The Characterization of Immunomodulatory and Remyelinating Agents on Microglial Polarization

Harriet O'Brien

ABSTRACT

Multiple Sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) characterized by destruction of the blood brain barrier (BBB), immune cell infiltration, and attack of the myelin sheath. As damage in MS is propagated by immune cells and involves extensive demyelination, addressing these two concerns is essential in the development of effective drug treatments. The diversification of microglia, the resident immune cell of the CNS, into activation states is more simply dichotomized into classically activated, pro-inflammatory M1 cells and alternatively activated, anti-inflammatory M2 cells. Tuftsin is an immunomodulating peptide known to promote the anti-inflammatory M2 switch of microglia by signaling through neuropilin-1 (Nrp-1) via the transforming growth factor beta (TGFβ) receptor pathway. DOCK, a molecular docking program, was used to virtually screen a library of FDA-approved compounds against Nrp-1 and revealed ritonavir as a ligand for the exact docking location as tuftsin. We show that ritonavir-mediated microglial activation, like tuftsin, shifts microglia to the M2 phenotype and through Nrp-1 signaling via the TGFβ pathway. As ritonavir is FDA-approved and tuftsin is not, these results have significant implications for streamlining the pipeline to clinical trials. Benztropine is known to enhance remyelination and has resulted in functional recovery in experimental systems of MS. Individually and in combined administration with tuftsin or ritonavir, benztropine polarizes microglia to the M2 phenotype. Although we show that both ritonavir and benztropine have significant dose-dependent neurotoxic affects, our findings support their therapeutic efficacy for MS treatment.

INTRODUCTION

Multiple sclerosis (MS) is a chronic, demyelinating autoimmune disease characterized by inflammation and axonal transection in the central nervous system (CNS) [1, 2]. Immune cell infiltration, attack of the myelin sheath, and destruction of the blood brain barrier (BBB) are affiliated with MS and other neurodegenerative diseases. The resulting inflammation is a consequence of progressive demyelination, axonal degeneration, and gliosis [3, 4]. Following neurological injury, the influx of peripheral macrophages to demyelinating regions is accompanied by the activation of microglia, derivatives of erythromyeloid precursors in the embryonic yolk sac [5-7].

Microglia are the resident innate immune cells of the CNS and play an important role as the sole unit of immunosurveillance. In terms of functionality and structure, microglia most closely resemble peripheral macrophages. Resting microglia, assuming a ramified morphology, survey the immune-privileged parenchyma for infectious or threatening agents and upon injury, activate, embodying an amoeboid shape [8]. Microglial activation has been implicated in the aggravation of several neurologic diseases. Neurological inflammation leads to both the activation of microglia and the migration of monocytes into the CNS [9, 10]. Microglial activation in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, contributes to tissue damage via the production of pro-inflammatory cytokines [11]. Thus, areas plagued by demyelination have a heightened presence of microglia [12]. Likewise, microglial depletion leads to the attenuation of EAE [13, 14] suggesting that microglia are a primary mediator of tissue damage in MS [15].

Diversification of activation states, or phenotypes, allow microglia to regulate cytotoxicity, repair, regeneration, and immunosuppression [16]. The spectrum of microglia phenotypes that occur in inflammatory lesions are more simply dichotomized into classically activated M1 cells and alternatively activated M2 cells [17, 18]. M1 microglia, associated with severe MS symptoms [19, 20], secrete pro-inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) [21]. M2 microglia, however, release anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGFβ) [22] and thus are associated with periods of recovery seen in MS patients [23]. As the phenotype of microglia in the CNS can be manipulated, promoting neuroprotective aspects of their polarization states is a promising avenue for treatment of demyelinating diseases [24].

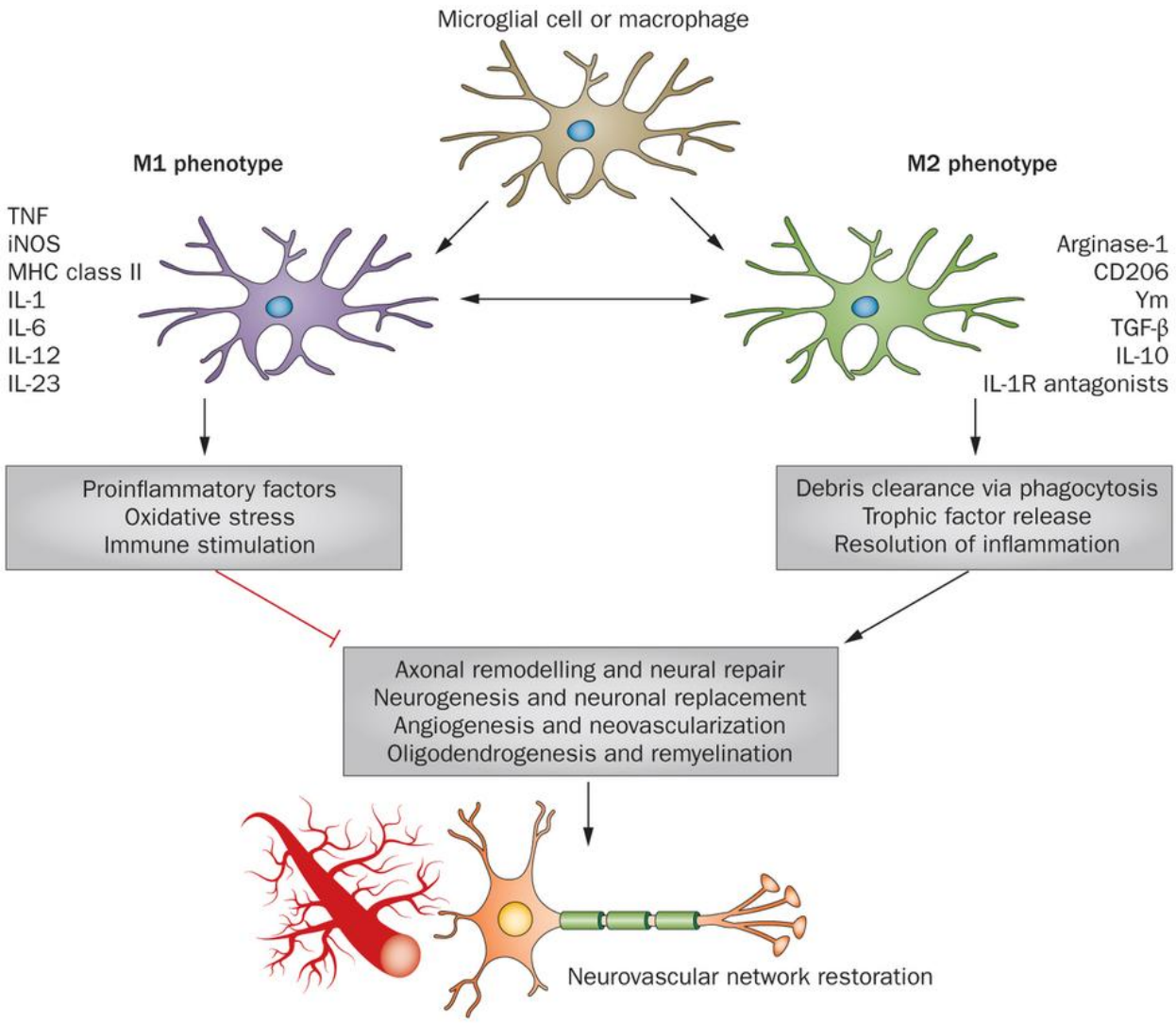

Figure 1. The phenotypic polarization of microglia at various stages after CNS injury has distinct roles in damage or restoration of the neurovascular network. M1 microglial populations express pro-inflammatory proteins that debilitate CNS restoration such as TNF, iNOS and IL-6. Contrastingly, M2 populations express proteins that improve repair and regeneration such as arginase-1, CD206 and IL-10. Abbreviations: IL-1R, IL-1 receptor; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor β; TNF, tumor necrosis factor; Ym, chitinase-like proteins. Figure reference: [25]

Tuftsin is a naturally occurring tetrapeptide (threonine-lysineproline-arginine) known to promote phagocytic activity in monocytic cells, all of which are thought to express tuftsin receptors [26]. The generally immunogenic response prompted by tuftsin, as well as its action on innate immune cells, particularly on microglia, make the peptide a viable candidate for MS immunotherapy. Tuftsin treatment is associated with anti-inflammatory effects within the CNS and the polarization of microglial populations to the M2 phenotype when exposed to excitotoxic

media *in vitro* [27]. The administration of tuftsin during EAE in wild-type mice has shown a decrease in severity of EAE symptoms and improved recovery through a significant reduction in demyelination [27]. Taken together, tuftsin promotes an overall anti-inflammatory shift in the immune environment, representing itself as a potential therapeutic for disease.

