Supplementary materials for:

Investigations into *SCAMP5*, a candidate lupus risk gene expressed in plasmacytoid dendritic cells

By Ghanem et al.

Materials and Methods

Human blood donors

The Genotype and Phenotype (GaP) Registry at The Feinstein Institutes for Medical Research provided de-identified blood from donors consented under an IRB-approved protocol (IRB# 09-081). The Committee for Participant Protection at The Feinstein Institutes for Medical Research approved this study (TAP0307.5.73). The GaP is a sub-protocol of the Tissue Donation Program (TDP) at Northwell Health and a national resource for genotype-phenotype studies (https://www.feinsteininstitute.org/robert-sboas-center-for-genomics-and-human-genetics/gap-registry/). For experiments comparing genotypes, subjects were selected on the basis of genotype from the Global Screening Array (GSA).

Patient and Public Involvement

Patients in our studies are actively interested in advancing knowledge of autoimmunity, even when they participate as controls in protocols such as the GaP. Thus, a Community Advisory Board comprised of diverse (ethnicity, age and gender) community members and study participants are consulted quarterly, and their input is integrated into the GaP study. Patients have not been involved in the design of the experimental work. There were no interventions in this study. The results will be communicated via publication and newsletters to participants.

FACS analysis and cell sorting

Fresh whole blood was drawn from healthy donors into sodium-heparin vacutainers (BD). PBMCs were extracted over Ficoll-Paque (GE Healthcare) using standard methods. PBMCs were then labeled as follows for cell sorting: CD19, CD3, CD4, CD8, CD56, CD14, CD16, CD304, CD303, CD11c, HLA-DR. Lymphocytes were sorted by gating on the lymphocyte population on light scatter: B cells (CD19+CD3-CD56-); CD4 T cells (CD3+CD4+); CD8 T cells (CD3+CD8+); NK cells (CD56+CD3-CD19-). Monocytes were sorted by gating on the monocyte population on light scatter: CD14 classical monocytes (CD14+CD16-); CD16 non-classical monocytes (CD16+CD14-). Dendritic cells were sorted by gating on the lineage negative population (CD19-CD3-CD56-CD16-): pDCs (CD304+CD303+); cDCs (HLA-DR+CD11c+). Cells were sorted into microfuge tubes on the BD FACSAria SORP cell sorter. Sorted cells were immediately pelleted by centrifugation and frozen dry at -80°C until RNA extraction for RT-PCR or lysate preparation for western blotting.

For intracellular staining of SCAMP5 and flow cytometric analysis, PBMCs were surface stained for CD304 (BDCA-4) followed by fixation and permeabilization using BD Cytofix/CytoPerm. Cells were then kept in BD Perm/Wash and stained for SCAMP5 using a rabbit polyclonal antibody (PA5-61269, ThermoFisher Scientific) followed by staining using anti-rabbit AF488. Cells were acquired using the BD Fortessa flow cytometer.

mRNA Expression

RNA was prepared from frozen cell pellets using the Qiagen RNeasy Micro Plus kit (Qiagen 74034). RNA was immediately reverse transcribed using Superscript IV VILO master mix at 50°C for 10 minutes (Invitrogen 11756050), or the SuperScript IV VILO Master Mix 'No RT' Control, using the Applied Biosystems Veriti thermal cycler. cDNA was then diluted in TE buffer and stored at -20°C pending further analysis. qPCR was performed using Taqman assays (ThermoFisher Scientific) and Taqman Gene Expression Master Mix (Applied Biosystems 4369016). qPCR reactions were performed on the Applied Biosystems Viia7 thermal cycler.

Protein expression studies

Lysates were prepared by resuspending cell pellets in 1X CHAPS lysis buffer supplemented with 1X HALT protease inhibitor (Thermo Scientific 78429). Lysates were incubated on ice for 30 minutes with periodic vortexing. LDS sample buffer was added to a final concentration of 1X and β-mercaptoethanol to a final concentration of 5%. Lysates were then directly loaded into a 10% Bis-Tris gel without boiling, as heating of lysates was found to aggregate SCAMP5 protein. Lysates were resolved at 200V for 45 minutes using 1X Novex BOLT running buffer. Gels were then equilibrated in 1X Novex Transfer buffer/20% methanol, followed by transfer onto 0.45uM PVDF membranes at 100V/350mA for 25 minutes. Blots were blocked for 60 minutes at room temperature using Licor Odyssey Blocking Buffer diluted 1:1 in PBS. Primary anti-SCAMP5 rabbit polyclonal antibody (PA5-61269) was diluted in staining buffer (Licor blocking buffer diluted 1:5 in PBS) at a dilution of 1:1,000 overnight at 4°C. After 3 washes in PBS Tween-20, secondary anti-rabbit HRP was added at a dilution of 1:10,000 in staining buffer for 2 hours at room temperature. After another 2 rounds of washing in PBS-T and a final wash in PBS, the blot was developed using chemiluminescent substrate and acquired using the BioRad Chemidoc Imager. The blot was reprobed for actin (Ab clone AC-15) as described for SCAMP5.

