Supplemental Figures for Cronk et al.,

"Methyl-CpG binding protein 2 is a critical regulator of macrophage gene expression and function"

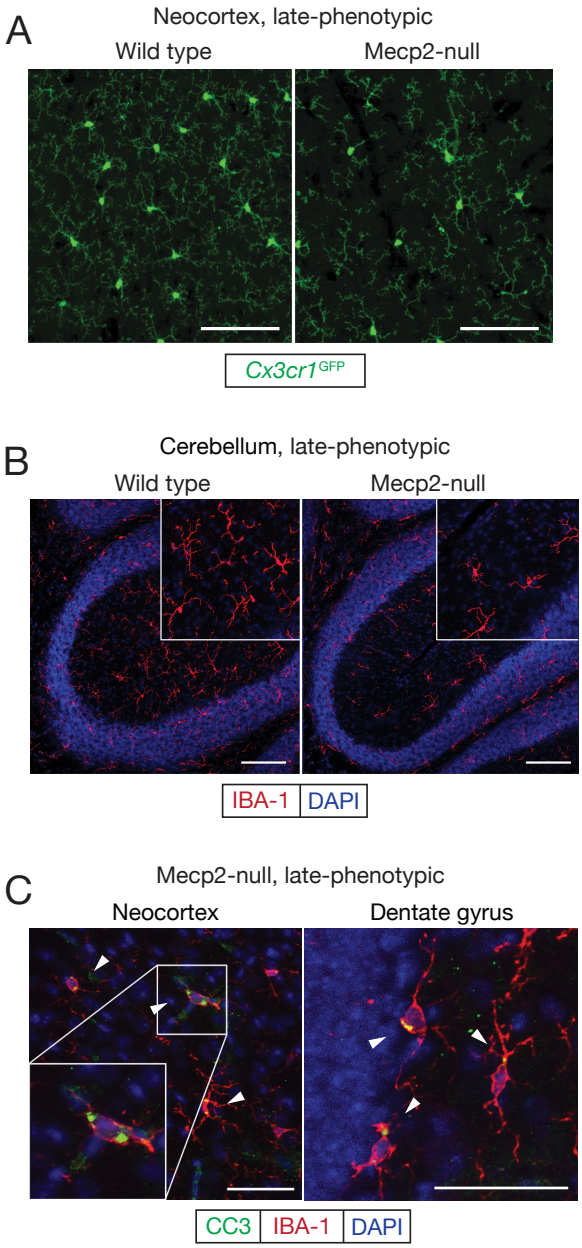

Figure S1, related to Figure 1.

Representative images of wild type and late-phenotypic Mecp2-null microglia.

(A, B) Representative images from *Cx3cr1*$^{GFP/+}$ or non-GFP (Iba1 staining) wild type and late-phenotypic Mecp2-null brains (scale bars: (A), 75 µm; (B), 120 µm). Images were cropped from larger images to allow for better visualization of microglial morphology.

(C) Iba1 and cleaved caspase 3 (CC3) staining in late-phenotypic Mecp2-null brains demonstrating CC3^{+} microglia (scale bar, 40 µm). Images were cropped from larger images to allow for better visualization of CC3 localization within microglia.

