**P2Y12 expression and function in alternatively activated human microglia**

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P2Y12 expression and function in alternatively activated human microglia

ABSTRACT

Objective: To investigate and measure the functional significance of altered P2Y12 expression in the context of human microglia activation.

Methods: We performed in vitro and in situ experiments to measure how P2Y12 expression can influence disease-relevant functional properties of classically activated (M1) and alternatively activated (M2) human microglia in the inflamed brain.

Results: We demonstrated that compared to resting and classically activated (M1) human microglia, P2Y12 expression is increased under alternatively activated (M2) conditions. In response to ADP, the endogenous ligand of P2Y12, M2 microglia have increased ligand-mediated calcium responses, which are blocked by selective P2Y12 antagonism. P2Y12 antagonism was also shown to decrease migratory and inflammatory responses in human microglia upon exposure to nucleotides that are released during CNS injury; no effects were observed in human monocytes or macrophages. In situ experiments confirm that P2Y12 is selectively expressed on human microglia and elevated under neuropathologic conditions that promote Th2 responses, such as parasitic CNS infection.

Conclusion: These findings provide insight into the roles of M2 microglia in the context of neuroinflammation and suggest a mechanism to selectively target a functionally unique population of myeloid cells in the CNS. Neurology.org/nn © 2015 American Academy of Neurology. Unauthorized reproduction of this article is prohibited.
likely facilitates the detection of nucleotides upon injury.\textsuperscript{12} It is interesting that loss of P2Y12 expression in microglia results in decreased process extension and migration following focal injury.\textsuperscript{12,13} In humans, the role of P2Y12 expression in microglia is unknown. Herein, we investigated the significance of P2Y12 expression in the context of human brain injury and repair. Our results confirm that P2Y12 is a unique marker of human microglia, increases following interleukin (IL)-4 and IL-13 activation, and mediates cell migration and inflammatory responses. Identifying P2Y12 as a molecule associated with the M2 tissue regenerative phenotype\textsuperscript{14-16} may help to discover novel therapeutic targets and mechanisms that promote CNS repair.

METHODS  

Standard protocol approvals, registrations, and patient consents. All institutional ethics approval was obtained according to Canadian Institutes of Health Research guidelines.

Human fetal and adult microglia isolation, culture, and polarization. Human fetal brain tissue was obtained from the Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY). Human adult microglia were isolated from healthy brain tissue of patients undergoing brain surgery for intractable epilepsy. Human fetal and adult microglia were cultured in 5% DMEM and 5% MEM, respectively, with 5% fetal bovine serum (FBS), penicillin/streptomycin, and glutamine. Cell polarization was performed over 48 hours, according to previously published methods.\textsuperscript{1,17} Briefly, microglia were polarized to the M1 phenotype using granulocyte macrophage colony-stimulating factor (GM-CSF) (5 ng/mL), interferon (IFN)–γ (20 ng/mL), and lipopolysaccharide (LPS) (serotype 0127:B8, 100 ng/mL). M2 cells were polarized using interferon–γ (20 ng/mL), and IL-13 (20 ng/mL).

Human MDM culture and polarization. Human peripheral blood mononuclear cells were isolated by Ficoll separation from the blood of healthy volunteers. CD14\textsuperscript{+} cells were positively selected using immunomagnetic bead selection (Miltenyi Biotec, Auburn, CA). Macrophage differentiation was performed over 5 days by culturing monocytes (5 \times 10^5 cells/mL) in RPMI supplemented with 10% FBS, M-CSF (25 ng/mL), penicillin/streptomycin, and glutamine. MDMs were polarized using the protocol described above.

Immunocytochemistry/immunohistochemistry. Immunocytochemistry was performed using a rabbit polyclonal anti-P2Y12 antibody\textsuperscript{10} (generated by Dr. H. Weiner, Harvard University, Cambridge, MA), a monoclonal anti-CD68 antibody (Dako, Burlington, Ontario, Canada; 1:100), and appropriate secondary antibodies. For human brain sections, slides were deparaffinized and antigen retrieval was performed using a 0.01 M sodium citrate buffer solution (pH 6.0) for 15 minutes in the microwave on high setting.

