

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

Engineered Mesenchymal Cells Improve Passive Immune Protection Against Lethal Venezuelan Equine Encephalitis Virus Exposure

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ABSTRACT

Mesenchymal stromal cells (MSCs) are being exploited as gene delivery vectors for various disease and injury therapies. We provide proof-of-concept that engineered MSCs can provide a useful, effective platform for protection against infectious disease. Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne pathogen affecting humans and equines and can be used in bio-warfare. No licensed vaccine or antiviral agent currently exists to combat VEEV infection in humans. Direct antibody administration (passive immunity) is an effective, but short-lived, method of providing immediate protection against a pathogen. We compared the protective efficacy of human umbilical cord perivascular cells (HUCPVCs; a rich source of MSCs), engineered with a transgene encoding a humanized VEEVneutralizing antibody (anti-VEEV), to the purified antibody. In athymic mice, the anti-VEEV antibody had a half-life of 3.7 days, limiting protection to 2 or 3 days after administration. In contrast, engineered HUCPVCs generated protective anti-VEEV serum titers for 21–38 days after a single intramuscular injection. At 109 days after transplantation, 10% of the mice still had circulating anti-VEEV antibody. The mice were protected against exposure to a lethal dose of VEEV by an intramuscular pretreatment injection with engineered HUCPVCs 24 hours or 10 days before exposure, demonstrating both rapid and prolonged immune protection. The present study is the first to describe engineered MSCs as gene delivery vehicles for passive immunity and supports their utility as antibody delivery vehicles for improved, single-dose prophylaxis against endemic and intentionally disseminated pathogens. Stem Cells Translational Medicine 2016;5:1–10

SIGNIFICANCE

Direct injection of monoclonal antibodies (mAbs) is an important strategy to immediately protect the recipient from a pathogen. This strategy is critical during natural outbreaks or after the intentional release of bio-weapons. Vaccines require weeks to become effective, which is not practical for first responders immediately deployed to an infected region. However, mAb recipients often require booster shots to maintain protection, which is expensive and impractical once the first responders have been deployed. The present study has shown, for the first time, that mesenchymal stromal cells are effective gene delivery vehicles that can significantly improve mAb-mediated immune protection in a single, intramuscular dose of engineered cells. Such a cell-based delivery system can provide extended life-saving protection in the event of exposure to biological threats using a more practical, single-dose regimen.

INTRODUCTION

The concept of engineering mesenchymal stromal cells (MSCs) to express therapeutically relevant biomolecules is an active area of research development. It is well known that MSCs have a low immunogenic profile [1–5], making them suitable gene therapy vectors for a range of indications and therapeutic molecules [6–16]. Drugs expressed by engineered MSCs have shown improved kinetics and therapeutic efficacy compared with direct administration of the same drug [17, 18]. Thus, engineered MSCs provide an attractive alternative to both direct injection of biologic agents, which could have inherent limitations in manufacturing, delivery, or halflife, and viral-based gene transfer strategies [19–21], which pose a high clinical risk for adverse effects and immune reactions [22, 23].

Important opportunities also exist to use strategically engineered MSCs as prophylactics against both endemic and weaponized pathogens. As evidenced in the 2014–2015 Ebola outbreak,

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manufacturing and delivery bottlenecks limit the real-world usefulness of prophylactic antibodies [24, 25]. However, we predicted that the protective window of a given antibody could be extended using engineered MSCs as an antibody delivery platform, whereby the cells constitute a renewable antibody source that compensates for the antibody's natural rate of degradation. Although in many therapeutic contexts, autologous cell therapy is ideal, only a stockpiled allogeneic source would be suitable for prophylactic use or in crisis situations. The advantage of using MSCs as the therapeutic agent is that they are considered immune-privileged or, at least, immune-evasive; thus, they can be used allogeneically, as shown in hundreds of registered MSC clinical trials. This approach requires a cell population that is amenable to genetic engineering, is facile to harvest and stockpile, and displays functional efficacy in vivo. We selected human umbilical cord perivascular cells (HUCPVCs), a rich source of MSCs [26-30] derived from Wharton's jelly that rapidly expand to clinically relevant numbers and is similar to bone marrow-derived MSCs [26, 27, 31]. HUCPVCs are particularly ideal for military applications, because they are obtained noninvasively from tissue classified as medical waste and are amenable to stockpiling in either native or engineered form [32].

For the present proof-of-concept study, we selected Venezuelan equine encephalitis virus (VEEV), an alphavirus endemic to South and Central America [33, 34], as our infectious disease model. The mosquito-borne virus is naturally transmitted to humans and equines through a reservoir of infected rodents [20], and VEEV outbreaks are increasingly being reported in the southern United States [34]. In humans, VEEV infection results in an acute, febrile illness that can progress to neurological symptoms, including encephalitis [35]. The most recent natural outbreak occurred in Columbia in 1995, affecting 75,000 people and resulting in more than 300 fatalities [36].

VEEV is also classified as a category B priority biodefense agent by the National Institute of Allergy and Infectious Diseases and the Centers for Disease Control and Prevention. Evidence from animal studies and documented accidental laboratory exposures indicates that aerosol exposure to VEEV is highly infectious and results in abrupt onset of symptoms 2–5 days after exposure [20, 37]. Moreover, VEEV is readily amplified to high titers in tissue culture and exhibits sufficient stability to be aerosolized and disseminated on a large scale [38]. Consequently, VEEV represents a significant biodefense threat.