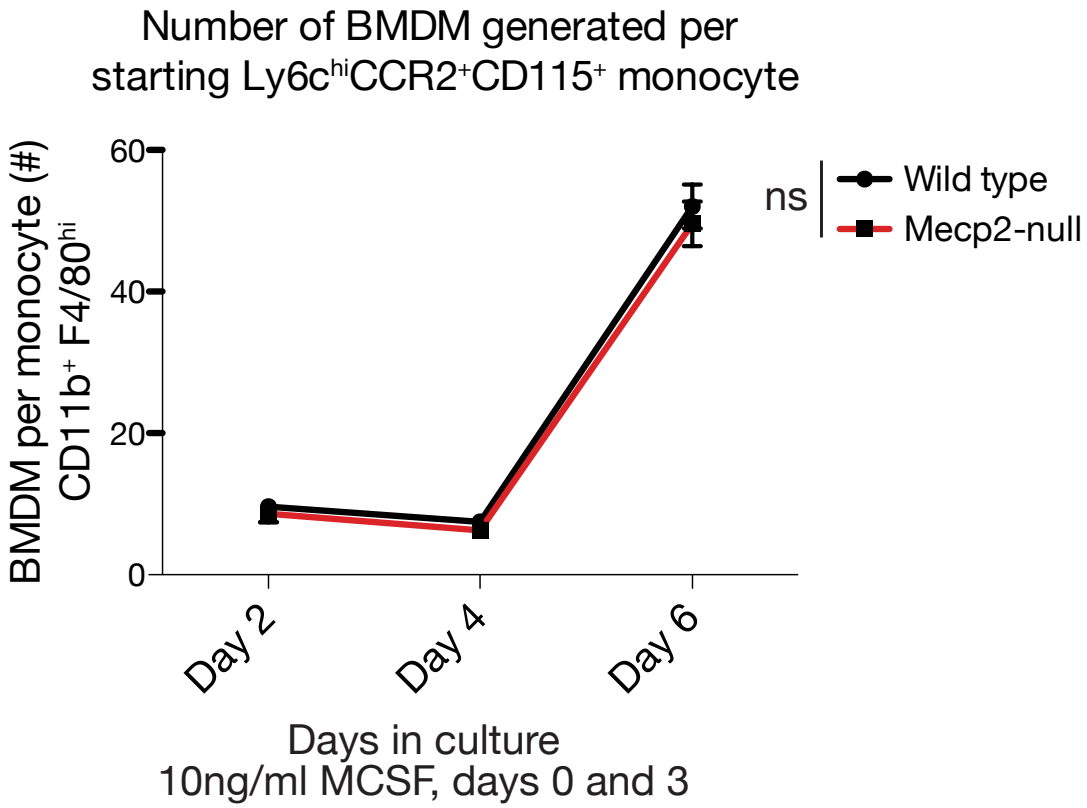

Figure S2, related to Figure 3. BMDM generated per monocyte in wild type and Mecp2-null. Wild type and Mecp2-null BMDM were generated as described. Briefly, equal numbers of live bone marrow cells were plated in 10ng/ml MCSF for 3 days, then given 50% additional volume of media containing 20ng/ml MCSF for an additional 3 days. Cell counts were obtained from cells generated from n=3 wild type and n=3 Mecp2-null mice on days 2, 4, and 6 during culture. Flow cytometric analysis of bone marrow prior to culture was performed to obtain the percentage of cells plated that were $Ly6C^{hi}CD115^{+}CD11b^{+}$ monocyte progenitors. This percentage was multiplied by the total number of cells plated to obtain the actual number of starting monocyte progenitors in each culture dish. The number of BMDM ($CD11b^{+}F4/80^{hi}$) counted on each day of development was divided by the number of starting monocytes to calculate the number of macrophages derived per monocyte.