Confocal microscopy of pDCs

For confocal analysis of SCAMP5 in pDCs, pDCs were isolated from PBMCs by immunomagnetic negative selection. Freshly isolated pDCs were cytospun onto charged glass slides. Cells were fixed and permeabilized by immersion in BD Ctyofix/Cytoperm, and simultaneously stained for SCAMP5 (rabbit polyclonal antibody PA5-61269) and Golgin-97 (ThermoFisher A-21270). A pre-immune rabbit polyclonal antibody was used as a negative control for SCAMP5 staining, (no staining was observed, data not shown). Cells were washed in BD Perm/Wash and stained using anti-rabbit AF488 and anti-mouse AF647. No. 1 coverslips were mounted using Prolong Antifade Glass with NucBlue and sealed using clear nail polish. After curing at 4°C for 30 minutes, images were acquired using the Zeiss LSM 880 confocal microscope.

Genomic analysis of the SLE association polymorphism rs2289583

Phased haplotypes were built from genotypes of rs2472300, rs6495122 and rs2289583 for 6,959 controls and 4,036 lupus cases (totaling 10,995 subjects and 21,990 haplotypes). Plink binary files were converted to a vcf file with plink2. Variants were phased using beagle 5.0 (v2018) using the hg37 genetic map. P values and odds ratios were calculated by comparing counts of each haplotype to the sum of other haplotypes.

Linkage disequilibrium (LD) statistics between SNPs were obtained using the 'LDpair Tool' on LD Link (https://ldlink.nci.nih.gov/?tab=ldpair) (Machiela and Chanock, 2015). LD statistics were derived from the European population.

Conditional analysis was carried out by multi-variable logistic regression, with the SNP to be conditioned as one of the independent variables, and phenotype being the dependent variable. The coefficients and the corresponding p-values for other SNPs, being other independent variables, are the association results conditional on the SNP to be conditioned.

Phenotyping of GaP Subjects on the basis of *SCAMP5* risk haplotype

The GaP Registry is a cohort of ~7,500 (and counting) genotyped healthy individuals who have agreed to recall on the basis of their genotype.[19] The GaP has been previously utilized for the study of endophenotypes regulated by various autoimmune risk variants and is available to the scientific community.[16 20 21] To assay for pDC phenotypes related to genotype at rs2289583, we chose from among this cohort. As the relationship of the causal polymorphism to rs2289583 has not yet been established, we sought to enrich for likely carriers of the causal allele by constructing SLE risk haplotypes inclusive of rs2289583. rs6495122 and rs2472300 are the next most significantly associated SNPs in the region and together along with rs2289583 they denote a risk haplotype.

Female subjects of childbearing age (here stipulated 18-40) homozygous for the risk or non-risk haplotypes were contacted for recall (subject characteristics in **Table S2**). 60CC whole blood was drawn from each donor into sodium-heparin vacutainers. Subjects were drawn in pairs, with one homozygous risk and one homozygous non-risk donor per day. Altogether, 9 risk:non-risk pairs were collected, with an additional 3 non-risk:non-risk pairs, over 12 discontinuous days of sampling.

Whole blood lysates were genotyped to confirm the fidelity of the recalled subject genotypes. Taqman genotyping was performed for rs2289583 on crude whole blood lysates: Frozen aliquots of whole blood were thawed and prepared for PCR using DNA Extract All Reagents Kit (Applied Biosystems 4403319). PCR was performed using a custom Taqman genotyping assay (assay ID: C__15881999_10) and TaqPath ProAMP Master Mix (Applied Biosystems A30865), with the VIC probe detecting the minor allele (A=risk) and the FAM probe detecting the major allele (C=non-risk). Genotypes were called on the Viia7 Real-Time PCR System (Applied Biosystems).

PBMCs were extracted over Ficoll-Paque using established methods. A small aliquot of PBMCs was kept for determination of baseline pDC frequency. pDCs were isolated using Miltenyi Biotec Human Plasmacytoid Dendritic Cell Isolation Kit II (130-097-415) in accordance with the manufacturer's protocol. A small aliquot of isolated pDCs was used for purity determination by staining for BDCA4 and BDCA2 **(Figure S6)**.

For pDC stimulation cultures, 3,000 pDCs were cultured per well of a round-bottom 96-well plate using Advanced RPMI (Gibco 12633012) with 5% FCS. Triplicate cultures were established per condition: no stimulation, imiquimod 4µg/mL (Invivogen), ODN-2216 2µM (Invivogen), or influenza virus 0.4HAU/mL (A/PR/8/34 H1N1, Advanced Biotechnologies Inc.).

RNA preparation, cDNA synthesis, qPCR, and western blotting on pDC pellets were performed as described above. IFN-α ELISAs were performed using MABTECH 3425-1H-6 in accordance with the manufacturer's protocol. QTL and ELISA experiments were performed in a single batch after all GaP donors had been collected.

