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Hematopoietic Stem Cells for Cancer Immunotherapy

Eric Gschweng^{1,2}, Satiro De Oliveira^{3,4}, and Donald B. Kohn^{1,2,3,4}

¹Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, David Geffen School of Medicine, Los Angeles, CA, USA

²Eli and Edythe Broad Center for Regenerative Medicine & Stem Cell Research University of California, Los Angeles, David Geffen School of Medicine, Los Angeles, CA, USA

³Department of Pediatrics, University of California, Los Angeles, David Geffen School of Medicine, Los Angeles, CA, USA

⁴The Jonsson Comprehensive Cancer Center, University of California, Los Angeles, David Geffen School of Medicine, Los Angeles, CA, USA

Abstract

Summary: Hematopoietic stem cells (HSCs) provide an attractive target for immunotherapy of cancer and leukemia by the introduction of genes encoding T-cell receptors (TCR) or chimeric antigen receptors (CARs) directed against tumor-associated antigens. HSCs engraft for long-term blood cell production and could provide a sustained source of targeted anti-cancer effector cells to sustain remissions. T cells produced *de novo* from HSCs may continuously replenish anti-tumor T cells that have become anergic or exhausted from *ex vivo* expansion or exposure to the intratumoral microenvironment. Additionally, transgenic T cells produced *in vivo* undergo allelic exclusion, preventing co-expression of an endogenous TCR that could mis-pair with the introduced TCR chains and blunt activity or even cause off-target reactivity. CAR-engineered HSCs may produce myeloid and NK cells in addition to T cells expressing the CAR, providing broader anti-tumor activity that arises quickly after transplant and does not solely require *de novo* thymopoiesis. Use of TCR- or CAR-engineered HSC would likely require cytoreductive conditioning to achieve long-term engraftment, and this approach may be used in clinical settings where autologous HSC transplant is being performed to add a graft-versus-tumor effect. Results of experimental and pre-clinical studies performed to date are reviewed.

Keywords

hematopoietic stem cells; immunotherapy; T-cell receptor; chimeric antigen receptor

T cells for TCR- and CAR-mediated immunotherapy: progress and limitations

Studies of immunotherapy using peripheral blood T lymphocytes modified with genes encoding T-cell receptors (TCRs) or chimeric antigen receptors (CARs) recognizing tumor-associated antigens have made great strides in our understanding of basic immunology and yielded excellent clinical responses in some settings (as discussed elsewhere in this issue). Pioneering work by Steven Rosenberg at the Surgery Branch of the National Cancer Institute (1, 2) paved the way for a plethora of studies that have improved the efficacy of this

Correspondence to: Donald B. Kohn, M.D. 3163 Terasaki Life Science Bldg. 610 Charles E. Young Drive Los Angeles CA 91356
Tel.: +1 310 794 1964 Fax: +1 310 206 0356 dkohn@mednet.ucla.edu.

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therapeutic approach utilizing *ex vivo* expanded antigen-specific tumor infiltrating lymphocytes reinfused into patients (1), as well as the engineering of peripheral blood T cells via viral gene transfer (2). Further work using CARs has recently shown great promise in several trials: engineering T lymphocytes with CARs directed against the B-lymphocyte antigen CD19 has led to complete remissions in a number of patients with advanced B-lineage malignancies (3–6). The potential of these therapies is indisputable, though limitations remain. Objective responses using TCR engineered T cells have been achieved in the majority of patients enrolled in trials: a measurable decrease in bulk tumor burden is often observed. However, the complete response rates have been quite low and the majority of clinical responses are short-lived with ultimate tumor relapse. This has been observed particularly with TCR-based approaches, though CAR-based approaches may also have some sub-optimal aspects as further studies are reported.

A major explanation for this sub-optimal outcome is the relatively limited *in vivo* survival or suppression or exhaustion of infused engineered T cells (7, 8). Expansion of T cells to an effective therapeutic bolus is achieved by *ex vivo* culture in supraphysiologic concentrations of IL-2, increasing the cell numbers by several orders of magnitude. It has been appreciated that driving cells to expand under these conditions ages the cells from a more naive and replicative phenotype to late-stage effectors. Characterized by the loss of markers CD45RA, CD62L, and CCR7, these cells have great cytotoxic capacity but greatly diminished regenerative potential. Experimental work in murine models (9) as well as non-human primates (10) has shown the improved anti-tumor efficacy of central memory phenotype cells (Tcm) versus late stage effectors (9). There are promising recent reports showing that including small molecule inhibitors or modulation of the cytokine milieu in which cells are expanded *ex vivo* makes possible the maintenance and generation of the more stem like T-cell populations known as stem cell memory (Tscm) cells, and that these cells are capable of a more sustained response by replenishing effectors (11) similar to the previous studies with Tcm cells. A clear benefit to the transfer of less mature, more stem-like cells is evident, likely due to the increased persistence and replenishing capability of these cells *in vivo*.

In accordance with this hypothesis, studies investigating T cells transduced with CARs seem to have greater persistence and activity than those modified with TCRs, potentially demonstrating improved efficacy. Scholler *et al.* (12) reported long-term follow-up of subjects who received T cells modified to express a CAR-like molecule targeted against HIV-infected cells, and even 10-15 years after a single infusion of modified T cells, these cells could still be detected in the peripheral blood of many subjects. Recent trials using CARs against CD19 have shown significant clinical efficacy and also observed persistence of the engineered T cells, although with a shorter follow-up time (3–6). The inclusion of costimulatory domains derived from CD28 or 4-1BB may lead to low level tonic signaling that sustains T-cell survival *in vivo*; alternatively, chronic exposure to the CD19 antigen as new B-cell precursors are produced in the marrow may induce survival of the CAR-modified cells (13).

