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Supplementary Materials for

IL-25 Causes Apoptosis of IL-25R–Expressing Breast Cancer Cells Without Toxicity to Nonmalignant Cells

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MATERIAL AND METHODS

Cell cultures

Nonmalignant human mammary epithelial MCF10A cells were cultured as described (*1*); human breast cancer cell lines (MCF7, MDA-MB361, T47D, ZR75, MDA-MB468, MDA-MB435-S, MDA-MB231, MDA-MD175-7, SKBR3, HS578T, HBL100 and HCC1937) and human embryonic kidney cells 293T were cultured as described (*2*) .

Microscopic imaging

Microscopic imaging of live cells was performed on a Zeiss Axiovert 200 M equipped with Hamamatsu Photonics K.K. Deep Cooled Digital Camera using Axiovision 4.5 software (Carl Zeiss) with phase I at 100X or phase II reflector at 200X magnification. Photomicrographs of histology specimens were taken with Zeiss Axioplan 2 Imaging platform and AxioVision 4.4 Software at 100X or 400X magnification.

Assessment of the cytotoxicity of CM from MECs

MCF10A cells were plated at $4x10^4$ cells in a 35mm-dish coated with 1ml Growth Factor Reduced Matrigel (BD Biosciences) and covered with 3ml growth medium supplemented with 2% Matrigel as described (*1*). After 15hrs CM (2.5ml) was collected and separated into soluble and pelleted fractions by centrifugation at 14,000 for 30min. The soluble fraction was size-fractionated with Centricon-10 and -50 units (Millipore) following the manufacturer's instruction; the pellet was resuspended in 400µl of growth medium. 400µl each of the following

fractions were obtained: 1) total CM, 2) pellet, 3) total supernatant, soluble fractions of 4) >50kDa, 5) 10-50kDa and 6) <10kDa. All the fractions were reconstituted with the essential growth factors and 2% Matrigel and applied to MCF7 or MCF10A cells seeded at 5000 cells/well in Matrigel-coated 8-well chamber slides. The collection/application of CM was performed every 12~15hrs for 1 week. To determine when differentiating MECs produce cytotoxic factors, the 10-50kDa fraction of CM was harvested at each day (days 0-6) from differentiating MCF10A in 3D lrECM using the above-mentioned condition. Different day fractions were individually applied to MCF7 cells plated at 1000 cells/well in Matrigel-coated 96-well plate. Fresh CM was applied every 24hrs. For cell number counting, cells were recovered from Matrigel after 1 week by digestion with dispase (BD Biosciences), and the viable cell numbers were measured using trypan blue exclusion method.

Sample preparation for mass spectrometric analysis

The cytotoxic fraction (10-50kDa, day 4) of CM from differentiating MCF10A cells and the soluble fraction of CM from MCF7 cells in 3-D laminin-rich ECM gels (day 4) were collected. Proteins in each medium were concentrated by trichloroacetic acid (TCA) precipitation and dissolved in boiling SDS sample buffer. Proteins were resolved by SDS-PAGE (10%) and visualized with Coomassie Blue staining. Gel slices (2mm thickness) were excised, de-stained with 25mM NH_4HCO_3 in 50% MeOH and digested with 50ng/ml trypsin in 50mM NH_4HCO_3 for 24 h at 37 °C. Peptides were extracted from gel slices with 3 volume of 50% acetonitrile, vacuum-dried and resuspended in 0.1% formic acid. Following sample clean up in C18 ZipTips (Millipore), peptides were eluted with 0.1% formic acid in 50% acetonitrile.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

The cytotoxic fraction (10-50kDa, day 4) of CM from differentiating MCF10A cells and the soluble fraction of CM from MCF7 cells in 3D matrix (day 4) were analyzed for LC-MS/MS spectrometry as described (*3, 4*). For LC-MS/MS analysis, the digests were first separated by cation exchange chromatography (polysulfoethyl A column, Nest Group) using a linear gradient between solvents A (5mM KH_2PO_4, 30% acetonitrile, pH 3) and B (solvent A with 350mM KCl) at a flow rate of 0.2 ml/min. Fractions were collected on the basis of UV absorbance (215 and 280nm) and desalted with C18 micro spin columns (Vivascience). LC-MS/MS was carried out by nanoflow reverse phase liquid chromatography (RPLC) (Ultimate LC Packings) coupled on-line to QSTAR XL tandem mass spectrometer (Applied Biosystems). RPLC was performed using a capillary column (75µm x 150mm) packed with Polaris C18-A resin (Varian Inc.), and the peptides were eluted using a linear gradient between solvents A (2% acetonitrile, 0.1% formic acid) and B (98% acetonitrile, 0.1% formic acid) at a flow rate of 250 nl/min. Each full MS scan was followed by three MS/MS scans where three most abundant peptide ions were selected to generate tandem mass spectra. Two LC-MS/MS runs were performed on the same sample to improve the dynamic range of mass spectrometric analysis.

Protein identification

For MS data analysis, monoisotopic masses (*m/z*) of peptide ions were obtained from the tandem mass spectra using Mascot script in Analyst QS version 1.1 software (Applied Biosystems) with the mass accuracy of ±200ppm. Certain chemical modifications (i.e., N-terminal acetylation or pyroglutamine, methionine oxidation, asparagine deamination, carbamylation of the N-terminus and lysine, phosphorylations of serine, threonine and tyrosine) were selected as variables during

the peptide search using Batch-Tag program in Protein Prospector version 4.25.0 software (UCSF). Both Uniprot and NCBInr public databases were queried to identify the proteins. Search Compare program in Protein Prospector was used to summarize the results including protein scores and discriminating score among peptide fragments. The result obtained for CM from differentiating MCF10A cells was compared to that for CM from MCF7 cells in 3-D cultures, and proteins present only in the former sample were identified. Proteins with the best score (>20) and discriminating score <6 were considered significant.

