Fig S1

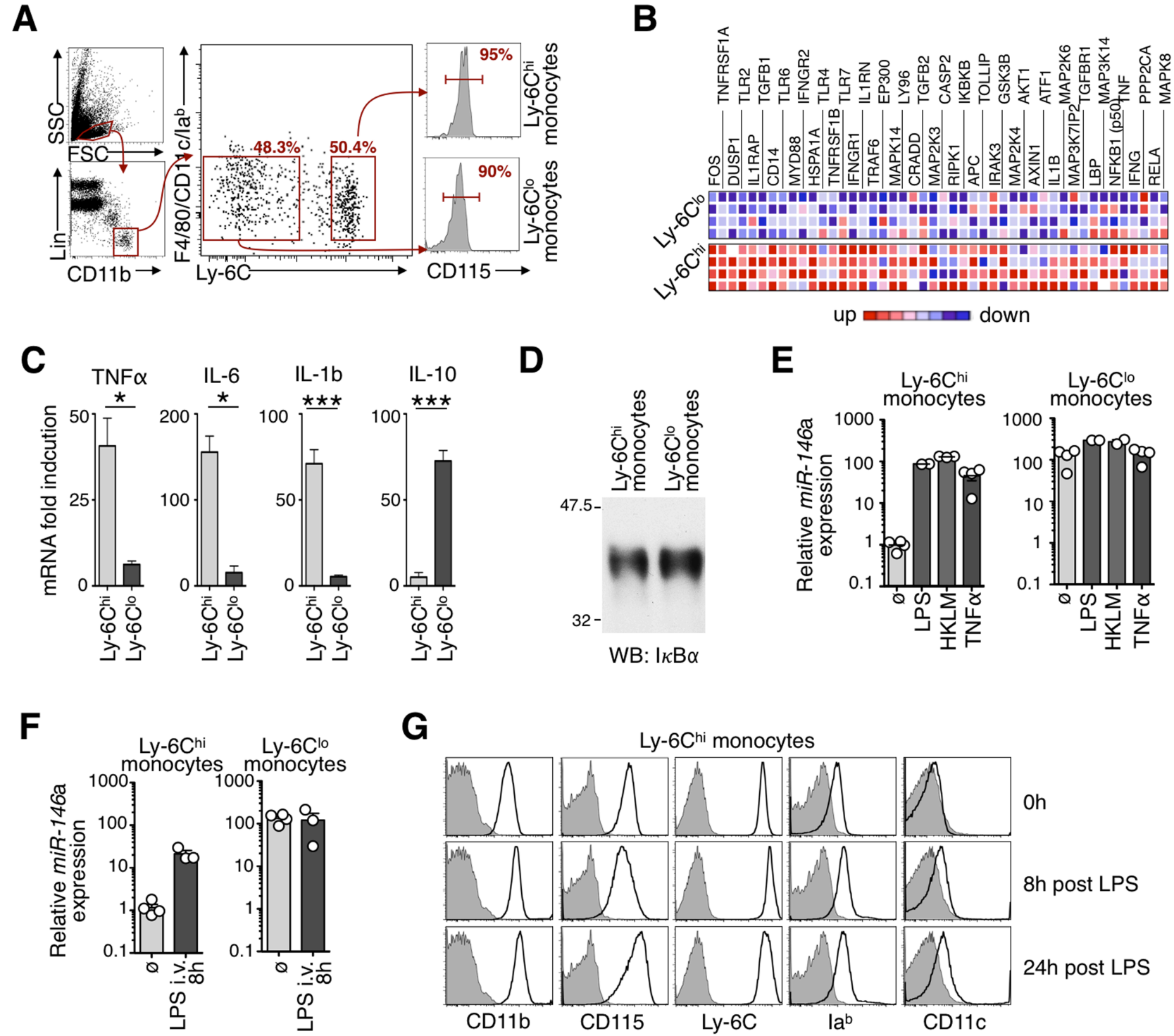

A
SSC
FSC
Lin
CD11b
F4/80/CD11c/Ia^b
Ly-6C
48.3%
50.4%
CD115
95%
90%
Ly-6C^hi monocytes
Ly-6C^lo monocytes
B
TNFRSF1A
TLR2
TGFB1
TLR6
IFNGR2
TLR4
TLR7
IL1RN
EP300
LY96
TGFB2
CASP2
IKBKB
TOLLIP
GSK3B
AKT1
ATF1
MAP2K6
TGFBR1
MAP3K14
TNF
PPP2CA
MAPK8
FOS
DUSP1
IL1RAP
CD14
MYD88
HSPA1A
TNFRSF1B
IFNGR1
TRAF6
MAPK14
CRADD
MAP2K3
RIPK1
APC
IRAK3
MAP2K4
AXIN1
IL1B
MAP3K7IP2
LBP
NFKB1 (p50)
IFNG
RELA
Ly-6C^lo
Ly-6C^hi
up
down
C
TNFα
IL-6
IL-1b
IL-10
*
*
***
***
mRNA fold indcution
50
25
0
200
100
0
100
50
0
100
50
0
Ly-6C^hi
Ly-6C^lo
D
Ly-6C^hi monocytes
Ly-6C^lo monocytes
47.5
32
WB: IκBα
E
Ly-6C^hi monocytes
Ly-6C^lo monocytes
Relative miR-146a expression
1000
100
10
1
0.1
ø
LPS
HKLM
TNFα
F
Ly-6C^hi monocytes
Ly-6C^lo monocytes
Relative miR-146a expression
1000
100
10
1
0.1
ø
LPS i.v. 8h
G
Ly-6C^hi monocytes
0h
8h post LPS
24h post LPS
CD11b
CD115
Ly-6C
Ia^b
CD11c

