

Gag-derived VLPs and the delivery of CRISPR-Cas9 system in Gene and Cell Therapies

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Abstract. Cancer had been an unsolved problem for decades that accounts for 375,400 cases in UK each year, with only a 50% survival rate of 10 or more years. With more recent advances in gene editing techniques like CRISPR-Cas9, immunotherapy was able to advance to better engineer T cells for adoptive T cell transfer therapies such as T cell receptor (TCR) therapy and chimeric antigen receptor T cell (CAR-T) therapy. For more efficient delivery of CRISPR-Cas9 system, several human immunodeficiency virus type 1 (HIV-1) as well as murine leukaemia virus (MLV) group-specific antigen (Gag)-based virus like particles (VLP)s were designed by either directly fusing the Cas9 mRNA, Cas9 protein, or sgRNA to either the N- or C-terminus of the Gag polyprotein or by inserting or replacing a part of the Gag polyprotein. The Gag polyproteins can then self-assemble, carrying their cargo and packaging them inside the VLP. All designs demonstrated a significant increase in cargo capacity and successful delivery of both Cas9 mRNA and Cas9 proteins or nucleases for T cell engineering, and this provides great potential for additional gene modifications in order to target specifically solid tumour due to their high efficiency and multiplexed editing nature.

Keywords: CRISPR-Cas9, Virus-like Particles, Immunotherapy.

1. Introduction

According to Cancer Research UK, cancer accounts for 375,400 cases within UK each year, with only 50% survival of 10 or more years, and is the second leading cause of death worldwide. Treatments against cancer had been an ongoing and rapidly developing field and it is of great importance to develop better therapeutics to improve cancer survival. The adaptive immune system plays a critical role in modulating tumour progression, where CD8⁺ cytotoxic T cells are the core components for antitumour response. Therefore, they had become a focus as a therapeutic target to engage the adaptive immune response to fight against cancer. However, cancer had evolved multiple mechanisms to escape immune surveillance by, for example, reduced antigen presentation, change in regulatory pathways, or via the recruitment of immunosuppressive cells [1].

Immunotherapy act by activating or suppressing the adaptive immune system in order to better target and eliminate cancer cells. Current most commonly used immunotherapies targeting T cells include immune checkpoint inhibition, cancer vaccines, and adoptive T cell transfer therapies [2]. Adoptive T cell transfer therapies currently uses either tumour-infiltrating lymphocytes (TILs) or engineered T cells to modulate the immune system, and both are highly promising methods for cancer treatment. More specifically, the latter one had been most extensively investigated in recent years, which includes engineered T cell receptor (TCR) therapy and chimeric antigen receptor T cell (CAR-T) therapy. These cells are traditionally engineered by viral transduction, whereas more recent work has focused on the potential of using gene editing tools with more advanced delivery methods for intracellular gene modifications.

The three most popular gene editing techniques are the transcription activator-like effector nucleases (TALENs), the zinc finger nucleases (ZFNs), and the clustered regularly interspaced short palindromic repeats (CRISPR) [3]. Among them, CRISPR-Cas9 plays a most crucial role due to its high efficiency and multiplexed editing, as well as its high flexibility since they can be redesigned to recognize almost any desired sequence [4]. The most common methods of current CRISPR-Cas9 delivery into cells include physical methods such as microinjection and electroporation, as well as chemical methods such as cell-penetrating short peptides, gold nanoparticles (AuNPs), and lipid-

based nanoparticles (LNPs), and biological methods such as viral vector foreign gene transduction, including the use of adeno-associated viruses (AAVs), lentiviruses (LVs), and adenoviruses (AVs) [5, 6]. For gene therapy specifically, the delivery of gene editing systems into target cells were mainly limited to viral transduction such as using adenoviral vectors (AVVs) but they may cause several problems [3]. Therefore, VLPs are thought to be more advanced delivery methods, which will be discussed in this review.

2. VLPs and the delivery of CRISPR-Cas9 system

VLPs are nanoparticles derived from viral envelopes that are highly similar to viruses but lack the genetic materials and are thus non-infectious and safe to use [7]. Their highly repetitive surface structures resemble pathogen-associated molecular patterns (PAMPs), which provide them with high immunogenicity [8]. What's more, VLPs can self-assemble into icosahedral structures with defined size of 20-200nm for efficient drainage into lymph nodes, and their defined chemical properties can be modified both interiorly and exteriorly by taking the advantage of several amino acids with different reactivities, for example lysine and cysteine [9,10]. Thus, VLPs are versatile and multifunctional drug and antigen delivery platforms that can be optimized not only by chemical modifications but also via genetic manipulations or direct evolution strategies to achieve their desired properties [10]. For more efficient delivery of the CRISPR-Cas9 system, VLPs are modified interiorly for higher cargo delivery capacity, whereas its exterior surface is designed to specify their targets, which are cells that are targeted for gene modification, or to increase cell uptake efficiency of VLPs. Methods of modifying both the interior as well as the exterior surfaces of the VLPs will be discussed in this review.

