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Activation-Specific Metabolic Requirements for NK Cell IFN-γ Production

Molly P. Keppel,* Nermina Saucier,* Annelise Y. Mah,*† Tiphanie P. Vogel,*‡ and Megan A. Cooper*§

There has been increasing recognition of the importance of cellular metabolism and metabolic substrates for the function and differentiation of immune cells. In this study, for the first time to our knowledge, we investigate the metabolic requirements for production of IFN-γ by freshly isolated NK cells. Primary murine NK cells mainly use mitochondrial oxidative phosphorylation at rest and with short-term activation. Remarkably, we discovered significant differences in the metabolic requirements of murine NK cell IFN-γ production depending upon the activation signal. Stimulation of NK cell IFN-γ production was independent of glycolysis or mitochondrial oxidative phosphorylation when cells were activated with IL-12 plus IL-18. By contrast, stimulation via activating NK receptors required glucose-driven oxidative phosphorylation. Prolonged treatment with high-dose, but not low-dose, IL-15 eliminated the metabolic requirement for receptor stimulation. In summary, this study demonstrates that metabolism provides an essential second signal for induction of IFN-γ production by activating NK cell receptors that can be reversed with prolonged high-dose IL-15 treatment. The Journal of Immunology, 2015, 194: 000–000.

Natural killer cells are innate immune lymphocytes that provide a first line of defense against infection, particularly viruses, and can recognize and kill tumor cells that have downregulated self-MHC or express activating ligands (1–3). NK cell effector functions can be triggered by inflammatory cytokines, such as IL-12, IL-15, and IL-18, or by engaging germline-encoded activating NK receptors whose ligands are displayed by infected and/or tumor cells (3–5). In response, NK cells produce inflammatory cytokines, principally IFN-γ, and kill target cells. Although many triggers of NK cell activation and subsequent NK cell effector responses have been well characterized, the metabolic fuels required to drive NK cell functional responses are largely unknown.

Metabolism is the biochemical process used by cells to break down fuels for energy production (i.e., ATP) or to generate critical biomolecules. There are two primary and overlapping metabolic pathways for generating ATP from metabolic fuels, anaerobic glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) (6). Glucose fuels both pathways and is first metabolized in the cytoplasm via glycolysis to produce two molecules of pyruvate, for a net yield of two ATP molecules. Pyruvate can then be converted to lactate (anaerobic metabolism) or, in the presence of oxygen, transported into the mitochondria to fuel OXPHOS for a net yield of 30+ ATP molecules. Other metabolic substrates that can fuel mitochondrial OXPHOS include fatty acids (fatty acid oxidation) and glutamine (glutaminolysis). Metabolic pathways, including glycolysis, are attractive pharmacologic targets for disease therapy, particularly cancers (7, 8). Furthermore, metabolic fuels are globally altered in many disease states such as metabolic syndrome and sepsis, and probably locally altered in certain microenvironments, including sites of infection and tumors (9–11). Thus, it is important to consider the effect of changes in metabolic fuels on the immune system.

Metabolic pathways in immune cells, including dendritic cells, neutrophils, and T cells, have been shown to be critical for cellular activation and differentiation (11–13). For example, differentiation of memory T cells is critically dependent on a switch in energy metabolism from primarily OXPHOS to glycolysis, whereas quiescent memory cells revert back to OXPHOS (11, 14). In CD8+ effector T cells, a glycolytic switch is required for synthesis of IFN-γ protein via the release of a posttranscriptional block in IFN-γ processing (15).

In this study, for the first time to our knowledge, we investigate the basic metabolic requirements for resting NK cell IFN-γ production via two different pathways, cytokine- and receptor-mediated activation. We hypothesized that NK cells would require metabolic fuels for activation and production of IFN-γ. Our results demonstrate that, unlike T cells, NK cells do not require a glycolytic switch for efficient IFN-γ production. Rather, we observed activation-specific metabolic requirements for NK cell IFN-γ production.

