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Supplementary Materials for

Migratory CD11b$^+$ conventional dendritic cells induce T follicular helper cell–dependent antibody responses

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at immunology.sciencemag.org/cgi/content/full/2/18/eaam9169/DC1)

Source data (Microsoft Excel format)

Supplementary Materials:

Materials and Methods

Mice. Age- and sex-matched 6- to 12-weeks-old mice were used in each experimental group. OTII [B6.Cg-Tg(TcraTcrb)425Cbn/J] mice purchased from Jax and were crossed onto the CD45.1 [B6.SJL-PtprcaPepcb/BoyCrl] mice from Charles river. *Dock8*$^{-/-}$ mice were generated as described previously (*1*). To generate conditional *Dock8*-deficient mice, guide RNAs (gRNA) were designed against intronic sequences flanking exons 10-14. Cas9-mediated double-stranded DNA breaks resolved by homology directed repair (HDR) with oligos containing loxP sites resulted in loxP insertion and floxing of exons 10-14. Cre mediated recombination results in ablation of intervening sequences containing exons 10-14, creating a frame shift and loss of DOCK8 protein expression. Cas9 targeting was performed as described in (*2, 3*) with some modifications (see below). To generate DC-*Dock8*$^{\Delta}$ mice, conditional *Dock8* mice were crossed with *CD11c*^{Cre} (Itgax-Cre) [B6.Cg-Tg(Itgax-Cre)1-1Reiz/J] mice purchased from Jax. The presence of *Dock8*^{flox} and *CD11c*^{Cre} transgene was confirmed by PCR. *Dock8* flox forward primer: GGGAGGAGAGAAGCCATGTT, reverse primer: GAAACATGGGCACCAAGG, CD11c Cre forward primer: ACTTGGCAGCTGTCTCCAAG, reverse primer: GCGAACATCTTCAGGTTCTG. Specific deletion of DOCK8 protein from dendritic cells was analyzed by western blot of sorted CD11c^{+} cells with an antibody against DOCK8 (Clontech Laboratories). *Batf3*$^{-/-}$ [129S-*Batf3*^{tm1Kmm}/J], *Ccr7*$^{-/-}$ [B6.129P2(C)-*Ccr7*^{tm1Rfor}/J], *Cxcr5*$^{-/-}$ (B6.129S2(Cg)-*Cxcr5*^{tm1Lipp}/J) and IRF4 flox [B6.129S1-*Irf4*^{tm1Rdf}/J] mice were purchased from Jackson Laboratories. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Generation of Cas9 mRNA and gRNAs. To generate Cas9 mRNA, the pCDNA3CAS9HA2XNLS plasmid was linearized with Xba I, phenol chloroform extracted, and then used as the template for *in vitro* transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). To generate gRNAs, single-stranded DNA oligos were used as templates. Sequences of oligos is shown below (bold sequence represents homology to *Dock8* intronic sequences):

5'-gRNA

AGCCACCTCATGCAGACCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC

TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT

3'-gRNA

CATTCTAGCTGAGTCCTGTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC

TAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT

A T7 promoter was added to each gRNA template by PCR amplification using the following primers:

5' F GaaattaatacgactcactatagggagaAGCCACCTCATGCAGACCTTGTTTTAGAGC

5'R AAAAAAGCACCGACTCGGTG

3'F Gaaattaatacgactcactatagggaga**CATTCTAGCTGAGTCCTGTG**GTTTTAGAGC

3'R AAAAAAGCACCGACTCGGTG

The T7-gRNA PCR products were column purified and used as the template for *in vitro* transcription using MEGAshortscript T7 kit (Life Technologies). Cas9 mRNA and the sgRNAs were purified using MEGAclear kit (Life Technologies) and eluted in RNase-free water. Donor oligos containing loxP sites were as follows.

5’ LOXP OLIGO

AG*G*A*GAGAAGCCATGTTTCATGTGCTCACAGCTCTTCTGCCCAGCAGCCACCTCA

TGCAGACCTTataacttcgtataatgtatgctatacgaagttatTGGAATAAATGATGAATGAGTGAAGGA

ACGAATGCTGGTGGTCAAGAACACTGCTCAGTGTC*T*G*G

3’ LOXP OLIGO

AA*C*C*CTTGCTTCCTCCCTCCTCCTCAGGTAACTCCGTTGTTAGCTGTGTCTCTTCA

GTGTCTTCTGCCAAGCTCACTGTGGATGACCATTCTAGCataacttcgtataatgtatgctatacgaagtt

atTGAGTCCTGTGTGGACTCTACCCCCAGGTACCTCTACCATCTACAAGTTGCTATGA

GAA*A*A*G

* = phosphorothioate modification

One-Cell embryo injection. Superovulated female C57BL/6 mice (4 week old) were mated to C57BL/6 stud males, and fertilized embryos were collected from oviducts. Cas9 mRNAs (100ng/μl) and gRNA (each 50ng/μl) and donor oligos (100ng/μl each) were resuspended in injection buffer (Tris-Cl, 5mM, pH 7.4; EDTA, 0.1mM, pH 8.0), and injected into the cytoplasm of fertilized eggs. Injected zygotes were transferred into uterus of pseudopregnant females. Resulting pups were genotyped by PCR and Sanger sequencing to confirm insertion of loxP sites flanking exons 10-14 using the following primers:

5’ F GGGAGGAGAGAAGCCATGTT

5’ R GAAACATGGGCACCAAGG

3’ F TTCAGTGTCTTCTGCCAAGC

3’ R GGGCCCTTTTCTCATAGCA

Correctly targeted mice were bred to C57BL/6 mice to confirm presence of loxP sites on the same chromosome. Mice were backcrossed with C57BL/6 mice for 3 generations before crossing to relevant Cre expressing lines.

Lung and skin dendritic cell analysis. Lungs were harvested from mice, minced and digested in RPMI containing fetal bovine serum (2%; Sigma), collagenase IV (0.5mg/mL; Sigma), collagenase D (0.5mg/mL: Roche) and DNase I (100units/mL; Sigma) for 40 minutes at 37°C and then RBC lysed. For skin digestion, tissue (1 cm^2) was removed after depilation, chopped into small fragments and incubated for 90 min at 37°C in 3 ml PBS containing Dispase (2.5 mg/ml, Roche) and DNase (5 μg/ml, Sigma) followed by incubation for 30 min at 37°C in collagenase (3 mg/ml; Worthington, Lakewood, NJ) and DNase (5 μg/ml). Following digestion, cell suspensions were filtered twice through nylon mesh (70 and 30 μm pore size) before staining for flow cytometric analysis.

