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Metabolic Reprogramming Supports IFN-γ Production by CD56^{bright} NK Cells

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Human NK cells can be classified into phenotypically and functionally distinct subsets based on levels of CD56 receptor. CD56^{dim} cells are generally considered more cytotoxic, whereas the CD56^{bright} cells are potent producers of IFN-γ. In this study, we define the metabolic changes that occur in peripheral blood NK cells in response to cytokine. Metabolic analysis showed that NK cells upregulate glycolysis and oxidative phosphorylation in response to either IL-2 or IL-12/15 cytokine combinations. Despite the fact that both these cytokine combinations robustly upregulated mammalian Target of Rapamycin Complex 1 in human NK cells, only the IL-2–induced metabolic changes were sensitive to mammalian Target of Rapamycin Complex 1 inhibition by rapamycin. Interestingly, we found that CD56^{bright} cells were more metabolically active compared with CD56^{dim} cells. They preferentially upregulated nutrient receptors and also differed substantially in terms of their glucose metabolism. CD56^{bright} cells expressed high levels of the glucose uptake receptor, Glut1 (in the absence of any cytokine), and had higher rates of glucose uptake compared with CD56^{dim} cells. Elevated levels of oxidative phosphorylation were required to support both cytotoxicity and IFN-γ production in all NK cells. Finally, although elevated glycolysis was not required directly for NK cell degranulation, limiting the rate of glycolysis significantly impaired IFN-γ production by the CD56^{bright} subset of cells. Overall, we have defined CD56^{bright} NK cells to be more metabolically active than CD56^{dim} cells, which supports their production of large amounts of IFN-γ during an immune response. ***The Journal of Immunology*****, 2016, 196: 2552–2560.**

Natural killer cells are lymphocytes with important roles in cancer and in the immune response to infection (1). Although NK cells are generally considered part of the classical innate immune system, evidence is emerging that they regulate and respond to the adaptive immune response and continue to function as effector cells. In humans, at least two functional and phenotypically NK cell subsets, defined based on expression levels of CD56, coexist in peripheral blood (reviewed in Ref. 2). Although there is some evidence that CD56^{bright} cells may be precursors of the more mature CD56^{dim} subset (3, 4), distinct phenotypic and functional differences between these subsets suggest that both are likely to play important roles during the NK cell immune response (2). In brief, CD56^{dim} cells predominate in peripheral blood and are strongly cytotoxic using a variety of mechanisms (5, 6). In contrast, the CD56^{bright} cells are predominantly found in secondary lymphoid organs and are not strongly cytotoxic (2, 7). They lack Killer cell Ig-like receptors, and only approximately half of them express CD16, an Fc receptor that enables Ab-dependent cellular cytotoxicity. CD56^{bright} cells have high levels of cytokine receptors (IL-2R, IL-12R, IL-18R) and are highly responsive to cytokines in terms of upregulating IFN-γ production (8–11). In fact, CD56^{bright} cells produce significantly more IFN-γ per cell than do CD56^{dim} cells (12). Understanding how CD56^{bright} cells are equipped for their specialized functions is important not only in terms of understanding the basic biology of these cells and their ontogeny as it relates to CD56^{dim} cells, but it will also inform optimal conditions for ex vivo culture of immunocompetent NK cells for immunotherapy.

Dynamically regulated cellular metabolism is now recognized as an important factor that contributes to a successful immune response (13). Metabolism is important to maintain energy homeostasis and to supply cells with the building blocks for macromolecular synthesis, but cellular metabolism can also directly influence immune cell function and differentiation (14, 15). Different immune cell subsets have very different metabolic demands that are accommodated by different types of glucose metabolism. Some lymphocytes predominantly use mitochondrial oxidative phosphorylation (OxPhos) to efficiently generate ATP, a process that requires oxygen. In contrast, effector lymphocyte subsets metabolize large amounts of glucose by aerobic glycolysis, a process in which glucose is metabolized to lactate in the presence of oxygen. This is a metabolic signature that is common to highly proliferative cells because it provides biosynthetic precursors for the synthesis of nucleotides, amino acids, and lipids. Aerobic glycolysis can also directly impact upon the functions of

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Abbreviations used in this article: ECAR, extracellular acidification rate; GnzB, granzyme B; MFI, mean fluorescence intensity; mTORC1, mammalian target of rapamycin complex 1; NBDG, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; OCR, oxygen consumption rate; OxPhos, oxidative phosphorylation; pS6, phosphorylated S6 ribosomal protein.

effector lymphocytes. We have recently reported that cytokines upregulate glucose metabolism in expanded murine NK cells and that rates of both glycolysis and OxPhos are increased in these activated cells (16). The mammalian target of rapamycin complex 1 (mTORC1) was important for both metabolic and functional changes in these activated NK cells, and elevated glycolysis directly affected multiple key NK cell effector functions. Because there are important differences between murine and human NK cells including potential for immunotherapy, divergent receptor systems, and intrinsic genetic and phenotypic variability (17, 18), we undertook experiments to investigate whether cytokines induced changes in metabolism in human NK cells and whether these affected their immune functions.

Materials and Methods

Cell isolation and cell culture

Blood samples were obtained from normal healthy donors from whom written consent had been obtained. PBMCs were isolated by Lymphoprep (Axis-Shield) gradient. For Seahorse experiments, NK cells were purified using an NK isolation Kit II (Miltenyi Biotec) as per manufacturer's instructions; purity was routinely >95% $CD56^{+}CD3^{-}$ NK cells.

Unless stated otherwise, 5×10^6 cells/ml PBMCs were incubated at 37°C for 18 h in RPMI 1640 GlutaMAX medium (Life Technologies, Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin (Invitrogen), and with IL-2 (500 U/ml) or IL-12 (30 ng/ml) and IL-15 (100 ng/ml). Where indicated, cells were cultured with or without the inhibitors rapamycin (20 nM) or oligomycin (40 nM), or cultured with 721.221 target cells for 4 hours, or galactose was substituted for glucose as previously described (16).

Flow cytometry analysis

Cells were stained for 30 min at 4°C with saturating concentrations of titered Abs CD56(HCD56/NCAM16.2) CD3(SK7/UCHT1) granzyme B(GB11); IFN-γ(B27), CD71(M-A172); CD69(L78); CD98(UM7F8); NKp44(p44-8.1); TRAIL(RiK-2); CD107a(H4A3) (eBioscience or BD Pharmingen). Analysis was performed using the gating strategy shown (Supplemental Fig. 1) and FlowJo software (Tree Star). Glut1 RBD ligand (Metafora Biosystems), glucose uptake assay using NBDG (Life Technologies), and phosphorylated S6 ribosomal protein (pS6) (Cell Signaling Technologies) staining were as previously described (16, 19).

Oxygen consumption rate and extracellular acidification rate measurement

XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure extracellular acidification rates (ECARs) and oxygen consumption rates (OCR) of purified NK cells as previously described (16).