RNA isolation and reverse transcription quantitative real-time PCR. Total RNA was isolated using standard Trizol protocols and DNase-treated (Qiagen, Germantown, MD). TaqMan quantitative real-time PCR was used to measure messenger RNA (mRNA) expression levels for all mRNAs. Relative gene expression data were calculated according to the \( \Delta \Delta C_t \) method.\textsuperscript{18}

Flow cytometry. Human microglia were collected by gently scraping and blocked in FACS buffer supplemented with 10% normal human serum and normal mouse IgG (3 μg/mL). Cells were then incubated at 4°C for 30 minutes with either a control isotype antibody directly conjugated with Alexa647 (BD Biosciences, Mississauga, Ontario, Canada) or a polyclonal rabbit anti-P2Y12 antibody (1:100) followed by an anti-IgG1-Alexa647 (1:100). After washing, cells were fixed in 1% formaldehyde and flow cytometry was performed using a FACSCalibur (BD Biosciences).

Ratiometric measurement of intracellular calcium. Microglia were loaded with fura-2 AM (5 μM, Molecular Probes, Life Technologies, Grand Island, NY) in Ringer solution containing NaCl (130 mM), KCl (5 mM), CaCl\textsubscript{2} (2 mM), MgCl\textsubscript{2} (1 mM), HEPES (10 mM), glucose (8 mM) (pH 7.4), and 1% bovine serum albumin (BSA) for 30 minutes at 37°C. Prior to imaging, microglia were washed and incubated for an additional 30 minutes. Fluorescence was measured at 510 nm and recorded with a high-resolution camera (CoolSNAP HQ2, Roper Scientific/Photometrics, Tucson, AZ). Pairs of 340 nm and 380 nm images were acquired and calculated using Metafluor 7.0 software (Molecular Devices, Sunnyvale, CA). The 340/380 nm emission ratios were proportional to intracellular calcium levels.

Cell migration assays. Migration assays were performed using Boyden-type 96-well plates according to manufacturer’s instructions (Neuro Probe, Gaithersburg, MD). Cells were suspended in media containing 0.5% BSA for 1 hour and pretreated with PSB0739 (10 μM) or vehicle (DMSO). ATP or ADP (1–300 μg/mL) was added to induce chemotaxis. Polycarbonate filters (10 μm [microglia] or 5 μm [monocytes] pore size) were placed in contact with media and cells were added (10^5 cells/mL) atop the filter. Following incubation (4 hours at 37°C), the filter surface was washed, fixed with 2% PFA, and stained using a 0.04% crystal violet.

Migration studies were also performed using microfluidic chambers with 10-μm diameter channels (1 mm in length) (ANANDA, McGill University, Montreal, Quebec, Canada). Microglia were pretreated with either PSB0739 (10 μM; R&D Systems, Minneapolis, MN) or vehicle for 1 hour and then plated (10^5 cells/mL) in the upper chamber. Media alone or media containing ADP (300 μM) was added to the lower chamber as a chemotactic agent. Following 3 days and supplementing ADP each day, cells were fixed, washed, and stained with DAPI and a FITC-conjugated CD68 antibody (1:40, BD Biosciences). Images were visualized using fluorescence microscopy (Leica, Wetzlar, Germany) and OpenLab software (PerkinElmer, Waltham, MA). The distance within the migration chamber for each nucleated cell was measured.

ELISAs. Human tumor necrosis factor (TNF) and IL-6 ELISAs were performed according to the manufacturer’s instructions (BD Biosciences). LPS activation (100 ng/mL) was used in all experiments as a positive control. All results are presented as percent of maximal response relative to ADP treatment (200 μM).

Statistics. All statistical analyses were performed using Prism 5.0 (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM. A one-way analysis of variance with Tukey post hoc
tests or unpaired Student t test was used to determine differences between the experimental conditions; p < 0.05 was considered statistically significant.

