Molecular Therapy
Methods & Clinical Development
Editorial

Good reasons for targeting SARS-CoV-2 by engineered extracellular vesicles

The possibility to inhibit pathogens by treatment with neutralizing antibodies and antibody fragments (passive immunization) has been discussed to a great extent in the case of SARS-CoV-2, mainly in the early phases of the pandemic, when a vaccine was not yet available.[1] Apart from sera collected from recovering patients, several nanobodies were selected. Nanobodies are small antigen-binding fragments (~15 kDa) that are derived from heavy chain only antibodies present in camelids and cartilaginous fishes.[2] Their tiny dimensions and elevated structural stability were exploited to prepare a spray formulation for topical treatment of the upper airways.[3–6]

In *Molecular Therapy Methods & Clinical Development*, Scott and colleagues illustrate a new application of nanobodies made possible by the modification of extracellular vesicles (EV).[7] In their article "Engineered extracellular vesicles directed to the spike protein inhibit SARS-CoV-2," they demonstrate that insertion of an anti-spike-specific nanobody into a portion of the major extracellular loop of CD63 (VHH72-CD63) enabled the EV (mainly small EV) to target the spike protein and exert a neutralizing effect on the antigen. This nanobody-exclusive application indicated that format does matter and raises the following questions: Do we need EV for passive immunization? Are there peculiarities of EV that no other macromolecule can provide?

Researchers who have dealt with EV know how cumbersome their purification and characterization are and how limited their yields usually are. These conditions render their production much more expensive than that of nanobodies and probably even monoclonal IgGs. Therefore, any advantage represented by EV delivery of therapeutics to the target antigen must compensate for the extra effort necessary for the preparation of such reagents. Scott and colleagues underline that EV can be used to transport a combined therapeutic payload. This is an interesting argument, as this strategy may lead to a curative application based on the use of multiple agents carried by EV, such as the neutralizing binders as well as other bioactive molecules. Phage display and *in vivo* biological screening are currently applied to explore the potential uses of EV to target specific sites. Studies have demonstrated that therapeutic EV enriched with bioactive ischemic myocardium-targeting peptides or cardiomyocyte specific peptides can be delivered to an inflamed, injured heart in a very precise manner, achieving superior efficacy outcomes.[8–10] Similarly, VHH72-CD63 might be targeted to the respiratory tract, exploiting synergistic effects of combining the neutralization effect with an anti-inflammatory "side effect," consistent with the pharmacological properties of mesenchymal stem cell-derived EV in COVID-19 patients.[11]

A further and conceptually different protective mechanism that would still require a thorough evaluation could be the possibility of obtaining antigen agglutination by means of EV, similar to what was proposed for nanobody multimers or self-assembling nanoparticles successfully targeting bacterial infections and neutralizing toxins.[12,13] EV are indeed nanoparticles carrying several copies of the active binder; their multivalent nature might offer the possibility to simultaneously scavenge several molecules of soluble antigen. This application might decrease the overall pathogen and/or toxin concentrations, while the resulting aggregates could activate phagocytosis and generate an immune response. Very recently, it has been shown that small EV improve the survival of mice infected with methicillin-resistant *Staphylococcus aureus* (MRSA) by serving as decoys for bacterial toxins.[14] Transferred small EVprotect host cells *in vitro* by serving as scavengers that can bind multiple toxins, and the capability of EV to specifically bind protein-containing structures has also been shown for viruses. CD4 molecules displayed on the surface of CD4+ T cell-derived EV can bind to envelope proteins of HIV-1, hindering virus interactions with target cells. Cocozza et al. suggested that EV carrying ACE2 are more efficient than soluble ACE2 as decoys for SARS-CoV-2 S protein-containing lentivirus, leading to a reduction of infectivity that correlates with the level of ACE2.[15] Another aspect that should be considered is that, apart from the replicative capacity, small EV share several biophysical and functional features with enveloped viruses (including the betacoronavirus). Like enveloped viruses, EV are nano-sized membranous particles consisting of cell-membrane proteins and lipids.[16] They use common cellular mechanisms such as the endosomal system as biogenesis pathways[17] and are very similar in size (small EV size range from 50 to 200 nm; virions being ~100–200 nm) and buoyant density (small EV 1.13–1.18 g/L versus enveloped viruses 1.16–1.18 g/L).[18] The aforementioned peculiarities confer, to small EVs, a similarity to enveloped viruses to such an extent that they have also been called "non-infectious defective viruses."[19] Most importantly, like in enveloped viruses, proteins and lipids expressed on the EV surface can interact with the plasma membrane of target cells, triggering biological functions and endocytosis of particles.[20,21] Thus, circulating EV in body fluids virtually recapitulate a systemic viral infection that may efficiently stimulate the immune system.

The latter point, however, should be carefully addressed before attempting to translate such an approach into the clinic. In general, the results by Scott and colleagues are encouraging, suggesting that EV may be a suitable tool to target viruses. VHH72-CD63 EV can effectively neutralize both pseudotyped viral vectors, compared with relevant controls including RBD-targeting S35 mAbs, recombinant bivalent VHH72, and COVID-19 convalescent plasma. Most importantly, they corroborate their data showing effectiveness against several variants of concern in live-virus assays. A second piece of

information that warrants further investigation would be optimizing the site for integrating novel functionality into CD63 tetraspanin. Indeed, the insertion of an antibody into the specific extracellular loop 2 (EX2.4) seems to be instrumental also for other virus-specific targets, such as scFv derived from a broadly neutralizing N6 antibody targeted to HIV gp160, thus making this specific scaffold a potentially universal site for EV functionalization.[7]

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