Aside from the reduced persistence and activity, another theoretical concern for the use of mature T cells as targets for anti-cancer TCRs is the potential for mis-pairing: that is, the pairing of endogenous cellular TCR chains with introduced transgenic chains. In this situation, T cells can be redirected, miss the intended cancer-antigen target, and instead be reactive to unpredictable moieties expressed on normal tissue. This has been demonstrated in a C57/Bl6 murine based study of immunotherapy using TCR α - and β -chains to engineer T cells via adoptive transfer (14). This system mimicked the clinical setting by including total body irradiation (TBI) and IL-2, and examined a variety of commonly studied TCR including OT-I, gp100, MART-1, and pmel-1. It was clearly demonstrated that mice developed symptoms of graft versus host disease (GvHD), and that the introduced T cell

chains, as a pair or individually, were necessary and sufficient to cause this outcome independent of IL-2 administration. It is important to note that the development of GvHD as a result of mis-pairing of an exogenous TCR introduced into mature T cells with an endogenous TCR chain has not been an observed problem in any clinical trial to date (15), though it remains a theoretical possibility.

The real or artifactual potential for induction of frank autoimmunity aside, the reduction of desired antigen-specific TCR surface expression due to mis-pairing poses another hurdle. A fraction of the transgenic TCR chains may be tied-up in pairs with endogenous TCR chains that do not possess the desired specificity, blunting their effectiveness for anti-tumor-specific activity by reducing the surface density of engineered TCR expression. As the surface density of TCR is correlated with activation (16, 17), the reduction of cancer antigen-specific TCR may render engineered cells incapable of mounting an effective immune response, nullifying therapeutic benefit. A number of approaches to reduce or eliminate the possibility of TCR mis-pairing have been described (18, reviewed elsewhere in this volume).

Hematopoietic stem cells for TCR- and CAR-mediated immunotherapy

Approaching T-cell immunotherapy by engineering of autologous hematopoietic stem cells (HSCs) with a pre-arranged anti-cancer-antigen TCR or CAR may offer a one-two punch, solving both the problem of effector cell persistence and TCR mis-pairing at the same time. HSCs are the most primitive of blood lineage cells. Residing predominantly in the bone marrow, HSCs produce all mature blood cells by proliferation and differentiation through a series of increasingly lineage-restricted progenitors. HSC transplantation has been a clinical practice for more than four decades to promote life-long hematological recovery after high doses of chemotherapy or radiation to treat malignancies, for marrow reconstitution in marrow failure states, such as aplastic anemia, and to treat genetic diseases of blood cells, by providing a source of normal stem cells that can produce the needed normal blood cell type. HSCs can be obtained for transplant from the bone marrow of a suitably matched donor, by leukopheresis of peripheral blood after mobilization by administration of pharmacological doses of cytokines such as G-CSF [peripheral blood stem cells (PBSCs)], or from the umbilical cord blood (UCB) collected from the placenta after delivery. The marrow, PBSCs, or UCB may be transplanted without processing, or the HSCs may be enriched by immune-selection with a monoclonal antibody to the CD34 surface antigen, especially if the HSC are to be modified by *ex vivo* gene transfer. Further enrichment of HSCs is an active area of research (19, 20) that will no doubt improve subsequent gene therapeutic approaches to immunotherapy.

Gene transfer and expression in HSCs has been under study for more than three decades (21). Vectors derived from viruses of the *Retroviridae* family, either γ -retroviral, such as murine leukemia viruses (MLV), lentiviral, such as the human immunodeficiency virus (HIV), or spumaviral, such as the human foamy virus (HFV), have been most effective for permanent gene insertion into the chromosomes of HSCs. This results in stable transmission to all progeny progenitors and mature blood cells. Typical clinical approaches to gene transduction of HSCs involves enrichment for the CD34⁺ T-cell fraction, culture in medium containing a cocktail of recombinant human cytokines including *c-kit* ligand, flt-3 ligand, and thrombopoietin to activate or pre-stimulate the HSC from quiescence for 1-2 days, and then exposure to the gene delivery vector for 1-2 days by its addition to the culture. At the completion of transduction, the cells are washed and formulated for either direct intravenous infusion or cryopreservation for transplantation at a later date. Using these current optimized methods, insertion of 1-3 copies of the delivered transgene may be achieved into the

majority of the HSCs, with preservation of their engraftment and multi-potent blood cell production capacity.

Expression of the delivered transgene is typically achieved by the use of a strong constitutive transcriptional control element such as a viral or cellular enhancer/promoter fragment. The transgene product may be transcribed in blood cells of all lineages produced from the HSCs, although other factors may modulate their expression. For example, the requirement for co-expression of the CD3 complex of proteins limits surface display of a transgenic TCR to T lymphocytes, even though the vector may be present and transcribed in all blood cells derived from a transduced HSC (notably, CARs are not limited by CD3 expression). Alternatively, some vectors are designed to use lineage-specific transcriptional control elements to restrict expression to specific cell types, such as erythroid, myeloid, etc.

The approach of using HSCs as the targets for TCRs or CARs is attractive for at least three reasons. First, as with approaches that use Tcm or Tscm as targets for gene modification, the regenerative nature of HSCs may provide a long lasting (potentially life-long) supply of effector T cells engineered against the antigen of interest. TCR- or CAR-modified HSCs will continuously produce T-lymphocyte progenitors that will undergo normal thymopoiesis and development, increasing the potential for development of immunological memory (Fig. 1). This is in contrast to mature T cells massively expanded *ex vivo* before infusion, with most having a finite life-span. Second, the off-target cytotoxicity or blunted surface expression of the engineered TCRs due to mis-pairing described above as a potential complication from transducing mature T cells may be eliminated by tackling the approach from HSCs that have not yet rearranged their germline TCR loci (22) if the transgenic TCR suppresses rearrangement of the endogenous TCR loci. This allelic exclusion would ensure the robust surface expression of an exclusive, antigen-targeted TCR. Third, it has been demonstrated that increasing the intensity of the pre-conditioning regimens used when administering tumor-infiltrating lymphocytes correlates with increasing clinical efficacy (23). Very high doses of conditioning (essentially cytoablative) mandate HSCs rescue of the patient, which provides an ideal setting for engineering a portion of the HSC graft for immunotherapy.

