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Imiquimod and interferon-alpha augment monocyte-mediated astrocyte secretion of MCP-1, IL-6 and IP-10 in a human co-culture system

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ABSTRACT

Toll-like receptor 7 (TLR7)-activation has been implicated as a significant mechanism of neuroinflammation triggered by ssRNA viruses. Infiltration of monocytes into the brain and astrocyte activation occurs during *in vivo* TLR7-mediated neuroinflammation. The objective here was to determine whether the TLR7 agonist, imiquimod, and interferon-alpha (IFN-α), promote monocyte-mediated astrocyte secretion of pro-inflammatory factors. Using a human primary co-culture system, we demonstrate that monocytes, together with imiquimod and IFN-α, promote astrocyte secretion of MCP-1, IL-6 and IP-10. Furthermore, TLR7-induced monocyte-derived IL-1β is critical for promoting the astrocyte response. Overall, this study provides a potential mechanism for TLR7-mediated neuroinflammation.

1. Introduction

A central feature of central nervous system (CNS) diseases, including viral encephalitis, is chronic neuroinflammation (Furr and Marriott, 2012). This CNS inflammation is characterized by activation of glial cells, including astrocytes and microglia, along with blood-derived immune cells (*e.g.* monocytes), all of which release an array of pro-inflammatory factors that lead to neuronal dysfunction and impaired cognition (Chitnis and Weiner, 2017; Colombo and Farina, 2016; Hong and Banks, 2015; Furr and Marriott, 2012; Terry et al., 2012). Blood-derived monocytes have been implicated in viral encephalitis due to their ability to be infected/activated in circulation, cross the blood-brain barrier and release inflammatory factors (cytokines, reactive species, virions and viral proteins) (Williams et al., 2014; Terry et al., 2012). The inflammatory factors produced by monocytes in the brain may have a profound impact on neuronal injury by promoting glial cell dysfunction, including astrocytes, leading to chronic pro-inflammatory cytokines/chemokines production and impaired glutamate control (Scutari et al., 2017; Gonzalez-Scarano and Martin-Garcia, 2005; Ton and Xiong, 2013; Williams et al., 2014; Kaul et al., 2001; Sofroniew, 2015; Andjelkovic et al., 2000). Thus, monocyte-astrocyte interactions have the potential to facilitate leukocyte infiltration, cytokine secretion and direct neuronal injury (Sofroniew, 2015; Chitnis and Weiner, 2017; Colombo and Farina, 2016).

Activation of toll-like receptor 7 (TLR7) by genomic ssRNA has been implicated as a major pattern-recognition receptor activated during viral-induced neuroinflammation (Furr and Marriott, 2012; Shastri et al., 2013; Lehmann et al., 2012). TLR7 is expressed in monocytes, as well as astrocytes and microglia, and receptor activation leads to cytokine secretion (Cros et al., 2010; Jack et al., 2005). A growing body of evidence has demonstrated a strong neuroinflammatory response with *in vivo* treatment of the TLR7 agonist, imiquimod. Specifically, Butchi

Abbreviations: ABM, astrocyte basal media; APC, allophycocyanin; BV-421, brilliant violet 421™; Cy, cyanine dye; DMEM, dulbecco's modified eagle medium; FITC, fluorescein isothiocyanate; FSC-A, forward scatter area; FSC-H, forward scatter height; GA-1000, gentamicin sulfate-amphotericin; LEAF, low endotoxin, azide-free; MFI, mean fluorescence intensity; Pac-Blue, pacific blue; PE, r-phycoerythrin; PerCP, peridinin chlorophyll protein complex; R837, imiquimod; RPMI, roswell park memorial institute; U251, U-251 MG cell line

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and colleagues have demonstrated a CNS inflammatory response in mice with intracerebroventricular inoculation of the TLR7 agonist, imiquimod. This response was characterized, in part, by astrocyte activation, as well as temporal induction of several pro-inflammatory factors including IL-1β, monocyte chemoattractant protein 1 (CCL2/MCP-1), IL-6 and IFN-γ-inducible protein 10 (CXCL10/IP-10) (Butchi et al., 2008; Butchi et al., 2011). Furthermore, topical administration of imiquimod (Aldara™) to mice, which enters the brain within 4 h post-treatment, also promoted a similar neuroinflammatory response profile (MCP-1, IL-6 and IP-10), which could be detected as early as 4 h post treatment and up to 3–5 days (Nerurkar et al., 2017). In addition, this study illustrated activation of both microglia and astrocytes (Nerurkar et al., 2017). Topical administration of imiquimod also was shown to promote immune cell infiltration into the brain, including monocytes (McColl et al., 2016), which would be speculated to exacerbate CNS inflammation. Of note, the TLR7-mediated pro-inflammatory CNS profile observed in these studies is similar to what is observed in patients who are infected with the ssRNA virus, human immunodeficiency virus (HIV), and display neuroinflammation and cognitive dysfunction (Tavazzi et al., 2014; Fischer-Smith et al., 2001; Anderson et al., 2015).

There are limited studies utilizing human-derived cells to investigate monocytes-glial cell interactions and whether monocytes, especially when activated through TLR7, promote glial cell activation and production of pro-inflammatory factors. Two reports have demonstrated that human monocytes/monocyte-derived macrophages (MDMs) promoted MCP-1 secretion by astrocytes when co-cultured (Andjelkovic et al., 2000; Muratori et al., 2010), while another demonstrated TNF-α and IL-1β production in co-cultures containing HIV-infected MDMs and astrocytes (Genis et al., 1992). However, it is unknown whether TLR7 activation alters human monocyte-mediated astrocyte secretion of MCP-1, in addition to other key immune factors (IL-6 and IP-10). Furthermore, the anti-viral cytokine, interferon-α (IFN-α), is produced in response to TLR7 activation and has been shown to be elevated in the cerebrospinal fluid (CSF) during viral infections (Uematsu and Akira, 2007; Ito et al., 2005; Rho et al., 1995; Perrella et al., 2001; Anderson et al., 2016; Ho-Yen and Carrington, 1987). IFN-α stimulates cells by binding the IFN-α receptor (IFNAR) (Ivashkiv and Donlin, 2014) and may induce specific inflammatory factors different than direct TLR7 activation. Therefore, the major objective of this study was to determine whether the TLR7 agonist, imiquimod, along with IFN-α, modulate monocyte-mediated astrocyte secretion of specific inflammatory factors (MCP-1, IL-6 and IP-10) using a human co-culture system. Furthermore, the readily available astrocyte line, U251, was used alongside primary astrocytes to determine whether it could serve as an initial alternative to primary astrocytes when examining monocyte-mediated astrocyte inflammatory responses.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM) (Catalog #: 12100046) and Roswell Park Memorial Institute (RPMI) 1640 (Catalog #: 31800–022) were purchased from Gibco™ by Life Technologies (Carlsbad, CA). DMEM was supplemented with 10% Fetal Bovine Serum (HyClone) (Thermo Fisher Scientific, Waltham, MA), 1% non-essential amino acids (Gibco™), 1 mM sodium pyruvate (Gibco™) and 100 U/mL Penicillin/100 μg/mL streptomycin (Gibco™). RPMI1640 was supplemented with 5% human AB serum (Sigma-Aldrich, St. Louis, MO) and 100 U/ml Penicillin/100 μg/mL streptomycin (Gibco™). Trypsin-EDTA (0.25%) was purchased from Gibco™. Universal Type I Interferon Alpha was purchased from PBL Assay Science (Piscataway Township, NJ) and imiquimod (R837) from InvivoGen (San Diego, CA). Golgi transport inhibitor (Monensin (2 μM)/Brefeldin A (3.0 μg/mL) in methanol) was purchased from Thermo Fisher Scientific. Antibodies purchased from BioLegend (San Diego, CA) included anti-CD45-Pacific Blue (clone: HI30, Cat. #: 304029), anti-CD14-Pe-Cy7 (M5E2, 301,814), anti-CD16-APC (3G8, 302,012), anti-CD56-PerCP (HCD56, 318,342), anti-CD57-PerCP/Cy5.5 (HNK-1, 359,622), anti-GFAP-BV 421 (2E1.E9, 644,710) anti-IL-6-APC (MQ2-13A5, 501,112), anti-IL-1β-FITC (JK1B-1, 508,206) and anti-MCP-1-PE (5D3-F7, 502,604). Anti-IP-10-PerCP-eFluor710 (4NY8UN, 46–9744-41) antibody was purchased from Thermo Fisher Scientific. For cytokine neutralization experiments, LEAF™ anti-human MCP-1 (2H5, 505,905), anti-human IL-6 (MQ2-13A5, 501,109), anti-human TNF-α (MAb1, 502,803), armenian hamster IgG (HTK888, 400,915), rat IgG1, κ (RTK2071, 400,413) and mouse IgG1, κ (MOPC-21, 400,123) were purchased from BioLegend. Anti-human IL-1β (4H5, mabg-hil1b-3) and mouse IgG1, κ (T8E5, mabg1-ctrlm) were purchased from Invivogen (San Diego, CA). Recombinant human IL-1β was purchased from BioLegend.

