Appendix A: Supplemental data for Zhang et al., Key role of CCR2-expressing macrophages in an inflammatory model of low back pain and radiculopathy.

Supplemental Figure A1

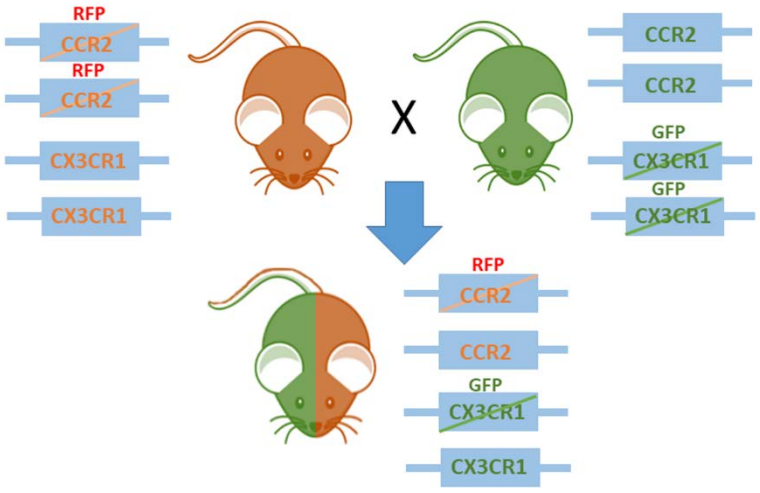


Figure A1: Breeding scheme for the reporter mice used in the study. To obtain mice with red and green fluorescent labeled macrophage subsets, the strain $Ccr2^{RFP/RFP}$ (left; Jackson Laboratory strain 017586, (Saederup et al., 2010)), in which CCR2, the receptor for CCL2, has been replaced at both loci by red fluorescent protein (RFP), was crossed with the strain $Cx3cr1^{GFP/GFP}$ (right; Jackson Laboratory strain 005582; (Jung et al., 2000)) in which the fractalkine receptor CX3CR1 is replaced at both loci by green fluorescent protein (GFP). The resulting progeny $CCR2^{+/RFP}$ $CX3CR1^{+/GFP}$ ("reporter" mice) have both RFP-labeled and GFP-labeled subsets of MØ, but have one normal copy of each of the 2 receptors(Saederup et al., 2010). The $CCR2^{RFP/RFP}$ parent strain was used for experiments requiring CCR2 knockout mice.

Supplemental figure A2:

