimulated with plate-bound anti-NK1.1 antibodies. The proportion of NK cells (CD4⁻CD8⁻NK1.1⁺DX5⁺) labeled with anti-CD107a (left) and intracellular anti-IFN γ antibody (right) is shown. Data from 6 independent experiments is shown (*P<0.05 by paired t-test). N=6 B) WT or DGK ζ KO LAK cells were co-cultured with YAC-1, RMA, or RMA-S cells at the indicated E:T ratios and % specific lysis was determined 4–6 hours later. C) WT or DGK ζ KO LAK cells were plated with or without YAC-1 cells at a 1:1 ratio for 24 hours. IFN γ content in the cell-free supernatants was determined by ELISA. One representative of N=3 independent experiments is shown for B and C.

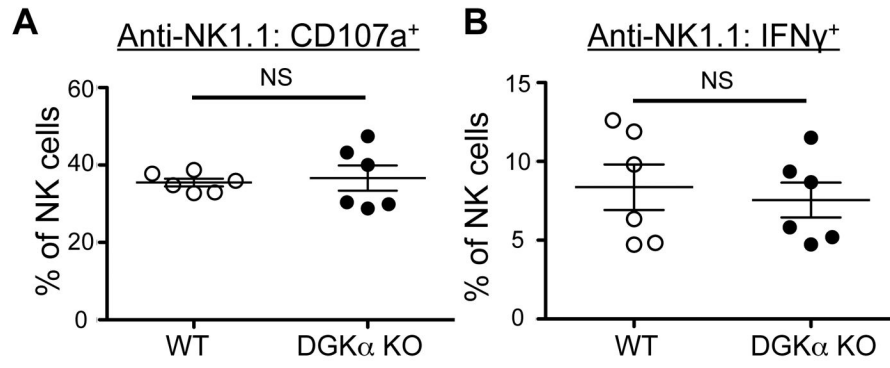


Figure 3. DGK α NK cells are not hyperresponsive compared to WT NK cells

Splenocytes from WT and DGK α KO mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with A) anti-CD107a and B) intracellular anti-IFN γ antibody is shown. NS = not significant by Student's t-test. Data shown are compiled from 2 separate experiments, N=6.

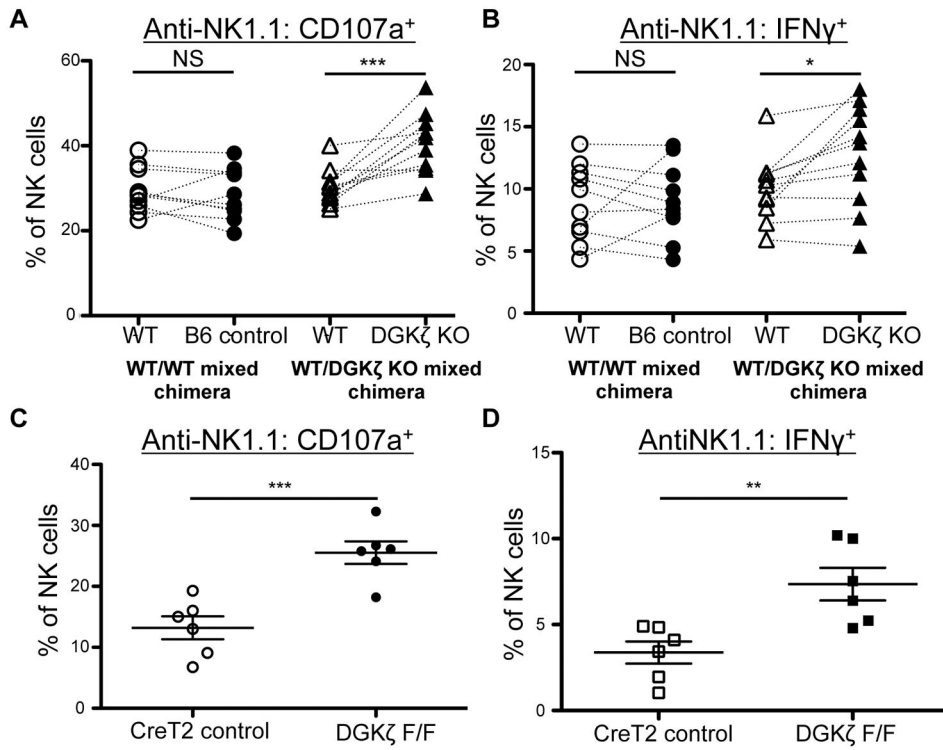


Figure 4. DGKζ KO NK cells are hyperresponsive in a cell-intrinsic and developmentally independent manner

A) Splenocytes from WT/WT control and WT/DGKζ KO mixed BM chimeras were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a and B) intracellular anti-IFNγ antibody is shown. Cells derived from either the B6 control BM or DGKζ KO bone marrow were paired with cells that were of WT competitor BM origin within the same mouse. N=11. C) Splenocytes from Tamoxifen-treated DGKζ^{F/F} Rosa26-STOP-Flox-YFP ERCreT2 or Rosa26-STOP-Flox-YFP ERCreT2 control mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of NK cells (CD3⁻DX5⁺NKp46⁺YFP⁺ lymphocytes) labeled with anti-CD107a and D) intracellular anti-IFNγ antibody is shown. N=6. *, ** and *** represent a statistical significance by paired t-test (A and B) or Student’s t-test (C and D) of p<0.05, p<0.01 and p<0.001 respectively. NS= not significant. All data shown in this figure is compiled from at least 3 independent experiments.

