Science Immunology

immunology.sciencemag.org/cgi/content/full/2/13/eaal1329/DC1

Supplementary Materials for

Dendritic cell–targeted lentiviral vector immunization uses pseudotransduction and DNA-mediated STING and cGAS activation

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Published 21 July 2017, *Sci. Immunol.* **2**, eaal1329 (2017)
DOI: 10.1126/sciimmunol.aal1329

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Materials and Methods

Antibodies and flow cytometry. Cells were stained with appropriate antibodies (Table S1) and analyzed on a MACSQuant analyzer (Miltenyi) using FlowJo software (TreeStar) with appropriate gating strategies (fig. S1). For dead cell staining, propidium iodine 1 μg ml^{-1} was added.

Cell lines. HEK293T/17 cells (ATCC) and 293T.DCSIGN cells (*1*) were cultured in DMEM with 10% (vol/vol) FBS. EL4 (C57BL/6J, H-2^b, thymoma) and E.G7 (derived from EL4 cells stably expressing one copy of chicken OVA cDNA) provided by L. Yang (UCLA) and GXR.CEM human lymphoblastoid $CD4^+$ T cells provided by B. Walker (Ragon Institute) were cultured in RPMI-1640 medium with 10% (vol/vol) FBS. All media was supplemented with 1% (vol/vol) penicillin and streptomycin (Gibco).

LV, VLP, and infectious HIV-1 production. A third generation HIV-based LV system was used with transfer vectors: FUGW encoding GFP (*1*) or FOVA encoding an invariant chain-OVA fusion construct (*72*). The packaging plasmids pMDLg/pRRE encoded *gag* and *pol* and pRSV-Rev encoded *rev*. The envelope plasmids were pVSV-G and pSVGmu (*1*). Introducing mutations G124A and P127A generated the fusion-defective VSV-G (*46*). The plasmid pNL4-3 (NIH AIDS Reagent Program) encoded infectious HIV-1. 293T cells were transfected using BioT (Bioland Scientific) according to manufacturer's instructions. To generate VLPs the LV transfer vector plasmid was omitted. Omitting the packaging plasmids generated capsid deficient vectors. Transfecting with the packaging plasmids generated bald particles (*7*). To generate VLPs carrying GFP or OVA, an expression plasmid encoding GFP (p.GFP) or p.OVA was included during transfection. Puc19 plasmid was transfected into 293T cells as a negative control. All viral supernatants were harvested at 36, 48, and 60 h post-transfection and filtered

through a 0.45-μm filter. The cell-free LV and VLP supernatants were additionally ultracentrifugated (Optima L-80 K preparative ultracentrifuge, Beckman Coulter) at 80,000 *g* for 90 min through a 20% (vol/vol) sucrose cushion. The pellets were then resuspended in an appropriate volume of cold PBS.

Quantification of vectors. 293T and 293T.DCSIGN cells were infected with LV and polybrene 5 μg ml^{-1} and analyzed at 48 h for GFP expression by flow cytometry to determine the infectious titer by the dilution ranges that exhibited a linear response. The concentration of gag was measured by p24 capture ELISA Kit (ImmunoDiagnostics) and GFP by GFP ELISA Kit (Abcam). The concentration of iOVA was determined using a sandwich ELISA with for coating onto 96-well plates using anti-CD74 5 μg ml^{-1} for coating of plates. The vectors were lysed in 0.5% (vol/vol) Triton X-100 and incubated on coated plates. The amount of captured OVA was determined using anti-chicken OVA 5 μg ml^{-1} and a horseradish peroxidase-conjugated goat-anti-mouse IgG (Bethyl Lab) at a dilution of 1:10,000. TMB Peroxidase Substrate System (KPL) was used and absorbance read at 450 nm.

Immunoblot analysis. Vector preparations were lysed in 1% Triton X-100 in PBS supplemented with HALT protease and phosphatase inhibitor cocktail (Life Technologies) and clarified by centrifugation. Aliquots of the lysate were mixed with non-reduced Laemmli's sample buffer and were resolved by 5–15% (wt/vol) SDS-PAGE. Antibodies to GFP, OVA, VSVG, and p24 were used (Table S1). To determine if proteins were inside the vector particles, samples were pre-treated with proteinase K 10 μg ml^{-1} (Qiagen) and incubated at 55 °C for 1 h, then the proteinase K was inactivated with PMSF 1mM (Sigma) before samples were lysed.

Fluorescent imaging. Cells were washed once with PBS, fixed using 4% (vol/vol) paraformaldehyde in PBS (10 min at 22 °C), and washed 4 times with PBS. Images were

collected using a fluorescence microscope (Axiovert 200m; Zeiss) equipped with three filter wheels (Lambda 10-3; Sutter Instruments), and a CCD camera (Evolution/Qimaging; Media Cybernetics). Images were collected using Image-ProPlus 5.1 software (Media Cybernetics). All data within each experiment was collected at identical imaging settings; relevant sets of images were adjusted only for brightness/contrast.

