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Original Article

Blocking PCNA interaction with NKp44 enhances primary natural killer cell-mediated lysis of triple-negative breast cancer cells

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Abstract: Among the innate immune cells, natural killer cells (NK) serve its role in cytolytic targeting against infected and cancerous cells. NK function is regulated by an intricate balance of signals from interactions between activating and inhibitory NK receptors and ligands expressed on target cells. As an immune evasion strategy, cancer cells, particularly triple-negative breast cancer cells (TNBCs), express ligands that interact with NK receptors to inhibit NK cell cytolytic function. Our studies have revealed that Proliferating Cell Nuclear Antigen (PCNA), normally expressed in the nucleus with DNA replication and repair roles, was present on the cell surface of TNBC cell lines MDA-MB-231, -436, and -468. To elucidate the function of cell surface PCNA, we blocked PCNA on TNBCs with antibodies which both disrupted interaction with NKp44 and enhanced lysis by primary NK cells. Furthermore, a combinational antibody treatment of TNBCs with α-LLT1 and α-PCNA antibodies augments NK-mediated lysis. These results together suggest that cell surface PCNA on TNBCs enables evasion from cytolytic killing by NK cells. Blocking PCNA-NKp44 interaction with antibodies may potentially open an additional avenue in treatment of TNBCs.

Keywords: PCNA, triple-negative breast cancer (TNBC), natural killer cells, NKp44, PCNA-NKp44 interaction

Introduction

As part of the innate immune response against tumors, viruses, and microbial infections, natural killer cells (NK), which comprise of 5 to 20% of circulating lymphocytes, utilize its cytotoxic functions to eradicate these targets [1, 2]. Upon activation, NK cells produce proinflammatory cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF-α), and initiate degranulation of perforin and granzymes onto the surface of target cells [3-6]. The ability of NK cells to activate itself, proliferate, and amplify its cytotoxic effector functions are regulated by an intricate balance of signals through its activating and inhibitory NK receptors interacting with ligands on surfaces of target cells [7]. This balance of signals from NK cell receptors interacting with ligands on target cells allows NK cells to distinguish between "self" healthy cells by recognizing a 'self-peptide' on the major histocompatibility complex-I (MHC-I) and inhibitory ligands from foreign, infected, or tumorigenic cells [8]. To enable recognition and killing of tumor and infected cells, NK cells utilize natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, and NKp46, which binds to activating ligands that are then transmitted through immunoreceptor tyrosine-based activating motifs (ITAMs) located in the cytoplasmic side of the receptor to induce cytotoxicity [7, 9].

Among the NCRs, NKp44 expression on NK cells promotes lysis of tumor cell lines by transducing signals through the ITAM containing accessory protein DAP12 which leads to release of cytolytic granules, TNF-α, and IFN-γ [9-11]. It has also been shown that NKp44 cytoplasmic tail contains a functional motif with a tyrosine sequence similar to immunoreceptor tyrosine-based inhibitory motif (ITIM) which inhibits NK

effector functions [10, 11]. Therefore, these studies suggest that engagement with NKp44 can either activate or inhibit NK function based on ligand specificity. Despite NK's utility to use NKp44 for killing target cells, cancer cells can also utilize NKp44 on NK cells to evade recognition [9, 12]. Prior studies have shown that ligand interaction with NKp44 led to inducing Fas-mediated apoptosis of NK cells [13].

Particularly, proliferating cell nuclear antigen (PCNA) has been shown to be a ligand that interacts with NKp44 and inhibit NK effector functions [12, 14]. Generally, PCNA is an accessory protein in the nucleus that contributes its roles in DNA replication, DNA repair mechanisms, chromatin remodeling, and cell cycle regulation [15, 16]. Beyond these general functions, overexpression of PCNA on cell surfaces of pancreatic, melanoma, lymphoma, and glioblastoma cells interacting with NKp44 inhibits NK activation and promotes tumor formation [14]. Of importance, Horton *et al.* have previously identified a novel interaction between Proliferating Cell Nuclear Antigen (PCNA) and Human Leukocyte Antigen I (HLA-I) on the surface of human diffuse B cell lymphoma cells [12]. This co-association of PCNA and HLA-I interacting with NKp44 had been shown to inhibit NK cell cytotoxic function and IFN-γ release against B cell lymphoma cells [12]. Furthermore, our lab demonstrated that blocking PCNA or NKp44 increased specific lysis of colorectal cancer cells by NK cells [17]. Therefore, this suggests that targeting cell surface PCNA on more invasive cancers, such as TNBCs, by blocking PCNA-NKp44 interaction to promote NK lysis can potentially be utilized as an additional immunotherapeutic strategy.