Statistical analysis

GraphPad Prism 6.00 (GraphPad Software) was used for statistical analysis. Data were tested and if a nonnormal distribution was found, a nonparametric test was used. If there were insufficient numbers to test normality, a nonparametric test was also used. In general, a nonparametric one-way ANOVA test was used with the Kruskal–Wallis post hoc test. A paired or unpaired Student *t* test was used as appropriate when there were only two data sets for comparison.

Results

$CD56^{bright}$ NK cells preferentially upregulate metabolism in response to cytokine

We have recently reported that murine NK cells upregulate metabolism in response to cytokine stimulation and that glycolysis

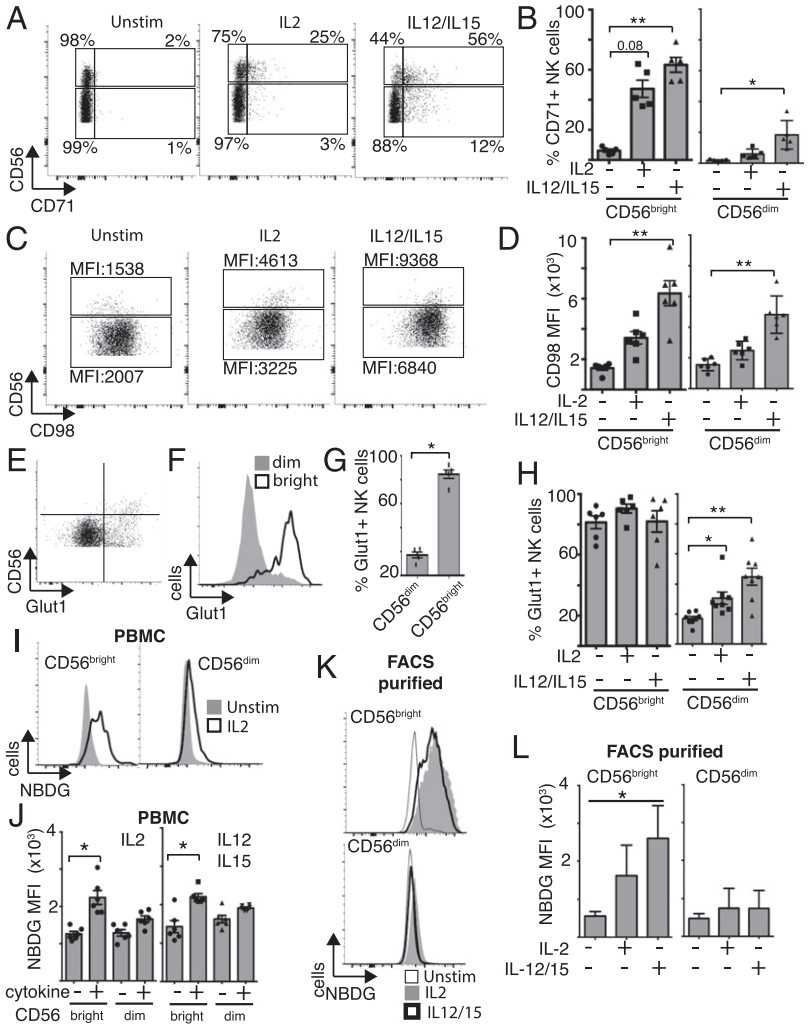

FIGURE 1. $CD56^{bright}$ NK cells are more metabolically active than $CD56^{dim}$ cells in response to cytokine. PBMCs stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. (**A** and **B**) Representative dot plots (A) and pooled data (B) (stratified into $CD56^{bright}$ and $CD56^{dim}$ subsets) of NK cells expressing CD71, the transferrin receptor, are shown. (**C** and **D**) Representative dot plots (C) and pooled data (D) (stratified into $CD56^{bright}$ and $CD56^{dim}$ subsets) of NK cells expressing CD98, a component of the L-amino acid transporter, are shown. (**E**) Representative dot plot of Glut1 expression on unstimulated NK cells is shown. (**F** and **G**) Representative histogram (F) and pooled data (G) of Glut1 expression on $CD56^{bright}$ and $CD56^{dim}$ NK subsets. (**H**) Glut1 expression on cytokine-stimulated $CD56^{bright}$ (*left*) and $CD56^{dim}$ (*right*) NK subsets. (**I** and **J**) Representative histograms (I) and pooled data (J) of glucose uptake by $CD56^{bright}$ and $CD56^{dim}$ NK subsets analyzed using the fluorescent glucose analog, NBDG. (**K** and **L**) $CD56^{dim}$ and $CD56^{bright}$ cells were sorted from freshly isolated PBMCs and stimulated overnight with IL-2 or IL-12/15, as described earlier. After 18 h, glucose uptake was measured using NBDG ($n = 3$). Data shows representative histograms (K) and pooled data (L). Data are mean ± SEM for six donors (A–D), five to eight donors (E–H), and three donors (I–L). Samples were compared using a nonparametric one-way ANOVA or a Student *t* test as appropriate. $*p < 0.05$, $**p < 0.01$. ns, not significant.

impacts on IFN-γ production by these cells (16). To investigate whether human NK cells behave in similar fashion, we examined cellular metabolism in human primary NK cells in response to either IL-2 or IL-12/15 combination. Nutrient receptor expression was increased in cytokine-stimulated NK cells. CD71, the transferrin receptor, was absent or expressed at very low levels on resting NK cells and increased in expression in response to cytokine (Fig. 1A). Interestingly, CD71 was preferentially upregulated on the CD56^{bright} subset of NK cells, but only on a subset of CD56^{dim} NK cells (Fig. 1A, 1B). CD98, a component of the L-amino acid transporter, was expressed on all NK cells, and expression levels increased in response to cytokine (Fig. 1C, 1D); however, cytokine-stimulated CD56^{bright} cells expressed more CD98 than CD56^{dim} cells.

In terms of glucose metabolism, we measured expression of Glut1, the glucose transporter thought to be primarily involved in supporting aerobic glycolysis in lymphocytes (20). Without cytokine, CD56^{dim} NK cells had relatively low levels of Glut1, but strikingly, CD56^{bright} cells expressed high levels of this glucose transporter (Fig. 1E–G). Because levels of Glut1 were high in the CD56^{bright} cells, this did not change substantially after cytokine stimulation. However, both IL-2 and IL-12/15 cytokine combinations significantly upregulated Glut1 on CD56^{dim} NK cells (Fig. 1H). Glucose uptake was measured using the fluorescent glucose analog 2-(*N*-(7-bitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (NBDG). Cytokine-stimulated CD56^{bright} NK cells, but not CD56^{dim} NK cells, significantly increased the rate of glucose uptake (Fig. 1I, 1J). These data suggest that CD56^{bright} cells have the machinery in place to facilitate a more rapid glucose uptake response after cytokine stimulation than their CD56^{dim} counterparts.