Fig S2

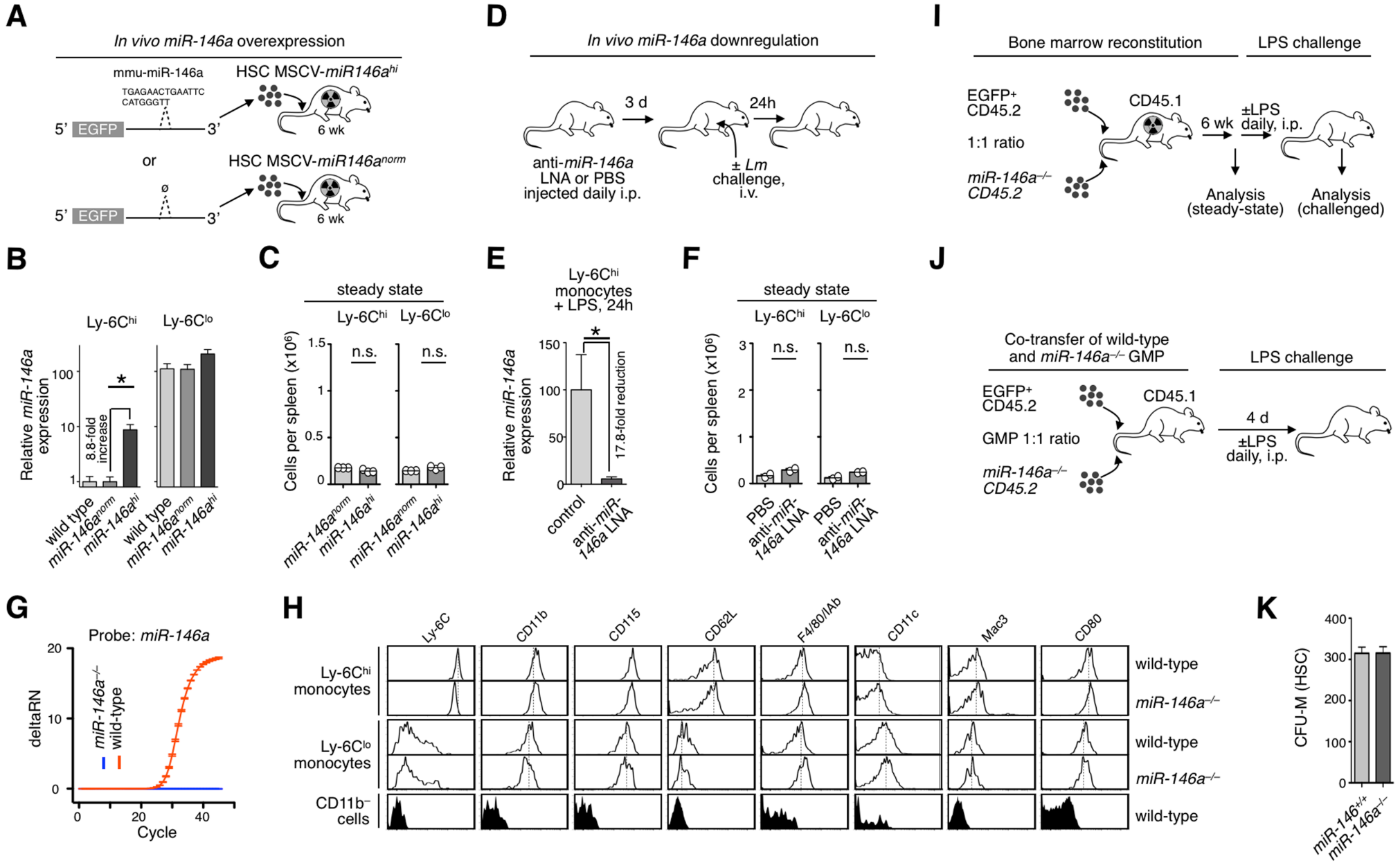

A
In vivo miR-146a overexpression
mmu-miR-146a
TGAGAACTGAATTC
CATGGGTT
5' EGFP 3'
HSC MSCV-miR146a^hi
6 wk
or
ø
HSC MSCV-miR146a^norm
6 wk
D
In vivo miR-146a downregulation
3 d
24h
anti-miR-146a LNA or PBS injected daily i.p.
± Lm challenge, i.v.
I
Bone marrow reconstitution
LPS challenge
EGFP+ CD45.2
1:1 ratio
miR-146a−/− CD45.2
CD45.1
6 wk
±LPS daily, i.p.
Analysis (steady-state)
Analysis (challenged)
B
Ly-6C^hi
Ly-6C^lo
Relative miR-146a expression
8.8-fold increase
wild type
miR-146a^norm
miR-146a^hi
C
steady state
Ly-6C^hi
Ly-6C^lo
Cells per spleen (x10^6)
n.s.
E
Ly-6C^hi monocytes + LPS, 24h
Relative miR-146a expression
17.8-fold reduction
control
anti-miR-146a LNA
F
steady state
Cells per spleen (x10^6)
PBS
anti-miR-146a LNA
J
Co-transfer of wild-type and miR-146a−/− GMP
LPS challenge
EGFP+ CD45.2
GMP 1:1 ratio
miR-146a−/− CD45.2
CD45.1
4 d
±LPS daily, i.p.
G
Probe: miR-146a
deltaRN
miR-146a−/−
wild-type
Cycle
H
Ly-6C
CD11b
CD115
CD62L
F4/80/IAb
CD11c
Mac3
CD80
Ly-6C^hi monocytes
Ly-6C^lo monocytes
CD11b− cells
wild-type
miR-146a−/−
K
CFU-M (HSC)
miR-146^+/+
miR-146a−/−

