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Potential Cancer Prevention and Treatment by Silencing the Killer Cell Immunoglobulin like Receptor Gene in Natural Killer Cells Derived from Induced Pluripotent Stem Cells

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Abstract

Cancer immunosurveillance is an important host protection process, monitoring the presence of irregular cells that could potentially transform into tumor cells, effectively clearing the body of transformed tumor cells at their earliest stages, and thus maintaining regular cellular homeostasis. Natural killer(NK) cells are effector lymphocytes of the innate immune system, playing a critical role in surveillance for tumor cells, while also eliminating virally infected cells. The significance of the anti-tumor role of NK cells was recently further verified by findings that immunosuppression in most cancer patients is not perceptible until late stages. NK cells express the low-affinity Fc-activating receptor, CD16, and the inhibitory receptor, killer cell immunoglobulin-like receptor (KIR). Consequently, activation of NK cells is determined by the balance of inhibitory and activating receptor stimulation. Here, we propose establishing an induced pluripotent stem cell (iPSC)-derived NK cell line with KIR gene knockout or knockdown as a possible regimen to treat and prevent cancer. We further postulate that an optimal mixture of NK iPSCs with and without *KIR* gene knockout, would reach a maximum antitumor activity, with minimal side effects. We also discuss the possible advantages of KIR-knockout NK iPSCs for adoptive immunotherapy in patients with cancer.

Keywords: Natural killer cell; Immunosurveillance; Killer cell immunoglobulin-like receptor; Induced pluripotent stem cells

Cancer Immunosurveillance and Evasion

Cancer immunosurveillance is an important host protection process, monitoring the presence of potentially transformable cells, which are then cleared at their earliest premalignant stages (i.e., prior to full transformation). Thereby maintaining normal physiological homeostasis [1] via "immunoediting" [2], by which the recognition and elimination of continuously arising, nascent transformed cells are carried out. However, some tumor cell variants escape immune surveillance, likely due to genetic instability, and undergo rapid phenotypic changes due to lymphocyte and cytokine selection pressure. Such tumor cells, capable of evading immune defenses and proliferation by anti-apoptotic and cell growth signal pathways, continue to expand, eventually leading to full-fledged malignancies.

Adoptive Immunotherapy (AIT)

Immunotherapy is a decades-practiced technique, based on adoptive immunotransfer (AIT) of *in vitro* modified tumor-specific lymphocytes [3-5], and has shown promise as an anti-cancer regimen. Most AIT approaches focus on T cells and in particular, cytotoxic T lymphocytes (CTLs) [6]. Major endeavors have now successfully increased the antitumor cell recognition and killing capacities by T cells from ex vivo cultures, often accompanied by preparative lymphodepletion and temporary ablation of the cancer patient's immune system. Considering the well-accepted fact that perceptible immunosuppression in most cancer patients is not seen until late stages, the antitumor activities of natural killer (NK) cells may hold even more promise [7-12].

NK cells are effector lymphocytes of the innate immune system, and play a critical role in tumor cell surveillance and elimination of virally infected cells. NK cells differ in adaptive immunity from T and B lymphocytes, which express T-cell antigen receptors (TCRs) and B cell surface immunoglobulin (Ig), respectively, and instead express the low-affinity Fc-activating receptor, CD16, and the inhibitory receptor, killer cell immunoglobulin-like receptor (KIR). Effective activation of NK cells is determined by the appropriate balance of stimulation of the inhibitory and activating receptors. The cytotoxic role of NK cells is mediated by cytoplasmic granule toxins, such as perforin and proteases, that disrupt the target cell membrane, resulting in either apoptosis or osmotic lysis. Compared to cytotoxic T lymphocytes in the adaptive immune system, which kill target cells expressing normal major histocompatibility complex (MHC) class I coupled with antigenic peptides [13], NK cells destroy compromised host cells (such as tumor or virus-infected cells) having down-regulated MHC class I molecule - missing self hypothesis, which states that normal tissue cells are not attacked by NK cells through recognition of their intact self MHC class I molecule by the NK cell KIR inhibitory receptor. By contrast, abnormal cells missing or with low expression of class I MHC are recognized and destroyed by NK cells through cooperation of CD16 and KIR. In addition to killing target cells, NK cells also secrete cytokines such as antiviral cytokine interferon gamma (IFNγ), the inflammatory cytokine tumor necrosis factor alpha (TNFα), and the immunosuppressor Interleukin-10 (IL-10), to regulate and maintain a moderate immune reaction.