2.1. HIV-1 Gag-derived VLPs as delivery system

Compared to traditional delivery methods using LV, AV, or AAVs, VLPs retain the high delivery efficiency of the traditional viral vectors but also have the potential to overcome problems such as viral DNA integration into the host genome since they lack genetic materials [4]. Another important feature associated with VLPs is their ability to transiently express or deliver CRISPR-Cas9 and sgRNA, which to a great extent limits detrimental off-target effects [11]. Today, the most popular type of VLPs designed for the delivery of the CRISPR-Cas9 system are assembled from the human immunodeficiency virus type 1 (HIV-1) group-specific antigen (Gag) precursor protein. Gag is a polyprotein that on its own is sufficient for VLP self-assembly, and its sequence contains a matrix protein (MA) responsible for membrane targeting, a capsid protein (CA) which mediates Gag-Gag interactions during viral envelope self-assembly, and a nucleocapsid protein (NC) vital for RNA packaging, as well as some other non-important peptides [4,12]. Some current methods of utilizing the Gag-derived VLPs to deliver CRISPR-Cas9 systems are summarized below, including the delivery of either Cas9 mRNA or Cas9 nucleases, where several interior modifications were done to increase the cargo capacity for increased delivery efficiency.

The cargo mRNAs delivered into target cells are used as template for transient expression of Cas9 nucleases, and its delivery is mostly achieved using pairs of RNA aptamers and RNA aptamer-binding proteins (ABPs). For example, a well-known high affinity interaction between the bacteriophage MS2 coat protein (MCP) and an MS2 aptamer had been utilized to load mRNAs into VLPs where the coat protein MCP is inserted to the Gag NC sequence, at the same time, multiple copies of MS2 aptamer are added to the 3' untranslated region of the cargo Cas9 mRNA [13,14]. This method significantly increased the cargo capacity from the original 2 copies of mRNAs packaged in LV vectors to up to 100 copies in Gag-based VLPs [4]. In another study, MCP also successfully replaced one of the two zinc finger domains (ZNF) on the NC for the same purpose of more efficient mRNA packaging [4].

Similar methods have also been developed for Cas9 nuclease delivery, where ABP is again inserted into the Gag sequence, forming Gag-ABP, whereas the aptamer is linked to the sgRNA [15]. The sgRNA adhered to the interior surface of the VLPs will then recruit Cas9 nucleases due to their high

affinity interactions and eventually form stable Cas9 RNPs [16]. In addition, more combinations of aptamer/ABP pairs can be used in addition to MS2/MCP, such as PP7/PCP, BoxB/ λ p22, and com/Com, which all have been demonstrated and proven effective by several studies [4]. During VLP assembly, the Gag-Cas9 sequence is co-expressed with sgRNAs, so that the sgRNAs are recruited to the inner surface to form Cas9 RNPs. This method had to date demonstrated packaging with highest efficiency under defined conditions, but to tune the delivery according to the requirement, further modifications are expected.

2.2. MLV Gag-derived VLPs as delivery system

In addition to the HIV-1 Gag derived VLPs, murine leukemia virus (MLV) VLPs have also been used to deliver Cas9 RNPs. One method was to fuse the Cas9 protein directly at the C-terminus of the MLV Gag polyprotein [17]. In the meantime, Pol was also co-expressed inside the VLP. Pol is also a polyprotein containing an integrase, a reverse transcriptase, and a protease, which are responsible originally used to transcribe the viral RNA genome into the host genomes to produce mature Gag proteins. Here, the protease co-expressed within the VLP is designed to cleave the linker between MLV Gag and Cas9 to release the RNP [17].

Another method was achieved by replacing NC with 2 copies of MCP, and at the same time linking 2 copies of MS2 to either the 3' untranslated region of the Cas9 mRNA or multiple functional positions on the sgRNA depending on the requirement to deliver mRNA or nuclease, and this method had demonstrated successful knockout of CXCR4 from a human T cell line [18].

3. CRISPR-Cas9 and cancer immunotherapy

To date, a variety of cancer treatment utilizing CRISPR-Cas9 have been developed to target multiple cancer cell lines, which have been demonstrated effective both in vitro and in vivo, leading to multiple therapeutic advancements [3]. However, most cancer cell engineering studies are still limited in vitro, whereas in vivo knockout of genes using CRISPR-Cas9 in mice were only demonstrated in a limited number of cases in colon cancer, melanoma, thyroid cancer, lung cancer, and multiple cases of lymphomas [3]. However, CRISPR-Cas9 has several limitations when directly targeting tumour cell lines, including the inability to tackle with large-scale chromosomal rearrangements, lack of precision due to off-target effects, slow performance in genetic knock-in and knock-out, and lack of efficient delivery systems. Advancements in delivery systems had already been discussed, where multiple interior modifications could be done to the Gag-based VLPs to increase the cargo capacity in delivering both the nuclease mRNAs and proteins, but reverting chromosome rearrangements and eliminating off-target effects seems to be impossible tasks, and they have long been major problems associated with CRISPR-Cas9 gene editing that may lead to devastating consequences [3].

