Supplementary Information for:

10.1073/pnas.2111234118

Post-translational modifications by ADAM10 shape myeloid antigen presenting cell homeostasis in the splenic marginal zone

Nathalie Diener[1], Jean-Fred Fontaine[2], Matthias Klein[3,4], Thomas Hieronymus[5], Florian Wanke[1], Florian C. Kurschus[1], Andreas Ludwig[6], Carl Ware[7], Paul Saftig[8], Tobias Bopp[3,4], Björn E. Clausen[1,4,9], and Ronald A. Backer[1,4,9]

[1] Institute for Molecular Medicine, Paul Klein Center for Immune Intervention, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany
[2] iOME Computational Biology and Data Mining, Johannes Gutenberg-University, Mainz, Germany
[3] Institute for Immunology, Paul Klein Center for Immune Intervention, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany
[4] Research Center for Immunotherapy (FZI), University Medical Center of the Johannes Gutenberg-University, Mainz, Germany
[5] Institute for Biomedical Engineering, Department of Cell Biology, University Medical Center, Aachen
[6] Institute of Pharmacology and Toxicology, Medical Faculty, RWTH Aachen University, Aachen, Germany
[7] Infectious and Inflammatory Diseases Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA.
[8] Institute of Biochemistry, Christian-Albrechts-University Kiel, Kiel, Germany
[9] These authors jointly directed this work

Ronald A. Backer
Email: r.backer@uni-mainz.de

Björn E. Clausen
Email: b.clausen@uni-mainz.de

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SI Materials and Methods

In Vivo Treatments

For LTβR agonist treatment, mice were treated with agonistic anti-LTβR antibody (clone 4H8) by intraperitoneal (i.p.) injection of 100μg of mAb every 3 days for 6 days. For FLT3L treatment, mice were i.p. injected with 10ug recombinant FLT3L (Creative Biomart Inc.) in 200ul PBS every day for 6 days. For BrdU pulse labeling, mice were injected i.p. with 1mg BrdU in 200ul phosphate buffered saline (PBS). BrdU incorporation was determined by flow cytometry 16h later using the BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. To discriminate between $CD11c^+$ conventional dendritic cells (cDC) in the splenic WP and RP, cDC were stained by injecting 1 μg anti-CD11c-APC (clone HL3) intravenously (i.v.) 4 min before the animals were sacrificed. Spleens were processed as described in the section '*Sample Preparation and Cell Sorting'*, and $CD11c^+$ cDC were stained *in vitro* with a different, noncompeting anti-CD11c antibody (clone N418).

Sample Preparation and Cell Sorting

Tissue was cut into grain size pieces and incubated in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium containing 200 U/ml Collagenase Type IV (Worthington Biochemical) and 0,5 U/ml DNaseI (Roche) while shacking at 37°C for 30 min or until digested. EDTA was added to a 10 mM final concentration, and the cell suspension was incubated for an additional 5 min at 4°C. Alternatively, for marginal metallophilic macrophages (MMM) isolation, spleens were cut into small pieces in polypropylene tubes with 0,75 ml of RPMI containing 4 mg/ml Lidocaine. Additionally, 0,25 ml of RPMI with 4 WU/ml Liberase TL (Roche) and 2 U/ml DNaseI (Roche) was added, and samples were incubated under continuous stirring at 37°C for 10-15 min until digested. Ice-cold RPMI plus 10% Fetal Calf Serum (FCS), 10 mM EDTA, 20 mM Hepes, and 50 μM 2-mercaptoethanol was added for an additional 5 min incubation at 4°C. To isolate skin cDC, ears were cut into small pieces and digested in 200 μl of digestion mix (400 U/ml Collagenase Type IV, 100 U/ml Hyaluronidase (Sigma-Aldrich) and 0,5 U/ml DNaseI) for 60 min at 37°C with continuous stirring. Red blood cells were lysed with ACK lysis buffer. Cells were washed once with PBS plus 10 mM EDTA and undigested material was removed by filtration. cDC were purified by positive selection with anti-CD11c Magnetic-activated cell sorting (MACS) microbeads (Miltenyi Biotec) according to the manufacturer's protocol. Cells were stained for CD4, CD8α, CD11c, MHCII, and viability dye and subsequently, cell sorting was performed with ARIA III cell sorter (BD Biosciences) in Hank's Balanced Salt Solution (HBSS) with 25 mM Hepes by gating on high expression of CD11c and MHCII and the absence or presence of CD4 and CD8α. Auto-fluorescent cells were excluded. The purity and viability of the sorted samples were determined by re-analysis on the cell-sorter and resulted in > 95% pure populations.

Bone Marrow-Derived DC (BM-DC) cultures

Cells were cultured in Iscove's modified Dulbecco's medium (IMDM, GE Healthcare) supplemented with 10% heat-inactivated FCS (Lonza), 200 U/ml penicillin, 200 μg/ml streptomycin (Gibco), and 50 μM β-mercaptoethanol (Invitrogen). BM-DC were generated either in the presence of FLT3L for 10 days of culture.