In this review, we discuss the known benefits and potential pitfalls associated with using HSCs for engineered immunotherapy and recent work focused on moving this technology to a clinical setting.

TCRs into HSCs: *Imperium sine fine dedi*

Stable retroviral or lentiviral introduction of pre-arranged cancer antigen-specific TCR or CAR to HSCs can provide an ongoing source of targeted T cells and, perhaps, other effectors. While infusions of TCR- or CAR-modified mature T cells have an anti-neoplastic impact within days to weeks, production of anti-tumor effector cells from engineered, transplanted HSCs may take longer, with myeloid and NK cells emerging within 1-3 weeks after transplantation and *de novo* T lymphopoiesis occurring only after several months. Though delayed, it is hypothesized that the stable engraftment of TCR or CAR-modified HSCs will lead to sustained production of targeted effector cells, potentially providing sustained anti-tumor activity.

It is well established that the transduction and transplant of HSC with a prearranged TCR can give rise to antigen-specific T cells in murine models. Early reports (24, 25) used retroviral vectors to deliver TCR α and TCR β chains to mouse bone marrow followed by transplantation into lethally irradiated recipients. Long term development of T cells expressing the introduced TCR was demonstrated, including in secondary recipients,

providing evidence of gene transfer to long-term HSCs. Later work showed that engineered T cells produced from TCR gene-transduced murine HSCs that were transplanted into lethally irradiated hosts were responsive to antigen stimulation by dendritic cells, developed a memory cell phenotype, and provided protection against *in vivo* tumor challenge (26, 27). Alajez *et al.* (28) used a novel fusion protein consisting of a single chain TCR that recognized a segment of the MUC1 tumor-associated protein in a non-MHC-restricted manner with the CD3 ζ chain intracellular signaling domain (in effect a chimeric antigen receptor) to transduce murine bone marrow which was transplanted into SCID mice. The recipients of the fusion protein-transduced marrow rejected MUC1 positive tumors more effectively than did controls.

Two studies examined the potential to produce T cells from transduced human HSCs using *in vitro* differentiation on the murine OP9-DL1 stromal cell line. Zhao *et al.* (29) transduced human UCB with retroviral vectors encoding TCR against relevant human tumor associated antigen peptides (NY-ESO-1 and p53) and directed their differentiation to T lymphocytes by co-culture on OP9-DL1 stromal cells. The T cells so produced displayed antigen-specific, HLA-restricted cytolytic activity. Van Lent *et al.* (30) used retroviral vectors to introduce the cDNA for human TCR α and TCR β chains recognizing peptides from either HA-2, CMV, or MART-1 into either human thymic T-lymphoid progenitor cells (CD34⁺CD1a⁻) or human UCB (CD34⁺CD38) progenitor/stem cells. Mature T cells were produced *in vitro* on OP9-DL1 stroma that possessed cytolytic activity with the antigen specificity of the introduced TCR. Interestingly, the introduction of only a TCR β chain greatly accelerated T lymphopoiesis, postulated to result from prolonged signaling from a preTCR complex formed with a pT α chain, whereas introduction of both the TCR β and α chains only moderately increased T-cell production, compared to cultures receiving only the TCR α chain or control non-transduced cultures.

TCR engineering of human HSCs to produce modified T cells *in vivo* was first shown using a model in which human thymic tissue is implanted into NOD/SCID/IL2Rg^{-/-} (NSG) immune-deficient mice, followed by injection of gene-modified human CD34⁺ cells (the BLT model). This study demonstrated the development of anti-HIV T cells after transfer of the genes encoding a TCR against an HIV-1 *gag* peptide. Functional, antigen-specific T effector cells were produced that were capable of killing HIV-infected cells (31). This same model was then used to generate MART-1 antigen-reactive human T-cell effectors capable of killing melanoma tumors in an antigen- and HLA-specific context after transplantation of human fetal liver-derived CD34⁺ HSCs transduced with the genes encoding a TCR to the melanocyte antigen MART-1 (32). One caveat to the BLT model is that the education of T cells in a human thymic organoid does not induce tolerance to the mouse host, generating self-reactive T cells that ultimately kill the humanized mouse via GvHD, limiting the duration of studies to short-term (33). Though efficacy can be demonstrated in this model in an antigen- and HLA-specific context, the examination of long-term provision of cells is hampered.

To address this issue, we used a transplant model where the clinically relevant human HSC sources of CD34-enriched UCB or PBSC are transplanted into sub-lethally irradiated NSG immune-deficient neonatal mice (Fig. 2). In a direct human HSC transplant to NSG neonates, developing human thymocytes are educated on the murine thymus. While this likely results in a reduced TCR repertoire compared with the BLT model, the mature T cells are host-tolerant, allowing long-term studies of these chimeric animals. Furthermore, using NSG hosts engineered to express human MHC molecules can allow the development of an HLA-restricted T-cell repertoire (34).

In NSG and NSG-HLA**A0201* mouse strains, we studied the development of human HSCs transduced with a TCR recognizing the *27-35*MART-1 nonamer. We demonstrated that both CD8⁺ and CD4⁺ T cells were produced that expressed the introduced TCR (22). The presence of the pre-arranged TCR in the developing thymocytes appeared to accelerate and boost CD8⁺ T-cell production, as had been observed *in vitro* by van Lent *et al.* (30). The T cells produced showed antigen-specific release of γ -interferon when tested *ex vivo* (22). Though the body of literature describing engineered immunity via HSCs is relatively young, proof of principle has been demonstrated showing the promise of this approach. Further work to classify the T-cell phenotypes developing in this model, and exploration of mouse models that mimic the human cytokine milieu and how they affect T-cell development and homeostasis in the context of engineered immunity via HSCs remain interesting arenas for exploration.

