PNAS

Supporting Information for

A backpack-based myeloid cell therapy for multiple sclerosis

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Extended Methods.

Dexamethasone Quantification
Dexamethasone concentrations were measured by HPLC-MS, using a similar method as previously reported (1). Briefly, dexamethasone standards and samples were dissolved in acetonitrile. Using an HPLC (Agilent 1290 Infinity II) with a single quadrupole mass analyzer detector (Agilent MSD XT), 5 uL of standard or sample was injected onto a 2.1 x 50 mm C18 column (Agilent Poroshell 120) using an isocratic 60:40 0.1% formic acid:acetonitrile containing 0.1% formic acid. Dexamethasone was detected at 393.3 Da in positive ion mode at a capillary voltage of 4000V. Drying gas was 300°C at 10 L/min, sheath gas was 250°C at 7 L/min, and a nebulizer pressure of 45 psig was used. Dexamethasone eluted at 1.6 min and was linearly detected between 0.25 – 1000 ng/mL.

Atomic Force Microscopy (AFM)
Backpacks (BPs) were adhered to glass slides, mounted on the AFM (JPK Nanowizard, Bruker) and imaged in Qi (single point contact) mode using All-In-One-Al cantilever D with a stiffness of ~40Nm^{-1}. 10µm X 10µm regions were scanned for quantifying BP topography, followed by a 2µm X 2µm scan on BP surface for probing stiffness. Topography and stiffness were recovered using JPK DP data processing software; stiffness was obtained by fitting corrected deflection curves to a Hertz model assuming a pyramid tip.

F(ab') Digestion
Anti-mouse CD45 antibody (Biolegend) was digested with pepsin using the Pierce™ F(ab')2 Preparation Kit (ThermoFisher) following the vendor's instructions to yield F(ab')2 fragments. The F(ab')2 fragments were purified by preparing an AminoLink™ Plus Immobilization Column (ThermoFisher) with Goat anti-Rat IgG Fc Secondary Antibody (ThermoFisher) coupled to the column following the vendor's instructions. The solution of F(ab')2 was incubated with the functionalized column for 30 minutes to allow Fc debris to bind to the column. The F(ab')2 solution was subsequently washed through the column and concentrated using Amicon Ultra-4 30 kDa Centrifugal Filters (MilliporeSigma) to a concentration of 0.5-2mg/mL. The purified CD45 F(ab')2 was reduced with an equal volume of 1.8mM DTT solution for 20 minutes and washed 4 times sequentially with Zeba desalting columns to yield CD45 F(ab') fragments with free thiol groups, as described previously (2).

EAE Induction
EAE was induced in female C57BL/6 mice (9-14 weeks) using the EK-2110 kit (Hooke Laboratories). Emulsion containing myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) and Complete Freund's Adjuvant was injected subcutaneously, 0.1mL/site on the upper and lower back for a total of 0.2mL/mouse. 2 and 24 hours after, a solution of pertussis toxin was prepared (1.1µg/mL) and injected intraperitoneally (0.1mL/dose, 1 dose per mouse).

EAE Scoring Rubric
7 days after EAE induction, mice were weighed and scored daily according to the following established rubric (3–5): 0- no obvious changes in motor function. 0.5- tip of tail is limp. 1.0- entire tail is limp; when picked up by base of tail, the whole tail drapes over finger from the base. 1.5- when picked up by base of tail, whole tail drapes over finger and when the mouse is dropped on a wire rack, at least one hind leg falls through consistently. 2.0- the animal has both a limp tail and weakness of one or both the hind limbs. When picked up by base of tail, the legs are now clasped and held close together. 2.5- Limp tail and dragging of both hind legs and/or complete paralysis of 1 hind limb at the hip; both hind legs still have some movement, but both feet flip during ambulation resulting in the tops of feet dragging. 3.0- Mouse has a limp tail and complete paralysis of both hind legs; animal can still right itself from lateral recumbence. 3.5- Limp tail, complete paralysis of hind legs, and when placed on its side, animal is unable to right itself from lateral recumbence. 4.0- Limp tail, complete hind leg paralysis, and partial front leg paralysis; animal is still alert and feeding when gel is placed nearby. 4.5- Complete hind and partial front leg paralysis, no movement around the cage; mouse is not alert. 5.0- moribund or death.

Single Cell Suspension Processing
For blood samples, the samples were lysed with ACK lysis buffer (3–5-minute incubation with 10-20x blood sample volume at room temperature) and the cells were collected by centrifugation at 300g for 5 minutes.

For brain and spinal cord samples, the organs were minced and incubated with enzyme solution from Multi Tissue Dissociation Kit 1 (Miltenyi) following vendor instructions for 60 minutes at 37°C. The suspension was triturated through 70µM cells strainers with cold PBS and pelleted at 300g for 10 minutes. Myelin and other debris were removed using density gradient separation with debris removal solution (Miltenyi) following manufacturer's instructions to yield the final myelin-free single cell suspension.

Antibodies

Cells were stained with the following antibodies for *in vivo* studies. Some samples were stained anti-CD45-Pacific Blue (BioLegend), anti-Ly6G-PeCY7 (BioLegend), anti-CD3- BUV737 (Invitrogen), anti-CD11b-APC-Fire 750 (BioLegend), anti-Ly6C-BV570 (BioLegend), anti-F4/80-AlexaFluor488 (BioLegend), and anti-CD19-BV750 (BioLegend) antbodies. Other samples were stained with anti-CD45-AlexaFluor700 (BioLegend), anti-CD3-APC/Cy7 (BioLegend), anti-CD4-BV510 (BioLegend), anti-CD8a-PeCy7 (BioLegend), anti-CD25-AF647 (BioLegend), anti-CD69, BV785 (BioLegend), anti-IL-17A-BV605 (BioLegend), anti-Tbet-BV421 (BioLegend), anti-FoxP3-PE-CY5 (BioLegend), anti-RORyT-AlexaFluor488 (R&D Systems), anti-IFNg–BUV737 (Invitrogen) antibodies. Other samples were stained with anti-CD45-PerCP-Cy5.5 (BioLegend), anti-F4/80-BV510 (BioLegend), anti-CD11b-BV750, (BioLegend), anti-CD80-Pacific Blue (BioLegend), anti-MHCII-APC (BioLegend), anti-CD86-BV605 (BioLegend), anti-CD206-PeCy7 (BioLegend), anti-Arg1–eFluor 450 (Invitrogen), anti-iNOS Pe-Cy7 (Invitrogen), anti-IL-10–APC-Cy7(BioLegend), and anti-IFNg-BUV737 (Invitrogen) antibodies.

References

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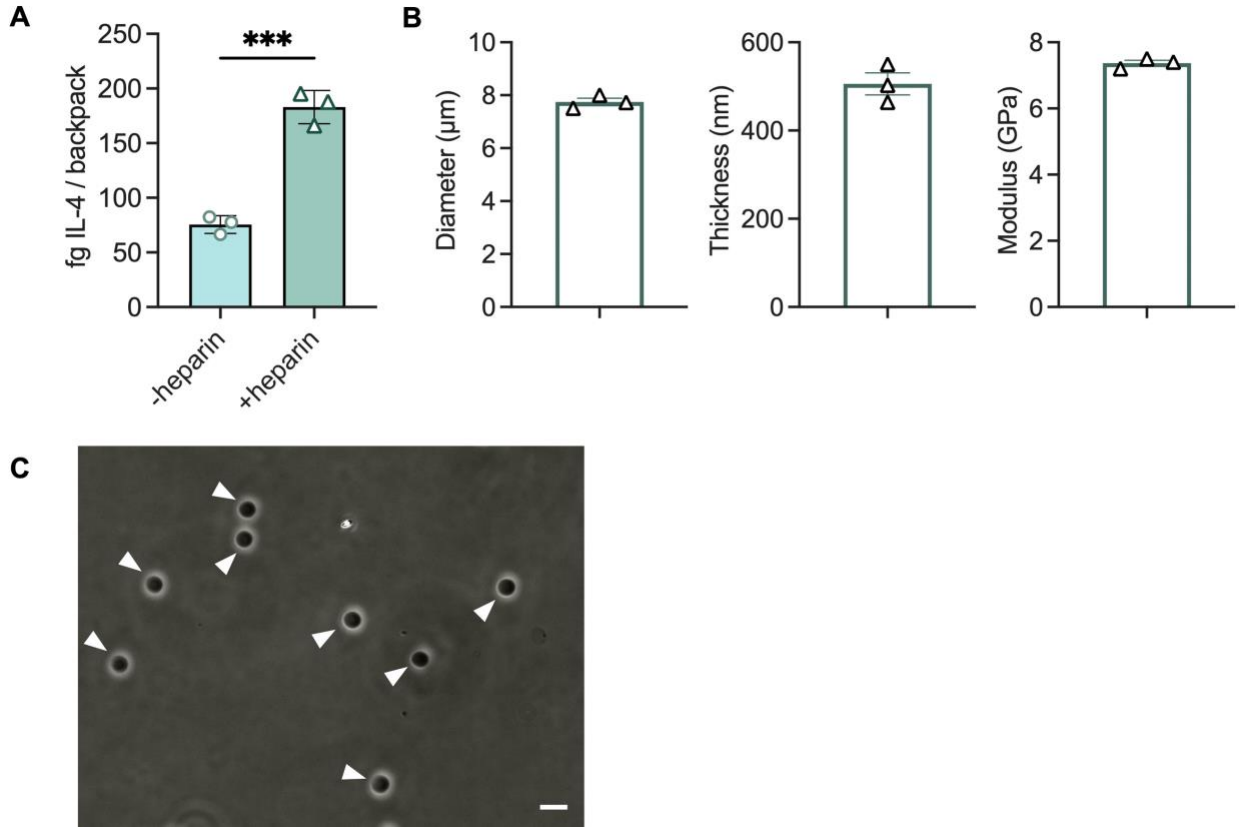