Immunization and detection of serum antibodies. Mice were immunized with 10μg NP16-OVA (Biosearch Technologies) and 1μg LPS (Invivogen) i.n or s.c. and boosted once on day 14 with 10μg NP-OVA alone. Sera from immunized mice were collected 7d post boost and ELISA for detecting NP4-specific IgG1, NP-4-specific IgG2c and NP16-specific IgM was performed. Briefly, 25μg/mL NP4-BSA or NP16-BSA was coated on 96 well Maxisorp immuno plates (Thermo Fisher Scientific) overnight. Plates were blocked with 1% BSA in PBS at 37^{o}C for 1h followed by addition of serially diluted serum samples and incubated at 37^{o}C for 2h. This was followed by incubation with biotinylated-anti-mouse IgG1 antibody (Clone A85-1; BD Biosciences) or anti-Mouse IgG2c HRP (Southern Biotech) or anti-Mouse IgM HRP (Southern

Biotech) at 37^{o}C for 1h. Strepavidin-HRP (Zymed) was added and incubated for 1h before developing the plate with chromogen, tetramethylbenzidine. Plates were read at 450nm on a microplate reader (Molecular Devices). Serum from mice immunized with NP-OVA in alum, or CFA or LPS were used as reference standards to calculate arbitrary units for IgG1, IgG2c and IgM respectively.

RNA-Seq analysis. RNA-Seq libraries were prepared with KAPA Stranded mRNA-Seq kit (Roche) according to manufacturer's instruction. First, poly A RNA was isolated from 100ng total RNA using oligo-dT magnetic beads. Purified RNA was then fragmented at 85C for 6 min., targeting fragments range 250-300bp. Fragmented RNA is reverse transcribed with an incubation of 25C for 10mins, 42C for 15mins and an inactivation step at 70C for 15mins. This was followed by second strand synthesis at 16C, 60mins. Double stranded cDNA fragments were purified using Ampure XP beads (Beckman). The dscDNA were then A-tailed, and ligated with illumina adaptors. Adaptor-ligated DNA was purified using Ampure XP beads. This is followed by 10 cycles of PCR amplification. The final library was cleaned up using AMpure XP beads. Sequencing was performed on Illumina NextSeq platform generating paired end reads of 76bp. Fragments were quasi-mapped to the mouse genome mm10 using salmon(*4*) at the gene level and differential expression analysis between CD103 and CD11b populations was performed using DESeq2 package in R(*5*). Differentially expressed genes with p-values <= 0.01 and fold-change >=2 were used for the pathway analysis with IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). RNA-Seq data was submitted to the Gene Expression Omnibus (*GEO*) database repository.

Relative gene expression analyses. RNA isolation and real-time PCR analysis were performed as described previously(*1*). Briefly, RNA was isolated from sorted BMDCs subsets using RNeasy Micro kit (Qiagen). cDNA was prepared using Oligo(dT) (Life Technologies), dNTP Mix (Life Technologies), and SuperScript III Reverse Transcriptase (Life Technologies). Real-time PCR was performed using SYBR Green Fast Universal Master Mix and Low ROX (Kapa Biosystems). cDNA expression was analyzed by the ΔCt (change in cycle threshold) method, and results were normalized to *Hprt* obtained in parallel reactions during each cycle and then expressed relative to control samples. The following primers were used: CXCL13: TCGTGCCAAATGGTTACAAA and GGTGCAGGTGTGTCTTTTGA; IRF4: CAATGTCCTGTGACGTTTGG and GGCTTCAGCAGACCTTATGC. CCR7: GTGGTGGCTCTCCTTGTCAT and GAAGCACACCGACTCGTACA; HPRT: CTGGTGAAAAGGACCTCTCG and TGAAGTACTCATTATAGTCAAGGGCA.

BMDC culture and viability analysis. Murine bone marrow cells were isolated from WT and *Dock8*$^{-/-}$ mice and cultured on non-tissue culture treated 10 cm dishes at a concentration of $0.5x10^6$ cells/mL in 10ml of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences), 1% Penicillin Streptomycin (Gibco), 1% L-Glutamine (Gibco), 1% HEPES (Gibco), 1% Sodium Pyruvate (Gibco), 50uM B-Mercapto-ethanol (Sigma) in presence of 10ng/mL of recombinant murine GM-CSF (PeproTech). Media was replaced on day 3 and 6. For the time course analysis, on day 7, WT and DOCK8-deficient BMDCs were harvested from dishes and plated in triplicates in non-tissue culture treated 12 well plates at a concentration of $1x10^6$ cells/mL. Cells were stimulated with LPS (100ng/ml, Sigma) for either 1 or 2 days or left untreated. For each day of the time course BMDCs were suspended in Phosphate Buffered Saline and stained for viability Ghost Dye-V510 (Tonbo Biosciences) for 10 min at room temperature

and were washed with PBS containing 2% Fetal Bovine Serum. Cells were then stained for CD11c, CD11b and MHC-II for 15 min at room temperature for FACS analysis as previously described. For the apoptotic and necrotic assay, BMDCs were suspended in Annexin V binding buffer containing FITC Annexin V and PI (Biolegend) for 15 min at room temperature before analysis.

Mixed bone marrow chimeras. CD45.1 congenic recipients were irradiated (1300 Rads in split doses of 650 each three hours apart). A total of 10^6 bone marrow cells at a ratio of 1:1 from CD45.1.2 ZBTB-DTR and CD45.2 DC-*Dock8*$^{\Delta}$ (or control mice) were given i.v. After 10 weeks of reconstitution, diphtheria toxin administration and OT-II T cell transfer was performed as described(*6*). Mice were immunized with 10μg OVA+ 1μg LPS i.n. Six days post immunization Tfh cells were analyzed as described above.

Influenza PR8 Infection model and antibody analysis. Influenza virus strain A/PR/8/34 (PR8; H1N1), used for all experiments, was propagated for 2-3 days at 35°C in the allantoic cavities of 10-day old fertilized chicken eggs (*7*). Harvested virus was purified by 10-50% sucrose gradient centrifugation, titered by MDCK plaque assay and stored at -80 °C. For inactivation, PR8 was treated with 0.1% formalin at 4 °C for 7 days, then incubated at 56 °C for 30 min. Viral protein concentration was determined using a Nanodrop. For all i.n. immunizations, mice were anesthetized by metafane and given 100 μg of virus protein in 50 μl of PBS at day 0, then boosted with 50ug of virus protein at day 7 and day 14. Serum samples were collected 21 days after primary immunization/7 days after the final boost. Immunized or naïve mice were challenged with 3000 PFU live PR8 (100 LD_{50}). Weight loss was monitored daily until 12 d post

infection. Mice with >25% weight loss or < 2 on the clinical disease scoring system, were euthanized**.** Influenza virus-specific antibodies were determined by ELISA as previously described (*8*). Briefly, formalin inactivated purified PR8 were coated on 96-well plates in carbonate buffer at 4°C overnight. After blocking, serial diluted serum samples were incubated at RT for 2hrs, and the plates were then incubated with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Results are shown as average OD value for each dilution