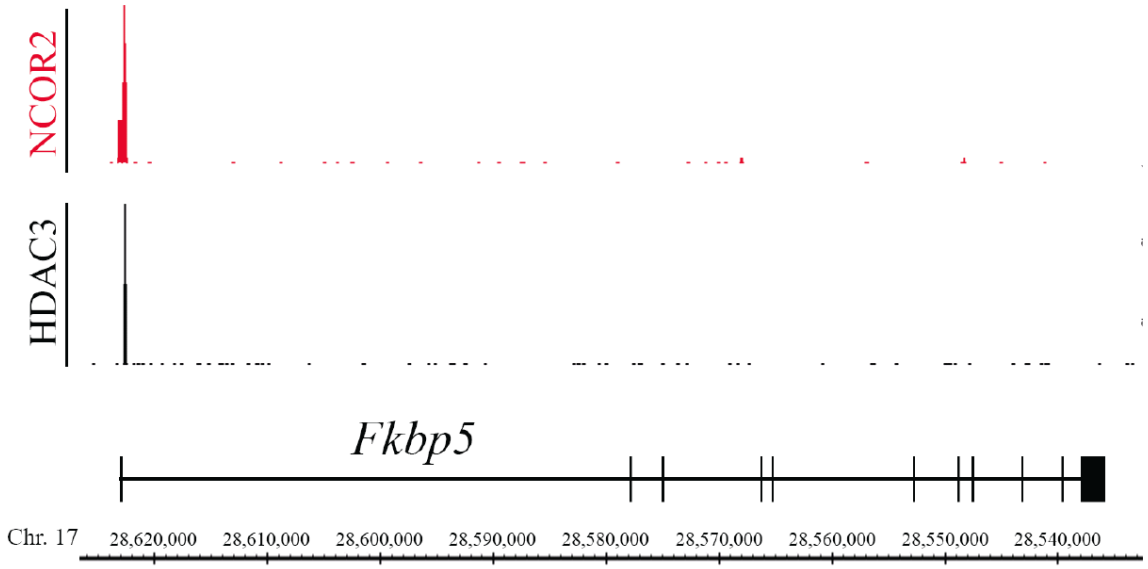

Figure S3, related to Figure 5. NCOR2 and HDAC3 occupancy profiles at the *Fkbp5* gene promoter. ChIP-Seq analysis demonstrates NCOR2 and HDAC3 binding profiles at *Fkbp5* gene promoter in wild type bone marrow derived macrophages. The genomic region surrounding the *Fkbp5* gene locus is shown, for which NCOR2 and HDAC3 occupancies are shown in red and black, respectively. Normalized counts, tag coverage per base per 10^6 sequenced reads, are indicated on the Y-axis. Data are representative of two experiments.

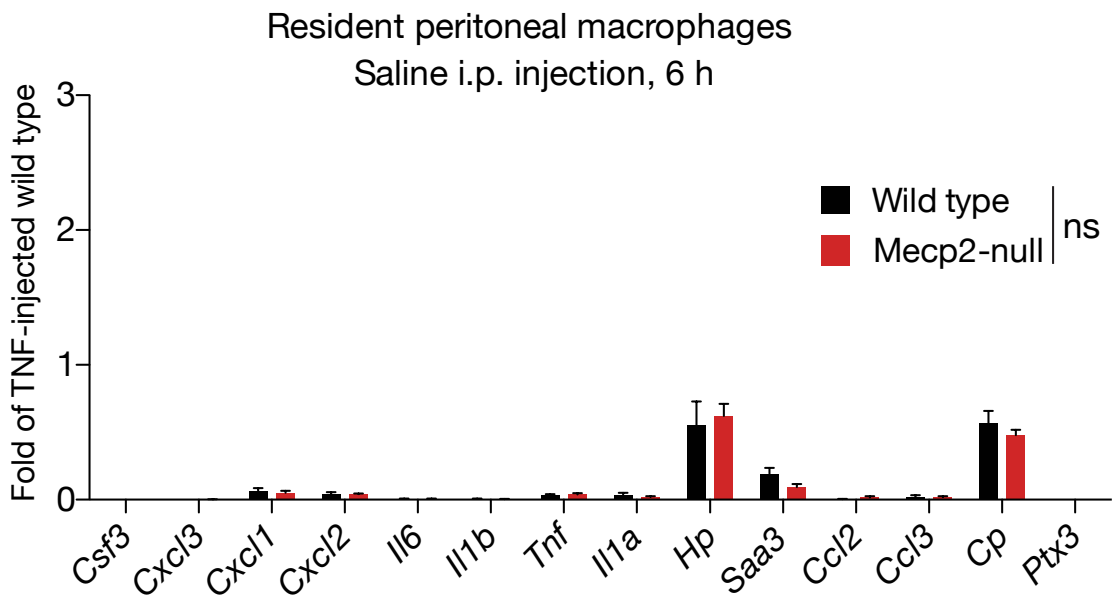

Figure S4, related to Figure 6. Intraperitoneal saline injection into Mecp2-null mice does not effect gene expression of TNF-induced transcripts. Mice were injected with 10µl saline per gram of mouse weight (equivalent volume to that injected in Figure 6B), allowed to respond for 6h, and then cells were collected by peritoneal lavage and positively selected for F4/80 via AutoMACS. RNA was collected and qRT-PCR performed. Gene expression was normalized to wild type expression after TNF treatment (see Figure 6B). No significant difference was detected by two-way ANOVA with Bonferroni post test, n = 3 mice per group. Error bars represent SEM.