Taqmas assays for eQTL experiments are as follows:

Gene	Assay ID
SCAMP5	Hs01547727_m1
PPCDC	Hs00222418_m1
RPP25	Hs00706565_s1
CSK	Hs01062581_m1
COX5A	Hs00362067_m1
18S rRNA	Hs03003631_g1
GAPDH	Hs02786624_g1
IRF7	Hs01014809_g1
POLR2A	Hs00172187_m1
IFNB1	Hs01077958_s1
IFNA2	Hs00265051_s1
IFNA10	Hs03406429_gH
GUSB	Hs00939627_m1

Taqman assays for intron-inclusion transcripts were custom generated by ThermoFisher and are described in the table below:

Intron interval name	qPCR assay ID	Chromosome 15 (Hg38)	
		Start position	End position
1-1		7,499,567	74,995,966
1-2		7,499,632	74,997,233
1-3		7,499,731	75,007,587
1-4		7,500,772	75,009,890
1-5		7,500,994	75,011,657
1-6	APEPXWT	7,501,168	75,011,791
2-1		7,501,184	75,012,676
3-1	APFVTGP	7,501,312	75,016,592
4-1	APGZK2M	7,501,674	75,017,652
5-1		7,501,797	75,018,393
6-1		7,501,853	75,018,788

Design of lentiviral expression constructs

SCAMP5-mKate2, c-mVenus, IFNsp-mVenus and mTagBFP2-clathrin were ordered as gene blocks from IDT. SCAMP5-mKate2 was cloned into the lentiviral backbone vector pLJM1-eGFP (Addgene #19319) using NheI and BstBI in place of eGFP. c-mVenus and IFNsp-mVenus were first cloned into the Gateway entry vector pENTR4 using NcoI and XhoI followed by recombination into the lentiviral Gateway destination vector pLenti CMVtight Puro DEST (Addgene #26430) using LR Clonase II. A lentiviral backbone vector expressing rtTA3 was obtained separately (Addgene #26429).

Lentiviral vectors were produced by co-transfection of lentiviral backbone vectors along with a lentiviral packaging mix (Invitrogen A43237) carrying the Rev, VSV-G and Gag/Pol genes using Lipofectamine 3000 into HEK 293T cells. Lentiviral media was collected 72 hours post-transfection, cleared through 0.22uM syringe filters and stored at -80c.

Transduction of HEK-293T cells with lentiviral particles

For SCAMP5-mKate2 expression, SCAMP5-mKate2 lentiviral particles were transduced into HEK-293T cells. Cells expressing mKate2 were sorted on the BD FACSAria and maintained in culture. For c-mVenus and IFNsp-mVenus expression, the respective lentiviral particles were co-transduced with rtTA3 into HEK-293T cells previously transduced with SCAMP5-mKate2, to generate cells constitutively expressing SCAMP5-mKate2 and either mVenus or IFNsp-mVenus in a doxycycline inducible system.

Live-cell confocal microscopy

HEK-293T cells expressing the desired transgenes were seeded onto 35mm poly-D-lysine coated dishes with No. 1 coverslip center pieces. Prior to imaging, the media was aspirated and replaced with FluoroBrite DMEM Media containing 10% FBS, with or without NucBlue for counterstaining of nuclei. Images were acquired on a Zeiss LSM880 Confocal microscope.

pDC total RNAseq

pDCs were isolated from 4 homozygous risk and 5 homozygous non-risk donors as described above. Yield and purities are presented in the table below. pDC pellets were immediately stored at -80c. RNA extraction was performed using Qiagen RNeasy Micro Plus kit (Qiagen 74034). Sequencing libraries were prepared with normalized inputs using TruSeq RNA Single Indexes Set A (Illumina Cat. # 20020492). Paired end sequencing reactions were performed on the Illumina NextSeq 500 sequencer using NextSeq 500/550 High Output Kit v2.5 (150 Cycles) (Illumina Cat. #20024907). FastQC was used to insure there was no adapter contamination and reads had acceptable quality scores. Intronic retention was quanitified using iREAD to the Gencode grch38 reference assembly.

Subject Genotype	pDC yield	pDC Purity
Non-risk / Risk	#	%Percent
3538	435,000	95.8
4752	61,272	67.4
5050	569,000	96.1
7406	217,000	94.1
2727	132,000	95.3
8640	567,000	96.5
8461	256,200	84.2
4855	229,000	81.2
8123	374,900	84.2

Data and Statistical Analyses

Unless otherwise indicated, raw data was tabulated in Microsoft Excel. ELISA standard curve interpolation, graph generation and statistical analyses were performed using GraphPad Prism. Flow cytometry data was analyzed using FlowJo. Confocal images were analyzed using ZenBlue. In all figures, numerical values are indicated for p-values <0.2, all other p-values denoted as *not significant.*

Fusion protein constructs

Scamp5-mKate2 protein sequence:

MAEKVNNFPPLPKFIPLKPCFYQDFEADIPPQHLSLTKRLYYLWMLNSVTLAVNLVGCLAWLIGGGGATNFGLAFLWLILFTPCSYVCWF
RPIYKAFKTDSSFSFMAFFFTFMAQLVISIIQAVGIPGWGVCGWIATISFFGTNIGSAVVMLIPTVMFTVVAVFSFIALSMVHKFYRGSGG
SFSKAQEEWTTGAWKNPHVQQAAQNAAMGAAQGAMNQPQTQYSATPNYTYSNEMGGGGPGGGGSVSELIKENMHMKLYMEG
TVNNHHFKCTSEGEGKPYEGTQTMRIKAVEGGPLPFAFDILATSFMYGSKTFINHTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTATQ
DTSLQDGCLIYNVKIRGVNFPSNGPVMQKKTLGWEASTETLYPADGGLEGRADMALKLVGGGHLICNLKTTYRSKKPAKNLKMPGVYY
VDRRLERIKEADKETYVEQHEVAVARYCDLPSKLGHR