Fig. S1. Material properties of backpacks. (A) Loading of IL-4 in backpacks with (+) and without (-) heparin was quantified by analyzing cumulative IL-4 release from backpacks over 14 days via ELISA; mean ± SD (n=3). (B) Backpacks were characterized via atomic force microscopy (AFM). Backpack diameter; mean ± SD (n=3); backpack thickness; mean ± SD (n=3); Young's modulus; mean ± SD (n=3). (C) Representative brightfield microscope image of resuspended backpacks in aqueous solution after 24 hours. Scale bar represents 10 µm.

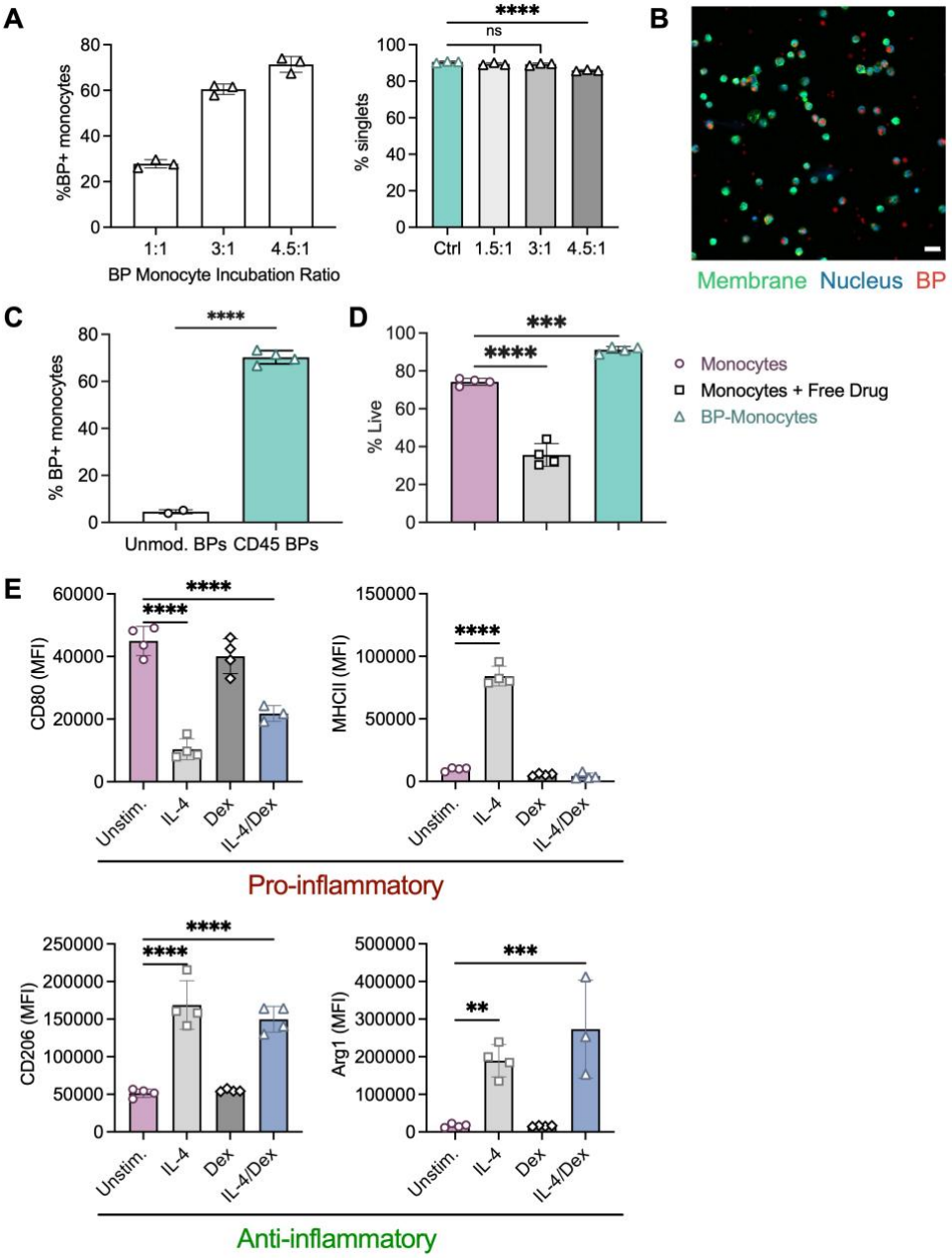

Fig. S2. **Backpack-monocyte design validation**. (A) CD45-functionalized backpacks were adhered to primary bone marrow murine monocytes. Left, percentage of monocytes with >1 backpack as a function of backpack:monocyte incubation ratio (assessed by flow cytometry); mean ± SD (n=3) Right, percent of singlets of all cells for monocytes alone (Ctrl.) or backpack-monocytes at increasing BP:Monocyte incubation ratios; mean ± SD (n=3). (B) Confocal micrograph of monocyte (membrane: green, nucleus: blue) with backpack (red). Scale bar represents 20μm. Data in A analyzed by two-tailed student's t test; **P<0.01. (C) Backpacks (BPs) were adhered to human blood-derived monocytes. Percentage of monocytes with >1 backpack (unmodified BP or CD45-modified BP), as determined by flow cytometry; mean ± SD (n=2-4). (D) Monocytes, monocytes with free IL-4/dexamethasone (20ng/mL IL-4, 1 μg/mL dexamethasone), or backpack-laden monocytes (BP-Mo.) were cultured for 72 hours and analyzed for viability via flow cytometry; mean ± SD (n=4). (E) Monocytes were plated for differentiation into macrophages with unstimulated media or media supplemented with IL-4 (10ng/mL), dexamethasone (Dex, 50ng/mL), or a combination of the two. After 48 hours, cells were harvested and analyzed for expression of pro-inflammatory (MHCII, CD80) and anti-inflammatory (CD206, Arg1) markers by flow cytometry; mean ± SD (n=4). Data in A were analyzed by two-tailed student's t test; ns, not significant, ****P<0.0001. Data in B, C analyzed by one-way ANOVA with Tukey's HSD test. **p<0.01, ***p<0.001, ****p<0.0001.

