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The human amniotic membrane derived MSC secretome restrains neuroinflammation and peripheral immune cell invasion of 3D neural tissue through conserved CXCL10 blockade and proteostatic rescue

Running title: Human amniotic secretome inhibits neuroinflammation

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Abstract

Chronic neuroinflammation is a convergent mechanism of neurodegenerative disease progression, yet therapies that restrain it without impairing central nervous system immune surveillance remain limited. The human amniotic membrane-derived mesenchymal stromal cell secretome (CM-hAMSC) was shown to modulate peripheral immunity, with demonstrated efficacy in several inflammatory models, but its action on the neuroinflammatory circuitry is poorly defined. Here, we systematically investigated the effects of CM-hAMSC across a nested hierarchy of complementary neuroinflammatory models, including: monolayer cultures of human cell line-derived neurons, astrocytes and microglia; 3D human tri-partite neurospheroids; a peripheral immune transwell interface; and microglia-free murine induced pluripotent stem cell-derived bi-partite neurospheroids, the latter of which was profiled by unbiased transcriptomics and proteomics. CM-hAMSC suppressed pro-inflammatory and cell death programs while preserving neuronal transcriptional programs associated with physiological function. Selectively modulated peripheral immune recruitment through suppression of CD4⁺ T-cell migration while sparing CD8⁺ T cells, prevented stress granule assembly in neurons and astrocytes, and markedly reduced syngeneic splenocyte invasion of inflamed 3D neural tissue. Integrated multi-omics revealed the inhibition of death and stress pathways and the concomitant upregulation of pro-survival pathways. Across four independent experimental systems, suppression of CXCL10 emerged as the most consistent mechanistic correlate of CM-hAMSC activity.

Keywords: human amniotic membrane-derived mesenchymal stromal cells; conditioned medium; secretome; neuroinflammation; 3D neurospheroid; CXCL10; microglia; stress granules; multi-omics; peripheral immune cell invasion.

Introduction

The expansion of global life expectancy has revealed a new medical challenge: neuroinflammatory and age-associated neurodegenerative diseases (NIDs) now constitute one of the heaviest burdens on health systems worldwide (Ransohoff 2016; Chitnis and Weiner 2017). Etiologies diverge across conditions: protein misfolding and aggregation in Alzheimer's and Parkinson's disease, demyelination in multiple sclerosis, motor-neuron loss in amyotrophic lateral sclerosis. Yet, pathological courses converge on a common axis of chronic, self-sustaining neuroinflammation (Ransohoff 2016; Chitnis and Weiner 2017; Heneka et al. 2015). Microglial reactivity, astrocytic dysfunction and the progressive recruitment of peripheral immune cells into CNS tissue act as downstream amplifiers of the initial insult and ultimately become the principal drivers of neuronal loss (Colonna and Butovsky 2017; Kwon and Koh 2020; Ransohoff 2016). Targeting this convergent inflammatory axis is therefore emerging as a complementary therapeutic strategy that may be applicable across etiologically diverse NIDs, alongside approaches aimed at disease-specific mechanisms. The therapeutic paradigm for this approach has shifted over the last decade, moving away from the original hypothesis of direct cell replacement toward trophic and immunomodulatory support delivered by cells or their acellular derivatives, such as their secretome or conditioned medium, including extracellular vesicles (Hoang et al. 2022; Galipeau and Sensébé 2018). Mesenchymal stromal cells (MSCs) from adult sources have dominated this field⁷, but perinatal tissues have progressively gained appeal due to their availability as biological waste, favorable ethical profile and rich, developmentally primed secretomes (Silini et al. 2020; Pozzobon et al. 2022; Silini et al. 2019). Among perinatal sources, MSCs isolated from the human amniotic membrane (hAMSCs) occupy a distinct niche. Their conditioned medium (CM-hAMSC) exerts broad immunomodulatory activity, including suppression of T-cell proliferation, CD8⁺ T cell maturation (Papait et al. 2023), inhibition of Th1/Th9/Th17 polarization, induction of regulatory T cells, attenuation of NK cytotoxicity and M1 to M2-like macrophage repolarization (Pianta et al. 2015; Magatti et al. 2017; Papait et al. 2022). These in vitro properties potentially mimic the physiological role of amniotic tissue in sustaining fetomaternal immune tolerance (Magatti et al. 2019). CM-hAMSC has shown efficacy in preclinical models of lung fibrosis, arthritis, inflammatory bowel disease and, more recently, Huntington's disease, where the administration reduced striatal atrophy and microglial activation in R6/2 mice (Cargnoni et al. 2014; Giampà et al. 2019; Parolini et al. 2014). Despite this well-characterized activity on peripheral immunity, the action of CM-hAMSC within the specialized microenvironment of the CNS remains fragmentary. Whether, and how, it engages the canonical cellular pillars of neuroinflammation, astrocytes and microglia, and how it interfaces with the chemokine-driven recruitment of peripheral immune cells has not been systematically dissected. Two technical barriers have historically limited progress on this question. First, the CNS immune microenvironment is heterocellular: reactive microglia amplify signals generated by neurons and astrocytes, and peripheral immune cells are recruited in response to integrated chemotactic gradients rather than to single-cell-type secretion (Kwon and Koh 2020; Prinz et al. 2011). Any monoculture readout of secretome activity therefore risks missing the ever-changing properties of the inflamed tissue. Second, until recently, it was rare to find accessible 3D models that both integrated the three canonical CNS cell types and remained amenable to pharmacological perturbation and immune cell recruitment assays. Recent advances in brain organoid technology have begun to close this gap, providing reproducible multicellular platforms that recapitulate cell-cell interactions across defined cell-type compositions (Kelley and Paşca 2022; Paşca 2018; Lancaster and Knoblich 2014).

Here, we leverage this methodological advance to dissect the action of CM-hAMSC across models of increasing complexity, within a framework that enables both the isolation of cell-type-specific contributions and the identification of the signaling pathways. We first profiled CM-hAMSC activity on 2D human neuron-like cells (NLCs) differentiated from SH-SY5Y cell line, astrocyte-like cells (ALCs) from U138 MG, and HMC-3 microglia. We then advanced to a human tri-partite neurospheroid model (tri-hNSPH) that integrates the three cell types. We characterized its culture medium as a chemotactic driver of peripheral blood mononuclear cell (PBMC) migration and phenotypic change. Finally, using a microglia-free murine iPSC-derived bipartite neurospheroid (bi-miPSC-NSPH)(Di Stefano et al. 2024), we dissected the transcriptomic and proteomic effects of CM-hAMSC on neurons and astrocytes independently of microglial influence. With this model, we further showed that CM-hAMSC protects inflamed neural tissue from autologous splenocyte invasion.

Overall, we demonstrated for the first time that CM-hAMSC selectively suppresses pathological microglial activation while preserving the physiological gene expression of neurons and reduces inflammatory cell stress. CXCL10 emerged as a conserved regulator of inflammation, linking the molecular responses tested on 2D monocultures to the effects observed on complex 3D human and murine models. These findings ultimately provide mechanistic grounding for CM-hAMSC as a promising cell-free therapeutic candidate in neuroinflammation treatment.

Materials and Methods

Cell culture and differentiation of human neural cell lines

All human cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, US). The neuroblastoma-derived SH-SY5Y cell line (CRL-2266; Lot 70055453) was maintained in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich; Cat. No. 56416C) supplemented 1:1 with Ham's F12 (EuroClone; Cat. No. ECB7502L), 10% heat-inactivated fetal bovine serum (FBS; EuroClone; Cat. No. ECS5000LH), 1% penicillin-streptomycin (P/S; EuroClone; Cat. No. ECB3001D) and 1% L-glutamine (EuroClone; Cat. No. ECB3000D). The human embryonic microglial cell line HMC-3 (CRL-3304; Lot 70054445) was cultured in EMEM supplemented with 15% FBS, 1% P/S and 1% L-glutamine. The glioblastoma-derived U138-MG cell line (HTB-16; Lot 70054708) was cultured in EMEM containing 10% FBS, 1% P/S and 1% L-glutamine.

For neuronal differentiation, SH-SY5Y cells were seeded onto Geltrex-precoated plates (Life Technologies; Cat. No. A1413202) at 3×10^4 cells/cm². After 3 days of expansion, cells were differentiated with 10 μ M all-trans retinoic acid (ATRA; Sigma-Aldrich; Cat. No. R2625) in Neurobasal medium (NB; Life Technologies; Cat. No. 21103049) supplemented with 1% B27 (Life Technologies; Cat. No. 17504-044) and 1% L-glutamine for 5 days with daily medium replacement. For astrocytic differentiation, U138-MG cells were seeded onto Geltrex-precoated plates at 8×10^3 cells/cm², allowed to expand for 24 h and then cultured for 3 days in growth medium supplemented with 2 mM sodium butyrate (Thermo Fisher Scientific; Cat. No. A11079).

Generation of 3D human tri-hNSPHs

Tri-hNSPHs were assembled by combining SH-SY5Y, U138-MG and HMC-3 cells at a 1:2:4 ratio at a total density of 1.2×10^4 cells per well in untreated U-bottom 96-well plates (Merck Life Science; Cat. No. CLS3788). Cells were seeded in NB supplemented with 1% B27 (Thermo Fisher

Scientific; Cat. No. A3582801) and 0.4% methylcellulose (Merck; Cat. No. M7027) in a final volume of 200 μ L/well. Tri-hNSPHs were maintained for 14 days on a rocking platform, with half-medium change every other day.

