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Investigating HLA-E Peptide Binding on Natural Killer Cells for Targeting Breast Cancer Stem Cells

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Abstract:

-Breast cancer stem cells (BCSCs) are a major challenge for many conventional therapies such as chemotherapy and radiotherapy. Natural killer cells are often mentioned in immunotherapy, but the need for activation makes NK cell immunotherapy less than satisfactory. Recent studies have found that the MHC HLA-E protein has an abundant peptide expression that can activate NK cells. This study will focus on using HLA-E-expressed peptides in MHC proteins to activate NK cells and develop new possibilities for immunotherapy.

Keywords:-component; natural killer cells; immunotherapy; breast cancer; Pmhc-HLA-E

1. Introduction

1.1 Breast cancer & Breast cancer stem cells (BCSCs)

As one of the deadliest malignancies in female disease, the proliferation of breast cancer groups is steadily escalating with each passing year [1]. The United States reported 1,777,566 new breast cancer cases in 2021. The precise mechanisms by which breast cancer starts are still being studied. Breast cancer stem cells (BCSCs) are the primary aggressor within tumors and are the main challenge in the development of breast cancer treatment [2]. BCSCs govern most tumor behaviors, including metastasis and resistance [1]. The conventional therapies currently on the market, chemotherapy, and radiotherapy, have proven that BCSCs show particular mechanisms for resisting those therapies [1]. High expression levels of aldehyde dehydrogenase-1 (ALDH1) and the ATP-binding cassette transporter G family (ABCG2), which are well-known as breast cancer resistance proteins, are the source of the therapy resistance. Those breast cancer resistance proteins contribute to the rapid efflux of cytotoxic drugs and metabolize chemotherapeutic drugs into non-toxic compounds [1].

Due to resistance to conventional therapy, it is generally accepted that the use of immunotherapy is preferred instead of other therapies to identify and target BCSC. Triple-negative breast cancer (TNBC) is a subtype of breast tumor with the highest BCSCs [3].

1.2 Natural killer cells (NK cells)

Natural killer (NK) cells are promising tools in cancer immunotherapy [4]. The function is driven by a balance between activating signals and inhibiting the signals [5]. NK cells express the CD3-CD56 marker after activation. With perforins and granzymes, peptides that are released by NK cells are able to eradicate cancerous cells [3]. Also, NK cells have

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beneficial cytotoxic effects, killing malignant cells/tumors. Furthermore, NK cells command the induction and development of the adaptive immune system against cancer. They show a great potential in immunotherapy. Immunotherapy for cancer as a leading strategy has been used widely in treating different kinds of cancer [5]. However, using NK cells in the treatment of solid breast cancer tumors has been unsatisfactory due to some limitations. NK cells require activation to imitate marker expression and cytotoxicity [3, 5]. CD94/NKG2C is an ITIM motif-associating activating receptor that is expressed by NK cells [3]. By stimulating it with a specific peptide, NK cells would be able to express the function to kill tumor cells.

1.3 .MHC-HLA-E Protein

HLA-E is a non-classical MHC protein in innate and adaptive immune recognition. The yeast-displayed peptide library testifies that in the context of HLA-E, over 500 high-confidence unique peptides expressed by HLA-E are capable of activating NK cells [6]. To start activation, peptides expressed by HLA-E will be recognized by CD94/NKG2C and bind on it. Using HLA-E for activation has the advantage that some signal peptides presented by MHC HLA-E have priority over the human proteome as a whole. Specifically, one of the peptides from HLA-E, VMAPRTLFL, shows comparable affinity to CD94/NKG2C [6].

1.4 Specific aspects

NK cell immunotherapy is still being explored. Instead of focusing on single NK cell therapy, the use of other proteins to activate NK cells to increase cytotoxicity and turn on expression markers has become a recent discovery. A combination of telomerase and NK cells has been shown to enhance the ability of NK cells to kill BCSCs [3]. Based on previous studies, the priority of using the combination of NK cells and peptides presented by HLA-E is strongly promising.

