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Pluripotent stem cell-derived natural killer cells for cancer therapy

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Abstract

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) provide an accessible, genetically tractable and homogenous starting cell populations to efficiently study human blood cell development. These cell populations provide platforms to develop new cell-based therapies to treat both malignant and non-malignant hematological diseases. Our group has previously demonstrated the ability of hESC-derived hematopoietic precursors to produce functional natural killer (NK) cells as well as an explanation of the underlying mechanism responsible for inefficient development of T and B cells from hESCs. hESCs and iPSCs, which can be reliably engineered in vitro, provide an important new model system to study human lymphocyte development and produce enhanced cell-based therapies with potential to serve as a "universal" source of anti-tumor lymphocytes for novel clinical therapies. This review will focus on the application of hESC-derived NK cells with currently used and novel therapeutics for clinical trials, current barriers to translation, and future applications through genetic engineering approaches.

Pluripotent stem cells to study blood development

For over 40 years, hematologists and oncologists have utilized transplantation of hematopoietic stem cells (HSC) to treat and cure hematologic malignancies [1]. HSC continue to be the only routinely used stem cells population for clinical therapies, though other stem cell-based therapies have been used in clinical trials. Since the derivation of human embryonic stem cells (hESCs) over a decade ago [2], numerous groups have successfully differentiated this pluripotent source to fully mature and functional subsets of each germ layer and hESCs remain one of the most promising cell sources for regenerative medicine. Phase I clinical trials using hESC-derived oligodendrocytes for spinal cord injury [3] have been approved by the United States Food and Drug Administration (FDA). Studies on derivation and differentiation of human induced pluripotent stem cells (iPSCs) are also rapidly advancing [4–7]. Therefore, the prospect to utilize hESC- and iPSC-derived hematopoietic products for diverse clinical therapies is not a distant prospect, but a reasonable expectation in the next few years [8].

Shortly after the original derivation of hESCs, we demonstrated hematopoietic development using an in vitro co-culture model and defined conditions [9]. These studies utilized co-culture of hESCs on irradiated stromal cells (serving as a microenvironment) and showed that during differentiation, hESC-derived cells acquired typical hematopoietic genes and surface antigen expression. Since these initial studies, we and others have further defined culture conditions to mediate derivation of almost all human blood lineages from hESCs [8]. Hematopoietic cells

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can be consistently produced from hESCs using two separate methods: stromal cell co-culture and embryoid body formation [10,11]. These hESC-derived hematopoietic precursor/progenitor cells can produce erythroid, myeloid, and lymphoid lineage cells in vitro [12–14]. However, use of hESCs to derive HSCs capable of long-term, multilineage engraftment when transplanted using in vivo models (such as immunodeficient mice) has been limited [15–19]. Several groups have demonstrated similar hematopoietic development from human induced pluripotent stem cells (iPSCs) [20–22]. These studies provide the intriguing possibility that iPSC-derived hematopoietic progenitors could be derived on a patient-specific basis and serve as the definitive example of personalized medicine.

Human induced pluripotent stem cells

Recently, the development of iPSCs has provided another platform to study human development. iPSCs can now be routinely derived from terminally differentiated somatic cells through expression of several transcription factors (typically OCT4, SOX2, KLF4, c-myc or Lin28) known to promote pluripotentcy [23–25]. iPSCs derived from mice undergo tetraploid complementation and demonstrate germ line chimerism- the most stringent test of pluripotency [26]. Human iPSCs, similar to hESCs, are capable of differentiating into mature cell types of all three germ layers [7,20,23,25,27]. The recent explosion of iPSC technology has led to successful derivation of iPSCs without integrating transgenes [28,29], a technology that may better enable clinical translation. iPSC technology also enables derivation of disease specific lines enabling in vitro study of diseases with natural genetic or biochemical defects [5,7,30–33]. Building on decades of studies done in mouse and human hematopoietic progenitors several groups have used hESCs, and now iPSCs, to study the mechanisms regulating blood cell development.

