Supplementary figures and methods

The JMJD3-IRF4 axis regulates M2 macrophage polarization and host responses against helminth infection

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Contents:

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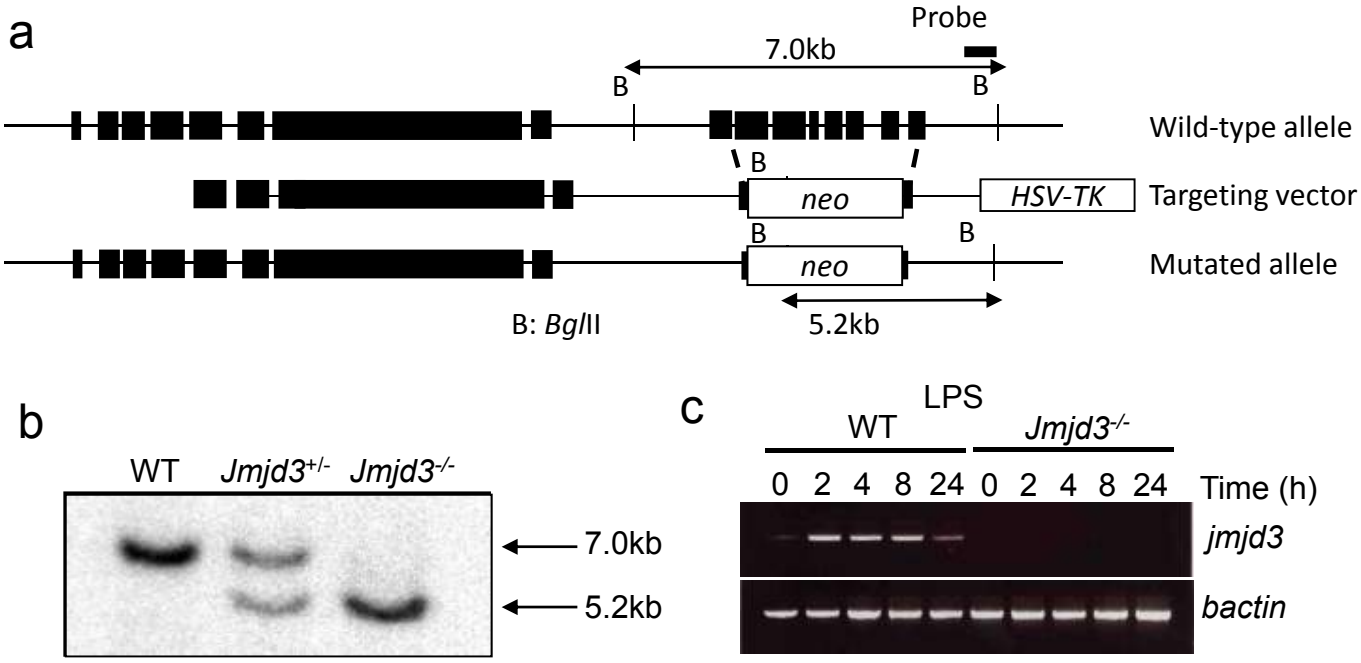

Supplementary Fig. 1 Generation of *Jmjd3*$^{-/-}$ mice. (a) Schematic representation of the mouse Jmjd3 gene, the targeting vector and the targeted allele. A targeting vector was designed to replace exon14 to exon21 containing the JmjC H3K27demethylase domain, with neomycin-resistance gene B.*Bgl* II (b) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extraceted from MEFs, digested with *Bgl* II, separated by electrophoresis and hybridized with the radiolabelled probe indicated in (a). A southern blot gave a single 7.0kb band for wild type(+/+), a 5.2kb band for homozygous (–/–) and both bands for heterozygous (+/–) mice. (c) RT-PCR analysis of RNA from Wild-type (WT) and *Jmjd3*$^{-/-}$ MEFs stimulated with LPS (1 μg/ml) for indicated periods. The RNAs were subjected to RT-PCR analysis for the expression of *Jmjd3* mRNA. The expression of β-actin gene was analyzed with the same RNA.

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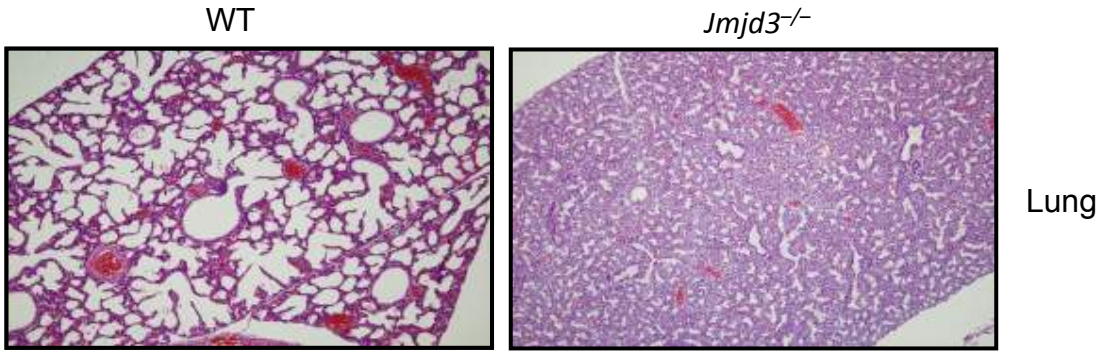
a

	WT	*Jmjd3*$^{+/-}$	*Jmjd3*$^{-/-}$	Total
Post -natal (%)	33 (42)	45 (58)	0 (0)	78 (100)

b



Supplementary Fig. 2 *Jmjd3*$^{-/-}$ mice show postnatal lethal phenotype due to premature development of lung tissues.

