Genome Editing and Engineering

Prof. Utpal Bora

Department of Bioscience and Bioengineering

Indian Institute of Technology, Guwahati

Module - 10

Engineered immune cells for Cancer therapy (II)

Lecture - 03

Cancer therapy (II) -Part B

Welcome to my course on Genome Editing and Engineering. We are discussing about engineered immune cells for cancer therapy in this lecture 2 part B. Let us start with off-the-shelf T-cell products. For the generation of an off-the-shelf T cell product, it is necessary to use methods that, number one, increase histocompatibility between donor and recipient cells and prevent the rejection of T cells by the host. Number two, inhibit the alloreactivity of the product, so that the adoptively transferred T cell do not exert cytotoxicity against recipient cells.

Alloreactivity due to MHCs. The expression of MHCs on the surface of allogenic T-cells causes their immediate rejection by the host immune system. The MHC1 presents peptides on most of the nucleated cells which are recognized by cytotoxic CD8 plus T-cells. The MHC class II presented peptides on only antigen presenting cells recognized by CD4 plus T-cells leading to activation and differentiation from effector cells.

T cells recognize non-self allogeneic MHC molecules resulting in activation and tissue rejection known as alloreactivity. Direct alloreactivity refers to intact, mismatched MHC expressed on allogenic cells and could be recognized with both naive and memory T cells. While indirect alloreactivity refers to polymorphic peptides derived from allogeneic MHC molecules and presented in the antigen binding group of self-MHC molecules. Alloreactivity due to endogenous TCRs. The endogenous TCRs of allogenic T cells may recognize the recipient's antigens as alloantigen, leading to GVHD.

In the TCR engineered T cells, the assembly of endogenous TCR alpha or beta chains with their transgenic counterparts may lead to the formation of mixed TCR complexes which might be self or alloreactive. One strategy to overcome these challenges is the elimination of endogenous TCR expression which leaves the control of T cell activation and proliferation to the transgenic TCR or CAR. For elimination, MHC and endogenous TCR expression genome editing has been attempted to avoid the allogenic rejection of the transferred T cells. Antitumor and alloreactive receptors expressed on chimeric antigen receptor and T cell receptor-engineered T cells. We can see three possible TCR complexes on TCR engineered T cells.

Number one, the endogenous TCR may exert alloreactive function. Number two, the transgenic (Tg) TCR containing transgenic alpha and beta TCR subunits is responsible for the anti-tumor activity. Number three, it is also possible that the combination of TG and endogenous subunits form a mixed TCR containing that results in potentially undesired function. Or we may have B, the CAR expression in these cells is responsible for anti-tumor activity providing the first and second activation signals through CD3-Zeta and co-stimulatory endodomains CD28 or 4,1BB respectively. However, CAR T cells also express the endogenous TCR complex that may react to the alloantigens.

Genome editing to produce off-the-shelf T cell products. We can use various platform technologies as ZFN, TELEN and CRISPR-Cas9 for production of the self T cell product. Genome editing technologies of various kinds can be applied to knock out the TRAC (T cell receptor alpha constant) and TIBC (T cell receptor beta constant) loci to prevent TCA expression and alloreactivity. The knockout of the genes such as B2M (beta-2-microglobulin), TAP1 (transfer associated with antigen processing 1), TAP2 (transporter associated with antigen processing 1), tapasin (TAP-associated glycoprotein) involved in mesohistocompatibility complex 1 and 2 expressions can provide T cells with universal histocompatibility. Apart from disrupting the endogenous TCR and MHC class 1 genes through the NHEJ pathway in genome-mediating, the strategy of HDR-based knock-in is also applied for T cell product generation.

Installation of transgenic TCR or CAR-T cells through HDR-mediated knock-in potential of CRISPR-Cas9. TCR and CAR can be inserted in between a locus of interest. For

instance, the introduction of TCR or CAR among tract locus in order to simultaneously knock out of endogenous TCR and establishment of recombinant TCR or CAR leads to homogeneous expression of a cassette and also the elimination of mutation probability which is common among vector integration methods. Universal CAR T-cells are now being manufactured by knocking out TCRN HLA-1 in allogenic T-cells.