Lymphocytes derived from human pluripotent stem cells

The interplay of transcription factors, cytokines, and tissue microenvironment in hematopoietic and, more specifically, lymphoid development has been well studied in mouse models. However, these successes have not been easily duplicated in human studies [34–36]. Within the adaptive immune system, our knowledge of B and T cells has advanced as one of the most well defined developmental paradigms. In contrast, many aspects of NK cell development and education, such as the underlying mechanisms driving newly-defined NK cell subsets to acquire effector function through a process of “licensing”, remain to be elucidated. Several reviews have focused on this important, growing area of research [37–40].

hESC-derived NK cells provide a genetically defined population to study NK cell development and overcome the high level of donor heterogeneity, such as when using peripheral blood NK (PB-NK) cells. Our group has demonstrated the ability of hESC-derived hematopoietic progenitor cells to produce functional NK cells in vitro [13,41]. hESC-derived NK cells express activating and inhibitory receptors similar to peripheral blood NK (PB-NK) cells and are highly efficient at direct cell-mediated cytotoxicity, antibody dependent cell-mediated cytotoxicity, and cytokine (IFN-γ) production [41]. hESC-derived NK cells provide a reliable developmental model to study human NK cells in vitro. Our studies of hESC-derived NK cells have also demonstrated in vivo function [42]. Using a tumor xenograft model, we showed complete tumor clearance with hESC-derived NK compared to UCB-derived NK cell or sham injected controls [42]. For cancer immunotherapy, preclinical mouse models such as this have been the cornerstone to translate novel therapies into the clinic. To date, several factors critical to the efficacy of NK cell-based therapies, including licensing, trafficking, and homing have yet to be well defined in humans. Therefore, hESC-derived hematopoietic progenitors provide both a novel system to study basic developmental processes, as well as an unlimited source of cells for cancer immunotherapy and could potentially serve as a universal source of lymphocytes for off-the-shelf therapy.

Current status of NK cells for clinical adoptive immunotherapy

For over two decades Rosenberg and colleagues at the NIH have been steadily advancing immunotherapy trials using tumor infiltrating lymphocytes (TILs), in combination with high-dose interleukin-2 (IL-2), for patients with metastatic melanoma, renal cell cancer, and other malignancies [43–45], They have repeatedly shown how ex vivo activation of cancer-killing lymphocytes can decrease tumor burden and significantly increase patient life-span. While a majority of adoptive immunotherapy trials have focused on T cells, there is growing interest in NK cell-based therapeutics following a seminal study by Ruggeri et. al. in 2002 [46]. Here, they demonstrated NK cell-mediated allo-reactivity could eliminate relapse, graft rejection, and protect against graft-vs-host disease (GVHD) for patients with acute myelogenous leukemia (AML) [46]. Another study by Miller and colleagues evaluated the in vivo efficacy of expansion for haplo-identical NK cells with interleukin 2 (IL-2) in a non-transplant setting. In an evaluation of 43 patients with advanced cancer (melanoma, renal cell carcinoma, and poor prognosis AML), IL-2 and a high-dose immunosuppressive regimen was shown to promote expansion of donor derived NK cells. This finding positively correlated to the serum concentrations of IL-15 (a key cytokine involved in NK cell development) induced by the high-dose immunosuppressive regimen. Intriguingly, 5 of 19 patients with poor-prognosis AML had a complete remission. Similar to the Ruggeri study, 4 out of the 5 patients in remission had an alloreactive NK cell receptor repertoire.

Recent studies identify several potential mechanisms attributing to both the successes and failures in NK cell based immunotherapy [47–50]. IL-2, used to expand T or NK cells in vitro and in vivo, also promotes the expansion of T regulatory cells (Tregs) which express the IL2 receptor alpha chain CD25. T regulatory cells have been shown to directly suppress both T and NK cell responses [51,52]. Additionally, it has not been well defined why NK cell immunotherapy works in AML but not other hematological or solid malignancies. Here, the ability to genetically modify NK cells for more direct recognition of tumor targets could enhance their therapeutic potential against a broader range of cancers. To date, most of this work has focused on antigen-specific T cells, with less emphasis placed on engineering NK cells. However, this type of gene therapy is difficult achieve in primary peripheral blood [53]-NKs and T cells, whereas high efficiency genetic modification is routinely feasible in hESC-derived NK cells.