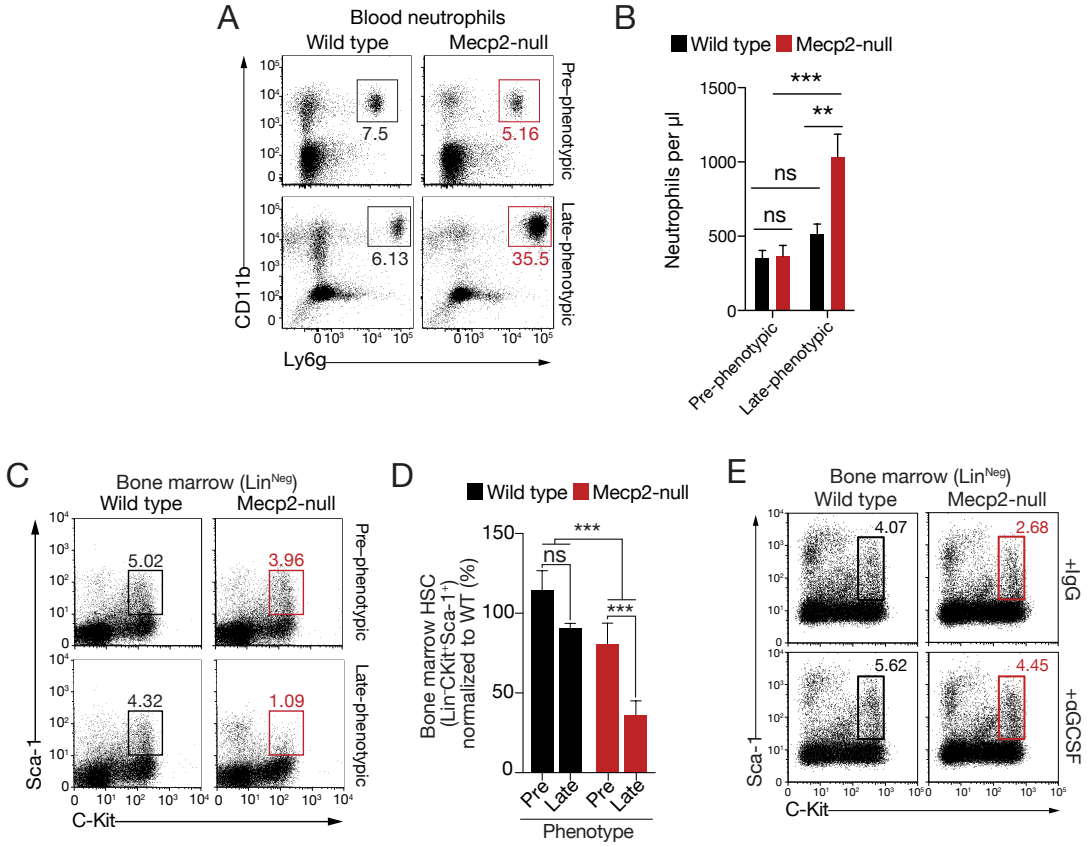

Figure S5, related to Figure 6. Mecp2-null mice have progressive neutrophilia and hematopoietic stem cell loss, which can be attenuated by anti-GCSF neutralizing antibodies.

(A, B) Neutrophil counts in Mecp2-null and age-matched wild types in pre- and late-phenotypic mice. Quantified by flow cytometry and total live cell counts from blood samples. (Two-way ANOVA with Bonferroni post-test; n = 6-9 mice per group; **, $p<0.01$; ***, $p<0.001$. Error bars represent SEM).

(C, D) Quantification of hematopoietic stem cells ($Lin^{-}C\text{-}Kit^{+}Sca\text{-}1^{+}$) in Mecp2-null and age-matched wild type mice in BM isolates as measured by flow cytometry in pre- and late-phenotypic mice ($Lin^{neg}CKit^{+}Sca\text{-}1^{+}$) (***, $p<0.001$; Two-way ANOVA with Bonferroni post-test. Error bars represent SEM).

(E) Flow cyotmetric plots of hematopoietic stem cells ($Lin^{-}C\text{-}Kit^{+}Sca\text{-}1^{+}$) in Mecp2-null and wild type mice treated with anti-GCSF neutralizing antibodies or isotype control.

Supplemental Movies

Movies were captured over 20 minutes at 1024x1024 and 22um depth, (Z depth 22um, step of 1um) and cropped in Imaris to a width of 800x800 pixels, subjected to the following two filters:
Background subtraction, filter width of 22um
Gaussian Filter, filter width of 0.01um
Processed files were saved as Quicktime movies.
Cx3cr1$^{GFP/+}$ (microglia; green), intravascular tracer (Qdot655; red)

Movie S1, related to Figure 1. Representative wild type microglia.

Movie S2, related to Figure 1. Representative Mecp2-null microglia from phenotypic mice.