Bulk RNA-Sequencing

Messenger RNA (mRNA) was purified from sorted splenic cDC subpopulations ($CD11c^+MHCII^+$ $CD8\alpha^+$ cDC1, $CD4^+$ cDC2, and $CD4^-$ cDC2, 3 mice/group) with the RNeasy Plus Micro Kit according to the manufacturer's instructions (Qiagen). mRNA was quantified with a Qubit 2.0 fluorometer (Invitrogen) and the quality was assessed on a Bioanalyzer 2100 (Agilent) with an RNA 6000 pico chip (Agilent). Barcoded mRNA-sequencing (seq) complementary DNA (cDNA) libraries were prepared from 50 ng of total mRNA using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra II RNA Library Prep Kit for Illumina according to the manual (New England BioLabs). Quantity was assessed using Invitrogen's Qubit HS Assay Kit and library size was determined using Agilent's 2100 Bioanalyzer HS DNA assay. Barcoded RNA-Seq libraries were onboard clustered using HiSeq Rapid SR Cluster Kit v2 using 8 pM and 59 base pair reads were sequenced on the Illumina HiSeq2500 using the HiSeq Rapid SBS Kit v2 (50 Cycle). The quality of raw sequence reads of all samples was analyzed with the FastQC tool (Babraham Bioinformatics). Raw sequence reads were mapped to the reference genome GRCm38 with Spliced Transcripts Alignment to a Reference (STAR) (1). Mapped reads were assigned to annotated features (Gencode M18) using the featureCounts program (2). Pairwise differential expression analysis between the experimental groups was performed with the DESeq2 package (3). Genes with corrected P-value (Benjamini and Hochberg's false discovery rate, FDR) <0.01 were considered differentially expressed. The data was subsequently analyzed for enrichment of GO terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using Ingenuity Pathway Analysis (IPA) version 01-13 (Qiagen Bioinformatics) and analyzed according to the manual. Bulk RNA sequencing data are available at Gene Expression Omnibus (GEO) under accession number GSE154767.

Whole Transcriptome Single-Cell RNA-Sequencing

Splenic cDC were enriched with CD11c-MACS beads (3 mice/group), and cells were subsequently labeled using the Mouse Immune Single-Cell Multiplexing Kit (BD Biosciences). Labeled cells were counted and captured using the BD Rhapsody Single-Cell Analysis System Instrument according to the provided guidelines (BD Biosciences). Whole transcriptome analysis (WTA) library prep and Sample Tag library prep were generated following the BD Rhapsody System mRNA WTA and Sample Tag Library Preparation Protocol (BD

Biosciences). Around 2200 single cells were sequenced to a depth of $250x10^6$ paired-end 100 nucleotide (nt) length reads per sample using the Illumina HiSeq2500 Sequencing System. The raw output data was preprocessed according to the Illumina standard protocol, and analyzed by the BD Rhapsody WTA Analysis pipeline. Subsequently, for visualization, the Partek Flow single-cell pipeline was used (Build version 9.0.20.0514, Partek Inc.). Contaminating non-cDC populations, based on a comparison of their gene expression profiles with immune cell transcriptome profiles reported by ImmGen Consortium, were removed from downstream analysis. Genes with an FDR step-up p≤0.001 and a fold-change 2 or ≤0,5 were considered differentially expressed. Single cell sequencing data are available at Gene Expression Omnibus (GEO) under accession number GSE154309.

Microarray Data Analysis

Robust Multichip Average (RMA)-normalized gene expression data of relevant samples have been downloaded from the GEO database: dataset GSE31551 (4), GSE45698 (5), and GSE15907 (6). Microarray probesets were annotated using the corresponding gene annotation file (GPL6246.annot) which was complemented with Ensembl IDs by using R's package mogene10stprobeset.db. Annotation of several probesets to the same Ensembl ID were resolved by keeping only the probeset associated with either the highest coefficient of variation (GSE15907), or highest absolute fold change and then highest average expression (GSE31551), or lowest FDR and then highest fold change (GSE45698). Ensembl IDs were used to compare to the RNA-seq data. Differential genes in GSE45698 were derived by the GEO2R tool (FDR <0.05 and absolute $\log_2$-fold-change >1). Differential genes for 2 arrays in GSE31551 were defined by an absolute $\log_2$-fold-change >1. To compare on a principal component analysis of our RNA-seq data to GSE15907, both datasets were normalized by within-sample and within-gene scaling, and only genes expressed in both datasets were used.

Transwell Migration Assay

CD11c MACS-purified cDC (10^5) were placed in a Transwell migration chamber (pore size 5 μm, Corning) and allowed to migrate at 37°C. The EBI2-ligand 7α,25-OHC, the S1PR1-ligand S1P or control medium was placed in the lower chamber at the indicated concentrations to induce chemotaxis. After 3 h, cells that migrated to the lower chamber were counted by flow cytometry using AccuCount Rainbow counting beads (Spherotech), and induced migration was calculated as the ratio of the number of cells migrating with/without chemoattractant. ADAM10 activity was blocked by the addition of the selective inhibitor GI254023X (GI4023) (Sigma Aldrich) at the start of the assay.

