Supporting Online Material for

Bifurcation of Toll-Like Receptor 9 Signaling by Adaptor Protein 3

Miwa Sasai, Melissa M. Linehan, Akiko Iwasaki*

*To whom correspondence should be addressed. E-mail: akiko.iwasaki@yale.edu

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This PDF file includes:

Supporting Online Materials

Materials and methods

Mice, viruses and reagents

Pearl mice, AP-3β$^{-/-}$ mice, and age and sex matched C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). AP-3δ subunit mutant 'mocha' mice were obtained from Dr. Victor Faundez (Emory University). Mice received i.v. tail vein injection of CpG-A (CpG 2216) (25 µg/mouse in 200 µl volume) and blood was collected at the indicated time points. All procedures used in this study complied with federal guidelines and institutional policies of the Yale Animal Care and Use Committee. CpG 2216 (CpG type-A) and CpG 1826 (CpG type-B) were purchased from TriLink BioTechnologies (San Diego, CA). Control GpC 2216 and poly U were synthesized by Yale Keck facility and Integrated DNA Technology (Coralville, IA), respectively. Poly I:C and LPS were purchased from Invivogen (San Diego, CA) and Sigma (St. Louis, MO) respectively. R848 was purchased from InVivogen. VSV was propagated and assayed on BHK cells as previously described (S*1*). Influenza virus type-A/PR8 strain (H1N1) was propagated and quantitated as previously described (S*2*).

Cell lines and primary cell culture

HEK293T cells were purchased from ATCC, and were maintained in DMEM supplemented with 10% FCS, penicillin/streptomycin (Invitrogen). RAW267.4 cells were purchased from ATCC and cultured in RPMI supplemented with 10% FCS, penicillin/streptomycin, 10 mM HEPES (Invitrogen) and 2-mercaptoethanol (Sigma). GFP-

fusion TLR9 expressing cells were generated by transduction with MSCV2.2 retrovirus, and TLR9-GFP positive cells were sorted by FACS Aria at Yale Sorting facility. Other retrovirally transduced cells were generated using the same method as TLR9-GFP stable RAW cells. Bone-marrow-derived pDC (S3), BM DC (S4) and BMM (S5) were prepared as previously described.

Plasmids

TLR9 sequence was amplified by PCR, and was cloned into a pEGFP-N1 plasmid (Clontech) to generate a carboxyl-terminus GFP-fusion expression construct. AP-3 μ3A and UNC93B sequences were amplified by PCR using mouse total bone marrow-derived cDNA as a template, then subcloned into pCR-Blunt (Invitrogen) and/or pEGFP-N1. Haemagglutinin-tagged TRAF3 was a kind gift from R. Medzhitov (S5). PH-domain of centaurin β2 (266-389 a.a.) was cloned by PCR to produce amino-terminus fusion protein with TRAF3-HA. Flag-tagged IRF7 in pcDNA3 vector was provided by R. Medzhitov. Retrovirus vectors containing TLR9-GFP, AP-3μ3A-Flag, UNC93B-HA, TRAF3-HA, and PH-TRAF3-HA were generated by subcloning from pEGFP-N1 or pCR-Blunt into pMSCV2.2. Lentivirus for YFP-IRF7 (S6) was a kindly gift from Dr. T. Taniguchi (Tokyo University). Lentivirus vectors containing TRAF3-HA or PH-TRAF3-HA were generated by subcloning from pMSCV2.2 into pCSII-EF-MCS vector.

Microscopy

Transduced BMMs were settled onto Alcian Blue-coated coverslips for 5 h. Cells were then stimulated with 3μM CpG-A, 1 μM CpG-B, 3 μM GpC, 2 μM Cy-5-labeled CpG-A or CpG-B (synthesized by Sigma) together with DOTAP (Roche, Indianapolis, IN) for 3 or 6 h, washed with PBS and fixed in 4% PFA. Cells were permeabilized and blocked with PS solution (1% goat serum, 0.05% saponin in PBS) for 15 minutes. Primary antibodies were diluted in PS as follows; mouse anti-GFP 1:2000 (Roche), rat anti-LAMP2 1:300 (gift from Dr. Ira Mellman) and rabbit anti-VAMP3 1:5000 (Synaptic Systems), rat anti-HA 1:20 (Roche). Calnexin antibody was a gift from Dr. Peter Cresswell (Yale University). In 100 μl volume, the antibodies were added to cells for 60 minutes. After washing with PS, secondary antibodies Alexa488 anti-mouse (Invitrogen), Cy3 anti-rat, Cy3 anti-rabbit, and Cy5 anti-rabbit (Jackson ImmunoResearch), were diluted at 1:400 in PS and added to cells for 30 minutes. Cells were washed with PS and then PBS. Coverslips were mounted on slides with Fluoromount G (Southern BioTech) and imaged using a Zeiss LSM 510 confocal microscope using 64x objective lens. Minimum of 15-20 cells were examined per slide.

ELISA

Protein level of IFNα present in the cell culture supernatant and serum was measured by ELISA as previously described (S7). Protein level of IL-12p40 present in cell culture supernatant and serum was measured using an ELISA kit (eBioscience) according to manufacturer's instructions.

Quantitative-PCR

RAW264.7 cells, BMM or Flt3L cultured pDCs were stimulated with DOTAP-A (3 μM), DOTAP-CpG-B (1 μM), R848 (1 μg/ml), DOTAP-poly U (20 μg/ml), LPS (200 ng/ml) or poly (I:C) (50 μg/ml) for indicated time periods. Total RNA was prepared using RNeasy kit (Qiagen), and then reverse-transcription was carried out with oligo (dT) primer using a Superscript II Kit (Invitrogen). Expression of the indicated genes following stimulation was measured by qRT-PCR using MX3000p (Stratagene).

