Supplemental material

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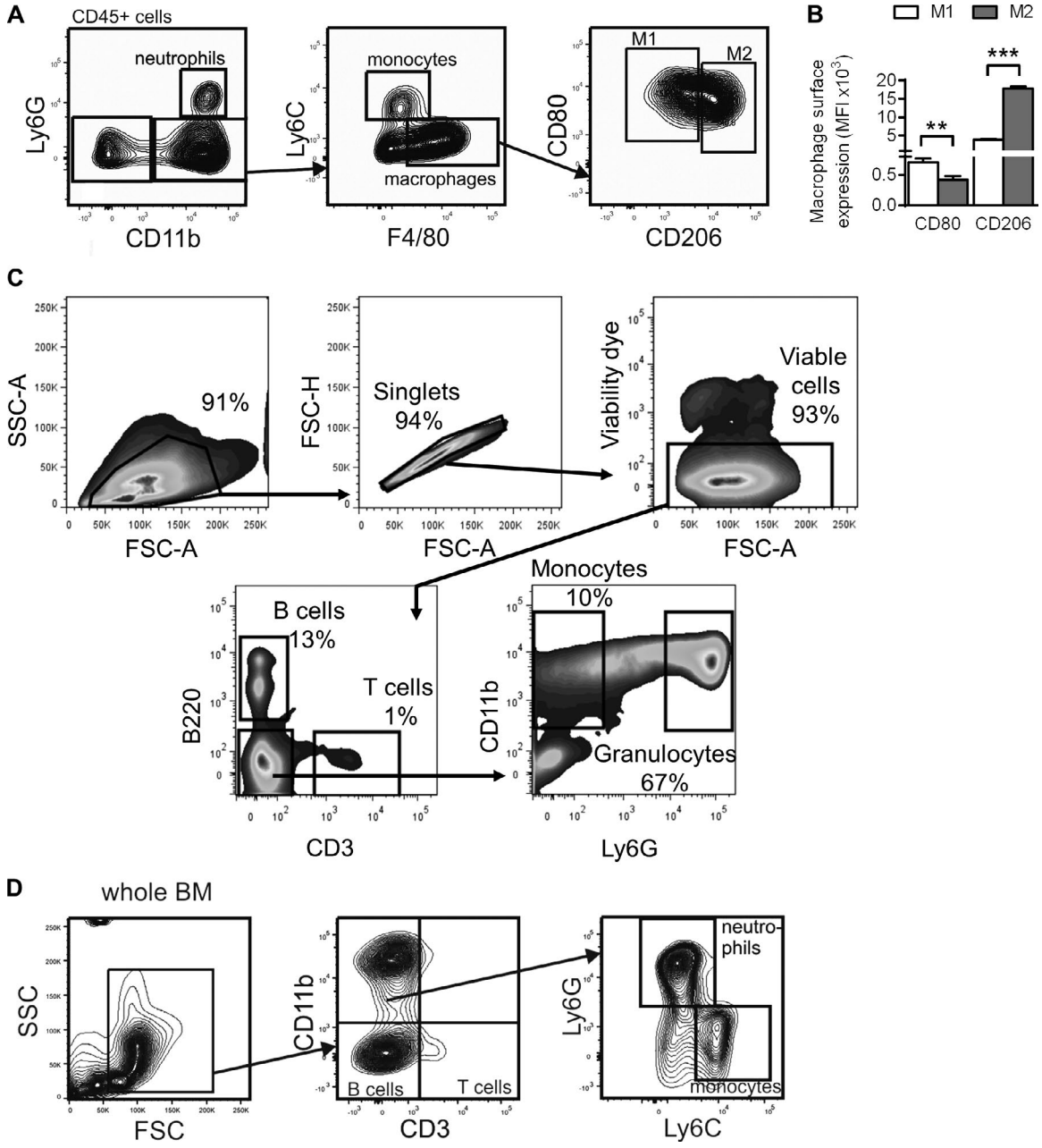


Figure S1. **Gating strategies for flow cytometry analyses. (A)** Infiltrated leukocytes were isolated from the ischemic thigh and calf muscles, 3 and 7 d after hindlimb ischemia induction. Live cells were gated on coexpression of CD45 and CD11b to identify myeloid cells; neutrophils (Ly6G$^+$), monocytes (Ly6G$^-$, Ly6C^{high}), macrophages (Ly6G$^-$, Ly6C^{low}, F4/80$^+$), M1-related macrophages (CD80^{high}, CD206^{low}), and M2-related macrophages (CD80^{low}, CD206^{high}). **(B)** Surface expression level of CD80 and CD206 in macrophages isolated from calf muscles after 3 d of hindlimb ischemia induction. n = 6 per group; error bars, SEM; **, P < 0.01; ***, P < 0.001 (Student's t test). **(C)** Gating strategy for flow cytometry–based isolation of BM-derived leukocytes. BM-derived cells were first gated on size and granularity followed by dye exclusion to identify live cells. Monocytes were defined as B220$^-$, CD3$^-$, CD11b$^+$, and Ly6G$^-$ and granulocytes as B220$^-$, CD3$^-$, CD11b$^+$, and