Materials and Methods

Mice and NK cell isolation

All mice were on the C57BL/6 background. Wild-type mice were purchased from the National Cancer Institute, and Rag-2−/−γ−/− mice were purchased from Taconic. Mice expressing the congenic CD45.1 receptor...
NK cell proliferation assays

NK cells were labeled with 1 μM CFSE or VioletTrace (Invitrogen) and cultured in 96-well plates with the indicated concentrations of murine IL-15. For in vivo proliferation, splenocytes from CD45.1^+ Rag-1^-/- mice (2-5 × 10^7/mouse) were labeled with CFSE and adoptively transferred by tail vein injection into congenic CD45.2^+ Rag-2^-/-γc^-/- hosts and assayed 3 d later.

Flow cytometric analysis and statistics

Flow cytometric analysis was performed on a Cytek-modified (Cytek Development) eight-color BD FACSscan or BD FACSaria Fusion (BD Biosciences). Analysis was performed using FlowJo software (Tree Star). Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software). Student’s paired t test was used to compare two matched groups, or ANOVA analysis was performed for more than two groups with a p value <0.05 considered significant.

Results

NK cells primarily use glucose-fueled OXPHOS at rest and with activation

To determine the basic metabolic profile of NK cells, we used an extracellular flux assay to measure OCR (a measure of OXPHOS) and ECAR (a measure of lactate and anaerobic glycolysis) of freshly isolated murine splenic NK cells (Fig. 1A, 1B). Baseline metabolic activity of resting splenic NK cells was relatively low, consistent with another recent report (17). At rest, NK cells preferentially use OXPHOS, as shown by the OCR:ECAR ratio. Short-term activation (4–6 h) with cytokines or Abs recognizing the activating receptors NK1.1 or Ly49D did not induce substantial changes in energy pathway usage. NK cell intracellular ATP was also stable following activation with IL-12 plus IL-18 or anti-NK1.1 (Fig. 1C), suggesting that these activation signals do not significantly increase or deplete ATP. Inhibition of OXPHOS with the ATP synthase inhibitor oligomycin, or inhibition of glucose metabolism by 2DG, a competitive inhibitor of glycolysis, significantly reduced ATP in activated NK cells (Fig. 1C). These results suggest that glucose is the primary OXPHOS fuel used during NK cell activation, because blockade of glucose metabolism reduced intracellular ATP to the same degree as global inhibition of OXPHOS. Consistent with this hypothesis, inhibition of fatty acids with etomoxir, a fatty acid oxidation inhibitor that blocks carnitine palmitoyltransferase-1, had no effect on NK cell ATP (Fig. 1C).

OXPHOS is required for receptor-stimulated NK cell IFN-γ

Because NK cells primarily use OXPHOS, we hypothesized that inhibition of this pathway might alter NK cell function. We tested the ability of fresh splenic NK cells to produce IFN-γ when stimulated with cytokines or activating receptors in the presence of increasing concentrations of oligomycin. NK cell survival was intact during all assays, but was significantly reduced if NK cells were cultured in the presence of oligomycin for >12 h (data not shown). Remarkably, treatment with 10 nM oligomycin almost completely abolished receptor-stimulated NK cell IFN-γ production, but had minimal effect on IL-12 plus IL-18–stimulated IFN-γ production (Fig. 2A). Inhibition of receptor-stimulated IFN-γ by oligomycin was dose dependent (Fig. 2B), whereas there was only minimal decrease in IFN-γ production by IL-12 plus IL-18–stimulated NK cells when cultured with up to 1 μM oligomycin (Fig. 2B and data not shown). The mean fluorescence intensity of IFN-γ in cytokine-producing NK cells was similar between untreated and treated cells, suggesting that metabolic inhibition did not alter strength of activation with IL-12 plus IL-18 (Fig. 2C). In addition, there was no decrease in NK1.1 expression with oligomycin that might account for the inability to stimulate NK cells via this receptor (data not shown).
Preservation of IFN-γ response with OXPHOS inhibition was unique to IL-12 plus IL-18 stimulation, because activation with another cytokine combination (IL-12 + IL-15) or with PMA and calcimycin, a stimulus that results in a percentage and strength of IFN-γ response similar to IL-12 plus IL-18, was impaired. However, the most profound defects in NK cell activation were in response to activating receptors, NK1.1 and Ly49D (Fig. 2D, normalized to maximum signal for each stimulus). Similar results were obtained with antimycin, an inhibitor of the electron transport chain and OXPHOS (data not shown), confirming that OXPHOS is critical for receptor-mediated activation of NK cells.

