Macrophages facilitate peripheral nerve regeneration by organizing regeneration tracks through Plexin-B2

Supplemental Material

1. Supplemental Figures
2. Supplemental Movie Legends
Supplemental Fig. S1. Plexin-B2 deficiency in macrophages does not affect myelin breakdown.

A) Images of the nerve bridge connecting proximal and distal nerve stumps at 5 days after sciatic nerve transection injury.

B, C) IF images and quantifications show similar breakdown and fragmentation of myelin basic protein (MBP) at 5 days after sciatic nerve transection injury in both control and Plexin-B2 cKO cohorts, indicating no changes in Wallerian degeneration by Plexin-B2 cKO in macrophages.
Supplemental Fig. S2. Perturbed organization of regenerating axons and Schwann cells in Plexin-B2 cKO animals after hemi-cut, but not crush sciatic nerve injury.

A) Schematic of hemi-cut sciatic nerve injury model and of the phenotype in Plexin-B2 cKO condition.

B) IF images show that at 7 days after hemi-cut sciatic nerve injury, regenerating axons (SCG10⁺) were aligned in the same orientation as the nerve axis in control mice, but were in disarray in Plexin-B2 cKO mice.

C) Quantification of the orientation of SCG10⁺ axons relative to the nerve axis after hemi-cut injury at 7 dpi. n=3 mice in each cohort. Unpaired two-tailed Student’s t-test. ***P<0.001. ns, not significant. Data represent means ± s.e.m.

D) Schwann cells (S100⁺) were aligned in same orientation as the nerve axis in control mice at 7 dpi after hemi-cut, but were in...
disarray in Plexin-B2 cKO mice.

E) Quantification of the orientation of S100+ cells after hemi-cut injury. n=3 mice in each cohort. Unpaired two-tailed Student’s t-test. ***P<0.001.

F) After crush sciatic nerve injury, regenerating axons (SCG10+) were aligned in a similar fashion in both control and Plexin-B2 cKO animals at 5 dpi. Dashed lines denote injury borders.

G) Quantification of the orientation of SCG10+ axons relative to the nerve axis after crush injury. n=3 mice in each cohort. Unpaired two-tailed Student’s t-test. ns, not significant.
Supplemental Fig. S3. Lack of Plexin-B2 in myeloid cells does not affect macrophage activation.

A) IF images of distal nerve stump after transection sciatic nerve injury show that macrophages (Iba1+) were organized along degenerating nerve tracks left behind by Wallerian degeneration (dotted white lines) in both control and Plexin-B2 cKO cohorts. Enlarged images of boxed areas are shown on right.

B) Quantifications of the orientation of Iba1+ cells relative to the nerve axis. n=3 mice in each cohort. Unpaired two-tailed Student’s t-test. ns, not significant. Data represent means ± s.e.m.

C) IF images of proximal and distal injury sites show similar fraction of macrophages (Iba1+) that express proliferative marker Ki67 and phagocytic marker CD68. Enlarged images of boxed areas are shown on right.

D) Quantifications of the fraction of Iba1+ cells that express Ki67 and CD68 in either proximal or distal injury sites. n=5 mice in each cohort. Unpaired two-tailed Student’s t-test. ns, not significant. Data represent means ± s.e.m.
Supplemental Fig. S4. Plexin-B2 cKO in macrophages causes matrix disarray, but has no effect on angiogenesis after sciatic nerve injury.

A) Second harmonic generation (SHG) microscopy images show disrupted collagen fiber alignment in the proximal injury site in Plexin-B2 cKO mice compared to controls at 5 days after sciatic nerve transection injury. Note that in the distal injury site, collagen fibers appeared similarly aligned along the nerve axis for both genotypes. Results from n=3 mice per group showed similar findings.

B) IF images show similar abundance and organization of highly engorged and tortuous blood vessels in the nerve bridge at 7 days after hemi-cut sciatic nerve injury. Results from n=3 mice per group show similar findings.
Supplemental Fig. S5. Regional differences of macrophage organization in nerve repair after sciatic nerve injury.

