Stimulation of NOD2 in Acute Myeloid Leukemia activates Natural Killer Cells and improves survival

Undergraduate Honors Research Thesis

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Abstract

Acute Myeloid Leukemia (AML) is a hematological malignancy of the myeloid lineage of immune cells in the blood and bone marrow. It is characterized by low five-year survival rates of 27.4%, and high incidence of relapse, indicating a need for novel therapeutic strategies. A signature of AML is the evasion of immune surveillance by Natural Killer (NK) cells, which are immune cells possessing anti-leukemic capabilities. NK cell activating receptors are downregulated in AML-NK cells, and these cells exhibit defective cytotoxic ability. Recently, our lab has found that treatment with MTP-PE (a synthetic ligand for NOD2 used for the treatment of osteosarcoma) along with IFNγ provided a survival benefit in a murine model of AML. Data from this study show that blood-derived NK cells isolated from mice treated with MTP-PE and IFNγ show greater levels of maturation, as indicated by markers CD27 and CD11b. Additionally, we saw increased expression of activation/degranulation markers, CD69 and CD107a, on healthy donor NK cells in in vitro cocultures with AML patient samples. This study is focused on elucidating the mechanism by which NK cell activation occurs when AML is treated with MTP-PE and IFNγ. Preliminary data suggests that ligand-receptor cell-to-cell contact between AML cells and NK cells is necessary to elicit the observed NK cell activation. In this study, we show that MTP/IFNγ treated AML cells strongly upregulate IL-15, an important cytokine involved in NK cell expansion and activation, as well as ligands of the receptor NKG2D. In AML patient samples, we have found the co-treatment to upregulate CD69 and CD107a on suppressed patient NK cells, both in peripheral blood and apheresis. We also observed expansion of NK cells in these patient samples. Further study is necessary to evaluate and understand the potential of MTP-PE and IFNγ to activate suppressed NK cells in Acute Myeloid Leukemia and improve anti-leukemic immunosurveillance.
Introduction

Human blood cells are distinguished into two lineages, each of which are composed of different cell types and serve a distinct function in mounting the immune response against pathogens. The myeloid lineage, which includes monocytes, granulocytes, erythrocytes, and platelets, are responsible for initiating the innate immune response, while the lymphoid lineage, which includes T cells, B cells, and Natural Killer (NK) cells, are generally associated with the adaptive immune response, though NK cells display more innate immune properties. The aberrant replication of these blood cells can manifest in leukemias or lymphomas. The uncontrolled division of myeloid cells originating in the bone marrow is known as Acute Myeloid Leukemia (AML).

AML is a highly heterogenous disease, characterized by a diverse range of genomic alterations which may occur at various stages of myeloid cell progression, which makes it a challenging disease to treat. In addition, it accounts for one third of all leukemias diagnosed and is one of the most common adult leukemia, with an incidence of over 20,000 cases in the United States alone. While advancement in the treatment of AML, spurred by an improved understanding of the molecular drivers of disease, has improved patient outcomes, there still exists a great need for novel therapies. Only 35-40% of patients aged 60 or younger and a dismal 5-15% of patients aged 60 or above survive for five or more years.

Current strategies for treatment are focused on initial induction therapy, of cytotoxic chemotherapy or hypomethylating agents, to achieve complete remission, followed by post-remission therapy. The most employed post-remission strategies to prevent relapse are the use of cytotoxic chemotherapies or allogenic hematopoietic stem cell transplantation. However, relapse remains a major clinical bottleneck, occurring in approximately half of all patients. Improving
patient immunosurveillance and clearance of cancer cells is potentially powerful therapeutic strategy to extend or prevent relapse. Further, many immunotherapeutic strategies have thus far been implemented to augment anti-leukemic adaptive immune functions, such as immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cell therapy.