**RESULTS** P2Y12 expression is significantly elevated in human microglia compared to MDMs. Compared to MDMs, P2Y12 expression was highly elevated in both fetal and adult microglia. Delta cycle threshold values were significantly lower in both human fetal and adult microglia compared with MDMs, indicative of greater expression (figure 1A). To confirm protein expression, co-immunocytochemistry was performed using anti-CD68 and anti-P2Y12 antibodies. Human microglia positively stained for P2Y12, while no staining was observed for MDMs (figure 1B). As a positive control, rat microglia positively stained for P2Y12, as previously described (figure e-1A at Neurology.org/nn).

Alternatively polarized “M2” human microglia significantly upregulate P2Y12 expression. To determine whether activation of human microglia influenced protein expression, co-immunocytochemistry was performed using anti-CD68 and anti-P2Y12 antibodies. Human microglia positively stained for P2Y12, while no staining was observed for MDMs (figure 1B). As a positive control, rat microglia positively stained for P2Y12, as previously described (figure e-1A at Neurology.org/nn).

![Figure 1](image-url)
expression of P2Y12, cells were polarized to either M1 (classical activation) or M2 (alternative activation). Compared to unpolarized microglia, P2Y12 mRNA expression was significantly increased in M2-polarized cells (10-fold change in adult microglia [figure 2A] and 4-fold change in fetal microglia [figure 2B]). Decreased expression was observed in M1-polarized cells, although results were not statistically significant. Flow cytometry experiments confirmed the increase in surface protein expression of P2Y12 whereby the mean fluorescence intensity was increased in M2-polarized adult microglia (D) and M2-polarized fetal microglia (E) compared to resting state. ADP-induced intracellular calcium responses clearly distinguish human microglia from macrophages and are enhanced under alternatively polarizing M2 conditions. To further validate the presence of functional P2Y12 receptors at the surface of human microglia, prominent intracellular calcium ([Ca^{2+}]_i) transients were observed in human fetal microglia following a short application of ADP (figure 3, A and B). Since ADP is also an agonist of P2Y1 and P2Y13, the P2Y12-selective antagonist PSB0739 was used to assess the selective contribution of P2Y12 to ADP-evoked [Ca^{2+}]_i responses. Treatment with PSB0739 (10 µM) suppressed ADP-induced [Ca^{2+}]_i transients (figure 3, A and B), confirming P2Y12 as the primary ADP receptor on human microglia. In contrast, ADP-induced [Ca^{2+}]_i responses were small or absent in human MDMs, yet they responded to ATP, likely through Gq-coupled P2Y2 receptors (figure 3, C and D). To measure across species, similar experiments were performed using rat microglia and produced similar transients (figure e-1, B and C). Differential ADP-evoked [Ca^{2+}]_i responses were also observed between unpolarized, M1-polarized, and M2-polarized human microglia. Compared to unpolarized microglia, transients were significantly larger in the M2-polarized cells (figure 3, E–G). No differences between unpolarized and M1-polarized microglia were observed (figure e-2). In both unpolarized and M2-polarized cells, ADP-induced [Ca^{2+}]_i responses were blocked by a selective P2Y12 antagonist. ADP-induced migration of human microglia is attenuated by a P2Y12 antagonist. To compare and measure how migration of human myeloid cells may be influenced by targeting P2Y12, microfluidic chambers and Boyden-type assays were used. In both assays, ADP induced a dose-dependent increase in migration. Microglia pretreated with PSB0739, a selective P2Y12 antagonist, significantly decreased migration (figure 4, A–C); this was further quantified using microfluidic chambers (mean distance of 317 µm compared to 532 µm in control; figure 4, D–F; figure e-3). No migratory...
responses were observed in peripheral-derived human monocytes (figure e-4).

ADP-induced inflammatory responses in human microglia are attenuated by a P2Y12 antagonist. To determine whether pharmacologic inhibition of the P2Y12 receptor could also influence inflammatory responses, human microglia were exposed to ADP in the presence or absence of PSB0739. In adult and fetal microglia, ADP induced a dose-dependent increase in TNF-α, with peak responses at 200 μM; no effect was observed with ATP (data not shown). Preexposure of PSB0739 for 1 hour resulted in a dose-dependent decrease in TNF-α following stimulation with ADP (figure 5, A and B). No effect was observed in MDMs (figure e-5). A 75% decrease in TNF-α levels was measured in adult and fetal microglia treated with 10 μM and 50 μM PSB0739, respectively. Decreased IL-6 expression was also measured; however, results were not statistically significant (figure 5, C and D).

