

Title: Intranasal brain delivery of regulatory T-cells (T-regs) and resolution of inflammation in Parkinson's Disease.

SCIENTIFIC NARRATIVE

IDEA

Neuroinflammation is a pathological feature in Parkinson's Disease (PD) (Appel), suggesting resolution of inflammation may have therapeutic benefits. Regulatory T-cells (T-regs) function by limiting the extent of immune responses and prevent autoimmunity (Jiang et al.). Requiring cell-cell contact to exert their suppressive effects, T-regs attenuate inflammation driven diseases in various models including nigrostriatal dopaminergic (DA) neurodegeneration in a mouse MPTP model of PD (Jiang et al., Reynolds et al.).

Recently, cells (mesenchymal stem cells) were successfully targeted to the rat brain via intranasal route producing promising results in the rat 6-OHDA lesion PD model (Danielyan et al.). This delivery method offers a safe and effective alternative to the traumatic surgical procedure of transplantation in the brain. IDEA: Intranasal targeting of T-regs to the brain may resolve neuroinflammation in PD, thus promoting the ideal conditions that encourage DA neuron protection and regeneration.

This approach revolutionizes the treatment of PD and uses a non-invasive brain delivery method of "therapeutic cells" (patients' T-regs). Brain-targeted T-regs are expected to attenuate inflammatory processes that exacerbate DA neurotoxicity. In the brain, T-regs are expected to exert their normal functions and respond to physiologic cues governing resolution of inflammation. Since the T-regs will be obtained from the same patient who receives them, cell rejection is not a factor; thus, this approach would be readily available to eligible PD patients.

HYPOTHESIS/OBJECTIVE

Hypothesis: Brain targeting of T-regs will effectively alter a diseased neuroenvironment to one promoting DA neuron protection and regeneration in preclinical PD rodent models.

Objective: In this study, our objective is to demonstrate brain targeting of T-regs by intranasal route and compare efficiency of delivery in normal mice and in 6-OHDA lesion mouse model by flow cytometry and fluorescence microscopy. *The behavioral, histopathological and neurobiochemical beneficial effects of this approach will be evaluated in a follow up study only if the results in this first study warrants these next steps.*

STUDY

All animal procedures will be carried out in accordance with InTouch BioSolutions LLC IACUC. Animal use protocol will undergo extensive review and approval prior to study initiation. An attending veterinarian is available to care for animals as needed. Personnel handling animals have received adequate training acceptable by IACUC requirements.

1. CD4+CD25+ T-reg isolation and in-vitro t-cell suppression assay
 - A) T-regs will be harvested from spleen and lymph nodes of the same age/sex/strain (female/10weeks-old/C57Bl/6) of mice to receive intranasal administration. T-regs will be isolated/enriched by negative selection using Miltenyi Biotec's separators and reagents (Miltenyi CD4+CD25+ Regulatory T Cell Isolation Kit, mouse). Cell purity will be

