

NK CELL CULTURE FOR IMMUNOTHERAPY OF SOLID CANCER: IN-VITRO CELL STUDIES

FIELD OF THE INVENTION

- 5 The present invention relates generally to immune cells, in particular natural killer (NK) cells for the apoptosis of solid cancer cells such as liver and breast cancer cells in vitro.

BACKGROUND OF THE INVENTION

- 10 Although several treatment options are available for cancer, the most existing methods involve mainly chemotherapy and/or radiation therapy. These treatments can negatively affect the standard of living for patients as many of such treatments have side effects. Immunotherapy utilising our body's immune system, such as T cells, B cells and NK cells, has been emerging as an alternate treatment option in recent years.
- 15 These treatments offer a great potential for a reduced risk of side effects and a greater tumoricidal or tumour-suppressive effect as they are more efficient in targeting tumour cells specifically.

NK cells are cytotoxic lymphocytes which plays an important role in the innate immune system. NK cells with this innate ability can fight against infections and tumour cells

20 without prior sensitization. The mechanism by which NK cells trigger target cell death is through the release of cytotoxic granules containing granzymes and perforin through receptor-mediated pathways. NK cells also play immunomodulatory functions by secreting chemokines and cytokines.

To achieve the translational therapeutic potential of NK cells against tumour cells the

25 poor expansion and proliferation of NK cells, priming and activating NK cells, infiltration and tracking of activated NK cells in the immunosuppressive tumour microenvironment should be addressed. Recently, nano/biomaterials have shown a great potential in controlling the challenges associated with NK cells, especially the expansion and proliferation. Thus, NK cell- based adoptive cell therapies have gained

30 rapid clinical significance.

Unlike other immune cells, which detect the major histocompatibility complex (MHC) presented on infected cell surfaces, which leads to cytokine release, leading to cell death through apoptosis or lysis, NK cells recognise infected or stressed cells in the absence of antibodies and MHC, thereby increasing the rate of immune reaction. This ability to kill cells without being activated through the MHC or antibody pathways has given NK cells the name of “natural killers”. NK cells target cells that are missing “self” MHC class 1 markers. This role is crucial as some infections, such as Influenza A infections, and many tumour cells, can reduce the expression of MHC markers. This allows them to avoid detection from other immune cells, such as cytotoxic T cells, that responds through the MHC interactions.

Another method of NK cell targeting is through the “induced-self” pathway. This occurs when a high level of MICA, MICB or ULBP/RAET1 family ligands are expressed. Healthy cells express these ligands in low levels. On the activation of either pathway, the NK cells secrete granzyme B and perforin. Perforin is a pore-forming glycoprotein that polymerises on the surface of the cell membrane, forming a channel on the cell membrane. NK cells and CD8⁺ T cells are known to secrete this protein. Granzyme B is a serine protease that cleaves aspartic acid residues in the P1 position. This enzyme enters the cell through the perforin-created channel, cleaving proteins in the cytoplasm. A wide range of non-cytotoxic cells are known to produce this enzyme, including basophils, mast cells and smooth muscle cells.

NK cells also secrete IFN- γ , a cytokine that activates macrophages and inflammatory responses. Together, granzyme B, perforin and IFN- γ may induce apoptosis of the target cell through macrophage activation, inflammatory and immunomodulatory responses, and hence producing an antitumour effect.

Exosomes are membrane-bound extracellular vesicles (EVs) produced in the endosomal compartment of most eukaryotic cells. Exosomes and EVs can be found in most biological fluids produced by multicellular organisms, including blood, urine, and cerebrospinal fluid. Exosomes and EVs are also released in vitro into the growth medium by cultured cells. Exosomes and EVs carry markers of cells of origin and have specialised functions in many physiological processes, including coagulation, intercellular signalling, and biological waste management. Therefore, there has been a growing interest in the clinical application of exosomes as biomarkers and cancer

therapies. NK cells, like many other eukaryotic cells, produce exosomes. Exosomes derived from NK cells have been suggested to express IFN- γ , Fas Ligand (FasL), a molecule that induces apoptosis on binding of its receptor, and multiple cytotoxic proteins that can induce apoptosis of target cells through multiple mechanisms. NK cell-derived exosomes may be effective in lysing malignant tumour cells and mediating a significant antitumour response against acute myeloid leukaemia and melanoma. NK cell-derived exosomes have, therefore, attracted great interest as a potential anticancer therapy.