(a) Genotyping analysis of live neonatal offsprings from the heterozygotes intercrosses.

(b) H&E staining of lung sections of wild-type and *Jmjd3*$^{-/-}$ mice. The data are representative of three individual mouse lungs.

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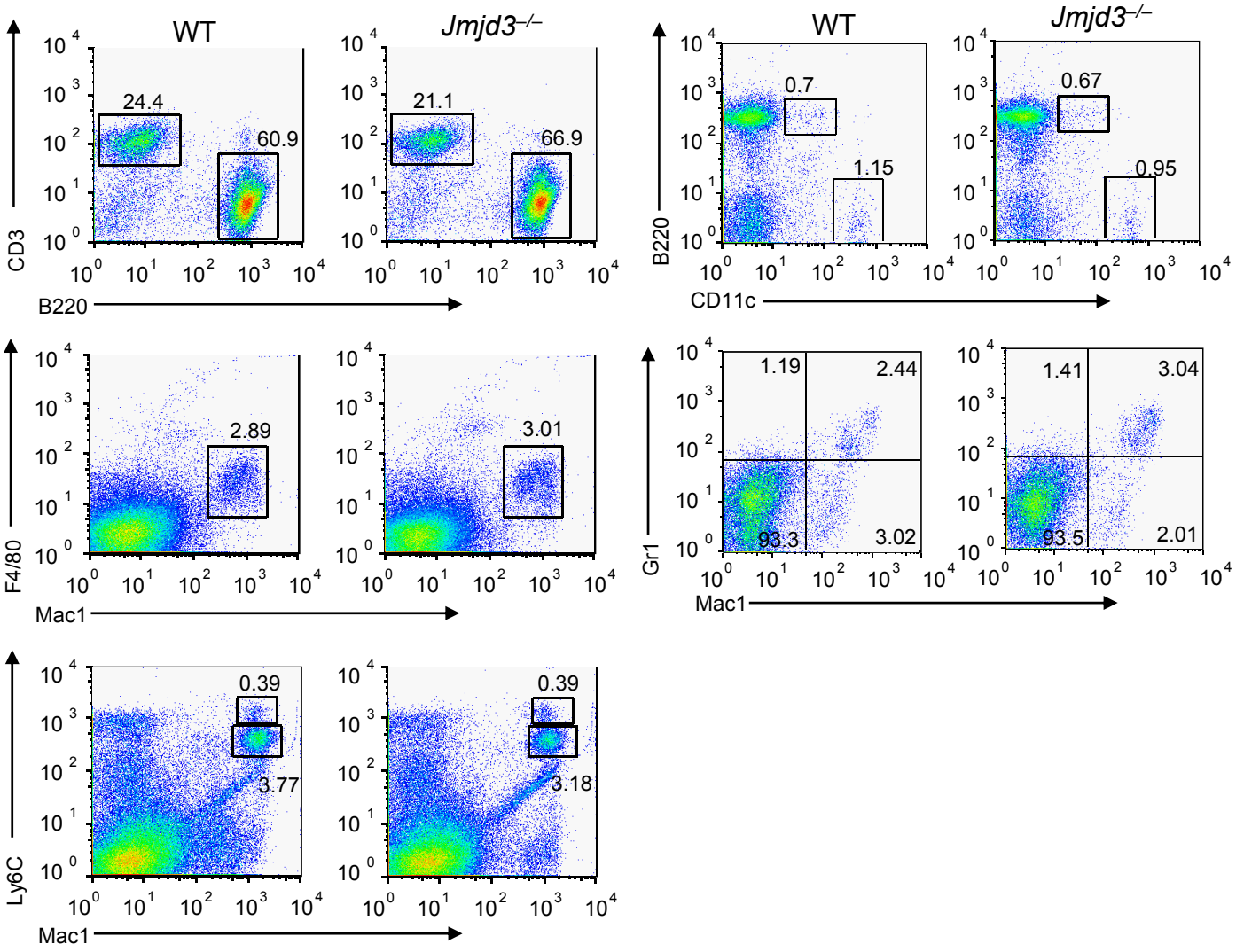
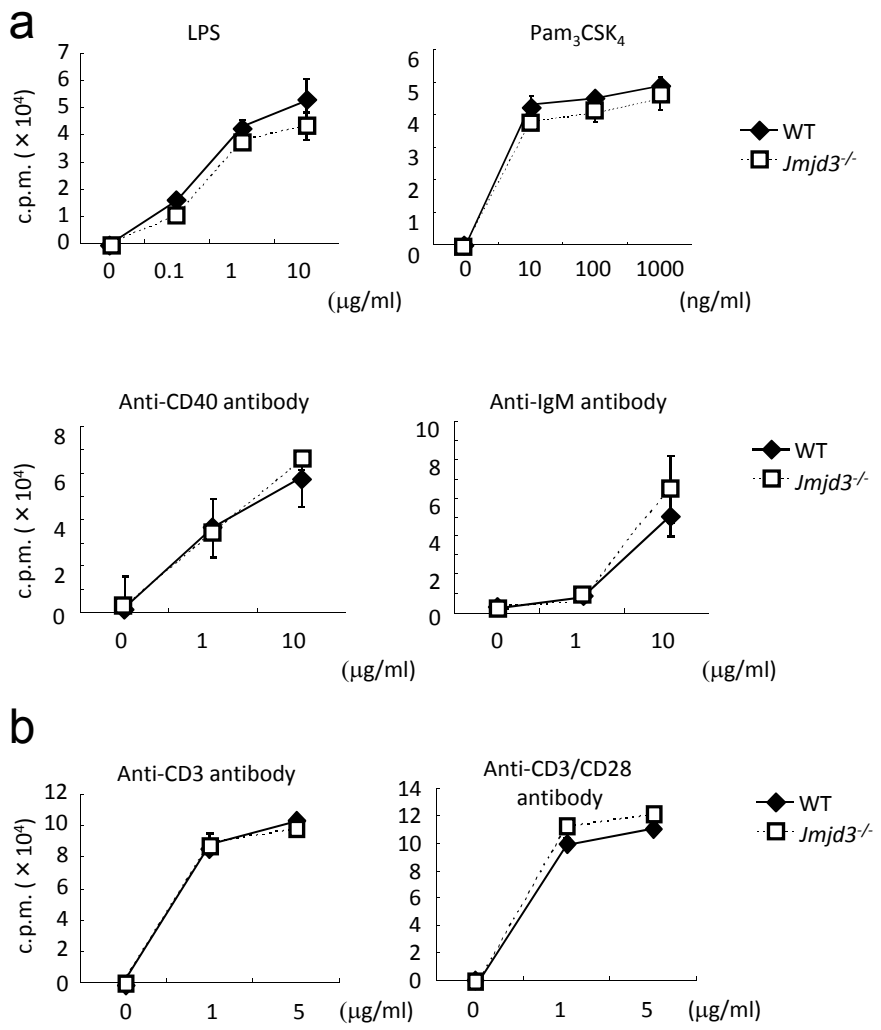


