Supplementary material for online publication

Supplementary Materials and Methods

Generation of bone marrow-derived dendritic cells (DC). Bone marrow (BM) cells were flushed from the tibias and femurs of BALB/c mice with culture medium composed of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), sodium pyruvate, 50 µM 2-mercaptoethanol (Sigma), 10 mM HEPES (pH 7.4), and penicillin/streptomycin (Invitrogen). After one centrifugation, BM cells were resuspended in Tris-ammonium chloride for 2 min to lyse red blood cells. After one more centrifugation, BM cells (1 x 10^6 cells/ml) were cultured in medium supplemented with 100 ng/ml recombinant mouse FLT3 ligand (R&D systems) in 6-well plates (Costar Corning). After 7 days, the non-adherent and loosely adherent cells were harvested with Versene, washed and transferred in 12-well plates (1.5 x 10^6 cells/plate) for cocultures with tumor cells.

In vitro phagocytosis assays. In 12-well plates, 25 x 10^6 adherent CT26 cells were labelled with Celltracker Green or Celltracker Orange (Calbiochem) and then incubated with drugs. In some experiments viable CT26 were coated with 2µl/10^6 cells of chicken anti-CRT antibody (ABR affinity bioreagents) or an isotype control for 30 min prior to washing and feeding to DCs. Alternatively, CT26 cells were coated with 2µg/10^6 cells of recombinant CTR protein on ice for 30 min and washed twice prior to addition to DCs. Cells were then harvested, labeled with CMTMR (Molecular Probes), washed three times with medium supplemented with FBS and cocultured with immature DC for 2 hours at a ratio of 1:1 and 1:5. At the end of the incubation, cells were harvested with versene, pooled with non-adherent cells present in the supernatant, washed and stained with CD11c-FITC antibody. Phagocytosis was assessed by FACS analysis of double positive cells. Phagocytic indexes refer to the ratio between values obtained at 4°C and values obtained at 37°C of co-incubation between DC and tumor cells.

In vivo phagocytosis assays. Under general anaesthesia with 4% isoflurane, 6 week old female BALB/c mice underwent intrasplenic injection of PKHA 26 (Sigma, 5 µM, 5 min, 37°C)-labelled 3 x 10^7 tumour cells. Two hours after injection, mice were sacrificed, and spleens were removed for dissociation with collagenase H (Roche). Cells were then filtered on a 40 µm mesh, resuspended in red blood cell lysis buffer, and washed. Splenocytes were incubated at RT with Pacific Blue-labelled CD11c antibody (Beckton Dickinson) for identification of DC. Phagocytosis was assessed by FACS analysis (LSR II, Beckton Dickinson) of double positive cells.

Fluorescence detection of cell surface CRT. CT26 cells (on a glass slide or in in 12-well plates) were first washed with FACS buffer (1x PBS, 5% fetus bovine serum, and 0.1% sodium azide) and then incubated with rabbit anti-mouse CRT antibody (1:100, Stressgen) in FACS buffer at 4 °C for 30 min. Cells reacted with anti-
rabbit IgG (H+L) Alexa fluor 488-conjugates (1:500) in FACS buffer at 4 °C for 30 min. After washing three times with FACS buffer, surface CRT was detected by cytofluorometric analysis on a FACS Vantage. In some experiments, cells were fixed with 4% paraformaldehyde, counterstained with Hoechst (2µM; Sigma), followed by fluorescence microscopic assessment with a Leica IRE2 microscope equipped with a DC300F camera.

**Immunoblot analyses.** Cells were washed with cold PBS at 4°C and lysed in a buffer containing 50 mM Tris HCl pH 6.8, 10 µg/mL sodium deoxycholate (SDS). Primary antibodies detecting CRT (dilution 1/2000, Stressgen), CD47 (dilution 1/500, BD Biosciences), eIF2α, eIF2α -P and PP1c α (dilution 1/2000, Cell Signaling Technology), GADD34 (dilution 1/2000, Abcam), were revealed with the appropriate horseradish peroxidase-labeled secondary antibody (Southern Biotechnologies Associates) and detected by ECL (Pierce). Anti-actin or anti-GAPDH (Chemicon) was used to control equal loading.

**Biotinylation of CT26 cell surface proteins.** Biotinylation and recovery of cell surface proteins were performed with a method adapted from Gottardi et al. and Hanwell et al. Briefly, 20 ×10⁶ CT26 cells grown on 75 cm² flask were placed on ice and washed three times with ice-cold PBS-Ca²⁺-Mg²⁺ (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂). Membrane proteins were then biotinylated by a 30-min incubation at 4 °C with NHS-SS-biotin 1.25 mg/ml (Pierce) freshly diluted into biotinylation buffer (10 mM triethanolamine, 2 mM CaCl₂, 150 mM NaCl, pH 7.5) with gentle agitation. CT26 cells were rinsed with PBS-Ca²⁺-Mg²⁺ + glycine (100 mM) and washed in this buffer for 20 min at 4 °C to quench unreacted biotin. The cells were then rinsed twice with PBS-Ca²⁺-Mg²⁺, scraped in cold PBS, and pelleted at 2,000 rpm at 4 °C. The pellets were solubilized for 45 min in 500µl of lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) containing protease inhibitors. The lysates were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatants were incubated overnight with packed streptavidin-agarose beads to recover biotinylated proteins. The beads were then pelleted by centrifugation, and aliquots of supernatants were taken to represent the unbound, intracellular pool of proteins. Biotinylated proteins were eluted from the beads by heating to 100 °C for 5 min in SDS-PAGE sample buffer before loading onto a 10% SDS-PAGE gel. To ensure the absence of leakage of biotin into the cells, we systematically verified the absence of the intracellular protein actin and GAPDH in biotinylated extracts.