Ly6G$^+$ cells. **(D)** Gating strategy for flow cytometry analysis of Transwell migration. BM-derived cells were first gated on size and granularity followed by expression of CD11b and CD3 to identify myeloid cells; neutrophils (CD3$^-$ CD11b$^+$ Ly6G^{high} Ly6C^{low}) and monocytes (CD3$^-$ CD11b$^+$ Ly6G^{low}, Ly6C^{high}).

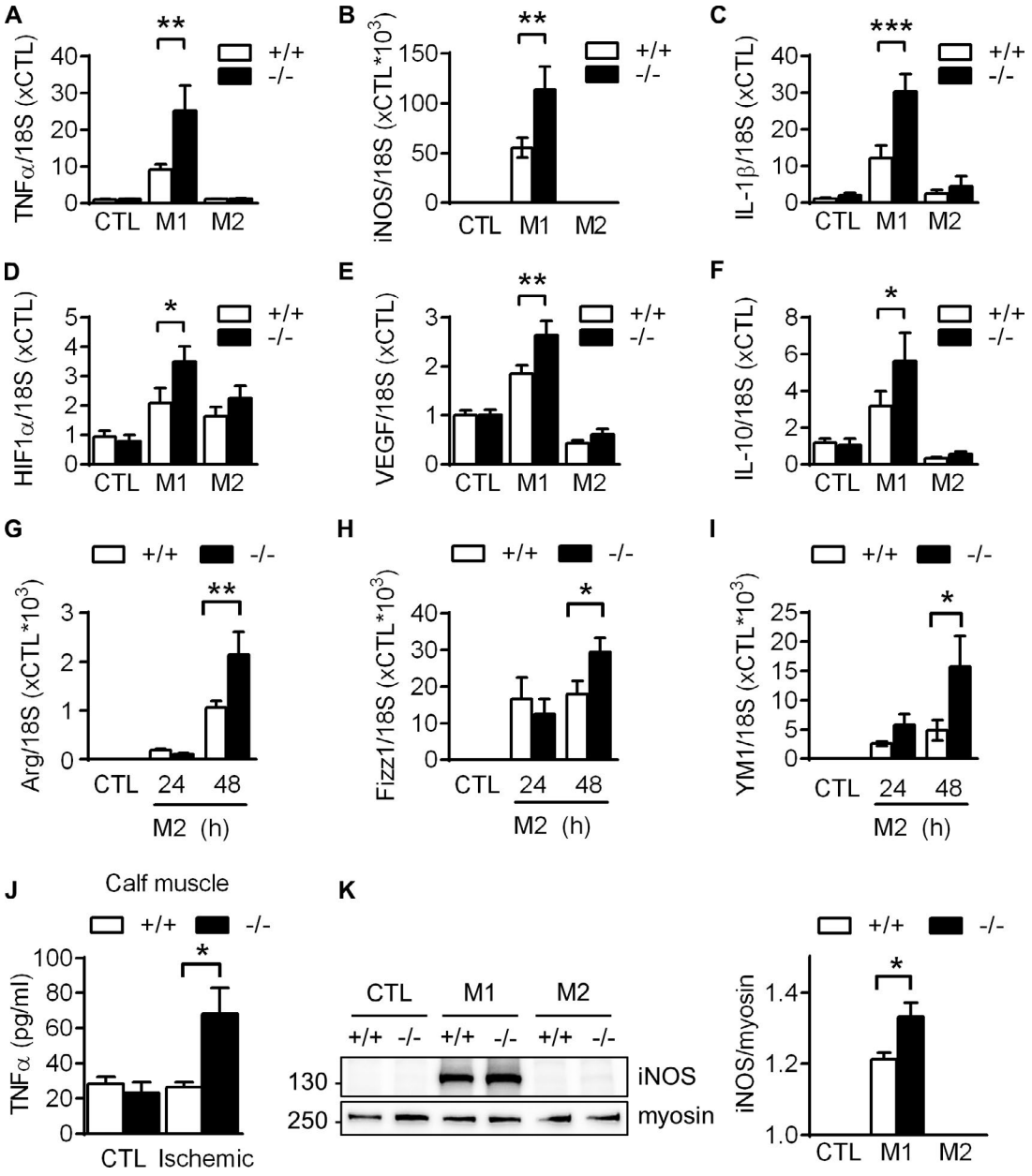


Figure S2. **VASP deletion increases M1 and M2 macrophage polarization in vitro. (A–F)** Expression of TNFα (A), iNOS (B), IL-1β (C), HIF-1α (D), VEGF (E), and IL-10 (F) in macrophages from WT (+/+) and VASP$^{-/-}$ (−/−) mice after in vitro polarization to M1 with LPS (10 ng/ml) and IFNγ (1 ng/ml) for 24 h or to M2 with IL-4 (25 ng/ml) for 24 h; *n* = 12 cell batches per group. **(G–I)** Expression of Arg (G), Fizz1 (H), and YM1 (I) in macrophages from WT (+/+) and VASP$^{-/-}$ mice after in vitro polarization to M2 with IL-4 (25 ng/ml) for 24 and 48 h; *n* = 6 cell batches per group. **(J)** VASP deletion increases TNFα protein levels in the ischemic calf muscle. 