Currently, no licensed vaccines or antiviral agents are available to protect humans against VEEV exposure. Two previously developed vaccines, TC-83 and C-84, have been used in humans under Investigational New Drug status but were not approved because of their high rate of reversion and poor immunogenicity, respectively [39]. More recently, the live-attenuated vaccine V3526 was shown to be unsafe for use in humans [40] despite its success in rodent and nonhuman primate animal models of VEEV infection [41-43]. To address the current lack of licensed medical countermeasures to combat VEEV infection, we previously developed a humanized antibody that neutralizes VEEV [44, 45]. This antibody fully protects mice from a lethal dose of VEEV when administered 24 hours before, or after, exposure [45]. Ideally, a single administration of the anti-VEEV antibody should protect military personnel or first-responders deployed to endemic regions or areas affected by intentional release, for several months-this is not possible using a single administered dose of the purified antibody.

In the present study, we tested the utility of HUCPVCs as a gene delivery platform to improve passive immunity against a pathogen. Compared with the purified anti-VEEV antibody, we show that a single intramuscular injection (IM) of anti-VEEVengineered HUCPVCs can generate, and maintain, higher circulating antibody titers. Most critically, pretreatment with engineered HUCPVCs significantly improved both survival and morbidity after exposure to a high lethal dose of highly virulent VEEV. Engineered HUCPVCs maintained the protective anti-VEEV titers for up to 38 days after implantation into mice and continued to secrete anti-VEEV antibody for more than 3 months. Thus, engineered HUCPVCs represent an attractive antibody delivery modality that improves the efficacy of already existing prophylactic countermeasures to infectious disease.

MATERIALS AND METHODS

Cell Culture

HUCPVCs [26] cryopreserved at passage 2 were provided by Tissue Regeneration Therapeutics, Inc. (TRT; Toronto, ON, Canada, http://www.verypowerfulbiology.com). HUCPVCs were thawed according to TRT's proprietary standard operating procedures. HUCPVCs were expanded in proliferation media comprising minimal essential medium- α GlutaMAX, with no riboxynucleosides or deoxynucleosides (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com), supplemented with 15% mesenchymal stem cell-fetal bovine serum (MSC-FBS; Thermo Fisher Scientific Life Sciences). At 70%–80% confluence, HUCPVCs were enzymatically detached from the culture vessel by brief incubation with TrypLE Select (Thermo Fisher Scientific Life Sciences) and reseeded at a density of 4,000 cells per cm². HUCPVC culture conditions were maintained at 37°C, 5% CO₂, and 80% relative humidity, with media replacement every 3–4 days.

For the in vivo studies, the HUCPVCs were engineered within 10 days of thawing and implanted 4 days after transduction with recombinant adenovirus. HUCPVCs were harvested by enzymatic detachment with TrypLE Select and washed once with Hank's balanced salt solution (HBSS), without calcium or magnesium (Thermo Fisher Scientific Life Sciences). After centrifugation, HUCPVCs were resuspended in an appropriate volume of HBSS to generate 150- μ I doses (75 μ I per thigh) and immediately injected.

Recombinant Adenovirus Constructs and Transduction

A single open reading frame, encoding the full-length, humanized anti-VEEV antibody, was previously cloned into a recombinant adenovirus serotype 5 vector (pAd5-anti-VEEV [44]) using the pAd-Easy system (Stratagene, Mississauga, ON, Canada, http:// www.agilent.com). Custom good laboratory practice-grade pAd5-anti-VEEV was produced by Norgen Biotek Corp. (Thorold, ON, Canada, http://www.norgenbiotek.com). A recombinant adenovirus encoding enhanced green fluorescent protein (pAd-eGFP; Vector BioLabs, Philadelphia, PA, http://www.vectorbiolabs. com) was used to optimize transduction protocols and to generate sham-engineered HUCPVCs for in vivo negative controls. Recombinant adenovirus expressing firefly luciferase (pAd5-luc; Vector BioLabs) was used to generate bioluminescent HUCPVCs for in vivo optical imaging.

HUCPVCs were transduced by 2-hour incubation with a minimal volume of recombinant adenovirus suspended in

nonproliferation medium comprising Dulbecco's modified Eagle's medium, high glucose, GlutaMAX (Thermo Fisher Scientific Life Sciences) supplemented with 2% MSC-FBS, without the addition of transfection mediators. Following viral transformation, engineered HUCPVCs were maintained in nonproliferating media.

Optimization of large-scale adenovirus-mediated transduction of HUCPVCs was performed by incubating confluent HUCPVCs, cultured in 150-cm² culture flasks, with a range of 1-400 pAd5-eGFP viral particle units per cell. The nuclei of cells were stained with Hoechst 33342 (Thermo Fisher Scientific Life Sciences). At 24 and 48 hours after transduction, the images were captured at \times 4 and \times 10 magnification with an EVOS digital inverted microscope (Advanced Microscopy Group, Burlington, ON, Canada, http://www.thermofisher.com). Transduction efficiency was determined by calculating the percentage of eGFPpositive cells using ImageJ, version 1.47 (National Institutes of Health, Bethesda, MD, http://www.imagej.nih.gov/ij). These results were validated by fluorescence-activated cell sorting (FACS) analysis. The percentage and relative intensity of eGFP-positive cells in lots of 250,000 engineered HUCPVCs were analyzed using a BD FACS Ariallu (BD BioSciences, Mississauga, ON, Canada, http://www.bdbiosciences.com), equipped with BD FACSDiva, version 6.1.3.

