Hsp70 in combination with IL-15 and PD-1 blocker interferes induction of cytotoxic NK cells in patients with relapsed acute myeloid Leukemia (AML)

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Abstract

Background: Natural killer cells are critical immune cells for AML targeting. However, little is known about the relationship between using checkpoint inhibitors and Hsp70 as NK cell activators and later immune responses to control AML. We aimed to investigate the antitumor effects of NK cells pre-treated with ex-vivo Hsp70, human PD-1 blocker, and IL-15. **Procedure:** The NK cells were isolated from patients-derived MNCs using MACS and activated using the different combinations of Hsp70, PD-1 blocker, and IL-15. Then their killing potential and the expression pattern of *PRF-1*, *PIK3CB*, *PD-1*, *AKT-1*, *FAS-L*, *TRAIL*, and *GER A & B* were estimated. **Results:** Our data revealed that the PD-1 expression was significantly reduced after NK cell activation with the different formulas of NK cell activators. Also, the expression of NKG2A was reduced, particularly in the IL-15 and IL-15 + PD-1 blocker treated groups, and adding the Hsp70 increased its expression. Moreover, the cytotoxic effect of NK cells increased in all groups, especially in IL-15 + PD-1 blocker group, in parallel with increasing in IFN-γ releasing, Granzymes, and perforin expression. All changes in IL-15 + PD-1 blocker groups were associated with the up-regulation of *PIK3CB* and *AKT-1* as key factors of NK cell activation. The presence of Hsp70 reduced IFN-γ releasing and down-regulation of *PIK3CB, AKT-1, Granzymes,* and *perforin*. **Conclusions:** We suggested that combining IL-15 and PD-1 blockers could enhance the killing potential of AML-NK cells. Moreover, Hsp70, in combination with IL-15 and PD-1 blocker, interferes activation of AML-NK cells through unknown mechanisms.

Title page:

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Abbreviations key:

NK Cell	Natural Killer Cell
AML	Acute Myeloid Leukemia
MNC	Mononuclear Cell
PD-1	Programmed Cell Death Protein 1
CD	Cluster of Differentiation
MACS	Magnetic Activation Cell Sorting
IL-15	Interleukin-15
BM	Bone Marrow
PD-L1	Programmed Cell Death Ligand-1
PBMCs	Peripheral Blood Mononuclear Cells
LDH	Lactate Dehydrogenase
IFN-γ	Interferon-gamma

ABSTRACT

Background: Natural killer cells are critical immune cells for AML targeting. However, little is known about the relationship between using checkpoint inhibitors and Hsp70 as NK cell activators and later immune

responses to control AML. We aimed to investigate the antitumor effects of NK cells pre-treated with ex-vivo Hsp70, human PD-1 blocker, and IL-15.

Procedure: The NK cells were isolated from patients-derived MNCs using MACS and activated using the different combinations of Hsp70, PD-1 blocker, and IL-15. Then their killing potential and the expression pattern of *PRF-1* , *PIK3CB* , *PD-1* , *AKT-1* ,*FAS-L* , *TRAIL* , and *GER A & B* were estimated.

Results: Our data revealed that the PD-1 expression was significantly reduced after NK cell activation with the different formulas of NK cell activators. Also, the expression of NKG2A was reduced, particularly in the IL-15 and IL-15 + PD-1 blocker treated groups, and adding the Hsp70 increased its expression. Moreover, the cytotoxic effect of NK cells increased in all groups, especially in IL-15 + PD-1 blocker group, in parallel with increasing in IFN-γ releasing, Granzymes, and perforin expression. All changes in IL-15 + PD-1 blocker groups were associated with the up-regulation of*PIK3CB* and *AKT-1* as key factors of NK cell activation. The presence of Hsp70 reduced IFN-γ releasing and down-regulation of*PIK3CB, AKT-1, Granzymes,* and *perforin* .

Conclusions: We suggested that combining IL-15 and PD-1 blockers could enhance the killing potential of AML-NK cells. Moreover, Hsp70, in combination with IL-15 and PD-1 blocker, interferes activation of AML-NK cells through unknown mechanisms.

Keywords: AML, NK cells, PD-1, Hsp70, Immunotherapy.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of disorders characterized by malignant clonal proliferation of myeloid blast cells in the bone marrow (BM) and peripheral blood, leading to cytopenia, infections, and bleeding [1,2]. Although many therapeutic interventions have been explored to treat patients with AML, chemotherapeutic regimens remain a crucial therapy component for these patients [3]. In the AML treatment chemotherapy guideline, the two antineoplastic agents, fludarabine and busulfan, are well-known. Fludarabine shows promising results in treating relapsed/refractory patients with AML; however, the neurotoxicity effect of this agent limits the use of high-dose fludarabine[4]. Despite its lower costs and acceptable efficiency over the decades, the significant side effects of busulfan, including mucositis of grade 2 or higher, led to the displacement of this agent with more expensive but secure monoclonal antibody imatinib[5]. To date, NK cell-based immunotherapy is one of the most current innovative immunotherapeutic techniques, unleashing the immunological suppression of NK cells to attack a variety of malignancies [6]. In AML, dysfunctional NK cells or immunosuppressive features of AML cells and their prognostic relevance justify using NK cell-based immunotherapy to restore impaired NK cell cytotoxicity against AML [7]. Meanwhile, treatment with busulfan and fludarabine inactive NK cells (PMID: 2933271) with unknown mechanism. Another defined mechanism that makes weak NK cells, as well as T cells in patients with AML, is overexpression of inhibitory immune checkpoint molecules such as programmed cell death ligand-1 (PD-L1) and PD-L2 up-regulated in blasts [8]. Furthermore, Heat shock protein 70 KDa (Hsp70) is an essential component of the protein folding system called chaperones and protects the cells from stress-induced damage [5]. Hsp70 as an antigenic peptide[9] can be used as a tumor-specific vaccine[10]. Furthermore, Hsp70 induces the release of pro-inflammatory cytokines from innate immune cells, increasing the expression of costimulatory molecules [11]. In addition, Hsp70 activates the NK cell cytotoxic effects against the Hsp70 representing tumor cells [12]. Despite promising outcomes resulting from investigations performed on PD-1 blocker base immunotherapy on solid tumors, the efficacy of this approach is not studied on hematologic malignancies. Therefore, in the present study, we assessed the combined effect of PD-1 blocker and Hsp70 on the activation of NK cells derived from patients with relapsed AML under treatment of Busulfan and fludarabine.