SCAMP5 sequence
Gl_4 linker
mKate2 sequence

mVenus protein sequences:

Sequence of mVenus with human IFN-a leader peptide [human codon-optimized]:

MALSFSLLMAVLVLSYKSICSLGCDLPVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLG
YGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITA
DKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

Sequence of untagged mVenus:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSA
MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQ
LADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

IFN-α leader peptide
mVenus

mTagBFP2-Clathrin protein sequence:

MVSKGEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIPDFFKQSFP
EGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNGPVMQKKTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIA
NAKTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEVAVARYCDLPSKLGHKLNSGLRSRAQASNSAVDMADDFGFFSSSE
SGAPEAAEEDPAAAFLAQQESEIAGIENDEGFGAPAGSHAAPAQPGPTSGAGSEDMGTTVNGDVFQEANGPADGYAAIAQADRLTQ
EPESIRKWREEQRKRLQELDAASKVTEQEWREKAKKDLEEWNQRQSEQVEKNKINNRASEEAFVKESKEETPGTEWEKVAQLCDFNP
KSSKQCKDVSRLRSVLMSLKQTPLSR

mTagBFP2 sequence
Linker peptide
Clathrin light chain B isoform A

Supplementary figures

Figure S1. Schematic of SCAMP5 topology. Generated using BioRender.

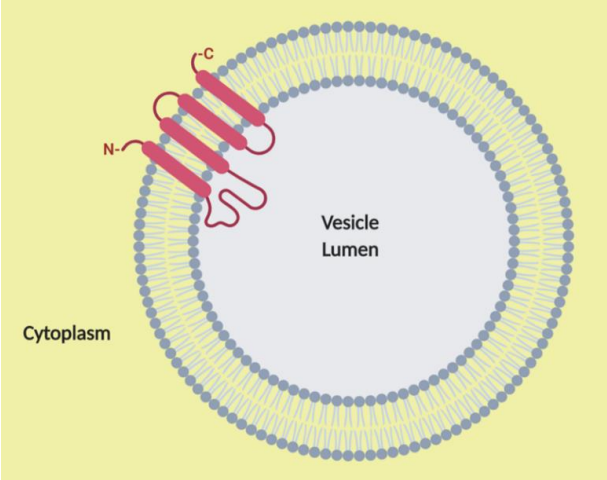
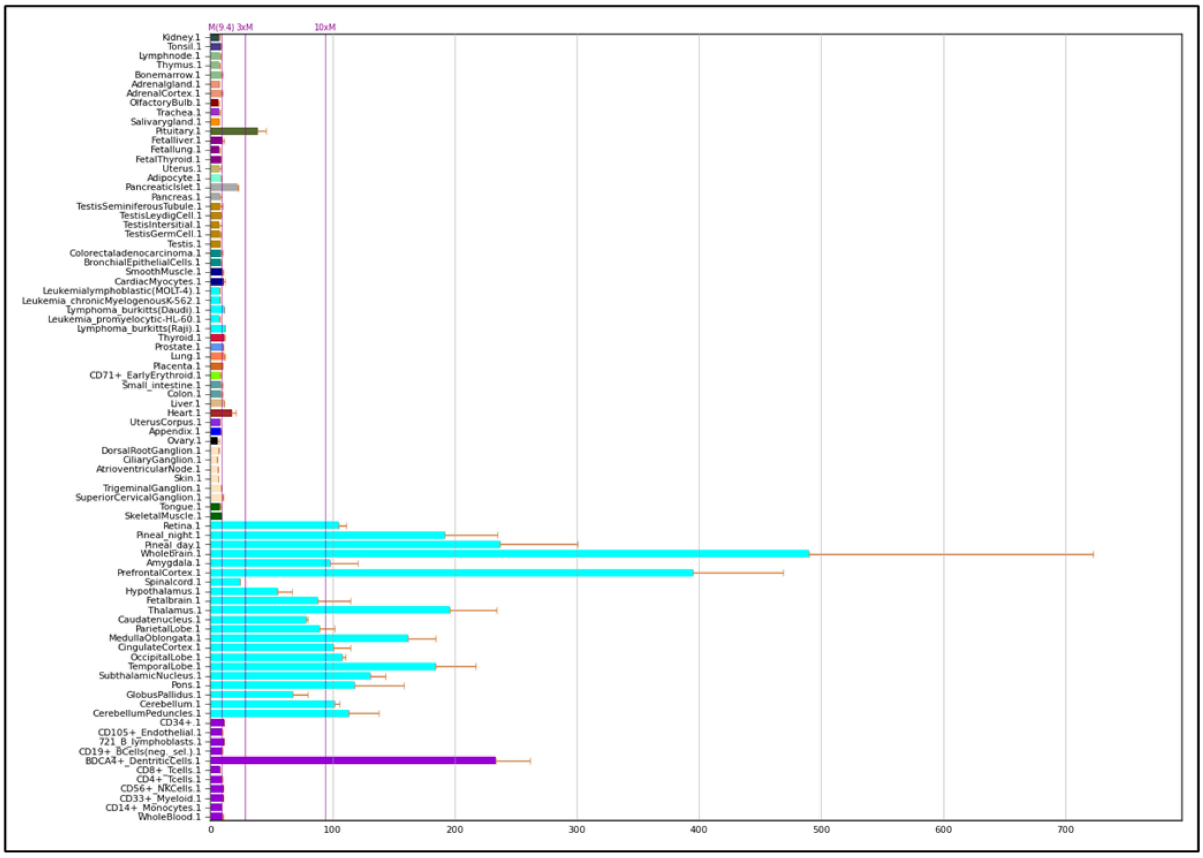


Figure S2.

