SUPPLEMENTAL FILE

METHODS

Materials. The culture medium used in all experiments was RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (all purchased from Life Technologies, Carlsbad, CA). For lymphocyte stimulation, phorbol 12-myristate acetate (PMA) and ionomycin calcium salt were purchased from Sigma-Aldrich (St. Louis, MO) while interleukin-4 (IL-4) was purchased from PeproTech (Oak Park, CA). Anti-Human LAG3-PE (3DS223H) and isotype control fluorophore-conjugated monoclonal antibodies were purchased from eBioscience Inc. and Biolegend Inc. (San Diego, CA). Antibodies used for signaling were purchased from Cell Signaling Technologies (Beverly, MA): anti-CD79A (#3351), anti-phospho-CD79A (Y182) (#5173), anti-CD19 (#3574), anti-phospho-CD19 (Y531) (#3571), anti-SYK (#2712), anti-phospho-SYK (Y525/526) (#2710), anti-Lyn (#2796), anti-phospho-LYN (Y507) (#2731), anti-PLCγ2 (#3872), anti-phospho-PLCγ2 (Y759) (#3874), anti-phospho-PKCα/βII (T638/641) (#9375), and anti-FLOT2 (#3244). Anti-PKCβ (Santa Cruz Biotechnology, Santa Cruz, CA sc-210), anti-ACTIN (AC-74) (Sigma-Aldrich, St. Louis, MO) and anti-LAG3 (17B4) (Novus, Littleton, CO) were purchased separately.

Cell culture. Lymphocytes isolated from HALP subjects were immortalized using Epstein Barr Virus (EBV) to generate B lymphocytes (University of North Carolina Lineberger Comprehensive Cancer Center Tissue Culture Facility, Chapel Hill, NC). EBV-transformed B lymphocytes were grown in suspension at density ~1-2 $x10^6$ cells per ml of complete RPMI 1640 media with L-glutamine, supplemented with 10% FBS and 1% Penicillin-Streptomycin. The media was changed twice a week or more often as needed prior to using cells for experiments. For all experiments listed with individual GG

or CC, GG is reference cell line 003 and CC is risk cell line 008 unless otherwise noted. Ramos cells (CRL-1596) were purchased from ATCC (Manassas, VA).

RNA-Sequencing. Total RNA was isolated from three subjects homozygous for the rs10846744 reference (GG) allele and three subjects homozygous for the rs10846744 risk (CC) allele and then subjected to full transcriptome sequencing using the Perkin Elmer next gen sequencing platform (RNA-Seq) (Perkin Elmer, Branford, CT). Bioinformatics was performed using Perkin Elmer Gene Sifter software program. The data was adjusted by selecting total map reads, quality reads >20, log transformation, and using Benjamini Hochberg to correct for multiple testing. RNA targets of interest were validated by real-time PCR and western blotting using standard methodologies. RNA-Seq was performed on the separate six cell lines under conditions where cells were cultured in serum (usual culture conditions) and following stimulation with phorbol esters (PMA 500 ng/ml), ionomycin (250 ng/ml), and IL-4 (100 U/ml) for 6 h.

LAG3 protein expression and functional studies. We used a number of assays to assess LAG3 protein expression and function in the six EBV-transformed B cell lines. *Flow cytometry*: Flow cytometry was performed on a 10-laser LSRII-B flow cytometer (Becton Dickson, Franklin Lakes, NJ) at the UConn Health Flow Cytometry Core. Dead cells were stained with Blue Dead Cell Stain Kit (Molecular Probes, Eugene, OR). To measure the response of LAG3 protein in stimulated B cells we first modified and optimized a protocol previously published by Smeland et al. (1). Cells were incubated with and without PMA (500 ng/ml), ionomycin (250 ng/ml) and IL-4 (100 U/ml) for varying time periods (0-4 h). Cells were labeled with LAG3-PE. The percentage of cell surface LAG3 protein expression was calculated by using only the live cell fraction and