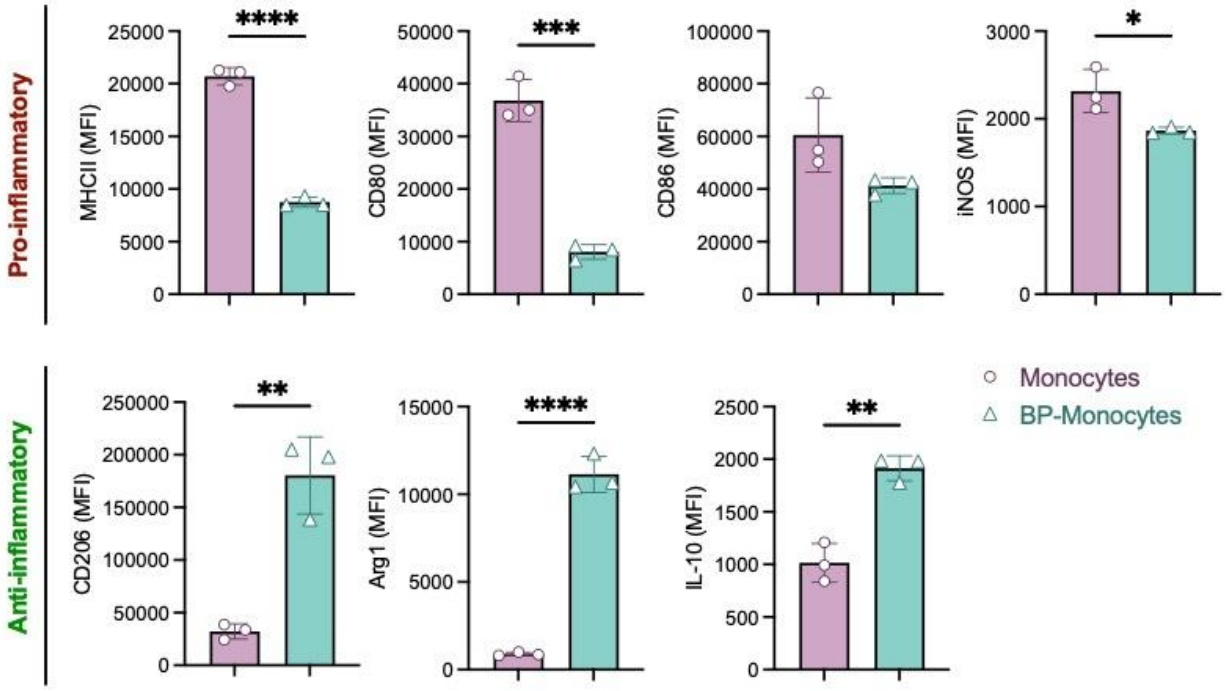

Fig S3. Backpacks polarize monocytes in unstimulated media over 48h. Monocytes or backpack-laden monocytes were cultured and analyzed for expression of pro-inflammatory (MHCII, CD80, CD86, and iNOS) and anti-inflammatory (CD206, Arg1, IL-10) markers via flow cytometry; mean ± SD (n=3). Data were analyzed by two-tailed student's t test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

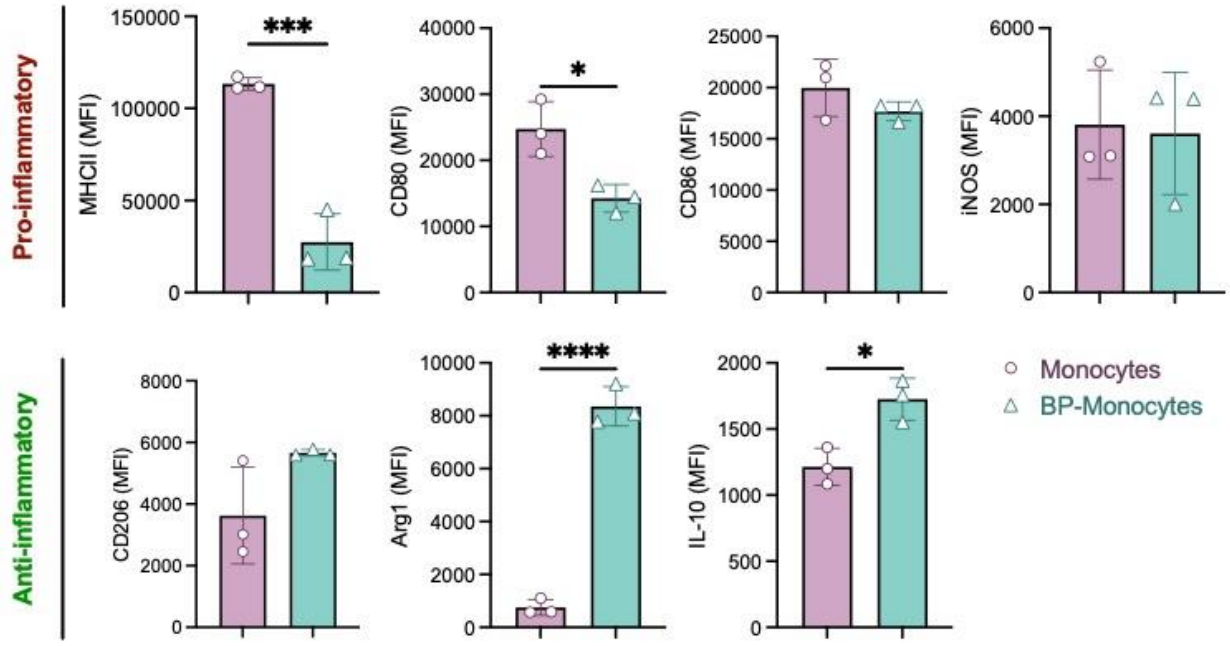

Fig S4. Backpacks polarize monocytes in pro-inflammatory media over 48h. Monocytes or backpack-laden monocytes were cultured in media supplemented with 1 ng/mL of IFNγ and analyzed for expression of pro-inflammatory (MHCII, CD80, CD86, and iNOS) and anti-inflammatory (CD206, Arg1, IL-10) markers; mean ± SD (n=3). Data were analyzed by two-tailed student's t test; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