A) IF image of the injury zones shows macrophage heterogeneity in different regions in regard to morphology, spatial organization, and alignment relative to regenerating axons. Vertical dashed lines denote injury borders.

B) Enlarged images of boxed areas in (A). In proximal nerve stump, macrophages (Iba1+) displayed elongated morphology and longitudinal alignment along regenerating axons (SCG10+). At the regenerating front in the nerve bridge, growth cones at the tip of regenerating axons were intimately associated with macrophages, both beginning to align in the direction of nerve axis (arrow). In distal nerve stump, macrophages were largely aligned along tracks left behind by Wallerian degeneration (white dashed lines).
Supplemental Fig. S6. Tissue disorganization in Plexin-B2 cKO mutant persists at 10 days after sciatic nerve transection injury.

A) IF images show that at 10 dpi after sciatic nerve transection, misguided regenerating axons (SCG10) were still present in Plexin-B2 cKO mice. Asterisks denote lesion center.

B) IF images for nerve fibers (NF-H⁺), Schwann cells (S100⁺), and macrophages (Iba1⁺) in sciatic nerve at 10 dpi after transection.

C) Quantifications show higher number of misaligned axons (NF-H⁺) and Schwann cells (S100⁺) in Plexin-B2 cKO mice as compared to littermate controls. n=3 mice in each cohort. Unpaired two-tailed Student’s t-test. *** P<0.001, ** P<0.01, ns, not significant.
Supplemental Fig. S7. Plexin-B2 deficiency does not affect macrophage proliferation, phagocytosis marker expression, and wound closure capacity in culture.

A) IF images of cultured bone marrow derived macrophages (BMDMs) show expression of myeloid markers CD11b and Iba1.

B) IF images demonstrate comparable expression of phagocytosis marker CD68 and proliferation marker Ki67 in BMDMs derived from Plexin-B2 cKO mice treated with or without 4-hydroxy-tamoxifen (OHT).

C) Quantifications of the fraction of Iba1⁺ cells that co-express Ki67 or CD68. n=6 independent cultures. Unpaired two-tailed Student’s t-test. Bar graphs represent means ± s.e.m.

D) Scratch wound assays show that Plexin-B2 ablation does not affect wound closure capacity of BMDMs.

E) Quantification of scratch wound assays. Analyses done with 6 images from n=3 independent cultures. Unpaired two-tailed Student’s t-test.
Supplemental Fig. S8. No effect of Plexin-B2 deletion in macrophages on spatial interaction with co-cultured Schwann cells; Plexin-B2 induction in BMDM is not contingent on neuronal contact.

A) Bone marrow-derived macrophages (BMDM) from Plexin-B2 cKO mice with or without 4-hydroxy-tamoxifen (OHT) treatment were co-cultured with IMS32 cells (Schwann cell line). Plexin-B2 deficiency did not appear to affect the spatial interactions
between macrophages (Iba1+) and Schwann cells (S100+).

B) DRG explants from adult mice were first cultured alone for 48 hours, and co-cultured with BMDMs from Plexin-B2 cKO mice (with or without OHT) for another 48 hours. Three randomly selected images were taken at the periphery of DRG explants where outgrowing axons were in contact with BMDMs. Axons are visualized by IF for tubulin β3 (Tuj1) and BMDMs by Iba1 IF. Arrows point at axon/BMDM crossing events. Quantifications show the fraction of BMDMs close to an axon (≤50 μm) that exhibited crossing events. n=12 axons for each condition, from three independent cultures. Unpaired two-tailed Student’s t-test. ***P<0.001.

C) IF images for Tuj1 show that DRG neurons extended similar neurite length when cultured for 72 hours in neuronal media mixed 1:1 with conditioned (cond.) media from control or Plexin-B2-deficient BMDMs. Basal fresh macrophage media without BMDM conditioning served as control.