Proliferation and Directed Differentiation Assays

Standard proliferation and differentiation assays were used to assess the effects of genetic engineering. A minimum of three HUCPVC lots were tested in each experiment, with unengineered and engineered HUCPVCs cultured in parallel. Proliferation was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay (Thermo Fisher Scientific Life Sciences) according to the manufacturer's instructions, with four samples from each cell population tested 24 hours and 7 days after seeding.

For directed differentiation assays, StemPro Chondrogenesis, Adipogenesis and Osteogenesis Differentiation Kits (Thermo Fisher Scientific Life Sciences) were used according to the manufacturer's instructions. A minimum of three HUCPVC lots were tested in each experiment, with unengineered and engineered HUCPVCs cultured in parallel. Uninduced cells were cultured and analyzed in parallel; spontaneous induction of the analyzed markers was not observed. Bright-field and phase contrast images of stained cultures were captured before dye extraction at $\times 10$ and $\times 20$ magnification using an Axiocam ERc 5s camera mounted to a Zeiss Axiovert 40CFL inverted microscope (Zeiss, Jena, Germany, http://www.zeiss.com).

Chondrogenic-specific proteoglycans were detected in chondrogenesis micromass cultures by staining with alcian blue (Sigma-Aldrich, Oakville, ON, Canada, http://www.sigmaaldrich. com). The cells were fixed for 20 minutes with 10% neutral buffered formalin (NBF; Fisher Scientific Ltd., Ottawa, ON, Canada, http://www.fishersci.ca), and then incubated with 1% alcian blue in 0.1 M hydrochloride (HCl; Sigma-Aldrich) overnight (3–7-day cultures) or for 1–2 hours (7-day or older cultures). The cultures were rinsed once with 0.1 M HCl, three times with phosphatebuffered saline (PBS) and kept in solution for imaging. Alcian blue staining was extracted by incubating overnight in 6 M guanidine HCl, with absorbance measured at 620 nm. Adipocytes were detected by Oil Red O (Sigma-Aldrich) staining. The cells were rinsed twice with PBS, fixed in 10% NBF, and gently rinsed again. The cultures were incubated for 5 minutes with 60% isopropanol (Sigma-Aldrich) and then for 30 minutes to 1 hour with freshly prepared Oil Red O working solution (three parts 0.3% Oil Red O in isopropanol to two parts dH_2O). The cultures were liberally washed with tap water and counterstained with Hematoxylin Gill number 3 (Sigma-Aldrich). Oil Red O dye was extracted from lipids by incubation with 100% isopropanol for 10 minutes by shaking, with absorbance measured at 510 nm. Induction of osteogenesis was assessed by alkaline phosphatase staining, according to the manufacturer's instructions (Leukocyte Alkaline Phosphatase kit 86R; Sigma-Aldrich). The cultures were counterstained with Hematoxylin Gill number 3.

Enzyme-Linked Immunosorbent Assay

For in vitro analyses, engineered HUCPVCs were seeded in 150-cm² culture flasks at a density of 2.5 million cells per flask, with media changes every 2-3 days. Total anti-VEEV IgG in the conditioned media was assayed by functional enzyme-linked immunosorbent assay (ELISA), as described previously [45]. In brief, microwell plates were incubated for 2 hours at room temperature with live VEEV-attenuated vaccine strain TC-83 suspended in $1 \times$ coating buffer. The plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and blocked with SuperBlock (PBS) Blocking Buffer (Pierce Biotechnology, Rockford, IL, http://www.thermofisher.com) according to the manufacturer's instructions. The samples were serially diluted in SuperBlock and incubated in antigen-coated microwells for 1 hour at room temperature. Following three PBS-T washes, anti-human IgG conjugated to horseradish peroxidase (Abcam, Toronto, ON, Canada, http://www.abcam.com) was diluted to 1:5,000 in SuperBlock and incubated for 30 minutes at 37°C. The plates were washed three times with PBS-T before the addition of 1-Step Ultra-TMB (Pierce Biotechnology) chromogenic substrate. The plates were incubated at room temperature, in the dark, for 10-15 minutes, with the reaction stopped by the addition of an equal volume of 2 M phosphoric acid (Sigma-Aldrich). Absorbance was read at 420 nm by a microplate autoreader (Molecular Devices, Toronto, ON, Canada, http://www.moleculardevices.com). Standard curves were generated for each assay using serially diluted humanized anti-VEEV IgG [44]. In addition to standard chromagen and zero blanks, conditioned media from unengineered HUCPVCs served as a negative control. The limit of detection, defined as 1.2 times the average absorbance of culture media from unengineered HUCPVC controls, was subtracted from the experimental samples. All samples were analyzed in triplicate at threefold serial dilutions ranging from 1:10 to 1:1350. Concentrations of antibody were extrapolated from a polynomial standard curve with a correlation coefficient of 0.996 (in vitro assay) or 0.999 or 1 (in vivo assays).

Serum titers of anti-VEEV IgG in mice treated with purified antibody or engineered HUCPVCs were quantified using the ELISA, as described. Blood samples were collected from a superficial tail vein at various time points, with the sera recovered by centrifugation. The limit of detection was generated for each microplate using serum obtained from mice injected with unengineered HUCPVCs and applied against experimental samples.