OXPHOS results in the production of reactive oxygen species (ROS), which have been shown to be signaling molecules, including for Ag-specific expansion of T cells and IL-2 production (18). Whereas oligomycin inhibits OXPHOS, it has also been shown to increase ROS (19). We therefore tested whether addition of ROS affected receptor-mediated NK cell activation. There was no change in the percentage of IFN-γ-positive NK cells after NK1.1 stimulation when the culture media was supplemented with ROS (Supplemental Fig. 1) with or without oligomycin. These data suggest that ROS do not inhibit or enhance receptor-mediated NK cell activation.

**FIGURE 1.** Metabolism of resting and activated NK cells. Extracellular flux assays were used to measure resting and activated NK cell OCR, a measure of mitochondrial OXPHOS, and ECAR, a measure of glycolysis. (A) Cytokine activation (4 h) or (B) receptor stimulation (6 h) did not significantly change OCR, ECAR, or the OCR:ECAR ratio. Results represent the mean ± SEM of triplicate wells from three independent experiments. (C) Intracellular ATP (pM/cell) after 6-h culture of NK cells with cytokines or plate-bound anti-NK1.1 in complete media (–) or with the metabolic inhibitors oligomycin (oligo, 1 μM), etomoxir (300 μM), and 2DG (50 mM). Statistics represent the comparison between stimulation alone versus stimulation with the indicated inhibitor for IL-12 plus IL-18 or anti-NK1.1–activated NK cells (one-way paired ANOVA). Results represent the mean ± SEM of triplicate wells from four independent experiments. *p ≤ 0.05, **p ≤ 0.01.
Glucose-dependent NK cell activation

Our findings suggest that metabolism functions as a second signal for receptor activation. We considered the possibility that the differences observed in IL-12 plus IL-18 versus receptor stimulation with metabolic inhibitors were due to the high number of IFN-γ-positive cells with this cytokine combination (>90% IFN-γ+) as compared with receptor stimulation (~30–40% IFN-γ+). NK cells were therefore cultured in low concentrations (1 ng/ml) of IL-12 and IL-18. This resulted in a similar percentage of IFN-γ-producing NK cells as anti-NK1.1 stimulation, but, similar to data with higher dose cytokines, there were no defects in IFN-γ production with oligomycin (Fig. 3A).

Because we observed minimal effect of OXPHOS inhibition, even with low-dose IL-12 plus IL-18 activation, we hypothesized...
that NK cells might require aerobic glycolysis to respond to this stimulus. However, culture of NK cells in glucose-free media resulted in minimal decreases in IFN-γ-producing NK cells with low-dose IL-12 plus IL-18, but significant defects with anti-NK1.1 (Fig. 3A) or anti-Ly49D (Supplemental Fig. 2). In contrast to OXPHOS inhibition, glycolytic inhibition more selectively inhibited receptor-stimulated IFN-γ because there were only modest decreases in PMA/calcimycin or IL-12 plus IL-15 activation (Supplemental Fig. 2). We tested the ability of NK cells to use other OXPHOS fuels by extracellular flux assay. Indeed, inhibition of glucose metabolism with 2DG resulted in decreased glycolysis (measured by ECAR) with compensatory increased nonglucose-fueled OXPHOS (measured by OCR) (Supplemental Fig. 2). However, inhibition of alternative OXPHOS fuels, fatty acids and glutamine, had minimal effect on receptor activation (Supplemental Fig. 3). These findings suggest that glucose is the primary OXPHOS fuel required for receptor activation.