**2D gel electrophoresis analysis and protein identification by mass spectrometry.** Purified proteins were precipitated using the Ettan 2-D clean up kit (GE Healthcare) were subsequently resuspended in urea buffer (7M urea, 2M thiourea, 2% Chaps, 1% SulfoBetaine SB3-10, 1% AmidoxSulfobetaine ASB14, 50mM DTT). For the first dimension of protein separation, isoelectric focusing (IEF) was performed using 18-cm immobilized nonlinear pH gradient strips (pH 3 to 10; GE Healthcare) on a IPGphor II electrophoresis unit (GE Healthcare). Proteins (100 µg) were loaded by in-gel rehydration for 9h, using low voltage (30V) then run using a program in which the voltage was set for 1 h at 100 V, 2 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, 2hrs, 2hrs voltage gradient 1,000-8,000V and 4 h at 8,000 V. Prior to the second-dimension electrophoresis, IPG gel strips were equilibrated for 10 min at room temperature in 1% dithiothreitol to reduce the proteins and sulphydryl groups were subsequently derivatized using 4% iodoacetamide (both solutions were prepared in 50 mM Tris [pH 8.8]-6
M urea-30% glycerol-2% SDS-2% bromophenol blue). Strips were transferred to 1.0-mm-thick 10% (wt/vol) polyacrylamide gels (20 by 20 cm), and the second-dimension gels were run at 50 μA for 6 h. Gels were stained with Sypro Ruby (BioRad) and visualized using a Typhoon 9200 scanner (GE Healthcare). The Investigator HT analyser (Genomic Solutions Inc) was used for matching and analysis of visualized protein spots among differential gels. Background subtraction was used to normalize the intensity value representing the amount of protein per spot.

Differentially expressed spots were excised from the gels with an automatic spot picker (Investigator ProPic, Genomic Solutions Inc.), placed in Eppendorf tubes, and destained by washing for 5 min with 50 μL of 0.1 M NH4HCO3. Then 50 μL of 100% acetonitrile were added and incubated for other 5 min. The liquid was discarded, the washing steps were repeated one more time and gel plugs were shrunk by addition of pure acetonitrile. The dried gel pieces were reswollen with 4.0 ng/μL trypsin (Promega, Madison, WI) in 50 mM NH4HCO3 and digested overnight at 37°C. Peptides were concentrated with ZipTip®μC18 pipette tips. Co-elution was performed directly onto a MALDI target with 1 μL of α-cyano-4-hydroxycinnamic acid matrix (5 μg/mL in 50% acetonitrile, 0.1% TFA). MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics. Spectra were acquired in positive MS reflector mode and calibrated either externally using five peaks of standard (ABI4700 Calibration Mixture) or internally using porcine trypsin autolysis peptide peaks (842.51, 1045.56 and 2211.10 [M+H]+ ions). Mass spectra were obtained from each sample spot by 30 sub-spectra accumulation (each consisting of 50 laser shots) in a 750 to 4000 mass range. Five signal-to-noise best peaks of each spectrum were selected for MS/MS analysis. For MS/MS spectra, the collision energy was 1 keV and the collision gas was air.

MS and MS/MS data were interpreted using the GPS Explorer software (Version 2.1, Applied Biosystems) which acts as an interface between the Oracle database containing raw spectra and a local copy of the MASCOT search engine (Version 1.8). Peptide mass fingerprints obtained from MS analysis were used for protein identification in Swiss Prot non-redundant database. All peptide mass values are considered monoisotopic and mass tolerance was set <50 ppm. Trypsin was given as the digestion enzyme, 1 missed cleavage site was allowed, methionine was assumed to be partially oxidized and serine, threonine and tyrosine partially phosphorylated. Mascot (Matrix Science) scores greater than 71 were considered to be significant (p<0.005). For MS/MS analysis, all peaks with a signal-to-noise ratio greater than 5 were searched against the Swiss Prot database using the same modifications as the MS database. Fragment tolerance less than 0.3 Da was considered.

**Preparation of cytoplasts.** Trypsinized CT26 cells were enucleated as described. Briefly, cells were treated in 2 ml of complete RPMI medium containing cytochalasin B (10μg/ml ; Sigma) and DNase I (80U/ml ; Sigma). Cell suspension was adjusted to a final concentration of 5x10⁶/ml and incubated at 37°C for 45 minutes before being layered onto a previously prepared discontinuous Ficoll (Pharmacia) density gradient (3 ml of 100%, in 1 ml of 90% and 3 ml of 55% Ficoll Paque layer containing 5μg/ml cytochalasin B and 40U/ml DNase I; gradients were prepared in ultracentrifuge tubes and pre-equilibrated at 37°C in a CO₂ incubator overnight). Gradients containing cell suspensions were centrifugated in a prewarmed SW41 Beckman rotor at 25 000 rpm for 20 minutes at 30°C. The cytoplasts-enriched fraction was collected from the interface between 90 and 100% Ficoll layers, washed in complete RPMI 1640 medium, and incubated at 37°C. The cells were incubated with...
MTX, CA, Sal and TA for the period of time indicated in the experiment. Then the cell surface CRT was detected (see materials and methods) and the viability was determined by with propidium iodine staining (2µg/ml, Sigma) for 5 min followed by cytofluorometric analysis. Alternatively cytoplasts were cocultured with immature DC for 2 hours at a ratio of 1:1 and 1:5. At the end of the incubation, cells were harvested with versene, pooled with non-adherent cells present in the supernatant, washed and stained with CD11c-FITC antibody. Phagocytosis was assessed by FACS analysis of double positive cells.