Concept Paper

Microglial Replacement and COURIER or SPIT for Neuronal Gene Editing

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Microglial Replacement and COURIER or SPIT for Neuronal Gene Editing

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Abstract: Adeno-associated viral (AAV) vectors can be used for gene delivery. AAV.CAP-Mac was recently developed; it can cross the blood-brain barrier and transduce cells throughout the CNS. However, some brain regions were not transduced in adult rhesus monkeys. Additionally, AAV vectors can only package 5 kb of DNA. Also, AAV vectors may be genotoxic and cytotoxic, especially at high doses. Finally, AAV vectors are very expensive to produce at sufficiently high titers for treatment. Off-the-shelf cell-based delivery systems wherein the cells can infiltrate the target tissue and be hyper-motile would be ideal. Also, mRNA-based gene editing would be a transient treatment, and thus be much safer.

Keywords: microglial replacement; adeno-associated viral (AAV) vectors; COURIER; SPIT; base editing; and prime editing

Introduction

The golden era of biotechnology ushered in a plethora of new tools for our toolkits, including precision gene editing via homologous recombination [1], base editing [2], prime editing [3–5], large serine recombinase-mediate gene insertion [6], and CRISPR transposases [7]. These have the potential to cure any genetic illness. With adeno-associated viral (AAV) vectors, gene therapy has been brought to bear in the human body, sometimes greatly improving function in patients [8]. However, full cures are generally not possible, and many genetic diseases remain untreatable currently. In the cases wherein effective treatments or cures are not attainable, it is not for a lack of *in vitro* tools, but rather our ability to deliver them to the cells around the body that need to be repaired.

Extravasation of the vector in the appropriate anatomical locales is the main issue here. AAV treatments seeking to transduce the liver are fairly straightforward [9]. This is because AAVs have a capsid diameter of ~25 nm, and the pores in the human vascular endothelium of the liver and spleen are fenestrated, leading to particles between ~8 nm-150 nm in diameter being delivered to those organs [10]. Similarly, injecting AAV vectors directly into the retina for treatment is a somewhat straightforward option [11]. Finally, diseases affecting white blood cells can be treated by genetically manipulating some of the patient's leukocytes *ex vivo*, expanding them, eliminating some proportion of the patient's hematopoietic stem cells [12], and then infusing the edited leukocytes back into the patient [13].

However, some genetic illnesses affect tissues throughout the body. Unfortunately - in terms of vector delivery, the diameter of the vascular endothelial pores in most regions may be between 5-12 nm [14]. The vascular endothelial pores at the blood-brain barrier are more like 1-2 nm in diameter. And particles ~5-8 nm or smaller are filtered out by the kidneys. While AAVs can utilize transcytosis to extravasate [15] in some anatomical locales, it is unclear if a single serotype or capsid variant can do so systemically. High doses can be cytotoxic as well [16].

Importantly, AAVs can only encode ~5 kb of DNA maximum, which is not enough to cure certain individuals with genetic disorders wherein long stretches of nucleotides are affected [17,18], [19–21] - e.g., at least some cases of Duchenne muscular dystrophy [22].

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According to Anzalone et al., "…in principle prime editing can correct up to ~89% of the 75,122 pathogenic human genetic variants in ClinVar…" [23]. New prime editing-based techniques can almost surely cover even more pathogenic human genetic variants [24]. However, multiple proteins, RNAs, or DNA molecules (or at least a single, large DNA molecule) may be required in particular cases if more complex molecular machinery must be employed - e.g., if heterochromatin is an issue. Also, the cost of producing sufficiently high titers of AAV vectors and other viral vectors for therapeutic purposes is at least currently very steep [25–27].

Intravenously injected lipid nanoparticle (LNP)-encapsulated CRISPR ribonucleoproteins (RNPs) have been successfully employed to treat genetic conditions affecting the liver and, to some degree, the lungs [28,29]. However, current LNP formulations at least do not effectively reach or target cells in the CNS, heart, or kidneys after intravenous injection [29,30]. Also, the larger the LNP - the less efficient it is at extravasation. Thus, it cannot encapsulate very large cargos unless targeted to the liver or spleen. Intrathecal and intracerebroventricular administration of RNPs - or direct intraparenchymal injection of RNPs into the striatum - will not result in widespread CNS editing [31].

Lastly, AAV.CAP-Mac was recently described, which can cross the blood-brain barrier and reach cells throughout the CNS [32]. However, some brain regions in the 17-year-old adult rhesus monkeys were not transduced [32]. Also, AAV therapies are currently expensive - and possibly genotoxic/cytotoxic [33]. Finally, it is possible that AAV.CAP-Mac may not work in humans as well as it did in non-human primates.