Immunofluorescence microscopy. Mice were immunized with 1.0μm yellow-green carboxylate fluorospheres (molecular probes) (500μg/mouse) and LPS (5μg/mouse) intranasally. 18-20 hours later MedLN were harvested, embedded in OCT Cryomount (Histolabs), flash-frozen in liquid nitrogen-chilled isopentane and subsequently cryo-sectioned into 8 μm thick sections. Tissue sections were fixed in 2% formaldehyde for 10 min, permeabilized with 0.1% Triton-X for 4 min and treated with Avidin-Biotin Kit (Biocare Medical) or with low protein IHC blocking buffer (eBioscience). Sections were stained for 1h at RT with primary antibodies mixed in PBS supplemented with 0.1% BSA. After washing, sections were stained with secondary antibodies anti-rabbit F(ab)2 (Jackson ImmunoResearch Laboratories) and streptavidin conjugates for 40 min at RT. Alternatively biotinylated Abs were pre-labeled using FlexiStain^{TM} (Kromnigon AB, Sweden). After washing, sections were mounted with ProLong Diamond Anti-Fade (Invitrogen). Anti-B220 (RA3-6B2) and CD11c (HL3) were purchased from BD Biosciences. Anti-CD3 (145-2C11) and CD103 (2E7) were purchased from Biolegend. Anti-CD11b (M1/70) was purchased from Affymetrix.

Images were acquired using Metasystem automated slide scanner (MetaSystems, Germany) equipped with SpectraSplit^{TM} filter system for extended multicolor imaging (*9*) (Kromnigon AB, Sweden) with a Zeiss Image Z.1 microscope, Plan-Apochromat 20x/0.8 objective (Carl Zeiss Microscopy, Germany) (Fig 6 B, C, G and Sup Fig 6 B, E, F). Alternatively, images were acquired using an LSM 880 system (Carl Zeiss Microscopy, Germany) with a Zeiss Image Z.1 microscope, Plan-Apochromat 40x/1,3 objective (Carl Zeiss Microscopy, Germany) (Fig 6 A, E, F and Sup Fig. 6 A, C, D). Brightness and contrast was adjusted using ImageJ (National institutes of Health, Bethesda, USA) or the Zen software (Black ed. v. 2,3, Carl Zeiss Microscopy, Germany). Bead^{+} cells were enumerated in a blinded fashion.

Flow cytometry. For staining surface antigens on DCs, mediastinal LN were minced and digested with collagenase IV (1 mg/mL; Sigma) for 40min at 37°C. For T cells, mediastinal LN were homogenized between frosted slides. Erythrocytes were lysed by briefly suspending cells in ACK lysis buffer. Cells were resuspended in 2% Fetal Bovine Serum in Phosphate Buffered Saline and incubated with fluorochrome-conjugated antibody cocktail for 30 min on ice. CCR7 staining was performed at 37°C and CXCR5 staining was performed at room temperature, both for 30 mins. For intracellular cytokine staining (ICCS), cells were restimulated with PMA (50ng/mL) and Ionomycin (1μg/mL) for 5 hours and Golgiplug (BD Biosciences) was added for the last 3 hours. ICCS was performed using BD ICCS kit as per manufacturer's instructions. Anti-B220 (RA3-6B2), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (N418), CD21/35 (7E9), CD23 (B3B4), CD24 (M1/69), CD45.1 (A20), CD45.2 (104), CD103 (2E7), CD326 (G8.8), CXCR5 (L138D7), GL7, PD1 (RMP1-30 and 29F.1A12), TCRβ (H57-597), IL-2 (JES6-5H4), MHC II (M5/114.15.2), Vα2 (B20.1), IFNγ (XMG1.2) mAbs were purchased from

BioLegend. Anti-CD44 (IM-7), CD95 (Jo2), CD45 (30-F11), CD25 (PC61), CXCR5 (2G8) and IgG1 (A85-1) mAbs were purchased from BD Biosciences. Anti-CCR7 (4B12) mAb was purchased from ThermoFischer Scientific. For total counts, a fixed volume of cell suspension was stained with CD45 (30-F11) for 20 mins at room temperature. To each sample, 25μl of CountBright™ Absolute Counting Beads (ThermoFischer Scientific) was added and immediately acquired on a flow-cytometer. Absolute counts of $CD45^+$ cells were calculated by the following equation: (Events acquired in $CD45^+$ gate/Events acquired in $Bead^+$ gate) x Absolute number of beads in each sample x Volume of sample. All flow-cytometry samples were acquired on LSRII (BD Biosciences) or MACSQuant (Miltenyi) flow cytometers and analyzed by FlowJo software (Version 9.3.2, TreeStar).

