TCR-modified cells

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Abstract

Background

With its roots in principles of basic immunology, synthetic biology and genetic engineering, the field of adoptive cell transfer (ACT) has quickly become one of the most promising and innovative approaches to treat cancer, viral infections and other immune-modulated diseases.

There are currently three main types of ACT using effector cells[1]: administration of tumour infiltrating lymphocytes (TILs), and gene transfer-based strategies relying on T-cell manipulation for expression of either chimeric antigen (Ag) receptors (CARs) - composed of antibody (Ab)-binding domains fused to T-cell signalling domains - or engineered T-cell receptor (TCR) α/β heterodimers.

Genetic modification of autologous T cells to target specific tumour antigens has been developed to overcome the consequences of immune tolerance and offers the possibility to endow the immune system with reactivities that are not naturally present. This approach has the additional benefit of rapid tumour eradication, which is usually observed with cytotoxic chemotherapy or with targeted therapies, and it contrasts to the delayed effects that are usually observed with vaccines and T-cell checkpoint therapies.

Lasting anticancer responses have been extensively reported for CARs targeting CD19 on chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and B-cell lymphoma[2], and the US Food and Drug Administration (FDA) has recently approved two genetically engineered CAR T-cell products, tisagenlecleucel (Kymriah)[3] and axicabtagene ciloleucel (Yescarta)[4], for clinical application. Although not having yet provided such a dramatic evidence of effectiveness, therapeutic TCR gene-modified T cells have also shown clinical activity and significantly reduce tumour burden[2]. Furthermore, they feature particularities that render them a more suitable approach for specific types of malignancies, including solid tumours.

This review will focus on the main characteristics of TCR gene-modified cells, their potential clinical application and promise to the field of ACT, basic manufacturing procedures and characterisation protocols, and overall challenges that need to be overcome so that redirection of TCR specificity may be successfully translated into clinical practice.

TCR structure and signalling

The TCR is a heterodimeric protein, typically consisting of an alpha (α) and a beta (β) chain, expressed on the cell surface as part of a complex with CD3 molecules. A minority of T cells can express an alternate receptor formed by gamma (γ) and delta (δ) chains (γδ T cells). TCR activation depends on the binding to a processed intracellular peptide presented by a major histocompatibility complex (MHC) molecule (the peptide-MHC antigen) on the target cells, followed by proper signal initiation and amplification, processes that involve an array of cell surface molecules.

Each αβ subunit contains variable (V) and constant (C) regions, with the latter being followed by a transmembrane region. Each V domain contains three loops which interact with the peptide-MHC antigen[5]. The αβ heterodimer lacks its own intracellular signalling domains. Therefore, it must associate with the six-subunit CD3 complex, which contains a total of ten immunoreceptor tyrosine-rich activation motifs (ITAMs) that are responsible for signal transduction[5]. Upon TCR binding to the peptide-MHC antigen, T-cell activation involves multiple other cell surface molecules that collectively contribute to initiate and amplify the signal.

TCR engagement is necessary but not sufficient for complete T-cell activation and function (i.e. proliferation, differentiation, persistence and cytokine release) and a second signal is required, which is provided by co-stimulatory molecules, such as CD28[6]. These molecules promote T-cell activation and survival through various signalling pathways involving additional protein kinases and anti-apoptotic factors.

T cells can be activated *in vitro* by binding of the TCR to as few as one peptide-MHC molecule[7–9], a feature that is partly accomplished by the action of CD4 and CD8 co-receptor molecules. CD4 is associated with T helper (Th) and regulatory (Treg) cells and recognition of class II MHC. CD8, on the other hand, is associated with cytotoxic T lymphocytes (CTLs) and recognition of class I MHC. The synergy of these co-receptors with the TCR is thought to be driven both by their ability to bind to invariant regions of MHC molecules and by the fact that, because their cytoplasmic tails are associated with particular protein kinases involved in ITAM-mediated signal transduction (e.g. Lck), these are brought in close proximity of the TCR/CD3 complex[10].

The ultra-sensitivity of the TCR system is what underlies its potential for targeting very low levels of intracellular antigens. Additionally, the ability to recognise almost any intracellular protein *via* the MHC system allows TCRs to target more antigens than can antibodies (or scFv-CARs), which recognise only cell surface antigens.

