Minicircle-Based Engineering of Chimeric Antigen Receptor (CAR) T Cells

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Abstract

Plasmid DNA is being used as a pharmaceutical agent in vaccination, as well as a basic substance and starting material in gene and cell therapy, and viral vector production. Since the uncontrolled expression of backbone sequences present in such plasmids and the dissemination of antibiotic resistance genes may have profound detrimental effects, an important goal in vector development was to produce supercoiled DNA lacking bacterial backbone sequences: *Minicircle* (MC) DNA. The *Sleeping Beauty* (SB) transposon system is a non-viral gene delivery platform enabling a close-to-random profile of genomic integration. In combination, the MC platform greatly enhances SB transposition and transgene integration resulting in higher numbers of stably modified target cells. We have recently developed a strategy for MC-based SB transposition of chimeric antigen receptor (CAR) transgenes that enable improved transposition rates compared to conventional plasmids and rapid manufacturing of therapeutic CAR T cell doses (Monjezi et al. 2016). This advance enables manufacturing CAR T cells in a virus-free process that relies on SB-mediated transposition from MC DNA to accomplish gene-transfer. Advantages of this approach include a strong safety profile due to the nature of the MC itself and the genomic insertion pattern of MC-derived CAR transposons. In addition, stable transposition and high-level CAR transgene expression, as well as easy and reproducible handling, make MCs a preferred vector source for gene-transfer in advanced cellular and gene

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therapy. In this chapter, we will review our experience in MC-based CAR T cell engineering and discuss our recent advances in MC manufacturing to accelerate both pre-clinical and clinical implementation.

Keywords
Sleeping Beauty · Minicircle · Transposition · Chimeric antigen receptor T cell · Immunotherapy

1 Introduction

1.1 MC DNA as Minimalistic Expression Cassette

MCs are derived from parental plasmid (PP) with antibiotic resistance marker, the gene of interest (GOI) and *ori*, as well as two special signal sequences right and left of the GOI. Through an intramolecular recombination process, the GOI (plus one of the recombination and purification sequence elements, SCAR) is cut out of that parental plasmid, circularized, and finally results in only the GOI and the signal sequence in a supercoiled (ccc) circular molecule.

Several recombinases have earlier been used so far to achieve this intramolecular recombination process: the integrase of bacteriophage lambda, the Cre recombinase from bacteriophage P1, the FLP recombinase of the yeast plasmid 2-μm circle, and the integrase of Streptomyces bacteriophage PhiC31 or the ParA resolvase from the multimer resolution system of the broad host range plasmid RK2 or RP4 (Bigger et al. 2001; Chen et al. 2003; Darquet et al. 1997; Jechlinger et al. 2004; Nehlsen et al. 2006) to separate the parental plasmid (PP) into a miniplasmid (MP) and a MC [a comprehensive overview was given by Schleef (2013)].

In earlier work, the ParA resolvase, a serine recombinase that mediates an intramolecular recombination between corresponding directly repeated resolution sites (Eberl et al. 1994; Smith and Thorpe 2002; Thomson and Ow 2006) in exclusively one direction to completion, is working successfully. Hence, a supercoiled monomeric MC is obtained (Jechlinger et al. 2004).

For successful MC production, an efficient silencing of recombinase expression before induction is important to avoid early recombination events leading to the displacement of the parental plasmid by the miniplasmid, that still contains the bacterial *ori*, hence replicating in bacteria. The P BAD/araC arabinose expression system (Bigger et al. 2001; Chen et al. 2003; Jechlinger et al. 2004) has been shown to be able to inhibit background expression of the recombinases in the noninduced state.

After successful recombination, the MC has to be isolated from a mixture of three types of circular DNA molecules: MCs, miniplasmids, and maybe residual

amounts of parental plasmids. This is done by a set of chromatography steps, including a tailor-made affinity chromatography step. The approach to selectively bind a sequence motif (identification sequence part of SCAR) with the purpose of separating this from a mixture of different DNAs (Gossen et al. 1993) led to the approach that we initially published in 2008 (Mayrhofer et al. 2008; Schleef and Schmeer 2011; Schleef et al. 2015).

Besides fulfilling requirements concerning product safety, MCs show a significantly higher efficiency with respect to gene expression levels and duration in vitro and in vivo, making them useful tools for future therapeutic applications (Kobelt et al. 2013; Chabot et al. 2013), and were recently presented the first time allowing the production of AAV particles, free of antibiotic resistance, and other prokaryotic sequence motifs.

