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Brain Cleanup as a Potential Target for Poststroke Recovery

The Role of RXR (Retinoic X Receptor) in Phagocytes

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Background and Purpose—Phagocytic cells, such as microglia and blood-derived macrophages, are a key biological modality responsible for phagocytosis-mediated clearance of damaged, dead, or displaced cells that are compromised during senescence or pathological processes, including after stroke. This process of clearance is essential to eliminate the source of inflammation and to allow for optimal brain repair and functional recovery. Transcription factor, RXR (retinoic-X-receptor) is strongly implicated in phagocytic functions regulation, and as such could represent a novel target for brain recovery after stroke.

Methods—Primary cultured microglia and bone marrow macrophages were used for phagocytic study. Mice with deleted RXR-α in myeloid phagocytes (Mac-RXR-$\alpha^{-/-}$) were subjected to transient middle cerebral artery occlusion to mimic ischemic stroke and then treated with RXR agonist bexarotene. RNA-sequencing and long-term recovery were evaluated.

Results—Using cultured microglia, we demonstrated that the RXR-α promotes the phagocytic functions of microglia toward apoptotic neurons. Using mice with deleted RXR-α in myeloid phagocytes (Mac-RXR-$\alpha^{-/-}$), we have shown that despite behaving similarly to the control at early time points (up to 3 days, damage established histologically and behaviorally), these Mac-RXR-$\alpha^{-/-}$ mice demonstrated worsened late functional recovery and developed brain atrophy that was larger in size than that seen in control mice. The RXR-α deficiency was associated with reduced expression of genes known to be under control of the prominent transcriptional RXR partner, PPAR (peroxisome proliferator-activated receptor)-γ, as well as genes encoding for scavenger receptors and genes that signify microglia/macrophages polarization to a reparative phenotype. Finally, we demonstrated that the RXR agonist, bexarotene, administered as late as 1 day after middle cerebral artery occlusion, improved neurological recovery, and reduced the atrophy volume as assessed 28 days after stroke. Bexarotene did not improve outcome in Mac-RXR-$\alpha^{-/-}$ mice.

Conclusions—Altogether, these data suggest that phagocytic cells control poststroke recovery and that RXR in these cells represents an attractive target with exceptionally long therapeutic window.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2020;51:958-966. DOI: 10.1161/STROKEAHA.119.027315.)**

Key Words: inflammation ■ macrophages ■ microglia ■ phagocytosis ■ retinoid X receptors

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Ischemic stroke is the leading cause of long-term neurological disability.[1] Multifactorial cell death pathways triggered by cerebral ischemia lead to brain cell death and destruction of a large mass of brain tissue.[2] This infarcted tissue, enclosed by the otherwise functional brain, acts as a reservoir for various cytotoxic and proinflammatory molecules that harm the adjacent healthy tissue, leading to augmented acute damage and secondary injury. In addition to adverse biochemical effects, the infarcted tissue forms a biological and physical barrier hampering neural reorganization, repair, and ultimately neurological recovery. Thus, to enable effective recovery, cellular debris and dead cells need to be cleared from the stroke-injured brain through a process involving phagocytosis. Microglia and infiltrating blood-derived macrophages (microglia/macrophages, MΦ) are the main phagocytes involved in this process, thus their proper function is fundamental to poststroke recovery.

For MΦ to engage in phagocytosis, they are regulated by various external (eg, cytokines, damage-associated molecular patterns) and internal (eg, PPAR [peroxisome proliferator-activated receptor]-γ; Nrf2 [nuclear factor erythroid-related factor 2], and scavenger receptors) factors provided by the local environment (eg, tissue injury) or pharmacological agents affecting these pathways.[3–8] Prophagocytic MΦ are associated with anti-inflammatory and trophic phenotype, which is often referred to as the reparative phenotype that plays a beneficial

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role in tissue repair.[9–13] Recent studies demonstrated that soon after ischemic injury, the reparative MΦ phenotype is transiently enhanced, in part, through release of IL (interleukin)-4 by the ischemia-primed neurons.[4] However, over time the abundance of the reparative MΦ declines, while the prevalence of proinflammatory, potentially harmful MΦ phenotype sharply increases.[4,14,15] The balance between the numbers of reparative versus harmful MΦ could play an instrumental role in phagocytosis-mediated cleanup and poststroke recovery.

RXR (Retinoid X receptor) is a ligand-dependent, transcription factor in the nuclear receptor superfamily.[16] It regulates metabolism and immune responses, including inflammation resolution.[8,17] The RXR-α isoform is uniquely abundant in macrophages, where it plays many essential functions, including tuning the expression of many genes associated with phagocytosis, including engulfment of myelin debris in a model of experimental autoimmune encephalomyelitis.[7,8,17] PPAR-γ is a transcriptional partner of RXR. They form the heterodimer RXR:PPAR-γ[18–20] that regulates target gene expression by binding to conserved DNA sequences, termed peroxisome-proliferator response elements. We and others have reported that the activation of PPAR-γ could polarize MΦ toward the reparative phenotype, with enhanced phagocytic function toward dead/damaged tissues and various cellular debris. This would lead to more efficient clearance of damage-associated cellular debris after ischemic stroke and intracerebral hemorrhage.[3,4,21] Although PPAR-γ–selective ligands are sufficient for PPAR:RXR dimerization and binding to peroxisome proliferator response element (to activate transcription), RXR activation by its selective agonist may lead to homodimerization and formation of heterodimers with other nuclear receptors.[8] Thus, RXR agonists could potently activate not only PPARγ[22–25] but also other transcriptional processes, for example, through liver x receptors,[26] which could potentially improve MΦ functions in stroke-affected brain.

