Materials and Methods

Animals

All experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the National Institutes of Health and the Committee and Animal Care at the New York University, University of Massachusetts, University of Colorado, and Massachusetts Institute of Technology. *Rorc(t)*^{FL} mice were generated as described in Fig. S7. *il17ra*^{KO} and *Rorc*^{Neo} mice were described elsewhere (*13, 45*). All C57BL/6 mice used in this study were obtained from Taconic (USA), because when these mice were obtained from different sources they often exhibited more variable immunological phenotypes (*46*), which may affect the penetrance and specific characteristics of the MIA phenotypes.

Generation of RORγ/γt conditional knockout mice

In order to develop a conditional knockout mouse line that removes both RORγ and RORγt in a Cre-dependent manner, we generated a targeting vector, from C57BL/6-derived BAC clone RP24-318 I7, in which two loxP sites flanked common exons 3-6. Cre-mediated deletion of exons 3-6 generates a frame shift mutation. Linearized targeting vector was then electroporated into albino C57BL/6 ES cells (CY2.4) in the gene targeting facility at the Rockefeller University. Homologous recombination was confirmed by Southern blot analyses with two different probes, as described in Fig. S7. To remove the neomycin resistance cassette, two ES cell lines with correctly targeted alleles were transiently electroporated with a Cre recombinase vector. ES cells with correct conditional alleles were confirmed by both Southern blot and PCR analyses and

subsequently injected into blastocysts at the NYU gene targeting facility. For generating Southern blot probes, we used the following primers (ROR5Pr3s 5'-CCCAGCAGGTAAATCAGTGGTTC-3' and ROR5Pr3a 5'-GCGGATAGAGCAAGGTCATTGG-3' for Probe A; ROR3Pr3s 5'-GTAACTGTGTTTATGACTCCCTGGC-3' and ROR3Pr3a 5'-CACTCTTTCTTGACATCTCCCCTTC-3' for Probe B). For PCR genotyping, the following primers were used (RORgflox1 5'-TTCCTTCCTTCTTCTTGAGCAGTC-3', RORgflox2 5'-CAGAAGAAAAGTATATGTGGCTTGTTG-3' for WT 166bps/Floxed 226bps and RORgflox3 5'-GGTCATTTACTGGACACCCTTTCC-3', RORgflox5 5'-GCTACACAGCAAAACCTTGTCTTGG-3' for WT 307bps/Floxed 384bps).

Maternal Immune Activation

Mice were mated overnight and females were checked daily for the presence of seminal plugs, noted as embryonic day 0.5 (E0.5). On E12.5, pregnant female mice were weighed and injected with a single dose (20mg/kg; i.p.) of poly(I:C) (Sigma Aldrich) or PBS vehicle. Each dam was returned to its cage and left undisturbed until the birth of its litter. All pups remained with the mother until weaning on postnatal day 21 (P21), at which time mice were group housed at maximum 5 per cage with same-sex littermates. For the IL-17 cytokine blockade experiment, monoclonal IL-17a blocking antibody (clone 50104; R&D) or isotype control antibody (IgG2a, clone 54447; R&D) were administered 6 h before maternal immune activation via i.p. route (500 μg/animal). For IL-6 cytokine injection into pregnant dams, carrier-free recombinant mouse IL-6 (R&D) was administered as a single dose (10 μg/animal; i.p.). For testing anti-IL17a therapeutic

effects, IL-17a blocking antibody or isotype control antibody (as described above) was administered 2 days after maternal immune activation (500 μg/animal; i.p.).