Animal Studies

The animal experiments were performed in accordance with Canadian Council on Animal Care-approved protocols and

institution standards of care. Eight- to 10-week-old female BALB/c^{nu/nu} mice (Charles River Laboratories, Gatineau, QC, Canada, http://www.criver.com) were housed in pathogen-free conditions and provided with sterilized water and irradiated chow ad libitum. Groups of five age-matched mice received an intramuscular injection of 2.5 million engineered HUCPVCs or 50 μ g of purified anti-VEEV antibody suspended in sterile saline. HUCPVCs were administered as 75- μ l injections to each thigh (150 μ l total per mouse). Challenge studies were performed in a biosafety level 3 (BSL3) containment suite using the Trinidad Donkey strain of VEEV (VEEV TrD). Under sodium pentobarbital anesthesia, the mice were intranasally inoculated with 1 imes 10⁴ VEEV TrD plaque-forming units (PFUs) suspended in 50 μ l of PBS. To maintain objectivity, clinical symptoms of infectivity (weight loss, piloerection, hunching, inactivity, excitability, and paralysis) were assessed blind by staff otherwise uninvolved in the study. The severity of clinical signs was measured on a scale of 1-4, with 0 indicating the absence of clinical symptoms. Any mouse scoring 4 was humanely euthanized. The mice were evaluated for 21 days after exposure. Only data from days 1-14 are reported, because no significant changes were observed between days 15 and 21.

Neutralization Assay for In Vitro Assessment of Anti-VEEV IgG Activity in Serum

For plaque reduction neutralization titer (PRNT) determination, the serum was pooled for each group selected for comparison. The pooled serum for the unchallenged mice on days 24, 31, and 38 days after treatment with engineered HUCPVCs was limited to samples positive for antibody. The serum was heat inactivated at 56°C for 30 minutes and then diluted in twofold serial dilutions and coincubated with diluted VEEV TC83 for 30 minutes at room temperature. The serum-virus mixture was applied to monolayers of VERO cells in six-well culture plates and incubated at 37°C for 1 hour. The mixture was replaced with Tragacanth Gum (Sigma-Aldrich) diluted in maintenance medium and then incubated for 2 days at 37°C. After 48 hours, the overlay was removed, the cells were fixed and stained with 0.3% crystal violet (Sigma-Aldrich) in HistoPrep (Thermo Fisher Scientific Life Sciences), and the plaques were counted. The assays were performed in duplicate.

Bioluminescence Imaging

Bioluminescence imaging was performed using the Xenogen In Vivo Imaging System Spectrum (PerkinElmer, Hopkinton, MA, http://www.perkinelmer.com), equipped with Living Image software, version 4.3.1. HUCPVCs were batch-engineered with pAd5luc at 200 multiplicity of infection (MOI), as described. Three mice were treated with a single dose of 2.5 million engineered HUCPVCs suspended in 75 μ l of HBSS and injected intramuscularly into the left hind limb. Eight minutes before imaging, the mice received an intraperitoneal injection of 150 μ l of D-luciferin Ultra salt solution (PerkinElmer). The mice were immobilized by isoflurane anesthesia delivered through a nose cone in the imaging chamber. Images were obtained immediately before cell injection and then periodically, starting at 2 hours after injection and up to 123 days after cell injection. For quantification, the scale intensity of the longitudinal images was normalized, and a region of interest (ROI) was selected according to the signal intensity. The ROI was kept constant and the total flux (photons emitted per second) measured. The study was terminated at 123 days before the loss of bioluminescence.

RESULTS

HUCPVCs are readily transduced using various methods [28, 31], including adenovirus, which does not alter the important immunological properties of MSCs [46]. Batch-scale transduction of up to five million cells per culture vessel was optimized using a recombinant adenovirus expressing enhanced green fluorescent protein (pAd5-eGFP). The transduction efficiency exceeded 98% using 200 MOI of viral particles per cell, as determined by microscopic analysis of unfixed cells and confirmed by counts of eGFPpositive cells using FACS (Fig. 1A, 1B). Varying eGFP intensity in cocultured cells was observed by FACS and fluorescent microscopy, suggesting that many cells can express multiple transgene copies or can express the transgene at different levels.

We had previously cloned the humanized anti-VEEV antibody gene into a single open reading frame to generate a recombinant serotype 5 adenovirus construct (pAd5-anti-VEEV) [44]. HUCPVCs were engineered with the recombinant pAd5-anti-VEEV at 200 MOI with no apparent toxicity. The effects of genetic engineering on the proliferation and multidifferentiation potential of HUCPVCs were assessed. Compared with unengineered cells from the same cord, the proliferation of HUCPVCs expressing anti-VEEV was reduced within 24 hours of seeding and stalled by day 7 (Fig. 2A, 2B). Reduced cell division in the engineered cells is advantageous, because the high proliferative capacity of HUCPVCs can limit the longevity of the episomal transgene [31], leading to potentially stochastic results. In directed differentiation assays, unengineered and antibody-secreting HUCPVCs induced equivalent levels of chondrogenic, adipogenic, and osteogenic lineage markers (Fig. 2C-2E). Early expression of these markers was also detected at the same time in the engineered and control cells (data not shown). Thus, HUCPVCs are phenotypically unaltered by adenovirus-mediated genetic engineering, despite constitutive expression of the exogenous antibody.