3 d after femoral artery excision, TNFα levels in the ischemic and CTL calf muscles were measured by cytometric bead array. *n* = 4 mice per group. (**K**) VASP deletion increases the iNOS protein levels in M1 macrophages in vitro. iNOS protein levels in BM-derived macrophages from WT and VASP$^{-/-}$ mice were analyzed by Western blot. Macrophages were polarized to M1 (10 ng/ml LPS + 1 ng/ml IFNγ) or M2 (25 ng/ml IL-4) or left untreated (CTL) for 24 h. *n* = 5 animals per group. Error bars, SEM; two-way ANOVA/Bonferroni; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

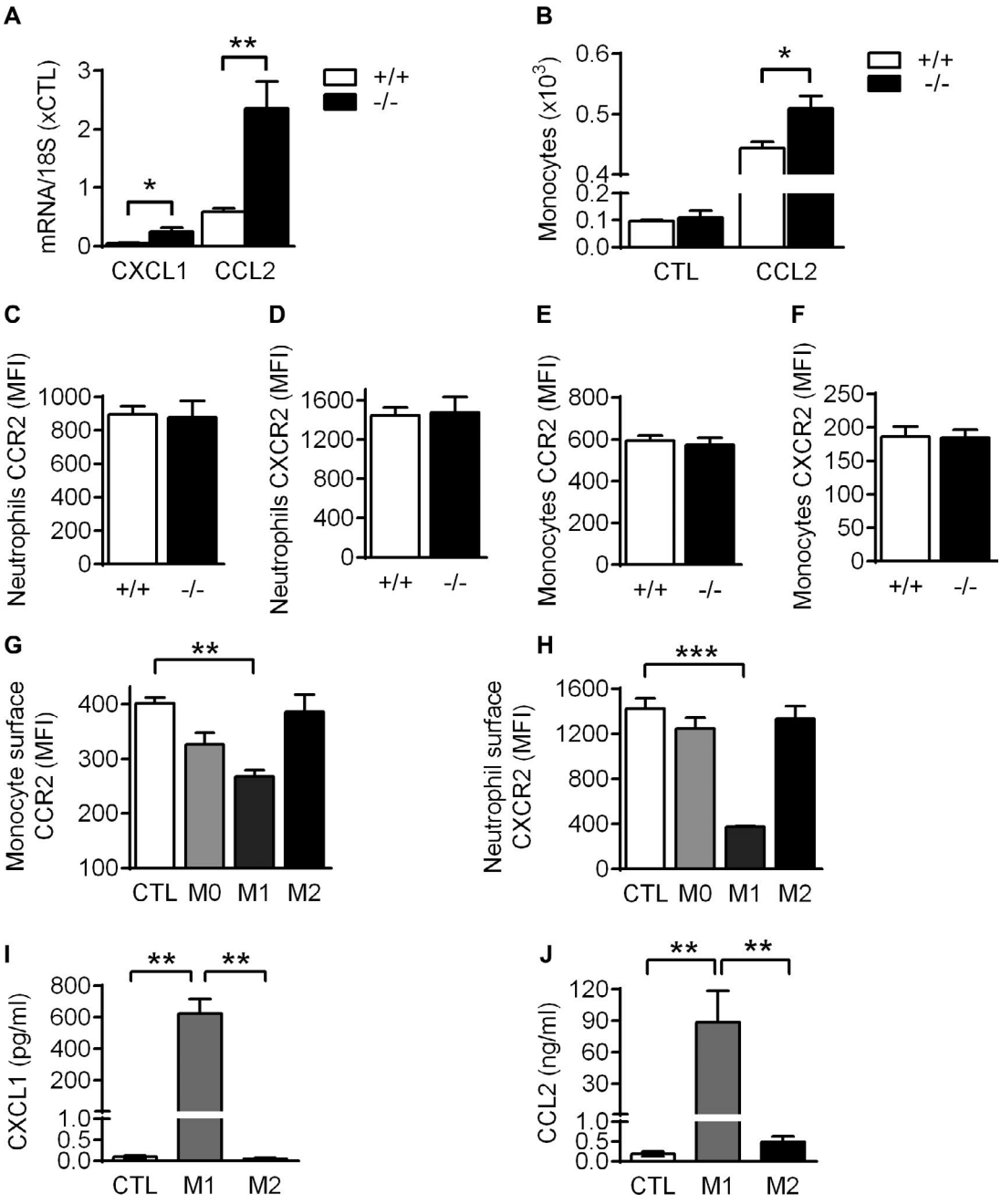


Figure S3. **Expression levels of chemokines and chemokine receptors. (A)** CXCL1 and CCL2 mRNA levels in ischemic calf muscles from WT (+/+) and VASP$^{-/-}$ (−/−) mice, 3 d after ischemia; *n* = 4 mice per group; Student's *t* test. **(B)** VASP deletion promotes chemotaxis in response to CCL2. Migration of WT (+/+) and VASP$^{-/-}$ (−/−) BM–derived monocytes through Transwell filters for 2 h using CCL2-supplemented RPMI medium as chemoattractant (50 ng/ml final concentration); *n* = 4; two-way ANOVA/Bonferroni. **(C–F)** Surface levels of CCR2 and