2.2. U251 and human primary astrocyte cell culture

The U-251 MG (U251) astrocyte cell line was purchased from Sigma-Aldrich (Cat #: 09063001) and passaged in supplemented DMEM according to manufacturer's direction. U251 cells were used between passages 4 and 10. Fetal-derived normal human astrocytes (NHA) isolated from the cerebral cortex were purchased from Lonza (Basel, Switzerland) and cultured according to manufacturer's direction in astrocyte basal media (ABM™) supplemented with SingleQuots™ Kit (rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and FBS). Media was changed every other day for passaging and plate seeding. NHA were used up to passage 4. Depending on the experiment, primary astrocytes from two or three donors were used (donor 1–3).

2.3. Peripheral blood mononuclear cell (PBMC) and monocyte isolation

PBMCs were isolated from human leukocyte packs (Gulf Coast Regional Blood Center, Houston, TX) of healthy donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA). Pan monocytes were isolated by negative selection (Human Pan Monocyte Isolation Kit from BioLegend or Miltenyi Biotec (Bergisch Gladbach, Germany)) per manufacturer's direction. The mean (± SD) monocyte purity for donors ($N = 49$) used in this report was 91.1 ± 5.4%.

2.4. Monocyte-astrocyte co-culture

One day prior to co-culture, U251 astrocytes were seeded at a cell density of 7×10^4 cells/well in a 24-well plate (3.5×10^4 cells/cm^2) in supplemented DMEM. The estimated cell density on the day of co-culture was 1×10^5 cells/well (as determined by optimization experiments). On the day of co-culture with U251 astrocytes, monocytes were purified as described above and resuspended in supplemented RPMI 1640. Normal human astrocytes (NHA) were seeded in supplemented ABM™ 3–6 days prior to co-culture at a cell density (based on doubling time) that would be an estimated 1×10^5 cells/well on the day of co-culture. For co-culture involving NHA, monocytes were placed in supplemented ABM™. Monocytes were placed at the appropriate concentration to establish a monocyte:astrocyte ratio of 1:20 with the exception of Fig. 1, where monocytes were placed at different concentrations for the differing cell ratios. Monocytes were added to the culture wells containing astrocytes at a volume of 500 μL. Overall, the total well volume was 500 μL with an astrocyte concentration of 2×10^5 cells/mL and monocyte concentration of 1×10^4 cells/mL. When appropriate, IFN-α and/or R837 were added to the co-culture at the concentration stated in the figure legends. The co-culture was incubated at 37 °C and 5% CO_2 for 20 h (h) and a Golgi block was added to culture for an additional 4 h. At 24 h, wells were washed with non-supplemented media and trypsinization was performed to remove astrocytes from plate. The flow cytometry procedure below was performed to measure cell viability and cytokine production. For particular

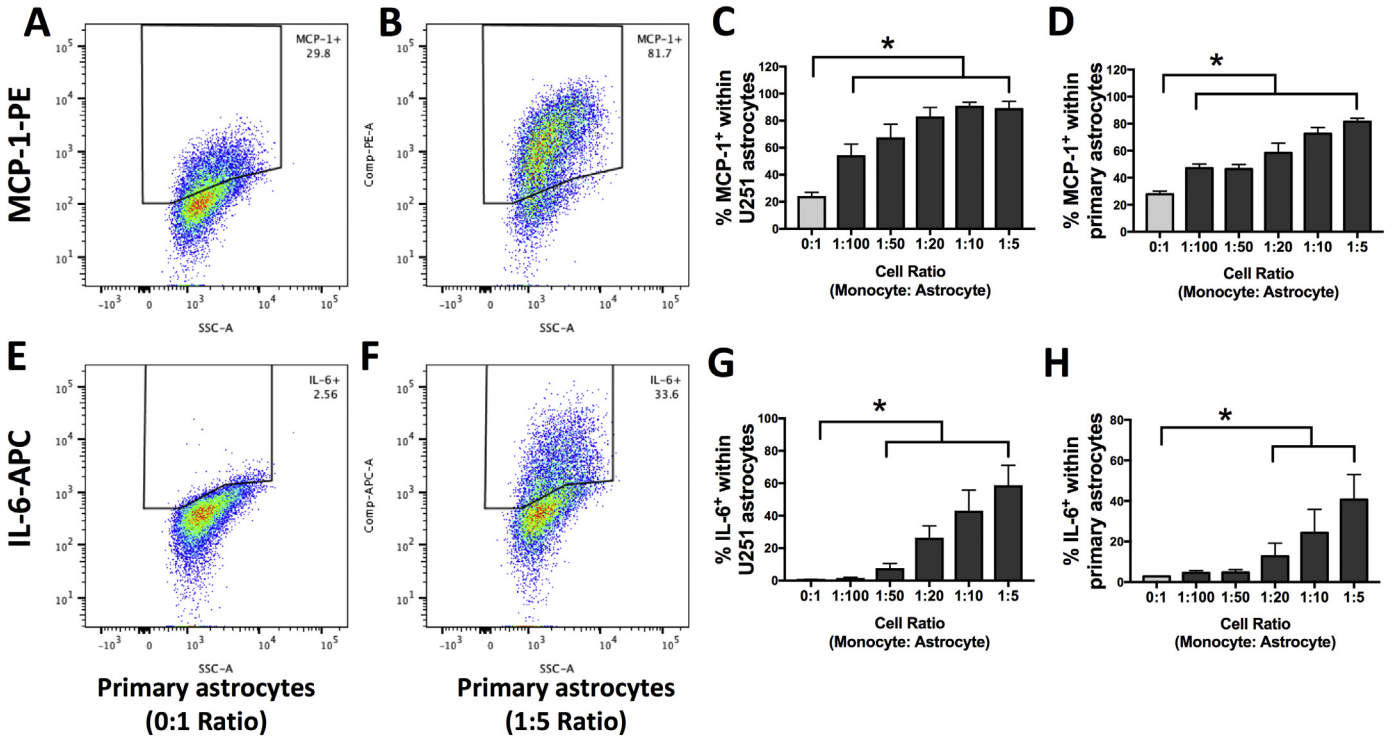

Fig. 1. Human primary monocytes induce human U251 and primary astrocytes to produce MCP-1 and IL-6. Human primary monocytes ($N = 5$ for C-D and N = 5 for G-H) were purified from healthy donors and co-cultured with U251 or primary astrocytes (donor 1). Astrocytes were cultured at 2×10^5 cells/mL and monocytes were added at various concentrations to establish monocyte:astrocyte ratios. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1 and IL-6. FSC-A and FSC-H were used to separate the astrocyte population from the monocytes. Panels A-B and *E*-F are flow cytometry plots of astrocyte production of MCP-1 and IL-6 comparing astrocytes alone (0:1 ratio) to a monocyte to astrocyte ratio of 1:5. For statistical analysis, dataset was first log transformed. Next, a one-way (C and G) or repeated measures (RM) (D and H) analysis of variance (ANOVA) with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0:1 ratio group ($p < .05$). Graphs are mean +/− SEM.

experiments, supernatants were collected at 20 h and stored at −80 °C. LEGENDplex™ (BioLegend) and/or ELISAmax™ technology was utilized to determine cytokine levels in supernatants per manufacturer's direction. Undetectable cytokine levels for specific samples were given a value of 0 pg/mL for graphing and statistical analysis.