Immunodepletion

The cytotoxic fraction (10-50kDa, days 3-5) of CM was harvested from differentiating MCF10A cells in 3-D culture. The medium was divided into six fractions (2ml each), and each fraction was clarified with 100μl of protein G sepharose beads at 4°C for 2hrs. One μg of antibody against BMP10, FGF11, ATIII, IL1F7, IL-25, VBP or p84 (Ctrl) was added to each fraction and incubated at 4ºC overnight. Antibody-protein complex was precipitated by 100μl protein A/G sepharose beads (1:1) at 4°C for 2hrs. The immunoprecipitates were washed in TEN buffer (10mM Tris-HCl (pH8.0), 0.25mM EDTA, 50mM NaCl) supplemented with 0.1% NP-40 and protease inhibitors, then analyzed by western blot. 1/20 of the immunodepleted fraction was also examined by western analysis, and depletion was repeated 3 times to ensure complete loss of a target protein. Depleted fractions were reconstituted with the essential growth factors and 2% Matrigel, then used to plate MCF7 cells seeded at 5000 cells/well in Matrigel-coated 8-well chamber slides. The fraction was applied every 24 hrs for 1 week. Cells were recovered from Matrigel, and the viable cell numbers were measured.

Stable cell lines for ATIII, IL1F7, IL-25 and VBP

Full-length cDNA clones of ATIII, IL1F7, VBP (pDR-LIB) and IL-25 (pPCR-Script/Amp) were obtained from ATCC. ATIII, IL1F7 and VBP cDNAs were excised at SmaI/XhoI sites and subcloned into EcoRV/XhoI sites of pcDNA3.1/Hyg vector (Invitrogen), while IL-25 cDNA was excised at HindIII/NotI and subcloned into pcDNA3.1/Hyg. 293T cells were transfected with the respective plasmid and selected with 70μg/ml Hygromycin B (Roche). The comparable expression levels of these proteins were confirmed by RT-PCR of the transcripts using primers shown in Table.S1 and western analysis of secreted proteins in the conditioned media. For determining the cytotoxic activity of each factor, 7ml of CM from 293T cells ($4x10^6$) was harvested, concentrated by 2 fold with Centricon-10 and normalized based on the total protein amount. The media were supplemented with growth factors plus 2% Matrigel and applied to MCF7 cells seeded at 5000 cells/well in Matrigel-coated 8-well chamber slides. Fresh CM was applied every 24 hrs for one week, and the viable cell numbers were counted. To generate a stable cell line for IL-25 purification, IL-25 cDNA was subcloned into BamHI site of pQCXIH retroviral vector (BD Biosciences). GP_2-293 packaging cells were transfected with pQCXIH/IL25 and pVSVG plasmids using Lipofectin, and retrovirus was harvested in the conditioned medium. 293T cells were infected with retrovirus using 8μg/ml polybrene, and the stable clones were selected with 70 μg/ml Hygromycin B (Roche). IL-25 retrovirus was generated to establish a stable 293T cell line as described (*5*).

Purification of secreted IL-25 by concanavalin A column followed by gel filtration column

CM was collected from a stable 293T cell line expressing IL-25, supplemented with protease inhibitors and loaded onto a column packed with concanavalin A-sepharose beads (CalBiochem)

pre-equilibrated with column buffer (10mM Tris (pH7.5), 150mM NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$). The column was washed with column buffer, and bound glycosylated proteins were eluted with 0.5M α-methyl mannose in column buffer. The eluates were pooled, concentrated with Centricon-10 and then separated by Superdex 200 gel filtration column (HR 10/30, 24mL; Amersham Pharmacia) using elution buffer (50mM Na_2HPO_4 (pH7.5), 50mM NaCl) at a flow rate of 0.4ml/min. Fractions were collected based on UV absorbance at 280nm and resolved on 10% SDS-PAGE for western analysis.

Purification of secreted IL-25 by indirect immunoaffinity chromatography

The IL-25 antibody was indirectly conjugated with agarose beads as follows. 1 mg of IL-25 antibody was dissolved in 2ml PBS and incubated with 1ml slurry of protein G- agarose (~50%) for 1h at RT. Beads were washed in 5ml sodium borate (0.2M, pH 9.0), resuspended in 5ml sodium borate supplemented with dimethylpimelimidate up to 20 mM, and incubated for 30 min at RT. The reaction was stopped by 5ml 0.2M glycine (pH 8.0); the supernatant was drained and replaced with a fresh 5ml 0.2M glycine. After 2h of incubation at RT, beads were suspended in 3ml PBS and transferred to a chromatography column. CM collected from a stable 293T cell line expressing IL-25 was supplemented with protease inhibitors and pre-cleared through a column pre-packed with 2 ml protein A/G (1:1) agarose. The flow-through was then loaded onto the immunoaffinity column at a flow rate of 1 drop (~ 20 µl)/sec. The resin was washed with 10ml each of PBS, wash buffer [10 mM Tris (pH 8.0), 140 mM NaCl, 0.025% NaN3, 0.5% Triton X-100, 0.5% sodium deoxycholate], and then sodium phosphate (pH 6.3) buffer [50mM sodium phosphate (pH 6.3), 0.1% Triton X-100, 0.5M NaCl]. The bound IL-25 protein was eluted in 20

ml glycine buffer [50 mM glycine (pH 2.5), 0.1% Triton X-100, 0.15M NaCl] , and neutralized with 4 ml 1 M Tris (pH 9.0), and concentrated with Centricon-30 unit.