CXCR2 receptors. FACS analysis (mean fluorescence intensity [MFI]) of surface CCR2 and CXCR2 chemokine receptor levels on WT (+/+) and VASP$^{-/-}$ (−/−) neutrophils (C and D) and monocytes (E and F); *n* = 7. **(G and H)** Surface levels of CCR2 and CXCR2 receptors after stimulation. FACS analysis (MFI) of WT monocyte surface levels of CCR2 (G) and WT neutrophil surface levels of CXCR2 (H) after 5 min stimulation with conditioned medium from control, M1 and M2 macrophages. *n* = 4 different cell batches; one-way ANOVA/Tukey. **(I and J)** CXCL1 and CCL2 concentration in conditioned medium from control, M1-, or M2-polarized macrophages. BM-derived macrophages from WT mice were polarized to M1 (10 ng/ml LPS + 1 ng/ml IFNγ) or M2 (25 ng/ml IL-4) or left untreated (control, CTL). 24 h later, the conditioned media were collected and CXCL1 (I) and CCL2 (J) concentrations were analyzed by cytometric bead array. *n* = 6; one-way ANOVA/Tukey. Error bars, SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

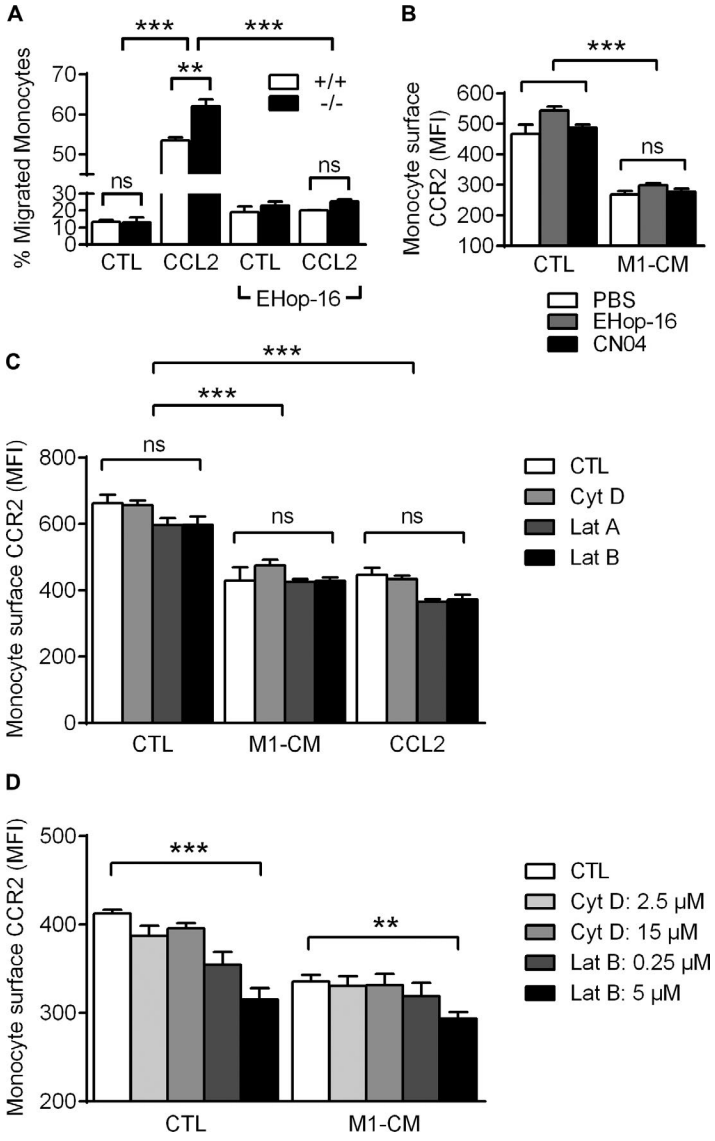


