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mTORC1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function

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The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of cellular metabolism and also has fundamental roles in controlling immune responses. Emerging evidence suggests that these two functions of mTORC1 are integrally linked. However, little is known regarding mTORC1 function in controlling the metabolism and function of NK cells, lymphocytes that play key roles in antiviral and antitumor immunity. This study investigated the hypothesis that mTORC1-controlled metabolism underpins normal NK cell proinflammatory function. We demonstrate that mTORC1 is robustly stimulated in NK cells activated in vivo and in vitro. This mTORC1 activity is required for the production of the key NK cell effector molecules IFN-γ, which is important in delivering antimicrobial and immunoregulatory functions, and granzyme B, a critical component of NK cell cytotoxic granules. The data reveal that NK cells undergo dramatic metabolic reprogramming upon activation, upregulating rates of glucose uptake and glycolysis, and that mTORC1 activity is essential for attaining this elevated glycolytic state. Directly limiting the rate of glycolysis is sufficient to inhibit IFN-γ production and granzyme B expression. This study provides the highly novel insight that mTORC1-mediated metabolic reprogramming of NK cells is a prerequisite for the acquisition of normal effector functions. ***The Journal of Immunology*****, 2014, 193: 4477–4484.**

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms two protein complexes, mTORC1 and mTORC2, with roles in regulating immunological systems. mTORC1, in particular, has emerged as a central regulator of immune responses. mTORC1 activity has been ascribed important roles in the regulation of a wide range of immune cells in both the innate and adaptive arms of immunity (1). It is becoming clear that mTORC1-regulated cellular metabolism is crucial to the immunoregulatory function of this kinase complex in immune cells. Indeed, new evidence is emerging that metabolism plays a fundamental role in dictating immune cell differentiation and function. In CTLs, mTORC1 activity is required to maintain the high rates of glycolysis that are essential to sustain normal migratory patterns and effector functions (2). mTORC1-regulated glycolysis is also linked to normal differentiation of effector CD4 T cells (3). Additionally, mTORC1 activity is involved in the initiation of lipid synthesis in TCR-stimulated CD8 T cells, a process that is required for blastogenesis and effective T cell activation (4). Similarly, in innate immune cells, the immunoregulatory functions of mTORC1 have been linked to metabolic regulation. By regulating the level of glycolysis in activated dendritic cells (DCs), mTORC1 controls DC survival and, ultimately, the T cell response (5). Therefore, immune cell metabolism is integrally linked to the important immunoregulatory functions of mTORC1.

Remarkably, the role played by mTORC1 in controlling NK cell metabolism and function has not yet been defined. NK cells are lymphocytes that bridge innate and adaptive immunity, and they have an important role in antiviral and antitumor immune responses. Although NK cells are critical in early immune responses, they also regulate the ensuing adaptive immune response through the release of cytokines that modulate the downstream immune response, notably IFN-γ and TNF-α, and by modulating DC numbers (6). More recently, it was appreciated that NK cells can undergo clonal expansion and, thus, function in parallel with the adaptive immune system. In particular, NK cells can be induced to express the high-affinity IL-2R in response to IL-12, thus allowing them to respond to low doses of T cell–derived IL-2 (7, 8). Indeed, IL-2 produced by T cells is critical for modulating NK cell activation in response to various pathogenic infections (7–9). Given our previous work linking mTORC1-regulated metabolism to normal CTL functions and the observation that the mTORC1 inhibitor rapamycin can disrupt NK cell effector functions (10–12), we hypothesized that mTORC1-regulated metabolism might be fundamental in the control of NK cell responses.

Indeed, the current study demonstrates that mTORC1 activity is essential for glycolytic reprogramming of activated NK cells and that this metabolic shift is a prerequisite for normal NK cell effector functions, such as the production of IFN-γ and increased expression of granzyme B.

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Abbreviations used in this article: DC, dendritic cell; 2DG, 2-deoxyglucose; ECAR, extracellular acidification rate; FSC, forward scatter; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Hex2, hexokinase 2; Ldha, lactate dehydrogenase a; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; NBDG, 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose; OCR, oxygen consumption rate; OxPhos, oxidative phosphorylation; poly(I:C), polyinosinic-polycytidylic acid; pS6, phosphorylated S6 ribosomal protein.

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Materials and Methods

Mice

C57BL/6J mice were purchased from Harlan (Bicester, U.K.) or Taconic Laboratories (Germantown, NY) and maintained in compliance with Irish Department of Health and Children regulations and with the approval of the University of Dublin's ethical review board or in accordance with institutional guidelines for animal care and use at Brown University.

In vivo NK cell activation with polyinosinic-polycytidylic acid

Mice were injected i.p. with 200 μg polyinosinic-polycytidylic acid [poly(I:C)] in PBS (InvivoGen), with or without 0.6 mg/kg rapamycin (blood levels 71–94 ng/ml) (Fisher), or with 1 g/kg 2-deoxyglucose (2DG; Sigma-Aldrich). Mice were sacrificed after 12 or 24 h, as indicated. Spleens were harvested, and NK cells were analyzed.

Cell culture

Splenocytes were isolated and cultured in IL-15 (25 ng/ml; PeproTech) at 37˚C for 5 d. On day 5, the cells were supplemented with IL-15 (25 ng/ml) and cultured for an additional 2 d. On day 7, cultured NK cells were stimulated for 18 h with IL-2 (20 ng/ml; National Cancer Institute Preclinical Repository) and/or IL-12 (10 ng/ml; Miltenyi Biotec) cytokines. Low-dose IL-15 (5 ng/ml) was added as a survival factor to unstimulated cultures or to those stimulated with IL-12 alone. Experiments were carried out in the presence or absence of 2DG (Sigma-Aldrich), rapamycin (20 nM; Fisher), and/or oligomycin (2 μM; Sigma-Aldrich) inhibitors. NK cells were MACS purified using an NK Cell Isolation Kit (Miltenyi Biotec) from day-7 cultures for biochemical analyses. Where indicated, NK cells were cultured in glucose-free medium supplemented with 10% dialyzed FCS (Fisher), 2 mM glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 1× concentration of MEM Vitamin cocktail (Life Technologies), 1× concentration of selenium/insulin/transferrin cocktail (Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 1% Penicillin/Streptomycin (Life Technologies) and with either glucose (10 mM) or galactose (10 mM).

