Hypothesis

Purging Autologous HSCs via Detection of Clonal Mutations

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Purging Autologous HSCs via Detection of Clonal Mutations

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Abstract: Allogeneic hematopoietic stem cell (HSC) transplants are usually used in cases where patients have active cancerous illness in their bone marrow, but allogeneic transplants can cause graft vs. host disease. If autologous HSCs could be purged of cancer cells, they could then be used for transplantation purposes without concern. A novel method of purging is discussed herein that does not require chemotherapy and is targeted for each patient. It could be applied either in vitro or *in vivo*.

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Introduction

Allogeneic hematopoietic stem cell (HSC) transplantation can be used to treat blood cancers, but it often leads to graft vs. host disease (GVHD)[1]. Advances in allogeneic transplantation may eventually enable clinicians to prevent GVHD, e.g., via OX40-targeting CAR T-cells [2] or OX40L–CAR-Tregs[3]. However, full suppression of GVHD may not be feasible for some time, and the use of autologous cells would still be ideal to avoid all possible short-term and long-term immunological issues. Fortunately, the mobilization of HSCs can now be done much more effectively than in the past[4]. However, the harvested patient cells could be contaminated to some extent with their tumor cells. A targeted method of purging HSCs could allow for safer autologous HSC transplantation[5].

If the patient's blood cancer cells in circulation, as well as the lymph nodes and bone marrow, are sequenced[6], clonal mutations can be identified - i.e., mutations that are contained in all of their cancer cells. To ensure a "signal", clonally mutated genes can be upregulated via targeted transcriptional activators. Then, the mutated RNA or proteins can be detected with molecular switches, and the cells can be eliminated using the effector module of said switches[7–9].

As described previously, expression of the transcriptional activators could be halted via detection of the clonally mutated transcript somewhere other than the mutation site[9]. This would prevent long-term activation of the gene in non-cancerous cells. This site could be recognized by an RNA-binding "proximity" switch based on Pumby modules[9,10].

Amplifying the signal of clonal mutations in non-coding regions - and targeting them - would also be possible in many instances[8,9].

This purging process can be done *ex vivo*, via mobilization and harvesting of patient HSCs, followed by transfection or transduction. It can also be done in vivo via mobilization of patient HSCs and dual adenoviral vector transduction[11]. In this in vivo system, one adenoviral genome expresses Flpe recombinase and a hyperactive transposase to circularize and randomly insert the transgene cassette in the second into the HSC genome.

A CRISPR transposase or twin prime editing components combined with a large serine recombinase could potentially be encoded by the first adenoviral vector that is used for integration of the second[12,13]. Given the large packaging space of a helper-dependent adenovirus (HDAd), these elements could also be encoded by the second vector itself - with no need for a first. Either way, they would enable targeted integration into a safe harbor locus instead of random integration. An episomal approach involving a scaffold/matrix attachment region (S/MAR) may also be possible for linear or circular DNA instead of integration[14,15].

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Purging Strategy

First, cancer cells in the patient's bloodstream, lymph nodes, and bone marrow would be sequenced to determine his or her clonal mutations[6]. After isolating mobilized CD_{34}^{+} cells from a patient's bloodstream[4], an ex vivo vector could target CD34 or CD90 for cell entry. CD90 is expressed by the subset of CD_{34}^{+} cells that are exclusively responsible for short- and long-term engraftment[16]. Here, the early enrichment strategy for CD_{34}^{+} HSCs containing the gene vector, either as an integrated element or S/MAR-containing plasmid, would rely on epitope base editing of the CD45 gene[17]. Epitope base editing would shield HSCs containing the therapeutic DNA from an antibody-drug conjugate (ADC) and be important for cancer therapy purposes[18–20]. A lentiviral vector would have ~8-10 kb of packaging space. A single integrating lentivirus[21], or a non-integrating lentivirus (NILV) with an S/MAR sequence[22], may suffice to briefly upregulate and target a clonally mutated gene, as well as base-edit CD45. A NILV that lacks an integrase gene would have a small amount more packaging space. Multiple lentiviral vectors could be employed if necessary - using a toxin-antitoxin or an antibiotic resistance approach. Base editing ribonucleoprotein complexes could also be transfected into the HSCs after purging. Regardless, however, an HDAd would have much more packaging space, i.e., ~36 kb. HSV-1 is another option, which would be able to package ~150 kb[23,24].