Because culture with cytokine can increase CD56 expression levels on human NK cells, we repeated the experiment with sorted CD56^{dim} and CD56^{bright} cells from PBMCs. Sorted NK cell subsets were stimulated overnight with cytokine, and glucose uptake (NBDG assay), as a direct readout associated with increased glycolysis, was measured. Although there were relatively modest increases in CD56^{dim} cells, CD56^{bright} cells increased glucose uptake dramatically, particularly in response to IL-12/IL-15 cytokine combination (Fig. 1K, 1L). These data directly demonstrate preferential glucose uptake in CD56^{bright} cells compared with CD56^{dim} NK cells.

mTORC1 activity is induced in cytokine-activated NK cells

Our previous research found that mTORC1 was activated in murine NK cells after stimulation with cytokine (16). Given that mTORC1 is a key metabolic regulator, we investigated whether mTORC1 was activated in human NK cells in response to these different cytokine combinations. Furthermore, in light of our data on metabolic changes in response to cytokines (Fig. 1), we stratified NK cells based on CD56 expression. mTORC1 activity in NK cells was determined by measuring pS6, a downstream target of mTORC1 signaling. pS6 levels were significantly elevated in both CD56^{bright} and CD56^{dim} NK cells in response to either IL-12/15 or IL-2 cytokine stimulations (Fig. 2A–D). These pS6 levels were substantially inhibited by the mTORC1 inhibitor, rapamycin, demonstrating that mTORC1 is active in these cells (Fig. 2A, 2C). Rapamycin treatment decreased the expression of CD71 in CD56^{bright} cells, the subset that predominantly expresses this surface receptor (Fig. 2E, 2F). In addition, rapamycin significantly abolished the IL-2 (Fig. 2G, 2H) and IL-12/15–induced (Fig. 2I, 2J) increases in CD98 expression in CD56^{bright} and CD56^{dim} NK cells. These data demonstrate that mTORC1 can regulate nutrient receptor expression on human NK cell, as previously seen in murine NK cells (16, 21).

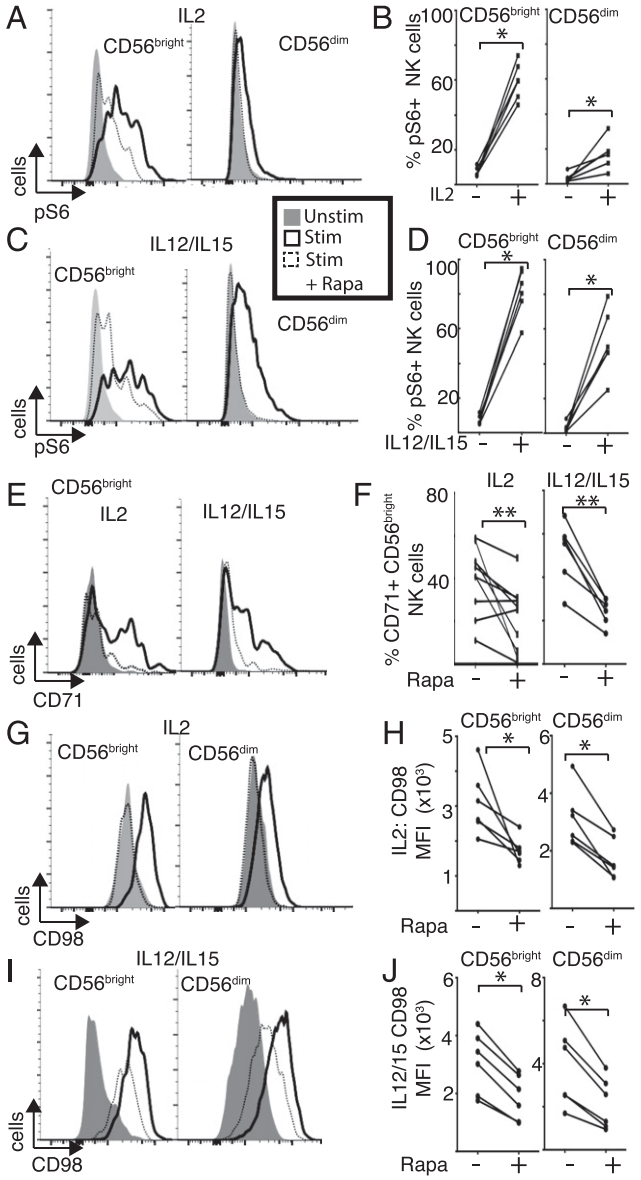

FIGURE 2. Cytokines activate mTORC1 in human NK cells, which controls nutrient receptor expression. PBMCs were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. Cells were then analyzed for pS6 levels by intracellular flow cytometry staining. (**A**–**D**) Representative histograms (A and C) and pooled data are shown for IL-2– or IL-12/15–stimulated NK cells stratified into CD56^{bright} and CD56^{dim} (B and D) subsets. (A and C) Some NK cells were treated with rapamycin for the final 20 min of stimulation at 37˚C to provide a negative control of pS6 levels in the absence of mTORC1 activity. (**E** and **F**) Representative histograms of IL-2– or IL-12/15–induced expression of CD71 expression on CD56^{bright} cells (E) and paired responses with or without rapamycin (F) are shown. (**G**) Representative histograms of IL-2–induced expression of CD98 expression on CD56^{bright} and CD56^{dim} NK cells and paired responses with or without rapamycin (**H**) are shown. (**I**) Representative histograms of IL-12/15–induced expression of CD98 expression on CD56^{bright} and CD56^{dim} NK cells and paired responses with or without rapamycin (**J**) are shown. Data are mean ± SEM of $n = 6$ donors (A–D), $n = 10$ and 6 donors for IL-2 and IL-12/15, respectively (E and F), and $n = 6$ donors (G–J). Samples were compared using either a one-way ANOVA followed by a Kruskal–Wallis test or a paired Student t test analysis as appropriate. $^*p < 0.05$, $^{**}p < 0.01$.

Cytokines upregulate glycolysis and OxPhos in human NK cells

Given the increased expression of Glut1 and glucose uptake in CD56^{bright} and a subset of CD56^{dim} NK cells, we undertook a detailed analysis of glucose metabolism. Because mTORC1 is well characterized in murine cells to be a key regulator of glucose metabolism (16, 22), we also investigated its importance in the metabolic changes observed. Purified NK cells were stimulated with cytokines overnight in the presence or absence of rapamycin, before metabolic analysis. One caveat of these analyses is that the high number of cells required precluded independent analysis of CD56^{bright} and CD56^{dim} subsets, and results reflect changes in the overall NK cell population. IL-2 and IL-12/15 stimulations both increased the rate of glycolysis in NK cells to similar levels (Fig. 3A–C). Although the observed ECAR values are quite low compared with those published for other activated lymphocytes (16, 21, 23), this likely reflects the heterogeneous nature of NK cells as discussed earlier. Interestingly, rapamycin inhibited increases in glycolysis induced by IL-2, but not by IL-12/15 stimulation (Fig. 3A–C).

