Enucleated ASCs: A novel delivery system for prolonged expression of therapeutic proteins after spinal cord injury

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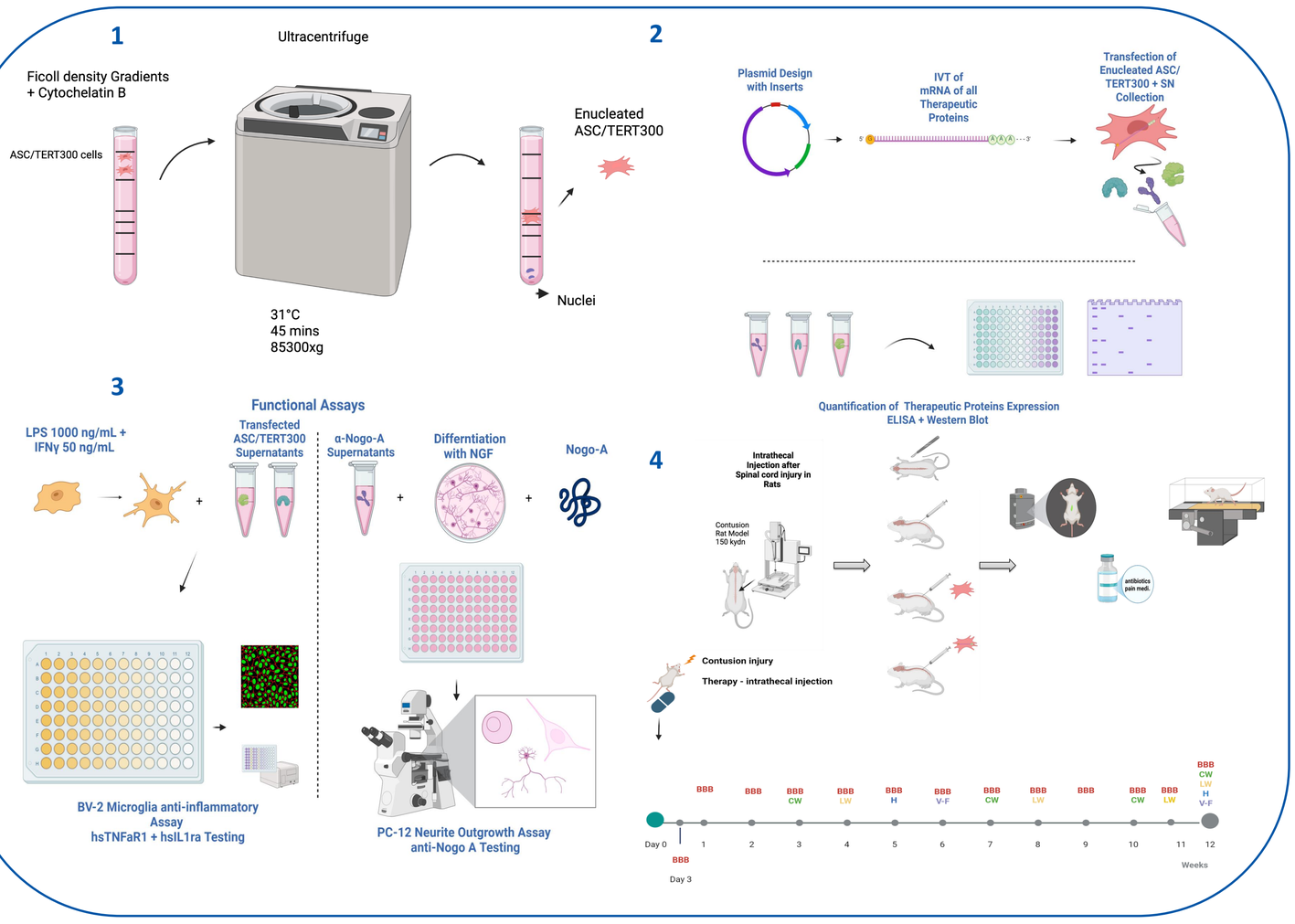
Background