Based on this record of successful (though limited) NK cell-based therapies against cancer, NK cells derived from hESCs or iPSCs now provide an intriguing starting point to expand this immunotherapeutic approach. Strictly considering cell number, the ability to create enough hESC-derived NK cells for adoptive immunotherapy (~10^7– 10^8 NK cells per patient) [54] is far more reasonable than the possibly insurmountable number of cells needed to generate one unit of RBCs (10^{12} RBCs per unit). Additionally, since the hESC-derived NK cells are void of any contaminating T or B cells, they could be positively selected on the basis of NK cell surface markers. This is in contrast to current strategies using peripheral blood NK cells that involve treatment of the aphaeresis product with CD3 beads (to deplete T cells) and CD20 beads (to deplete B cells and prevent “passenger lymphocyte syndrome”).

hESC-derived NK cells also have their own barriers to translation. The reliable derivation of hematopoietic progenitors from hESCs is dependent on stromal cell co-culture models. The use of mouse stoma provides a barrier to clinical translation, though not insurmountable if a master cell bank of the stromal cells is produced. The traditional “feeder-free” system of embryoid body (EB)-mediated differentiation of hESCs/iPSCs may be utilized, though tends to be more variable in differentiation into hematopoietic progenitor cells. Alternative methods such as “spin-EBs” [55] have standardized this approach and have the potential to provide the large numbers of cells needed for clinical trials [56]. By aggregating undifferentiated hESCs through centrifugation, spin EBs provide a uniform and reproducible method to produce

hematopoietic progenitors in the absence of murine stroma [57]. More studies are needed to determine if this is an effective means to generate fully functional NK cells similar to those developed on stroma.

hESC-derived NK cells have been extensively characterized and have defined effector functions parallel to PB-NKs, which are now routinely used for clinical trials [42]. Compared to UCB-derived NK cells, hESC-derived NK cells have superior ability to kill tumor cells both in vitro and in vivo [58]. In fact, UCB-derived NK cells are less functionally mature, and their use in clinical trials has fallen out of favor. Our studies demonstrate hESC-derived NK cells are similar to PB-NKs in several ways. Like PB-NK cells, hESC-derived NK cells express a number of NK cell effector proteins on their surface (Fc receptor, NKG2D, NKp46, Fas ligand, TRAIL, and KIR family members). They also have potent anti-tumor effects both in vitro and in vivo as hESC-derived NK cells completely clear the CML tumor K562 in vivo [42]. Because the use of NK cells has shown encouraging results in patients with AML [42], new clinical trials have been initiated using NK cells to treat other hematologic and solid tumors.

With the seemingly effective clinical use of PB-NK cells [47,54], there is often a question of what advantage hESC or iPSC-derived NK cells could provide. To answer this, it is important to consider the relative ease it is to genetically-engineer hESCs (or iPSCs) [59–62]. In this manner, we have begun studies to express antigen specific receptors that could provide an enhanced cellular therapeutic capable of specifically recognizing and killing tumor cells. We hypothesize the genetically-modified hESC or iPSC-derived NK cells could dramatically increase the tumor cell populations amenable to this type of immunotherapy. Additionally, autologous NK cells can be derived from iPSCs. Presumably, these iPSC-derived cells would not require significant chemotherapy to be given to the patient, as is done in part to promote in vivo survival of the allogeneic NK cells that have been used in clinical trials. Third, it may also be possible to utilize hESC or iPSC-derived NK cells to treat infectious diseases such as HIV where autologous peripheral blood lymphocytes would not be a good option due possible infection of donor cells. Indeed, several studies suggest NK cells play a role in anti-HIV immunity [62–64].