Flow cytometric analysis

Cells (1×10^6–3×10^6 cells) were stained for 30 min at 4˚C with saturating concentrations of the following Abs: eFluor 450 NK1.1 (PK136), eFluor 660 NKp46, PerCP–eFluor 710 NKp46 (29A1.4), PE NKp46 (29A1.4), FITC CD3 (145-2C11), FITC TCR-β, allophycocyanin TCR-β (H57−597), PE-Cy7 CD69 (H1.2F3), PerCP-Cy5.5 CD69 (H1.2F3), allophycocyanin-Cy7 CD25 (PC61), allophycocyanin CD71 (R17217), PE CD98 (RL388), allophycocyanin IFN-γ (XMG1.2), PE-Cy7 IFN-γ (XMG1.2), and PE-Cy7 granzyme B (NGZB) (all from eBioscience or BD Pharmingen). Live cells were gated according to their forward scatter (FSC)-A and side scatter; single cells were selected based on FSC-W and FSC-A, and NK cells were identified as $NKp46^+$, $NK1.1^+$, $CD3^-$. For intracellular cytokine staining, endocytosis was blocked using GolgiPlug (BD Pharmingen) for 4 h. Cells were then fixed and permeabilized using Cytofix/Cytoperm reagent (BD Pharmingen), as per the manufacturer's instructions. Data were acquired on a FACSCanto, LSR Fortessa, or FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (TreeStar).

Phospho-S6 ribosomal protein intracellular staining

For in vivo experiments, cells were fixed and stained, as described previously (13), using PE anti–phospho-S6 ribosomal protein Ser 235/236 (eBioscience). For in vitro experiments, cells were fixed and stained, as described previously (14), using anti–phospho-S6 ribosomal protein Ser 235/236 (Cell Signaling Technologies) and PE-conjugated donkey anti-rabbit Ig G (Jackson ImmunoResearch).

Western blot analysis

Cells were lysed (1×10^7/ml) in Tris lysis buffer containing 10 mM Tris (pH 7.05), 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 10% glycerol, 0.5% Triton, 1 μM DTT, and protease inhibitors. Lysates were centrifuged (4˚C, 16,000 × *g* for 10 min), separated by SDS-PAGE, and transferred to nitrocellulose membrane. Blots were probed with Abs recognizing phospho-Akt^{S473} phospho-S6 ribosomal $protein^{S235/236}$, phospho-$S6K^{T389}$, phospho-$GSK3\alpha/\beta^{S21/9}$, and total Akt (Cell Signaling Technologies).

Quantitative real-time PCR

Cultured NK cells were purified by magnetic bead sorting using an NK Cell Isolation Kit (Miltenyi Biotec) prior to stimulation. RNA was extracted using the RNeasy RNA purification mini kit (QIAGEN), according to the manufacturer's protocol. Purified RNA was reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences). Real-time PCR was performed in triplicates in a 96-well plate using iQ SYBR Green–based detection on a ABI 7900HT Fast Real-Time PCR System. For the analysis of mRNA levels, the derived values were normalized to RpLp0 mRNA levels using the following primers: Rplp0 forward: 5′-CATGTCGCTCCGAGGGAAG-3′, Rplp0 reverse: 5′-CAGCAGCTGGCACCTTATTG-3′, lactate dehydrogenase a (Ldha) forward: 5′-CTGGGAGAACATGGCGACTC-3′, Ldha reverse: 5′-ATGGCCCAGGATGTGTAACC-3′, Glut1 forward: 5′-GGAATCGTCGTTGGCATCCT-3′, Glut1 reverse: 5′-CGAAGCTTCTTCAGCACACTC-3′, hexokinase 2 (Hex2) forward: 5′-TCGCCTGCTTATTCACGGAG-3′, Hex2 reverse: 5′-CCATCCGGAGTTGACCTCAC-3′, Ifng forward: 5′-ACGCTACACACTGCATCTTG-3′, and Ifng reverse: 5′-GTCACCATCCTTTTGCCAGTTC-3′.

Oxygen consumption rate and extracellular acidification rate measurement

An XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of NK cells cultured under various conditions. In brief, purified NK cells were adhered to a CellTaq (BD Pharmingen)–coated XF 24-well microplate (Seahorse Bioscience) at 750,000 cells/well, 1.25×10^7 cells/ml. Sequential measurements of ECAR and OCR, following addition of the inhibitors (Sigma-Aldrich) oligomycin (2 μM), rotenone (100 nM) plus antimycin (4 μM), and 2DG (30 mM), allowed for the accurate calculation of oxygen consumption due to oxidative phosphorylation (OxPhos) and acidification due to glycolysis.

Glucose uptake

A total of 3×10^6 splenocytes or 0.5×10^6 cultured NK cells was washed and incubated at 37˚C for 15 min in glucose-free media supplemented with 10% dialyzed FCS (Fisher), 2 mM glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 1× concentration of MEM vitamin cocktail (Life Technologies), 1× concentration of selenium/insulin cocktail (Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 1% Penicillin/Streptomycin (Life Technologies). Splenocytes and cultured NK cells were incubated at 37˚C for an additional 2 or 1 h, respectively, in supplemented glucose-free media containing the fluorescently labeled glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Life Technologies) at a final concentration of 50 μM before analysis using flow cytometry.

Statistical analysis

GraphPad Prism 6.00 for Macintosh (GraphPad) was used for statistical analysis. A one-way ANOVA test was used throughout with the Tukey test for multiple comparisons. A Student *t* test was used when only two data sets were compared. For comparison of relative IFN-γ mean fluorescence intensity (MFI) values, a one-sample *t* test was used to calculate *p* values, with the theoretical mean set to 1.00.