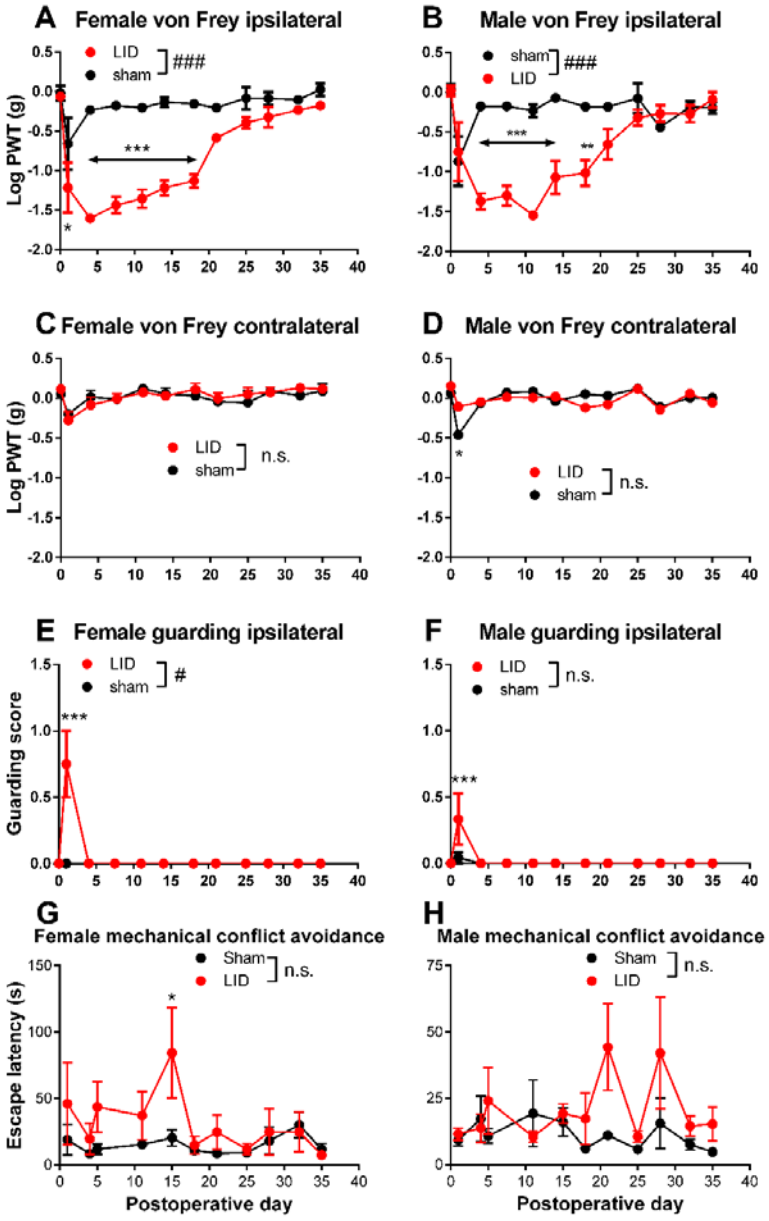
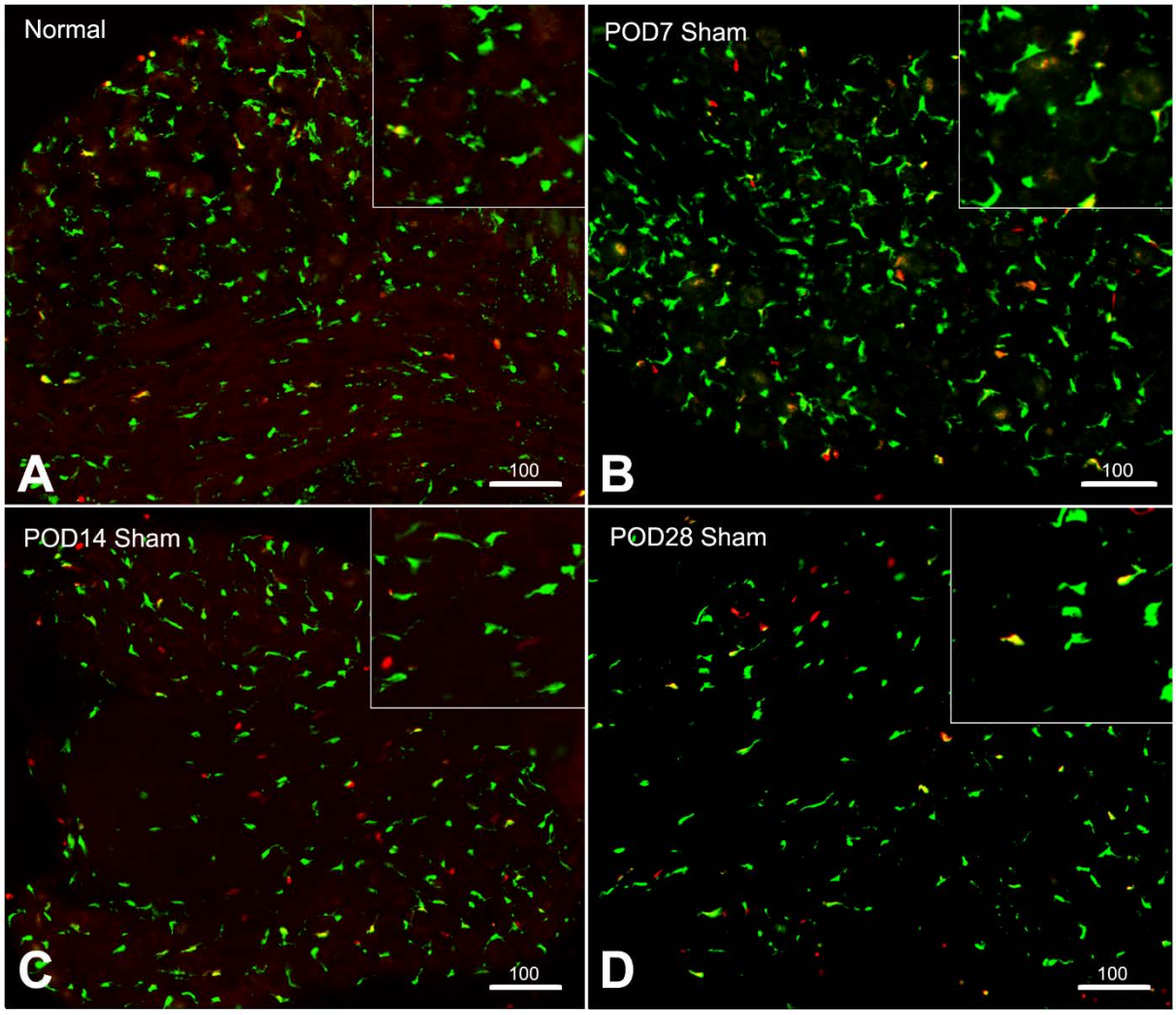