Tuftsin binds to neuropilin-1 (Nrp-1), a single-pass transmembrane protein that is upregulated in activated microglia and is associated with neuroprotection post-injury [28, 29]. Following binding, tuftsin signals via the canonical TGFβ signaling pathway by which the drug exerts its anti-inflammatory effects [30]. This signaling cascade upregulates microglial secretion of anti-inflammatory cytokines and subsequently down-regulates the secretion of pro-inflammatory cytokines. While the tuftsin-mediated polarization of microglia to the anti-inflammatory phenotype is favorable, tuftsin does not constitute an efficient therapeutic compound, as it is a small peptide. A virtual screen (detailed in Materials and Methods) identified a refined subset of FDA-approved [31] compounds predicted to bind to Nrp-1. Ritonavir, an antiretroviral drug, was ranked with the highest predictability to potentially bind to Nrp-1 next to nicotinamide adenine dinucleotide (NADH).

Ritonavir, an HIV-1 protease inhibitor, is a current treatment for HIV infection and a viable candidate for reposition as an anticancer agent [32]. Recent studies show that the antineoplastic effects of ritonavir include inhibition of inflammatory cytokine production, proteasome activity, cell proliferation, and cell viability [33]. Given the virtual screen prediction, we explored whether or not ritonavir, like tuftsin, would induce the shift of microglia to the M2 phenotype. To the extent of our knowledge and after thorough examination of literature, the characterization of ritonavir's effects on microglial polarization has yet to be researched.

Benztropine, an anticholinergic that is FDA-approved for treatment of Parkinson's disease, Parkinsonism, and dystonia, shows a significant decrease in the clinical severity of EAE when administered alone or in combination with additional immunosuppressive treatments for MS [34]. Evidence from the cuprizone-induced mouse model for demyelination both *in vivo* and *in vitro*, and EAE adoptive transfer experiments, indicated that the efficacy of this drug stems from direct enhancement of remyelination rather than immunosuppression. Benztropine induces the differentiation of oligodendrocyte precursor cells (OPCs) to mature oligodendrocytes required for remyelination. Disease remission of MS is largely dependent on the migration of OPCs to sites of injury and the consequent differentiation to mature cells [35-37]. As benztropine

has not been associated with immunosuppression, its effect on microglial polarization has not been characterized *in vitro*.

For the treatment of MS, minimizing ongoing damage by reducing inflammation while promoting recovery through increased remyelination would address both avenues of long-term damage, and thus an OPC differentiation-inducing drug would be most effective if introduced clinically as part of a combination therapy with an immunosuppressive drug [34]. Benztropine, with proven drug administration that has led to functional recovery in EAE and a decrease in clinical severity of remission phases and relapse, could accompany tuftsin, a drug known for its immunosuppressive characteristics, as a viable candidate for a combinatorial treatment. Likewise, ritonavir, an FDA-approved drug characterized as a potential immunosuppressive agent, could play a role similar to that of tuftsin in a combined treatment therapy. We examined the effectiveness of tuftsin and ritonavir in polarizing microglia to the M2 phenotype individually and while being administered with benztropine at different of concentrations.

MATERIALS AND METHODS

Virtual Screen In collaboration with another laboratory, DOCK, a molecular docking program, was used to screen the Zdd library (all FDA approved compounds) against Nrp-1 to isolate potential drug leads. After a protein data bank (PDB) structure of Nrp-1 was found, the downloaded file was prepared by removing excesses including repeat chains, water molecules, ions, and crystallography artifacts (creating the dockprep file). The receptor file was saved with and without hydrogen atoms, as well as with and without charges assigned to each atom. SPHGEN (Sphere Gen) was a program used to generate spheres that represent locations where ligands can be docked. A box was generated to serve as a boundary for docking ligands. Docking and energetic calculations were done against the grid, a three-dimensional grate whose points represent electrostatic and hydrophobic charges.

N9 microglia cultures Immortalized N9 microglia (obtained originally from the American Type Culture Collection (ATCC)) were plated in N9 medium (Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), antibiotic-antimycotic) in a 10 cm cell culture plate. After confluence, cells were rinsed with Hank's Balanced Salt Solution (HBSS), treated with 5mL of Tryspin/EDTA (Sigma) to re-suspend cells adherent to the plate, and incubated for five minutes at 37°C. Following partial cell detachment, Trypsin was deactivated by adding 5mL of

N9 medium to the plate. The remaining adhered cells were detached manually by pipetting. The medium containing the floating microglia was collected and centrifuged at 500 G for 5 minutes, following which the supernatant was aspirated and the cell pellet resuspended in 10mL of N9 microglia medium. Microglia were counted on a hemocytometer and plated at a density of 2.5 x 10^4 cells per coverslip, each of which were placed in 12-24 well tissue culture plates. After adherence, cells were treated both individually and in combination with the following: tuftsin (served as an M2-inducing control), benztropine, and ritonavir at various concentrations. Lipopolysaccharide (LPS) at 100 ng/mL served as a control to induce both the M1 and M2 shift in microglia. Neuronal conditioned media (NCM) was utilized in a 1:1 ratio with N9 medium in some experiments to mimic an *in vivo* excitotoxic environment.

Primary Microglia and Neuronal Conditioned Media The neuronal conditioned media (NCM) and microglia were derived from cortices of mice that were euthanized for a different experimental purpose and would otherwise have been discarded. These cells and conditioned media were generated as previously described [27]. Primary microglia were utilized for experimentation after ten days *in vitro.* The cells were plated at a density of 2.0 x 10^4 cells per coverslip in primary microglial medium (DMEM + 1% FBS). Before seeding, the media was centrifuged and resuspended to remove debris. NCM was added to the culture medium at a 1:1 ratio to induce excitotoxic injury. Cells were treated as described above for N9 cells.

Immunofluorescence Cells used for immunofluorescence were fixed for 30 minutes at room temperature in 4% paraformaldehyde (PFA). Following three 15 minute PBS rinses, the cells were blocked in serum of the host of the secondary antibody (3% NGS, 1% TritonX-100). The cells were then left at room temperature for an hour with anti-iNOS (1:500; BD Biosciences) and anti-arginase 1 (1:500; BD Biosciences). Following three five-minute phosphate-buffered saline (PBS) rinses, cells were incubated with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 555 conjugated secondary antibodies for 1 hour at room temperature. After an additional three five-minute PBS rinses, coverslips were mounted using Fluoromount-G with DAPI (Southern Biotech, USA). N9 microglia were visualized with a Nikon Zeiss LSM 510 confocal microscope while primary microglia were visualized with an inverted epifluorescent microscope (Zeiss Axiovert 200M).

Live/Dead Cell Viability Stain N9 microglia were cultured accordingly and treated with varying concentrations of ritonavir (1 uM, 5 uM, 10 uM, 25 uM). After 16-20 hours, microglia were treated with 1 μg/mL propidium iodide (PI) and incubated at 37°C for 15 minutes. Live cells were evaluated under an inverted epifluorescent microscope (Zeiss Axiovert 200M). Due to the visibility of both the microglia and PI infiltration through the microscope lens, picture locations were predetermined by gridding each well into four quadrants.

Immunoblotting N9 microglia were lysed in 50 mM Tris-HCl (pH 8) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, and protease inhibitor cocktail (Sigma-Aldrich) in 1:1000 concentration. After 15 minute incubation at room temperature, the lysates were centrifuged for debris removal at 13,000 RPM for 10 minutes at 4°C. The supernatant was aspirated. The extracts were separated on a reducing 10% SDS-PAGE gel and blotted to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore). Membranes were incubated for 16-20 hours at 4°C with p-Smad2/3 (1:1000) or Smad2/3 (1:1000) primary antibodies and for 1 hour at room temperature with HRP-conjugated anti-rabbit secondary (1:2500). Membranes were visualized with ECL (Pierce) on film.

Immunofluorescence Quantification Images from immunofluorescence were quantified using ImageJ, an open source image processing program developed by the National Institutes of Health (NIH). The presence of iNOS (M1) and arginase-1 (M2) positive cells that displayed Iba-1, a microglial immunofluorescence marker, were counted and determined quantification.