Results

mTORC1 activity is required for NK cell IFN-γ production in vivo

NK cells are activated in vivo in response to viral infections. Poly(I:C), a synthetic dsRNA analog, is often used as a mimic of viral infection (15). To investigate whether NK cell activation in vivo is associated with increases in mTORC1 activity, mice were injected with poly(I:C), and splenocytes were isolated for analysis. There was a dramatic increase in phosphorylated S6 ribosomal protein (pS6), a readout of mTORC1 signaling, in NK cells analyzed 12 and 24 h after poly(I:C) injection (Fig. 1A, 1B). In contrast, poly(I:C) did not increase pS6 in T cells (Fig. 1A). To assess the importance of mTORC1 activity in poly(I:C)-activated NK cells, rapamycin was coadministered with poly(I:C) in mice. Rapamycin did not affect NK cell activation, as measured by CD69 expression (Fig. 1C), but it had a significant effect on IFN-γ production in splenic NK cells (Fig. 1D, 1E). Inhibition of mTORC1 did not have a global effect on cytokine production because poly(I:C)-stimulated increases in TNF-α expression were not affected by rapamycin treatment (Fig. 1F, 1G). When poly(I:C) was

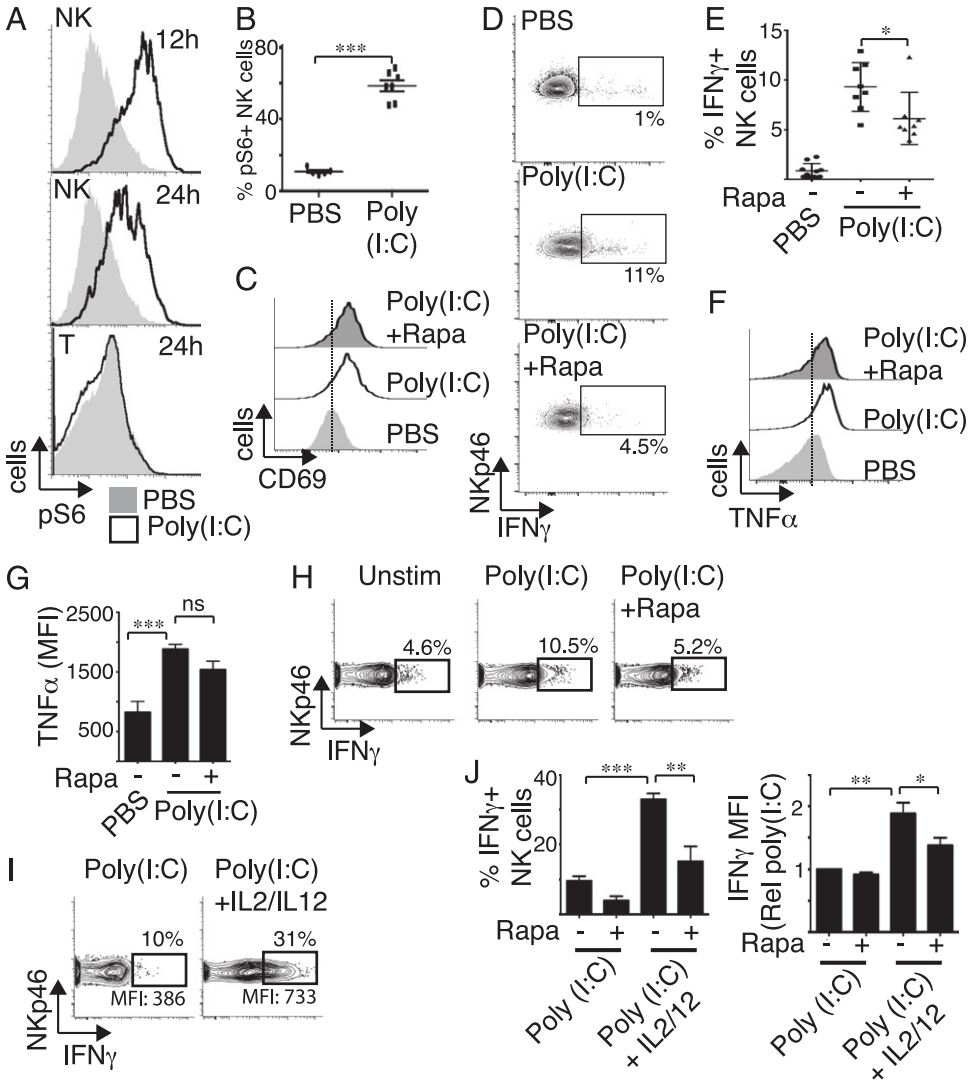

FIGURE 1. mTORC1 activity is required for IFN-γ production in NK cells in vivo. (**A**–**E**) Mice were administered PBS or 200 μg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection. Spleens were harvested after 12 or 24 h, and NKp46$^+$ TCRβ$^-$ NK cells (A and B) or TCRβ$^+$ T cells (A) were analyzed by flow cytometry for levels of pS6. (C–**G**) Spleens were harvested after 24 h for analysis. NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells were analyzed by flow cytometry for CD69 expression (C), IFN-γ production (D and E), and TNF-α expression (F and G). (**H**–**J**) Splenocytes were stimulated ex vivo with poly(I:C), with or without IL-2/IL-12 and with or without rapamycin, or were left untreated for 18 h, and IFN-γ production, frequency, and MFI were analyzed in NK1.1$^+$ NKp46$^+$ CD3$^-$ cells. Data are mean ± SEM or are representative of 5–10 mice for each condition from two separate experiments (A–E), four mice for each condition (F and G), or three separate experiments (H–J). *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$.

administered to splenocytes in vitro, IFN-γ production was similarly increased in NK cells in an mTORC1-dependent manner (Fig. 1H).

It was apparent that poly(I:C) does not result in T cell activation in vivo or in vitro, as measured by pS6 and CD69 levels (Fig. 1A, data not shown). Given the evidence that NK cell activation can be closely linked to T cell responses and the observations that T cell–derived IL-2 and the innate cytokine IL-12 are important for optimal NK cell activation in response to various infections (7, 8), the effect of supplementing poly(I:C)-stimulated splenocytes with IL-2 and IL-12 was investigated. Poly(I:C) plus IL-2/IL-12–stimulated NK cells had significantly enhanced levels of IFN-γ production compared with poly(I:C) stimulation alone, both in terms of the frequency of IFNγ$^+$ NK cells and the amount of IFN-γ protein expressed per NK cell, as measured by the MFI of IFN-γ staining (Fig. 1I, 1J). Importantly, these elevated levels of IFN-γ were inhibited by rapamycin treatment (frequency and MFI) (Fig. 1J). Thus, mTORC1 activity is important for IFN-γ production in NK cells activated in response to innate stimuli and to the T cell–derived cytokine IL-2.

