Modeling Immune-mediated Neuropathic Pain Using Human iPSC-Derived Sensory Neuron and Macrophage Co-culture Systems

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Abstract

OBJECTIVE/RATIONALE: Between 10-20% of the global adult population experience chronic pain, placing significant financial burdens on health care systems and global economies. However, in the field of pain drug discovery, researchers have been unable to deliver new non-opioid compounds to improve pain management and reduce the personal and societal impacts of chronic pain. Since the onset of chronic and neuropathic pain results from direct and indirect mechanisms following injury, the significance of immune cell-mediated inflammation in neuropathic pain has recently gained increasing interest. When stimulated, macrophages secrete both pro-nociceptive inflammatory cytokines, promoting neuropathic pain, and anti-inflammatory cytokines which can mitigate and resolve pain. Elucidating sensory neuron/macrophage interactions may identify novel targets for pain therapeutics to broaden treatment strategies.

Human induced pluripotent stem cell (iPSC)-derived sensory neurons together with human iPSC-derived macrophages provide a biologically relevant co-culture system to recapitulate immune-mediated neuropathic pain in vitro. In this study we demonstrate methods for culturing human iPSC-derived sensory neurons with isogenic iPSC-derived macrophages.

METHODS/RESULTS: Human iPSC-derived sensory neurons and iPSC-derived macrophages from the same donor 01279 were from FUJIFILM CDI. Neurons were characterized to identify the expression of nociceptor ion channels and receptors (NaV1.8, TRPV1). Cells were used in functional calcium assays to show neuronal excitability upon treatment with capsaicin (4 µM), ATP (10 µM). Similarly, iPSC-derived macrophages were shown to be functionally naïve and able to respond to pro- or anti-inflammatory stimulation. We next demonstrate that iPSC macrophages can be cultured in sensory neuron medium and retain phagocytic and cytokine function. To model peripheral neuroinflammation we cultured iPSC sensory neurons with unstimulated or LPS-stimulated iPSC macrophages and evaluated the effect of each condition on responses to sensory stimuli using a fluorescent calcium assay. We also investigated differences in sex responses to iPSC macrophages by comparing co-cultures with sensory neurons from male or female donors.

CONCLUSIONS: These data demonstrate the utility of iPSC-derived sensory neurons and macrophage co-cultures for generating human-relevant, high-throughput approaches to study mechanisms of immune-mediated chronic and neuropathic pain.

Keywords: Pain models, iPSC, and Inflammatory Pain

Human iPSC-derived Macrophages

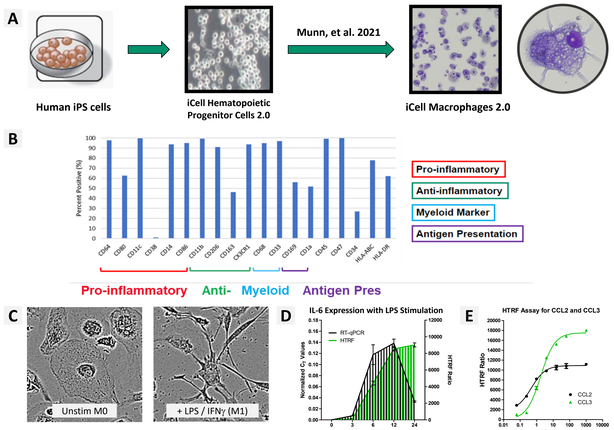

Figure 1. Characterization of iCell Macrophages 2.0. (A) iCell Mac 2.0 are differentiated starting from human iPSC through an intermediate cell type <iCell Hematopoietic Progenitor Cells 2.0> following a published protocol (Munn, et al. 2021). **(B)** Flow cytometry analysis to demonstrate that Mac 2.0 express both pro- and anti-inflammatory proteins, as well as myeloid and antigen presentation markers. **(C)** Examples of macrophage morphology in the unstimulated (M0) naïve state vs. cells that were stimulated overnight with LPS and IFNγ (100 and 50 ng/mL, respectively). **(D)** Timing of IL-6 gene (**black**) and protein (**green**) expression following LPS stimulation. **(E)** HTRF assay data quantifying cytokine release of CCL2 (monocytic chemotactic protein 1; MCP-1) and CCL3 (macrophage inflammatory protein-1 alpha; MIP-1α) upon dose-response of LPS.