TCRs *vs* CARs

As mentioned above, TCRs recognise processed peptides presented by MHC molecules. In practical terms, this means that target antigens can be derived from the entire protein composition of tumour cells, including intracellular proteins, and also from a number of non-surface antigens of virally infected cells and tumours associated with viral infection, such as hepatitis-associated hepatocellular carcinoma, papilloma virus-associated cervical cancer, and Epstein-Barr virus-related malignancies[11]. This is in contrast to CARs, whose potential targets are cell surface antigens expressed at higher densities on cancer cells, densities which are highly dependent upon the specific target[5]. Additionally, a single peptide-MHC molecule has been shown to be sufficient to elicit T-cell activation, whereas previously published studies investigating the density of CD20 required to activate T cells expressing a CD20-specific CAR showed that a minimum of 200 molecules/cell were required in order for cytokine release to be achieved[12,13].

Another fundamental advantage of TCRs over CARs is their ability to target cancer mutagenomes, which is of particular importance in the context of solid tumours. Although patient T cells are tolerant to peptides derived from self-proteins, point mutations in tumour cells resulting in single amino acid changes can elicit robust T-cell responses. There are two mechanisms whereby point mutations can generate immunogenic epitopes to which patient T cells are not tolerant[14]. First, mutations may generate novel TCR contact residues thereby producing immunogenic neoepitopes or, alternatively, they may create novel HLA-binding residues resulting in the presentation of peptides in tumour cells that are absent in normal tissues. Because of recognition of linear peptide sequences, TCRs can potentially target the mutational landscape associated with cancer development. In contrast to TCRs, point mutations largely escape antibody recognition and thus CAR targeting because the vast majority of mutated proteins are intracellular and because antibodies are less effective in the specific recognition of point mutations in otherwise unaltered self-proteins[14].

Clinical application of TCR gene-modified cells

Overviews have been previously published on TCR gene therapy clinical trials, which, to date, have been mostly limited to MHC-I-restricted candidates, more specifically, HLA-A*0201, which is found in approximately 45% of Caucasian people[15–17]. A summary of these trials is provided in Table 1.

In 2006, Rosenberg *et al* reported for the first time that metastatic melanoma patients treated with lymphocytes genetically engineered to express a TCR specific for a melanocyte-differentiating antigen (MART-1), featured long-term persistence of infused cells and tumour regression in 2 of 17 patients[18]. Subsequent studies further demonstrated that TCR gene-modified T cells were generally safe, well tolerated, and had the potential to be effective therapeutically in cancer patients[19,20], whilst also highlighting the potential for adverse events[20].

Since these early studies, identification of TCR genes encoded to recognise epitopes expressed by human tumours and improvements in TCR gene transfer technology have allowed for normal T-cell antigen specificity redirection and targeting of a variety of antigens and malignancies, including gp100,

p53, carcinoembryonic antigen (CEA), cancer–testis antigen (CTA) family members (e.g. NY-ESO-1, MAGE-A3 and MAGE-A4), and viral protein family members[11,21,22].
Importantly, all completed and ongoing TCR gene therapy trials target tumour-associated antigens that are also expressed to various extents in normal tissues. This physiological expression of TCR-targeted antigens poses the risk of "on-target" immune pathology, such as that observed with TCR-targeting of MART-1, gp100 and CAE (Table 1), which is further discussed below. To better identify potential target antigens, *in silico* proteome searches may be performed, which analyse target peptides for structural uniqueness[23,24]. This type of analysis could potentially identify "off-target/off-tumour" toxicities associated with self-peptide/MHC antigens that could pose problems with cross-reactivity[5].

Manufacturing procedures

We are currently manufacturing TCR engineered T cells to GMP using a retroviral vector that specifically incorporates a number of safety features for clinical application. Retroviral vectors were the first viral vectors used for clinical application and are still used as gene-transfer vehicles in about 20% of the current clinical trials[25]. The wide usage of retroviral vectors is due to their broad cell tropism, efficient integration and stable gene expression in target cells. In addition, they can be consistently manufactured at relatively low cost.

