Supplemental Information

A requirement for mast cells in malignant pleural effusion

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SUPPLEMENTAL TABLES

Table S1, related to Figure 2A. Transcripts overrepresented in Lewis lung carcinoma and MC38 colon adenocarcinoma cells compared with B16F10 melanoma cells. Mean adenocarcinoma/melanoma differential gene expression (ΔGE) from two independent microarray analyses of cancer cell global gene expression using mouse gene ST 1.0 and 2.0 (mGST1, mGST2). Gene symbols in red font were further examined in this study.

Gene symbol	Gene name	ΔGE mGST1	ΔGE mGST2	ΔGE mean
Ptgs2	prostaglandin-endoperoxide synthase 2	33.47	103.66	68.57
Ly6a	lymphocyte antigen 6 complex, locus A	36.90	94.30	65.60
Spp1	secreted phosphoprotein 1	59.60	45.21	52.41
S100a6	S100 calcium binding protein A6 (calcyclin)	51.29	46.96	49.12
Ccl2	chemokine (C-C motif) ligand 2	26.57	67.39	46.98
Nid1	nidogen 1	13.06	58.03	35.54
Ly6e	lymphocyte antigen 6 complex, locus E	58.51	10.84	34.68
Pla2g7	phospholipase A2, group VII	31.15	38.05	34.60
Hist1h1b	histone cluster 1, H1b	15.49	48.03	31.76
Apobec3	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3	12.73	44.27	28.50
Pxdn	peroxidasin homolog (Drosophila)	28.80	27.28	28.04
Prrx1	paired related homeobox 1	21.03	33.06	27.05
Il1r1	interleukin 1 receptor, type I	15.31	37.87	26.59
Stambpl1	STAM binding protein like 1	13.83	36.92	25.37
Gatm	glycine amidinotransferase	33.03	17.37	25.20
Emp2	epithelial membrane protein 2	14.39	35.95	25.17
F2r	coagulation factor II (thrombin) receptor	16.63	31.87	24.25
Apcdd1	adenomatosis polyposis coli down-regulated 1	27.54	19.60	23.57
S100a4	S100 calcium binding protein A4	23.03	17.71	20.37
Bgn	biglycan	15.76	23.72	19.74
Gpr149	G protein-coupled receptor 149	13.98	24.02	19.00
Dkk2	dickkopf homolog 2 (Xenopus laevis)	15.47	22.21	18.84
Ptgs1	prostaglandin-endoperoxide synthase 1	23.60	12.62	18.11
Vcam1	vascular cell adhesion molecule 1	13.17	22.29	17.73
Htra1	HtrA serine peptidase 1	15.10	19.14	17.12
Vcan	versican	17.54	16.38	16.96
Ptges	prostaglandin E synthase	22.57	11.30	16.94
Ptprn	protein tyrosine phosphatase, receptor type, N	15.34	17.36	16.35
Slc14a1	solute carrier family 14 (urea transporter), member 1	15.75	16.84	16.30
Hspb8	heat shock protein 8	13.59	18.37	15.98
Dhrs9	dehydrogenase/reductase (SDR fam.) memb. 9	14.08	17.60	15.84
Flnc	filamin C, gamma	12.76	18.19	15.48
Flrt2	fibronectin leucine rich transmembrane protein 2	12.92	17.57	15.25
Ltbp1	latent TGF beta binding protein 1	13.04	16.78	14.91
Thbs2	thrombospondin 2	17.49	10.14	13.82
Itga3	integrin alpha 3	11.91	14.58	13.25
Axl	AXL receptor tyrosine kinase	13.74	11.02	12.38
Il18rap	interleukin 18 receptor accessory protein	11.32	11.23	11.28
Kcnn	potassium intermediate/small conductance calcium-activated channel, subf. N, member 4	10.60	11.04	10.82

Table S2, related to Figure 2A. Transcripts overrepresented in B16F10 melanoma cells compared with Lewis lung carcinoma and MC38 colon adenocarcinoma cells. Mean melanoma/adenocarcinoma differential gene expression (*Δ*GE) from two independent microarray analyses of cancer cell global gene expression using mouse gene ST 1.0 and 2.0 (mGST1, mGST2).

Gene symbol	Gene name	*Δ*GE mGST1	*Δ*GE mGST2	*Δ*GE mean
Tyrp1	tyrosinase-related protein 1	211.95	139.32	175.64
Dct	dopachrome tautomerase	199.29	131.38	165.33
Pmel	silver	96.20	128.08	112.14
Slc45a2	solute carrier family 45, member 2	87.47	50.22	68.84
Syt4	synaptotagmin IV	63.58	74.06	68.82
Mlana	melan-A	61.03	59.64	60.34
Plagl1	pleiomorphic adenoma gene-like 1	67.68	24.95	46.31
Glrb	glycine receptor, beta subunit	37.91	47.74	42.83
Vgll3	vestigial like 3 (Drosophila)	47.77	18.13	32.95
Bace2	beta-site APP-cleaving enzyme 2	26.74	38.56	32.65
Rab38	RAB38, member of RAS oncogene family	38.95	24.84	31.90
Gjc3	gap junction protein, gamma 3	10.27	47.33	28.80
Rasgrp3	RAS, guanyl releasing protein 3	20.02	36.00	28.00
Tyr	tyrosinase	11.24	40.94	26.09
Cdh19	cadherin 19, type 2	19.26	31.69	25.48
Ankfn1	ankyrin-repeat and fibronectin type III domain1	22.89	25.59	24.24
Gpm6a	glycoprotein m6a	25.34	22.18	23.76
Mc1r	melanocortin 1 receptor	19.61	27.20	23.40
Nrcam	neuron-glia-CAM-related cell adhesion molecule	16.74	27.25	22.00
Dmxl2	Dmx-like 2	15.19	28.54	21.87
Fndc3c1	fibronectin type III domain containing 3C1	18.97	24.11	21.54
Tspan10	tetraspanin 10	13.11	29.05	21.08
Itga4	integrin alpha 4	27.07	12.76	19.91
Sytl2	synaptotagmin-like 2	14.59	21.37	17.98
Prkcq	protein kinase C, theta	23.21	12.60	17.91
Pde11a	phosphodiesterase 11A	18.16	17.40	17.78
Lama4	laminin, alpha 4	21.39	14.07	17.73
Tspan6	tetraspanin 6	10.81	24.13	17.47
Slc35f1	solute carrier family 35, member F1	21.65	12.41	17.03
Robo1	roundabout homolog 1 (Drosophila)	10.17	21.77	15.97
Rab27a	RAB27A, member RAS oncogene family	11.22	19.91	15.56
Tspan12	tetraspanin 12	15.54	13.68	14.61
Mcoln3	mucolipin 3	11.06	17.64	14.35
Mlph	melanophilin	10.55	17.92	14.24
Sirpa	signal-regulatory protein alpha	15.21	13.16	14.19
4932411E22Rik	RIKEN cDNA 4932411E22 gene	10.45	17.31	13.88
Cntfr	ciliary neurotrophic factor receptor	11.17	16.30	13.73
Cyp2j6	cytochrome P450, fam. 2, subf. j, polypept. 6	10.58	16.31	13.44
Myo7a	myosin VIIA	11.47	15.32	13.39
Ckb	creatine kinase, brain	10.06	16.41	13.24
Pax3	paired box gene 3	12.38	13.53	12.95
Pkia	protein kinase inhibitor, alpha	12.14	13.11	12.63
Snca	synuclein, alpha	10.28	14.94	12.61
Chchd10	coiled-coil-helix-coiled-coil-helix domain 10	11.81	12.79	12.30
Gpr143	G protein-coupled receptor 143	13.21	11.27	12.24
Nr4a3	nuclear receptor subfamily 4, gr. A, member 3	13.22	10.47	11.84
Dock3	dedicator of cyto-kinesis 3	12.19	10.77	11.48
C530028O21Rik	RIKEN cDNA C530028O21 gene	10.69	12.23	11.46
Plp1	proteolipid protein (myelin) 1	11.00	10.32	10.66
Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene 3	10.63	10.22	10.43