Colony formation assay

Breast cancer cells (MCF7, MDA-MB468, SKBR3 and T47D) at 1000/well and MCF10A cells at 500/well were seeded in 6-well plates in triplicate and maintained for 24hrs. Designated amounts of IL-25 were diluted in elution buffer to 100μl and then in 900μl growth medium to culture cells. After 10 days cells were stained with 2% Methylene Blue in 50% EtOH, and the numbers of colonies were counted.

Tumor inhibition assay in nude mice

Animal experiments were performed under federal guidelines and approved by Institutional Animal Care and Use Committee at UCI. Exponentially growing MDA-MB-468 cells at $10x10^6$ in 100μl of DMEM plus 5% Matrigel were injected into the mammary fat pad of 6-8 week old athymic female BALB/c-nude mice (nu/nu) (Charles River Laboratories). After tumors grew to $80mm^3$ (day 10), mice were randomized into control (n=7) and experimental groups (n=8) to receive on site injection of vehicle (elution buffer, 50μl) or IL-25 (300ng, 50μl) once every day for 31 days. Mouse body weights and tumor volumes were measured twice weekly. Student's t test was used to determine *p*-value as indicated in the figure legend. At the end of experiments, mice were sacrificed and subjected to pathological examinations of different organs, including intestines, kidney, heart, lungs, liver and stomach, to test the presence of systemic inflammation. Tumor xenografts were excised for histological examinations. For the safety test, IL-25 (1.5μg)

was injected into the tail veins of female C57 mice (vehicle: n=3; IL-25: n=5), and signs for systemic stress (e.g., lethargy and weight loss) were monitored daily.

Breast tumor samples

Patient cohort 1: All cases were invasive ductal carcinomas, which account for more than 90% of archived breast cancer samples in National Taiwan University Hospital. The grades of these tumors were 17 grade 1, 40 grade 2, and 12 grade 3 according to Nottingham Histologic Score. 38 were stage 1, 20 stage 2, and 11 stage 3 according to AJCC staging system. Patient cohort 2: All tumor cases were invasive ductal carcinomas between stages II and III according to Scarf-Bloom-Richardson system. Normal tissues were obtained from reduction mammoplasty samples. These tissues were provided by the Cooperative Human Tissue Network (NCI).

Histology and immunohistochemistry

Dissected tumors were fixed in 4% paraformaldehyde overnight and embedded in paraffin with a tissue processor. 4-5µm sections were deparaffinized and hydrated. Tumor xenografts were stained with hematoxylin and eosin, while human breast tumor specimens were processed for immunohistochemistry. Antigen retrieval was performed in 0.01 M citric buffer at 100°C for 10 minutes. After being blocked in 3% H_2O_2 and nonimmune horse serum, the slides were reacted with a monoclonal antibody against human IL-25R (GeneTex CAT # GTX13653; 1:100 dilution) at 4°C overnight and incubated with link antibodies, followed by peroxidase conjugated streptavidin complex (LSAB kit, DAKO Corp.) The peroxidase activity was visualized with diaminobenzidine tetrahydroxychloride solution (DAB, DAKO) as the substrate. The sections were lightly counterstained with hematoxylin. The positivity of staining was determined by

Allred Immunohistochemistry Scoring system (*6*), and more than 5% tumor cells stained in each section (score >2+) was defined as positive by a trained pathologist. The survival curve of patients was obtained by Kaplan-Meier analysis using XLSTAT-Life Version 2007.4 software. Statistical significance was assessed by using the log-rank test. A second set of breast tissue samples (10 normal and 20 malignant) were prepared as frozen sections (8-10 μm), fixed in 4% paraformaldehyde for 20 min, stained with a different IL-25R antibody (R&D Systems CAT # MAB1207; 1:50 dilution). NOTE: The manufacturer (GeneTex) of the IL-25R antibody clone used for staining paraffin sections as described immediately above replaced it with another clone; however, our testing showed that this replacement clone did not have the same immunoreactivity as the first clone listed under the same catalog number and thus we chose not to use this second clone from GeneTex. We found that by western blot, IP, detection of IL-25R in knock-down and overexpression experiments the results obtained using the IL-25R antibody available from R&D Systems were interchangeable with those obtained using the first clone of GeneTex antibody. The only relevant difference being that the R&D antibody could not be used for IHC on paraffin-embedded sections, and that is why the IHC analysis using the R&D antibody was performed using frozen sections.

IL25R, FADD or TRADD siRNA treatment

Breast cancer cells or nonmalignant MCF10A cells were plated at 3.5×10^5/60mm dish and maintained for 24 hrs. Cells were transfected with 400pmol of annealed siRNA for IL-25R, FADD or TRADD (Table S1, Qiagen) using Oligofectamine (Invitrogen) according to manufacturer's instruction. Luciferase siRNA was used as a non-specific control.