Fig S3

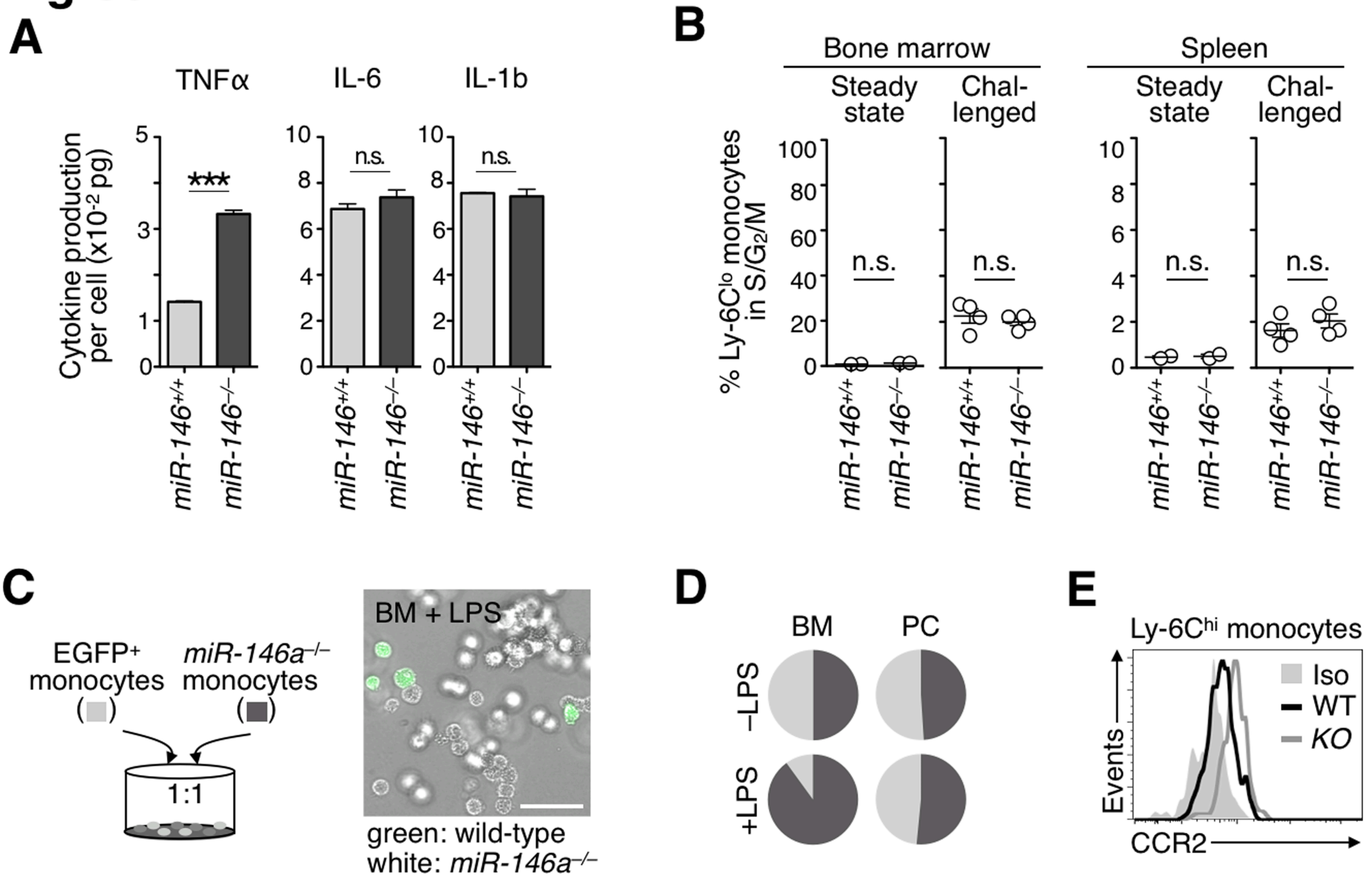
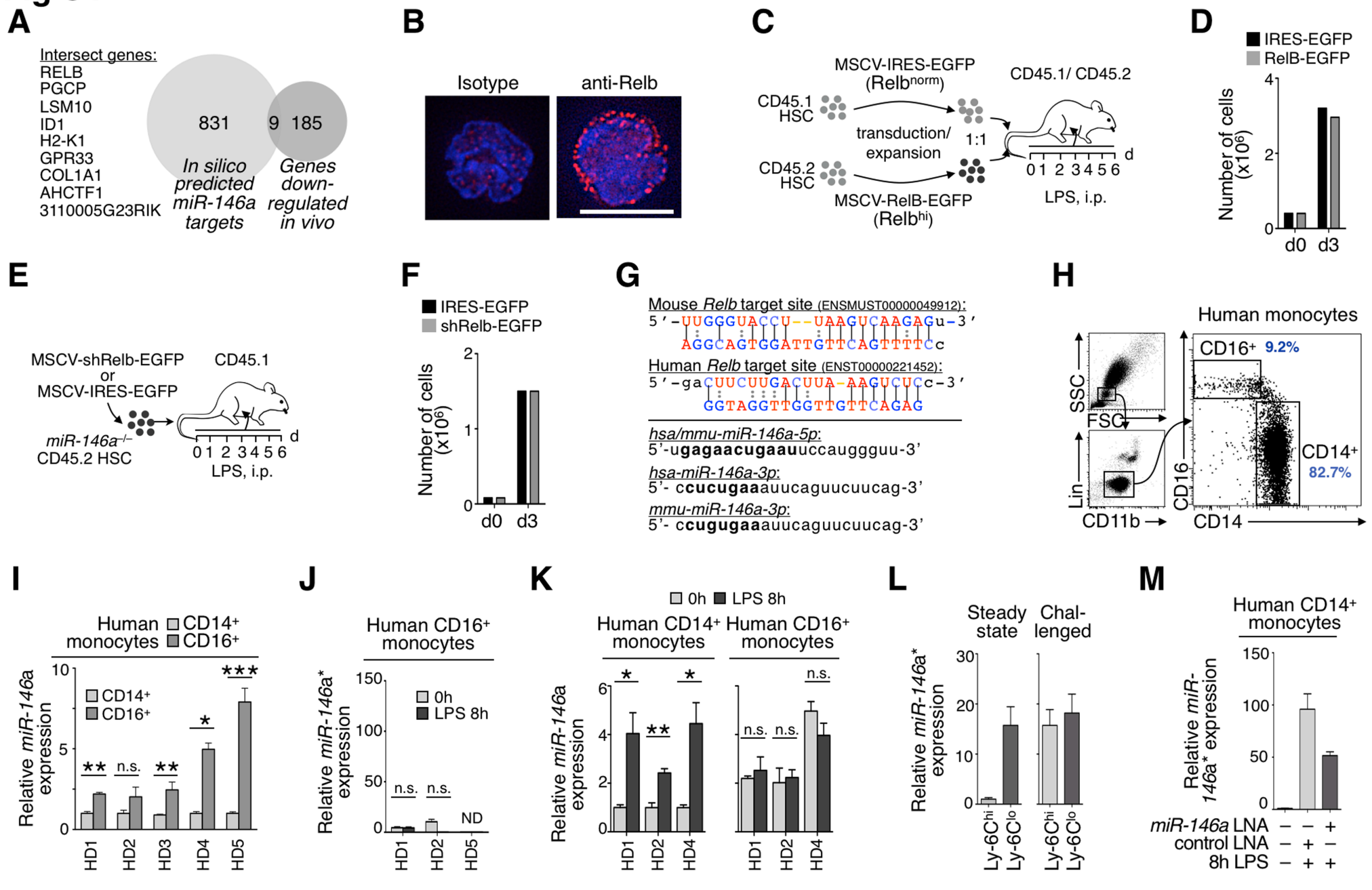