Isolation, expansion and phenotypic characterization of hAMSCs

Human placentas were processed immediately after collection following the institutional ethics protocol (informed consent obtained from all donors). Amniotic membrane fragments were isolated as described by Silini et al. (Tcw et al. 2017) and digested at 37°C for 9 min with 2.5 U/mL dispase (VWR; Cat. No. 734-1312), then transferred to RPMI-1640 (EuroClone; Cat. No. ECB9006LX10) supplemented with 10% FBS, 1% P/S and 1% L-glutamine. Fragments were subsequently treated with 0.94 mg/mL collagenase A (Roche; Cat. No. 10103586001) and DNase I (Roche; Cat. No. 11284932001) for 2.5–3 h at 37°C. Cell suspensions were centrifuged, filtered through 100- μ m cell strainers (EuroClone; Cat. No. GR542000) and expanded to passage 1 at 1×10^4 cells/cm² in Chang medium D (Irvine Scientific; Cat. No. 12401360) with 1% L-glutamine. Only preparations with >98% CD13⁺CD90⁺, <2% CD45⁺ and <2% CD324⁺ were used.

Production and functional validation of CM-hAMSC

hAMSCs were cultured for 5 days at 5×10^5 cells/well in 500 μ L DMEM/F12 1:1 (EuroClone; Cat. No. ECM0090L) with 1% L-glutamine and 1% P/S in 24-well plates. Conditioned medium was harvested, centrifuged at 300g, filtered through 0.2- μ m sterile filters (Sartorius Stedim; Cat. No. 17761) and stored at –80°C. Each experimental batch pooled CM-hAMSC from at least 3 donors to minimize donor-dependent variability. Functional validation was performed by assessing CM-hAMSC capacity to inhibit PBMC proliferation after T-cell receptor stimulation with 0.125 μ g/mL OKT3 (Janssen-Cilag; Cat. No. L04AA02). 1×10^5 PBMCs/well were co-cultured with 10, 50 or 100 μ L of CM-hAMSC in a final volume of 150 μ L for 72 h, and proliferation was quantified by EdU incorporation (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit; Thermo Fisher; Cat. No. C10420). Only CM-hAMSC batches exhibiting dose-dependent inhibition were used in subsequent experiments.

Generation of murine iPSC-derived bi-partite neurospheroids (bi-miPSC-NSPHs)

Bi-partite murine iPSC-derived neurospheroids (bi-miPSC-NSPHs) were generated from a previously established and validated C57BL/6 CX3CR1-eGFP⁺/– CCR2-RFP⁺/– murine iPSC line, as described by (Di Stefano et al. 2024; Quarta et al. 2019; Quarta et al. 2021; Van Breedam et al. 2022). Bi-miPSC-NSPHs were individually cultured in U-bottom 96-well plates and grown for 4.5 weeks in 200 μ L/well of neural differentiation (ND) medium composed of DMEM/F12 and Neurobasal-A (Gibco; Cat. No. A2477501) media at a 1:1 ratio, supplemented with 0.5% N2 (Gibco; Cat. No. 17502048), 1% B27 and 100 U/mL–100 μ g/mL P/S.

Pro-inflammatory stimulation and CM-hAMSC treatments

For tri-hNSPHs, inflammation was induced with a cocktail of IFN γ (50 ng/mL; BD Biosciences; Cat. No. 554616), TNF α (5 ng/mL; Miltenyi Biotec; Cat. No. 130-094-014) and IL-1 β (10 ng/mL; Miltenyi Biotec; Cat. No. 130-093-895). CM-hAMSC was delivered alone or in combination with the cytokines at 50% of total culture volume. After 3 days, tri-hNSPHs were subjected to a 24-h starvation in serum-free NB for multiplex and ELISA assays, or in RPMI-1640 (EuroClone; Cat.

No. ECB9006) with 5% FBS, 1% P/S and 1% L-glutamine for transwell migration assay. Bi-miPSC-NSPHs were stimulated with a pro-inflammatory cytokine cocktail consisting of TNF α (10 ng/mL), IL-1 β (10 ng/mL) and IFN γ (50 ng/mL). For the splenocyte invasion assay, LPS (1 μ g/mL; Sigma-Aldrich) was added to the cytokines. CM-hAMSC was administered either at 50% of the total culture volume or as a dose-response series of 3%, 6.25%, 12.5%, 25% and 50%. After 72 h, culture supernatants were collected and stored at -80°C for downstream cytokine analysis, while Bi-miPSC-NSPHs were either fixed for immunofluorescence analysis or snap-frozen for omics analyses.

Quantitative real-time PCR

Cells were lysed in RLT buffer and stored at -80°C . Total RNA was isolated with the EZ1 RNA Cell Mini Kit (Qiagen; Cat. No. 959034) on the BioRobot EZ1 Advanced XL Workstation. cDNA was synthesized with iScript Advanced (Bio-Rad; Cat. No. 1725038), pre-amplified with SsoAdvanced PreAmp Supermix (Bio-Rad; Cat. No. 1725160) and qRT-PCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; Cat. No. 1725274) on a CFX96 system. Data were analyzed with Bio-Rad CFX Maestro v2.2. Primer sequences are reported in **Table 1**.

Immunofluorescence and stress granule (SG) quantification

2D cells were fixed in 10% neutral buffered formalin (Diapath; Cat. No. F0043) for 30 min. Tri-hNSPHs and bi-miPSC-NSPHs were fixed for 1 h, embedded in OCT and cryosectioned at 10 μm on a Leica CM1950 cryostat. Samples were permeabilized (0.2% Triton X-100, 1 h) and blocked with normal goat serum (Invitrogen; Cat. No. 31872) for 1 h. Primary antibodies were incubated overnight at 4°C , followed by fluorophore-conjugated secondaries for 90 min at 37°C . Autofluorescence was quenched with TrueVIEW (Vector Laboratories; Cat. No. 84000) for tri-NSPHs, nuclei counterstained with 0.1 $\mu\text{g/mL}$ DAPI (Invitrogen; Cat. No. D1306), and slides mounted with VECTASHIELD mounting medium (Vector Laboratories; Cat. No. H-1000) or in ProLong R Gold antifade reagent (Thermo Fisher Scientific; Cat. No. P36930). Imaging was performed on a Leica MICA platform. High-resolution images were acquired either using the Nikon CSU-W1 SoRa confocal microscope with 40 $\times$ (water) objective in normal confocal mode and NIS-Elements software (Nikon) or using Zeiss LSM710 confocal laser scanning microscope and ZEN 2010 B SP1 software (Zeiss). Stress granule (SG) formation was assessed by immunofluorescent detection of G3BP1 and PABPC1. Images were analyzed in ImageJ using the SG-counter plugin with the following settings: smooths = 30, post-subtraction smooths = 3, threshold = 2000, minimum particle size = 1, maximum particle size = 1000 and circularity = 0.6. The percentage of SG-positive cells, number of SGs per cell and relative SG size per cell were quantified. All primary antibodies are commercially available and their specificity has been extensively validated by their respective manufacturers and in previous peer-reviewed literature ²³, (Bufi et al. 2026a). Primary and secondary antibodies used for human and mouse samples are enlisted in **Table 2**.

Multiplex bead-based cytokine immunoassay and ELISA

Cytokines were quantified in 5 independent samples of tri-hNSPH culture supernatants per condition using BD CBA Flex Sets (BD Biosciences) on a FACSymphony A3 with FCAP Array v3.0. TNF α was measured with the Human TNF α CytoSet kit (Invitrogen; Cat. No. CHC1753). CXCL10 levels in bi-miPSC-NSPH culture supernatants were quantified using the Mouse

CXCL10 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. For CXCL10 ELISA experiments, 8 independent samples were analyzed per condition. Cytokine panel is shown in **Table 3**.

Flow cytometric phenotyping

HMC-3 were enzymatically detached while PBMCs were simply collected from transwell lower chambers. Cells were stained with fixable viability dye eFluor 780 (Thermo Fisher; Cat. No. 65-0865-14), then with fluorochrome-conjugated anti-human antibodies (**Table 4**) for 20 min at 4°C. For intracellular staining, permeabilization was carried out with BD Cytofix/Cytoperm (BD Biosciences; Cat. No. 554714). Samples were acquired on a FACSymphony A3 with FACSDiva v8.0 and analyzed with FlowJo v10.8.0. hAMSC characterization used the ISCT panel (CD13, CD73, CD90, CD105, SSEA-4, CD45, CD324)(Dominici et al. 2006).

PBMC isolation and transwell migration assay

PBMCs were separated from buffy coats by density gradient centrifugation (Histopaque; Merck; Cat. No. 10771), cryopreserved in FBS with 10% DMSO (Merck; Cat. No. D2650) and stored in liquid nitrogen. Migration was performed using 24-well hanging inserts with 5- μ m pores (Merck Millipore; Cat. No. PTMP24H48). 600 μ L of tri-hNSPH supernatant was placed in the lower chamber; 1×10^6 PBMCs were seeded in the upper insert in 500 μ L RPMI 1640 with 5% FBS, 1% P/S and 1% L-glutamine. Cells were allowed to migrate for 2 or 5 h. Migrated cells were counted (Bürker chamber, Trypan blue exclusion) and processed for flow cytometric phenotyping.

Splenocyte isolation and invasion assay with bi-miPSC-NSPHs

Surplus C57BL/6 mice were group-housed, with a maximum of 5–6 animals per cage, and euthanized in compliance with national regulations. They were used exclusively as healthy donors for the ex vivo isolation of splenocytes. As no in vivo experimental intervention or allocation to treatment groups was performed, randomization and blinding at the animal level were not applicable to this study. Splenocytes were isolated by mechanical dissociation of dissected spleens through 70- μ m cell strainers, followed by erythrocyte lysis using NH₄Cl buffer. For invasion assay, 1×10^6 splenocytes were seeded onto untreated, inflamed or CM-hAMSC primed bi-miPSC-NSPHs and co-cultured for 10 days with half-medium change every other day. CM-hAMSC was administered either as a single 50% dose or as three repeated 25% doses on day 0, day 3 and day 5.