METHODS

Patients

Nine patients diagnosed with relapsed non-M3 AML were treated with fludarabine and busulfan at the Blood and bone Marrow Transplantation center in Taleqani hospital (Tehran, Iran) between 2019 and 2022

were joined in this study. All patients signed informed consent before entering the study, and the advantage of their admission was explained verbally and in writing. All procedures in the present study were performed following the relevant guidelines and regulations of the Royan Institute and approved by the Institutional Review Board and Ethics Committee of the Royan Institute, Tehran, Iran (approval no. IR.ACECR.ROYAN.REC.1400.055). The main biological characteristics of the patients are in Table 1.

Cell culture and Reagents

MNCs were harvested from Peripheral blood using Ficoll-Hypaque density gradient centrifugation. The Ficoll-Paque centrifugation is done as per the manufacturer's protocol. The KG-1 cell line was purchased from Royan Institute Cell Bank (Tehran, Iran). KG-1 cells were cultured in RPMI-1640 containing 10% FBS (GIBCO, Cat. No: 26140-079), 3 mM L-glutamine (GIBCO, Cat. No: 25030-024), 1% Pen/Strep antibiotics (GIBCO, Cat. No: 15070-063), and a humidified atmosphere of 5% CO_2 at 37 °C.

Immunophenotyping of cells

Peripheral Blood Mononuclear Cells (PBMCs) derived from Patients with AML, were stained with FITC anti-human CD335 (NKP46; Cat. No: 331922), PE anti-human CD159a (NKG2A; Cat. No: 142803), PerCp/Cyanine5.5 anti-human CD337 (NKP30; Cat.No:325216), PE anti-human CD314 (NKG2D; Cat. No: 320806), and BD Simulates CD3/CD16$^+$CD56 (Cat. No: 342403) to evaluate the abundance, phenotype and function of NK cells by BD FACS Calibur and analyzed in FlowJo software Ver.10.6.1. All fluorescence-labeled antibodies were acquired from Biolegend (USA).

NK Cell Isolation, cultivation and cytotoxicity assay

NK cells were isolated from MNCs of patients with AML before the initiation of induction chemotherapy (n = 9) by selecting CD56$^+$ cells using magnetic bead selection (Miltenyi Biotech, USA). The purity of the isolated cells was calculated and confirmed by flow cytometry and specific antibody against CD56$^+$. Then, NK cells were divided into five groups (NK cells without any factor, NK cells that received Human IL-15, NK cells that received IL-15 and PD-1 blocker, NK cells received IL-15 and Hsp70, and the last group of NK cells received IL-15, PD-1 blocker and Hsp70). These NK cell groups from patients with AML were assessed for their capacity to kill NK cell-sensitive KG1 cells. The KG-1 cell line was inactivated using Mitomycin C (20 μg/ml; 2×10^6cells per well) to inhibit their proliferation, then they were labeled with Calcein AM for 45 minutes of incubation at 37 °C in 5% CO2. The labeled cells were washed with PBS$^-$, and resuspended in X-X IVO medium with 10% FBS. KG1 cells were co-cultured at an effector: target (E: T) ratio of 10:1, which incubated for 24 hours at 37 °C in 5% CO2. Cytotoxicity level was evaluated with calcein/propidium iodide (PI) staining by flow cytometry technique. The control groups consisted of tumor cells grown in the same media without NK cells exposure.

ΙΦΝ-γ προδυςτιον ανδ ΛΔΗ ρελεασε εαλυατιον

Human Lactate dehydrogenase (LDH) and Interferon-gamma (IFN-γ) ELISA Kits were used to test LDH release from the KG1 cell line, and IFN-γ production by NK cells, following the manufacturer's procedure. Briefly, 100 μL per well of standard solutions or samples was divided into aliquots in duplicate into a precoated 96-well plate. After discarding the plate content, 100 μL/well of biotinylated anti-human LDH and IFN-γ antibodies were added to each well. After rinsing three times with PBS, a prepared ABC working solution (100 μL/well) was added to each well. Finally, after washing with PBS$^-$, a prepared stop solution of 100 μL/well was added to each well, and the plate was read at 450 nm in a microplate reader.

RNA isolation and qRT-PCR

Identic to the manufacturer's procedure, whole cellular RNA (1 μg) was extracted from cells using TRIzol reagent, then assessed the quantity and quality of RNA samples on Nanodrop and gel electrophoresis. Only RNA samples with RNA Integrity Numbers (RIN) > 6 were included in the analyses. Then reverse transcriptional reaction was conducted to obtain cDNA by Prime Script RT Master Mix. According to the manufacturer's instructions, cDNA was synthesized from 1 μg of total RNA with an RT-for-PCR kit

(Takara Bio, Inc., Otsu, Japan). Primers (PRF1, PIK3B, PD-1, AKT-1, FAS-L, TRAIL, GERA & B) were designed and certified using NCBI-Primer BLAST. Specimens were duplicated from three independent trials; β2-microglobulin RNA levels were employed as an internal reference for all experiments. The relative expression levels of genes were calculated using the 2^{-CT} methods.

Statistical analysis

The data were presented as mean ± SEM, and the statistical analysis was performed using GraphPad Prism software (version 9). The Two-tailed Student t-test or paired Student t-tests examined the statistical significance of the two groups' comparisons and one-way or two-way ANOVA analysis when comparing more than two groups. P-values less than 0.05 were considered statistically significant.