Use of hESC and iPSC-derived NK cells for cancer therapeutics

The remainder of this review, depicted in Figure 1, will focus on expression of genes for specific proteins to create NK cells with more potent ability to kill tumor cells. We will summarize some of the possible avenues for NK cell based therapy derived from hESCs and/or iPSCs either alone or in combination with current practices.

Perhaps the most promising direction for hESC-based immunotherapies is to engineer hESCs or iPSCs to express chimeric antigen receptors (CARs) capable of directing cytotoxic lymphocytes to tumor sites. CARs typically contain an extracellular domain, derived from the Fab (antigen-specific) portion of an antibody that has high specificity to recognize a tumor antigen. The external domain is genetically linked to a transmembrane domain and an intracellular signaling domain [65]. Upon antigen binding, the CAR initiates a signaling cascade leading to release of cytotoxic granules. Several iterations of CARs have utilized distinct signaling and co-stimulatory domains in different combination to optimize function. Recently, Carpenito et al. showed greater numbers of co-stimulatory domains can increase the half-life of engineered lymphocytes in vivo. This advance lead to increased tumor clearance in a tumor xenograft model [66].

Decreased in vivo half-life of transferred cells is considered one of the major obstacles hindering successful adoptive transfer therapies. Restifo and colleagues have shown using naïve, “younger” populations of T lymphocytes results in greater in vivo expansion and tumor clearance in mice. This finding correlates to longer telomeres in this cell population and fits

the hypothesis that limited replicative potential causes rapid decline of infused cells. Dudley and Rosenberg have adopted this strategy to expanding tumor infiltrating lymphocytes (TILs) ex vivo for use in clinical trials [67]. They also find that younger TILs express high levels of co-stimulatory molecules and have longer telomeres. This work suggests that since hESCs and iPSCs have persistent telomerase activity [2,68], the hESC and/or iPSC-derived lymphocytes may survive better in vivo and provide enhanced anti-tumor activity. However, it is not currently known whether hESC-derived NK cells have longer or shorter telomeres than PB-NKs or how this would change throughout in vitro expansion. Although CARs provide antigen-specific recognition and proliferation of lymphocytes, their function in young or mature populations has also yet to be determined.

While CAR-based therapies against cancer (and potentially infectious disease) is intriguing, this system needs to be advanced with caution in light of recent reports of CAR-engineered lymphocytes leading to serious adverse events when infused into patients [69,70]. Although these adverse events may dampen some of the enthusiasm for CARs, they reiterate the importance for in depth preclinical testing to define tissue specific expression of antigens recognized by CARs. CAR-specific T cells activated upon contact with antigen produce no inhibitory signal if they encounter the antigen on normal tissue. In a manner similar to decreased GVHD in NK cell adoptive transfer trials, one could hypothesize hESC-derived NK cells and PB-NKs would remain more tolerogenic than T cells, as the infused NK cells would still co-express inhibitory receptors recognizing “self”. Alternatively it may be difficult to inhibit this overriding activation signal, therefore second generation CARs that have fewer intracellular activation domains, could in fact be safer for clinical trials.

An alternative approach for hESC/iPSC-derived NK cells would be to express tumor-specific (T cell receptors) TCRs. NK cells express all of the components needed to signal through the TCR [71], making them a suitable platform to utilize ectopic TCR expression. Studies by the Rosenberg group [42] provide direct evidence for the potential of adoptive immunotherapy as a successful treatment for lethal malignancies. Although their initial studies used tumor infiltrating lymphocytes from actual patient cancers, more recently they have used T cells engineered with T cell receptors (TCRs) specific for tumor associated antigens (TAA) [72]. While this group is able to modify T cells from individual patients, this expensive and cumbersome process is not feasible for multi-institutional study and routine clinical application. Another disadvantage to using TCRs is the restrictive nature of specific major histocompatibility (MHC) molecules. The use of HLA-A2 restricted TCRs covers roughly 30–40% of the Caucasian population but is not an optimal TCR-MHC combination for each individual or malignancy. The ideal cellular source would function independent of MHC haplotype but remain specific for tumor antigen. Engineering hESCs to express antigen specific receptors could provide an enhanced cellular therapeutic capable of specific recognition and killing of tumor cells. As NK cells express key CD3 chains (ζ and ε) required for TcR signaling and activation, engagement of TcR-expressing NK cells will lead to functional cytolytic activity [73].