1.2 Non-viral Gene-Transfer Through *Sleeping Beauty* Transposition

SB transposons enable close-to-random integration into the genome of mammalian (Izsvak et al. 2010; Ivics and Izsvak 2011; Aronovich et al. 2011; Swierczek et al. 2012). SB was derived from an ancient, inactive transposon from several fish genomes. After various modifications, a hyperactive transposase (SB100) could be developed that yields enhanced stable gene-transfer in several human cell types including T cells (Ivics et al. 1997; Mates et al. 2009). The potential to use SB-mediated transposition to integrate CAR and T cell receptor transgenes in human T cells has been intensively investigated (Izsvak et al. 2010; Swierczek et al. 2012; Peng et al. 2009; Field et al. 2013; Huang et al. 2008; Jin et al. 2011; Singh et al. 2008, 2013, 2014). Nevertheless, transfection of conventional plasmid DNA to provide transposon and transposase typically resulted in low gene-transfer rates and extensive T cell toxicity, and hence, long ex vivo culture times were required to generate therapeutic doses of gene-modified T cells.

The SB gene-transfer strategy is based on mobile genetic elements, the transposons, containing a gene of interest flanked by inverted terminal repeats (IR/DR), and a transposase that binds to the IR/DR and mobilizes the transposon for integration into the target genome through a cut-and-paste mechanism. Those two elements are typically encoded on two separate vectors. Alternatively, the transposase can be encoded by an RNA molecule, while the transposable element (the gene of interest—GOI—to be integrated into the genome of the target cell) is still encoded on a DNA molecule. As initially presented by Izsvak et al. (2010), the structure of this transposon molecule is influencing the transposition efficacy. While the proximal sequences between the IR/DR are to be transposed, the other (distal) sequences between the IR/DRs (in other words the residual molecule "behind" the IR/DR) are remaining outside the genome of the modified cell and are expected to be subject of degradation.

1.3 Principles of CAR Design

Adoptive immunotherapy with gene-engineered tumor-reactive T cells expressing a transgenic T cell receptor (TCR) or synthetic CAR is emerging as a powerful and potentially curative treatment of malignant diseases. CARs are fusion proteins comprised of an extracellular antigen-binding domain, most commonly a single-chain variable fragment (scFv) of variable heavy (VH) and variable light (VL) chains of a monoclonal antibody, and an intracellular signaling module. To link the extracellular and intracellular portion, various spacer and transmembrane (TM) domains are used that anchor the receptor on the T cell surface. The signaling module of first-generation CARs contained only the CD3ζ chain (Signal 1), and second- and third-generation CARs include one or two (respectively) costimulatory domains, e.g., CD28, 4-1BB, OX40, or ICOS, to provide Signal 2 which is critical for optimal T cell stimulation and induction of an effective immune response (reviewed in: Turtle et al. 2012).

A key difference of CAR recognition, compared to the TCR, is the ability of CARs to bind to surface molecules on target cells. Thus, CAR recognition does not depend on the intracellular processing of antigens and presentation of immunogenic peptides on HLA (human leukocyte antigen) molecules. Another important aspect is that CARs as synthetic molecules can be equipped with targeting domains that bind not only to proteins but also to a broad range of potential tumor targets such as carbohydrates, gangliosides, proteoglycans, and also heavily glycosylated proteins, expanding the panel of antigens that can be targeted on tumor cells.

1.4 Gene-Transfer Strategies and Clinical Experience with CAR T Cells

There are two principle strategies for expressing the CAR transgene in T cells: (i) transiently, e.g., by transfection of CAR-encoding mRNA, which provides a window of activity in the range of several days and is self-limiting by the fading expression of receptor and dilution of the mRNA as the T cells start to proliferate (Beatty et al. 2014) and (ii) permanently by stable gene-transfer with viral or non-viral vectors. The majority of pre-clinical and clinical work with CAR T cells is performed using stable gene-transfer, and the overwhelming majority of investigators is using gamma-retroviral (RV) and lentiviral vectors (LV) for gene-transfer (reviewed in: Ramos et al. 2014). The use of non-viral gene-transfer by SB-mediated transposition for CAR T cell engineering has been explored but was limited by very low gene-transfer rates and significant toxicity to T cells after transfection of conventional plasmids to insert SB transposase and CAR transposon (Singh et al. 2008, 2013; Field et al. 2013). We have recently shown that both challenges can be addressed by the use of MCs to encode SB transposase and transposon (Monjezi et al. 2016). Table 1 provides a comparison of key features of LV- and SB-mediated gene-transfer.