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Fig. S1

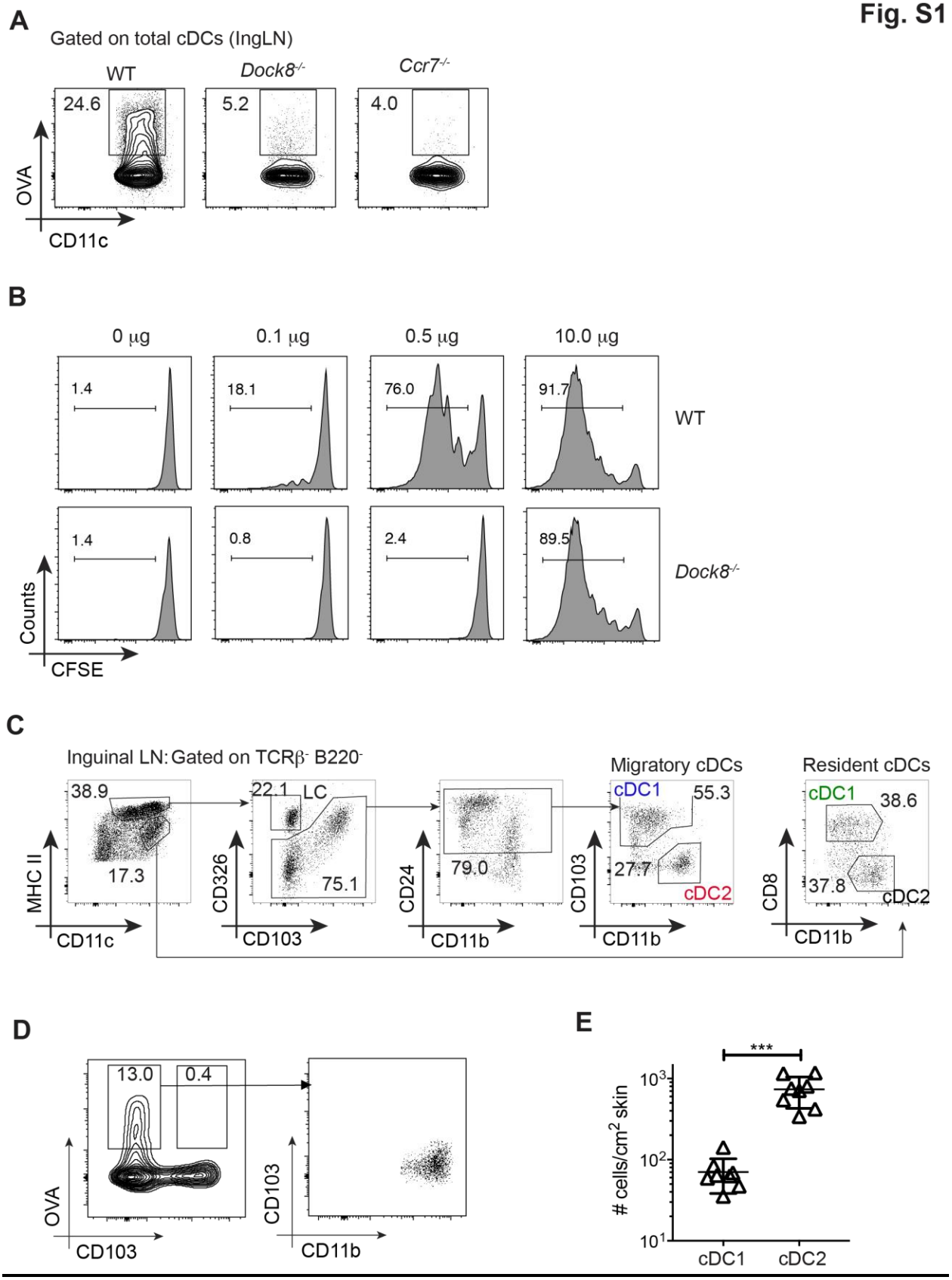

A
Gated on total cDCs (IngLN)
WT
Dock8-/-
Ccr7-/-
24.6
5.2
4.0
OVA
CD11c
B
0 μg
0.1 μg
0.5 μg
10.0 μg
1.4
18.1
76.0
91.7
WT
1.4
0.8
2.4
89.5
Dock8-/-
Counts
CFSE
C
Inguinal LN: Gated on TCRβ- B220-
Migratory cDCs
Resident cDCs
38.9
17.3
MHC II
CD11c
22.1
LC
75.1
CD326
CD103
79.0
CD24
CD11b
cDC1
55.3
27.7
cDC2
CD103
CD11b
cDC1
38.6
37.8
cDC2
CD8
CD11b
D
13.0
0.4
OVA
CD103
CD103
CD11b
E
***
cells/cm2 skin
10^3
10^2
10^1
cDC1
cDC2

Fig. S1. Skin migratory cDC2s transport most of the antigen to draining LNs after subcutaneous immunization.

(A) In contrast to WT mice, few antigen$^+$ DCs were observed in the inguinal LNs of either *Dock8*$^{-/-}$ or *Ccr7*$^{-/-}$ mice. Frequency of OVA$^+$ DCs in inguinal LNs of WT, *Dock8*$^{-/-}$ and *Ccr7*$^{-/-}$ mice 18h post s.c. immunization with 50μg/flank OVA Alexa647 and 1μg/flank LPS. Representative data from 4 independent experiments with 3 mice per group.

(B) Higher s.c. antigen doses overcome T cell activation defect in *Dock8*$^{-/-}$ mice. WT and *Dock8*$^{-/-}$ recipient mice were adoptively transferred with 10^6 CFSE-labeled OTII T cells and immunized s.c. with 0-10μg OVA and 1μg LPS and analyzed for T cell proliferation 3d later. Representative data from 2 independent experiments with 2 mice per group.

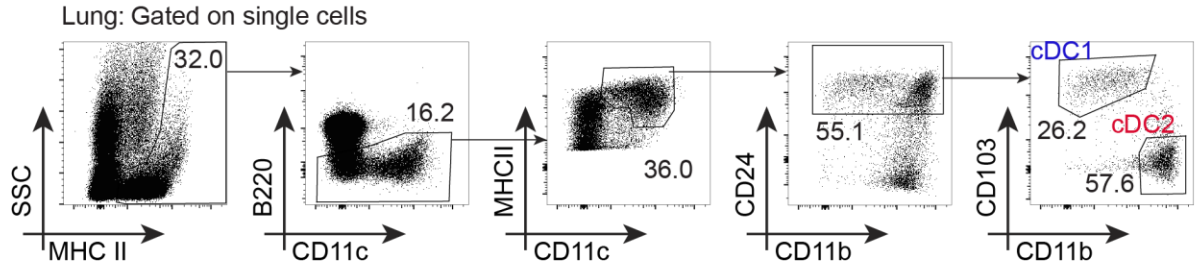
(C) Gating strategy for identifying LN-resident, migratory cDC subsets and Langerhans cells (LC) in inguinal LNs.

(D) Flow cytometry plot from (Fig. 1C) demonstrating that CD103- migratory cDCs from the IngLNs of immunized mice containing antigen are CD11b+ cDC2s.

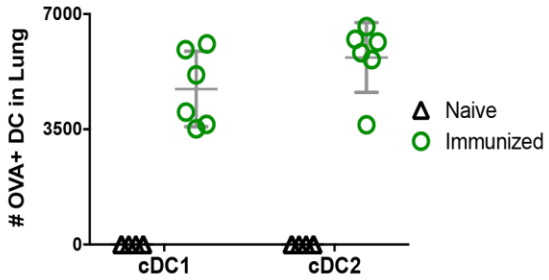
(E) Number of cDC1 (CD103$^+$CD64$^-$MHCII$^+$CD11c$^+$) and cDC2 (CD11b$^+$CD64$^-$MHCII$^+$CD11c$^+$) in digested skin from naïve mice pooled from 4 separate experiments. Mean (±s.d.) ***P<0.001.

Fig. S2

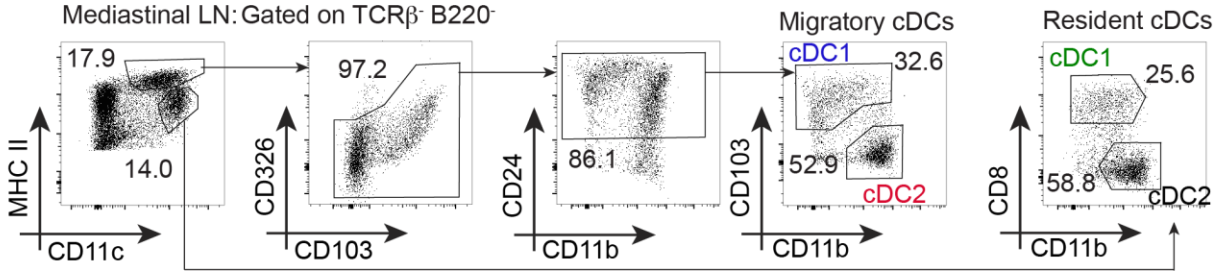
A


Lung: Gated on single cells
32.0
SSC
MHC II
16.2
B220
CD11c
MHCII
36.0
CD11c
CD24
55.1
CD11b
cDC1
26.2
cDC2
57.6
CD103
CD11b