Cell preparation, Flow cytometry, ELISA

Embryos at each implantation site were dissected in ice-cold HBSS containing Ca^{2+} and Mg^{2+} (Gibco). Myometrium was first peeled off of the decidua and embryos were discarded. Dissected decidual and placental tissues were then minced and enzymatically dissociated in HBSS containing 0.28 Wunsch units (WU)/mL Liberase (Roche) and 30 μg/mL DNase I (Roche) for 30 min at 37°C with intermittent mixing. Digested tissues were washed in PBS containing 5 mM EDTA and 5% fetal bovine serum and then incubated again in the same buffer for 15 min at 37°C prior to filtration through a cell strainer. After separation on a discontinuous 40% & 80% Percoll gradient, the mononuclear cell fraction was treated with ACK lysis buffer (Lonza). Mononuclear cells ($1x10^6$ cells/mL) were cultured for 24 h with or without phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) in T cell media: RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS (Hyclone), 50 U penicillin-streptomycin (Invitrogen), 2 mM glutamine, and 50 μM β-mercaptoethanol. Cell culture supernatant was used for ELISA analyses. Unstimulated cells were used to prepare total RNA for qPCR analyses. For flow cytometry, cells were incubated for 5 h with PMA, ionomycin and GolgiStop (BD). Intracellular cytokine staining was performed according to the manufacturer's protocol (Cytofix/Cytoperm buffer set from BD with Pacific Blue-conjugated CD4, FITC or PerCP-Cy5.5-conjugated CD8, APC-

Cy7-conjugated TCR-β, PE-Cy7-conjugated anti-IL-17a, PE-conjugated anti-IFN-γ, PE-Cy7-conjugated anti-CD25 and PE-conjugated Foxp3 (eBioscience). LSR II (BD Biosciences) and FlowJo software (Tree Star) were used for flow cytometry and analysis. Dead cells were excluded using the Live/Dead fixable aqua dead cell stain kit (Invitrogen). For ELISA with sera and placenta/decidua extract, IL-6 (Ebioscience), IL-17a, TNF-α, IL-1β, IFN-β (Biolgened), and IL-10 (BD) were measured according to the manufacturer's protocol.

Ultrasonic vocalizations

On postnatal day 7~9, both male and female mice were removed from the nest and habituated to the testing room for 15 minutes (separate of dam). After the habituation period, mouse pups were placed in a clean 15 cm glass pyrex high wall dish. Mouse pup ultrasonic vocalizations (USVs) were then detected for 3 min using an UltraSoundGateCM16/CMPA microphone (AviSoft) in the sound attenuation chamber under stable temperature and light control (15 lux), and recorded with SAS Prolab software (AviSoft). USVs were measured between 33-125 kHz. USVs were scored as contiguous if gaps between vocalizations were <.02 msec. For certain USV tests, Ultravox software (Noldus information Technology, USA) was used. An amplitude filter was used to eliminate extraneous peripheral noise (i.e. HVAC). Due to the unreliability of automated USV scoring, all pup USVs were measured and confirmed manually by observers blind to the experimental conditions.

Three-chamber social approach

8~12-week-old male mice were tested for social behavior using a three-chamber social approach paradigm. Experimental mice were habituated for 1 h in separate clean holding cages and then introduced into a three-chamber arena with only empty object-containment cages (circular metallic cages, Stoelting Neuroscience) for a 10-min acclimation phase in two 5-min sessions in a 3-4 h period. The following day the mice were placed in the center chamber (without access to the left and right social test areas) and allowed to explore the center area for 5 min. After this exploration period, barriers to adjacent chambers were removed, allowing mice to explore the left and right arenas, which contained a social object (unfamiliar C57BL/6 male mouse) in one chamber and an inanimate object (plastic toy) in the other chamber. Experimental mice were given 10 min to explore both chambers and measured for approach behavior as interaction time (i.e. sniffing, approach) with targets in each chamber (within 2 cm, excluding non-nose contact or exploration). Sessions were video-recorded and object exploration time and total distance moved were analyzed using the Noldus tracking system. A social preference index was calculated as the percentage of time spent investigating the social target out of the total exploration time of both objects. The analysis was conducted with investigators blind to the treatments and genotypes of subjects. Arenas and contents were thoroughly cleaned between testing sessions. Multiple social targets from different home cages were used for testing to prevent potential odorant confounds from target home cages.

