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ORIGINAL ARTICLE

Comparative transcriptomic analysis of macrophages treated with a combination of ROCK pathway inhibitors

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Abstract

Background: At present, there is no therapy for the long-term (chronic) rejection of transplanted organs. This condition leads to tissue fibrosis and occlusion of the blood vessels. **Aim:** The overall goal of the current research is to identify a clinically applicable therapy for chronic rejection in transplanted organs. Our previous study showed that inhibitors of the RhoA/Rock pathway, such as Rezurock and fingolimod, prevent chronic rejection in rodent transplantation models, with Rezurock being superior in reducing fibrosis. **Materials and methods:** In this study, we analyzed the effect of a Rezurock and fingolimod combination on the transcriptome of mouse peritoneal macrophages and protein expression in both mouse and human macrophages. **Results:** The Rezurock/fingolimod combination resulted in the differential expression of 4,855 genes (2,477 downregulated and 2378 upregulated). Downregulated genes were related to fibrotic pathways, extracellular matrix, blood vessel development, cell adhesion, and cytokine production. Protein expression analysis showed that Rezurock/fingolimod treatment had a significantly stronger effect on the expression of pentraxin 3, chemokine (C-C motif) ligand 2, C-C motif chemokine receptor 2, and transforming growth factor beta 1 in mouse macrophages, and was much more effective in reducing the expression of Notch1 and Rho-associated coiled-coil kinase 2 in human macrophages compared to individual treatments. **Conclusion:** Rezurock/fingolimod treatment not only affects fibrotic pathways but also downregulates genes related to cell cycle progression and cytokine production and disrupts macrophage recruitment signaling. These findings indicate that Rezurock, alone or in combination with other immunomodulators, may be a promising candidate for clinical therapy targeting chronic rejection.

Keywords: Rezurock; Fibrosis; Chronic rejection; Macrophage; Ras homolog family member A/Rho-associated coiled-coil kinase pathway

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1. Introduction

Although short-term survival rates of transplanted organs have reached satisfactory levels, long-term survival remains a major post-transplantation challenge. According to the International Report on Organ Donation and Transplantation Activities from Global Observatory on Donation and Transplantation for 2022,[1] and published data,[2] approximately 157,000 solid organ transplants were performed worldwide, including kidney (~102,090); liver (~37,436), heart (~8,988), lung (~6,784), pancreas (~2,026), and small bowel (~170). However, around 70% percent of transplant recipients experienced symptoms of organ rejection within 10 years post-transplantation. Current treatment approaches depend on organ type; however, the most common treatments include immunosuppressants and corticosteroids, which primarily target the immune response and reduce inflammation.[3,4] The primary histological features in biopsies of chronically rejected organs are macrophage-driven narrowing or occlusion of the vessels and tissue fibrosis.[3,5] Because fibrosis is an important hallmark of chronic rejection, we hypothesize that targeting macrophages could become a new, efficient approach in post-transplant treatment. In our quest for clinically applicable anti-chronic rejection therapy, we showed that in a rodent cardiac transplantation model, the pharmacologic inhibition of the Ras homolog family member A (RhoA)/Rho-associated coiled-coil kinase (ROCK) pathway or macrophage-specific RhoA knockout eliminates, through changes in the actin cytoskeleton, macrophage infiltration of the allograft and inhibits chronic rejection.[6,7] After testing several commercially available RhoA/ROCK inhibitors, we found that Rezurock—Food and Drug Administration (FDA)-approved for the treatment of chronic graft-versus-host disease—is superior in inhibiting fibrosis in the mouse cardiac transplantation model.[8] Molecularly, RhoA/ROCK signaling mediates cytoskeletal remodeling during cell migration and polarization of macrophages and additionally affects the proliferation rates of immune cells.[9] Therefore, we believe that inhibitors of the RhoA/ROCK pathway could represent a novel approach for post-transplant treatment.

Here, we studied the effects of Rezurock and fingolimod combination on gene and protein expression in mouse and human macrophages. Fingolimod, primarily recognized as a nonselective functional antagonist of sphingosine-1-phosphate receptors, is FDA-approved for multiple sclerosis treatment. However, our previous work demonstrated that fingolimod also inhibits the RhoA/ROCK pathway, although it is much less effective in inhibiting fibrosis than Rezurock.[9] Our transcriptomic analysis reveals that combined Rezurock/fingolimod treatment downregulates genes associated with cell cycle progression, metabolic and cytokine production pathways, and dysregulates signaling pathways involved in macrophage recruitment. Overall, our findings suggest that Rezurock/fingolimod, in combination, holds therapeutic potential in preventing the chronic rejection-related functions of macrophages in organ transplantation.

2. Materials and methods

2.1. Mice handling

All experiments were performed according to The Methodist Hospital Research Institute's animal care and use standards, based on the National Institute of Health (NIH) guidelines outlined in the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 85-23 Revised 1985). The Institute also mandates compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*.

2.2. Isolation and culture of peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of C57BL/6J mice (n = 25) obtained from Jackson Laboratory, Bar Harbor, USA and cultured as previously described.[10]

2.3. RAW 264.7 macrophage culture

RAW 264.7 cells (ATCC, Manassas, USA) were maintained and cultured as previously described.[10]

2.4. Isolation and culture of peripheral blood mononuclear cells from human blood and differentiation of monocytes into macrophages (human monocyte-derived macrophages)

Blood (500 mL) was purchased from the blood bank. Fifty-milliliter stem cell tubes (SepMate™ Peripheral Blood Mononuclear Cell [PBMC] Isolation Tubes, STEMCELL Technologies, Vancouver, BC, Canada) were filled to the 15 mL mark with Ficoll-Paque Plus (Cytiva, Marlborough, MA 01752, USA). After slowly adding blood to the 40 mL mark, the tubes were centrifuged at 700 × g for 15 min at room temperature (RT). The white PBMC layer was transferred to the 50 mL tubes, and after adding phosphate-buffered saline, centrifuged at 500 × g for 10 min at RT. Cells were cultured in RPMI 1640 medium (11875-093, Gibco, Waltham, Massachusetts, USA. Supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units/mL), and macrophage colony-stimulating factor (Peprotech 300-25, Cranbury, New Jersey 08512,

USA) at 50 ng/mL, at 37°C and 5% carbon dioxide. A total of 2×10^7 cells were seeded in each T75 flask. After 24 h, the medium was changed to remove non-adherent cells, leaving only monocytes (adherent cells). Cells were differentiated into macrophages (human monocyte-derived macrophages [HMDM]) over 6 days. On day 6, the HMDM were treated with inhibitors.

2.5. Treatment with inhibitors

At 70% confluency, cells were treated for 24 h with Rezurock (HY-15307, MedChem Express, United States of America [USA]) at a concentration of 10 μM, fingolimod (S5002, Selleckchem, USA) at 300 nM, or Rezurock and fingolimod in combination. For HMDM, Rezurock alone or in combination with fingolimod was used at a concentration of 5 μM. Dimethyl sulfoxide (DMSO; Sigma, USA) was used as a control, and its volume matched the volume used for the highest drug concentration to ensure consistent DMSO levels across all samples.

2.6. RNA isolation

Mouse peritoneal macrophages were pelleted and sent to Active Motif, Inc. (USA) for RNA isolation, library preparation, and sequencing analysis.

2.7. RNA sequencing and analysis

Next-generation sequencing was performed using the Illumina platform, and Venn diagrams were generated as described previously.[10] Genes selected for the Metascape Gene Ontology (GO) analysis had a $p \leq 0.05$ and $\log_2$ fold change‖ > 0. All genes considered differentially expressed in the treated samples, compared to the control, had a $p \leq 0.05$ and ‖$\log_2$ fold change‖ > 0. For RNA sequencing (RNA-seq) analysis, two biological replicates were used for each treatment condition. In our gene expression analysis, sequencing quality was ensured by filtering raw reads to obtain clean reads. This was followed by alignment to the reference genome using HISAT2 and STAR tools. Gene expression levels were normalized using the "Fragments Per Kilobase of exon per Million mapped reads" method, and principal component analysis plots were used to visualize sample clustering and assess potential batch effects. All samples were processed under identical conditions to minimize technical variability.