One advantage to implementing tumor-specific TCRs in NK cells is reduced competition for TCR binding between endogenous and exogenous α/β subunits. A recent study found aberrant pairing of transgenic and endogenous α/β subunits can create a repertoire of self-reactive lymphocytes resulting in autoimmune pathology [74]. Another major advantage in using TCRs stems from years of epitope-discovery and TCR generation. This provides an assortment of optimal tumor-specific TCRs suitable for preclinical testing. TCR expressing hESC-derived NK cells would need to see antigen in the context of the correct MHC, but could be designed (using different ES cell lines) to cover a larger repertoire of MHC molecules and used as a universal source of anti-tumor lymphocytes for HLA matched patients.

In a simpler model, unmodified hESC-derived NK cells could be used in combination with current practices. Some of the most successful immunotherapies in human cancer are monoclonal antibodies (mAbs) [75]. Over the past decade, identification and manufacturing of monoclonal antibodies (e.g. anti-CD20, anti-VEGF, anti-HER2, anti-CD52) has been a major focus of both the academic and private sectors. We now have a better understanding of their in vivo function and have tailored therapies to accommodate many specific disease mechanisms. Of the many modifications made to mAbs over the years, most importantly is the decreased immunogenicity by making chimeric or humanized forms. Also, several groups identified the isotypes (typically IgG1) that are most stable, have greatest affinity for the Fc receptor, and increased ability to fix complement. One of the major cell types needed for antibody dependent cellular cytotoxicity (ADCC) are NK cells. Our studies have shown that surface expression of the FcR on hESC-derived NK cells parallels that of PB-NKs and can also mediate ADCC [13].

Although this remains to be tested in vivo, we hypothesize combination therapeutics with monoclonal antibodies that target tumors (or other desired cell populations) and hESC-derived NK cells would add an additional mechanism to kill human tumors, without the potential risks of using CARS. Another approach has been the development of agonists of the apoptosis inducing proteins Fas ligand and TRAIL [76]. Because hESC-derived NK cells also express high levels of both of these ligands, one can begin to synthesize the synergy and application of utilizing hESC-derived NK cells in combination therapeutics to treat a wide variety of malignancies.

Development of T cells and B cells from human pluripotent stem cells

Yet another approach to advance hESCs and iPSCs toward these clinical applications includes using hESC- or iPSC-derived hematopoietic progenitors to directly differentiate antigen-specific T cells. However, compared to development of NK cells, production of T cells from hESCs has proven to be a difficult task, despite the close developmental relationship between T and NK cells [77]. Notably, one group used the SCID-hu mouse system (immunodeficient mouse engrafted with human fetal thymus and liver tissues) to study T cell development in vivo [78]. In this study, hESC-derived NK cells could be identified, though no more than 1% hESC-derived $CD3^+$ (T cell marker) cells developed. In contrast, hematopoietic progenitors isolated from UCB typically produce greater than 10 times the number of T cells than the hESC-derived progenitor cells. In vitro models using OP9 stroma expressing the NOTCH ligand Delta-like 1 (OP9-DL1) provide a step-wise differentiation model to study the distinct stimuli modulating T cell development [79]. Timmermans and colleagues demonstrated that dissection of hematopoietic zones (found to be rich in $CD34^{high}43^{low}$ hematopoietic progenitors) lead to increased production of T cells from hESCs [80]. This highly labor intensive and inefficient process depends on both the hESC line used and variability between serum lots. Using this and other model systems (including fetal thymic organ culture), our lab identified molecular mechanisms (*ID* gene expression) likely to promote NK cell development at the expense of T and B cells [77]. We found that hESC-derived hematopoietic progenitors are unable to produce T cells when co-cultured with OP9-DL1 stroma, and instead produce NK cells. Also, functional B cell development from hESCS or iPSCs has not been achieved, likely due toward expression of *ID*-family genes in the hESC/iPSC-derived progenitor cells. When cultured in parallel to hESCs, cord blood $CD34^+$ hematopoieitic progenitor cells routinely produce B cells, phenotypically marked by progression through pro-B ($CD34^+19^+45^+$), pre-B ($CD34^-19^+45^+$preBCR), immature ($CD19^+45^+IgM^+$) and mature B cell ($CD19^+21^+45^+IgM/D^+$) stages. Although T and B cells would provide an important cellular source for in vitro and in vivo study, NK cells remain the most defined and effective hESC-derived blood population studied to date. They not only serve as an important developmental model but hold potential as a valuable cellular source for adoptive immunotherapy. More rigorous in vitro and in vivo