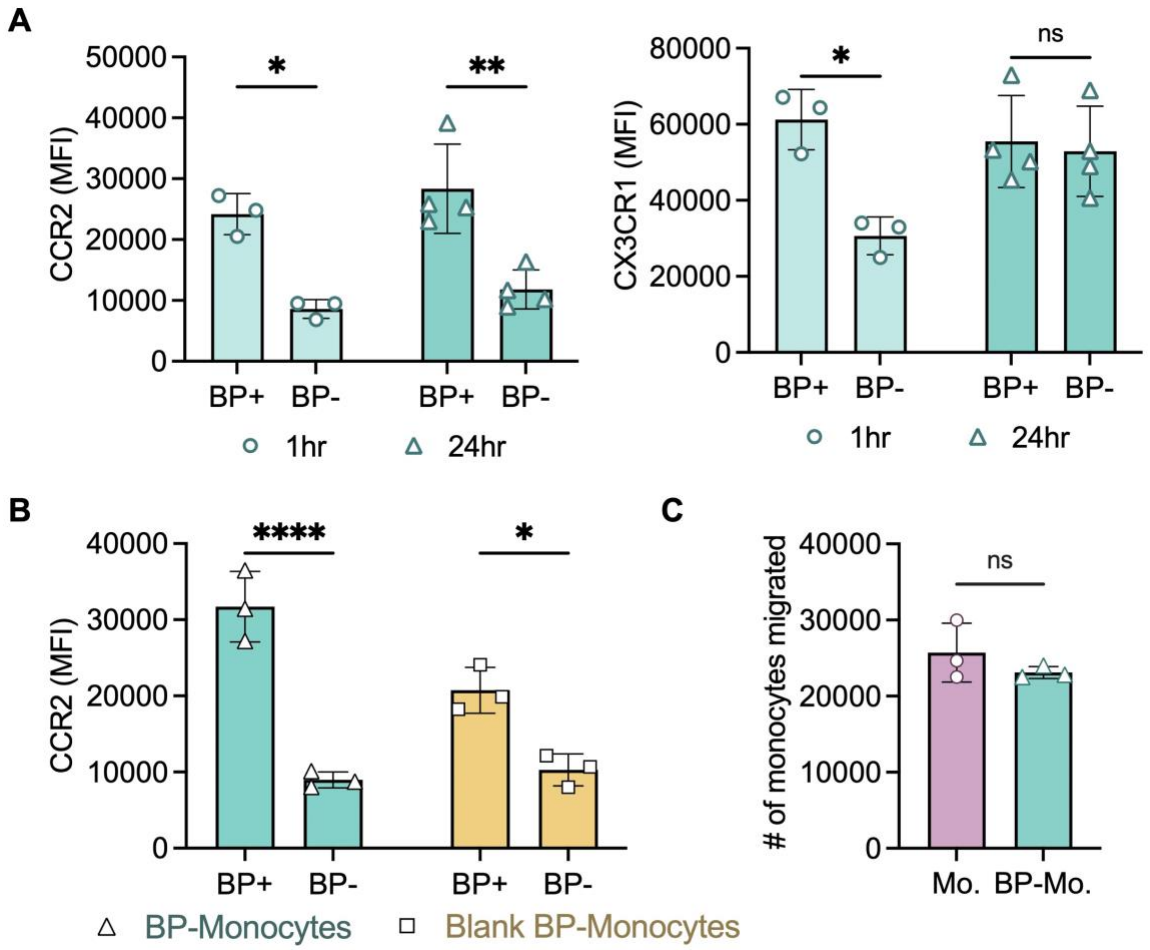

Fig. S5. Characterization of backpack design for promoting monocyte migration. (A) Chemokine receptor expression (CCR2, CX3CR1) of the backpack+ and backpack- subpopulations of backpack monocytes at 1 and 24 hr, quantified by flow cytometry; mean ± SD (n=3-4). (B) Chemokine receptor expression (CCR2) of the backpack+ and backpack- subpopulations of backpack-monocytes (monocytes with drug-loaded backpacks) and monocytes with unloaded backpacks at 24 hours; mean ± SD (n=3). (C) Migration of backpacks adhered to primary human monocytes was assessed using a Transwell assay, with endothelial cells seeded on 5µm inserts, and media containing 10ng/mL CCL2 added to the lower chamber. 200k monocytes or backpack-monocytes were added into the upper chamber. The number of monocytes or backpack-monocytes in the lower chamber after 24 hours was counted; mean ± SD (n=3). Data in A, B, were analyzed by two-tailed student's t test; ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data in C analyzed by two-tailed student's t test; ns, not significant.

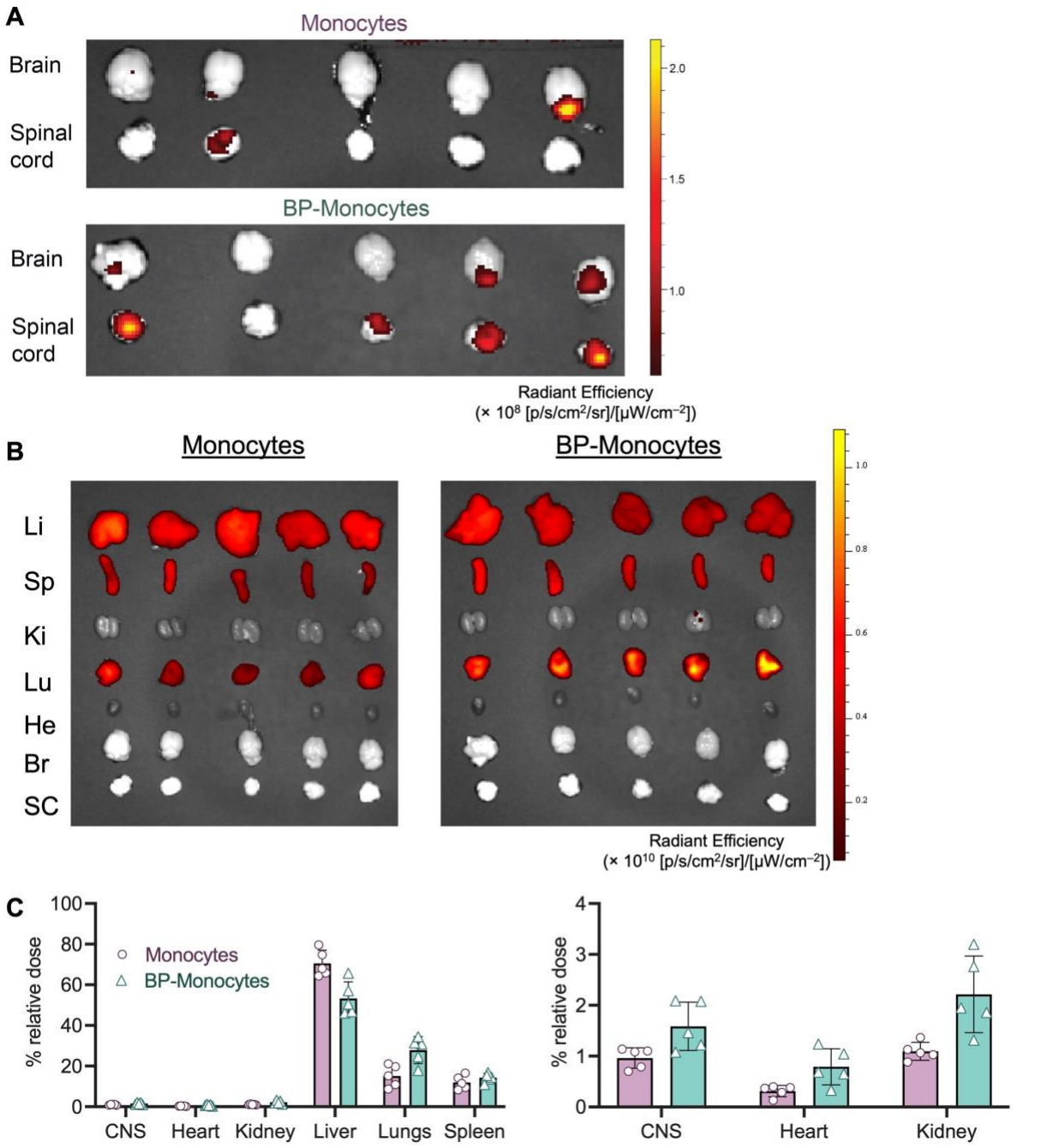

Fig. S6. Biodistribution characterization of monocytes and monocyte backpacks 24hours after administration. EAE was induced in female C57BL/6J mice. Mice were administered 3x10^6 monocytes or backpack-laden monocytes (BP-monocytes) intravenously via tail vein on day 11, at the onset of clinical signs, and sacrificed 24 hours after. Monocytes were labeled with DiR 750 prior to injection. (A) *In vivo* imaging system (IVIS) imaging of fluorescence signal (DiR 750) from adoptively transferred cells in CNS (brain and spinal cord). (B) IVIS imaging of fluorescence signal (DiR 750) from adoptively transferred cells in organs (Liver, Li; Spleen, Sp; Kidney, Ki; Lungs, Lu; Heart, He; Brain, Br; Spinal cord, SC). (C) Fluorescence quantification of relative accumulated dose for monocytes and backpack-monocytes; mean ± SD (n=5).

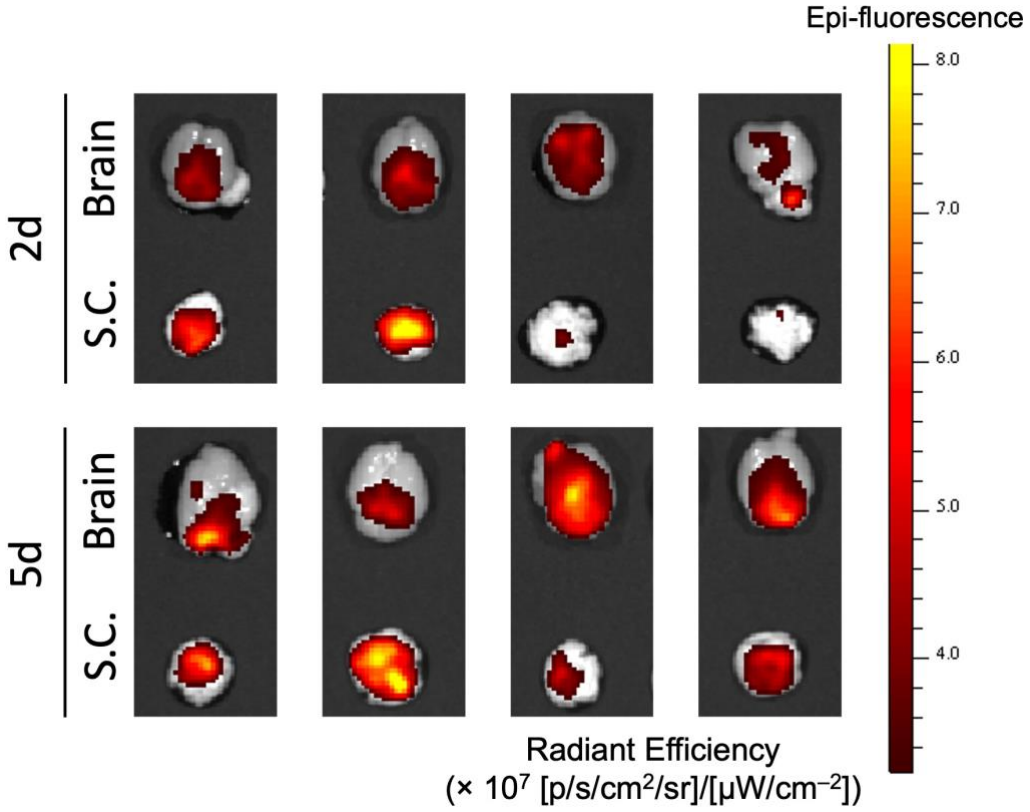

Fig. S7. Backpack-monocytes persist in the CNS of EAE for up to 5 days. EAE was induced in female C57BL/6J mice. Mice were treated with monocytes or backpack-monocytes at the onset of clinical signs on Day 11 (i.v., tail vein), with the adoptively transferred monocytes stained with DiR 750. The mice were sacrificed after 2 days or 5 days, and the CNS was harvested. *In vivo* system imaging (IVIS) of brain and spinal cord displaying DiR 750 signal.