NK Cells' Application Dilemma and NK-92 Cell Line Development [14-19]

As an evolutionary response to immunosurveillance, most tumor cells still express certain levels of self MHC class I to escape the "missing cell" mechanism of NK cell recognition, while also expressing tumor antigens with weak immunity or "drift" of antigenic determinant epitopes. Thus these tumor cells are able to evade attack by cytotoxic CD8+ T cells, the effector lymphocytes that facilitate adaptive immunity. Some tumor cells have also evolved to "shed" decoy soluble ligands, including natural killer group 2 and member D (NKG2D) [20], to neutralize NKG2D receptor on NK cells, thus avoiding the death cascade. Since NK cells recognize target cells expressing non-self-human leukocyte antigen (HLA), autologous NK cell transplantation has not shown any antitumor effects, due to the surviving tumor cells cloaking themselves from NK cell recognition through the above mechanisms. Moreover, current NK cell-based therapies are constrained by the necessity to isolate sufficient numbers of NK cells from donors, as well as by the need to achieve acceptable efficiencies of ex-vivo expansion of NK cells. Consequently, as an adoptive anticancer therapy, the application of NK cells has been limited. Since the NK-92 cell line was established in 1996 from a patient who had a rare NK cell lymphoma type, it was shown to share features and characteristics of natural killer cells used in adoptive immunotherapy [21]. Thus, *ex vivo* cultured NK cells present another attractive therapeutic option by their consistent and vigorous elimination of tumor cells, because if cancer cells maintain their MHC phenotype, they may go unnoticed by NK cells, but not by NK-92 cells, which do not express the KIRs that negatively regulate NK cell activity through interaction with self-MHC. Consequently, at present, NK-92 cells have been undergoing clinical trials and their antitumor activities have been observed in patients with advanced tumors, such as renal cancer, lung cancer, and melanoma [22-24].

Potential Application of iPSC-derived, KIR-Knockout NK Cells for Cancer Treatment and Prevention

As a potential anticancer strategy, we propose establishing an induced pluripotent stem cell (iPSCs)-derived NK cell line having a knocked-out or siRNA-silenced *KIR* gene. The antitumor mechanism of this iPSCs-derived, *KIR* gene-silenced NK cells will share the similar characteristics with NK-92 cell line (Figure 1). IPSCs can be derived from mature adult somatic cells by genetic reprogramming to an embryonic stem cell–like state by enforced expression of pluripotency-related genes and transcriptional repression of differentiation-related genes [25]. Since iPSCs can be derived directly from adult tissues, they not only bypass the need for donors, but can be made in a patient-matched manner, which means that each individual could have their own pluripotent stem cell line to which they are immunotolerant [26]. In comparison to the NK-92 cell line that is derived from cancer cells, KIR gene silencing in NK iPSCs is much safer and could provide unlimited supplies of autologous cells (Figure 2).This individual-specific cell line could be readily accepted by patients. Consequently, NK iPSCs as an immunotherapeutic perspective have been currently reported [27]. Here, we postulate that titration of NK iPSCs with and without KIR gene silencing could create an optimal mixture for maximum antitumor activity, with minimum side effects, due to an appropriate balance of tolerance and killing of KIR-possessing and non-possessing cells, respectively. Validation of this hypothesis could be carried out in mice models. Moreover, we speculate that the optimized mixture of NK iPSCs could be applied to prevent cancer occurrence in high-risk populations by potentiating tumor immunosurveillance, while also usable as an alternative adjuvant therapy for cancer patients who cannot tolerate chemotherapy or radiotherapy after surgery, as a neoadjuvant therapy before surgery to prevent metastasis of the primary tumor due to the operation itself, or by combination with systemic chemotherapy or irradiation to eradicate residual malignant tumor cells. IPSC-derived NK cells also represent an ideal long-term maintenance regimen for preventing cancer recurrence, due to their ease of manipulation and lack of side effects. Similar to the NK-92 cell line, NK iPSCs with or without KIR gene silencing could be genetically engineered to express other proteins of interest, including chimeric antigen receptors (CARs) that target multiple tumor-associated antigens, and the high affinity Fc receptor FcγRIII (CD16), thus enhancing NK cell antitumor specificity and killing abilities [28-32]. KIR gene-silenced NK iPSCs from specific patients could form a cell line pool for the development of a NK cell line for adoptive immunotherapy and maximum tumor killing efficacy. If preparation of autologous cell line is difficult or its effectiveness is not obvious, comparisons could be made for each cell line based on age, gender, health status, geographical distribution, etc. This approach could allow optimization of the percentage of contribution of different factors for establishing an effective and efficient NK iPSC cell line, in addition to allowing detection of different KIR gene knockdown efficiencies, based on the antitumor activity of specific lines.