RESULT

The Expression pattern of PD-1 on NK, NKT, and T cells in non-M3 relapsed AML

Autologous NK cell therapy is accessible for cancer immunotherapy. However, the main question is whether these NK cells have enough ability to overcome cancer cells. Therefore, we evaluated the expression pattern of PD-1 as an inhibitor marker on NK cells in MNC derived from whole blood samples of seven patients with AML who treated by Fludarabine and Busulfan. Immunophenotyping of whole blood of patients determined that 63.25 +- 5.3% of total lymphocytes were T cells, of which 15.93+-2.76% were PD-1 positive. Instead, NK ($CD56^+/16^+CD3$) cells were about 12.3+-4.18% of total lymphocytes with 7.05 +- 1.14% expression of PD-1, and NKT ($CD56^+/16^+CD3^+$) cells were 6.02+-2.68% of total lymphocyte with 11.47+- 3.28% PD-1 expressing cell (**Fig. 1**).

NK cells Activation by IL-15, Hsp70 and PD-1 blocker

To assess the effect of IL-15, Hsp70, and PD-1 blocker as activation mediators for NK cell therapy, $CD56^+$ cells were isolated from nine patients with non-M3 relapsed AML using manual magnetic cell sorting (MACS). Totally, 80.22+- 2.54 % of purified cells were NK ($CD56^+/16^+CD3^-$) cells, 16 +- 1.85 % were NKT ($CD56^+/16^+CD3^+$) cells and lower than 1.82 +- 0.25 % of them were T cells ($CD56^-/16^-CD3^+$) (**Fig. 2A**).

In the next step, purified cells were incubated with different components, including IL-15, PD-1 blocker, and Hsp70, which were reported to be essential for their expansion and activation**(Fig. 2B)** . The results displayed that the number of PD-1 expressing NK cells and the mRNA level of PD-1 was significantly reduced in all groups treated with IL-15 in combination with Hsp70 and PD-1 blocker, compared to inactive and IL-15 treated group **(Fig. 3A)** . Meanwhile, the expression of NKG2A, as an inhibitory receptor, was reduced in those groups that received in PD-1 blockers. The results indicated that Hsp70 in combination with IL-15 can promote the expression of NKG2A **(Fig. 3B)** . Looking at NKP30 and NKP46 as activator receptors signified that their level reduced in those groups that activated with PD-1 blocker, Hsp70, and their combinations compared to inactive NK cells and IL-15 treated group **(Fig. 3C and 3D).**

The different activator combinations' effect in NK cell-mediated cytotoxicity

As mentioned earlier, the combination of IL-15, Hsp70, and PD-1 blocker reduced the expression pattern of both activatory and inhibitory receptors of the patient's NK cells. Nevertheless, the main question is how these components affect the cytotoxic potential of NK cells. Our results indicated that although cytotoxicity of treated NK cells on the KG1 cell line, as target cells, enhanced in all treated groups, it was dominant in groups with PD-1 blocker in their formulation **(Fig. 4A and 4B).** Meanwhile, the rate of LDH released from KG-1 cells co-cultured with NK cells only exhibited elevation in IL-15 + Hsp70 + PD-1 blocker group that was not significant **(Fig. 4C).** As the lactate dehydrogenase (LDH) assay is used in NK cell cytotoxicity assessment against tumor cells, its release is detected at 4 hours post activation [13]. For the LDH release assay, we removed condition media after 24 hours, but our result did not show a significant difference between groups that may be associated with the NK and KG1 cells co-culture time which incubated over 4 hours. Interferon gamma (IFN-γ) is another factor that its released enhanced post activation of NK cells. IFN-γ

level significantly increased in IL-15 + PD-1 blocker treated group compared with inactive NK cells ($p<0.03$, **Fig. 3D**). Also, we found that in those groups that Hsp70 was in their formulation, the secretion of IFN-γ significantly reduced **(Fig. 3D)** . The reason for this variation may back to use of fludarabine in these patients. Actually it has been reported that fludarabine increases the secretion of interferon, and therefore, after using the protein, its secretion no longer shows an increase [14].

Expression of granzyme A and B in different formulations of NK activators

Granzymes are derived from serine proteases and expressed in cytotoxic T cells and NK cells [15]. GZMA and GZMB are carried out and stored in the particular granules of resting NK cells and released after activation [16]. Therefore, we assessed the expression of GZMA and GZMB in different groups of activated NK cells from seven patient with AML. The results indicated that IL-15 + Hsp70 + PD-1 blocker group upregulated the expression of both GZMA and GZMB ($p<0.01$, **Fig. 4E and 4F**). Moreover, the lytic granules of NK cells contain the pore-forming protein perforin that, after the formation of immune synapses, creates a pore in the membrane of the target cell; finally, granzymes may enter the target cell cytoplasm and cleave several substrates, leading to apoptosis via the intrinsic pathway [17]. our result also showed that expression of PRF-1 in NK cells exposed to IL-15 + Hsp70 + PD-1 blocker was increased compared to all other groups **(Fig. 4G).**

Changing in the regulatory system of NK cells in patients with AML post activation *in vitro*

Fas ligand and TRAIL as death ligands on the surface of NK cells can activate target killing by attaching death receptors on the target cell, which activates NK cell cytotoxicity through caspase-8 dependent pathway extrinsic apoptosis [18]. We expected that Fas ligand expression upregulated in activated groups. However, its over-activated when the NK-cells were treated with IL-15 + Hsp70 ($p<0.0001$,**Fig. 5A**). Similarly, TRAIL expression also increased in IL-15 + Hsp70 group but it was just significant to IL-15 + PD-1 blocker + Hsp70 group ($p<0.0089$, **Fig. 5B**). Studies showed that PI3K–AKT–mTOR pathway is the main pathway in regulating the development, differentiation, and activation processes of immune cells like NK cells [19]. Also, PIK3CB, as a subunit of (phosphoinositide 3-kinase) PI3K, has a crucial role in NK cell cytotoxicity [20]. In the assessment of the AKT-1 gene, it seems that the PD-1 blocker in combination of IL-15 has the potential to increase its expression levels (**Fig. 5C**). Also, the increased expression level of PIK3CB in the IL-15 + PD-1 blocker treated NK cells was significant in comparison to other groups ($p<0.01$, **Fig. 5D**) excluding the IL-15 + PD-1 blocker + Hsp70 group which was not significant ($p>0.05$,**Fig. 5D**).