Here, we explored the role of RXR-α in MΦ as a factor involved in regulating MΦ function in promoting brain tissue clearance and neurological functional recovery after ischemic stroke.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. All animal studies followed the guidelines outlined in Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Animal Welfare Committee of the UT-Health. All studies were performed using a randomization (coin toss) approach, and all the analyses were performed by investigators blinded to the treatment assignments (animals were coded for the group allocation). Animals were fed a standard rodent diet and housed in standard cages on a 12-hour inverted light-dark cycle. Experiments included male and female animals at ages of 4 to 6 months. Behavioral analyses were conducted from the hours of 10:00 AM to 4:00 PM.

See the Materials in the online-only Data Supplement for detailed description of all the methods.

Mac-RXR-α$^{-/-}$ Mice

We used conditionally disrupted RXR-α mice in myeloid cells. The experimental mice were progeny of LyzM-Cre^{+}/RXRα$^{-/-}$ mice crossed with RXR-α^{LoxP}. The genotypes of mice were age- and sex-matched littermates, either RXR-α^{LoxP} wild-type control (LyzM-Cre^{-}/RXR-α^{LoxP}) or Mac-RXR-α$^{-/-}$ knockout (LyzM-Cre^{+}/RXR-α^{LoxP}).[27]

Molecular Signaling and Genotyping

The sequence of polymerase chain reaction primers used to genotype LyzM-Cre mice are included in the supplement and follow earlier report.[27]

Administration of RXR Agonist

For in vitro experiments, microglia were preincubated for 24 hours with 0.5 μmol/L bexarotene (Sigma) and then exposed to dead neurons (DN) to assess phagocytosis. Dimethyl sulfoxide (DMSO; 0.1%) was used for vehicle control. For in vivo studies, bexarotene in 3% DMSO was administered intraperitoneally at 5 mg/kg, first at 24 hours after surgery and then once a day for a total of 7 days. DMSO (3%) was used for vehicle control.

Ischemia Model in Mice

Transient (60 minutes) focal ischemia was induced by unilateral middle cerebral artery (MCA)/common carotid artery occlusion (CCAo).[4] We experienced no mortality.

Brain Atrophy Volume Measurement

The postischemic atrophy infarction volume was measured using indirect method as previously described.[4]

Neurological Deficits Measurement

Behavioral tests in mice were conducted in a quiet and low-lit room by an experimenter blinded with respect to the treatment groups. A battery of behavioral tests, including foot fault, postural flexing, and corner turn, and arrive at a combination score, as reported.[4] All animals survived to the terminal end point.

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction

The RNA extraction and SYBR Green-based quantitative reverse transcription-polymerase chain reaction were performed as we described.[3,4,28] The sequences of primers are listed in Table I in the online-only Data Supplement. The expression fold-change was calculated using the delta-delta Ct method.

Primary Brain Glial Culture and Microglia Isolation

The cortical cultures were prepared as we described.[4] After a total of 14 to 21 days in culture, the microglia were harvested by shaking the co-cultures and plated at a density of 1.4×10^5 cells/mL.

Bone Marrow-Derived Macrophages Isolation

Bone marrow was harvested from 2- to 4-month-old mice and processed as describe.[29]

Phagocytosis Assay for DN

We assessed phagocytosis of DNs as earlier reported.[30] Briefly, DNs were generated using γ irradiation. DNs were added to the microglia or bone marrow-derived macrophage cultures at 50:1/DNs:microglia/bone marrow-derived macrophage for 1.5 hours. Phagocytosed DNs were visualized using neuronal-specific class III β-Tuj1 (tubulin) antibody and the amount of the Tuj1^{+} neurons per phagocyte was analyzed on still microscope images with ZEN blue edition software (Zeiss).

RNA-Sequencing and Analysis for Microglia

Cultured microglia from Mac-RXR-α$^{-/-}$ and RXR-α^{LoxP} mice were incubated with 0.5 μmol/L bexarotene or 0.1% DMSO for 24 hours. We used 2×10^6 cells for RNA extraction. RNA-sequencing, including library construction and data analysis, was performed by Novogene Inc. The clean reads were mapped to the reference genome using STAR software, and the mapping results were visualized with the

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Integrative Genomics Viewer. Differential expression analysis was performed using DESeq2 R package; Enrichment analysis, including gene ontology enrichment and Kyoto encyclopedia of genes and genomes pathway, was done using clusterProfiler software.

Statistical Analysis

All data are expressed as mean±SEM. All statistical analyses were performed using the GraphPad Prism 7 and InStat. Repeated-measures 2-way ANOVA followed by Tukey post hoc test was used to evaluate differences among groups at different time points in behavioral tests. Two-way ANOVA followed by Tukey post hoc test was used to analyze data with 2 grouping variables. Remaining data were analyzed using 1-way ANOVA followed by Tukey post hoc test. Nonpaired *t* test was used when 2 groups are compared.

Results

Gene Profile of RXR-α-Deficient Microglia Suggest Altered Phagocytic and Reparative Functions

We have reported that PPAR-γ promotes phagocytic activities and reparative capacities of microglia,[3,4] suggesting that RXR-α a transcriptional partner of PPAR-γ, may (co)regulate microglia' contribution to brain repair after stroke. To probe this notion, we now performed gene profile analysis of microglia from conditional RXR-α knockout mice (Mac-RXR-$\alpha^{-/-}$), with RXR-α deletion selectively targeting myeloid phagocytes (Figures I and II in the online-only Data Supplement), in presence or absence of RXR agonist, bexarotene. RXR-α^{LoxP} mice were used as control.