In addition, innate immune inflammatory responses have also been studied for their anti-cancer effects. Toll-like receptors (TLRs) are a particular class of pattern recognition receptors (PRR) primed to sense pathogen-associated molecular patterns, and the stimulation of these receptors in several cancers has been found to induce inflammation and cancer cell death. In fact, agonists for TLR3, -4, -7/8, and -9 are currently being evaluated for therapeutic potential in many clinical trials for treatment of malignancies. Another category of PRRs are Nucleotide-binding and oligomerization domain NOD-like receptors (NLRs). These molecules oligomerize in response to the sensation of foreign molecular patterns, and initiate effector signaling cascades. Thus, they share a common molecular arrangement, consisting of a C-terminal leucine-rich repeat (LRR) with ligand recognition functions, a central nucleotide-binding and oligomerization domain (NBD), and an N-terminal effector-initiating domain. The downstream functions of NLRs are various and key in the categorization of these receptors. The two most prominent subcategories of NLRs, which vary at their effector N-terminus, are NLRC and NLRP. NLRC proteins have one or more CARD (caspase-recruitment) domains, while NLRP proteins have pyrin domains, which are best associated with activation of the inflammasome.

Here, we explore the therapeutic stimulation of NOD2, a particular cytosolic NLRC receptor which senses the bacterial cell wall motif muramyl dipeptide (MDP), for the treatment of Acute Myeloid Leukemia. MDP binds the LRR domain of NOD2, and initiates auto-oligomerization of the molecule. The consequent CARD-CARD interactions result in the
recruitment and activation of RIP2 kinase, which when active, results in various cellular processes that relieves the critical inflammatory transcription factor NF-κB to translocate to the nucleus and initiate the transcription of various pro-inflammatory genes, causing cellular functions such as cytokine and growth factor release, apoptosis via caspase-1 activation and autophagy processes. Furthermore, NOD2 is primarily expressed in cells of the myeloid lineage, including monocytes, macrophages, and myeloid-dendritic cells, making it an ideal target for initiating anti-leukemic inflammation in myeloid cancer.

Synthetic derivatives of the natural NOD2 agonist, MDP, have been developed for therapeutic use, including MDP-Lys(L18) and MTP-PE (muramyl tripeptide phosphatidylethanolamine). The latter, which contains an alanine and lipophilic dipalmitoylphosphatidylethanolamine group, allows for greater *in-vivo* bioactivity due to its increased ability to permeate cell membrane or retain in liposomes that are readily phagocytosed by macrophages or monocytes. MTP-PE, otherwise known as mifamurtide, has been approved for therapeutic use in the treatment of non-metastatic osteosarcoma in the European Union, but remains an investigational drug in the United States. In osteosarcoma, MTP-PE activated circulating macrophages and consequently other immune cells, causing anti-tumor effects that resulted in higher overall survival and disease-free survival in clinical trials. Our lab conducted a pre-clinical study to study the effects of MTP-PE on Acute Myeloid Leukemia and found that MTP-PE alone could not elicit anti-tumor effects. However, priming the cellular response with treatment of anti-viral cytokine, IFN-γ, elicited synergistic effects in initiating AML cell apoptosis, and the combination therapy significantly lowered disease burden and improved overall survival in a murine model of AML. Furthermore, we observed increased maturation of NK cells in this murine model and enhanced cytotoxic activity against leukemic cells.
NK cells are innate immune lymphoid cells which act against virally infected or cancerous cells. NK cell activation is mediated by a complex network of activating and inhibitory receptors, which respond to molecules expressed by malignant cells or healthy cells, respectively, on their cell surface. AML disease canonically inhibits NK cell populations, through the establishment of immunosuppressive microenvironments or through impairing the mechanisms by which NK cells recognize cancer cells for elimination. Conversely, high NK cell frequency in circulation, cytolytic activity, and IFN\(\gamma\) secretion potency are all prognostic factors associated with improved survival outcomes and decreased relapse rates. Thus, a potential immunotherapeutic strategy to prevent relapse would be to activate NK cells.