DISCUSSION

Reflective of the current study revealed several key findings that help us better understand the role of Hsp-70 and PD-1 blocker-based therapy in patients with AML. Immune checkpoints, including PD-1 and CTLA-4, are novel targets for cancer immunotherapy and made a promising tool in the path of solid cancer treatment [21,22]. PD-1/PD-L1 inhibition has been demonstrated in multiple myeloma patients to improve NK cell-mediated tumor lysis [23,24]. Despite the favorable outcomes of blocking such immune checkpoints, the results were not well studied in hematological cancers. meanwhile, many malignant cells over-expressed heat shock proteins, including Hsp70 and Hsp90, which indicate their crucial role in malignant progression[25,26]. Regarding AML, the overexpression of Hsp70 is identified [27,28], however, its role is controversial[28,29]. Several studies have reported that Hsp70 can induce cytotoxic activity in NK cells against tumors, including melanoma and glioblastoma [30,31]. We depicted that pre-treatment of NK cells with Hsp70 and PD-1 inhibitor may stimulate their anti-tumor effects. Our results indicated that the percentage of NK cells in patients with non-M3 relapsed AML who received fludarabine and busulfan was about 12.3±4.18% of total lymphocytes similar to normal people[32]. However, 7.05 ± 1.14% of them expressed PD-1 in NK cell population that higher than PD-1 expression in normal NK cells (2-5%) [14,33]. The main question of the present study was to evaluate the combinatory effect of Hsp70 and PD-1blocker to activate NK cells derived from patients with non-M3 relapsed AML. We found that the expression of PD-1 significantly reduced when NK cells activated with a different formula of IL-15, Hsp70, and PD-1 blocker (**Fig. 3A**). However, the activatory and expanded clones in groups that received IL-15 + PD-1 blocker seemed to be more than other groups (**Fig. 2B, Fig.**

3B and 3**C**). NKG2A, in cooperation with PD-1, exerts its inhibitory activity on NK cells through binding to classical and non-classical MHC class I molecules[34]. Our results exhibited that the expression of surface NKG2A in the NK cells treated in IL-15 and IL-15 + PD-1 blocker groups, considerably decreased (**Fig. 3B**) and its expression was increased with adding the Hsp70 to activation media. It is worth mentioning that IL-15 is recommended to be involved in PD-1 blocking and NK cell activation through activation of the PI3K/AKT/mTOR signaling pathway [35], as also observed in our data. The over-expression of NKG2A also was reported by Fehniger et al. study, which showed induction of cytotoxicity in NK cells in parallel to increasing NKG2A when the cells were treated with IL-2/TKD (TKDNNLLGRFELSG; a 14-mer Hsp70 subunit) [36]. Here our results showed a significant increase in the expression level of PIK3CB and AKT-1, the downstream molecules of NKG2D and critical regulator of NK cell activation, in the presence of IL-15 + PD-1 blocker. However, we did not detect any changes in the level of NKG2D positive NK cells, and a significant reduction was observed in NKP30 and even NKP46 post treatment of cells by Hsp70 or PD-1 blocker and both of them (**Fig. 3C** and **D**). Meanwhile, the cytotoxic potential of NK cells enhanced in groups that received PD-1 blocker, which was concomitant with increasing in releasing of interferon gamma (IFN-γ) and upregulation of granzyme A/B and PRF1[16,37-40].

Although, several studies reported that a combination of Hsp70 and PD-1 blocker might over-activate NK cells in Fighting cancer cells[41,42]. But our results presented that the presence of Hsp70 as an activatory factor in the combination of IL-15 for increases the ambiguity of data. For example, Hsp70 in activating media caused an increase in the NKG2A positive cells, reduced IFN-γ releasing, and reduced the expression of FAS-L and TRIAL. All uncertainties may back to AML-NK cells as a source of NK cells and even the undefined role of Hsp70 these patient's specific cells [43,44]. Therefore, based on the results presented in this study, we suggested that the combination of IL-15 and PD-1 blocker can reactive AML-NK cells, increasing their killing ability against tumor cells, enhanced the key factors in NK cell function and increase releasing of IFN-γ, granzymes as well as perforin. Moreover, we clearly showed that Hsp70 acts as a disruptive factor to induce cytotoxic NK cells when combined with IL-15 and PD-1 blocker.

Conflict of Interest statement

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest.

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Ethical approval and consent to participate

All procedures in the present study were performed in accordance with the relevant guidelines and regulations of the Royan Institute for Stem Cell Biology and Technology and approved by the Institutional Review Board and Ethics Committee of the Royan Institute, Tehran, Iran (approval no. IR.ACECR.ROYAN.REC.1400.055).

Authors' contributions

JF contributed to the conceptualization of the study, data collection, and was a major contributor to writing the manuscript. AHa revised the manuscript and supervised the study. MA, and FG contributed to data collection and data analysis. AHe revised the manuscript. MS and ME contributed to the conceptualization of the study, revised the manuscript, and supervised the study. All authors read and approved the final manuscript.

References

1. Ghazawi FM, Le M, Cyr J, et al. Analysis of acute myeloid leukemia incidence and geographic distribution in Canada from 1992 to 2010 reveals disease clusters in Sarnia and other industrial US border cities in Ontario. *Cancer* . 2019;125(11):1886-1897.

2. Zhang C, Lam SS, Leung GM, et al. Sorafenib and omacetaxine mepesuccinate as a safe and effective treatment for acute myeloid leukemia carrying internal tandem duplication of Fms-like tyrosine kinase 3. *Cancer* . 2020;126(2):344-353.

3. Kantarjian HM, Jabbour EJ, Garcia-Manero G, et al. Phase 1/2 study of DFP-10917 administered by continuous intravenous infusion in patients with recurrent or refractory acute myeloid leukemia. *Cancer* . 2019;125(10):1665-1673.

4. Jackson GH. Use of fludarabine in the treatment of acute myeloid leukemia. *The Hematology Journal: the Official Journal of the European Haematology Association* . 2004;5:S62-7.

5. Rasor B, Dickerson T, Zhao Q, et al. Comparison of fixed dose reduced-intensity conditioning with fludarabine and busulfan to PK-guided busulfan AUC (FluBu4K) in hematopoietic stem cell transplant for AML/MDS. *Leukemia & Lymphoma* . 2021;62(4):944-951.