Microglial Replacement

The small molecule CSF1R inhibitor, PLX5622, potently depletes microglia [34]. PLX3397 is an FDA approved and also potently depletes microglia in mice and non-human primates. It may do so in humans as well, although dosing. and replace them in a fairly harmless manner [35]. I thought it might be possible to edit induced pluripotent stem cell-derived macrophages (iPSC-Macs) [36] *ex vivo*, and then infuse them intrathecally or intravenously to replace a patient's microglia with ones that can employ your COURIER system.

Normally, the remaining population of microglia or peripheral monocytes might outcompete iPSC-Macs that are infused into the bloodstream [37,38], but an inhibitor-resistant CSF1R variant has been developed [39]. Thus, constant selection is possible - which should enable non-invasive microglial replacement.

Also, while microglia sample their surrounding microenvironment constantly with protrusions that extend and retract, they may not move around much from place to place [40,41]. To address this potential issue, random migration of the iPSC-Mac-derived microglia in the CNS could be induced or enhanced by inhibiting LRRK2 [42]. Other methods of inducing hyper-motility are also possible [43,44].

For gene editing purposes, the iPSC-Macs could also be "off-the-shelf" [45]. After treatment, a small molecule like a rapamycin analog that can penetrate the blood-brain barrier (BBB) can be administered to eliminate them via caspase-9 activation [46], and the patient's hematopoietic stem cells (HSCs) would repopulate the microglia.

Neuronal Gene Editing

Base editing of the SMN2 gene in neurons is a viable strategy for spinal muscular atrophy [47], [48]. In this case, the COURIER cargo could be an mRNA molecule encoding a zinc finger base editor. However, they currently have too much off-target activity [49]. TALE base editors exist, as well [50]. Alternatively, the cargo could be a self-amplifying RNA (saRNA) vector [51]. An saRNA vector could enable the use of a CRISPR base editor, wherein a subgenomic promoter effectively replicates an sgRNA module but weakly replicates the proteinaceous component of the base editor [52,53]. As larger RNA molecules were packaged less efficiently in COURIER, but dual delivery was possible, a trans-amplifying RNA (taRNA) vector could be employed [54]. In either case, the RNA-dependent RNA polymerase could be inhibited somehow in the edited microglia.

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With saRNA and taRNA vectors, there may be some considerations with superinfection exclusion and possibly gene dosage compensation [55,56].

As opposed to an AAV vector, this process might be too slow for SMA1 patients. However, as described in [47], clinicians could co-administer the antisense oligonucleotide drug nusinersen to extend the therapeutic window.

Two other CNS genetic illnesses that microglial replacement could potentially help with are Tay-Sachs, i.e., prime editing to remove a four-base duplication [57]. From what I saw, Tay-Sachs may have a somewhat longer therapeutic window than SMA1. Also, I think Huntington's disease can be mitigated by base editing - at least in some instances [58].

Secreted Particle Information Transfer (SPIT) could also be exported for gene editing [59]. However, mRNA export may be more efficacious, as opposed to RNP secretion. Also, SPIT utilizes viral capsid proteins, which would probably be potently immunogenic.

COURIER Immunogenicity

As Horns et al. mentioned in their COURIER article, the protein nanocages may not really cause an immune response. Low-dose dexamethasone could possibly be sufficient to prevent undue inflammation from all of the COURIER components. Dexamethasone has been used to counter excessive immune responses in patients with SARS-CoV-2 [60].

If not, there are other strategies. The innate immune response to dsRNA generated from vector replication could possibly be attenuated by the expression of the MERS-CoV ORFa protein [61]. If cyclic induction is required over a long period of time, the adaptive immune response to the gene editing components may need to be attenuated as well. First, the vector could encode a deimmunized dCas9 protein [62]. Second, it may help if the vector were to incorporate multiple, tandem miR-142-3p binding sites in the dCas9 mRNA 3'UTR [63]. Third, the vector could express the SARS-CoV-2 ORF6 protein, which inhibits the MHC class I pathway [64].

Of course, adding more elements requires a more packaging space. If necessary, one could theoretically minimize the size of the required elements. Or, as was also mentioned in the COURIER article, alternative nanocage architectures could tune cargo capacity.

Testing This *In Vitro*

There is a 2023 *Nature* paper that describes a method of integrating microglia into neuronal organoids by co-culturing said organoids with iPSC-Macs [65]. The iPSC-Macs take on a more microglia-like identity, as *in vivo* when transplanted into the brains of mice. These "microglia-sufficient" organoids could possibly be used to test the gene editing approach in addition to traditional 2D culture.

Conclusion

Microglial replacement in combination with COURIER or SPIT could enable cheap and effective CNS gene editing.

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