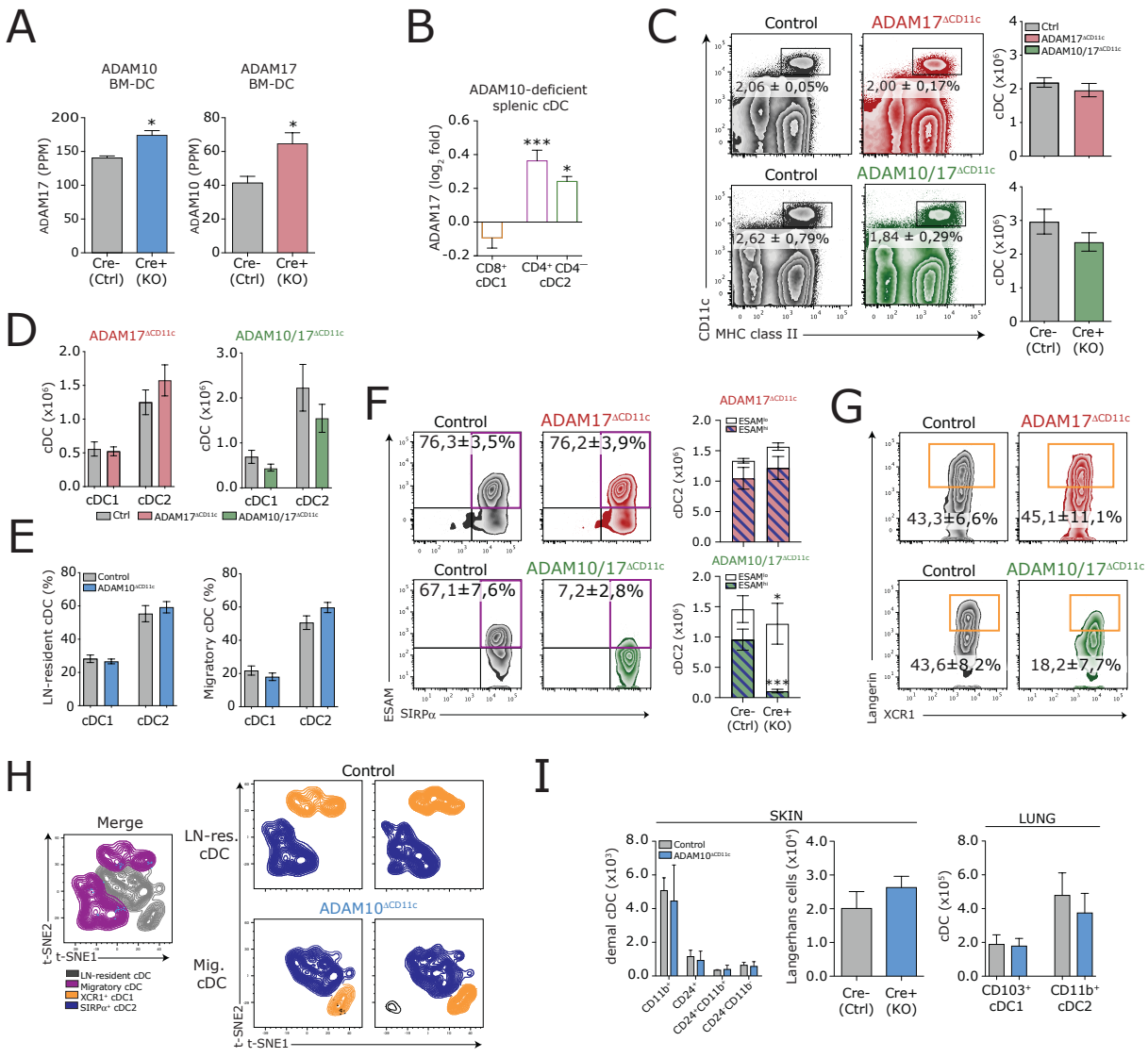


Figure S1: cDC1 and cDC2 subsets in lymphoid and non-lymphoid organs of CD11c-specific ADAM10- and/or ADAM17-deficient mice. (A) ADAM17 (left) and ADAM10 (right) protein expression on CD11c$^+$ MACS-purified GM-CSF-derived BM-DC from ADAM10-deficient and ADAM17-deficient mice, respectively, determined by liquid chromatography-mass spectrometry. **(B)** Log_2-fold-change of *Adam17* mRNA between ADAM10-deficient/control bulk-sorted splenic CD8α^+ cDC1 (orange) and CD4$^+$ cDC2 (purple) and CD4$^-$ cDC2 (green). **(C)** Frequencies (flow cytometry plots) and absolute numbers (bar graphs) of CD11c$^+$MHCII$^+$ cDC in ADAM17$^{\Delta CD11c}$ (top) and ADAM10/17$^{\Delta CD11c}$ (bottom) mice. **(D)** Absolute numbers of XCR1$^+$ cDC1 and SIRPα^+ cDC2 subsets in spleens of control versus ADAM17$^{\Delta CD11c}$ and ADAM10/17$^{\Delta CD11c}$ mice. **(E)** Absolute numbers of XCR1$^+$ and SIRPα^+ LN-resident cDC1 and cDC2 subsets (left) and XCR1$^+$ and SIRPα^+ CD11c$^+$MHCII^{hi} LN-migratory cDC subsets (right) in control versus ADAM10$^{\Delta CD11c}$ mice. **(F)** Flow cytometry plots show average frequencies of splenic ESAM^{hi} SIRPα^+ cDC2 in control, ADAM17$^{\Delta CD11c}$, and ADAM10/17$^{\Delta CD11c}$ mice. Bar graphs represent absolute cell counts of ESAM^{hi} and ESAM^{lo}