Marble burying test

One week following the social approach task, male mice were acclimated for 0.5-1 h in separate clean holding cages. Mice were placed in a testing arena (arena size: 16”X8”X12”, bedding depth: 2”) containing 20 glass marbles, which were laid out in four rows of five marbles equidistant from one another. At the end of a 15-min exploration period, mice were gently removed from the testing cages and the number of marbles buried was recorded. A marble burying index was scored as 1 for marbles covered >50% by bedding, 0.5 for ~50% covered, or 0 for anything less.

Intraventricular cytokine injection

At E14.5, uterine horns of pregnant mice were exposed by a caudal ventral midline incision (<2 cm). Each uterine horn was exteriorized carefully and each fetus was identified. Recombinant mouse IL-17a cytokine (R&D, 0.6 μL of 2 ng/μL), IL-6 (R&D, 0.6 μL of 10ng/μL) or saline together with the dye Fast Green (Sigma, 0.3 mg/mL) was injected (3-4 μL) into the third ventricle of each embryo by a pulled micropipette. After injection of all embryos, the uterus was replaced within the abdomen and the cavity was lavaged with warm sterile saline.

Gender genotyping

Genomic DNA was extracted from tail tips of each embryo. For gender discrimination of each embryo, PCR was carried out using *sry* (sex-determining region of the Y chromosome) gene specific primers: 5’-ACAAGTTGGCCCAGCAGAAT-3’, and 5’-GGGATATCAACAGGCTGCCA-3’.

Immunohistochemistry

Fetal brains of male embryos were dissected and fixed with 4% paraformaldehyde in PBS for 6h at 4 ^{o}C. Adult brains of male offspring were perfused and fixed with 4% paraformaldehyde in PBS for overnight at 4 ^{o}C. The brains were removed and sectioned at 50-μm thickness with a Leica VT1000S vibratome (Leica, USA). Slices were permeabilized with blocking solution containing 0.4% Triton X-100, 2% goat serum, and 1% BSA in PBS for 1 h at room temperature, and then incubated with anti-TBR1 (ab31940, Abcam), anti-SATB2 (ab51502, Abcam), and anti-CTIP2 (ab18465, Abcam) antibodies overnight at 4 ^{o}C. The following day, slices were incubated with fluorescently conjugated secondary antibodies (Invitrogen, USA) for 1 h at room temperature, and mounted in vectashield mounting medium with DAPI (Vector laboratories). Images of stained brain slices were acquired using a confocal microscope (LSM710; Carl Zeiss) with a 20X objective lens; all image settings were kept constant. Spatial locations of the patches were registered based on their distance from the midline of the brain. These cortical malformations were quantified using cropped images containing the malformations, or the corresponding region in WT brains. The region of interest (300x300 μm^2) was divided into 10 equal laminar blocks representing different depths of the cortical plate. SATB2-, TBR1-, or CTIP1-positive cells were counted using Image J software. Signal intensity in each image was normalized relative to the total signal intensity.

Real-Time PCR

Total RNA was extracted from the cerebral cortex of E14.5 fetal brain of male embryo (RNase plus mini kit, Qiagen) as well as from the decidua- and the placenta-derived mononuclear cells and reverse transcribed into cDNA using oligodT (ProtoScript first strand cDNA synthesis kit, NEB) according to the manufacturer's instructions. mRNA levels of target genes (*il17ra*, *il17rc*, *il17a* and *il6*) were quantified with a Real-Time PCR System (CFX connect Real-Time PCR, Bio-Rad) using fluorescent SYBR Green technology (Bio-Rad). Real-Time PCR was performed on 2 μL of cDNA synthesized from 200 ng of total RNA. Changes in relative gene expression normalized to *gapdh* or *actin* levels were determined using the relative threshold cycle method based on the Cont-PBS group. The detailed nucleotide sequences are shown as follows:

iIl17ra 5'-CCACTCTGTAGCACCCCAAT-3' and 5'-CAGGCTCCGTAGTTCCTCAG-3'; *il17rc* 5'-GGTACTGTCCCCAGGGGTAT-3' and 5'-GAGGCCGGTTTTCATCTCCA-3'; *il17a* 5'- CTCCAGAAGGCCCTCAGACTAC -3' and 5'- AGCTTTCCCTCCGCATTGACACAG -3'; *il6* 5'-ACACATGTTCTCTGGGAAATCGT -3' and 5'-AAGTGCATCATCGTTGTTCATACA -3'; *actin* 5'- GGCTGTATTCCCCTCCATCG -3' and 5'- CCAGTTGGTAACAATGCCATGT -3'; *gapdh* 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'