In addition to the potential life-long provision of effector cells, the engineering of HSCs with exogenous TCRs is an attractive solution to the issue of TCR mis-pairing. While inclusion of a number of clever designs to the TCR construct can minimize this undesired consequence, the HSC approach is capable of sidestepping this pitfall entirely due to the mechanism of allelic exclusion. Early in T-cell development, the endogenous TCR β and TCR α genes undergo V(D)J rearrangement via the VDJ recombination complex of DNA modifying enzymes. Upon successful rearrangement of one allele, the recombination of the remaining germ-line allele is inhibited (at least for TCR β). This ensures that only a single α and single β chain will be expressed in a given T cell, conferring a single MHC-specific antigenic reactivity (the same allelic exclusion mechanism is present in B cells, where it was discovered) (35).

Work by our group at UCLA has demonstrated genetically (22) and molecularly (36) that the expression of a prearranged TCR upstream of T-cell development by the engineering of human HSCs does indeed induce allelic exclusion as in normal development. Deep sequencing of the TCRV β CDR3 region in T cells derived from HSCs engineered to express a transgenic TCR specific for the *27-35*MART-1 peptide and differentiated into T cells in the NSG mouse showed only the introduced T-cell rearrangement, whereas T cells lacking expression of the introduced TCR contained a broad array of TCRV β gene rearrangements (22). In line with this finding, quantitative analysis of TREC, RNA spectratyping, and TCRV β chain expression by flow cytometry demonstrated allelic exclusion in MART-1⁺ T cells when compared to MART-1⁻ T cells in a BLT model (36). In the latter study, while V β family member surface staining clearly demonstrated exclusion, spectratyping of RNA showed enrichment, but not exclusive expression, of the transgenic TCR chain. This result may suggest leakiness of allelic exclusion in this model or perhaps post-transcriptional silencing of only one rearranged TCR loci resulting in surface expression an exclusive V β chain. These somewhat contradictory findings may highlight differences in HSC progenitors from fetal liver versus UCB, or of the thymopoietic processes in BLT versus the NSG models; a comprehensive study examining engineered immunity induced allelic exclusion at the DNA, RNA, and protein levels would be informative.

More recently, we have performed studies using a lentiviral vector carrying the cDNA for a human TCR recognizing the 157-165 peptide from the NY-ESO-1 tumor-associated antigen (37). This work has focused on the use of human PBSC as a clinically relevant HSC source compared to fetal liver or UCB, as PBSCs will be the cell source used for an upcoming trial of HSC based immunotherapy at UCLA. While higher doses of CD34⁺ PBSCs are needed to effectively engraft the NSG mice compared to what was used with UCB (1×10^6 versus $1-3 \times 10^5$, respectively), we have observed the production of T cells expressing the anti-NY-ESO-1 TCR in mice transplanted with transduced PBSCs. Extensive immunophenotypic characterization of T cells produced *in vivo* and expressing the TCR transgene is in progress.

In contrast to the TCR against MART-1 that appeared on both CD4⁺ and CD8⁺ T cells, expression of the NY-ESO-1 TCR has been restricted to CD8⁺ T cells, indicating that the specific TCR plays an instructive role in T-cell development (Fig. 3).

The NY-ESO-1 TCR lentiviral vector also carries the modified herpes simplex thymidine kinase gene (sr39TK) that functions as both a PET reporter as well as a potential suicide gene (see below) (38). The transferred TCR proteins will only be displayed on the surface of T cells produced from the transduced HSCs due to the requirement for co-expression of CD3, limiting detection of total gene modified cells using a TCR assay based approach such as binding to MHC-tetramer. However, the sr39TK reporter gene will be expressed at high levels from the vector's MSCV LTR promoter in cells of all hematopoietic lineages. This reporter allows *in vivo* imaging to be performed to examine sites of hematopoiesis, visualizing the gene-modified progeny of the transduced CD34⁺ cells *in vivo* in the NSG recipients. Following administration of an [¹⁸F]-FHBG PET probe (200-250uCi) to the mice, we have observed hematopoietic niches including the long bones, sternum, spleen, vertebrae, ribs, as well as the thymus as hot spots of reporter signal, indicative of engrafted, transduced human HSCs and their cell progeny in NSG hosts (E.G., manuscript in preparation).

As central tolerance limits the development of self-reactive T cells, and cancer antigens targeted in T-cell therapy are frequently self-expressed antigens (39). As such, the isolation of self-reactive TCRs is quite rare and, when present, the TCR affinity is accordingly low (40). This principle potentially poses a challenge for the TCR-modified HSC approach. Mature T cells have already passed the stage of development where auto-reactive cells have been deleted, and the introduction of a self-reactive TCR into these cells is possible. However, in a pre-thymic stem/progenitor cell destined for thymic education and surveillance, auto-reactivity may result in deletion of thymocytes expressing self-reactive TCR.

An elegant approach to circumvent this limitation has been demonstrated in the mouse (41). In this study, the investigators determined that microRNA miR-181 family members were expressed transiently at high levels during thymopoiesis, with peak expression occurring at the double-positive thymocyte stage, and falling upon maturation and migration of the T cells to the periphery. They designed a vector carrying a conalbumin-specific TCR in which the vector transcript was made sensitive to suppression by endogenous miR-181a by inclusion of repetitive miR-181a target sequences following the transgene. In a murine model of HSC transplant to B10.D2 mice, known to negatively select this specific TCR, only transplants containing the TCR vector with the miR-181a target sequences resulted in the development of engineered mature TCR-expressing T cells that survived through thymic selection. Importantly, peripheral T cells expressing the conalbumin-specific TCR expanded when the animals were vaccinated with antigen-pulsed dendritic cells, and *ex vivo* functional assays showed responsiveness in an antigen dose-dependent fashion. This latter point demonstrates the feasibility for use of this approach for other potentially self-reactive TCRs (or CARs) in the clinical setting, as modulation of the TCR through development allowed self-reactive T-cell development yet did not ablate mature cell function. It would be of interest to know if the downregulation of TCR expression coincident with high levels of miR-181a during development will abrogate allelic exclusion and allow endogenous TCR rearrangement to occur, thereby permitting potential TCR mis-pairing in T cells expressing endogenous and exogenous TCR genes.