RNA-sequencing, label-free proteomics and Ingenuity Pathway Analysis

Bi-miPSC-NSPHs from control, stimulated and stimulated + CM-hAMSC conditions were processed for transcriptomic and label-free proteomic analysis, according to previously established procedures (Di Stefano et al. 2024). For RNA sequencing, each sample consisted of two pooled bi-miPSC-NSPHs. The bi-miPSC-NSPH RNA-seq dataset included $n = 4$ control, $n = 4$ stimulated and $n = 5$ stimulated + CM-hAMSC biological replicates. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and RNA integrity was assessed before library preparation and paired-end sequencing. Reads were aligned to the mouse reference genome GRCm39 using STAR v2.7.10b, and gene counts were generated using FeatureCounts. Differential gene expression analysis was performed using DESeq2 and edgeR in the BigOmics Playground platform. Genes with adjusted $p < 0.05$ and absolute log₂ fold change > 1 were considered differentially expressed. For

label-free proteomics, nine bi-miPSC-NSPHs were pooled per experimental condition (50µg final protein concentration). Proteins were processed using filter-aided sample preparation (FASP) protocol, digested with 0.5 µg/µl trypsin (and analyzed in analytical replicate by nanoLC-MS/MS using an Ultimate 3000 UPLC system coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Raw data were processed using Proteome Discoverer v2.4.0.305 (Thermo Fisher Scientific). Differentially expressed proteins (DEPs) were filtered according to Di Stefano and colleagues (Di Stefano et al. 2024). Differentially expressed genes and proteins were integrated into a combined matrix and analyzed using Ingenuity Pathway Analysis. Diseases and Biofunctions and Canonical Pathways were considered predicted to be activated or inhibited using z-score thresholds of ≥ 2 or ≤ -2 , respectively. RNA-seq data are available under BioProject accession PRJNA989029, and proteomics data are available under PRIDE: accession number PXD080752.

Statistical analysis

No statistical methods were used to predetermine sample sizes. Sample sizes were selected based on effect sizes and inter-replicate variability observed in previous studies using the same experimental models and readouts, together with preliminary data (Bufi et al. 2026a; Di Stefano et al. 2024; Papait et al. 2023; Pianta et al. 2015; Papait et al. 2022; Van Breedam et al. 2022). The number of independent biological replicates proved fully adequate to achieve robust statistical significance. All experiments using human cell lines were repeated five times with independent CM-hAMSC mixes. Normality was assessed by Shapiro-Wilk testing with Q-Q plot inspection; homoscedasticity was assessed using the Brown-Forsythe test. For normally distributed data normalized to the control group, a one-sample t-test was used to determine significant deviations of treated conditions against the reference value. Comparisons among the remaining experimental groups were conducted using a one-way ANOVA test followed by Tukey's post-hoc test. When homoscedasticity was not confirmed, Brown-Forsythe and Welch ANOVA followed by Dunnett's post-hoc test was utilized. Non-normal qRT-PCR data were log₂-transformed and presented as $-\Delta\Delta Ct$. Outliers were identified by ROUT (Q=1%) and excluded from the analyses. Hierarchical clustering and heatmap visualization was made with ClustVis (<https://biit.cs.ut.ee/clustvis/>) with z-score row scaling, correlation distance for clustering and Ward's method for linkage algorithm. Fluorescence intensity was quantified with FIJI (v2.9.0) on ≥ 5 spheroids per condition. For PBMC migration, 6 donors were combined with 3 CM-hAMSC batches and data were analyzed by two-way ANOVA with Tukey's post-hoc test. For SG analysis, staining was repeated twice on three biological replicates; data were analyzed by two-way ANOVA followed by Dunnett's post-hoc test. All analyses were performed with GraphPad Prism v10.5.0. For figure 6, CD45 fluorescence intensity was quantified in total, border and core areas: the border region was defined as the outer 100 µm of the sphere, while the remaining inner area was defined as the core. Cryosections from four bi-miPSC-NSPHs per condition were stained twice, and CD45⁺ splenocyte invasion was quantified in four different areas per bi-miPSC-NSPH. Statistical analysis was performed using JMP Pro Version 16. Data were log-transformed and analyzed using a linear mixed-effects model followed by Tukey's post hoc test. Normality of residuals and homoscedasticity were visually assessed.

Results

Establishment of human 2D monocultures and the 3D tripartite human neurospheroid (tri-hNSPH) model

To investigate CM-hAMSC activity in models of increasing complexity, we employed a standardized set of human neural platforms that we recently described (Buñi et al. 2026a). Briefly, SH-SY5Y and U138-MG cell lines were differentiated into neuron-like cells (NLCs) and astrocyte-like cells (ALCs), respectively, following previously optimized protocols (Buñi et al. 2026a). Five-day exposure to 10 μ M all-trans retinoic acid (ATRA) drove SH-SY5Y toward a neuronal morphology with robust β 3-tubulin upregulation and loss of Nestin, while sodium butyrate treatment induced S100B⁺ astrocytic features in U138-MG cells (*Supplementary Figure S1*). These were combined with HMC-3 microglia in a 1:2:4 ratio in methylcellulose-supplemented neurogenic medium, generating tri-partite human neurospheroids (tri-hNSPHs) that, after 14 days, displayed a reproducible architecture with a central S100B⁺ astrocytic core, peripheral β 3-tubulin⁺ neurons and widespread Iba-1⁺ microglia (*Supplementary Figure S1*). This system represents a simplified yet functional neural-glial-immune unit compatible with high-throughput pharmacological and immune cell migration assays (Kelley and Paşca 2022; Paşca 2018; Xu et al. 2021).

CM-hAMSC restrains pathological pathways of inflamed NLCs, while preserving the physiological expression of homeostatic genes

We first asked how CM-hAMSC modifies the transcriptional response of neurons to a canonical inflammatory trigger. Differentiated NLCs were exposed to CM-hAMSC alone, a pro-inflammatory cocktail of IL-1 β , TNF α and IFN γ , or the combination. Unstimulated NLCs served as basal controls. Gene expression was profiled by qRT-PCR across a panel covering inflammatory signaling and neuron-specific regulatory pathways (**Figure 1A**). CM-hAMSC strongly suppressed the cytokine-induced upregulation of pro-inflammatory and cell death-related transcripts, *TNFA*, *FASR*, *iNOS*, *CXCL10*, *CCL5* and *NF- κ B*, restraining the neuronal contribution to tissue inflammation (**Figure 1B**). In parallel, CM-hAMSC upregulated a set of neuron-specific transcripts associated with synaptic plasticity and neurotrophic support: *NPTX1*, *COX2*, *BDNF* and *CCL2* (**Figure 1C**). This divergent modulation is biologically meaningful: *NPTX1* mediates structural synaptic remodeling and activity-dependent synaptic pruning (Chang et al. 2010); *BDNF* is a neurotrophin supporting neuronal survival, neurotransmitter biosynthesis and neurogenesis (Park and Poo 2013); and *COX2* and *CCL2*, while framed as pro-inflammatory, are constitutively expressed in excitatory neurons. Indeed, they contribute to PGE₂-dependent synaptic plasticity and to neuromodulation of presynaptic excitability, respectively (Kaufmann et al. 1996; Zhou et al. 2011). Other analyzed genes displayed no relevant transcriptional changes (*Supplementary Fig. S2*). The same experimental design applied to ALCs yielded only modest responses: transcripts associated with pro-resolving astrocytic phenotypes were not significantly modulated (*Supplementary Fig. S3*), and among pro-inflammatory markers only *CXCL10* reached statistical significance (**Figure 1D**).