then subtracting the percent isotype staining values from the percent staining values for cells treated with monoclonal LAG3 antibodies. The flow data was analyzed suing FlowJo software version 10 (FlowJo LLC, Ashland OR USA). *Cytokine secretion into the medium*: Levels of interleukin 10 (IL-10) and tumor necrosis factor-α (TNFα) were measured in media aliquots isolated from cells cultured under basal and stimulated conditions for varying time periods (0-4 h) by multiplex (Milliplex; Millipore, Temecula, CA) on Luminex 200, using XMAP technology, values represented are fold changes of LAG3 (pg/ml) of the treated condition divided by the unstimulated (basal) control. *Western blotting*: We used western blotting to measure total and phosphorylated expression of the following proteins known to be involved in downstream signaling in stimulated B cells: p-CD79A, p-CD19, p-SYK p-LYN, p-PLCγ2, and p-PKCβ. In some experiments we also stimulated cells with CD40 ligand (CD40L) (200 ng/ml) for 2h and then isolated whole cell lysates for western blotting (Figures S4 and S5). Cells were solubilized with 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na deoxycholate, 150 mM NaCl, 1mM PMSF, 5 mM NaF, 1 mM Na3VO4, 1 mM β-Glycerophosphate, 10 mM Na4P2O7, 2 mM EDTA and Complete protease inhibitor cocktail (Roche Diagnostics Corp., San Francisco, CA). After 30 minutes of incubation on ice, lysates were clarified by centrifugation (10,000 g) for 15 minutes at 4°C and supernatants were collected. Protein concentrations were determined using a BCA assay, and equal amounts were subjected to SDS/linear gradient PAGE following solubilization in Laemmli sample buffer. Gel-resolved proteins were subsequently electrotransferred to PVDF membranes via wet tank transfer, which were blocked with 5% nonfat milk prior to antibody incubation. Membranes were then incubated overnight at 4°C first with antibodies to

phospho-proteins, then total proteins. Antibody–antigen complexes were identified by chemiluminesence (ECL+System; Amersham Biosciences, Piscataway, NJ). Anti-ACTIN was used as a loading control. Phospho-proteins were normalized to corresponding total proteins using Image Studio Lite 4.0 for quantification (Licor, Lincoln, NE). Proteins were sized using kaleidoscope prestained standards (Bio-Rad, Hercules, CA).

Lipid raft isolation. In order to assess expression of LAG3 protein in the plasma membrane lipid raft compartment, lipid raft membranes were isolated using 500 mM sodium carbonate (pH 11.0) and sucrose density centrifugation. The sucrose gradient method was performed essentially as described previously (2) with modifications. Cells ($1x10^8$) were washed with ice-cold PBS and resuspended with 500 mM sodium carbonate, pH 11.0 (2) containing phosphatase and protease inhibitors (1mM PMSF, 5 mM NaF, 1 mM Na3VO4, 1 mM β-Glycerophosphate, 10 mM Na4P2O7, 2 mM EDTA and Complete protease inhibitor cocktail (Roche Diagnostics Corp., San Francisco, CA). The solution was further homogenized with ten strokes in a Wheaton dounce homogenizer. For the discontinuous sucrose gradient, 300 µL of cleared supernatant was mixed with 300 µl of 85% sucrose and transferred to the bottom of a 2.2 ml Beckman centrifuge tube. The diluted lysate was overlaid with 1 ml 35% sucrose and finally 600 µl 5% sucrose. The samples were ultracentrifuged in a Beckman tabletop centrifuge at 70,000 g for 20 h at 4°C. Following centrifugation, gradients were portioned into 10, 220 µl fractions. Fractions 1-3 were pooled (combined fraction 1 on blot). To determine the location of lipid rafts and distinct proteins in the discontinuous sucrose gradient, 40 µl of the raft fractions (4 and 5 of the sucrose gradient, 2 and 3 on

blot) and non-raft fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted.

Overexpression and silencing of LAG3 protein assays. We used two experimental approaches to determine if the presence or absence of LAG3 protein was causal in altering downstream signaling pathways. We first overexpressed LAG3 protein in risk (CC) cells by transfecting them with lentiviral vectors expressing GFP tagged full-length human LAG3 cDNA. Our second approach was to use specific short-hairpin shRNA vectors to silence LAG3 expression in reference (GG) cells. In both experimental systems, western blot analysis was used to determine overexpression or silencing of LAG3. *Lentiviral transfection and transduction*: LAG3-GFP inserted into the pReceiver-Lv122 overexpressing vector, shRNA-LAG3 inserted into the psi-LVRH1MP RNAi silencing vector, scrambled shRNA, and lentiviral Mock GFP control vectors were obtained from GeneCopoeia (Rockville, MD). Four shRNA to LAG3 were screened for selection of the plasmid with the most efficient knockdown. Lentivirus was generated by using Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia, Rockville, MD). Briefly, 2.5 µl of each individual lentiviral plasmid and 5.0 µl of EndoFectin Lenti reagent were added in Opti-MEM I, to form the DNA-EndoFectine complex. Twenty minutes after incubating the complex at room temperature, the DNA-EndoFectine complex was added to the dish with HEK 293 in DMEM with 10% FBS and incubated in 5% CO_2 at 37°C overnight. The culture medium was replaced with fresh DMEM with 5% FBS and continued to be incubated. The viral-containing culture medium was collected 48 hr post-transfection and concentrated after filtration. For transduction with lentivirus, $1x10^6$ of EBV-transformed B lymphocytes in 1.5 ml of complete media were seeded in a