A
TNFα
IL-6
IL-1b
Cytokine production per cell (x10-2 pg)
***
n.s.
n.s.
miR-146+/+
miR-146–/–
B
Bone marrow
Spleen
Steady state
Chal-lenged
% Ly-6Clo monocytes in S/G2/M
n.s.
C
EGFP+ monocytes
miR-146a–/– monocytes
1:1
BM + LPS
green: wild-type
white: miR-146a–/–
D
BM
PC
–LPS
+LPS
E
Ly-6Chi monocytes
Iso
WT
KO
Events
CCR2

Fig S4


A
Intersect genes:
RELB
PGCP
LSM10
ID1
H2-K1
GPR33
COL1A1
AHCTF1
3110005G23RIK
831
9
185
In silico predicted miR-146a targets
Genes down-regulated in vivo
B
Isotype
anti-Relb
C
MSCV-IRES-EGFP (Relb^norm)
CD45.1 HSC
CD45.2 HSC
transduction/ expansion
1:1
MSCV-RelB-EGFP (Relb^hi)
CD45.1/ CD45.2
0 1 2 3 4 5 6 d
LPS, i.p.
D
IRES-EGFP
RelB-EGFP
Number of cells (x10^6)
d0 d3
E
MSCV-shRelb-EGFP or MSCV-IRES-EGFP
CD45.1
miR-146a^-/- CD45.2 HSC
0 1 2 3 4 5 6 d
LPS, i.p.
F
IRES-EGFP
shRelb-EGFP
Number of cells (x10^6)
d0 d3
G
Mouse Relb target site (ENSMUST00000049912):
Human Relb target site (ENST00000221452):
hsa/mmu-miR-146a-5p:
5'-ugagaacugaauuccauggguu-3'
hsa-miR-146a-3p:
5'- ccucugaaauucaguucuucag-3'
mmu-miR-146a-3p:
5'- ccugugaaauucaguucuucag-3'
H
Human monocytes
CD16+ 9.2%
CD14+ 82.7%
SSC
FSC
Lin
CD11b
CD16
CD14
I
Human monocytes CD14+ CD16+
Relative miR-146a expression
HD1 HD2 HD3 HD4 HD5
J
Human CD16+ monocytes
0h
LPS 8h
Relative miR-146a* expression
n.s.
ND
K
0h LPS 8h
Human CD14+ monocytes
Human CD16+ monocytes
Relative miR-146a expression
L
Steady state
Chal-lenged
Relative miR-146a* expression
Ly-6C^hi Ly-6C^lo
M
Human CD14+ monocytes
Relative miR-146a* expression
miR-146a LNA
control LNA
8h LPS

SUPPLEMENTAL INFORMATION

1. Extended Experimental Procedures

Mice

All experiments used age-matched animals of the C57BL/6 background. Some co-adoptive transfer and/ or co-culture experiments used C57BL/6-Tg(UBC-GFP)30Scha/J (Ub-GFP) mice and/ or B6.SJL-Ptprc^{a} Pepc^{b} / BoyJ (CD45.1) mice.

Monoclonal antibodies (mAbs), flow cytometry and cell sorting

The following monoclonal antibodies were used for mouse experiments: From BD Biosciences: PE-conjugated anti-CD90 (clone 53-2.1), PE-conjugated anti-B220 (clone RA3-6B2), PE-conjugated anti-CD49b (clone DX5), PE-conjugated anti-NK1.1 (clone PK136), PE-conjugated anti-Ly-6G (clone 1A8), PE-conjugated anti-CD11c (clone N418), APC-conjugated anti-CD11b (clone M1/70), APC-Cy7-conjugated anti-CD11b (clone M1/70), PE-Cy7-conjugated anti-F4/80 (clone BM8), Alexa Fluor 700-conjugated anti-CD11c (clone HL3), APC-conjugated anti-CD117 (clone 2B8), APC-Cy7-conjugated anti-CD16/32 (clone 2.4G2), PE-Cy7-conjugated anti-Sca1 (clone D7), Alexa Fluor 700-conjugated anti-CD34 (clone RAM34), FITC-conjugated anti-CD34 (clone RAM34), APC-conjugated anti-CD45.1 (clone A20), PerCP-Cy5.5-conjugated anti-CD45.2 (clone 104), biotin-conjugated anti-CD115 (clone AFS98), PerCP-conjugated streptavidin; from BD Pharmingen: PE-conjugated Ter119 (clone TER-119), PE-conjugated anti-CD19 (clone 1D3), PE-conjugated anti-CD11b (clone M1/70), APC-conjugated anti-mouse TNFα, FITC-conjugated anti-mouse CD62L (clone MEL-14). From R&D: APC-conjugated anti-mouse TNFα. From eBioscience: APC-conjugated EpCAM (clone G8.8), PE-conjugated CD207 (Langerin) (clone eBioL31). For cell cycle analysis FxCycle violet stain ('DAPI') was used (Invitrogen).

For mouse monocyte staining the lineage ('Lin') antibody mix contained the following PE-conjugated antibodies: anti-Ter119, anti-CD90, anti-B220, anti-CD19, anti-CD49b, anti-NK1.1, anti-Ly-6G. The gating strategy for mouse monocytes is described in Fig S1a. For progenitor staining the lineage ('Lin') antibody mix included the following PE-conjugated antibodies: anti-CD90, anti-B220, anti-CD19, anti-CD49b, anti-NK1.1, anti-Ly-6G, anti-CD11b, anti-CD11c, and anti-CD127. Hematopoietic stem cells (HSC) were defined as: Lin^{-} c-Kit^{+} Sca1^{+}. Granulocyte and macrophage progenitors (GMP) were defined as: Lin^{-}, CD117^{+}, Sca-1^{-}, CD34^{+}, CD16/32^{+}. Langerhans cells were defined as CD207^{+}, EPcam^{+}, CD11c^{+}, CD11b^{int}, MHCII^{int} (Alvarez et al., 2008). Splenic DC were defined as CD11c^{+}, CD11b^{+}, MHCII^{+} and either CD8^{+} or CD8^{-}.

The following monoclonal antibodies were used for human experiments: from R&D Systems: FITC conjugated antibodies specific for lineage markers: anti-CD3e (clone ucht1), NKp46 (clone 195314), for CCR2 staining: anti-mCCR2-PE (clone FAB5538P), Rat IgG 2B-PE (clone ICO13P); from BD Biosciences: anti-CD15 (clone hi98), anti-CD19 (clone hib19), and anti-CD56 (clone mem188); PE conjugated anti-CD11c (clone b-ly6); anti-CD68 (clone y1/82a); PerCP conjugated anti-CD14 (clone mfp9); APC-conjugated anti-CCR2 (clone 48607); anti-HLADR (clone 1243); PECy7-conjugated anti-CD16 (clone 3g8); APC-conjugated anti-CD11b (clone icrf44); biotinylated anti-CD120a (clone mabtnfr1-b1); and anti-CD115 (clone 12-3a3-1b10). The gating strategy for human monocytes is described in Fig S4h. For isolation of human monocyte subsets citrate-blood of healthy volunteers was subjected to red-blood cell lysis for 3 min with ACK red blood cell lysis buffer.