Table S3, related to Figure 5A. Transcripts significantly induced or suppressed in bone marrow-derived mast cells by Lewis lung adenocarcinoma-conditioned media. Differential gene expression (*Δ*GE) between Lewis lung carcinoma (LLC)-conditioned media-treated BMMC and non-conditioned media (DMEM)-treated BMMC (n = 2) assessed by microarray (mouse Gene ST2.0, Affymetrix, Sta.Clara, CA). A positive *Δ*GE indicates induction and a negative *Δ*GE suppression by adenocarcinoma-conditioned media. Selected cut-offs were $P < .05$ and ΔGE > 2.

Gene symbol	Gene name	P[a]	xΔGE[b]
Cd68	CD68 antigen	0.034	+5.70
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	0.010	+4.28
Il1b	interleukin 1 beta	0.027	+4.00
Gm19585	predicted gene, 19585	0.019	+3.58
Tpsab1	tryptase alpha/beta 1	0.040	+3.48
Gzmc	granzyme C	0.022	+3.18
F11r	F11 receptor	0.045	+2.83
Gm14047	predicted gene 14047	0.003	+2.48
LOC73899|LOC100503558	uncharacterized LOC73899|LOC100503558	0.009	+2.38
Tpm4	tropomyosin 4	0.036	+2.37
Eno2	enolase 2, gamma neuronal	0.040	+2.24
Klf10	Kruppel-like factor 10	0.019	+2.13
Ebi3	Epstein-Barr virus induced gene 3	0.033	+2.11
Ifitm1	interferon induced transmembrane protein 1	0.019	+2.01
Fdps	farnesyl diphosphate synthetase	0.016	-2.23
Sc4mol	sterol-C4-methyl oxidase-like	0.016	-2.25
Insig1	insulin induced gene 1	0.028	-3.13

[a]*P*, Paired Student's t-test probability value; [b] *Δ*GE, difference in gene expression between BMMC treated with LLC-conditioned media and BMMC treated with non-conditioned media. Gene symbols in ***bold*** type were induced in an adenocarcinoma-restricted fashion. *Tnfrsf9* was induced by all tumor-conditioned media.

Table S4, related to Figure 5A. Transcripts significantly induced or suppressed in bone marrow-derived mast cells by MC38 colon adenocarcinoma-conditioned media. Differential (>2-fold) gene expression (ΔGE) between MC38 colon adenocarcinoma-conditioned media-treated BMMC and non-conditioned media (DMEM)-treated BMMC (n = 2) assessed by microarray (mouse Gene ST2.0, Affymetrix, Sta.Clara, CA). A positive ΔGE indicates induction and a negative ΔGE suppression by adenocarcinoma-conditioned media.

Gene symbol	Gene name	P[a]	xΔGE[b]
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	0.033	+4.19
Cd68	CD68 antigen	0.020	+3.86
Tpsab1	tryptase alpha/beta 1	0.045	+3.47
Pde7b	phosphodiesterase 7B	0.046	+2.44
Gm19585	predicted gene, 19585	0.012	+2.27
Il1b	interleukin 1 beta	0.047	+2.20
Mrgprb13	MAS-related GPR, member B13	0.001	-2.06

[a]*P*, Paired Student's t-test probability value; [b] ΔGE, difference in gene expression between BMMC treated with MC38-conditioned media and BMMC treated with non-conditioned media. Gene symbols in ***bold*** type were induced in an adenocarcinoma-restricted fashion. *Tnfrsf9* was induced by all tumor-conditioned media.

Table S5, related to Figure 5A. Transcripts significantly induced or suppressed in bone marrow-derived mast cells by B16F10 melanoma-conditioned media. Differential (>2-fold) gene expression (*Δ*GE) between B16F10 melanoma-conditioned media-treated BMMC and non-conditioned media (DMEM)-treated BMMC (n = 2) assessed by microarray (mouse Gene ST2.0, Affymetrix, Sta.Clara, CA). A positive *Δ*GE indicates induction and a negative *Δ*GE suppression by adenocarcinoma-conditioned media.

Gene symbol	Gene name	P[a]	xΔGE[b]
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	0.019	+4.19
Procr	protein C receptor, endothelial	0.036	+2.41
Adssl1	adenylosuccinate synthetase like 1	0.045	+2.13
Nox1	NADPH oxidase 1	0.0004	+2.09

[a]*P*, Paired Student's t-test probability value; [b] *Δ*GE, difference in gene expression between BMMC treated with B16F10-conditioned media and BMMC treated with non-conditioned media. Gene symbols in ***bold*** type were induced in an adenocarcinoma-restricted fashion. *Tnfrsf9* was induced by all tumor-conditioned media.

