## SUPPLEMENTARY MATERIALS AND METHODS

### Tumor cell lines

The RBL-5 lymphoma cells transformed by Rauscher lymphoma virus were obtained by Dr. Ion Gresser (Villejuif, Paris). The EL-4 lymphoma cell line, obtained from American Type Culture Collection (ATCC, TIB-39). These cell lines were maintained in RPMI 1640 medium (Lonza), supplemented with 10% heat-inactivated FCS (EuroClone), 2 mM L-glutamine, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin (Lonza) and 0.05 mM 2-mercapto-ethanol. EG.7-OVA cells (EG7; obtained from ATCC, CRL-2113), were derived by transfection of EL4 cells with a plasmid carrying the chicken ovalbumin -OVA- and neomycin phosphotransferase -G418 resistance- genes and were cultured in similar medium supplemented with 0.4 mg/ml G418 (Calbiochem). OVA expression on MHC-I molecules of EG7 cells was routinely checked by flow cytometry. B16-F10 melanoma cell line (obtained from ATCC; CRL-6475) was maintained in IMDM complete medium. Each cell line was routinely tested for morphology, growth curve and absence of Mycoplasma, and maintained in culture for no more than 5 passages from thawing.

### Reagents

Mafosfamide (4-sulfoethylthio-cyclophosphamide L-lysine, Niomech –IIT GmbH) was used at 10μM and added to the cell cultures for a minimum of 4h. CTX (Sigma) was dissolved in 0.15 mol/L NaCl and filter sterilized. High titer mouse IFN-I ($1.5x10^6$ U/mg protein) was produced from the C243-3 cell line following a method adapted from Tovey et al. (2). Briefly, confluent cells were primed by the addition of 10 U/ml of IFN-I in MEM enriched with 10% FCS and 1mM sodium butyrate. After 16 hours of culture at 37° C, C243-3 cells were infected with Newcastle Disease Virus (multiplicity of infection of 1) in MEM + 0.5 % FCS + 5mM theophylline. Eighteen hours post-infection, culture supernatant was collected and adjusted to pH 2.0 at 4°C. IFN was concentrated and partially purified by ammonium sulfate precipitations and dialysis against PBS.

All IFN-I preparations were further subjected to dialysis for 24 hr at 4°C against 0.01 M perchloric acid and then against PBS, before testing them for any possible residual toxicity on a line of L1210 cells resistant to IFN-I. These partially purified IFN-I preparations were endotoxin-free, as assessed by the *Limulus* amebocyte assay. IFN-I titers were assayed by inhibition of the cytopathic effect of vesicular stomatitis virus on L929 cells and proved to be constituted of approximately 75% IFN-β and 25% IFN-α, as evaluated by neutralization assays using mAbs to IFNs. A mock preparation not containing IFN-I served as control for all assays.

**Mice and in vivo treatments**

Six- to 7-week-old specified pathogen-free female C57BL/6, OT-1 (Charles River), and $IFNAR^{-/-}$ mice on a B6 background (kindly provided by Dr U. Kalinke, Langen, Germany) were housed in the Animal Facility of the Istituto Superiore di Sanita` and manipulated in accordance with the local Ethical Committee guidelines. EG7 ($5x10^6$) or RBL-5($8x10^5$) tumor cells were implanted s.c. into the right flank of mice. On day 10 or 14 of tumor growth respectively, mice were treated i.p. with CTX (100 mg/kg) or saline. In some experiments, mice were subsequently exposed to 4 peritumoral injections of type I IFN ($10^5$ IU), given daily starting from day 1 post CTX. Tumor growth was monitored by using a calliper.