TCRs have the advantage over CARs of being able to recognize a wider variety of tumor-associated antigens, not limited to those that are expressed on the cell surface. Thus, the use

of HSCs as a vehicle to continuously produce fresh TCR-modified T cells may provide an additional means to extend the activity of this approach.

Driving CARs into HSCs

As with gene modification of HSCs with TCRs, modification of HSCs with genes encoding CARs brings the prospect of long-term production of immune effector cells targeted to tumor-associated antigens. One major potential advantage with CARs is that their expression will not be limited to T cells, as is the case with TCR genes introduced into HSCs, since the surface display of CARs does not require CD3 co-expression. Thus, CARs may be expressed on multiple hematopoietic lineages (T, NK, and myeloid), amplifying the potential graft-versus-cancer activity (21, 42–45). While *de novo* production of T cells from transplanted HSCs may be slow and limited in adult recipients, myeloid and NK cells should be produced rapidly after transplant, yielding an early source of anti-tumor immunity (42–44). It is expected that CAR-bearing T cells would be the most efficient cytotoxic effector cells against tumor cells produced by CAR-modified HSCs, due to their longer lifespan, greater activation and proliferation properties, and potential for generation of immunological memory. Results from studies assessing CAR-modified NK and myeloid cells indicate that NK cells have similar antigen-specific cytotoxicity to T cells (42, 43, 46). However, the continued generation of large numbers of CAR-bearing neutrophils, monocytes and NK cells may constitute a powerful anti-cancer barrier, thanks to their tissue homing, large circulating pool in peripheral blood, and vascular diapedesis. Myeloid and NK cells do not depend on thymopoiesis to become active cytotoxic effectors and will exponentially amplify the anti-tumor immune response directed by CAR. The multi-lineage expression of CARs, associated with potent engineered antigen-specific cytotoxicity, makes the CAR modification of HSC a very promising cancer immunotherapy approach to be explored.

Furthermore, CAR-modified HSCs can be infused in the context of the standard medical procedure of hematopoietic stem cell transplantation (HSCT), when the chemotherapy conditioning regimen would create optimal conditions for the efficacy of this approach, favoring engraftment of gene-modified cells, as well as decreasing tumor burden and decreasing the risks of immunogenicity of the CAR molecules. Recently, a clinical trial has been opened infusing CD19-specific CAR-modified T cells in patients with high-risk B-lineage malignancies undergoing autologous HSCT using UCB, to add potential graft-versus-leukemia effects to the relatively naive T cells contained within the graft (47). In this setting, CAR modification of a portion of the donor HSC may add additional anti-leukemic effects.

Studies performed by the research group at Cell Genesys, Inc. in the mid-1990s were the first efforts to explore the potential for using HSCs as the cell platform for expressing a CAR-like molecule (42, 43). They produced a CAR-like fusion protein between the extracellular domain of human CD4 and the intracellular CD3 ζ chain, termed a universal receptor (UR), to direct cytolytic effector cells to kill cells infected with HIV-1 through binding of the CD4 domain with HIV-1 gp120 displayed on the surface of the HIV-infected cell. This UR had been shown to arm T cells to kill HIV-infected cells and was advanced to several clinical trials into peripheral blood T cells, where it showed some evidence of *in vivo* anti-HIV effects (42, 48, 49).

Moving towards a stem cell approach, this same investigative group studied the activity of the UR introduced into murine bone marrow HSCs, which was then transplanted into recipient mice. They demonstrated that it led to production of effective cytolytic cells, using a leukemia cell line transduced to express HIV-1 gp120 as a surrogate target for the CD4/CD3 ζ fusion construct. Expression of the UR was observed on leukocytes of multiple

lineages derived from the transduced HSCs, including granulocytes, monocytes, and NK cells. A direct demonstration that non-T cell effectors (myeloid and NK cells) were responsible for the cytolytic activity was obtained by transducing the UR gene into bone marrow from *SCID* mice (that would be genetically incapable of producing T lymphocytes), which still led to elimination of the gp120-expressing target cells *in vivo* after transplant (42). They also reported introduction of the UR into human blood NK cells and into human CD34⁺ cells that produced CAR-targeted myeloid cells upon *in vitro* differentiation and again demonstrated specific cytolytic activity (43).

In a later paper, these authors showed the insertion of the same CD4/CD3 ζ fusion gene into marrow HSCs from normal mice led to production of UR-expressing myeloid, NK, and B cells, but a complete absence of transgenic T cells, with a blockade to production of UR-expressing T cells occurring during the T1→T2 transition of thymopoiesis. This observation raises the specter that intrathymic negative selection of cells expressing a self-reactive CAR or TCR may prevent their maturation to mature T cells. However, the finding may be specific to this UR with a CD4 extracellular domain, because the UR-expressing T cells were not eliminated when the transduced HSC were transplanted into MHCII gene knockout mice, suggesting that it was a specific interaction between murine MHCII and human CD4 that triggered their elimination (50).