SIRPα^+ cDC2 in these mice. **(G)** Average frequencies of splenic Langerin$^+$ XCR1$^+$ cDC1 in control, ADAM17$^{\Delta CD11c}$, and ADAM10/17$^{\Delta CD11c}$ mice. **(H)** t-SNE representation of pLN cDC from control and ADAM10-deficient mice. pLN-resident cDC (black) and migratory cDC (purple) are further subdivided into XCR1$^+$ cDC1 (orange) and SIRPα^+ cDC2 (aubergine) subsets. **(I)** Absolute numbers of CD11c$^+$MHCII$^+$ dermal DC subsets (top left), Langerhans cells (CD11c$^+$MHCII$^+$CD11b$^+$Epcam$^+$CD103$^-$ cells, top right) in the skin, and CD103$^+$ cDC1 and CD11b$^+$ cDC2 subsets among CD11c$^+$MHCII$^+$ cells in the lung of control and ADAM10$^{\Delta CD11c}$ mice. *p<0,05 (Student's *t*-test); proteomics and bulk RNA-seq data both represent 3 control and 3 ADAM10-deficient samples.

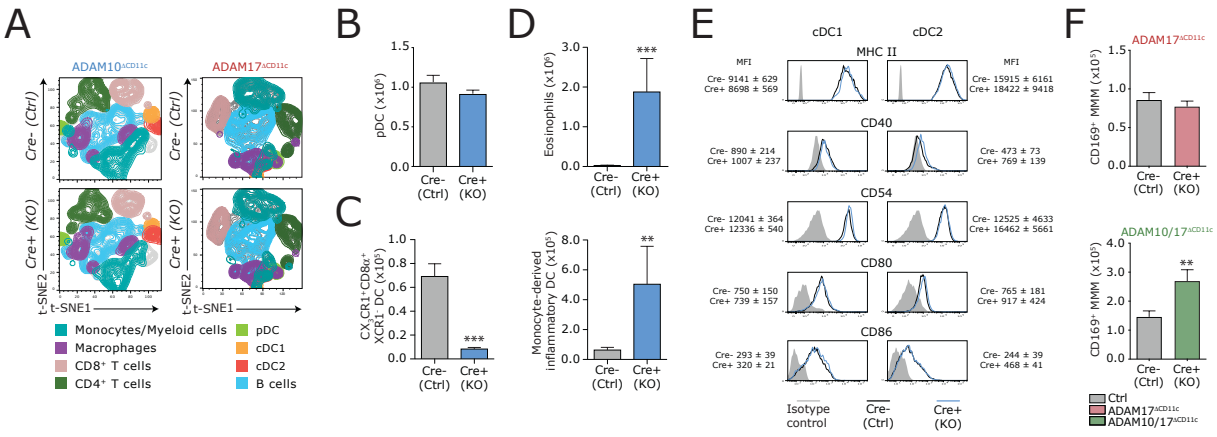
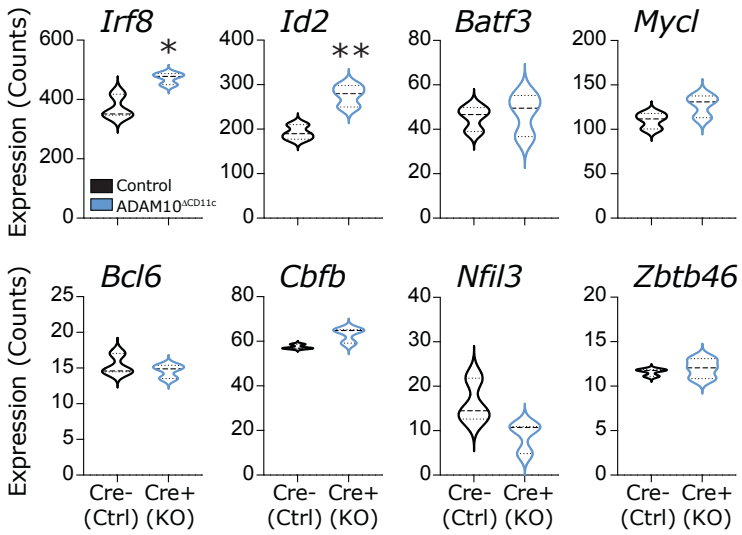