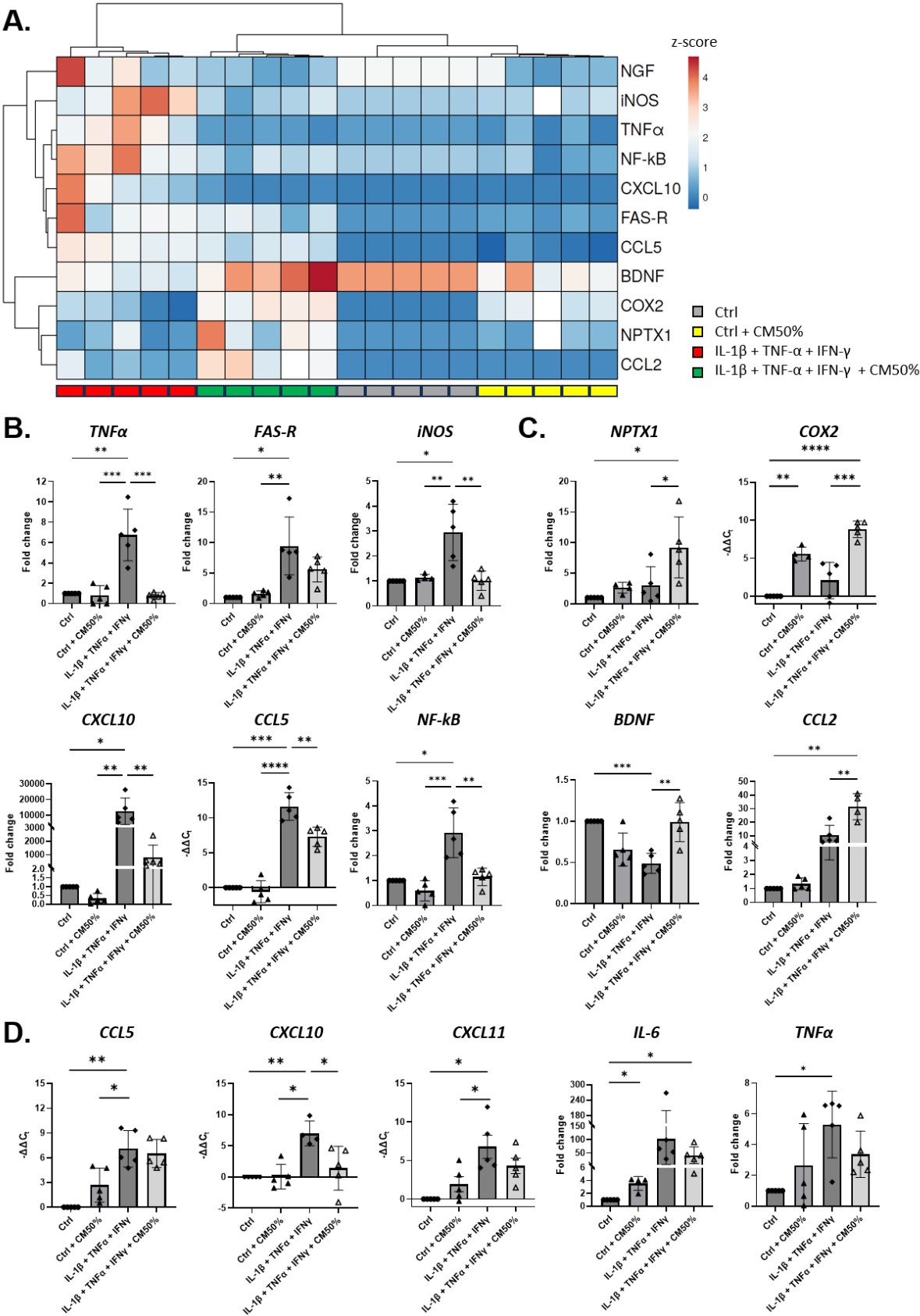


Figure 1. CM-hAMSC modulates inflammatory and neuroprotective gene expression in NLCs. (A) Schematic representation of the qRT-PCR data for modulated gene panel used to assess inflammatory and neuron-specific tran-

scriptional changes in NLCs under Ctrl, CM-hAMSC (“CM50%”), inflammatory (IL-1 β + TNF α + IFN γ), or combined conditions. **(B)** CM-hAMSC strongly attenuates cytokine-induced upregulation of pro-inflammatory and cell death-related transcripts (*TNF α* , *FAS-R*, *iNOS*, *CXCL10*, *CCL5*, *NF- κ B*). **(C)** CM-hAMSC selectively enhances the expression of transcripts associated with synaptic plasticity and neurotrophic support (*NPTX1*, *COX2*, *BDNF*, *CCL2*). **(D)** qRT-PCR of ALCs showing limited responsiveness to CM-hAMSC, with statistical significance only for *CXCL10*. Data shown as mean $\pm$ SD, n = 5; statistical significance was determined by a one-sample t-test and by one-way ANOVA with Tukey’s post-hoc test. Outliers were identified with ROUT method (Q=1%) and excluded from the analysis. *p < 0.03; **p < 0.02; ***p < 0.0002; ****p < 0.0001.

CM-hAMSC restricts M1-like microglial activation while preserving phagocytic competence

Microglia are the primary driver of CNS innate immunity and the main source of pro-inflammatory cytokines during neurodegeneration (Heneka et al. 2015; Colonna and Butovsky 2017). To test CM-hAMSC activity on the microglial compartment, HMC-3 cells were cultured under conditions mirroring those used for NLCs and ALCs and subjected to the same treatments. CM-hAMSC alone did not alter basal gene expression, indicating absence of pro-inflammatory effects on resting microglia. Inflammatory stimulation, by contrast, produced a robust upregulation of classical M1-like markers (*C3*, *IFN γ* , *MMP9*, *COX2*, *CCL2*, *CCL5*, *CXCL9*, *CXCL10*, *CXCL11* and *IL-6*), which CM-hAMSC co-treatment significantly attenuated across the entire panel (**Figure 2A–B**). *IL-18* was the only pro-inflammatory transcript that was modestly upregulated rather than suppressed, suggesting a selective rather than generalized immunomodulatory action. Additional analyzed genes did not vary significantly (*Supplementary Fig. S4*).

To interrogate the functional consequences of this transcriptional shift, we examined a panel of microglial markers by flow cytometry. Among the proteins profiled (*Supplementary Fig. S5*), only HLA-DR and CD68 were expressed by HMC-3. Inflammatory stimulation strongly upregulated HLA-DR, reflecting the induction of antigen-presenting capacity. On the other hand, CM-hAMSC treatment brought HLA-DR close to basal levels, indicative of restrained antigen-presenting activity. Strikingly, CD68 expression remained unchanged across all conditions, demonstrating that CM-hAMSC does not impair microglial phagocytic competence (**Figure 2C**).

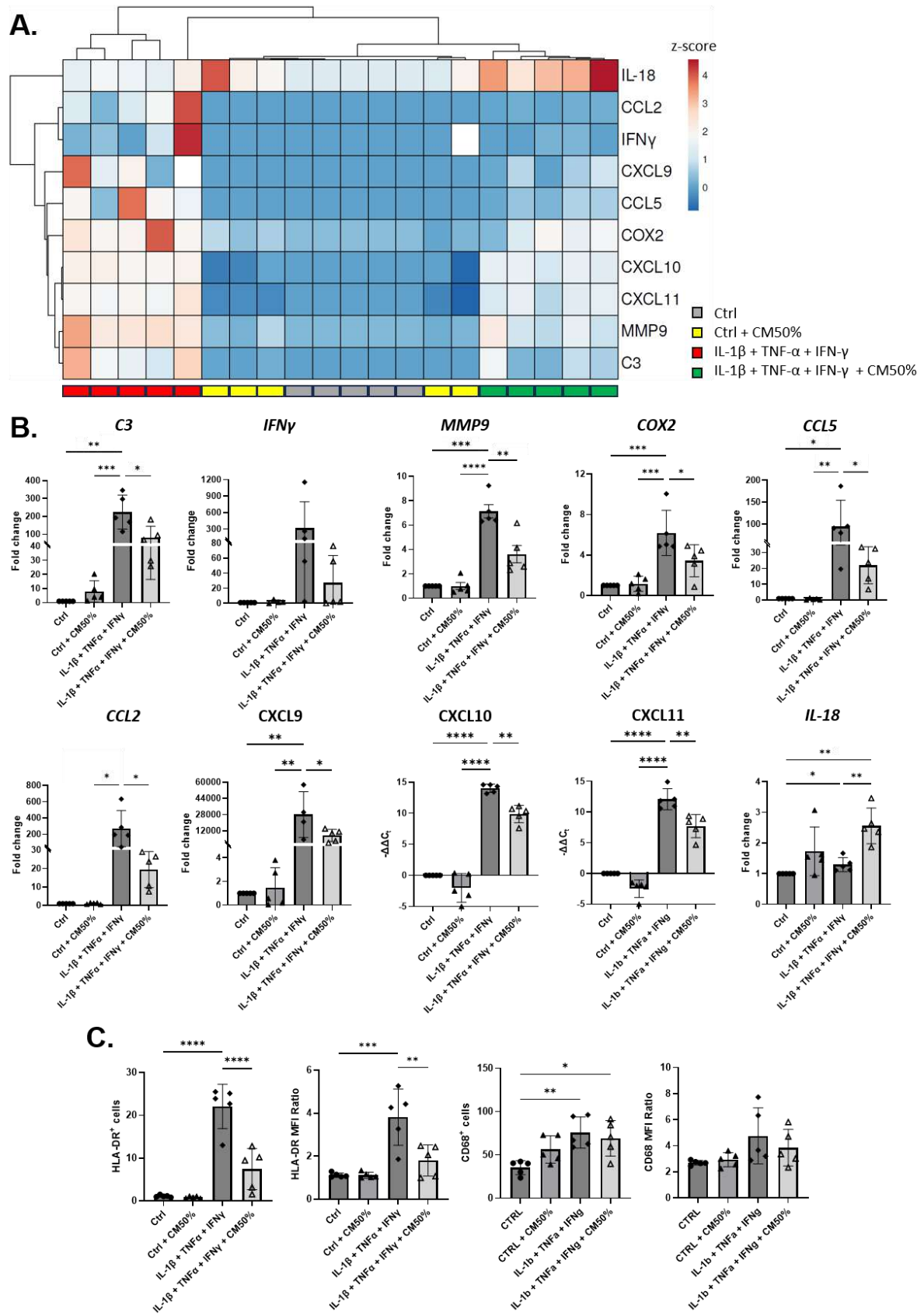


Figure 2. CM-hAMSC restricts cytokine-induced inflammatory activation in HMC-3 microglia. (A) Heatmap of the qRT-PCR panel used in HMC-3 across Ctrl, CM-hAMSC ("CM50%"), inflammatory or combined conditions. (B) Inflammatory stimulation induces robust transcriptional upregulation of classical M1-like markers (*C3*, *IFN* γ ,

MMP9, COX2, CCL5, CCL2, CXCL9, CXCL10, CXCL11, IL-6), significantly attenuated by CM-hAMSC co-treatment. IL-18 shows a modest increase in CM-hAMSC-treated cells. **(C)** Flow-cytometric analysis of HLA-DR and CD68 showing CM-hAMSC-mediated impairment of antigen-presenting activity (HLA-DR) with preservation of phagocytic competence (CD68). Data shown as mean $\pm$ SD, n = 5; statistical significance was determined by a one-sample t-test and by one-way ANOVA with Tukey's post-hoc test. For panel C, statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test. Outliers were identified with ROUT method (Q=1%) and excluded from the analysis. *p < 0.03; **p < 0.02; ***p < 0.0002; ****p < 0.0001.