Retroviral vector design

As a heterodimer, TCRs differ from CARs in that two chains need to be expressed rather than just one to redirect specificity. This results in larger constructs and potentially non-uniform chain expression[11]. The presence of endogenous TCRs also allows for the opportunity of chain mispairing between endogenous and introduced alpha and beta chains, with a TCR-transduced T-cell therefore having the potential to express four distinct TCRs, only one of which is desired. Because these interactions may reduce the level of expression of the introduced TCR and lead to novel, unpredictable and potentially dangerous target specificities, it is important that vector design aims at maximising the level of introduced TCR expression whilst preventing any potential serious adverse events.
A schematic representation of the retroviral vector construct is shown in Figure 1. To help promote efficient translation and surface assembly of the introduced receptor without altering the TCR sequence itself[11], TCR alpha and beta chains were codon optimised and linked *via* an internal self-cleaving porcine teschovirus 2A sequence. A leader sequence (LS) was also incorporated, from which all "start" codons were removed, thereby decreasing the risk of possible protein/peptide production and reduce the likelihood of homologous recombination with endogenous retroviral sequences[26]. To enhance gene expression and minimise mispairing with endogenous TCR chains, an additional cysteine residue[27] was engineered into each one of the constant regions.
Furthermore, because human-murine hybrid TCRs have been described not only to preferentially pair and effectively compete for human CD3 molecules but also to mediate higher levels of cytokine secretion *in vitro*[28,31] when introduced into primary human T cells[28–30], both alpha and beta constant regions were replaced with murine sequences.

Transduction protocol and in process controls

Manufacture of T cells genetically engineered to express a specific TCR is initiated from density gradient-purified peripheral blood mononuclear cells (PBMCs). T-cell activation is first carried out in gas-permeable cell bags using clinical grade microbeads conjugated with anti-CD3 and anti-CD28 antibodies for 48 hours. One round of transduction is then performed with the retroviral vector in bags pre-coated with RetroNectin, which promotes co-localization of the retroviral vector with the target cells to enhance transduction efficiency. Activated cells are exposed to the viral supernatant for up to 72 hours, after which activating beads are removed, and the cells washed and resuspended in cell culture medium for an overnight incubation. At the end of the 6-day production run the cells are cryopreserved for later administration after QC release. Purification of TCR-transduced T cells is not

performed, and bulk TCR transduced cells are therefore administered to the patients. This is because this procedure aims at keeping the *in vitro* manipulation and culture period to a minimum in order to preserve maximum T-cell function.
This semi-closed, small-scale manufacturing platform takes a maximum of 6 days, successfully supports an ongoing clinical trial (NCT02988258) and can be easily adapted for other clinical trials involving the transduction and expansion of either autologous or donor T cells.
All donors are screened for infectious disease markers as per 2006/17/EC, and the cells are monitored throughout the procedure to collate information on sterility, viability, and cell count. Appropriate bead removal is confirmed by microscopy. A sample of the final TCR-transduced product is withheld to test for replication-competent retrovirus (RCR) in case of reported related serious adverse events.

Release testing of the product
An appropriate set of practical and scientifically defendable release criteria is essential to guarantee the products' integrity, consistency and efficacy. The underlying principle for release criteria is to provide adequate testing to ensure the product's identity (which, in the case of T-cell products, is commonly assessed by flow cytometry analysis), purity (i.e. absence of microbeads and non-T cells or other contaminants), safety (i.e. no bacterial/fungal and/or mycoplasma contamination and lack of RCR and endotoxin) and potency (which, in the case of genetically-modified products, may simply correspond to a minimum viability and transduction efficiency for products in early stage trials)[25].
Release testing of our product includes assessment of sterility (no bacterial contamination assessed by Bactec automated-based methods and Gram stain), viability (>80%), cell count (dose-dependent) and evaluation of transduction efficiency (5-70%).
To determine the percentage of T cells effectively expressing the introduced TCR, transduced cells are stained with antibodies against CD3, CD8, CD4 and the Vβ chain of interest (i.e. the Vβ chain included in the retroviral vector construct). However, the introduced Vβ chain may also be used by some endogenous TCRs that are not specific for the intended target. To determine the percentage of T cells expressing endogenous Vβ, non-transduced T cells (which are kept as negative controls alongside the transduced ones throughout manufacture but are not exposed to the retroviral vector) are also stained with antibodies against CD3, CD8, CD4 and Vβ. Transduction efficiency is obtained by subtracting the percentage of Vβ-expressing cells in the negative control from the overall frequency of Vβ+ cells in the transduced population.
Staining with anti-murine constant beta (mCb) chain antibodies and/or HLA-A*0201/peptide tetramers or multimers, which identify only the T cells expressing the introduced TCR, may also be used to assess transduction efficiency. However, tetramer staining is based on low affinity interactions that might be susceptible to small changes in the density of the TCR ligand and, as such, it is common for TCR-transduced T cells to bind tetramer poorly immediately after transduction, despite displaying antigen-specific effector function on stimulation with cognate antigen. This may be because T-cell activation involves multiple receptor/ligand interactions, including ligation of the TCR, CD8 co-receptor, co-stimulatory molecules such as CD28 and accessory molecules, which may render this activation pathway less susceptible to small reductions in the amounts of TCR expressed by the responding T cells.
Release of the cell product for infusion is handled through the issuance of a certificate of analysis (CoA) summarising the characteristics of the product and the tests performed. The CoA also details the release specifications and results of each test, including the method used and acceptable range of results[25].