Traumatic spinal cord injury (SCI), often caused by falls or motor vehicle accidents, leads to irreversible sensory and/or motor impairment due to neuronal loss, demyelination, and chronic inflammation. The secondary stage of SCI involves inflammation-induced tissue degeneration, leukocyte infiltration due to increased vascular permeability, and glial scar formation at the injury site; ultimately hindering regeneration and causing permanent damage. Adipose-derived stem/stromal cells (ASCs) are a potential cell-based therapy for the secondary SCI stage, offering immuno-modulatory properties and relatively simple harvesting. Enucleation of these stem cells provides the advantages of the therapeutic cell properties and cargo-loading abilities without permanent engraftment in the host, owing to their short life span.

Objectives

In this project, we aim to apply the enucleated ASCs as a cargo delivery vehicle in an in vivo spinal cord injury model. By loading the enucleated ASCs with mRNA of neuro-regenerative (anti-NogoA antibody) and anti-inflammatory proteins (human soluble interleukin-1 receptor antagonist/tumor necrosis factor α receptor 1). As a potential therapy technique, targeting the secondary inflammatory response post spinal cord injury.

Methodology



Results - Protein Expression

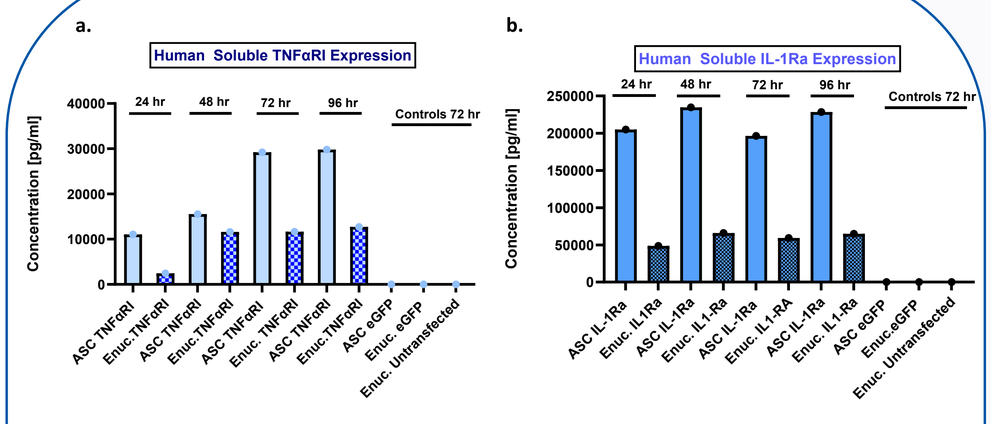


Figure 1. Protein expression levels of TNFαR1 and IL1ra over time. Total expression was assessed at 24, 48, 72, and 96 hours after mRNA transfection with hsTNFαRI of intact and enucleated ASC/TER300 in (a) MesenCult ACF Plus growth media or (b) hsIL-1ra using ELISA. Supernatants of untransfected cells and GFP-transfected cells were used as a negative control. Results are presented as mean ± SD. n=3

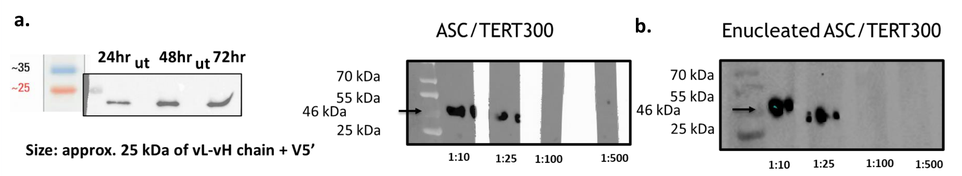


Figure 2. Expression of anti-Nogo A scFv. (a) Expression of single chain variable part (scFv) of anti-Nogo-A 24, 48, and 72 hours post mRNA transfection of ASC/TER300 (~23 kDa) with untransfected cell supernatant as a negative control, shown in a Western blot. (b) Binding of scFv-a-Nogo-A to recombinant Nogo-A peptide ~46 kDa is shown on a Western blot as well, with different dilutions (1:10, 1:25, 1:100, and 1:500) of intact and enucleated ASC/TERT300 cells supernatant 72 hours after transfection used as a primary antibody, with anti-V5 tag as secondary antibody.