Another major challenge in NK cell immunotherapy is immunosuppression by the cancer cells. MHC Class I-expressing cancer cells exhibit immunosuppressive abilities, avoiding immune pathways by many immune cells. To overcome this upregulation of MHC-I, ex vivo manipulation of NK cell receptor expression has emerged as a promising tool to overcome the immune response inhibition.

Culture and expansion of NK cells posed great challenges in prior art. Earlier studies have used a perfusion bioreactor system for the expansion of NK cells in vitro. This bioreactor system is a complex and expensive method. Accordingly, there is a need for a facile and inexpensive cell culture method, utilizing a novel cell culture medium. Furthermore, there is a need for sufficient cell numbers and a high purity, which depends on different factors (e.g., starting material, additives like cytokines and the culture system). It would be a further advantage if NK cells could be prepared by environmentally friendly additives like natural compounds and economically inexpensive method.

SUMMARY OF THE INVENTION

The present invention provides a method of culturing and expanding PBMC-derived NK cells as a novel therapeutic platform for solid cancers such as liver and breast cancer cells.

In one aspect, there is provided a cancer cell-targeting immune cell, wherein the cancer cell-targeting immune cell is used for the apoptosis of cancer cells, wherein the immune cells are natural killer (NK) cells, and cancer cells are liver cancer and breast cancer cells.

According to one embodiment, the cancer cell-targeting NK cell is derived from a peripheral blood mononuclear cell (PBMC) suspension of a healthy donor cell. According to another embodiment, the cancer cell-targeting exosome is derived from NK

- 5 In another aspect, there is provided a method for culture and expansion of NK cells. The method includes preparation of cell targeting NK cell in a complete cell culture medium as described herein in a culture containing base medium, natural compounds, and a polymer.

10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the inverted microscope images of PBMC-isolated spherical shaped, non-clustered NK cells (a, b); and a histogram (c) illustrating the cell size and number of cells within the expected size of NK cells (6-10 μm), according to various embodiments.

- 15 FIG. 2 depicts the microscopic observation of (a) control liver cancer HEPG2 cells on day 1 and (b) control liver cancer HEPG2 cells on day 7; (c) cocultured NK cells and HEPG2 cells on day 1; (d) cocultured NK cells and HEPG2 cells on day 7, according to various embodiments.

- 20 FIG. 3 depicts the cell counting histograms for (a) control liver cancer HEPG2 cells counted on day 13.; (b) cocultured group (HEPG2 after NK cell treatment) counted on day 15.

FIG. 4 depicts the microscopic observation of (a, b) control MDA-MB-468 cells observed on day 15; (c, d) treated group: MDA-MB-468 ($\sim 1.7 \times 10^6$ cells) with NK cells ($\sim 1.3 \times 10^6$), according to various embodiments.

- 25 FIG. 5 depicts the cell counting histograms for (a) control breast cancer MDA-MB-468 cells counted on day 15; (b) cocultured group (MDA-MB-468 after NK cell treatment) on day 15, according to various embodiments.

DETAILED DESCRIPTION OF THE INVENTION

Immunotherapy constitutes increasingly an important area of cancer research. In particular, NK cells have been emerging as promising candidates for cancer immunotherapy, especially due to their ability to fight cancer through efficient targeting of tumour cells.

Although the concept of deriving NK cells from PBMC is known in prior arts, the cell growth is a difficult task. Our inventive step is a novel method of culturing NK cells in a cell culture medium containing different substances. Limited research has been done on culturing healthy donor derived PBMCs and NK cells.