CM-hAMSC suppresses the integrated inflammatory response of 3D tri-hNSPHs without inducing cytotoxicity

Having established cell-type-specific responses in 2D monocultures, we asked whether CM-hAMSC retains its activity in a heterocellular 3D context where cell-cell interactions amplify inflammatory signaling. CM-hAMSC administration to unstimulated tri-hNSPHs did not alter the baseline secretion of any analyzed mediator, including IFN γ (**Figure 3A–B**), CXCL9, CXCL10, CXCL11, CCL2, CCL5, IL-1 β and TNF α (**Figure 3C**), confirming that the secretome is not pro-inflammatory per se on 3D neural tissue. Inflammatory stimulation, by contrast, triggered a robust and coherent secretion of all these chemokines, validating the tri-hNSPH system as a functional 3D inflammation platform. CM-hAMSC co-treatment reduced the secretion of every measured cytokine and chemokine, including the intracellular pool of IFN γ visualized in situ (**Figure 3A**), demonstrating that the immunomodulatory activity is maintained from 2D monocultures to 3D co-cultures.

An unexpected observation emerged during these experiments: microscopic inspection revealed a partial disassembly of the outer tri-hNSPH layers following CM-hAMSC exposure. We hypothesized that this reflected the well-documented enrichment of matrix metalloproteinases (MMPs) in the amniotic secretome (Muntiu et al. 2023; Marassi et al. 2024). To test this causally, CM-hAMSC was pre-treated for 90 min with 100 μ M minocycline, a tetracycline-derivative with broad MMP-inhibitory activity (Garrido-Mesa et al. 2013). Under these conditions, tri-hNSPH outer layers were preserved, confirming MMP activity as the mechanistic driver of the structural effect (**Figure 3D**). Finally, immunofluorescence staining for cleaved caspase-3 showed the absence of appreciable cell death across all conditions (**Figure 3D**), excluding a cytotoxic component of CM-hAMSC action.

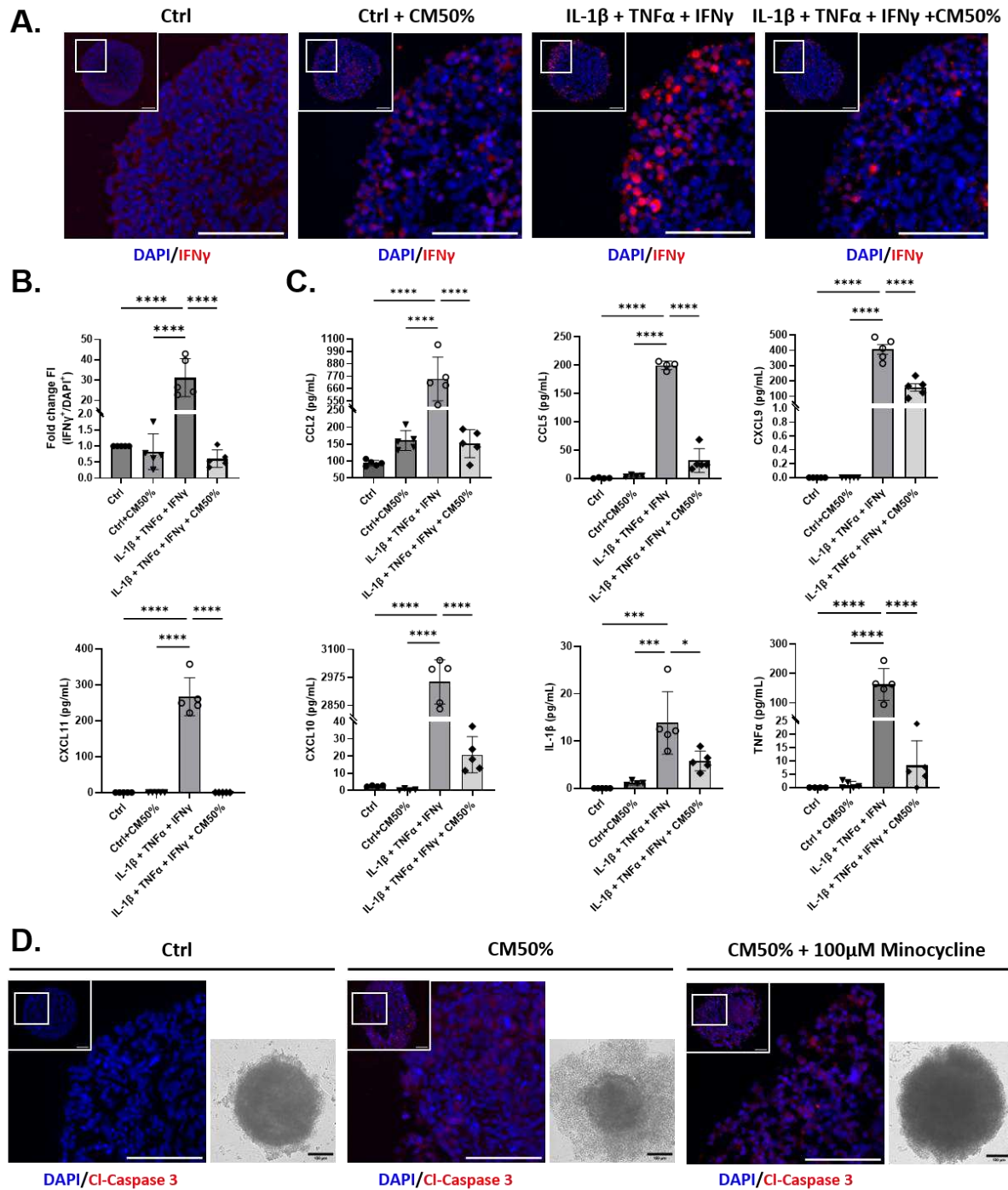
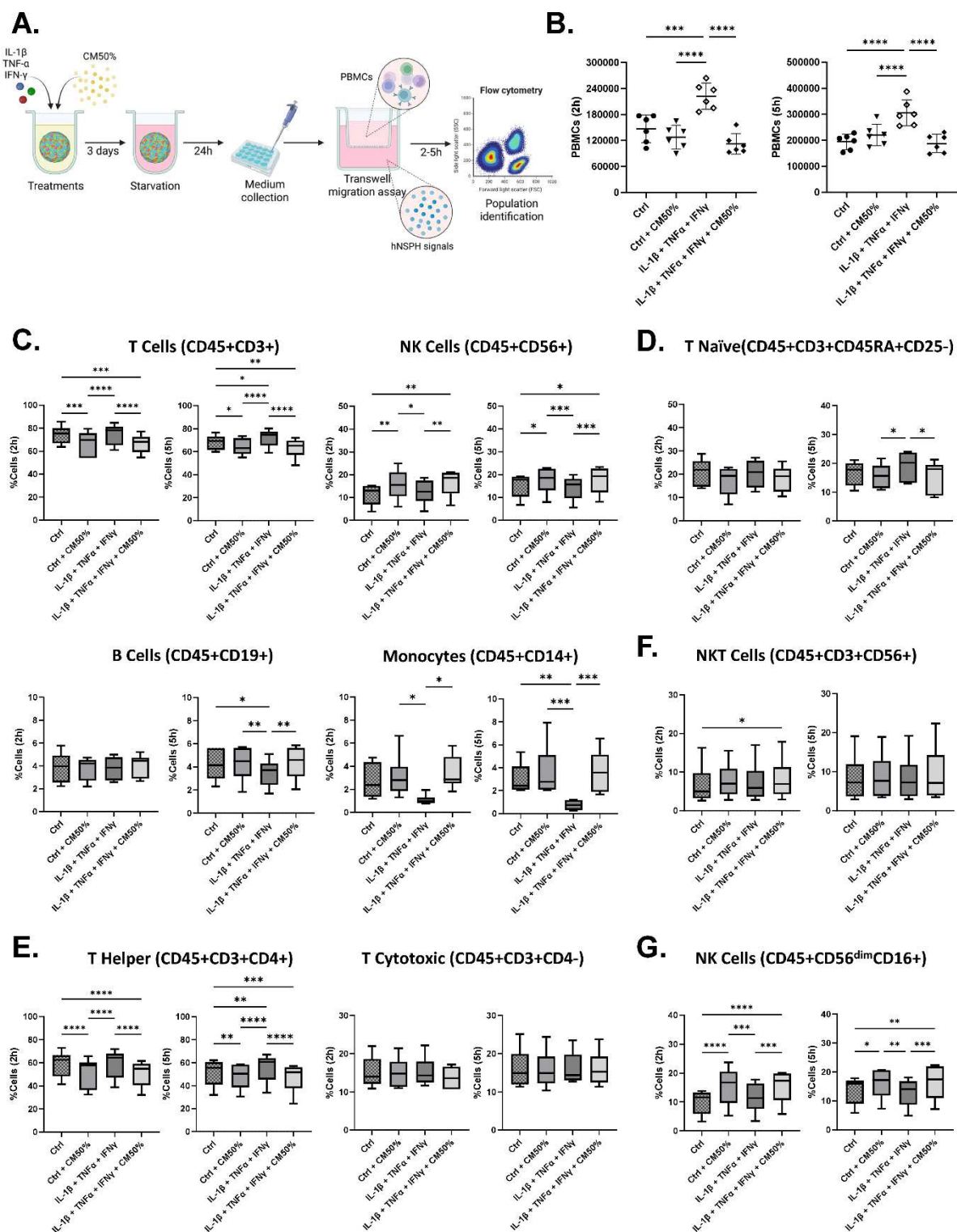


Figure 3. CM-hAMSC suppresses cytokine-induced inflammatory responses in 3D tri-hNSPHs without affecting viability. (A) Representative IFN γ immunofluorescence of cryosectioned tri-hNSPHs across conditions. (B) Quantification of IFN γ fluorescence intensity. (C) Multiplex bead-based immunoassay profiling of secreted CCL2, CCL5, CXCL9, CXCL10, CXCL11, IL-1 β and TNF α showing a robust cytokine induction which is significantly attenuated by CM-hAMSC. (D) Minocycline pre-treatment of CM-hAMSC preserve tri-hNSPH outer-layer integrity and cleaved caspase-3 immunofluorescence staining confirms the absence of cell death. Data are shown as mean $\pm$ SD, n = 5. Statistical significance for panel B was determined by a one-sample t-test and by one-way ANOVA with Tukey's post-hoc test. For panel C, the statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test. Outliers were identified with ROUT method (Q=1%) and excluded from the analysis. *p < 0.03; **p < 0.02; ***p < 0.0002. Scale bars: 100 μ m. *p < 0.03; ***p < 0.002; ****p < 0.0001.