Immunoprecipitation

HEK293T cells were transfected with indicated expression vectors by LipofectAMINE 2000 reagent (Invitrogen). Transfected HEK293T cells were collected 24 h after transfection and lysed in buffer containing 20 mM Tris (pH 7.5), 1% NP-40, 150 mM NaCl, and cocktail of protease inhibitors (Roche Diagnostics). Cell lysates were clarified by incubating with protein G sepharose (GE Healthcare) at 4°C for 1 h, and then precipitated using anti-Flag M2 mouse mAb (Sigma) and protein G sepharose for 12 h at 4°C. For immunoprecipitation using RAW264.7 cells, cells were treated with 1 mM dithiobis (succinimidyl proprionate) (DPS) for 30 min at room temperature. DPS was neutralized with 10 mM Tris (pH 7.5), for 15 min, and then cells were lysed in lysis buffer. After immunoprecipitation using anti-Flag mAb and three washes with lysis buffer, the proteins that were bound to the sepharose were separated from proteins G using excess Flag-peptide (Sigma), and reimmunoprecipitated using anti-GFP mAb for 6 hrs at 4 °C. After washing with lysis buffer three times, the proteins bound to the beads were eluted by boiling in SDS sample buffer. Eluted proteins were separated by electrophoresis on 7.5% polyacrylamide

gel, and transferred onto PVDF membrane. The membrane was incubated with mouse anti-GFP mAb (Santa Cruz), rabbit anti-HA pAb or mouse anti-Flag M2 mAb (Sigma), followed by incubation with HRP-conjugated secondary Ab against IgG of appropriate species (BioSource). The peroxidase activity was detected by ECL Western Blotting Detection Reagents (GE Healthcare).

Lentivirus mediated gene transduction in BMM

Lentivirus vectors pMDLg/pRRE (packaging plasmid) and pCMV-VSV-G-RSV-REV (combined Rev and VSV-G expression plasmid) and the construct containing the gene of interest were co-transfected into HEK293 T cells. Infectious lentiviruses in culture supernatants were harvested 48 h after transfection. BM cells were cultured with lentiviruses and were differentiated into BMM as previously described (S5). Cells were stimulated with the indicated DOTAP-CpG for 12 h and analyzed.

Flow Cytometry

Cells from bone marrow, inguinal LN and spleen were isolated form WT and *Ap3b1*$^{-/-}$ mice. Single cells suspensions from each organ were pretreated with anti-Fc receptor Ab (eBioscience) at 4 °C for 10 min. These cells were stained with fluorochrome-labeled antibodies specific for mouse CD11c, CD11b, MHC classII, CD8α, B220, Siglec-H (eBioscience) or PDCA-1(Miltenyi Biotec, Germany) at 4 °C for 15 min with FACS buffer (2% FBS, 2 mM EDTA, 0.1% NaN_3 in PBS). Dead cells were excluded using AmCyan staining (Invitrogen).

To detect intracellular cytokines, bone marrow cells were stimulated with CpG-A (3 μM) for 8 hrs and in the presence of 5 μg/ml Brefeldin-A (Sigma) for the last 3 hrs. Cells were pretreated with anti-Fc receptor Ab, then stained with indicated Abs. Subsequently cells were fixed and permiabilized using Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C. Cells were stained with 2.5 μg/ml FITC- conjugated RMMA-1(rat mAb against IFN-α) (PBL) and 1 μg/ml PE - conjugated anti-TNF-α (eBioscience) for 30 min at 4 °C. Multiparameter analyses were performed on a flow cytometer (LSRII or LSRII Green, BD) and were analyzed using FlowJo software (Tree Star, Inc.).

Statistical Analysis

Normally distributed continuous variable comparisons were performed using two-tailed unpaired *t*-test and one-way ANOVA followed by Tukey's post test comparison using Prism software.

Supporting References

S1. J. M. Lund *et al.*, *Proc Natl Acad Sci U S A* 101, 5598 (Apr 13, 2004).

S2. T. Ichinohe, H. K. Lee, Y. Ogura, R. Flavell, A. Iwasaki, *J Exp Med* (Jan 12, 2009).

S3. A. D'Amico, L. Wu, *J Exp Med* 198, 293 (Jul 21, 2003).

S4. A. Sato, M. M. Linehan, A. Iwasaki, *Proc Natl Acad Sci U S A* 103, 17343 (Nov 14, 2006).

S5. J. C. Kagan *et al.*, *Nat Immunol* 9, 361 (Apr, 2008).

S6. K. Honda *et al.*, *Nature* 434, 1035 (Apr 21, 2005).

S7. J. Lund, A. Sato, S. Akira, R. Medzhitov, A. Iwasaki, *J Exp Med* 198, 513 (Aug 4, 2003).

Supporting Figures

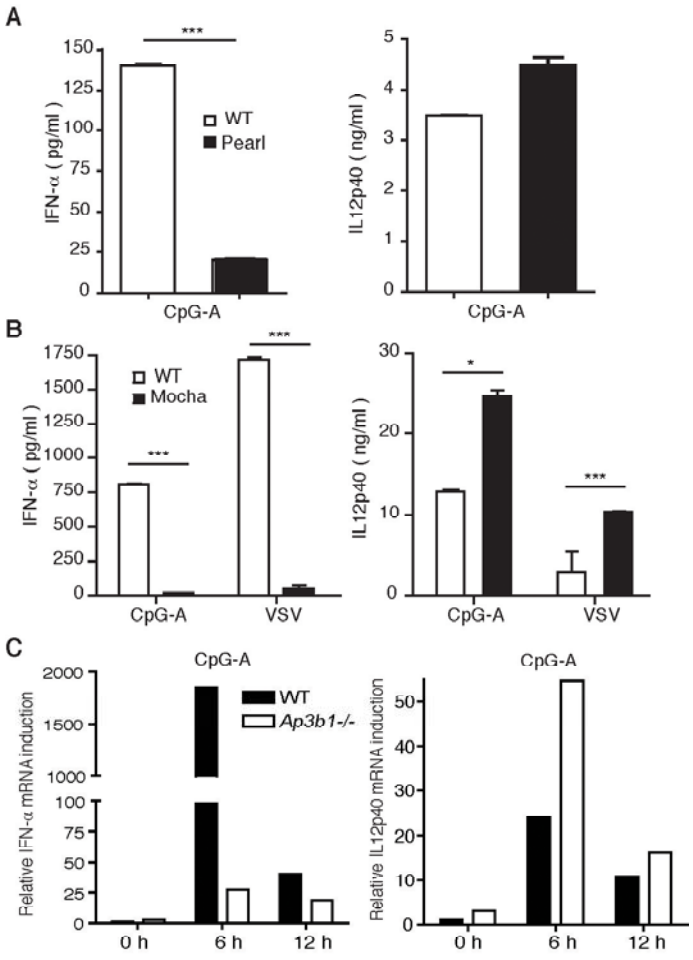

Figure S1

Type-I IFN, but not IL-12p40, production is impaired in AP-3 mutant pDCs.