The present invention provides a cancer cell-targeting NK cells, wherein the cancer cell-targeting NK cell is derived from a peripheral blood mononuclear cell (PBMC) suspension of a healthy donor. First, the PBMC suspension is incubated with an enzyme that can cleave DNA into phosphor-dinucleotide and small oligonucleotide fragments. The enzyme can be one of a specific endonuclease such as deoxyribonuclease I (DNase I, encoded by DNASE1). This DNase can reduce or prevent the clumping of cryopreserved cell suspensions. These enzymes are often used in synthetic biology or in genetic engineering to make recombinant DNA into eukaryotic cells (J Venter, H Smith, C Hutchison - US Patent App. 11/635,355, 2007; L Young, HO Smith, DG Gibson - US Patent 10,577,629, 2020).

According to various embodiments, the filtered PBMCs may be suspended in a buffer, for example, a phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+} ions) and the NK cells may be isolated using a NK cell isolation kit employing an immunomagnetic separation. The size of isolated NK cells ranges between 6-10 μm .

According to various embodiments, the separated NK cells may be directly cultured in a complete serum-free cell culture, wherein the medium contains a base medium, supplementary materials and/or coating materials. The terms "supplementary material" and "coating material" referred herein can be used interchangeably and can be any kind of non- toxic and biodegradable nanomaterials known in the art, for example cationic polymers such as polydopamine (Liebscher, J. European Journal of Organic Chemistry 2019 (31-32): 4976- 4994).

This polymer is a good coating material for all type of surfaces due to its abundant catechol moieties and amine groups. Nanoparticles may also be used for activating NK cells. (Uthaman, S., et al. ACS Applied Materials & Interfaces 2022).

5 The cell growth medium may contain components such as non-essential amino acids, sodium pyruvate, lipoic acid, vitamin B12, biotin, and ascorbic acid. Natural compounds may include curcumin, ginseng extract, etc. which may enhance the anticancer activity of NK cells.

10 According to various embodiments, the supplementary material in the cell culture medium may contain curcumin and ascorbic acid (vitamin C), whereby natural oil (e.g., gingelly oil) and ethanol may be used to extract curcumin; may exhibit a great promise in anticancer activity of NK cells.

According to various embodiments, NK cell-derived exosomes may offer advantages and may be directly used as a cancer cell-targeting NK cells for breast and liver cancer cell lines.

15 According to various embodiments, NK-derived exosomes may be used in a combination therapy for treating breast cancer and liver cancer, hepatocellular carcinoma (HCC).

20 In various embodiments, a method is provided for the targeting and killing of a cancer cell. The method can include at least one cancer cell-targeting immune cell (e.g., NK cell) to kill the targeted cancer cell as described herein. The method can further include at least one staining agent (e.g., fluorescent dye or fluorescent nanoparticle) to detect the targeted cell.

25 It should be noted that modification and variation of the inventions herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

Other examples of immune cells that may be used according to the present invention include, but are not limited to, NK-92 cells, and T-cells.

Other embodiments are within the following claims and non-limiting examples.

EXAMPLES

Example 1

Isolation of NK cells from PBMCs

5 NK cells were isolated from PBMCs of a healthy donor using magnetic particles-based cell separation method. First, a peripheral blood mononuclear cell (PBMC) suspension from whole blood was prepared. The PBMC suspension was then incubated with DNase I solution at 100µg/ml and filtered through a 37µm cell strainer. The cells were then resuspended in a buffer D-PBS (without Ca²⁺ and Mg²⁺) and the NK cells were isolated using a direct human NK cell isolation kit protocol employing an
10 immunomagnetic separation.

PBMC-isolated NK cells were spherical shaped, and non-clustered within the expected size range of NK cells (6-10 µm). This method allowed for the separation of NK cells with a good recovery efficiency. The initially observed high viability count of NK cells (98%, 1.91 x 10⁶ cells/ml) was found to be retained for over 3 weeks (FIG.
15 1).

Example 2

Culture and expansion of NK cells

PBMC-derived NK cells were cultured in a 37°C incubator for 1-2 weeks. The
20 complete cell culture medium contained an immuno-culture base medium (serum-free), supplementary materials and coating materials (e.g., cationic polymers, non-toxic and biodegradable nanomaterials, or natural compounds).