Table 1 Summary of key features of viral vectors and SB MC vector used as gene-transfer vehicles

Gene delivery vector	Viral vectors	*Sleeping Beauty* MC vector
Host range	Broad	Broad
Gene-transfer efficiency	High	High
Transgene expression stability	Long-term	Long-term
Immunogenicity	Yes	NA
Genotoxicity/insertional mutagenesis	Yes	NA
Vector integration site profile	Bias to oncogenes/exons/highly expressed genes, 3 % to GSH	Close to random, 23 % to GSH
Transgenic capacity size	Low to high (<4 kbp to >30 kbp)	Not known upper limit
Storage and handling	Special caution required	No special caution required
Cost of production	High	NA

NA Data not available
GSH Genomic safe harbor

A critical issue in cancer immunotherapy is to identify target antigens that allow selective (or preferential) elimination of tumor cells while sparing normal tissues. The clinical development of CAR T cell therapy is most advanced in hematologic malignancies, i.e., B cell leukemia and lymphoma with CARs targeting the B cell marker CD19. An anticipated but acceptable side effect of CD19-CAR therapy is the depletion of normal B cells. Coexpression of "safety switches," e.g., a truncated epidermal growth factor receptor (EGFRt) depletion marker (Wang et al. 2011) or inducible caspase-9 (iCasp9) suicide genes (Di Stasi et al. 2011), can be used to delete CAR T cells after a therapeutic window to prevent, terminate, or mitigate undesired effects or toxicity of CAR T cells.

Most of currently reported pre-clinical and clinical studies with CAR T cells employ bulk CD3+ T cells that contain a random composition of naïve and memory CD8+ killer, CD4+ helper, and potentially even CD4+ regulatory T cell subsets. All of these T cell subsets are not only phenotypically, but also functionally distinct, and their frequency differs substantially between individuals. Thus, there is significant product to product heterogeneity in cell composition when CAR T cell is prepared from a bulk CD3+ T cell population (Brentjens et al. 2011). This heterogeneity complicates the analysis and interpretation of data obtained in pre-clinical experiments and in the clinical setting, as essentially every patient is receiving a cell product of different potency and with different attributes in pharmacokinetic and -dynamic. Therefore, we prefer to perform CAR gene-transfer into defined T cell subsets (in the easiest case, separately into CD8+ killer and CD4+ helper T cells), analyze their in vitro function separately in our pre-clinical models, and formulate CAR T cell products with defined CD8+ and CD4+ subset

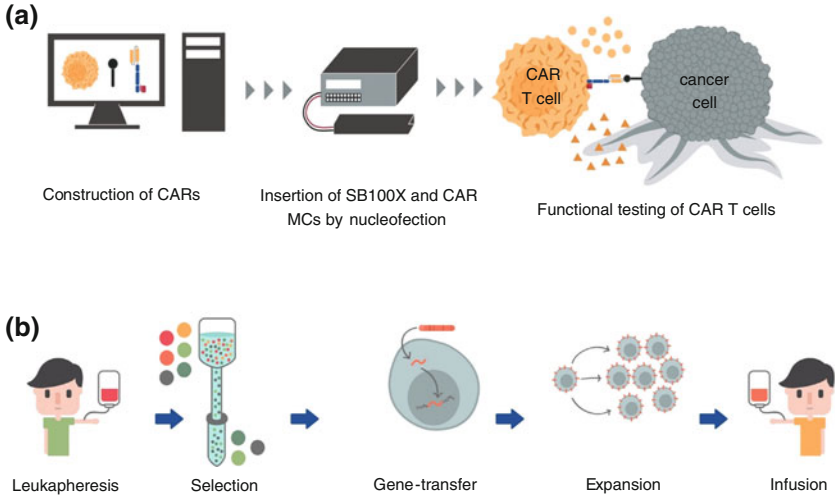

Fig. 1 **a** CARs are designed using specialized computer software. MCs encoding the genetic information for the CAR are introduced into T cells using a nucleofector. CAR T cell effector functions such as cytolytic activity, cytokine secretion, and proliferation are tested in pre-clinical models. **b** To prepare CAR T cells, white blood cells are harvested in a process called leukapheresis and appropriate T cell subsets (e.g., CD8+ killer and CD4+ helper T cells) are purified. Gene-transfer is performed using MC-encoded SB100X transposase and CAR transposon. CAR T cells are expanded prior to administration to patient

composition for in vivo studies and clinical applications Fig. 1a. We have recently shown that this strategy is advantageous to the use of bulk CD3+ T cells and have successfully translated this concept into the clinical setting (Sommermeyer et al. 2016; Turtle et al. 2016). The CAR T cell manufacturing process is summarized in Fig. 1b.