The role for mTORC1-controlled cellular metabolism in dictating NK cell function was supported by the observation that NK cells producing IFN-γ had increased metabolic activity. A comparison of poly(I:C)-activated cells revealed that IFN-γ–producing NK cells were larger, based on FSC, than those not producing IFN-γ (Fig. 2A). Additionally, in the presence of rapamycin, the induced increased cell size of poly(I:C)-activated NK cells was impaired (Fig. 2B, 2C). A requirement for the increased cellular growth associated with blastogenesis is the provision of essential nutrients, including the key fuel source glucose. Consistent with this, the larger NK cells in poly(I:C)-treated mice had elevated rates of glucose uptake, as measured using the fluorescent 2DG analog NBDG. They also had increased expression of important nutrient receptors, including CD71 (transferrin receptor) and CD98 (a component of the L-amino acid transporter) (Fig. 2D). Rapamycin treatment inhibited the induction of elevated glucose uptake and the increased expression of CD71 and CD98 in NK cells (Fig. 2E). Thus, mTORC1 signaling in activated NK cells is required to allow NK cells to upregulate nutrient uptake for normal blastogenesis, as well as for the induction of IFN-γ expression.

mTORC1 controls glycolytic metabolism in activated NK cells

The data suggested that mTORC1-dependent production of the proinflammatory cytokine IFN-γ in activated NK cells is linked to the metabolic activity of NK cells. Proinflammatory immune cells tend to be characterized by elevated levels of glucose uptake and increased rates of glycolysis, metabolizing glucose primarily to lactate rather than through OxPhos in the mitochondria (16). To investigate whether changes in cellular metabolism were associated with the proinflammatory functions of activated NK cells, we

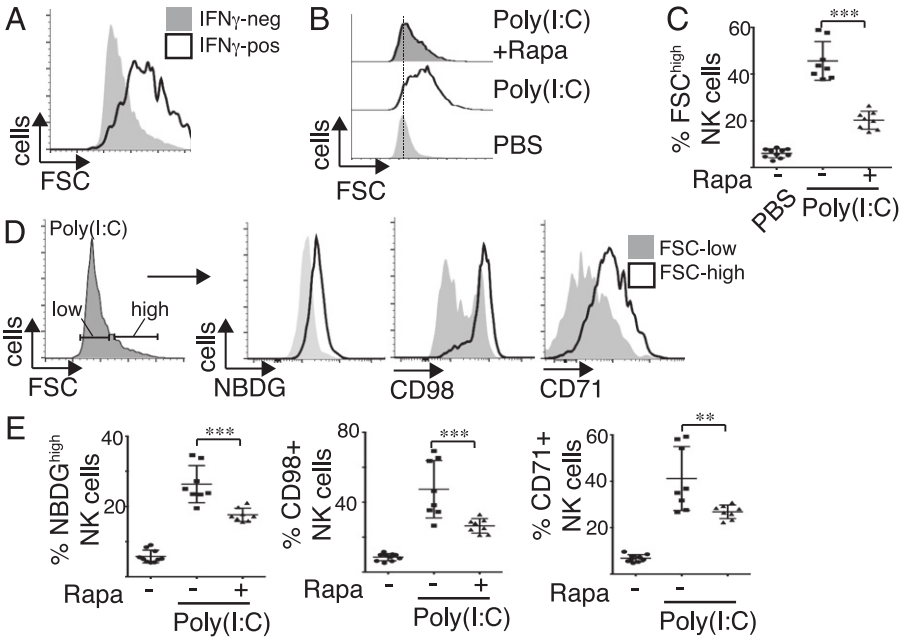

FIGURE 2. Rapamycin treatment prevents normal blastogenesis of NK cells activated in vivo by poly(I:C). (**A**–**E**) Mice were administered PBS or 200 μg poly(I:C) alone or in combination with rapamycin (0.6 mg/kg) by peritoneal injection, and spleens were harvested after 24 h. Splenocytes were isolated, and NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells (or NK1.1$^+$ NKp46$^+$ TCRβ$^-$ NK cells for NBDG experiments) were analyzed by flow cytometry. (A) FSC of NK cells was compared for IFNγ$^+$ and IFNγ$^-$ NK cells from poly(I:C)-treated mice. Differences in NK cell size (B) and the frequency of FSC^{high} NK cells (C) in each treatment group were analyzed. (D) Poly(I:C)-stimulated NK cells were segregated based on cell size (*left panel*), and small versus large NK cells were analyzed for levels of glucose uptake (NBDG) and CD98 and CD71 expression (*right panels*). (E) Analysis of the frequency of NK cells with high levels of NBDG and expression of CD71 and CD98. Data are mean ± SEM or are representative of 8–10 mice for each condition from two separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

performed detailed metabolic analysis of purified NK cells following cytokine stimulation, measuring rates of glycolysis and OxPhos. Due to the numbers of cells required for these analyses, NK cells were first expanded for 7 d from isolated splenocytes (17). This was achieved using low-dose IL-15, a cytokine required for DC-mediated NK cell priming in vivo (18–20). Indeed, this culture process provided large numbers of "primed" NK cells that responded robustly to subsequent stimulation, with distinct increases in cell size, CD69 expression, and IFN-γ production, as expected (Supplemental Fig. 1).

Purified IL-15–cultured NK cells were stimulated with IL-2 and/or IL-12, and rates of glycolysis and OxPhos were measured. IL-2 stimulation increased the rate of glycolysis (measured as ECAR) 2-fold. Alone, IL-12 had no effect on the rate of glycolysis, but the combination of IL-2 and IL-12 resulted in a synergistic increase in NK cell glycolysis (Fig. 3A). The synergistic action of IL-2 and IL-12 reflected IL-12–induced expression of the high-affinity IL-2R subunit CD25 (Fig. 3B) (7, 8), which facilitates enhanced IL-2 signaling, and substantially elevated mTORC1 activity (Fig. 3C). Consistent with increased glycolysis, IL-2/IL-12–stimulated NK cells had increased levels of glucose uptake (Fig. 3D). IL-2/IL-12 stimulation also increased the rate of OxPhos (measured as OCR) in NK cells (Fig. 3E). Although increases in both glycolysis and OxPhos were observed in NK cells following cytokine stimulation, overall there was a shift in the balance of NK cell metabolism from OxPhos to glycolysis (Fig. 3F). These data clearly demonstrate that NK cells undergo distinct metabolic reprogramming following cellular activation and highlight a key role for the adaptive cytokine IL-2 in promoting elevated levels of NK cell glucose uptake and glycolysis.