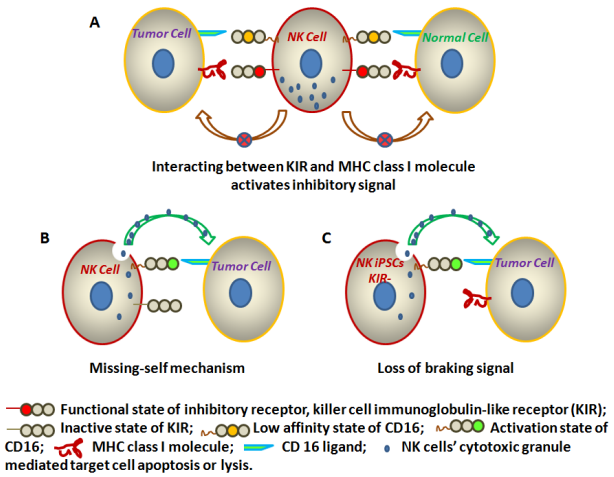

Figure 1. Schematic diagram indicating the recognition and killing of NK cells on target cells through interacting of KIR receptor and MHC class I molecule.

A. Activation of inhibitory signal through interaction of inhibitory receptor, killer cell immunoglobulin-like receptor (KIR) on the surface of NK cells and MHC class I molecule on the normal cells or tumor cell expressing the MHC I. B. Indicating the tumor cell lacking MHC I molecule is attacked by NK cell through the mechanism of missing-self. C. Showing the tumor cell is destroyed by KIR-silencing NK iPSCs due to the loss of braking signal.

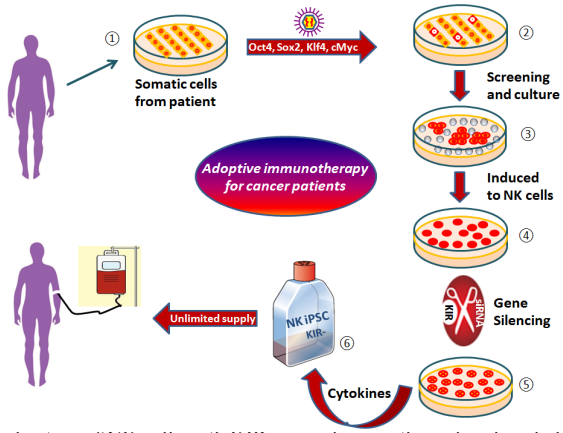

Figure 2. A flow chart of preparation and application of NK cells with KIR gene silencing through induced pluripotent stem cells. ① Isolate and culture peripheral blood mononuclear cells of cancer patient. ② Transfect stem cell-associated transcription factor genes into the cultured somatic cells from patient by retroviral or lentiviral vector. The cells with white ring indicate the cells expressing the exogenous genes. ③ Harvest and culture the cells according to manipulating procedures of embryonic stem cells (ES cells) using mitotically inactivated mouse embryonic fibroblasts (MEFs) as feeder cells (gray cells). ④ Expand iPSC clones and differentiate into NK cell line. ⑤ Silence KIR gene of NK cells by gene knocking out or small interfering RNA (siRNA). ⑥ Culture and activate the NK cell with KIR gene silencing at presence of cytokines and transfuse back to the patients in a manner of unlimited supply.

Note: Technically, the tandem order of genetic reprogramming ② and gene silencing ④ can be switched. The cytokines such as interleukin 2 (IL-2) and other factors can be added before transfusion to further enhance the killing effect of iPSC-derived *KIR* gene-silenced NK cells on tumor cells.