First, the genome mapping results of RXR-α confirmed the successful deletion of RXR-α exon 4 in microglia from Mac-RXR-$\alpha^{-/-}$ mice (Figure IC in the online-only Data Supplement). Next, the analysis of differential gene expression showed limited differences in gene expression between microglia from Mac-RXR-$\alpha^{-/-}$ and RXR-α^{LoxP} mice, with only a few differentially expressed genes (Figure 1A). However, after exposure to the bexarotene, RXR-α^{LoxP} microglia showed a total of 386 differentially expressed gene (Figure 1B), and this induction of gene expression by bexarotene was reduced (20 differentially expressed gene) in Mac-RXR-$\alpha^{-/-}$ microglia (Figure 1C). Gene ontology enrichment analysis of RXR-α^{LoxP} microglia showed that bexarotene enhanced signaling pathways included lipid transport and metabolism (Apoe, Abca1 [ATP-binding cassette, subfamily A], and Srebf2 [sterol regulatory element binding protein-2]), tissue repair, and importantly, phagocytosis (CD36, Axl [AXL tyrosine kinase], Mertk [mer tyrosine kinase], and Tgm2 [transglutaminase 2]; Figure 1D). The extent of activation of these RXR-mediated signaling pathways by bexarotene was diminished in Mac-RXR-$\alpha^{-/-}$ microglia. In addition, activation of RXR with bexarotene suppressed migration, proliferation, and leukocyte activation pathways (cathepsin c, Cx3cr1, and CD86) in RXR-α^{LoxP} microglia (Figure 1D). These results provide useful insights into the role of RXR activation in microglial phagocytic and reparative processes. Furthermore, the results of Kyoto encyclopedia of genes and genomes pathway analysis showed that activation of RXR with bexarotene-enhanced PPAR:RXR signaling pathways (Figure III in the online-only Data Supplement), confirming the important role of PPAR in RXR signaling in microglia.

Loss of RXR-α Impairs Phagocytic Capacity of MΦ

The optimal brain remodeling and poststroke recovery necessitates effective removal of proinflammatory infarcted tissue from the affected brain, process that requires MΦ and could be regulated by RXR. Thus, our next step was to determine if RXR improves the phagocytic capacity of microglia and macrophages. We harvested the microglia and bone marrow-derived macrophages from Mac-RXR-$\alpha^{-/-}$ mice and RXR-α^{LoxP} and exposed them to mouse apoptotic neurons (target of phagocytosis) to establish an index of phagocytosis, based on the amount of internalized neurons per phagocyte (Figure 2). We found that, compared to control cells, the phagocytic capacity of Mac-RXR-$\alpha^{-/-}$ microglia (Figure 3A and 3B) and macrophages (Figure 3C and 3D) were significantly compromised, confirming that RXR-α deficiency is detrimental for phagocytosis. In agreement with the promoting role of RXR, in a parallel experiment we showed that activation of RXR with bexarotene-augmented phagocytic efficacy of microglia and macrophages in an RXR-α-dependent fashion (Figure 3).

These results suggest that RXR-α in both microglia and macrophages is important for efficient phagocytosis.

In Ischemia-Affected Brain, Selective Deletion of RXR-α in MΦ Is Associated With Reduced Expression of Genes That Control Reparative Functions of Phagocytes

To probe the role of RXR in MΦ after ischemic stroke, we subjected Mac-RXR-$\alpha^{-/-}$ mice and RXR-α^{LoxP} (littermate control) mice to a transient MCA/CCA occlusion. By measuring the infarct volume at 3 days after stroke, we established that Mac-RXR-$\alpha^{-/-}$ and the RXR-α^{LoxP} mice have indistinguishable infarctions (8.98±3.19 versus 8.10±2.81 mm3 for Mac-RXR-$\alpha^{-/-}$ and RXR-α^{LoxP}, respectively; P=0.78, n=6/group), suggesting that RXR-α in MΦ does not modulate susceptibility to ischemia during the acute stages of injury.

However, MΦ are well-known for their reparative functions, properties that are controlled by RXR and could be important during repair after stroke. Granulocytes express negligible levels of RXR-α[31] (also Figure II in the online-only Data Supplement) and as such were not considered as factor contributing to the outcome in this study.

Thus, to gain more insight into this process, we performed the gene expression profiling in ischemia-affected hemisphere at 3 days after MCA/CCAo in Mac-RXR-$\alpha^{-/-}$ and RXR-α^{LoxP} mice. Since the important role of RXR-α in MΦ includes transcriptional control of lipid metabolism and phagocytic pathways, we probed for genes reflecting these functions, including LPL (lipoprotein lipase), CD36, and CD206 (Figure 4). When compared to RXR-α^{LoxP}, Mac-RXR-$\alpha^{-/-}$ mice showed reduced expression of genes encoding for (1) growth factors that modulate reparative processes and tissue remodeling, for example, bFGF and VEGF (vascular endothelial growth factor) and (2) scavenger receptors that modulate phagocytosis, for example, CD36, CD163, CD206, CD204, and ABCA1, which are also regarded as biomarkers for a reparative phenotype of MΦ (Figure 4). One limitation of this experiment is that it does not selectively analyze MΦ but the whole brain. However, similarities in gene expression profile between the cultured microglia and whole brain (eg, genes signifying phagocytosis) suggest strong contribution of MΦ toward whole-brain profile. Overall, this data suggests the important role of RXR-α