Soft agar assay

One % agar was mixed with the equivalent volume of 2x DMEM plus 20% FBS and 2% penicillin/streptomycin. One ml of the agar solution was poured into a 35 mm plate in triplicate and solidified. 0.7% agar solution equilibrated to 40°C was mixed with 2x growth medium and breast cancer cells at 4000 cells/ml and poured onto the base agar at 1 ml/plate. The solidified agar was covered with 500µl growth medium and maintained in 37°C humidified incubator for 2 weeks. The plates were stained with 0.01% crystal violet for 30 min, and colonies were counted under dissecting microscope.

Immunoprecipitation

Nine mg of whole cell lysates were collected in 3ml of Triton lysis buffer (25mM Tris (pH7.6), 150mM NaCl, 1% TritonX-100) supplemented with protease and phosphatase inhibitors. The lysate was divided into three fractions (3mg protein/1ml each), and each fraction was clarified with 50µl of protein G sepharose beads at 4°C for 2hrs. Two µg of antibody against p84 (Ctrl), IL-25R or FADD (Cell Signaling) was added to each fraction and incubated at 4ºC overnight. Antibody-protein complex was precipitated by 50µl protein A/G sepharose beads at 4°C for 2hrs and washed in TEN buffer with 0.1% NP-40 and protease inhibitors. Immunoprecipitates were resolved on 12% SDS-PAGE and detected by western analysis.

IL25R expression constructs

The expression construct of human IL-25R in pCMV6-XL5 vector was obtained from OriGene. The wild-type IL-25R was subjected to mutagenesis to generate a siRNA-resistant mutant (RM),

a deletion mutant at TRAF6 binding domain (ΔTRAF6) or death domain-like region (ΔDD) by using a corresponding primer pair (Table S1). Correct mutagenesis was validated by sequencing.

IL17B shRNA construction. Forward and reverse oligonucleotides (see supplementary Table S1 for the sequences) were annealed to generate a double-stranded DNA oligonucleotide, which in turn was ligated into *Bam*H1/*Eco*R1 site of pGreen puro lentiviral vector (System Biosciences). For control, luciferase shRNA vector was used (System Biosciences). Lentivirus production and transduction of target cells were conducted following the guideline by the manufacturer. Briefly, lentivirus vector and packaging plasmid mix (System Biosciences) were transfected into 293FT cells (Invitrogen) using Lipofectamine® 2000. After 48 hours, medium was harvested, filtered and used to infect target cells with the addition of polybrene (10 μg/ml). After 24 hours medium was replaced. At 72 hours post-infection puromycin (0.5 μg/ml) was added for selection and maintained throughout the culturing period.

Cell proliferation assay. Cells were plated at a density of 1 x 10^3 cells per well in 96-well plates in DMEM plus 10% FBS and incubated at 37°C. Twenty-four hours before each time point, the medium was replaced. At each time point, MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich) was added to cells to the final concentration of 1.6 mg/ml, and the reaction was incubated at 37°C for 4 hours. Then, the medium was removed and the precipitated reaction product was dissolved in MTT solvent (4 mM HCl, 0.1% NP-40 in isopropanol). The absorbance was measured at 570 nm.

Invasion assays. Cells at 2.5 X 10^4 were placed on the top of a thin Matrigel layer in 24-well invasion chamber (BD Biosciences) according to the manufacturer's protocol. After 48 hours, cells were stained with 0.2% methylene blue in 50% ethanol. Samples were prepared in triplicate and invaded cells were counted on at least 3 different fields on the trans-well filters. Statistical analysis was performed using an unpaired t-test. A p-value <0.05 was considered to be significant.