2.8. Cell lysis and western blots

Control, Rezurock-, fingolimod-, and Rezurock/fingolimod-treated RAW 264.7 and HMDM macrophages were lysed in radioimmunoprecipitation assay cell lysis buffer (#9806, Cell Signaling, USA) supplemented with 1× protease inhibitor (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, #11836170001, Roche, USA) and 1× phenylmethanesulfonyl fluoride (#8553, Cell Signaling, USA), and prepared for Western blotting as described previously.[10] Western blot bands were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as a loading control (protein of interest vs. GAPDH), and each experiment was repeated three times to reduce technical noise. Statistical analysis was performed to ensure data significance.

2.9. Antibodies

The following primary antibodies were used: ROCK2 (8236s, Cell Signaling, USA), Notch1 (3608s, Cell Signaling, USA), pentraxin 3 (PTX3; PA5-36156, Invitrogen, China), collagen Type1 (14695-1-AP, Proteintech, USA), chemokine (C-C motif) ligand 2 (CCL2; MA5-17040, Invitrogen, China), C-C motif chemokine receptor 2 (CCR2; MA5-42780, Invitrogen, China), and transforming growth factor beta 1 (TGF-β1; 21898-1-AP, Proteintech, USA), all at 1:1,000 dilution. ROCK1 antibody (ab199899, Abcam, USA; currently discontinued; https://www.abcam.com/en-us/products/unavailable/rock1-antibody-c-terminal-ab199899) was used at 1:2,000 dilution, and GAPDH (14C10, Cell Signaling, USA) at 1:3,000 dilution. For secondary antibodies, anti-rabbit immunoglobulin G (IgG; 7074P2, Cell Signaling, USA) and anti-mouse IgG (7076, Cell Signaling, USA) were used at 1:5,000 dilutions.

2.10. Statistics

For western blotting (protein expression) analysis, three biological replicates were used to ensure statistical power, as described previously.[10] Differentially expressed genes (DEGs) were selected using a p-value cutoff of < 0.05, and a $\log_2$ fold-change > 0. These thresholds were applied when generating volcano plots for each treatment group.

The protein expression changes after drug treatment were consistent with the transcriptomic findings, supporting the reliability of the DEG selection. Pathway enrichment and GO analyses were carried out using the Metascape platform based on DEGs with $p \leq 0.05$ and a $\log_2$ fold change > 0. Results were interpreted using bar plots and clustering analyses to identify significantly enriched biological processes. RNA-seq data were analyzed using DESeq2, which includes internal normalization and applies the Wald test followed by Benjamini–Hochberg correction to control the false discovery rate (FDR). All pathway enrichment analyses (e.g., GO and Kyoto Encyclopedia of Genes and Genomes [KEGG]) were likewise corrected for multiple hypothesis testing using FDR-adjusted p-values. Statistical significance thresholds (adjusted p<0.05) were applied consistently across all analyses.

3. Results

In all our analyses, we purposely used non-activated (M0) macrophages. M0 (or naïve) macrophages are in a resting state and serve as precursors to polarized macrophages. It is known that the transcription and protein expression profiles of activated macrophages depend on the type (direction) of polarization (M1 vs. M2), and particularly on the specific type of activator. In addition, our previous research showed that the response of M0 macrophages to RhoA/ROCK inhibition is very similar to that of M2 macrophages but differs from the response of M1 macrophages. Both M0 and M2 macrophages exhibit high levels of RhoA messenger RNA (mRNA), whereas M1 macrophages express RhoA mRNA at approximately three times lower levels.[11] Thus, to obtain "basic" or naïve transcriptomic and proteomic data, we chose to use non-activated (M0) macrophages.

3.1. RNA sequencing

We performed RNA sequencing of mouse peritoneal macrophages after combined treatment with Rezurock and fingolimod. The data were then compared with our previously published results from individual treatments with Rezurock or fingolimod in mouse peritoneal macrophages.[10] A schematic representation of the workflow is shown in Figure 1. Figure S1A displays the distribution of control (DMSO-treated), Rezurock-only, and Rezurock/fingolimod-treated macrophages. Figure S1B shows a box plot representing gene expression distribution in control (DMSO-treated) and Rezurock/fingolimod-treated macrophages. Some of the data presented in the Supplements were already published in our previous publication.[10] All genes considered differentially expressed compared to the control (DMSO-treated) cells had a $p \leq 0.05$ and $\|\log_2 \text{fold change}\| > 0$. Pathways associated with these DEGs were identified using Metascape.

3.2. Effect of Rezurock/fingolimod combination on mouse peritoneal macrophages

The combination treatment of macrophages with Rezurock and fingolimod resulted in the differential expression of 4,855 genes (2,477 downregulated and 2,378 upregulated) compared to the DMSO control (Figure 2A). In the volcano plot, green dots represent upregulated genes, whereas red dots represent downregulated genes. The distribution of the DEGs is depicted in the heatmap (Figure 2B). The downregulated genes were related to the cell cycle, DNA metabolic processes, neutrophil degranulation, chromosome organization, leukocyte proliferation, and cytokine production (Figure 2C; Tables S1 and S2). The upregulated genes were associated with protein processing, membrane trafficking, Rho GTPase signaling, autophagy, Golgi organization, neuron projection development, and histone modification (Figure 2D; Tables S3 and S4).

Our previously published work showed that Rezurock is superior to fingolimod in modulating the transcriptome profile of mouse peritoneal macrophages and in regulating fibrosis pathway-related proteins in both mouse macrophages and human monocyte-derived macrophages.[10] Here, we show that 1,751 genes were shared between Rezurock-treated macrophages and the combination of Rezurock/fingolimod, 726 genes were downregulated only after the combination treatment,

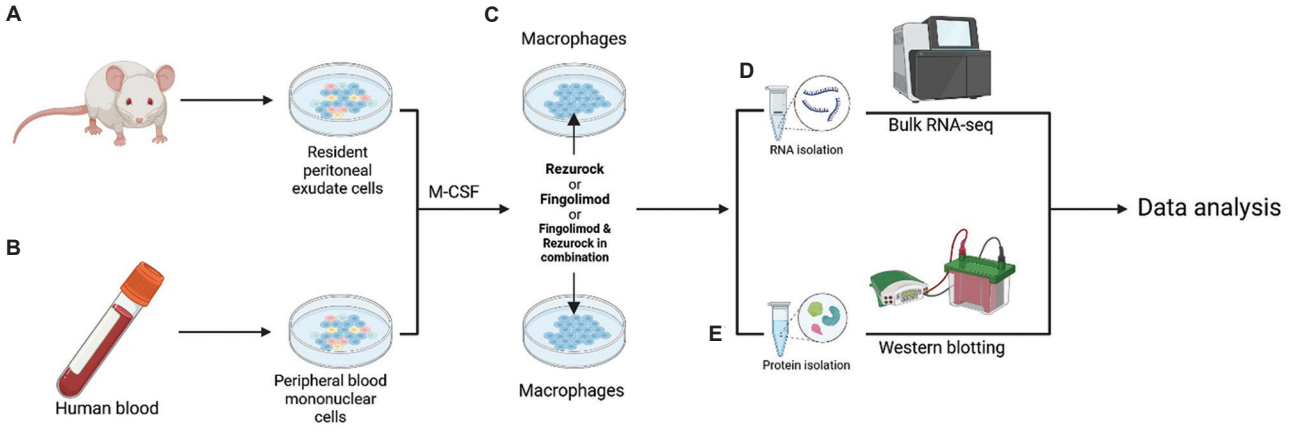

Figure 1. Experimental workflow for macrophage isolation, drug treatment, and downstream analysis. Resident peritoneal exudate cells were harvested from the peritoneal cavity of mice (A), while mononuclear cells were isolated from human blood samples (B). In both cases, cells were cultured in the presence of M-CSF to induce macrophage differentiation. Once differentiated, macrophages were treated with Rezurock, fingolimod, or a combination of both drugs to assess their individual and combined effects (C). Following treatment, RNA was isolated for bulk RNA sequencing to evaluate gene expression changes (D), and protein was extracted for western blotting to assess alterations in protein levels (E).
Abbreviation: M-CSF: Macrophage colony-stimulating factor.