Flt3L-pDCs from Pearl (AP-3β1 mutant) (A), and from Mocha (AP-3δ mutant) (B) mice were stimulated with CpG-A (3 μM) (A, B) or VSV (MOI 10) (B) for 24 hrs. Levels of IFN-α (top) and IL-12p40 (bottom) were measured by ELISA. (C) Flt3L-pDCs from WT and *Ap3b1*$^{-/-}$ mice were stimulated with CpG-A for the indicated duration. Levels of mRNA expression were determined by qRT-PCR. *, $p < 0.05$. ***, $p < 0.001$. Results are means ± SEM (n = 3).

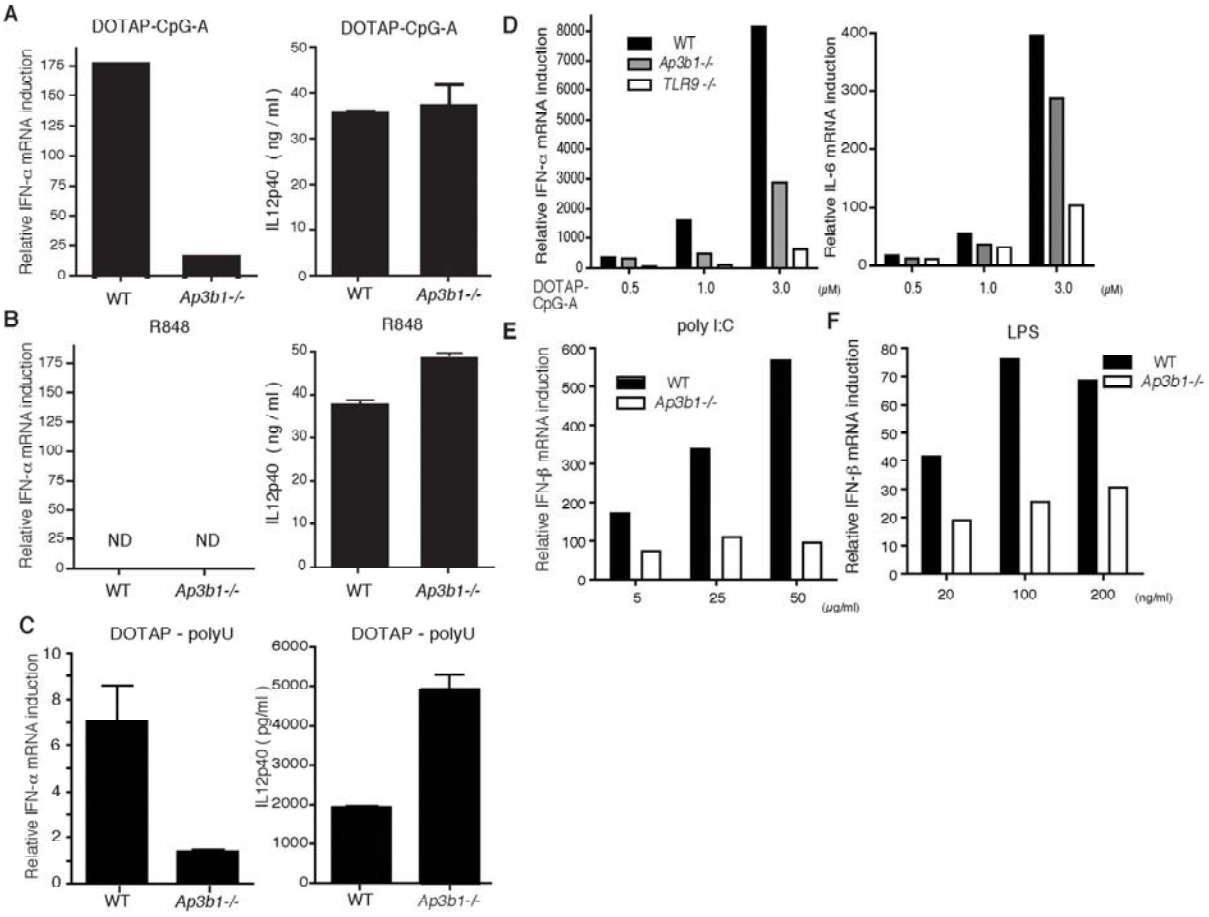

Figure S2

Type I interferon, but not IL-12p40, production is impaired in *Ap3b1*$^{-/-}$ BM DCs and BMMs following endosomal TLR stimulation.

BM DCs from WT and *Ap3b1*$^{-/-}$ mice were stimulated with DOTAP-CpG-A (3 μM) (A), R848 (1 μg/ml) (B) or DOTAP-poly U (20 μg/ml) (C). Levels of IFN-α (left) and IL-12p40 (right) were measured by quantitative RT-PCR (6 h) or by ELISA (24 h). (D-F) BMMs from WT and *Ap3b1*$^{-/-}$ mice were stimulated with the indicated concentrations of DOTAP-CpG-A (D), poly (I:C) (E) or LPS (F) for 6 h. The induced mRNA levels of IFN-α (D, left), IL-6 (D, right) and IFN-β (E, F) were measured by qRT-PCR. Results of qRT-PCR are representative of two independent experiments. Results of ELISA are means ± SEM (n=3) and are representative of two independent experiments.