2 Methodologies of SB-Minicircle and CAR T Cell Manufacturing

2.1 Construction of Transposable MC Vectors

The existing and functionally evaluated plasmid encoding SB100, also known as SB100X (Ivics et al. 1997), was used to purify a restriction fragment to transfer into the parental plasmid (PP) in a way that after recombination of MC induction, the SB100 expression cassette is located on the resulting MC (Fig. 2a). This was carried out by inserting a BglII restriction fragment of the plasmid pcDNA3.1-CMV-SB100, carrying the SB100 expression cassette, into the BglII restriction site of the parental plasmid PP11 published earlier (Schleef et al. 2015). The resulting pP11.SB100 contains the SB100 expression cassette and a sequence tag for the

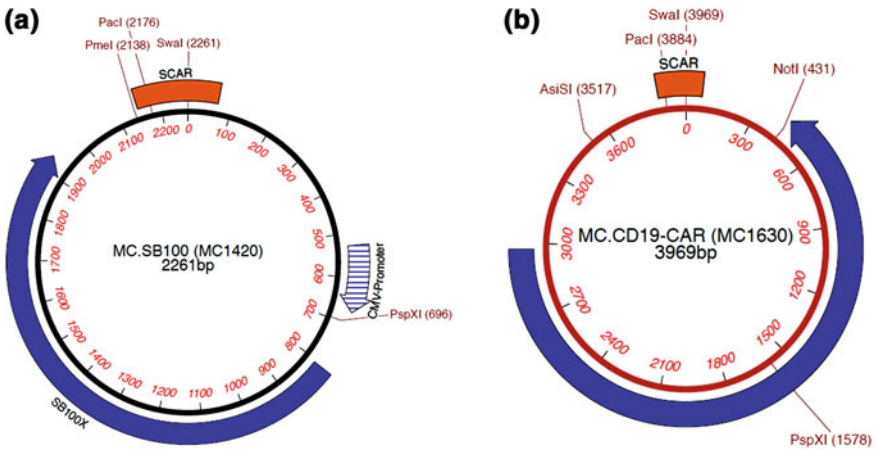

Fig. 2 *Minicircle* DNAs for SB100 (**a**) and for CD19-CAR (**b**), consisting only of the GOI plus one of the recombination and purification sequence elements (SCAR) as a supercoiled (ccc) circular molecule

subsequent purification of MC.SB100, flanked by the recombination sequences. Importantly, no backbone sequences (e.g., bacterial origin of replication and selection marker) are contained within this area. Such sequences are positioned outside the framed area and will finally end up on the miniplasmid as by-product and are removed during affinity chromatography. The resulting MC and miniplasmid both contain the recombination sequence. The remaining nucleotides of recombination and tag sequences on the MC are called "SCAR" (sequence for chromatography, affinity, and recombination) since they constitute the only small portion on the MC that is neither the part of the expression cassette nor the GOI.

A CD19-CAR-encoding MC was derived from a parental pT2HB_CD19-CAR plasmid and obtained by inserting the restriction fragment with the CD19-CAR gene into the PP in a way that also in this case, the resulting MC carries the CD19-CAR gene (Fig. 2b). This was carried out by transferring the Eco53kI and SalI restriction fragment of pT2HB_CD19-CAR into the parental plasmid PP11 (see above) resulting in a parental plasmid pP11.CD19-CAR. This PP was also subject to recombination to obtain MC and miniplasmid as described in detail above.