Among the most invasive cancers, triple-negative breast cancer (TNBC) lacks expression of estrogen (ER), progesterone (PR), and human epidermal growth factor-2 (HER-2) receptors, which presents a major clinical challenge due to its subpar response to hormonal treatments [19]. Importantly, prior studies have demonstrated that TNBCs utilize mechanisms to specifically evade NK immune recognition and response by upregulating expression of Lectin-Like Transcript-1 (LLT1) to interact with NKR-P1A, a natural cytotoxicity receptor (NCR) expressed on activated NK cells [20, 21]. Thus, the paradigm of inhibitory ligands expressed on cancer cells and its functions to evade NK surveillance has yet to be fully understood, which warrants further study on different types of cancers in order to develop effective NK immunotherapeutic strategies.

For this study, we postulated that TNBCs express PCNA on its cell surface to evade NK-mediated killing through interaction with NKp44. We have observed higher expression and abundance of cell surface PCNA on human TNBC cell lines than non-tumorigenic breast cells. Furthermore, blocking surface PCNA with anti-human PCNA antibodies have enhanced lysis by primary NK cells. In addition, we seek to determine if combinational targeting of multiple ligands on TNBCs can further increase lysis of TNBCs. Since it has been previously shown that blocking LLT1 on human TNBCs has increased NK-mediated killing, we simultaneously blocked interaction of PCNA, HLA-I, and LLT1 with respective NK receptors to assess further amplification of TNBC lysis [20]. Hence, we conclude that inhibiting surface PCNA-NKp44 interaction renders TNBCs highly susceptible to killing by NK cells, which can introduce a potential avenue to future treatment of patients with TNBC and other invasive cancers.

Materials and methods

Cell lines and culture

Non-tumorigenic human cell line MCF10A and human TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were acquired from American Type Culture Collection (ATCC). MDA-MB-231 and MDA-MB-436 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Life Technologies), and 1X penicillin-streptomycin (Life Technologies). MDA-MB-468 cells were cultured in 4+ Roswell Park Memorial Institute 1640 complete medium (RPMI, Life Technologies) containing 15% FBS and 1X penicillin-streptomycin. MCF10A cells were grown in Medium 171 (Life Technologies) containing Mammary Epithelial Growth Supplement (Life Technologies). Cells were cultured in sterile culture flasks in a 37°C 5% CO_2

humidified incubator and grown to 80-90% confluence before passage. Passaging was performed by aspirating the cells carefully with 1X PBS with 10 mM ethylene-diamine-tetracetic acid (EDTA) or Trypsin-EDTA (Life Technologies).

Isolation of primary natural killer cells from peripheral blood mononuclear cells

With approval by the University of North Texas Health Science Center Institution Review Board, whole blood samples were first collected from healthy individuals with consent. Using the method of density centrifugation with Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO), peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated whole blood samples. Per manufacturer's protocol, primary NK cells were isolated from PBMCs by using a NK cell isolation kit (Miltenyi Biotec, San Diego, CA), which uses a magnetic column of the MACS Miltyenyi Biotec Separator to collect the flow-through fluid containing purified NK cells. Human primary NK cells were cultured in 4+ RPMI complete media supplemented with 15% FBS and 50 U/ml of recombinant human interleukin-2 (Peprotech).

Flow cytometry

PCNA cell surface expression was analyzed by flow cytometry. MCF10A, MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells were grown to 80-90% confluence on sterile culture flasks before harvesting with 1X PBS-EDTA for collection. Cells were not permeabilized in order to analyze membrane expression of PCNA. To minimize non-specific binding of antibodies, cells were first treated with human IgG Fc fragment (Rockland, Inc.). After blocking, cells were surface stained with anti-human PCNA-PE antibodies (5 μl per 10^6 cells; BioLegend, Clone #PC10) or isotype control mouse anti-human IgG_{2a}-PE antibodies (Biolegend, Clone #MOPC-21, San Diego, CA) in the dark at 4°C for 30 minutes. After staining, cells were washed with 1X PBS with BSA to remove unbound antibodies. Cells were analyzed by the Beckman Coulter Cytomics FC500 Flow Cytometer in the University of North Texas Health Science Center Flow Cytometry Core Facility. FlowJo v10 software (FlowJo, LLC, Ashland, OR) was utilized for data analysis to assess percentage of events that are $PCNA^+$.

Subcellular protein fractionation

Cells were cultured up to 80-90% confluency in a cell culture flask before harvesting by aspirating with 1X PBS containing 10 mM EDTA. Approximately one million cells of each cell line were then harvested and centrifuged into a pellet. Subcellular protein fractionation was utilized to isolate membrane, cytoplasmic, and nuclear proteins in cell lines being tested according to manufacturer's protocol using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Waltham, MA). Isolated proteins were used for western blot analysis.