Metabolic analysis of IL-2/IL-12–stimulated NK cells in the presence of rapamycin revealed that mTORC1 activity was required for elevated levels of glycolysis but not OxPhos (Fig. 3G, 3H). This reflects that mTORC1 signaling is required to promote the expression of the glucose transporter, Glut1, and key rate-limiting glycolytic enzymes, Hex2 and Ldha (Fig. 3I). Given that the alternative mTOR complex, mTORC2, also was described to control cellular glycolysis through regulating the activity of the Akt serine/threonine kinase (21) and the reports that prolonged rapamycin treatment can destabilize the mTORC2-signaling complex (22), it was important to demonstrate that the observed metabolic effects of rapamycin in NK cells were not due to altered mTORC2 activity. Therefore, the activity of mTORC2 was investigated by immunoblot analysis of IL-2/IL-12–stimulated NK cells in the presence or absence of rapamycin. The data show that, in cytokine-activated NK cells, rapamycin treatment had no effect on the phosphorylation of the mTORC2 substrate Akt on serine 473 or on Akt kinase activity, as measured by the phosphorylation of the Akt substrate GSK3 (Fig. 3J). In contrast, the ATP competitive mTOR inhibitor AZD-8055, which targets both mTORC1 and mTORC2, prevents the phosphorylation of Akt on serine 473 and inhibits Akt-mediated phosphorylation of GSK3. Therefore, mTORC1, and not mTORC2, activity in NK cells is critical for the glycolytic reprogramming that occurs following cytokine stimulation.

mTORC1-maintained glycolysis in NK cells is required for IFN-γ production and granzyme B expression

To address whether mTORC1-controlled metabolism underpins the immunoregulatory functions of this kinase in NK cells, we investigated the levels of IFN-γ production in cultured NK cells treated with IL-2/IL-12, with or without rapamycin (Fig. 4A, 4B) and found them to directly correlate with measured rates of glycolysis (Fig. 3G). Additionally, IL-2/IL-12–mediated upregulation of granzyme B expression in NK cells was inhibited by rapamycin (Fig. 4A, 4B), demonstrating that the mTORC1 pathway also was

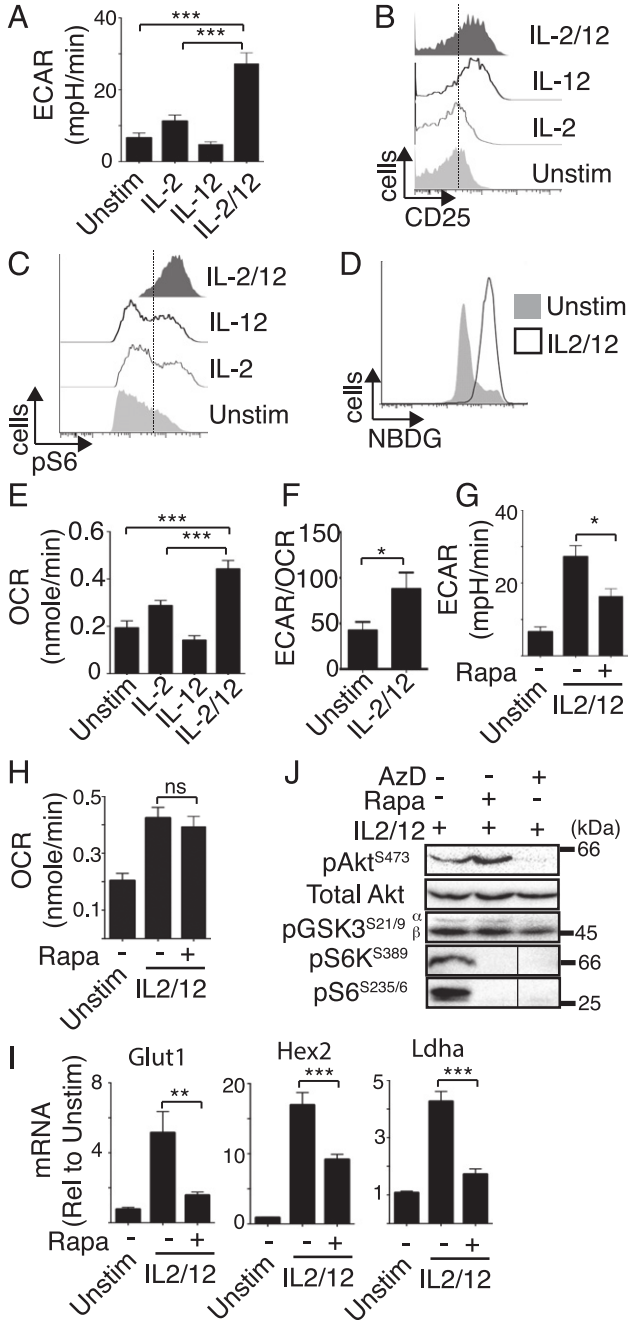

FIGURE 3. mTORC1 is required for metabolic reprogramming of activated NK cells. Analysis of the rate of glycolysis (ECAR) (**A**), CD25 expression (**B**), mTORC1 activity as measured by pS6 levels (**C**), glucose uptake (NBDG) (**D**), and OxPhos (OCR) (**E**) in cultured NK cells stimulated for 18 h with IL-2, IL-12, or IL-2 plus IL-12 or left unstimulated. (**F**) Ratio of glucose utilization for glycolysis to OxPhos in NK cells stimulated with IL-2/IL-12 or left unstimulated or 18 h. Cultured NK cells were stimulated for 18 h with IL-2/IL-12, with or without rapamycin (20 nM), or were left unstimulated and analyzed for the rates of glycolysis (**G**) and OxPhos (**H**), as well as the expression of Glut1, Hex2, and Ldha mRNA (**I**). (**J**) Immunoblot analysis of cultured NK cells stimulated with IL-2/IL-12 for 18 h, with or without rapamycin (20 nM) and with or without AZD-8055 (1 μM). Data are mean ± SEM or are representative of three to five experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

required for granzyme B expression. To determine whether mTORC1-controlled glycolysis accounts for the effect of rapamycin on the expression of IFN-γ and granzyme B, experiments were designed to directly limit the rate of glycolysis in cytokine-activated NK cells. As a first approach, glycolysis was limited using suboptimal doses of the glycolytic inhibitor 2DG, as described previously (23). Cultured NK cells were activated in the presence of low doses of 2DG (0.5–1 mM). Although NK cells upregulated the expression of CD69 normally, IFN-γ production and granzyme B expression were greatly diminished (Fig. 4C, 4D).