Table S6, related to Methods. Antibodies used for these studies

Method[a]	Target	Provider[b]	Catalog #	Dilution	Conjugate[c]
IF, FC	cKIT	eBioscience	12-1171-83	1:200, 0.1 μg/10^6 cells	PE
IF	CD68	AbD Serotec	MCA1957 A488T	1:100	FITC
IF	IL-1β	Abcam	ab9722	1:100	-
HIS, IF	PCNA	Abcam	ab2426	1:2000, 1:200	-
IF	GFP	Santa Cruz	sc-8334	1:200	-
FC	Sca1	Biolegend	108106	0.1 μg/10^6 cells	FITC
FC	Lin (CD3ε)	eBioscience	13-0031-82	0.1 μg/10^6 cells	biotin followed by streptavidin-APC or streptavidin-PerCP
FC	Lin (B220)	eBioscience	13-0452-82	0.1 μg/10^6 cells	
FC	Lin (CD11b)	eBioscience	13-0112-82	0.1 μg/10^6 cells	
FC	Lin (TER-119)	Biolegend	116204	0.1 μg/10^6 cells	
FC	Lin (CD19)	eBioscience	13-0191-85	0.1 μg/10^6 cells	
FC	Lin (Gr1)	eBioscience	13-5931-82	0.1 μg/10^6 cells	
FC	Lin (CD8a)	eBioscience	13-0081-85	0.1 μg/10^6 cells	
FC	CD25	eBioscience	12-0251	0.1 μg/10^6 cells	PerCP
FC	CD45	Biolegend	103116	0.1 μg/10^6 cells	APC-Cy7
FC	F4/80	Biolegend	123128	0.1 μg/10^6 cells	PerCP
FC	CD11b	eBioscience	12-0112-82	0.1 μg/10^6 cells	PE
FC	CD11c	eBioscience	17-0114-82	0.1 μg/10^6 cells	APC
WIB	GFP	Santa Cruz	sc-9996	1:500	-
WIB	RelA	Santa Cruz	sc-372-G	1:500	-
WIB	IκBα	Santa Cruz	sc-371	1:500	-
WIB	β-actin	Santa Cruz	sc-47778	1:500	-
WIB	RelB	Cell Signaling	4954	1:1000	-
WIB	IKKα	Cell Signaling	2682	1:1000	-
WIB	IKKβ	Cell Signaling	2684	1:1000	-
WIB	histone 3	Cell Signaling	9175	1:1000	-
WIB	Rabbit anti-goat IgG	Santa Cruz	sc-2922	1:5000	HRP
WIB	Goat anti-rabbit IgG	Southern	4030-05	1:8000	HRP
WIB	Goat anti-mouse IgG	Biotech	1030-05	1:8000	HRP
IF	donkey anti-rabbit & anti-mouse IgG	Invitrogen	A21206 A21202	1:1000	Alexa 488
IF	donkey anti-rabbit & anti-mouse IgG	Invitrogen	A10042 A10037	1:1000	Alexa 568

[a]Application: HIS, histology; IF, immunofluorescence; FC, flow cytometry; WIB, Western immunoblotting.
[b]Providers*:* eBioscience, San Diego, CA; AbD Serotec, Kidlington, UK; Abcam, Cambridge, UK; Santa Cruz Biotechnology, San Diego, CA; Cell Signaling, Danvers, MA; Southern Biotech, Birmingham, AL; Invitrogen, Carlsbad, CA.
[c]Conjugates*:* FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein; APC, allophycocyanin; HRP, horse radish peroxidase.

Table S7, related to Methods. PCR primers used for these studies

Method[a]	Primer	Sequence	Amplicon length
qPCR	*GusbF*	TTACTTTAAGACGCTGATCACC	165 bp
qPCR	*GusbR*	ACCTCCAAATGCCCATAGTC	
qPCR	*Cd68F*	GACCTACATCAGAGCCCGAG	90 bp
qPCR	*Cd68R*	GAATGTCCACTGTGCTGCCT	
qPCR	*Tpsab1F*	TGCTGAAACTCACAAACCCT	139 bp
qPCR	*Tpsab1R*	GGCAGGTTTACACCATTGTC	
qPCR	*Il1bF*	TTTGACAGTGATGAGAATGACC	162 bp
qPCR	*Il1bR*	AATGAGTGATACTGCCTGCC	
qPCR	*Il1r1F*	TGGAAGTCTTGTGTGCCCTT	150 bp
qPCR	*Il1r1R*	GCCACATTCCTCACCAACAG	
qPCR	*NfkbiaF*	AGCAAATGGTGAAGGAGCTG	112 bp
qPCR	*NfkbiaR*	AAGTGCAGGAACGAGTCTCC	
qPCR	*NfkbibF*	CTGAACCTGAGGACGAGGAC	115 bp
qPCR	*NfkbibR*	GTTGTCGGTTTTGGCTCCTG	
qPCR	*ChukF*	AACCAGCCTCTCAGTGTGTT	106 bp
qPCR	*ChukR*	CTGGATGCAAATGGTCCTTCA	
qPCR	*IkbkbF*	CAGTGCCTGTGACAGCTTAC	115 bp
qPCR	*IkbkbR*	TTGCTCCTTCACAGTGTCCT	
qPCR	*Ccl2F*	CTACAAGAGGATCACCAGCAG	145 bp
qPCR	*Ccl2R*	TTCTGATCTCATTTGGTTCCGA	
qPCR	*Cxcl5F*	AGGAGGTCTGTCTGGATCCA	117 bp
qPCR	*Cxcl5R*	CACTGGCCGTTCTTTCCAC	
qPCR	*Stat3F*	AATGGAAATTGCCCGGATCG	134 bp
qPCR	*Stat3R*	TCTGCTGCTTCTCTGTCACT	
qPCR	*MycF*	CTCGAGCTGTTTGAAGGCTG	138 bp
qPCR	*MycR*	CGCAGATGAAATAGGGCTGT	
qPCR	*Cdkn1aF*	TCTGAGCGGCCTGAAGATTC	133 bp
qPCR	*Cdkn1aR*	GGGCACTTCAGGGTTTTCTC	
qPCR	*Notch1F*	TGAAGAACGGAGCCAACAAG	147 bp
qPCR	*Notch1R*	GCAATCGGTCCATGTGATCC	
qPCR	*CrebbpF*	CAGTGAATCGCATGCAGGTTT	147 bp
qPCR	*CrebbpR*	GAACTGAGGCCATGCTGTTC	
qPCR	*Hdac1F*	ACGACGAATCCTATGAAGCCA	148 bp
qPCR	*Hdac1R*	GCGTGTCCTTTGATGGTCAG	
CL	*IkbkbF*	ATGAGCTGGTCACCGTCCCTCCCAACCC	2274 bp
CL	*IkbkbR*	TCAGTCACAGGCCTGCTCCAGGC	

[a]Application: RT, reverse transcriptase-polymerase chain reaction; qPCR, quantitative (real-time) PCR; CL, cloning;

SUPPLEMENTAL MOVIES

Supplemental Movie S1. Spontaneously moving bone marrow-derived mast cell from red fluorescent mouse (high power). BMMC were obtained from *mT/mG* mouse after 4 weeks of culture with interleukin-3 and KIT ligand, were placed in DMEM drops onto uncovered glass slides, and were observed using time-lapse fluorescence microscopy on a SP5 confocal microscope at magnification x 600 (Leica, Heidelberg, Germany).

Supplemental Movie S2. Spontaneously moving bone marrow-derived mast cells from red fluorescent mouse (low power). BMMC were obtained from *mT/mG* mouse after 4 weeks of culture with interleukin-3 and KIT ligand, were placed in DMEM drops onto uncovered glass slides, and were observed using time-lapse fluorescence microscopy on a SP5 confocal microscope at magnification x 400 (Leica, Heidelberg, Germany).