Figure A2: Lack of strong sex differences for effect of local inflammation ("LID") of the dorsal root ganglion (DRG) on pain behaviors in mice. Data presented in Figure 1 are shown separated by sex. Baseline behaviors were measured 1 day prior to surgery, the value is plotted on day 0. On day 0, the L4 DRG was inflamed with 3 µl of zymosan in incomplete Freund's adjuvant. The control group received sham surgery with no zymosan injection. A, B, von Frey threshold on the ipsilateral side; C, D, von Frey threshold on the contralateral side; E, F, guarding score (maximum score is 3) on the ipsilateral side (no contralateral guarding was observed); G, H, latency to enter chamber with lattice of pins at 5 mm

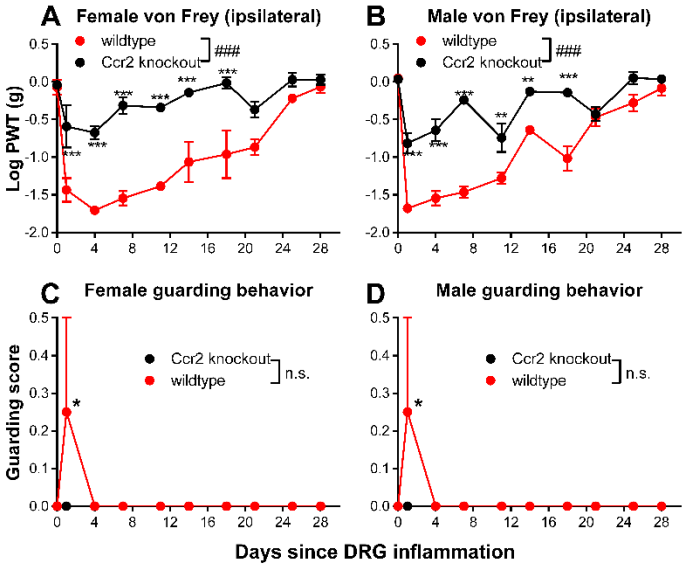
height; D, #, p<0.05; ##, p<0.01; ###, p<0.001; significant overall effect for the sham vs. LID factor, n.s., not significant (2 way repeated measures ANOVA); $F_{(1,6)}$ = 115.7 (A); 64.9 (B);0.153, not significant (n.s.), p = 0.82, (C); 0.012, n.s., p = 0.91 (D); 2.19, n.s., p = 0.19 (E); 3.147, n.s., p = 0.13 (F); 3.15, n.s., p = 0.13 (G); and 2.57, n.s., p = 0.16 (G). In panels A, B, E, and F the interaction effect was also significant. *, p<0.05; **, p<0.01; ***,p<0.001; significant difference between the groups at the indicated time point (Bonferroni's multiple comparisons posttest). N = 4 mice/group, wildtype, 4 of each sex. Same data is shown in Figure 1 with the 2 sexes combined. Analysis of the combined data using sex as a factor with 3-way ANOVA confirmed no significant effect of the sex factor for the ipsilateral von Frey test ($F_{(1,12)}$= 24.1, p = 0.72), contralateral von Frey test (F = 1.4, p = 0.25), ipsilateral guarding score (F = 1.4, p = 0.26), or escape latencies (F = 1.7, p =0.21).