evaluation will be necessary for successful translation of hESC-derived NK cells into clinical trials.

Conclusion

Hematopoietic cell-based therapies are routinely utilized as an effective means to cure many patients with hematologic malignancies such as leukemia, lymphoma, and myeloma. Antitumor immunotherapy using T cell and NK cell-based therapies have demonstrated the promising therapeutic potential of harnessing the cellular immune system to fight human cancer. Despite these advances, many patients still succumb to these malignancies. Creating lymphocytes capable of recognizing specific tumor antigens is a high priority in the field and most approaches have focused on generating HSCs or T cells containing transgenic receptors (TCRs or chimeric receptors) [44,81,82]. While T-cell immunotherapy has been extensively applied and shown promising results in patients with renal cell carcinoma and malignant melanoma, others have successfully utilized adoptive NK cell transfers for patients with chemotherapy refractory AML. The use of NK cells for other malignancies has not been successfully applied and a better understanding of basic NK cell biology is needed to advance their use in clinical trials. NK cells derived from human embryonic stem cells provide a genetically tractable system to study both NK cell development and function. Also, hESC-derived NK cells could provide a universal source of lymphocytes for "off-the-shelf" cancer therapeutics. hESCs would provide an allogeneic source for NK cell adoptive transfer therapies with known KIR-ligand mismatch and optimal KIR alloreactivity. iPSCs derived on a patient specific basis for a variety of therapies is an attractive option, though currently remains a labor intensive, time consuming process. The ability to modify hESC and iPSC-derived NK cells with tumor-specific receptors holds promise for utilizing them against a wider variety of malignancies. Further in depth preclinical investigation is essential to propel hESC-NK cells into phase I clinical trials. Although current methodologies using murine stroma are not optimal for clinical translation, new strategies could provide the quantity of NK cells needed for clinical trials. Eventually, clinical translation of hESC-derived NK cells will need to be advanced with appropriate pre-clinical and clinical trials.