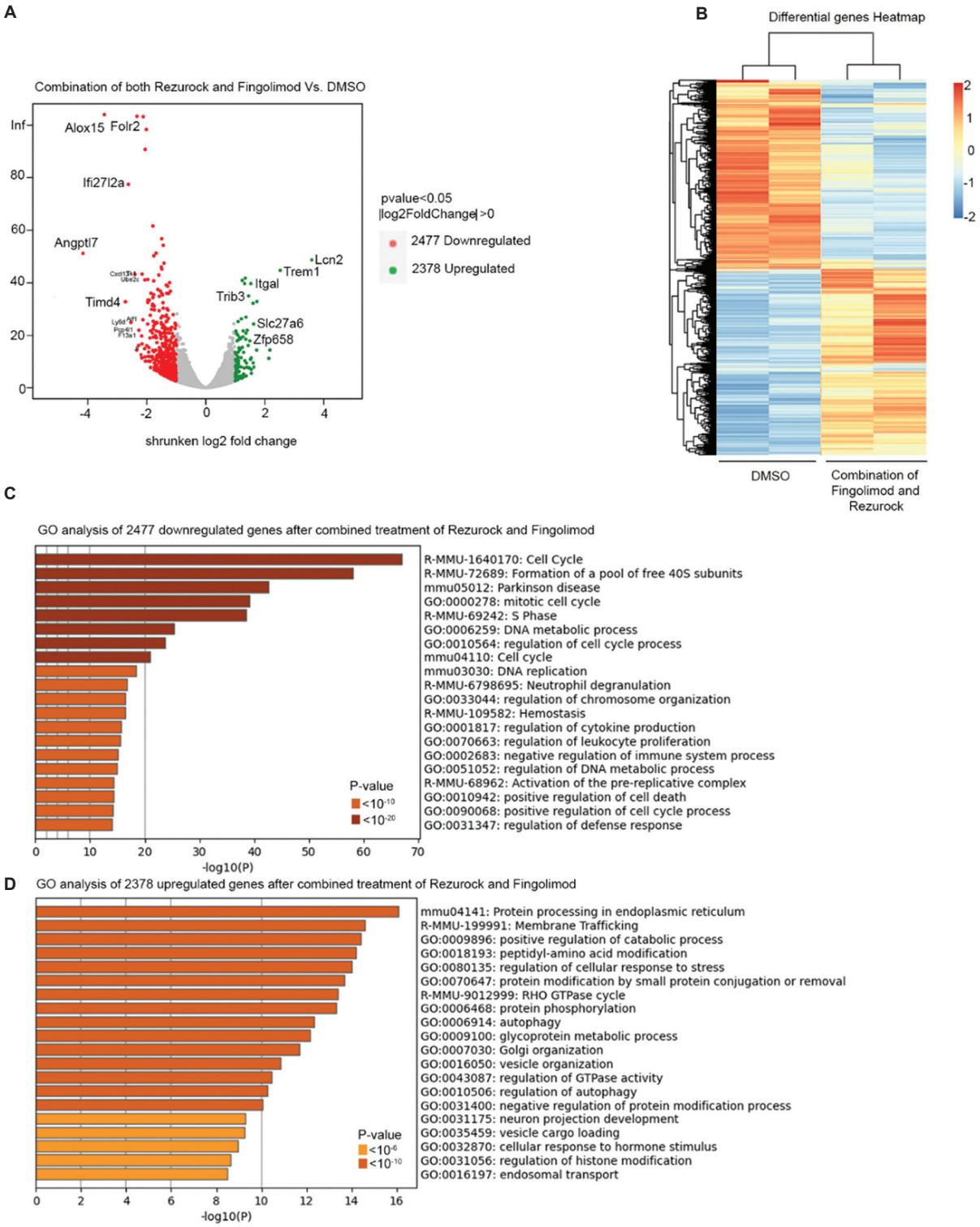

Figure 2. Distribution of DEGs and associated biological pathways in mouse macrophages treated with the Rezurock/fingolimod combination compared to control (DMSO-treated) macrophages. (A) Volcano plot showing DEGs in Rezurock/fingolimod-treated macrophages versus DMSO-treated controls (p<0.05 and $\|\log_2$ fold change$\|$ > 0). (B) Heatmap illustrating the distribution of DEGs between DMSO- and Rezurock/fingolimod-treated mouse macrophages. (C) GO analysis of downregulated genes in the Rezurock/fingolimod combination treatment. (D) GO analysis of upregulated genes in the Rezurock/Fingolimod combination treatment.

Abbreviations: DEGS: Differentially expressed genes; DMSO: Dimethyl sulfoxide; GO: Gene ontology; Rho: Ras homolog family member.

whereas 310 genes were downregulated exclusively in Rezurock-treated macrophages (Figure 3A and B). The genes downregulated after combination treatment were related to extracellular matrix organization, blood vessel development, neutrophil degranulation, cell–cell adhesion, and cytokine production (Figure 3C; Table S5). The downregulated genes specific to Rezurock treatment were involved in lipid metabolism, atherosclerosis, hemostasis, cytokine signaling, neutrophil signaling, lipid localization, and regulation of the actin cytoskeleton (Figure 3D; Table S6). The genes downregulated in both Rezurock and combination treatments were related to the cell cycle, translation, chromosome organization, regulation of cellular stress, adaptive immune response, and chromosome segregation (Figure 3E; Table S7). Among the upregulated genes, 357 were unique to Rezurock treatment (Figure 4A and B), 821 were uniquely upregulated after Rezurock/fingolimod treatment, and 1,554 genes were shared between the two treatments. The genes upregulated only after combination treatment were related to Golgi vesicle transport, histone modification, the Rho GTPase pathway, protein modification, DNA methylation, and the mitogen-activated protein kinase signaling pathway (Figure 4C; Table S8). The genes shared between Rezurock and combination treatments were associated with protein processing in the endoplasmic reticulum (ER), metabolic processes, autophagy, GTPase activity, and membrane trafficking (Figure 4D; Table S9). The genes upregulated only in Rezurock-treated macrophages were related to ER-to-Golgi transport, lysozyme pathway, fatty acyl-coenzyme A synthesis, membrane lipid metabolism, protein localization to the ER, neutrophil degranulation, and apoptotic signaling (Figure 4E; Table S10).

In summary, treatment with Rezurock/fingolimod had a more pronounced effect on the mouse macrophage transcriptome than individual treatment with either Rezurock or fingolimod.[10] The shared 87 downregulated genes were mostly related to antigen processing, cytokine production, chemokine signaling, regulation of leukocyte differentiation, cell activation, macrophage markers, G alpha signaling, and interleukin (IL)-2 production (Figure 5A-C; Table S11). Among the upregulated genes, 35 were shared by all three treatment groups (Figure 5D and E). These 35 genes were associated with protein folding, adipogenesis, transcription, immunoglobulin production, and cell–cell adhesion (Figure 5F; Table S12).