In Situ Hybridization

E14.5 male embryos from PBS or poly(I:C)-treated mothers were collected in ice-cold PBS and subsequently fixed in 4% paraformaldehyde for 4 h at 4 ℃. Isolated brains were dehydrated in 30% sucrose/PBS solution overnight, and then embedded in Tissue Tek

O.C.T. compound (Sakura Finetek, Torrance, CA). The blocks were sectioned at 16-µm thickness using a cryostat (Leica). Fluorescent *in situ* hybridization was performed using a branched cDNA probe with amplification technology (ViewRNA ISH Tissue Assay kit, Panomics, Santa Clara) according to the manufacturer's protocol. Briefly, the sections were rehydrated and treated with proteinase K for 20 min at 40 ^{o}C, followed by re-fixation in 4% paraformaldehyde for 5 min. IL-17Ra and Gapdh probes were applied to the sections and incubated for 6 h at 40 ^{o}C. The probes were designed based on the NCBI reference mRNA sequence: *il17ra* (NM_008359) and *gapdh* (NM_008084).

Statistics

Statistical analyses were performed using Prism or SPSS. ANOVAs were followed by Tukey or Holm-Sidak corrections. All data are represented as mean +/- SEM. Sample sizes were estimated using post-hoc power analyses from similar previously conducted studies (*32, 47*).

Fig. 3

USV statistics: $F(3,121)=48.55$, $p<.0001$

Post-hoc (Tukey)

PBS,Cont-IgG vs PBS, anti-IL-17a $p=.9878$

PBS,Cont-IgG vs Poly(I:C), Cont-IgG $p<.0001$

PBS,Cont-IgG vs. Poly(I:C), anti-IL-17a $p=.8899$

PBS, anti-IL-17a vs Poly(I:C), Cont-IgG $p<.0001$

PBS, anti-IL-17a vs Poly(I:C), anti-IL-17a $p=.6938$

Poly(I:C), Cont-IgG vs Poly(I:C), anti-IL-17a $p<.0001$

Social Interaction statistics: $F(3,62)=15.16$, $p<.0001$

Social vs Inanimate (within group)

PBS,Cont-IgG ;Social vs. PBS,Cont-IgG ;Inanimate p<.0001

PBS, anti-IL-17a ;Social vs. PBS, anti-IL-17a ;Inanimate p=.0021

Poly(I:C), Cont-IgG ;Social vs. Poly(I:C), Cont-IgG ;Inanimate p=.1764

Poly(I:C), anti-IL-17a ;Social vs. Poly(I:C), anti-IL-17a ;Inanimate p=<.0001

Social Interaction across groups (between groups)

Antibody blockers F(1,62)=10.48, p=.0019, Treatment F(1,62)=6.764, p=.0116, Interaction F(1,62)=27.59, p<.0001.

PBS,Cont-IgG vs Poly(I:C),Cont-IgG p<0.0001

PBS,Cont-IgG vs PBS,anti-IL17a p=.5241

PBS,Cont-IgG vs Poly(I:C),anti-IL17a p=.967

PBS,anti-IL-17a vs Poly(I:C),Cont-IgG p<0.001

Poly(I:C),Cont-IgG vs Poly(I:C);anti-IL-17a p<.0001

PBS,anti-IL-17a vs Poly(I:C),anti-IL-17a p=.2285

Marble Burying statistics: F(3,61)=62.02, p<.0001

Post-hoc (Tukey)