Addition of low quantities of biodegradable polymers such as polydopamine or natural compounds such as curcumin extracted from the natural oil (e.g., gingelly oil) to the
25 culture medium showed a high viability count of NK cells (99-100%), with a large number of expanded cells (5-10 x 10⁶ cells/ml) cultured for 1-2 weeks.

Example 3

Coculturing of liver cancer HEPG2 cells and NK cells

First, PBMC-derived NK cells and HEPG2 liver cancer cells were cultured separately in a 37°C 5% CO₂ incubator for a specified duration (1-2 weeks). The control HEPG2 cells were grown in a medium containing Eagle's MEM and 10% FBS. NK cells (cultured in a complete medium as illustrated in Example 2) were added to HEPG2 cells, and its effects were studied over a period of 15 days.

PBMC-derived NK cell culture supernatant (3 ml) was centrifuged for 10 min @ 100 g. The pellet was resuspended in 9 ml of NK cell complete growth medium and added to a flask of HEPG2 cells in a different ratio of HEPG2 to NK cells.

Microscopic observation of control liver cancer HEPG2 cells showed that HEPG2 cells were attached with the expected size range of 10-20 µm (FIG. 2a, b).

In the cocultured group, a smaller (~6-10 µm) cells were observed to be NK cells. HEPG2 cells were found to be attached with the expected size range (~10-20 µm). Thus, a good interaction between NK cells and HEPG2 was noted (FIG. 2c,d).

The histogram depicting the number of cells versus the cell size indicated that the average live control liver cancer cell size of 15.60 µm falls within the expected size range of 10-20 µm. The viability checked on a cell counter for control liver cancer counted on day 13 illustrated that approximately, 94% of cells (7.85×10^4 /ml) HEPG2 cells were viable (FIG. 3a).

In the cocultured group (HEPG2 after NK cell treatment) on day 15, it was found that approximately, 71% of cells (2.62×10^4 /ml) HEPG2 cells were dead. The histogram depicting number of cells versus cell size indicated that the majority of dead HEPG2 cells falls within the size range of 10.4 to 12.4 µm (FIG. 3b).

Example 4

Coculturing of breast cancer MDA-MB-468 cells and NK cells

First, PBMC-derived NK cells and MDA-MB-468 breast cancer cells were cultured separately in a 37°C 5% CO₂ incubator for a specified duration (1-2 weeks). The control MDA-MB-468 cells were grown in a medium L-15 with 10% FBS. Next, NK cells were added to MDA-MB-468 cells, and its effects were studied over a period of 15 days.

PBMC-derived NK cell culture supernatant (3 ml) was centrifuged for 10 min @ 100 g. The pellet was resuspended in 9 ml of NK cell complete growth medium (NK cells cultured in a complete medium as illustrated in Example 2) and added to a flask of MDA-MB-468 cells in a 1:1 ratio of MDA-MB-468 to NK cells.

- 5 The microscopic observation of control MDA-MB-468 cells observed on day 15 revealed that the cells were healthier and confluent with cell sizes within the range of 20~30 μm (FIG. 4a, b).

- The cocultured cells [MDA-MB-468 ($\sim 1.7 \times 10^6$ cells) with NK cells ($\sim 1.3 \times 10^6$)], were incubated in a 37°C 5% CO₂ incubator for a specified duration (1-2 weeks) and
10 observed under an inverted microscope (FIG. 4c,d). In the cocultured group, breast cancer cells appeared to be dying, with cell rounding observed for 90~95% of cells. Two sets of cells - NK cells with size of 6~7 μm and MDA-MB-468 cells with size of 20~30 μm can be seen.

- The histogram depicting number of cells versus cell size indicated that the average
15 live control breast cancer cell size of 15.76 μm falls within the expected size range of 10-20 μm . The viability checked on a cell counter for control breast cancer MDA-MB-468 cells counted on day 15 illustrated that approximately, 91% of cells ($2.09 \times 10^5/\text{ml}$) were viable (FIG. 5a).