2.5. Transwell assay

Transwell®-Clear Inserts (24-well plate format) with a 0.4 μm pore size (Catalog #: 3470) were purchased from Corning (Corning, NY). The co-culture was performed as above with the exception of the total well volume being 700 μl. The lower chamber consisting of astrocytes at a volume of 600 μl while the upper chamber contained monocytes at a volume of 100 μl. The final cell concentration of monocytes and astrocytes was kept the same (astrocyte: 2×10^5 cells/mL and monocyte: 1×10^4 cells/mL).

2.6. Cytokine quantification in monocyte supernatants

To determine the cytokines produced by monocytes, the co-culture procedure above was performed in the absence of astrocytes (monocytes only). Supernatants were collected at 20 h and stored at −80 °C. LEGENDplex™ (BioLegend) technology was utilized to determine cytokine levels in supernatants per manufacturer's direction. Data analysis was performed using the LEGENDplex™ software. Undetectable cytokine levels for specific samples was given a value of 0 pg/mL for graphing and statistical analysis.

2.7. Cytokine neutralization assay

For cytokine neutralization, the co-culture procedure above was performed with the addition of neutralization antibodies targeting human MCP-1, IL-6, TNF-α and IL-1β. The neutralization antibodies were used at a concentration of 1 μg/mL. The isotype control for each respective neutralization antibody was added to culture at the same concentration (1 μg/mL).

2.8. Intracellular IL-1β production by monocytes

Purified monocytes (1×10^6 cells/mL) were left untreated (NS) or stimulated with IFN-α or R837 for 16 h and a Golgi block was added for 4 h. The flow cytometry procedure below was used to measure intracellular IL-1β protein expression.

2.9. Flow cytometry

Staining buffer (PBS, 1% BSA, 0.1% NaN_3) was used to wash cells in between staining and fixing steps. Cells were first incubated with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific) and then with staining buffer containing 20% human AB serum to block Fc receptors. BD Cytofix™ (BD Biosciences, San Jose, CA) was used to fix cells. For intracellular staining, cells were stained with antibody in BD Perm/Wash™ (BD Biosciences). Fixed cells were analyzed on a BD Canto II™ (BD Biosciences). For intracellular staining, a protein transport inhibitor containing Monensin and Brefeldin A (Thermo Fisher Scientific) was added to cell culture 4 h prior to harvesting of cells for analysis. Data analysis was performed using FLOWJO v10 software. Monocyte surface staining to determine purity was performed

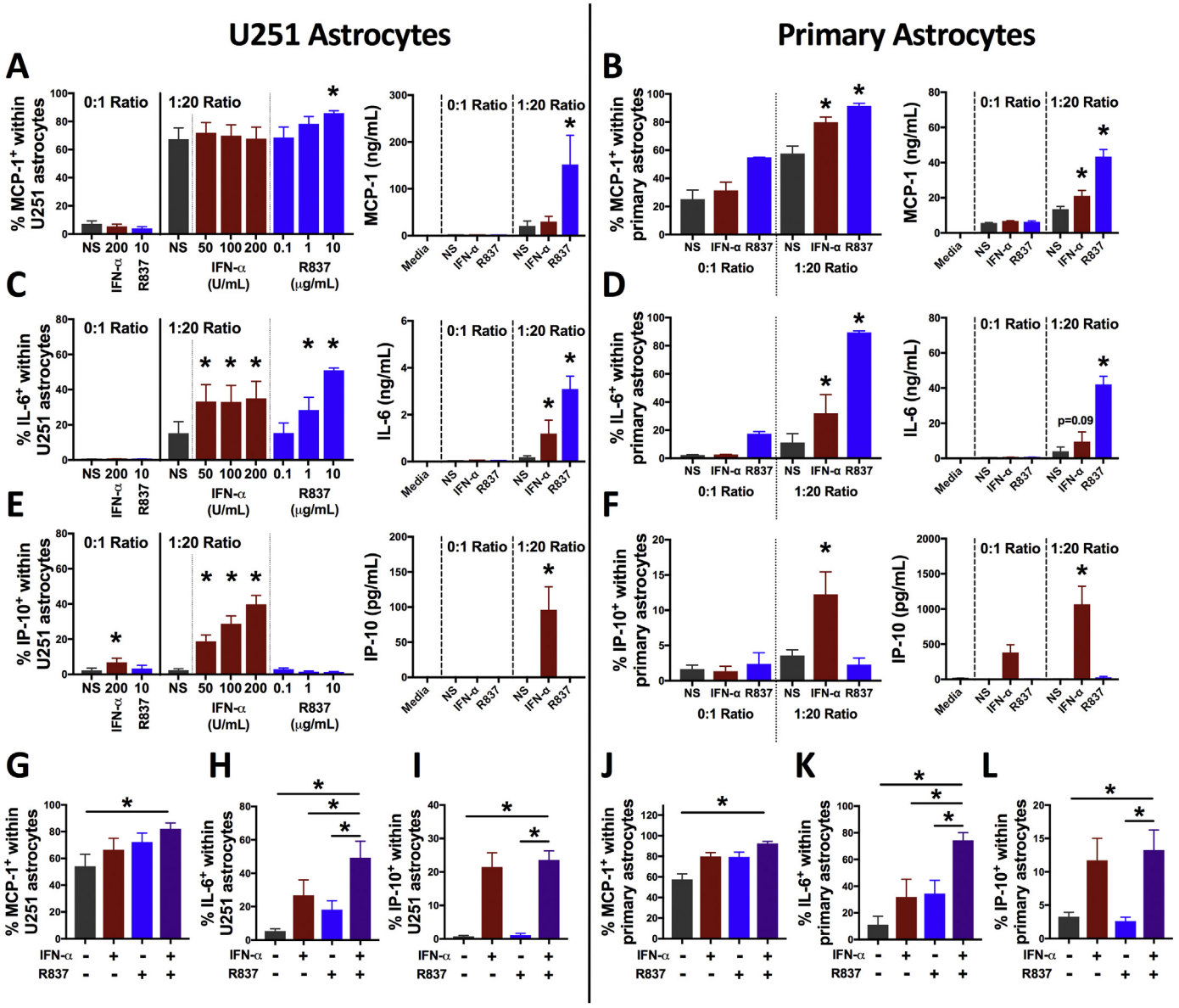

Fig. 2. Imiquimod/R837 treatment alone and with IFN-α, enhances monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10. (A-F) Human primary monocytes were co-cultured with U251 or primary astrocytes (donor 1) at a ratio of 1:20 (monocyte:astrocyte) and stimulated with IFN-α (50, 100 and 200 U/mL) or R837 (0.1, 1 and 10 μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10. The right panels in A-F are supernatant levels of cytokines in co-culture measured *via* LEGENDplex™ and/or ELISAmax™. The N values for monocytes isolated from human donors was $N = 9$ and $N = 3$ for left and right panels, respectively, in A, C, E, and N = 5 for right and left panels in B, D, F. (G-L) IFN-α (100 U/mL) and R837 (1 μg/mL) were added alone and in combination to the co-culture and astrocyte production of MCP-1, IL-6 and IP-10 was determined ($N = 6$ for G-I and N = 5 for J-L). For A-F, dataset was log transformed and a RM ANOVA with a Dunnett's multiple comparisons post-test was performed. For G-L, dataset was log transformed and a RM ANOVA with a Tukey's post-test was performed. For A-F, * denotes a statistical difference from NS of the respective ratio group ($p < .05$). Graphs are mean +/− SEM.

immediately after isolation using anti-CD14, CD16, CD45, CD56, and CD57. Single cells were first gated based on CD45 expression ($CD45^+$). Within the $CD45^+$ population, monocytes were defined as $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate) and $CD14^{+/-}CD16^+$ (non-classical) but lacking CD56/CD57 expression. For analysis of co-culture experiments, astrocytes were gated apart from monocytes based on forward-scatter area and height (FSC-A and FSC-H) and optimization experiments verified that the astrocytes were $GFAP^+$ (Supplementary Fig. 1A-B). An additional optimization experiment was performed using the leukocyte marker CD45 to show no monocyte contamination in the gating strategy utilized (Supplementary Fig. 1C-D). The percent of $MCP\text{-}1^+$, $IL\text{-}6^+$ or $IP\text{-}10^+$ cells within the astrocyte population was reported in the figures. The mean fluorescent intensity (MFI) within the respective positive cells for the corresponding protein was calculated. The gates set for each protein were based on isotype controls.