12-well plate and 500 µl of virus suspension was added. The cells were incubated at 37°C for 72 h. To assess the effect of either overexpressing LAG3 in lymphocytes with the risk (CC) allele or silencing LAG3 in lymphocytes with reference (GG) allele on the downstream signaling pathway, transduced cells were stimulated with and without phorbol esters (PMA) (500 ng/ml), ionomycin (250 ng/ml) and IL-4 (100 U/ml) cocktail for 2 h and then processed for western blotting to assess phosphorylation of downstream signaling proteins.

Plasma or soluble lymphocyte activation gene 3 assay. Plasma LAG3 protein ELISA kits were purchased from RayBiotech, Inc. (Norcross, GA) and LAG3 was measured per the manufacturer's instructions with optimization. Briefly, aliquots of fasting plasma samples stored at -80^{o}C from 150 HALP and 5623 MESA subjects were thawed, diluted 3-fold, and then 100 µl were used for duplicates per sample for plasma LAG3 measurement. This ELISA has not been validated for clinical use.

Table S1. Intra-chromosomal transcriptionally regulated gene transcripts by RNA-Seq.

Downregulated	Fold-change	Upregulated	Fold-change
Lymphocyte activation gene3	5	Carboxypeptidase M	4
Aldehyde dehydrogenase 1 family member L2	4	Tescalcin	4
Inhibin beta C	3	Glycosyltransferase 1 domain containing 1	3
WD repeat domain 6	3		
Fatty acyl coA reductase	2		

Table S2. Inter-chromosomal transcriptionally regulated gene transcripts by RNA-Seq. (full list available in public repository, chromosome=Chrom.)

Downregulated	Fold-change	Upregulated	Fold-change
Major histocompatibility complex, class II, DQ beta 2; (Chrom. 6)	82	Neuroblastoma breakpoint family, member 3; (Chrom. 1)	34
Major histocompatibility complex, class II, DQ beta 2 , DQ alpha 2;(Chrom. 6)	24	Mesoderm specific transcript; (Chrom. 7)	16
Olfactory receptor, family 3, subfamily A, member 2; (Chrom. 17)	13		
UL16 binding protein 1; (Chrom. 6)	10		
Leucine zipper, putative tumor suppressor 1, (Chrom. 8)	9		

Table S3. Association of plasma LAG3 in MESA with CAC and cIMT

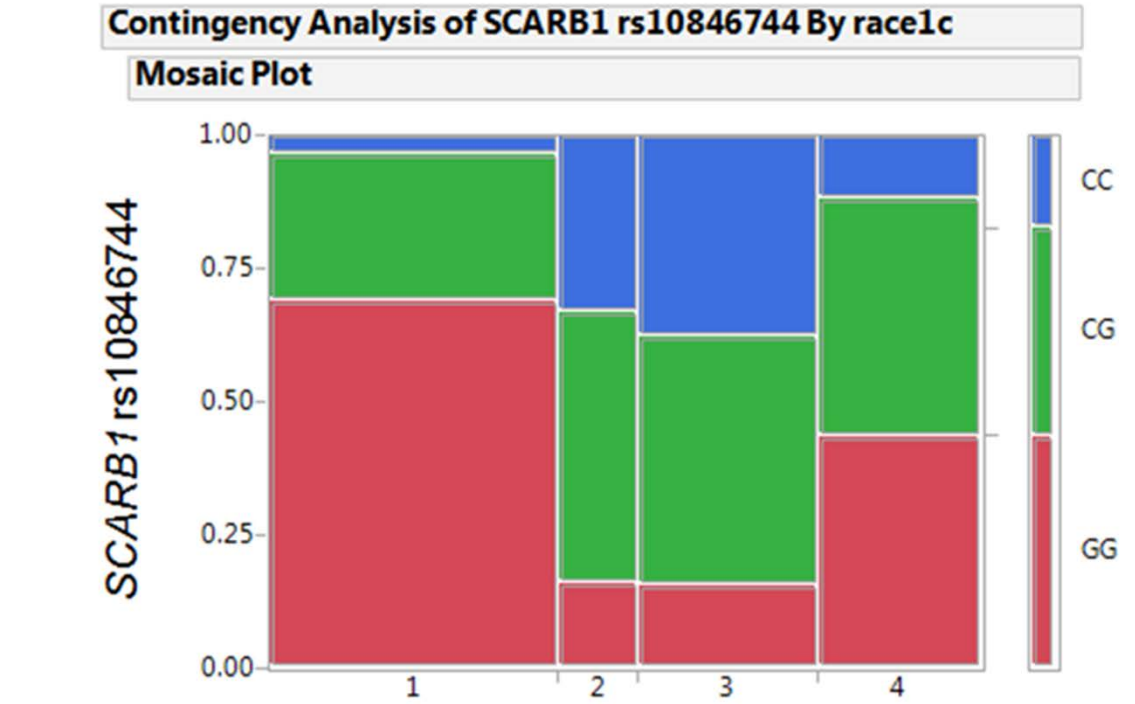
Main outcome*	Group	N	Beta	SE	P-value
	White	1155	-0.028	0.025	0.258
	African American	564	0.007	0.029	0.824
	Hispanic	494	-0.030	0.064	0.644
	Chinese	227	-0.132	0.079	0.095
CAC scores (log scale)	Meta-analysis	2440	-0.020	0.018	0.250
	White	2044	0.002	0.002	0.333
	African American	1284	0.001	0.002	0.545
	Hispanic	1087	0.007	0.004	0.130
	Chinese	558	-0.0001	0.006	0.992
Common IMT (log scale)	Meta-analysis	4973	0.002	0.001	0.062