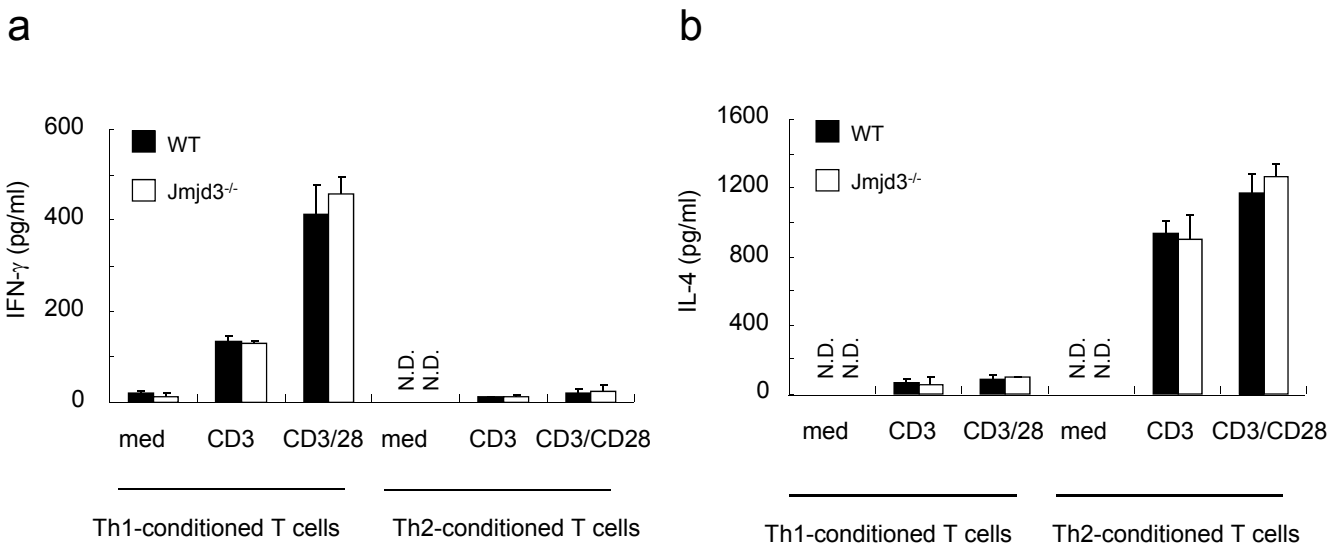
Supplementary Fig. 3 Flow cytometric analysis of *Jmjd3*$^{-/-}$ mouse splenocytes.

Splenocytes from WT and *Jmjd3*$^{-/-}$ mice were stained with indicated antibodies and were analyzed by flow cytometry. Numbers indicate the percentages of cells in the quadrants or squares. Results are representative of two to three independent experiments.