Figure S4. **Impact of Rac1 and actin dynamics on CCR2 internalization. (A and B)** CCR2 receptor internalization is largely independent of Rac1 activity. (A) CCL2-induced (50 ng/ml) migration of WT (+/+) and VASP$^{-/-}$ (−/−) BM-derive monocytes through Transwell filters in the presence or absence of Rac1 inhibitor EHop-16 (10 µM). (B) Surface levels of CCR2 chemokine receptor (mean fluorescence intensity [MFI]) in BM-derived monocytes after stimulation with M1 macrophage conditioned medium (M1-CM) for 5 min without or with 1-h preincubation with the Rac1 inhibitor EHop-16 (10 µM) or the Rac1 activator CN04 (1 µg/ml). *n* = 4 different mice per group. **(C and D)** Impact of actin inhibition on CCR2 surface levels. Surface levels of CCR2 chemokine receptors (MFI) in BM-derived monocytes after stimulation with M1 macrophage conditioned medium (M1-CM) or CCL2 (50 nM) for 5 min, with or without 30-min preincubation with cytochalasin D (Cyt D, 10 µM), latrunculin A (Lat A, 0.5 µM) or latrunculin B (Lat B, 0.5 µM; C); or cytochalasin D (2.5 or 15 µM) or latrunculin B (0.25 or 5 µM; D). *n* = 4 different mice per group. Error bars, SEM; **, P < 0.01; ***, P < 0.001; ns = nonsignificant (two-way ANOVA/Bonferroni).