1.5 Research limitations

In addition, many peptides expressed by HLA-E show good binding to NK cells, but due to the large population. The HLA-E presented NK receptor-recognized peptide has remained poorly characterized [6]. Many other types of signaling peptides remain to be discovered and have the potential for NK cell activation. Identifying the peptides that could bind the NK cell activating receptor is challenging.

While it is not possible to confirm whether VMAPRTLFL is a top priority for NK cell activation, its affinity with the NK cell activation receptor has exceeded that of the human proteome, which has research value. By using the peptide VMAPRTLFL, which is presented by HLA-E to bind on the NK cell activating receptor CD94/NKG2C, NK cells will operate successfully to activate higher cytotoxicity and express markers that destroy cancer cells. There is no doubt that immunotherapy is a promising area of research. Compared with single NK cell immunotherapy, HLA-E demonstrated that VMAPRTLFL peptide activation and enhancement of NK cells could become a new strategy for immunotherapy, which can focus on improving immunotherapy and even have the possibility of fundamentally curing breast cancer.

1.6 Hypothesis

Since the recognition of peptides VMAPRTLFL which are expressed by MHC-HLA-E can be used to stimulate the NK cell activating receptor CD94/NKG2C. The stimulation of NK cells with an increasing ratio set and treatment duration with HLA-E bound to VMAPRTLFL can kill primary BCSCs. In this way, cytotoxicity of NK cells will be activated through the expression of CD3-CD56, and the size of BCSC xenograft tumors will reduce.

2. Material & Methods

2.1 Cell source

The breast cancer cell line MDA-MB-231 is known for having a particularly high proportion of breast cancer stem cells (BCSCs), with approximately 90% exhibiting the CD44+CD24− phenotype [7].

2.2 MTT Assay

BCSC were cultured in a medium at 37 °C and 5-6.5% CO2 for 3 h. The cells were inoculated into microplates with a density of 5 × 104 cells/ well, and 200μl medium containing NK cells, MHC HLA-E were co-cultured with NK cells at effector: target (E:T) of 1:1, 5:1, 10:1, 50:1, 100:1 in a final volume of 200μl. NK cells, which are treated with PBS buffer, served as a negative control group. NK cells with Taxol served as a positive control group. Culture at 37 °C, 5-6.5% CO2 for 24, 48 and 72h. After culturing, 30μL of MTT solution labeling reagent was added to each well and incubated for 4 h. 100μl Solubilization solution was added to each well. The absorbance of the sample was determined in the microplate reader after ensuring that the violet formazan crystal was fully dissolved. Depending on the filter configuration of the enzyme labeler used, a wavelength between 550- 600 nm is selected to determine the absorbance value of the formazan product [3].

2.3 Apoptosis Assay

BCSCs (1 × 106 cells) were incubated in a T25 flask for 48 hours. After incubating, MHC HLA-E was co-cultured with NK cells at an effector: target (E: T) of 1:1, 5:1, 10:1in a final volume of 200µl. The different ratios were added in a T25 flask and cultured. 2µl Taxol served as a positive control group, and the negative control group was not treated with any drugs. The apoptotic cells floating in the supernatant were collected and washed twice with PBS and centrifuge (670×g, 5 min, room temperature). Each precipitate was suspended in PBS (400µl). 2 µl Annexin V-FITC was mixed with the cells and incubated for 15-20 minutes. After Annexin V incubation, 2 µl of propidium iodide (PI) was added. The cells were incubated at room temperature for 5-10 minutes to fully stain the PI and avoid aggregation. Every 12,24,48,72 hours, results were recorded using flow cytometry for observation [3].

Flow cytometry was used for analysis and cells were divided into different quadrants. Quadrant 1 contains cells that were PI negative and Annexin V negative (not stained), which were considered healthy cells. Quadrant 2 contained cells that were PI negative and Annexin V positive (stained by Annexin V), which were considered viable apoptosis cells [3]. Quadrant 3 contained cells that were PI-positive and Annexin V-positive (stained by both PI and Annexin V), which were considered viable apoptosis cells. Quadrant 4 contained cells that were PI positive and Annexin V negative (stained by PI), which were considered dead cells or cells that were mechanically damaged.