Table S4. Prevalence of CHD-h and all-cause mortality in MESA participants with HDL-C ≥ 60 mg/dl

Outcomes	White		Chinese		African American		Hispanic	
	Yes	No	Yes	No	Yes	No	Yes	No
All-cause mortality	70	571	10	120	58	333	22	195
CHD-hard	25	616	3	127	15	376	8	209

Figure S1.

Genotypic Frequency of *SCARB1* rs10846744 in MESA participants



Tests

N	DF	-LogLike	RSquare (U)
5604	6	820.56024	0.1417

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	1641.120	<.0001*
Pearson	1539.531	<.0001*

1=Caucasian
2=Chinese-American
3=African-American
4=Hispanic

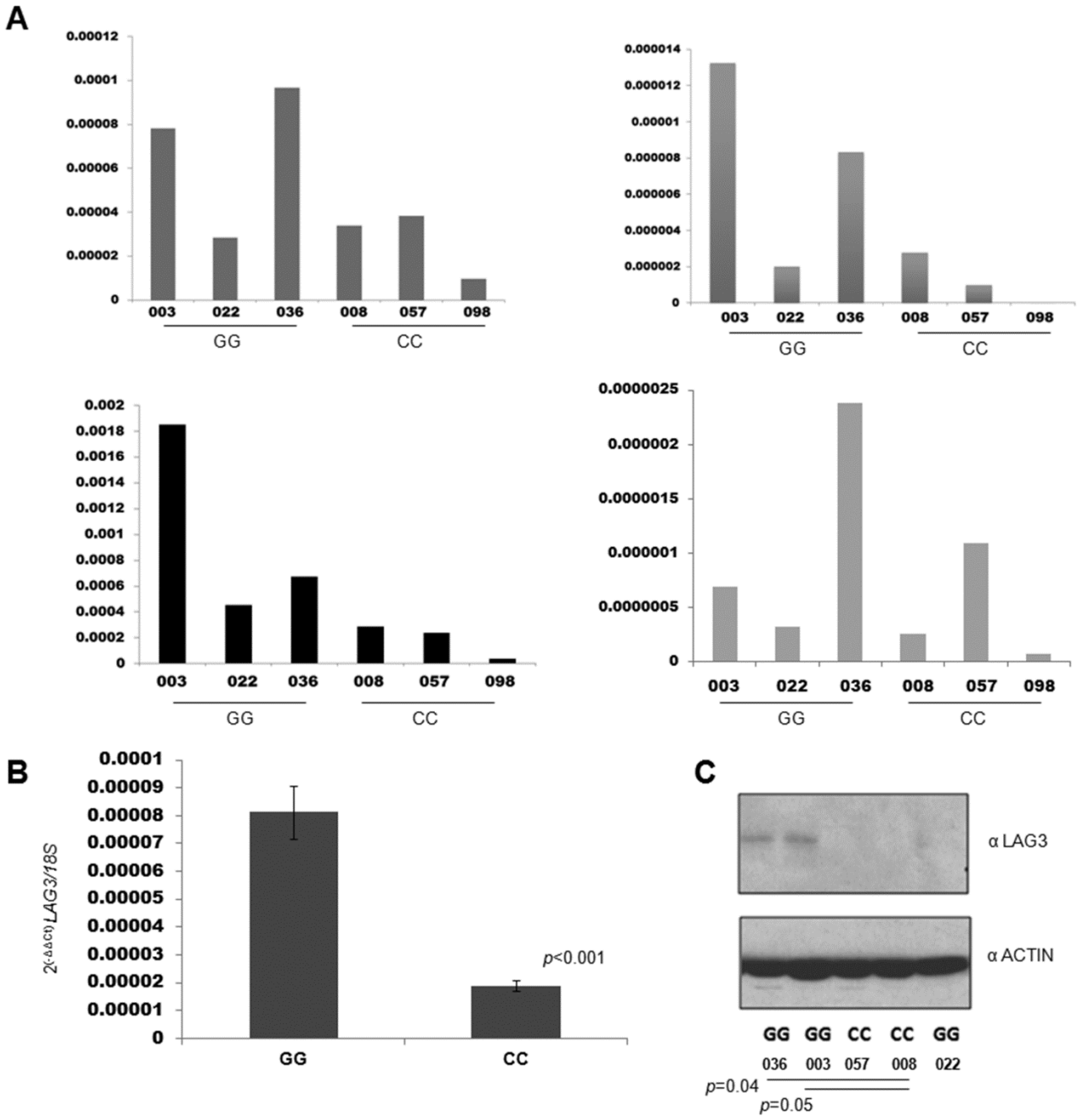


Figure S2. ***LAG3*** **RNA and LAG3 protein expression in reference (GG) and risk (CC) expressing cells. (A)** Using real-time PCR, individual cell lines from reference (GG) and risk (CC) cells were tested for *LAG3* RNA expression and values were normalized to the endogenous control *18S*. **(B)** *LAG3* RNA expression from 4 combined experiment data sets (N=3 reference (GG) allele combined and N=3 risk (CC) allele combined; mean ± standard error). **(C)** LAG3 protein was measured by western blotting. LAG3 protein levels were significantly lower in risk (CC-008 and CC-057 cell lines) as compared with reference (GG-036 cell line) [*p*=0.04] and the reference (GG-003 cell line) [*p*=0.05]. There were no significant differences found between reference GG-022, risk CC-008 and risk CC-057 cell lines. A two-sided Student's t-test was used to analyze results and the blot is representative of one of three independent experiments. *P* values less than 0.05 were considered significant. The reference (GG) cells are identified as GG-003, GG-022, and GG-036. The risk (CC) cells are identified as CC-008, CC-057, and CC-098.