3.3. Effect of Rezurock/fingolimod combination on the fibrosis pathway-related proteins

Rezurock prevents fibrosis more effectively than other RhoA/ROCK inhibitors. It downregulates genes involved in fibrosis and collagen deposition pathways. Table 1 lists some of the genes downregulated by Rezurock and the Rezurock/fingolimod combination, but not by fingolimod alone. We previously published the expression data of these fibrosis-related proteins (some selected from Table 1 and others from independent fibrosis studies) following treatment with Rezurock or fingolimod separately.[10] Here, we compared the expression of fibrotic pathway-related proteins in control (DMSO-treated), Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination-treated treatment.

We evaluated the expression levels of ROCK1, ROCK2, Notch1, PTX3, collagen Type I, CCL2, CCR2, and TGF-β1 in RAW 264.7 mouse macrophages (Figure 6). We found that the expression of ROCK1, ROCK2, Notch1, and collagen Type I was similar across Rezurock alone, fingolimod alone, and the Rezurock/fingolimod combination treatments. However, for PTX3, CCL2, CCR2, and TGF-β1, the combination treatment had a much stronger inhibitory effect compared to either drug alone (Figure 6). We also analyzed the protein expression of ROCK1, ROCK2, and Notch1 following Rezurock, fingolimod, and combination treatment in HMDMs (Figure 7). All three treatments reduced the levels of these proteins. However, the Rezurock/fingolimod combination was significantly more effective at reducing Notch1 and ROCK2 expression in HMDMs than either individual treatment.

In addition, we performed GO enrichment analysis of *Stat3*/*Stat5*-related pathways among DEGs in macrophages treated with Rezurock, fingolimod, or their combination (Figure 8; Tables S13-S15). Macrophages treated with either drug, or the combination, exhibited differential modulation of immune-related pathways. GO enrichment analysis revealed significant changes in cytokine signaling and *Stat3*/*Stat5*-associated processes, with the strongest enrichment observed following combination treatment (Figure 8A-C). Heatmap analysis showed more pronounced transcriptional changes following Rezurock treatment compared to fingolimod alone, while the combination treatment induced the most extensive modulation, suggesting enhanced macrophage reprogramming (Figure 8D-F).

4. Discussion

Chronic rejection of transplanted organs remains incurable, and long-term organ survival rates continue to be unsatisfactory.[3,4] Existing therapies primarily focus on inhibiting T-cell proliferation and activation to prevent graft rejection.[3,4] However, we and others hypothesize that targeting macrophages could offer additional therapeutic benefits, as they are well-established contributors to

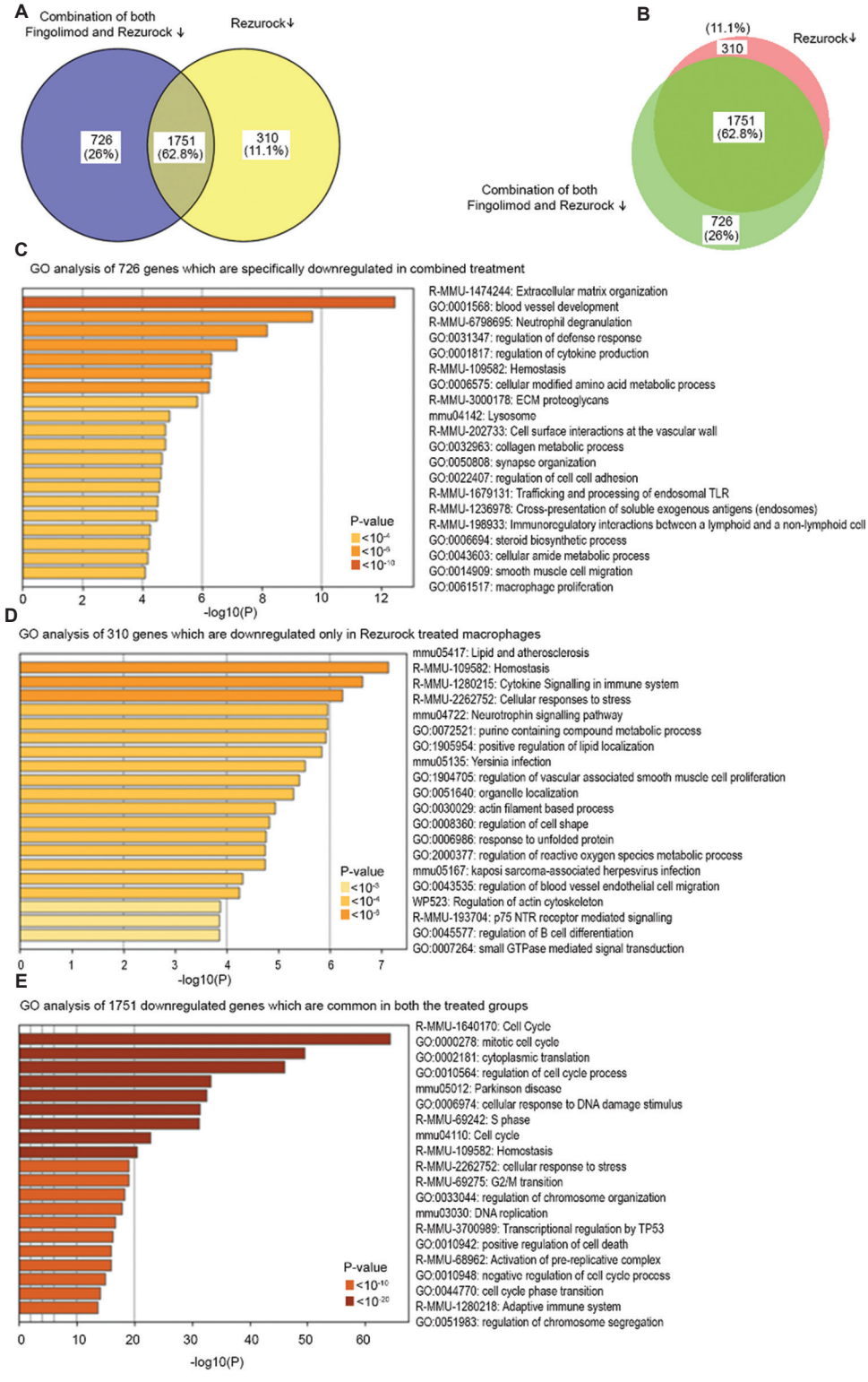

Figure 3. Comparison of downregulated genes between Rezurock/fingolimod combination treatment and Rezurock-only treatment in mouse macrophages. (A and B) Venn diagrams showing the overlap of downregulated genes in Rezurock/fingolimod combination treatment and Rezurock-only treatment. (C) GO analysis of downregulated genes specific to the Rezurock/fingolimod combination treatment. (D) GO analysis of downregulated genes specific to the Rezurock-only treatment. (E) GO analysis of downregulated genes common to both Rezurock/fingolimod combination and Rezurock-only treatments. Abbreviations: GO: Gene ontology; ECM: Extracellular matrix; NTR: Non-catalytic tyrosine-phosphorylated receptor; TP53: Tumor protein 53.