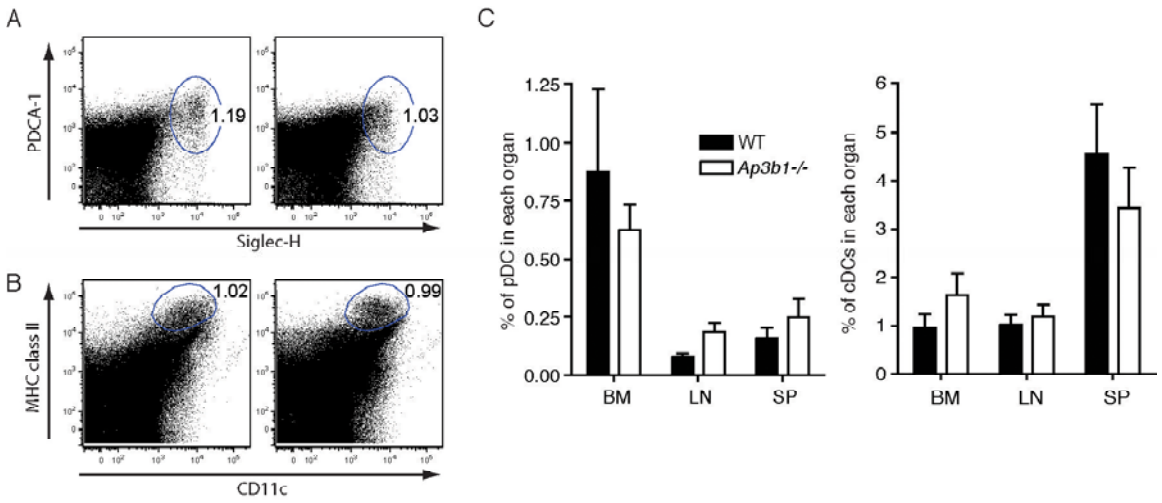

Figure S3

In vivo development of pDCs and cDCs is intact in *Ap3b1*$^{-/-}$ mice

Single cell suspensions from the spleen, bone marrow and lymph nodes (inguinal) were stained with antibodies against CD11c, Siglec-H and MHC class II. Dead cells were excluded by AmCyan staining. Number of pDCs (Siglec H^{+}, CD11c^{int}, MHC class II^{+}, left) and cDCs (Siglec H^{-}, CD11c^{+}, MHC class II^{hi}, right) are depicted for each organ. The data are average of cell percentages ± SEM (n=4).

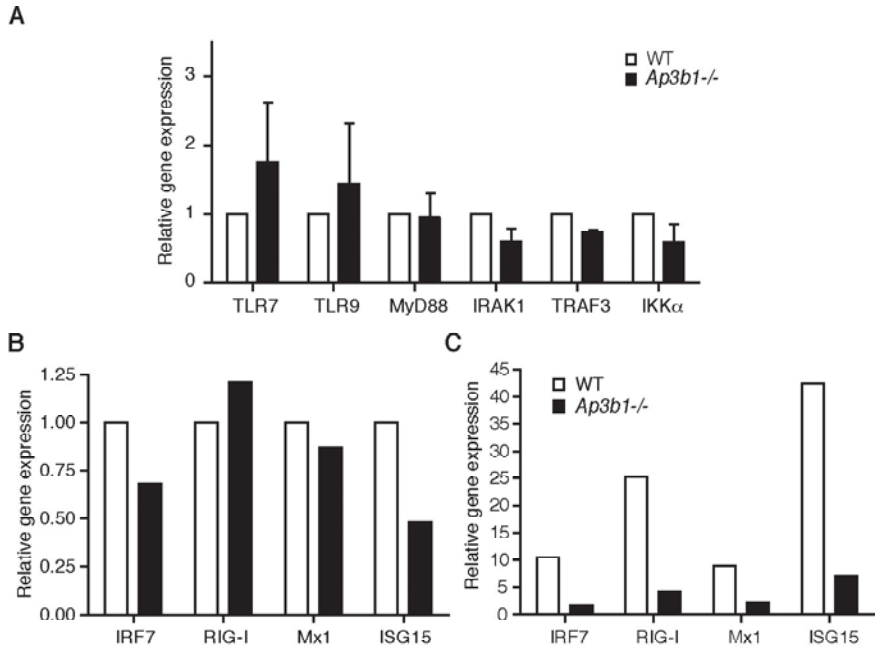

Figure S4

Expression of TLRs, signaling molecules and IFN-responsive genes in *Ap3b1*$^{-/-}$ pDCs.
FLt3L-pDCs from WT (empty) and *Ap3b31*$^{-/-}$ (filled) mice were cultured for 7 days. (A) The expression levels of TLR7, TLR9, MyD88, IRAK1, TRAF3, IKKα were measured by quantitative RT-PCR at steady state condition. Results are mean ± SEM (n = 3) and are representative of three independent experiments. For each gene, indicated levels are relative to expression levels in WT cells. (B, C) The expression levels of IFN-responsive genes, IRF7, RIG-I, Mx1 and ISG15, were analyzed by quantitative RT-PCR from Flt3L-pDCs at steady state (B) or after stimulation with CpG-A (3 μM) for 12 h (C). The results are representative of three independent experiments.

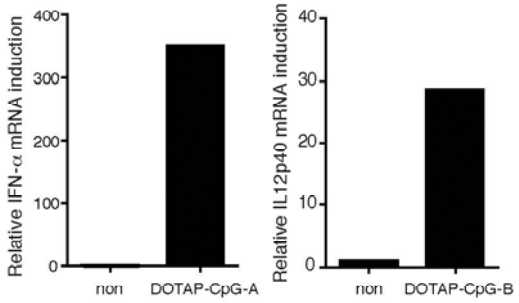

Figure S5

TLR9 fused to GFP at the C-terminus (TLR9-GFP) retains signaling capacity.

BMMs from *TLR9*$^{-/-}$ mice were transduced with TLR9-GFP retrovirus for 2 days. Cells were stimulated with DOTAP-CpG-A (left: 3 μM) or DOTAP-CpG-B (right: 1 μM) for 12 h. The induction mRNA levels of IFN-α (left) or IL12p40 (right) were measured by quantitative RT-PCR. Results of qRT-PCR are representative of two independent experiments.