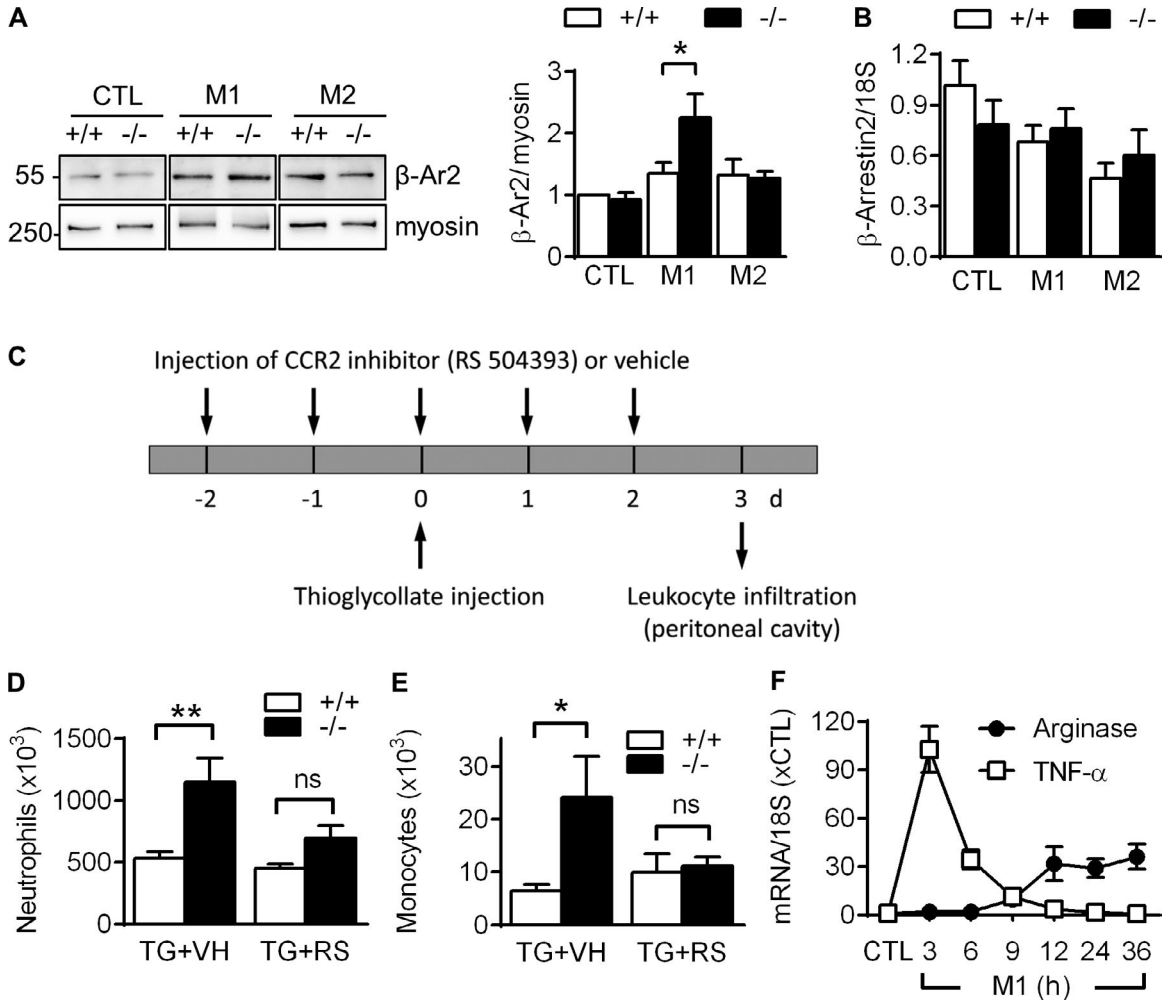
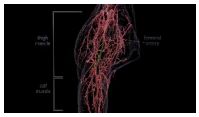
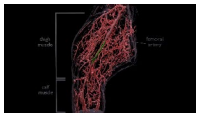


Figure S5. **(A and B) VASP deletion increases β-arrestin 2 protein but not mRNA levels in M1 macrophages.** BM-derived macrophages from WT (+/+) and VASP$^{-/-}$ (−/−) mice were polarized to M1 (10 ng/ml LPS + 1 ng/ml IFNγ) or M2 (25 ng/ml IL-4) or left untreated (CTL), and β-arrestin 2 protein (A) and mRNA (B) levels relative to myosin or 18S rRNA were determined by Western blotting and qPCR, respectively. *n* = 6. **(C–E)** CCR2 inhibition blocks the increased leukocyte infiltration into the peritoneal cavity of VASP$^{-/-}$ mice. **(C)** Time protocol showing the daily injection of the CCR2 inhibitor RS504393 (+RS; 4 mg/kg per day) or vehicle (+VH) from 2 d before until 2 d after thioglycollate (TG) injection to induce peritonitis. Leukocyte infiltration into the peritoneal cavity was analyzed by FACS 3 d after TG injection. **(D and E)** Quantification of leukocyte numbers isolated from peritoneal cavities 3 d after TG injection; neutrophils (D), monocytes (E). *n* = 6 individual animals per group. **(F)** Expression levels of early and late response genes after induction of M1 polarization. mRNA levels of TNF-α (early response of transcription) and arginase (late response of transcription) in WT macrophages were measured by qPCR at the indicated time points after induction of M1 polarization with 10 ng/ml LPS and 1 ng/ml IFNγ; *n* = 9 different cell batches from WT mice. Error bars, SEM; *, P < 0.05; **, P < 0.01; ns = nonsignificant (two-way ANOVA/Bonferroni).



Video 1. **μCT images of a representative WT (+/+) mouse, 21 d after induction of hindlimb ischemia, were used to generate a 3D video of collateral vessel formation in the thigh muscle.** The excised femoral artery is indicated in green; the corkscrew-like collaterals are shown in blue.



Video 2. **μCT images of a representative VASP$^{-/-}$ (−/−) mouse, 21 d after induction of hindlimb ischemia, were used to generate a 3D video of collateral vessel formation in the thigh muscle.** The excised femoral artery is indicated in green; the corkscrew-like collaterals are shown in blue.