Figure S2: The splenic lymphoid and myeloid immune cell network in ADAM10$^{\Delta CD11c}$ mice. (A) Splenic lymphoid and myeloid immune cell network in ADAM10$^{\Delta CD11c}$ (left) and ADAM17$^{\Delta CD11c}$ (right) mice. The t-SNE algorithm (18.000 cells, randomly selected from 3 Cre-negative and 3 Cre-positive mice) was used to depict different populations, with immune cell populations as color dimension. Monocytes were identified by flow cytometry as CD11b^{+}CD45^{+}CD64^{+}Ly6G^{-}MHCII^{-} cells, macrophages as CD11b^{+}CD11c^{+}CD45^{+}CD64^{+}Ly6G^{-} MHCII^{+} cells, T cells as CD3^{+}CD45^{+}TCRβ^{+} with CD4 or CD8 expression (CD4+ T cells and CD8+ T cells, respectively), pDC as CD11c^{int}CD45^{+}CD45R^{+}CD317^{+}Siglec-H^{+}, cDC as CD3^{-} CD11c^{+}CD19^{-}CD45^{+}MHCII^{+} with XCR1 or SIRPα expression (cDC1 and cDC2, respectively) and B cells as CD3^{-}CD19^{+}CD45^{+}cells. **(B)** Quantification of total CD11c^{int}CD45^{+} CD45R^{+}CD317^{+}Siglec-H^{+} pDC in spleen of control and ADAM10$^{\Delta CD11c}$ mice as determined by flow cytometry. **(C)** Absolute numbers of CX$_3$CR1^{+}CD8α^{+}XCR1^{-} DC in spleen of control and ADAM10$^{\Delta CD11c}$ mice as determined by flow cytometry. **(D)** Quantification of eosinophils (top; Lin^{-}CD11b^{+}CD11c^{-}Gr-1^{-}NK1.1^{-}SSC^{hi} cells) and inflammatory DC (bottom; CD11c^{+}MHCII^{+}CD11b^{+}CD64^{+}FcεR1^{+}F4/80^{+}Ly6C^{+} cells) in spleens of control and ADAM10$^{\Delta CD11c}$ mice. **(E)** Expression of activation markers on control and ADAM10-deficient splenic cDC subsets. Plots are gated on CD11c^{+}MHCII^{+} cells and subsequently for XCR1^{+} cDC1 (left panel) and SIRPα^{+} cDC2 (right panel). Values represent mean frequencies ± SEM of indicated markers on control and ADAM10-deficient cells. **(F)** Quantification of total MMM numbers in spleen of control, ADAM17$^{\Delta CD11c}$, and ADAM10/17$^{\Delta CD11c}$ mice. MMM were characterized as living CD45^{+} autofluorescent CD169^{+} cells. **P<0,01, ***P<0,001 (Student's *t*-test). Bar graphs represent pooled data from more than 3 experiments (n=3-5 mice/experiment).

A



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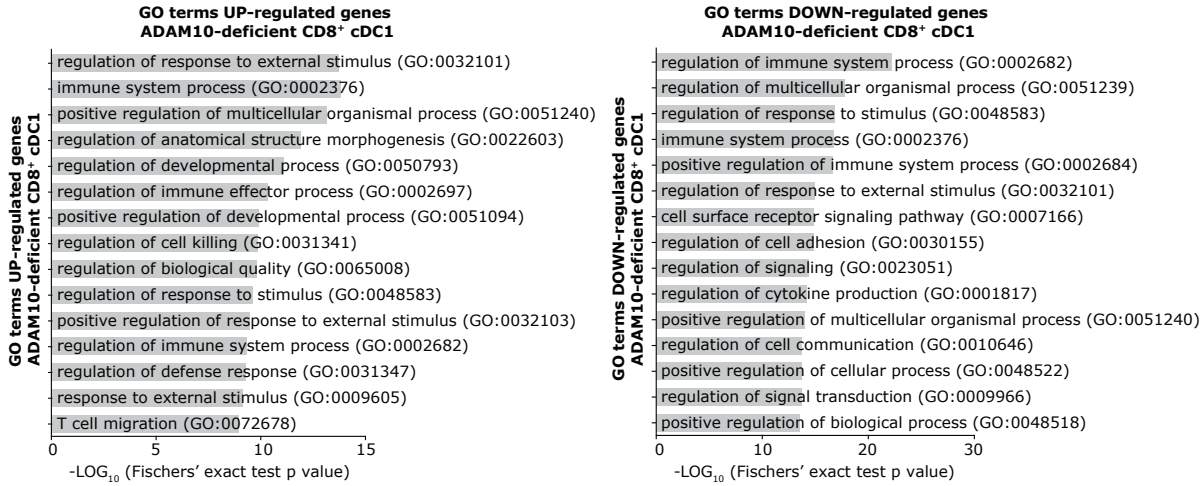

Figure S3: cDC1-defining transcription factor expression and ADAM10-regulated pathways in control vs. ADAM10-deficient cDC1. (A) Expression of transcription factors that are associated with cDC1 development in bulk sorted splenic CD11c$^+$MHCII$^+$ CD8α^+ cDC1 (expression in Reads Per Kilobase Million). **(B)** GO enrichment analysis associated with up-regulated (left) and down-regulated (right) genes in ADAM10-deficient/control CD8$^+$ cDC1. Bulk RNA-seq data represents 3 control and 3 ADAM10-deficient samples. *p<0,05, **P<0,01 (Student's *t*-test).