**IL-12 plus IL-18 stimulation is metabolism independent**

Having confirmed that IL-12 plus IL-18 stimulation is independent of glucose metabolism or OXPHOS at low- or high-dose stimulation, we next evaluated whether cytokines, but not receptors, induce metabolic plasticity and allow NK cells to efficiently use either glycolysis or mitochondrial OXPHOS. To test this, NK cells were cultured with inhibitors of both glycolysis and OXPHOS. Because ATP is required for cell survival, NK cells did not live when cultured in the presence of both oligomycin and 2DG (data not shown). Therefore, NK cells were activated with low concentrations of IL-12 plus IL-18 in the presence of two different doses of the glycolytic inhibitor 2DG (10 or 50 mM) plus etomoxir in glutamine-free media to simultaneously block glycolysis and the other two major mitochondrial OXPHOS fuels (Fig. 3B). Consistent with the hypothesis that NK cells can produce IFN-γ relatively independent of metabolism, NK cell IFN-γ production with IL-12 plus IL-18 was completely preserved when both glycolysis and each of the other major OXPHOS fuels, fatty acids or glutamine, were blocked (Fig. 3B). By contrast, inhibition of glycolysis and the other major fuels of OXPHOS led to a significant impairment in anti-NK1.1–mediated NK cell activation (Fig. 3C), which was not significantly different from oligomycin or glucose-free media alone (Fig. 3A, 3C).

We next tested whether IL-12 plus IL-18 induced a glycolytic switch upon treatment with oligomycin that was absent in receptor-stimulated NK cells. Oxygen consumption (OCR) decreased and glycolysis (ECAR) increased with oligomycin treatment, as expected (Fig. 4A–D). However, there was no difference in OCR or ECAR readings from oligomycin-treated NK cells with or without stimulation (IL-12 plus IL-18 or NK1.1). Similarly, the OCR: ECAR ratios of oligomycin-treated cells stimulated with IL-12 plus IL-18 or anti-NK1.1 were equivalent (Fig. 4E). Together, these data suggest that IL-12 plus IL-18 does not induce significant metabolic plasticity that would account for the observed metabolism-independent activation.

**IL-12 plus IL-18 activation of NK cell IFN-γ production is transcriptionally regulated**

To determine whether OXPHOS inhibition affected receptor-mediated transcription of *Ifng*, we measured levels of transcript in IL-12 plus IL-18 or anti-NK1.1–stimulated NK cells with or without oligomycin at different time points by quantitative RT-PCR (Fig. 5A, 5B). There was delayed transcription of *Ifng* in low-dose IL-12 plus IL-18–stimulated NK cells with oligomycin, which normalized by 4 h (Fig. 5A). However, whereas the percentage and mean fluorescence intensity of IFN-γ–producing NK cells were similar between low-dose IL-12 plus IL-18 and anti-NK1.1–activated NK cells at 6 h (Fig. 5C and data not shown), receptor stimulation resulted in very little upregulation of *Ifng* transcript (Fig. 5B). The low levels of *Ifng* transcription were delayed with oligomycin in NK1.1-activated NK cells, and, similar to cytokine stimulation, normalized by 4 h. These data suggest that production of IFN-γ protein is transcriptionally regulated with cytokine activation, but posttranscriptionally regulated with receptor stimulation. Consistent with this hypothesis, IL-12 plus IL-
18–stimulated NK cells had delayed production of IFN-γ protein with oligomycin, which normalized by 6 h. By contrast, there was very little production of IFN-γ protein with oligomycin treatment of anti-NK1.1–stimulated NK cells, even at 6 h, when transcript levels of Ifng were the same. Thus, although oligomycin had a similar effect on Ifng transcription for both stimuli (i.e., delayed upregulation), there are significant differences in the transcriptional and posttranscriptional control of IFN-γ protein production between the two stimuli.