2.10. Recombinant IL-1β treatment of astrocytes

U251 or primary astrocytes were seeded into 24-well plates as described in co-culture section above. Astrocytes were treated with varying concentrations of recombinant IL-1β (noted in each experiment) for 20 h and a Golgi block was added for 4 h. Additional experiments on astrocytes were performed with IL-1β treatment (20 pg/

mL) in combination with IFN-α or R837. Two independent experiments were performed in triplicates for U251 cells. For primary cells, one independent experiment was performed for two separate biological replicates.

2.11. Statistical analysis

Statistical analysis was performed using Prism 7 and 8 (GraphPad, San Diego, CA). The experimental data was graphed as the mean +/− SEM. The statistical tests performed for each experiment are indicated in the figure legends.

3. Results

3.1. Human primary monocytes induce human U251 and primary astrocytes to produce MCP-1 and IL-6

To determine whether monocytes were able to promote astrocyte production of specific inflammatory factors, purified monocytes from human donors were cultured with U251 or primary astrocytes at cell ratios (monocyte:astrocyte) ranging from 1:100 to 1:5, and astrocytes were measured for intracellular MCP-1, IL-6 and IP-10. As shown in flow cytometry plots in Figs. 1A-B, monocytes at a 1:5 ratio (monocyte:astrocyte) strongly increased the percent of astrocytes producing MCP-1 compared to astrocytes cultured alone (0:1 ratio). Furthermore, the astrocyte MCP-1 response increased in a monocyte-dependent manner, with comparable responses observed between U251 and primary astrocytes (Fig. 1C-D). Interestingly, MCP-1 production by astrocytes (U251 and primary) was significantly increased at the lowest ratio (1:100) tested compared to astrocytes alone (0:1 ratio) (Fig. 1C-D). The percent of astrocytes producing IL-6 also increased in a monocyte-dependent manner, with a significant increase in IL-6$^+$ astrocytes detected at a 1:50 ratio for U251 and 1:20 for primary astrocytes when compared to astrocytes alone (0:1 ratio) (Fig. 1E-H). IP-10 production by astrocytes was not induced by monocytes (Supplementary Fig. 2). Astrocytes from a second donor were used to verify these findings and similar trends were observed in MCP-1 and IL-6 production; however, the percent of MCP-1$^+$ astrocytes were significantly increased at the 1:20 ratio when compared to astrocytes alone (Supplementary Fig. 3A and 3B).

3.2. Imiquimod/R837 treatment alone and with IFN-α, enhances monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10

The next objective was to determine whether imiquimod/R837 and IFN-α treatment of the co-culture affected monocyte-mediated astrocyte production of MCP-1, IL-6 and/or IP-10. R837 treatment of the co-culture containing U251 or primary astrocytes increased the percent of MCP-1$^+$ astrocytes compared to co-culture without stimulation (NS) (Fig. 2A-B). This observation was paralleled with an increase in supernatant MCP-1 in the R837-stimulated co-culture (Fig. 2A-B). IL-6 production by astrocytes was also augmented by R837-treatment of the co-culture, as evidenced by an increase in IL-6$^+$ astrocytes as well as elevated supernatant IL-6 compared to the NS-co-culture (Fig. 2C-D). It is notable that R837 treatment of astrocytes alone displayed a trending increase in the percent of MCP-1$^+$ and IL-6$^+$ cells (two technical replicates), suggesting that R837 is capable of directly stimulating primary astrocyte production of these factors (left panel, Fig. 2B and 2D). R837 had minimal to no effect on astrocyte production of IP-10 in the co-culture, which was observed with both U251 and primary astrocytes (Fig. 2E-F, Supplementary Fig. 3E and 4C). After extending the co-culture period to 48 and 72 h, R837 promoted a minor, but statistically significant increase in the percent of astrocyte expressing IP-10 at the 48 h time point (Supplementary Fig. 5A). Furthermore, a similar effect was observed on supernatant IP-10 levels, as a minor increase was observed at 48 as well as 72 h (Supplementary Fig. 5B). The R837-mediated effects observed in Figs. 2B, D and F were also evident in co-cultures containing primary astrocytes from two additional donors (Supplementary Fig. 3C-E and 4A-C).

Recombinant IFN-α treatment, in combination with monocytes, had no effect on the percent of astrocytes expressing MCP-1 or supernatant MCP-1 in the U251 co-culture compared to co-culture without stimulation (NS) (Fig. 2A). However, IFN-α did augment the production of IL-6 and IP-10 (% and supernatant) in U251 containing co-cultures (Fig. 2C and E). In co-cultures containing primary astrocytes from three separate biological donors, IFN-α induced MCP-1, IL-6 and IP-10 production by astrocytes compared to the control (NS) co-culture, evidenced by an increase in the percent positive astrocytes as well as supernatant levels of these factors (Fig. 2B, D and F, Supplementary Fig. 3C-E and 4A-C). It is noteworthy that although not statistically significant, the IFN-α treated co-culture using the second astrocyte donor displayed increases in MCP-1 and IL-6 (Supplementary Fig. 3C-D). Interestingly, direct addition of IFN-α addition to astrocytes alone (0:1 ratio) also resulted in an increase in the percent of IP-10$^+$ U251 astrocytes (left panel, Fig. 2E) and in supernatant IP-10 levels of primary astrocyte co-cultures (right panels in Fig. 2F, Supplementary Fig. 3E and 4C), suggesting that IFN-α is directly acting on the astrocyte.

To determine the combined impact of R837 and IFN-α, the co-culture was treated with R837 (1 μg/mL) and IFN-α (100 U/mL) alone, and in combination. In co-cultures containing either U251 or primary astrocytes from three separate donors, there was an additive effect on the percent of astrocytes expressing IL-6 with R837 and IFN-α together, compared to when treated individually (Fig. 2H and K, Supplementary Fig. 3G and 4E). No additive effect was observed on the percent of MCP-1$^+$ or IP-10$^+$ astrocytes in co-culture containing U251 cells or for two of the three primary astrocyte donors (Fig. 2G, I, J and L, Supplementary Fig. 3F, 3H, 4D and 4F).

3.3. Astrocyte production of MCP-1, IL-6 and/or IP-10 in response to monocytes alone and in combination with IFN-α or R837 is primarily mediated through soluble factors

To determine whether monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10 was driven through cell-cell contact or secreted soluble factors, Transwell®-Clear Inserts (0.4 μm pore size) were utilized to separate the monocytes and astrocytes. U251 astrocytes were in the bottom chamber and monocytes were placed in the upper chamber. As shown in Fig. 3A-B, monocyte-mediated astrocyte production of IL-6 and MCP-1, regardless of activation, was similar between normal wells (N) and transwells (T). However, there was a significant decrease in monocyte-mediated astrocyte production of IP-10 (IP-10$^+$ cells) when stimulated with IFN-α in transwells compared to normal wells (Fig. 3C). Overall, these data demonstrate that soluble factors released from monocytes are promoting astrocyte production of IL-6 and MCP-1, and both soluble factors and cell-cell contact are promoting IP-10 production.