Torica et al. developed specific universal T-cells for tumor-related antigens from a donor to meet the need for patient-specific T-cells to transmit them to multiple receptors. This was performed by ZFN gene editing technique in CD19 specific CAR T cells to eliminate the expression of endogenous TCRs for the prevention of GVHD. Poirot et al were the first to knock out endogenous TCR by TALEN-mediated disruption of T cell receptor alpha constant chain in lentiviral transduced CD19 CAR T cells. This platform was further entered into clinical studies on two infants with relapsed or refractory CD19 B-cell acute lymphoblastic leukemia.

Equim et al. have exploited CRISPR-Cas9 to knock out the TCR locus and insert CD19-specific CAR in this locus concurrently by designing a guide RNA that targets the 5' end of the first track axon. They observed a regular curve expression in T cells, increased potency of T cells and decreased terminal differentiation and exhaustion in the mouse model of acute myeloid leukemia. This figure presents the summary of CRISPR-Cas9 editing strategies to generate optimally potent and widely available CAR T cell products. In here we can see CRISPR-9 editing can be used to develop allogenic CAR T-cell therapies which will ameliorate many of the current issues associated with autologous CAR T-cell products like host compatibility, FDA approved and cell quantity and quality concerns, time consuming and costly manufacturing process, the latter two has to be addressed. And there has to be unlimited pool of healthy donors.

Reduced cost in manufacturing timeline will ease patient disease. GVHD and rejection should not be there. And healthy donor allogeneic T cells will address using CRISPR-Cas9 technology. B, removal of the endogenous TCA by targeting tract via CAR T genes. Knock-in addresses histocompatibility barriers associated with third-party cell products derived from unrelated donors.

You can see here CRISPR-Cas9-mediated site-specific knock-in of CAR constructs. CAR directed to track locus. There is non-random integration unlike traditional antiviruses. Uniform CAR expression at tract locus with endogenous promoter. No donor TCA-induced alloreactivity and low level of tonic signaling.

Enhancing CAR-T cell function by genome editing. Immunosuppressive tumor microenvironment and T cell exhaustion due to role of co-inhibitory molecule such as PD-1, CTLA-4, lymphocyte antigen, LAC-3, and TIM-3 are responsible for dysfunction of T cells. Genome editing tools have been applied to disrupt these inhibitory genes to enhance a CAR-T cell function. CRISPR-Cas9-mediated PD-1 depletion was proven to augment the ability of CAR-T cells in killing tumor cells in vitro and clearing of PD-L1c tumor xenografts in vivo.

In addition to co-inhibitory genes, diacylglycerol kinase ablation in CAR T cells resulting in improvement of anti-tumor immunity has been reported. Knocking out granulocyte macrophage colony stimulating factor gene was demonstrated to enhance CAR T cell functions as well as reduce the risk of cytokine release syndrome and inflammation. Studies have also confirmed that knocking down the endogenous TGF-B receptor 2 in CAR T cells with CRISPR-Cas9 technology could decrease the exertion of CAR T cells and increase solid tumor killing efficacy both in vitro and in vivo. Representative targets of CAR T cells engineered by CRISPR-Cas9 system. So, we can see here the various genes like Lactree PD-1, DGK, ZM-CSF, TGF-BR2 and the target cells like anti-CD19 CAR T cells, anti-CD19 CAR T cells, anti-EGFR V3 CAR T cells, anti-CD19 CAR T cells, anti-mesothelin CAR T cells respectively.

And the cancer cell lines corresponding to each target gene are listed like RAGI in case of LAG-3, K562 in PD-1, U87MG glioblastoma cell line for DGK. NAL-M6, GM-CSF, then CRL-5826 for TGF-BR2. And in all these cases, mostly CRISPR-Cas9 is used and for the case of PD-1-DGK, CRISPR-Cas9 RNP has been deployed. Mostly electroporation is the method of delivery except in the case of GM-CSF where lentiviral vectors has been used. And you can see the results corresponding to every delivery method and the target genes and target cells.