B


OVA+ DC in Lung
7000
3500
0
cDC1
cDC2
Naive
Immunized

C


Mediastinal LN: Gated on TCRβ- B220-
17.9
14.0
MHC II
CD11c
97.2
CD326
CD103
CD24
86.1
CD11b
Migratory cDCs
cDC1
32.6
52.9
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CD103
CD11b
Resident cDCs
cDC1
25.6
58.8
cDC2
CD8
CD11b

D

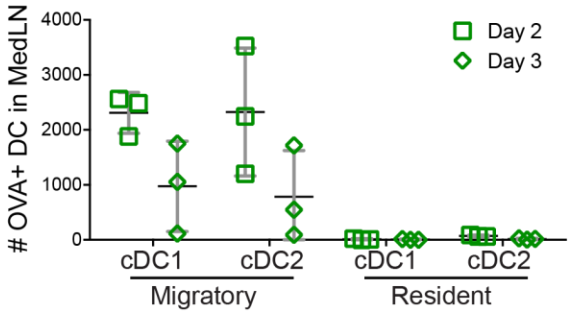
OVA+ DC in MedLN
4000
3000
2000
1000
0
Day 2
Day 3
cDC1
cDC2
cDC1
cDC2
Migratory
Resident

Fig. S2. Intranasal immunization targets antigen to both migratory cDC subsets.

(A) Gating strategy for identifying migratory and LN-resident cDC subsets in lungs.

(B) Absolute counts of OVA^+ migratory cDCs in the lungs of WT mice (as gated in Fig. S2A) 18h post i.n. immunization with 50μg/mouse OVA Alexa647 and 1μg/mouse LPS. Each symbol represents an individual mouse; mean (±s.d.). Representative data of 2 individual experiments with 4-6 mice/group.

(C) Gating strategy for identifying migratory and LN-resident cDC subsets in mediastinal LNs

(D) Few $antigen^+$ resident cDCs are observed in LNs out to 3 days following intranasal immunization. Absolute counts of OVA^+ cDC subsets in the MedLN of WT mice (as gated in Fig. S2C) 2 days and 3 days post i.n. immunization with 50μg/mouse OVA Alexa647 and 1μg/mouse LPS. Each symbol represents an individual mouse; mean (±s.d.). 3 mice/group; 2 independent experiments.

Fig. S3

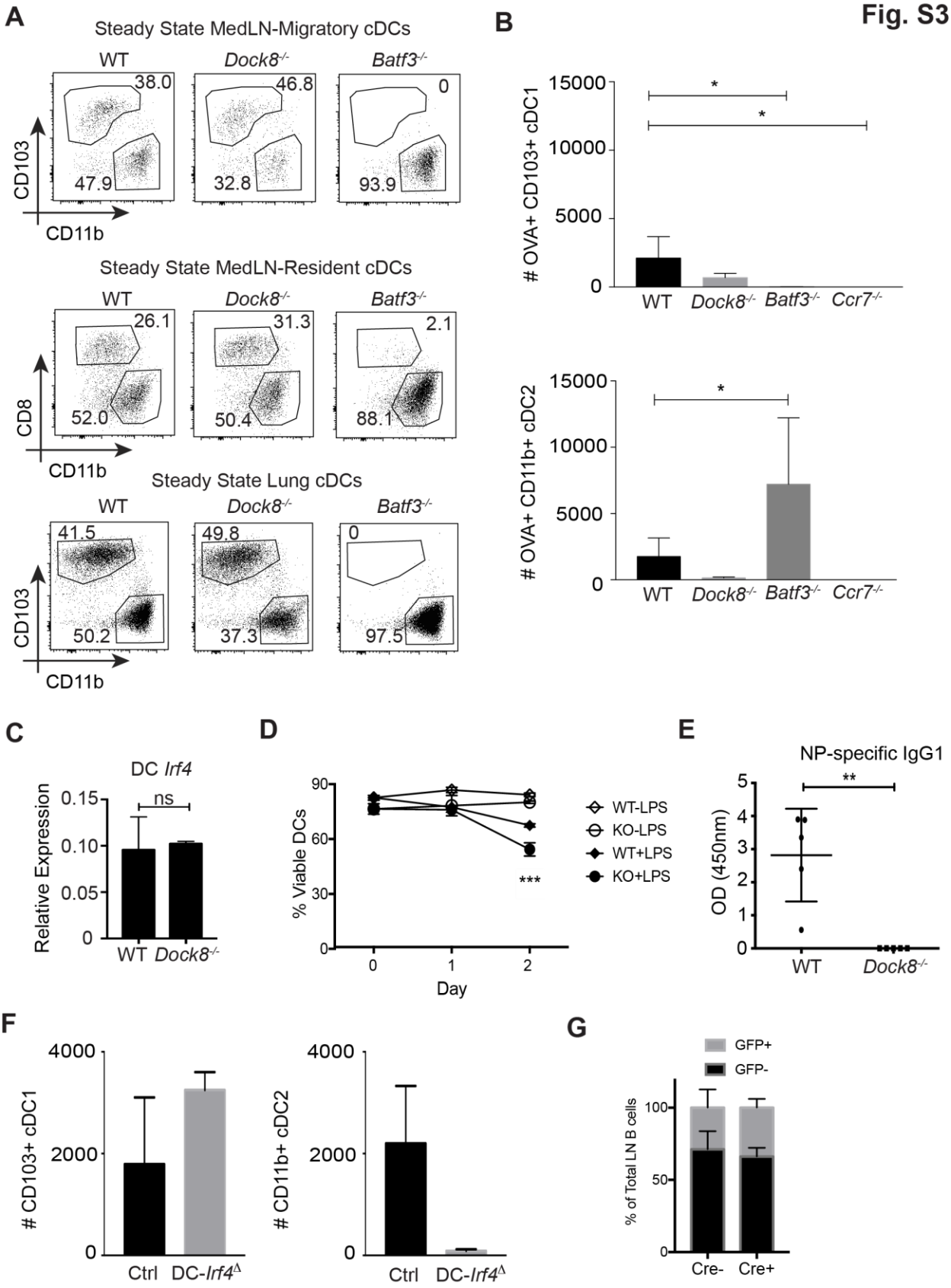
A
Steady State MedLN-Migratory cDCs
WT
Dock8-/-
Batf3-/-
38.0
46.8
0
47.9
32.8
93.9
CD103
CD11b
Steady State MedLN-Resident cDCs
26.1
31.3
2.1
52.0
50.4
88.1
CD8
CD11b
Steady State Lung cDCs
41.5
49.8
0
50.2
37.3
97.5
CD103
CD11b
B
OVA+ CD103+ cDC1
15000
10000
5000
0
*
*
WT
Dock8-/-
Batf3-/-
Ccr7-/-
OVA+ CD11b+ cDC2
15000
10000
5000
0
*
WT
Dock8-/-
Batf3-/-
Ccr7-/-
C
DC Irf4
Relative Expression
0.15
0.10
0.05
0
ns
WT
Dock8-/-
D
% Viable DCs
90
60
30
0
***
0
1
2
Day
WT-LPS
KO-LPS
WT+LPS
KO+LPS
E
NP-specific IgG1
OD (450nm)
5
4
3
2
1
0
**
WT
Dock8-/-
F
CD103+ cDC1
4000
2000
0
Ctrl
DC-Irf4Δ
CD11b+ cDC2
4000
2000
0
Ctrl
DC-Irf4Δ
G
GFP+
GFP-
% of Total LN B cells
100
50
0
Cre-
Cre+