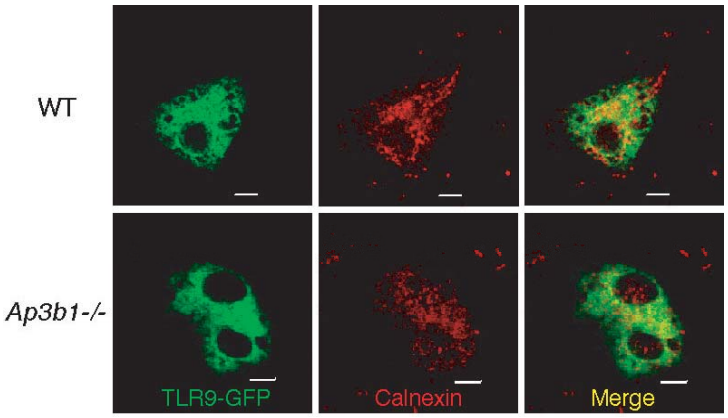

Figure S6

TLR9-GFP localization at steady state.

BMMs from WT and *Ap3b1*$^{-/-}$ mice were transduced with TLR9-GFP retrovirus for 2 days. Cells were seeded on glass slide, and then were fixed with 4% paraformaldehyde. Confocal images are of macrophages stained with anti-GFP (green) and anti-calnexin (ER marker) (red). The data are representative of three independent experiments. (Scale bars, 5 μm.)

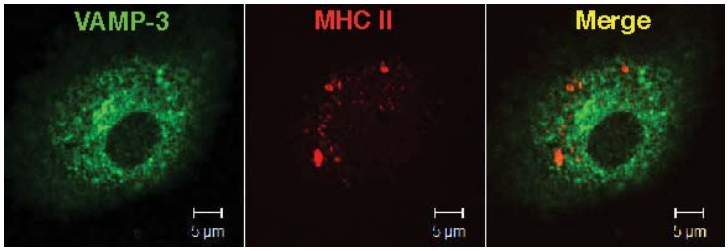

Figure S7

VAMP3 does not co-localize with MHC class II in DCs.

BM DCs from WT mice were seeded on glass slide and were fixed with 4 % paraformaldehyde. Cells were stained with anti-VAMP3 (green) and anti-MHC-II (red) antibodies. The data are representative of two independent experiments. (Scale bars, 5 µm.)

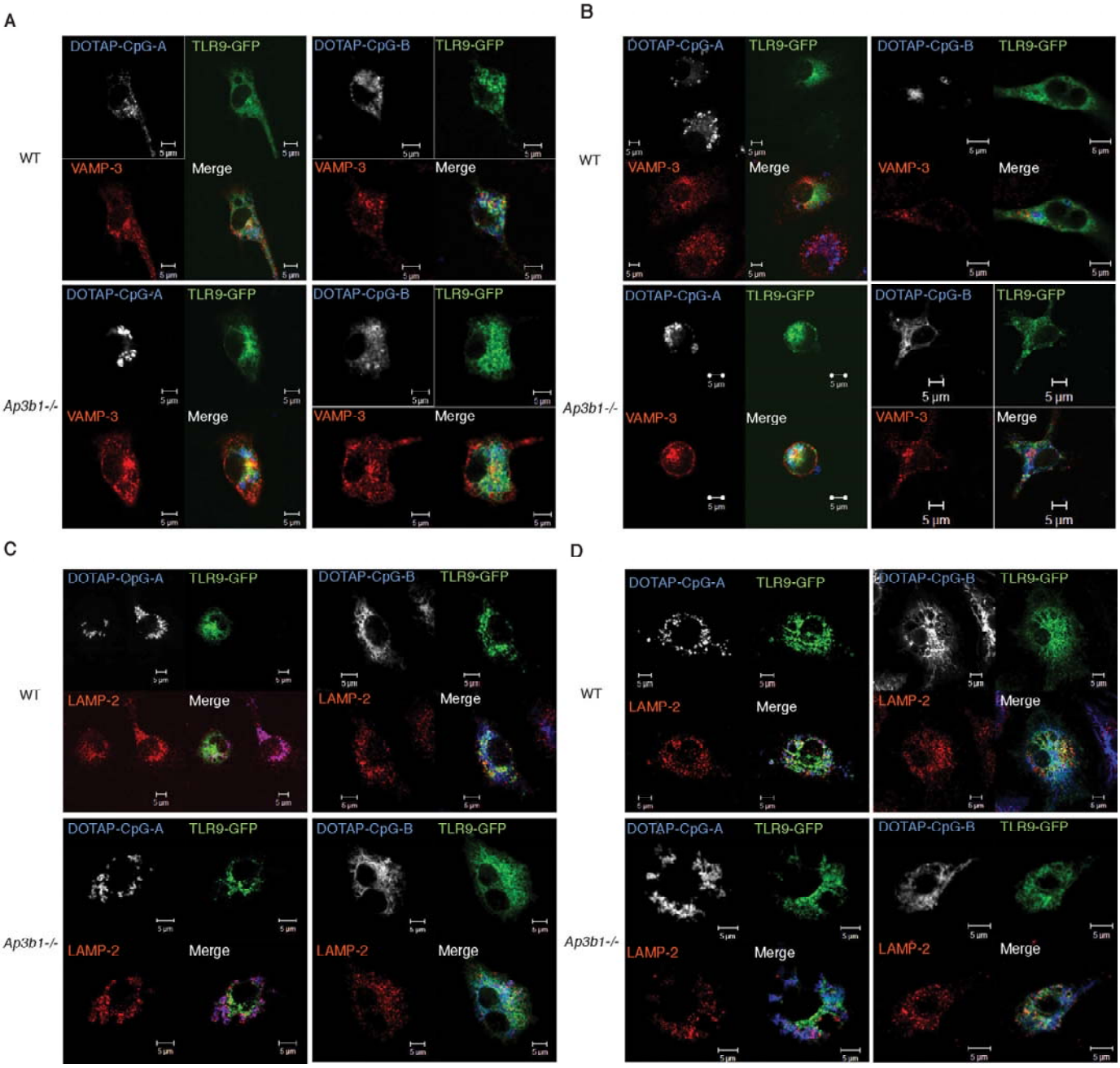

Figure S8

Intact trafficking of CpG-A and CpG-B in *Ap3b1*$^{-/-}$ macrophages.