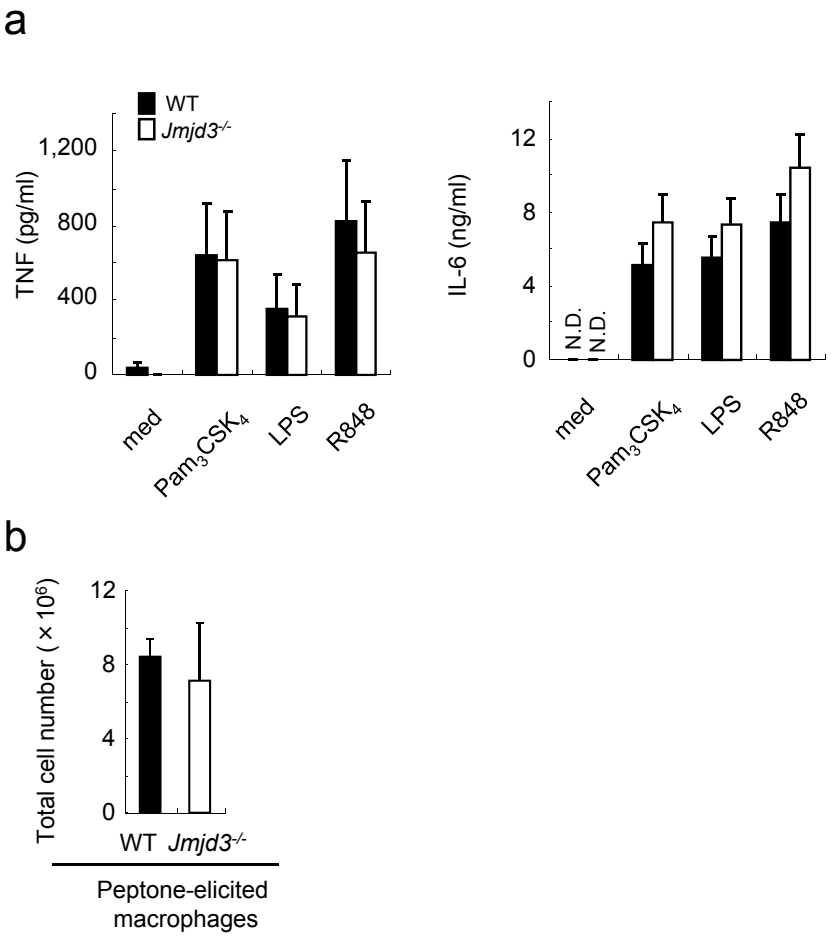
Supplementary Fig. 4 Normal proliferation of B cells and T cells in response to mitogens and antigen receptor stimuli in *Jmjd3*$^{-/-}$ mice.

(a) Splenic B cells obtained from WT and *Jmjd3*$^{-/-}$ mice were stimulated with indicated concentrations of LPS, Pam_3CSK_4, anti-CD40 antibody and anti-IgM antibody for 48h. Proliferation was determined by ^{3}H-thymidine incorporation.
(b) Splenic T cells obtained from WT and *Jmjd3*$^{-/-}$ mice were stimulated with indicated concentraions of anti-CD3 antibody and anti-CD3/CD28 antibody for 48 h. Proliferation was determined by ^{3}H-thymidine incorporation. The results are representative of three independent experiments (error bars, s.d.).



Supplementary Fig. 5 Normal production of IFN-γ and IL-4 in response to TCR stimulation in Th1 and Th2 cells in *Jmjd3*$^{-/-}$ mice.

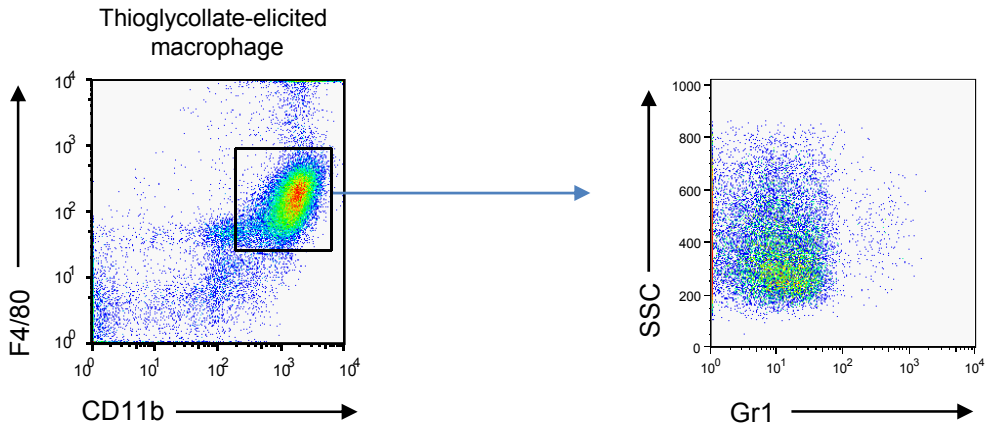
Splenic T cells obtained from WT and *Jmjd3*$^{-/-}$ mice were cultured under Th1 conditions (10 μg/ml anti-CD3 antibody, 10 μg/ml IL-12 and 10 μg/ml neutralizing anti-IL-4 antibody) or Th2 conditions (10 μg/ml anti-CD3 antibody, 1000 U/ml IL-4 and 10 μg/ml neutralizing anti-IFN-γ antibody) for 5days. Then the cells were harvested, washed 3 times and stimulated with anti-CD3 antibody (10 μg/ml), and co-stimulation with anti-CD3 antibody (10 μg/ml) and anti-CD28 antibody (10 μg/ml) for 24 hours. The concentrations of IFN-γ (a) and IL-4 (b) and in the culture supernatant were measured by ELISA.