In all mouse experiments single cell suspensions of bone marrow, blood and spleen were treated for 3 min with ACK red blood cell lysis buffer and passed through a 40 µm nylon cell strainer (BD Falcon). To isolate cells from the peritoneal cavity 10 ml of ice cold PBS containing 2 mM EDTA were injected with a 19 gauge needle on a BD 10 ml control syringe (BD Biosciences) trough the surgically exposed peritoneum. To isolate Langerhans cells the skin of healthy C57BL/6 mice was cut into small pieces and incubated for 30 min in a 0.1% collagenase solution in RPMI (Cellgro Mediatech, Inc.) supplemented with 2.5U/ml dispase in a rotating shaker at 37°C. After addition of DNAseI the samples were incubated for another 30 min at 37°C followed by a thorough mincing process through a 40 µm nylon cell strainer (BD Falcon).

In all cell labeling experiments, single cell suspensions were labeled for 30 min at 4°C with appropriate antibodies in PBS supplemented with 1% fetal bovine serum (FBS). For intracellular TNFα staining, cells were stimulated in complete medium (RPMI (Cellgro Mediatech Inc.), 10% FBS (Stem Cell Technologies), 100 U Penicillin/Streptamycin (Cellgro Mediatech Inc.)) for 4 h at 37°C in a cell culture incubator (37°C, 5% CO_2) with either 100 ng/ml LPS (serotype O55:B5) (Sigma) or with $5x10^8$/ml HKLM (heat killed *Lm)* (Invivo Gen). Golgi-plug (1 µl/ml) (BD Biosciences) was added after 1 h into the reaction. For intracellular Relb staining, 50 µl of peripheral blood was initially treated with ACK red blood cell lysis buffer and cultured at 37°C for 8 h in complete medium with 100 ng/ml LPS or kept on ice. Samples were stained for appropriate cell surface markers, fixed and permeabilized for intracellular staining using the BD cytofix cytoperm kit and the BD Phosflow II kit (both BD Biosciences) according to manufacturer's protocols. Cells were either stained over night with a rabbit polyclonal anti-Relb antibody (sc-226, Santa Cruz) at a dilution of 1:400 or with a matching isotype control (sc-2027, Santa

Cruz), or remained unstained (FMO control). Intracellular staining was revealed using an APC cross-linked goat-anti-rabbit IgG mAb (Invitrogen) for 2 h.

In all experiments the number of cells was defined as the total number of cells per organ multiplied by the percentage of each cell type identified by flow cytometry (LSRII; BD Biosciences). Data were analyzed with FlowJo v.8.8.7 (Tree Star, Inc.). All cell sorting was done on a BD FACS Aria (FACS Aria, BD Biosciences). Mouse and human monocytes were defined as described before (Cros et al., 2010; Swirski et al., 2009).

Bone Marrow Chimeras And Adoptive Cell Transfer

For generation of bone marrow chimeras, recipient mice received a single dose of 950 rad 4 h prior to i.v. injection of $1x10^6$ lineage-depleted donor bone-marrow cells per mouse. For fate mapping experiments, non-irradiated recipients received $4\text{-}10x10^4$ GMP or retrovirally transduced and *in vitro* expanded HSC i.v.. For *in vivo* short-term recruitment assays, $1x10^6$ Ly-$6C^{hi}$ wild-type (Ub-GFP, CD45.2) and *miR-146a*$^{-/-}$ (CD45.2) monocytes were adoptively co-transferred i.v. into i.p. LPS-challenged recipients (CD45.1). The number of cells recruited in the peritoneal cavity was counted 6 h later.

Gene expression arrays, analysis according to MIAME guidelines and use of online repositories and databases

miRNA expression profiling was performed at the Broad Institute using an Illumina Mouse v1 MicroRNA expression bead chip. Total RNA of splenic monocyte subsets sorted from C57BL/6 mice was isolated using the mirVana miRNA isolation kit (Ambion). For each subset three replicates were generated, which consisted of monocytes pooled from 10 spleens. RNA quantity and quality was assessed as described (Swirski et al., 2009) using the Bioanalyzer total RNA micro-chips (Agilent) and a Nano-drop spectrophotometer (Thermo Scientific). Raw data were exported and normalized using the Bead Studio v3.2 software (Illumina). Further statistical analysis of expression values was performed in Excel (Microsoft).

Microarray gene expression data for steady-state and challenged monocyte subsets were obtained from previously published sources and normalization of the obtained raw data files was performed using Gene Pattern (http://www.broadinstitute.org/cancer/software/genepattern/) (Reich et al., 2006). Gene expression data of monocytes isolated from the peritoneal cavity of *Listeria monocytogenes* (*Lm*) challenged mice have been published by (Auffray et al., 2007) and (Cros et al., 2010) and can be accessed on the Array Express database (http://www.ebi.ac.uk/arrayexpress/) under the accession E-MEXP-2545 and in the

Gene Expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession GSE8294. Expression data for steady-state Ly-6C^{hi} and Ly-6C^{lo} blood monocytes as well as the microRNA expression data were newly generated for this study and have been submitted to the GEO database where they can be accessed under the accession number GSE32364.

The steady state blood Ly-6C^{hi} monocyte expression data have been previously published (Swirski et al., 2009). For these data microarray normalization and analysis was performed as described (Swirski et al., 2009) or using Genepattern (Reich et al., 2006).

Gene Set Enrichment analysis (GSEA) (Subramanian et al., 2005) was used to identify a signature of inflammatory genes differentially expressed in steady-state monocyte subsets. Analysis of functional categories used a library of higher order terms of biological processes derived from the Panther database (Mi et al., 2007) and tested this library against genes that were found to be differentially expressed among monocyte subsets (Benjamini-Hochberg $p<0.05$).