**Immunofluorescent labelling**

The following mAbs were used: anti-CD8α-PE, anti-CD45R/B220-PE, anti–CD40-biotin, anti-CD11c, which was used in either PE-, FITC-, APC or biotin-conjugated form, anti-CD80-biotin, anti-CD86-biotin, anti–$H2D^b$-biotin, anti–$I\text{-}A^d/I\text{-}E^d$-biotin, anti-CD31-APC (all from BD Pharmingen) anti-CRT-biotin (Cell Signalling Technology) and anti-MHC-I-OVAp-biotin (eBioscience). Biotinylated mAbs were detected with streptavidin-PerCP (BD Pharmingen), streptavidin Alexa-Fluor 488 (Invitrogen) or streptavidin Tricolor (Caltag). Stained cells were

analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson). Viable cells were selected for analysis based on forward- and side-scatter properties.

**DC isolation and Bromodeoxyuridine (BrdU) labelling**

The DC were isolated from secondary lymphoid organs as described (3). Briefly, cell suspensions were digested in DNase/Collagenase and subsequently centrifuged on Nycodenz (1.077 g/ml; Life Technologies), density-gradient. The low-density fraction was collected, washed, and used for phenotypic analysis. At the same day of CTX administration, naive or tumor-bearing mice were injected i.p. with 1 mg of BrdU (Sigma) dissolved in saline and filter sterilized to ensure immediate availability of the precursor. Mice were then continuously given BrdU (0.8 mg/ml) in drinking water. The DC were isolated at various times as described above and surface stained with anti-CD11c, anti-CD8α, and anti-CD45R/B220. The cells were then intracellularly labelled with FITC-conjugated anti-BrdU mAb (Becton Dickinson), as previously described (3). The stained DC were then analyzed by FACS for the number of BrdU-positive cells in each gated DC subset.

**Bone marrow DC precursor's analysis and culture**

Bone marrow cells were collected at various times post CTX treatment and surface stained for detection of DCP with the following panel of mAbs: biotin-conjugated antibodies to lineage (Lin) markers CD3ε, CD4, CD8, NK1.1, CD19, F4/80, Ter119 and I-$A^d$/I-$E^d$ (BD Pharmingen) followed by Streptavidin Tricolor, anti-CD11c-FITC and anti-CD45/B220-PE. In some experiments cells were labelled with anti-Flt3/CD135-PE combined with anti-CD11c-FITC and Lin cocktail to which biotin anti-CD11b and anti-CD45/B220 were added, and then analysed FACS. For detection of cell proliferation, BM cells were labelled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 10 minutes at 37°C and then cultured in complete IMDM medium containing 10 ng/ml rmGM-CSF (Peprotech). At various times of differentiation, BM-DC were

collected, labelled with PE-conjugated anti-CD11c and analysed by FACS for CFSE dilutions and CD11c positivity.

**Analysis of tumor-infiltrating DC**

EG7 tumors were excised from CTX-treated or saline-treated mice at various times. For flow cytometric analysis, the tumor mass was cut into small fragments, then digested in type III collagenase-containing medium (1 mg/ml) for 30 min at room temperature in agitation, followed by EDTA (0.1 M, pH 7.2) for an additional 5 min. The homogenate was then passed through a cell strainer and the resulting cell suspension was labelled with the following mAbs: anti-CD11c-APC, anti-CD3-PE, anti-CD19-PE, anti-I-$A^d$/I-$E^d$-biotin, followed by streptavidin PerCP. Since EG7 tumors did not express I-$A^d$/I-$E^d$ and CD11c molecules, tumor-infiltrating DC were detected as $CD3^-CD19^-CD11c^+I\text{-}A^+$ cells. For confocal laser-scanning microscopy (CLSM), since tumors of CTX-treated mice at day 7 p.i. were too small for sectioning, we could perform the analysis only at day 3. Briefly, tumor tissues were snap frozen and embedded in Optimal Cutting Temperature compound (OCT; Tissue-Tek) and stored at − 80 °C. Frozen sections (5 µm thick) were fixed in cold acetone and stained with the following mAbs and isotype: anti-CD11c-FITC, anti-I-$A^d$/I-$E^d$-biotin, anti-CD86-biotin, anti-MHC-I-OVAp-biotin, mouse IgG followed by streptavidin-Alexa Fluor 594 (Invitrogen). Nuclei were visualized by DAPI (1µg/ml). CLSM observations were performed with a Leica TCS SP2 AOBS apparatus, using 405, 488, 594 nm excitation spectral laser lines appropriately tuned by acousto-optical tunable filter. Signals from different fluorescent probes were taken in sequential scan settings, and co-localization was detected in yellow (pseudo-color). Several fields were analyzed for each labeling condition.