Supplementary Fig. 6 Cytokine responses to TLR ligands and number of macrophages in peptone-elicited PEC from wild-type and *Jmjd3*$^{-/-}$ mice.

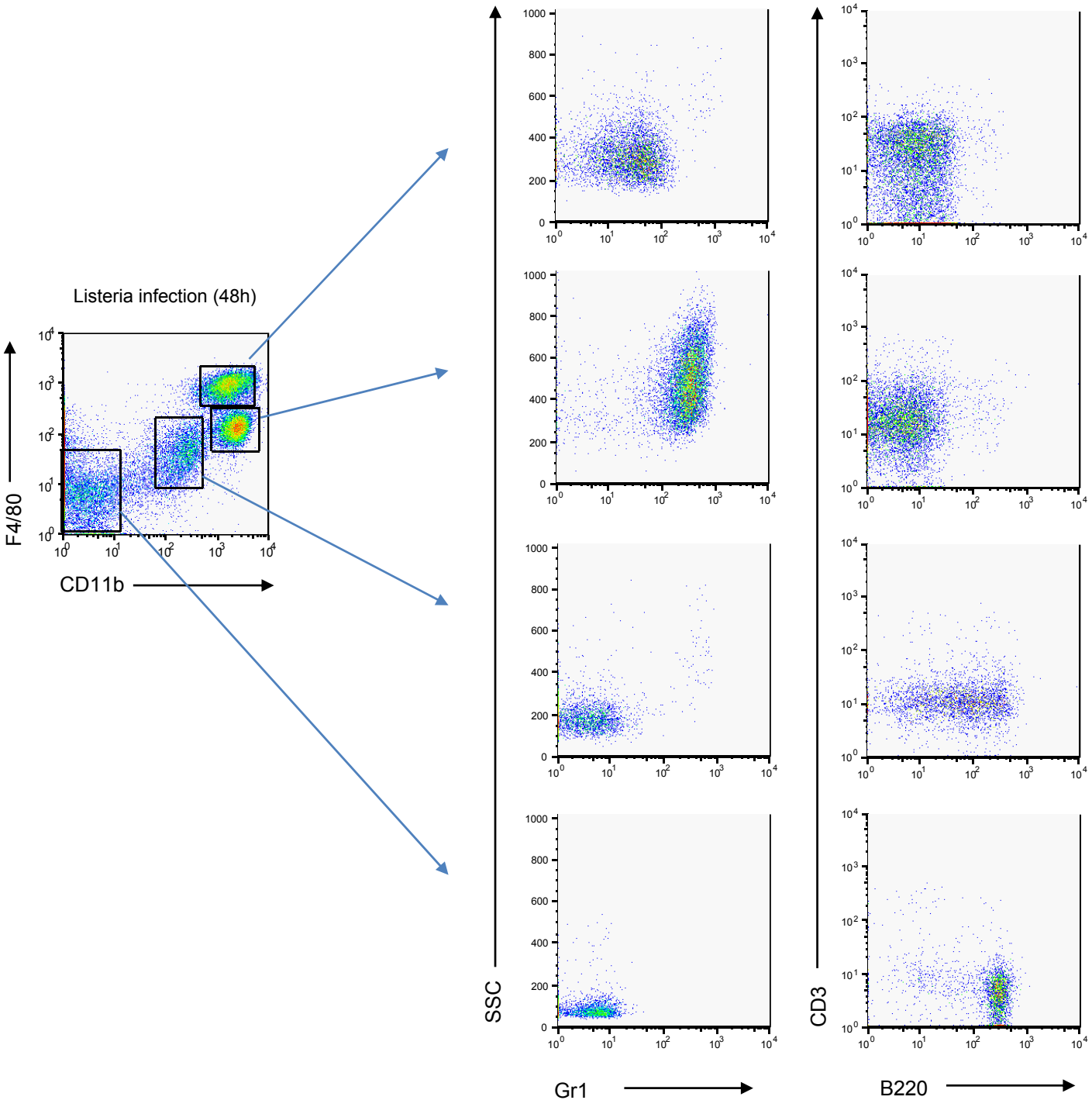
(a) PEC were prepared from wild-type and *Jmjd3*$^{-/-}$ chimeras 4 days after intraperitoneal administration of 10% peptone. Then the cells were stimulated with the indicated TLR ligands and production of TNF and IL-6 was measured by ELISA.

(b) Total numbers of F4/80^{+}CD11b^{+} macrophages in peptone-elicited PEC were counted in WT and Jmjd3-$^{-/-}$ chimeras. The data are representative of two independent experiments.



Supplementary Fig. 7 The expression levels of Gr1 in F4/80^{+}CD11b^{+} thioglycollate-elicited macrophage.