Short-term cytokine priming cannot compensate for the absence of an OXPHOS signal for receptor activation

To determine whether IL-12 or IL-18 could prime cells to respond to receptors independent of metabolism, fresh NK cells were pretreated with different combinations of cytokines, followed by activation with anti-NK1.1 with or without oligomycin (Fig. 6A). All cells were cultured with IL-15 (10 ng/ml low dose or 100 ng/ml high dose) to maintain survival. Although the baseline IFN-γ production with NK1.1 stimulation was higher with...
cytokine priming, there was still a defect in activation in the presence of oligomycin (Fig. 6A). Similar results were obtained when cytokines were added at the start of receptor stimulation (data not shown). Thus, these results suggest that receptor-mediated activation of NK cells requires a metabolically derived second signal that is not required for, or stimulated by, IL-12 or IL-18 activation.

NK cell metabolic requirements for receptor stimulation are reversed with prolonged IL-15 priming

IL-15 is critical for NK cell differentiation and survival and also primes NK cells for cytotoxicity and in vivo effector functions (4, 20–22). Long-term treatment with high-dose IL-15 or IL-2 differentiates lymphokine-activated killer cells that have enhanced antitumor responses (23). We hypothesized that one mechanism by which IL-15 might enhance and prime NK cell function is by altering the metabolic requirements for NK cell activation. Indeed, following 72-h culture with 100 ng/ml IL-15, NK cells had no defect in IFN-γ production with OXPHOS inhibition in response to cytokine, receptor, or PMA and calcimycin activation (Fig. 6B). A small difference in receptor stimulation without glucose persisted, and activation continued to be independent of fatty acid oxidation or glutamine (Fig. 6C). High-dose, 100 ng/ml, IL-15 was required to induce metabolism-independent activation, as lower-dose (10 ng/ml) IL-15 had no effect on the metabolic requirement for receptor activation (Fig. 6D). Short-term stimulation was not sufficient to induce this effect, because there was no change in the metabolic requirements after overnight treatment with 100 ng/ml IL-15 (Fig. 6A), and at least 48 h of stimulation was required (data not shown).

One major difference between stimulation with 10 versus 100 ng/ml IL-15 is that cells proliferate only with the higher dose (data not shown). Thus, it is possible that the changes in metabolic requirements were due to proliferation and not IL-15 treatment. However, when comparing IFN-γ production by the least-divided versus most-divided NK cells, as marked by CFSE dilution, both populations produced normal amounts of IFN-γ with oligomycin, and, in fact, we consistently observed higher IFN-γ production in undivided cells (Fig. 6E). Furthermore, adoptive transfer of CFSE-
labeled splenocytes into alymphoid Rag2−/− γc−/− hosts and induction of homeostatic proliferation had no effect on the metabolic requirement for IFN-γ production (Supplemental Fig. 4). We also tested whether the TLR3 analog poly(I:C), which upregulates dendritic cell IL-15/IL-15rα (4), could prime NK cell metabolic independence. However, defects persisted in receptor stimulation of metabolically inhibited NK cells after in vivo activation overnight with poly(I:C) (Supplemental Fig. 4D), although to a lesser degree than naive NK cells.

Finally, we evaluated whether treatment with high-dose IL-15 affected NK cell metabolism. Indeed, NK cells significantly up-regulated OXPHOS and glycolysis as measured by OCR and ECAR (Fig. 6F) after culture in high-dose IL-15, consistent with another recent study (17). Although both metabolic pathways increased, there was a decreased dependence on OXPHOS in IL-15–treated NK cells, as measured by a decreased ratio of OCR:ECAR.

Discussion
Metabolism is critical for a wide array of cellular functions and has been increasingly recognized to be important for immune cell function (12). To our knowledge, this represents the first study to investigate the metabolic fuels used by fresh NK cells for production of IFN-γ. Remarkably, we demonstrate that a metabolism-driven second signal is required for receptor-mediated, but not IL-12 plus IL-18–stimulated, activation of NK cell IFN-γ.