Human iPSC-derived Sensory Neurons

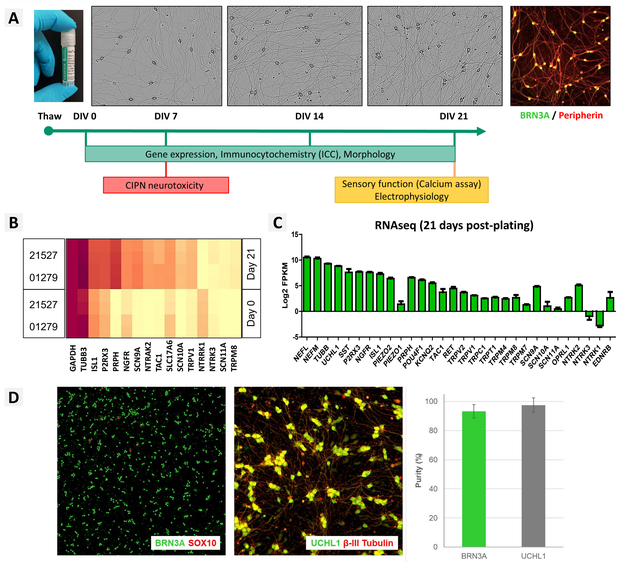

Figure 2. Characterization of iCell Sensory Neurons. (A) Cryopreserved iCell Sensory Neurons were thawed and cultured on Geltrex-coated plates and imaged at weekly timepoints. Cells display neuronal morphology with a bright cell body and extensive neurite networks. The timeline demonstrates when cells are ready for testing in various applications. **(B)** RNA from both donors of iCell Sensory Neurons (01279=male, 21527=female) was collected at DIV 0 and DIV 21. qPCR shows consistent expression of pan-neuronal genes (TUBB3) and sensory neuron-specific genes (P2RX3, TRPV1, NaV1.7) across donors with increased expression at DIV 21. **(C)** RNAseq collected at DIV 21 corroborate qPCR data and confirm expression of additional neural markers (NEFL, NEFM) and other sensorineural TRP channels, Na channels (SCN), and mechanoreceptor receptors (PIEZO1/2). (D) BRN3A and UCHL1 expression quantified at day 7 by ICC illustrates the high purity and consistency of iCell Sensory Neurons across multiple lots (n=13).