In the first case, LAG-3 knockout CAR T cells display comparable effective functions of two standard CAR T cells. In the second case, PD-1 disruption augmented anti-tumor ability of CAR T cells. In the third case, DGK knockout rendered CAR T cells resistant to soluble immunosuppressive factors. In the fourth case, ZM-CSF knockout CAR T cells exhibited decreased expression of GM-CSF with normal function and enhanced anti-tumor activity. In the last case, TGF-BR2 edited CAR T cells had better in vivo elimination of tumor cells with an increased proportion of memory T cell substrates.

Loss of function screening. Loss of function screening is another application of CRISPR in which effect of T cell function on cancer immunotherapy in presence or absence of a gene is investigated and the results may be used for development of further immunotherapeutic approaches. By adopting such study by Seyfried et al, it was revealed that the knockout of some T cell regulatory proteins such as SOCS1, TCEB2, RASA2 and CBLB improves both proliferation and anti-cancer capability of modified T cells. To elucidate immunomodulatory impacts of combinatorial role of anti-cancer on cytotoxic properties of effector T cells and to explore the mechanism of CAR T cell cytotoxicity, a detailed in vitro loss of function screening by means of CRISPR was conducted recently.

And this figure is obtained from Miri 2020. CRISPR-cas9 mediated cytokine modulation. This modulate cytokine signaling via genome editing during CAR T cell activation and expansion have the potential to bolster anti-tumor activity and enhance T cell persistence and or reduced toxicity. CRISPR-cas9-based gene editing combined with viral or non-viral DNA delivery permits simultaneous biallelic or sequential gene targeting to engineer T-cells with expression cassettes in a site-specific manner. Cytokine-encoding DNA cassettes can be knocked into targeted genomic loci, placing these genes under the control of specific promoters for temporal control of expression.

For example, IL-15 has been knocked into the IL-13 gene locus, thus placing IL-15 expression under control of the endogenous IL-13 promoter as IL-13 is highly active upon T cell activation. This creates an inducible T cell specific IL-15 activation switch. IL-15 act as an immunomodulatory in cancer treatment by inducing the differentiation and proliferation of T, B and natural Achilla cells. Some genes encoding cytokines such as GM-CSF and IL-6 drive neurotoxicity and cytokine release syndrome.

Knocking out of these genes with CRISPR-Cas9 editing has the potential to produce an optimally potent and durably persistent cell product while reducing adverse events associated with aberrant cytokine production. GM-CSF knockout CAR-T cells maintain normal functions and increased antitumor activity in vivo and potentiate improved overall survival compared to conventional CD-19 CAR-T cells. Genetic knockdown or ablation of the IL-6 gene also has the potential to ameliorate CRS-like toxicity in leukaemia-bearing mice. Clinical study on GM-CSF-KO by CRISPR-Cas9. In a clinical study of one patient with non-Hodgkin lymphoma and two patients with multiple myeloma treated with CAR-T, AIL-6 IL-1 RA with GM-CSF TCR KO along with GM-CSF IL-6 and IL-1 which were responsible for neurotoxicity and cytokine release syndrome in CAR T cell therapy was targeted together with CRISPR Cas9.

A novel platform was developed to reduce cytokine toxicity consisting of CRISPR-edited KO of GM-CSF and autonomous co-expression of IL-6 and IL-1 blockers in CART, referred as CAR-T aIL-6 IL-1 array with GM-CSF-TCR-KO. A single guide RNA sequence was designed for targeting the PAM motifs located in the first region of GM-CSF gene. In A, we can see the scheme of CRISPR-edited GM-CSF-KO cart cell secreting IL-6 and IL-1 blockers. Patient derived T-cells were engineered with CRISPR-Cas9 RNP for GM-CSF-TCR-KO and transduced with lentivector encoding second generation 41-BB-Zeta CAR and anti-IL-6-SGFV and IL-1 array for blocking IL-6 oblique IL-1 signaling. In B, we can see the constructive lentifactors for co-expressing CAR and cytokine blockers.