NK cells have the ability to rapidly produce large amounts of IFN-γ protein within hours of activation. Surprisingly, NK cells were relatively metabolically inactive at baseline and had no significant increase in their OXPHOS or glycolysis after short-term activation, as measured by an extracellular flux assay. However, despite no increased metabolic activity with stimulation, inhibition of glucose/glycolysis or global OXPHOS inhibition resulted in near-complete abrogation of receptor-stimulated IFN-γ production. The receptors tested in this study, NK1.1 and Ly49D, both partner with ITAM-bearing adapters (24), suggesting that OXPHOS is a requisite second signal for ITAM-mediated IFN-γ production. Inhibition of other OXPHOS fuels, including glutamine or fatty acid oxidation, had no effect on receptor-stimulated IFN-γ production, and glucose appears to be the primary fuel required to drive OXPHOS.

The activation-specific metabolic requirements for NK cell IFN-γ production shown in this work are quite distinct from T cells, which require glycolysis, but not mitochondrial OXPHOS, for production of IFN-γ in response to receptors, PMA and ionomycin, or IL-12 plus IL-18 (15, 25). In this study, we discovered a major difference in the transcriptional upregulation of Ifng between cytokine- and receptor-stimulated NK cells. It is well described that NK cells constitutively express Ifng transcript, but not protein (26). Thus, it is likely that one mechanism by which metabolic inhibition impairs receptor-stimulated, but not IL-12 plus IL-18–stimulated, IFN-γ protein production is by inhibition of translation of pre-existing transcript. In CD4 T cells, glycolysis was shown to be important for the posttranscriptional processing of IFN-γ due to consumption of the glycolytic enzyme GAPDH, which was otherwise bound to the 3' untranslated region of IFN-γ transcript (15). However, in contrast to our findings in this work, inhibition of OXPHOS with oligomycin had no effect on receptor-stimulated T cell production of IFN-γ (15). Thus, consumption of GAPDH is unlikely to explain our observed dependence on both glycolysis and OXPHOS for NK cell receptor-stimulated IFN-γ production. The results in this work suggest distinct cell-specific regulation of IFN-γ protein production by innate and adaptive effector cells. However, one caveat to this conclusion is that NK cells were studied directly ex vivo in this work, whereas prior work with T cells was performed after in vitro culture/activation; thus, it is possible that activation alters T cell metabolic requirements for IFN-γ production.

Prolonged culture of NK cells with high-dose IL-15 led to increased NK cell metabolism and particularly glycolysis, as measured by a decrease in the OCR:ECAR ratio. Cells cultured for >48 h in high-dose IL-15 no longer required OXPHOS for receptor-stimulated IFN-γ production, an effect that was dose dependent and did not require proliferation. Interestingly, IL-15–stimulated NK cells still demonstrated some dependence on glucose for IFN-γ production. Perhaps this is a reflection of their overall increased dependence on glycolysis for cellular metabolism, or, alternatively, an indication that they have switched to a regulatory mechanism more similar to T cell control of IFN-γ posttranscriptional processing. Alterations of the metabolic requirements of NK cell activation with IL-15 are clinically relevant, because there are ongoing clinical trials of IL-15 treatment of NK cells prior to adoptive immunotherapy, as well as administration of IL-15 for cancer therapies targeting NK cells (27). Our data would suggest that, in addition to priming and expansion of NK cells, IL-15 may also impart NK cells with enhanced functionality in metabolically deprived locations such as tumor microenvironments.

IL-15 is known to prime NK cell effector functions, including cytokine production and cytotoxicity (4, 20). Recently, the Walzer laboratory demonstrated that high-dose IL-15 activates mTOR, which stimulates NK cell glucose uptake, proliferation, and cytolytic responses (17). Similarly, Nandagopal et al. (28) reported that IL-15–induced mTOR is important for NK cell IFN-γ production when costimulated with this cytokine. mTOR is an important metabolic regulator of T cell function and upregulates glucose uptake and glycolysis in activated T cells (29). Another recent study also demonstrated a dependence on glycolysis, but not OXPHOS, for IL-12 plus IL-2–stimulated IFN-γ production by NK cells cultured in IL-15 for 7 d, and implicated IL-15–induced mTOR for glycolytic programming of NK cells (30). Thus, IL-15–induced mTOR may represent a potential mechanism whereby NK cells cultured with high-dose IL-15 upregulate glycolysis. However, it remains unclear how prolonged high-dose IL-15 signaling and increased glycolysis alter the OXPHOS requirement for receptor-mediated signaling, as shown in this work.