Results – Protein Functionality Anti-Inflammatory Assay

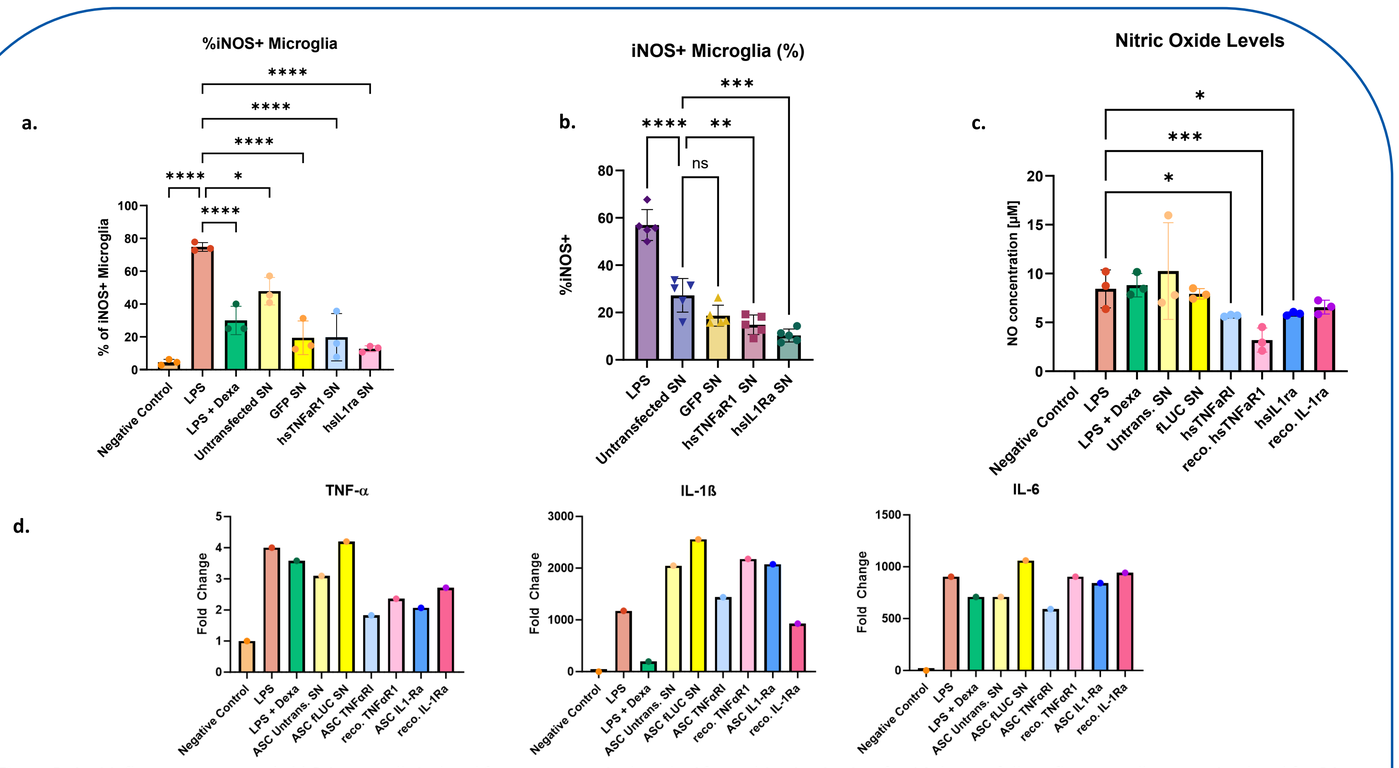


Figure 3. Anti-inflammatory assay. (a,b) Primary cells isolated from rat pup spinal cords. After cultivation in vitro for 14 days or full confluency, cells were stimulated for 24 hours (LPS 100 ng/mL and 20 ng/mL IFN-γ), then treated for 24 hours. Treatment groups: 72 hours supernatants of ASC/TERT300 after mRNA transfection with hsTNFaR1 and hsIL-1ra, and untransfected or GFP-transfected ASCs as a control. Negative control was not stimulated. Cells were fixed with 4% PFA and immuno-stained for GFAP as an astrocyte marker, Iba1 as a microglia marker, and iNOS as an activated microglia marker. Lower activation of microglia is shown in all treated groups in comparison to activation only (a) and treatment with hsIL1ra and hsTNFaR1, with less activation in comparison to untransfected SN treatment (b). (c) BV-2 microglial cell line was stimulated with conditioned medium from LPS (1 µg/mL) stimulated cells for 24 hours and treated with the same treatment groups. Nitric oxide (NO) secretion levels (µM) are measured as an indication of the inflammatory state. Data presented as mean ± SD (c). Statistical significance was determined using one-way ANOVA with Dunnett´s test; *p < 0.05 was considered statistically significant. n=3 (a,c). n=5 (b). (d) qPCR was performed with RNA collected of BV-2 cells pelleted 6 hours after LPS stimulation and treatment with supernatants as mentioned in c . N=5 was pooled for higher RNA yields. Results normalized to GapDH as housekeeping gene.

Results- Protein Functionality Neurite Outgrowth Assay