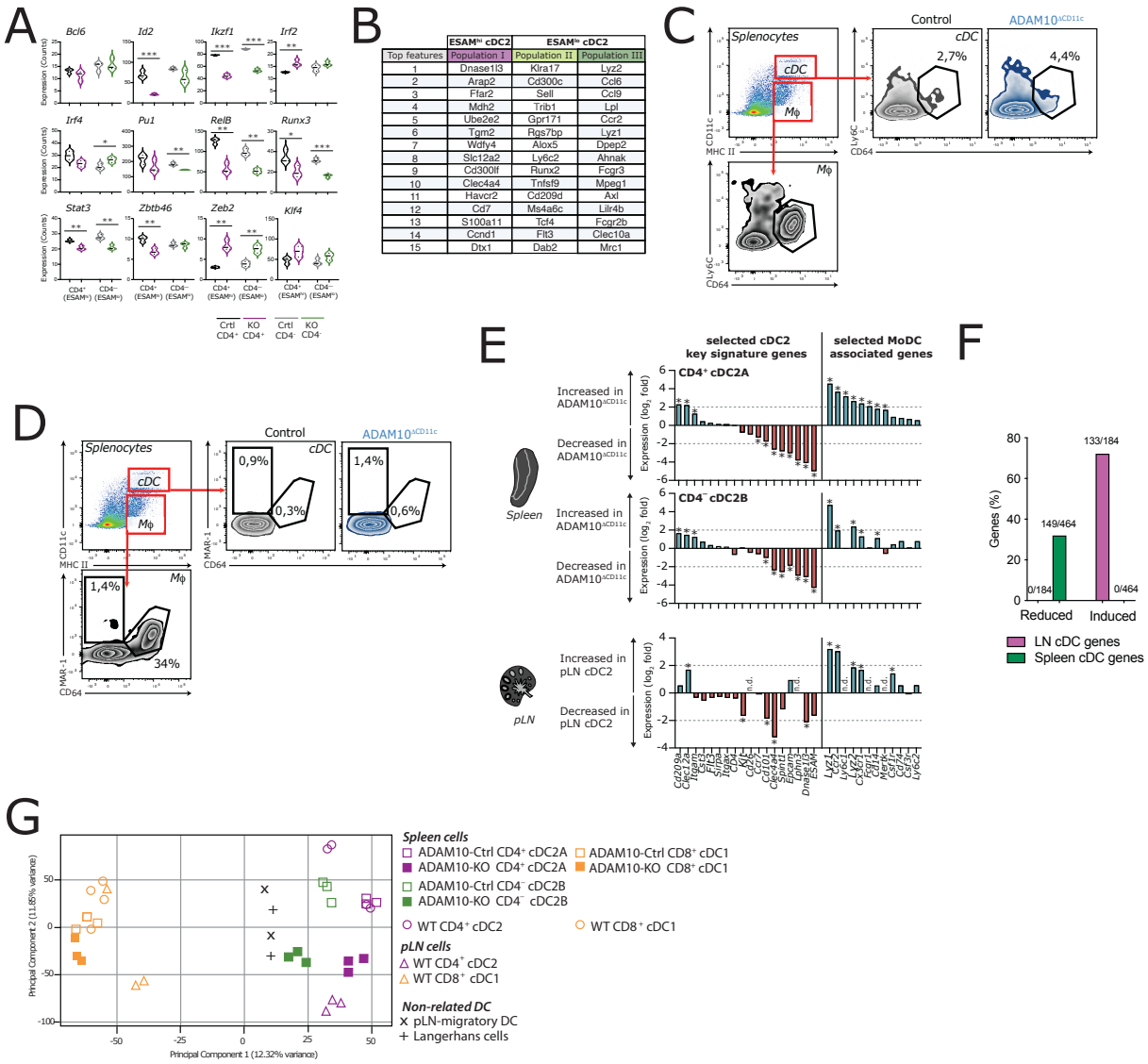

Figure S4: Phenotypic and transcriptional profiling of the emerging ESAM^{lo} cDC2B in ADAM10$^{\Delta CD11c}$ mice. (A) Expression of transcription factors that are associated with cDC2 development in bulk sorted splenic CD11c^{+}MHCII^{+} CD4^{+} cDC2A and CD4^{-} cDC2B (expression in Reads Per Kilobase Million). **(B)** Top 15 most significant gene expression features between the three cDC2 population I-III. Frequency of Ly6C^{+}CD64^{+} DC **(C)** and MAR-1^{+}CD64^{+} DC **(D)** in spleen of control and ADAM10$^{\Delta CD11c}$ mice. DC were pregated as CD11c^{hi}MHCII^{+} cells. Gating strategy is based on the expression of either Ly6C+CD64 or MAR-1+CD64 on WT macrophages (CD11c^{int}MHCII^{+} cells). FACS plots show one representative mouse/group. **(E)** Top; log_2-fold-change between ADAM10-deficient/control splenic CD4^{+} and CD4^{-} cDC2 for selected cDC2 key signature genes and Mo-DC-associated genes. Bottom; log_2-fold-change of same selected genes between WT pLN-resident and WT splenic cDC2. WT pLN and WT spleen gene expression data sets are retrieved and accessible as GEO GSE15907 [1]. **(F)** Frequency of spleen-cDC2-specific (gene set of 464 genes, green) or pLN-cDC2-specific (gene set of 184 genes, purple) genes with significantly lower expression (Reduced) or higher

expression (Induced) in ADAM10-deficient cDC2 than in their wild-type counterparts (adjusted P value, <0.05). WT pLN and WT spleen gene expression data sets are retrieved and accessible as GEO GSE15907 [1]. **(G)** Principal component analysis plot of bulk RNA-seq data of differentially expressed genes in splenic CD8α^+ cDC1, CD4$^+$ cDC2 and CD4$^-$ cDC2 of control and ADAM10$^{\Delta CD11c}$ mice, and of published microarray data of WT splenic and WT LN-resident CD8α^+ cDC1 and CD4$^+$ cDC2 (*as in E*). Bulk RNA-seq and SC-seq-WTA data both represent 3 control and 3 ADAM10-deficient samples. *p<0,05, **P<0,01, ***P<0,001 (Student's *t*-test).