Because glucose uptake is a relatively accurate measure of levels of glycolysis, we used NBDG uptake to confirm, on a single-cell basis, the requirement for mTORC1 signaling for glucose metabolism in NK cell subsets. Rapamycin treatment abolished the elevated levels of glucose uptake observed in IL-2–stimulated CD56^{bright} NK cells (Fig. 3D, 3E). Although the increase in glucose uptake in IL-2–stimulated CD56^{dim} NK cell was minimal, rapamycin nonetheless significantly decreased these rates. However, there were no changes in IL-12/15–induced glucose uptake in either CD56^{dim} or CD56^{bright} subset following rapamycin treatment (Fig. 3F, 3G). These data argue for an important role for mTORC1 signaling in promoting elevated levels of glucose uptake and glycolysis in the CD56^{bright} subset of NK cells after IL-2, but not IL-12/15, cytokine stimulation.

The maximum glycolytic rates for cytokine-stimulated NK cells were also determined. Cytokines increased the glycolytic capacity up to 5-fold, indicating increased expression of glycolytic machinery, a process termed glycolytic reprogramming (Fig. 3H). Although both IL-2 and IL-12/15 increased the glycolytic capacity equivalently, IL-2 but not IL-12/15–stimulated glycolytic capacity was significantly decreased by rapamycin treatment.

The rate of mitochondrial OCR, which represents OxPhos levels, was also measured. Both cytokine stimulations increased the OCR (Fig. 3I). However, changes in OxPhos were insensitive to rapamycin. Taken together, these data show that cytokine-activated NK cells undergo metabolic changes to increase both rates of glycolysis and OxPhos. However, it appears that IL-2–induced glycolysis is regulated in part by mTORC1, whereas IL-12/15–induced glycolysis is independent of mTORC1.

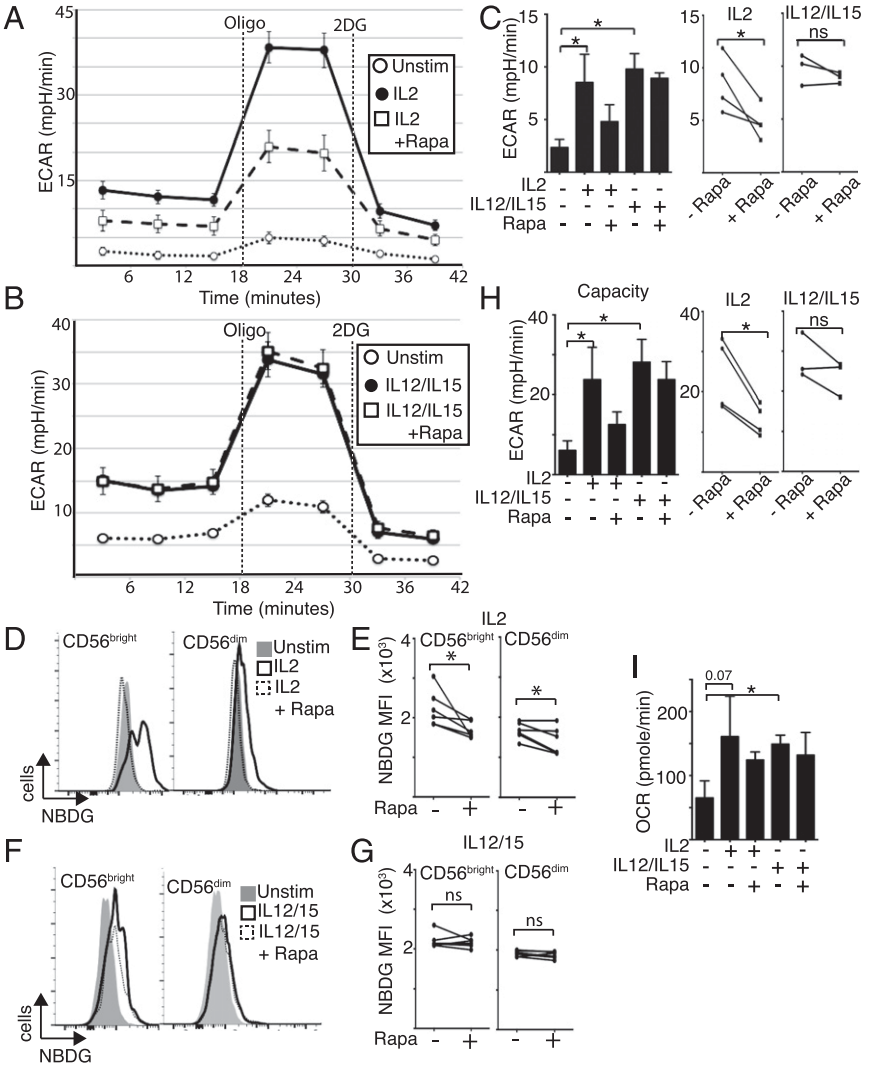

FIGURE 3. Human NK cells upregulate glycolysis and OxPhos in response to cytokine. NK cells were purified and stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added as indicated. Detailed metabolic analysis was performed using the Seahorse extracellular flux analyzer. (**A**–**C**) Representative traces and pooled data for the ECAR in response to IL-2 (A and C; $n = 4$ donors in quadruplicate) or IL-12/15 (B and C; $n = 3$ donors in quadruplicate) with or without rapamycin are shown. (**D** and **E**) Representative histograms of IL-2–induced glucose uptake (NBDG) by CD56^{bright} and CD56^{dim} NK cells (D) and individual paired responses with or without rapamycin (E, $n = 6$). (**F** and **G**) Representative histograms of IL-12/IL15–induced glucose uptake (NBDG) by CD56^{bright} and CD56^{dim} NK cells (F) and individual paired responses with or without rapamycin (G, $n = 6$). (**H**) Pooled data for the glycolytic capacity of cells are shown for IL-2 ($n = 4$ donors in quadruplicate) and IL-12/15 ($n = 3$ donors in quadruplicate) in the presence or absence of rapamycin. (**I**) Pooled data for OCR are shown for IL-2 ($n = 4$ donors in quadruplicate) and IL-12/15 ($n = 3$ donors in quadruplicate). Data are mean ± SEM. Samples were compared using either a one-way ANOVA followed by a Kruskal–Wallis post hoc test or a nonparametric paired Student t test analysis as appropriate. $^*p < 0.05$. ns, not significant.

mTORC1 is not required for short-term cytokine-induced effector functions in human NK cells

Having observed differences in the requirements for mTORC1 for short-term metabolic reprogramming in IL-2 and IL-12/15–stimulated NK cells, we investigated whether mTORC1 is required for NK cell effector functions induced by these cytokines in this time frame. Both IL-2 and IL-12/15 induced robust expression of the activation Ag CD69 on NK cells. The activating natural cytotoxicity receptor NKp44 and death receptor, TRAIL, were preferentially expressed on CD56^{bright} NK cells in response to cytokine as expected (24, 25). None of these responses were inhibited by rapamycin (Fig. 4A–C). In terms of effector functions, we measured granzyme B (GnzB) and CD107a degranulation as markers associated with NK cell cytotoxicity. GnzB is expressed in all NK cells; the CD56^{dim} subset constitutively contained more GnzB, but upon cytokine activation, both IL-2 and IL-12/15 induced a strong upregulation of GnzB in all CD56^{bright} NK cells to levels comparable with CD56^{dim} (Fig. 4D, 4E). Inclusion of rapamycin did not affect GnzB upregulation in CD56^{bright} cells (Fig. 4D, 4E). Similarly, although cytokines potently caused NK cells to degranulate in the presence of target cells, this was independent of mTORC1 (Fig. 4F).