DNA analysis by PCR and fluoremetry. Virus and VLP samples were inactivated/lysed by heating to 95 °C for 15 min. To determine whether DNA was carried within the particles, virus and VLP samples were pre-treated with DNase I (Sigma) at a final concentration of 0.1 mg ml^{-1} at room temperature for 10 min, and then the DNase I was inactivated with EDTA 0.625 mM at 70 °C for 10 min, before inactivation/lysis. To show that DNase I degradation was complete, DNase I was not inactivated by EDTA prior to lysis. PCR amplifications from samples were carried out in 0.2-mL thin-walled reaction vessels in the Eppendorf Mastercycler proS. REDExtract-N-Amp PCR ReadyMix (Sigma) was used per manufacturer instructions to obtain readily visible PCR products after 35 amplification cycles (30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C) with the appropriate primer sequences **(**Table S2**)**. The amplification products were electrophoresed on 2% (wt/vol) agarose gel with subsequent ethidium bromide staining. DNA from vector preparation was extracted using a genomic DNA extraction kit (ThermoFisher Scientific). Double-stranded and single-stranded DNA was measured from extracted DNA using the Quantifluor dsDNA and ssDNA system (Promega).

DNA deep sequencing. Total DNA from 200 µl of vector preparation was fragmented using Qsonica Q800R sonicator to the average size of ~200 bp, the fragments were end repaired and A-tailed, followed by adaptor ligation and PCR. DNA libraries were constructed using the Nextera XT DNA Sample Preparation Kit and Nextera XT Index Kit (Illumina). Libraries were

quantified with Quibit and the insert size distribution was assessed with 2100 BioAnalyzer (Agilent). All libraries were sequenced on Illumina HiSeq2500. Reads were aligned to the human genome version hg19 and the plasmid DNA sequence maps using BWA.

HIV passaging. Human PBMCs (UCLA Center for AIDS Research Virology Core Lab) were cultured in PHA 5 μg ml^{-1} (Sigma) with IL-2 5 ng ml^{-1} (Peprotech) cells at 1×10^6 cells ml^{-1} and infected with NL4-3 virus supernatant (50 ng p24) and incubated at 37 °C for 1 d. Infected cells were washed and incubated again at 37 °C for 2 d. Uninfected cells were concurrently treated in an identical manner. Cell-free supernatant was collected and filtered through a 0.22 μm filter. Aliquots of cell-free supernatants were stored at –80 °C.