Statistical Analysis Statistical analysis was performed using one-way ANOVA followed by a Bonferroni test for multiple comparisons within a group. Comparisons between two groups were analyzed with t-tests and indicated in the figure legends. For all figures, $P<0.05$ was the threshold for significance and denoted as *. Significances of $p<0.01$ and $p<0.001$ were marked by ** and ***, respectively. Results were represented as average with error bars indicating the standard error of the mean. For all experiments, n refers to the number of biological replicates used for each condition.

RESULTS

A virtual screen identified ritonavir as a potential binder to Nrp-1 Upon activation, microglia can polarize to either a pro-inflammatory (M1) or anti-inflammatory phenotype (M2)

[17, 18]. While M1 microglia are associated with neurodegeneration, M2 microglia are associated with immunosuppression that can control disease progression. Tuftsin induces said immunosuppression through signaling via Nrp-1 and the canonical TGFβ signaling pathway [30]. A virtual screen of all FDA-approved compounds against Nrp-1 identified ritonavir (ZINC03944422) as having a potential ligand for Nrp-1 in the exact docking location as tuftsin. This *in silico* prediction set the precedent for further experimentation to characterize, first, whether or not ritonavir-treated microglia would produce a shift to immunosuppression.

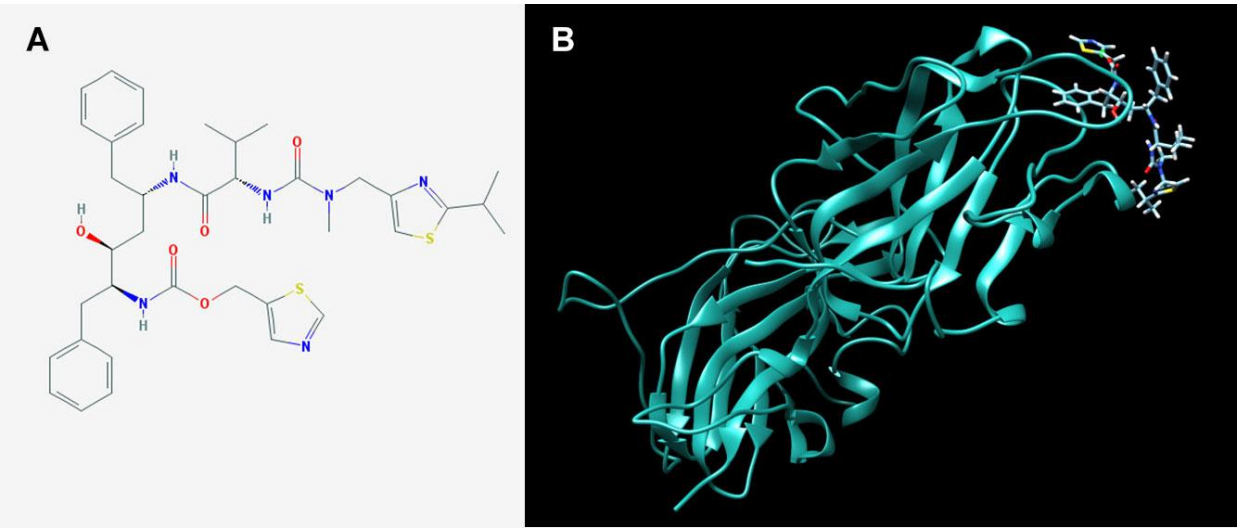

Figure 2. (**A**) The molecular structure of the compound, ritonavir, selected in the virtual screen [49] (**B**) The binding mode of ritonavir to Nrp-1 was identified by running a library containing a subset of FDA-approved compounds against the grid. Nrp-1 is represented by the ribbon model and the binding site is located at the pharmacophore model of ritonavir. (Image generated in Chimera)

Polarization of ritonavir-treated N9 microglia to the M2 phenotype The ability of ritonavir to mediate M2 microglial polarization was examined at a variety of concentrations using the N9 mouse microglial cell line. We treated N9 microglial cells for 16-20 hours with 1, 5, 10 and 25 μM ritonavir, selecting concentrations based on literature in other fields [38]. For comparison, we included media control, 100 μg/mL tuftsin (M2 control) and 100 ng/mL LPS (M1 and M2 control). Cells were imaged for the presence of M1 and/or M2 phenotypes with staining of iNOS and arginase-1, respectively. At all concentrations tested, ritonavir polarizes microglia to the M2 phenotype as indicated by high percentages of arginase-1 positive cells and the near-absence of iNOS positive cells.

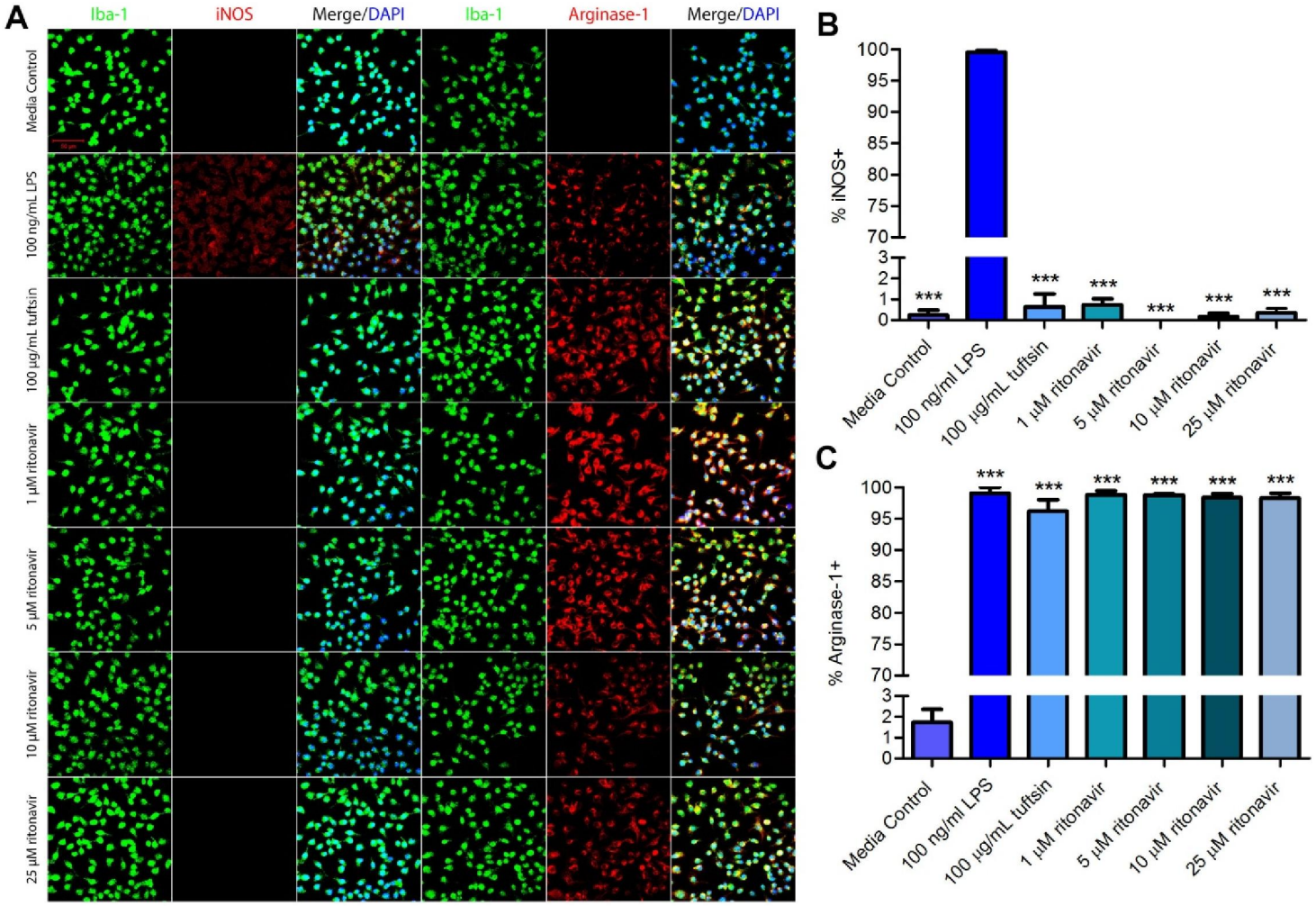

Figure 3. N9 microglial cells were treated with varying concentrations of ritonavir and M1/M2 controls (LPS/tuftsin) as described in Materials and Methods. Cells were fixed and stained for Iba-1 (FITC, green), arginase-1 (rhodamine, red) and iNOS (rhodamine, red) to visualize the polarization or lack thereof of the microglia to either the M1 (iNOS) or M2 (arginase-1) phenotype (**A**). Percentages of iNOS+ and arginase-1+ cells were counted for ritonavir and control drugs and were quantified in (**B**). n=3; data are mean ± SE (n=3); ***, $p<0.001$; *** denotes significant difference from 100 ng/mL LPS control (**B**) and denotes significant difference from Media Control (**C**). Top left image (**A**); scale bar (red) reads 50µm.