PBS,Cont-IgG vs PBS, anti-IL-17a p=.5084

PBS,Cont-IgG vs Poly(I:C), Cont-IgG p<.0001

PBS,Cont-IgG vs. Poly(I:C), anti-IL-17a p=.9847

PBS, anti-IL-17a vs Poly(I:C), Cont-IgG p<.0001

PBS, anti-IL-17a vs Poly(I:C), anti-IL-17a p=.6691

Poly(I:C), Cont-IgG vs Poly(I:C), anti-IL-17a p<.0001

Fig. 4

USV statistics: F(5,97)=8.936, p<.0001

Post-hoc (Holm-Sidak)

WT (PBS) vs. WT (IC) p<.001

HET (PBS) vs. HET (IC) p<.05

KO (PBS) vs. KO (IC) p=.062

WT (PBS) vs. HET (PBS) p=.538

HET (PBS) vs. KO (PBS) p=.216

KO (IC) vs. WT (IC) p=.012

WT (PBS) vs. HET (IC) p=.002

HET (IC) vs. KO (IC) p=.062

KO (PBS) vs. WT (IC) p<.001

WT (PBS) vs. KO (PBS) p=.852

HET (IC) vs. KO (PBS) p<.001

WT (PBS) vs. KO (IC) p=.248

HET (PBS) vs. WT (IC) p=.001

HET (PBS) vs. KO (IC) p=.876

HET (IC) vs. WT (IC) p=.876

Social Interaction statistics: F(5,117)=6.904, p<.0001

Social vs Inanimate (within group)

WT-PBS	p<.0001
WT-IC	p>.9999
HET-PBS	p<.0001
HET-IC	p>.9999
KO-PBS	p=.0001
KO-IC	p<.0001

Social Interaction across groups (between groups)

Genotype F(2,117)=1.1547, p=.2172, Treatment F(1,117)=15.27, p=.0002, Interaction F(2,117)=4.842, p=.0095.

WT (PBS) vs. WT (IC) p=.0004

HET (PBS) vs. HET (IC) p=.0359

KO (PBS) vs. KO (IC) p=.9999

WT (PBS) vs. HET (PBS) p>0.9999

HET (PBS) vs. KO (PBS) p=.9822

HET (PBS) vs KO (IC) p=.9961

WT(IC) vs. HET (IC) p=.9999

KO (IC) vs. WT (IC) p=.0049

WT (PBS) vs. HET (IC) p=.0139

HET (IC) vs. KO (IC) p=.0714

KO (PBS) vs. WT (IC) p=.0381

WT (PBS) vs. KO (PBS) p=.9607

HET (IC) vs. KO (PBS) p=.1929

WT (PBS) vs. KO (IC) p=.9878

HET (PBS) vs. WT (IC) p=.0029

Distance moved (between groups)

Genotype F(2,113)=0.2697, p=.7641, Treatment F(1,113)=0.6454, p=.4234, Interaction F(2,113)=0.054, p=.9476.

WT (PBS) vs. WT (IC) p=.9677

HET (PBS) vs. HET (IC) p>.9999

KO (PBS) vs. KO (IC) p=.9980

WT (PBS) vs. HET (PBS) p=.9819

HET (PBS) vs. KO (PBS) p=.9988

HET (PBS) vs KO (IC) p>.9999

WT (IC) vs. HET (IC) p=.9996

KO (IC) vs. WT (IC) p>.9999

WT (PBS) vs. HET (IC) p=.720

HET (IC) vs. KO (IC) p=.9999

KO (PBS) vs. WT (IC) p=.9983

WT (PBS) vs. KO (PBS) p=.9997

HET (IC) vs. KO (PBS) p=.9893

WT (PBS) vs. KO (IC) p=.9722

HET (PBS) vs. WT (IC) p>.9999

Marble Burying statistics: F(5,114)=13.90, p<.0001, Genotype F(2,114)=7.542, p<.0001, Treatment F(1,114)=9.598, p=.0025, Interaction F(2,114)=16.40, p<.0001.