Supplemental Movie S3. Bone marrow-derived mast cell (BMMC) degranulation induced by recombinant mouse (rm) osteopontin (SPP1). BMMC were obtained from wild-type mice after 4 weeks of culture with interleukin-3 and KIT ligand, were placed in 50 μL DMEM drops onto uncovered glass slides, and were observed using time-lapse phase-contrast microscopy on a SP5 confocal microscope at magnification x 600 (Leica, Heidelberg, Germany). rmSPP1 was added (50 μl, 30 ng/ml) after 10 sec video time.

SUPPLEMENTAL METHODS

Reagents

Anti-mouse CCL2 and CCL12 neutralizing antibodies, as well as IgG2a control antibody were kindly provided by Oncology Discovery Research, Janssen R&D LLC (Spring House, PA) (37); rmCCL2 was from Peprotech (London, UK); rmIL-1β, rm IL-3, and rmKITL were from Immunotools (Friesoythe, Germany); rmSPP1 and rmTPSAB1 were from R&D Systems (Minneapolis, MN); C48/80 and Evans' blue were from Sigma-Aldrich (St. Louis, MO); and imatinib mesylate was from Selleckchem (Houston, TX).

Cells

LLC, B16F10, A549, and SKMEL2 cells (NCI Tumor Repository, Frederick, MD) and MC38 cells (gift from Dr. Barbara Fingleton, Vanderbilt University, Nashville, TN) (38) were cultured at 37^0C in 5% CO_2-95% air using DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell lines were tested biannually for identity (by the short tandem repeat method) and for *Mycoplasma Spp.* (by PCR). For *in vivo* injections, cells were harvested using trypsin, incubated with Trypan blue, counted as described elsewhere, and injected through a left intercostal space, as described elsewhere (25, 26, 38, 47, 48). Only 95% viable cells were used *in vivo*.

Animals

C57BL/6 (#000664), *cKit^{Wsh}* (#005051), *NOD/SCID* (#001303), *CAG.Luc.eGFP* (#008450), *CAG.eGFP* (#003291), *mT/mG* (#007676), *Lyz2.Cre* (Ref. 39; #004781), and *Dta* (Ref. 40; # 009669) mice from Jackson Laboratories (Bar Harbor, MN), as well as *Cpa3.Cre* (gift from Dr. Hans-Reimer Rodewald, Heidelberg, Germany) (15) and *Il1b-/-* mice (gift from Dr. Yoichiro Iwakura, Tokyo University of Science, Tokyo, Japan) (44), were bred at the Center for Animal Models of Disease of the University of Patras. Experiments were approved *a priori* by the local Veterinary Administration and were conducted according to Directive 2010/63/EU (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF). Experimental mice and littermate controls were sex-, weight (20-25 g)-, and age (6-12 week)-matched; both male and female mice were used.

Cancer models and drug treatments

For MPE generation, mice received 150,000 murine or 1,000,000 human cancer cells intrapleurally and were sacrificed after 14 or 30 days, respectively. For pleural lavage, 1 mL normal saline was injected intrapleurally and was withdrawn starting after 30 seconds waiting time. Both techniques are described elsewhere (25, 26, 38, 47, 48). Imatinib mesylate (1 mg/kg in 100 μL PBS) or PBS (100 μL) were given daily intraperitoneally. Anti-mouse CCL2, CCL12 (a murine CCL2 ortholog), and IgG2a control antibodies were delivered intraperitoneally at 50 mg/kg every three days (37, 38).

Human samples

Pleural fluid was obtained during diagnostic thoracenteses in patients with MPE due to lung cancer (n = 14), breast cancer (n = 6), and malignant pleural mesothelioma (n = 4), as well as patients with CHF treated at Institution #6 between January 2006 and December 2008. Detailed clinical, cytologic, biochemical, and biologic data were available for these patients, including a semi-quantitative radiologic score of their effusion size and aspirate measurements, within the framework of a large clinical protocol. Samples from Institutions #9 and #10 were diagnostic MPE cytologic specimens from 20 patients with lung adenocarcinoma aspirated between June and August 2013. Diagnosis and sample handling were done as described elsewhere (25, 26, 38, 47, 48). All protocols abided by the Helsinki Declaration, were approved *a priori* by the local hospital ethics committees and by all patients via written informed consent.

Cytology & histology

Pleural fluid cytocentrifugal specimens (5 x 10^4 cells each) and cells cultured on glass slides were fixed with 4% paraformaldehyde or with Mota's fixative (8% lead acetate, 4% glacial acetic acid, and 50% ethanol in distilled water) for 5 minutes and were stained with May-Gruenwald-Giemsa or toluidine blue (0.05% for 5-15 minutes). Alternatively, cells were labeled with the indicated antibodies (Supplemental Table S6) and counterstained with hematoxylin or Hoechst 33258 (Sigma-Aldrich, St. Louis, MO; dilution 1:5000) with or without MC granule-labeling avidin (Vector Labs, Burlingame, CA). Distinct cell types were enumerated as a percentage of 500 cells on the slide. For MC counting, 10,000 cells per slide were counted. For histology, lungs with pleural tumors or whole thoraces fixed in 4% paraformaldehyde overnight and desalted in EDTA for two weeks (only whole thoraces) were embedded in paraffin or in OCT (Sakura, Tokyo, Japan) and were stored at -80oC. 5-μm paraffin or 10-μm-cryosections were mounted on glass slides. Sections were stained with toluidine blue or labeled using the indicated antibodies (Supplemental Table S6) and counterstained with Hoechst 33258 or with the Envision color development system (Dako, Carpinteria, CA). Immunoreactivity was quantified as described previously (25, 26, 38, 47, 48). Bright-field and fluorescent microscopy were carried out using either an AxioObserver D1 (Zeiss, Jena, Germany) inverted microscope or an SP5 (Leica, Heidelberg, Germany) confocal microscope.

BMMC

Bone marrow cells were flushed from *C57BL/6*, *cKit*^{Wsh}, *CAG.Luc.eGFP*, *CAG.eGFP*, *mT/mG* and *Il1b-/-* femurs and tibias using full DMEM, and were simply cultured in full culture media (the same used for cancer cell line cultures), supplemented with rmIL-3 with or without rmKITL (100 ng/mL each). Non-adherent cells were passaged twice-weekly for four to six weeks, as described elsewhere (31).

Flow cytometry

After NH_4Cl red blood cell lysis, pleural cells or BMMC were suspended in PBS 2% FBS, stained with the indicated antibodies (Supplemental Table S6) for 20 minutes, fixed in 1 % paraformaldehyde for 10 minutes, analyzed on a FACSCalibur cytometer (BD Biosciences, Alameda, CA), and data were examined using FlowJo software (FlowJo, Ashland, OR).