A) *SCAMP5* expression across human tissues determined using microarray. (BioGPS)
http://biogps.org/#goto=genereport&id=192683



B) *SCAMP5* expression across circulating human leukocyte subsets as determined by RNA-seq. (Human Protein Atlas)
https://www.proteinatlas.org/ENSG00000198794-SCAMP5/blood

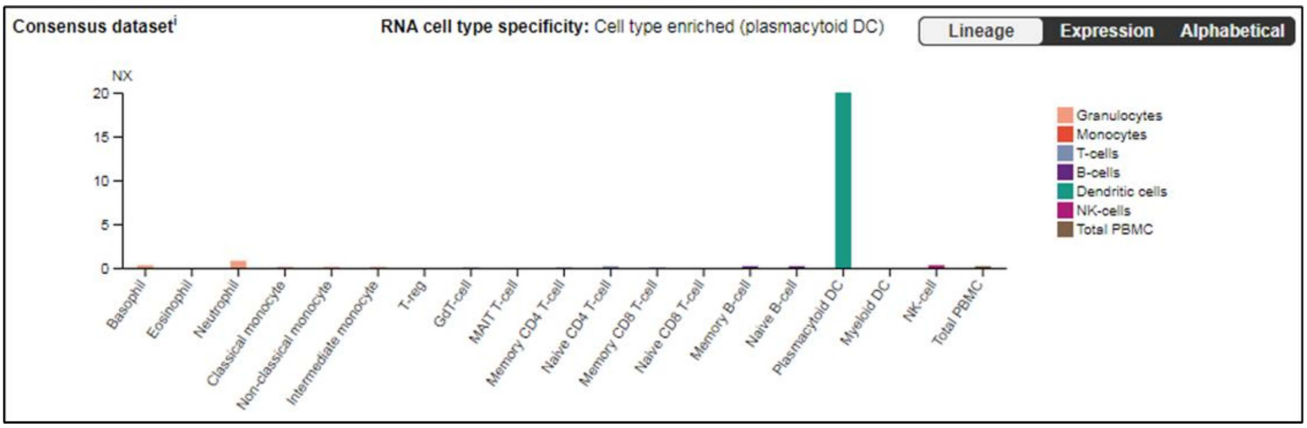


Figure S3. Comparative expression in pDCs of genes neighboring *SCAMP5* from donors homozygous for the risk or non-risk haplotypes about rs2289583. Quantitative RT-PCR for *CSK, COX5A, RPP25* and *PPCDC* transcript from pDCs normalized individually to 18s rRNA, *GAPDH* and *IRF7*. Comparisons between genotypes by Mann-Whitney test. *ns= not significant.*