Supplemental Tables

Table S1, related to Figure 5. Gene set Signatures
Shown is the list of custom gene sets of hypoxia and glucocorticoid transcriptional signatures. These signatures were derived from the indicated public available databases.

Table S2, related to Figure 5. Transcriptional signatures enriched in *Mecp2*-null cells
Shown is the summary of gene set enrichment analysis (GSEA) that indicate the statistical scores of the enrichment of hypoxia, glucocorticoid and TNF-induced transcriptional signature genes in the transcriptome of Mecp2-null microglia cells.

Table S3, related to Figure 5. Glucocorticoid Signature Genes Up-regulated in *Mecp2*-null cells
Shown is the list of the Glucocorticoid Signature Genes that are up-regulated in microglia and peritoneal macrophages isolated from *Mecp2*-null mice.

Supplemental Experimental Procedures

Flow cytometry

Single cell suspensions were prepared by passing cells through a 70 μm filter prior to staining. Cells were pelleted and resuspended in flow cytometry buffer (0.01 M PBS, 1% BSA, 2 mM EDTA, 0.1% sodium azide) containing 300 mg/ml mouse IgG as a blocking agent. Cells were then stained with the appropriate dilution of antibody or fluorescent label for 30 m at 4º C in the dark. In general, antibodies were obtained from eBioscience or BD. CCR2-FITC was obtained from R&D Systems. Viability dye and anti-Ly6g antibodies were obtained from Biolegend. After staining, cells were washed in 3 ml flow cytometry buffer, pelleted, and fixed in either 1% PFA or, for intranuclear staining, cells were prepared via manufacturer's instructions using the eBioscience Foxp3/Transcription Factor Staining Buffer Set. Cells were stained with either 1:1000 Chicken-anti-MeCP2 antibody (Millipore) or rabbit-anti-Mecp2 (a kind gift from the laboratory of Gail Mandel) in permeabilization buffer (eBioscience) for 30 m at 4ºC in the dark. Cells were washed in 3 ml permeabilization buffer, pelleted, and then stained with the appropriate fluorescently-labeled secondary antibody (Invitrogen) at 1:1000 in permeabilization buffer for 30 m at 4ºC in the dark. Cells were washed in 3 ml permeabilization buffer, pelleted, and resuspended in 0.01 M PBS for analysis on a flow cytometer.

Preparation of brain samples

Mice were perfused intracardially with 0.01 M PBS containing 5 u/ml heparin until thoroughly exsanguinated to minimize peripheral immune cell contamination. The entire

brain was then removed and meninges and choroid plexus carefully separated and discarded from the rest of the brain tissue. The brain was then minced gently using forceps and scalpel to begin dissociation of the tissue. Each brain was placed in 5 ml HBSS (containing Mg and Ca), 2 mg/ml papain, 50 u/ml DNASE-I (Sigma) and Glutamax (Invitrogen) in a 15 ml tube. Samples were then incubated at 37º C for 15 m, followed by gentle trituration five times up and down with a 5 ml plastic tissue culture pipette. Samples were again incubated at 37º C for 15 m, followed by a second gentle trituration five times up and down with a 5 ml plastic tissue culture pipette. Samples were incubated at 37º C for 15 m a third time, followed by gentle trituration ten times with a 9" heat-polished glass Pasteur pipette. At this point, cells were well dissociated. Each 15 ml tube was filled to the top with DMEM/F12/10% FBS and filtered gently through a 70 μm cell strainer. Cells were then pelleted at 290 RCF, 10 m, with a slow brake. Supernatant was removed and cells were used for flow cytometric analysis or RNA collection.

Preparation of brain suspension for RNA collection

After initial processing to create single-cell brain suspensions, samples were resuspended in 2.1 ml of 1.122 g/ml isotonic Percoll (Sigma) + 7.9 ml PBS. This mixture was underlayed with 2 ml 1.088 g/ml isotonic Percoll. The samples were then centrifuged for 30 m at 1159 RCF at RT, slow start, and no brake. Myelin debris at the top of the gradient was then removed. The liquid (but not the pellet) was then transferred to a 50 ml conical tube. The liquid was washed with DMEM/F12/10% FBS, pelleted, and then labeled with microglia $CD11b^+$ magnetic selection beads (Miltenyi). AutoMACS sorted

cells isolated by this method were typically about 85% pure $CD45^{lo}CD11b^{+}$ microglia. RNA was collected by RNeasy mini kit (Qiagen).