In this study, we explore whether NK cells can be activated from their suppressed state via MTP-PE and IFN\(\gamma\) treatment in human patient samples, and seek to understand the mechanism by which NOD2 stimulation contributes to NK cell maturation and cytotoxic activity. Our lab has previously found that NK cells alone do not respond to MTP-PE and IFN\(\gamma\) treatment, but also require co-incubation with AML cells. Further, we have found that direct contact between treated AML cells and NK cells is necessary to elicit activation upon the combination treatment. Thus, we hypothesize that MTP-PE and IFN\(\gamma\) causes AML cells to upregulate NK activating markers on their cell surface, such as Il-15 and NKG2DL, which compel NK cells to react cytotoxically in effect to eliminate the cancer cells. Elucidating the mechanism of this cell-to-cell signaling and investigating its potential to prevent relapse and activate suppressed NK cells in human patients is the primary purpose of our study.
Materials and Methods

Reagents:
Mifamurtide (MTP-PE) was purchased from MedChemExpress LLC (Monmouth Junction, NJ) and Sigma-Aldrich (St. Louis, MO) and resuspended in water to a stock concentration of 2µg/µl, and 10µg/µl, respectively. Recombinant human IFNγ were purchased from R&D Systems (Minneapolis, MN) and reconstituted to a stock concentration of 200µg/mL.

Peripheral Blood Mononuclear Cell (PBMC) isolation
Blood from AML patients was obtained under written informed consent in accordance with protocol approved by the institutional review board of The Ohio State University. The blood was diluted equal parts in PBS, layered upon Ficoll, and centrifuged at 1400 rpm for 30 minutes with minimal acceleration/deceleration. Afterwards, the separated peripheral blood mononuclear cell fraction was visually demarcated and was aspirated for further processing.

CD56+ Positive Selection
NK cells were isolated using EasySep Human CD56 Positive Selection Kit II for human (STEMCELL Technologies, Vancouver, Canada) in accordance with manufacturer instructions. PBMC were incubated with CD56+ selection cocktail for 10 minutes in polystyrene round bottom tubes. Next, RapidSpheres™ were vortexed for 30 seconds and incubated with the PBMC at a concentration of 50µl/mL, for 5 minutes. Finally, the tube was placed in EasySep™ Magnet and incubated three times, discarding supernatant, and replacing with MACS buffer between incubations. The remaining NK cells were resuspended in 20% FBS RPMI media, previously prepared.

Cell Culture Treatments
Aphaeretic WBCs and peripheral blood from AML patients were obtained under written informed consent in accordance with protocol approved by the institutional review board of The Ohio State University. Samples were stored in liquid nitrogen in 20% FBS and 10% DMSO prior to use. Upon thawing, cells were washed and cultured in RPMI Medium 1640 (Life Technologies) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), and 56 U/ml/56 µg/ml penicillin/streptomycin (Invitrogen) at 37°C in 5% CO₂. MTP-PE was used at a working concentration of 10µg/mL and IFNγ was used at 10ng/ml. Cells were incubated after treatment for either 24 hours or 36 hours.