3.4. Monocytes display differing cytokine/chemokine secretion profiles when cultured without stimulation, with IFN-α or R837

To determine potential monocyte-secreted factors that stimulate astrocyte production of MCP-1, IL-6 and IP-10, monocyte monocultures were treated with IFN-α or R837 under the same conditions as the co-culture (monocytes only). Supernatants were analyzed for several known monocyte-derived inflammatory factors (MCP-1, TNF-α, IL-1β, IFN-γ, IL-6 and IP-10) were quantified using LEGENDplex™. Untreated monocytes (NS) produced minimal but detectable amounts of TNF-α, IL-1β, IL-6 and MCP-1, compared to media alone (Fig. 3D). R837 stimulation enhanced production of IL-1β (5.94-fold), IL-6 (5.78-fold) and TNF-α (1.59-fold), compared to NS monocytes, while IFN-α only induced monocyte production of MCP-1 (3.61-fold) (Fig. 3D). IFN-γ was

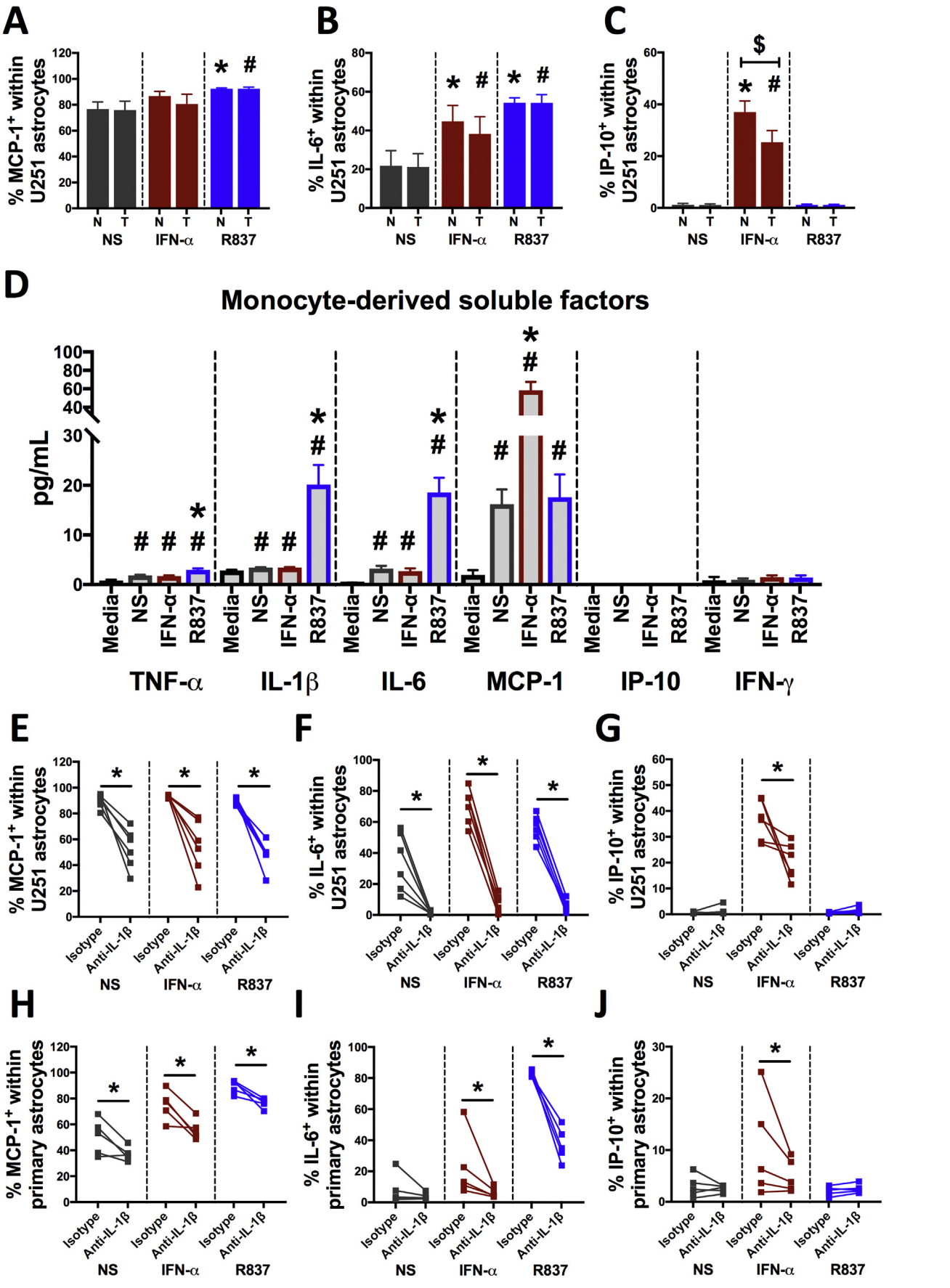

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Fig. 3. IL-1β is a major factor governing the monocyte-mediated astrocyte response to R837. (A-C) Human monocytes (N = 9) were co-cultured with U251 astrocytes at a ratio of 1:20 (monocyte:astrocyte) in either normal wells (N) or 0.4 μm transwells (T). Co-culture was stimulated with IFN-α (100 U/mL) or R837 (10 μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. (D) Human monocytes (N = 9) were cultured alone at 1×10^4 cells/mL. Monocytes were either left untreated (NS), treated with IFN-α (100 U/mL) or R837 (10 μg/mL). Monocytes were cultured for 20 h and supernatants were collected. LEGENDplex™ was used to measure supernatant levels of MCP-1, TNF-α, IL-1β, IFN-γ, IL-6 and IP-10. (E-J) Human primary monocytes (N = 6 for E-G and N = 5 for H-J) were co-cultured with astrocytes (U251 or primary donor 1) at a ratio of 1:20 and stimulated with IFN-α (100 U/mL) or R837 (10 μg/mL). Anti-IL-1β was added to the co-culture at a concentration of 1 μg/mL. An isotype (1 μg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. For A-C, a RM two-way ANOVA with a Sidak's multiple comparisons post-test was performed. * denotes a statistical difference from the NS group in normal (N) wells ($p < .05$) and # denotes a statistical difference from the NS group in transwells (T). $ denotes a statistical difference between normal wells and transwells. For D, a log transformation was performed if there was unequal variance (F test). Then an unpaired t-test was performed to determine significant differences between media and each treatment. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed to determine differences between NS and either IFNα or R837-treated monocytes. # denotes a statistical difference between media control. * denotes a statistical difference from the NS ($p < .05$). For E-J, a paired t-test was performed to determine significant differences between isotype control and anti-IL-1β group. A log transformation was performed for H-J prior to the paired t-test. * denotes a statistical difference from the isotype control ($p < .05$). Graphs are mean +/− SEM.

not induced by monocytes regardless of the stimulation and IP-10 was not detectable (Fig. 3D).

3.5. IL-1β is a critical soluble factor involved in monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10 in response to R837 treatment

To determine whether the cytokines (MCP-1, IL-1β, IL-6 and TNF-α) produced by monocytes had an impact on monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10, neutralizing antibodies targeting these factors were added to the co-culture. In the R837-stimulated co-cultures containing U251 or primary astrocytes, anti-IL-1β treatment suppressed the percent of astrocytes producing MCP-1 and IL-6 as well as substantially decreased the levels (MFI) of these factors within the positive cells compared to the isotype control (Fig. 3E-F and 3H-I, Supplementary Fig. 6A-B, 6D-E, 7A-B, 7D-E, 8A-B and 8D-E). Interestingly, IL-1β neutralization of U251 cells displayed a greater suppression of in MCP-1 and IL-6 compared to primary astrocytes. In the R837-stimulated co-culture, IL-1β neutralization promoted a 47% and 90% decrease in the percent of MCP-1^+ and IL-6^+ U251 astrocytes as compared to 14% (% MCP-1^+) and 55% (% IL-6^+) reduction in primary astrocytes (Fig. 3E-F and 3H-I). The reduction in levels (MFI) of each protein with IL-1β neutralization was comparable between U251 and primary astrocytes in the R837-stimulated co-culture (Supplementary Fig. 8A-B and 8D-E).