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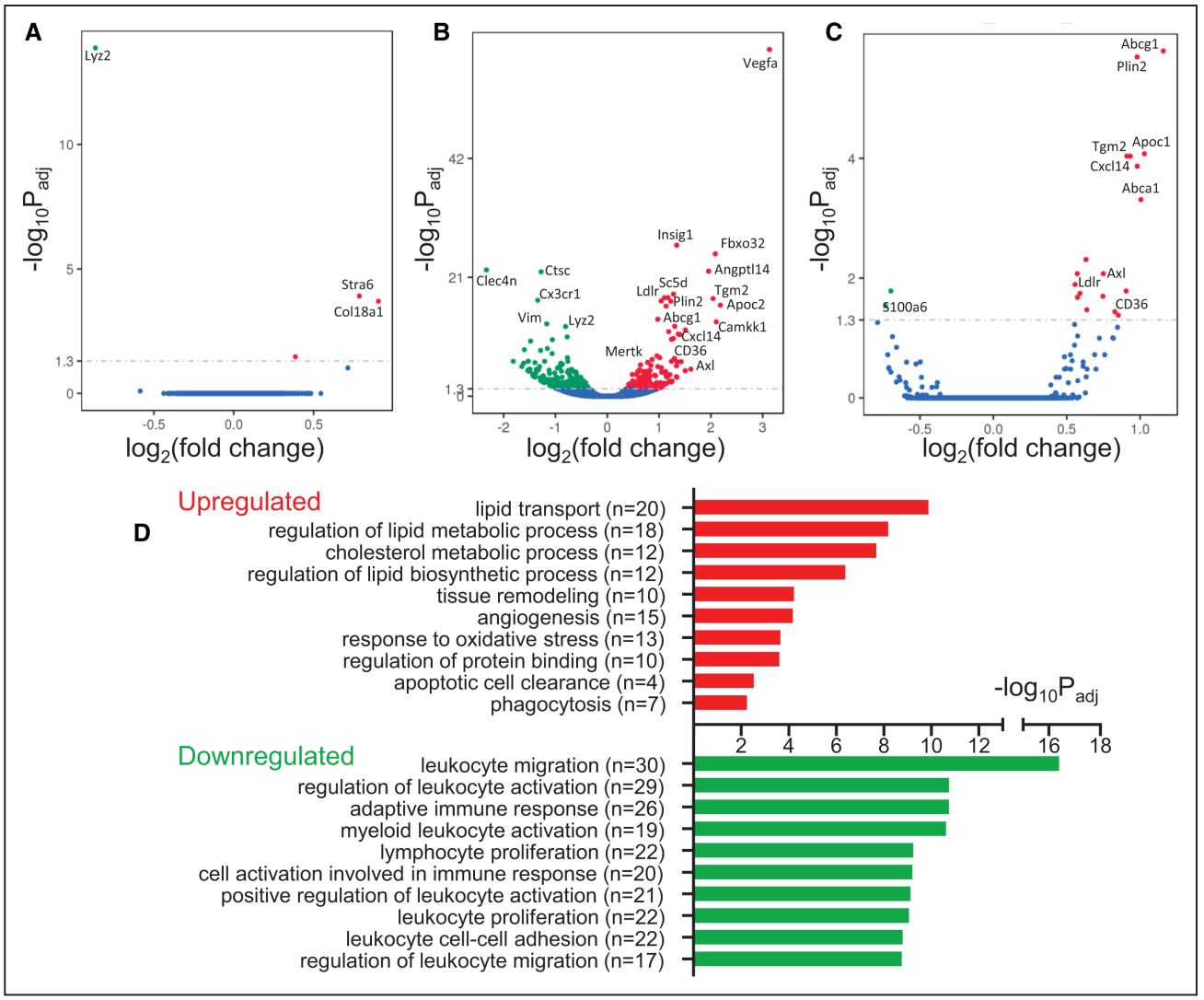

Figure 1. Diminished reparative phenotype of microglia from Mac-RXRα$^{-/-}$mice; RNA-seq analysis. **A**, Mac-RXR-α$^{-/-}$ vehicle compared to RXR-α^{LoxP} vehicle with 4 differentially expressed genes (DEGs); (**B**) RXR-α^{LoxP} BEX (bexarotene) compared to RXR-α^{LoxP} vehicle with 386 DEGs, and (**C**) Mac-RXR-α$^{-/-}$ BEX compared to Mac-RXR-α$^{-/-}$ vehicle with 20 DEGs. **D**, Gene ontology enrichment analysis showed pathways that were significantly upregulated and downregulated by RXR (retinoic X receptor) agonist BEX in RXR-α^{LoxP} microglia. Primary cultured microglia from Mac-RXR-α$^{-/-}$ and RXR-α^{LoxP} mice were incubated with 0.5 μmol/L BEX or vehicle control (0.1% dimethyl sulfoxide [DMSO]) for 24 h before RNA isolation. Each RNA sample was extracted from 2×10^6 cells. Three samples were analyzed in each RXRα^{LoxP} group and 2 samples in each Mac-RXRα$^{-/-}$ group. n indicates the number of genes.

in MΦ in modulating phagocytosis and reparative processes in the stroke-affected brain.

RXR-α in MΦ Assist in Recovery After Focal Cerebral Ischemia

As demonstrated above, RXR-α deficiency in MΦ does not affect acute ischemic damage. However, the gene profile analyses and analysis of phagocytic activities, suggest that RXR-α in MΦ could promote the reparative phenotype of MΦ important in long-term recovery after stroke.