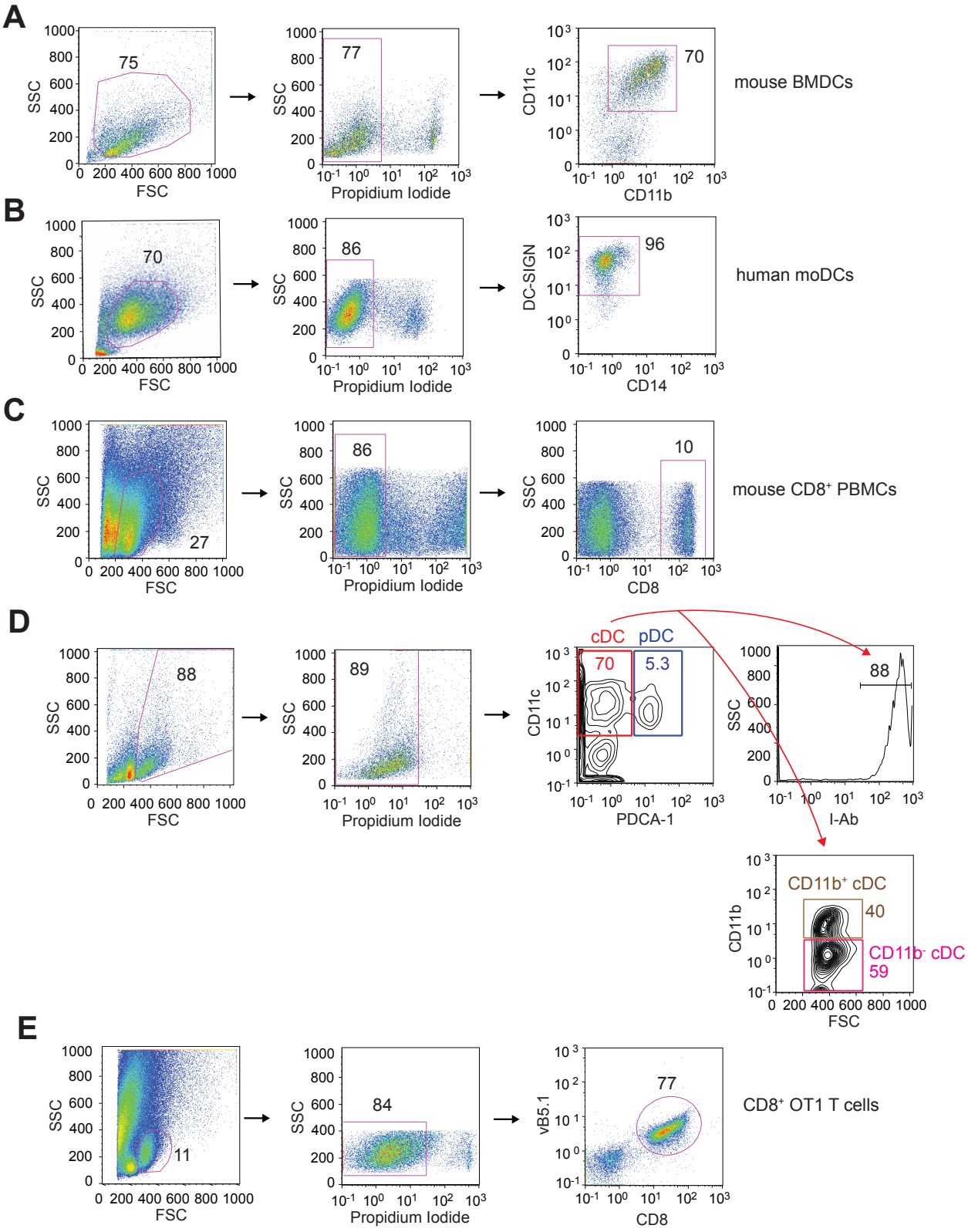

Fig. S1. Flow cytometry gating strategies. (**A-E**) Flow cytometric analysis of mouse BMDCs (A), human moDCs (B), mouse CD8$^+$ PBMCs (C), mouse DCs isolated from lymph nodes (D), and mouse CD8$^+$ OT1 T cells (E). Numbers adjacent to the gates reflect the percentage of cells within the gates.