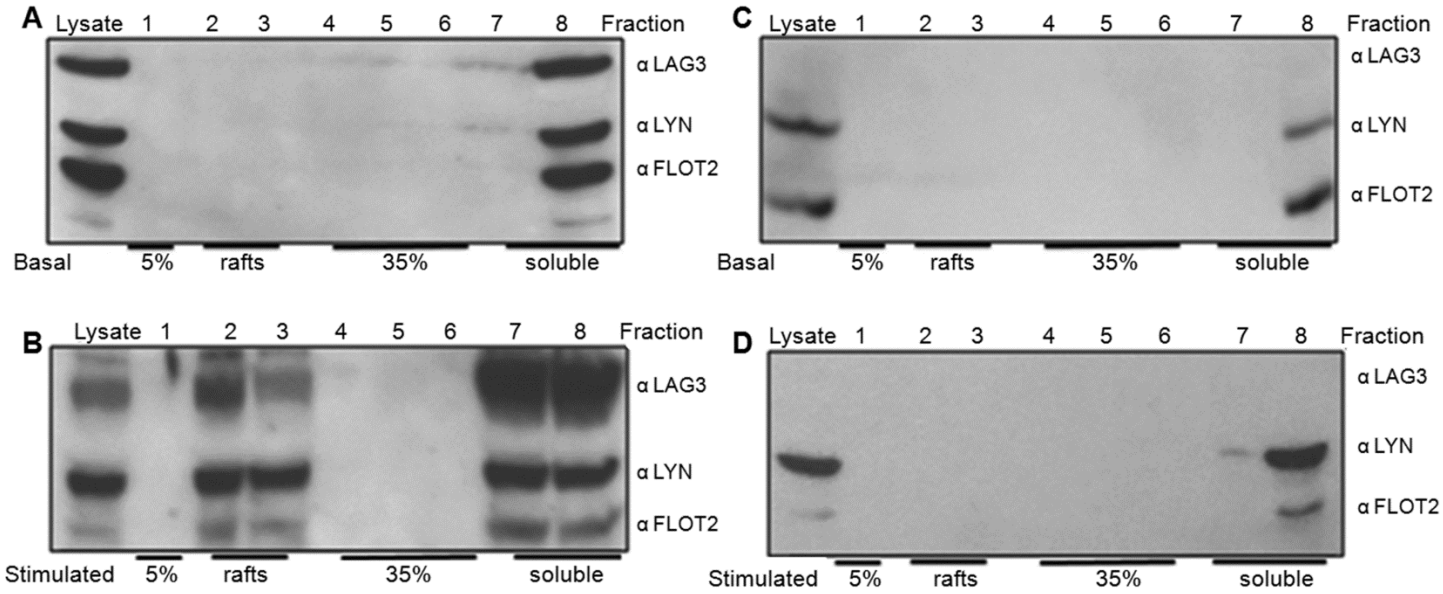


Figure S3. LAG3 protein expression is significantly lower in lipid rafts of rs10846744 risk (CC) expressing cells. Lipid rafts were isolated from Epstein Barr Virus (EBV)-transformed B cells under basal or stimulated conditions (phorbol ester, PMA 500 ng/ml, ionomycin 250 ng/ml, and IL-4 100 U/ml) using a modified three-step sucrose density gradient for sodium carbonate-extracted homogenized lysates. Expression of the following targets was determined by immunoblotting using specific antibodies: LAG3; LYN and FLOT2 (Flotillin) (both lipid raft markers). Expression of LAG3 protein (normalized to FLOT2) localized to the lipid raft fractions of the stimulated reference (GG) cells **(B, lanes 2-3)** as compared with the basal condition **(A, lanes 2-3)**, N=3, p=0.03. LAG3 protein was not detected in the risk (CC) cells whether under basal **(C, lanes 2-3)** or stimulated conditions **(D, lanes 2-3)**. A two-sided Student's t-test was used to analyze results and the blot is representative of one of three independent experiments. *P* values less than 0.05 were considered significant.