**Real-time quantitative Polymerase Chain Reaction (qPCR)**

RNA was extracted from using the Total RNA Purification Plus Kit (Norgen Biotek, Thorold, ON, Canada). RNA was reverse transcribed using the High-capacity cDNA Reverse Transcriptase kit (Applied Biosystems, NY) and then quantified by quantitative real-time PCR (qPCR) using Power SYBR Green Master Mix (Applied Biosystems, Grand Island, NY). GAPDH was used as a reference gene for normalizing target genes. The following human primers (Invitrogen, CA) were utilized: GAPDH (forward primer, 5’-ACTTTGGTATCGTGGGAAGGACT-3’; reverse primer, 5’-GTAGAGGGAGGATGATTTCT-3’), MICA (forward primer, 5’-AGGGTTTCTTGGAGTACCA-3’; reverse primer, 5’-GGTCTCTCTGTCACCAGTCTTAA-3’), MICB (forward primer, 5’-TGGAGACTCAAGAATCGACGT-3’; reverse primer, 5’-CTGCATAGCGATAGTGTG-3’), CD112 (forward primer, 5’-CATTGGCGAGTTGCCACC-3’; reverse primer, 5’-GCCACTGTCTGAGGGTCTT-3’), CD155 (forward primer, 5’-TGGAGGTGACGCATGTCG-3’; reverse primer, 5’-GTGTTGGACTCCGAATGCTG-3’), AICL (forward primer, 5’-TACCAATCGTTTGAGGATG-3’; reverse primer, 5’-CTGCAAATCCATTTTCTTTCG-3’),
CD48 (forward primer, 5'-GGCAGGCTCAGACTTGATCC-3'; reverse primer, 5'-GTAGGTGTGCTGTTGCTCCTTTC-3'), B7-H6 (forward primer, 5'-"CAGGAGAGTACCAGTGAGG-3'; reverse primer 5'- CATGCCCACTTGTCCAGCAAA-3'), NKp44L (forward primer, 5'- AAATCCAACAGTTATCCCCACC-3'; reverse primer 5'- CGCATAGGGCAACACAATGTA-3'), HLA-A (forward primer, 5'-AAAAGGAGGGAGTTACACTCAG-3'; reverse primer 5'- GCTGTGAGGACACATCAGAG-3'), HLA-BW4 (forward primer, 5'- CAGGCAGGTGCAGTCCAGG-3'; reverse primer 5'- CAGCCGTACATGCCCTGGAG-3'), HLA-E (forward primer, 5'- TTCCGAGTGAGCTCCTGGAC-3'; reverse primer 5'- GTGCCTAGGGCAACTGGCCTG-3'), DAP-12 (forward primer, 5'- TCATGGGGAGTTGCTCATTGTAATAC-3').

Data are presented as fold change from untreated of the relative copy number (RCN), calculated as $2^{-\Delta Ct} \times 100$, where $\Delta Ct$ is the Ct(target) – Ct(GAPDH).

**Enzyme Linked Immunosorbent Assay (ELISA)**

Supernatants from cell cultures treated with/without 10 μg/ml MTP-PE and/or 10 ng/ml IFNγ and were analyzed at 24h using Human Granzyme B DuoSet ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer instructions.

**Flow Cytometry**

Acute Myeloid Leukemia apheresis and PBMC samples were treated with/without 10 μg/ml MTP-PE and/or 10 ng/ml IFNγ and were stained for flow cytometry analysis. Cells were collected 24 hours after treatment and washed twice in PBS. To prevent nonspecific binding of the primary antibodies, cells were blocked with Chrompure Human IgG, whole molecule (Jackson ImmunoResearch, West Grove, PA) at 10 μg/ml for 15 minutes on ice. Next, cells were incubated
on ice for 30 minutes with either a panel of markers for myeloid cancer cells or a panel of markers for NK cells. The NK panel consisted of Alexa Fluor® 488 anti-human CD69 Antibody, PE/Dazzle™ 594 anti-human CD107a (LAMP-1) Antibody, Brilliant Violet 421™ anti-human CD314 (NKG2D) Antibody, and. The AML panel consisted of FITC mouse anti-human CD14 Antibody, APC anti-human ULBP-1 Antibody, PE/Cyanine7 anti-human MICA/MICB Antibody, PerCP anti-human IL-15 Monoclonal Antibody. For some samples, a viability marker to exclude dead cells was also used (Live/dead Blue; Thermo Fisher Scientific). After staining, cells were fixed with 2% paraformaldehyde in PBS and acquired using the LRS Fortessa flow cytometer, at the Flow Cytometry Shared Resources Core at The Ohio State University.

**Statistical Analyses**

Analyses and graphs for all figures were completed in GraphPad Prism Version 9 (GraphPad Software, San Diego, CA) using a Tukey’s multiple comparisons test. Results were qualified to be statistically significant if the p-value ≤ 0.05.
Results

MTP-PE and IFNγ potentiate cytotoxic activity of healthy donor NK cells against AML apheresis samples