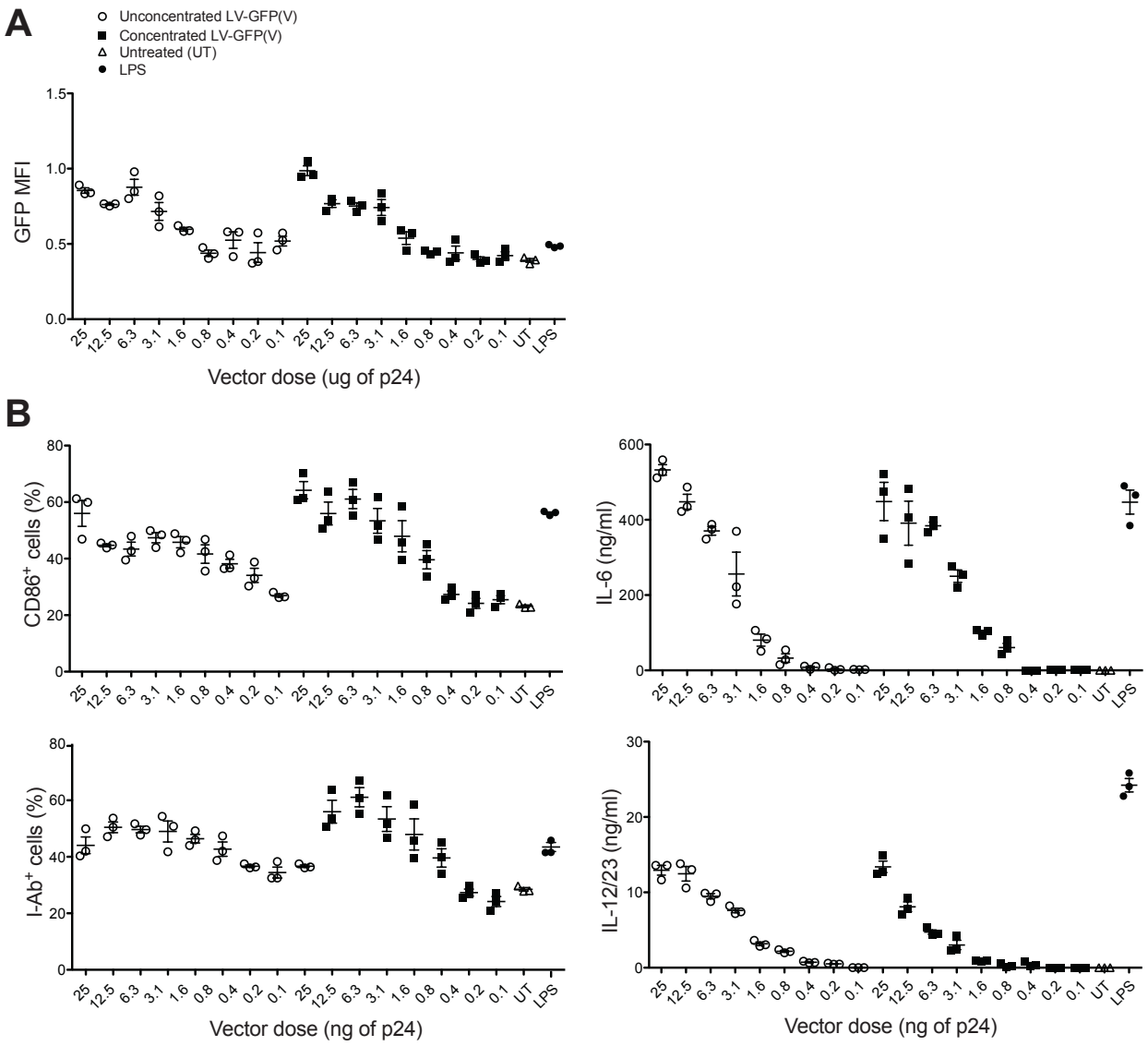

Fig. S2. LV-mediated GFP expression and activation of BMDCs are dose-dependent. BMDCs (2E6 cells/ml) were treated with serial dilutions of LV-GFP(V) in 600ul total volume. (**A**) Expression of GFP by BMCDs was measured by flow cytometry 24 h post-LV treatment. (**B**) The percentages of $CD86^+$ and $I\text{-}Ab^+$ cells was measured by flow cytometry and cytokine secretion of IL-6 and IL-12/23 in the supernatant at 24 h post-LV

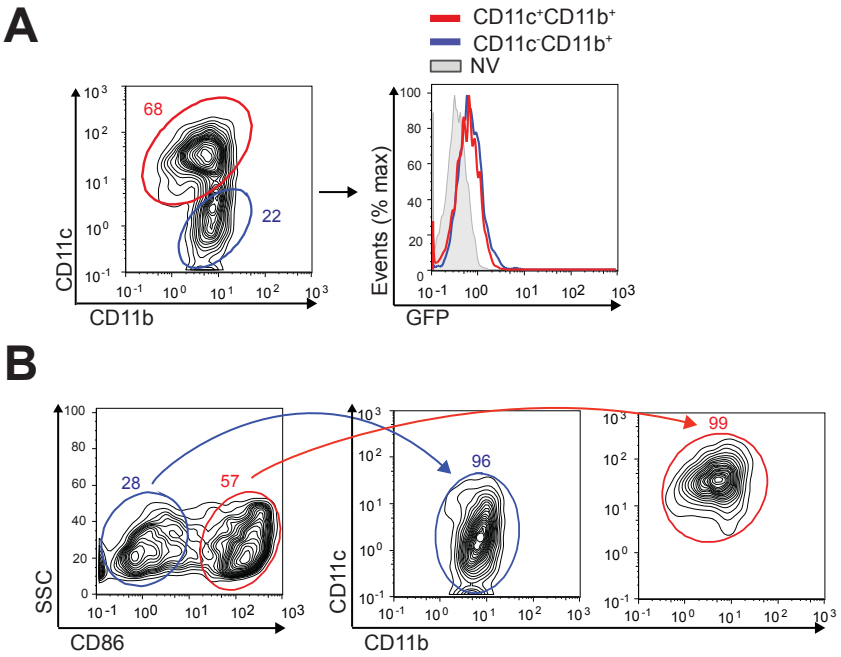

Fig. S3. Mouse bone marrow-derived CD11c$^+$CD11b$^+$ cells are pseudotransduced and activated. (**A, B**) Bone marrow cells from wild-type mouse were cultured in GM-GSF for 8 days and treated with LV-GFP(V) and analyzed by flow cytometry 24 h post-LV treatment. FACS plot showing CD11c and CD11b expression of cells (A, left). FACS histogram showing GFP expression of the gated CD11c$^+$CD11b$^+$ and CD11c$^-$CD11b$^+$ populations (A, right). Cells were then gated based on expression of activation marker CD86 (B, left). CD86$^+$ and CD86$^-$ were analyzed for expression of CD11c and CD11b (B, right).