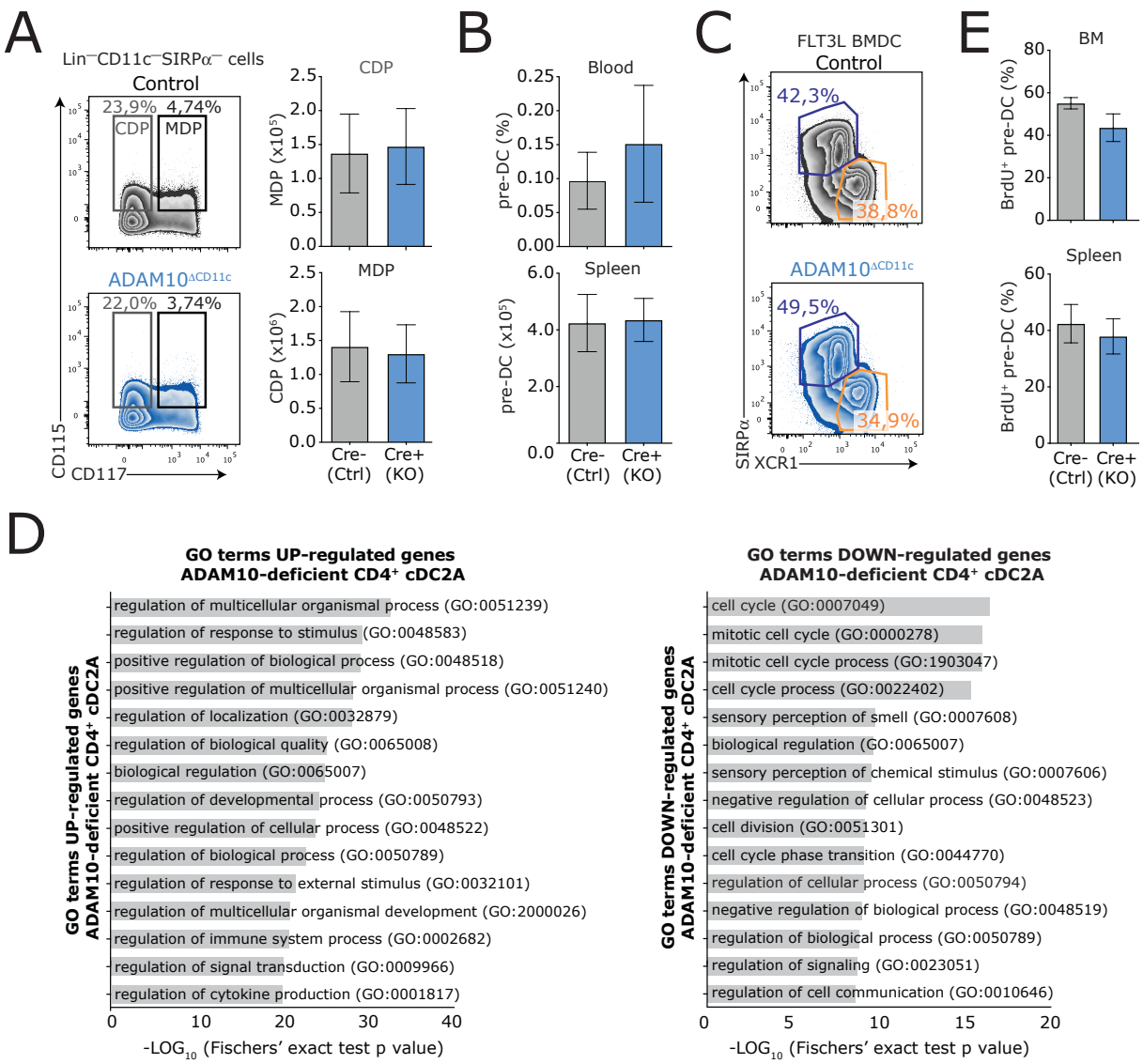

Figure S5: Commitment, development, and proliferation of cDC2 in control and ADAM10$^{\Delta CD11c}$ mice. (A) Quantification of bone marrow MDP and CDP numbers in control and ADAM10$^{\Delta CD11c}$ mice. MDP and CDP are characterized by the expression of either CD115 or CD117 on Lin$^-$CD11c$^-$SIRPα^-FLT3$^+$ cells, respectively. **(B)** Lin$^-$CD11c$^+$MHCII$^-$FLT3$^+$SIRPα^{int} pre-DC numbers in blood (top) and spleen (bottom) of control and ADAM10$^{\Delta CD11c}$ mice. **(C)** Composition of FLT3L-treated BM-DC cultures, as analyzed for the frequencies of SIRPα^+ (purple gate) and XCR1$^+$ (orange gate) subsets among control (top) and ADAM10-deficient (bottom) CD11c$^+$MHCII$^+$ cells. **(D)** Go enrichment analysis associated with up-regulated (left) and down-regulated (right) genes in ADAM10-deficient/control CD4$^+$ cDC2A. **(E)** Control or ADAM10$^{\Delta CD11c}$ mice were injected with BrdU. Proliferating cells were detected by BrdU-incorporation 12 h later. Bar graphs indicate the frequency of BrdU positive cells among pre-DC in BM (top) or spleen (bottom). Data is pooled from 3 experiments (n=3-6 mice/experiment), mean frequencies ± SEM. Bulk RNA-seq data represents 3 control and 3 ADAM10-deficient samples.