Western blot

Membrane, cytoplasmic, and nuclear protein samples of each cell line isolated from the subcellular protein fractionation method were loaded on the Novex 4-12% Bis-Tris Gels (Life Technologies) for gel electrophoresis. Proteins separated on the gel were then transferred onto a nitrocellulose membrane using the iBlot Gel Horizontal Transfer Device (Invitrogen). After transfer, the nitrocellulose membrane was then blocked with 5% fat-free milk in 1X Tris-buffer saline (TBS) at room temperature for 60 minutes before incubation with mouse anti-human unconjugated PCNA antibody (BioLegend) in 5% fat-free milk with 0.05% Tween-20 overnight at 4°C. Upon completion of overnight incubation, membrane was washed 0.05% Tween-20 and incubated with horseradish peroxidase-linked (HRP) anti-mouse secondary antibody at room temperature for 2 hours before imaging.

^{51}Cr release assay

Cells were grown to 80-90% confluency in sterile flasks before harvesting by aspirating with 1X PBS containing 10 mM EDTA. After harvesting, target cells were labeled with radioactive isotype ^{51}Cr (Perkin Elmer, Waltham, MA) for 90 minutes in the 37°C 5% CO_2 incubator. Target cells were then either incubated with 1 μg of mouse anti-human PCNA antibody, 1 μg of goat anti-human LLT1 antibody (ThermoFisher Scientific), 1 μg of anti-human HLA-I antibody, or 1 μg of mouse anti-human IgG_{2a} isotype control for 30 minutes to allow antibody binding to ligand. Primary NK cells were treated with human Fc fragment to block all CD16a recep-

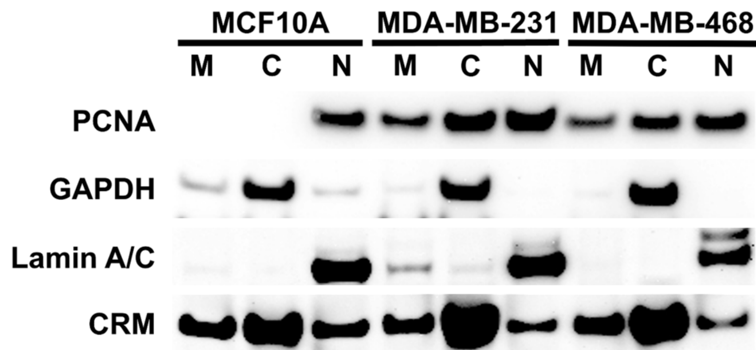

Figure 1. Elevated presence of cell surface and cytosolic PCNA on TNBC cell lines. To assess for differences in PCNA protein abundance in cell membrane (M), cytoplasm (C), and nucleus (N), proteins of normal mammary MCF10A cells and TNBC cell lines MDA-MB-231 and MDA-MB-468 were extracted by subcellular protein fractionation. Western blot analysis was performed on protein extracts from each location for detection of PCNA with lamin A/C, GAPDH, and cross-reacting material (CRM) as loading controls for nuclear, cytoplasmic, and membrane proteins, respectively. CRM was detected due to α-Lamin A/C antibodies recognizing cross-reactive protein products.

tors to prevent NK cells' antibody-dependent cellular cytotoxicity (ADCC) from being induced. After antibody incubation, target cells were incubated with NK cells at three effector-to-target ratios of 25:1, 5:1, and 1:1 (NK-to-5000 target cells) for 4 hours at 37°C in a 96-well plate. Upon completion of incubation, cells were centrifugated and the supernatants were collected. Gamma counting was performed with a Wizard 2470 (Perkin Elmer, Waltham, MA). Percent specific lysis was calculated as [(experimental release-spontaneous release)/(maximum release-spontaneous release)] × 100. Percent specific lysis of target cells treated with antibodies targeting each ligand were compared to target cells treated with isotype antibody.

Results

Elevated presence of PCNA on the cell surface of triple-negative breast cancer cells