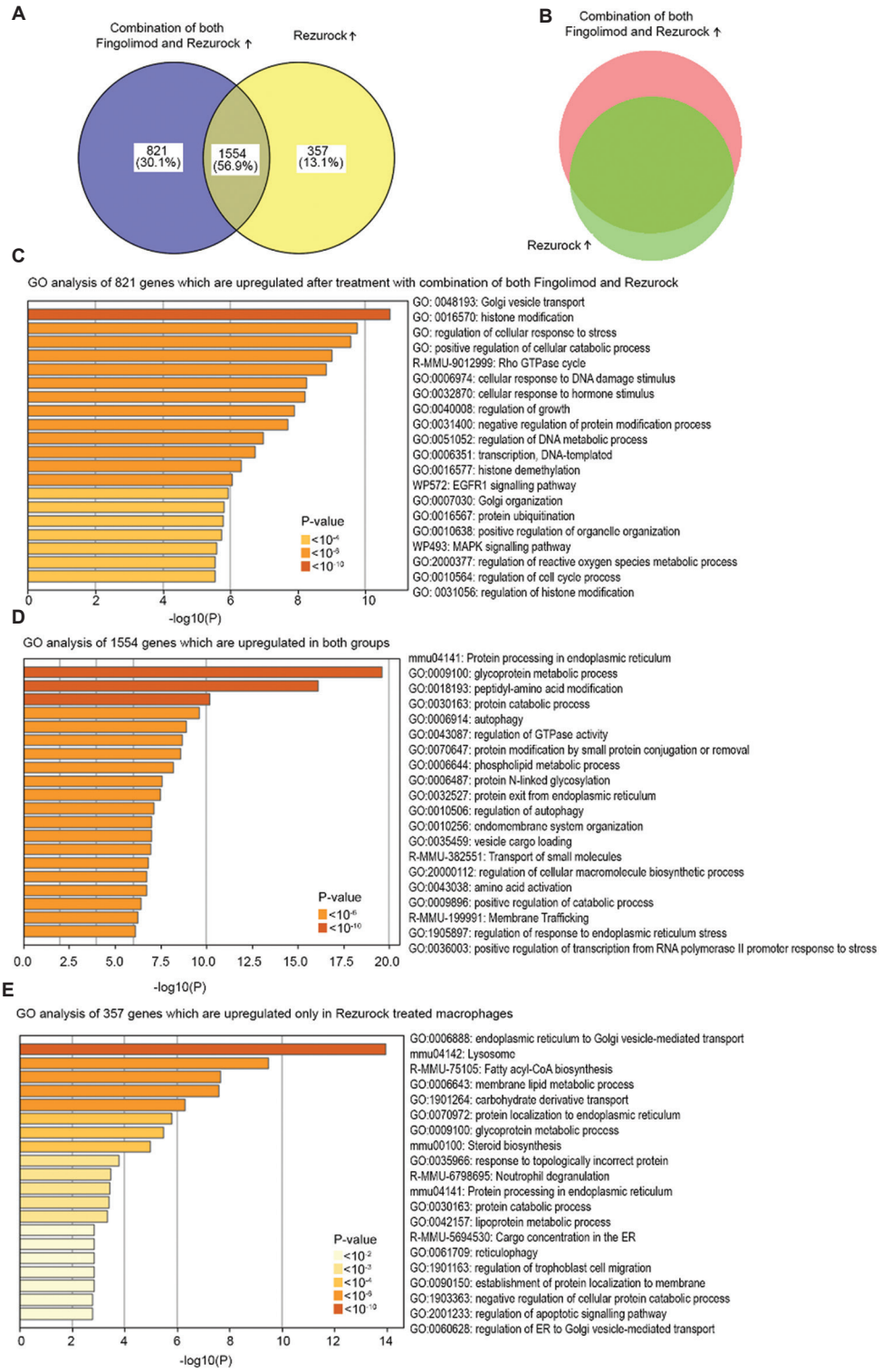

Figure 4. Comparison of upregulated genes between Rezurock/fingolimod combination treatment and Rezurock-only treatment in mouse macrophages. (A and B) Venn diagrams comparing upregulated genes between the Rezurock/fingolimod combination treatment and Rezurock-only treatment. (C) GO analysis of upregulated genes specific to the Rezurock/fingolimod combination treatment. (D) GO analysis of upregulated genes common to both the Rezurock/fingolimod combination and Rezurock-only treatments. (E) GO analysis of upregulated genes specific to the Rezurock-only treatment.
Abbreviations: GO: Gene ontology; CoA: Coenzyme A; ER: Endoplasmic reticulum; MAPK: Mitogen-activated protein kinase; Rho: Ras homolog family member.

Table 1. Fibrotic pathway-related genes downregulated by Rezurock treatment, either alone or in combination with fingolimod, in mouse macrophages

Gene name	Role in fibrosis	Fold change in fingolimod-treated macrophages	Fold change in Rezurock-treated macrophages[a]	Fold change in Rezurock/ fingolimod combined treatment in macrophages	Protein expression level (relative to control sample=1)		
					Fingolimod treatment	Rezurock treatment	Rezurock/ fingolimod combined treatment
Cxcl13	Prognostic biomarker of idiopathic pulmonary fibrosis	-	−2.23	−2.58	-	-	-
Cxcl10	A profibrotic factor	-	−2.02	−2.14	-	-	-
Ptx3	Associated with fibrotic lesions and collagen deposition	-	−1.92	−2.68	0.51	0.15	0.08
Wnt4	Mainly associated with renal fibrosis	-	−1.52	−2.72	-	-	-
Tgfb3	Regulates cytokine-stimulating fibrosis	-	−1.18	−1.62	-	-	-
Tcf19	Associated with pulmonary fibrosis	-	−1.07	−1.97	-	-	-
Ccr2	Associated with hepatic and pulmonary fibrosis	-	−1.04	−1.19	0.36	0.19	0.17
Notch1	Enhances protein expression in pulmonary fibrosis	−1.03	-	-	0.54	0.38	0.29
Cxcl12	CXCR4 drives tissue fibrosis through binding its specific ligand of CXCL12	-	−0.63	−1.94	-	-	-
Cxcl14	Recruits fibroblasts to the sites of fibrosis	-	−0.61	−0.84	-	-	-
Mapkapk2	Plays an essential role in the cell migration of neutrophil, macrophage, leading to fibrosis	-	−0.50	−0.35	-	-	-
Pak1	A profibrotic factor	-	−0.42	−0.52	-	-	-
Smad3	Promotes renal fibrosis by binding to the promoter region of collagens to trigger their production	-	−0.40	−0.37	-	-	-
Ccl2	Associated with monocyte/macrophage inflammatory response, angiogenesis, collagen synthesis, myofibroblast differentiation, and fibroblast recruitment	-	−0.32	−0.48	0.96	0.29	0.17
Pdgfb	Plays a key role in the expansion of myofibroblasts by stimulating their proliferation, migration, and survival	-	−0.31	-	-	-	-
Pdgfbra	Associated with connective tissue growth, leading to a progressive fibrosis phenotype	-	-	−1.49	-	-	-
Timp3	Localized to fibroblastic foci, extracellular matrix, and important mediator of lung fibrogenesis	-	−0.72	-	-	-	-
Rock1	Associated with pulmonary fibrosis	-	-	-	0.49	0.42	0.47
Rock2	Associated with pulmonary fibrosis	-	-	-	0.53	0.35	0.4
Col1	Activated in fibrosis	-	-	-	0.38	0.44	0.41
Tgfb1	Activated and upregulated in fibrosis	-	-	-	0.67	0.42	0.25

Note: [a]Some information presented in Table 1 was previously published.[10]
Abbreviations: CXCL12: C-X-C motif chemokine ligand 12; CXCR4: C-X-C chemokine receptor Type 4.

vessel occlusion and fibrosis progression after organ transplantation.[5,6] In response to chemoattractants released by damaged or inflamed allograft tissues, macrophages infiltrate the transplanted organ and secrete various profibrotic factors, which promote fibroblast activation and differentiation into myofibroblasts. This interaction contributes to extracellular matrix accumulation, potentiating fibrosis and eventually leading to graft dysfunction and chronic rejection over time.[5,6] In our previous studies, pharmacological inhibition of the RhoA/ROCK pathway effectively abrogated chronic rejection after heart transplantation by preventing macrophage