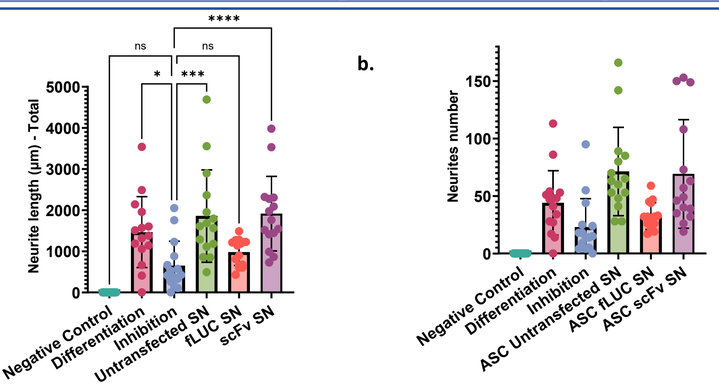


Figure 4. Neurite outgrowth assay with PC-12 cells. PC-12 cells were seeded in Collagen type-I coated plates for 24 hours in full growth media (RPMI, 10% horse serum (HS), 5% FCS, 1% p/s) overnight. Day 1 changed to differentiation media with 1% HS, 1% p/s in RPMI with 100 ng/ml NGF for 2 hours. Nogo-A was added for inhibition and treatment with supernatants of ASC/TERT300 72 hours after mRNA transfection with scFv anti-Nogo-A or fLuc-transfected ASCs as a control. Cells were monitored for 6 days post-differentiation, with media change every 48 hours (+ treatments). A significant recovery of neurite elongation is shown after scFv anti-Nogo A-containing supernatant in comparison to Nogo-A inhibition of the differentiation of PC12 to neurites (a). The differentiation (by neurite numbers) is another parameter to support the effect of treatment (b). Statistical significance was determined using one-way ANOVA with Dunnett´s test; *p < 0.05 was considered statistically significant. n=5

Conclusions

1. Expression of anti-inflammatory therapeutic proteins after mRNA transfection was proven and quantified in intact and enucleated ASC/TERT300.
2. scFv anti-Nogo-A antibody was successfully expressed in intact and enucleated ASC/TERT300.
3. Binding of anti-Nogo-A to recombinant Nogo-A peptide is shown.
4. In vitro functional assays for testing the therapeutic proteins were established.
5. Therapeutic effects of the proteins are shown in the anti-inflammatory assay.
6. Neurite outgrowth assay for anti-Nogo-A shows inhibition of Nogo-A activity.