To determine whether engineered HUCPVCs can secrete therapeutically useful quantities of the anti-VEEV antibody in vitro, culture supernatant was harvested from the engineered cells every 3-4 days, and the antibody levels were quantified by functional ELISA. Four days after transduction, one million engineered HUCPVCs secreted 6–10 μ g of anti-VEEV IgG daily (Fig. 3), increasing to approximately 28 μ g/day by day 7 (Fig. 3). This rate could be maintained for 21 days in culture, depending on the cell lot and despite stalled proliferation. The inherent variability in sustained antibody production after day 7 was observed in HUCPVCs isolated from different donors, although reproducible results were obtained using individual cell lots (Fig. 3). In a previous study, administration of 50 μ g of purified anti-VEEV IgG per mouse protected the mice against VEEV exposure [45]. Less than 25 μ g of this injected antibody reached the circulation (Fig. 4A); thus, we predicted that an approximate dose of two million engineered HUCPVCs would generate protective serum anti-VEEV titers in mice within 24 hours of administration. If the engineered HUCPVCs persisted, they could potentially regenerate protective titers daily for up to 3 weeks (Fig. 3) and replenish the antibody lost by natural degradation.

We next assessed the level and duration of circulating anti-VEEV produced by engineered HUCPVCs in vivo. IM injection is the most practical route of administration in combat theaters;



Figure 1. HUCPVCs are engineered at high efficiency. Scaled-up transduction efficiency of HUCPVCs with a recombinant adenovirus encoding eGFP reporter at 200 multiplicity of infection. **(A):** Twenty-four hours after transduction, nearly all the cells were positive for eGFP. Nuclei were stained with Hoechst 33324 (blue) (original magnification \times 10). **(B):** Fluorescence-activated cell sorting analysis of 250,000 cells in **(A)** verified that 98.6% of cells were GFP-positive. The GFP-negative population is shown in blue. Abbreviations: eGFP, enhanced green fluorescent protein; FITC-A, fluorescein isothiocyanate A; HUCPVCs, human umbilical cord perivascular cells.



Figure 2. In vitro characterization of engineered HUCPVCs. **(A, B):** HUCPVCs engineered with the anti-VEEV transgene showed reduced proliferative capacity compared with unengineered HUCPVCs in 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assays. **(A):** Within 24 hours, proliferation was stalled in the engineered HUCPVCs. **(B):** By day 7, the unengineered cells had more than doubled and the number of engineered cells had not significantly increased after seeding. **(C-F):** Differentiation potential of HUCPVCs was retained after engineering with the anti-VEEV transgene. **(C):** Chondrogenesis micromass culture. Cells positive for the proteoglycan marker alcian blue were evident 7 days after induction (original magnification $\times 10$). **(D):** Adipogenesis. Lipid vesicles stained with Oil Red O were visible 7 days after induction. **(E, F):** Osteogenesis. Alkaline phosphatase-positive cells (violet) were detected 7 **(E)** and 14 **(F)** days after induction (original magnification $\times 20$). **(F):** Nuclei were counterstained with hematoxylin (blue). Abbreviations: HUCPVCs, human umbilical cord perivascular cells; VEEV, Venezuelan equine encephalitis virus.

thus, we developed our animal model using this scheme. In vivo testing was performed using athymic (nude) mice, because MSCs are not suitable for xenogeneic grafting despite being nonalloreactive in humans [47]. Nude mice received an IM injection of 2.5 million antibody-secreting HUCPVCs per mouse 4 days after genetic engineering. The control groups received

50 μ g of purified anti-VEEV antibody per mouse intramuscularly or 2.5 million sham HUCPVCs engineered with eGFP per mouse. No adverse effects were observed after IM administration of the cells or antibody. The serological profiles of circulating anti-VEEV were generated from blood samples taken at various time points. The purified anti-VEEV antibody

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Figure 3. Engineered HUCPVCs can produce therapeutically useful quantities of anti-VEEV antibody in culture. Culture supernatants were harvested every 3-4 days from HUCPVCs engineered with the anti-VEEV transgene. Functional humanized anti-VEEV IgG was quantified in the conditioned media by enzyme-linked immunosorbent assay, using live VEEV strain TC-83 as antigen. Results from triplicate experiments for HUCPVCs harvested from two representative umbilical cords (white and black) are shown. The rate of antibody secretion was consistent between HUCPVC lots for the first 7 days after transduction, increasing to approximately 28 μ g/million cells per day. Inherent variability in the capacity of donor-specific HUCPVCs to sustain antibody synthesis is evident after this point, although reproducible results were obtained using cells from the same lot. The peak rate of anti-VEEV IgG synthesis could be maintained for up to 21 days after engineering (dark gray). Abbreviations: HUCPVCs, human umbilical cord perivascular cells; VEEV, Venezuelan equine encephalitis virus.

administered by direct injection exhibited a serum half-life of 3.7 \pm 0.5 days (Fig. 5).

The passively administered purified anti-VEEV immediately began to deteriorate (Fig. 4A). In contrast, the serum antibody titers of mice receiving engineered HUCPVCs increased for up to 10 days after implantation (Fig. 4A). The predicted protective titers were maintained for up to 38 days after implantation in 40% of mice, despite an apparent decline in antibody synthesis. Intriguingly, serum antibody was still detected at day 109 in 10% of the mice, albeit at marginal levels (Fig. 4A). The rate of antibody decay in the mice treated with HUCPVCs expressing anti-VEEV never fit the half-life profile for the passively transferred antibody. Together, these findings indicate the successful engraftment of a small population of engineered HUCPVCs, which resulted in sustained antibody synthesis and that the cells are not quiescent but retain metabolic activity in vivo.