In addition, we investigated whether mTORC1 signaling is required for NK cell production of IFN-γ in response to cytokine. Rapamycin treatment did not affect the frequency of either CD56^{dim} or CD56^{bright} NK cells producing IFN-γ in response to IL-12/15 stimulation (Fig. 5A–C). Although there was a trend toward decreased IFN-γ production per CD56^{bright} NK cell, as determined by the mean fluorescence intensity (MFI) of IFN-γ^{+} NK cells, with rapamycin treatment, this effect was not statistically significant (Supplemental Fig. 2A). Although IL-2 is not a potent stimulus for the production of IFN-γ in CD56^{dim} or CD56^{bright} NK cells, rapamycin inhibited IL-2–induced IFN-γ production in both NK cell subsets, in terms of the frequency of NK cells producing IFN-γ (Fig. 5A–C). As seen with IL-12/15 stimulations, there was a trend toward decreased IFN-γ per CD56^{bright} NK cells, although this was not statistically significant (Supplemental Fig. 2A). Thus, these data identify a discrete role for mTORC1 signaling in IFN-γ production in response to IL-2, but not IL-12/15, cytokine stimulation.

Finally, based on the observation that CD56^{bright} NK cells upregulated general metabolic markers and produced more IFN-γ, we hypothesized that the CD56^{dim} NK cells that produce IFN-γ are those that have induced a metabolic response. Indeed, when IL2 or IL-12/15–stimulated CD56 ^{dim} NK cells were stratified based on IFN-γ production, it was clear that NK cells that were producing IFN-γ were more metabolically active and had significantly more CD71 expression (Fig. 5D, 5E) than those that were not producing any cytokine.

Elevated OxPhos is required for cytokine-induced NK cell function

We next investigated whether the increases in NK cell metabolism associated with cytokine stimulation were important for NK cell effector functions. Cytokine stimulation induced a pronounced increase in cellular OxPhos, suggesting that increased ATP synthesis is important in these activated cells (Fig. 3). To determine the importance of OxPhos, we included the ATP synthase inhibitor, oligomycin, in our experiments. We used a relatively low dose of oligomycin that limits the rate of mitochondrial ATP synthesis and OxPhos (Supplemental Fig. 2B) without causing an energy crisis in the cells, an approach previously described (by others) (26). Oligomycin treatment did not inhibit cytokine-induced GnzB expression in NK cell subsets (Fig. 6A, 6B). However, oligomycin inhibited degranulation induced by IL-2 in both CD56^{dim} and CD56^{bright} subsets, but inhibition of degranulation induced by IL-12/15 cytokines was observed only in the CD56^{dim} subset (Fig. 6C, 6D).

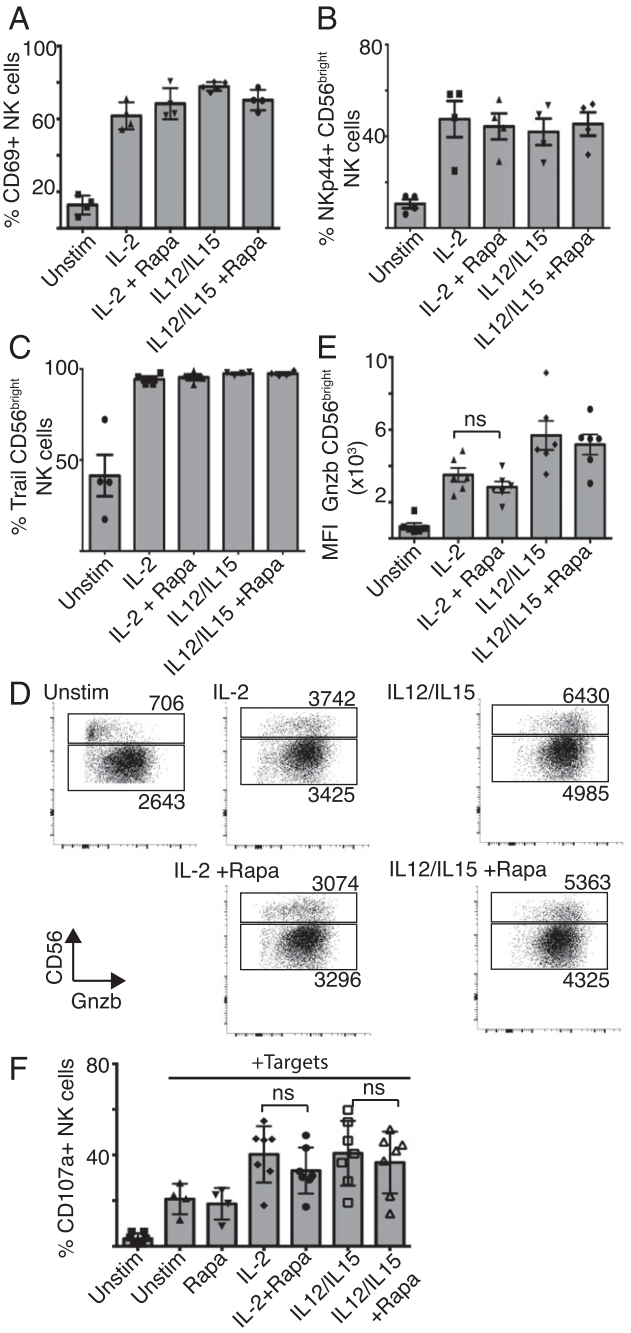

FIGURE 4. Cytokine-induced effector functions of human NK cells at 18 h are independent of mTORC1. PBMCs were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. (**A**) Expression of the CD69 surface Ag on NK cells is shown. (**B** and **C**) Frequency of expression of NKp44 (B) or TRAIL (C) on CD56^{bright} NK cells. (**D** and **E**) Representative dot plots of GnzB (D) and pooled data (E) of GnzB expression (MFI) on CD56^{bright} NK cells. (**F**) NK cell degranulation as determined by CD107a positivity after incubation with target cells is shown. Data are mean ± SEM from four donors (A–C), six donors (D and E), and four to seven donors (F). Samples were compared by a one-way ANOVA followed by a Kruskal–Wallis post hoc test. ns, not significant.