In vivo, mobilized HSCs could be targeted by the dual adenoviral vectors that can bind $CD34^{11}$. Here, CD45 base editing would also be effected. Display of a "self-peptide" based on CD47 on the adenoviral capsids[25,26] may preclude or reduce the need for innate immune suppression. Foamy viruses can also be intravenously administered, as they are resistant to human serum inactivation. However, like lentiviruses, they have less packaging space than adenoviruses (i.e., ~12-13 kb)[27].

The ex vivo vector or adenoviral genome-derived transgene cassette would encode zinc finger activators, transcription activator-like effector activators, or CRISPR-mediated transcriptional activation modules that upregulate the expression of the patient's clonally mutated genes. The "negative feedback" switches would also be expressed to ensure limited transcriptional upregulation in non-cancerous cells.

Either one would additionally encode molecular switches that can recognize the patient's clonally mutated mRNA sequences and respond via activation of a toxin module that eliminates the cell[7–9]. One example of a type of switch that could accomplish this is the reprogrammable ADAR sensor "RADAR", a system developed recently that can even detect certain point mutations in mRNA[28,29]. "Craspase", an RNA-guided protease, could be utilized as well. Here, the RNA cleavage capacity of Craspase should be abolished, using a "stay-on" variant[30]. Craspase could detect clonal point mutations in general, as less than 4 mismatches in the cognate target RNA 3' end precludes Craspase proteolytic activity[31]. If necessary, synthetic mismatches could perhaps be used to imbue point mutation specificity - as with "SHERLOCK"[32].

For clonal mutations in gene promoters, the altered sequence could be used as a means of selectively upregulating the downstream gene in cancer cells and downregulating it in normal cells. The difference in expression levels could be exploited for selective lysis of the blood cancer cells. For large (non-deletion) mutations in promoters at least, downregulation of the gene in normal cells may not be necessary. Clonal mutations in intergenic DNA could be recognized directly by DNA-binding proteins, and the signal could be amplified using a zymogen cascade.

When using a vector with more packaging space, a temporal promoter cascade[33] could be employed. Using this cascade, the vector could enact purging for a long period of time prior to CD45 base editing, helping to ensure that no cancer cells are CD45 epitope-edited prior to purging and somehow lose or silence however many copies of the therapeutic DNA vector they contain - in a manner independent of cell division. It is possible that synthetic genetic circuitry will be needed to account for cell division in the temporal purging process[34]. Assuming integration occurs rapidly enough, an integrating vector would account for potential cancer cell division after epitope editing but prior to purging, as well as ensure that as many healthy HSCs as possible are epitope-edited. Expression of the purging and CD45 base editing components could be made dependent on a small molecule, however, to allow time for integration. It may also be possible to automatically promote

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gene expression only after integration. A promoter specifically recognized by an encoded transcription factor could be generated via fusion of the 5′ end of the integrated construct and the host safe harbor locus DNA - wherein the TATA box and transcriptional start site are included in the vector DNA, but the upstream sequence formed from integration is recognized by a synthetic transcription factor. Similarly, an S/MAR-containing vector would initially have to become established, i.e., bind to a chromosome. Gene expression could be inhibited at first for an experimentally determined period of time to allow establishment to occur. A small, non-immunogenic protein could be transcribed upstream of the S/MAR, to ensure the vector DNA is not lost.

For the in vivo approach, protein engineering approaches could shelter transduced HSCs from the adaptive immune system[35,36]. Alternatively, MHC class I and natural killer cell inhibitor proteins could be expressed by the vector during this process[37]. Moreover, zinc finger base editors may be less immunogenic than TALE or CRISPR base editors[38,39]. The base editor module, or its protein component in the case of CRISPR, could be regulated by a recently developed, tetracycline-regulated RNA switch, as RNA elements would be non-immunogenic[40].

The genetic material required for purging and CD45 epitope editing should be removed or destroyed at the end of treatment. Along these lines, an S/MAR-containing plasmid could be lost via small molecule-inducible transcriptional inhibition of read-through into the S/MAR region[41]. Alternatively, it could be targeted at multiple locales by an inducible CRISPR/Cas9 module. An integrated vector could be removed via a recombinase[42] that is expressed or activated upon small molecule administration. Ideally, if the vector is to be integrated into the host genome, it would be integrated into a safe harbor locus and excised without a scar after treatment[43].