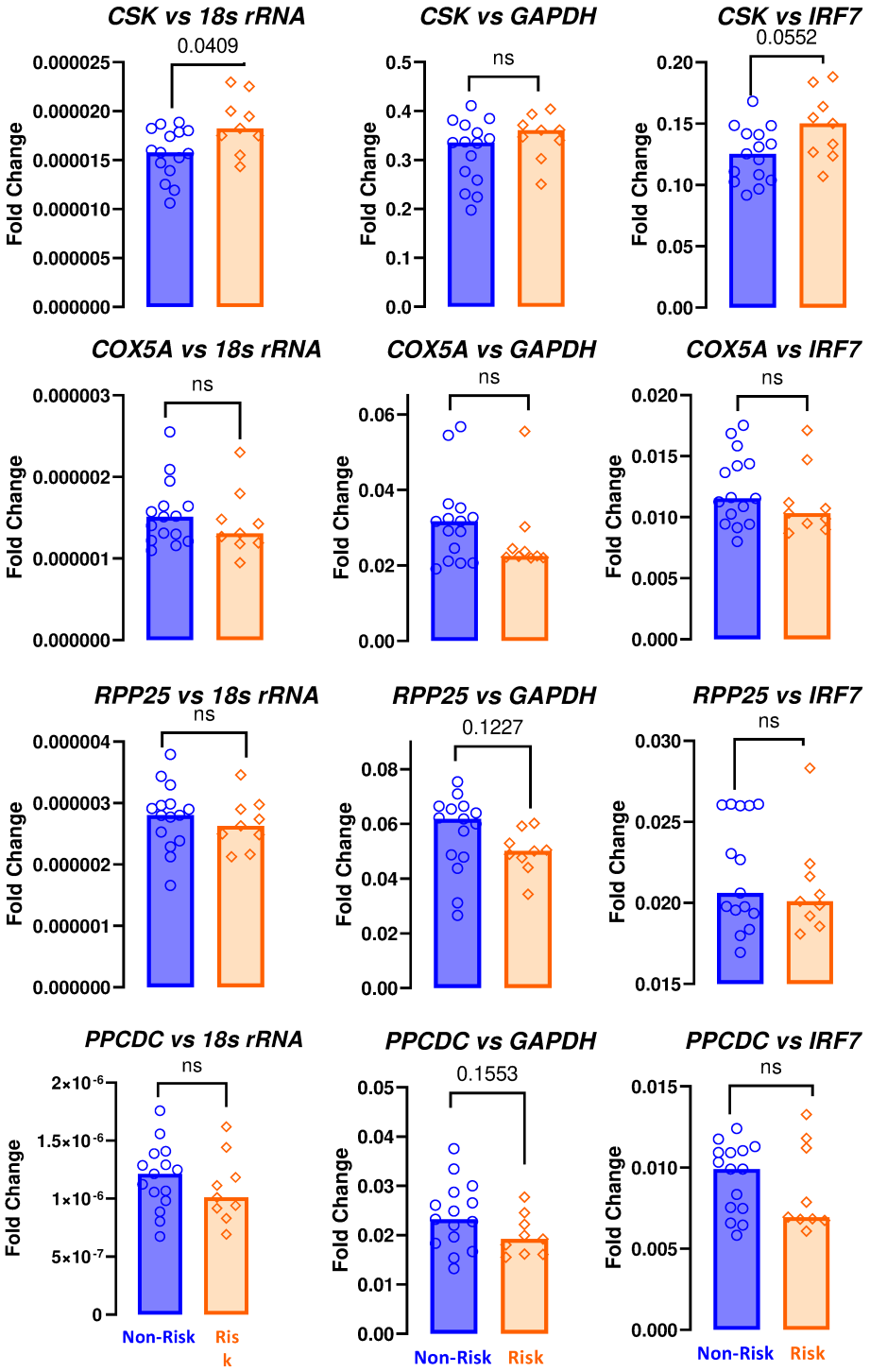


Figure S4. Expression of *SCAMP5* intron sequences. A) A schematic diagram of the *SCAMP5* gene (ENST00000425597) shows the location of the reported expressed intronic segments, and the identifier assigned to each. **B)** RNAseq FPKM values of *SCAMP5* intronic segments. **C)** Relative expression of the three mostly highly expressed *SCAMP5* intron segments, relative to total expression of *SCAMP5* (as measured by quantitative RT-PCR assay spanning the exon 2 to exon 3 boundaries of intron 2 of the *SCAMP5* gene). The 'No RT' control cDNAs were also included and no amplification was observed for any assay within the 40 cycles tested, indicating that the intron expression measured here was from mRNA and not amplification of contaminating genomic DNA. Each data point represents one subject (for qRT-PCR each data point represents the mean of technical triplicates). Bars show the median expression values.