2.4 Xenografic assay

2×10^6 BCSCs were orthotopically injected in female nude mice. Mice kept the same diet as before the injection. Tumors were monitored by ultrasound, measuring the size of the tumors every 24 hours and the tumor growth curve was recorded. After 7 weeks of tumor formation, MHC HLA-E were co-cultured with NK cells at an effector: target (E: T) of 1:1, 5:1, 10:1in a final volume of 200µl, 20µl PBS and 20µl Taxol were injected respectively. The mice were sacrificed and weighed after 7 weeks after injections [12].

3. Statistical analysis

Data analysis was conducted utilizing GraphPad Prism software. The outcomes are presented as the mean value with the standard deviation. To ascertain significant disparities among the groups, a one-way analysis of variance (ANOVA) was implemented, following Tukey's post hoc test (denoted by an asterisk for P values less than 0.05). All the experiments were repeated 5 times to improve reliability.

4. Result

Table 1. The pMHC-HLA-E Combination Effects on Immune Response and Xenograft Tumor Outcomes

Combination of possible results (CR)	pMHC-HLA-E increases cytotoxicity by MTT?	pMHC-HLA-E increases apoptosis by annexin V/PI FACS?	pMHC-HLA-E decreases xenograft tumor size by weight?	Supporting the hypothesis?
CR1	+	+	+	Full
CR2	+	+	-	Partially
CR3	+	-	+	Partially
CR4	-	+	+	Partially
CR5	-	-	+	Partially
CR6	-	+	-	Partially
CR7	+	-	-	Partially
CR8	-	-	-	Fully Contradicts

In Table 1, the sign "+" represents that the experiment offered a statistically significant result. The result shows a great similarity with the Taxol positive control group and differences with the PBS negative control. The sign "-" represents that the experiment offered a non-statistically significant result or a result not in the hypothesis direction. The result shows a great similarity with the PBS negative control group and differences with Taxol positive control group. Or the result was in a totally different direction, which would violate the hypothesis.

CR1: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed a light

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color and contained significantly fewer numbers of viable, metabolically active BCSCs. The microplate with Taxol showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with PBS showed a darker solution than the others. A higher ratio of NK cells binding with pMHC HLA-E presents a clearer MTT solution.

The results of this apoptosis assay observed that more BCSCs were stained in the NK cells pMHC HLA-E treatment than with the Taxol treatment. After flow cytometry analysis, BCSCs that were PI negative ad Annexin V negative (quadrant 1) were significantly less than the BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and Taxol treatments have a lighter weight than the tumor from the mice without any treatment.

CR2: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed a light color and contained significant numbers of viable, metabolically active BCSCs. The microplate with Taxol showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with PBS showed a darker solution than the others. A higher ratio of NK cells binding with pMHC HLA-E presents a clearer MTT solution.

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In xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and without treatments have a heavier weight than the tumor from the mice with Taxol treatment.

CR3: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed a light color and contained significant numbers of viable, metabolically active BCSCs. The microplate with Taxol showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with PBS showed a darker solution than the others. A higher ratio of NK cells binding with pMHC HLA-E presents a clearer MTT solution.

The results of this apoptosis assay observed that less or no BCSCs were stained in the NK cells pMHC HLA-E treatment as with the PBS treatment. Taxol treatment contained more BCSCs stained with PI and Annexin V. After flow cytometry analysis, BCSCs that were PI negative and Annexin V negative (quadrant 1) were more than BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and Taxol treatments have lighter weight than the tumor from the mice without any treatment.

CR4: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed without significant differences or dark color. Some or many active BCSCs were observed. The microplate with PBS showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with Taxol showed a lighter colored solution than the others.

The results of this apoptosis assay observed that more BCSCs were stained in the NK cells pMHC HLA-E treatment than with the Taxol treatment. After flow cytometry analysis, BCSCs that were PI negative ad Annexin V negative (quadrant 1) were significantly less than the BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and Taxol treatments have lighter weight than the tumor from the mice without any treatment.

CR5: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed without significant differences or dark color, and some or many active BCSCs were observed. The microplate with PBS showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with Taxol showed a lighter colored solution than the others.