To determine if TNBCs display elevated PCNA at the cell surface, subcellular protein fractionation was performed on non-tumorigenic mammary MCF10A cells and TNBC MDA-MB-231 and MDA-MB-468 cells. PCNA protein extracted from the membrane, cytoplasm, and nucleus of these cell lines were then analyzed by western blot analysis (**Figure 1**). The results revealed that both TNBC cell lines displayed higher abundance of PCNA at the cell surface than MCF10A cells, suggesting that membrane PCNA may be a viable target for blocking interaction with NKp44 that could enhance killing of TNBCs while sparing non-tumorigenic cells. Furthermore, both TNBC cell lines have also displayed elevated cytosolic PCNA than MCF10A, which recent studies have revealed that cytosolic PCNA played an important role in inhibiting apoptosis and promoting cell survival in both neutrophils and acute myeloid leukemia [22-24]. To further determine if PCNA is a cell surface ligand to target for enhancing lysis against TNBCs, flow cytometry analysis was performed on TNBC cell lines and normal MCF10A cells to quantify and compare surface PCNA expression. After blocking Fc receptors or Fc-receptor ligands, cells were stained with PE-fluorophore conjugated PCNA antibodies (PCNA-PE) or with IgG_{2a}-PE antibodies as an isotype control (**Figure 2A**). We have found that TNBC MDA-MB-231 and MDA-MB-436 cells had significantly higher percentage of cells with cell surface PCNA present compared to both TNBC MDA-MB-468 cells and non-tumorigenic MCF10A at 13.2% and 22.2% versus 8.3% and 8.5%, respectively (**Figure 2B**). Thus, these results suggest that cell surface PCNA may be a possible target of interest that will allow NK cells to specifically kill TNBCs while sparing normal healthy breast cells.

Inhibiting PCNA-NKp44 interaction promotes NK-mediated lysis against TNBCs

For assessing the function of cell surface PCNA, we utilized a chromium-release cytotoxicity assay with primary human NK cells isolated from peripheral blood mononuclear cells from healthy donors. TNBC cells lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were radiolabeled with ^{51}Cr and treated with 1 μg of anti-PCNA antibodies or IgG_{2a} antibodies as an isotype control. The labeled cells treated with antibodies were co-incubated with primary NK cells for 4 hours at effector-to-target ratios

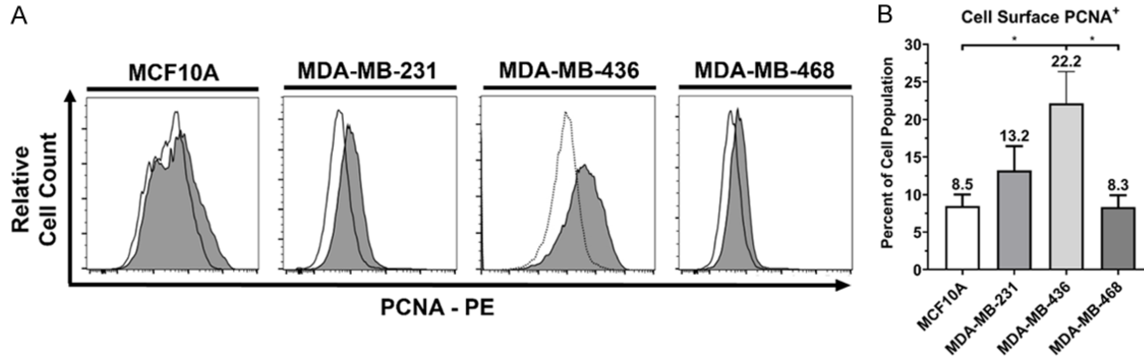

Figure 2. TNBCs exhibit higher presence of PCNA at the cell surface than non-tumorigenic cells. Presence of PCNA at the cell surface of TNBC MDA-MB-231, TNBC MDA-MB-436, TNBC MDA-MB-468 cell lines, and non-tumorigenic MCF10A cell lines were determined by flow cytometry analysis. A. Representative histograms from flow cytometry analysis displaying cells stained with anti-PCNA-PE antibodies (gray shade) and cells stained with isotype control IgG_{2a}-PE antibodies (dotted line). B. Cumulative data displaying mean percentage of cells that are positive for surface PCNA (n=6-11 independent experiments). **P<0.05, compared to each cell line by student paired two-tailed t-test.*