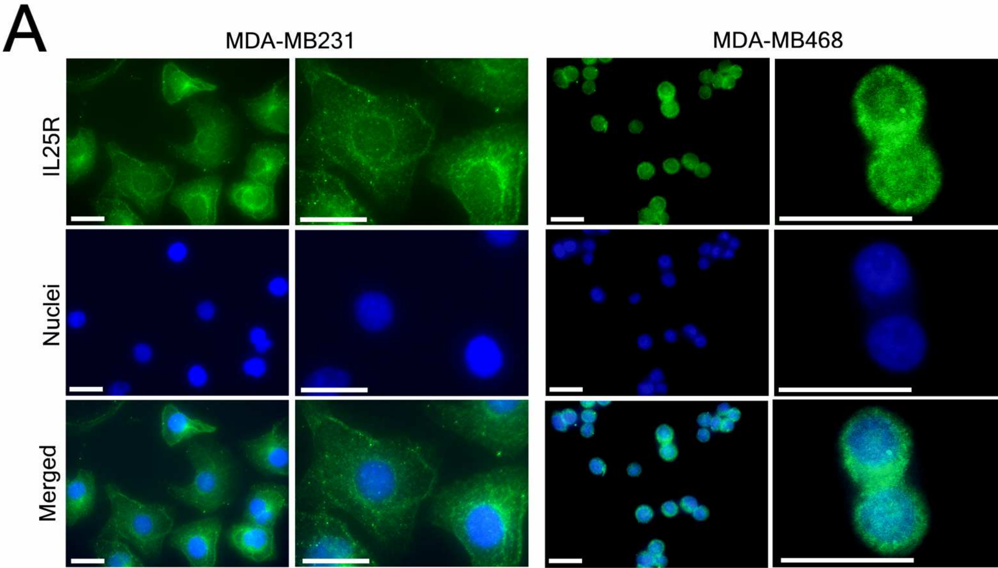
Supplementary data

Table S1. Oligonucleotide sequences used in this study**.**

Name	F/R	Sequence
RT-PCR Primers		
ATIII RT	F	5’-GCT TTT GCT ATG ACC AAG CTG-3’
	R	5’-TGC TTC ACT GCC TTC TTC ATT-3’
IL1F7 RT	F	5’-AAA CCC GAA GAA ATT CAG CAT-3’
	R	5’-CCC ACC TGA GCC CTA TAA AAG-3’
IL25 RT	F	5’-TTC CTA CAG GTG GTT GCA TTC-3’
	R	5’-CGC CTG TAG AAG ACA GTC TGG-3’
mIL25 RT	F	5’-ATG TAC CAG GCT GTT GCA TTC TTG-3’
	R	5’-CTA AGC CAT GAC CCG GGG CC-3’
VBP RT	F	5’-AAT CAA GGC TCA GCA ATC TCA-3’
	R	5’-CAT CTT TGT TTG TGG GCA ACT-3’
IL25R RT	F	5’-AGA GGC CTT CCA GAC TCA GAC-3’
	R	5’-AAA CCC GAT GAT AGT GCT GTG-3’
IL17B	F	5’-CCT CAC AAC CTG CTG TTT CTT-3’
	R	5’-ACA GCT GCA AGT TGA CCT CAC-3’
α-tubulin	F	5’-TGA CCT GAC AGA ATT CCA GAC CA-3’
	R	5’-GCA TTG ACA TCT TTG GGA ACC AC-3’
m18S	F	5’-TCG GAA CTG AGG CCA TGA TT-3’
	R	5’-CCT CCG ACT TTC GTT CTT GAT T-3’
mE-cadherin	F	5’-CGA GAG AGT TAC CCT ACA TA-3’
	R	5’-GTG TTG GGG GCA TCA TCA TC-3’
siRNA		
IL25R	F	5’-r(CGC GAG CUU CAG UGG UGA U)dTdT-3’
	R	5’-r(AUC ACC ACU GAA GCU CGC G)dTdT-3’
FADD	F	5’- r(GAA GAC CUG UGU GCA GCA U)dTdT-3’
	R	5’-r(AUG CUG CAC ACA GGU CUU C)dTdT-3’
TRADD	F	5’- r(GGU CAG CCU GUA GUG AAU C)dTdT-3’
	R	5’-r(GAU CA CUA CAG GCU GAC C)dTdT-3’
shRNA		
IL17B	F	5’-GAT CCG TGG AGG AGT ATG AGA GGA ACT TCC TGT CAG ATT CCT CTC ATA CTC CTC CAC TTT TTG-3’
	R	5’-AAT TCA AAA AGT GGA GGA GTA TGA GAG GAA TCT GAC AGG AAG TTC CTC TCA TAC TCC TCC ACG-3’
Mutagenesis		
IL25R ΔTRAF6[1]	F	5’-ATT AAG GTT CTT GTG GTT TA*C ATA TG*T TTC CAT CAC ACA ATA-3’
	R	5’-TAT TGT GTG ATG GAA A*CA TAT G*TA AAC CAC AAG AAC CTT AAT-3’
IL25R ΔDD[2]	F	5’-TGG CAG AAA AAG AAA ATA GCA *GAG CTC* GCA GCA GAC AAA GTC GTC TTC CTT-3’
	R	5’-AAG GAA GAC GAC TTT GTC TGC TGC *GAG CTC* TGC TAT TTT CTT TTT CTG CCA-3’
IL25R RM[3,4]	F	5’-CAG AAG AAA CAA ACG CGG *GCT AGC* GTG GTG ATT CCA GTG ACT GGG-3’
	R	5’-CCC AGT CAC TGG AAT CAC CAC *GCT AGC* CCG CGT TTG TTT CTT CTG-3’

[1]Unique NdeI restriction site generated is shown in italics.
[2]Unique SacI restriction site generated is shown in italics.
[3]Unique NheI restriction site generated is shown in italics.
[4]Nucleotides substituted without changing the encoded amino acids are shown underlined.