Post-hoc (Tukey, corrects for multiple comparisons)

WT (PBS) vs. WT (IC) p=.0008

HET (PBS) vs. HET (IC) p=.0015

KO (PBS) vs. KO (IC) p=.0507

WT (PBS) vs. HET (PBS) p=.9996

HET (PBS) vs. KO (PBS) p=.9388

HET (PBS) vs. KO (IC) p=.3196

WT (IC) vs HET (IC) p=.9963

KO (IC) vs. WT (IC) p<.0001
WT (PBS) vs. HET (IC) p=.0015
HET (IC) vs. KO (IC) p<.0001
KO (PBS) vs. WT (IC) p=.0494
WT (PBS) vs. KO (PBS) p=.8569
HET (IC) vs. KO (PBS) p=.0483
WT (PBS) vs. KO (IC) p=.6361
HET (PBS) vs. WT (IC) p=.0006

Fig 5

USV statistics: the Student's *t*-test
PBS vs. IL17a p<.0001

Social Interaction statistics: F(1,28)=28.65, p<.0001
Social vs Inanimate (within group)
PBS ;Social vs. PBS ;Inanimate p=.0002
IL-17a ;Social vs. IL-17a ;Inanimate p=.015

Marble Burying statistics: the Student's *t*-test
PBS vs. IL17a p<.0001

Fig 6

USV statistics: F(2,58)=97.05, p<.0001
Post-hoc (Tukey)
PBS,Cont-IgG vs Poly(I:C), Cont-IgG p<.0001
PBS,Cont-IgG vs. Poly(I:C), anti-IL-17a p<.0001
Poly(I:C), Cont-IgG vs Poly(I:C), anti-IL-17a p<.0001

Social Interaction statistics: F(2,36)=21.62, p<.0001
Social vs Inanimate (within group)
PBS,Cont-IgG ;Social vs. PBS,Cont-IgG ;Inanimate p<.0001
Poly(I:C), Cont-IgG ;Social vs. Poly(I:C), Cont-IgG ;Inanimate p=.0064
Poly(I:C), anti-IL-17a ;Social vs. Poly(I:C), anti-IL-17a ;Inanimate p=.0255

Marble Burying statistics: F(2,36)=120.5, p<.0001

Post-hoc (Tukey)

PBS,Cont-IgG vs Poly(I:C), Cont-IgG p<.0001

PBS,Cont-IgG vs. Poly(I:C), anti-IL-17a p=.0121

Poly(I:C), Cont-IgG vs Poly(I:C), anti-IL-17a p<.0001

Fig S8.

Social Interaction statistics:

Social vs Inanimate (within group)

HET-PBS t(9)=3.858, p=.004

WT-IC t(6)=0.450, p=.669

HET-IC t(23)=3.622, p=.001

KO-IC t(27)=8.573, p<.001

Social Interaction (between groups)

F(3,65)=3.544, p=.019; Genotype F(2,68)=4.848, p=.011, Treatment F(1,69)=2.305, p=.134,

HET (PBS) vs. WT (IC) p=.135

HET (PBS) vs. HET (IC) p=.433

HET (PBS) vs. KO (IC) p=.998

WT (IC) vs. HET (IC) p=.636

WT (IC) vs. KO (IC) p=.042

HET (IC) vs KO (IC) p=.113

Fig S10.

USV statistics: the Student's *t*-test

PBS vs. IL17a p=.0002

USV statistics: F(2,44)=24.59, p<.0001

Post-hoc (Tukey)

PBS, Cont-IgG vs IL-6, Cont-IgG p<.0001

PBS, Cont-IgG vs IL-6, anti-IL-17a p=.0741

IL-6, Cont-IgG vs. IL-6, anti-IL-17a p<.0001

Supplementary Text

Author contribution

C.A.H., H.W., G.B.C., Y.S.Y., J.R.H. and D.R.L. designed the experiments and/or provided advice and technical expertise. G.B.C., Y.S.Y., H.W., S.K., H.K., C.A.H. and J.R.H., performed the experiments. S.V.K. generated RORγt conditional mouse lines. J.R.H., G.B.C., Y.S.Y., H.W., C.A.H. and D.R.L. wrote the manuscript with input from the co-authors.