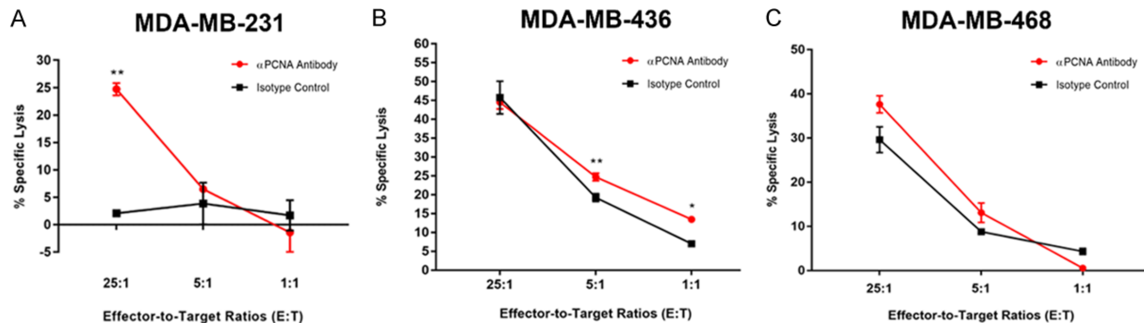

Figure 3. Disrupting cell surface PCNA-NKp44 interaction increased lysis by NK cells. TNBC (A) MDA-MB-231, (B) MDA-MB-436, and (C) MDA-MB-468 cells were radiolabeled with ^{51}Cr before blocking with Fc fragment. After blocking, cells were either treated with 1 µg of anti-PCNA antibodies (red line) or with IgG2a isotype control antibodies (black line) for 30 minutes. Radiolabeled cells were then co-incubated with primary human NK cells (already blocked with Fc fragment) at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 4 hours. Percent specific lysis was quantified and calculated. Assays were performed in triplicates and error bars represent standard deviations. **P<0.05, **P<0.01, compared to isotype control within each E:T ratio by student paired t-test.*

(E:T) of 25:1, 5:1, and 1:1 (**Figure 3**). To prevent antibody-dependent cell-mediated cytotoxicity from occurring with Fc receptors and Fc ligands, both TNBCs and NK cells were treated with human Fc fragment.

At 25:1 E:T ratio, there was a statistically significant increase in lysis of MDA-MB-231 cells treated with anti-PCNA antibodies over isotype control (**Figure 3A**). At this ratio, 24.7% of cells with PCNA blocked were killed by NK cells compared to 2.1% in cells with isotype antibody. A significant difference was not observed at the 5:1 and 1:1 E:T ratios. Additionally, another TNBC cell line MDA-MB-436 cells with surface PCNA blocked were killed by NK cells at both 5:1 and 1:1 E:T ratios at 24.7% and 19.3%, respectively (**Figure 3B**). Although there was a slight increase in MDA-MB-468 cells killed by NK cells when cell surface PCNA was blocked at 25:1 and 5:1 E:T ratios, there was no statistical difference in percent specific lysis between cells treated with anti-PCNA antibodies versus cells with isotype antibodies (**Figure 3C**). Therefore, blocking cell surface PCNA on TNBCs prevents interaction with NKp44 on NK cells and led to enhanced lysis by NK cells. This data suggests that PCNA on the surface of TNBCs serves its role in inhibiting NK cell function as an immune evasion strategy.

Combinational blocking of PCNA, HLA-I, and LLT1 further amplifies NK-mediated killing

In our prior study, we demonstrated that TNBCs displayed higher presence of surface LLT1 than

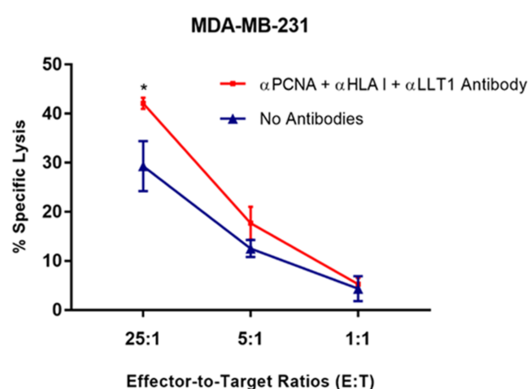

Figure 4. Blocking LLT1, PCNA, and HLA-I on TNBCs augmented NK-mediated lysis. TNBC MDA-MB-231 cells was radiolabeled with ^{51}Cr before blocking with Fc fragment. Cells were either untreated as a negative control (black line) or simultaneously treated with a combination of 1 μg of anti-LLT1, anti-PCNA, and anti-HLA-I antibodies (red line) for 30 minutes. Cells were co-incubated with Fc-blocked primary human NK cells derived from PBMCs at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 4 hours. Percent specific lysis was subsequently quantified and calculated. Assay was performed in triplicates and error bars indicate standard deviations. **P<0.05 compared to no antibodies within each E:T ratio by student paired t-test.*