To overcome these problems, one most prominent solution is to directly target immune cells to stimulate stronger antitumour responses instead of directly targeting tumour cells. Currently, the two most popular candidate of engineered immune cells both take the advantage of the important role of CD8⁺ cytotoxic T cells in fighting cancer, in which they are genetically manipulated to target specific cancer neoantigens [1]. These two types of engineered T cells, CAR-T cells and TCR-T cells, both demonstrated great potential for cancer therapies, but several problems remain. Most of the currently used viral vector delivery increases the risk of genomic damage, which may contribute to the stimulation of immune system to target the engineered cells, eventually resulting in T cell exhaustion. However, non-viral methods such as the use of nanoparticles and microparticles requires electroporation for the delivery of gene editing tools into the cell, which limits this process only in vitro.

In general, problems such as on-target off-cancer toxicity, reduced T cell fitness after infusion, limited ability for penetration into solid tumour, reduced effect due to TME, and cytokine storms, remains to be unsolved problems associated with engineered T cell therapies on solid tumours [19, 20].

To overcome these problems, several new strategies have been developed to improve CAR T cell immunotherapies. For example, to increase antigen specificity, there had been extensive screening for neoantigens, which are antigens that are present on only cancer cells but not normal cells [19]. However, most of these proteins are intracellular and are difficult to be targeted by CAR T cells. In order to overcome this, further T cell engineering can be done to increase the affinity of antigen binding, to target multiple antigens for stronger immune response, and to limit CAR T cell activities only at the cancer site, etc. [19]. For these kinds of engineering, VLPs are ideal delivery tools for the CRISPR-Cas9 system.

Recently, a cell-type specific strategy using HIV-1 Gag-derived VLPs as a delivery system for CRISPR-Cas9 to engineer T cells was developed by Hamilton et al [21]. The designed Gag-based VLPs were formed by fusing the Cas9 protein directly to the C-terminus of the Gag polyprotein. Upon fusion with the T cells, a HIV-1 Pol encoded protease mediates the separation of Cas9 by cleaving a linker sequence designed and inserted between the Gag and the nuclease. This method had successfully demonstrated efficient gene editing using the Gag-based VLPs, with the advantages of transient editing which limits the off-target effects, and the ability for cell-type specific delivery due to VLP surface modifications such as altering the surface glycoproteins, providing them with greater specificity under various applications. In addition, they are not electroporation based, meaning that they have the potential to be delivered in vivo in future applications.

In addition, VLPs have the ideal size and PAMP-like surface geometry for recognition specifically by dendritic cells (DCs), therefore, they also have their unique potential for delivering gene editing tools specifically to DCs [22]. By improving the precision of targeting specific cell types and by inducing transient Cas9 delivery or expression, a higher safety profile is expected. Current work on engineered DC cells have been extensively focusing on enhancing tumour-associated antigen (TAA) presentation, increasing DC lymph node migration, overcoming immunosuppression, and enhancing immune cell recruitment to sites of malignancy [23]. Currently, most DC engineering had been done using the traditional viral vectors, but the use of Gag-based VLPs is of great potential and should be focused during future research.

4. Conclusions

VLPs, have demonstrated its potential as an effective delivery method in a variety of aspects such as drug and vaccine delivery, and here we demonstrate that they are also the ideal and rapidly developing tool to deliver gene editing systems into target cells with high specificity. The use of CRISPR-Cas9 mediated gene editing and its delivery is a rapidly evolving field of research. In order to engineer T cells, LV and electroporation were for a long time the most widely used method for only in vitro delivery, and they may cause problems of DNA incorporation into genome in terms of LV transduction and massive cell death in terms of electroporation.

Gag-derived VLPs are currently the most used type of VLPs. Current Gag-based delivery methods are all demonstrated to be effective but lack direct comparison since different loci and cell types are used during the experiment. It is important for future investigation to compare them systematically for more rational decision on which method to use based on different requirements. It is also important to characterize and compare the effects of different methods on cargo capacity and VLP assembly. For example, by modifying the NC sequence, it is reported to have a reduction of VLP production, possibly because the VLP self-assembly is impaired due to the removal of ZNF2, which was reported to decrease VLP production by up to 10-fold [24]. In addition, delivery of mRNA to mammalian cells were demonstrated to be more successful than sgRNA delivery overall but lacks direct comparison under controlled conditions.

In general, it can be assumed that cells that can be infected by lentivirus can also be targeted by VLPs since they have the same pseudo typed envelope. Since many antigens are currently available to be targeted by LV vectors, there is great potential for VLPs to target the majority of current targets but with higher specificity and efficiency.

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