Supplemental Figure A3



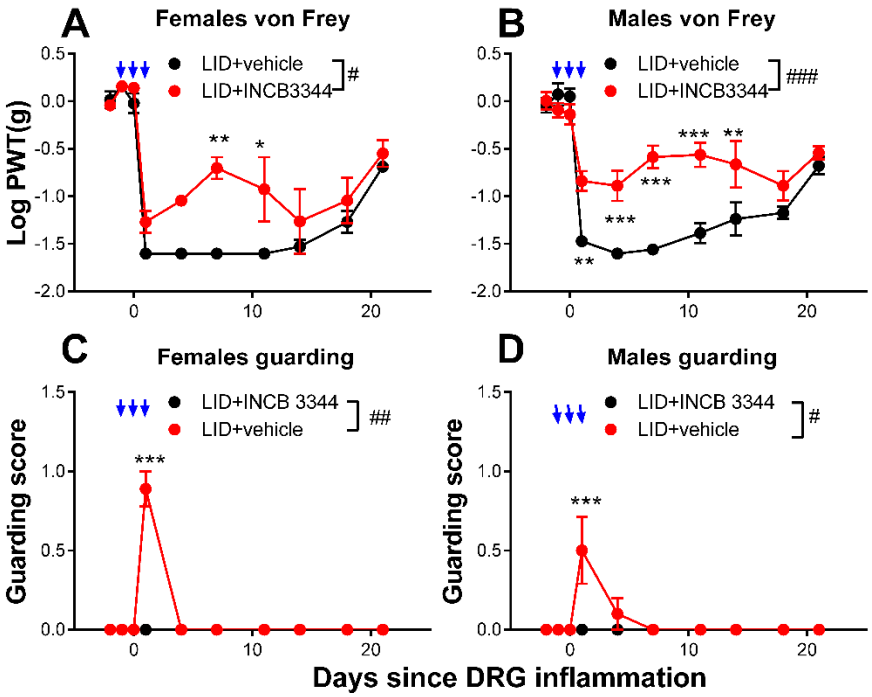
Supplemental figure A3. Density of *Cx3cr1*$^{+/GFP}$ and *Ccr2*$^{+/RFP}$ MØ in cellular areas of the DRG in normal DRG and sham operated DRG from experiment shown in Figure 2. DRG sections were obtained from normal DRG (A) or at postoperative day (POD) 7 (B), 14(C) and 28 (D) after sham control operation for DRG inflammation on POD0. An example of DRG from a POD4 sham operated animal and summary data are shown in Figure 2. RFP and GFP signals are shown merged. Inset is 2x.