<u>The importance of potency assays</u>
According to the IHC guideline 6QB, potency is the quantitative measure of biological activity using a suitable quantitative biological assay (also called potency assay or bioassay), based on the characteristic of the product which is linked to its relevant biological properties[32]. It constitutes a quality attribute for any biological product, and the implementation of relevant assays is often at the

centre of many challenges and discussions amongst developers and regulators throughout product development. Potency assessment is important, not only as for manufacturing as a tool to assess product quality and consistency, but also for clinical development, as it helps predict the product's clinical efficacy by creating a link to the dose.
A review on potency assay development for cellular therapy products has been previously published[33]. For cell-based immunotherapy products[34,35], including those containing genetically modified cells[36], the development of adequate potency assays may be complicated by multi-antigen formulations and the inherent variability of the starting material; therefore, a combination of methods may be advisable for appropriate functional characterisation. To estimate the potency of transduced cells, biological tests should be applied to determine the functional properties achieved by the genetic modification. Potency can be expressed as a combination of several parameters including the number of genetically modified cells, gene copy number, expression level of the transgene and the product activity level, as shown to be efficacious in clinical studies[36].
One of the requirements included in Directive 2003/63/EC (Annex I, part IV) is that human somatic cell therapy medicinal products are made of a defined number of viable cells. Cell viability is, therefore, an important parameter of product integrity and may be used as an in-process control after manipulation of certain cell characteristics[35]. Cell viability may also be an important element of the potency of cell-based products, but it should be linked to other measures of potency that demonstrate the potential for biological activity of the product, such as quantitative antigen expression or biological activity as measured in the bioassay[35].
For genetically modified cells, the presence and expression of the transgene allows for more targeted potency assay development than unmodified cells, for which potency evaluatio strategies can rely on a large variety of markers and biomolecules.
In vitro assays allow the measurement of biochemical/physiological responses at the cellular level. Such assays are generally suitable as a direct measure of the biological activity for characterisation when they correlate with the intended therapeutic effect. Measurable biological activities are, for example, *in vitro* lysis of target cells by tumour-specific (CD8) T cells, *in vitro* cytokine production by specific cells and co-stimulatory capacity of DCs. Indirect measures of potency can also be used, provided that a correlation between the surrogate and the defined biological activity has been demonstrated (eg: determination of cell surface markers, activation markers, secretion of factors, expression of a single gene product or protein expression patterns)[35].

Alternative manufacturing procedures

Development and GMP manufacture of TCR-engineered cells are highly dependent on the type of product required, the most suitable method of gene transfer, and the overall intended scale of the procedure. The sections below describe some alternative approaches to the design and production of TCR-modified cells.