BMMs from WT and *Ap3b1*$^{-/-}$ were transduced with TLR9-GFP retrovirus for 2 days. Cells were seeded on glass slide over-night. Cells were stimulated with Cy5-labeled CpG-A (2 µM) or CpG-B (2 µM) coupled to DOTAP for 3 h (A, C) and 6 h (B, D), then were fixed with 4 % paraformaldehyde. Confocal images represent cells that were stained with antibodies to GFP (green) and VAMP3 (A, B) or LAMP2 (C, D) (red). The data are representative of two independent experiments. (Scale bars, 5 µm.)

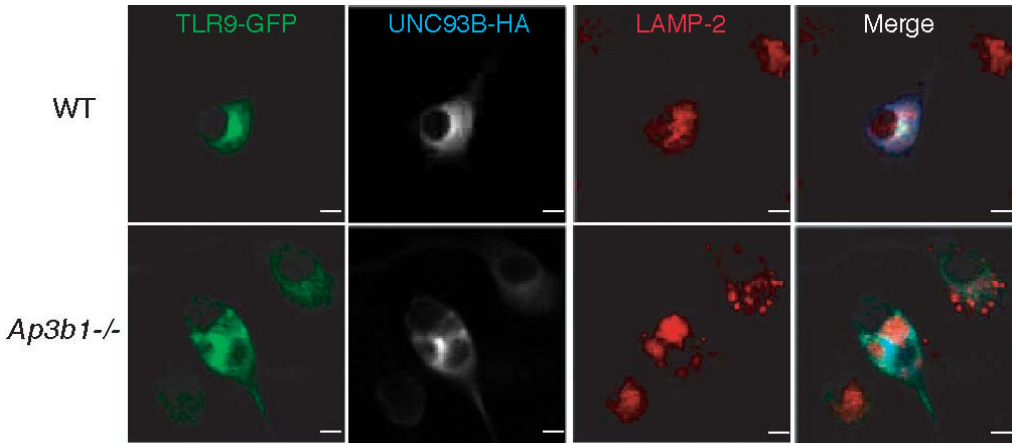

Figure S9

UNC93B fails to localize to LAMP2^{+} compartment in *Ap3b1*$^{-/-}$ cells.

BMM from WT and AP-3$^{-/-}$ mice were retrovirally transduced with TLR9-GFP and UNC93B-HA. Cells were stimulated with DOTAP-CpG-A for 6 h, and analyzed for TLR9-GFP (green), UNC93B (white/blue) and LAMP2 (red). The data are representative of four independent experiments. Scale bars, 5 µm.

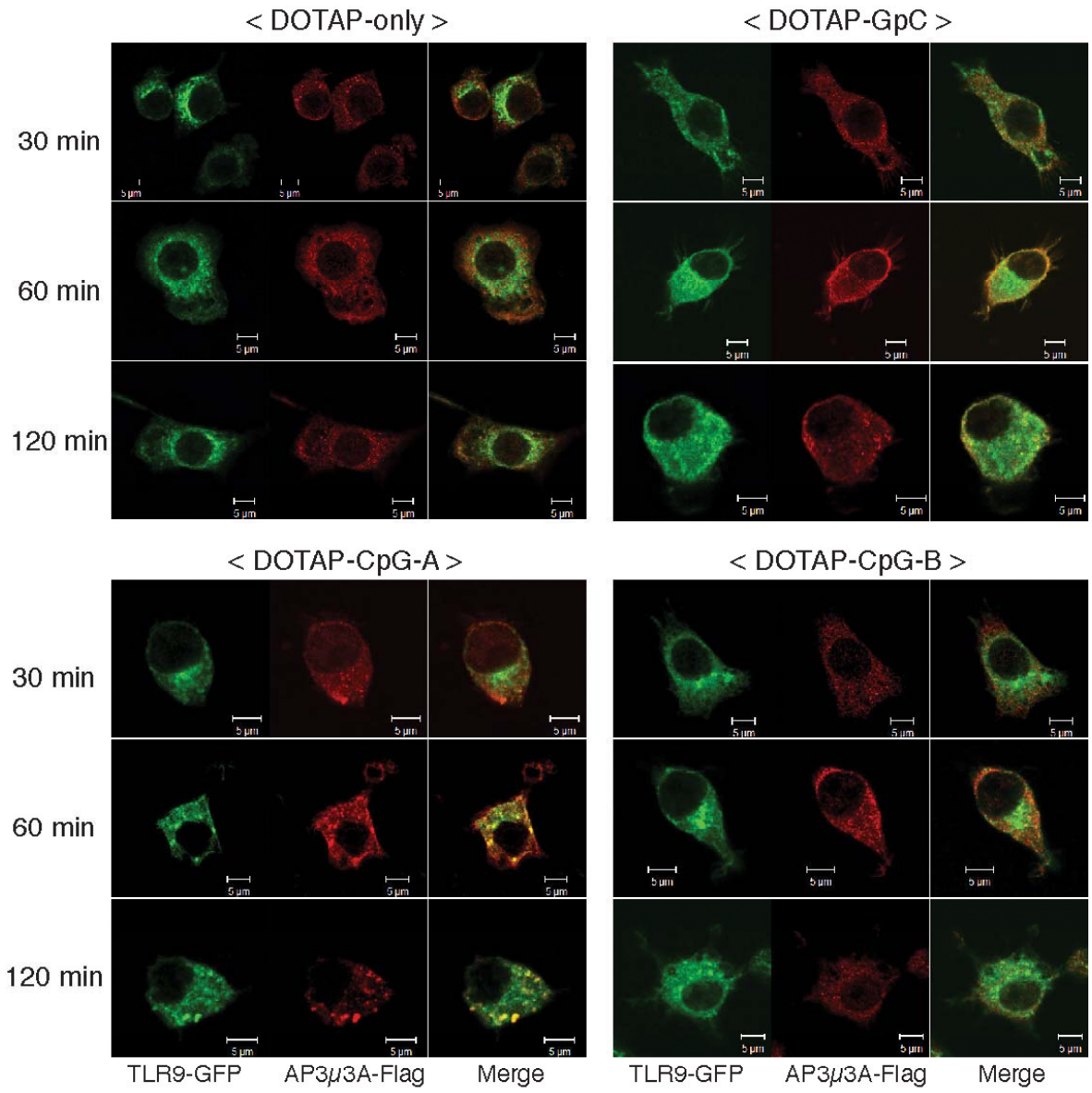

Figure S10

AP-3 forms a complex with TLR9 specifically in response to DOTAP-CpG-A stimulation.