Supplemental Figure A4



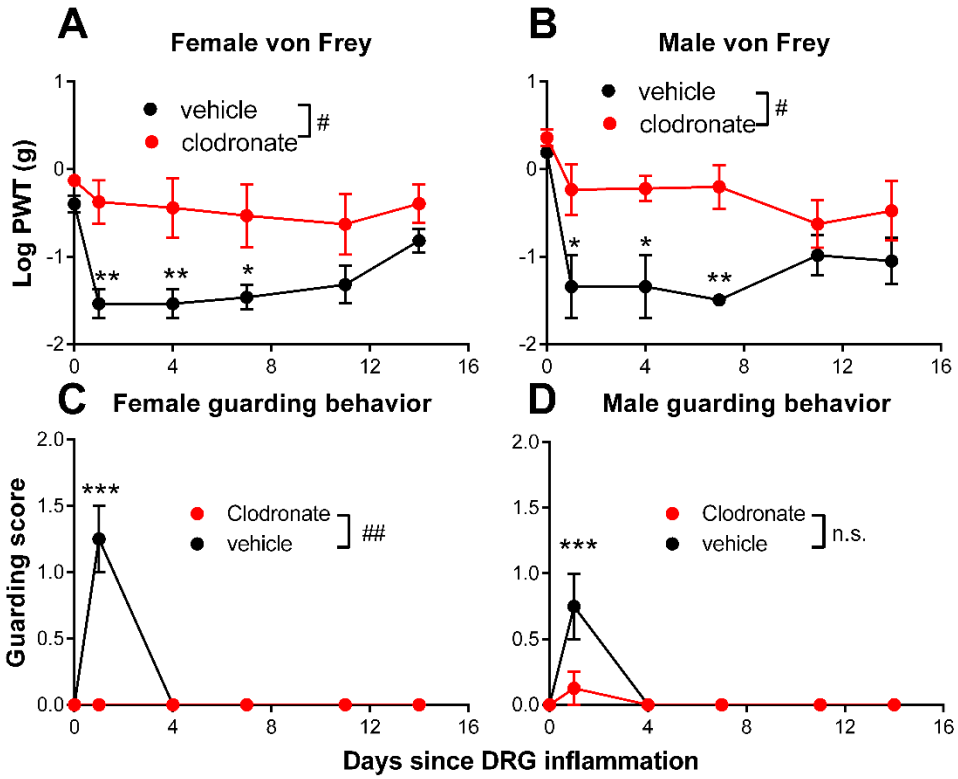
Supplemental Figure A4. Lack of sex differences for effect of CCR2 knockout on pain behaviors induced by local inflammation ("LID") of the dorsal root ganglion (DRG). Data presented in Figure 4 are shown separated by sex. Baseline behaviors were measured on 2 days prior to surgery, the average value is plotted on day 0. On day 0, the L4 DRG was inflamed with 3 µl of zymosan in incomplete Freund's adjuvant. The control group received sham surgery with no zymosan injection. A, B, von Frey threshold on the ipsilateral side; C, D, guarding score (maximum score is 3) on the ipsilateral side, ###, $p<0.001$; significant overall effect for the genotype factor, n.s., not significant (2 way repeated measures ANOVA); $F_{(1,6)} = 47.1$ (A); 166.5 (B);1.00, n.s., $p = 0.36$, (C); and 1.0, n.s., $p = 0.45$ (D). In panels A and B the interaction effect was also significant. *, $p<0.05$; **, $p<0.01$; ***,$p<0.001$; significant difference between the groups at the indicated time point (Bonferroni's multiple comparisons posttest). N = 4 mice/group, wildtype, 4 of each sex. Same data is shown in Figure 4 with the 2 sexes combined. Analysis of the combined data using sex as a factor with 3-way ANOVA confirmed no significant effect of the sex factor for the von Frey test ($F_{(1,12)} = 0.09$, $p = 0.77$) or the guarding score ($F = 0.0$, $p = 1.0$).