Vector systems

Amongst the different types of gene vector systems available, retroviral and lentiviral vectors have become state-of-the-art technology for lymphocyte gene transfer.
Lentiviral vectors share some similarities with their retroviral counterparts, such as mediation of efficient gene transfer, high level of transgene expression and broad tropism, whilst introducing a number of advantageous characteristics like the ability to transduce non-dividing cells and safer chromosome integration profile[25], and have also been successfully used to engineer hematopoietic stem cells for the treatment of a number of conditions[37–40].
The sleeping beauty (SB) transposon/transposase system is a relatively new technology in the gene therapy field. This is a double plasmid-based methodology, where one plasmid is the transposon encoding the gene of interest (e.g. CAR or TCR), and the second plasmid expresses the transposase that enables the insertion of the transgene into TA dinucleotide repeats. Plasmids are introduced into

T cells by electroporation, and transfected cells subsequently expanded on artificial antigen-presenting cells[41,42]. Advantages of using the SB system include the increased simplicity of clinical-grade plasmid manufacture and the cost effectiveness due to lesser safety testing requirements when compared to cell products genetically modified with retroviral or lentiviral vectors[25].

Construct design

As discussed, the ability to redirect T cells to recognise a specific antigen is not enough to ensure an effective immunotherapy, and therefore antigen recognition needs to be coupled with efforts to ensure engineered T-cell expression and function towards the desired the target whilst limiting off-target or off-tumour recognition. T cells should also be able to persist long-term and be able to traffic to and accumulate at the target site. Additionally, optimal modified T cells should exhibit robust, multi-functional immune responses, resist mechanism of anergy, exhaustion and immunosuppression, and be susceptible to deletion on demand to diminish potential toxicity issues[11].

The following sections describe some additional specific modifications that can be introduced in vector constructs to try and maximise engineered T-cell effectiveness.

Engineered TCR expression, affinity and function. Affinity and expression levels of therapeutic TCRs are two key parameters that determine how much antigen is needed for the triggering of T-cell function. Besides the already discussed codon optimization, introduction of an additional disulphide bond between the TCR chains, and the introduction of murine residues into the constant region domains, several other engineering strategies may be employed to prevent mispairing and further enhance both the level of TCR expression on the T-cell surface and antigen-specific effector functions. These include 1) provision of additional CD3 molecules[43], 2) addition of leucine zippers at the end of intracellular tails[44], 3) altering of TCR glycosylation[45], and 4) substitution of particular TCR residues[46]. Extensive comparison has been performed between poorly and strongly expressed human TCRs and key residues have been identified, which affect the level of surface expression. Interestingly, these residues are outside the complementary determining regions of the variable domains and are therefore accessible to replacements without affecting T-cell specificity[2].

Endogenous TCR knock-down. The ability to suppress the endogenous TCR repertoire during the process of T-cell engineering to redirect antigen specificity is also important to improve effectiveness of the introduced TCR and allow the safe use of third-party or allogeneic T-cell donors[47]. Gene editing tools such as CRISPR-Cas9, TALENs and MegaTAL nucleases are all currently being evaluated for their ability to reliably and efficiently edit primary human T cells, and recently published work has described a strategy to simultaneous knock down the endogenous TCR beta chain in recipient T cells using CRISPR/Cas9 while transducing a cancer-reactive TCR of choice[48]. This TCR replacement strategy resulted in markedly increased surface expression of both transgenic αβ and γδ TCRs, which in turn translated to a stronger, and more polyfunctional response of engineered T cells to their target cancer cell lines. Additionally, the TCR-plus-CRISPR-modified T cells were up to a thousand-fold more sensitive to antigen than standard TCR-transduced Tcells or conventional model proxy systems used for studying TCR activity[48].

Additional strategies for endogenous TCR downregulation include the use of small interfering RNA (siRNA)[49] or designed zinc-finger nucleases[50].

Single-chain TCRs. A novel approach to alleviate mispairing has been demonstrated by recent reports using single-chain TCRs[11]. One group engineered a recombinant TCR consisting of a single-chain Valpha-Vbeta-Cbeta and a Calpha chain, which only paired with each other and not the endogenous TCR[51]. Other groups have used a stabilised Valpha-Vbeta single-chain TCR linked to intracellular signalling domains to elicit functional activation of T cells in the absence of co-receptors and to circumvent mispairing with endogenous TCRs[52–54].