NK cells kill malignant cells by the targeted release of cell lytic molecules such as granzyme and perforin, or through the binding of cognate death-receptors on target cells, such as TRAIL-TRAIL or Fas-FasL. Release of Granzyme B and expression of TRAIL on NK cell surface are important metrics to study NK cell activity. To verify whether crosstalk between NK cells and AML cells was critical to NK cell activation by MTP-PE and IFNγ, NK cells were isolated from healthy human peripheral blood, and treated with the combination of drugs, either with or without AML apheresis cells in co-culture. We found that significant elevation in levels of granzyme B after treatment only occurred when NK cells were in co-culture with AML cells. NK cells alone did not respond to the treatment, with similarly basal levels of granzyme B as observed in the untreated co-culture group. These results are presented in Figure 1. In addition, we performed flow cytometry on NK cells in the same model system, and found upregulation of TRAIL on NK cells upon MTP-PE and IFNγ treatment only with AML cells in co-culture.
Figure 1. *MTP-PE and IFNγ potentiate cytotoxic activity of healthy donor NK cells against AML apheresis samples*

NK cells isolated from healthy donor peripheral blood, Acute Myeloid Leukemia patient apheresis samples, and a one to one co-culture of the two groups were treated at 1x10^6 total cells/mL with MTP-PE (10μg/mL) and IFNγ (10ng/mL). After 24 hours, supernatant was collected and analyzed using Human Granzyme B DuoSet ELISA following manufacturer instructions. (n=2)
MTP-PE and IFNγ activate NK cells in AML Apheresis and PBMC samples

Having shown that NK cells isolated from healthy donors could be activated to exhibit cytotoxic activity against AML cells upon MTP-PE and IFNγ treatment, we next sought to determine the potential of this co-treatment in activating canonically suppressed NK cells from AML patients. We treated AML PBMC samples with MTP-PE and IFNγ and performed flow cytometry for the following activation markers, CD69 and CD107a and NKG2D expressed on NK cells. We found modest but not statistically significant upregulation of CD69, CD107a, and NKG2D in the MTP-PE and IFNγ treated groups, as compared to the single treatments and untreated. The data suggests a trend towards NK cell activation even within the immunosuppressive environment of AML PBMs, but additional experimental replicates are necessary to have conclusory evidence. The experiment was repeated with AML apheresis samples, which have significantly fewer NK and immune cells than PBMs. Cells were analyzed by flow cytometry at both 24 and 36 hours after MTP-PE and IFNγ treatment, thereby improving the opportunity for detection of activation markers in the minimal NK cell population in patient apheresis. At both timepoints, CD69 expression trended towards upregulation in both the IFNγ and MTP-PE and IFNγ treatment groups, compared to MTP-PE alone and untreated. However, CD107a and NKG2D expression was unaffected by the treatments, and had great variability between replicates, at both timepoints. These data are shown in Figure 2.
Figure 2. MTP-PE and IFNγ activate NK cells in AML Apheresis and PBMC samples

A) AML PBMC patient samples were treated with MTP-PE (10µg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours. Activation marker expression on NK cells was measured and analyzed via flow cytometry (n=3). B) AML apheresis patient samples were treated with MTP-PE (µg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours (n=3) or 36 hours (n=5). Activation marker expression on NK cells was measured and analyzed via flow cytometry.
MTP-PE and IFNγ upregulate transcription of NK activating ligands in AML apheresis samples

Having determined that juxtacrine signaling between AML cells and NK cells mediates activation by MTP-PE and IFNγ, we intended to determine the specific molecular interactions responsible for the observed effect. A panel of potential ligands responsible for activation was curated based upon thorough review of the existing literature, and mRNA expression of these molecules upon treatment by MTP-PE and IFNγ was quantified via quantitative real-time PCR. We found nearly 40-fold upregulation of the surface bound cytokine, IL-15, in the MTP-PE and IFNγ treated groups. Ligands for the receptor NKG2D, MICA and MICB were significantly upregulated in the combination treated cells, whereas ULBP1 showed no such effect, with the MTP-PE single treatment showing the highest average expression. Ligands for the NK cytotoxicity receptors were also studied, including B7-H6 (the ligand for NKp30), AICL (the ligand for NKp80), and NKp44L (the ligand for NKp44). AICL showed significant upregulation with the combination treatment, though this effect was similarly recapitulated by the IFNγ treatment alone. NKp44L similarly showed statistically significant upregulation in the combination, with similar effects between both individual treatments. CD48, the ligand for activating receptor 2B4 on NK cells, trended towards being upregulated by the combination treatment in an super additive manner, but this was not statistically significant. Finally, a ligand for the receptor DNAM-1, CD112, was profoundly upregulated with the combination treatment as compared to both the untreated and single treatments, indicating synergistic effects. These results are shown in Figure 3.
**Figure 3. MTP-PE and IFNγ upregulate transcription of NK activating ligands in AML apheresis samples**