Identification of new *miR-146a* targets

To identify new candidate *miR-146a* target mRNAs we used the MicroCosm Targets database (version 5) (Kozomara and Griffiths-Jones, 2011) (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). We compared the expression profiles of these candidate targets in Ly-6C^{hi} and Ly-6C^{lo} monocytes that were isolated from the peritoneal cavity either 2 h or 8 h after challenge with *Lm* (Auffray et al., 2007). The time points were convenient because Ly-6C^{hi} monocytes did not detectably up-regulate *miR-146a* at 2 h but did so at 8 h. 831 of the 957 potential candidate genes could be mapped to the microarray used to analyze the monocyte subsets. Among those, we screened for genes that 1) were significantly down-regulated (Benjamini-Hochberg adjusted $p<0.001$) in Ly-6C^{hi} monocytes from the 2 h to 8 h time point (i.e. concomitantly with *miR-146a* induction in these cells) and 2) were expressed at lower levels in Ly-6C^{lo} cells at both time points (i.e. in line with constitutive *miR-146a* expression in these cells).

Quantitative Real-Time PCR

RNA used for miRNA expression analysis was isolated with the mirVana miRNA isolation kit (Ambion). cDNA was generated using the Taqman microRNA reverse transcription kit (Applied Biosystems). Expression of miRNA was determined using Taqman miRNA specific probes against *has/mmu-miR-146a*, *hsa-miR-146a** and *mmu-miR-146a*,* and expression was normalized using *snoRNA55* snRNA endogenous control probes (Applied Biosystems). Total RNA used for mRNA expression analysis was isolated using the RNAeasy or RNAeasy micro kits (Qiagen) and cDNA was generated using the Taqman

high capacity reverse transcription kit (Applied Biosystems). Expression of mRNAs was determined using the following gene specific Taqman probes: Human RELB (Hs00232399_m1), mouse Relb (Mm00485672_m1), mouse TNFα (Mm00443258_m1), mouse IL-6 (Mm00446190_m1), mouse IL-1b (Mm01336189_m1) and mouse IL-10 (Mm00439614_m1) (Applied Biosystems). Relative gene expression was determined using a probe against 18s rRNA as endogenous control.

Western blot

Monocytes were lysed in RIPA lysis buffer (20 mM Tris pH8.0, 50 mM NaCl, 0.5% Na-deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), protease inhibitor cocktail (complete tablet, Roche)). 5 μg proteins were loaded on a 10% SDS-polyacrylamide gel for electrophoresis (1 hour, 150 V). Transfer (1 hour, 100 V) was performed on a PVDF membrane. After blocking in PBS-0.5% tween 5% milk, the membrane was incubated over night at 4°C with a rabbit anti-IκBα antibody (Santa-Cruz sc-371, 1/1000 dilution in blocking solution). Anti-rabbit secondary antibodies coupled to peroxydase were added for 45 minutes at room temperature (Jackson ImmunoResearch, 1/5000 in blocking solution). Chemiluminescence was used for detection (Amersham ECL Plus).

Immunofluorescence and image quantification

Immunofluorescence staining of p65 and Relb was performed on fixed and permeabilized monocytes. Cells were permeabilized and blocked in 4% goat serum in PBS containing 0.2% Triton X-100 for 30 minutes at room temperature. The incubation with primary antibodies was performed at 4°C over night. For murine p65 staining a rabbit polyclonal anti-p65 antibody (sc-372, Santa Cruz) was used at a dilution of 1:100. Intracellular p65 localization was revealed using an Alexa Fluor 555-conjugated goat-anti-rabbit IgG mAb (Invitrogen).

For murine and human Relb staining a rabbit polyclonal anti-Relb antibody (sc-226, Santa Cruz) was used at a dilution of 1:400. Intracellular Relb localization was revealed using a biotinylated anti-rabbit IgG (1:100, Vector Laboratories, Inc.) followed by streptavidin-Texas Red (1:100, GE Healthcare). The slides were coverslipped using a mounting medium with DAPI (H-1200, Vector Laboratories, Inc.) to identify cell nuclei. Isotype control staining was performed using a rabbit IgG antibody (sc-2027, Santa Cruz).

Images were captured with an Applied Precision Delta Vision Olympus IX71 inverted microscope, using a 60X Oil Olympus objective with 1.6x optical zoom, Photometrics CoolSNAP HQ2 camera cooled to −30°C and softWORX software controller. Images passed through deconvolution routine using point

spread functions based on that microscope. The same acquisition conditions were applied to all captured images.

To quantify cytoplasm → nucleus p65 and Relb protein translocation, high-resolution images were manually segmented in Image J (http://rsbweb.nih.gov/ij/) into nuclear and cytoplasmic regions based on DAPI nuclear staining. Protein signals in cytoplasmic vs. nuclear regions were quantified using the measurement tool of ImageJ.

***In vitro* monocyte transmigration assays**

Splenic Ly-6C^{hi} monocytes obtained from Ub-GFP wild-type and *miR-146a* knockout mice ($2x10^5$ each) were mixed and placed on a matrigel-coated 8 μm pore size PET membrane of transwell assay plates (BD Biocoat, BD) and incubated in a humidified incubator at 37°C, 5% CO_2 for 1 h, allowing the cells to attach to the matrigel. Migration was induced by addition of complete medium (RPMI, 10% FCS, 100U/ml Pen/strep, 2mM L-Glu) supplemented with recombinant murine MCP-1 (10 ng/ml) (Peprotech) to the lower compartment. Wells with media alone served as control to assess non-specific transmigration. Cell transmigration was assessed after 2 h by flow cytometry as the percentage of GFP^+ (wild-type) and GFP^- (knockout) cells in the bottom chamber.

Colony forming cell assay

FACS-sorted HSC obtained from wild-type or *miR-146a*$^{-/-}$ mice were cultured in complete methylcellulose-based medium (MethoCult GF M3434; StemCell Technologies) in 6-well plates to determine the number of myeloid colony-forming units (CFU). Colony numbers and morphology were assessed after 10 days of culture.

Bacterial Colony forming unit (CFU) assays

Mice were infected i.v. with $3x10^3$ live *Listeria monocytogenes* (*Lm*). Spleens were collected 24 h later and homogenized in 30 ml ice cold PBS. Serial dilutions of single cell suspensions were plated on Brain Heart Broth Agar (Fluka). To assess colonies the plates were incubated over night at 37°C. For each biological replicate at least 3 plates at 2 different dilutions were assessed.