Multiphoton microscopy

Microglia were imaged through thinned skull. Briefly, mice were anesthetized with isoflurane and the skull thinned using a dental drill and microblade. Multiphoton imaging was performed using a Leica TCS SP8 multiphoton system equipped with a Coherent Chameleon Ti:Sapphire laser. GFP was excited with a wavelength of 880 nm. Images were collected from a z-series with a depth of approximately 20 microns with a step size of 1 micron over 20 microns. Movies were generated and image analysis was performed using ImageJ.

Immunohistochemistry

Mice were euthanized and perfused with 5 u/ml heparinized PBS followed by 4% PFA in 0.01 M PBS. Organs were removed and post-fixed in 4% PFA in 0.01 M PBS at 4ºC (2 days for brain). Tissue was then cryoprotected by equilibrating in 30% sucrose, followed by slicing on a cryostat. Brain slices were labeled free-floating; spleen slices were mounted onto gelatin-coated charged slides. Meninges were fixed with acetone/ethanol within the skull cap *in situ* at -20C for 20min, washed with PBS, stained, carefully removed and whole mounted on slides for imaging. Tissue was blocked for 1 h RT in 0.01 M PBS containing 0.1% Triton X-100 and 10% of the appropriate serum for secondary antibody staining . Slices were then incubated for 24 h (or 48 h for MeCP2 staining) in the dark at 4º C using the indicated antibodies in 0.01 M PBS containing

0.01% sodium azide, 0.5% Triton X-100 and 2 mg/ml BSA. Antibodies used were 1:1000 Chicken-anti-MeCP2 (Millipore), 1:300 Goat-anti-Iba1 (Abcam), 1:300 Rat-anti-CD169 (Biorad), 1:300 Rabbit-anti-CC3 (Cell Signaling), 1:100 Rabbit-anti-CD163 (Santa Cruz), 1:200 Rat-anti-F4/80 (AbD Serotec), and 1:100 a647-anti-CD31 (BD Biosciences). After staining, slices were washed with 0.01 M PBS 3 times for 5 m on a shaker. Tissue was then incubated for 1 h at RT with the appropriate fluorescent secondary antibodies (Invitrogen) at 1:1000 in the same buffer used for primary antibody staining. Slices were then stained for 1 m in 1:20,000 DAPI diluted in PBS, washed with 0.01 M PBS 3 times for 5 m on a shaker, and then mounted on slides with Aquamount (Thermo Scientific) for microscopy.

Sholl analysis

Sholl analyses were made using the Linear Sholl Method Plugin for ImageJ. At least 40 microglia were analyzed for each region, with Sholl shells established in 5 μm intervals, 10 μm from the soma center to the longest observed process length. Briefly, representative images from at least three 40 μm sections of a given brain region were used in n = 3 mice for each group. Slices were stained 1:300 with Goat-anti-Iba1 (Abcam) followed by secondary staining at 1:1000 in Chicken-anti-Goat antibody conjugated to Alexa Fluor 647 (Invitrogen). Z stacks were obtained using a Leica SP8 confocal system to profile entire microglia in the Z direction. Z planes were taken every μm, with max projections utilized for Sholl analysis and detection of soma size.

Quantification of meningeal macrophages

After staining (see ***Immunohistochemistry*** supplemental methods section) 1.35mm^2 images of meninges stained with CD163, F4/80, CD31, and DAPI were taken with a Leica SP8 confocal microscope and blinded for quantification. The total number of F4/80$^+$CD163$^+$ and F4/80$^+$CD163$^-$macrophages were counted per 1.35mm^2 area and used for analysis.

Intestine preparation for flow cytometry

Mice were perfused with heparinized PBS and the small intestine was removed (duodenum, jejunum and ileum), and then slit along the entire length longitudinally to expose the interior. Intestines were washed 3 times in a petri dish containing HBSS + 5% FBS + 2 mM EDTA to remove fecal contents. Intestines were then placed in 20 ml HBSS + 5% FBS + 2 mM EDTA at 37ºC on a shaker at 250 RPM for 20 m, two times. Small intestines were drained through a nylon mesh and finely minced, then placed in 20 ml HBSS + 1.5 mg/ml collagenase VIII (Sigma) + 40 u/ml DNAse-I (Sigma) for 15 m on a shaker at 200 RPM. The resulting suspensions were vortexed for 20 seconds and then passed through a 70 μm cell strainer into a 50 ml conical tube. The conical tube was then filled to the top with HBSS + 5% FBS + 2 mM EDTA and centrifuged at 4ºC, 425 RCF, for 5 m. The supernatant was decanted and the pellet was resuspended in HBSS + 5% FBS + 2 mM EDTA. Resuspended pellets were centrifuged at 4ºC, 425 RCF, for 5 m. The supernatant was decanted and the pellet was resuspended in flow cytometry buffer (0.01 M PBS, 1% BSA, 2 mM EDTA, 0.1% sodium azide).