Constructs & Transfections

For RNA interference, random (sh*C*), anti-*Ccl2* (sh*Ccl2*), or anti-*Spp1* (sh*Spp1*) shRNA 64-mers were cloned into the *pSuper.retro.puro* backbone (Oligoengine, Seattle, WA). shRNA sequences were AGCTTTTCCAAAAA-target-TCTCTTGAA-target reverse complement-GGG and target sequences were sh*C*: CTGTCTATCGAAGAATGGG; sh*Ccl2*: TGTGAAGTTGACCCGTAAA; and sh*Spp1* GCTTATGGACTGAGGTCAA (25, 41). The *Ccl2* and *β-gal* expression vectors have been described elsewhere (25). All new plasmids were deposited with Addgene (http://www.addgene.org/). A newly engineered pMIGR1-based (Addgene ID 27490) bicistronic retroviral expression vector was generated by replacing the eGFP sequences with the puromycin resistance gene (Addgene ID 58250). *Ikbkb* cDNA was cloned via RT-PCR from MC38 total RNA using specific primers (Supplemental Table S7) and was subcloned into peGFP-C1. *Egfp* and *Egfp.Ikbkb* cDNAs were subcloned into the new retroviral expression vector (Addgene ID's 58249 and 58251, respectively). Cells were transfected with 5 μg DNA by the standard calcium phosphate procedure and either studied transiently or stable clones were generated by puromycin selection.

Adoptive bone marrow transplants and bone marrow-derived mast cell transfer

For adoptive bone marrow replacement (Figures 5, A and B and 8E), mice received ten million intravenous bone marrow cells 12 hours after total-body irradiation (1100 Rad), as described elsewhere (35). One mouse in each experiment was not engrafted (sentinel) and was observed till moribund between days 5 and 15 post-irradiation. For adoptive BMMC transfer for purposes of MC pulse and chase studies (Figure 5), irradiated *C57BL/6* chimeras engrafted with $cKit^{Wsh}$ bone marrow (39) received pleural tumor cells at day 30 post-transplant, followed by same-day 5 x 10^5 iv *CAG.Luc.eGFP* or *CAG.eGFP* BMMC, and $cKit^{Wsh}$ mice without prior irradiation received 8 x 10^5 sc *CAG.Luc.eGFP* BMMC followed by next-day pleural tumor cells. For intrapleural BMMC delivery (Figure 8A), naïve *C57BL/6* mice received 10^5 intrapleural BMMC, with or without B16F10 cells. For functional BMMC give-back studies (Figure 11, G and H), *C57BL/6*, $cKit^{Wsh}$, and *Il1b-/-* mice received 2.5 x 10^5 sc BMMC.

Bioluminescence imaging

Cells and mice were serially imaged on a Xenogen Lumina II and data were analyzed using Living Image v.4.2 (Perkin-Elmer, Waltham, MA), after addition of 300 μg/mL D-luciferin to culture media or delivery of 1 mg intravenous D-luciferin (47, 48).

ELISA

CCL2, SPP1, IL-1β and IL-1α levels of cell culture supernatants, of cell-free MPE, and sera were determined using dedicated murine and human ELISA kits according to the manufacturer's instructions (Peprotech, London, UK and R&D, Minneapolis, MN).

Vascular Permeability Assays

Mice with MPE received i.v. 0.8 mg Evans' blue and were killed after one hour for determination of MPE levels of the albumin-binding dye (48). Intradermal injections of test molecules (1.5 ng/50 μL PBS), cell-free MPE (50 μL), or cancer cell-conditioned media (50 μL) performed at different spots of the shaved dorsal mouse skin were followed by immediate Evans' blue injections as above, euthanasia, skin inversion and imaging after one hour. The surface area of dye leak was determined using Fiji academic freeware (http://fiji.sc/Fiji), as described elsewhere (25).

Real-time qPCR, and microarray

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) followed by RNAeasy (Qiagen, Hilden, Germany), was reverse transcribed using Superscript III (Invitrogen), and qPCR was performed using SYBR Green Master Mix in a StepOnePlus cycler (Applied Biosystems, Carlsbad, CA). Reverse transcriptase-PCR primers are given in Supplemental Table S7. For microarray, cells cultured in triplicate independent wells for each cell line-condition were subjected to RNA extraction as above. Five μg pooled RNA was tested for RNA quality on an ABI2000 bioanalyzer (Agilent Technologies, Sta. Clara, CA), labeled, and hybridized to GeneChip Mouse Gene 1.0 or 2.0 ST arrays (Affymetrix, Sta. Clara, CA). For analysis, the Affymetrix Expression Console (parameters: annotation confidence, full; summarization method: iter-PLIER include DABG; background: PM-GCBG; normalization method: none) was used, followed by normalization of all arrays together using a Lowess multiarray algorithm. Intensity-dependent estimation of noise was used for statistical analysis of differential expression. Microarray data are available at the GEO database (http://www.ncbi.nlm.nih.gov/geo/; Accession ID: GSE58190).

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer [25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS]. Nuclear and cytoplasmic extracts were prepared using the dedicated NE-PER kit (Thermo Scientific, Walthal, MA) and analyzed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by electroblotting to PVDF membranes (Millipore, Billerica, MA). Membranes were incubated with primary antibodies at the indicated concentrations followed by HRP-conjugated appropriate secondary antibodies (Supplemental Table S6), and were visualized by enhanced chemiluminescence (Millipore).

Cellular assays

Tumor cell proliferation in response to IL-1β or BMMC-CM was determined using MTT reduction (Promega, Madison, WI). Mast cell migration was studied in

Boyden chambers with 8.0 µm pore size (Millipore) using a method modified from Kitaura et al (36). Equal numbers of cancer cells were cultured in the lower and of bioluminescent BMMC in the upper chambers. After 48 hr the upper chambers were removed and the bioluminescent signal of transmigrated BMMC was measured using bioluminescent imaging. MC histamine content was measured by a microplate-adapted o-phthalaldehyde method (58). For this, BMMC exposed to various stimuli for varying time-intervals were lysed in ultrapure water by osmotic shock and six freeze-thaw cycles. O-phthalaldehyde was added and fluorescence was determined on a Victor 3 plate reader (Perkin Elmer, Waltham, MA) using specific parameters (excitation: 360 nm; emission: 450 nm). Glycine, which contains one -NH_2 group, was used for standard curve generation and histamine quantification. All cellular experiments were done at least thrice, while one representative experiment is shown.