Supplementary Figures



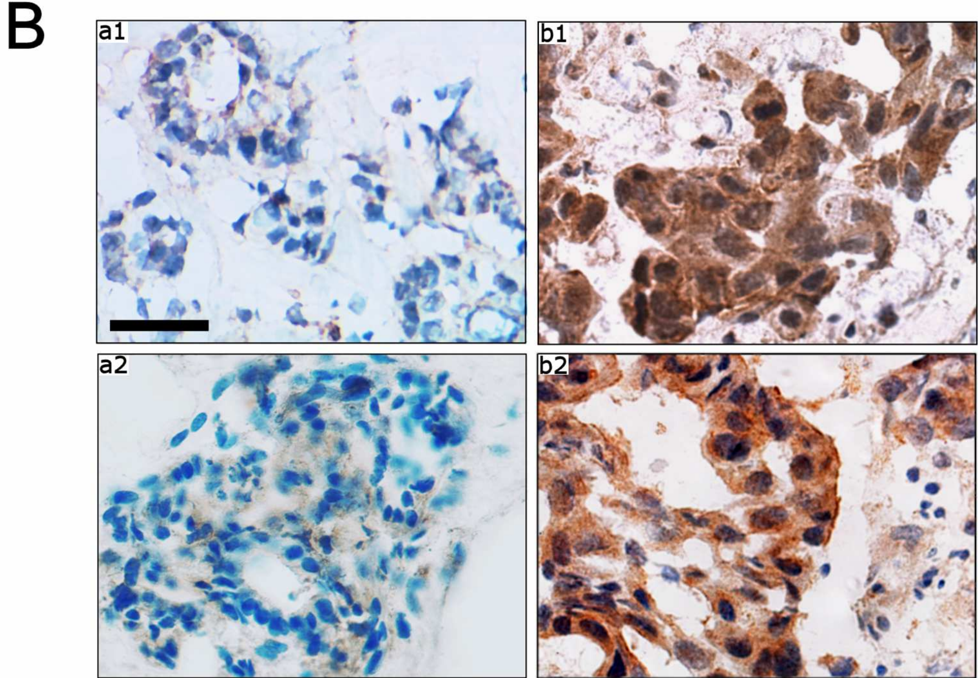


Fig. S1. Strong IL-25R staining in breast cancer cells as detected by a second IL-25R antibody. **(A)** Cultured MDA-MB231 and MDA-MB468 breast cancer cells were immunostained against IL-25R with a monoclonal antibody from R&D Systems and subjected to immunofluorescent imaging. Note the accentuated staining at cell membranes as well as Golgi. **(B)** Frozen sections of normal human breast tissue (left, N=10) vs. breast cancer tissue (right, N=20) sections were immunostained against IL-25R with the same antibody used in **(A)** and subjected to immunohistochemical imaging. The staining specificity of this antibody is the same as that observed by the antibody from GenTex; however, the membrane staining shown here is frozen sections is not as accentuated or consistent as that shown using IHC on paraffin sections (**Fig. 5H**). Scale bar: 50μm.

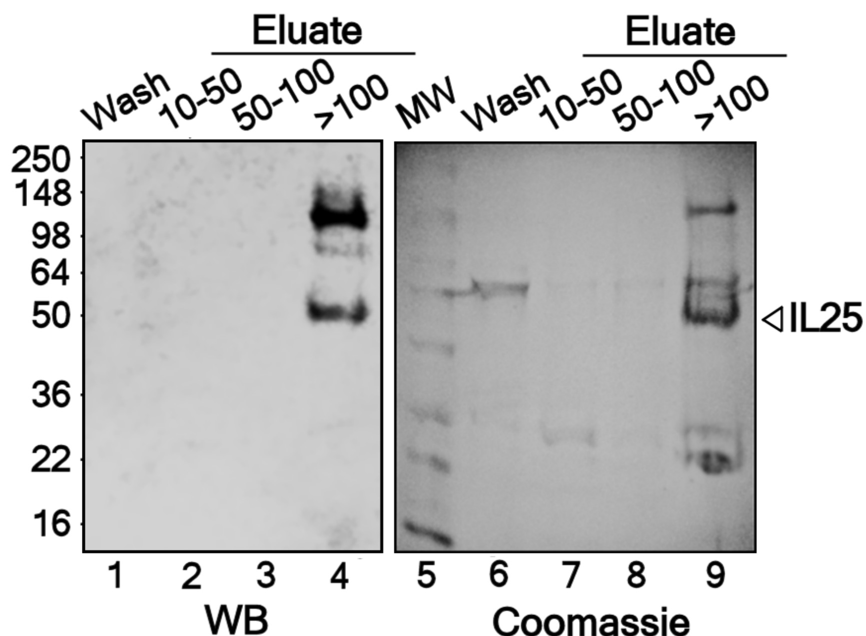


Fig. S2. Indirect immunoaffinity purification of IL-25. (Left): Western blot of IL-25 purified by acidic (low pH) elution. Conditioned medium from IL-25 expressing 293T cells was passed through Anti-IL25 antibody-protein G-agarose column, and the column was washed with pH 8 and then pH 6.3 buffers. The bound protein(s) were eluted by a low pH buffer (pH 2.5) containing glycine and then neutralized immediately. The eluate was size-fractionated by a series of centrifugal filters (10, 50 and 100 kDa cut-offs.) Native Il-25 was enriched in the fraction >100 kDa, possibly due to multimerization**.** (Right): The SDS-PAGE for samples corresponding to those analyzed by western blot (left) after staining with Coomassie dye. The IL-25 protein eluted in the >100 kDa fraction is fairly pure (>50%; 45~50 kDa as a monomer) with the approximate concentration of ~80 ng/µl (lane 9).