6. Ciurea SO, Kongtim P, Soebbing D, et al. Decrease post-transplant relapse using donor-derived expanded NK-cells. *Leukemia* . 2022;36(1):155-164.

7. Martín-Antonio B, Suñe G, Perez-Amill L, Castella M, Urbano-Ispizua A. Natural killer cells: angels and devils for immunotherapy.*International journal of molecular sciences* . 2017;18(9):1868.

8. Xu J, Niu T. Natural killer cell-based immunotherapy for acute myeloid leukemia. *Journal of Hematology & Oncology* . 2020;13(1):1-20.

9. Castellino F, Boucher PE, Eichelberg K, et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. *The Journal of experimental medicine* . 2000;191(11):1957-1964.

10. Udono H, Srivastava PK. Heat shock protein 70-associated peptides elicit specific cancer immunity. *The Journal of experimental medicine* . 1993;178(4):1391-1396.

11. Asea A, Kraeft S-K, Kurt-Jones EA, et al. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature medicine* . 2000;6(4):435-442.

12. Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels R. Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. *The Journal of Immunology* . 1997;158(9):4341-4350.

13. Yang L, Shen M, Xu LJ, et al. Enhancing NK cell-mediated cytotoxicity to cisplatin-resistant lung cancer cells via MEK/Erk signaling inhibition. *Scientific reports* . 2017;7(1):1-13.

14. Niu C, Li M, Zhu S, et al. PD-1-positive Natural Killer Cells have a weaker antitumor function than that of PD-1-negative Natural Killer Cells in Lung Cancer. *International Journal of Medical Sciences* . 2020;17(13):1964.

15. Ewen C, Kane K, Bleackley R. A quarter century of granzymes.*Cell Death & Differentiation* . 2012;19(1):28-35.

16. Chung YM, Khan PP, Wang H, et al. Sensitizing tumors to anti-PD-1 therapy by promoting NK and CD8+ T cells via pharmacological activation of FOXO3. *Journal for immunotherapy of cancer* . 2021;9(12)

17. Ambrose AR, Hazime KS, Worboys JD, Niembro-Vivanco O, Davis DM. Synaptic secretion from human natural killer cells is diverse and includes supramolecular attack particles. *Proceedings of the National Academy of Sciences* . 2020;117(38):23717-23720.

18. Zhu Y, Huang B, Shi J. Fas ligand and lytic granule differentially control cytotoxic dynamics of natural killer cell against cancer target. *Oncotarget* . 2016;7(30):47163.

19. Ali AK, Nandagopal N, Lee S-H. IL-15–PI3K–AKT–mTOR: a critical pathway in the life journey of natural killer cells. *Frontiers in immunology* . 2015;6:355.

20. Ruiz-Garcia R, Vargas-Hernandez A, Chinn IK, et al. Mutations in PI3K110D Cause Impaired NK Cell Function Partially Rescued by Rapamycin Treatment. SPRINGER/PLENUM PUBLISHERS 233 SPRING ST, NEW YORK, NY 10013 USA; 2017:S4-S4.

21. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell–mediated antitumor immunity. *Nature medicine* . 2003;9(5):562-567.

22. McDermott DF, Atkins MB. PD-1 as a potential target in cancer therapy. *Cancer medicine* . 2013;2(5):662-673.

23. Clara JA, Childs RW. Harnessing natural killer cells for the treatment of multiple myeloma. Elsevier; 2022:

24. Costa F, Marchica V, Storti P, Malavasi F, Giuliani N. PD-L1/PD-1 axis in multiple myeloma microenvironment and a possible link with CD38-mediated immune-suppression. *Cancers* . 2021;13(2):164.

25. Albakova Z, Armeev GA, Kanevskiy LM, Kovalenko EI, Sapozhnikov AM. HSP70 multi-functionality in cancer. *Cells* . 2020;9(3):587.

26. Murphy ME. The HSP70 family and cancer. *Carcinogenesis* . 2013;34(6):1181-1188.

27. Reikvam H, Hatfield KJ, Ersvaer E, et al. Expression profile of heat shock proteins in acute myeloid leukaemia patients reveals a distinct signature strongly associated with FLT3 mutation status–consequences and potentials for pharmacological intervention. *British journal of haematology* . 2012;156(4):468-480.

28. Ryningen A, Ersvaer E, Oyan AM, et al. Stress-induced in vitro apoptosis of native human acute myelogenous leukemia (AML) cells shows a wide variation between patients and is associated with low BCL-2: Bax ratio and low levels of heat shock protein 70 and 90. *Leukemia research* . 2006;30(12):1531-1540.

29. Chant ID, Rose PE, Morris AG. Susceptibility of AML cells to in vitro apoptosis correlates with heat shock protein 70 (hsp70) expression. *British journal of haematology* . 1996;93(4):898-902.

30. Lobinger D, Gempt J, Sievert W, et al. Potential role of Hsp70 and activated NK cells for prediction of prognosis in glioblastoma patients. *Frontiers in Molecular Biosciences* . 2021;8:669366.

31. Elsner L, Flugge PF, Lozano J, et al. The endogenous danger signals HSP70 and MICA cooperate in the activation of cytotoxic effector functions of NK cells. *Journal of cellular and molecular medicine* . 2010;14(4):992-1002.

32. Rakova J, Truxova I, Holicek P, et al. TIM-3 levels correlate with enhanced NK cell cytotoxicity and improved clinical outcome in AML patients. *Oncoimmunology* . 2021;10(1):1889822.

33. Liu Y, Cheng Y, Xu Y, et al. Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. *Oncogene* . 2017;36(44):6143-6153.

34. Backstrom E, Kristensson K, Ljunggren HG. Activation of natural killer cells: underlying molecular mechanisms revealed. *Scandinavian Journal of Immunology* . 2004;60(1-2):14-22.

35. Moynagh PN. IL-15 in autoimmunity and cancer: O-tu-b or not O-tu-b? *Nature Immunology* . 2019;20(7):780-782.

36. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. *Blood, The Journal of the American Society of Hematology* . 2001;97(1):14-32.