Thus, to test this hypothesis, we subjected Mac-RXR-α$^{-/-}$ and RXR-α^{LoxP} mice to MCA/CCAo and then monitored the animals' neurological deficit over 28 days, the time necessary to achieve infarcted tissue clearance. We found that the neurological deficit at 28 days was significantly worse in Mac-RXR-α$^{-/-}$, as compared to RXR-α^{LoxP} mice (Figure 5A and 5B), signifying a beneficial role of MΦ RXR-α during the recovery process. Responses were similar for male and females (Figure IV in the online-only Data Supplement). After completing the behavioral assessment at day 28, we also measured brain atrophy (missing tissue) volume and established that Mac-RXR-α$^{-/-}$ had larger atrophy than the RXR-α^{LoxP} mice (Figure 5C; gray versus black), especially among male mice (Figure V in the online-only Data Supplement), suggesting that RXR-α in MΦ is needed to limit maturation/progression of brain atrophy. To emphasize the relevance of brain atrophy volume, at the end of the experiment (day 28), we revealed a positive correlation between the atrophy volume and neurological deficit (which was similar for both genotypes; Figure 5D). This provided new and important information: histological damage at 4 weeks accurately predicts functional outcome. Also, these results suggest that approaches aimed at activating RXR-α may represent a target for improving stroke recovery.

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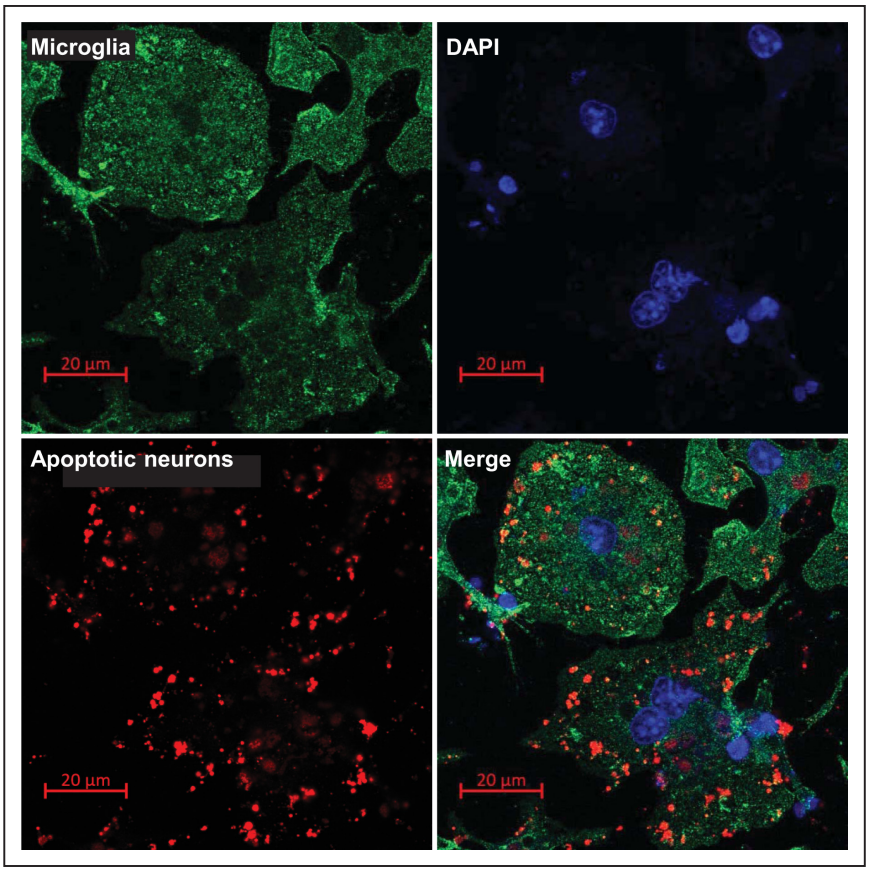

Figure 2. Microglia phagocytose apoptotic neurons. Representative micrograph illustrating primary cultured microglia (CD68⁺ cells/green) phagocytosing apoptotic neurons (neuronal tubulin 1/Tuj1⁺/red), used for establishing the phagocytosis index. DAPI indicates 4′,6-diamidino-2-phenylindole.

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Bexarotene Improves Poststroke Recovery After Focal Cerebral Ischemia With a 1-Day Therapeutic Window

Our next experiment investigated RXR-α as a therapeutic target for poststroke recovery. Since our in vivo experiment with Mac-RXR-α$^{-/-}$ suggests that RXR-α plays a role during the postacute recovery process, we tested the therapeutic relevance of RXR activation to poststroke recovery. We used clinically translational bexarotene to activate RXR. We subjected Mac-RXR-α$^{-/-}$ and the control mice to MCA/CCAo and then administered bexarotene at 24 hours after stroke and then daily for 7 days. We found that control animals receiving bexarotene showed the most robust neurological recovery, with a significant improvement seen at day 14 after the stroke that persisted until day 28, the end of the experiment (Figure 5A; red versus black lines). The beneficial effects of bexarotene on recovery were not detected on day 3 and 7, suggesting again that bexarotene acts through mediating processes involved in secondary injury or repair. Notably, we did not see beneficial effects of bexarotene on poststroke recovery in Mac-RXR-α$^{-/-}$ mice (Figure 5A and 5B; blue versus gray lines), suggesting that at least in part, the role of bexarotene in recovery is through modulation of RXR-α activity in MΦ.

After concluding behavioral assessments (day 28), we measured the brain atrophy to determine the tissue loss at the site of ischemic injury. In agreement with the neurological assessment, bexarotene-treated control mice demonstrated reduced brain atrophy volumes (Figure 5C; red versus black). The beneficial effect of bexarotene on brain tissue preservation was lost in Mac-RXR-α$^{-/-}$ mice.

We did not detect major differences in any measured responses between male and female mice (Figures IV and V in the online-only Data Supplement).