RAW264.7 cells expressing TLR9-GFP and AP-3μ3A (Flag) were stimulated with DOTAP-GpC (3 μM), DOTAP-CpG-A (3 μM), DOTAP-CpG-B (1 μM) or DOTAP alone for the indicated time periods. The cells were stained and analyzed for TLR9-GFP (green) and AP-3μ3A (red). (Scale bars, 5 μm.) The data are representative of two independent experiments.

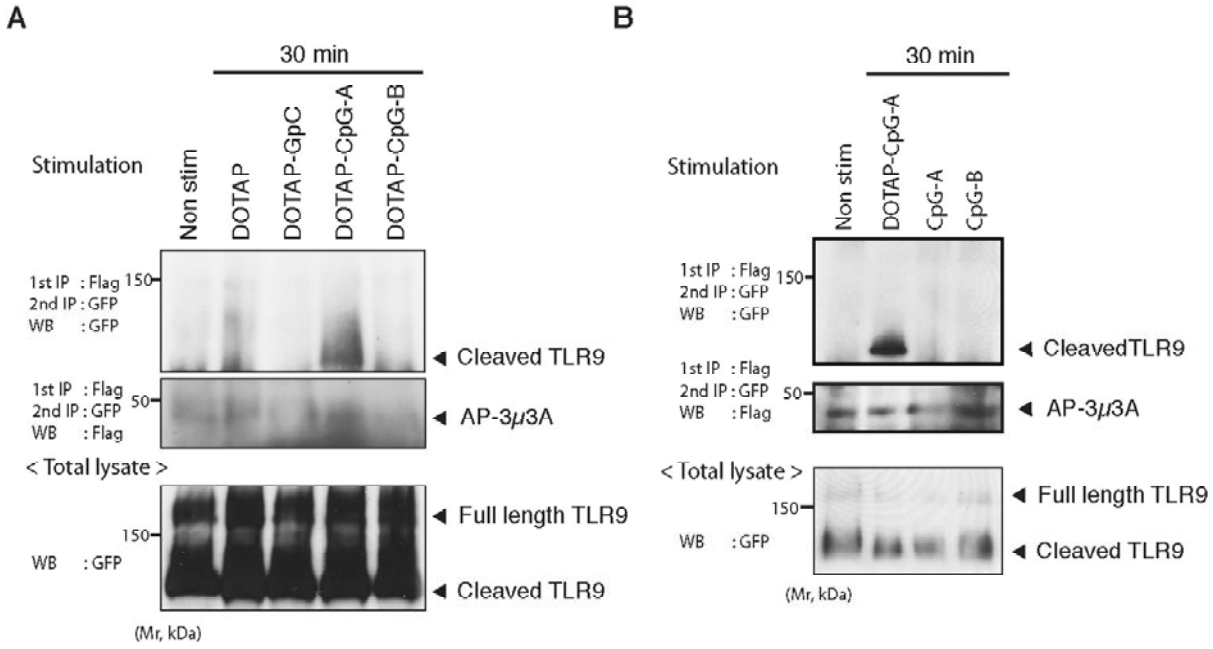

Figure S11

Cleaved TLR9 interacts with AP-3 following DOTAP-CpG-A stimulation.

RAW264.7 cells transduced with a combination of retroviruses encoding TLR9-GFP and/or AP-3μ3A-Flag were stimulated with DOTAP-GpC (3μM), DOTAP-CpG-A (3μM), DOTAP-CpG-B (1 μM) or DOTAP alone (A), or with DOTAP-CpG-A (3μM), CpG-A alone (3 μM) or CpG-B alone (1 μM) (B) for 30 min. Cell lysates were prepared and analyzed either directly (bottom) or after immunoprecipitation with an anti-Flag antibody and immunoblotting with an anti-GFP antibody (top).

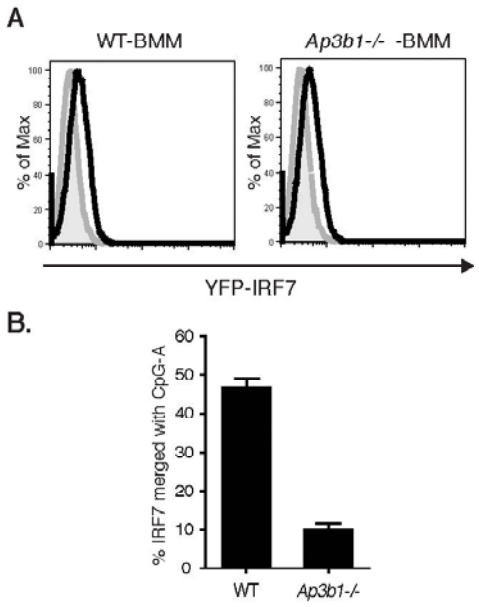

Figure S12

IRF7 localizes to LysoTracker^{+} CpG-A-containing vesicles

BMMs from WT and *Ap3b1*$^{-/-}$ mice were transduced with YFP-IRF7 lentivirus for 4 days. The levels of YFP-IRF7 were measured by flow cytometry (A). Gray lines indicate non-transduced cells and dark lines indicate YFP-IRF7 transduced cells. (B) The percentages of the cells in which the YFP-IRF7 merged with CpG-A at 6 h after addition of DOTAP-CpG-A-Cy5 were analyzed by confocal microscopy as described in Figure 4A. The data are representative of two independent experiments (A). The data in (B) are the average of two independent experiments in which n = 26 (WT) and n = 21 (*Ap3b1*$^{-/-}$) cells were included in the analysis.