37. Hurkmans DP, Basak EA, Schepers N, et al. Granzyme B is correlated with clinical outcome after PD-1 blockade in patients with stage IV non-small-cell lung cancer. *Journal for immunotherapy of cancer* . 2020;8(1)

38. Bai R, Cui J. Burgeoning Exploration of the Role of Natural Killer Cells in Anti-PD-1/PD-L1 Therapy. *Frontiers in Immunology* . 2022:2253.

39. Calvo T, Reina-Ortiz C, Giraldos D, et al. Expanded and activated allogeneic NK cells are cytotoxic against B-chronic lymphocytic leukemia (B-CLL) cells with sporadic cases of resistance. *Scientific reports* . 2020;10(1):1-14.

40. Davis Z, Felices M, Lenvik T, et al. Low-density PD-1 expression on resting human natural killer cells is functional and upregulated after transplantation. *Blood advances* . 2021;5(4):1069-1080.

41. Shevtsov M, Pitkin E, Ischenko A, et al. Ex vivo Hsp70-activated NK cells in combination with PD-1 inhibition significantly increase overall survival in preclinical models of glioblastoma and lung cancer. *Frontiers in Immunology* . 2019;10:454.

42. Zeng Y, Lv X, Du J. Natural killer cell-based immunotherapy for lung cancer: Challenges and perspectives. *Oncology Reports* . 2021;46(5):1-14.

43. Thomas X, Campos L, Le Q-H, Guyotat D. Heat shock proteins and acute leukemias. *Hematology* . 2005;10(3):225-235.

44. Reikvam H, Nepstad I, Sulen A, Gjertsen BT, Hatfield KJ, Bruserud O. Increased antileukemic effects in human acute myeloid leukemia by combining HSP70 and HSP90 inhibitors. *Expert opinion on investigational drugs* . 2013;22(5):551-563.

Legends:

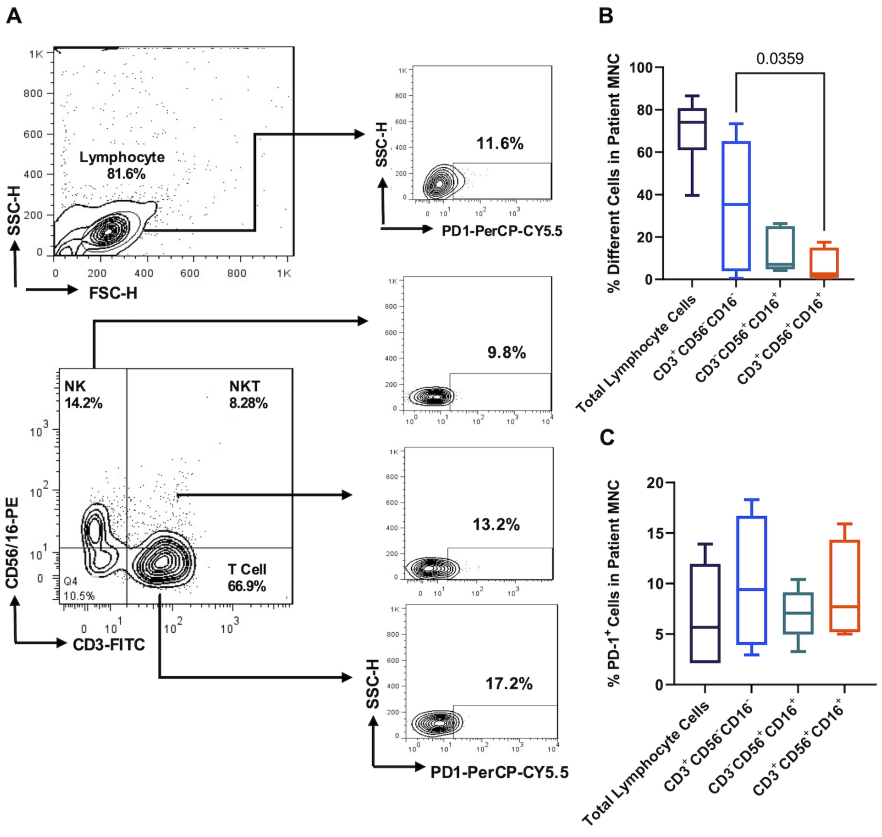
Figure 1 The PD-1 expression on T, NKT and NK cells derived from MNCs of patients with non-M3 relapsed AML. A. Schematic presentation of different cell types and characterization of PD-1 expressing cell types in patients' MNCs. **B.** Quantification demonstration of different cells in the patients' MNCs. **C** . PD-1 expressing cell types graph illustrate 11.6% of total lymphocyte cells express PD-1 receptor and 9.8% of $CD56^+$ and $CD16^+$ cells, known as NK cells, express PD-1 receptor. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis performed using unpaired two-way Anova. (n=7, *$p<0.05$, **$p<0.01$, and ***$p<0.001$). CD: cluster of differentiation; MNC: mononuclear cells; PD-1: Programmed cell death protein.