non-tumorigenic MCF10A cells [20]. Additionally, we found that disrupting LLT1-NKRP1A interaction with antibodies and siRNA-mediated knockdown of *LLT1* led to increased lysis of TNBCs by NK cells [20]. Furthermore, another study demonstrated that an association of surface PCNA with Class I Human Leukocyte Antigen (HLA-I) forms an inhibitory ligand for NKp44 which results in inhibiting NK cytotoxicity against target cells [12]. Since our studies have shown that blocking LLT1-NKRP1A and PCNA-NKp44 interaction between TNBCs and NK cells have enhanced NK-mediated lysis, we tested a combinational antibody treatment strategy by simultaneously targeting LLT1, PCNA, and HLA-I to determine if this augments NK cytolytic killing against TNBCs. Therefore, using MDA-MB-231 cells, we performed a chromium-release cytotoxicity assay with primary human NK cells on TNBCs treated with 1 μg of each anti-LLT1, anti-PCNA, and anti-HLA-I antibodies. Radiolabeled TNBCs were then co-incubated with primary NK cells at 25:1, 5:1, and 1:1 E:T ratios for 4 hours (**Figure 4**). Interestingly, we found that a higher percentage of TNBCs treated with LLT1, PCNA, and HLA-I simultaneously blocked were killed by NK cells than TNBCs without any ligands blocked. A statistically significant increase in killing was observed at 25:1 E:T ratio where 42.1% of TNBCs with all three ligands blocked were lysed compared to 29.3% in cells without ligands blocked. Even though there was not a statistical difference observed at the 5:1 E:T ratio, an increase in killing of TNBCs with all three ligands blocked was still observed. At 5:1 E:T ratio, 17.7% of TNBCs with ligands blocked were lysed as compared to 12.5% of cells without antibody treatment. Therefore, our findings indicate that combinational antibody targeting of LLT1 and PCNA associated with HLA-I on the surface of TNBCs had further augmented NK cytolytic lysis against TNBCs over treatment with anti-PCNA or anti-LLT1 alone.

Discussion

Among the subtypes of breast cancers, TNBC is known to be one of the most invasive cancers which renders patients to have poor prognosis with limited treatment options that are not as effective for driving towards remission [19, 25, 26]. Due to TNBCs' absence of ER, PR, and HER2 receptors, hormonal treatments, such as tamoxifen, fulvestrant, and aromatase inhibitors, aimed to either suppress ER, PR, and HER2 production levels or modify and block these receptors have not shown much success in clinical treatment against TNBC [24]. Hence, the use of radiation therapy, neoadjuvant or adjuvant chemotherapy treatment, and surgery are more common to treat TNBC [19, 27, 28]. Although TNBC patients exhibited higher response to an initial round of chemotherapy treatment, the higher number of recurrence cases after the first round of chemotherapy demonstrates that tumors in these patients exhibit resistance to the effects of these therapies [29]. Therefore, these limitations in conventional treatments presses the need to utilize immunotherapeutic options that would maximize the capability of innate immune cells to effectively eradicate TNBCs. Specifically, utilizing NK-based immunotherapy has several advantages in that NK cells can easily and quickly expand in numbers and possess direct cytotoxic mechanisms against cancerous cells [28]. Among the mechanisms, NK cells utilize its activating and inhibitory receptors on its surface to interact with ligands on the surface of target cells to distinguish between self-healthy

cells and cancerous cells. Despite this capability, cancer cells can upregulate ligands, such as LLT1 and CS1, on its cell surface that will interact with inhibitory receptors on NK cells that can inhibit NK cytotoxic functions [12, 17, 18, 20, 30, 31].

A prior study from our group by Horton *et al*. established that PCNA associates with HLA-I on the surface of B lymphoma cells to inhibit NK effector function through interaction with NK receptor NKp44 [12]. Additionally, our group identified that blocking surface PCNA, NKp44, or LLT1 increased NK-mediated lysis of colorectal cancer cells [17]. Since PCNA has been shown to inhibit NK cell function, we investigated to determine if TNBCs evade NK cells by upregulating PCNA on the cell surface to interact with NKp44 on primary human NK cells. In this study, through flow cytometry, we identified that TNBC cell lines have higher presence of PCNA on the cell surface than normal breast cells, which suggest that NK cells can target TNBCs while sparing healthy cells. Since PCNA is involved in DNA replication process in the nucleus as an accessory protein, we performed subcellular protein fractionation and have shown that TNBCs have higher cell surface PCNA than normal cells.

Interestingly, our subcellular protein fractionation revealed that TNBC cell lines have a higher abundance of PCNA in the cytoplasm than normal breast cells. Beyond the established role of PCNA in the nucleus in DNA-related processes and cell proliferation, recent studies have demonstrated that PCNA localized in the cytoplasm have anti-apoptotic function in both neutrophils and blasts from patients with acute myeloid leukemia (AML) [22, 23]. Witko-Sarsat *et al*. showed that mature neutrophils express high levels of cytosolic PCNA and is associated with procaspases -3, -8, -9, and -10 to prevent their activation [22]. Furthermore, it has been revealed that as neutrophils undergo granulocyte differentiation PCNA was redistributed from the nucleus to the cytoplasm and cytosolic PCNA decreases during apoptosis [22]. In addition, overexpression of cytosolic PCNA renders neutrophils more resistant to apoptosis, which could provide a possible insight into the role of cytosolic PCNA in enhancing TNBCs survival against NK cells and other innate immune cells [22]. In context of cancer, cytosolic PCNA localization was found in primary leukemic cells from patients with AML, which favored AML cell survival by mediating the Warburg effect through promoting glycolysis [23]. Since we observed elevated cytosolic PCNA in TNBCs, PCNA may have a possible dual function at the cell surface where it interacts with NKp44 to inhibit NK function and in the cytoplasm where it promotes cancer cell survival possibly by inhibiting activation of caspases and increasing glycolysis. This dual function of PCNA, which needs further studies, may explain how TNBCs survive and become resistant to chemotherapies and further highlights it as a target of interest in NK-based immunotherapeutic strategies.