Discussion

Our hypothesis was that MΦ-mediated brain cleanup (eg, phagocytosis-mediated removal of dead tissue) may play essential role in poststroke recovery. To test this hypothesis, we took advantage of a well-established role of RXR-α as key regulator of phagocytic activities of myeloid MΦ and the use of lysozyme M-Cre to delete RXR-α in these cells. The neutrophils express RXR-α at the negligible level[31] (and Figure II in the online-only Data Supplement) and as such were not considered as contributor to the process. Using in vitro phagocytosis assay, we determined that microglia and macrophages respond similarly to RXR modulation, thus we did not differentiate between these cells, regarding their contribution to poststroke recover. Using reversible ischemia model, a model that in the era of thrombolysis and thrombectomy has major translational value,[32] we established that Mac-RXR-α$^{-/-}$ mice have worse poststroke recovery, as compared to control mice, despite similar level of the initial damage (up to 3 days post-ictus), based on the infarct volume and neurological deficit assessment. In agreement with a beneficial role for RXR during the poststroke recovery phase, repetitive activation of RXR with the clinically relevant RXR-activating agent bexarotene, initiated as late as 24 hours after the stroke onset, improved the recovery rate, as assessed with validated sensory-motor tests.[4] Our in vitro functional data with MΦ in culture indicate that one of the important roles RXR-α plays in MΦ is to enhance phagocytosis,[8,17] a process that is responsible

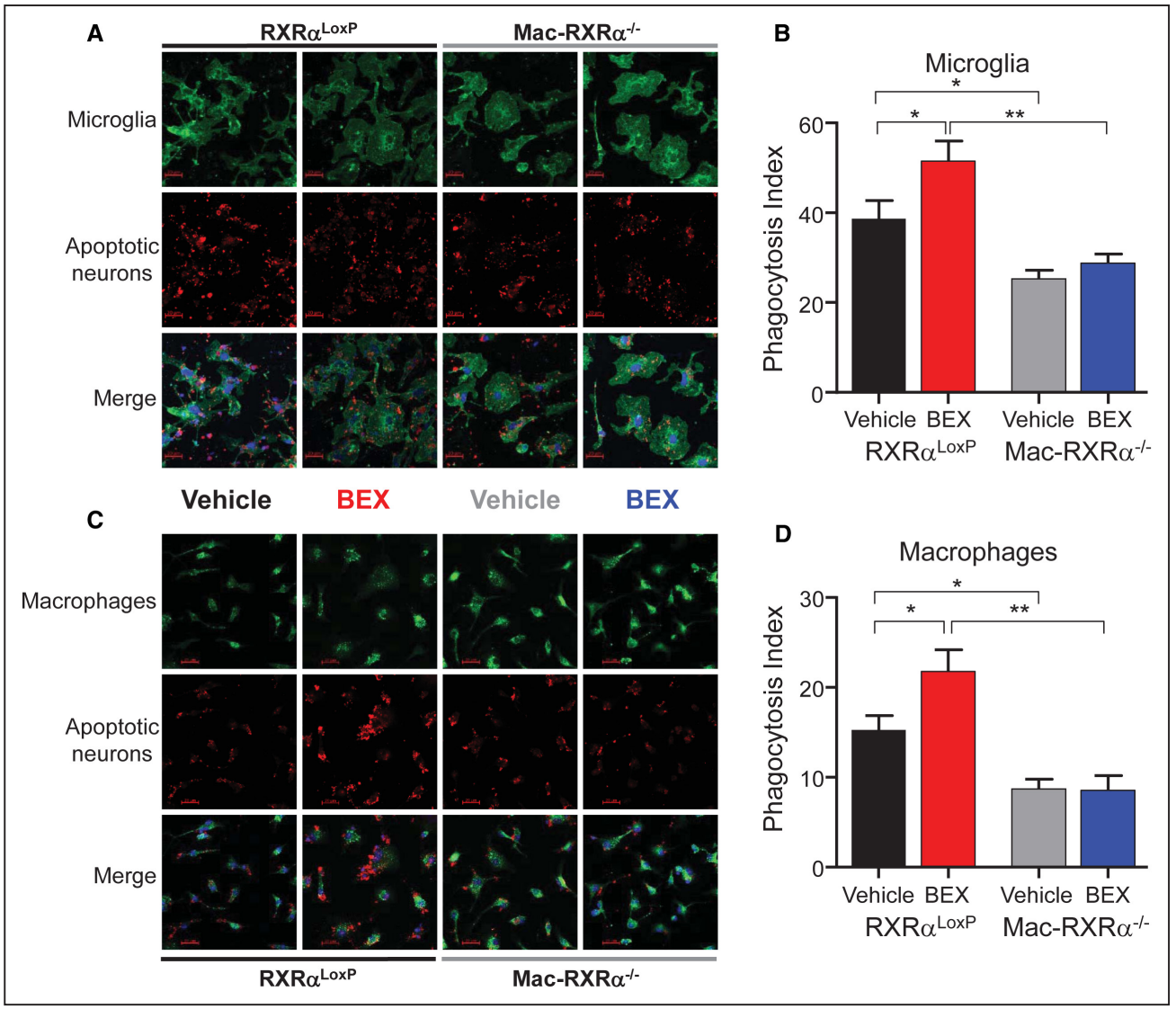

Figure 3. Loss of RXR (retinoic X receptor)-α in microglia or macrophages impairs phagocytosis. The primary microglia (**A** and **B**) in culture or bone marrow-derived macrophages (BMM; **C** and **D**) were harvested from RXR-α^{LoxP} and Mac-RXR-$\alpha^{-/-}$ pup brains and treated with 0.1% dimethyl sulfoxide (DMSO; vehicle) or bexarotene (BEX, 0.5 μmol/L). Twenty-four hours later, cells were exposed to mouse apoptotic neurons. Phagocytic index was determined at 1.5 h by measuring the amount of engulfed apoptotic neurons per individual phagocyte, based on $Tuj1^+$ pixel count in each analyzed cell. The boundary of phagocyte was establish using CD68 immuno-labeling. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). **B** and **D**, Bar graphs demonstrating the phagocytic index. The data are expressed as mean±SEM (n=50 microglial cells/condition). *P<0.05; **P<0.01. Two-way ANOVA followed by pairwise comparison.