Peritoneal macrophage collection for RNA-seq

Mice were euthanized via CO_2 and the skin over the peritoneum was removed. The peritoneal cavity was filled with sterile 0.01M PBS containing 2% FBS, and immediately clamped shut with forceps. Filled peritoneal cavities were manually agitated for 1 m, followed by collection of peritoneal lavage fluid via syringe. Cells were then pelleted, and depleted of non-macrophages using biotin-anti-CD3, biotin-anti-B220, and biotin-anti-Ly6g antibodies followed by incubation with streptavidin magnetic beads (Miltenyi) and negative depletion via AutoMACS, which resulted in ~90% pure peritoneal macrophages ($CD11b^{+}F4/80^{+}$). RNA was collected by RNeasy mini kit (Qiagen).

Processing and analysis of blood samples

After euthanasia blood was collected via the retinal artery after removal of the eye. For cell counts, a drop of blood was collected in a heparinized tube followed immediately by counting of a 2 μl aliquot diluted in 38 μl of an acridine orange and propidium iodide solution to label and enumerate live nucleated cells (leukocytes) on a Nexcelom Cellometer Auto 2000. Blood was additionally collected for flow cytometric analysis. For flow cytometry, blood samples were RBC lysed using ACK lysis buffer (5 m incubation followed by wash in 0.01 M PBS). RBC-lysed samples were then labeled for flow cytometric analysis as described previously. Counts were obtained by calculating the product of the whole blood cell count measured at the time of blood collection and the percentage of each cell population observed by flow cytometry.

Clodronate and DiI liposome injection

Clodronate and DiI-labeled liposomes were prepared by a reverse phase evaporation technique as previously described (Faustino et al., 2011) with minor modifications (200 mg/ml initial clodronate concentration; DOPC:Cholesterol 20:10 mg/ml final lipid concentration). Unentrapped clodronate was removed by centrifugation in a microfuge. Liposome size adjustment was achieved by differential centrifugation. Wild type and Mecp2-null littermates, 6-7 weeks old, were injected via tail vein with 100 µl clodronate containing liposomes diluted with sterile saline to 200 µl total volume. After 48 h, mice were injected via tail vein with 100 µl DiI liposomes diluted with sterile saline to a 200 µl total volume. Mice were sacrificed on day 5 post-clodronate liposome injection and analyzed for monocyte reconstitution and maturation into Ly6c^{lo} resident monocytes.

Chromatin immunoprecipitation DNA sequencing (ChIP-Seq) analysis

For ChIP-Seq analysis formalin-fixed cells were processed as described (Litvak et al., 2012). BMMs were cross-linked for 10 min. in 1% paraformaldehyde, washed, lysed and processed for ChIP using anti-rabbit IgG Dynabeads (Invitrogen) pre-conjugated with antibodies against either HDAC3 (sc-11417) (Santa Cruz Biotechnology) or NCOR2 (PA1-843) (Thermo Scientific). The purified ChIP DNA was prepared for sequencing with the Illumina ChIP-Seq Sample Prep kit and processed in according to the manufacturer's protocol. The ChIP-Seq data was aligned to the mouse genome (NCBI37/mm9; July 2007) using the ELAND alignment software (Illumina). Regions where the ChIP signals were enriched relative to the normal rabbit serum (NRS) control were determined as described (Litvak et al., 2012; Ramsey et al., 2010). We used a false

discovery rate of less than 1%. Integrated Genome Browser (IGB) (Nicol et al., 2009) was used to display the ChIP-Seq data.