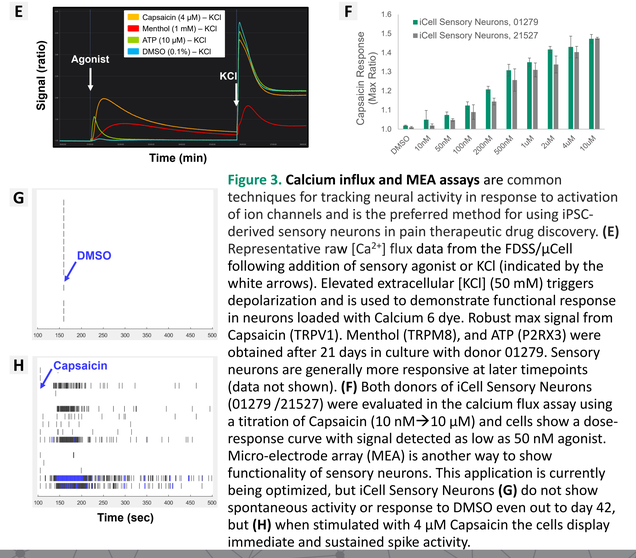

Figure 3. Calcium influx and MEA assays are common techniques for tracking neural activity in response to activation of ion channels and is the preferred method for using iPSC-derived sensory neurons in pain therapeutic drug discovery. **(E)** Representative raw [Ca^{2+}] flux data from the FDSS/µCell following addition of sensory agonist or KCl (indicated by the white arrows). Elevated extracellular [KCl] (50 mM) triggers depolarization and is used to demonstrate functional response in neurons loaded with Calcium 6 dye. Robust max signal from Capsaicin (TRPV1). Menthol (TRPM8), and ATP (P2RX3) were obtained after 21 days in culture with donor 01279. Sensory neurons are generally more responsive at later timepoints (data not shown). **(F)** Both donors of iCell Sensory Neurons (01279 /21527) were evaluated in the calcium flux assay using a titration of Capsaicin (10 nM→10 µM) and cells show a dose-response curve with signal detected as low as 50 nM agonist. Micro-electrode array (MEA) is another way to show functionality of sensory neurons. This application is currently being optimized, but iCell Sensory Neurons **(G)** do not show spontaneous activity or response to DMSO even out to day 42, but **(H)** when stimulated with 4 µM Capsaicin the cells display immediate and sustained spike activity.

Tissue-resident Macrophages Interact with Sensory Neurons

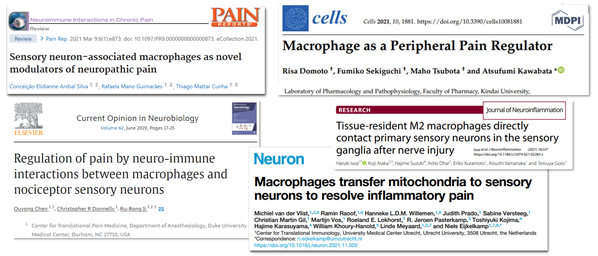

Figure 4. Sensory Neuron-associated macrophages are tissue-resident cells that interact directly with sensory neurons in the peripheral nervous system. These cells play a critical role in various physiological processes, including pain regulation, tissue repair, inflammation, infection, auto-immunity, and nerve degeneration/regeneration. Many of these concepts are covered by well-written review articles and backed by numerous peer-reviewed publications in the field.

Viability and Function of Mac 2.0 in Sensory Neurons Medium

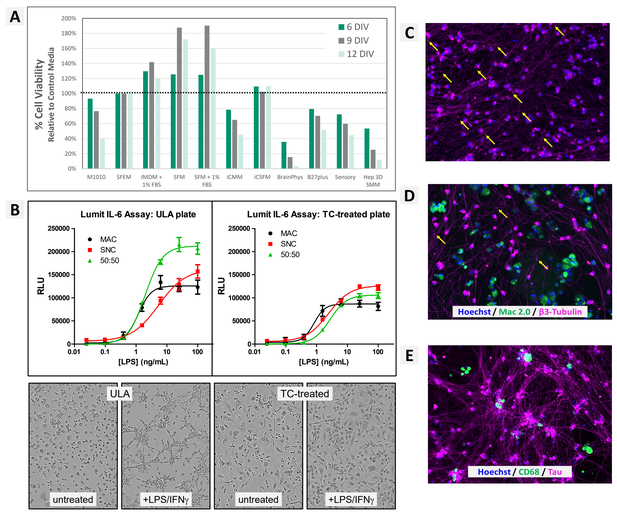

Figure 5. Establishing co-culture conditions with iCell Macrophages 2.0 and iCell Sensory Neurons is more complicated than just mixing two different cell types together and requires numerous considerations. In this first experiment, **(A)** iCell Mac 2.0 were plated in 96-well format and that day were exchanged into various media formulations, primarily existing maintenance media for other iCell products from FUJIFILM CDI. Cell viability was assessed by CellTiter-Glo® 2.0 assay (measures ATP) at different timepoints in culture (DIV 6, 9, and 12). The number of viable cells was normalized to macrophages in their control media and each condition is expressed as a % cell viability relative to control. iCell Mac 2.0 in complete iCell Sensory Neurons Medium maintained 50-70% viability out to DIV 12. **(B)** Macrophage functionality was next evaluated using a cytokine release assay to measure IL-6 (Lumit™ by Promega). Cells were plated on either ultra-low attachment (ULA) or standard tissue culture (TC)-treated plates. Additionally, cells were cultured in either control media for iCell Mac 2.0 (**MAC**, in black), complete media for iCell Sensory Neurons (**SNC**, red), or a mixture of the two media (**50:50**, green) for 72 h. Importantly, iCell Mac 2.0 retained functional cytokine release under all conditions and appeared to yield the strongest response when in "50:50 media" and cultured on ULA plates. These results are being examined further. iCell Sensory Neurons were cultured in the **(C)** absence or **(D)**, **(E)** presence of iCell Mac 2.0 prior to fixing and staining. iCell Mac 2.0 are inherently green auto fluorescent but can also be stained with anti-CD68 antibody. Yellow arrows in image C point to cellular debris often present after plating a mono-culture of sensory neurons. When co-cultured with iCell Mac 2.0 (panel D), these cells amazingly clean up the cellular debris, likely through phagocytosis. More experiments are ongoing to improve our understanding of the balance of survival and function in co-culture.