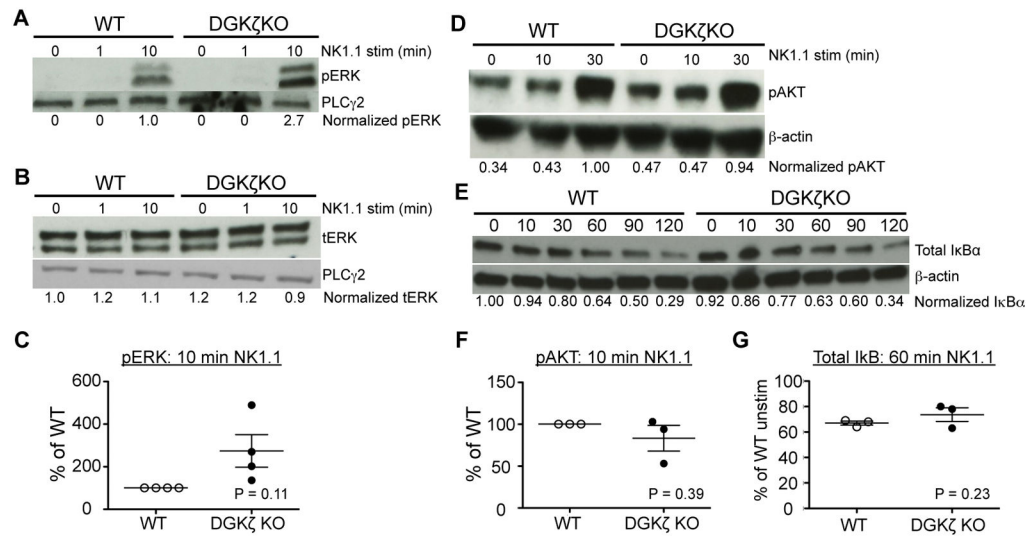


Figure 5. DGKζ KO NK cells display enhanced ERK, but not AKT or NFκB, signaling downstream of activating receptor stimulation

A) Fresh splenic NK cells were left unstimulated or stimulated with anti-NK1.1 antibody for the indicated duration. Cell lysates were analyzed for pERK and B) total ERK by Western blot analysis. C) Compiled data showing the relative levels of pERK in WT versus DGKζ KO NK cells (after quantification against loading controls) at 10 min post-stimulation are plotted. D) Lysates from anti-NK1.1-stimulated LAK cells were analyzed for pAKT or E) IκBα by Western blot analysis. F) Compiled data showing the relative levels of pAKT in WT versus DGKζ KO NK cells (after quantification against loading controls) at 10 min post-stimulation are plotted. G) The relative levels of total IκB in WT versus DGKζ KO NK cells after 60 min of stimulation with anti-NK1.1 antibodies, as compared to unstimulated cells (and after quantification against loading controls) are plotted. Quantification of protein against loading controls was performed using ImageJ software, and quantified protein levels were then normalized against either unstimulated (B and D) or stimulated (A and C) WT NK cells. Total PLCγ2 or β-actin was used as a loading control. Data shown is representative of at least 3 independent experiments. Statistical analysis was performed by (E, F) Student's t-test or (G) paired t-test.

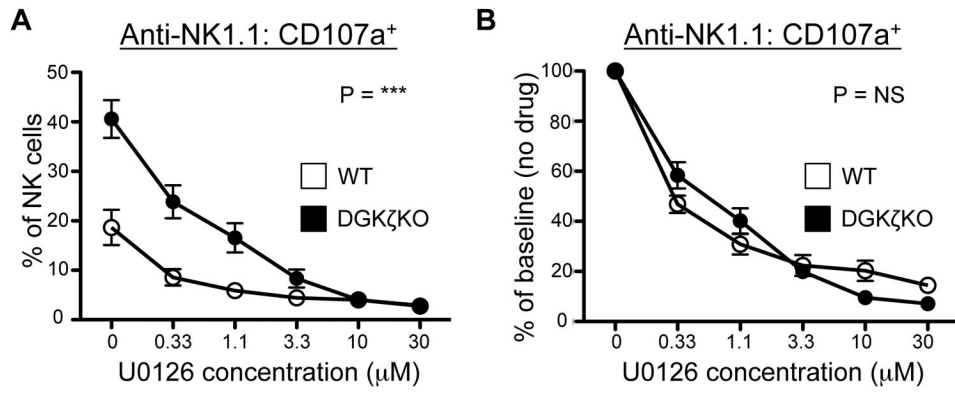


Figure 6. Enhanced ERK signaling is associated with the hyperresponsiveness of DGK ζ KO NK cells