- Maximum breast cancer cells killing efficiency of ~86% by NK cells were noted (FIG.
20 5b). Approximately, 86% of cells ($6.28 \times 10^4/\text{ml}$) MDA-MB-468 cells were dead. The histogram depicting number of cells versus cell size indicated that most dead MDA-MB-468 cells falls within the size range of 12.5 to 20 μm .

CLAIMS

1. An immune cell for cancer therapy, wherein the immune cell is a natural killer (NK) cell used directly to kill cancer cells.
2. The immune cell of claim 1, wherein the NK cells are derived from a healthy
5 donor of peripheral blood nuclear cells (PBMCs).
3. The immune cell of claim 2, wherein the PBMC suspension is incubated with an enzyme.
4. The immune cell of claim 2, wherein the NK cells are separated using the NK cell isolation kit through an immunomagnetic separation.
- 10 5. The immune cell of claim 4, wherein the size of isolated NK cells ranges between 6-10 μm .
6. The immune cell of claim 5, wherein the NK cells are cultured in a simple serum-free immuno-culture base medium.
7. The immune cell of claim 4, wherein the NK cells are cultured in a complete
15 medium containing supplementary coating material.
8. The supplementary coating material of claim 7, wherein said coating material comprises one or more components.
9. The supplementary coating material of claim 8, wherein said one or more components are cationic polymers or natural compounds.
- 20 10. The supplementary coating material of claim 9, wherein said cationic polymer is polydopamine.
11. The supplementary coating material of claim 9, wherein said natural compounds are curcumin, vitamin C, and natural oil (e.g., gingelly oil).
12. A method of culture and expansion of NK cells comprising the steps of: (i)
25 preparing an immuno-culture serum-free medium; (ii) preparation of a coating polymer; and (iii) preparation of a natural curcumin extracted from the natural oil (e.g., gingelly oil), wherein said coating polymer and natural compound act as a good supplementary material to the base culture medium.

13. The immune NK cells of claim 12, wherein the NK cells exhibit a high viability (up to 100%), and a high expansion number.
14. The immune cell of claim 1, wherein the NK cells are derived from a 50-year-old white male patient with rapidly progressive non-Hodgkin's lymphoma of PBMCs.
- 5 15. The immune cell of claim 14, wherein the NK cells are an interleukin-2 (IL-2) dependent NK-92 cell.
16. NK-92 cells of claim 15, wherein the NK-92 cells are grown in a medium containing IL- 2.
17. The cell growth medium of claim 16, wherein the culture medium is a myeloid
10 -based long-term culture.
18. The cell growth medium of claim 17, wherein the culture medium contains Fetal bovine serum (FBS).
19. The cell growth medium of claim 17, wherein the culture medium contains components such as non-essential amino acids, sodium pyruvate, lipoic acid, vitamin
15 B12, biotin, and ascorbic acid.
20. A method of tumoricidal activity of NK cells, wherein the tumour cells are liver cancer and breast cancer cells.
21. The cancer cells of claim 20, wherein liver cancer cells are HEPG2 cells.
22. The cancer cells of claim 20, wherein breast cancer cells are MDA-MB-468
20 cells.
23. A method of coculturing of liver cancer HEPG2 cells and NK cells, wherein 71% of said HEPG2 cells are dead by the anticancer activity of said NK cells.
24. A method of coculturing of breast cancer MDA-MB-468 cells and NK cells, wherein 86% of said MDA-MB-468 cells are dead by the anticancer activity of said NK
25 cells.
25. A method of tumoricidal activity of exosomes, wherein said exosomes are derived from NK cells.

Abstract

**NK CELL CULTURE FOR IMMUNOTHERAPY OF SOLID CANCER: IN-VITRO
CELL STUDIES**

5

The invention generally refers to an immunotherapeutic platform such as natural killer (NK) cells, wherein the expansion of NK cells in vitro is a facile and inexpensive cell culture method, utilizing a novel cell culture medium and components, and methods of using NK cells with a high cytotoxicity toward solid tumour cell lines in vitro, thus
10 offering a high clinical potency.

(FIG 1)