Ritonavir-activated N9 microglia exposed to excitotoxic media exert an M2 shift Neuronal conditioned media (NCM) are prepared from primary cortical neurons exposed to the excitatory neurotransmitter glutamate and mimic the excitotoxic environment encountered by microglia *in vivo* during MS due to dying neurons. A tuftsin/NCM combinatorial treatment has been previously reported to exert an anti-inflammatory M2 shift in primary microglia [30]. Given the efficacy of ritonavir individually in N9s and its proposed similarity to tuftsin in terms of binding targets, we examined the effects of ritonavir/NCM treatment at varying concentrations. Data indicate no shift to the M1 phenotype, as evident in (**A**). Percentages of M2 microglia are similar

to percentages of non-NCM ritonavir treatments with the exception of 1 µM ritonavir. This dose is noticeably lower, though not statistically significant compared to 1 µM ritonavir without NCM (two-tailed t-test; ns, p=0.2823), nor is it statistically different compared to higher doses. All concentrations, however, are significantly different compared media control.

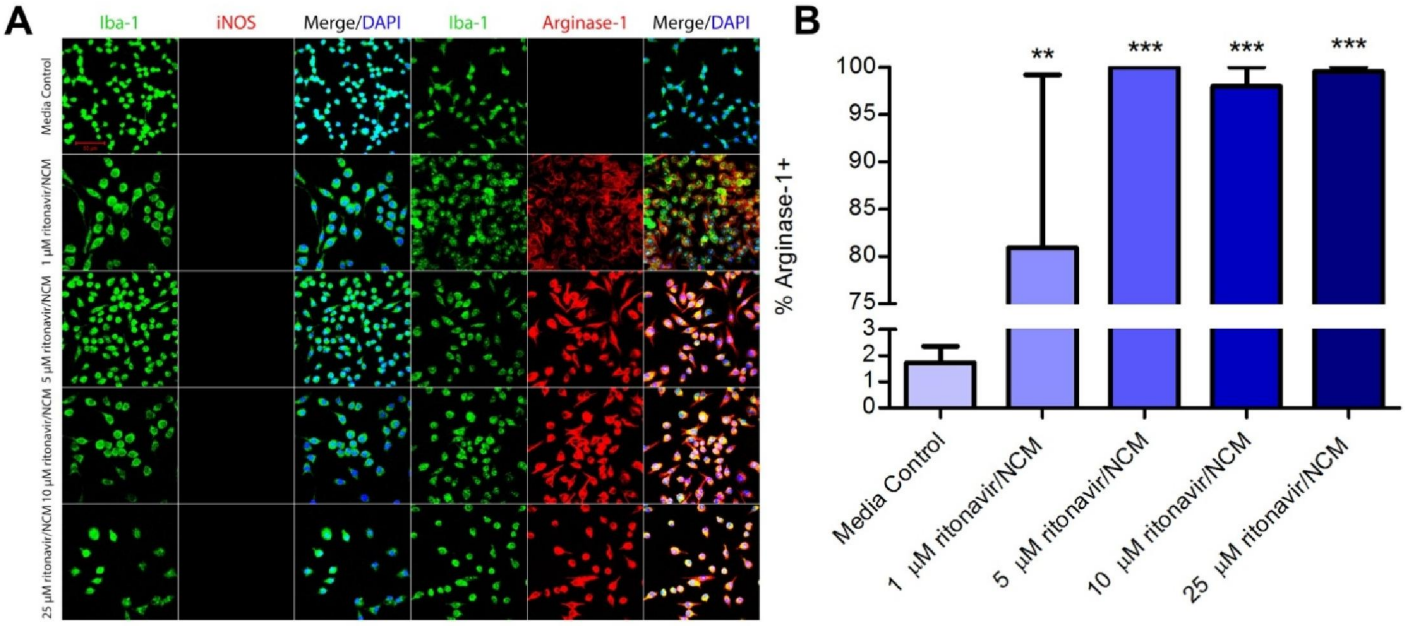

Figure 4. N9 microglial cells were treated with varying concentrations of ritonavir with NCM as described in Materials and Methods. Cells were fixed and stained for Iba-1 (FITC, green), arginase-1 (rhodamine, red) and iNOS (rhodamine, red) to visualize the polarization or lack thereof of the microglia to either the M1 (iNOS) or M2 (arginase-1) phenotype (**A**). Percentages of iNOS+ and arginas-1+ cells were counted for ritonavir/NCM and media control, and were quantified in (**B**). n=2-3; data are mean ± SE (n=2-3); **, p<0.01;***, p<0.001; ** and *** denote significant difference from Media Control. Top left image (**A**); scale bar (red) reads 50µm.

Benztropine polarizes N9 microglia to the M2 phenotype As benztropine is known to enhance remyelination, we examined its effect on the efficacy of tuftsin in inducing immunosuppression via microglial polarization to the M2 phenotype (Fig. 5). Benztropine does not hinder tuftsin-mediated M2 polarization and, in fact, also polarizes microglia to the M2 phenotype. All concentrations and treatment combinations display extremely high percentages of arginase-1+ cells (M2). Levels of iNOS+ cells (M1) are generally low, however, not non-existent with the exception of 1.5 µM benztropine and 5 µM benztropine + 100 µg/mL tuftsin. In fact, the percentage of M1 cells after treatment with 5 µM benztropine is significantly greater than Media Control, 1.5 µM benztropine, and 5 µM benztropine + 100 µg/mL tuftsin. As the percentage of M1 cells increases with increasing doses, it is likely that higher doses of benztropine are toxic.