WT or DGK ζ KO splenocytes were stimulated with plate-bound anti-NK1.1 antibody in the presence of various concentrations of the MEK inhibitor U0126. A) The proportion of NK cells (CD3⁻DX5⁺NKp46⁺) incorporating anti-CD107a antibody is shown. B) The percentage of responding cells as compared to no drug control (% of baseline) is plotted. N=3–5 per concentration. *** = P<0.001, NS= not significant by 2 way-ANOVA. Data shown is compiled from at least 3 independent experiments.

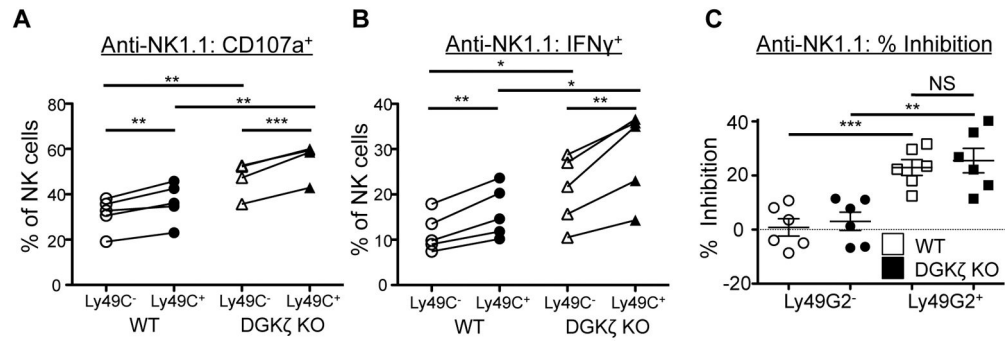


Figure 7. DGKζ deficiency does not affect NK cell inhibitory receptor function

A) Splenocytes from WT and DGKζ KO mice were stimulated with plate-bound anti-NK1.1 antibody. The proportion of Ly49C⁺ versus Ly49C⁻ NK cells (CD3⁻DX5⁺NKp46⁺) labeled with anti-CD107a and B) intracellular anti-IFNγ antibody (right) is shown. Data shown is compiled from 2 independent experiments, N=5. C) Splenocytes from WT and DGKζ KO mice were stimulated with plate-bound anti-NK1.1 antibody in the absence or presence of anti-Ly49G2 antibody. The percentage inhibition of degranulation (as measured by anti-CD107a labeling) in Ly49G2⁻ or Ly49G2⁺ NK cells by the addition of the anti-Ly49G2 antibody is shown by scatter plot. Data shown is compiled from 2 independent experiments, N=6.

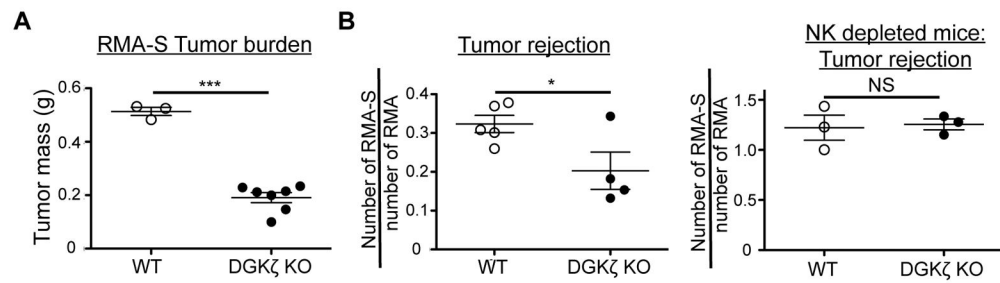


Figure 8. DGK ζ KO NK cells are more effective at effectively clearing TAP-deficient tumor cells
 A) RMA-S tumor cells were injected s.c. into WT or DGK ζ KO mice. 12 days later, the tumors were harvested and weighed. One representative of 2 independent experiments is shown. B) NK cell-sufficient (left) or NK cell-depleted (right) WT or DGK ζ KO mice were injected i.v. with a mixture of CFSE-labeled RMA and Celltrace Violet-labeled RMA-S tumors. 18 hours later, splenocytes were analyzed for the presence of residual tumor cells via flow cytometry. The ratio of RMA-S versus RMA within each WT or DGK ζ KO mouse was calculated and shown as a scatter plot. One representative of 2 independent experiments is shown. *, ** and *** represent a statistical significance by Student's t-test of $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. NS = not significant.