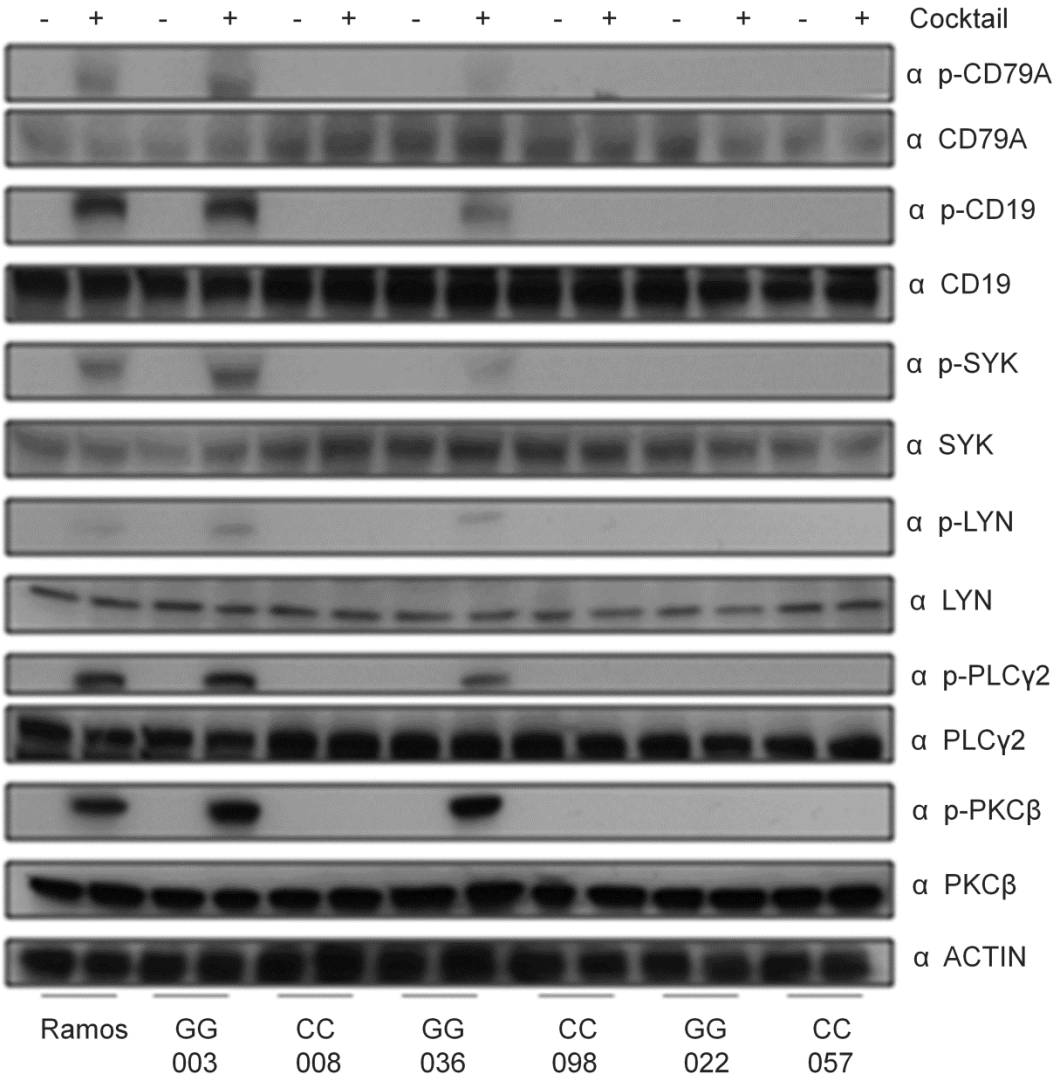


Figure S4. B cell receptor (BCR) cell signaling for reference (GG) and risk (CC) cells under stimulated conditions with phorbol ester, PMA/ionomycin+IL-4 for 2 h. Whole cell lysates were isolated from Epstein Barr Virus (EBV)-transformed B lymphocytes expressing the reference (GG) or risk (CC) allele under basal or cocktail stimulated (phorbol ester 500 ng/ml, ionomycin 250 ng/ml, and IL-4 100 U/ml) conditions for 2 h. Reactions were terminated by the addition of an equal volume of lysis buffer and blotted with the indicated total and phospho-antibodies. Experiments were performed from cell lines derived from HALP subjects expressing the reference (GG) or risk (CC) allele for a total of 6 independent cell lines (N=3 for the reference [GG] cells and N=3 for the risk [CC] expressing cells)**.** A two-sided Student's t-test was used to analyze results and the blot is representative of one of three independent experiments. *P* values less than 0.05 were considered significant.