Thioglycollate-elicited macrophages from wild-type mice were analyzed by flow cytometry using indicated Abs.



Supplementary Fig. 8 Listeria infection induced macrophages as well as neutrophils to peritoneal cavity .

Intraperitoneal infection of listeria recruited F4/80^{+}Mac1^{+} macrophages (Gr1^{-}) F4/80^{dull}Mac1^{+} neutrophills (Gr1^{+}) to the peritoneal cavity after 48 hours in wild-type and Jmjd3$^{-/-}$mice.

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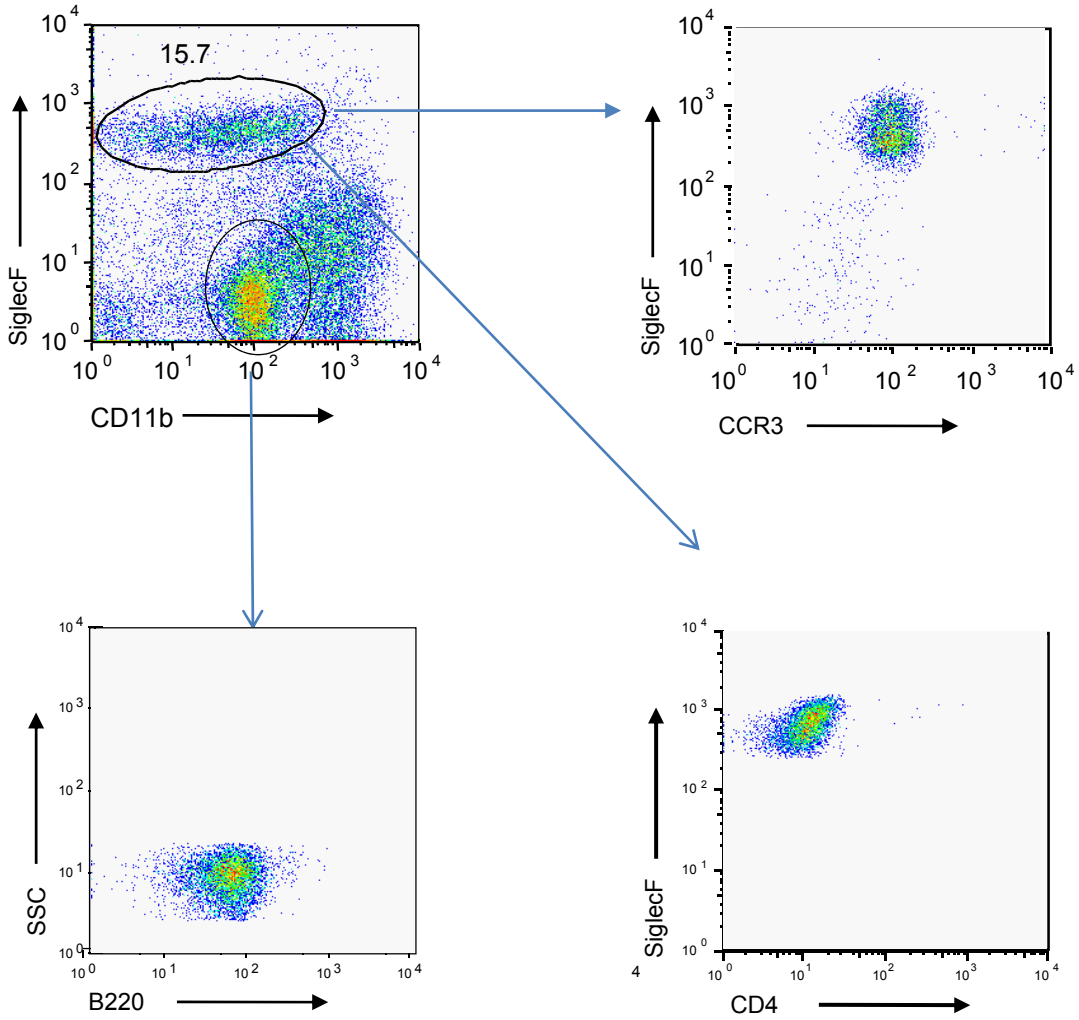
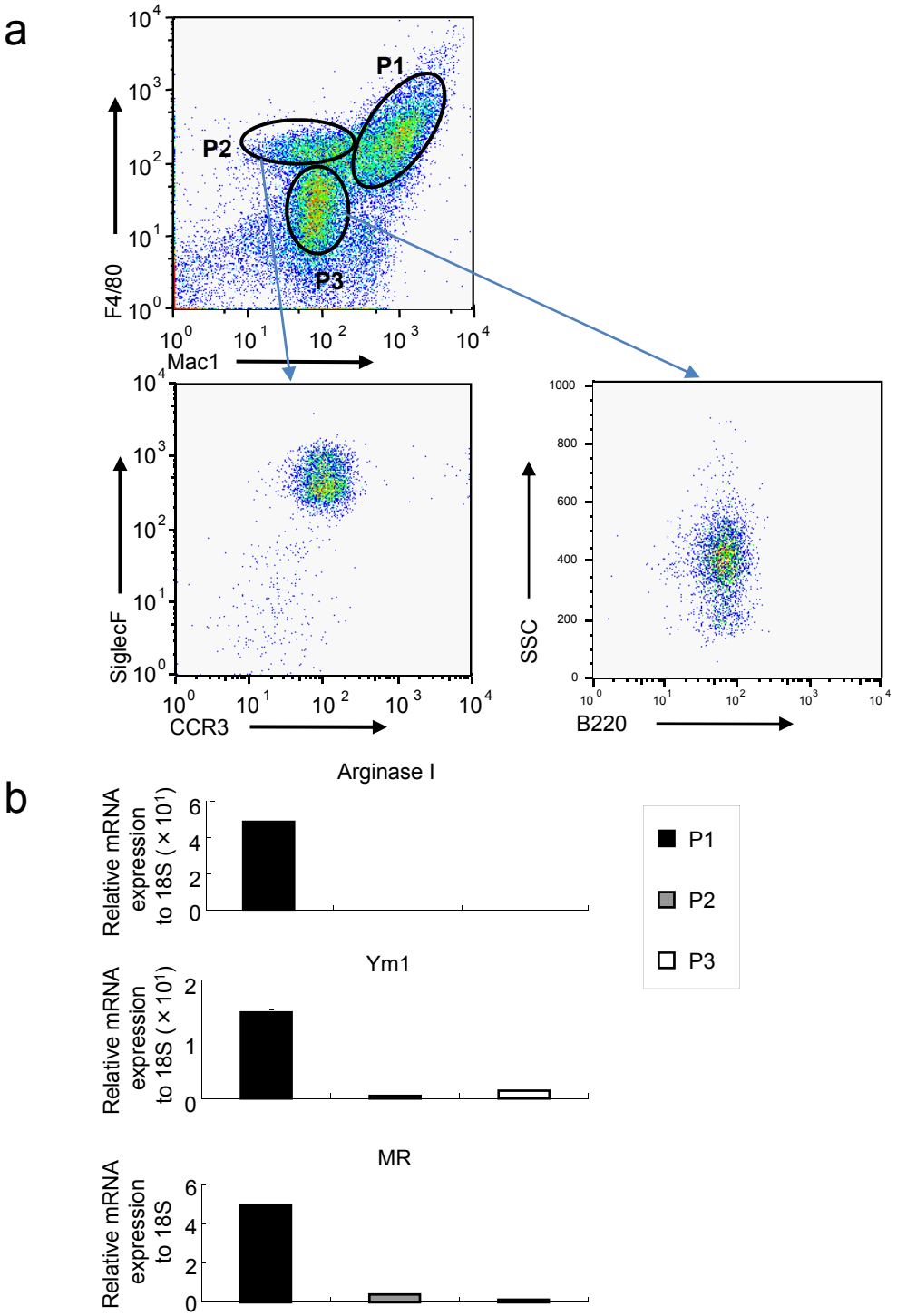