In exploring the role of the intracellular signaling domain, they also compared the CD3 ζ domain, used for TCR signaling in T cells, to the analogous domain from the Fc receptor- γ (Fc γ R1), which would normally be used in myeloid and NK cells (43). CD3 ζ and Fc γ R chains share the immunoreceptor tyrosine-based activation motif (ITAM), and both trigger activation pathways leading to target cytolysis, phagocytosis, and effector cell degranulation. In both murine and human T, myeloid, and NK cells, the UR with the CD3 ζ domain consistently displayed greater activity than one with the Fc γ R domain, which may be attributed to the presence of three ITAMs in the CD3 ζ domain but only one in the Fc γ R domain (42, 43). Other studies have reported similar findings on the greater activity of the CD3 ζ domain over the Fc γ R domain in T cells (42, 43, 51–54).

The optimal intracellular signaling domains for CAR to be used via HSCs are not fully known. The development of CAR activating moieties have mostly focused on the activation of T cells, with CD3 ζ being the most commonly used moiety (51, 55), as the terminal portion of the CAR construct, by itself (first generation CAR) or following the inclusion of different co-stimulatory domains (second and third generation CARs) (56, 57). Some knowledge has been obtained on the molecular events of the T-cell activation triggered by CARs, generally observing that the same activation pathways activated by TCR stimulation are involved. It has been demonstrated that CARs in T cells leads to immunological synapse formation (58, 59), calcium influx, and activation of the phosphatidylinositol and tyrosine kinase pathways (60, 61).

Although it is generally agreed that costimulation is required for efficacy in cancer immunotherapy approaches (4, 47, 56, 62, 63), to this moment it is still not fully resolved which costimulatory molecule produces the best results in CAR-mediated T-cell activation (nor for myeloid and NK cells). It has been shown that CAR signaling mediates cell activation in neutrophils, monocytes, and NK cells, besides T cells, with either the Fc γ R or CD3 ζ activation moieties (42, 43, 46, 64). Activation of NK cells has been shown to require costimulation, with successful antigen-specific activation only achieved with second-generation CAR (46, 64). It was found that the 41BB costimulatory domain was most effective when CARs were introduced into mature human NK cells (64–66). Our group and others have shown that first generation CARs successfully activated neutrophils and

monocytes (42, 43), and their antigen-specific cytotoxicity did not differ from that triggered by second generation CARs (DeOliveira *et al.*, manuscript in revision).

Gene modification of HSCs requires stable transgene integration, ideally delivering one transgene copy per HSC (21). We have consistently achieved 40-50% of transduction efficiency of human HSCs with CARs at an average of 1-3 copies/cell using lentiviral vectors with internal enhancer/promoters from the MND LTR. Higher transduction rates may not be warranted, as it would not be desired to completely devote the immune system to mono-specific anti-tumor effector cells; fortunately, for the immunotherapeutic applications, such high level engraftment of gene-modified human HSCs has not been achieved in clinical trials for genetic diseases where complete gene correction of all engrafted HSCs may be clinically useful.

CAR-modified HSCs undergo normal differentiation and proliferation, and their cell progeny are morphologically and functionally normal (DeOliveira *et al.*, manuscript in revision). We have successfully engrafted anti-CD19 CAR-modified human CD34⁺ HSC in NSG mice, with CAR-bearing cells detected in all hematopoietic lineages (Fig. 4) in bone marrow, spleen, and peripheral blood. Notably, the absence of human CD19⁺ B cells suggests active cytotoxicity by the anti-CD19 CAR expressing cells, as B lymphocytes are normally the most common human cells to develop in this model.

With this initial progress towards modification of HSCs with CARs, several questions remain unresolved. The relative benefits of adding the activity of CAR-armed myeloid and NK cell effectors to the T-cells' army remain to be determined. It also remains to be determined whether the presence of the CAR will have adverse effects on *de novo* T-cell production, by causing negative selection, whether the CAR will suppress endogenous TCR gene rearrangement by allelic exclusion, as has been shown with TCRs, and whether the diverse CAR-expressing leukocytes will cause unique toxicities. Ongoing studies in the NSG mice may address some of these questions and initial clinical trials may provide more answers.

Suicide genes to eliminate TCR/CAR modified HSC grafts

With the engineering of HSCs comes the risk of insertional oncogenesis, as has occurred in several clinical trials in which γ -retroviral vectors were used to transduce autologous HSCs that were transplanted for patients with primary immune deficiency diseases (67). The introduction of these retroviral vectors, containing long terminal repeats with strong promoter/enhancer elements, into the susceptible genome of HSCs is capable of activating proximal cellular *proto*-oncogenes, and inducing cell proliferation that can progress to frank transformation. While the potential for this to occur in the setting of adult patients who would likely not have the high level of engraftment and proliferation seen in young immune deficiency patients, it remains a hypothetical risk. The long-term production of targeted effector cells, an attractive benefit of using HSC for immunotherapy, also poses potential problems if there is toxicity, either on-target/off-tissue toxicity, such as sustained suppression of CD19⁺ B cells, or the previously mentioned off-target reactivity possible by the introduction of a self-reactive TCR.

To alleviate these risks, the inclusion of a suicide gene to ablate gene-modified cells has been undertaken in a number of studies. The most widely studied suicide gene is derived from the herpes simplex virus thymidine kinase (HSV-TK) gene, which confers sensitivity to the anti-viral nucleoside analogue ganciclovir (68–73). Other suicide genes have been produced using fusion proteins that express FAS signaling moieties or caspase components under the post-transcriptional control of small molecule ligand (74–77). These latter genes

are attractive in that they do not carry the immunogenicity demonstrated by the sr39TK construct, though they do not allow PET functionality to visualize modified cell tracking. The presence of a suicide gene in the TCR or CAR vector would allow cells that are over-proliferating or causing toxicity to be eliminated by administration of the pro-drug or inducing agent.