Anti-IL-1β treatment of the NS- and IFN-α-stimulated co-cultures containing U251 cells resulted in decreased astrocyte production of MCP-1 and IL-6 (% and MFI), while IP-10 production (% and MFI) was decreased specifically in the IFN-α-stimulated co-culture (Fig. 3E-G, Supplementary Fig. 8A-C). A similar response was observed in IFN-α-stimulated co-cultures containing primary astrocytes, in which anti-IL-1β decreased astrocyte production (% and/or MFI) of MCP-1, IL-6 and IP-10 in at least two of the three primary astrocyte donors tested (Fig. 3H-J, Supplementary Fig. 6A-F, 7A-F and 8D-F). However, anti-IL-1β treatment of non-stimulated co-cultures containing primary astrocytes from three astrocyte donors displayed minimal to no impact on MCP-1 or IL-6, which differs from the observations in the co-cultures containing U251 cells (Fig. 3H-I, Supplementary Fig. 6A-B, 6D-E, 7A-B, 7D-E and 8D-E).

TNF-α neutralization resulted in minor yet significant impairment in the percent of MCP-1^+ (NS and IFN-α), IL-6^+ (IFN-α), and IP-10^+ (IFN-α) U251 astrocytes (Supplementary Fig. 9A-C). However, in primary astrocytes (both donors), anti-TNF-α treatment of either U251 or primary astrocyte co-cultures displayed minimal to no effect on astrocyte production of MCP-1, IL-6 or IP-10 (Supplementary Fig. 6G-I, Supplementary Fig. 9D-F). Additionally, no inhibition was observed with anti-IL-6 or MCP-1 treatment in any of the three co-culture conditions containing U251 cells (Supplementary Fig. 10).

To confirm monocyte production of IL-1β (shown in Fig. 3D), intracellular staining for IL-1β was performed on control (NS), IFN-α and R837-stimulated monocyte monocultures. IL-1β expression was detected, albeit at lower levels, in monocytes left unstimulated (NS) compared to isotype control (Supplementary Fig. 11A, B, C and F). Monocytes stimulated with IFN-α did not induce IL-1β (% and MFI) compared to NS monocytes, while R837 treatment resulted in a robust increase in the percent and levels (MFI) of IL-1β within monocytes (Supplementary Fig. 11A-F).

To determine whether astrocytes were producing IL-1β, primary astrocytes from both donors were cultured alone and stimulated with R837 or IFN-α and supernatant IL-1β was measured. Regardless of stimulation, minimal to no IL-1β production was observed by astrocytes (Supplementary Fig. 12A). To identify whether monocytes could potentially induce astrocyte production of IL-1β, monocytes were co-cultured with primary astrocytes (1:5 ratio), stimulated with IFN-α or R837 and measured for intracellular IL-1β. In the NS and IFN-α-treated co-cultures, only monocytes were positive for IL-1β (26–27% of monocytes) while < 1.5% of astrocytes were IL-1β$^+$ (Supplementary Fig. 12B). For the R837-treated co-culture, both populations had detectable IL-1β, with roughly 70% of the monocytes and 10% of the astrocytes expressing IL-1β (Supplementary Fig. 12B).

3.6. Replacement of monocytes with recombinant IL-1β in the control, R837 and IFN-α cultures, promotes a similar profile of MCP-1, IL-6 and IP-10 production by astrocytes

To determine if IL-1β alone (at concentrations comparable to that observed in Fig. 3D) could promote astrocyte production of MCP-1, IL-6 and IP-10, U251 and primary astrocytes were treated with recombinant IL-1β at various concentrations (0.5, 1, 5, 10, 20, 50 and 100 pg/mL) and intracellular MCP-1, IL-6 and IP-10 production was measured. Recombinant IL-1β induced astrocyte production of MCP-1 and IL-6 in a concentration-dependent manner (Fig. 4A-B and 4D-E). A significant increase in the percent of MCP-1^+ astrocytes was detected at concentrations as low as 0.5 and 1 pg/mL of IL-1β for U251 and primary astrocytes, respectively, when compared to 0 pg/mL (Fig. 4A and D). A detectable increase in IL-6 production was observed starting at 5 pg/mL of IL-1β for both U251 and primary astrocytes (Fig. 4B and E). IL-1β had no effect on IP-10 production for both U251 and primary astrocytes (Fig. 4C and F). Similar trends were observed with the second primary astrocyte donor (Supplementary Fig. 13A-C). One notable difference between astrocyte donors was the percent of astrocytes producing IL-6 in response to IL-1β. For instance, the percent of astrocytes producing IL-6 in response to 100 pg/mL of IL-1β in astrocyte donor 1 was 56% (Fig. 4E) *versus* 28% in the second donor (Supplementary Fig. 13B).

To determine whether IL-1β treatment could mimic the actions of monocytes in the IFN-α- and R837-stimulated co-cultures, astrocyte monocultures were treated with recombinant IL-1β in combination with R837 or IFN-α. The concentration of IL-1β added (20 pg/mL) to culture was selected based on its comparable profile to monocytes (1:20 ratio) at promoting astrocyte production of MCP-1 and IL-6. As noted in Fig. 2, direct stimulation of primary astrocytes with R837 promotes a minor, but significant increase in the percent of astrocytes expressing