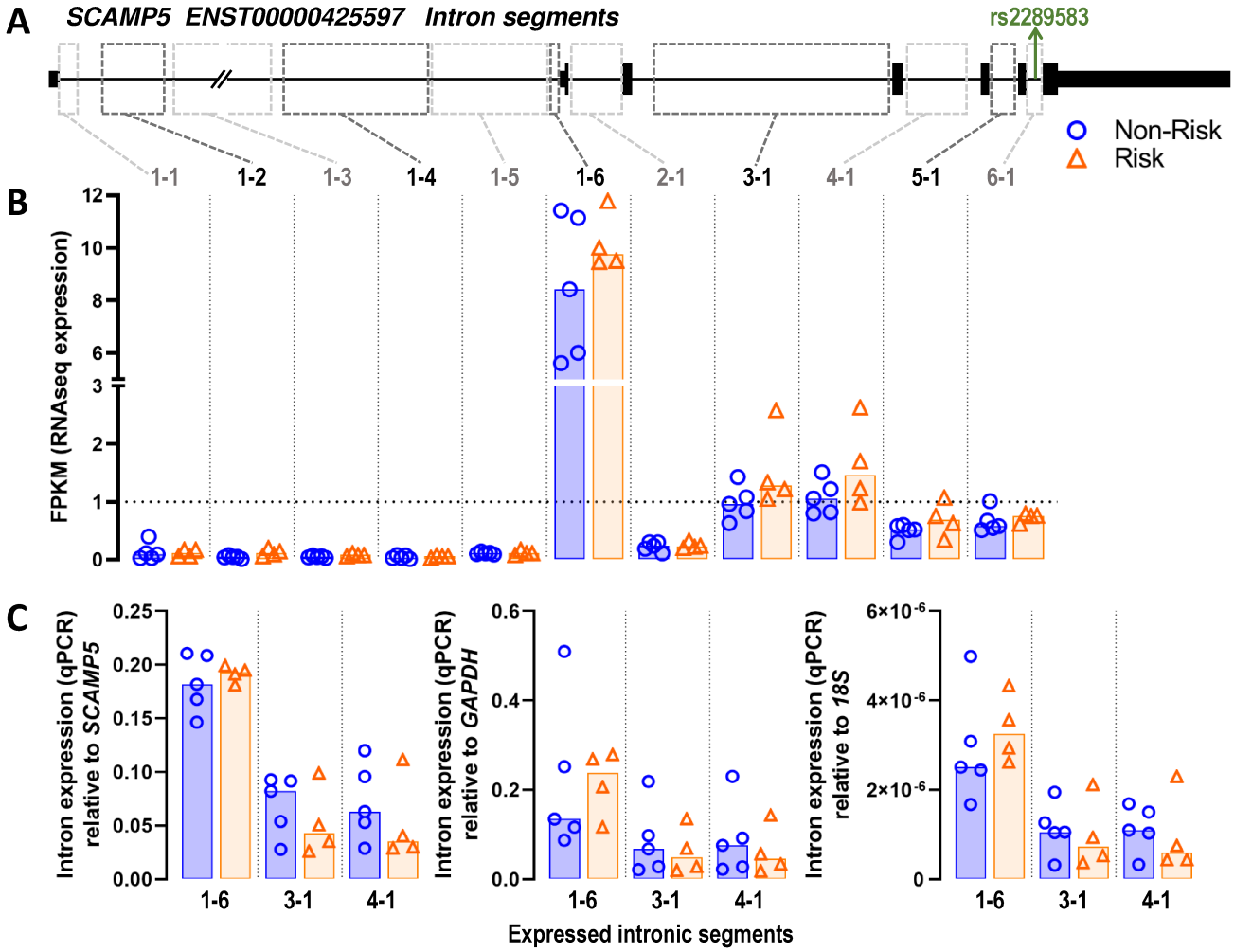


Figure S5. BDCA4 KO using CRISPR in primary human pDCs. pDCs were isolated to near purity and electroporated with Cas9 particles complexed with non-targeting or *BDCA4* gRNA A or B. Cells were cultured for 72hrs and stained for viability and BDCA4 expression prior to acquisition by flow cytometry.

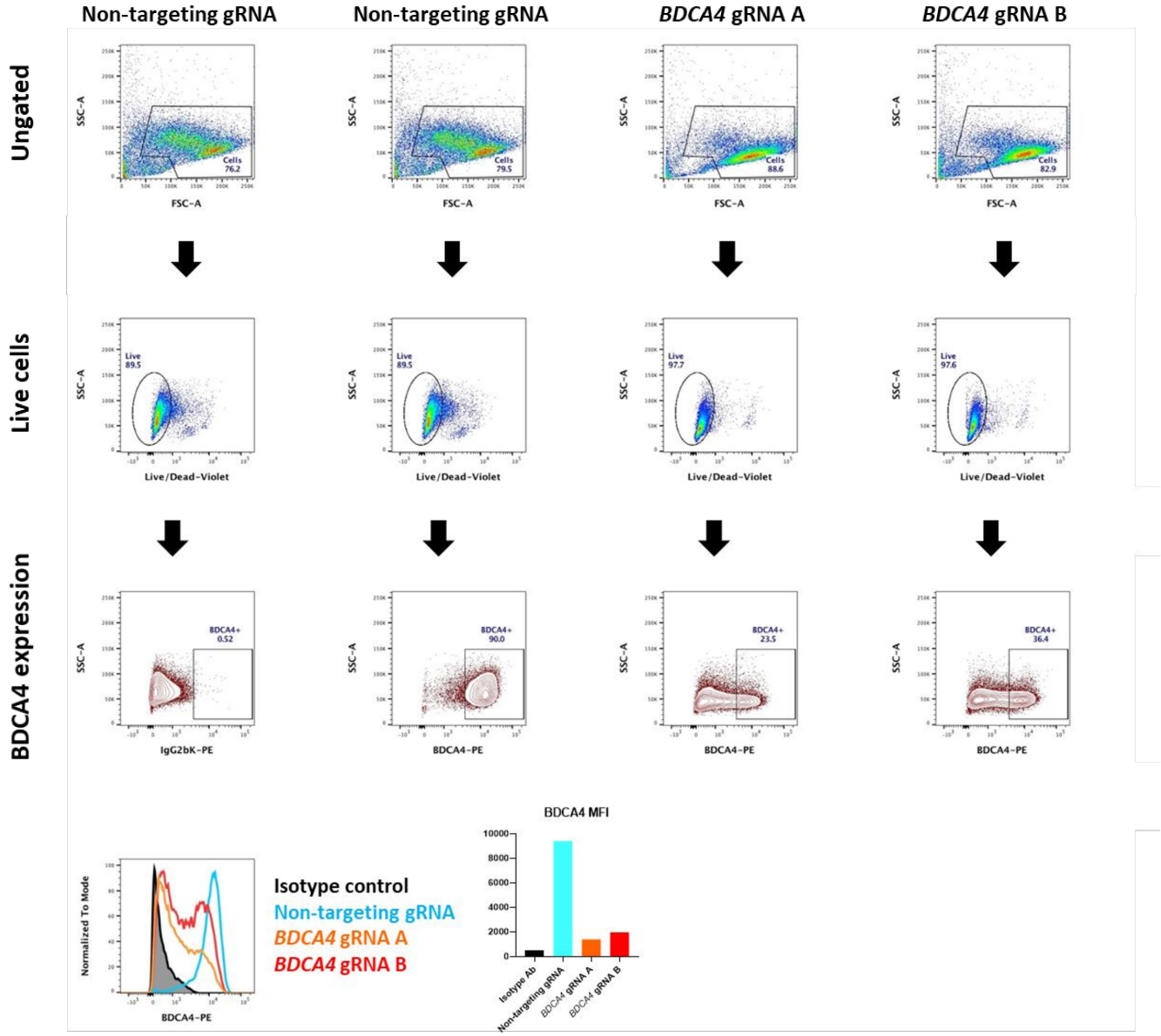


Figure S6. Representative pre- and post-isolation purity of pDCs from recalled GaP subjects.