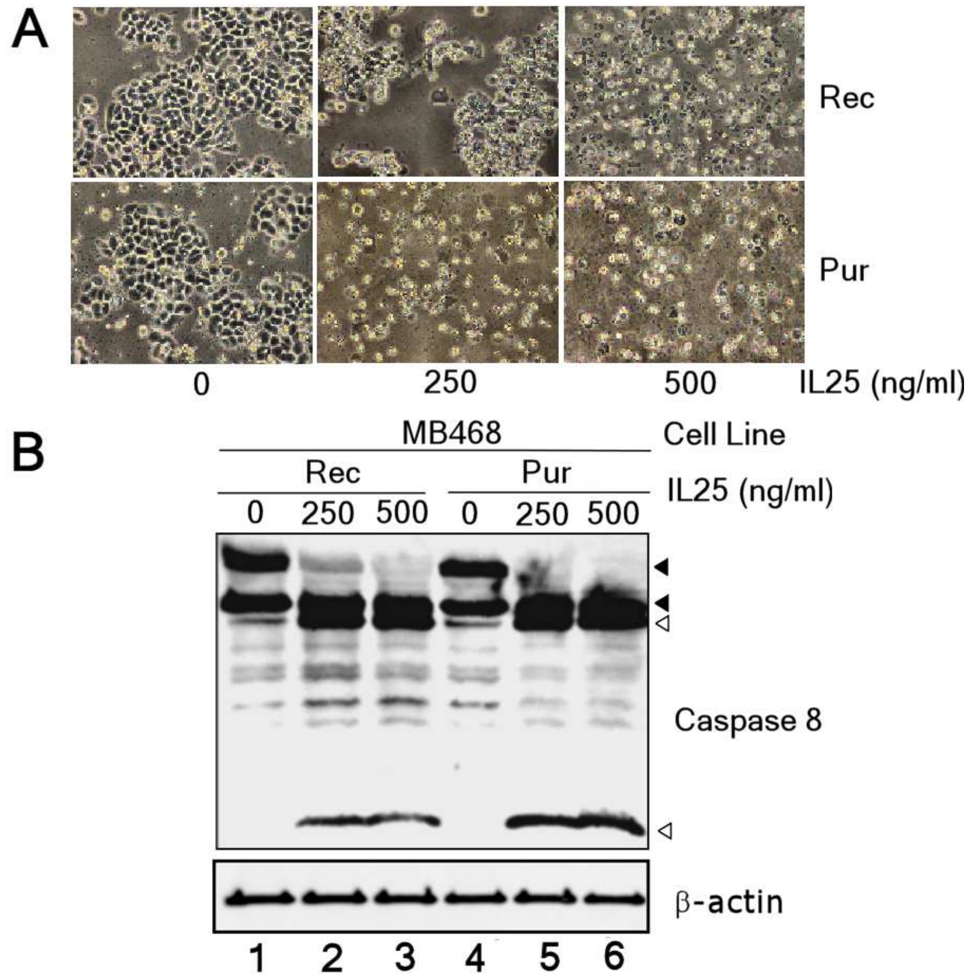


Fig. S3. Cell-killing activity of recombinant versus purified human IL-25 protein on breast cancer cells in conventional cultures.

(A) Morphology of MDA-MB468 breast cancer cells after treatement with *E.*coli-generated (Rec, R&D Systems) vs. mammalian cell-generated, purified (Pur, see Methods) IL-25 protein at designated concentrations (0, 250, or 500 ng/ml) for 24h. Note the extensive apoptosis of cells treated with both batches of IL-25.

(B) Caspase-8 activation in MDA-MB468 breast cancer cells treated as in **Fig. S3A**. Note the slightly higher degree of caspase 8 activation (cleavage product: white arrows in the upper panel) by treatment with IL-25 purified from mammalian cells. Black arrowhead: noncleaved protein; white arrowhead: cleaved protein.