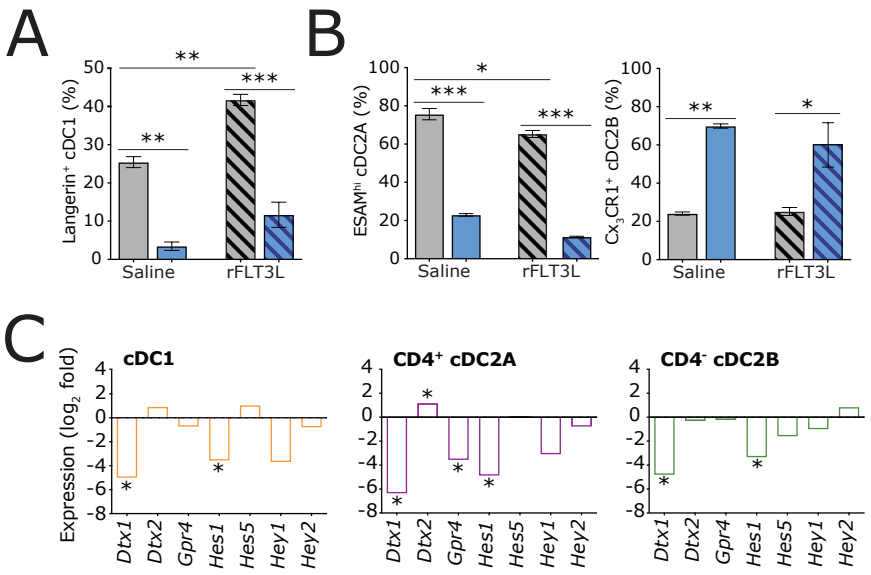

Figure S6: Splenic cDC subsets upon FLT3L treatment and expression of Notch2 signaling-associated genes in cDC subsets of control vs. ADAM10-deficient mice. (A) Frequency of splenic Langerin$^+$ cDC1 (left) in saline and rFLT3L treated control and ADAM10$^{\Delta CD11c}$ mice. **(B)** Frequencies of splenic ESAM^{hi} cDC2A and CX$_3$CR1$^+$ cDC2B in saline and rFLT3L treated control and ADAM10$^{\Delta CD11c}$ mice. **(C)** Log$_2$-fold-change between ADAM10-deficient vs. control for selected Notch2 target genes in bulk sorted splenic CD11c$^+$MHCII$^+$ CD8α^+ cDC1 (left), CD4$^+$ cDC2A (middle) and CD4$^-$ cDC2B (right). Bulk RNA-seq data represents 3 control and 3 ADAM10-deficient samples *p<0,05, **P<0,01, ***P<0,001 (Student's *t*-test).

Table S1: Monoclonal antibodies for flow cytometry

ANTIBODY	CLONE	SOURCE
CD3	145-2C11	Biolegend
CD4	GK1.5	Biolegend
CD8a	53-6.7	Biolegend
CD11b	M1/70	eBioscience
CD11c	N418	Biolegend
CD11c	HL3	BD Biosciences
CD19	6D5	Biolegend
CD24	M1/69	Biolegend
CD40	3/23	Biolegend
CD45pan	30-F11	BD Biosciences
CD45R (B220)	RA3-6B2	Biolegend
CD49b	DX5	eBiosciences
CD54	YN1/1.7.4	Biolegend
CD64	X54-5/7.1	Biolegend
CD68	FA-11	BD Biosciences
CD80	160-10A1	BD Biosciences
CD86	GL1	BD Biosciences
CD95 (FAS)	Jo2	BD Biosciences
CD103	2E7	Biolegend
CD115	AFS98	Biolegend
CD117 (c-Kit)	2B8	Biolegend
CD135 (FLT3)	A2F10.1	BD Biosciences
CD169 (Siglec-1)	SER-4	eBioscience
CD172a (Sirpa)	P8a	eBioscience
CD205	DEC205	Biolegend
CD207 (Langerin)	eBioL31	eBioscience
CD209b (Sign-R1)	22D1	eBioscience
CD317 (PDCA-1)	eBio927	eBioscience
CD371 (Clec12a)	5D3/CLEC12A	Biolegend
Cx3CR1	SA011F11	Biolegend
DCIR2	33D1	Biolegend
ESAM	1G8/ESAM	Biolegend
F4/80	BM8	Biolegend
FCeRIa	MAR-1	Biolegend
GR-1	RB6-8C5	BD Biosciences
IRF4	3E4	Biolegend
LT-bR	eBio3C8	eBioscience
Ly6C	AL-21	BD Biosciences
Ly6G	1A8	BD Biosciences
MerTK	M1/69	BD Biosciences
MHC class II	5/114	eBioscience
NK1.1	PK136	Biolegend
Notch2	HMN2-35	Biolegend
RelB	C-19	Santa Cruz
RORgt	B2D	eBioscience
Siglec-F	E50-2440	BD Biosciences
Tbet	eBio4B10	eBioscience
XCR1	ZET	Biolegend
Annexin V		Immunotools
7AAD		BD Biosciences
fixable viability dye		eBioscience

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