Supplementary Fig. 9 Cell types recruited to peritoneal cavity in response to Chitin administration.

PEC were prepared from wild-type mice 48 h after intraperitoneal administration, and flow cytometry analysis was performed. Siglec-F^{+} cells also expressed CCD3, but not CD3. CD11b^{int}SiglecF^{-} cells also expressed B220.

The data are representative of two independent experiments.



Supplementary Fig. 10 Chitin-elicited macrophages, but not eosinophils and B cells, highly expressed mRNA encoding M2 macrophage markers.

(a) Intraperitoneal administration of chitin recruited F4/80^{+}Mac1^{+} macrophages (P1), Siglec-F^{+}CCR3^{+} eosinophils (P2) and Mac1^{int}B220^{+}CD5^{-} B cells (P3) to the peritoneal cavity after 48 hours in wild-type mice. (b) Total RNA prepared from each population (P1, P2 and P3) were extracted and subjected to QPCR analysis for the expression of indicated M2 markers. The data are representative of two independent experiments.

Supplementary methods

Microarray analysis

Total RNA was isolated from wild-type and *Jmjd3*$^{-/-}$ M-BMM using TRIzol RNA isolation kit (Invitrogen) and further purified using an RNeasy kit (Qiagen). Biotinylated cDNA was synthesized from 100 ng total RNA with the Ovation biotin RNA amplification and labeling systems (Nugen) according to the manufacturer's protocol. The product was purified using an DyeEx 2.0 spin kit (QIAGEN), fragmented, and hybridized to Affymetrix mouse expression array A430 2.0 microarray chips, according to the manufacturer's protocol (Affymetrix). Staining, washing and scanning of Affymetrix mouse Genome 430 2.0 microarray chips was done following the manufacturer's instructions. Robust multichip average (RMA) expression values were calculated using R and the Bioconductor affy package. For each probe the changes in expression between wild-type and *Jmjd3*$^{-/-}$ samples were defined as the difference between $\log_2$ values for wild-type and *Jmjd3*$^{-/-}$ M-BMM. Genes were assigned the values of their corresponding probe(s). In cases where a gene was associated with multiple probes the average value was taken.