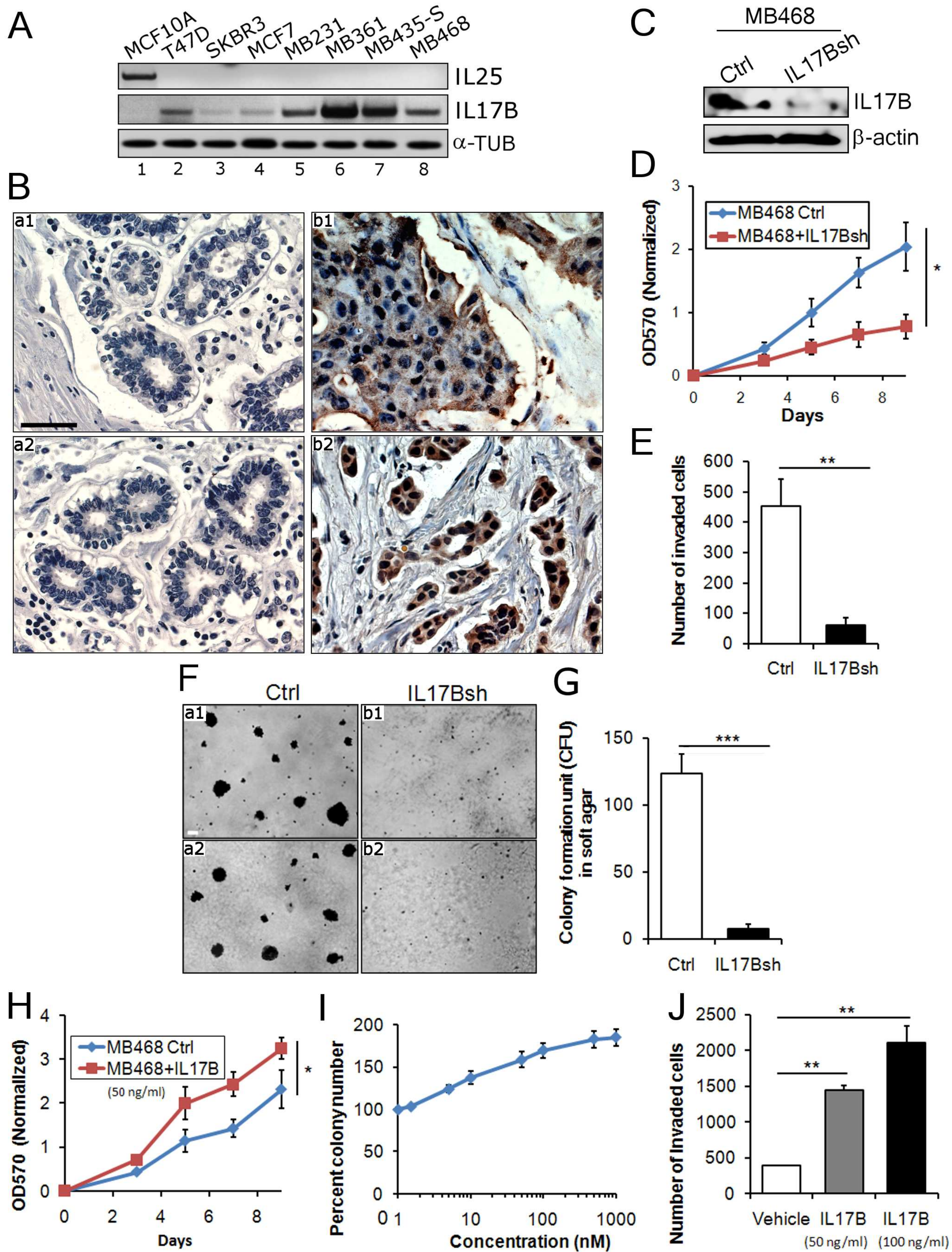

A
MCF10A
T47D
SKBR3
MCF7
MB231
MB361
MB435-S
MB468
IL25
IL17B
α-TUB
1 2 3 4 5 6 7 8
B
a1
b1
a2
b2
C
MB468
Ctrl
IL17Bsh
IL17B
β-actin
D
MB468 Ctrl
MB468+IL17Bsh
OD570 (Normalized)
Days
E
Number of invaded cells
Ctrl
IL17Bsh
F
Ctrl
IL17Bsh
G
Colony formation unit (CFU) in soft agar
Ctrl
IL17Bsh
H
MB468 Ctrl
MB468+IL17B
(50 ng/ml)
OD570 (Normalized)
Days
I
Percent colony number
Concentration (nM)
J
Number of Invaded cells
Vehicle
IL17B (50 ng/ml)
IL17B (100 ng/ml)

Fig. S4. IL-17B is up-regulated in a subset of breast cancer cell lines and tissues and is important for their growth and invasive potentials.

(**A**) Opposing expression patterns of IL-25 and IL-17B in nonmalignant MECs (MCF10A) and breast cancer cells detected by RT-PCR. α-tubulin (α-TUB) serves as an internal loading control.

(B) Specimens of nontumorous human breast tissue (a1-2) vs. human breast cancer (b1-2) were immunostained with a monoclonal antibody against IL-17B. IL-17B was upregulated in 30% of breast cancer specimens examined (12/40) while being undetectable in normal tissues (0/18). Scale bar: 50 μm.

(**C**) Reduction of IL-17B in MDB-MB468 breast cancer cells with specific shRNA, in relation to control. The result was determined by western blot.

(**D**) Reduction of IL-17B in MDA-MB468 cells impaired the proliferation rate, as determined by MTT assay. *P-value < 0.05. N=12.

(E) Reduction of IL-17B in MDA-MB468 cells impaired the invasion rate, as determined by Matrigel invasion assay. **P-value < 0.01. N=4.

(**F**) Reduction of IL-17B in MDA-MB468 cells impaired anchorage-independent growth in soft agar. Scale bar: 50 μm.

(G) The number of colonies formed on in soft agar as in **(F)** were quantified and compared between control and IL-17Bsh-treated cells. ***P-value < 0.001. N=6.

(**H**) Ectopic addition of recombinant IL-17B protein (50 ng/ml) to the culture medium promoted the proliferation rate of MDA-MB468 cells, as determined by MTT assay. *P-value < 0.05. N=12.

(I) Ectopic addition of recombinant IL-17B protein to the culture medium at different concentrations promoted the clonogenic potential of MDA-MB468 cells. N=6.

(J) Ectopic addition of recombinant IL-17B protein to the culture medium at different concentrations (50 or 100 ng/ml) promoted the invasiveness of MDA-MB468 cells. $^{**}P$-value < 0.01. N=4.

Supplementary References

1. J. Debnath, S. K. Muthuswamy, J. S. Brugge, Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30**, 256 (2003).
2. S. Furuta, X. Jiang, B. Gu, E. Cheng, P. L. Chen, W. H. Lee, Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells. *Proc. Natl. Acad. Sci. U S A* **102**, 9176 (2005).
3. X. Wang, C. F. Chen, P. R. Baker, P. L. Chen, P. Kaiser, L. Huang, Mass spectrometric characterization of the affinity-purified human 26S proteasome complex. *Biochemistry* **46**, 3553 (2007).
4. R. J. Chalkley, P. R. Baker, L. Huang, K. C. Hansen, N. P. Allen, M. Rexach, A. L. Burlingame, Comprehensive analysis of a multidimensional liquid chromatography mass spectrometry dataset acquired on a QqTOF mass spectrometer: 2. New developments in protein prospector allow for reliable and comprehensive automatic analysis of large datasets. *Mol. Cell Proteomics* **4**, 1194 (2005).
5. S. Furuta, J. M. Wang, S. Wei, Y. M. Jeng, X. Jiang, B. Gu, P. L. Chen, E. Y. H. P. Lee, W. H. Lee, Removal of BRCA1/CtIP/ZBRK1 repressor complex on ANG1 promoter

leads to accelerated breast tumor growth contributed by prominent vasculature. *Cancer Cell* **10**, 13 (2006).

6. D. C. Allred, J. M. Harvey, M. Berardo, G. M. Clark, Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod. Pathol.* **11**, 155 (1998).