CM-hAMSC inhibits PBMC recruitment by inflamed 3D neural tissue and selectively reshapes the phenotype of migrating immune cells

Recruitment of peripheral immune cells into inflamed CNS tissue is a late but critical amplifier of neurodegeneration (Chitnis and Weiner 2017; Kwon and Koh 2020). To characterize the conditioned medium of tri-hNSPHs, supernatants from each treatment condition were collected after partial starvation and used as chemotactic stimuli in transwell migration assays with freshly isolated human PBMCs. Migrated cells were counted and phenotyped by flow cytometry at 2 and 5 h (Figure 4A). Supernatants from inflamed tri-hNSPHs robustly enhanced PBMC migration at both time points, confirming the establishment of a chemotactically active inflammatory microenvironment. Strikingly, supernatants from tri-hNSPHs co-treated with CM-hAMSC and the inflammatory cocktail reduced PBMC migration below basal levels at 2 h and restored it to baseline at 5 h (Figure 4B).

Phenotypic profiling revealed a cell-type-selective reshaping of the migrated populations (Figure 4C). CD3⁺ T lymphocytes were the most markedly affected compartment, their recruitment falling below basal levels at both time points under combined inflammatory stimulation and CM-hAMSC treatment. CD56⁺ NK cells and CD14⁺ monocytes displayed the opposite pattern, with increased migration toward CM-hAMSC-treated supernatants. B-cell migration was largely unaffected at 2 h and followed the NK/monocyte trend at 5 h. Subset analysis revealed preferential recruitment of naïve T cells under inflammatory conditions and full normalization upon CM-hAMSC treatment (Figure 4D). Among T-cell subsets, CD4⁺ T helper cells recapitulated the suppression observed for total CD3⁺ cells, whereas CD8⁺ cytotoxic T cells were unaffected (Figure 4E). NKT cells showed minimal modulation (Figure 4F), while within the NK compartment the cytotoxic CD56^{dim}CD16⁺ subset emerged as the principal contributor to the increased NK migration (Figure 4G).



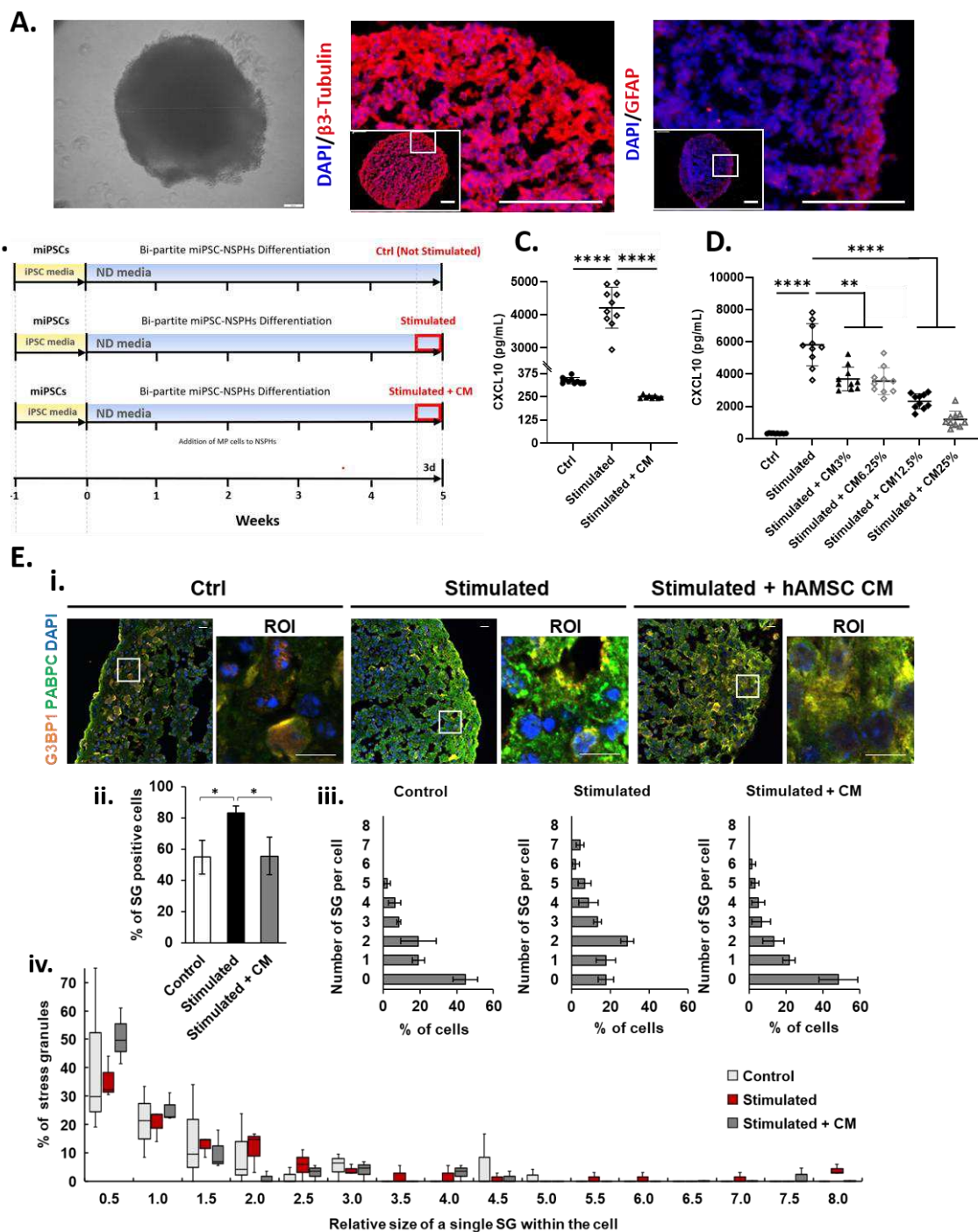
508 **Figure 4. CM-hAMSC inhibits neuroinflammation-induced PBMC recruitment and selectively reshapes the**
509 **phenotype of migrated immune cells. (A)** Experimental workflow: supernatants from tri-hNSPHs (Ctrl, CM-
510 hAMSC ("CM50%"), inflammatory and combined) are used as chemotactic stimuli in transwell migration assays.
511 PBMCs are quantified and phenotyped at 2 h and 5 h. **(B)** Total PBMC migration: CM-hAMSC reduces inflammatory-
512 driven recruitment at both time points. **(C)** Phenotypic distribution of migrated PBMCs showing selective reduction

in CD3⁺ T cells with relative enrichment of CD56⁺ NK cells and CD14⁺ monocytes. **(D)** Naïve T cells are preferentially recruited under inflammation and normalized by CM-hAMSC. **(E)** CD4⁺ T helper cells are strongly suppressed while CD8⁺ cytotoxic T cells are unaffected. **(F)** NKT cells show minimal modulation. **(G)** CD56^{dim}CD16⁺ NK cells account for most of the increased NK migration under CM-hAMSC treatment. 6 PBMC donors were mixed to 3 CM-hAMSC batches; n=6. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. Scale bars: 100 μ m. *p < 0.03; **p < 0.02; ***p < 0.002; ****p < 0.0001.

CM-hAMSC suppresses inflammatory signaling and cellular stress in isogenic bi-miPSC-NSPHs

To further characterize CM-hAMSC activity in a controlled 3D neural environment, we employed our previously established isogenic murine induced pluripotent stem cell derived bi-partite NSPH model (bi-miPSC-NSPHs) composed of β 3-tubulin⁺ neurons and GFAP⁺ astrocytes (**Figure 5A**). Compared with the human 2D and tri-hNSPH experiments described above, this platform provides a genetically uniform neural tissue-like system, allowing us to assess whether the immunomodulatory and stress-protective effects of CM-hAMSC could be reproduced in an independent, isogenic 3D neural model. Upon pro-inflammatory challenge with TNF α , IL-1 β and IFN γ , bi-partite NSPHs exhibited robust inflammatory activation, as evidenced by a strong induction of CXCL10 secretion. Quantitative ELISA analysis further demonstrated that CM-hAMSC treatment suppressed CXCL10 production not only at the standard dose of 50% CM-hAMSC (**Figure 5B-C**), but also in a dose-dependent manner, with significant inhibitory effects observed at concentrations as low as 3% CM-hAMSC (**Figure 5D**). Beyond extracellular mediators, cytokine stimulation triggered extensive assembly of stress granules (SGs), visualized by cytoplasmic clustering of the canonical markers G3BP1 and PABPC1 (**Figure 5E**). Importantly, CM-hAMSC treatment markedly impeded SG formation, resulting in a strong decline in the percentage of SG-positive neural cells and a reduction in granule burden, with fewer SGs per cell (**Figure 5E**). These findings indicate that CM-hAMSC attenuates inflammation-induced chemokine release, translational arrest and proteostatic stress in the neuronal-astrocytic compartment.