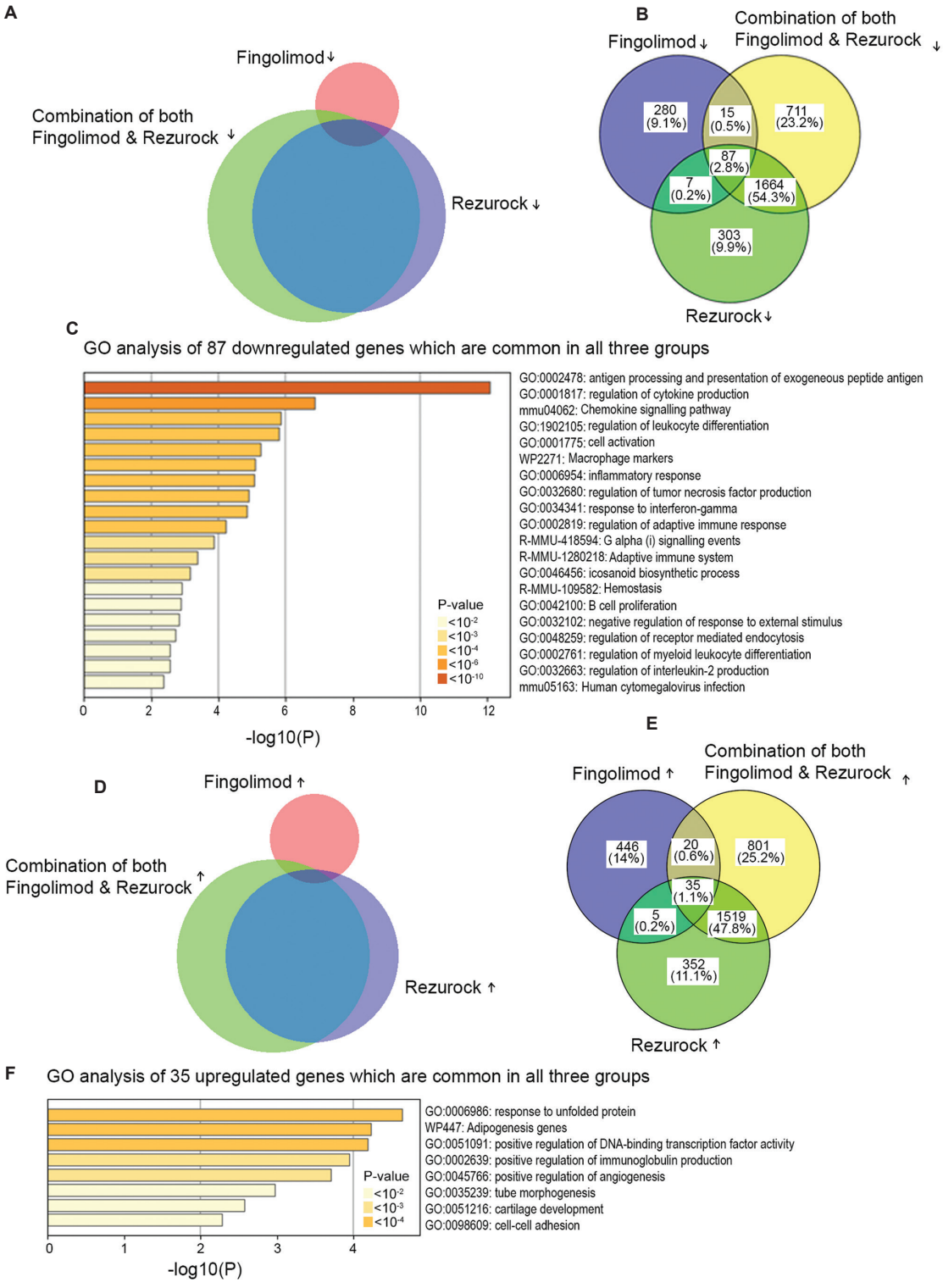

Figure 5. Comparison of differentially expressed genes in Rezurock/fingolimod combination treatment and Rezurock- or fingolimod-only treatments in mouse macrophages. (A and B) Venn diagrams comparing downregulated genes among Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination treatments. (C) GO analysis of downregulated genes common to all three treatment groups. (D and E) Venn diagrams comparing upregulated genes among Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination treatments. (F) GO analysis of upregulated genes common to all three treatment groups.
Abbreviation: GO: Gene ontology.

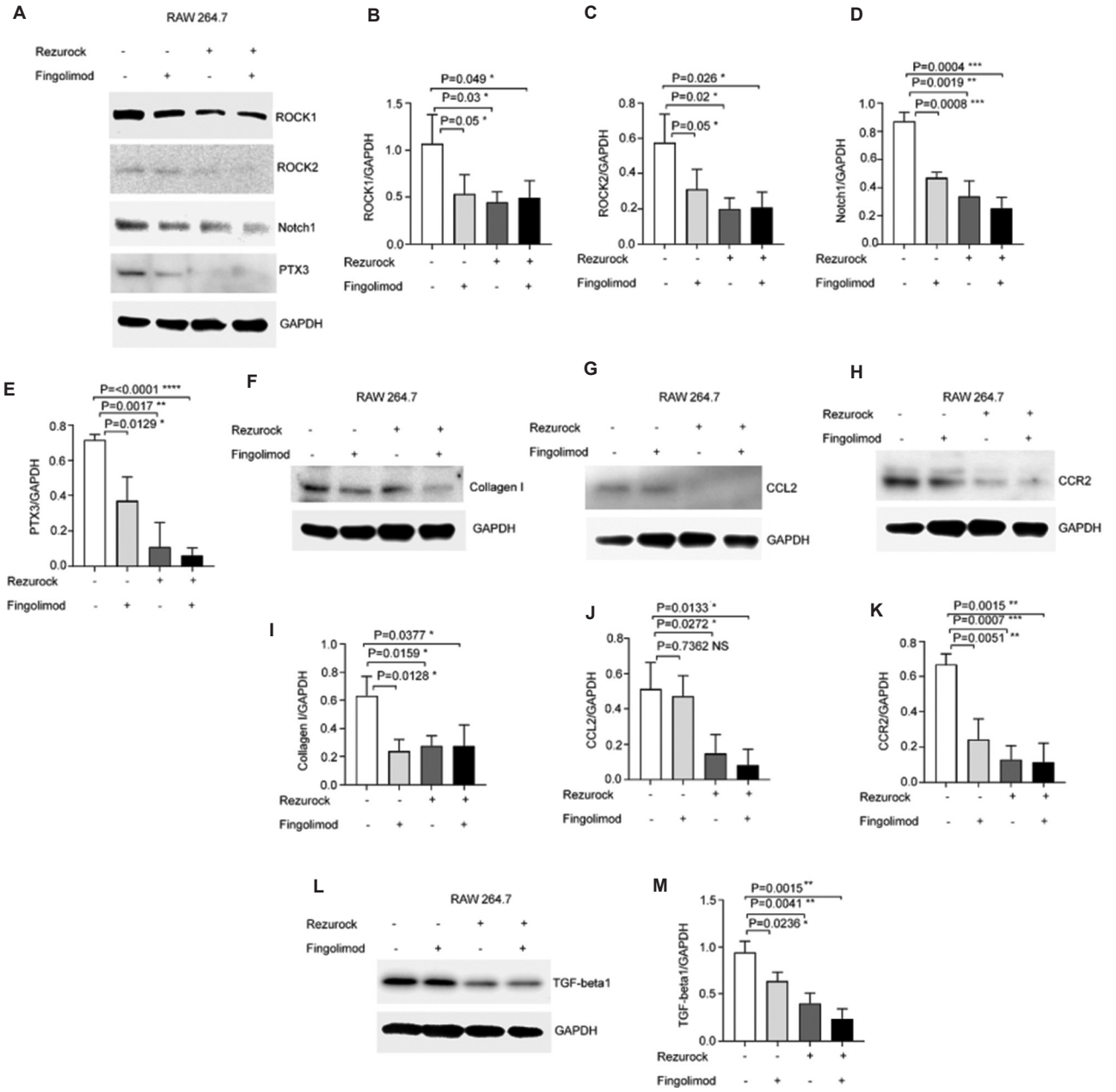

Figure 6. Effect of Rezurock and fingolimod treatment alone or in combination on fibrosis pathway-related protein expression in RAW 264.7 cells. (A, F-H, L) Western blot analysis of ROCK1, ROCK2, Notch1, PTX3, collagen Type I, CCL2, CCR2 and TGF-β1, respectively. GAPDH was used as a loading control. (B-E, I-K) Graphical representations of three independent western blot experiments corresponding to each protein.
Notes: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.
Abbreviations: CCL2: Chemokine (C-C motif) ligand 2; CCR2: C-C motif chemokine receptor 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PTX3: Pentraxin 3; ROCK: Rho-associated coiled-coil kinase; TGF-β1: Transforming growth factor beta 1.