Fig. S3. *Dock8* deficiency is not associated with impaired lung or MedLN DC development or survival.

(A) Analysis of DC subsets at steady state in WT, *Dock8*$^{-/-}$ and *Batf3*$^{-/-}$ mice. Flow cytometry dot plots show the frequency of migratory cDCs (upper panels) and LN-resident cDCs in MedLNs (middle panels) and cDCs in the lungs (lower panels).

(B) Absolute counts of OVA^{+} migratory cDC subsets in the MedLN of WT, *Dock8*$^{-/-}$, *Batf3*$^{-/-}$ and *Ccr7*$^{-/-}$ mice (as gated in Fig. S2C) 18h post i.n. immunization with 50μg/mouse OVA Alexa647 and 1μg/mouse LPS. Pooled analysis from 2 independent experiments with a total of 3-5 mice/group.

(C) Relative expression of *Irf4* mRNA from WT and *Dock8*$^{-/-}$ derived unstimulated BMDCs normalized to *hprt*. Error bars = s.d. of 3 biological replicates.

(D) LPS mediated maturation does not induce greater cell death in *Dock8*$^{-/-}$ DCs. Frequency of viable (PI^{-}AnnexinV^{-}) bone marrow derived DCs (CD11c^{+} MHCII^{hi}) from WT and *Dock8*$^{-/-}$ mice stimulated in vitro with 100ng/mL LPS for 0, 1 and 2 days.

(E) *Dock8*$^{-/-}$ mice have impaired antibody responses to inhaled antigen. WT and *Dock8*$^{-/-}$ mice were immunized i.n. with 10μg NP16-OVA with 1μg LPS and boosted with 10μg NP16-OVA. NP-4 specific serum IgG1 from WT or *Dock8*$^{-/-}$ mice 21d post immunization is shown. Serum dilution 1:3000 and each symbol represents an individual mouse.

(F) Absolute counts of OVA^{+} migratory cDC subsets in the MedLN of DC-*Irf4*$^{\Delta}$ and control mice (*Irf4*$^{fl/fl}$ *Itgax-Cre*$^{-}$) 18h post i.n immunization with 50μg OVA-AF647 and 1μg LPS (as in Fig 2F). Pooled analysis from 2 independent experiments with a total of 3-4 mice/group.

(G) GFP expression, marking *Irf4* deletion, in MedLN B cells from immunized DC-*Irf4*$^{\Delta}$ and control (Cre-negative) mice.

Data representative of 2-4 individual experiments with 2-5 mice/group. Graphs indicate the mean (±s.d.). *P<0.05, **P<0.01, ***P<0.001.

Fig. S4

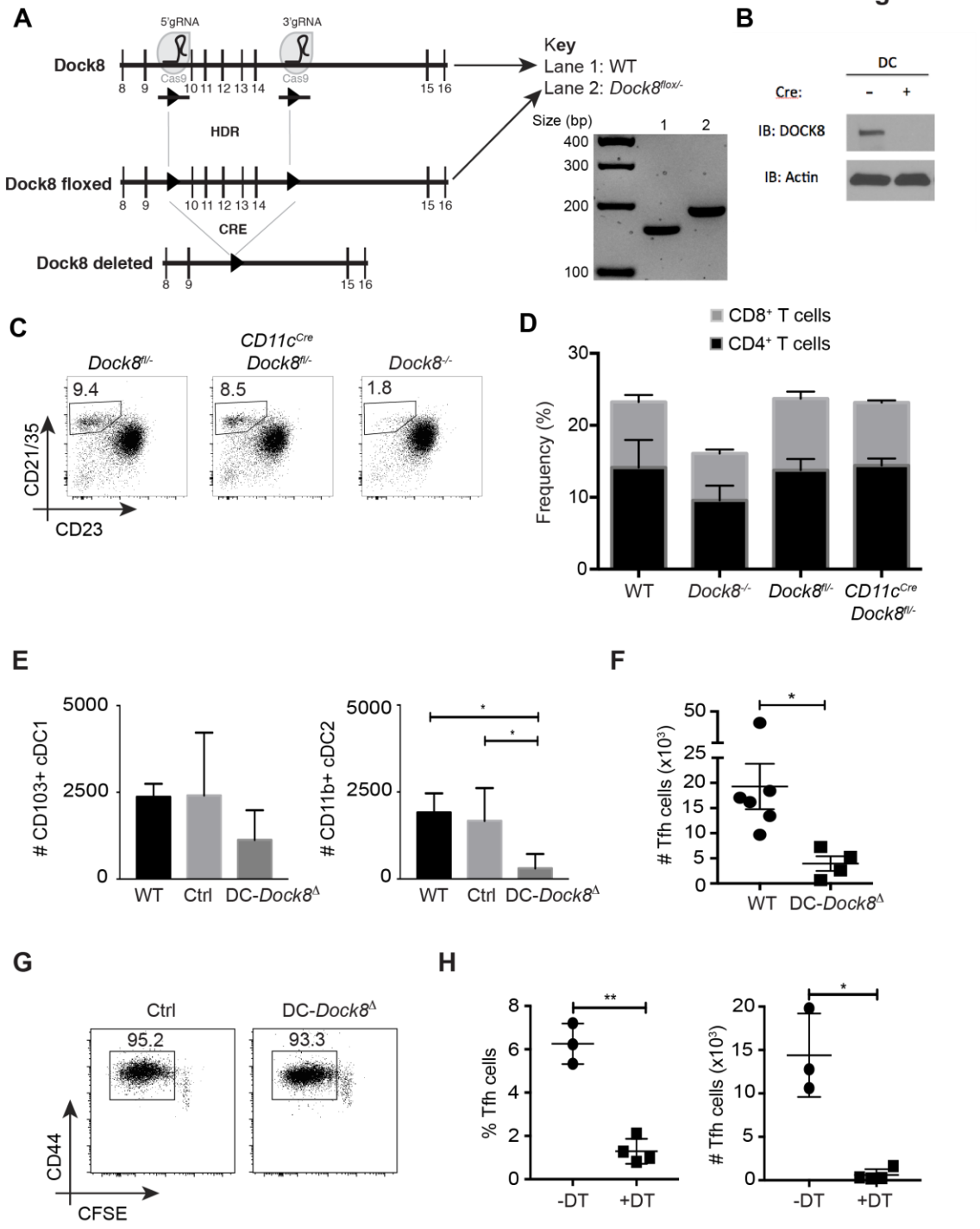

Fig. S4. Generation and characterization of *DC-Dock8*$^{\Delta}$ mice.