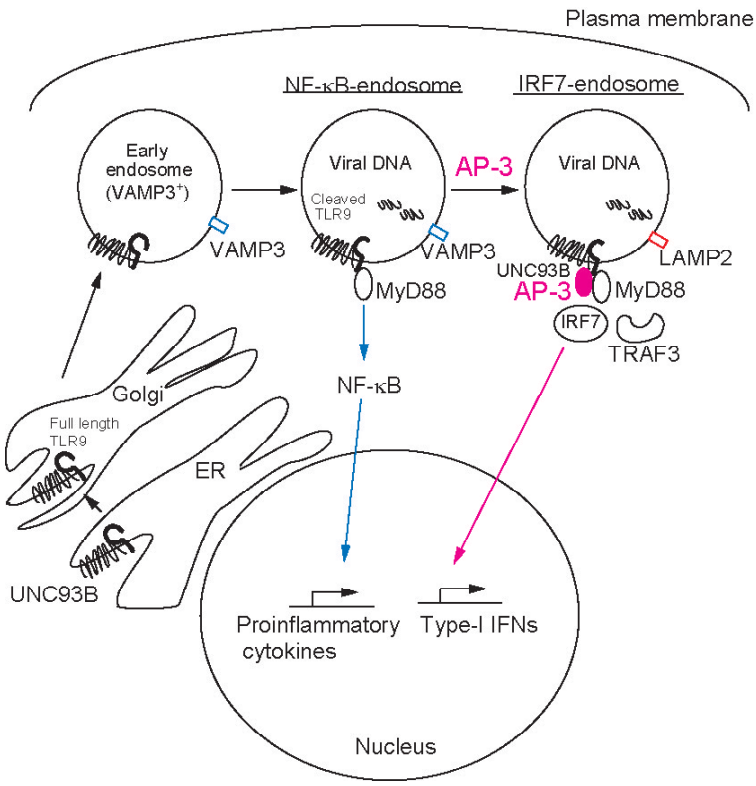

Figure S13

Schematics of compartmentalization of TLR9 signaling.

TLR9 synthesized in the ER traffics through the Golgi to enter the early endosome, a process facilitated by UNC93B. As the endosome matures and acidifies, TLR9 is cleaved, and becomes competent for signaling to activate pro-inflammatory cytokine genes (NF-κB endosome). The AP-3 complex interacts with cleaved TLR9 and facilitates its trafficking to the LAMP2^{+} LRO, whereby TLR9 can engage molecules such as TRAF3 and IRF7 and induce transcription of IFN genes (IRF7-endosome).

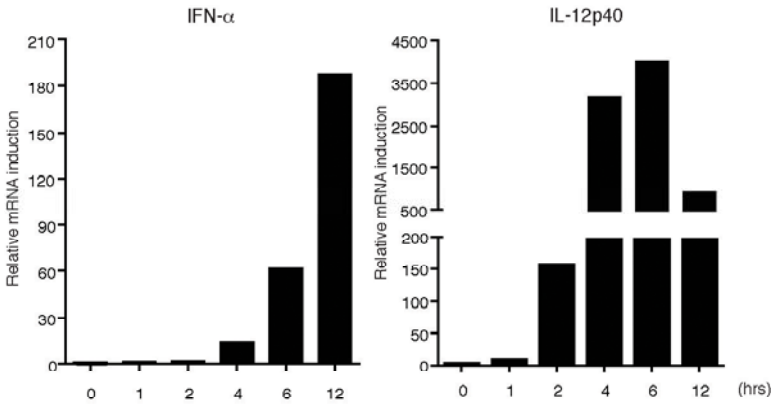

Figure S14

Kinetics of cytokine induction following DOTAP-CpG-A stimulation

BMMs were stimulated with DOTAP-CpG-A (3 μM) for the indicated time periods. The induction of IFN-α (left) and IL-12p40 (right) were analyzed by qRT-PCR.
The data are representative of two independent experiments.

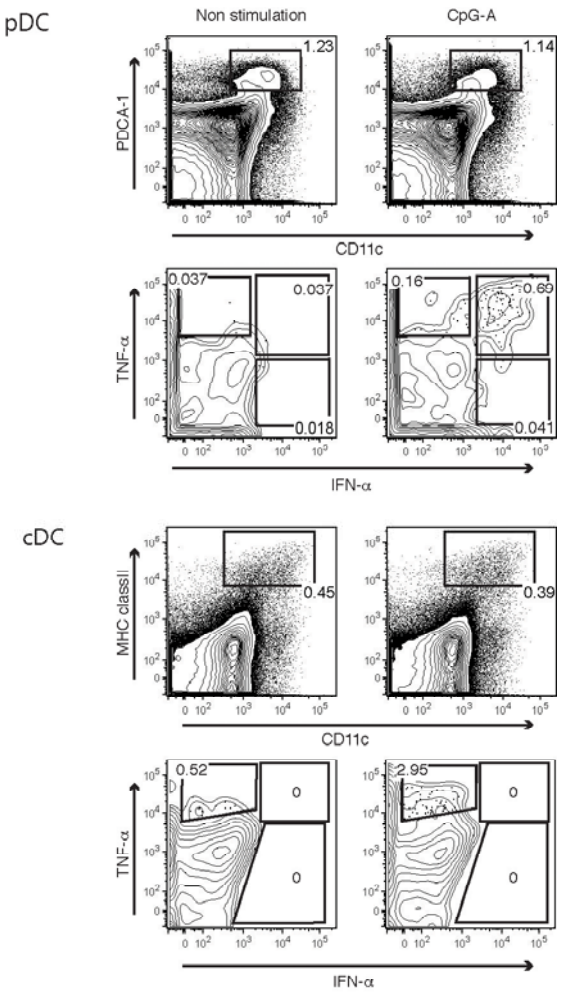

Figure S15

IFN-α secretion only occurs in pDCs concomitantly producing TNF-α

Total BM cells from WT mice were stimulated with CpG-A (for 3 μM) for 8 hrs in the presences of Brefeldin A. Cells were stained with antibodies against the indicated markers, IFN-α and TNF-α. The data are representative of two independent experiments.