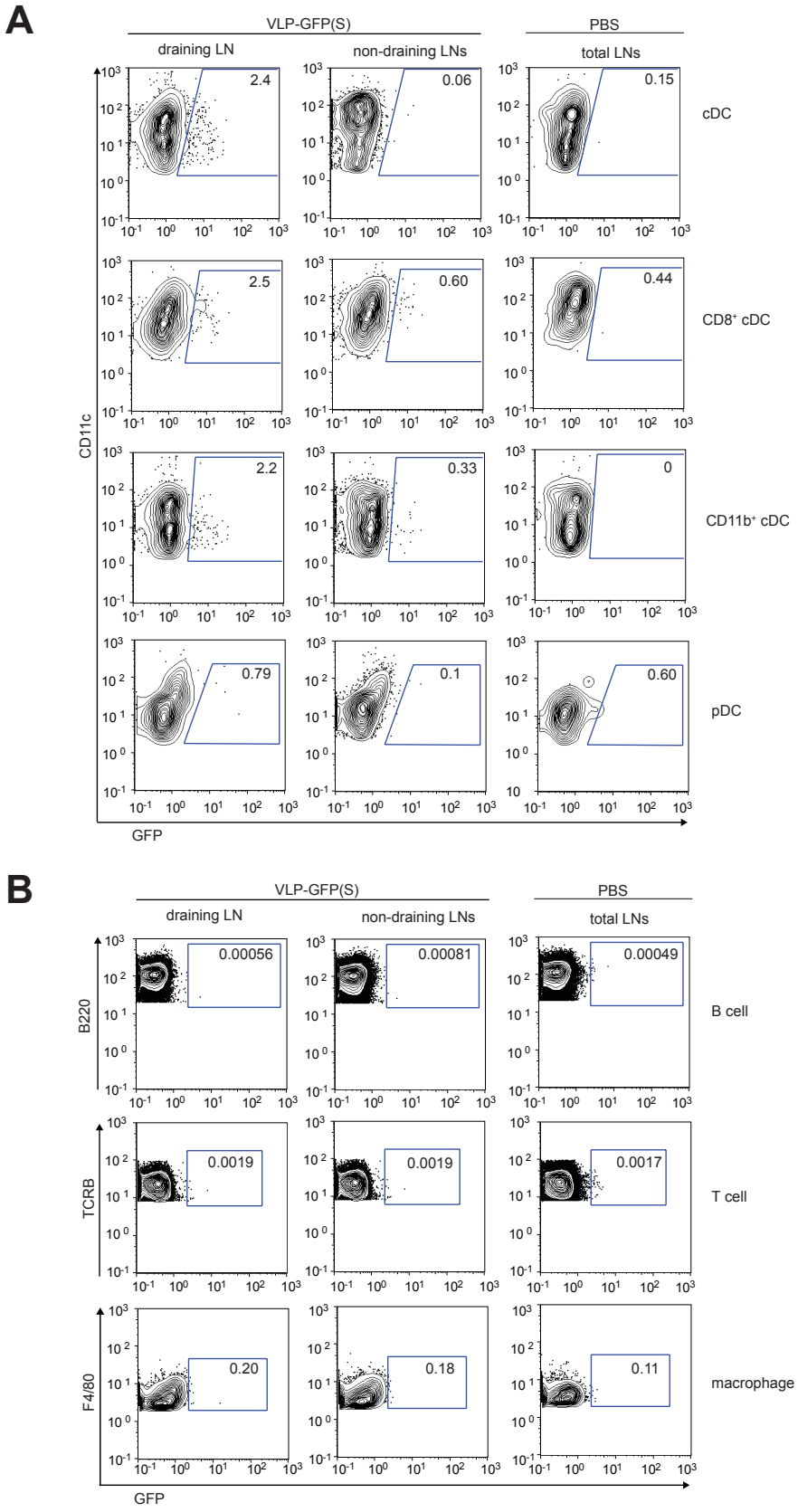

Fig. S4. Mouse cDCs are pseudotransduced *in vivo*. VLP carrying GFP pseudotyped with SVGmu (100ng of p24) was injected subcutaenously into the R hindleg of wild-type mice. Control mice received equal volumes of PBS. Right inguinal draining lymph node and non-draining lymph nodess (left inguinal, axillary, and cervical) were harvested 24 h post-injection. (**A**) DC subsets were gated (fig. S1D) and analyzed for GFP expression. (**B**) B cells, T cells, and macrophages isolated from lymph nodes were also analzyed for GFP expression.