(A) Targeting details of introducing *loxP* sequences flanking exons 10-14 of *Dock8* using CRISPR/Cas9 genome editing technology. Guide RNAs (gRNA) were designed against

intronic sequences flanking exons 10-14. *In vitro* transcribed Cas9 mRNA and gRNAs were injected into fertilized oocytes to generate *Dock8*$^{f/+}$ mice as previously described (*10*). Resulting offspring were sequenced to confirm insertion. Inset shows increase in amplicon size of *Dock8* genotyping PCR following insertion of *loxP* sequences.

(B) Confirmation of DC specific deletion of *Dock8* with western blot analysis of BMDCs generated from *Dock8*$^{fl/-}$ (Cre^{-}) and DC-*Dock8*$^{\Delta}$ (Cre^{+}) mice.

(C) Frequency of splenic marginal zone B cells in *Dock8*$^{fl/-}$, DC-*Dock8*$^{\Delta}$ and *Dock8*$^{-/-}$ mice.

(D) Frequency of splenic CD4^{+} and CD8^{+} T cells in WT, *Dock8*$^{-/-}$, *Dock8*$^{fl/-}$ and DC-*Dock8*$^{\Delta}$ mice.

(E) Absolute counts of OVA^{+} migratory cDC subsets in the MedLN of WT, control (*Dock8*$^{fl/-}$) and DC-*Dock8*$^{\Delta}$ mice 18h post i.n immunization with 50μg OVA-AF647 and 1μg LPS (as in Fig 3A). Pooled analysis from 2 independent experiments with 3-6 mice/group.

(F) Number of OTII Tfh cells in MedLN from mice immunized as in (Fig. 3D).

(G) Recipient control (*Dock8*$^{fl/-}$ *Itgax-Cre*$^{-}$) and DC-*Dock8*$^{\Delta}$ mice were adoptively transferred with CFSE-labeled OTII T cells and immunized i.n with 10μg OVA and 1μg LPS and analyzed for T cell proliferation 3d later. Data representative of 2 independent experiments with 2-3 mice/group.

(H) Mixed bone marrow chimeras were generated with DC-*Dock8*$^{\Delta}$ (Cre^{+}) and Zbtb46-DTR donors. OTII T cells were transferred and groups of mice received either Diphtheria toxin (DT) to deplete WT DCs or PBS (-DT). OVA/LPS was delivered i.n. as in (Fig. 3C) and % and number of Tfh cells of transferred OTII cells was measured in MedLN on day 6. Data representative of 2 independent experiments with 3-4 mice/group. *P<0.05, **P<0.01.

Fig. S5

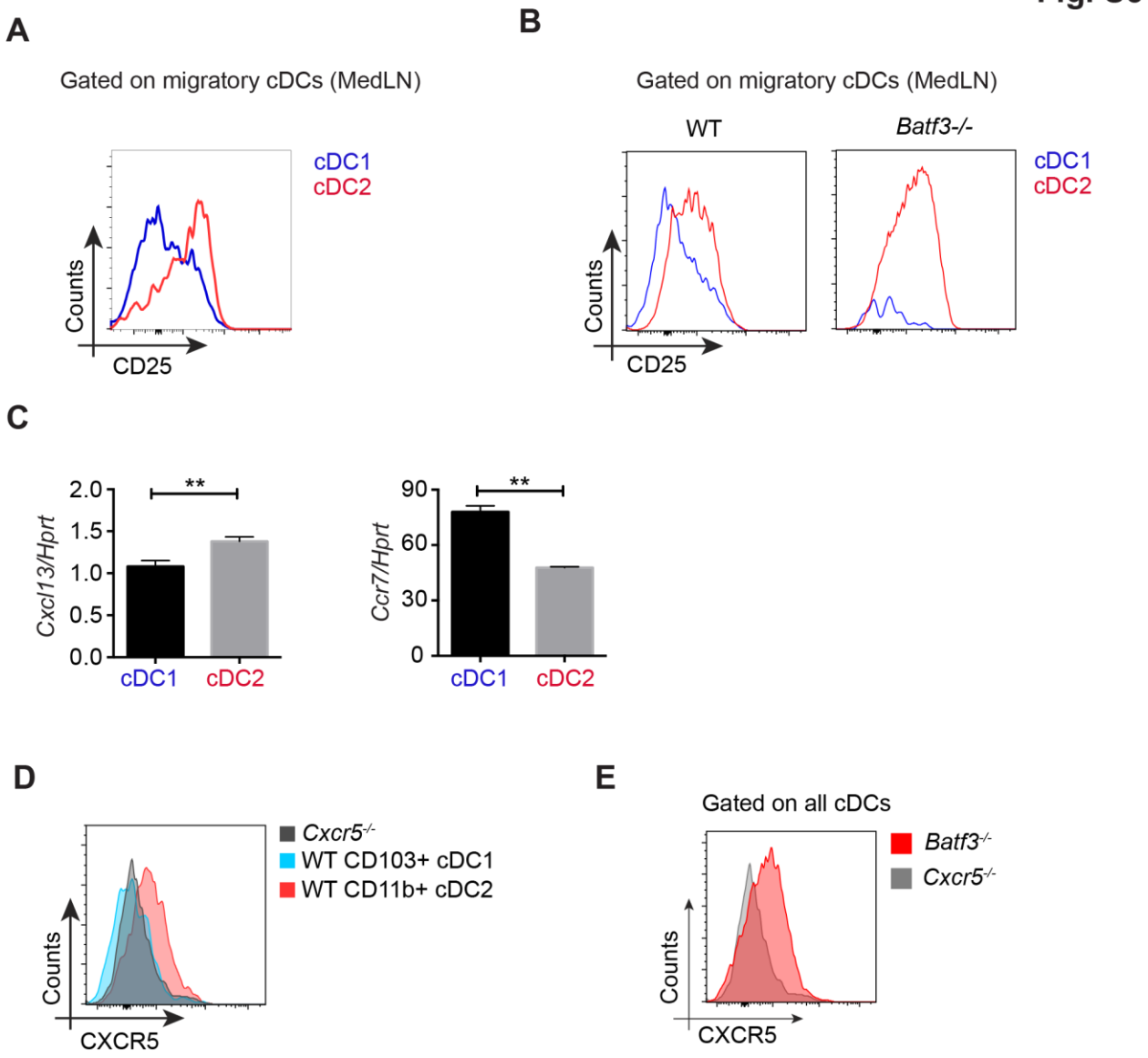

Fig. S5. cDC2 characteristics suggest a unique localization within MedLNs.