Multiplexed CRISPR-Cas9 in CAR-T cell therapy. Multiplexed CRISPR-Cas9 ethylene can be used to enhance the anti-tumor efficacy and improve the safety of autologous or allogenic CAR T cell products. CRISPR-Cas9-mediated precision editing of clonal master iPSCs, has the potential to generate a renewable cell source that can be repeatedly used to mass produce homogeneous, optimally potent, best-in-class, universal CAR-T cell products in a cost-effective manner. Successful multiplex CRISPR-Cas9 editing of CAR-T cells has been done with targeting inhibitory genes and those encoding death receptors such as CD95-FAS to prevent TME-mediated inhibition of apoptosis. Track PDCD1 and B2M triple knockout CAR T cells have demonstrated robust anti-tumor

function in in-vitro and in-vivo models incorporating CD19 and prostate stem cell antigen-directed CARs.

The first in-human trial of multiplex CRISPR-Cas9-edited T-cells with a transgenic TCR specific for tumor-associated antigen NY-ESO1 in patients with myeloma and sarcoma was carried out. Transfer of gene-edited TCR-engineered T-cells into patients resulted in durable engulfment with edits at all three genomic loci. Gene editing for universal CAR T-cells. Gene editing has been attempted to overcome allo-recognition, which otherwise limits allogenic T cell therapies. Previously, T cells modified using Talen and expressing chimeric antigen receptor against CD19 have been used to treat refractory relapsed B-cell acute lymphoblastic leukemia in infants.

CRISPR RNA electroporation to disrupt adenogenesis TCRN B2 microglobin genes for disruption of major histocompatibility complex class I in T cells was transduced with a lentiviral CAR vector, but editing and transiting effects were unlinked in a previous study. Georgiasis et al developed an initial proof of concept of for generation of universal T cells expressing chimeric antigen receptors against CD19 target antigens combined with transient expression of DNA targeting nucleases to disrupt the T cell receptor alpha constant chain as reported by Geodiasis in 2018. A self-inactivating SYN lentiviral platform that coupled transgene expression with CRISPR editing effects for efficient and homogeneous T cell modification was designed. Incorporation of a Pol-3 promoter and single-guide RNA sequence into the 30LTR of a U3-deleted third-generation lentiviral vector generated a self-duplicating CRISPR expression cassette.

A region immediately proximal to 30 repeat regions was selected to preserve reverse transcription mediated duplication to the 50LTR, resulting in a provial form with both 50 and 30 flanking terminal CRISPR elements. For targeting of endogenous TCR expression an sgRNA sequence targeting, the TRAC locus was placed under the control of the human Pol III promoter, U6, followed by a sgRNA sequence specific for *S. pyogenes* Cas9, which was delivered separately as mRNA by electroporation. The lentiviral vector encoded a chimeric antigen receptor under the control of an internal human phosphoglycerate kinase promoter. Terminal CRISPR-CAR coupled interval vector configuration.

Schematic of CAR-19 expression for the targeted killing of CD19 plus B cells with simultaneous CRISPR mediated TCR disruption, where ideally all T cells are CAR-19 plus and TCR are shown in figure A. In figure B, we can see the self-inactivating lentiviral plasmid configuration, coupling terminal track, CRISPR guide RNA and CAR-19 transgene expression, and a human Pol III promoter as the RNA CRISPR, cited in the deleted unique region, proximal to repeat elements of the three prime long-terminal This figure is obtained from Geodesis et al. 2018 and for full details, kindly refer to the reference cited at the end of this lecture. Following reverse transcription as shown in Figure C, 3' LTR elements including Pol III sgRNA cassette duplicate to the 5' LTR of the proviral vector. In Figure D, we can see the single guide RNA expressed from both 5' and 3' LTR vector elements forms ribo-nucleoprotein complexes with Cas9 following electroporation of Cas9 mRNA and this effect is restricted to the transduit populations only.