- evaluated by flow cytometry (BD FACSCalibur, Cellquest Pro Software) using the Mouse Treg Flow™ Kit by Biogend.
- B) Suppressive capacity of T-regs will be evaluated as per Invitrogen's validated method and according to a modified procedure by Grauer et al.; Briefly, purified CD4+CD25- will be stained with CFSE¹ (Invitrogen) and stimulated with Dynabeads® (Invitrogen) coated with anti-mouse CD3 for 4 days in culture (humidified incubator, 5% CO₂ at 37⁰ Celsius) in the presence of CD4+CD25+ Treg cells in a 1:1 ratio. On day 4, CFSE-labeled cell proliferation will be assessed by flow cytometry. 64% of the CFSE-labeled cells are expected to divide under normal conditions while only 13 % of the cells are expected in the presence of functional CD4+CD25+ T-reg cells.
- 2) CFSE labeling of CD4+CD25+ T-reg and intranasal administration (modified protocol based on Danielyan et al.)
- A) T-regs will be labeled with CFSE as per manufacturer's recommendation (Invitrogen) and staining confirmed by flow cytometry. Cells will be washed 3Xs with sterile PBS and resuspended in sterile PBS.
- B) Intranasal cell or vehicle (PBS) application will be performed after a short anesthesia (isoflurane). 2.5 x 10⁵ CFSE-labeled T-regs will be administered in 20 microliter of sterile PBS by intranasal route [10 microliter per nostril] Control: equal volume vehicle (PBS). To enhance delivery, prior to vehicle or cell treatment, animals may receive 100U hyaluronidase (Sigma-Aldrich) dissolved in 20 microliter of sterile PBS by intranasal route [5 microliter (2Xs for each nostril)]. After 4 hours, animals will be euthanized by CO₂ asphyxiation followed by cervical dislocation.
- 3) Enumeration of brain associated CFSE-labeled T-regs by flow cytometry (modified protocol based on Nitcheu et al. and Grauer et al.)
The brain from euthanized mice will be harvested and immediately dissected on an ice-cooled block into olfactory bulb, cortex, striatum, cerebellum, remaining tissue (with brain stem), the tissues weighed and dissociated by Miltenyi Biotec gentleMACS² following recommended directions to achieve a single cell suspension as described in Pennartz et al. and in this video: <http://www.jove.com/details.stp?id=1267>. CFSE positive T-regs in dissociated neural tissue single cell suspension will be labeled with PE-anti-CD3 (BD Biosciences) and CFSE+CD3+ cells detected and enumerated by flow cytometry.
- 4) 6-OHDA lesion mouse model
10 weeks old female C57Bl/6 mice will be anesthetized with 2.5% isoflurane carried in 100% oxygen. The animal will be placed within a Kopf stereotaxic frame with nose and ear bars specially adapted for mouse. The surgical area will be shaved and wiped with 70% alcohol. A 1cm long mid-line scalp incision will be made and a hole 1 mm in diameter will be drilled into left and/or right skull at the stereotaxic coordinates (AP=0.5, L=2.3 and V=-3.2). 8 microgram of 6-OHDA (Sigma-Aldrich) dissolved in 2 microliter of 0.2% L-ascorbic acid in saline will be delivered unilaterally into striatum with a 5 microliter Hamilton syringe connected to a 30 gauge needle at the rate of 0.5 microliter/min. The needle will be slowly removed after a 5min delay. The incision will be closed by a wound clip and disinfectant applied externally. The animals will be allowed to recover before they are administered treatment by intranasal route.

5) Evaluation of brain serial sections and enumeration of CFSE-labeled T-regs by fluorescence microscopy.

Mice will be euthanized by CO₂ exsanguination and transcardially perfused with 0.9% saline (room temperature) for 1 min followed by ice-cooled 40 ml 4% paraformaldehyde/0.1 M phosphate buffer. The whole brain will be post-fixed in the same paraformaldehyde solution for 24 hrs, transferred to 25% sucrose/0.1 M phosphate buffer, freeze-sectioned at 35 micrometer in the coronal plane through the entire mouse brain. Sections will be evaluated for CFSE-positive T-regs by fluorescence microscopy using a conventional fluorescence microscope.

IMPACT

This first study will evaluate conditions that promote T-reg targeting to the brain by intranasal route. Clear evidence of targeting of T-regs to the brain would warrant examining this approach in PD rodent models evaluating DA neuroprotective and regenerative events and assessment of improvements in behavioral, histopathological and neurobiochemical (e.g., levels of dopamine and inflammatory mediators) tests. Methods to enrich for antigen specific T-regs (e.g., alpha-synuclein specific) for use in relevant PD rodent models (e.g., AAV/a-synuclein model) will also be examined. Clear evidence of behavioral and histopathological benefits would warrant subsequent steps toward examining this approach in the clinical testing.

¹CFSE; Carboxyfluorescein succinimidyl ester is a fluorescent cell-staining dye that remains inside the cell upon labeling. ²gentleMACS; gentleMACS is a tissue dissociator.

LITERATURE CITATIONS

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Pennartz, S. et al., *Journal of Visualized Experiments* **29**, (2009)

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