The persistence of transgene-expressing HUCPVCs after IM injection was unexpected; therefore, we used in vivo optical imaging to validate this finding. Consistent with the anti-VEEV antibody studies, HUCPVCs were batch-engineered with recombinant adenovirus encoding firefly luciferase (pAd5-luc) at 200 MOI and implanted by IM injection in the left hind limb. Luciferase-expressing cells were detected at the site of injection at least 123 days after transplantation (Fig. 4B; supplemental online Fig. 1). The implanted HUCPVCs appeared to remain in situ in healthy animals, because bioluminescence was not detected at remote sites.

The serological data suggest that engineered HUCPVCs could extend protection against VEEV exposure, perhaps for as long as 38 days after prophylactic treatment. We directly tested this prediction in a challenge study, in which the mice were intranasally infected with a lethal dose of the highly virulent VEEV TrD in BSL3



Figure 4. Engineered HUCPVCs can prolong therapeutic levels of circulating anti-VEEV antibody. (A): Mice treated with 2.5 million engineered HUCPVCs (red) per mouse exhibited significantly higher levels of functional serum anti-VEEV antibody compared with passive transfer of 50 μ g of purified antibody per mouse (blue). Predicted protective titers were maintained for up to 38 days in 40% of mice, and low levels of antibody were still detected at day 109 in 10% of mice. Positive serum titers were never observed from the sham-treated group receiving HUCPVCs engineered with recombinant adenovirus encoding enhanced green fluorescent protein (data not shown). Circles represent individual mice from duplicate studies (n = 5 mice per study). Horizontal bars represent the average from both studies. (B): Bioluminescence for individual mice captured by optical imaging over 123 days and quantified as total flux (photons per second). No photon emissions were detected before injection (day 0). Nude mice received 2.5 million HUCPVCs, engineered with recombinant adenovirus expressing firefly luciferase in the left hind-limb muscle. Intense bioluminescence was detected 2 hours after transplantation. After an initial decline in bioluminescence to day 7, the bioluminescence was relatively maintained until at least day 123, indicating successful engraftment and continued transgene expression. Migration of cells away from the site of injection was not detected. Abbreviations: HUCPVCs, human umbilical cord perivascular cells; IM, intramuscular; VEEV, Venezuelan equine encephalitis virus.

containment. Mice were pretreated with either 50 μ g of purified antibody or 2.5 million antibody-secreting HUCPVCs, 24 hours or 10 days before challenge. The control groups were pretreated with saline (untreated) or HUCPVCs engineered with pAd5eGFP (sham HUCPVCs). Clinical signs of infection were assessed blind using a five-point scale reflecting weight loss and clinical symptoms; the mice were humanely euthanized if the score was 4. The efficacy of the humanized anti-VEEV antibody was previously demonstrated in wild-type mice receiving a low dose of 30–50 PFUs of VEEV TrD [45]. However, in the present study, the mice were challenged with a 200-fold higher dose of 10,000 PFUs per nude mouse. The dose increase was necessitated



Figure 5. The purified anti-VEEV antibody has a half-life of 3.7 days in nude mice. The average serum titers from four studies (n = 19 mice total) are shown. Blood samples were taken from the mice on days 1, 3, 7, 10, and 17 after IM injection of 50 μ g of purified anti-VEEV antibody per mouse. The serum half-life best fits an exponential rate of decay of 3.7 \pm 0.5 days. Abbreviations: IM, intramuscular; VEEV, Venezuelan equine encephalitis virus.

by the immune-compromised mouse model, in which the absence of T cells likely impedes the rapid hyperinflammatory response that produces lethal encephalitis in wild-type mice.

Although 50 μ g of purified anti-VEEV fully protected mice against 30–50 PFUs of VEEV [45], it was insufficient to protect against 10,000 PFUs delivered intranasally (Fig. 6A). In contrast, pretreatment with engineered HUCPVCs 24 hours or 10 days before exposure fully protected the mice from intranasal VEEV exposure (Fig. 6A). The protection can be attributed to successful HUCPVC-mediated synthesis of anti-VEEV IgG in vivo, because the mice pretreated with sham HUCPVCs were unprotected and followed the survival curve of untreated mice (Fig. 6A). The mice pretreated with engineered HUCPVCs not only survived the challenge but also exhibited considerably less morbidity than did the mice pretreated with the antibody alone (Fig. 6B).

The serological data (Fig. 4A) suggest that mice pretreated with engineered HUCPVCs might continue to be protected 24, 31, or 38 days after administration. To reduce the requirement for animals, we tested this hypothesis in vitro. The VEEV-neutralizing activity of pooled serum obtained from mice pretreated with anti-VEEV-expressing HUCPVCs immediately before lethal challenge, or 21, 31, and 38 days after administration, was assayed by PRNT [48]. VEEV-neutralizing antibody titers (PRNT₅₀) are expressed as the highest dilution of serum producing a 50% or greater reduction in plaque count compared with the negative controls in which virus was incubated with diluent. Average serum titers were calculated from individual serum titers obtained previously by functional ELISA (Fig. 4A). The serum from mice that remained asymptomatic in the lethal challenge study had considerably more neutralizing activity (PRNT₅₀, 5,120) than did the mice that exhibited clinical signs of infection but eventually recovered (PRNT₅₀, 3,840; Table 1). Serum obtained from unchallenged mice 21 days after administration of engineered HUCPVCs had equivalent neutralizing activity to the challenged mice that recovered (Table 1), suggesting that mice would still be protected 21 days after engineered cell treatment. Although less potent, significant neutralizing activity was still detected in serum obtained 31 and 38 days after transplantation of HUCPVCs expressing anti-VEEV (Table 1). These data suggest that mice could be partially protected from lethal exposure to VEEV for up to 38 days after prophylactic treatment with engineered HUCPVCs.