D) IF images show comparable expression level of Plexin-B2 in BMDMs cultured for 48 hours in normal media, 1:1 mix with conditioned media from DRG neurons, or co-cultured with DRG neurons (Thy1-GFP+). To collect conditioned media, DRG neurons were plated at high density in 4-well chamber slides (10,000 cells / chamber) in 500 µl media per chamber; media supernatant was collected after 48 hours, and added 1:1 to BMDM basal media. As a negative control, note absence of Plexin-B2 IF signals in Plexin-B2-deficient BMDMs. Right, quantifications of Plexin-B2 fluorescence intensity in IBA1+ BMDM. n=3 wells per condition. One-way ANOVA; ***P<0.001.

E) BMDMs were exposed to lipopolysaccharides (LPS; 10 ng/ml) or normal media (Ctrl) for 48 hours. Fluorescence intensity of Plexin-B2 was quantified from 3 wells each, total of 40 cells (+LPS) and 42 cells (Ctrl). Unpaired two-tailed Student’s t-test; ***P<0.001.
Supplemental Fig. S9. Expression of Plexins in injured peripheral nerve.

A) Single cell RNA-seq data from transected sciatic nerve at 3 dpi from Kalinski et al., 2020 was re-analyzed for UMAP embedding and clustering to reveal different cell types. Macrophages and Schwann cell clusters are highlighted.

B) Feature plots of expression levels of Plexin genes in injured nerve. Plexin-B2 is highly expressed in macrophages.
Supplemental Fig. S10. Expression of Semaphorins in injured peripheral nerve.
A) Feature plots for Semaphorin genes. *Sema4b* and *Sema4c* are mainly expressed in Schwann cells, and *Sema4d* in macrophages.
B) IF staining validates upregulation of Sema4D in macrophages in injured sciatic nerve.
Supplemental Fig. S11. Expression of Semaphorins in DRG neurons after nerve injury.

A) Single cell RNA-seq data (Smart-seq2 platform) from Wang et al., 2021 of DRG neurons in control and sciatic nerve injury condition at 14 dpi was re-analyzed for Semaphorin gene expression (FPKM, fragments per kilobase per million reads). Bar graphs indicate expression levels of Semaphorin genes in DRG neurons (n=202 cells for control, n=277 for injury), error bars indicate s.e.m. Sema4b and Sema4f are the main Semaphorins expressed in DRG neurons, both were upregulated after injury. Unpaired Student’s t-test. *P<0.05, **P<0.01, ***P<0.001.

B) Violin plots of Atf3 expression in different subclusters of DRG neurons after sciatic nerve injury (SNI) based on re-analysis of scRNA-seq data (10X Genomics platform) from Wang et al., 2021. Note that Injury-induced types 1, 2, 3 of DRG neurons exhibit robust induction of Atf3 at 2d or 14 d after SNI.

C) Time course analyses show robust induction of Sema4b and Sema4f in each of the three injury-induced subtypes of DRG neurons at the indicated time points after injury as compared to control condition with no injury.
Supplemental Movie Legends

**Supplemental Movies S1&S2. Contact inhibition of locomotion (CIL) between macrophages and extending axons, which requires Plexin-B2. Related to Figure 5E, F.**

Regenerating DRG neurons from Thy-1CreER/GFP reporter mice were plated onto BMDM cultures. In control condition (Movie S1), macrophages displayed a higher probability of contact repulsion when encountering elongating neurites from GFP+ DRG neurons (arrows), whereas in Plexin-B2 cKO condition (Movie S2), mutant macrophages frequently ignored and migrated across neurites (arrows).

Time of movie: 24 hours total, 30 min between each frame.

**Supplemental Movies S3&S4. Parallel alignment of growth cones with wildtype macrophages post-collision, but not PB2 cKO macrophages. Related to Figure 5G, H.**

DRG neurons from Thy-1CreER/GFP reporter mice were plated onto BMDM cultures. When macrophages collided with growth cones at the tip of elongating neurites in control conditions (Movie S3), they tended to change orientation post-collision, in a manner that would lead to parallel alignment of growth cone and macrophage (arrows). In the Plexin-B2 cKO condition (Movie S4), mutant macrophages exhibited a higher probability of ignoring growth cones and continued their migratory trajectory, leading to random crossings of macrophages and growth cones (arrows).

Time of movie: 24 hours total, 30 min between each frame.