**Homing of tumor-infiltrating DC to LN.**

At day 7 post-CTX or saline, mice were injected intratumorally with 50 µl FITC Isomer 1 (1% solution dissolved in a solvent of acetone-dibutylphtalate,1:1; Sigma). Three days later (day 10 p.i.)

draining inguinal, axillary, or, as a control, controlateral LN were excised and homogenized. The resulting cell suspensions were labelled with anti-CD11c APC and analysed by FACS. Migratory DC were detected as $CD11c^{+}FITC^{+}$.

**Detection of apoptosis and assessment of cell death immunogenicity**

For *in vitro* detection of apoptosis, EG7 or EL4 cells were either UV irradiated or treated with MAFO (10 μM) and then analysed for Annexin V/Propidium Iodide (PI) labelling at various times of incubation. Apoptosis occurred at 48h of culture in MAFO-treated tumor cells and at 18h in UV-irradiated cells (data not shown). For *in vivo* apoptosis detection, FLIVO assay was used. Briefly, green-fluorescent FLIVO™ reagent (FAM-VAD-FMK, Immunochemistry Technologies) was first dissolved in DMSO (50 μl) and then diluted in sterile injection buffer according to the manufacturer's instructions and immediately injected via the lateral tail vein of mice at specified time points after CTX treatment. Thirty minutes after FLIVO injection, mice were sacrificed and the tumor mass removed. Flow cytometry of cell suspensions and CLSM analysis of tumor tissue sections were performed. For immunogenicity of cell death *in vitro*, $5 \times 10^4$ EG7 cells either MAFO-treated or UV-irradiated were plated in 96-well plates and analyzed for surface CRT and CD31 expression. Briefly, anti-CRT-biotin was added to the cell culture medium. After 4, 18, and 24h EG7 cells were harvested, washed twice in cold PBS and incubated for 30 min with streptavidin Alexa-Fluor 488. For CD31 expression, EG7 cells were harvested, washed twice with cold PBS and incubated for 30 min with anti-CD31-APC, or control isotype, diluted in cold blocking buffer. Samples were analyzed by FACS, by gating on PI-negative cells. Live EG7 cells served as controls for all assays. To test the release of DC-activating signals by EG7 dying cells, supernatants from MAFO treated or UV irradiated tumor cells were collected after 48h and 18h culture, respectively, reflecting the times of apoptosis occurrence, and added to naïve splenic DC. Eighteen hours later, DC were surface stained for CD11c, CD8α, CD40, CD80, and MHC-I and analysed by FACS. DC activation was further assessed by release of IL1-β and IL-6 in culture

supernatants using quantikine ELISA kits (R&D Systems). For *in vivo* assessment of immunogenic apoptosis, EG7 cells were programmed for cell death by either MAFO treatment or UV irradiation and injected s.c. ($30\times10^6$) 4 hours later into one flank of C57BL/6 mice. One week later, mice were challenged with live tumor cells ($5\times10^6$) by s.c. injection into the opposite flank. Tumor growth was monitored.