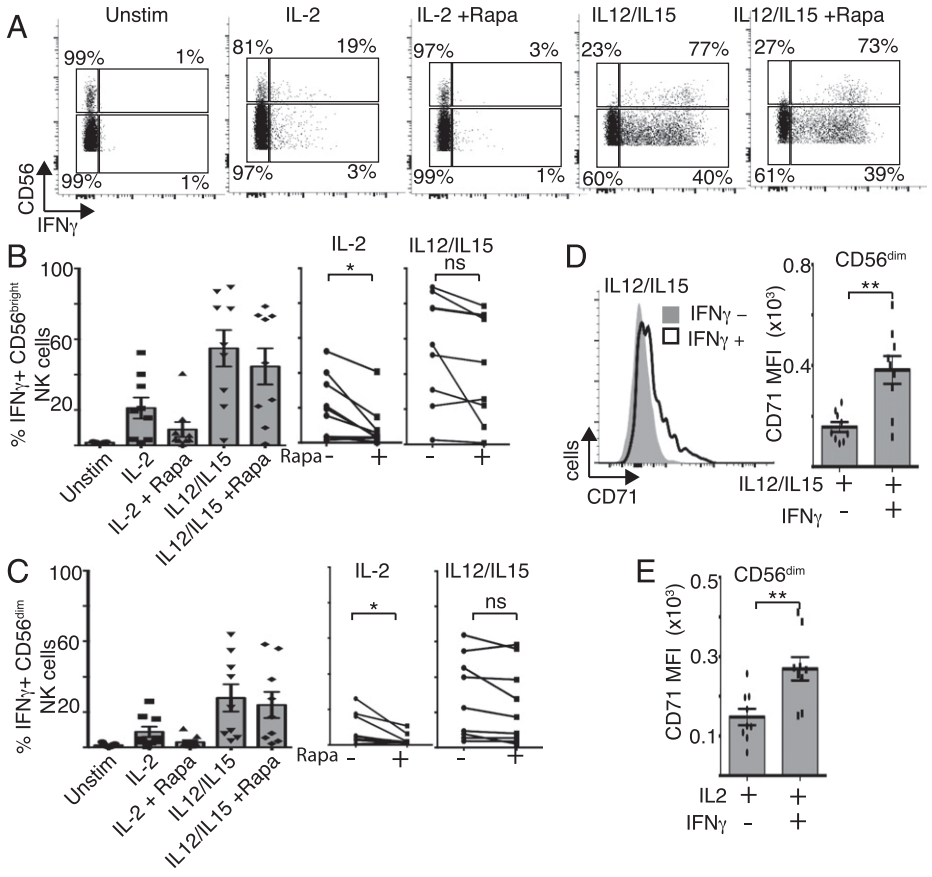

FIGURE 5. NK cell subsets that produce cytokines are metabolically active. PBMCs were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Rapamycin (20 nM) was added where indicated. (**A**) Representative dot plots of IFN-γ production by NK. (**B** and **C**) Frequency of IFN-γ–producing $CD56^{bright}$ (B) and $CD56^{dim}$ (C) NK cells is shown (*left*) with individual paired responses with or without rapamycin (*right*). (**D**) $CD56^{dim}$ cells were stratified based on IFN-γ^+ staining. The expression of CD71 on these different subsets was then analyzed, and a representative histogram (*left panel*) and pooled data (*right panel*) are shown. (**E**) $CD56^{dim}$ NK cells were analyzed as in (D) and pooled data are shown. Data are mean ± SEM from nine donors. Paired Student *t* test was used to compare the data. $*p < 0.05$, $**p < 0.01$. ns, not significant.

Elevated OxPhos was also required for the IFN-γ response in IL-12/15–stimulated NK cells. Oligomycin treatment resulted in a reduced frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells producing IFN-γ in response to IL-12/15 stimulation (Fig. 6E). In addition, the level of IFN-γ produced per cell, by $CD56^{bright}$ subset (the main subset that produces IFN-γ), was also reduced by oligomycin treatment (Fig. 6F). The relatively small IL-2–induced IFN-γ response was not affected by oligomycin treatment (Supplemental Fig. 2C). Overall, oligomycin inhibited most NK cell functions supporting the tenet that increased ATP production caused by mitochondrial OxPhos is important for fueling NK cell functions.

Elevated rates of glycolysis are essential for maximal IFN-γ production

We have previously reported that glycolysis is important for IFN-γ production in murine NK cells (16). To investigate whether glycolysis is important for human NK cell effector functions, we carried out experiments in the presence of galactose as an alternate carbon fuel source to glucose that cannot support elevated glycolysis (16, 26). Inhibiting glycolysis in this way had minimal inhibitory effects on NK cell degranulation or GnzB induction in $CD56^{bright}$ or $CD56^{dim}$ cells (Fig. 7A–D). In terms of IFN-γ production, limiting the rate of glycolysis had no effect on IL-2–stimulated IFN-γ production (Fig. 7E, 7F). However, when the rate of glycolysis was limited in the IL-12/15–stimulated NK cells, there was a trend toward a decreased frequency of $CD56^{bright}$ NK cells making cytokine (Supplemental Fig. 2D) with a significant reduction in the amount of IFN-γ being produced per cell (Fig. 7E–G). Although the IFN-γ response is variable in donor PBMCs, the relative amounts of IFN-γ in galactose cultured cells were significantly decreased compared with those cultured in glucose. Thus, these data argue that elevated glycolysis in human NK cells is required for maximal IFN-γ responses.

Discussion

NK cells in humans can be divided based upon expression levels of CD56 Ag into phenotypically and functionally distinct subsets of cells. $CD56^{dim}$ cells account for up to 90% of peripheral blood NK cells, whereas $CD56^{bright}$ cells are predominantly tissue resident and are found in secondary lymphoid tissues and other organs (12). Although there is evidence to suggest that $CD56^{bright}$ cells are precursors of $CD56^{dim}$ cells (3, 4), they can also mediate strong independent immunological effects through the production of large amounts of IFN-γ. To our knowledge, this study is the first to define the metabolism of human NK cells and identify that these important NK cells subsets in peripheral blood have distinct metabolic phenotypes. Our data show that cytokines can robustly activate NK cell metabolism. Detailed metabolic analysis showed that cytokines upregulate glycolysis and OxPhos in NK cells. Perhaps more important was the observation that $CD56^{bright}$ cells in peripheral blood were different from $CD56^{dim}$ cells in terms of their metabolism. $CD56^{bright}$ cells were much more glycolytic and preferentially upregulated metabolism in response to cytokines compared with $CD56^{dim}$ cells. $CD56^{bright}$ cells efficiently upregulated the CD71 and had higher expression of CD98 and higher glucose uptake. Furthermore, these $CD56^{bright}$ cells are primed to become more metabolically active as they express higher levels of cytokine receptors (6) and have higher basal expression of Glut1, allowing them to rapidly take up glucose after activation. These changes in metabolism allow $CD56^{bright}$ cells to meet the biosynthetic and energy demands associated with the production of large amounts of IFN-γ very rapidly upon activation. Indeed, glycolytic rates have been directly linked to the production of IFN-γ in murine NK cells and T cells because glycolytic enzymes can directly modulate IFN-γ mRNA translation (16, 26). Thus, $CD56^{bright}$ cells are metabolically prepared for their functions as