In our studies using the NY-ESO-1 TCR lentiviral vector also carrying the sr39TK gene (described above), we examined its potential as a suicide gene to eliminate the cell products of the TCR-transduced human HSCs in the NSG mice. Upon administration of ganciclovir (50 mg/kg IP \times 5 days), the transduced cells were completely eliminated from the mice, as indicated by both repeat PET scans, FACS analysis for NY-ESO-1 tetramer staining, and PCR of blood, spleen, thymus, and bone marrow for the lentiviral psi element (not shown). The ability to eliminate the engineered graft is attractive in case of a serious adverse event, and remains an important safety switch for inclusion in HSC-based approaches to engineered immunity.

Clinical considerations for use of TCR/CAR-modified HSCs

Sustained HSC engraftment requires pre-transplant conditioning with agents that have myeloablative or at least marrow cytoreductive activity, such as total body irradiation or busulfan. These may be used at sub-myeloablative doses (e.g. 200 cGy TBI or 4 mg/kg busulfan) for marrow cytoreduction with minimal acute toxicity. In contrast, the lymphodepleting agents typically used prior to T-cell immunotherapy (e.g. fludarabine, cyclophosphamide) are not significantly myeloablative and would not be expected to increase HSC engraftment. The need for cytoreductive conditioning as preparation for HSC immunotherapy would make application in disease settings where autologous or allogeneic HSCT is already used (e.g. leukemia and lymphoma) as most logical first choices.

The clinical application of TCR or CAR therapies via HSCT holds several challenges. Adult HSCs are generally more myeloid skewed than those from younger people, which may limit the numbers of T cells that can be produced from the transplanted, engineered HSCs (78). Additionally, the adult thymus is certainly much less active, undergoing involution with advancing age and the potential for *de novo* thymopoiesis is diminished, although some activity remains (79, 80). Prior treatments, such as chemotherapy or radiation therapy, may also decrease thymic function. These potential limits to new T-cell development emphasize the potential benefits derived from non-T-cell effectors that CAR-modified HSC may produce.

Summary

The potential advantages and proof-of-principles have been provided for approaches to cancer immunotherapy using TCR- or CAR-modified HSCs. It remains to be proven in clinical trials whether these approaches will lead to additive efficacy to those realized using mature T cells as targets. Certainly the two approaches may be complementary when applied together in some settings. Clinical trials are being developed that will provide some initial assessments.

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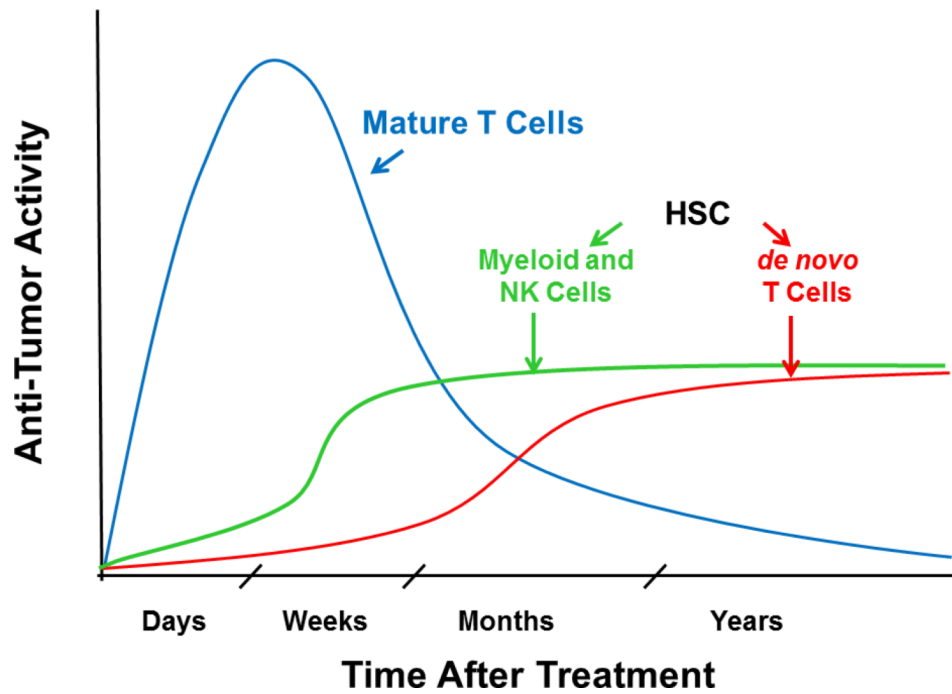


Fig. 1. Theoretical time course of anti-tumor activity

The theoretical anti-tumor activity produced in patients is depicted following a single infusion of TCR- or CAR-transduced mature T cells (blue), and following stable engraftment of TCR- or CAR-transduced HSCs, yielding myeloid and NK cells expressing CARs (green) within weeks of transplant and *de novo* T cells expressing TCRs or CARs (red) within months.