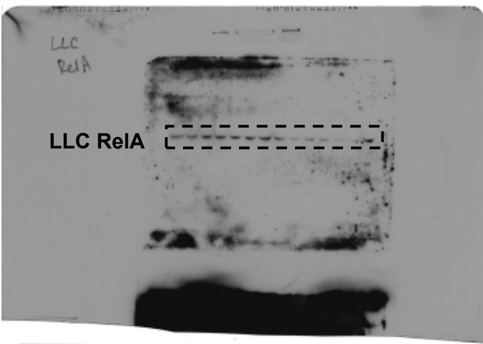
Statistics

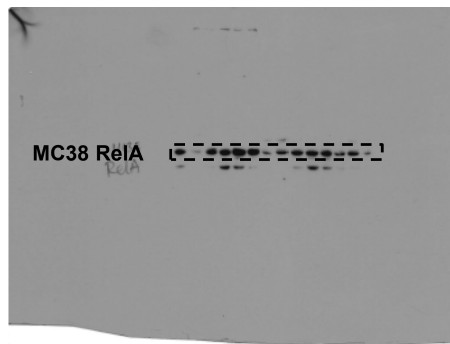
Sample size was calculated using power analysis on G*power academic freeware (59), assuming $\alpha = 0.05$, $\beta = 0.8$, and $\rho = 0.3$ (http://www.gpower.hhu.de/). No data were excluded from analysis. Animals were allocated to different treatments by alternation and transgenic animals were enrolled case-control-wise. Data were collected by at least two blinded investigators from samples coded by a non-blinded investigator. All data were examined for normality of distribution by Kolmogorov-Smirnof test. Normally and not normally distributed values are given as mean ± SD and median ± interquartile range, respectively. Sample size (n) always refers to biological and not technical replicates. Differences in means between two or multiple groups were examined, respectively, by two-tailed Student's t-test or one-way ANOVA with Bonferoni post-tests, and in medians between two or multiple groups by Mann-Whitney U-test or Kruskal-Wallis test with Dunn's post-tests, as appropriate. Two-way ANOVA with Bonferoni post-tests was employed for comparison of the effects of two parameters on outcome. Correlations were done using Pearson's R or Spearman's ρ, as appropriate. All P values are two-tailed and were considered significant when <.05. All statistical analyses were done and plots were created using Prism v5.0 (GraphPad, La Jolla, CA).

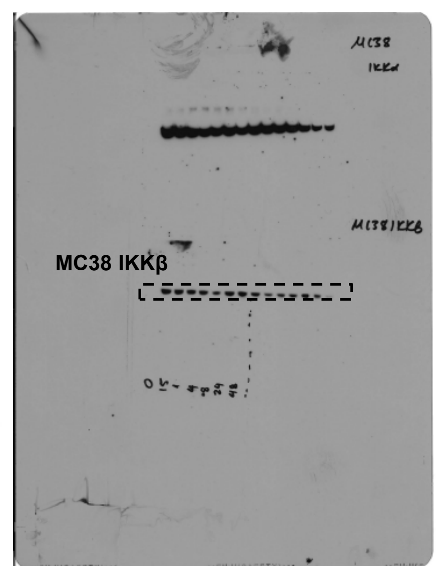
Study approval

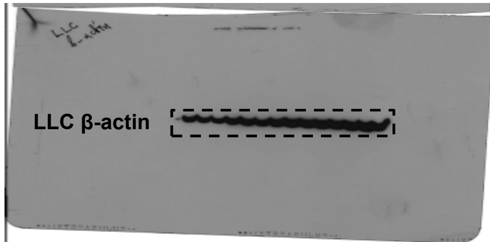
All animal experiments were approved a priori by the Veterinary Administration of Western Greece according to a full and detailed protocol (approval # 276134/14873/2). Human studies were approved a priori by the Ethics Committee of the General Hospital of Athens Evangelismos (Athens, Greece; approval #379-7/12/2006 and extension #323-4/12/2012).

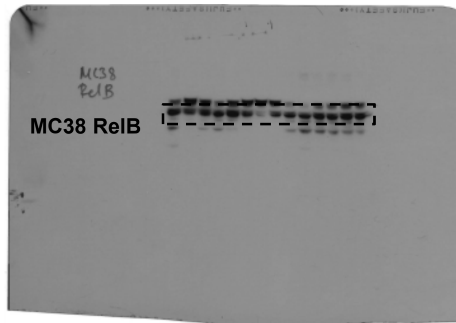
Immunoblots shown in Figure 13A. Dashed lines indicate blot areas shown in the main Figure.