Design of retroviral vectors for gene transfer and shRNA delivery

To generate a *miR-146a* expression vector, a 410 bp-long stretch of mouse chromosome 11 containing the murine *miR-146a* coding sequence was amplified from chromosomal DNA of C57BL/6 mice. The 5' oligo added a BglII site and the 3' oligo added a XhoI site to the sequence, which were used to clone the

construct directly downstream of an EGFP expression cassette into an MSCV2.2 based retroviral vector backbone. To ectopically express Relb in HSC a fully sequenced murine Relb (accession AK156767) cDNA clone was obtained from Open Biosystems and cloned again into the multiple cloning site upstream of an IRES in a MSCV2.2-IRES-EGFP retroviral vector using oligos introducing a 5' XhoI site before the start codon and a 3' EcoRI site after the stop codon respectively. shRNAs were designed and cloned according to a strategy described previously (Dickins et al., 2005). The 97-nucleotide oligomer used to generate the shRNA targeting the Relb locus was: 5'-TGCTGTTGACAGTGAGCGCTCCACATGGAATCGAGAGCAATAGTGAAGCCACAGATGTATTGCTCTCGATTCCATGTGGATTGCCTACTGCCTCGGA-3'. The fidelity of the PCR amplifications, cloning and oligo syntheses were confirmed by sequencing.

Generation of retroviral particles and retroviral transduction

To generate retroviral particles 293T cells were co-transfected with the respective retroviral vector and the EcoPak packaging vector (Gavrilescu and Van Etten, 2007) using the TransIT-LT1 transfection agent (Mirus). The transfection medium was replaced after 24 h and viral supernatants were collected 24 h thereafter, filtered through a 0.22 µm syringe filter and used directly for transfection of HSC or cell lines. HSC were obtained by FACS from lineage negative bone marrow of wild-type mice or *miR-146a*$^{-/-}$ mice. 0.5-1x10^5 HSC were plated into a retronectin-coated (Takara) well of a 6 well plate and transduced over night with 200µl retroviral supernatant in1ml stem cell media supplemented with 4 µg/ml polybrene (American Bioanalytical Inc.). The media composition consisted of RPMI (Mediatech Inc.) supplemented with 10% FCS (stem cell grade, Stem Cell Technologies), mSCF 1 µg/ml (Peprotech), TPO 0.5 µg/ml (Peprotech), Flt3-L 0.5µg/ml (Peprotech), IL-3 0.2 µg/ml (Peprotech), Pen/Strep 100 U/ml (Mediatech Inc.), L-Glutamine 2mM (Mediatech Inc.). The cells were subsequently expanded over 3 days in fresh stem cell medium *in vitro*. Transduction efficiency (usually >80%) was assessed prior to injection based on EGFP expression by flow cytometry and specific expression or silencing of the gene of interest was assessed by Taqman real-time PCR using gene specific primers.

***In vivo* and *in vitro* LNA–mediated *miR-146a* silencing**

Locked nucleic acid (LNA)–mediated *miR-146a* silencing *in vivo* was performed as described previously (Starczynowski et al., 2010). LNAs specific against *miR-146a* and controls were obtained from Exiqon. Mice received 25 mg/kg i.p. injections of anti-*miR-146a* LNA in PBS for 3 consecutive days prior to inflammatory challenge. For *in vitro* silencing experiments monocytes were transfected with 100 nM of anti-*miR-146a* LNA or a scrambled LNA control using the TransIT LT-1 transfection agent (Mirus).

3' UTR reporter assays

A 320 bp genomic fragment corresponding to the murine *Relb* 3'UTR region of transcript (ENSMUST00000049912) bearing the predicted binding sequence (AGGCAGTGGATTGTTCAGTTTTCc) for *miR-146a* was obtained by PCR from total C57BL/6 DNA and the sequence was confirmed by sequencing. The fragment was cloned into the pmirGLO Dual-Luciferase miRNA target vector (Promega). A second reporter vector was cloned with a mutated seed region (TGGGAGACCTTTGAAGTCTTTAGC) but otherwise identical sequence to the 320bp genomic fragment. The reporter vectors were transfected into NIH3T3 cells stably expressing either a *miR-146a* or an empty control vector using the TransIT-LT1 transfection agent (Mirus). The efficiency of *miR-146a*-mediated gene silencing was determined 24 h post transfection using the pmirGLO Dual-luciferase assay system (Promega) on a Safire2 plate reader (Tecan).

2. Supplemental Figure Titles and Legends

Figure S1 (related to Figure 1)

a) Identification of murine blood monocyte subsets by flow cytometry. 'Lin' is defined as [Ter119, CD90.2, NK1.1, DX5, B220, CD19, Ly-6G].

b) GSEA Leading-Edge analysis of molecular signatures acquired by microarray gene expression analysis comparing blood Ly-6C^{hi} vs. Ly-6C^{lo} monocyte subsets.

c) Quantification of TNFα, IL-6, IL-1b and IL-10 mRNA induction in blood monocyte subsets 8 h after LPS challenge (n=3-4). Fold induction is expressed relative to non-stimulated monocytes for each subset.

d) Western blot of IκBα in FACS-sorted splenic Ly-6C^{hi} and Ly-6C^{lo} monocytes that were processed identically to cells used for experiments in this study. 1x10^6 cells of each subset were sorted into complete medium, pelleted in a cooled centrifuge at 300g for 5 min and flash frozen for western blot analysis.

e) *miR-146a* expression in monocyte subsets 8 h after *in vitro* exposure to LPS, HKLM or TNFα. Expression is relative to Ly-6C^{hi} monocytes at time 0 h (n=2-4).

f) *miR-146a* induction in monocytes *in vivo* 6 h after i.v. LPS injection. Splenic reservoir monocytes were isolated from spleens of normal or LPS-challenged mice by FACS and *miR-146a* was quantified using Taqman real-time PCR. Expression is relative to Ly-6C^{hi} monocytes in steady-state (n=4 animals for steady-state group and n=3 animals for challenged group).

g) Phenotype of sorted Ly-6C^{hi} monocytes upon *in vitro* stimulation with LPS for 8 h and 24 h. The culture conditions were identical to those used to measure LPS dependent *miRNA-146a* induction (Fig 1f,

S1e, f). Histograms are representative of at least 3 independent experiments.
Data are presented as mean±SEM. (* $p<0.05$, *** $p<0.001$, Student's t-test).