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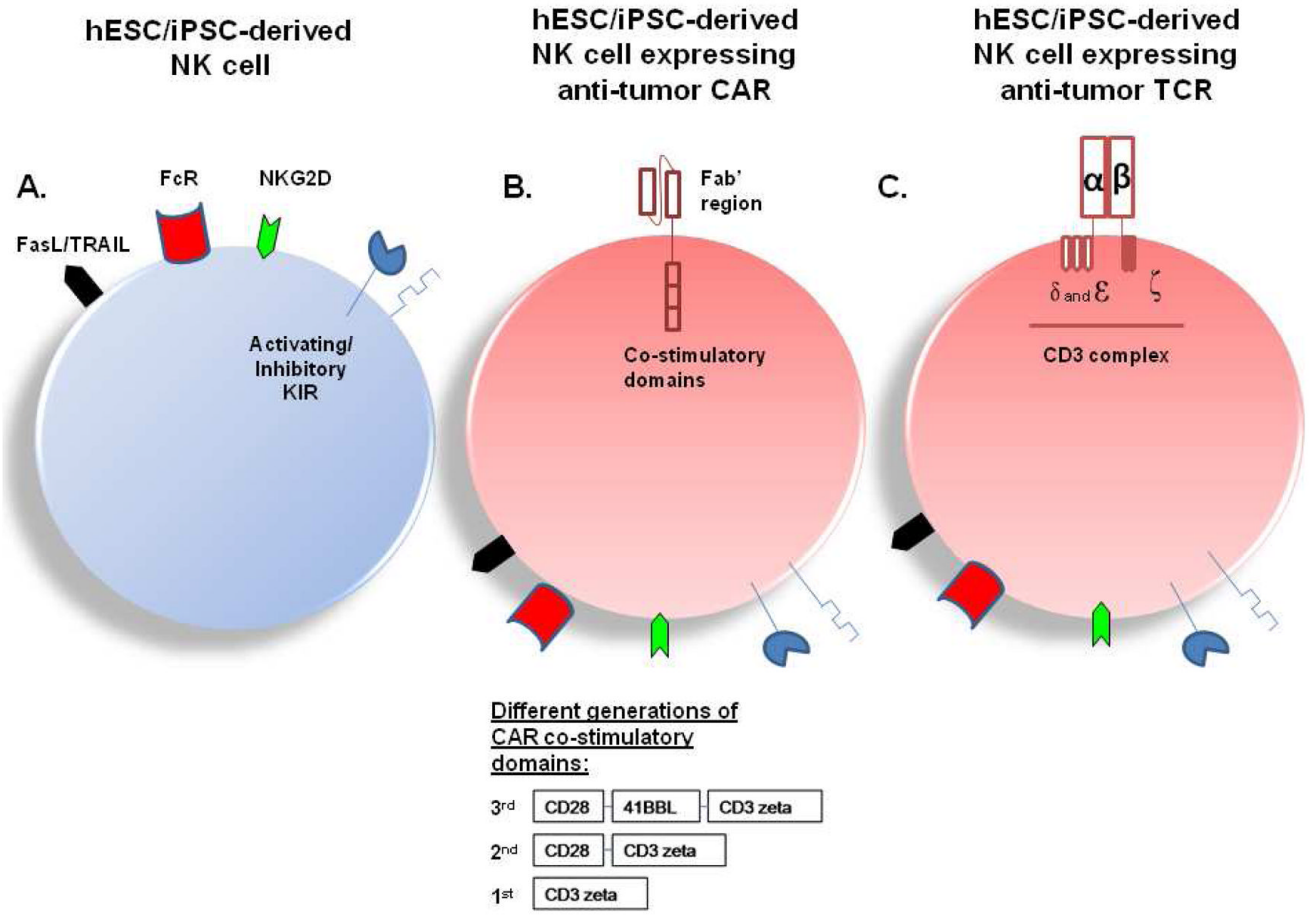

Figure 1.

Models of proposed hESC/iPSC-derived NK cells suitable for clinical trials against cancer. A) Unmodified hESC or iPSC-derived NK cells express a variety of NK cell effector molecules at high levels, including: Fc receptor (CD16), Fas ligand (FasL), TRAIL, NKp46, NKp44, NKG2D, and killer immunoglobulin-like receptors (KIRS). B) hESC and iPSC-derived NK cells can be modified with chimeric antigen receptors (CARs) for specific tumor antigens. Schematic of important functional domains of CARs is illustrated. Each individual co-stimulatory domain combination is fused to an antigen-specific recognition motif on the surface of the cell (e.g. Fab' portion of an antibody). C) hESC and iPSC-derived NK cells can be modified with cloned T cell receptors (TcRs) for specific tumor antigens. As NK cells express the CD3 chains (ζ and ε) required for TcR signaling and activation, engagement of TcR-expressing NK cell will lead to functional cytolytic activity.