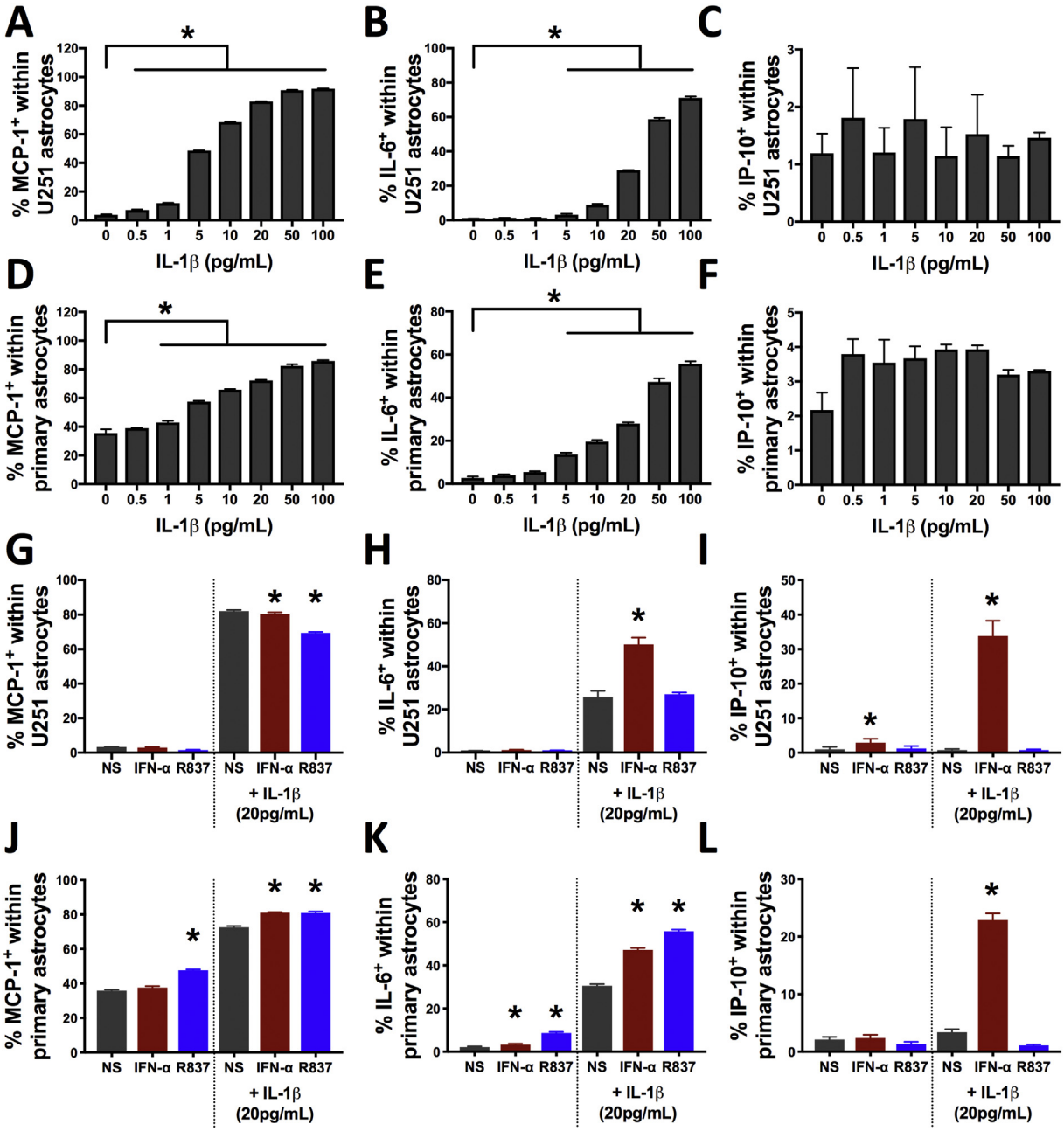

Fig. 4. Replacement of monocytes with recombinant IL-1β in the control, R837 and IFN-α cultures, promotes a similar profile of MCP-1, IL-6 and IP-10 production by astrocytes. (A-F) Astrocytes (U251 and primary donor 1) were treated with varying concentrations of recombinant IL-1β (0, 0.5, 1, 5, 10, 20, 50 and 100 pg/mL). (G-L) Astrocytes were left untreated (NS), treated with IFN-α (100 U/mL) or R837 (10 μg/mL) alone or in combination with IL-1β (20 pg/mL). Cells were incubated for 20 h and a Golgi block was added for 4 h. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed for A-L. * denotes a statistical difference from 0 pg/mL for A-F and the respective NS treatment group for G-L. Graphs are mean +/− SEM.

MCP-1 compared to control (NS) astrocytes (Fig. 4J, Supplementary Fig. 13D and 14A), demonstrating direct activity of R837 on the primary astrocytes. Treatment of U251 cells in combination with both IL-1β and R837 decreased the percent of MCP-1$^+$ astrocytes compared to IL-1β alone, while having no effect on IL-6 or IP-10 production (Fig. 4G-I). By contrast, IL-1β + R837 treatment of primary astrocytes increased the percent of IL-6$^+$ astrocytes compared to IL-1β alone, which was observed in 2 of 3 astrocyte donors examined (Fig. 4K, Supplementary Fig. 13E and 14B). IL-1β + R837 treatment of primary astrocytes had minimal to no effect on MCP-1 and IP-10 production compared to IL-1β alone (Fig. 4J and L, Supplementary Fig. 13D, 13F, 14A and 14C). Further, IL-1β + IFN-α treatment enhanced IL-6 production (%) by astrocytes (U251 and primary) compared to IL-1β alone, and IP-10 production was induced (Fig. 4H, I, K and L, Supplementary Fig. 13E-F and Supplementary Fig. 14B–C), showing similarity to the IFN-α-treated co-cultures. Minor effects were observed with IFN-α on the percent of MCP-1$^+$ astrocytes (U251 and primary astrocytes) compared to IL-1β alone (Fig. 4G and J, Supplementary Fig. 13D and 14A).

3.7. The intermediate monocyte population positively associates with U251 astrocyte production of IL-6

As there are three subsets of human monocytes (classical, intermediate and non-classical) (Wong et al., 2012), we determined whether

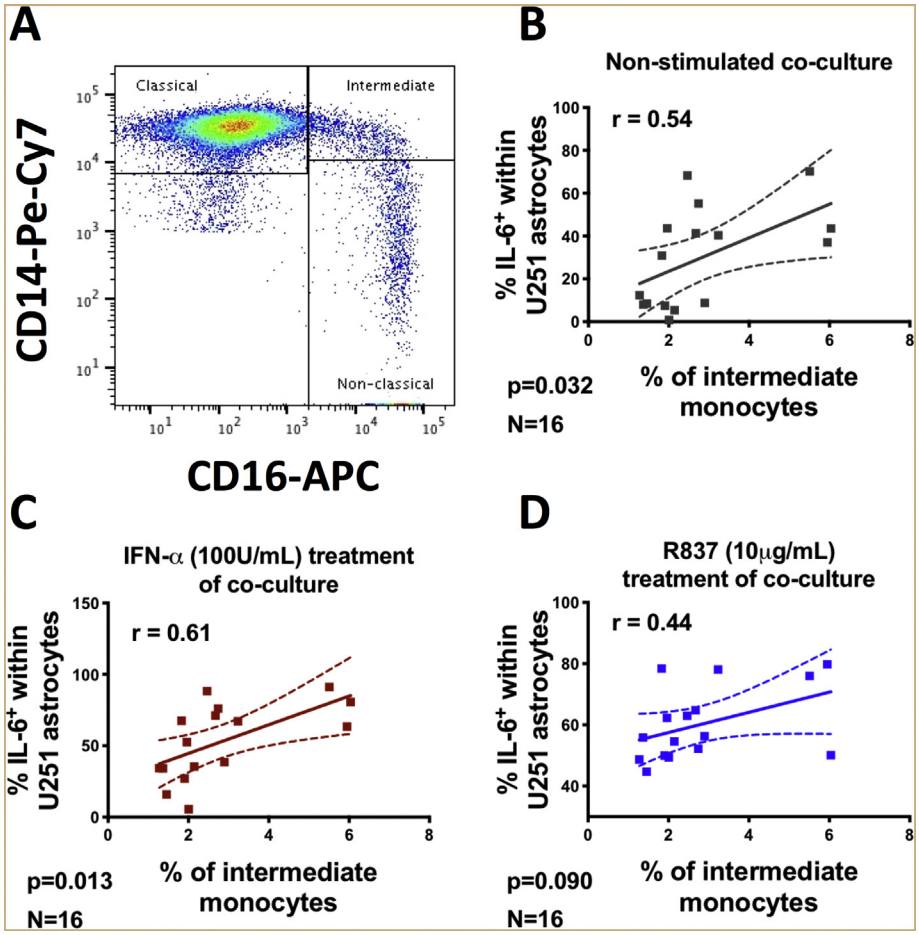

Fig. 5. The intermediate monocyte population positively correlates with U251 astrocyte production of IL-6. Human primary monocytes (N = 16) were co-cultured with U251 astrocytes at a ratio of 1:20 and stimulated with IFN-α (100 U/mL) or R837 (10 μg/mL). Cells were co-cultured for 24 h, with a Golgi blocker added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10. Extracellular staining of CD14 and CD16 was performed on an aliquot of monocytes prior to co-culture. The percent of each monocyte population was determined. A correlation analysis (Prism 7, GraphPad) was performed between monocyte subsets and U251 astrocyte production of MCP-1, IL-6 or IP-10.

there was an association between specific monocyte subsets and astrocyte production (U251) of IL-6, IP-10 or MCP-1. Fig. 5A demonstrates the flow cytometry gating strategy used to separate each monocyte subset. The percent of each subset was determined within the monocyte population. Then the overall percent within the whole monocyte preparation was determined, which took into account the overall purity of the sample. Interestingly, the percent of intermediate monocytes exhibited a significant positive correlation with the percentage of IL-6$^+$ astrocytes in the NS (r = 0.54, p = .032) and IFN-α-treated (r = 0.61, p = .013) co-cultures, while a trending correlation (r = 0.44, p = .09) was observed with R837 treatment (Fig. 5B-D).

4. Discussion

As circulating monocytes have the potential to exacerbate several neurological conditions, including viral encephalitis (Williams et al., 2014; Feng et al., 2011; Mishra and Yong, 2016; Terry et al., 2012), it is important to investigate the role of monocytes on the brain-resident cell inflammatory responses. Previous reports have identified monocyte infiltration into the brain during *in vivo* imiquimod (TLR7)-induced neuroinflammation, along with increased astrocyte reactivity and cytokine production (Butchi et al., 2008, Nerurkar et al., 2017, McColl et al., 2016, Butchi et al., 2011). Of note, several inflammatory factors were induced in the brain of imiquimod-treated mice, including MCP-1, IL-6 and IP-10, which were observed across multiple studies (Butchi et al., 2008, Nerurkar et al., 2017, McColl et al., 2016, Butchi et al., 2011). Here we utilized a human co-culture system to determine if imiquimod and IFN-α enhance monocyte-mediated astrocyte secretion of MCP-1, IL-6 and IP-10; as this may be a potential mechanism explaining the observations seen *in vivo*. Furthermore, the ability of monocytes to induce astrocyte secretion of these factors will facilitate a positive feedback loop of immune cell infiltration, including monocytes (MCP-1) and T cells (CXCL10), which was observed up to 5-days post imiquimod treatment (Sofroniew, 2015, McColl et al., 2016, Deshmane et al., 2009, Liu et al., 2011).