infiltration into the allografts and reducing collagen deposition.[11,12] In line with our findings, other studies have shown that pharmacological inhibition of ROCKs attenuates bleomycin- and radiation-induced pulmonary fibrosis by regulating macrophage polarization.[13]

Our current research aimed to investigate changes in gene expression patterns in macrophages following combined treatment with the RhoA/ROCK inhibitors Rezurock and fingolimod. We recently demonstrated that fingolimod, when administered alongside an early T-cell

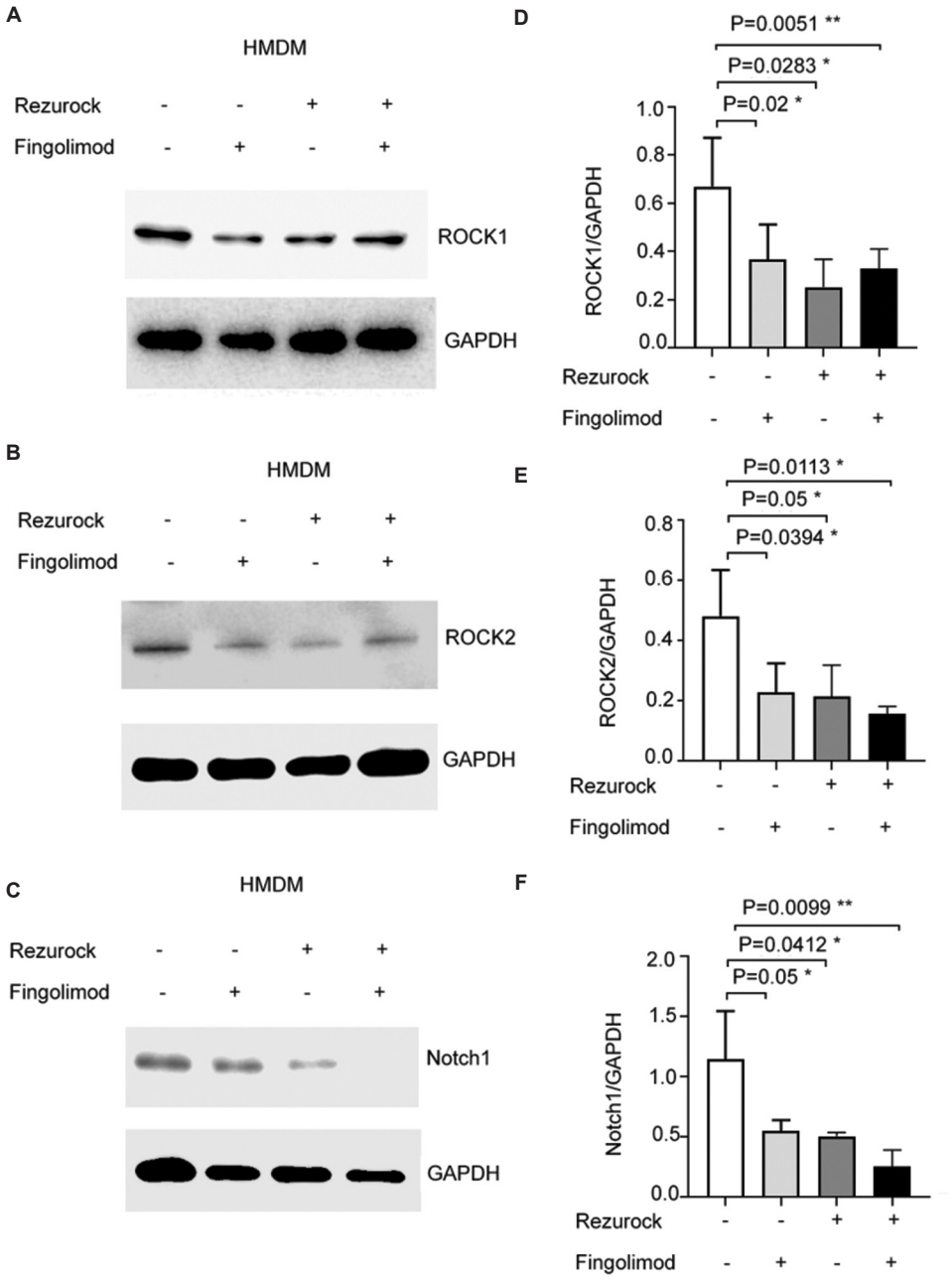

Figure 7. Effect of fingolimod and Rezurock treatment alone or in combination on fibrosis pathway-related protein expression in HMDM. (A-C) Western blots showing the expression of ROCK1, ROCK2, and Notch1, respectively. GAPDH was used as a loading control. (D-F) Graphical representations of three independent Western blot experiments corresponding to each protein.
Notes: *p≤0.05; **p≤0.01; ***p≤0.001.

response inhibitor, prevented macrophage infiltration into allografts, reducing vessel occlusion and fibrosis.[8] In addition, peritoneal macrophages treated with fingolimod exhibited downregulation of pathways involved in cell–cell adhesion and cellular defense mechanisms.[10,14] Rezurock, a selective ROCK2 inhibitor, was superior in inhibiting allograft fibrosis and suppressed pathways related to cell cycle progression, DNA replication, adaptive immune responses, and organelle assembly. Both drugs also shared commonly downregulated pathways associated with cytokine production and chemokine signaling.[10,12]

In our present and previous studies,[10] GO analysis revealed that Rezurock and fingolimod alone downregulated cell cycle- and immune-related pathways in macrophages.