2.2 Manufacturing of MC DNA

The production of the two MC DNAs for SB100 and for CD19-CAR used in this publication (performed as a service of PlasmidFactory, Bielefeld, Germany) was carried out in 2 major production steps: the microbial cultivation in a bioreactor and the purification by specific chromatographic steps. The cultivations of *E. coli* cells carrying the respective parental plasmids were carried out at 37 °C in a MBR bioreactor (MBR BIO REACTOR, Switzerland) with 5 L, pH adjusted to 7.0 with

2 M sodium hydroxide solution and 2 M phosphoric acid. The airflow rate was fixed at 5 L/min. The oxygen concentration of 60 % was controlled by varying the stirrer speed. LB medium was used without addition of any antibiotics and free of any substance deriving from any animal source. The bioreactor was inoculated with 50 mL of an *E. coli* K12 culture transformed with the parental plasmid PP and grown in LB medium (as pre-culture) for approximately 15 h. Recombinase expression was induced at an OD_{600} » 4 by adding L-arabinose. After an additional 1 h of growth, cells were harvested by centrifugation, frozen, and purified.

The recombination product (MC and miniplasmid) was further purified after the primary recovery as presented earlier (Mayrhofer et al. 2008) with a modified non-commercial proprietary chromatography matrix obtained from PlasmidFactory (Bielefeld, Germany). The specific binding of MC DNA was optimized with different ionic strength and pH values and resulted in a highly purified product as shown below.

2.3 Engineering of CAR T Cells

CD8+ killer and CD4+ helper T cells were isolated from Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood by centrifugation over Ficoll-Paque. Distinct T cell subsets were obtained by negative selection using magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany) or reversible Streptamers® that mark the target cell population but can subsequently be released with biotin to yield quasi-untouched T cells (IBA, Göttingen, Germany). The purity and yield of the T cell population was determined by flow cytometry using conjugated mAbs specific for CD3, CD4, and CD8. 7-AAD (BD Biosciences, San Jose, CA) was used to discriminate dead from live cells.

Although SB transposition can be directly performed in freshly isolated T cells, we observed that gene-transfer rates are substantially higher, and the subsequent expansion of T cells much more productive, if prior T cell activation is performed. T cells can be activated through anti-CD3/CD28 stimulation using Dynabeads (ThermoFisher, Waltham, MA) for 2 days prior to SB transposition or by culture on anti-CD3/CD28 mAb-coated plates. T cells were propagated in RPMI-1640 medium supplemented with 10 % human serum, glutamine, 100 U/mL penicillin–streptomycin, and 50 U/mL recombinant human IL-2.

Transfection of SB100 and CAR-encoding MC can be accomplished by electroporation. We performed nucleofection using a 4D nucleofector (Lonza, Cologne, Germany): 1–2 × 10e6 of activated T cells were nucleofected with 1 µg of each transposon and transposase conventional plasmids or equimolar amount of their corresponding MC DNA vectors in 20 µl of P3 primary cell line buffer based on manufacturer's protocol (Lonza). T cell viability was monitored by trypan blue staining. The EGFRt transduction marker that is encoded *in cis* with the CAR in the SB transposon was utilized to detect and enrich CAR+ (i.e., EGFRt+) T cells prior to expansion with irradiated CD19+ feeder cells and functional testing (Hudecek et al. 2013, 2015). Functional analyses aim at documenting the specificity of

CAR T cells for the targeted tumor antigen and the ability to eliminate tumor cells. Functional testing is preceded and accompanied by careful phenotypic analysis of the CAR T cell product by flow cytometry including CD3, CD4, and CD8, expression of the CAR transgene (using the EGFRt marker).

2.4 Functional Testing of CAR T Cells

In vitro characterization of CAR T cells typically focuses on the 3 cardinal effector functions—cytolytic activity, cytokine production, and proliferation. The specific cytolytic effect against relevant target cells, i.e., CD19+ lymphoma cell lines, and CD19− control targets, was analyzed in a 4-h bioluminescence-based cytotoxicity assay (Brown et al. 2005). The assay was set up in triplicate wells of a 96-well plate at effector:target cell ratios ranging from 20:1 to 1:1, using 5000 target cells per well. The specific lysis of CD19+ target cells was calculated using the standard formula (Brown et al. 2005). The production of cytokines such as IFNg and IL-2 was analyzed in supernatants that were removed from cocultures of 50,000 CAR-transduced and control untransduced T cells with target cells (effector:target cell ratio: 4:1 to 2:1) after 20 h of incubation. Cytokines were quantified by ELISA or multiplex cytokine analysis. Antigen-dependent induction of CAR T cell proliferation was evaluated by CFSE dye dilution after 72 h of coculture of effector and target cells, and finally the viability of CAR T cells was analyzed by 7-AAD staining.