Blood Cancer Elimination Strategy

Base editing of the CD45 gene in the patient's HSCs would allow anti-CD45 chimeric antigen receptor (CAR) T-cells to be intravenously administered and eliminate blood cancer cells in the patient's body[44]. Autologous CAR T-cells should be purged as well, even for non-T–cell malignancies - in order to be maximally safe. (Lineage switching is possible.) Genetically edited allogeneic CAR T-cells, alone or combined with allogeneic OX40L–CAR-regulatory T-cells (Tregs), could at least eventually negate human leukocyte antigen (HLA)-mismatching issues - allowing for "off-the-shelf" immune cells[2,3,37,45]. Off-the-shelf cells from a healthy donor would preclude the issue of tumor cell contamination. The off-the-shelf immune cells could be eliminated after treatment and re-administered later if necessary[46]. The off-the-shelf immune cells should have an epitope-edited CD45 gene themselves to avoid fratricide (or simply cytotoxicity in the case of OX40L–CAR-Tregs). CD45 epitope editing would also allow for selection of CAR-transduced or transfected cells prior to in vitro expansion - when the off-the-shelf cells are initially generated through genetic alteration or if a new allogeneic donor is needed for HLA-matching or one or more other reasons. For acute myeloid leukemia, instead of CD45 epitope editing, a combination of FLT3, CD123, and KIT epitope editing can be exploited[47]. An ADC or immunotoxin targeting CD45 could be used instead of anti-CD45 CAR T-cells[48]. It was demonstrated that the base edited CD45 gene product for the ADC cross-referenced here[17] was more biophysically optimal than the one generated earlier for CAR T-cell targeting[44]. However, repeated injections may be required for ADCs and immunotoxins, as opposed to autologous CAR T-cells, which can persist in circulation for long periods of time.

Discussion

The purging strategy described in this piece could ensure that anti-CD45 immunotherapy involving base editing of patient HSCs can be employed to treat blood cancer without generating $CD34^+$ cancer cells resistant to the therapy.

If a patient has no clonal mutations, a small set of mutations that together are present in all of their cancer cells can be targeted. If so, both in vitro and in vivo purging and CD45 editing may require the use of an intracellular bacterium for packaging space reasons. A facultative intracellular

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bacterium would be best for facile production at high titer. Outside of growth media with a specific small molecule in it, the bacterium would only be able to replicate in host CD_{34}^{+} cells - and would be restrained by a quorum sensing molecule like AI-2[49].

A herpes simplex virus type 1 amplicon vector that is integrated[50] or contains an S/MAR sequence[51] could also be used here - or potentially a vaccinia virus that replicates up to a certain copy number (restrained via an encoded antisense RNA[52]). The vaccinia virus has 25 kb of packaging space[53]. However, I am uncertain if RNAs and proteins of the size required for treatment at least could diffuse out of and into the cytoplasmic vaccinia viral factories. Also, preventing xenophagy of the virus prior to mutation detection may be difficult. Doing so for intracellular bacteria may be easier, as they have essentially unlimited packaging space and thus can express a wide variety of deubiquitinating enzymes and other proteins that prevent xenophagy.

In the near future, multiplexed epitope editing of universal leukocyte markers (including CD45) could potentially be performed to avoid the survival of escape variants. Another way to multiplex would be to use a diphtheria toxin (DT) resistance marker, which would protect the transplanted HSCs from a DT-based immunotoxin[54,55].

Clonal mutation targeting could also be directly used for the treatment of blood cancers via an oncolytic or regular gene vector. All white blood cells could be targeted by using one or possibly more universal leukocyte markers as entry receptors for the vector. In healthy blood cells, the vector could replicate to low copy number and be secreted in a way that preserves cell viability. If it detects a clonal mutation, it would replicate excessively and lyse the cell or just lyse it. Vector replication or cytotoxic protein expression could even be induced automatically or via small molecule in cancer cells after waiting for a period time to allow for infection of all the blood cancer cells. This would be useful to reach blood cancer cells in lymphatic organs, such as the lymph nodes, i.e., by "hitching a ride" within healthy or cancerous, extravasating leukocytes.

Again, if the patient has no clonal mutations, a small set of mutations that together are present in all of their cancer cells could be targeted - and an intracellular bacterium may be required for this. A facultative intracellular bacterium with robust flagellar, extracellular movement may be ideal in this context. Outside of growth media with a specific small molecule in it, the bacterium would only be able to replicate in host CD_{45}^{+} cells, and would be limited by quorum sensing unless the set of subclonal mutations are detected and excessive replication is automatically induced immediately or after a waiting period - or is exogenously induced via small molecule after a waiting period. Alternatively, the vaccinia virus could potentially be harnessed here.

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