P2Y12 is highly expressed in CD68+ cells during parasitic brain infection. Previous reports have demonstrated that parasitic helminth infections induce a robust Th2 response. Considering Th2 cytokines are known to promote an M2-like cell phenotype, we hypothesized that increased P2Y12 expression could be observed in a Th2-driven CNS inflammatory condition such as *Schistosoma mekongi*. Neuropathologic assessment demonstrated the presence of chronic granulomata within the brain parenchyma consisting of multinucleated giant cells surrounding spaces containing chitinous helminthic tissue and ova. Surrounding infiltrates consisted of macrophages, lymphocytes, and plasma cells (figure 6A). A marked reactive astrocytic response was seen in the adjacent parenchyma. Within the granulomas, immunohistochemistry experiments showed the presence of several CD68+/CD163+ (M2 marker) cells staining positive for P2Y12 (figure 6, B and C).

**DISCUSSION** In the inflamed CNS, activated resident microglia and blood-derived macrophages are a heterogeneous population of cells that can differentially contribute to pathophysiologic mechanisms related to injury and repair. Despite direct in vitro evidence supporting these observations, the ability to reliably distinguish these cell types in situ remains challenging. Several recent publications have used novel genomic and proteomic technologies to assign unique molecular signatures to different myeloid cell populations. In a recent study, detailed flow cytometry and immunohistochemical analyses were performed using both naïve and experimental autoimmune encephalomyelitis mice chimeras (CX3CR1-GFP+/+—wildtype), whereby peripheral monocytes were distinguishable from microglia. In vivo, these experiments demonstrated that resident GFP-microglia expressed P2Y12, whereas recruited GFP+ monocytes did not. In the
present study, we have validated P2Y12 as a receptor that is exclusively expressed on human microglia. Using human fetal and adult microglia, we report that P2Y12 expression is enhanced under alternatively activated (M2; IL-4 & IL-13) conditions, mediates cell migration, and participates in eliciting acute proinflammatory response toward danger-associated molecules that are released during CNS injury.

P2Y12 is a G\textsubscript{i/o}-coupled purinergic receptor that was initially identified on platelets and is responsible for platelet activation during the blood clotting process.\textsuperscript{2} P2Y12 antagonists have therefore been developed as antithrombotic agents to reduce the risk of heart attack and stroke in high-risk patients. Beyond platelets, P2Y12 expression was first described in rodent microglia\textsuperscript{26} and has been implicated in cell activation and migration, and participates in eliciting acute proinflammatory response toward danger-associated molecules that are released during CNS injury.

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In our experiments, we confirmed that P2Y12 is highly expressed on human microglia compared to MDMs (figure 1). A direct comparison of P2Y12 expression between fetal and adult microglia revealed that expression was greater in fetal cells than adult microglia, which is consistent with results demonstrating that P2Y12 expression declines with age.\textsuperscript{29} In response to ADP, P2Y12 receptor signaling induces a rapid release of [Ca\textsuperscript{2+}], which activates several downstream signaling pathways.\textsuperscript{30} Using a selective P2Y12 antagonist, PSB0739, we demonstrated that ADP-induced [Ca\textsuperscript{2+}] is dramatically abrogated in human microglia. In MDMs, ADP did not induce an increase in [Ca\textsuperscript{2+}], thus validating our initial observations that P2Y12 expression was minimal to negligible in blood-derived myeloid cells (figure 3). In assays designed to measure P2Y12-dependent migration of human myeloid cells to ADP, an important signal that promotes microglial migration and recruitment, we observed that human microglia dose-dependently migrate toward ADP. This response was blocked with preexposure to the P2Y12 antagonist (figure 5).