AML apheresis patient samples were treated with MTP-PE (10μg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours. mRNA transcript was measured and analyzed through quantitative real-time PCR. (n=8)
MTP-PE and IFNγ upregulate NK activating ligands in AML Apheresis and PBMC

Above, we show that MTP-PE and IFNγ significantly upregulate mRNA expression of key NK activating ligands in AML apheresis cells. However, this data does not verify the production of protein products, which are necessary for NK activation by binding their cognate receptor and causing downstream effects. We performed flow cytometry analysis on treated patient apheresis and PBMC samples using fluorescence-conjugated antibodies targeted to a select group of NK activating ligands: IL-15, and NKG2D ligands ULBP1 and MICA/B. Results (Figure 4) showed a trend towards IL15 upregulation in the patient apheresis samples at 36 hours and 24 hours, but not in the patient PBMC samples. ULBP1 and MICA/B did not show any marked trends across either patient sample type or timepoint of collection.
Figure 4. MTP-PE and IFNγ upregulate NK activating ligands in AML Apheresis and PBMC

A) AML PBMC patient samples were treated with MTP-PE (10µg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours. Activating ligand expression on AML cells was measured and analyzed via flow cytometry (n=3). B) AML apheresis patient samples were treated with MTP-PE (10µg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours (n=3) or 36 hours (n=5). Activating ligand expression on AML cells was measured and analyzed via flow cytometry.
MTP-PE and IFNγ may expand NK cell frequency in AML PBMC

In addition to NK cell activation, NK cell frequency in peripheral blood is also associated with improved outcomes in patients. In addition to studying NK cell activation in AML PBMC, we also studied the NK cell population through CD56+ gating. Notably, out of 9 total AML patient samples, one patient showed a significant expansion of NK cells with the combination treatment. NK cells composed roughly 2% of total lymphocytes in the untreated and single treated groups, compared to 10% in the MTP-PE and IFNγ. Patient characteristics were obtained from the Leukemia Tissue Bank, and was identified to be XYZ.

Figure 5. MTP-PE and IFNγ may expand NK cell frequency in AML PBMC

AML PBMC U-11-0891 patient sample was treated with MTP-PE (10μg/mL) and IFNγ (10ng/mL) at 3x10^6 cells/mL for 24 hours. NK cells population frequency was calculated by staining with Super Bright 600, CD56 (NCAM) Monoclonal Antibody, and analyzing using FlowJo software (FlowJo LLC, Ashland, Oregon). (n=1)
Discussion

The development of NK cell therapies offers significant potential to improve the lives of Acute Myeloid Leukemia patients. The immunosuppressive microenvironment of AML and the evasion of immune escape by leukemia blasts pose major impediments to potent immunosurveillance \(^{16}\). Here, we identified a novel method to activate and potentially expand NK cells in acute myeloid leukemia through NOD2 stimulation and sought to elucidate the signaling mechanism inducing the activation.

Previously, we identified that NK cells are matured and activated in a murine model of AML synergistically upon treatment with MTP-PE and IFN\(\gamma\). However, this may not properly recapitulate the potential for consistent activation across highly heterogeneous AML human patient samples. In this study, we show that NK cells exhibit signals of activation in both AML apheresis samples and PBMC samples \textit{in-vitro}, as referenced by upregulation of markers CD107a and CD69. Additional replicates are needed to reach statistical significance, but there is a clear trend towards activation. This data was constrained by multiple factors, including the minimal basal NK cell frequency in the low volume patient samples, the low viability in frozen AML patient samples and consequent inability to study the cell-cell interactions for longer time points. In the future, we will consider using AML fresh blood samples to overcome these barriers.