Following reverse transcription and duplication of the hybrid DU3-sgRNA delivery of Cas9 mRNA resulted in targeted TRAC locus cleavage and allowed the enrichment of highly homogeneous CAR plus TCR populations by automated magnetic separation. Molecule analysis including NGS, WGS, and Digenome-seq verified on target specificity with no evidence of predicted off-target events. Robust anti-leukomic effects were demonstrated in humanized immunodeficient mice and were sustained longer than by conventional CAR plus TCA plus T cells. Terminal track (TT) CAR T cells offered a possibility of a pre-manufactured non-HLA-meshed CAR cell therapy.

Challenges of using CRISPR-Cas9 in immunotherapy. In spite of its tall premises, CRISPR-Cas9 system, many safety and efficacy issues impede its translation to clinical applications. Most of the CRISPR-engineered T cells for clinical trials were transduced by electroporation, which might result in cell damage and impeding T cell proliferation via ex vivo, which is not desirable as ACT-based cancer immunotherapies are dependent on adequate T cells for refusion. Therefore, more safe and efficient delivery ways such as viral vectors or alternatively direct delivery in situ or in vivo should be explored. The immunogenicity of Cas9 proteins may be another challenge that constrain the clinical translation of CRISPR Cas9 system.

Anti-SaCas9 (Cas9 from *S. aureus*) and anti-SpCas9 (Cas9 from *S. pyogenes*) antibodies were detected in 78% and 58% of donors respectively. Moreover, 78% and 67% of donors possess T cells against Sa-Cas9 and Sp-Cas9 protein respectively. It showed that there were pre-existing adaptive immune responses to Cas9 proteins in human which may cause adverse effects when treating patients with CRISPR-Cas9 system. However, no potential rejections were noted in clinical trials.

Some research suggests that the pre-existing immune responses to Cas9 proteins do not appear to be an obstacle to clinical application of CRISPR-Cas9 system. The risk of off-target effects resulting from non-specific cutting and further leading to unwanted mutations remain a major obstacle to translation of CRISPR-Cas9 system to clinical therapeutics use.

The status of clinical trials. Several anti-CD19 CAR T cell constructs have been investigated and responses differ extensively among various studies in patients with hematological malignancies, especially refractory or relapsed acute lymphocytic leukaemia. In 2017, the Food and Drug Administration granted regular approval of axicaptagene ciloleucal or Yescarta as a therapeutic option to treat pediatric/young adult B lymphoblastic leukaemia and adult diffuse large B cell lymphoma. Yescarta is a CD19-specific cortisol mainly exploited for the treatment of adult patients with relapsed or refractory large BCL following two or more lines of systematic treatment.

In July 2020, Tecartus was approved for the treatment of adult patients with mantle cell lymphoma. In February 2021, Breyanzi, the fourth CAR T-cell therapy for adults with relapsed and refractory large B-cell lymphoma was approved by US FDA. CAR T-cell therapy is more restricted in solid tumors than in hematological malignancies as CAR T-cells are circulated to the bloodstream and lymphatic system and thereby have more interaction with blood tumor cells. The first clinical trial testing, the safety and feasibility of CRISPR-Cas9-indicated TCR T-cells in patients with refractory cancer was reported in 2020. The safety and efficacy of CRISPR-Cas9-indicated universal CAR T-cells in vivo needs to be further tested in clinical studies.

Currently, 8 relevant clinical trials are growing on as shown in this table. The clinical trials based on the use of CRISPR-Cas9 technology. To provide more effective universal CAR T cells registered in the clinical trials.gov as of June 2021 are all according to the conditions NHL, solid tumor, BCL, solid tumor and BCL and the CAR use is CAR-CD19 in the first two cases and the fourth case, as well as 20, 22 in the fourth case and also the sixth case.

For case of solid tumors, mesothelin is being deployed. So, the target locus is accordingly listed against the condition and you can see the various phases they are in. In the case of BCL2, it has gone to phase 2 and the location of the various clinical trials mostly in China except USA for the second case and the participant numbers are ranging from around 10 to 80 in case of various conditions and you can see the various NCT number as listed against the trials. So, these are some of the references which you may consult or go through for getting more details of the various concepts and diagrams that has been presented here in this particular lecture. Thank you for your patient hearing.