Figure 5. CM-hAMSC directly restrains inflammation and cellular stress in a 3D iPSC-derived murine neurospheroid model. **(A)** Representative brightfield and immunofluorescence images of bi-miPSC-NSPHs containing β 3-tubulin⁺ neurons and GFAP⁺ astrocytes. Nuclei are counterstained with DAPI **(B)** Schematic representation of bi-miPSC-NSPH treatments with inflammatory cytokines and CM-hAMSC. **(C-D)** ELISA quantification of CXCL10 in supernatants showing dose-dependent abrogation of inflammation-induced CXCL10, with 50% CM-hAMSC in C and 3-25% CM-hAMSC in D. **(E)** SG quantification in bi-miPSC-NSPHs upon inflammatory stimulation and CM-hAMSC treatment. **(i)** G3BP1 and PABPC1 immunofluorescence images showing that CM-hAMSC prevents the assembly of stress granules induced by pro-inflammatory cytokines. **(ii)** Quantification of SG⁺ cell percentage; **(iii)** number of SGs per cell, and **(iv)** relative SG size per cell. ELISA data are shown as mean $\pm$ SD, n = 10. Statistical significance for panel C and D was determined by Brown-Forsythe and Welch ANOVA followed by Dunnett's post hoc test. Outliers were identified with ROUT method (Q=1%) and excluded from the analysis. Data for SG quantification are shown as mean $\pm$ SD and the statistical significance was determined by two-way ANOVA followed by Dunnett's post hoc test. Scale bars: 100 for overview images and 20 μ m for ROI images. *p < 0.03; ****p < 0.0001.



Multi-omic profiling and functional assays identify CM-hAMSC as a suppressor of inflammatory stress responses and splenocyte infiltration

To place these observations in a broader molecular context, transcriptomic and label-free proteomic datasets from control, stimulated and CM-hAMSC-treated bi-miPSC-NSPHs were integrated and analyzed using Ingenuity Pathway Analysis. Inflammatory stimulation increased activation of pathways associated with immune signaling, cellular stress, cell death and senescence (**Figure 6A**). In CM-hAMSC-treated NSPHs, this profile was attenuated, with reduced activation scores for several inflammatory and stress-associated pathways, with relative preservation of survival- and cell-viability-associated programs (**Figure 6A**). These multi-omic data indicate that CM-hAMSC effects extend beyond CXCL10 suppression and include broader modulation of stress-, death- and

survival-associated molecular programs in inflamed NSPHs. Finally, we evaluated whether these molecular and cellular effects were associated with reduced immune-cell infiltration into the 3D neural tissue. Bi-miPSC-NSPHs were co-cultured with syngeneic C57BL/6 splenocytes under control (Ctrl), inflammatory (stimulated) and CM-hAMSC-treated conditions. CD45⁺ immune-cell infiltration was readily observed in this 3D co-culture system under both control and inflammatory conditions, with signal detected at the outer border and within the spheroid core (**Figure 6B**). Importantly, CM-hAMSC reduced CD45⁺ immune-cell presence across the total NSPH area, including both border and core regions. This effect was observed after both a single 50% CM-hAMSC dose and repeated 25% CM-hAMSC dosing (**Figure 6B**). Together, these findings demonstrate that CM-hAMSC suppresses inflammatory chemokine production, cellular stress and immune-cell infiltration in an isogenic 3D neural model.

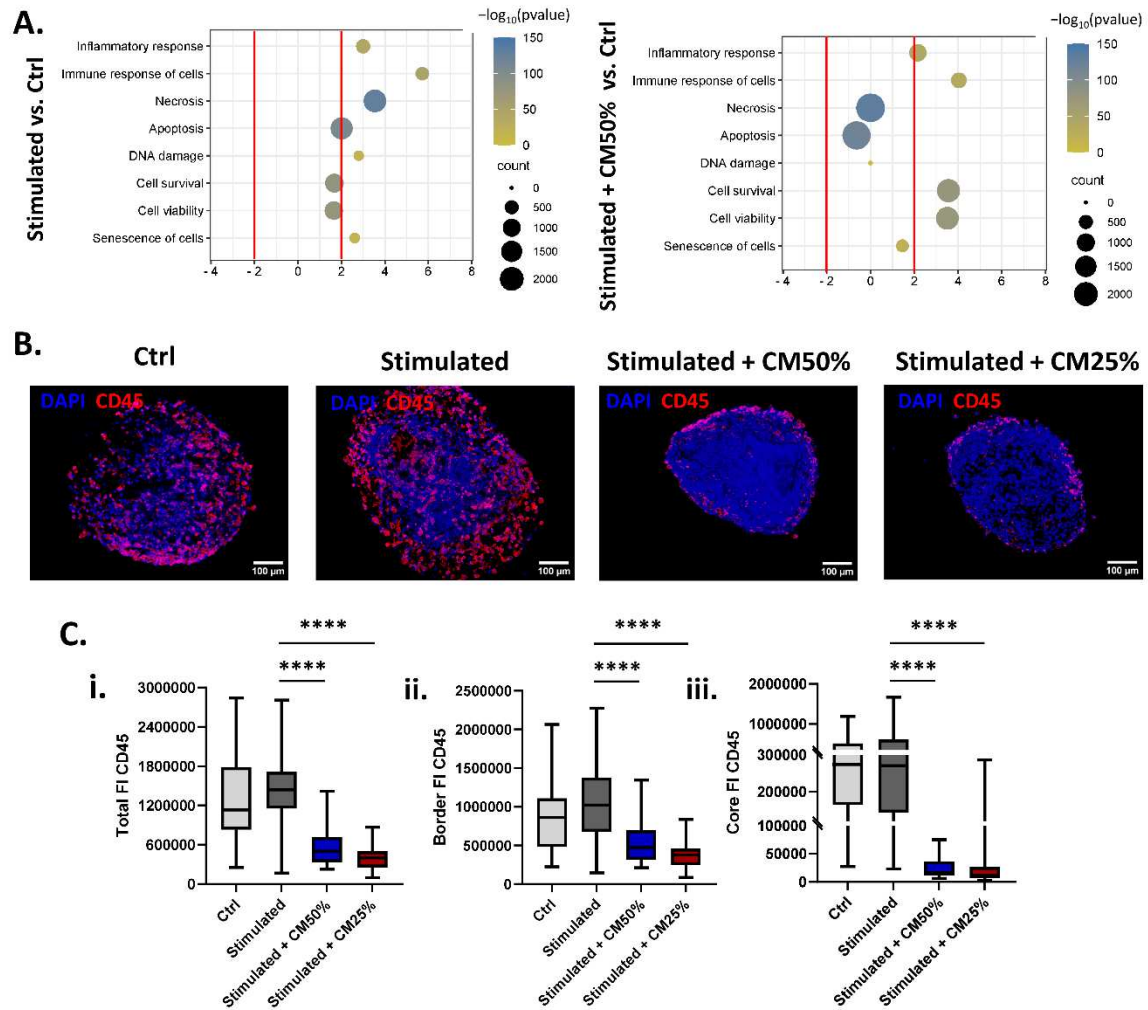


Figure 6. CM-hAMSC suppresses inflammatory stress responses and splenocyte infiltration in bi-miPSC-NSPHs.

(A) Ingenuity Pathway Analysis bubble plot integrating DEGs (RNA-seq) and DEPs (label-free proteomics) from bi-miPSC-NSPHs comparing Stimulated vs Ctrl (left panel) and Stimulated + CM-hAMSC (“CM50%”) vs Ctrl (right panel) conditions. Bubble position indicates activation z-score; bubble size indicates molecule count; color indicates $-\log_{10}(p\text{-value})$. Red vertical lines indicate z-score thresholds of ± 2 . CM-hAMSC treatment partially normalized pathways related to cell death, cellular stress and inflammatory signaling, with relative preservation of survival- and cell-viability-associated programs. (B) Representative CD45 immunofluorescence stainings of bi-miPSC-NSPHs co-cultured with syngeneic C57BL/6 splenocytes for 10 days in basal (Ctrl), inflamed (Stimulated) and inflamed + CM-hAMSC (CM50% and CM25%) conditions. (C) Quantification of CD45 fluorescence intensity in (i) total, (ii) border and (iii) core bi-miPSC-NSPH areas. Statistical significance was determined by a linear mixed effects model followed by Tukey’s post hoc test. Scale bars: 100 μm . **** $p < 0.0001$. RNA-seq: BioProject PRJNA989029. Proteomics: accession number PXD080752.

Discussion

In this study we demonstrate that CM-hAMSC suppresses pathological microglial activation while preserving transcriptional programs associated with synaptic plasticity and neuronal survival. This cell-type selectivity is central for its therapeutic relevance: broad anti-inflammatory strategies suppress both homeostatic and pathological immune activity (Heneka et al. 2015; Kwon and Koh 2020), and this lack of discrimination may contribute to their limited clinical efficacy. The preservation of phagocytic capacity alongside suppression of HLA-DR antigen presentation in microglia confirms that CM-hAMSC reduces inflammatory amplification without impairing the phagocytosis required for clearance of misfolded proteins and apoptotic debris (Colonna and Butovsky 2017; Giampà et al. 2019; Kettenmann et al. 2011).