Additionally, we observed the upregulation of several significant NK markers through mRNA transcript. IL-15 is known to promote NK and CD8+ T cell activity and is under clinical evaluation for its potential to treat hematological malignancies. The molecule can be both secreted and membrane-bound, which is consistent with our preliminary evaluation that contact with NOD2 stimulated AML cells is necessary for activation \(^{24}\). It has been reported in a study
by Carson et al. that LPS-activated monocytes signal to NK cells *via* production of IL-15, and that this axis of signaling is critical to IFNγ production by NK cells, a proxy of cytotoxic activity. Thus, it could be hypothesized that a similar mechanism of bacterial infection mimicry may induce NK activating effects in myeloid leukemia cells. Indeed, we found a significant upregulation of mRNA transcript across a variety of heterogeneous AML patients upon MTP-PE and IFNγ. Notably, IL-15 signaling to NK cells upregulates other activating NK cell receptors, such as NKG2D, and may prime NK cells for elevated sensitivity to malignant cells. However, additional study is necessary to determine if there is indeed an upregulation of IL-15 on cancer cell surface upon co-treatment and elucidate this interaction’s significance through neutralization of IL-15/Il-15R.

We also observed upregulation of mRNA transcript for NKG2D ligands, MICA and MICB. NKG2D is a major receptor responsible for NK cell activation and has been studied for AML immunotherapy - CAR-T cells targeting NKG2D have shown promising response rates in clinical trials. By upregulating the NKG2D ligands endogenously in AML cells through MTP-PE and IFNγ treatment, we offer a potent strategy for NK cell recognition and clearance. In fact, NKG2D ligand deficiency has been found to define leukemia stem cells, which are the most resistant cancer cells to chemotherapy and are chiefly responsible for refractory leukemic disease. Utilizing NOD2 stimulation to upregulate the expression of NKG2D ligands may selectively improve innate immunosurveillance of leukemia stem cells, directly contributing to relapse prevention. However, our flow cytometry analysis was not concordant with our transcript data, and MICA/B did not show significant upregulation. Additional replicates are necessary to validate the upregulation of these ligands by MTP-PE and IFNγ. In addition, we intend to optimize dilution of the isotype control used in flow cytometry, which exhibited excessive
fluorescence and contributed to incorrect trends in analysis when MFIs were normalized to UT across samples.

In the future, we intend to further validate the expression of the NK activating ligands in AML cells upon MTP-PE and IFNγ treatment, by flow-cytometry, as well as verify NK cell activation in AML patient samples by CD69 and CD107a expression. We also aim to interrogate the various NK-AML interactions we identified in Figures 3 and 4, by neutralizing NKG2D or IL-15R with blocking antibodies and observing NK cell activation. Finally, we aim to study the effect of MTP-PE and IFNγ in a humanized murine model of AML, to aptly recreate the complex immune dynamics of AML in humans and offer closer insights into the potential of this therapeutic paradigm for AML patients.
In conclusion, our studies show that NOD2 stimulation has potential to prevent relapse and improve survival outcomes through the activation of suppressed Natural Killer (NK) cells in Acute Myeloid Leukemia (AML). We show that MTP/IFNγ treated AML cells strongly upregulate IL-15, an important cytokine involved in NK cell expansion and activation, as well as ligands of the receptor NKG2D. In AML patient samples, we have found the co-treatment to upregulate CD69 and CD107a on suppressed patient NK cells, both in peripheral blood and apheresis samples. We also observed significant expansion of NK cells in one of these patient samples. Further study is necessary to evaluate and understand the clinical potential of MTP-PE and IFNγ treatment for NK cell activation. The potential for MTP-PE and IFNγ treatment for AML is significant and its two-fold effects are novel, as it initiates both caspase-1-dependent apoptosis in AML cells, while simultaneously activating NK cells to improve immunosurveillance and anti-leukemic cytotoxicity.
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