Galactose is a carbon fuel source that is metabolized by glycolysis following conversion to glucose-6-phosphate through the Leloir pathway (24). However, the rate at which galactose is converted to glucose-6-phosphate is slow and acts to limit the overall rate of glycolysis. Therefore, a second complementary approach to limit the rate of glycolysis in activated NK cells involved culturing NK cells in galactose rather than glucose. Metabolic analysis confirmed that galactose did not support elevated rates of glycolysis; however, the cells had normal rates of OxPhos (Fig. 4E). Galactose-cultured NK cells upregulated CD69 normally in response to IL-2/IL-12, but IFN-γ production and granzyme B expression were substantially reduced (Fig. 4F, 4G). Importantly, IL-2/IL-12–stimulated NK cells cultured in glucose or galactose had equivalent levels of mTORC1 activity, as measured by pS6 levels (Fig. 4H), demonstrating that glycolysis acts downstream of mTORC1 in the regulation of NK cell function. Therefore, the data demonstrate that increasing glycolytic metabolism in activated NK cells is a prerequisite for normal proinflammatory NK cell function.

Elevated levels of glycolysis correlate with the frequency of activated NK cells producing IFN-γ (Fig. 4), as well as with the amount of IFN-γ protein expressed per IFNγ$^+$ NK cell, as determined by the MFI of IFN-γ staining (Fig. 5A). In CD4 T cells, a mechanism was described in which the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (Gapdh) has an additional role outside glycolysis and can destabilize IFN-γ mRNA to control the levels of IFN-γ protein produced per T cell (25). Because these dual functions for Gapdh appear to be competitive, this model predicts that increasing the rate of glycolysis in NK cells would result in IFN-γ mRNA stabilization and increased IFN-γ protein levels. Indeed, rapamycin treatment and the resultant decrease in glycolysis levels correlate with decreased IFN-γ mRNA in activated NK cells (Fig. 5B). To test whether glycolytic enzymes, such as Gapdh, can modulate IFN-γ expression in NK cells, we used the pharmacologic ATP synthase inhibitor oligomycin to increase the rate of NK cell glycolysis. NK cells were activated with IL-2/IL-12 in the presence of rapamycin for 14 h before the addition of oligomycin for an additional 5 h, and the levels of IFN-γ protein (MFI) were measured. The addition of oligomycin to NK cells activated in the presence of rapamycin resulted in a 2.5-fold increase in the rate of glycolysis (Fig. 5C) to a level equivalent to that of IL-2/IL-12–stimulated NK cells in the absence of rapamycin (Fig. 3A). These elevated rates of glycolysis had no effect on the MFI of IFN-γ expression in IFNγ$^+$ NK cells (Fig. 5D). Therefore, acutely restoring elevated rates of glycolysis does not restore the levels of IFN-γ protein expression in NK cells. This argues that glycolytic regulation of IFN-γ mRNA stability, akin to that described by Chang et al. (25), does not have a substantial impact upon IFN-γ protein expression levels in activated NK cells. Therefore, it is likely that there are alternative mechanisms linking the rate of glycolysis to the amount of IFN-γ protein expression in activated NK cells.

To determine whether elevated levels of glycolysis are required for NK cells to produce IFN-γ in vivo, mice were injected with poly(I:C) in the presence or absence of the glycolytic inhibitor 2DG. This inhibitor is preferentially taken up by cells with high levels of Glut1 expression and elevated levels of glucose uptake, such as activated NK cells, as well as by other activated immune