for brain cleanup and inflammation resolution through removal of damage-associated cellular debris. Also, our RNA-sequencing data suggest the importance of RXR-α in phagocytic and reparative phenotype activation. Although the phenotypic difference between RXR-α-deficient and proficient microglia is limited in the absence of stimulus, activating RXR with bexarotene significantly increases expression of genes involved in lipid transport and metabolism, tissue remodeling, and phagocytosis in RXR-α-proficient (and not deficient) MΦ. All of these pathways are essential for cleanup and repair processes in brain tissue after stroke. Furthermore, the PPAR-γ signaling pathway is upregulated in RXR-α-proficient but not RXR-α-deficient microglia after exposure to bexarotene, suggesting an eminent role for PPAR-γ in RXR-α transcriptional regulation. We and others have previously demonstrated that MΦ-mediated cleanup is, in part, under the control of PPAR-γ, a transcriptional partner of RXR, which in MΦ regulates the expression of several scavenger receptors, molecules that are critical for debris clearance during phagocytic engulfment.[17,33] Examples include CD36, Axl, and Mertk, proteins that we and others have implicated in the postintracerebral hemorrhage cleanup process.[3,34] Thus, one possible scenario is that RXR-mediated prophagocytic effects are achieved through activation of transcriptional activities of the RXR:PPAR-γ complex through direct RXR activation. Indeed, this cooperative interaction appears to be the likely scenario. By using primary rat microglia (data not included), we documented that activation of RXR with bexarotene effectively augmented PPAR-γ agonist (rosiglitazone)-induced phagocytic activity. In a more direct experiment, we showed that PPAR-γ antagonist could reverse bexarotene-augmented microglia-mediated

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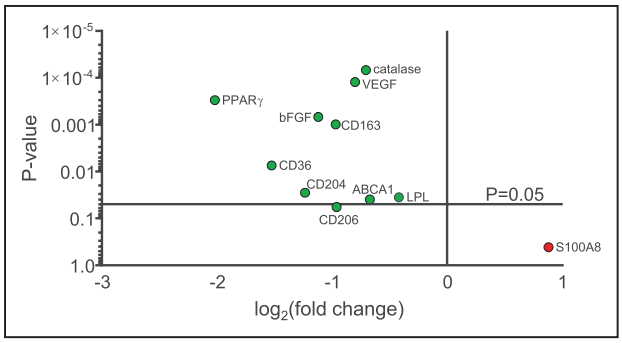

Figure 4. In ischemia-affected brain, RXR (retinoic X receptor)-α deletion in microglia/macrophages (MΦ) leads to reduced expression of genes that signify reparative functions of microglia. Gene expression profile (by quantitative reverse transcription-polymerase chain reaction [RT-qPCR]) for the indicated genes in ischemia-affected ipsilateral cortices of RXR-α^{LoxP} and Mac-RXR-$\alpha^{-/-}$ mice, 3 d after middle cerebral artery (MCA)/CCAo. Gene expression fold-change was calculated using delta-delta Ct method with RXR-α^{LoxP} mice as baseline and GAPDH as reference gene. N=6 mice/group. VEGF indicates vascular endothelial growth factor.

phagocytosis (data not included). Finally, our targeted analyses of the gene expression profile in the ischemia-affected brain tissue from Mac-RXR$\alpha^{-/-}$ mice, suggest that the loss of RXR-α in MΦ coincided with the reduced expression of prototypic PPAR-γ-regulated genes, such as lipoprotein lipase, CD36, and catalase, as well as PPAR-γ itself. Collectively, this data indicate that the inhibition (knockout) or stimulation (bexarotene) of RXR in microglia is mirrored by similar responses of PPAR-γ, suggesting that under our experimental conditions, the activity of RXR in MΦ is effectively coupled to PPAR-γ.

Various cellular mechanisms are engaged in the regulation of phagocytic/endocytic functions conducted by MΦ. One such essential mechanism, especially in relation to damage associated with the septic form of inflammation such as that caused by ischemia-induced damage, is through expression of various scavenger receptors. These membrane-associated proteins are intrinsic to various forms of MΦ and play key roles in the postinjury tissue cleanup and repair.[35,36] We established here that the RXR-α deficiency in MΦ is associated with the reduced expression of several scavenger receptors in the brains of mice subjected to MCA/CCAo. These changes, as measured 3 days after the stroke, included reduced expression of CD36 (the defining member of class B scavenger receptors with a well-established role in efferocytosis), CD163 (scavenger receptor for hemoglobin:haptoglobin complexes and other cell degradation products[37,38]), CD206 (mannose receptor that is involved in engulfment of several mannose-bearing serum glycoproteins[39]), and CD204 (SR-A/class A scavenger receptor involved in various phagocytic/endocytic functions[40]). It has to be emphasized that these gene expression changes were unlikely related to the extent of initial ischemic damage, as Mac-RXR-$\alpha^{-/-}$ and the control mice demonstrated indistinguishable infarct volume and behavioral deficit at the time point when gene analysis was performed. It has to be independently noted that the above scavenger receptors are also often recognized as biomarkers for the reparative phenotype of MΦ. This is often referred to as the M2 phenotype, MΦ with stronger phagocytic capabilities, antioxidative activities, and able to more effectively generate anti-inflammatory cytokines and trophic factors.[13]