CM-hAMSC upregulated *COX2* and *CCL2* in neurons while downregulating both in microglia. Although *COX2* and *CCL2* are commonly associated with inflammation, their roles in neurons are distinct. *COX2* is constitutively expressed in excitatory neurons and drives *PGE₂* synthesis required for synaptic plasticity and long-term potentiation (Kaufmann et al. 1996; Yang and Chen 2008). *CCL2* acts as a neuromodulator of presynaptic excitability rather than as an inflammatory signal (Zhou et al. 2011). Their upregulation in neurons by CM-hAMSC therefore likely reflects the maintenance of physiological neuronal signaling programs rather than induction of a pro-inflammatory state. The simultaneous downregulation of both factors in microglia, in which the detrimental role is well documented, confirms that CM-hAMSC acts in a cell-type-specific manner and suggests that CM-hAMSC does not act as a broadly suppressive anti-inflammatory agent.

U138-MG is a glioblastoma-derived line, and its limited transcriptional response to CM-hAMSC (only *CXCL10* was significant) most likely reflects this origin: glioblastoma cells carry extensive genomic alterations that disrupt the inflammatory signaling pathways through which CM-hAMSC acts. Primary or iPSC-derived astrocytes mount a substantially broader response to inflammatory stimuli (Liddelow et al. 2017; Tcw et al. 2017). The action of CM-hAMSC on astrocytes could therefore be underestimated here, and validation in primary or iPSC-derived human astrocytes, ideally within vascularized or BBB-containing assembloids (Prinz et al. 2011; Dao et al. 2024), will be required to determine the full extent of CM-hAMSC activity on astrocytes. The observation that *CXCL10* suppression is significant even in this model confirms the consistency of this specific effect.

CM-hAMSC suppressed the full cytokine and chemokine output of tri-hNSPHs, including the intracellular pool of IFN γ detected in situ. These findings indicate that the immunomodulatory effects observed in 2D monocultures are retained in a more complex three-cell-type 3D environment. The absence of cleaved caspase-3 rules out a cytotoxic mechanism, distinguishing CM-hAMSC from secretomes that reduce cytokine output simply by killing the producing cells (Galipeau and Sensébé 2018).

A separate structural effect was also observed. CM-hAMSC caused MMP-dependent remodeling of the outer cell layers of tri-hNSPHs, which was prevented by minocycline co-treatment. This is consistent with the high MMP content of amniotic membrane-derived preparations, where MMPs are physiologically active during gestation (Magatti et al. 2019; Marassi et al. 2024; Muntiu et al. 2023). Minocycline-mediated prevention of this remodeling is consistent with an MMP-dependent mechanism, and suggests that selective MMP depletion, by fractionation or routine minocycline co-administration, should be further evaluated to separate structural from immunomodulatory activity (Galipeau and Sensébé 2018; Ferreira et al. 2018).

In the PBMC migration assay, CM-hAMSC selectively reduced the recruitment of CD3⁺CD4⁺ T-helper cells while leaving CD8⁺ cytotoxic T-cell migration unaffected. This is consistent with prior reports of MSC-mediated Th17 suppression without impairment of cytotoxic responses (Galipeau and Sensébé 2018) (Pianta et al. 2015; Baron and Storb 2012). This selectivity could be relevant to the neuroinflammatory context: CD4⁺-driven Th1/Th17 activity is a principal driver of neuronal damage in multiple sclerosis and experimental autoimmune encephalomyelitis (Prinz et al. 2011; Chitnis and Weiner 2017), whereas CD8⁺ cytotoxicity is required for pathogen surveillance and thus should be preserved in any long-term immunomodulatory strategy. The observation that CM-hAMSC increased CD56^{dim}CD16⁺ NK cells and CD14⁺ monocytes could be a consequence of reduced T-helper migration rather than direct innate immune recruitment: as the dominant CD4⁺ gradient is suppressed, the weaker chemotactic signals acting on innate populations become proportionally more apparent, despite an overall reduction in total cell migration. This selective suppression of CD4⁺ helper drive with preservation of cytotoxic surveillance recapitulates the established paradigm of MSC-mediated immunomodulation (Galipeau and Sensébé 2018; Pianta et al. 2015; Baron and Storb 2012; Buñi et al. 2026b) and is particularly desirable in a NID context, in which dampening of inflammation must not compromise pathogen clearance capacity.

The bi-miPSC-NSPH (Di Stefano et al. 2024) allowed us to test how CM-hAMSC acts directly on neurons and astrocytes. Three independent readouts support a common conclusion. First, CXCL10 secretion was suppressed dose-dependently across 3–50% CM-hAMSC concentrations, with significant inhibition already at 3%. This low effective concentration is notable and could inform dose selection in prospective in vivo studies. Second, CM-hAMSC prevented the inflammation-induced formation of stress granules in both neurons and astrocytes. SG accumulation has been causally linked to pathological protein aggregation in ALS, frontotemporal dementia (FTD) and related disorders (Wolozin and Ivanov 2019; Mateju et al. 2017; Markmiller et al. 2018). Their suppression in the absence of cell death indicates that CM-hAMSC targets the cellular stress directly, rather than acting by reducing cell viability. Third, transcriptomic and proteomic profiling showed inhibition of cell death and stress-associated pathways alongside upregulation of pro-survival gene expression (Cargnoni et al. 2014). Finally, single (50%) and repeated (25% × 3) dosing produced equivalent protection against splenocyte invasion of inflamed tissue, showing that continuous exposure is not required. This could reflect prolonged retention of secreted factors within the tissue, or induction of a cellular state that is maintained after the stimulus is withdrawn.

CXCL10 was the most consistently downregulated soluble mediator across all four experimental systems examined. As a principal ligand for CXCR3, CXCL10 promotes Th1-skewed CD4⁺ T-cell trafficking (Groom and Luster 2011; Koper et al. 2018), and is elevated in the CSF and lesions of patients with multiple sclerosis, Alzheimer’s disease and ALS (Groom and Luster 2011; Krauthausen et al. 2015). Its suppression by CM-hAMSC was observed across two-dimensional monoculture models, three-dimensional human neurospheroid cultures, murine co-culture systems, and a lymphocyte transmigration assay, indicating a reproducible effect across experimental contexts. This convergence provides a mechanistic link between the attenuation of PBMC migration and the reduction in splenocyte invasion. These findings suggest that CXCL10 suppression contributes substantially to the immunomodulatory activity of CM-hAMSC. From a translational perspective, CXCL10 could satisfy several criteria for a pharmacodynamic biomarker (Buñi et al. 2025). It is detectable in the CSF, its suppression by CM-hAMSC is concentration-dependent

across a clinically relevant dose range, and it is directly coupled to a quantifiable immune recruitment outcome. Identifying a soluble, functionally linked biomarker of this kind addresses a recognized gap in the characterization of MSC-derived secretome preparations, for which validated pharmacodynamic endpoints remain lacking.

Conclusions

These findings build on prior work demonstrating CM-hAMSC activity in neuroinflammation (Giampà et al. 2019; Parolini et al. 2014) and the immunomodulatory properties of amniotic membrane-derived preparations (Silini et al. 2019; Pianta et al. 2015; Magatti et al. 2017; Papait et al. 2022). The present study provides a systematic evaluation of CM-hAMSC activity across the principal cellular constituents of neuroinflammation, encompassing individual CNS cell types, integrated three-dimensional neural tissue constructs, and the peripheral immune interface. The neurospheroid platforms employed here were selected to prioritize experimental reproducibility and pharmacological tractability rather than developmental fidelity, and the findings generate discrete, testable hypotheses amenable to validation in more physiologically complex systems, including iPSC-derived and vascularized organoid models (Kelley and Paşca 2022; Paşca 2018; Lancaster and Knoblich 2014; Xu et al. 2021; Cakir et al. 2019).

Several limitations warrant consideration. The human CNS cellular components were derived from immortalized cell lines, which likely attenuate the magnitude of CM-hAMSC activity relative to primary tissue, particularly in astrocytes (Liddelow et al. 2017; Tcw et al. 2017). Further validation in primary or iPSC-derived human neural cells is therefore required. CM-hAMSC is a compositionally heterogeneous preparation, and the attribution of specific biological activities to defined soluble mediators remains unresolved. Systematic fractionation and component-level functional testing represent a necessary and experimentally feasible next step toward mechanistic characterization. Additionally, neither neurospheroid model incorporates a functional blood-brain barrier, and the immune recruitment assays employed capture early-phase transmigration events. Collectively, these findings indicate that CM-hAMSC exerts reproducible, cell-type-selective immunomodulatory activity across complementary in vitro systems, support CXCL10 as a candidate pharmacodynamic target and soluble biomarker of secretome activity, and provide a structured experimental framework for subsequent evaluation in disease-specific in vivo models.

Declarations

Ethics approval and consent to participate

All animal procedures were performed in compliance with the European guidelines for the care and use of laboratory animals (Directive 2010/63/EU) and were approved by the Committee on Animal Care and Use at the University of Antwerp, Belgium (Approval No: 2021-09).

All procedures involving human samples were conducted in accordance with the principles of the Declaration of Helsinki. Human term placentas were obtained from healthy donors following vaginal delivery or cesarean section after written informed consent was provided. The protocol was approved by the local Institutional Ethics Committee [Comitato Etico Provinciale di Brescia, Italy; approval number NP 2243, January 19, 2016].

Clinical trial number: Clinical trial number: not applicable.

Consent For Publication

All authors approve the manuscript and give their consent for submission and publication.

Data and code availability

Raw RNA-sequencing data generated and analysed in this study are deposited in the NCBI BioProject database under accession number PRJNA989029. Label-free mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository: accession number PXD080752. All other data supporting the findings of this study are available from the corresponding author upon reasonable request. Any additional custom code used for data analysis is available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author contributions

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