Figure S2 (related to Figure 2)

a) Experimental approach to constitutively overexpress *miR-146a* in monocytes *in vivo*.

b) Quantification of *miR-146a* expression using a specific Taqman primer and monocytes isolated from bone marrow chimeras generated as shown in Fig S2a.

c) Quantification of monocyte subsets in spleen of steady state bone marrow chimeras expressing either a control or a *miR-146a* overexpressing vector.

d) Experimental approach to silence *miR-146a* with anti-*miR-146a* LNA.

e) Validation of anti-*miR-146a* LNA mediated knockdown of *miR-146a*. Isolated anti-*miR-146a* treated or control Ly-6C^{hi} monocytes were incubated for 24 h in LPS containing media to promote the induction of *miR-146a*. Quantification was performed using a Taqman specific probe against the mature *miR-146a* sequence.

f) Quantification of monocyte subsets in spleen of resting mice treated with PBS (control) or anti-*miR-146a* LNA.

g) Confirmation of presence or absence of *miR-146a* in wild-type (red) or *miR-146a*$^{-/-}$ (blue) mice using a Taqman probe specific for mature miR-146a. Total RNA of $1x10^6$ bone marrow cells of each group was isolated and cDNA of mature miRNA transcripts was generated using specific RT primers.

h) Phenotypic characterization of monocyte subsets in blood of wild-type or *miR-146a*$^{-/-}$ mice. Data are representative of 3 independent experiments.

i) Experimental approach for LPS challenge of mice reconstituted with wild-type and *miR-146a*$^{-/-}$ bone marrow. The same approach was used for thioglycollate challenge, with the exception that mice received only one injection of thioglycollate i.p. and were sacrificed 24 h later.

j) Experimental approach for fate mapping donor wild-type and *miR-146a*$^{-/-}$ GMP in LPS-challenged recipient mice.

k) Colony-forming capacity of sorted hematopoietic stem cells of wild-type and *miR-146a*$^{-/-}$ bone marrow. Cells were cultured in methylcellulose media for 10 days in presence of 10 ng/ml M-CSF.
Data are presented as mean±SEM. (* $p<0.05$, Student's t-test).

Figure S3 (related to Figure 3)

a) Cytokine production of sorted wild-type and *miR-146a*$^{-/-}$ splenic macrophages (defined as [Ter119, CD90.2, NK1.1, DX5, B220, CD19, Ly-6G]$^-$ CD11b$^+$ Ly-6C^{lo} [F4/80,IAb]^{int} after *in vitro* LPS stimulation. Cytokine production is expressed per cell (n=3).

b) Quantification of cell cycle status of Ly-6C^{lo} monocytes in wild-type or *miR-146a*$^{-/-}$ animals in steady state (n=2) or after 4 consecutive days of LPS injection i.p. (n=4) in bone marrow and spleen.
c) *In vitro* co-culture of wild-type (EGFP^{+}, WT) and *miR-146a*$^{-/-}$ (EGFP^{-}, KO) Ly-6C^{hi} monocytes isolated from bone marrow or peritoneal cavity. Cells were cultured for 7 d in M-CSF conditioned medium in presence or absence of LPS. Merged fluorescence/differential interference contrast image depicts Ly-6C^{hi} derived cells on d 7. Green overlay: Ub-EGFP wild-type cells. Scale bar: 50 μm.
d) Flow cytometry quantification of co-culture experiments. Pie charts indicate ratios between wild-type (light grey) and *miR-146a*$^{-/-}$ (dark grey) cells at d 7. Bone marrow (BM) derived *miR-146a*$^{-/-}$ cells significantly outnumbered WT cells on d 7 ($p<0.001$) (n=7).
e) Flow cytometry analysis of Ccr2 expression in wild-type (WT) and *miR-146a*$^{-/-}$ (KO) monocytes. Matching isotype control for the anti-Ccr2 antibody is shown in solid grey. Plots are representative for n=8 animals per group. Data are presented as mean±SEM. (*** $p<0.001$, Student's t-test).

Figure S4 (related to Figure 4)
a) Venn-diagrams showing the number of *in silico*–predicted *miR-146a* target genes (left) and the number of genes, which are downregulated in Ly-6C^{hi} monocytes during inflammatory challenge and maintained at lower levels in Ly-6C^{lo} cells *in vivo* (right). 9 intersect genes are identified on the left.
b) Immunofluorescence staining of resting mouse Ly-6C^{hi} monocytes with a matching isotype control or anti-Relb antibody. Scale bar 10 μm.
c) Experimental strategy for ectopic expression of Relb in monocytes.
d) *In vitro* expansion of bone marrow HSC modified with control IRES-GFP or Relb-EGFP vector. Data show cell counts on d0 (time of retroviral transduction) and d3.
e) Experimental strategy for shRNA–mediated *Relb* silencing in *miR-146a*$^{-/-}$ cells.
f) *In vitro* expansion of bone marrow HSC modified with control IRES-GFP or shRelb-EGFP vector. Data show cell counts on d0 (time of retroviral transduction) and d3.
g) Top: Mouse *miR-146a (miR-146a-5p)* and human *miR-146a* (miR-146a-3p)* target sites in the Relb 3' UTR as predicted by miRbase (MicroCosm Targets Version 5). Below: sequences of mature hsa/mmu-*miR-146a-5p* and the alternative processing isoform in human (*hsa-miR-146a-3p*) and in mouse (*mmu-miR-146a-3p*).
h) Identification of human blood monocyte subsets by flow cytometry. 'Lin' is defined as [CD56, CD3e, CD15, NKp46, CD19]. Monocytes were further refined as CD115^{+} and HLA-DR^{+}.
i) Differential expression of *miR-146a* in peripheral blood monocyte subsets of 5 healthy donors (HD). Real-time PCR was performed using mature *miR-146a* specific Taqman primers. Expression is relative to the CD14^{+} CD16^{-} subset. Each reaction was performed in triplicates.

j) Quantification of *miR-146a** induction in human $CD16^{+}(CD14^{-})$ monocytes 8 h after LPS challenge using a specific Taqman probe for the mature form of the miRNA transcript. Expression is expressed relative to 0h.
k) *miR-146a* expression in human monocyte subsets analyzed either *ex vivo* or 8 h after LPS challenge. Each reaction was performed in triplicates.
l) Quantification of *miR-146a** in steady state and LPS challenged mouse monocyte subsets using a specific Taqman probe for the mature processing isoform.
m) Anti-*miR-146a* LNA mediated knockdown in human $CD14^{+}(CD16^{-})$ monocytes. The cells were isolated from blood of a healthy donor by FACS (according to the strategy depicted in S4h), transfected with either anti-*miR-146a* LNA or a control LNA, and challenged for 8 h with LPS.
Data are presented as mean±SEM. (* $p<0.05$,** $p<0.01$, *** $p<0.001$, Student's t-test).

3. Supplemental References

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