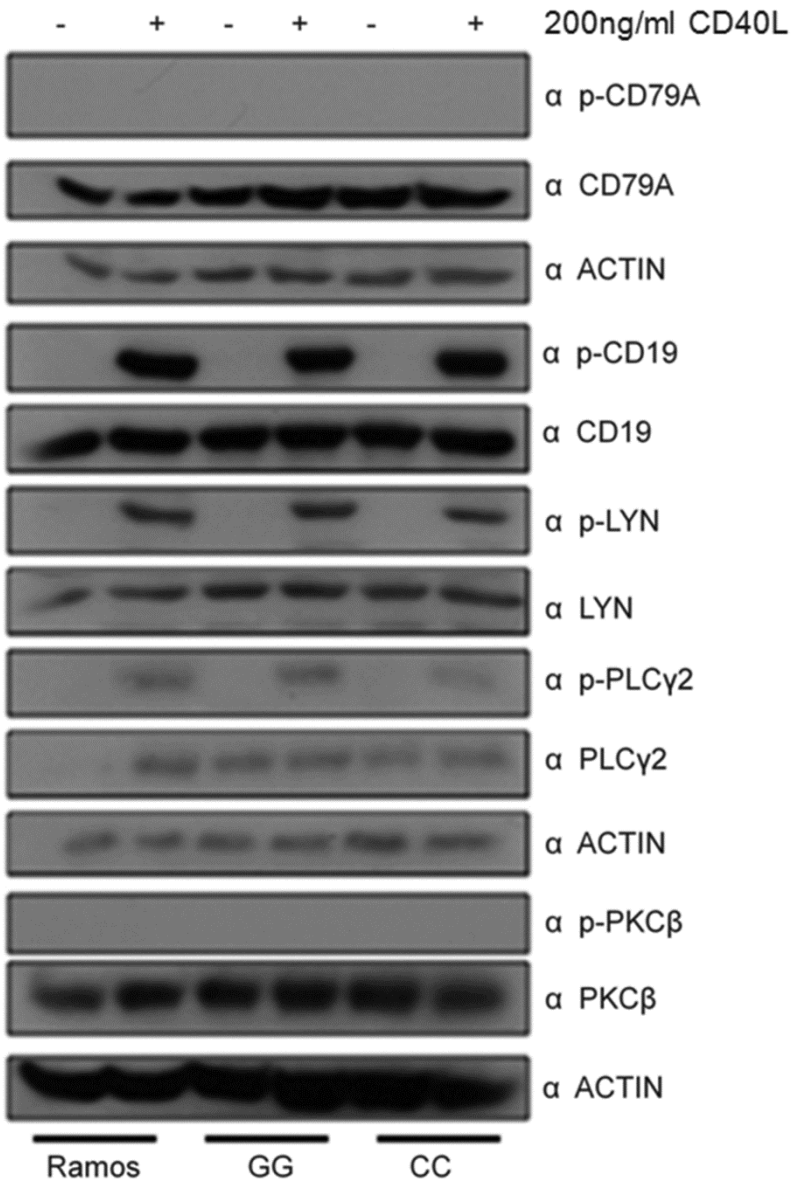


Figure S5. Activation of B cells by CD40 cell signaling is independent of BCR signaling for reference (GG) and risk (CC) cells under CD40L stimulated conditions for 2 h. Whole cell lysates were isolated from Epstein Barr Virus (EBV)-transformed B cells expressing the reference (GG) or risk (CC) allele under basal or cocktail stimulated (phorbol ester, PMA 500 ng/ml, ionomycin 250 ng/ml, and IL-4 100 U/ml) conditions for 2 h. Reactions were terminated by the addition of an equal volume of lysis buffer and blotted with the indicated total and phospho-antibodies. Western blot represents data from one experiment.

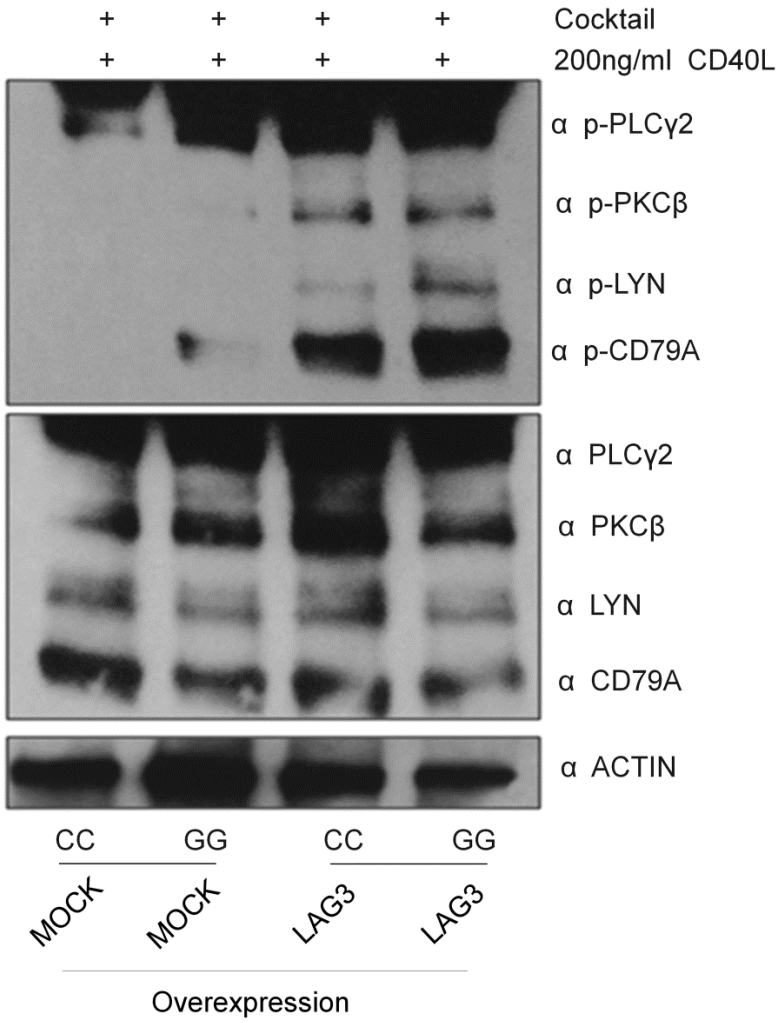


Figure S6. Restoration of phospho-CD79A and phospho-PKCβ B cell receptor signaling in the risk (CC) cells overexpressing LAG3 protein. Whole cell lysates from either reference (GG) or risk (CC) allele cells were isolated from Epstein Barr Virus (EBV)-transformed B cells expressing either the mock GFP or the lentiviral LAG3-GFP in cocktail (phorbol ester 500 ng/ml, ionomycin 250 ng/ml, and IL-4 100 U/ml) and CD40L stimulated conditions for 2 h. Western blot represents data from one experiment.

References

1. Smeland EB, Blomhoff HK, Funderud S, Shalaby MR, Espevik T. Interleukin 4 induces selective production of interleukin 6 from normal human B lymphocytes. *J Exp Med* 1989;170(4):1463-1468.
2. Ostrom RS, Insel PA. Methods for the study of signaling molecules in membrane lipid rafts and caveolae. *Methods Mol Biol.* 2006;332:181-91.