**Protein extraction and western blotting**

Proteins were extracted from live, UV-irradiated or MAFO-treated tumor cell lines and assayed by western blot, as described previously (2). Briefly, $12x10^6$ tumor cells were incubated for 30min on ice in lysis buffer (50mM Tris HCl, pH 8.0, 120mM NaCl, 0.25% Nonidet P40, 0.1% SDS) (Sigma) containing the protease inhibitors PMSF, aprotinin, leupeptin and pepstatin (Roche) each at a final concentration of 10 ng/mL, and 1mM DTT (Sigma). In addition, supernatants from cultured tumor cells were collected. A quantity of 40 μg of each protein sample was loaded onto a 10% SDS-PAGE gel. Loading of supernatants was performed by normalization to cell numbers. Following separation, proteins were blotted onto a nitrocellulose membrane (Whatman). Membranes were blocked with 5% non-fat dry milk in PBS-0.5% Tween 20, and then probed with rabbit anti-mouse HMGB1 polyclonal antibody (2 mg/ml; Abcam) or anti-mouse β-tubulin monoclonal antibody (Sigma), followed by HRP-conjugated anti-rabbit IgG antibody or anti-mouse IgG antibody (Amersham Pharmacia Biotech). Immunoreactive protein bands were detected by using the ECL detection kit (Amersham Pharmacia Biotech). Protein expression was quantitated by densitometry using ImageJ software (http://rsbweb.nih.gov/ij/).

**Phagocytosis of apoptotic EG7 tumors and cross-priming of CD8 T cells by DC**

For uptake analysis, EG7 cells were labelled with 1μM CFSE and either cultured with 10μM Mafosfamide for 48 h or UV irradiated and left in culture for 18h for apoptosis to occur. Apoptotic cells ($4x10^6$/ml) were co-cultured with naïve splenic $CD11c^+$ DC at a 4:1 ratio for 18h in the

presence of type I IFN ($5x10^3$ U/ml) or mock. DC were then separated from apoptotic cells by Nycodenz centrifugation, surface stained and analysed by FACS. For proliferation assays, DC were loaded with apoptotic EG7 (EG7-DC) or EL4 cells (EL4-DC) by 18h co-culture with type I IFN or mock, then immunofluorescently sorted by using a FACSAria and cultured at graded numbers with $10^5$ OT-1 $CD8^+$ T cells. DC loaded with soluble OVA (0.2 mg/ml) served as positive control for OT-1 stimulation. $^3$H-Thymidine incorporation was measured at the third day of culture. Ag-specific IFN-γ production by CD8 T cells was assessed by ELISPOT assay using reagents and methods as recommended by the manufacturer (Mabtech AB). Briefly, OT-1 cells ($5x10^4$) and EG7-DC or EL4-DC ($10^5$) were co-cultured for 2 days on anti-IFN-γ Ab pre-coated 96-well polyvinylidene fluoride-bottomed plates (Millipore) in triplicates. IFN-γ spot forming cells were detected using BCIP/NBT substrate (Sigma) and visualized using an ELIscan reader (A.EL.VIS).

**Total RNA extraction and qRT-PCR**

Total RNA was extracted from 2-$10x10^6$ BM-DCs or tumor tissue by using Trizol reagent (Invitrogen). Messenger RNA was reverse transcribed by means of Verso cDNA kit (Thermo Scientific). Quantitative real-time reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed using Sensimix Plus SYBR kit containing the fluorescent dye Sybr Green (Quantace). Forward and reverse primers (Supplementary Table II) were purchased from Primm. The conditions of real-time PCR reaction were as follows: 15 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C (45 cycles). PCR products were continuously measured by means of an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, USA). Quality and specificity of amplicons in each sample was detected by dissociation curve analysis. Triplicates were performed for each experimental point. For quantization, threshold cycle ($C_T$) values were determined by the Sequence Detection System software (Applied Biosystems), and ΔCT was obtained by subtracting $C_T$ of reference gene, β-actin, to $C_T$ of target gene. Gene expression was presented as relative amount of mRNA normalized to β-actin and was calculated as $2^{-\Delta Ct}$ , a modification of the $2^{-\Delta\Delta Ct}$ method (4).

**Statistical analysis**

Levels of significance for comparison between samples were determined by the two-tailed Student's t-test. P values minor to 0.05 were considered statistically significant.

**References**

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