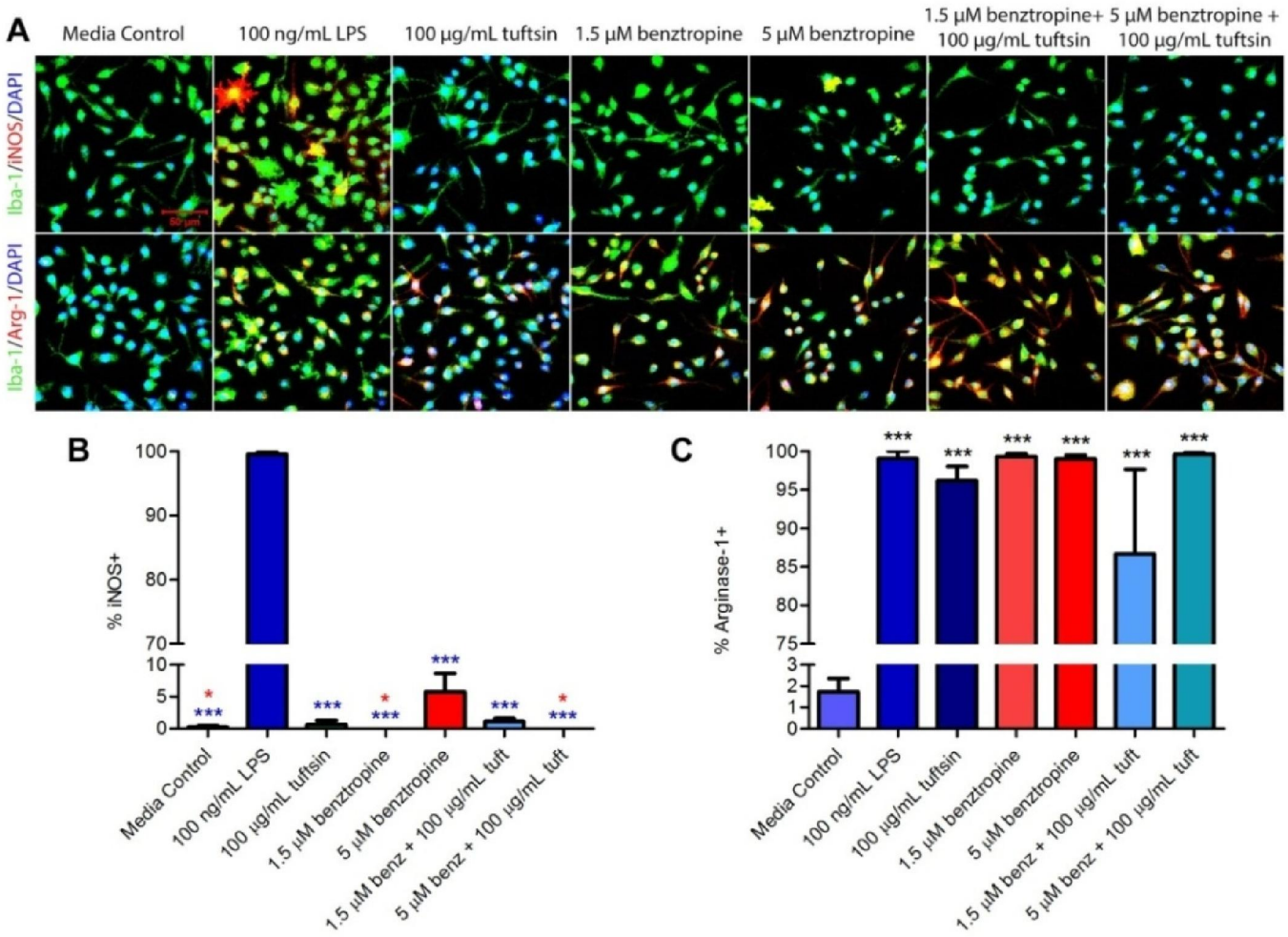

Figure 5. N9 microglial cells were treated with benztropine (at concentrations of 1.5 uM and 5uM) and tuftsin, both individually and in combination, as described in Materials and Methods. Cells were fixed and stained as previously stated (**A**). Percentages of iNOS+ and arginas-1+ cells were counted for benztropine and control drugs and were quantified in (**B**). n=3; data are mean ± SE (n=3); *, $p<0.05$;***, $p<0.001$; * and *** denote significant difference from 5 µM benztropine and 100 ng/mL LPS, respectively. Top left image (**A**); scale bar (red) reads 50µm.

Individual and combinatorial treatments of tuftsin, ritonavir, and benztropine polarize N9 microglia to the M2 phenotype An OPC differentiation-inducing drug, such as benztropine, would likely be administered as a combination therapy with an immunosuppressive agent. We therefore evaluated the efficacy of benztropine when combined with tuftsin, a known immunosuppressive drug, and ritonavir, described as an immunosuppressive drug as evidenced by our results. All combinations have displayed the efficacy of each drug in M2-microglial polarization alone and in combined treatments of benztropine with immunosuppressive agents.

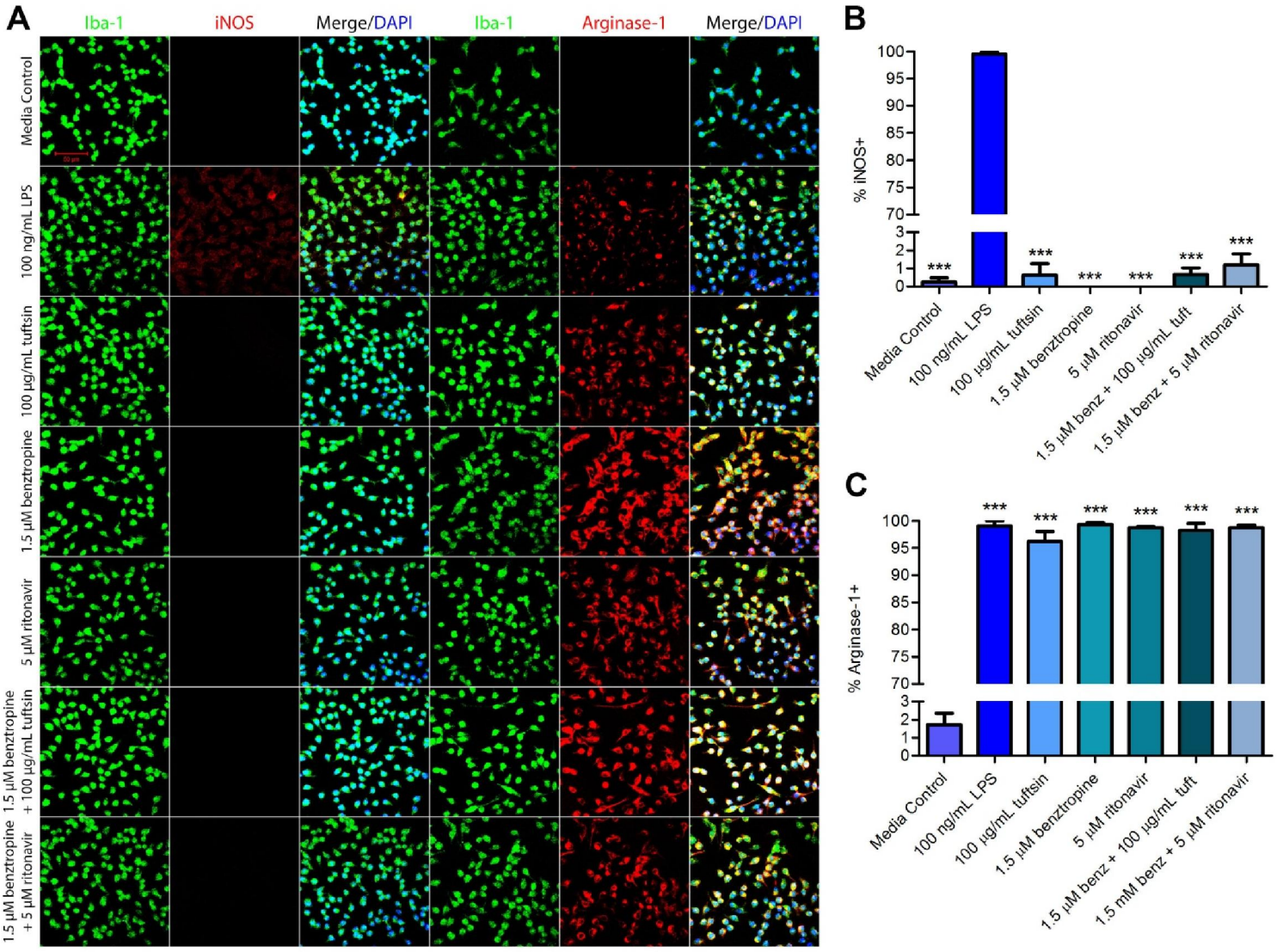

Figure 6. N9 microglial cells were treated with varying concentrations of ritonavir and M1/M2 controls (LPS/tuftsin) as described in Materials and Methods. Cells were fixed and stained for Iba-1 (FITC, green), arginase-1 (rhodamine, red) and iNOS (rhodamine, red) to visualize the polarization or lack thereof of the microglia to either the M1 (iNOS) or M2 (arginase-1) phenotype (**A**). Percentages of iNOS+ and arginas-1+ cells were counted for all treatments and were quantified in (**B**). n=3; data are mean ± SE (n=3); ***, $p<0.001$; *** denotes significant difference from 100 ng/mL LPS control (**B**) and denotes significant difference from Media Control (**C**). Top left image (**A**); scale bar (red) reads 50µm.

Ritonavir-activated primary microglia polarize to the M2 phenotype

While the N9 microglial cell line is a common, useful line for preliminary *in vitro* experimentation on microglia, N9s have developed morphological and biochemical characteristics that differ from those of primary microglia cells as a result of their immortalization [39]. Although they share the same expression of cell surface markers, phagocytose, and secrete cytokines following activation, amplified treatment-mediated responses are often a result of their increased adherence and proliferation [40]. After examining the

efficacy of ritonavir in polarizing N9 microglia to the anti-inflammatory (M2) phenotype, we sought to explore whether or not ritonavir would induce the same shift in primary microglia cells. While N9s have shed statistically significant light on the direction of cell polarization, study of primary microglia will better simulate the effects of ritonavir in a biological system.

Primary microglia were fixed and stained for the presence of M1 and M2 markers. Paralleling the results in ritonavir-treated N9 microglia, arginase-1+ cells predominate especially compared to iNOS+ cells which are non-existent in this experiment, with the exception of the M1 control.