(A to B) Flow cytometry histograms shows an overlay of CD25 expression on migratory cDC subsets in MedLNs of WT (A-B) and *Batf3*$^{-/-}$ mice (B), 18h post i.n. immunization with 1µg LPS.

(C) Quantitation of *Cxcl13* and *Ccr7* mRNA normalized to *Hprt* from sorted migratory cDC subsets from mediastinal LN (MedLN) of WT mice 18h post i.n. immunization with 1µg LPS. ***P*<0.01.

(D) Flow cytometry histograms shows an overlay of CXCR5 expression on migratory cDC subsets of WT and all cDCs of *Cxcr5*$^{-/-}$ mice, 18h post i.n. immunization with 1µg LPS (MedLN analyses).

(E) Flow cytometry histograms shows an overlay of CXCR5 expression on all cDCs in MedLNs of *Batf3*$^{-/-}$ and *Cxcr5*$^{-/-}$mice, 18h post i.n. immunization with 1µg LPS.

Data representative of 2-4 independent experiments and 2-3 mice/group.

Fig. S6

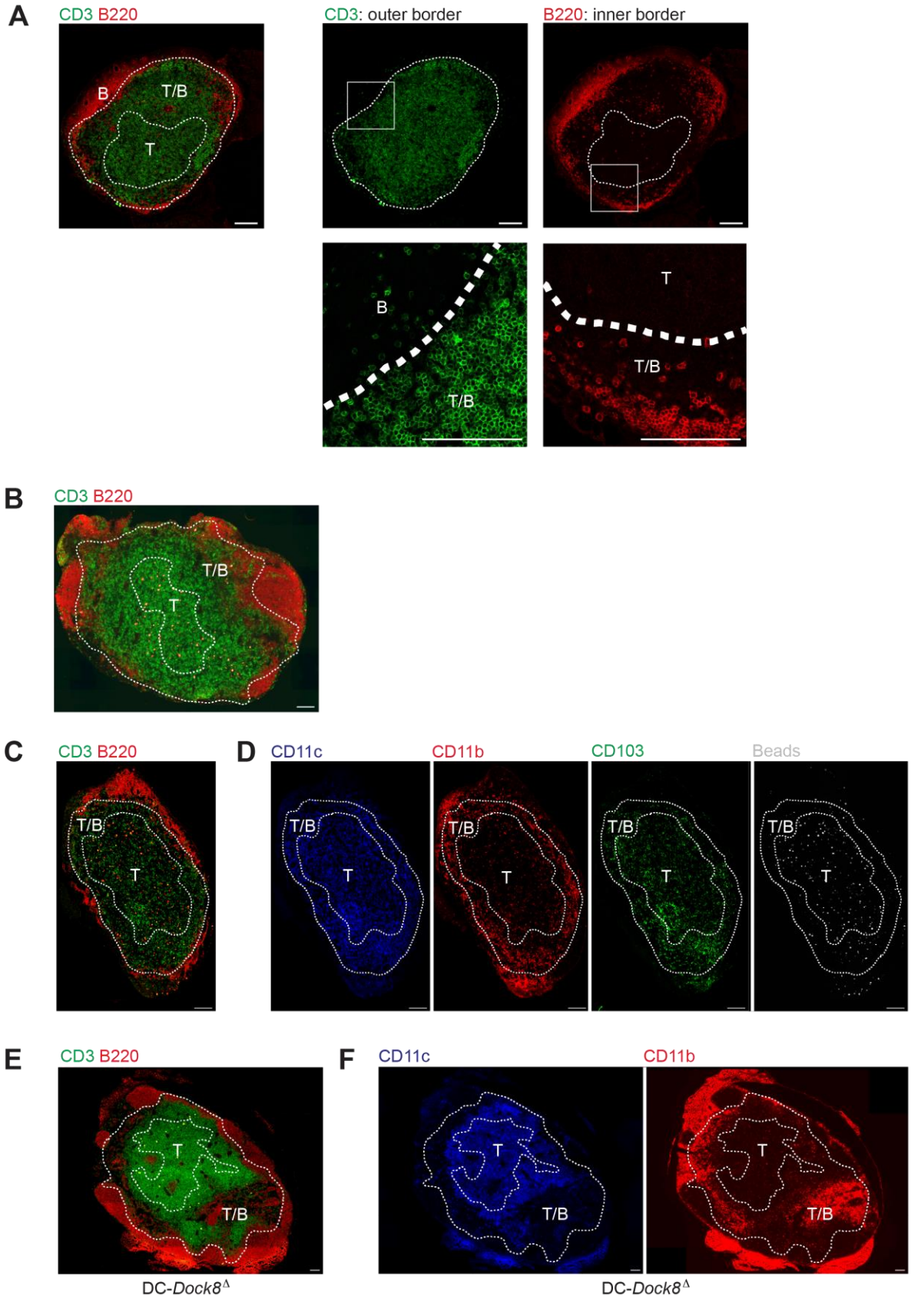
A
CD3 B220
B
T/B
T
CD3: outer border
B220: inner border
B
T/B
T
T/B
B
CD3 B220
T/B
T
C
CD3 B220
T/B
T
D
CD11c
CD11b
CD103
Beads
T/B
T
E
CD3 B220
T
T/B
DC-Dock8Δ
F
CD11c
CD11b
T
T/B
DC-Dock8Δ

Fig. S6. Definition of T cell zone and T-B border in MedLN.

(A) Immunofluorescence image used to define T cell zone (T) and T-B border (T-B) in WT MedLN as in Fig 6A. Upper panels show overlays and single stains for T cell ($CD3^+$) and B cell ($B220^+$) areas while lower panels show close-ups of defined borders.

(B) Immunofluorescence image used to define T cell zone and T-B border in WT MedLN as in (Fig 6B-C).

(C) Immunofluorescence image used to define T cell zone and T-B border in WT MedLN as in (Fig 6E-F).

(D) Immunofluorescence from multi-stained samples showing the channels for CD11c, CD11b, CD103 and beads, used to define migratory cDC subsets shown in Fig 6E-F.

(E) Immunofluorescence image used to define T cell zone and T-B border in DC-*Dock8*$^{\Delta}$ *(Dock8*$^{fl/-}$ *ItgaxCre*$^+$*)* MedLN as in (Fig 6G).

(F) Immunofluorescence from multi-stained samples showing the channels for CD11c and CD11b, used to define migratory cDC subsets shown in (Fig 6G).

Data is representative of 2-3 independent experiments and 2-4 mice per group. (Scale bars= 100μm).