Targeted gene delivery

Apart from cell separation prior to transduction, methods of targeted gene deliver have been developed, which may facilitate the transduction of a specific subpopulation. It is the case of

previously published work describing a lentiviral-derived vector delivering genes exclusively and specifically to CD8+ T cells[55]. This technology relies on engineered glycoproteins of measles virus (MV), which are the hemagglutinin (H) and fusion (F) proteins, both incorporated into the envelope membrane of lentiviral particles. Cell-type specificity is provided through a single-chain antibody (scFv) that recognizes a cell-surface antigen selectively expressed on the cell type of interest fused to an engineered H protein, which is blinded for its natural receptors CD46 (complement regulatory protein) and CD150 (signalling lymphocyte activation molecule, SLAM). The extension of this technology to other target cell types of interest relies on the availability of suitable scFv. These must not only be specific for the target cell but also have to be efficiently expressed on the surface of the packaging cells as H protein fusion to become readily incorporated into vector particles[55].

Closed systems and automated platforms

One of the main challenges in growing cells for immunotherapy, particularly when large numbers of cells are required, is the development of a manufacturing process that is stable and reproducible. With this in mind, fully closed system platforms have been developed, which help standardise manufacturing procedures and reduce the risk of contamination.

An example of such a system is the CliniMACS Prodigy®, which offers an automated all-in-one solution for cell processing and has been widely used for the production of CAR T cells[56–58]. Unfortunately, the current yield of this fully automated and closed system ranges from only 1-5x10^9cells, which is sufficient to treat patients enrolled on most CAR T-cell protocols for haematologic malignancies, but inadequate for many TCR T-cell protocols, which may require up to 100x10^9 cells. Although only partially closed, a large-scale manufacturing process using modular systems and semi-automated devices has been recently described, which resulted in highly functional clinical-grade TCR-transduced T cells[59].

Safety and efficacy

Safety and efficacy of TCR-transduced T cells have been extensively evaluated in early phase clinical trials, and most products have been observed to be safe. However, there are intrinsic risks to the administration of these products, which may considerably upsurge with all the technical advances leading to increased efficacy of TCR-engineered T-cell-based therapies.

Cytokine release syndrome (CRS). Highly proliferative T cells can lead to CRS, which may range from high fever and myalgias to unstable hypotension and respiratory failure. A key insight into CRS came with the observation that, in addition to the expected effector cytokines such as interferon (IFN)-gamma, interleukin (IL)-6 can be quite elevated during the exponential phase of CAR T-cell therapy[59]. CRS is directly and possibly causally related to a complementary toxicity, which is macrophage activation syndrome[60]. These insights have resulted in a therapeutic option for severe CRS, which is IL-6 blockade using the IL-6 receptor antagonist tocilizumab[1].

Off-tumour/On-target toxicity. In addition to CRS, which is unrelated to the antigenic specificity of the product, there are toxicities that directly result from the activity of the engineered T cells, which are unable to distinguish between normal cells and cancer cells that express the targeted antigen. Previously published results have shown lethal toxicities in two patients, which were related to T cells engineered to express a TCR targeting melanoma-associated antigen (MAGE)-A3 cross-reacting with a peptide from the muscle protein Titin[61,62]. Strategies such as peptide scanning and the use of more complex cell structures are recommended in preclinical studies to mitigate the risk of off-target toxicities in future clinical investigations[61]. Paradoxically, the use of lower affinity TCR for tumour-related antigens may reduce off-tumour activity due to the lower levels of antigen expression on normal cells.

Neurologic toxicity. Emergence of neurologic symptoms, which are varied but self-limiting, including delirium, dysphasia, akinetic mutism, and seizures, has also been reported after infusion of T cells

engineered with an HLA-A2-restricted MAGE-A3-specific TCR[61]. This was probably due to a TCR-mediated inflammatory response that resulted in neuronal cell destruction and raises caution for clinical applications targeting MAGE-A family members with highly active immunotherapies.

Given the possibility of adverse events with the use of TCR-engineered T cells, it is beneficial to preserve the ability to eradicate the transferred cells, if needed[11]. For this purpose, there are strategies that aim at turning off antigen receptor expression or eliminating engineered cells after transfer by incorporating certain "suicide genes" into the transgene[63]. Another common approach incorporates caspase 9 under an inducible promoter (iC9) to initiate apoptosis of transduced cells[63].

Conclusion

References

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