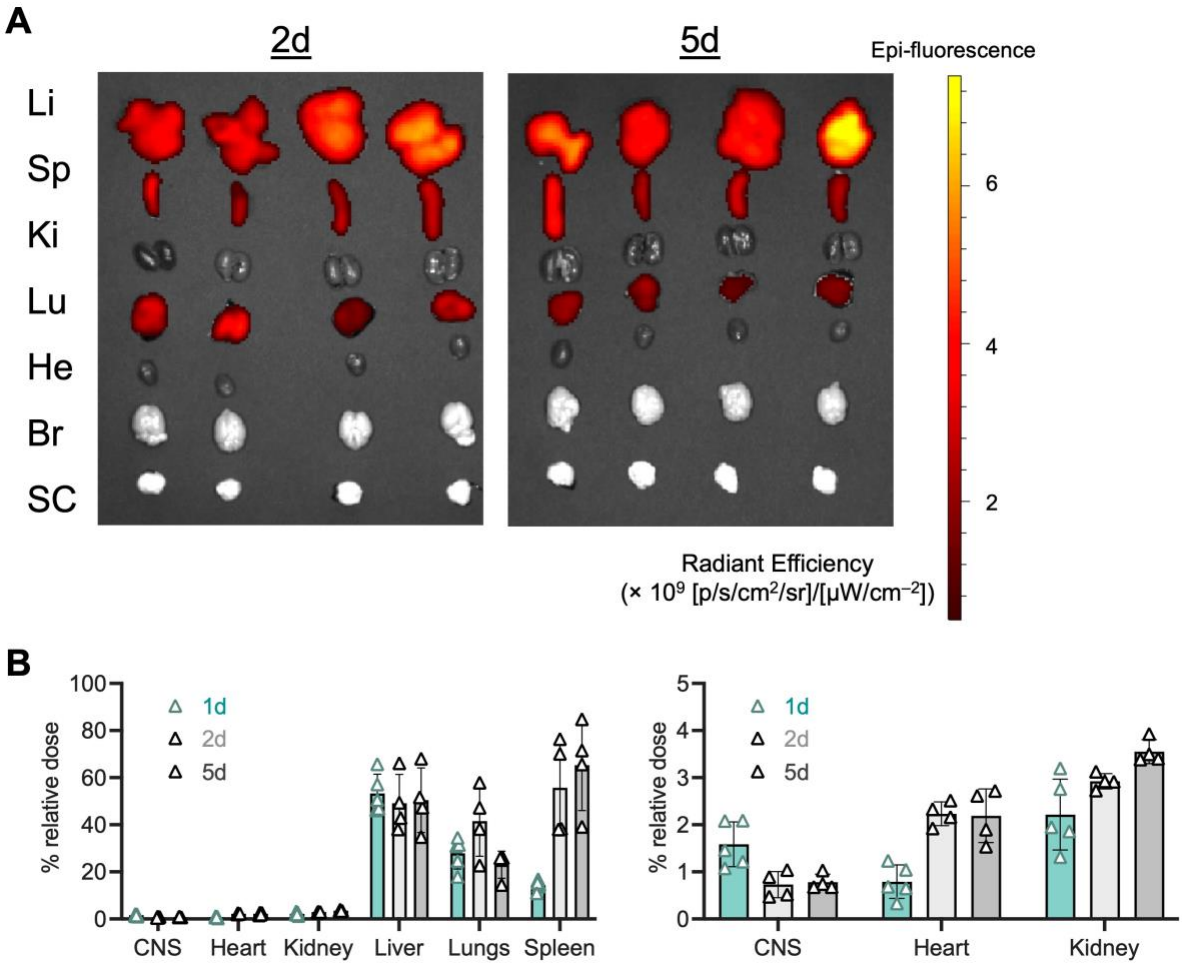

Fig. S8. Biodistribution characterization of monocytes and monocyte backpacks two and five days after administration. EAE was induced in female C57BL/6J mice. Mice were treated with monocytes or backpack-monocytes at the onset of clinical signs on Day 11 (i.v., tail vein), with the adoptively transferred monocytes stained with DiR 750. The mice were sacrificed after 2 days or 5 days, and the CNS was harvested. (A) IVIS imaging of fluorescence signal (DiR 750) from adoptively transferred cells in organs (Liver, Li; Spleen, Sp; Kidney, Ki; Lungs, Lu; Heart, He; Brain, Br; Spinal cord, SC). (B) Fluorescence quantification of relative accumulated dose for monocytes and backpack-monocytes; mean ± SD (n=4-5).

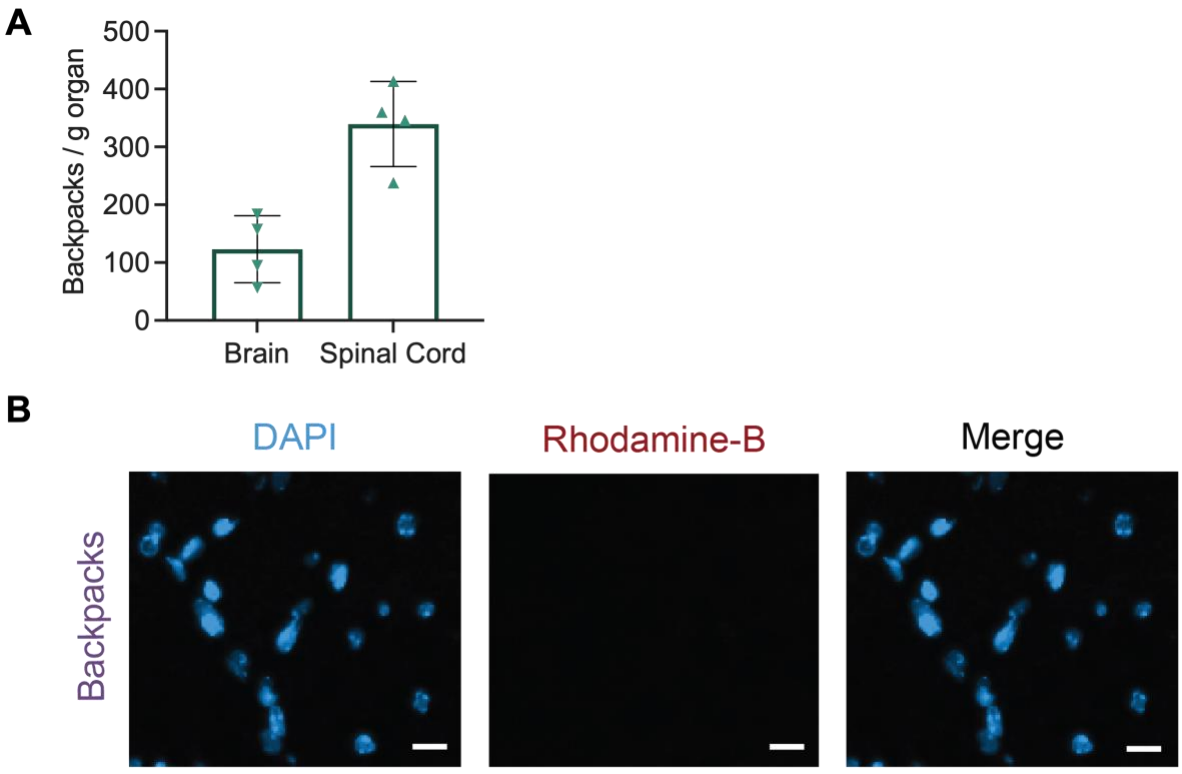

Fig. S9. Free backpacks do not substantially accumulate in the CNS of EAE mice. EAE was induced in female C57BL/6J mice. EAE mice were administered $3x10^6$ free backpacks intravenously via tail vein on day 11, at the onset of clinical signs. Mice were sacrificed 24 hours after for CNS harvest. (A) After single cell suspension processing of the brain and spinal cord, infiltrating backpacks per mass organ was quantified via flow cytometry; mean ± SD (n=4). (B) Fluorescence imaging of lumbar spinal cord stained for DAPI (nucleus, blue) and Rhodamine-B (backpacks, red). Scale bar represents 10µm.