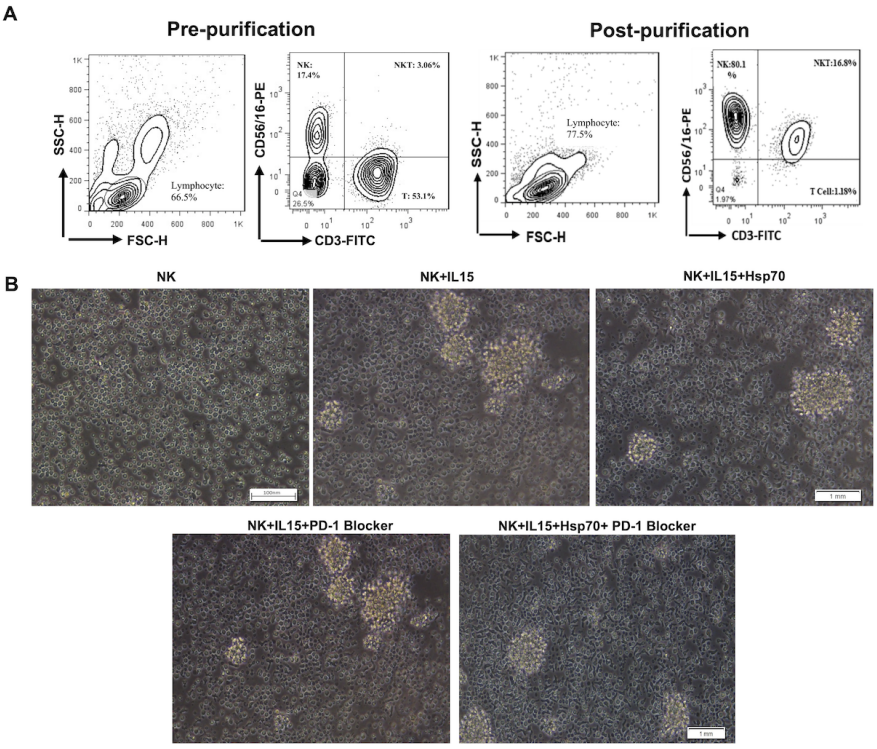
Figure 2 NK cell isolation and activation in different component. A. Purification of NK cells before isolation showed about 17% NK cells which showed more than 80% NK cells after isolation with MACS, less than 2% T cells, and 16% NKT cells. **B.**Morphologically, NK cells in different components, including IL-15, Hsp70, and PD-1 blocker, displayed round clones with the ability of expansion and single activated NK cells, (n=7). Images were obtained with a 20x objective, the magnification used for morphological assessment (scale = 100 μm).

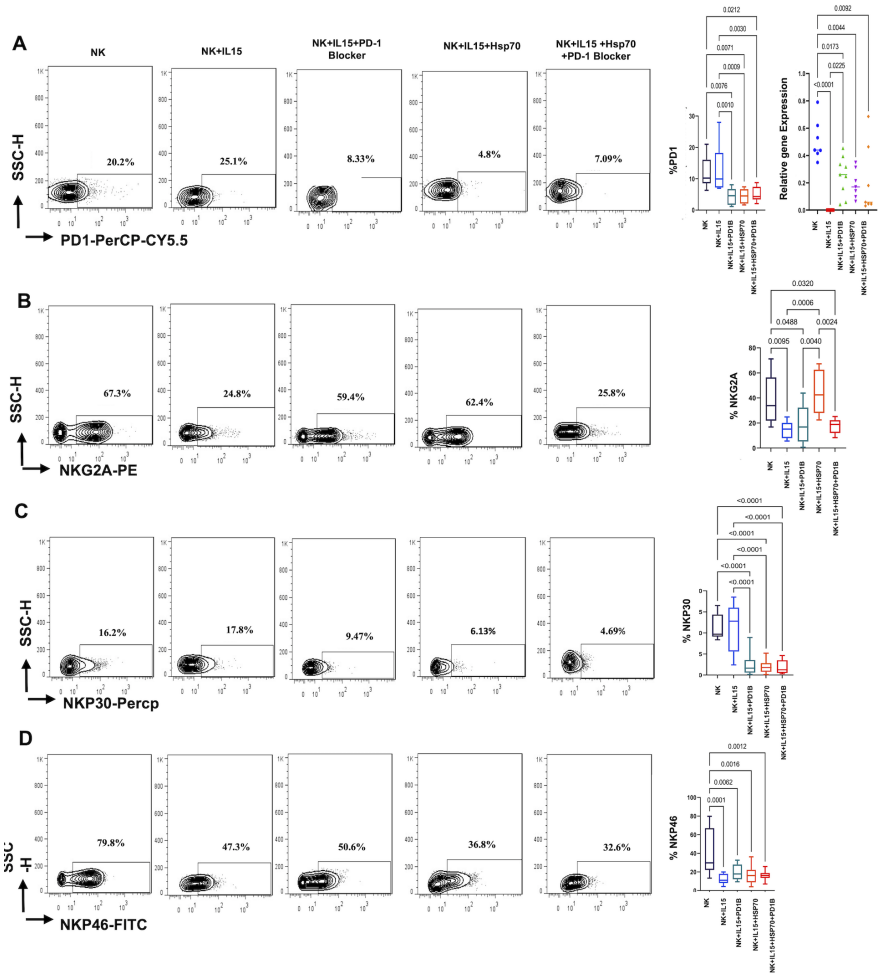
Figure 3 The expression of activatory and inhibitory receptors in different activation NK cells . Purified NK cells were activated in presence of IL-15, Hsp70 and PD-1 blocker for 24 hours **A.** The expression of PD-1 was assessed at mRNA and protein level. The results indicated of higher reduction of PD-1 in those group that received Hsp70, PD-1 blocker and their combination than IL-15 or in active NK cell, (n=9, $p<0.02$). **B.** The expression of NKG2A was significantly reduced in all groups (n=9, $p<0.04$) except combination of IL-15 and Hsp70 (n=9, $p>0.05$). **C.**The expression of NKP30 and D. NKP46 as activator receptors reduced in all combinatorial groups post activation (n=9, $p<0.006$), which means that their expression was higher in inactivated NK cells. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis performed using unpaired two-way Anova. (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