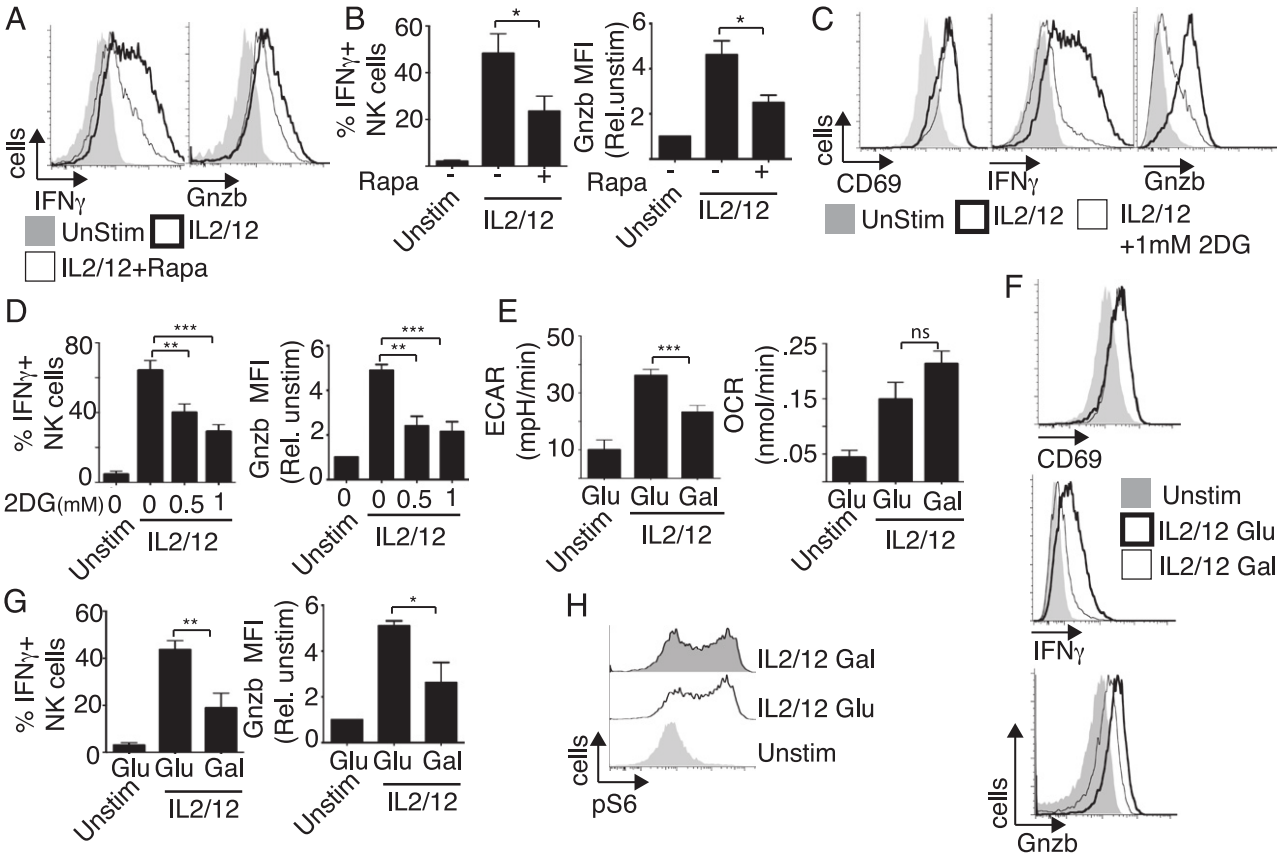

FIGURE 4. Elevated rates of glycolysis are required for normal NK cell effector functions. (**A** and **B**) Cultured NK cells were left unstimulated or treated with IL-2/IL-12, with or without rapamycin, for 18 h and analyzed by flow cytometry for IFN-γ and granzyme B (Gnzb) expression in NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells. Cultured NK cells were either left unstimulated or treated with IL-2/IL-12, with or without 2DG, at the stated concentrations for 18 h and were analyzed by flow cytometry for CD69 (**C**) and IFN-γ and Gnzb (C and **D**) expression in NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells. Cultured NK cells were either left unstimulated in media containing 10 mM glucose or treated with IL-2/IL-12 in media containing glucose (10 mM) or galactose (10 mM) for 18 h and analyzed for rates of glycolysis and OxPhos (**E**), as well as by flow cytometry for levels of CD69 (**F**), IFN-γ and Gnzb (F and **G**), and pS6 (**H**) in NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells. Data are mean ± SEM or are representative of three to five experiments (A–D, F, and G), eight replicates from two separate experiments (E), or three separate experiments (H). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

cell subsets. Administration of 2DG did not affect poly(I:C)-mediated NK cell activation, as measured by CD69 expression (Fig. 6A); however, activated NK cells were smaller in size and had lower rates of glucose uptake in the presence of 2DG, consistent with decreased glycolysis (Fig. 6B–D). Crucially, in vivo 2DG administration significantly reduced IFN-γ production by activated NK cells (Fig. 6E), which is consistent with the observed effect of glycolytic inhibition in ex vivo NK cells (Fig. 4D–G). It is clear that in vivo inhibition of glycolytic cells did not globally disrupt NK cell function, because poly(I:C) induced the expression of TNF-α to equivalent levels, independent of the presence or absence of 2DG (Fig. 6F).

The data presented in this article demonstrate that mTORC1 activity is required for glycolytic reprogramming in activated NK cells and that this is a prerequisite for the acquisition of normal effector functions, such as IFN-γ production and increased granzyme B expression.

Discussion

This study reveals that mTORC1-regulated metabolic reprogramming is central to its immunoregulatory functions in NK cells. As mTORC1 activity was previously demonstrated to be required for elevated levels of glycolysis in activated CD4 Th17 and CD8 cytotoxic T cells (2, 3), our current data extend the characterization of mTORC1 as a key regulator of glycolytic metabolism in an innate lymphocyte subset. Indeed, the metabolic changes described in this study to accompany NK cell activation are similar to those that occur in proinflammatory effector T cell subsets (2, 3, 26, 27). In activated T cells, elevated glycolysis has been linked to normal effector functions, including granzyme B expression by activated CD8 T cells, and inhibition of glycolysis disrupts the expression of IFN-γ in activated CD4 T cells (23, 25). Both activated NK cells and effector T cells, which cannot engage in elevated glycolysis, can maintain energy homeostasis through OxPhos, but it is clear that oxidative metabolism alone is not sufficient to allow for cell growth or to sustain normal effector functions of activated lymphocytes. Therefore, a common theme is emerging that suggests that glycolytic metabolic reprogramming is an essential step for lymphocytes to successfully acquire proinflammatory effector functions.

The exact mechanism linking glycolysis to the expression of these effector molecules is not clear. In CD4 T cells, Gapdh can bind to the AU-rich 3′ untranslated region of IFN-γ mRNA, resulting in mRNA destabilization (25). Consistent with a role for glycolytic enzymes, such as Gapdh, in regulating IFN-γ mRNA in NK cells, limiting the rate of glycolysis decreased the levels of IFN-γ protein expressed by IFN-γ–producing cells. One prediction of this model is that acutely increasing the rate of glycolysis would result in stabilization of IFN-γ mRNA and an increase in IFN-γ protein expression. However, in our experiments, the increased rates of glycolysis that resulted from acute oligomycin treatment had no effect on IFN-γ protein levels, arguing that glycolytic enzymes, such