This study has also determined the function of cell surface PCNA on TNBCs. Based on a prior study that surface PCNA associates with HLA-I to inhibit NK function by interacting with NKp44, we hypothesized that cell surface PCNA functions as an inhibitory ligand that spares TNBCs from NK-mediated lysis. We tested this by blocking surface PCNA with anti-PCNA antibodies to prevent interaction with NKp44 and have found enhanced killing of TNBCs by primary NK cells, independent of antibody-dependent cell-mediated cytotoxicity mechanism with CD16 Fc receptors. By disrupting the PCNA-NKp44 interaction between TNBCs and primary NK cells, inhibitory signals to NK cells from this interaction are blocked allowing NK cells to favor towards a net overall activation of NK cytolytic function.

Since there are multiple inhibitory ligands expressed on TNBCs that can together provide a net inhibitory signal to NK cells, combinational antibodies targeting multiple inhibitory ligands may provide another immunotherapeutic route that can elicit NK anti-tumor functions [32]. We have previously demonstrated that LLT1 was upregulated on TNBCs and blocking LLT1-NKRP1A interaction has enhanced NK-mediated lysis against TNBCs [20]. Therefore, we performed a combinational antibody treatment simultaneously targeting LLT1, PCNA, and HLA-I and have observed higher percentage of TNBCs killed than targeting LLT1 or PCNA alone [20]. This data illustrates that combinational antibody therapy targeting both LLT1 and PCNA to augment NK activity could be utilized as a strategy. Additional *in vitro* and *in vivo* murine stud-

ies studying the use of combinational antibody targeting these two ligands will be needed to further confirm enhanced killing against TNBCs and its application in a TNBC mouse model. Considering the need to develop alternative treatment strategies for TNBC, targeting cell surface PCNA as well as other inhibitory ligands will activate NK cells to lyse tumor cells. A clear understanding of inhibitory ligands on TNBCs will allow development of additional immunotherapeutic treatments for patients with difficult-to-treat TNBC.

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Disclosure of conflict of interest

None.

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References

[1] Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008; 9: 495-502.

[2] Langers I, Renoux VM, Thiry M, Delvenne P and Jacobs N. Natural killer cells: role in local tumor growth and metastasis. Biologics 2012; 6: 73-82.

[3] Kiessling R, Klein E, Pross H and Wigzell H. “Natural” killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol 1975; 5: 117-121.

[4] Kiessling R, Klein E and Wigzell H. “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol 1975; 5: 112-117.

[5] Zhang Y and Huang B. The development and diversity of ILCs, NK cells and their relevance in health and diseases. Adv Exp Med Biol 2017; 1024: 225-244.

[6] Abel AM, Yang C, Thakar MS and Malarkannan S. Natural killer cells: development, maturation, and clinical utilization. Front Immunol 2018; 9: 1869.

[7] Lanier LL and Phillips JH. NK cell recognition of major histocompatibility complex class I molecules. Semin Immunol 1995; 7: 75-82.

[8] Morvan MG and Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer 2016; 16: 7-19.

[9] Horton NC and Mathew PA. Nkp44 and natural cytotoxicity receptors as damage-associated molecular pattern recognition receptors. Front Immunol 2015; 6: 31.

[10] Cantoni C, Bottino C, Vitale M, Pessino A, Augugliaro R, Malaspina A, Parolini S, Moretta L, Moretta A and Biassoni R. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J Exp Med 1999; 189: 787-96.

[11] Campbell KS, Yusa S, Kikuchi-Maki A and Catina TL. NKp44 triggers NK cell activation through DAP12 association that is not influenced by a putative cytoplasmic inhibitory sequence. J Immunol 2004; 172: 899-906.

[12] Horton NC, Mathew SO and Mathew PA. Novel interaction between proliferating cell nuclear antigen and HLA I on the surface of tumor cells inhibits NK cell function through NKp44. PLoS One 2013; 8: e59552.

[13] Poggi A, Massaro AM, Negrini S, Contini P and Zocchi MR. Tumor-induced apoptosis of human IL-2-activated NK cells: role of natural cytotoxicity receptors. J Immunol 2005; 174: 2653-60.