Both microglia and macrophages are dependent on distinct activation signals that are required to induce different functional properties, often referred to as M1 and M2 polarization. While rodent and human macrophages can exhibit a spectrum of activation phenotypes both in vivo and in vitro,\textsuperscript{31} it should be noted that tissue-resident myeloid cells (e.g., microglia) are a distinct myeloid cell subset that
Figure 5  ADP-induced inflammatory responses require P2Y12 receptors in human microglia

Increasing concentrations of ADP (5, 50, and 200 μM) increased tumor necrosis factor (TNF) and interleukin (IL)-6 release in human adult microglia (A, C) and fetal microglia (B, D), with maximal response reached at 200 μM after 6 hours. A 1-hour pretreatment with a P2Y12 receptor antagonist (PSB0739) dose-dependently decreased TNF and IL-6 cytokine expression induced by ADP (200 μM) as measured by ELISAs. Error bars represent mean ± SEM; n = 4/condition; **p < 0.01 compared to ADP (200 μM) treatment alone.

requires further investigation with respect to how different modes of activation can influence their cellular properties. For this reason, we have investigated how IL-4 and IL-13 can lead to a microglia phenotype, which is similar in an in situ microenvironment known to express high levels of these cytokines. Previously, we have extensively profiled different activation states of human microglia and characterized several different phenotypic markers (e.g., gene, microRNA, and protein) and functional properties.1,5 In vitro, M1 microglia (IFN-γ & LPS stimulated) are implicated as potential mediators of tissue injury, whereas M2 (IL-4/13) cells can produce anti-inflammatory molecules and have been reported to significantly mediate repair mechanisms in animal models of stroke,32 spinal cord injury,18 and demyelination.17 Under proinflammatory conditions, in vitro and in situ studies have demonstrated that P2Y12 expression is decreased in rodent microglia and results in the inability to migrate toward an ADP gradient.28 In postmortem samples from the cerebral cortex of patients with multiple sclerosis (MS), P2Y12 expression is absent in microglia/macrophages within the lesion,33 but it is expressed in cells surrounding brain lesions. This decrease in P2Y12 expression correlated with the extent of demyelination. In our study, we confirmed that in the active MS lesion, P2Y12 was not expressed (not shown), but it was observed in CD68+ cells in areas adjacent to the lesion (figure e-6A). P2Y12 was not expressed in other glial cells such as astrocytes (figure e-6B).

Our in vitro and in situ observations prompted additional studies to examine how a T<sub>H</sub>2/M2 environment might affect P2Y12 expression in situ. In vivo studies have convincingly shown that parasitic infection can promote both T<sub>H</sub>2 and M2-polarized...
phenotypes and can attract the migration of M2 macrophages to parasite eggs and chitin. Using a rare case of human *S. mekongi* infection of the brain, we confirmed that P2Y12+/CD163+ M2 cells were present in regions circumscribing helminthic ova (figure 6). It is interesting that previous associations have been made between parasite infection and disease-relevant immune responses in MS. In MS, patients infected by helminths have been shown to harbor decreased proinflammatory T-cell responses and increased inflammation-resolving regulatory B cells producing IL-10, brain-derived neurotrophic factor, and nerve growth factor. In addition, compared to uninfected patients, clinical outcome measures are improved in helminth-infected patients with MS.

In this report, we have confirmed that P2Y12 is selectively expressed on human microglia and can be used as a marker to distinguish CNS-resident microglia from blood-derived myeloid cells. P2Y12 expression on microglia is dependent on different modes of activation such that an inflammatory environment decreases expression whereas a Th2/M2 microenvironment increases expression. In response to their endogenous ligand, ADP, human microglia rapidly increase \([\text{Ca}^{2+}]_i\), release proinflammatory cytokines, and display significant migratory ability. Application of a selective P2Y12 receptor antagonist was demonstrated to reverse these functions. Our findings provide insight into how the functional properties of microglia differ from peripheral-derived macrophages in the inflamed CNS. These properties are in part controlled by their relative level of P2Y12 expression, which may provide a novel targeting strategy for treating CNS inflammatory conditions.

**AUTHOR CONTRIBUTIONS**

Craig S. Moore: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data, statistical analysis, study supervision. Ariel Ase: drafting/revising the manuscript, analysis or interpretation of data, acquisition of data, statistical analysis. Angham Kinsara: study concept or design, analysis or interpretation of data. Vijayaraghava T.S. Rao: study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data, statistical analysis, study supervision. Mackenzie Michell-
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DISCLOSURE

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