The results of this apoptosis assay observed that less or no BCSCs were stained in the NK cells pMHC HLA-E treatment as with the PBS treatment. Taxol treatment contained more BCSCs stained with PI and Annexin V. After flow cytometry analysis, BCSCs that were PI negative and Annexin V negative (quadrant 1) were more than BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In the xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and Taxol treatments were lighter weight than the tumors from the mice without any treatment.

CR6: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed without significant differences or dark color, and some or many active BCSCs were observed. The microplate with PBS showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with Taxol showed a lighter colored solution than the others.

The results of this apoptosis assay observed that more

BCSCs were stained in the NK cells pMHC HLA-E treatment than with the Taxol treatment. After flow cytometry analysis, BCSCs that were PI negative ad Annexin V negative (quadrant 1) were significantly less than the BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In the xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and without treatments have heavier weight than the tumors from the mice with Taxol treatment.

CR7: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed a light color and contained significant numbers of viable, metabolically active BCSCs. The microplate with Taxol showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with PBS showed a darker solution than the others. A higher ratio of NK cells binding with pMHC HLA-E presents a clearer MTT solution.

The results of this apoptosis assay observed that less or no BCSCs were stained in the NK cells pMHC HLA-E treatment as with the PBS treatment. Taxol treatment contained more BCSCs stained with PI and Annexin V. After flow cytometry analysis, BCSCs that were PI negative and Annexin V negative (quadrant 1) were more than BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In the xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and without treatments have heavier weight than the tumors from the mice with Taxol treatment.

CR8: By obtaining this result, the microplate with NK cells binding pMHC HLA-E treatment showed without significant differences or dark color, and some or many active BCSCs were observed. The microplate with PBS showed the same result as the microplate with NK cells (binding with pMHC HLA-E). The microplate with Taxol showed a lighter colored solution than the others.

The results of this apoptosis assay observed that less or no BCSCs were stained in the NK cells pMHC HLA-E treatment as with the PBS treatment. Taxol treatment contained more BCSCs stained with PI and Annexin V. After flow cytometry analysis, BCSCs that were PI negative and Annexin V negative (quadrant 1) were more than BCSCs that were stained with at least PI or annexin V (quadrant 2/3/4).

In xenografic assay, the tumors from the mice with NK cells binding pMHC HLA-E and without treatments have heavier weight than the tumor from the mice with Taxol treatment.

Possible results for the variables of ratio sets and treatment duration

With a higher ratio and duration of co-culturing the NK cells with MHC HLA-E, the cytotoxicity was shown to be greater. With the increase of time, the cytotoxicity of NK cells and the apoptosis rate of BCSCs gradually reached a peak.

The higher ratio of co-culturing NK cells with MHC HLA-E and longer duration, the lower cytotoxicity showed. Perhaps the cytotoxicity would remain unchanged. With the increase of time point, the cytotoxin of NK cells and apoptosis rate of BCSCs gradually decreased or unchanged. Furthermore, it didn't make any contribution to reducing tumor sizes

5. Discussion

CR1: This result supports the hypothesis. The pMHC HLA-E increased the cytotoxicity of NK cells, which caused the BCSCs death [6]. Without activation, NK cells could not function with cytotoxicity. The number of cells killed by the cytotoxin rose with the increasing ratio set. pMHC HLA-E also increased the apoptosis rate of BCSCs. Physically, by observation of tumor weight changes, activated NK cells effectively reduced the weight of the tumor. What is more, the ratio set of NK cells after activating with pMHC HLA-E had a positive feedback on cytotoxicity. The direct cytotoxic effect of NK cells leading to cell death was confirmed. NK cells are proven to be valuable as breast cancer drugs from multiple perspectives [3, 6].

CR2: The result partially supports the hypothesis. The first two experiments proved that NK cells were cytotoxic after activation and could kill BCSCs cells, but the weight of the cells in the final mouse model experiment was heavier than that of the positive control. The failure may be due to the high content of BCSCs in the mouse model, which was easier to perform compared to a lower content of MTT. Increasing the number of NK cells activated by pMHC HLA-E may provide a new opportunity. There is still potential for NK cells to be used as drugs to treat breast cancer, but testing for quantification needs to be increased [6].