Hypothesis of Carcinogenesis and Adoptive Immunotherapy

Normal somatic cells having transforming potential occur perpetually throughout the human body, due to UV radiation, carcinogen exposure, or infection with specific viruses [33], but are routinely eliminated by our normal immune system in a timely fashion. However, this homeostasis can be broken once the number of these oncogenic cells ata primary site exceeds the monitoring capacity of the immune system during distinct stages of life. Moreover, such transformation likely needs some time duration, during which immune defense mechanisms are weakened. This period of time allows these premalignant cells to escape effective attack by the innate immune system and transform into atypical cells. If the weak immune defense status continues, these atypical offending cells have an increased probability to form in situ neoplasms, surrounded by a tumor-sustaining microenvironment [34], resulting in some of these cells progression into cancer. Thus, the adaptive immune system fails, precluding complete clearance of abnormal cell by the immune system (even a recovering immune system). The remaining activity of the immune system determines the length of a dormancy period, during which gradually increased tumor load finally and fully overcomes immune system defensive mechanisms, whereby patient symptoms start appearing,

and the tumor cell cluster from the primary location starts metastasizing. A potential technique to cure cancer and eradicate residual tumor cells could be to re-establish patients' immune systems, an approach that has been already verified by successful cases of AIT following temporary immune system ablation. However, such preparative lymphodepletion approaches are traumatic, and not all the patients can withstand such harsh procedures. Consequently adoptive immunotherapy strategies have generally endeavored to genetically engineer cell lines expressing specific proteins or peptides, and tolerate possible evolutionary advantages of naturally occurring or hereditary genetic mutations. An example of this is thalassemia red blood cells carrying a hemoglobin gene mutation that may confer a degree of protection against malaria, especially when caused by plasmodium falciparum [35,36]. We suggest that we could also engineer genetically ablated cell lines to treat more serious diseases, such as an iPSC-derived *CCR5* gene (C-C chemokine receptor type 5)-silenced CD4+ T cell to treat AIDS (acquired immune deficiency syndrome) patients. As a co-receptor of CD4 molecule, which is used by the human immunodeficiency virus (HIV) to enter its target cells, the silencing of CCD5 expression will make CD4+ T cells resistant to HIV attack. This has been verified by a few individuals carrying a mutation known as CCR5-Δ32 in the *CCR5*gene, protecting them against HIV infection [37], and this characteristic has being adopted to treat or for long-term control of HIV using stem-cell transplantation technology [38-40]. Here we envision a promising possibility of establishing iPSC-derived KIR gene-silenced NK cells for cancer prevention, treatment, and impeding the recurrence of cancer.

Application Prospect of Induced Pluripotent Stem Cells

The establishment of induced pluripotent stem cells (iPSCs) from adult somatic cells is breakthrough progress in life sciences. To date, iPSCs can be directly derived from human peripheral blood mononuclear cells (PBMCs), for example, T lymphocytes or B lymphocytes [41-43]. These pluripotent stem cells retain their characteristic rearranged T-cell receptor (TCR) genes or immunoglobulin (Ig) genes, and can be induced to differentiate again into functioning T-cells or B-cells, which means we can expect the production of biologics through an iPSCs expression system, especially, monoclonal antibodies against different pathogens, tumor antigens, and some inflammatory factors [44-46]. The stem cell antibodies will possess the advantages of both monoclonal and polyclonal antibodies by mixing individual iPSCs clones expressing different antibody repertoires, preserving native and complete post-translational modifications. The development of stem cell monoclonal antibodies will potentially relieve the shortage of some biologics, such as for the passive immune component of post-exposure prophylaxis (PEP) of rabies, which at present uses human rabies immunoglobulin (HRIG) from plasma of immunized donor subjects [47]. This also provides a new strategy to prevent and treat emerging and zoonotic infectious diseases of high-consequence [48] through establishing specific iPSCs secreting monoclonal antibodies of interest.

The other potential attractive application of iPSCs is human genetic disease modeling and drug development [49,50]. These patient-derived pluripotent stem cells will exhibit defective cell behaviors not observed in the healthy iPSCs, providing insights into the pathophysiology of the disease. Drug screening based on an individual's genetic background embodying single nucleotide polymorphisms (SNPs) such as sickle-cell anemia [51] and cystic fibrosis [52] will produce profound significance on the pharmacogenetics, providing opportunities for personalized medicine.

Disclaimer and Financial Interest Disclosure

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC. The authors have no relevant affiliations or financial involvement with any organization or entity in the manuscript. No whiting assistance was utilized in the production of this manuscript.

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