Supplemental Figure A5

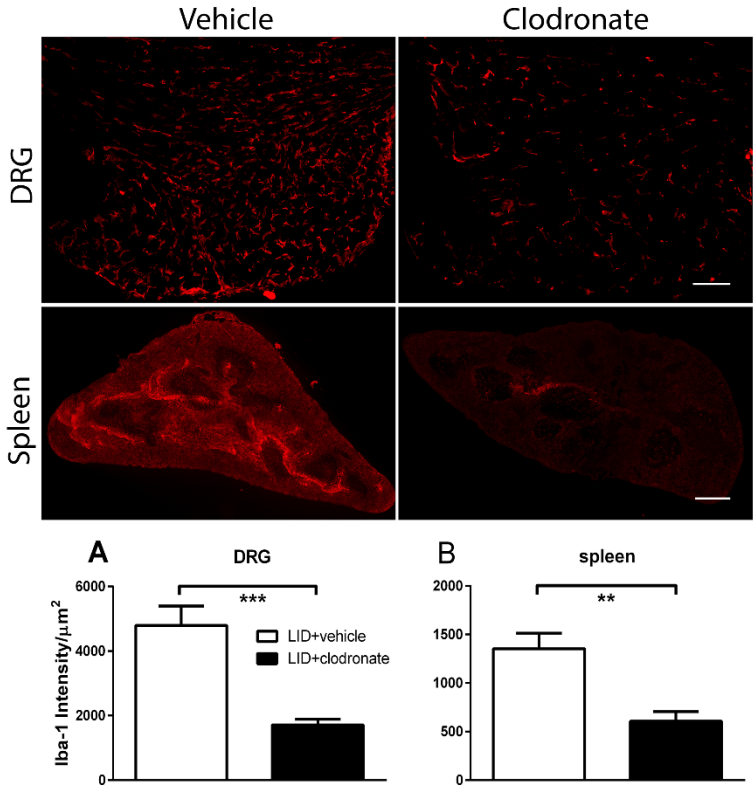


Supplemental Figure A5. Lack of strong sex differences for effect of INCB3334 pretreatment on pain behaviors induced by local inflammation ("LID") of the dorsal root ganglion (DRG). Data presented in Figure 5 are shown separated by sex. Baseline behaviors were measured on 2 days prior to and just prior to the DRG inflammation surgery on day 0. INCB3334 or vehicle was injected intravenously on days -1, 0, and 1 (blue arrows; behaviors measured 2 hours later). On day 0, the L4 DRG was inflamed A, B, von Frey threshold on the ipsilateral side; C, D, guarding score (maximum score is 3) on the ipsilateral side. #, $p<0.05$; ##, $p<0.01$; significant overall effect for the clodronate vs. vehicle factor, n.s., not significant (2 way repeated measures ANOVA); $F_{(1,4)} = 10.6$ (A); $F_{(1,8)} = 68.1$ (B); $F_{(1,4)} = 64.0$, (C); and $F_{(1,8)} = 5.7$ (D). In all panels the interaction effect was also significant. *, $p<0.05$; **, $p<0.01$; ***,$p<0.001$; significant difference between the groups at the indicated time point (Bonferroni's multiple comparisons posttest). N = 3 female and 5 male mice/group, wildtype. Same data is shown in Figure 7 with the 2 sexes combined. Analysis of the combined data using sex as a factor with 3-way ANOVA confirmed no significant effect but a trend towards significance of the sex factor for the von Frey test ($F_{(1,12)} = 4.7$, $p = 0.0505$) and no significant effect of sex on the guarding score ($F = 0.7$, $p = 0.41$).

Supplemental Figure A6



Supplemental Figure A6. Lack of sex differences for effect of clodronate pretreatment on pain behaviors induced by local inflammation ("LID") of the dorsal root ganglion (DRG). Data presented in Figure 6 are shown separated by sex. Baseline behaviors were measured 1 day prior to surgery, the value is plotted on day 0. Liposomal clodronate was injected on day -2. The control group received vehicle liposome injection. On day 0, the L4 DRG was inflamed. A, B, von Frey threshold on the ipsilateral side; C, D, guarding score (maximum score is 3) on the ipsilateral side, #, $p<0.005$; significant overall effect for the clodronate vs. vehicle factor, n.s., not significant (2 way repeated measures ANOVA); $F_{(1,6)} = 7.7$ (A); 12.5 (B); 25.0 (C); and 5.0, n.s., $p = 0.07$ (D). In panels A, B, and D the interaction effect was also significant. *, $p<0.05$; **, $p<0.01$; ***,$p<0.001$; significant difference between the groups at the indicated time point (Bonferroni's multiple comparisons posttest). N = 4 mice/group, wildtype. Same data is shown in Figure 6 with the 2 sexes combined. Analysis of the combined data using sex as a factor with 3-way ANOVA confirmed no significant effect of the sex factor for the von Frey test ($F_{(1,12)} = 1.0$, $p = 0.33$) or the guarding score ($F = 1.0$, $p = 0.34$)



Supplemental Figure A7. Effect of pretreatment with liposomal clodronate on Iba-1-signal in the DRG (top) and spleen (middle). Wildtype mice received either intravenous liposomal clodronate or vehicle control injections. 2 days later, the DRG were inflamed. DRG (top, scale bar 20 µ) and spleen sections (middle; scale bar 200 µ) were obtained 4 days after inflammation and Iba1 measured (red signal). **, $p<0.01$; ***, $p<0.001$; significant difference between vehicle and clodronate treated groups, t-test. N = 4 – 6 wildtype mice per group, both sexes.

References

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., Littman, D.R., 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol 20, 4106-4114.

Saederup, N., Cardona, A.E., Croft, K., Mizutani, M., Cotleur, A.C., Tsou, C.L., Ransohoff, R.M., Charo, I.F., 2010. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. PLoS One 5, e13693.