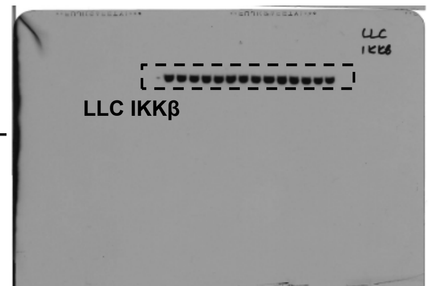


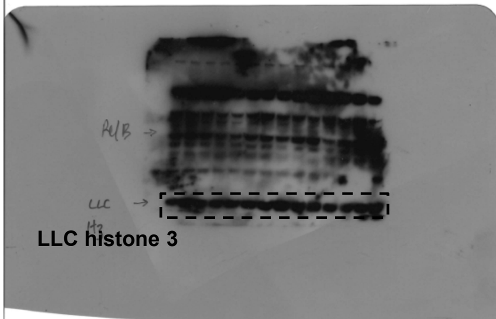


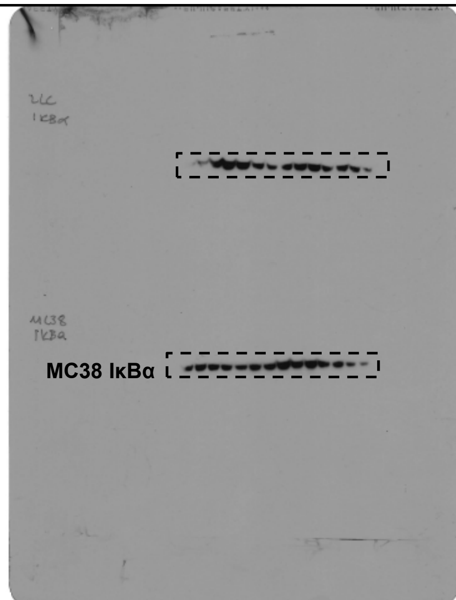


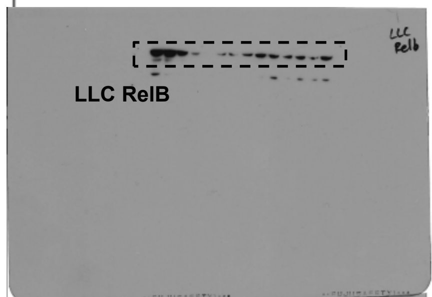


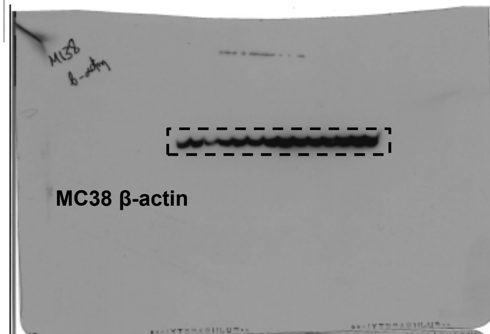


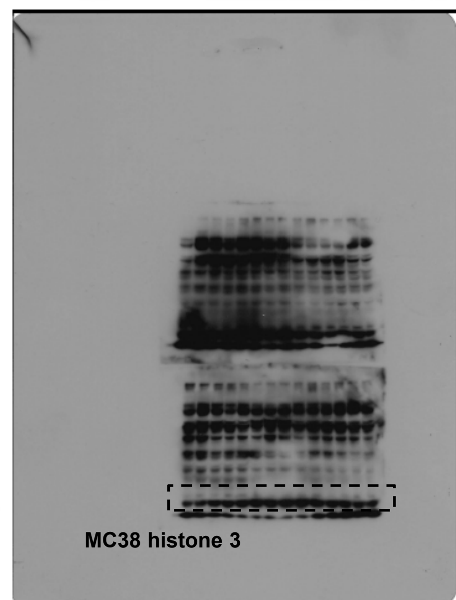


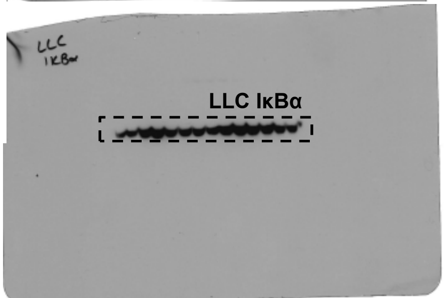


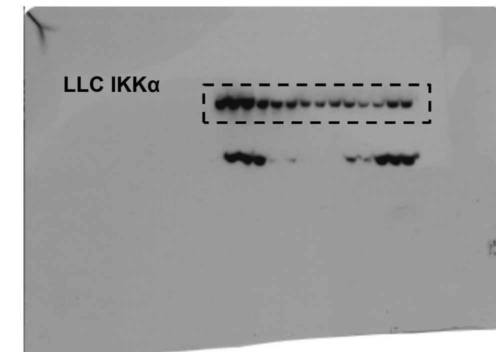


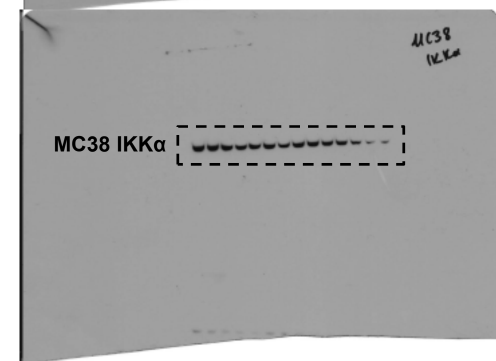




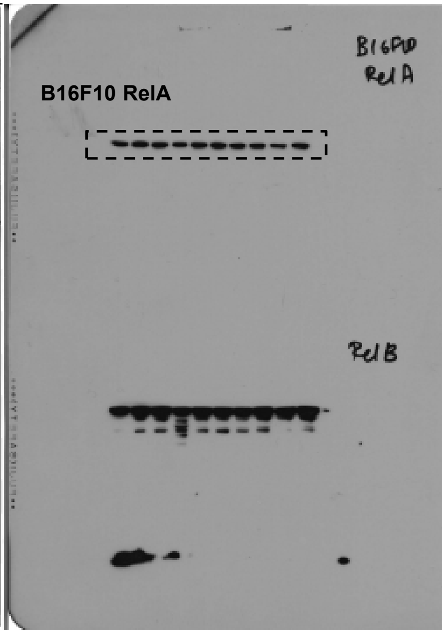


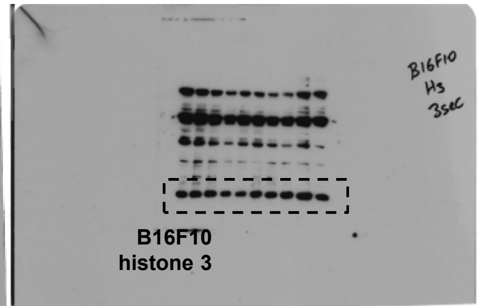


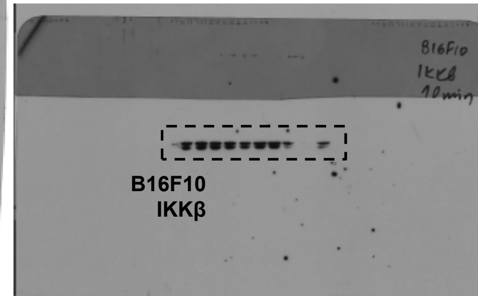


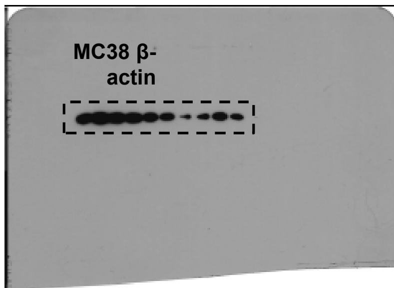


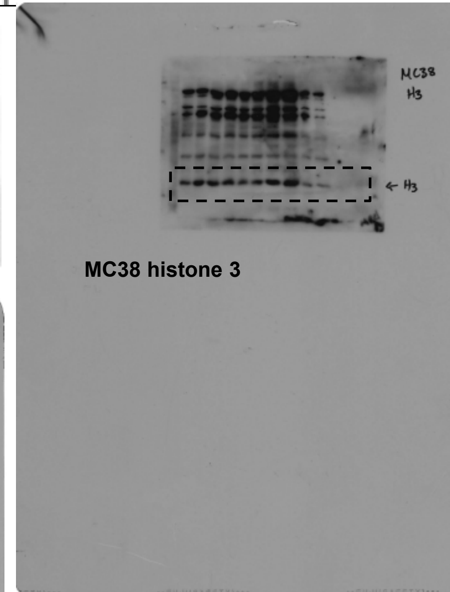
Immunoblots shown in Figure 13B. Dashed lines indicate blot areas shown in the main Figure.