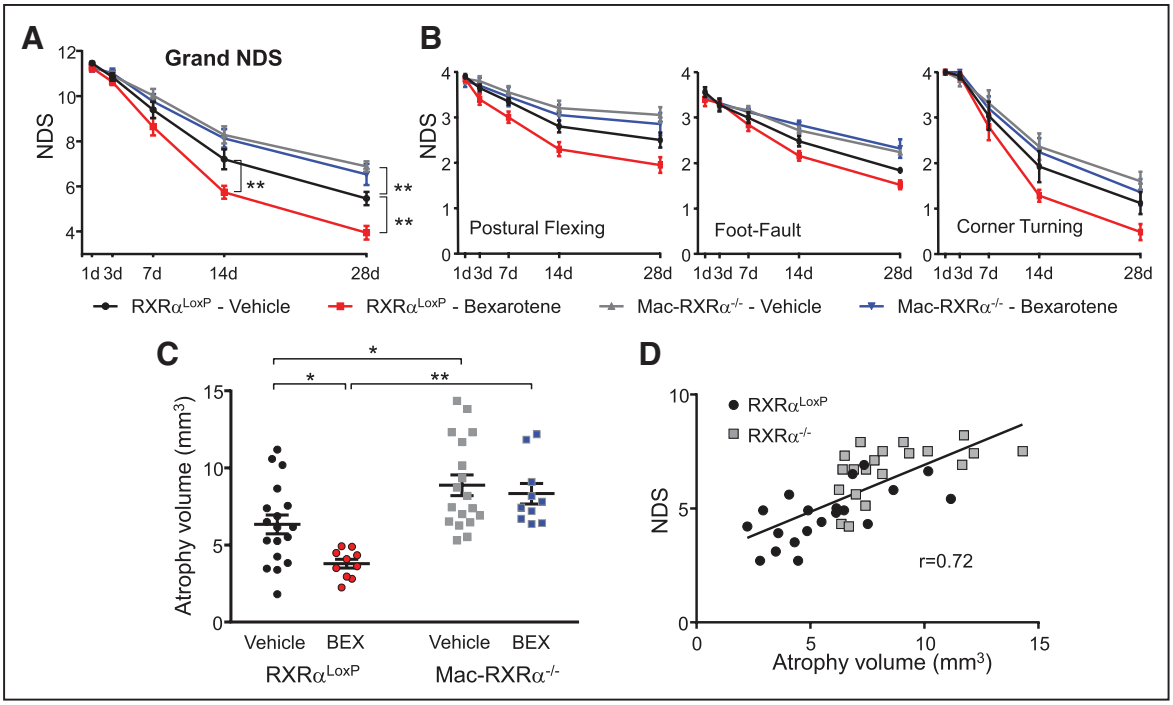

Figure 5. Neurological deficit outcomes and brain atrophy volumes 28 d after middle cerebral artery (MCA)/common carotid artery occlusion (CCAo). The Mac-RXR-$\alpha^{-/-}$ and RXR-α^{LoxP} mice were subjected to 60 min of MCA/CCAo. Bexarotene (BEX), 5 mg/kg, or vehicle was injected intraperitoneally, starting at 24 h after MCA/CCAo and then daily for 7 d. The composite neurological deficit scores (Grand NDS) (**A**), and the individual NDS including postural flexing, foot fault, and corner turning (**B**) on day 1 through day 28 are demonstrated. Data are expressed as mean±SEM (n=10/group). $^{**}P<0.01$. Repeated measure 2-way ANOVA followed by pairwise comparison. **C**, The brain atrophy volume at the site of ischemic injury at day 28 was measured. Data are expressed as mean±SEM (n=18 in each vehicle group and n=10 in each BEX group). $^{*}P<0.05$, $^{**}P<0.01$. Two-way ANOVA followed by pairwise comparison. **D**, Correlations between brain atrophy volumes and neurological deficit scores at day 28 were calculated by Pearson coefficient *r* (r=0.725, $P<0.001$).

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A reason for why the atrophy volume at 4 weeks after stroke was larger in Mac-RXR-$\alpha^{-/-}$ mice as compared to the control mice cannot be clearly deduced from the experimental data collected in this study. We know that the initial stroke-induced injury was similar in the control and Mac-RXR-$\alpha^{-/-}$ mice. Thus, it is justified to assume that the differences in lesion size could be due to pathogenic processes that took place at some later stages of ischemic lesion maturation. Also, since these changes occurred as a result of modification primarily limited to MΦ, we assume that at least one of the MΦ functions that are under control of RXR is causally related to differences in the ischemic outcome in these animals. As we discussed earlier, one key function of MΦ is to conduct phagocytosis-mediated cleanup to achieve a better and faster elimination of dying cells and cellular debris, the main source of cytotoxicity and deleterious inflammation. In agreement with the existing data,[7,8] our in vitro experiments suggest that RXR indeed plays an important role in optimizing the phagocytic function of MΦ, and as such RXR activation (eg, with bexarotene) after stroke could enhance the cleanup and reduce the injury to the brain tissue caused by toxic byproducts of ischemic injury. Finally, since the behavioral deficit across the genotypes showed a positive correlation with the brain atrophy volume, we think that it is unlikely that the sole neuronal plasticity (independent of the total lesion size) within the perilesional brain tissue could account for the behavioral improvement. This once again suggests that the cleanup process could account for a key mechanism associated with functional outcome.

In conclusion, we propose that RXR could represent an attractive and clinically relevant target for improving poststroke recovery through modification of MΦ.

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Disclosures

None.

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