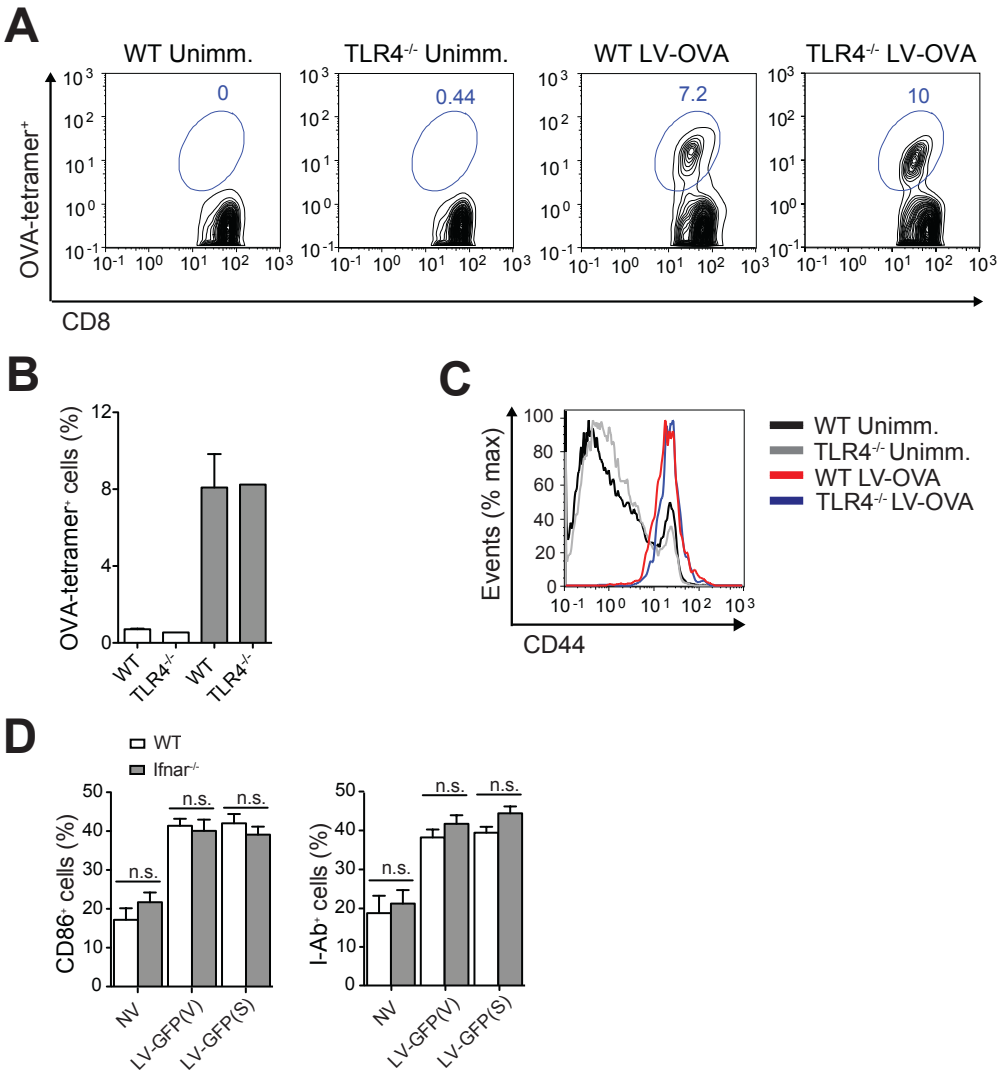

Fig. S5. LV activation of DCs is independent of TLR4 and type I IFN signaling. (**A-C**) TLR4$^{-/-}$ (n=2) and wild-type mice (n=4) were immunized with DC-targeting LV-OVA. Unimminzed TLR4$^{-/-}$ (n=2) and wild-type mice (n=4) received equal volumes of PBS. Two week post-immunization, spleens were harvested and CD8^{+} T cells analyzed by flow cytometry. FACS plots showing OVA-tetramer expression of CD8^{+} T cells (A). Graph depicts percentages of OVA-tetramer^{+} CD8^{+} T cells from the spleen of immunized and unimmunized mice (B). FACS histogram plot show CD44 expression of OVA-tetramer^{+}CD8^{+} T cells from immunized mice and naive CD8^{+} T cells from unimmunized mice (C). (**D**) BMDCs from mice deficient in Type I IFN receptor were treated with LV-GFP(V) or LV-GFP(S) and analyzed at 24 h for expression of CD86 and I-Ab by flow cytometry. n.s.=not significant, $P > 0.05$; (unpaired Student's t-test).