For pre-clinical in vivo studies, xenograft models in immunodeficient NSG mice (NOD-Scid-gc$^{-/-}$) are well established and have been demonstrated to provide clinically relevant data on antitumor efficacy, as well as on CAR T cell engraftment and persistence. We performed analyses in NSG mice that were engrafted with 0.5–1 × 10e6 firefly-luciferase transduced Raji lymphoma cells. Raji was inoculated by tail vein injection and mice treated with CAR-modified or unmodified T cells on day 7, when systemic lymphoma had developed. The antitumor response was analyzed by serial bioluminescence imaging after administration of D-luciferin substrate (IVIS Lumina, Perkin-Elmer, Waltham, MA) (Hudecek et al. 2013, 2015; Monjezi et al. 2016).

3 Antitumor Function of Minicircle Engineered CAR T Cells

3.1 Characterization of MC DNA

The plasmid and MC DNA used to perform the experiments presented here were characterized by a comprehensive QC. The content of LPS-endotoxin was determined at <20 E.U./mg by the use of a Kinetic-QCL kit (Lonza), the DNA concentration was adjusted to 1 mg/mL, and the DNA was dissolved in water for injection (WFI). CGE and agarose gel date could demonstrate that the products each were pure and predominantly a homogenous supercoiled MC DNA (Fig. 3).

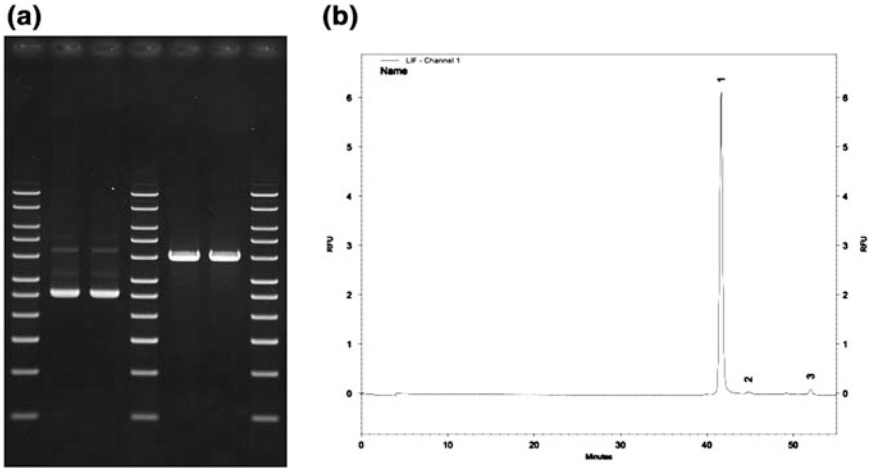

Fig. 3 **a** 0.8 % agarose gel electrophoresis stained with ethidium bromide after gel run (1 V/cm) of the circular, supercoiled, monomeric MC.CD19-CAR (lanes 2 and 3) and the linearization product (lanes 5 and 6; PacI digest resulting in a 3969 bp fragment). 1 kbp ladder in lanes 1, 4, and 7 (PlasmidFactory, item no. MSM-865-50). **b** Capillary gel electrophoresis of the circular, supercoiled (ccc), monomeric MC MC.CD19-CAR (peak 1; 97.3 %). Peak 2 (1.2 %) and peak 3 (1.5 %) are traces of relaxed circular (rc) and open circular (oc) molecules

3.2 Gene-Transfer Rate and Integration Profile

We have recently shown that high-level, stable gene-transfer can be accomplished by cotransfection of MC-encoded SB100 and CD19-CAR in both CD8+ and CD4+ T cells (Monjezi et al. 2016). Stable gene-transfer rates of >50 % can routinely be accomplished with MCs and are typically slightly higher in CD4+ compared to CD8+ T cells. Importantly, the gene-transfer rate after cotransfection of MCs is significantly higher compared to corresponding conventional plasmids, and the process less toxic, most likely due to the lower amount of DNA that is introduced into T cells. Whether or not transient gene expression can be obtained by transfection of transgene-encoding MC alone (i.e., without SB100X) depends on the amount of MC that is being transfected and varies considerably between transgenes.