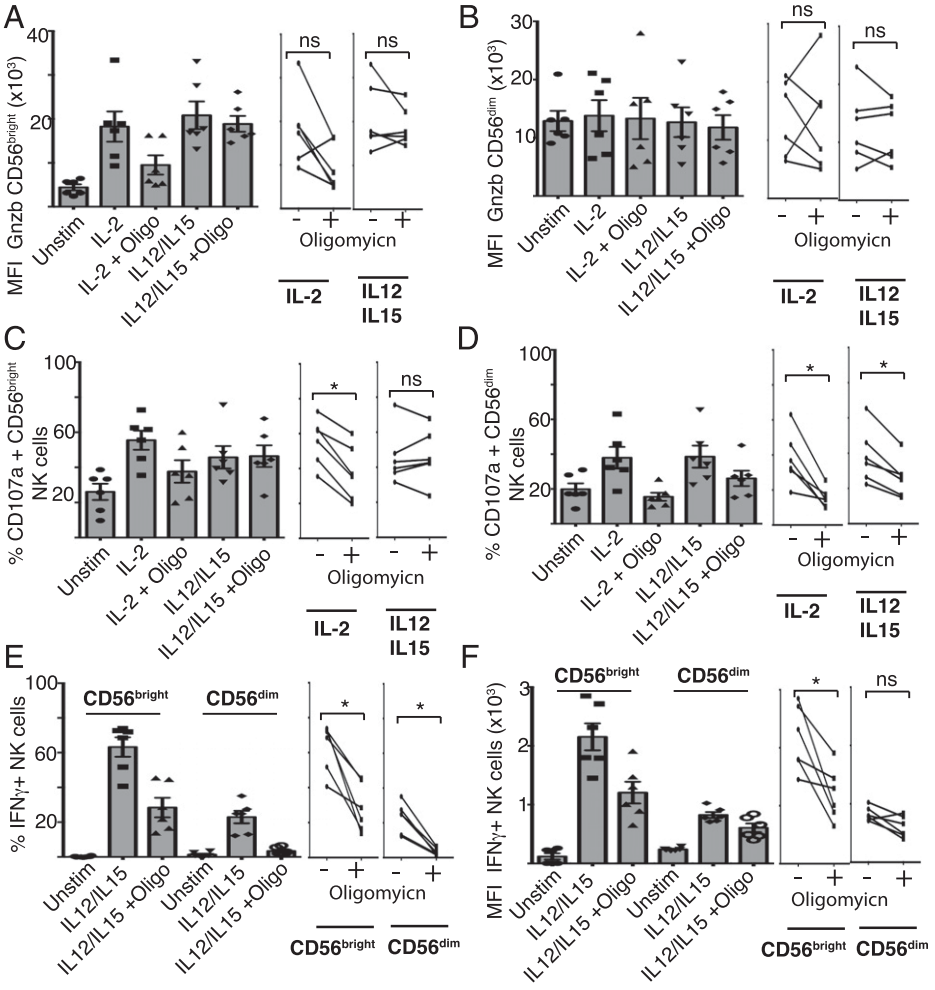

FIGURE 6. OxPhos is required for NK cell effector functions. PBMCs were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Oligomycin (40 nM) was added for the duration of cultures where indicated. (**A** and **B**) GnzB expression (MFI) in $CD56^{bright}$ (A) and $CD56^{dim}$ NK cells (B) is shown (*left*), with individual paired responses with or without oligomycin (*right*). (**C** and **D**) NK cell degranulation as determined by CD107a expression after incubation with target cells is shown (*left*) for $CD56^{bright}$ (C) and $CD56^{dim}$ cells (D), with individual paired responses with or without oligomycin (*right*). (**E** and **F**) Frequency of IFN-γ–producing NK cells (E) and MFI of IFN-γ^+ NK cells (F) is shown for $CD56^{bright}$ and $CD56^{dim}$ cells (*left*), with individual paired responses with or without oligomycin (*right*). Data are mean ± SEM, from six donors. Paired Student *t* test was used to compare the data. $*p < 0.05$. ns, not significant.

rapid responders postinfection. However, it is known that most $CD56^{bright}$ cells are found in secondary lymphoid tissues and in organs such as the uterus where they may play additional roles to their antiviral functions (reviewed in Ref. 2). Although our data suggest that $CD56^{bright}$ cells in the peripheral circulation are more metabolically responsive to cytokine, it will be interesting to confirm whether this is also the case for tissue-resident NK cells.

In contrast, $CD56^{dim}$ NK cells are heterogenous in their metabolic response to cytokine. Only a subset of these cells upregulate CD71, Glut1, and glucose uptake in response to cytokine. This might be explained by the fact that the principle function for $CD56^{dim}$ NK cells is cytotoxicity, and although cytokine can increase NK cell cytotoxicity, freshly isolated NK cells are able to kill target cells without any additional stimulation. Indeed, they are primed for this function with high constitutive levels of GnzB. Therefore, there is not a substantial biosynthetic burden on cytokine-stimulated $CD56^{dim}$ cells. However, it should be noted that a subset of $CD56^{dim}$ NK cells also make substantial amounts of IFN-γ, and our data support that these cells also upregulate their metabolism to deal with the associated increased biosynthetic demands as $CD56^{dim}$ NK cells that produced IFN-γ had increased surface expression of CD71. Therefore, $CD56^{dim}$ IFN-γ–producing NK cells and $CD56^{bright}$ NK cells both increase nutrient receptor expression and nutrient uptake.

The data show that elevated levels of OxPhos are particularly important for the function of human NK cells after short-term IL-12/15 or IL-2 cytokine stimulation. OxPhos is important to fuel efficient ATP synthesis that is required for activated NK cell function. OxPhos can also support cellular biosynthesis as it facilitates the conversion of glutamine, and other fuels, into precursors for biosynthetic pathways (14). In general, activated lymphocytes tend to make more ATP through glycolysis, decreasing their reliance on OxPhos. In this way, glycolytic lymphocytes can maintain ATP homeostasis when OxPhos is repressed, as might occur at hypoxic inflammatory sites or within hypoxic tumors. Nonetheless, this does not seem to be the case for 18-h cytokine-stimulated human NK cells, which need OxPhos for normal function. However, recent research suggests that the timescale for metabolic reprogramming of murine and human lymphocytes may be different. In human T lymphocytes, glycolytic reprogramming may occur over the course of 72 h as opposed to the 24 h required in murine T cells (23, 27). Therefore, cytokine-stimulated human NK cells may further upregulate glycolytic metabolism beyond the 18 h observed in this study, which might be predicted to decrease their reliance on OxPhos. Nevertheless, over the time course relevant for early innate immune responses, it is clear that elevated OxPhos is critical for optimal NK cell responses. It will be of interest to study NK cell metabolism and function in the context of NK cells that function alongside the adaptive immune response.