[14] Rosental B, Brusilovsky M, Hadad U, Oz D, Appel MY, Afergan F, Yossef R, Rosenberg LA, Aharoni A, Cerwenka A, Campbell KS, Braiman A and Porgador A. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. J Immunol 2011; 187: 5693-702.

[15] Maga G and Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 2003; 116: 3051-3060.

[16] Stoimenov I and Helleday T. PCNA on the crossroad of cancer. Biochem Soc Trans 2009; 37: 605-613.

[17] Malaer JD and Mathew PA. Role of LLT1 and PCNA as natural killer cell immune evasion strategies of HCT 116 cells. Anticancer Res 2020; 40: 6613-6621.

[18] Sun Y, Malaer JD and Mathew PA. Lectin-like transcript 1 as a natural killer cell-mediated

immunotherapeutic target for triple negative breast cancer and prostate cancer. J Cancer Metastasis Treat 2019; 2019: 80.

[19] Bianchini G, Balko JM, Mayer IA, Sanders ME and Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol 2016; 13: 674-690.

[20] Marrufo AM, Mathew SO, Chaudhary P, Malaer JD, Vishwanatha JK and Mathew PA. Blocking LLT1 (CLEC2D, OCIL)-NKRP1A (CD161) interaction enhances natural killer cell-mediated lysis of triple-negative breast cancer cells. Am J Cancer Res 2018; 8: 1050-1063.

[21] Knab VM, Gotthardt D, Klein K, Grausenburger R, Heller G, Menzl I, Prinz D, Trifinopoulos J, List J, Fux D, Witalisz-Siepracka A and Sexl V. Triple-negative breast cancer cells rely on kinase-independent functions of CDK8 to evade NK-cell-mediated tumor surveillance. Cell Death Dis 2021; 12: 991.

[22] Witko-Sarsat V, Mocek J, Bouayad D, Tamassia N, Ribeil JA, Candalh C, Davezac N, Reuter N, Mouthon L, Hermine O, Pederzoli-Ribeil M and Cassatella MA. Proliferating cell nuclear antigen acts as a cytoplasmic platform controlling human neutrophil survival. J Exp Med 2010; 207: 2631-2645.

[23] Ohayon D, De Chiara A, Chapuis N, Candalh C, Mocek J, Ribeil JA, Haddaoui L, Ifrah N, Hermine O, Bouillaud F, Frachet P, Bouscary D and Witko-Sarsat V. Cytoplasmic proliferating cell nuclear antigen connects glycolysis and cell survival in acute myeloid leukemia. Sci Rep 2016; 6: 35561.

[24] Gregório AC, Lacerda M, Figueiredo P, Simões S, Dias S and Moreira JN. Therapeutic implications of the molecular and immune landscape of triple-negative breast cancer. Pathol Oncol Res 2018; 24: 701-716.

[25] Foulkes WD, Smith IE and Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010; 363: 1938-1948.

[26] Yeh J, Chun J, Schwartz S, Wang A, Kern E, Guth AA, Axelrod D, Shapiro R and Schnabel F. Clinical characteristics in patients with triple negative breast cancer. Int J Breast Cancer 2017; 2017: 1796145.

[27] Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M, Cutter D, Darby S, McGale P, Taylor C, Wang YC, Bergh J, Di Leo A, Albain K, Swain S, Piccart M and Pritchard K. Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet 2012; 379: 432-444.

[28] Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, Bonnefoi H, Cameron D, Gianni L, Valagussa P, Swain SM, Prowell T, Loibl S, Wickerham DL, Bogaerts J, Baselga J, Perou C, Blumenthal G, Blohmer J, Mamounas EP, Bergh J, Semiglazov V, Justice R, Eidtmann H, Paik S, Piccart M, Sridhara R, Fasching PA, Slaets L, Tang S, Gerber B, Geyer CE Jr, Pazdur R, Ditsch N, Rastogi P, Eiermann W and von Minckwitz G. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. Lancet 2014; 384: 164-172.

[29] Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 2013; 13: 714-726.

[30] Malaer JD and Mathew PA. CS1 (SLAMF7, CD319) is an effective immunotherapeutic target for multiple myeloma. Am J Cancer Res 2017; 7: 1637-1641.

[31] Malaer JD, Marrufo AM and Mathew PA. 2B4 (CD244, SLAMF4) and CS1 (CD319, SLAMF7) in systemic lupus erythematosus and cancer. Clin Immunol 2019; 204: 50-56.

[32] Mentlik James A, Cohen AD and Campbell KS. Combination immune therapies to enhance anti-tumor responses by NK cells. Front Immunol 2013; 4: 481.