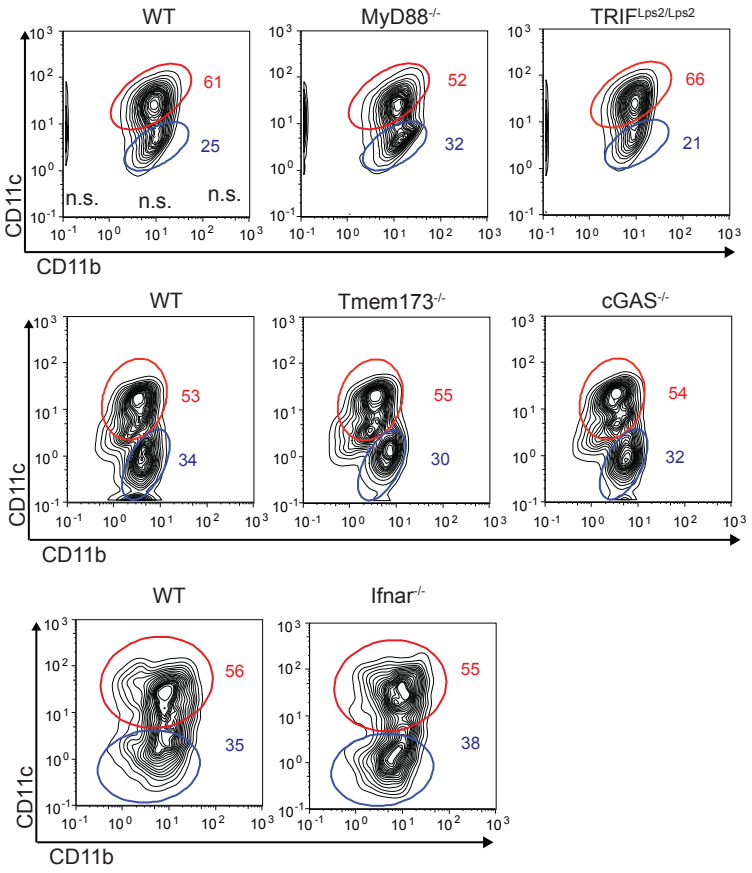

Fig. S6. Wild-type and mutant bone marrow-derived CD11c^{+}CD11b^{+} cells are generated in GM-CSF culture. Bone marrow cells from wild-type and mutant mice were cultured in 100ng ml^{-1} GM-CSF for 8 days. FACS plot showing CD11c and CD11b expression of these BM-derived cells.

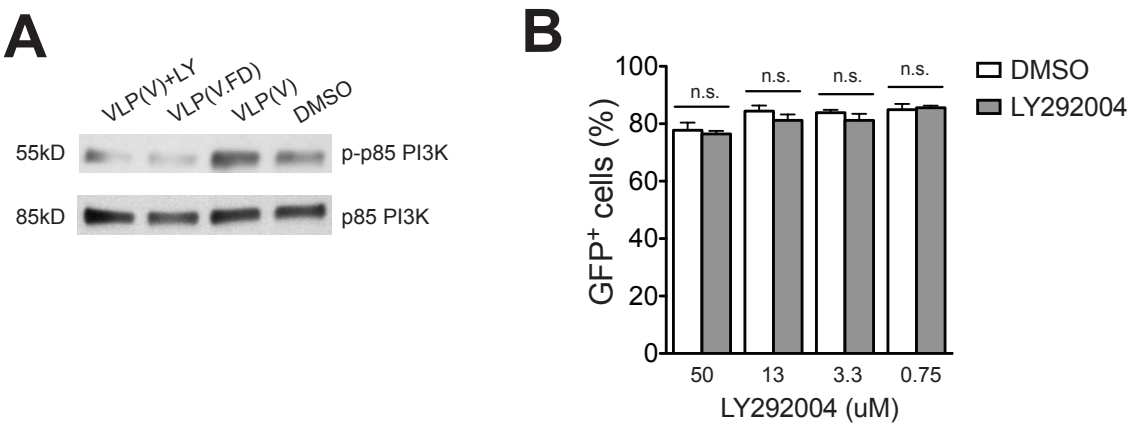

Fig. S7. VSV-G viral fusion activates PI3K. (**A**) Wild-type BMDCs were treated with VLP(V) with or without 50uM LY292004 or fusion defective VLP(V.FD) and analyzed at 2.5 h by Western blot for phosphorylated p85 subunit of PI3K and total p85. (**B**) 293T cells were incubated with LY2902004 1 h prior to treatment with LV-GFP(V) and then analyzed 48 h later for GFP expression by flow cytometry. n.s.=not significant, $P > 0.05$; (unpaired Student's t-test).