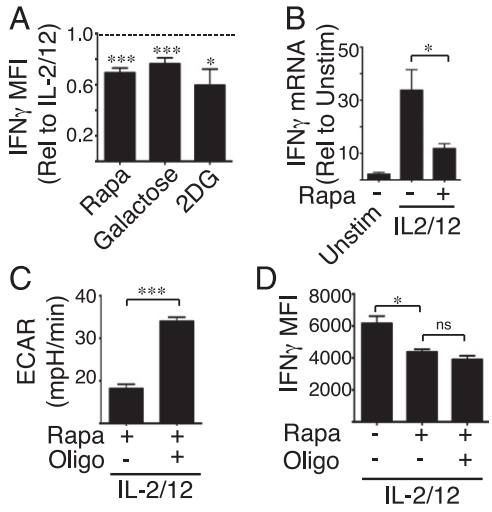

FIGURE 5. Elevated NK cell glycolysis is required for maximal IFN-γ protein expression. (**A**) IL-2/IL-12–stimulated cultured NK cells were treated, with or without rapamycin and with or without 2DG, in the presence of glucose or galactose, as in Fig. 4. The data show the relative levels of IFN-γ MFI in NK cells, with decreased levels of glycolysis compared with the relevant IL-2/IL-12 control (dotted line). (**B**) IFN-γ mRNA expression in cultured NK cells that were left unstimulated or stimulated with IL-2/IL-12, with or without rapamycin, for 18 h. (**C**) Rate of glycolysis (ECAR) in cultured NK cells stimulated with IL-2/IL-12 plus rapamycin before and after the inhibition of ATP synthase with the addition of oligomycin (2 μM). (**D**) Cultured NK cells were activated with IL-2/IL-12 for 18 h, with or without rapamycin, or were activated with IL-2/IL-12 for 14 h before the addition of oligomycin for five additional hours. Data are mean ± SEM from 4–12 experiments (A) or 3 experiments (B–D). $*p < 0.05$, $***p < 0.001$.

as Gapdh, do not have a significant impact upon IFN-γ expression in IL-2/IL-12–activated NK cells. Although our data demonstrate that the levels of granzyme B protein expression are also linked to the rate of glycolysis in NK cells, unlike IFN-γ mRNA the 3′ untranslated region of granzyme B mRNA does not contain an AU-rich region. This further suggests that mechanisms independent of the regulation of mRNA stability are likely to be involved in controlling effector protein expression levels. Together, these data show that mTORC1-regulated glycolysis is critical for promoting IFN-γ expression in activated NK cells, and it also regulates the protein levels of the key NK cell effector molecules IFN-γ and granzyme B and, thus, will have a significant impact upon NK functional outputs.

This study highlights a crucial role for signaling through the high-affinity IL-2R, CD25, in promoting elevated rates of NK cell glycolysis and concomitant enhanced NK cell effector functions. It is becoming clear that NK cells act in parallel with T cells of the adaptive immune system during immune responses to diverse immunological challenges (6–8). Although it is clear that T cells are not essential for NK cell responses (28–31), our research suggests that the adaptive response may enhance NK cell effector function, through the actions of the T cell–derived cytokine IL-2 in promoting elevated NK cell glycolysis. Additionally, given that a key mechanism through which regulatory T cells control NK cell responses is through sequestering IL-2 (9, 32), it seems likely that regulatory T cell–mediated suppression of NK cell effector functions involves decreased IL-2–stimulated levels of NK cell glycolysis. Therefore, the data presented in this article promote the idea that T cell–controlled IL-2 availability may be a factor that impacts upon the regulation of NK cell metabolism and function.

We described a new regulatory axis that is required for the acquisition of normal NK cell effector functions. This has widespread implications for our understanding of NK cell immune responses to viral infection, tumors, and other inflammatory situations. In lymphocytes, mTORC1 is an acute sensor of the immune microenvironment. Among other things, it monitors the availability of nutrients, such as glucose and amino acids (33, 34). Sites of inflammation can become nutrient depleted as a result of competition for nutrients among the large numbers of infiltrating immune cells. Indeed, virally infected cells increase rates of glucose uptake and glycolysis, thereby limiting the levels of glucose available for immune cells in the local microenvironment (35–37). Equally, malignant cells within solid tumors have hugely elevated rates of nutrient uptake and promote nutrient deprivation within the tumor microenvironment (38, 39). Therefore, these environments that are important to the immunological functions of NK cells can limit mTORC1 activity, resulting in reduced glucose uptake and glycolysis in activated NK cells that would ultimately disrupt proinflammatory NK cell functions. Indeed, NK cells isolated from human solid tumors have been found to be defective in their proinflammatory functions, including IFN-γ production and cytotoxicity (40–42).

In summary, the current study demonstrates that normal effector functions of activated NK cells are reliant on successful mTORC1-dependent metabolic reprogramming that increase rates of glucose uptake and glycolysis. This regulatory axis has important implications for NK cell responses within infection and tumor microenvironments.

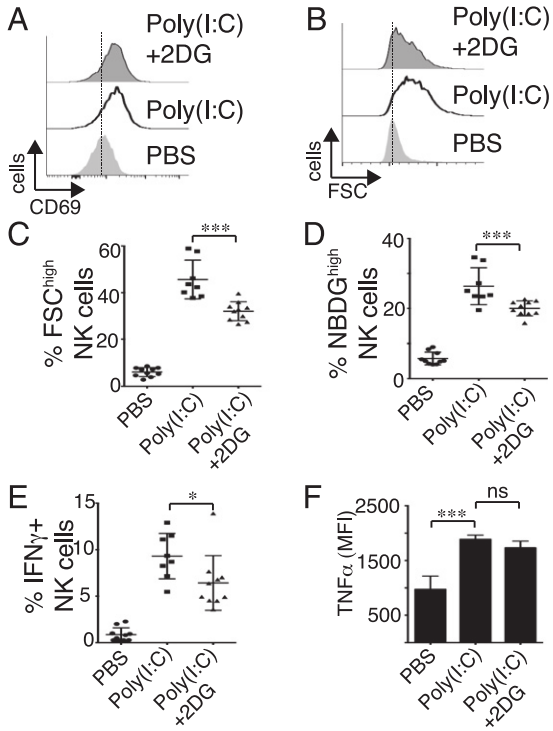

FIGURE 6. Disruption of NK cell glycolysis in vivo inhibits NK cell growth and effector function. (**A**–**F**) Mice were administered PBS or 200 μg poly(I:C) alone or in combination with 2DG (1 g/kg) by peritoneal injection, and spleens were harvested after 24 h. Splenocytes were isolated, and NK1.1$^+$ NKp46$^+$ CD3$^-$ NK cells (or NK1.1$^+$ NKp46$^+$ TCRβ$^+$ NK cells for NBDG experiments) were analyzed for CD69 expression (A), cell size (B and C), glucose uptake (NBDG) (D), IFN-γ (E), and TNF-α production (F). All data are mean ± SEM or are representative of 8–10 mice for each condition from two separate experiments. $*p < 0.05$, $***p < 0.001$.

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Disclosures

The authors have no financial conflicts of interest.

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