An important finding was that cytokine combinations drive different metabolic changes in NK cells. The responsiveness of NK cells to IL-2 suggests that NK cells are regulated by T cell–derived cytokines and function in parallel with the adaptive immune response at time scales beyond the initial few days of infection (28). Indeed, there is a growing literature to support this, including the demonstration of activated NK cells persisting for up to 2 mo post hantavirus infection (29). In various vaccine and infection studies,

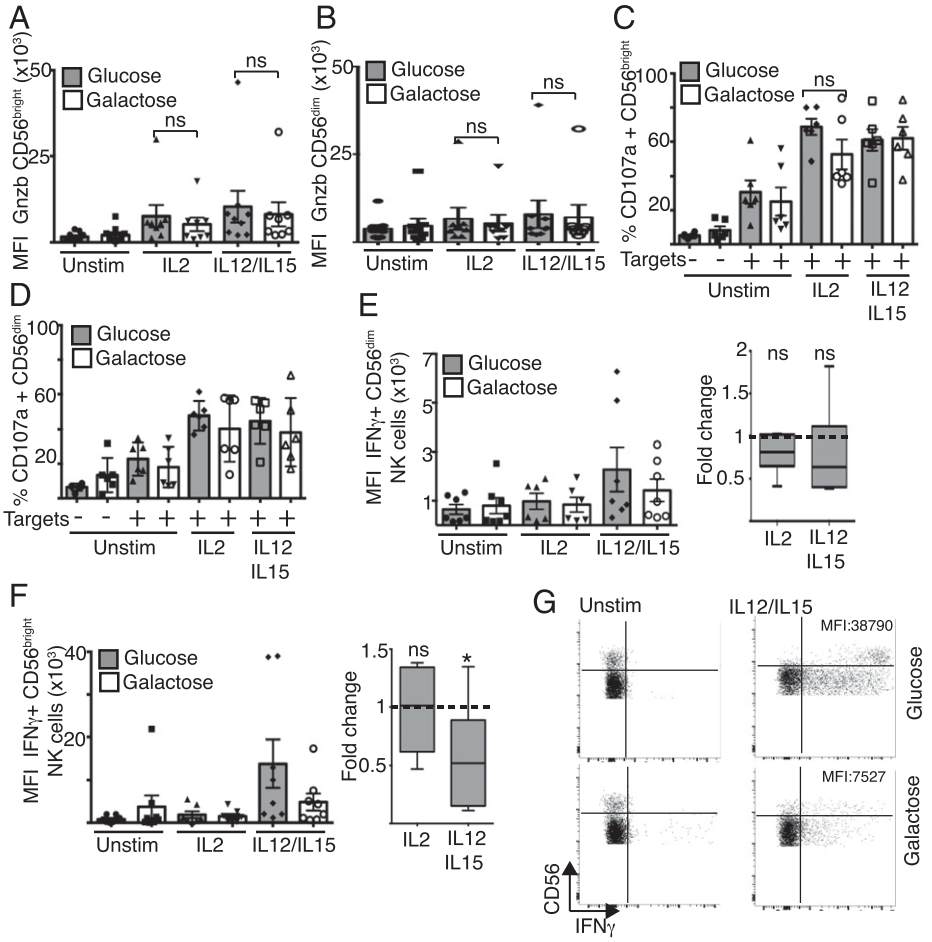

FIGURE 7. Glycolysis required for maximal IFN-γ production by CD56^{bright} NK cells. PBMCs were stimulated for 18 h with either IL-2 (500 U/ml) or IL-12 (30 ng/ml)+IL-15 (100 ng/ml), or left unstimulated. Cultures were carried out in glucose-replete medium or medium in which galactose (10 mM) replaced glucose. (**A** and **B**) GnzB expression (MFI) in CD56^{bright} (A) and CD56^{dim} cells (B) is shown. (**C** and **D**) NK cell degranulation as determined by CD107a positivity after incubation with target cells is shown for CD56^{bright} (C) and CD56^{dim} cells (D). (**E**) IFN-γ expression (MFI) in IFN-γ^{+} CD56^{dim} cells is shown (*left*) with the fold change, glucose versus galactose (*right*). (**F**) IFN-γ expression (MFI) in IFN-γ^{+} CD56^{bright} cells is shown (*left*) with the fold change, glucose versus galactose (*right*). (**G**) Representative dot plots IFN-γ production in IL-12/15–stimulated NK cells. Data are mean ± SEM of six donors. After testing for normal distribution, the fold changes in IFN-γ MFI caused by galactose were compared with a one-sample *t* test against a theoretical mean set to 1.00. $^{*}p < 0.05$. ns, not significant.

NK cell production of IFN-γ at later time points has been shown to be dependent on T cell–derived IL-2 (30–32). In our hands, IL-2 potently activated human NK cell cytotoxicity and had a much more modest effect on IFN-γ production. Both IL-12/15 and IL-2 upregulated glycolysis and glycolytic capacity to similar levels. However, a distinct difference was observed in terms of the mechanisms involved. IL-2–induced increases in glycolysis and glycolytic capacity were mTORC1 dependent, whereas the IL-12/15–induced responses were not. These data argue that IL-2 has the potential to drive mTORC1-dependent changes to NK cell glucose metabolism, as we have previously observed with cytokine-expanded murine NK cells and for murine T cells (16, 22). However, there are also examples in murine and human lymphocytes where mTORC1-independent mechanisms promote increased glycolytic metabolism (33, 34). Our study suggests that NK cells can engage both mTORC1-dependent and -independent mechanisms in response to distinct cytokine stimulations. Based on our data, we suggest that there might be temporal changes in NK cell metabolism that are dependent on the nature of cytokines present. Cytokines such as IL-12/15 may upregulate NK cell metabolism and functions for immediate and potent responses, of which IFN-γ is particularly important. In contrast, although IL-2 can drive a more modest short-term IFN-γ response, it can potentially drive a more sustained NK cell activation that allows NK cells to function in parallel to the adaptive immune response over a longer time period. In this context, controlling NK cell metabolism through mTORC1 potentially provides an additional level of control over NK cell function. This is because mTORC1 activity is acutely sensitive to conditions in the immune microenvironment, most notably the availability of nutrients. Microenvironmental control of mTORC1 and NK cell metabolism and function may be an important mechanism for appropriately limiting NK cells functioning as part of adaptive immune responses to avoid excessive immunopathology.

Disclosures

The authors have no financial conflicts of interest.

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