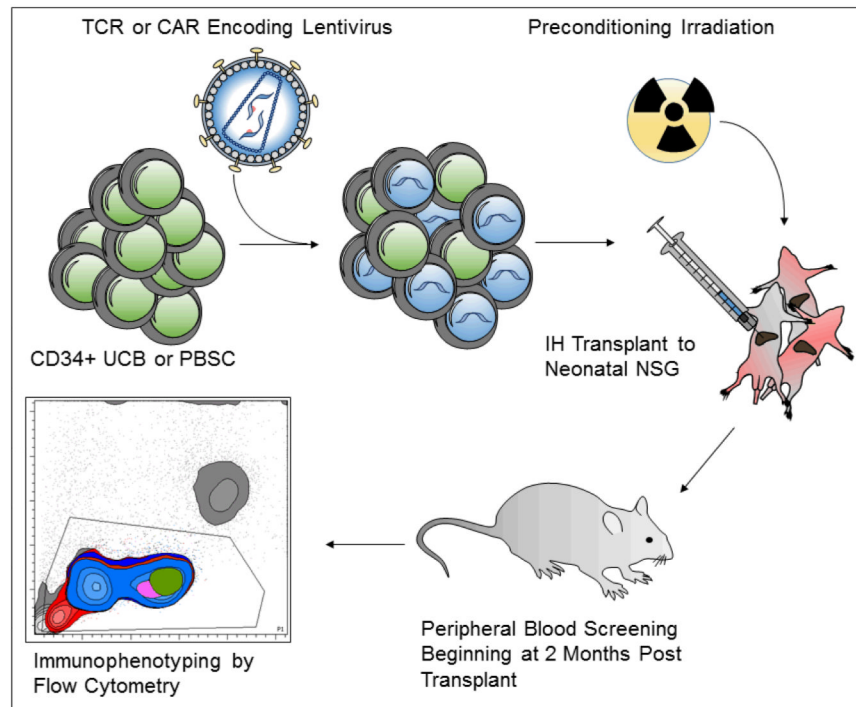


Fig. 2. *In vivo* xenograft model to study the use of hematopoietic stem cells for cancer immunotherapy

Umbilical cord blood (UCB) or peripheral blood stem cells (PBSCs) are processed to purify the CD34⁺ population enriched for HSCs. Cells are pre-stimulated and transduced with a concentrated lentiviral vector encoding a TCR or CAR. Gene-modified cells are transplanted via intra-hepatic (IH) injection to pre-conditioned (150 cGy) NSG neonatal mice. Beginning at 2 months post-transplantation, immunophenotypic analysis of peripheral blood is performed by polychromatic flow cytometry.

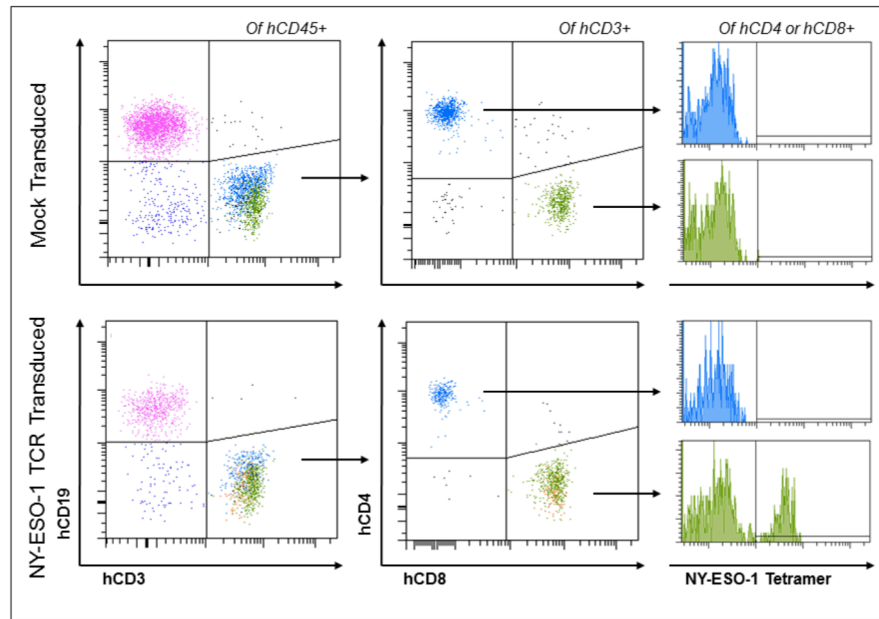


Fig. 3. Immunophenotypic analysis of humanized mice

NSG mice transplanted with either mock-transduced and NY-ESO-1-TCR vector-transduced PBSC reconstitute a human immune system *in vivo*, as evidenced by human CD19⁺ B cells and both CD4⁺ and CD8⁺ human CD3⁺ T cells. CD8⁺, NY-ESO-1 tetramer-binding effector T cells that developed from gene-modified HSCs are detectable (bottom right).

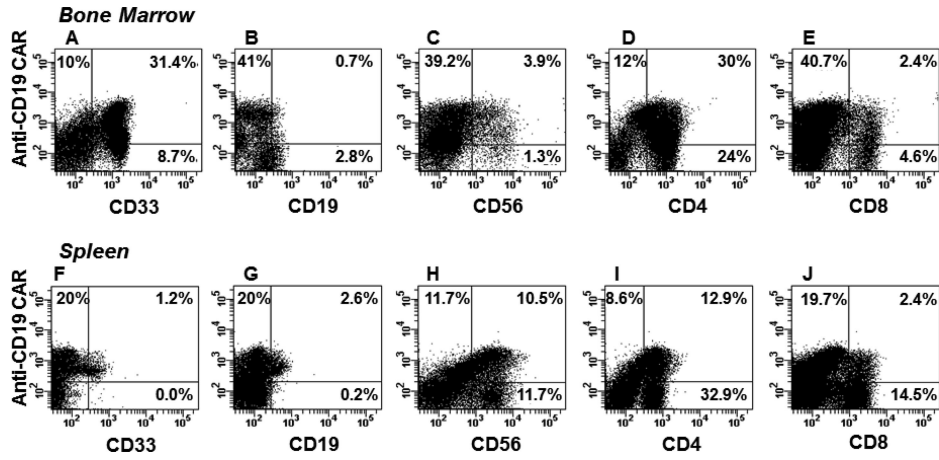


Fig. 4. CD19-specific CAR-bearing cells in humanized NSG mouse
Representative flow cytometry dot plots from a humanized mouse engrafted with CAR-modified human HSCs are depicted. (A-E). Cells from bone marrow were analyzed after staining with a FITC-labeled anti-IgG Fc to detect CAR (Y axis) versus antibodies against the human surface markers CD33 (myeloid cell marker), CD19 (B-cell marker), CD56 (NK cell marker), CD4 and CD8 (T-cell markers), represented on X axis. (F-J). Cells from spleen of the same mouse stained using same antibody mix. Bold numbers in quadrants represent percentages from total human CD45 population in each sample.