3D Co-culture Model Optimization

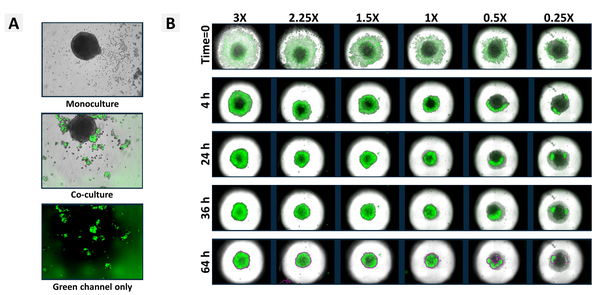

Figure 6. Exploring iCell Sensory Neuron 3D co-culture is an approach to potentially enhance function and more accurately model neuroinflammation. **(A)** iCell Sensory Neurons were seeded into ultra-low attachment (ULA) spheroid-forming plates (20K cells/well) and iCell Mac 2.0 (auto-fluorescent green cells) were added 4 days later. Co-cultures were maintained in a 50:50 mixture of the recommended maintenance media for each respective cell type. This experiment used a ULA plate that did not support uniform spheroid co-culture formation. **(B)** Time-course using live-cell imaging analysis on the Incucyte SX5 (from time=0 when iCell Mac 2.0 were added) examined how different ratios of cells (MAC:SNC) impact co-culture in 3D. **(C)** The average green object mean intensity for each condition plotted over time and illustrates that the number of green macrophages stays consistent during the expt.

Organ-on-a-Chip: Cell Type Compartmentalization

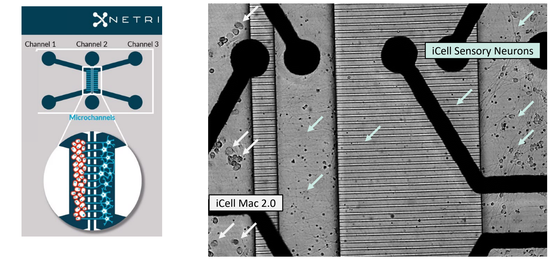

Figure 7. Modeling peripheral neuroinflammation using micro-engineered biomimetic devices, also known as "organ-on-a-chip". Systems like the DuaLink™ microfluidic device from Netri can compartmentalize cell types and allow control of microenvironments. We added iCell Sensory Neurons and iCell Mac 2.0 together in DuaLink, demonstrating proof-of-concept immuno-nerve pathway co-culture. Further work on this system should help to study the bidirectional communication and between peripheral sensory neurons and macrophages and enable cell-specific drug discovery for neuroinflammatory pain within a complex, multi-cellular model.

Summary and Future Directions

iCell Sensory Neurons (C1259 and C1261) and iCell Macrophages 2.0 (C1193) are newly launched human iPSC-derived products from **FUJIFILM Cellular Dynamics**. Studying their interactions in co-culture can improve models for inflammatory pain.

In this research we show:

- Media optimization is required to establish co-culture, with 50:50 media showing the best function and viability of both cell types.
- iCell Macrophages 2.0 can clear cellular debris from sensory neuron cultures.
- iCell Sensory Neurons can be cultured as 3D neurospheres.
- Macrophages incorporate and persist in 3D neurospheres with cell density and medium optimization.

More foundational tests are currently being performed in our laboratories and we will pass along our learnings to the community in the form of **iCell® Lab Notes** and **Application Protocols**.

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