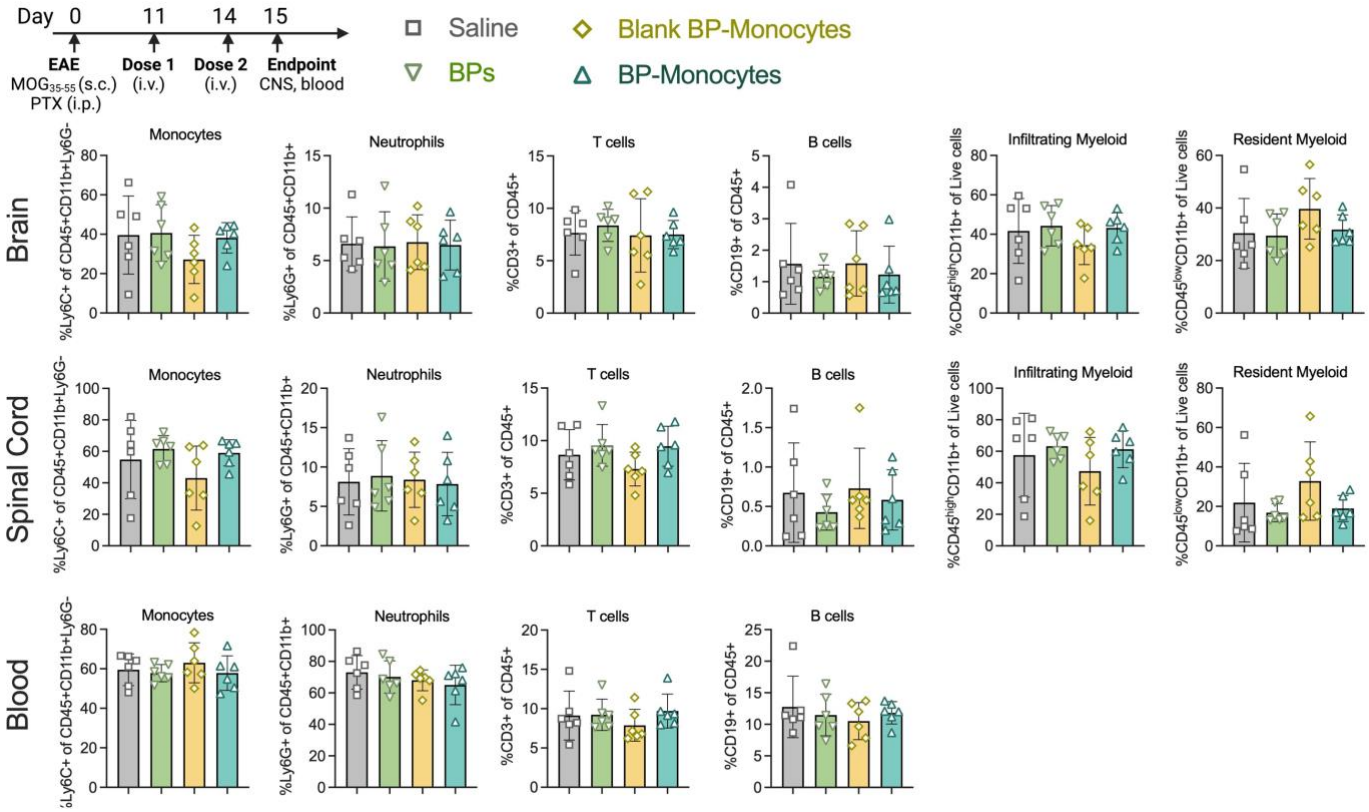

Fig. S10. General immune cell abundances in CNS and blood at Day 15. EAE was induced in female C57BL/6J mice. Mice were administered $3x10^6$ backpacks (BPs), blank backpack-carrying monocytes, BP-monocytes, or saline intravenously via tail vein on days 11 and 14 and sacrificed on day 15. The CNS and blood were harvested, processed into single cell suspensions, and analyzed via flow cytometry for immune cell populations. Percentages of monocytes (%$Ly6C^+$ of $Ly6G^-CD11b^+CD45^+$), neutrophils (%$Ly6G^+$ of $CD11b^+CD45^+$), T cells (% $CD3^+$ of $CD45^+$), B cells (%$CD19^+$ of $CD45^+$), infiltrating myeloid cells ($CD45^{high}CD11b^+$ of live cells), resident myeloid cells ($CD45^{low}CD11b^+$ of live cells); mean ± SD (n=6). Statistical analysis: one-way ANOVA with Tukey's HSD test.

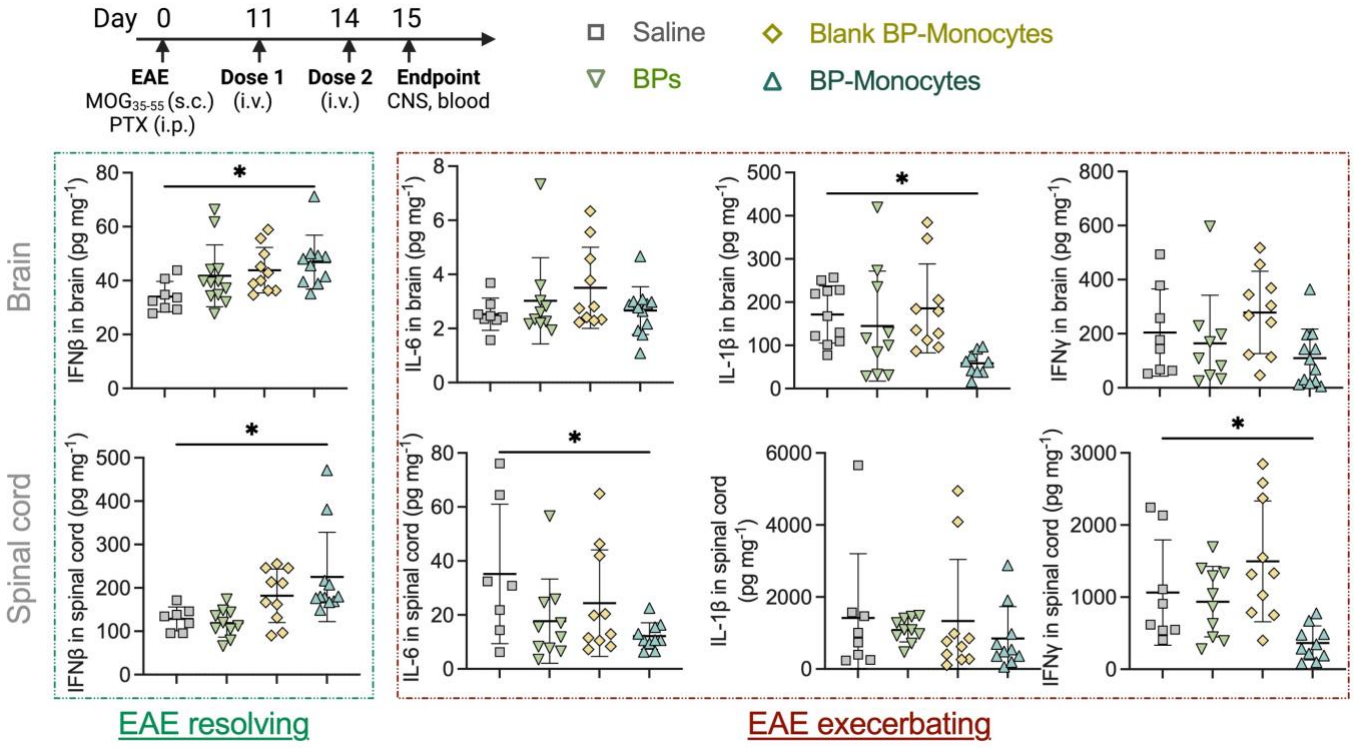

Fig. S11. Cytokine content in brain and spinal cord at Day 15. EAE was induced in female C57BL/6J mice. Mice were administered $3x10^6$ backpacks (BPs), blank backpack-carrying monocytes, BP-monocytes, or saline intravenously via tail vein on days 11 and 14 and sacrificed on day 15. The CNS was harvested the tissue homogenate was analyzed for concentrations of anti-/pro-inflammatory mediators at day 15; mean ± SD (n=7-10). Statistical analysis: one-way ANOVA with Tukey's HSD test; ns, not significant, *P<0.05.