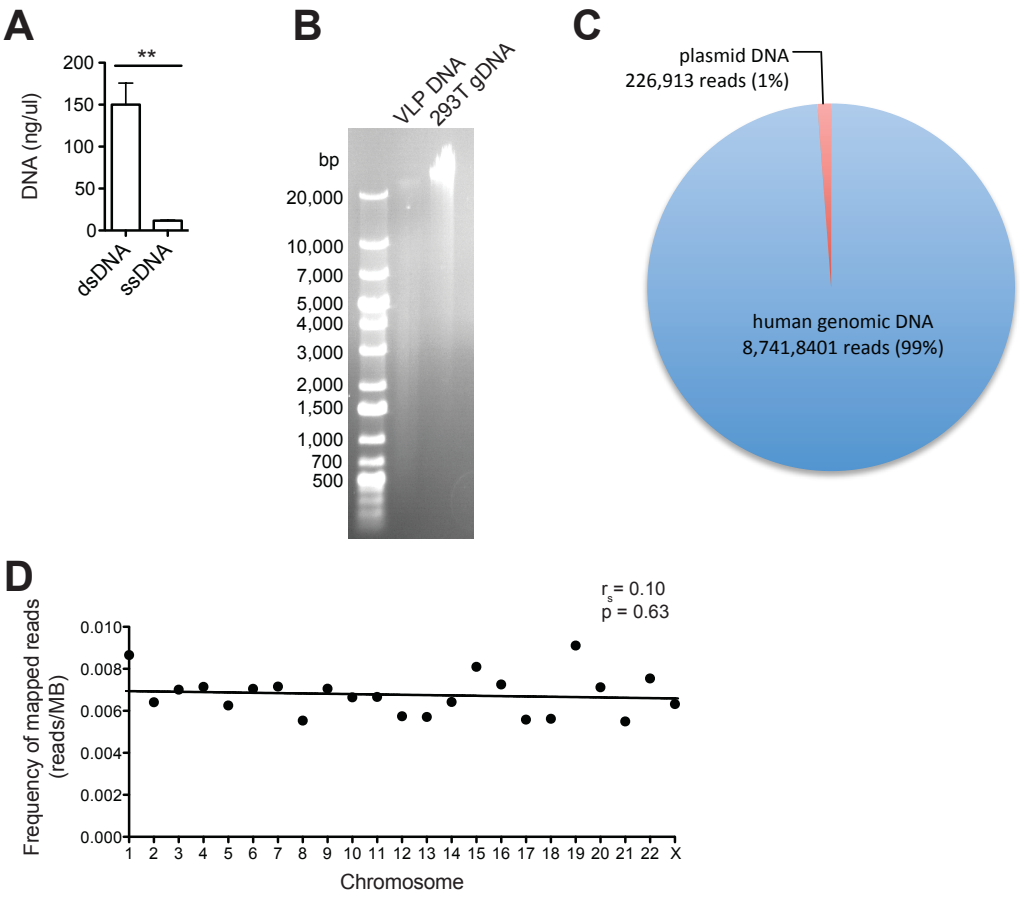

Fig. S8. Nonviral DNA in vector particle is primarily dsDNA, fragmented, and human genomic in origin. (**A**) Extracted DNA from LV preparation was analyzed for concentration of double-stranded and single-stranded DNA by fluoremtory-based assay. (**B**) Extracted DNA from LV prepation and from 293T cells was analzed by gel electrophoresis using EtBr staining. (**C, D**) Extracted DNA from LV prepations was deep sequenced and the reads mapped to the human genome (hg19) or plamid maps. Frequency and total mapped reads were quantified by origin (C). Frequency of reads and human chromosome number were subjected to Spearman's rank correlation analysis (D).

Table S1. Antibodies used in this study.

Antibody	Company	Clone/Cat No.
anti-human CD14	Biolegend	HCD14
anti-human CD86	Biolegend	IT2.2
anti-human DC-SIGN	Biolegend	9E9A8
anti-human HLA-DR	Biolegend	L243
anti-mouse B220	Biolegend	RA3-6B2
anti-mouse TCRB	Biolegend	H57-597
anti-mouse CD4	Biolegend	Gk1.5
anti-mouse CD8	Biolegend	53-6.7
anti-mouse CD11b	Biolegend	M1/70
anti-mouse CD11c	Biolegend	N418
anti-mouse CD44	Biolegend	IM7
anti-mouse CD62L	Biolegend	MEL-14
anti-mouse CD69	Biolegend	H1.2F3
anti-mouse CD74	BD Biosciences	ln-1
anti-mouse CD86	Biolegend	GL-1
anti-mouse F4/80	Biolegend	BM8
anti-mouse I-Ab	Biolegend	AF6-120.1
anti-mouse PDCA-1	Biolegend	129C1
anti-GFP	Santa Cruz Biotechnology	8334
anti-ovalbumin	Abcam	ab1221
anti-p24	Fitzgerald	10R-H120b
anti-phospho-p85 PI3K	Cell Signaling Technology	4228
anti-p85 PI3K	Cell Signaling Technology	19H8
anti-VSVG	Sigma-Aldrich	P5D4
EasySep™ Mouse Pan-DC Enrichment Kit	Stem Cell Technology	19763
SIINFEKL H-2Kb tetramer	NIH Tetramer Facility	

Table S2. Primer sets used in this study.

Target	*Forward sequence*	*Reverse Sequence*	*Notes*
VSV-G	TGAAGTGCCTTTTGTACTTAGCCTTTTTATTC	ACCAGCGGAAATCACAAGTAGTGACC	
amp^R	ACCAGCGGAAATCACAAGTAGTGACC	AAGCCATACCAAACGACGAGCG	
AluYd6 (human)	GAGATCGAGACCACGGTGAAA	TTTGAGACGGAGTCTCGTT	(*53*)
ACTB (human)	CATGTACGTTGCTATCCAGGC	ATTACCCACTCCCGACCCG	Primerbank ID 501885a1