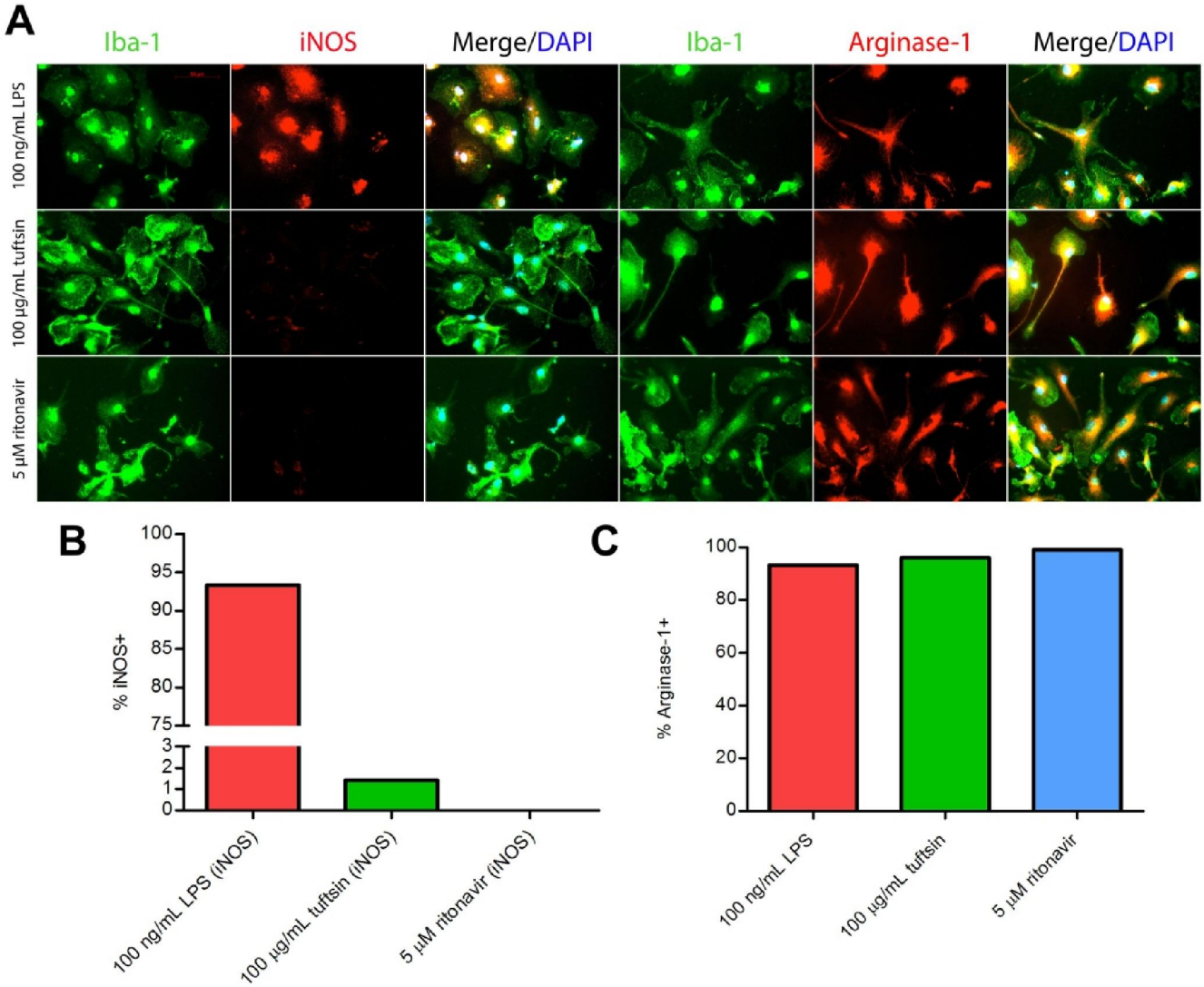

Figure 7. Primary microglial cells were treated with 5 µM ritonavir and M1/M2 controls (LPS/tuftsin) as described in Materials and Methods. Cells were fixed and stained for Iba-1 (FITC, green), arginase-1 (rhodamine, red) and iNOS (rhodamine, red) to visualize the polarization or lack thereof of the microglia to either the M1 (iNOS) or M2 (arginase-1) phenotype (**A**). Percentages of iNOS+ and arginas-1+ cells

were counted for ritonavir and control drugs and were quantified in (**B**). Top left image (**A**); scale bar (red) reads 50µm.

Dose dependent death of ritonavir-treated N9 microglia

Ritonavir-related neurotoxicity has been reported at higher doses in previous studies pertaining to the safety of drugs used for HIV-therapy [41]. Noticeable decrease in the cell density of N9 microglia with high-dose ritonavir treatment prompted our examination of cell viability with propidium iodide (PI) staining. As a 50 µM concentration of ritonavir appeared to kill the vast majority of cells, we discontinued the administration of this dose. The dose dependent toxicity evident in (Fig. 8, A) determined the concentrations of potentially-viable doses to examine cell death. Propidium iodide is a membrane impermeant, DNA stain that is generally excluded from viable cells and permeates those with compromised membranes. Thus, the notable difference between PI levels in 25 µM compared to all other concentrations affirms the hypothesis that ritonavir kills N9 microglia at doses of 25 µM or higher.

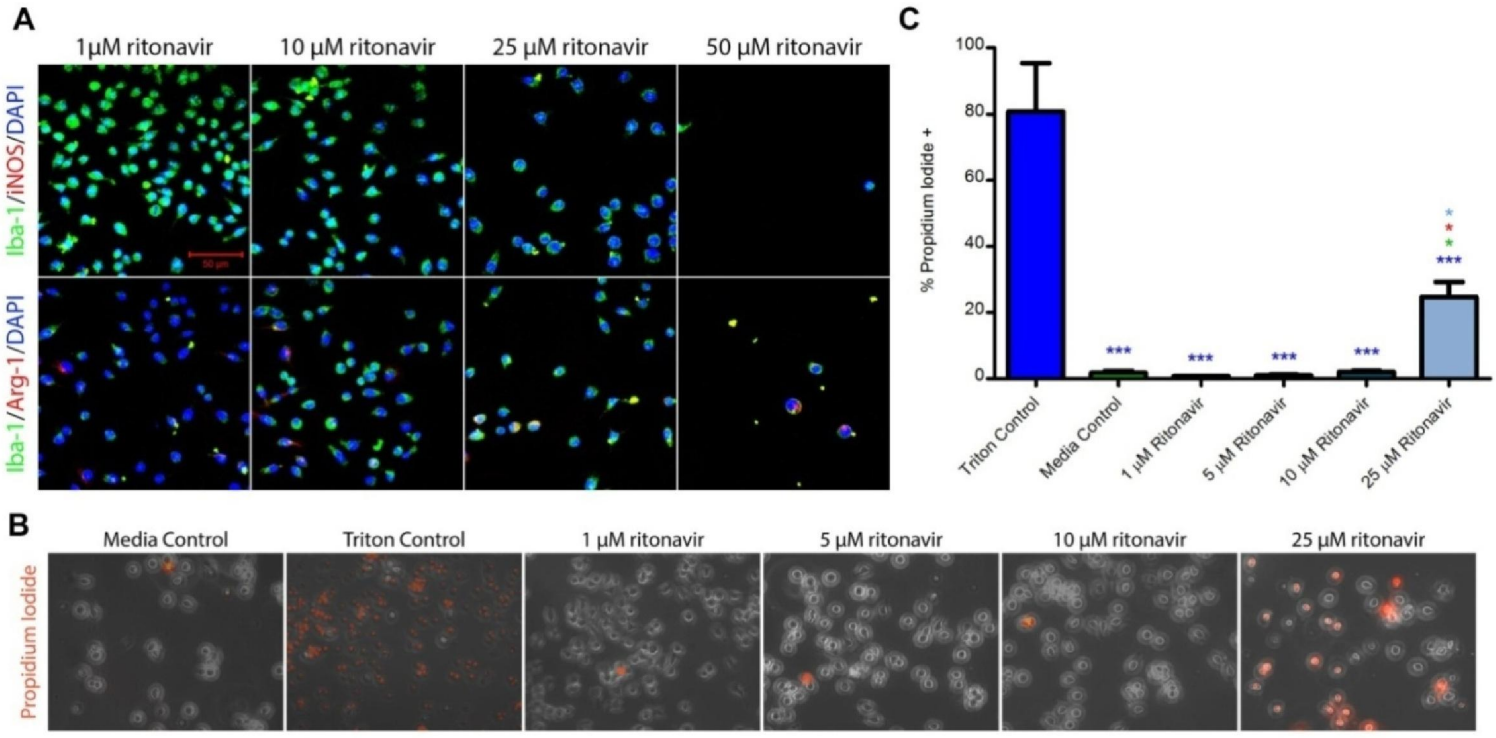

Figure 8. N9 microglial cells tested initially for the presence of M1/M2 cells showed noticeable cell death (**A**). N9 microglial cells were treated with varying concentrations of ritonavir and triton (cell-death control) as described in Materials and Methods. Sixteen to twenty hours after ritonavir treatment, cells were treated with PI and evaluated live to visualize PI infiltration (cell death) (**B**). Percentages of propidium iodide+ cells were counted for ritonavir and triton/media control and were quantified in (**C**). n=3-4; data are mean ± SE (n=3-4); *, $p<0.05$; ***, $p<0.001$; ***, *, *, and * denote significant

difference from Triton control, Media Control, 1 μM ritonavir, and 5 μM ritonavir, respectively. Top left image (**A**); scale bar (red) reads 50μm.