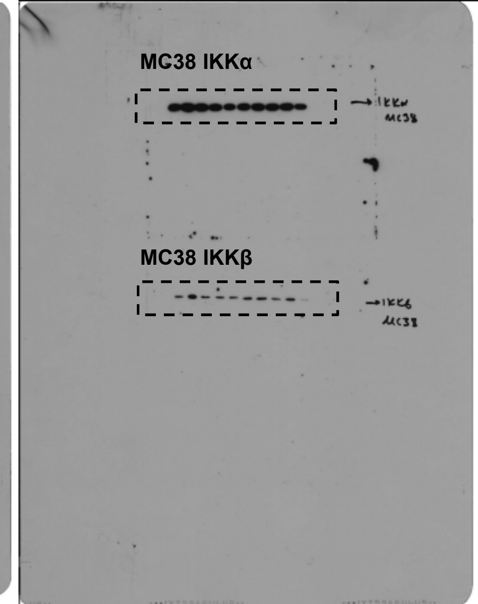


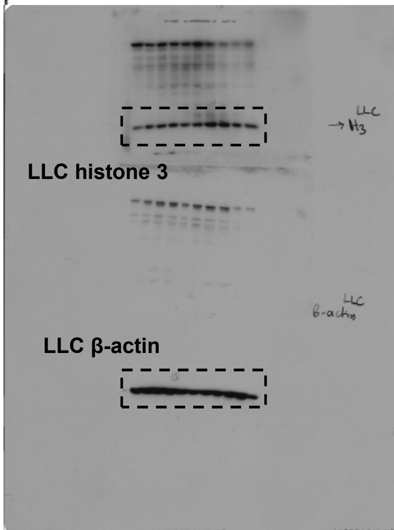


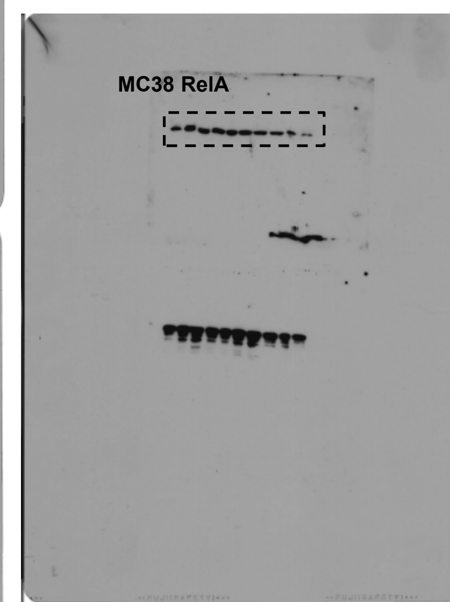


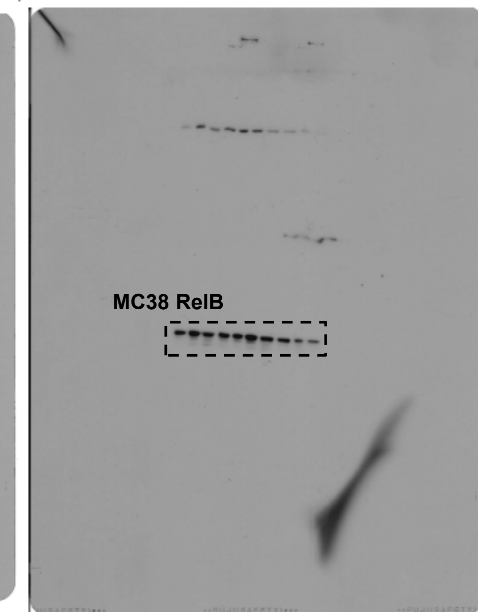








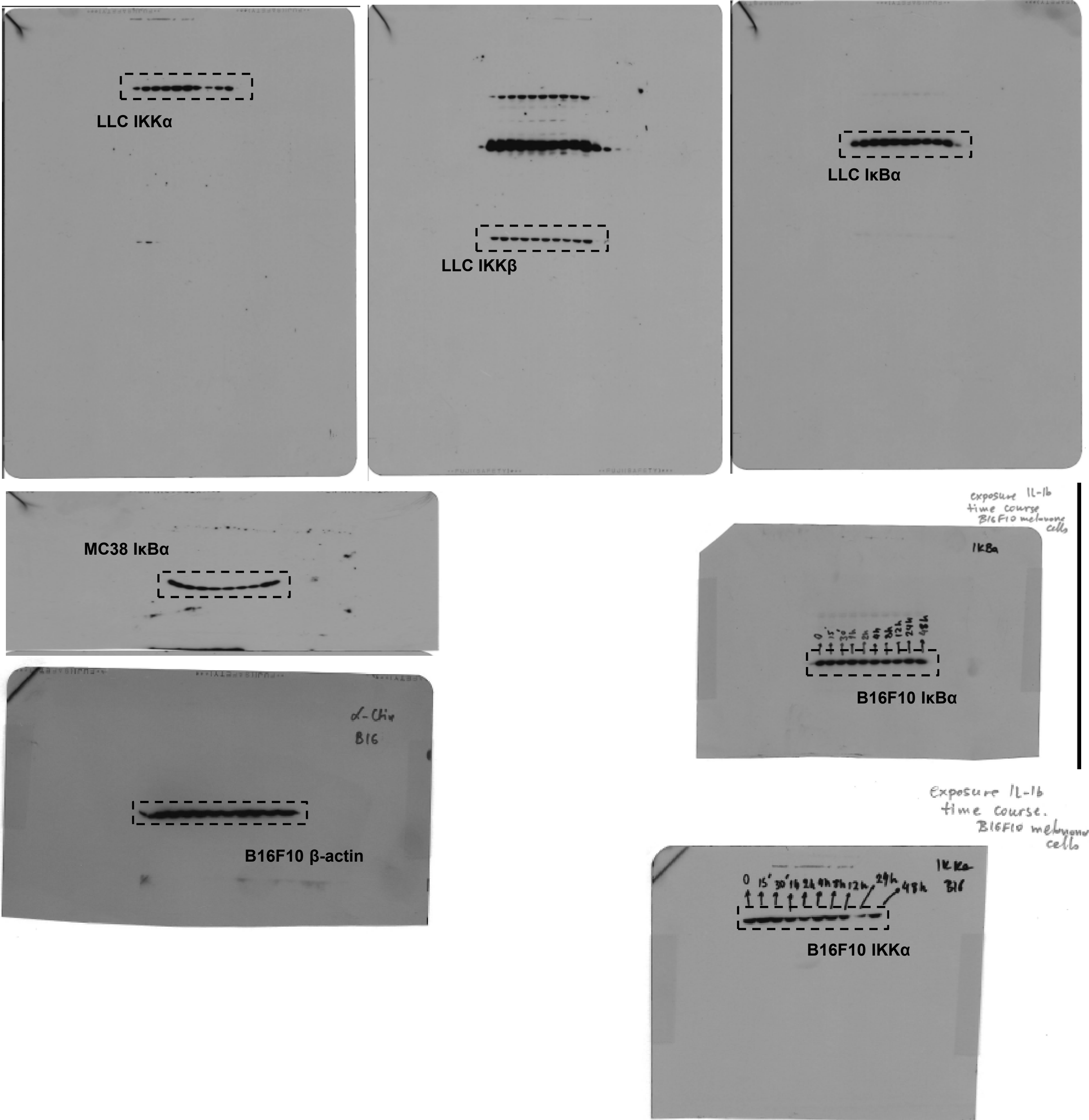




LLC RelA

LLC RelB

Immunoblots shown in Figure 13B continued. Dashed lines indicate blot areas shown in the main Figure.



Immunoblots shown in Figure 14B. Dashed lines indicate blot areas shown in the main Figure.