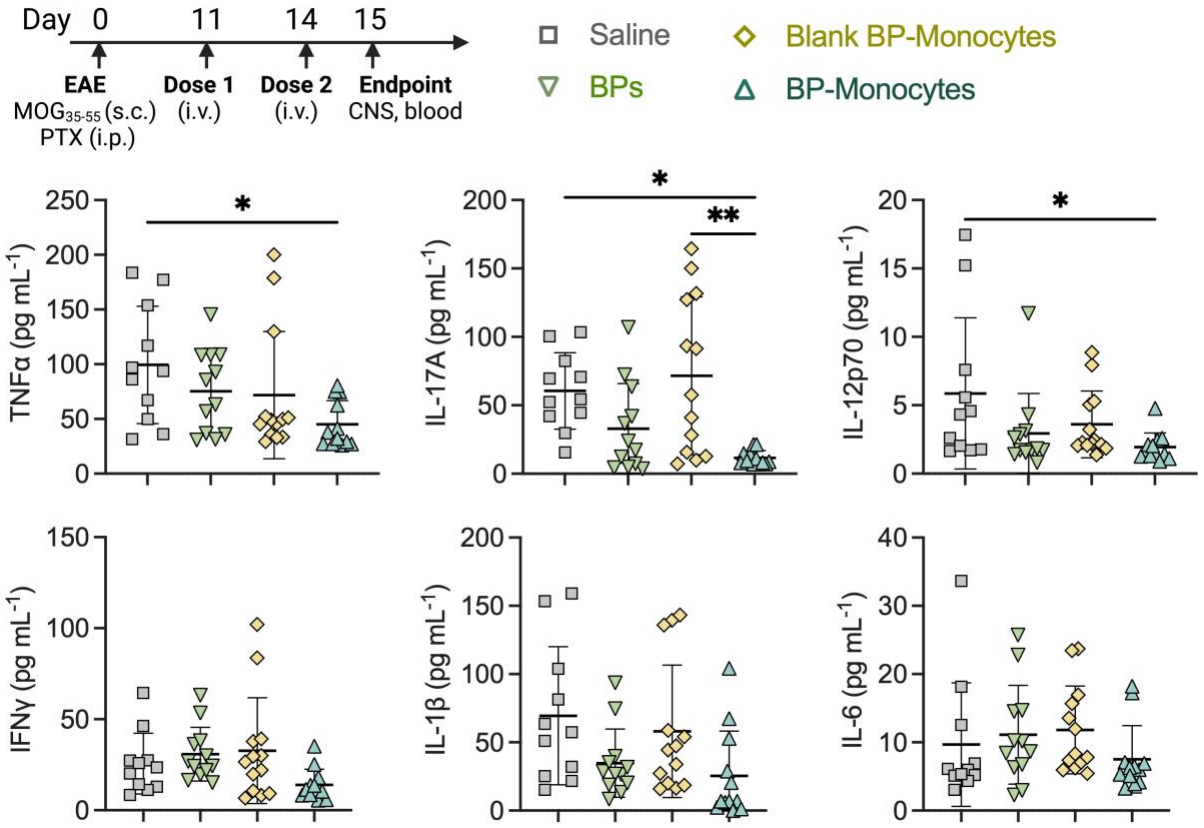

Fig. S12. Serum cytokine content at Day 15. EAE was induced in female C57BL/6J mice. Mice were administered $3x10^6$ backpacks (BPs), blank backpack-carrying monocytes, BP-monocytes, or saline intravenously via tail vein on days 11 and 14 and sacrificed on day 15. Blood was drawn, processed into serum, and analyzed for concentrations of pro-inflammatory mediators at day 15; mean ± SD (n=10-13). Statistical analysis: one-way ANOVA with Tukey's HSD test; ns, not significant, *P<0.05.

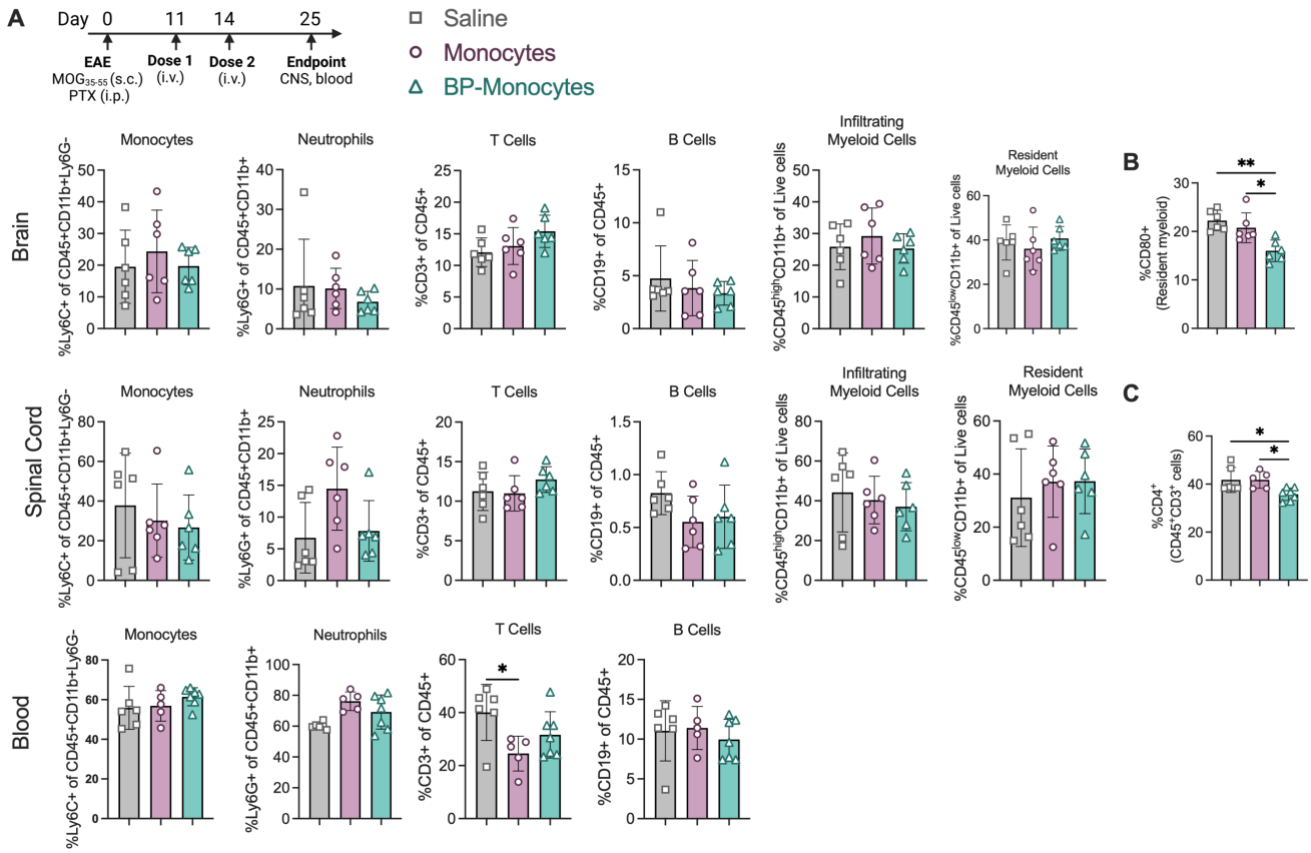

Fig. S13. General immune cell abundances in CNS and blood at Day 25. EAE was induced in female C57BL/6J mice. Mice were administered $3x10^6$ monocytes, backpack (BP)-carrying monocytes or saline intravenously via tail vein on days 11 and 14 and sacrificed on day 25. The CNS and blood were harvested, processed into single cell suspensions, and analyzed via flow cytometry for immune cell profile. A) Percentages of monocytes (%Ly6C$^+$ of Ly6G$^-$CD11b$^+$CD45$^+$), neutrophils (%Ly6G$^+$ of CD11b$^+$CD45$^+$), T cells (% CD3$^+$ of CD45$^+$), B cells (%CD19$^+$ of CD45$^+$), infiltrating myeloid cells (CD45^{high}CD11b$^+$ of live cells), resident myeloid cells (CD45^{low}CD11b$^+$ of live cells); mean ± SD (n=5-7). B) %CD80$^+$ of resident myeloid cells of the brain (CD45^{low}CD11b$^+$); mean ± SD (n=6). C)%CD4$^+$ T cells (of CD3$^+$CD45$^+$ T cells) of the blood; mean ± SD (n=6). Statistical analysis: one-way ANOVA with Tukey's HSD test; ns, not significant, *P<0.05, **P<0.01.