Ritonavir may signal through the canonical TGFβ signaling pathway

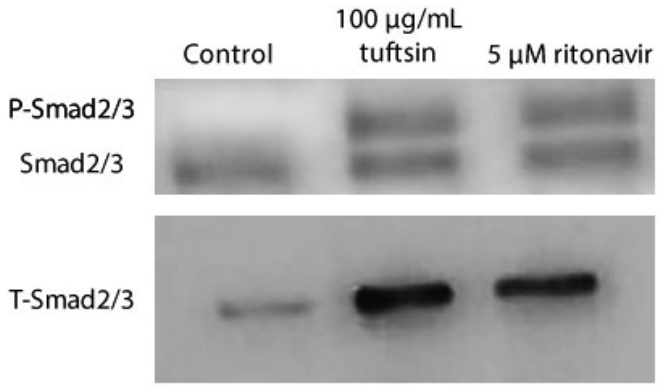

Figure 9. A western blot was performed on N9 microglia exposed to 100 μg/mL tuftsin and 5 μM ritonavir. The blot was probed for total Smad2/3 (T-Smad2/3) and phospho-Smad2/3 (P-Smad2/3).

TGFβ, an anti-inflammatory cytokine, signals through two independent pathways: the canonical signaling pathway, associated with Smad2/3 phosphorylation, and the non-canonical pathway, associated with signaling through PI3k, Akt, or Erk [42]. The canonical signaling pathway has been affiliated with the attenuation of inflammatory immune responses; however, the non-canonical pathway has been shown to antagonize, and even reduce canonical signaling [42, 43].

Immunoblots examining phosphorylated-Smad2/Smad3 (p-Smad2/3) levels were performed to determine whether ritonavir signals through the canonical TGFβ pathway. N9 microglial cells were treated with ritonavir and tuftsin, known to signal through the canonical pathway. Phosphorylation levels were evaluated qualitatively, relative to the media control. Compared to the absence of p-Smad2/3 in media control, the presence of nearly identical levels in tuftsin and ritonavir may indicate that ritonavir, like the virtual screen predicted, binds to Nrp-1 in the exact binding location as tuftsin.

DISCUSSION

In this study, ritonavir is shown to function exactly as tuftsin does with respect to the induction of an anti-inflammatory switch in microglia. Previous work has reported tuftsin's capability in promoting this M2 shift by signaling exclusively through Nrp-1 via the canonical TGFβ signaling pathway [27, 30]. A virtual screen was performed that ranked ritonavir as a likely candidate to bind in the binding pocket location identical to that of tuftsin on Nrp-1. Assuming

the prediction is accurate, ritonavir, as tuftsin does, may polarize microglia to the anti-inflammatory phenotype. In this report N9 and primary microglial polarization to the M2 phenotype by both tuftsin and ritonavir are shown via immunofluorescent imaging. As tuftsin is known to promote the M2 shift of microglia in the EAE model of multiple sclerosis [27], its purpose in this study was primarily comparative but solidified work in previous literature. The effect of ritonavir on microglial polarization, however, is nonexistent in current literature to the extent of our knowledge.

It is notable that in past work on primary microglia, tuftsin treatment alone has not promoted the M2 shift [27]. Consistent with the notion that microglia are often activated in a "two-hit" series [44, 45], tuftsin promotes the M2 shift in primary microglia only when exposed to excitotoxic media (NCM). Contrastingly, N9 microglia readily polarize to the M2 phenotype upon tuftsin-mediated activation without NCM. Ritonavir's role in microglial polarization was first characterized without, then with NCM in the N9 microglial cell line to understand the direction in which the cells would polarize, if at all. Our findings suggest significantly high percentages of M2 positive cells, especially compared to M1, upon ritonavir-mediated activation. While this is true for the N9 microglial cell line and most likely an accurate representation of its shift, this line can lead to an amplified response to drug treatment [39]. To ascertain ritonavir's effect in a manner more similar to a living system, primary microglia cells were derived directly from mice. Results from primary microglia show that, like the N9s, ritonavir does polarize microglia to the anti-inflammatory phenotype.

Evidence of an anti-inflammatory shift prompted further investigation of the manner by which ritonavir induces this response. Signaling through Nrp-1 and the canonical TGFβ pathway is associated with Smad2/3 phosphorylation which has been linked with the inhibition of immune responses [46, 47]. Ritonavir has been shown to potentially signal through the canonical TGFβ signaling pathway as shown by the presence of p-Smad2/3. These data, if replicated and confirmed, would support the prediction that ritonavir binds to Nrp-1 at the same docking location as tuftsin. Unlike tuftsin, ritonavir is a small molecule and is already FDA-approved [31] and would therefore be a more appealing candidate for immunosuppressive therapy.

Benztropine does not favor either M1 or M2 phenotypes *in vivo*, both in literature [34] and currently within our lab. This does not correspond with the clear M2-polarization that resulted

from benztropine treatment of N9 microglia *in vitro* in this experiment. This could be a consequence of the fact that we used the N9 microglial cell line. To clarify the effects of this drug *in vitro*, experimentation on primary microglia is necessary. Benztropine has, however, been shown to induce the differentiation of OPCs to mature oligodendrocytes and would be ideal for combinatorial therapy with immunosuppressive agents. Incorporation of benztropine in EAE treatment regimens with approved immunosuppressive drugs has resulted in enhanced functional recovery and decreases the concentrations of both drugs required to achieve efficacy [34]. Our results confirm that benztropine does not modify the ability of either tuftsin or ritonavir to promote the M2 shift *in vitro*. Thus, they would be appealing options for a combined MS treatments.

Successful translation of these findings to MS patient populations would require preclinical and clinical evaluation. Benztropine has significant dose-dependent neurological and psychiatric side effects [48]. At the higher concentration used in this experiment (5 μM), benztropine had high percentages of M1 cells that significantly differed from the 1.5 μM dose treatment. Similarly, ritonavir has been previously noted to have dose-dependent neurotoxic affects; however, the specific effect on microglia had not been previously characterized. Our findings support dose-dependent neurotoxicity and suggest that doses higher than 25 μM will be cytotoxic.

Our work is novel as the characterization of ritonavir on microglial polarization has not been reported in literature. Similarly, the polarization of benztropine-treated microglia *in vitro* has, to the best of our knowledge, not been investigated either. Thus, the combinatorial treatments of tuftsin or ritonavir with benztropine presents new evidence that benztropine, a remyelinating drug, does not hinder the effects of these immunosuppressive drugs. Additionally, our findings suggest that these drugs should be administered in lower doses as their neurotoxicity is dose-dependent.

A number of additional *in vitro* techniques could prove to which receptors the drugs bind and the pathways through which they signal. To confirm polarization of microglial cells to the particular M2 phenotype that releases the pro-inflammatory cytokine TGFβ, ritonavir can be examined for its ability to promote the release of TGFβ through an ELISA assay. Further, EG00229 is an inhibitor that blocks tuftsin's binding on Nrp-1 [30] and would in theory prevent a ritonavir-mediated M2 microglial shift. Using this inhibitor would verify whether or not ritonavir shifts to

an immunosuppressive response via Nrp-1 binding. The clinical effects of benztropine on EAE, an animal model of MS, and its effect on microglia are being examined concurrently. Ritonavir, on the other hand, has not been assessed in *in vivo* EAE study, a step that would determine its potential efficacy in the attenuation of EAE behavioral outcomes. Because both drugs (benztropine and ritonavir) are already FDA-approved, the potential for their drug repurposing to neurodegenerative diseases like MS will ultimately be determined by the replication of these findings.