Figure 6. Prophylactic treatment with antibody-secreting HUCPVCs confers protection against lethal exposure to VEEV. (A): Survival curve. Pretreatment with HUCPVCs expressing anti-VEEV, either 24 hours or 10 days before exposure (red and maroon, respectively), protected mice against lethal VEEV exposure. Similar pretreatment with 50 μ g of purified antibody was only partially protective when given 1 day (dark blue) or 10 days (light blue) before exposure. Pretreatment with sham-engineered HUCPVCs (white) had no benefit and closely recapitulated the survival curve of untreated mice (black). (B): Morbidity scores. Pretreatment with HUCPVCs expressing anti-VEEV reduced the frequency and severity of clinical signs of infection. Clinical symptoms were objectively scored on a scale from 0 (blue; normal weight and behavior) to 4 (red; \geq 35% weight loss and/or severe signs of infection). The mice were terminated if the score was 4. Untreated and sham-treated mice exhibited significant clinical symptoms by day 6, followed by rapid mortality within 3 days. Mortality was delayed in mice pretreated with purified antibody; however, most of the mice eventually succumbed. Mice pretreated with engineered HUCPVCs not only survived the challenge but also exhibited only mild, if any, clinical signs of infection and weight loss. Collective data from duplicate studies are shown (n = 5 mice per group per study), with the exception of mice that failed to recover from anesthesia after intranasal inoculation. Abbreviations: Eng., engineered; HUCPVCs, human umbilical cord perivascular cells; PE, pre-exposure; VEEV, Venezuelan equine encephalitis virus.

Table 1. Serological VEEV-neutralizing activity implies prolonged protection against lethal VEEV exposure by pretreatment with engineered HUCPVCs

Variable	PRNT ₅₀ ^a	Average serum titer (µg/ml)
Challenged mice (asymptomatic)	5,120	62.3
Challenged mice (recovered)	3,840	24.5
Day 21	3,840	27.1
Day 31	2,560	20.4
Day 38	2,560	15.3

^aThe highest dilution of serum producing a 50% or greater reduction in plaque count compared with negative controls in which virus was incubated with diluent.

Abbreviations: HUCPVCs, human umbilical cord perivascular cells; PRNT, plaque reduction neutralization test; VEEV, Venezuelan equine encephalitis virus.

DISCUSSION

The importance of targeted monoclonal antibodies (mAbs) for immune protection has long been overshadowed by the easy appeal of broad-spectrum drugs [24]. However, the site-specific nature of mAbs makes them invaluable tools in many contexts, including the fight against multidrug-resistant pathogens. mAbs are a critical component of the catalog of medical countermeasures against infectious agents but have largely failed to reach the clinic in this context owing to manufacturing costs, the challenge of timely (presymptomatic) administration of effective doses, and the multiple dosing regimens often required to maintain efficacy. Despite the successful development of mAbs for the treatment of autoimmune diseases and cancer, and the wealth of published data describing development of mAbs targeted against infectious pathogens, still only two mAbs have been licensed for infectious disease [24].

The cell-based modality for delivery of medical countermeasures we have described offers several advantages over current paradigms. For example, the plant-produced countermeasure for Ebola virus, ZMapp, is a cocktail of three synergistic mAbs [49] that had limited usefulness during the Ebola outbreak because sufficient quantities could not be manufactured in a timely fashion [25]. In animal models, the protective efficacy of ZMapp was further enhanced by coadministration of Ad-vectored interferon- α [50, 51]. In contrast, the efficacy of implanting cocktails of engineered HUCPVCs for osteogenic repair has already been shown [32]; thus, we propose this paradigm as an alternative approach to deliver single-dose, synergistic cocktails of antibodies and adjuvants. The cell-based modality also lends itself to future applications in personalized medicine, because cocktails of pre-engineered cells could be designed and administered at the point of care.

The HUCPVC cell-based delivery platform successfully improved the prophylactic efficacy of an existing medical countermeasure, the humanized anti-VEEV antibody, and dramatically reduced both morbidity and mortality in recipients. Although we used athymic nude mice, they can still mount an adaptive antibody response against human antigens [52] and thereby truncate the half-life of the humanized antibody. We have shown in the present study that the serum half-life of anti-VEEV in nude mice is only slightly longer than previously observed in wildtype mice [45] (3.7 vs. 2.25 days, respectively). Because humanized antibodies typically exhibit a prolonged half-life of up to 28 days after human administration [53], such a reduced rate of antibody turnover creates a scenario in which fewer engineered HUCPVCs could sustain protective antibody titers for a longer period of time in humans. This species-related immune response might, in part, explain the differences reported by Bartholomew et al. [54] in the secretory activity of human MSCs delivered intramuscularly into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (28 days) or subcutaneously, within immune-isolatory devices, in baboons (137 days). Thus, it is plausible to expect that protective anti-VEEV serum titers might be sustained for a longer period of time by administering engineered HUCPVCs to humans.

An empirical prediction of the required IgG dose to protect or treat humans infected with VEEV is not possible, because the number of viral particles required to make a patient sick is currently unknown. However, if one assumes the dosing of anti-VEEV IgG would be similar to the 15 mg/kg per month dosing of the commercial anti-respiratory syncytial virus antibody [55] and that the half-life is 2 weeks, the average monthly titer would be 7.5 mg/kg. As shown in Figure 3, one million engineered HUCPVCs can secrete 25 μ g of IgG per day (0.75 mg/month). Therefore, 10 million HUCPVCs could be expected to produce 7.5 mg antibody per month in a human. Such a cell dose is considerably lower than the commonly used clinical dose of 100 million MSCs per adult.