CR3: The result partially supports the hypothesis. This experimental result confirmed the cytotoxicity of NK cells activated by pMHC HLA-E and successfully reduced tumor weight. However, there was no significant or no increase in apoptosis rate. The operation error during the centrifugation might have led to mechanical damage of cells, which was in the fourth quadrant and was not recorded as early or late apoptosis, resulting in a low statistical apoptosis rate.

CR4: The result partially supports the hypothesis. The NK cells binding with pMHC HLA-E showed no changes in

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cytotoxicity, but it increased the apoptosis rate of BCSCs and decreased tumor weight. This indicates that pMHC HLA-E activates NK cells to a certain extent, but NK cells kill BCSCs not through cytotoxins but other functions. This is going to be a very interesting topic to study and bring a whole new way of thinking about immunotherapy. A new approach for the use of NK cells will be discovered.

CR5: The result partially supports the hypothesis. The result showed that NK cells binding with pMHC HLA-E didn't change the cytotoxicity or apoptosis rate. However, the tumor did show a weight reduction. Due to the in vivo experiment, other immune cells in the mice may have responded to the injected drug. Perhaps the mice's immune cells could also react with NK cells or pMHC HLA-E and bind with each other and activate their own immune defense mechanisms [8].

CR6: The result partially supports the hypothesis. NK cells increased apoptosis rate but did not increase cytotoxicity or decrease tumor weight. This indicates that pMHC HLA-E activates NK cells to a certain extent, but NK cells kill BCSCs not through cytotoxin, but after activation, other functions of NK cells cause the increase of apoptosis rate of BCSCs. NK cell therapy may trigger stress responses in cancer cells, such as endoplasmic reticulum stress or oxidative stress, which can activate apoptosis pathways within cells [9].

CR7: The result partially supports the hypothesis. NK cells were detected with increased toxicity but failed to induce apoptosis or reduce tumor weight. Depending on the ratio set of NK cell therapy, it may be too small to kill BCSCs despite the increased toxicity [10]. For subsequent apoptosis experiments, the dose should be increased and repeated to further determine whether the increased cytotoxicity of NK cells after activation by pMHC HLA can be used to kill BCSCs.

CR8: The result is totally against the hypothesis. This experimental result is completely contrary to the hypothesis. The cytotoxicity of NK cells after activation could not kill BCSCs. This may be because NK cells themselves are derived from the human immune system, and BCSCs extracted from TNBC patients have established toxic tolerance against NK cells. pMHC HLA-E and NK cell binding could not enhance cytotoxicity and could not break through the resistance of BCSCs itself, resulting in failure to kill BCSCs [11].

Discussion of the possible results for the variables of ratio set and treatment duration

The change in the ratio and duration could have effects on the experimental results. The higher ratio and longer duration will increase the NK cell toxicity and apoptosis rate for BCSCs. It was further determined that the drug had the potential to cure cancer. The higher ratio and longer duration will decrease the NK cell toxicity and apoptosis rate for BCSCs, which was totally against the hypothesis. The experimental ratio set might too low to produce an observable change.

The increased ratio of NK cell therapy and duration also could have no feedback on cytotoxicity, apoptosis, or tumor size. The data is not statistically significant [10, 11]. The ratio set in the experiment may be too high, and overactivation may lead to the depletion of immune cells, reducing the effectiveness of the immune response.

6. Conclusion

BCSCs from TNBC were treated with a specific immune therapy due to their potential to cure breast cancer. However, using NK cells as a single immunotherapy often yields unsatisfactory results. However, binding NK cells with VMAPRTLFL, a peptide expressed by MHC HLA-E, resulted in enhanced cytotoxicity and the killing of BCSCs. By employing the MTT assay, apoptosis assay, and xenograft assay, the different assays assessed the drug's effects by comparing the changes induced by actual therapy. After discussing the outcomes of various combinations, future experiments might further investigate the potential of using NK cells as vectors to activate immunotherapies designed to induce apoptosis in cancer cells through the use of diverse proteins.

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