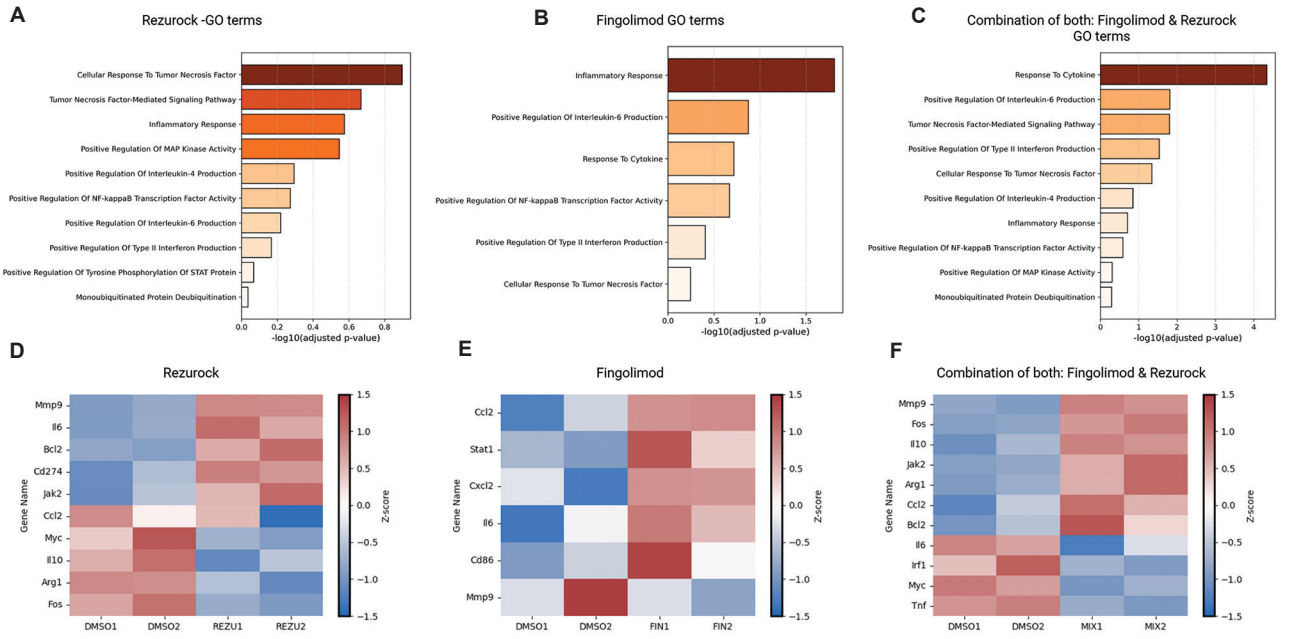

Figure 8. Stat3/Stat5-driven immune modulation by Rezurock, fingolimod, and their combination in macrophages. Gene Ontology enrichment analysis of Stat3/Stat5-related pathways among differentially expressed genes in macrophages treated with Rezurock (A), fingolimod (B), or their combination (C). Heatmaps showing the expression of selected Stat3/Stat5 downstream genes and inflammatory markers in macrophages treated with Rezurock (D), fingolimod (E), and the combined treatment (F).
Abbreviation: Stat: Signal transducer and activator of transcription.

Notably, Rezurock also downregulated actin assembly pathways, suggesting that it affects macrophage migratory properties, which are crucial for allograft infiltration.[10] These findings are supported by another research showing that ROCK2 downregulation reduces macrophage motility.[15] In addition, Rezurock was shown to inhibit TNF secretion and macrophage migration, thereby impeding liver fibrosis in mice.[16] The combination of Rezurock and fingolimod further downregulated genes related to cell cycle progression. Transcriptome analyses of patients with fibrotic lungs have shown that the most upregulated pathways in alveolar macrophages are associated with the mitotic cell cycle and migration.[17,18] Thus, by inhibiting macrophage proliferation, the combined treatment may contribute to reduced fibrosis. We also observed that the combined treatment upregulated signaling pathways broadly related to cellular homeostasis and protein quality control, such as protein processing in the ER and membrane trafficking. In addition to the significant downregulation of cell cycle-related genes, the combination treatment also downregulated profibrotic pathways such as extracellular matrix organization and collagen metabolic processes, as well as immune-related pathways involved in defense responses and cytokine production.

We validated our transcriptomic findings through western blot analysis. As expected, we observed downregulation of ROCK1 and ROCK2 after both single and combined treatments in RAW 264.7 cells and human monocyte-derived macrophages. In addition, we found reduced Notch1 expression following Rezurock or fingolimod treatment, with the combined Rezurock/fingolimod treatment further enhancing this effect in both cell types. The downregulation of Notch1 attenuates hepatic fibrosis by preventing M2 macrophage polarization, thus reducing the profibrotic activity of these cells.[19] Consequently, the combined treatment may slow fibrosis progression. We also observed significant downregulation of inflammation-related proteins, particularly PTX3, CCL2, and its receptor CCR2, after individual treatments. However, CCL2 expression was not significantly affected by fingolimod alone. The combined treatment further enhanced the downregulation of these proteins in RAW 264.7 cells. PTX3, primarily produced by macrophages in atherosclerotic lesions, is associated with chronic rejection characteristics such as inflammation, endothelial dysfunction, and vascular remodeling.[20,21] Elevated PTX3 expression has also been detected in bleomycin-induced fibrotic lungs, highlighting its involvement in fibrosis progression.[21]

CCL2 acts as a chemoattractant for monocytes and macrophages, recruiting them from the bone marrow to sites of inflammation. CCR2, predominantly expressed in leukocytes, monocytes, and macrophages, serves as

the primary receptor for CCL2.[22-24] Elevated CCL2 levels have been observed in lung biopsies from patients with idiopathic pulmonary fibrosis, while CCR2 has been implicated in promoting hepatic fibrosis in mice.[25,26] Our data suggest that the combined treatment effectively blocks the CCL2/CCR2 axis, preventing macrophage infiltration into the transplanted organ by reducing both the recruitment signals from macrophages within the allograft and the responsiveness of bone marrow-derived macrophages to these signals.

Finally, we found that both drugs, when administered individually, significantly reduced the expression of key profibrotic proteins, including collagen I and TGF-β, in RAW 264.7 macrophages. While the combined treatment downregulated collagen I to a similar extent as the individual treatments, it further enhanced the downregulation of TGF-β. Extensive collagen deposition is a well-established marker of fibrotic tissues,[27,28] whereas TGF-β secreted by macrophages promotes the fibroblast-to-myofibroblast transition.[29,30]

Current immunosuppressive regimens in solid organ transplantation are largely similar, with only minor adjustments based on organ-specific immune tolerance and risk of rejection.[31-34] The cornerstone therapy typically includes a calcineurin inhibitor (CNI), an antiproliferative agent, and corticosteroids, with some variation in induction therapy.[31-34] CNIs block IL-2 transcription by inhibiting calcineurin, thereby disrupting T-cell proliferation.[35] Antimetabolites suppress lymphocyte proliferation by inhibiting purine synthesis and thus DNA replication.[36] Corticosteroids exert broader effects by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cell activation, leading to reduced cytokine production, adhesion molecule expression, and antigen presentation.[37] However, because these regimens do not target the fibrotic activity of macrophages, they are effective for acute rejection but do not reduce or prevent chronic rejection.

It is worth noting that, beyond pharmacological interventions aimed at preventing transplant rejection, non-pharmacologic methods, such as the application of magnetic nanoparticles or magnetic devices, have emerged as promising novel approaches.[38-40] A recent study showed that functionalized magnetic nanoparticles could eliminate donor-specific antibodies (DSAs) from saline, blood, and plasma of healthy donors and sensitized patients. DSAs, such as antibodies directed against donor class I human leukocyte antigens (e.g., HLA-A), remain a major barrier to kidney transplant success.[41,42] Another possible approach is the magnetic manipulation of the macrophage actin cytoskeleton to prevent their infiltration into transplanted organs.[43-46]

5. Conclusion

We showed that combined treatment with FDA-approved ROCK2 inhibitors—Rezurock and fingolimod—reprograms macrophages isolated from mice and humans, shifting them from a highly proliferative, profibrotic state toward a less fibrotic phenotype with reduced activity. Our data also indicate that Rezurock, by being superior in preventing fibrosis and enhancing the effect of another ROCK inhibitor, represents a promising strategy for preventing chronic rejection, as it targets critical profibrotic pathways in macrophages. Furthermore, transcriptomic analysis revealed significant downregulation of cell cycle-related pathways, indicating that this combination therapy could benefit not only post-transplant patients but also individuals with autoimmune and chronic inflammatory disorders, where reducing macrophage proliferation is crucial for disease management.

We must recognize that in the future, pharmacologic intervention will probably not be the only option for alleviating organ rejection. With the advent of new technologies, such as magnetic nanoparticles that remove harmful antibodies or magnetic devices that affect immune cell trajectories, novel approaches may emerge for managing both acute and chronic rejection of transplanted organs.

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Conflict of interest

Malgorzata Kloc is Editor-in-Chief of this journal, but was not involved in any way, directly or indirectly, in the editorial and peer-review process for this paper. All other authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

All experiments were performed according to The Methodist Hospital Research Institute animal care and use standards, as outlined in the *Guide for the Care and Use of Laboratory Animals* (DHHS publication No. [NIH] 85-23 Revised 1985). The Institute also mandates concordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institute of Health *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*. The use of mice was approved by The Houston Methodist Research Institute Institutional Animal Care and Use Committee (IACUC; Protocol number #S00007095).

Consent for publication

Not applicable.

Availability of data

Data from this study are available from the corresponding author on reasonable request.

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