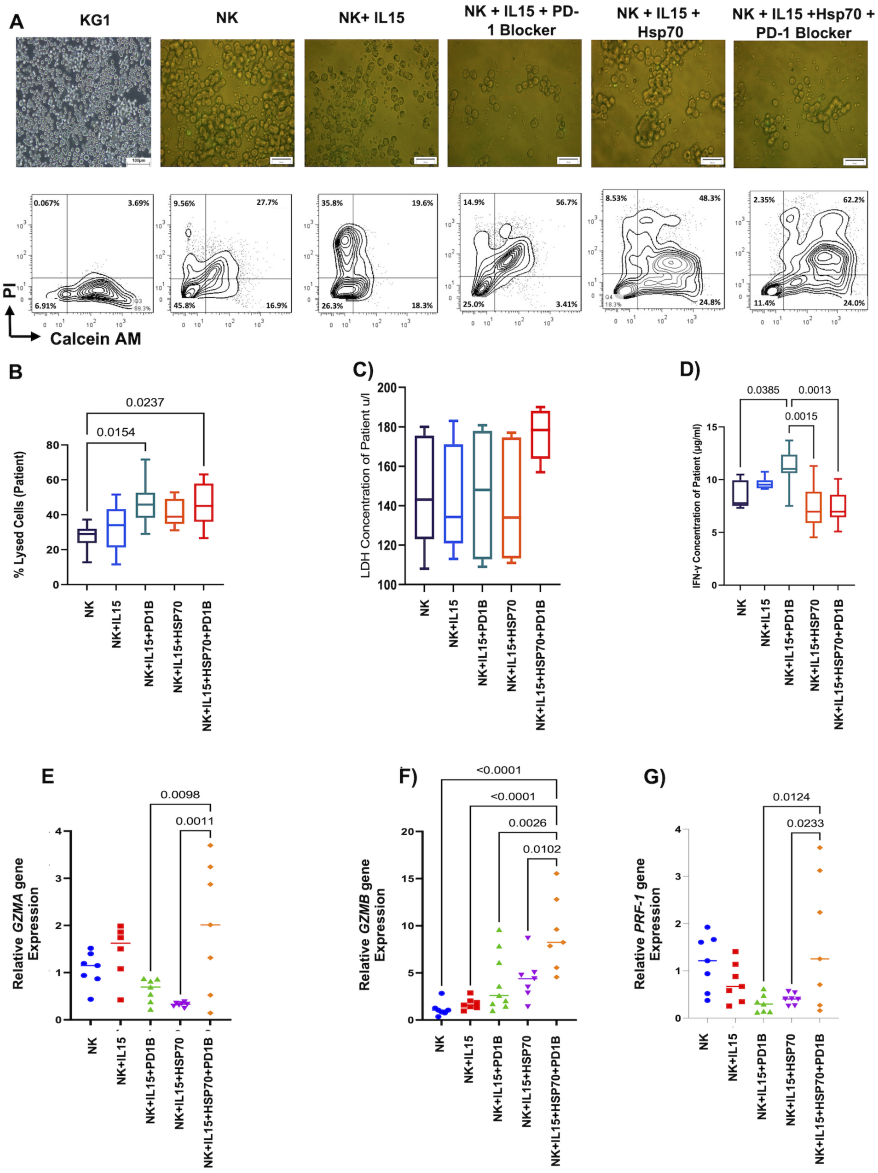
Figure 4 The effect of different activator combinations on NK cell-mediated cytotoxicity. A. Morphological illustration and flowcytometry results of different activator combinations' effect in NK cell-mediated cytotoxicity on KG-1 cell line. Images were obtained with a 40x objective, the magnification used for morphological assessment (scale =50 μm). **B.** NK cell cytotoxicity potential increased in all groups compared to the inactive group. However, it was significant in those groups that had PD-1 blocker in their formulation, (n=9, $p<0.02$). **C.** The LDH released from KG-1 cells co-cultured with NK cells only increased in IL-15 + Hsp70 + PD-1 blocker group that was not significant (n=7, $p>0.05$). **D.**IFN-γ level in the group that received IL-15 + PD-1 blocker significantly increased compared to other groups ($p<0.03$). Although, it was not significant in comparison to IL-15 treated group (n=7, $p>0.05$). **E** . *GZMA* and **F.***GZMB* expression level was increased in IL-15 + Hsp70 + PD-1 blocker group compared to other groups (n=7, $p<0.01$).**G.** *PRF-1* was higher in IL-15 + Hsp70 + PD-1 blocker treated group (n=7, $p>0.02$). Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis performed using unpaired two-way Anova. (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

Figure 5 NK cell gene regulation after activation in patients with AML. A. *Fas ligand* expression upregulated in activated groups. However, it was over-activated when the NK-cells were treated with IL-15 + Hsp70 (n=7, $p<0.0001$). **B.** The expression of *TRAIL* gene was upregulated in IL-15+Hsp70 treated NK cells which is just significant compared to IL-15+ Hsp70 + PD-1 blocker (n=7, $p<0.008$). **C.** *AKT-1* expression level showed a significant increment in the IL-15 + PD-1 blocker treated NK cells compared to other groups (n=7, $p<0.01$). **D.** The expression level of *PIK3CB* enhanced in the IL-15+PD-1 blocker treated NK cells but as shown in the plot there is no significance with IL-15 + Hsp70 + PD-1 blocker group (n=7, $p<0.01$). Statistical analysis performed using unpaired two-way Anova. (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).









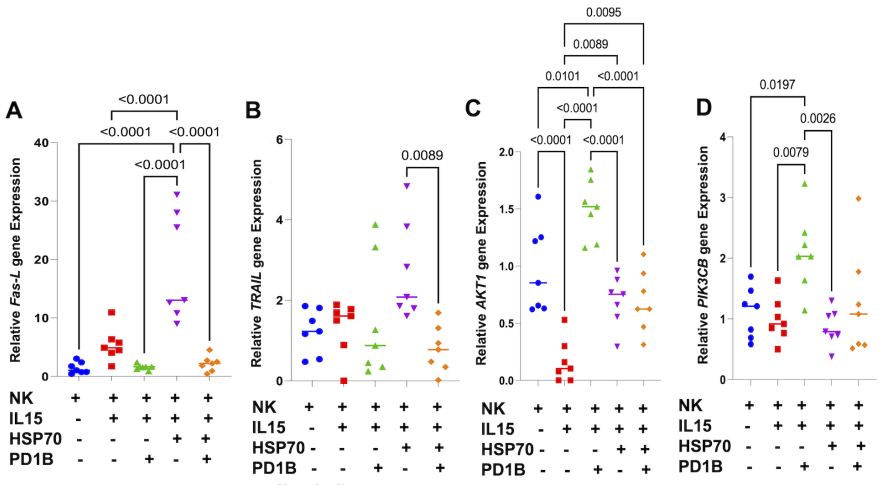


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TABLE 1.docx available at https://authorea.com/users/503127/articles/582916-hsp70-in-combination-with-il-15-and-pd-1-blocker-interferes-induction-of-cytotoxic-nk-cells-in-patients-with-relapsed-acute-myeloid-leukemia-aml