Quantitative real-time PCR

For measurement of the expression of mRNA transcripts total RNA was collected by TRIzol (Invitrogen) for BMDM and resident peritoneal macrophage assays. RNA was collected by Rneasy mini kit (Qiagen) for qRT-PCR of acutely isolated microglia. RNA was reverse-transcribed and analyzed by real-time PCR with TaqMan Gene Expression assays (Applied Biosystems) using the following TaqMan primers: *Cxcl2* (Mm00436450_m1); *Cxcl3* (Mm01701838_m1); *Cyr61* (Mm00487499_g1); *Ddit4* (Mm00512504_g1); *Hif3a* (Mm00469375_m1); *Tnf* (Mm00443258_m1); *Il6* (Mm00446190_m1), *Fkbp5* (Mm00487406_m1), *Il1b* (Mm00434228_m1), *Csf3* (Mm00438335_g1), *Ptx3* (Mm00477268_m1), *Cp* (Mm01289313_m1), *Saa3* (Mm00441203_m1), *Hp* (Mm00516884_m1), *Ccl3* (Mm00441259_g1), *Cxcl1* (Mm04207460_m1), *Ccl2* (Mm00441242_m1), and *Il1a* (Mm00439620_m1). Data were acquired using a ViiA™ 7 Real-Time PCR System (Applied Biosystems) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) and were normalized to the expression of *Eef1a1* mRNA transcripts (encoding eukaryotic translation elongation factor 1 α1) in individual samples for analysis performed in the Litvak lab. Studies performed in the Kipnis lab were normalized to *Hprt* transcripts (Mm01545399_m1) and collected using a CFX384 Real-Time PCR Detection System (Bio-Rad).

Normoxia vs. Hypoxia treated BMDM

Day 7 L929 derived wild type and Mecp2-null BMDMs were grown in either a normoxic tissue culture incubator at 21%-O2, 5%-CO_2 or a humidified hypoxic chamber at 1%-O_2, 5%-CO_2 (Invivo2 200 Hypoxia workstation Biotrace INC) for 24 h. mRNA was isolated for qPCR analysis.

TNF stimulated BMDM

Day 7 L929 derived wild type and Mecp2-null BMDMs were grown in either a normoxic tissue culture incubator at 21%-O2, 5%-CO_2. Purified TNF (10 ng/ml) (eBioscience) was added to the media and incubated for 6 h. mRNA was isolated for qRT-PCR analysis.

Analysis of BMDM proliferation per monocyte

400,000 bone marrow cells from wild type or Mecp2-null counterparts (n = 3 per group) were cultured with 10ml media and anti-anti (Invitrogen) and 10ng/ml MCSF (eBioscience) in 10cm petri dishes, with a second dose of 20ng/ml MCSF in 5ml media added on day 3 of culture. Cells were grown in DMEM/F12 with 10% FBS and anti-anti (Invitrogen). Number of macrophages generated per starting monocyte was calculated by multiplying the percentage of $Ly6c^{hi}CCR2^{+}CD115^{+}$ monocytes in the initial bone marrow by the starting number of total bone marrow cells (400,000). At the indicated time points, the number of macrophages generated from a single plate was counted and divided by the starting number of monocytes to arrive at the number of macrophages generated per monocyte.

Peritoneal macrophage collection for intraperitoneal TNF injection experiments

For intraperitoneal TNF experiments, a protocol optimized for the collection of TNF-treated peritoneal macrophages was used. TNF, carrier free (eBioscience) or an equivalent amount of saline was injected into the peritoneum at a dose of 50μg/kg (1μg in a 20g mouse). After 6 h, mice were euthanized via CO_2 and the skin over the peritoneum was removed. The peritoneal cavity was filled with sterile 0.01M PBS containing 2mM EDTA, and immediately clamped shut with forceps. Filled peritoneal cavities were manually agitated for 1 m, followed by collection of peritoneal lavage fluid via syringe. Cells were then pelleted, and positively selected for $F4/80^+$ macrophages using F4/80-PE (eBioscience) followed by incubation with anti-PE magnetic beads (Miltenyi) and positive selection via AutoMACS, which resulted in ~70-80% pure peritoneal macrophages ($CD11b^+F4/80^+$). RNA was collected by TRIzol (Invitrogen).

GCSF neutralizing antibody treatment

Treatment began at 6-7 w in either Mecp2-null or wild type controls. Injections with either 5 mg/kg GCSF neutralizing antibody (clone 67604, R&D Systems), or Rat IgG1 isotype control (clone 43414, R&D Systems) were performed every 48 h. For flow cytometric analysis, mice were analyzed one to two weeks after the start of treatment.

Supplemental References

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