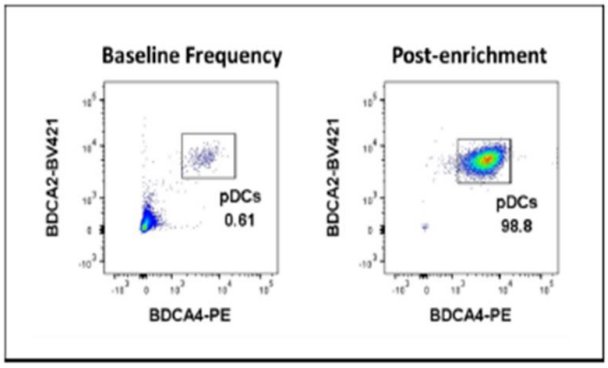


Table S1. Construction of an SLE risk haplotype about rs2289583.

	rs2472300	rs6495122	rs2289583*	Number of subjects		Frequency			
				Controls	SLE	Controls	SLE	P value	OR (95% CI)
Non-risk	G	C	C	6,324	3,404	45.4%	42.2%	$2.5x10^{-6}$	0.88 (0.83 - 0.93)
Risk	A	A	A	2,508	1,645	18.0%	20.4%	$1.8x10^{-5}$	1.16 (1.09 - 1.25)
	G	A	C	1,953	1,137	14.0%	14.1%	0.92	1.00 (0.93 - 1.09)
	A	A	C	1,286	780	9.2%	9.7%	0.30	1.05 (0.96 - 1.15)
	G	C	A	957	548	6.9%	6.8%	0.82	0.99 (0.88 - 1.10)
	G	A	A	545	355	3.9%	4.4%	0.084	1.13 (0.98 - 1.29)
	A	C	C	300	174	2.2%	2.2%	1.00	1.00 (0.83 - 1.21)
	A	C	A	45	29	0.3%	0.4%	0.72	1.11 (0.70 - 1.77)
				13,918	8,072				

Table S2. Characteristics of GaP subjects recalled for pDC isolation.

Subject	Sex Assigned at Birth:	Race	Medical Conditions	SCAMP5 Haplotype	pDC Frequency Pre-Enrichment	pDC yield	pDC purity
3333	Female	Caucasian	None	Non-Risk	0.60%	4.55E+05	97.00%
3538	Female	Caucasian	Hypothyroidism	Non-Risk	0.40%	3.18E+05	97.70%
4315	Female	Caucasian	None	Non-Risk	0.44%	2.18E+05	95.10%
4752	Female	Caucasian	Allergies	Non-Risk	0.11%	3.55E+04	86.10%
4855	Female	Caucasian	Eczema	Non-Risk	0.39%	2.35E+05	96.50%
4892	Female	Caucasian	Allergies, Asthma	Non-Risk	0.36%	8.50E+04	97.40%
5121	Female	Caucasian	Allergies	Non-Risk	0.75%	4.43E+05	95.00%
5135	Female	Caucasian	Psoriasis	Non-Risk	0.73%	2.10E+05	98.20%
5444	Female	Caucasian	Allergies	Non-Risk	0.44%	1.45E+05	95.80%
6845	Female	Caucasian	None	Non-Risk	0.64%	2.56E+05	98.60%
7101	Female	Caucasian	None	Non-Risk	0.78%	3.00E+05	92.70%
7153	Female	Caucasian	None	Non-Risk	0.13%	1.56E+05	93.20%
7170	Female	Caucasian	Allergies, Asthma, Arthritis	Non-Risk	0.41%	1.20E+05	94.50%
7644	Female	Caucasian	Arthritis, Diabetes Type 1, Psoriasis	Non-Risk	1.94%	5.85E+05	98.60%
7738	Female	Caucasian	Allergies, Hypothyroidism	Non-Risk	0.53%	1.60E+05	97.70%
2727	Female	Caucasian	None	Risk	0.25%	1.13E+05	93.70%
3425	Female	Caucasian	None	Risk	0.74%	4.20E+05	97.80%
5050	Female	Caucasian	Allergies	Risk	0.62%	3.13E+05	98.80%
5181	Female	Caucasian	None	Risk	0.63%	2.32E+05	97.10%
5451	Female	Caucasian	None	Risk	0.44%	2.19E+05	98.60%
6257	Female	Caucasian	Celiac Disease	Risk	0.61%	9.18E+04	96.70%
6335	Female	Caucasian	None	Risk	0.46%	2.23E+05	97.70%
7450	Female	Caucasian	None	Risk	0.79%	3.00E+05	97.70%
7992	Female	Caucasian	None	Risk	0.68%	4.40E+05	98.00%

Videos 1-4. Intracellular trafficking of SCAMP5-bearing vesicles in transduced HEK cells.

Videos 5-7. Time-lapse imaging of c-mVenus, IFNsp-mVenus and SCAMP5 in transduced HEK cells.