In summary, these findings demonstrate that NK cell IFN-γ responses are dictated by the metabolic environment in an activation-specific manner. This suggests that NK cell function in vivo will be affected by the availability of metabolic fuels and that drugs targeting metabolism, such as glycolytic inhibitors, will impact NK cell activation. The finding that prolonged high-dose IL-15 alleviates the metabolic requirement for receptor-mediated NK cell stimulation suggests a potential mechanism to reverse the metabolic dependence of receptor activation. Additional investigation into the glucose-driven OXPHOS signal that is required for receptor signaling, and how this signal interacts with known receptor signaling pathways, will be important (31). Finally, it will be interesting to explore the metabolic requirements for other NK cell functions, including cytotoxicity, licensing, and generation of memory NK cells (32–34).

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Disclosures
The authors have no financial conflicts of interest.
References


Supplemental Figure 1. Addition of reactive oxygen species does not alter receptor-mediated NK cell activation. Enriched splenic NK cells were stimulated with plate-bound anti-NK1.1 for 6h and NK cell production of IFN-γ measured by intracellular flow cytometry. A) NK cells were activated in the presence of oligomycin (100nM) and/or H$_2$O$_2$ (1µM). NK cell survival was intact with this optimized dose of H$_2$O$_2$, but cell death was observed with increasing concentrations of H$_2$O$_2$ (not shown). B) Stimulation of NK cells in the presence of oligomycin and/or galactose (Gal, 500µM) plus galactose oxidase (GAO, 0.045U/mL) which generates H$_2$O$_2$ (reference 18).
Supplemental Figure 2. Glucose is required for receptor-stimulated IFN-γ and inhibition of glucose metabolism leads to upregulation of OXHPOS. (A) NK cells were stimulated for 4-6h with anti-Ly49D, PMA + calcimycin (PMA+CA), or IL-12+IL-15 in complete (--) or glucose-free (GF) media and IFN-γ measured by flow cytometry. **p≤0.007. Results represent the mean +/-SEM of 3 independent experiments. (B-D) Freshly isolated enriched murine splenic NK cells were assayed for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by extracellular flux assay before and after addition of the glycolytic inhibitor 2DG (50mM, shown by the arrow). Results represent the mean +/- SEM of 3 replicate wells at each timepoint and are representative of 4 independent experiments.
Supplemental Figure 3. Fatty acid oxidation or L-glutamine are not required for receptor-stimulated IFN-γ production. Enriched NK cells were stimulated for 6hr with receptors (anti-NK1.1 or anti-Ly49D) (A) in the presence or absence (--) of etomoxir, a fatty acid oxidation inhibitor, or (B) with limiting concentrations of L-glutamine. *p<0.05; **p≤0.007. Results represent the mean +/-SEM of 3 independent experiments.
Supplemental Figure 4. Metabolic in inhibition of NK cell receptor-stimulated IFN-γ after in vivo proliferation or activation with poly(I:C). (A-C) CFSE-labeled splenocytes from Rag-1−/− mice were adoptively transferred into Rag-2−/-g-/- hosts and harvested 3 days later. (A) Representative flow plot of Ly5.1+ adoptively transferred NK cells (NKp46+) and CFSE dilution. (B & C) NK cells were stimulated with anti-NK1.1 in the absence or presence of oligomycin (oligo, 100nM). Results represent the mean +/-SEM of 8 mice from 3 independent experiments. (D) Wt mice were treated with poly(I:C) (black bars) or control PBS (white bars). Splenocytes were stimulated 14-16h later with plate-bound anti-NK1.1 without or with oligomycin (1nM, 10nM, or 100nM) or in glucose free media (GF). Results represent the mean +/-SEM of 7 independent experiments with 2-3 mice per group. Statistics represent comparison between anti-NK1.1 versus each inhibitor for control-treated or poly(I:C) treated splenocytes. *p<0.05, **p≤0.01, ****p≤0.0001.