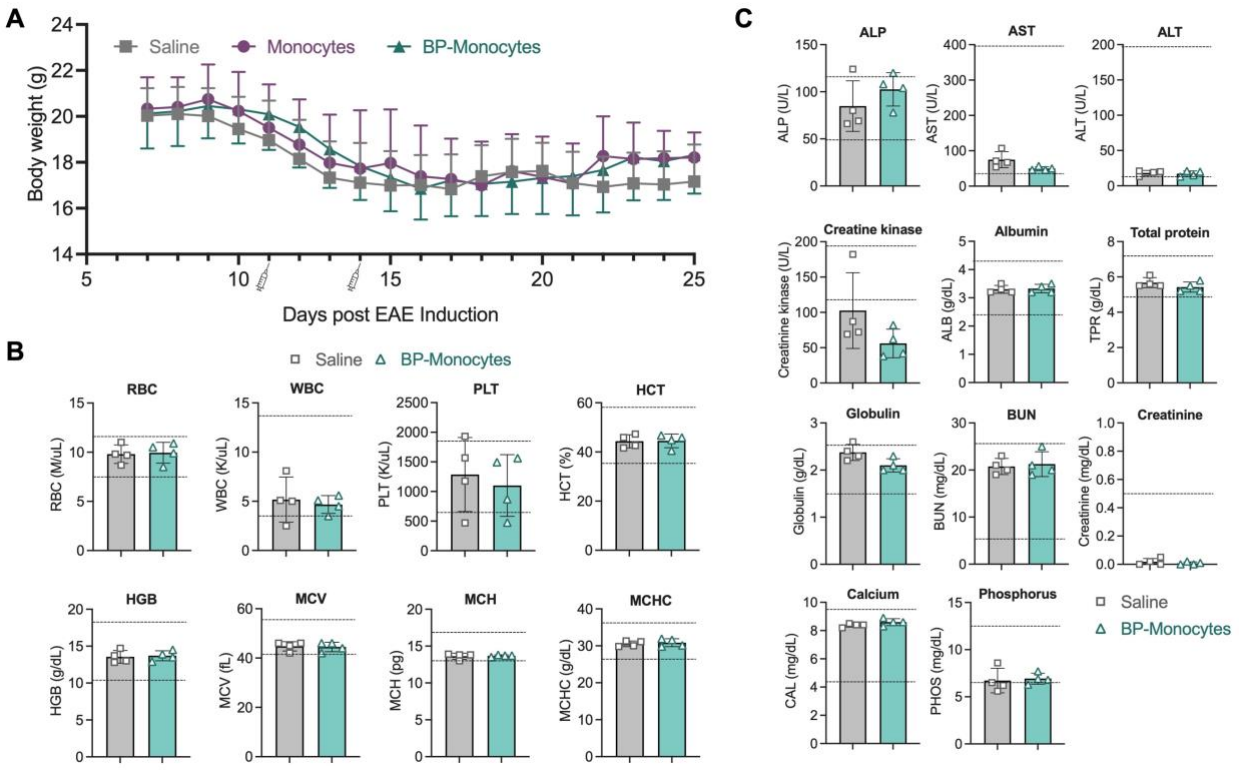

Fig. S14. Safety assessment of backpack-monocyte treatment. EAE was induced in female C57BL/6J mice. Mice were administered $3x10^6$ monocytes, backpack (BP)-carrying monocytes or saline intravenously via tail vein on days 11 and 14 and monitored until day 25. (A) Body weight over time following two doses of treatment from Figure 5B; mean ± SE (n=11-14). (B). Hematology analysis of EAE mice treated with saline or monocyte backpacks. No significant differences between saline group and monocyte backpack group were detected. RBC: red blood cell, WBC: white blood cell, PLT: platelet, HCT: hematocrit, HBG: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration; mean ± SD (n=4). (C) Serum chemistry analysis of EAE mice treated with saline or monocyte backpacks. No significant differences between saline group and monocyte backpack group were detected. ALP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BUN: blood urine nitrogen; mean ± SD (n=4). Data were analyzed by two-tailed student's t test. Dashed Lines indicate established normal range.

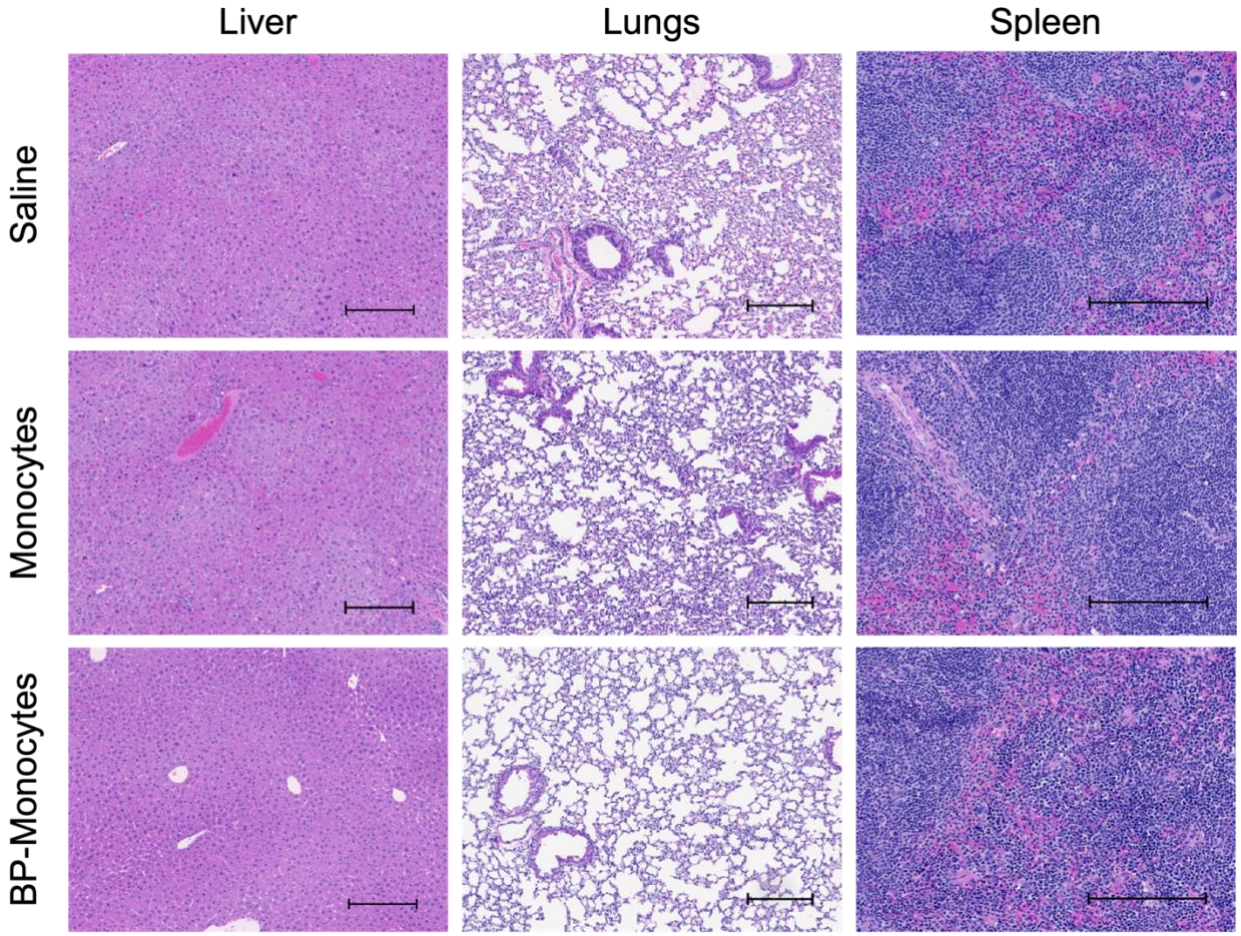

Fig S15. H&E of major mouse organs following different treatments in EAE model. Mice received same doses as in efficacy study (Main Fig. 5). Organs of mice were analyzed by H&E staining. Representative of n = 3 biologically independent animals per group. Scale bar = 200μm.