The recombinant replication-defective pAd5 system is ideal for engineering HUCPVCs with a genetic payload on a large scale. HUCPVCs were engineered with the anti-VEEV transgene by simple incubation with the recombinant adenovirus, without the addition of transduction mediators, resulting in nearly 100% gene transfer efficiency. We observed similar transduction efficiencies using recombinant pAd5 vectors encoding other therapeutic molecules and reporter genes ([32]; L.R. Braid, unpublished data), which abrogates the need to screen out untransformed cells. MSCs derived from other sources such as bone marrow are more refractory to transduction by recombinant adenovirus [56, 57], making the HUCPVC population particularly well-suited for this gene transfer paradigm.

Ex vivo transduction of the cells is an important aspect of the engineered cell platform; the recipient is never exposed to the live adenovirus that, in large doses, has been shown to elicit adverse effects in clinical trials [22, 23]. Moreover, the transgene remains episomal, mitigating the concerns associated with integrationbased gene therapy approaches, including insertional mutagenesis, position effects on transgene expression, and uncontrolled propagation of the transgene. Consistent with other reports that the inherent properties of MSCs such as immune-modulation are unchanged by adenoviral-mediated gene transduction [28, 31, 46], engineered HUCPVCs retained their differentiation plasticity in our study. However, our in vitro analyses revealed that HUCPVCs isolated from different umbilical cords had variable intrinsic capacity to sustain anti-VEEV synthesis, which correlated with reduced biological activity in other assays (unpublished data). However, the current protocols for HUCPVC isolation, cryopreservation, and expansion in serum-free conditions have reduced such variability. Taken together with the documented high-proliferative capacity, ex vivo stability, and availability of HUCPVCs from biological waste material, these observations indicate the high suitability of the HUCPVC-mediated gene transfer modality for scale-up and clinical applications.

Finally, we documented cell engraftment exceeding 3 months after IM implantation. In subsequent studies, which will be reported later, we have documented cell survival in excess of 5 months, using smaller doses of 1 and 0.5 million bioluminescent cells. Histological examination of biopsy specimens of the injection site showed cells between the muscle fibers, exhibiting an undifferentiated morphology and no evidence of other mesenchymal tissue lineage differentiation (L.R. Braid, unpublished data). Such persistence is a consequence of IM implantation, as it is not observed when cells are administered by other routes. Our results corroborate those described by Liu et al. [17], who reported peak circulating levels of human soluble tumor necrosis factor receptor II (hsTNFR) on day 7 after IM injection of engineered MSCs into NOD/ SCID mice, followed by declining hsTNFR titers to day 30. Using luciferase as a bioluminescent tracker, Vilalta et al. [58] similarly reported that adipose-derived MSCs survived 8 months in situ following IM implantation, and Nakabayashi et al. [59] has also shown robust IM dwell time of allogeneic MSCs in rats. The survival of engineered HUCPVCs following IM implantation potentiates a single dose treatment modality better suited for military field contexts than the current requirement for intravenous infusion of prophylactic antibodies or stem cell treatments in a clinical setting. We anticipate that future refinement of the engineering and transplant protocols will further extend the window of protection demonstrated here. For example, preliminary data indicates that a lower dose of cells may generate more stable serum titers, effectively elevating antibody levels at later time points (L. Braid, unpublished data). Together with the rapid decline in IgG (Fig. 4A) and luciferase (Fig. 4B) expression in the initial days after transplantation, this observation suggests that an optimal dose that facilitates diffusion of cells throughout the muscle and thus is adequately supported by the vasculature immediately after transplantation will minimize the initial loss of cells and stabilize dosing.

CONCLUSION

The present proof-of-concept study identified HUCPVC-mediated antibody gene therapy as a novel technology for immediate and sustained passive immunity against a significant bio-warfare threat. This cell-based modality offers advantages over traditional administration of purified antibody, including single-dose, IM implantation for extended immune protection against infectious disease. Given the success of this pilot study, we expect that HUCPVC-mediated gene therapy will provide a broad-spectrum solution for stealth delivery of therapies for a range of applications and biologic agents, including delivery of additional medical countermeasures for biological and chemical defense purposes.

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AUTHOR CONTRIBUTIONS

L.R.B.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; W.-G.H.: conception and design, collection and/or assembly of data, manuscript review, final approval of manuscript; J.E.D.: conception and design, provision of study material or patients, manuscript writing, manuscript review, final approval of manuscript; L.P.N.: conception and design, collection and/or assembly of data, manuscript review, final approval of manuscript; data, manuscript review, final approval of manuscript; L.P.N.: conception and design, collection and/or assembly of data, manuscript review, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

L.R.B. is chief executive officer and chief scientist of Aurora Bio-Solutions, Inc., has a research contract from the Department of National Defence awarded to Aurora BioSolutions Inc., has stock options from Tissue Regeneration Therapeutics, Inc. (TRT), and has received honorarium from TRT for providing expert testimony to support U.S. patent 9,005,599. J.E.D. is an officer and a shareholder of TRT, which provided the cells for the study, and receives inventor royalty payments and has stock and stock options in TRT. The other authors indicated no potential conflicts of interest.

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