REFERENCES

1. Mor, F., M. Kantorowitz, and I.R. Cohen, *The dominant and the cryptic T cell repertoire to myelin basic protein in the Lewis rat.* Journal of Neuroscience Research, 1996. **45**(6): p. 670-679.

2. Trapp, B.D., et al., *Axonal transection in the lesions of multiple sclerosis.* N Engl J Med, 1998. **338**(5): p. 278-85.

3. Dhib-Jalbut, S., *Pathogenesis of myelin/oligodendrocyte damage in multiple sclerosis.* Neurology, 2007. **68**(22 Suppl 3): p. S13-21; discussion S43-54.

4. Guo, M.F., N. Ji, and C.G. Ma, *Immunologic pathogenesis of multiple sclerosis.* Neurosci Bull, 2008. **24**(6): p. 381-6.

5. Henderson, A.P., et al., *Multiple sclerosis: distribution of inflammatory cells in newly forming lesions.* Ann Neurol, 2009. **66**(6): p. 739-53.

6. Kierdorf, K., et al., *Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways.* Nat Neurosci, 2013. **16**(3): p. 273-80.

7. Alliot, F., I. Godin, and B. Pessac, *Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain.* Brain Res Dev Brain Res, 1999. **117**(2): p. 145-52.

8. David, S. and A. Kroner, *Repertoire of microglial and macrophage responses after spinal cord injury.* Nat Rev Neurosci, 2011. **12**(7): p. 388-99.

9. Gehrmann, J., Y. Matsumoto, and G.W. Kreutzberg, *Microglia: intrinsic immuneffector cell of the brain.* Brain Res Brain Res Rev, 1995. **20**(3): p. 269-87.

10. Guillemin, G.J. and B.J. Brew, *Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification.* J Leukoc Biol, 2004. **75**(3): p. 388-97.

11. Benveniste, E.N., *Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis.* J Mol Med (Berl), 1997. **75**(3): p. 165-73.

12. Bauer, J., et al., *Phagocytic activity of macrophages and microglial cells during the course of acute and chronic relapsing experimental autoimmune encephalomyelitis.* J Neurosci Res, 1994. **38**(4): p. 365-75.

13. Huitinga, I., et al., *Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages.* J Exp Med, 1990. **172**(4): p. 1025-33.

14. Brosnan, C.F., M.B. Bornstein, and B.R. Bloom, *The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis.* J Immunol, 1981. **126**(2): p. 614-20.

15. Sriram, S. and M. Rodriguez, *Indictment of the microglia as the villain in multiple sclerosis.* Neurology, 1997. **48**(2): p. 464-70.

16. Shechter, R. and M. Schwartz, *Harnessing monocyte-derived macrophages to control central nervous system pathologies: no longer 'if' but 'how'.* J Pathol, 2013. **229**(2): p. 332-46.

17. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. **25**(12): p. 677-86.

18. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective.* Annu Rev Immunol, 2009. **27**: p. 451-83.

19. Steinman, L., *Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system.* Cell, 1996. **85**(3): p. 299-302.

20. Steinman, L., *Multiple approaches to multiple sclerosis.* Nat Med, 2000. **6**(1): p. 15-6.

21. Bernardino, L., et al., *Modulator effects of interleukin-1beta and tumor necrosis factor-alpha on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures.* J Neurosci, 2005. **25**(29): p. 6734-44.

22. Rott, O., B. Fleischer, and E. Cash, *Interleukin-10 prevents experimental allergic encephalomyelitis in rats.* Eur J Immunol, 1994. **24**(6): p. 1434-40.

23. Mikita, J., et al., *Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration.* Mult Scler, 2011. **17**(1): p. 2-15.

24. Butovsky, O., et al., *Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis.* J Clin Invest, 2006. **116**(4): p. 905-15.

25. Hu, X., et al., *Microglial and macrophage polarization-new prospects for brain repair.* Nat Rev Neurol, 2015. **11**(1): p. 56-64.

26. Nishioka, K., et al., *The chemical synthesis of the phagocytosis-stimulating tetrapeptide tuftsin (Thr-Lys-Pro-Arg) and its biological properties.* Biochim Biophys Acta, 1973. **310**(1): p. 230-7.

27. Wu, M., et al., *Tuftsin promotes an anti-inflammatory switch and attenuates symptoms in experimental autoimmune encephalomyelitis.* PLoS One, 2012. **7**(4): p. e34933.

28. Agudo, M., et al., *Regulation of neuropilin 1 by spinal cord injury in adult rats.* Mol Cell Neurosci, 2005. **28**(3): p. 475-84.

29. Majed, H.H., et al., *A novel role for Sema3A in neuroprotection from injury mediated by activated microglia.* J Neurosci, 2006. **26**(6): p. 1730-8.

30. Nissen, J.C., D.L. Selwood, and S.E. Tsirka, *Tuftsin signals through its receptor neuropilin-1 via the transforming growth factor beta pathway.* J Neurochem, 2013. **127**(3): p. 394-402.

31. Schouten, J.T., *FDA approves 2 new protease inhibitors: ritonavir (Norvir) and Crixivan (Indinavir sulfate). Food and Drug Administration.* STEP Perspect, 1996. **8**(1): p. 7-8.

32. Shim, J.S. and J.O. Liu, *Recent advances in drug repositioning for the discovery of new anticancer drugs.* Int J Biol Sci, 2014. **10**(7): p. 654-63.

33. Chow, W.A., C. Jiang, and M. Guan, *Anti-HIV drugs for cancer therapeutics: back to the future?* Lancet Oncol, 2009. **10**(1): p. 61-71.

34. Deshmukh, V.A., et al., *A regenerative approach to the treatment of multiple sclerosis.* Nature, 2013. **502**(7471): p. 327-32.

35. Franklin, R.J. and C. Ffrench-Constant, *Remyelination in the CNS: from biology to therapy.* Nat Rev Neurosci, 2008. **9**(11): p. 839-55.

36. Franklin, R.J., *Why does remyelination fail in multiple sclerosis?* Nat Rev Neurosci, 2002. **3**(9): p. 705-14.

37. Kremer, D., et al., *The complex world of oligodendroglial differentiation inhibitors.* Ann Neurol, 2011. **69**(4): p. 602-18.

38. Hu, J., et al., *Effects of combined alcohol and anti-HIV drugs on cellular stress responses in primary hepatocytes and hepatic stellate and kupffer cells.* Alcohol Clin Exp Res, 2015. **39**(1): p. 11-20.

39. Rawji, K.S. and V.W. Yong, *The benefits and detriments of macrophages/microglia in models of multiple sclerosis.* Clin Dev Immunol, 2013. **2013**: p. 948976.

40. Stansley, B., J. Post, and K. Hensley, *A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease.* J Neuroinflammation, 2012. **9**: p. 115.

41. Huisman, M.T., et al., *Assessing safety and efficacy of directed P-glycoprotein inhibition to improve the pharmacokinetic properties of saquinavir coadministered with ritonavir.* J Pharmacol Exp Ther, 2003. **304**(2): p. 596-602.

42. Zhang, Y.E., *Non-Smad pathways in TGF-beta signaling.* Cell Res, 2009. **19**(1): p. 128-39.

43. Tian, M., J.R. Neil, and W.P. Schiemann, *Transforming growth factor-beta and the hallmarks of cancer.* Cell Signal, 2011. **23**(6): p. 951-62.

44. Hains, L.E., et al., *Pain intensity and duration can be enhanced by prior challenge: initial evidence suggestive of a role of microglial priming.* J Pain, 2010. **11**(10): p. 1004-14.

45. O'Leary, F.M., et al., *Injury-induced GR-1+ macrophage expansion and activation occurs independently of CD4 T-cell influence.* Shock, 2011. **36**(2): p. 162-9.

46. Prud'homme, G.J. and Y. Glinka, *Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity.* Oncotarget, 2012. **3**(9): p. 921-39.

47. Rahimi, R.A. and E.B. Leof, *TGF-beta signaling: a tale of two responses.* J Cell Biochem, 2007. **102**(3): p. 593-608.

48. Acri, J.B., B.K. Siedleck, and J.M. Witkin, *Effects of benztropine on behavioral and toxic effects of cocaine: comparison with atropine and the selective dopamine uptake inhibitor 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-propyl)-piperazine.* J Pharmacol Exp Ther, 1996. **277**(1): p. 198-206.

49. National Center for Biotechnology Information. PubChem Compound Database; CID=392622, https://pubchem.ncbi.nlm.nih.gov/compound/392622