Chromatin immunoprecipitation-sequencing (ChIP-seq)

M-BMM were prepared and 5×10^6 macrophages were fixed with 10% formaldehyde for crosslink histones to DNA directly. Cells were washed twice using ice cold PBS containing complete mini and then lysis buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl (pH=8.1)) was added. After harvesting cells into a conical tube, sonication (TOMY UR-20P) was performed to obtain the DNA fragments. Fragment lengths peaked between 150 and 300 bps. An antibody against histone H3K27me3 (Millipore 07-449) was pre-bound overnight to 80μl of Dynabeads (VERITAS) in PBS/BSA 0.5%. Beads

were then added to lysates and incubation was allowed to proceed overnight. Beads were washed once with High salt immune complex wash buffer (Millipore) and Low salt immune complex wash buffer (Millipore), seven times using LiCl immune complex wash buffer (Millipore) and once in TE containing 50mM NaCl. DNA was eluted in elution buffer (1%SDS 10mM EDTA, 50mM Tris-HCl (pH=8.1)) and cross links reversed by incubation overnight at 65℃. After RNase A and Proteinase K treatment, DNA was then purified via ethanol precipitation using Etachinmate (Nippongene). Yields were ~100 ng/5$\times 10^6$ macrophages. Two hundred ng of DNA was used for the fragment library. P1 and P2 adaptor ligation and all subsequent steps were performed on all samples according to the SOLiD 3 Plus System Fragment Library Preparation protocol. Templated bead generation for each library was performed according to the SOLiD 3 Plus System Templated Beads Preparation Guide full-scale protocols. Each sample was deposited on 6 spots of the slide at a target bead density of 160-200 K beads/panel. High throughput sequencing was performed using the SOLiD 3 Plus System and analysis of 50 bp reads was carried out.

Analysis of ChIP-Seq data

For each ChIP-Seq dataset, uniquely mapped ChIP-Seq tags with at most 4 mismatches were considered for further analysis. Locations of tags were shifted by +100 bps from their 5' ends, in order to reflect the center of the original DNA fragment corresponding to each tag.

1. Overall H3K27 methylation in different regions of genes in M-BMM

To obtain an overall picture of the H3K27me3 distribution, we collected the tags mapped to transcribed regions (based on the genomic-wide July 2007 mm9 RefSeq mouse gene annotations in the UCSC database [44], http://genome.ucsc.edu/), and their upstream and downstream regions (30kb each). Upstream and downstream regions were separated into 30 bins of 1kb each, and transcribed regions were separated into 50 bins

of equal size. ChIP-Seq tags mapped to each bin were counted for both wild-type and *Jmjd3*$^{-/-}$ M-BMM. The ratio of tag counts in samples versus unimmunoprecipitated control data was calculated for each bin.

2. Correlation between ChIP-Seq data and gene expression data.

For each gene *x*, the number of tags mapped within the transcribed regions was counted for both ChIP-Seq experimental samples and control samples (Cmod (x) and Ccontrol (x), respectively). The intensity of histone modification was calculated as follows:

$$Intensity\ (x) = \log 2\left(\frac{C_{\mathrm{mod}}\ (x)}{C_{control}\ (x)} * \frac{N_{control}}{N_{\mathrm{mod}}} \right)$$

where Nmod and Ncontrol are the total counts of tags uniquely mapped in the mouse genome. The correlation between histone modification and gene expression was assessed by Pearson's correlation.

Additionally, 16,090 RefSeq genes were classified into 20 bins by sorting the genes by their expression change. The average value of the modification intensity was calculated in each bin.

3. Assessment of H3K27me3 status in individual genes.

Significant tag peaks were defined using threshold tag counts based on an estimated false discovery rate (FDR) of 1e-6. The FDR was determined for each dataset separately in the following way. The genomic sequence was divided into bins of size 1 kb, in steps of 500 bps. The distribution of real tag counts per bin was obtained for each data set, and the distribution of random tag counts was estimated by randomly mapping the same number of tags as in the real data over the entire genome. From these two distributions, for each tag count p the FDR was calculated as

$FDR_p = R_p / O_p$

where R_p stands for the ratio of random peaks with ≥ p tags, and O_p for the ratio of observed (real) peaks with ≥ p tags. The threshold for significant tag peaks was set as the lowest tag count where FDR is ≤ 1e-6. This resulted in threshold tag counts of 18 for

wild-type and *Jmjd3*$^{-/-}$ M-BMM H3K27me3. Any bin with a tag count equal to or higher than these thresholds was regarded as significant.

Genes were classified based on the presence or absence of significant tag peaks for H3K27me3 in wild-type and *Jmjd3*$^{-/-}$ M-BMM in the region around their transcription start site. For each RefSeq gene, TSS locations were taken from the UCSC database, and the -5 kb to +1 kb region was divided into bins of size 1kb, in steps of 500 bps. Tag counts for each bin were counted. We defined three classes as follows: class 1 genes have one or more significant peaks in wild-type; class 2 genes have no significant peaks in both wild-type and *Jmjd3*$^{-/-}$; class 3 genes have one or more significant peaks in *Jmjd3*$^{-/-}$, but not in wild-type.

Reference

44. Rhead, B. *et al.* The UCSC Genome Browser database: update 2010. *Nucleic Acids Res* **38**, D613-619 (2010).