Genome-wide insertion site analysis is an approach to assess genotoxicity associated with genetic modification. We analyzed insertion sites of CD19-CAR transposons that had been mobilized from MCs to determine whether there was a preference for integration into distinct sites of the genome. For CD19-CAR transposons, mobilized from MCs as described in this chapter, we detected a close-to-random integration profile, without preference for highly expressed or

cancer-related genes (Monjezi et al. 2016). Thus, the previously described, favorable integration pattern of SB was well maintained, even though the gene-transfer rate with MCs was substantially higher than in previous studies with conventional plasmid DNA. Criteria have been defined that identify "genomic safe harbor" (GSH) where integration is neither expected to cause genotoxicity, nor malignant transformation (Sadelain et al. 2011). A comprehensive GSH analysis disclosed a significantly higher percentage of GSH integrations had occurred with SB transposons after mobilization from MCs compared to LV integrants. These attributes make SB transposition from MCs the most effective and safest stable gene-transfer strategy known to date.

3.3 Functional Characterization of CAR T Cells

CAR T cells that were engineered with MC-based SB transposition displayed specific and very potent effector functions in vitro and in pre-clinical models in vivo (Monjezi et al. 2016). Importantly, CAR expression and CAR T cell antitumor function were stable over several weeks and multiple rounds of expansion, confirming that mobilization of the CAR transposon from the MC had resulted in stable integration and high-level transgene expression without gene silencing. We have also demonstrated in vitro and in vivo that the antitumor function of CAR T cells that we engineered by SB transposition from MCs was equally potent as with CAR T cells that were generated from the same donors by lentiviral gene-transfer (Monjezi et al. 2016). This is encouraging, and efforts to obtain clinical validation for the potency and safety of adoptive therapy with MC-engineered CAR T cells are ongoing.

The function of CAR T cells is influenced by the copy number of the CAR gene in their genome. The current paradigm is that higher gene copy number leads to higher expression of CAR protein and potentially better function, although an upper threshold may be reached where the expression of the CAR on the T cell surface and availability of adaptor proteins that propagate CAR signaling are saturated, or where the signal of the CAR is getting too strong and induces activation-induced cell death (AICD). We have shown that the number of CD19-CAR transposons in T cells that were transfected with MC-encoded SB100 transposase and CAR transposon correlates with the expression of CAR and EGFRt protein and is well balanced between CD8+ and CD4+ T cells, and in a similar range as the number of LV integrants after viral gene-transfer (Monjezi et al. 2016). Further, a comprehensive integration site analysis of CAR transposons demonstrated that a significantly higher proportion of integrations had occurred in GSH that is not expected to cause genotoxicity or malignant transformation (Monjezi et al. 2016). Intriguingly, the use of MC DNA for CAR gene-transfer provides the opportunity to use titrated amounts of MC during gene-transfer until a gene copy number has been reached that is optimal with regard to gene-transfer rate and CAR T cell function and satisfactory for regulators with regard to transposon copy number and number of GSH integrations.

4 Future Perspectives

4.1 GMP *Minicircle* Manufacturing

A prerequisite for clinical utilization of MCs in Germany and Europe is the ability of GMP manufacturing to satisfy regulatory requirements, even if the MC DNA is not directly injected in patients but rather used as a gene-transfer tool to modify T cells ex vivo as described here. Once their GMP manufacturing has been established, MCs will provide a tool that enables cost-effective, exportable manufacturing of CAR T cells, rapid evaluation of novel concepts in CAR T cell therapy in the academic setting, as well as scalable manufacture of validated CAR technologies for large patient cohorts to improve the outcome of prevalent hematologic and solid tumor malignancies.

Further, a process for *high-quality* grade MC production (Schmeer and Schleef 2014) will be established that will be used for subsequent viral vector or RNA production, since full GMP is often requested but in fact not necessary for these applications.

4.2 Clinical Implementation

As of July 2016, all clinical trials of CAR T cell therapy that reported clinical efficacy have used RV or LV to accomplish CAR gene-transfer (Maude et al. 2014; Davila et al. 2014; Turtle et al. 2016; Ramos et al. 2014). The results of clinical trials that employed CAR T cells that were generated by non-viral gene-transfer with SB transposition from conventional plasmids were rather disappointing; however, the lack of efficacy may have been caused by reasons related to the design of the specific CAR construct that was used in these trials and T cell composition of the CAR T cell products and be unrelated to the gene-transfer strategy that has been employed. It has been shown that CAR design and composition of CAR T cell products profoundly affect efficacy (Hudecek et al. 2013, 2015; Sommermeyer et al. 2016).

The use of non-viral gene-transfer and MCs to deliver SB transposase and CAR transposons into T cells is conceptually attractive and has significant potential to become the new gold standard in this field.

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