In the present study, non-stimulated monocytes promoted astrocyte production of MCP-1 and IL-6 at cell ratios as low as 1:100 and 1:20 (monocyte:astrocyte), respectively, suggesting that a low-level of monocyte infiltration into the CNS can likely promote astrocyte inflammatory responses. Furthermore, imiquimod/R837 activation of monocytes strongly enhanced astrocyte secretion of MCP-1 and IL-6. However, R837 had a minimal impact on IP-10 production in the co-culture, which contrasts with the induction observed *in vivo* (Butchi et al., 2008, Nerurkar et al., 2017, McColl et al., 2016, Butchi et al., 2011). IFN-α, which is produced in response to TLR7 stimulation (Ito et al., 2005; Uematsu and Akira, 2007), was in fact, able to induce astrocyte production of IP-10, as well as MCP-1 and IL-6. The results presented here suggest that IFN-α is acting directly on the astrocyte, plausibly through binding to the IFNAR (Wang et al., 2012), as recombinant IL-1β + IFN-α induces IP-10 and IL-6 production, compared IL-1β treatment alone. Furthermore, combining R837 and IFN-α in the co-culture promoted an additive effect on astrocyte production of IL-6, suggesting an exacerbation of the astrocyte inflammatory response when the stimuli are present together.

The mechanism by which R837-stimulated monocytes promote astrocyte activation was shown to be through soluble factors as cell-cell contact was not required, which is in contrast from a previous report (Andjelkovic et al., 2000). The discrepancy could be due to multiple differences in experimental conditions including different astrocyte sources, monocyte:astrocyte ratios or stimulation (*e.g.* R837). However,

IFN-α-induced astrocyte production of IP-10 was mediated, in part, through cell-cell contact.

The major monocyte-derived soluble factor responsible for astrocyte production of MCP-1 and IL-6 in R837-stimulated co-cultures was identified to be IL-1β, which is in agreement with a previous report (Andjelkovic et al., 2000). By contrast, we did not observe involvement of TNF-α, which was also previously reported (Andjelkovic et al., 2000). In addition, an association was observed in the co-culture between the level of intermediate monocytes and U251 astrocyte production of IL-6, which is consistent with the current view of intermediate monocytes being a major source of IL-1β (Cros et al., 2010; Yang et al., 2014) We also demonstrate that recombinant IL-1β induced astrocyte production of MCP-1 and IL-6 in a similar manner as the monocyte, which has been documented elsewhere (Aloisi et al., 1992; Andjelkovic et al., 2000). The substantive observation from the present study is the sensitivity of human astrocytes to IL-1β concentrations as low as 1-5 pg/mL, as it demonstrates that inflammatory responses in the brain can be initiated by remarkably low amounts of IL-1β. Interestingly, neutralization of IL-1β in co-cultures containing primary astrocytes did not fully suppress MCP-1 and IL-6 production suggesting that other mechanisms are contributing to the astrocyte response, which could include other monocyte-derived factors not examined here, such as IL-1α (Kasahara et al., 1998), as well as a direct effect of R837 on the astrocyte population.

For the majority of endpoints in this study, all three of the primary astrocyte donors responded similarly, especially in the R837-stimulated co-culture. However, a few differences were observed as identified in the results. For example, IFN-α stimulation of co-cultures containing primary astrocytes from two of the three donors displayed an augmentation in MCP-1 and IL-6, while the second donor exhibited a non-significant increase in these factors. The differences observed here may be attributed to factors such as astrocyte heterogeneity (Miller, 2018; Schitine et al., 2015).

There were limitations in this study, including the source of primary astrocytes used, which were fetal-derived, as adult primary astrocytes were not accessible. There is a possibility for varying inflammatory responses between fetal and adult astrocytes in terms of monocyte and IL-1β activation. However, a previous study demonstrated IL-1β-mediated activation of adult human astrocytes to release pro-inflammatory factors (Oh et al., 1999), suggesting that adult astrocyte responses to monocytes and IL-1β would be similar to the fetal astrocyte responses reported here. Also, as physiologically relevant ratios of monocyte:astrocytes aren't well established during viral-mediated neuroinflammation and is likely dependent on a number of factors, the ratios utilized in this report may not precisely reflect what is observed *in vivo*. In addition, the co-culture presented here consisted of only monocytes and astrocytes, despite other cell types, including microglia, pericytes, brain microvascular endothelial cells (BMVECs) and differentiated macrophages, being present during *in vivo* neuroinflammation and interacting with monocytes (Abbott et al., 2006; Chitnis and Weiner, 2017). In fact, IL-1β has been shown to induce inflammatory responses in pericytes and BMVECs (Jansson et al., 2014, O'Carroll et al., 2015), suggesting that monocyte-derived IL-1β also impacts the function of these cell types. Future studies will be needed to evaluate the role of imiquimod/R837 on other cell-cell interactions. Lastly, despite being freshly isolated human monocytes, once present in the *in vitro* co-culture conditions, these cells have the potential to shift to a macrophage phenotype due to increased basal activation (Coccia et al., 1999).

5. Conclusion

This study used a human co-culture system that included primary monocytes and either U251 or primary fetal astrocytes to expand the current body of knowledge on monocyte-astrocyte interactions, with emphasis on monocyte-mediated astrocyte inflammatory responses by TLR7 activation. Specifically, we demonstrate that monocytes, in combination with imiquimod and IFN-α, induce astrocyte production of MCP-1, IL-6 and IP-10, which are key factors involved in leukocyte recruitment and TLR7-mediated CNS inflammation. In addition, monocyte-derived IL-1β was identified to be a primary factor governing the astrocyte response, highlighting a potential mechanism of TLR7-mediated neuroinflammation. These findings together with the *in vivo* observation of monocyte influx into the CNS with peripheral imiquimod administration (McColl et al., 2016), suggest that single-stranded RNA viruses, through TLR7 activation of monocytes in the periphery and CNS, are capable of inducing or augmenting CNS inflammation. This potential TLR7-mediated mechanism may have important implications in neuroinflammation and cognitive dysfunction observed in infections such as chronic HIV, hepatitis C and influenza (Hong and Banks, 2015; Gonzalez-Scarano and Martin-Garcia, 2005; Huckans et al., 2009; Grover et al., 2012; Hosseini et al., 2018; Jurgens et al., 2012). In addition, this study demonstrates the effectiveness of the astrocyte line, U251, as a surrogate for primary astrocytes in this co-culture model, as similar conclusions could be drawn between the two. Lastly, the co-culture system developed here could serve as a useful tool for examining monocyte involvement in various neuroinflammatory diseases (*e.g.* Alzheimer's, Parkinson's and Multiple Sclerosis) (Feng et al., 2011; Grozdanov et al., 2014; Mishra and Yong, 2016), and may also serve as a useful assay for drug development.

Ethics approval and consent to participate

Leukocyte packs and fetal astrocytes from consenting human donors were purchased from Gulf Coast Regional Blood Center and Lonza, respectively, according to each company's policy.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

None of the authors present on this paper report any competing interests.

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Author contributions

MDR was central to the origination and completion of this study. He performed the literature search, developed and executed the experimental approach, and wrote the manuscript. **RBC** contributed to the development of the experimental design and manuscript. **RBC** was responsible for running the samples on the flow cytometer. **AB** and **SS** contributed to the execution of experiments requested during peer review. **AA** contributed to the development of the discussion and provided critical analysis of the manuscript. **NEK** participated in discussions that were the basis for investigating the role of monocytes on astrocyte inflammation. **NEK** also contributed to the development of the experimental design, interpretation of results and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.576969.

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