Separation and Culture of PBMC

PBMC were isolated from citrate blood by density-gradient centrifugation in Leukosep separation tubes (PAA Laboratories GmbH, Austria). After repeated washing with PBS containing 50,000 IU/liter heparin (Liquemin N 25000; Roche, Germany) cells were adjusted to a concentration of 10^6/ml and seeded as triplicates in 96-well round bottom microtiterplates at a ratio of 100,000 PBMC feeder cells (pre-irradiated with 30Gy) to 50,000 PBMCs. RPMI 1640 (Biochrom) containing 10% rabbit serum (heat-inactivated and sterilized at 600Gy), 5% HEPES buffer, and 1μg/ml penicillin/streptomycin was used as culture medium and incubation performed in a humidified incubator at 37°C under 5% CO2.

Tissue-lysates were freshly prepared in cold phosphate buffer (50mM) using a glass homogenizer. The suspension was filtered with a filtertip (pore size, 1.2μm) to adjust fragment size. Protein concentration was measured photometrically according to Bradford and was adjusted to 1mg/ml, followed by sterilization at 600 Gy.

Stimulation of PBMC and T Cell Proliferation Assay

The lysate-loaded feeder cells were added to the native PBMCs for T cell stimulation. At day 5 after coculture, the positive controls received 10ng/ml PHA and, at day 6, 10 μCi methyl-[3H]thymidine/well (Amersham, UK) were added followed by cell harvesting after another 16 h (Cell-Harvester, Inotech, Switzerland) and measurement of tritium incorporation into DNA using a β-counter (BASreader 5148; Fuji, Duesseldorf, Germany) and AIDA software (Raytest, Berlin,Germany).

The resulting data were used to calculate the stimulation index (SI) for each experimental group considering [^{3}H]thymidine incorporation ratios of tumor lysate to normal liver lysate ≥3.0 as proof of specific activation (17). Data sets conforming to $SI_{tumor\ lysate} \neq 0$ and $SI_{PHA/liver} > 3.0$ were considered as true results.

VX2 cytotoxicity assay

VX2 tumor cells were purified on Percoll as described above and seeded into whitebottom microtiter plates using RPMI 1640 without phenol (containing 20% FBS, 0.5% pencillin/streptomycin). 10,000 VX-2 target cells were incubated with 1,000 effector cells in a final volume of 200µl in round-bottom 96-well microtiter plates. To obtain humoral cytoxicity, 10,000 target cells were incubated with 200µl rabbit plasma. After incubation for 4 h at 37°C, 100µl of supernatant were harvested and stored at –20°C for further analysis. Tumor cell lysis was quantified by an adenylate kinase (AK) release assay using a luciferase assay (ToxiLight Kit, Lonza, Verviers, Belgium). 20µl of supernatant were incubated with AK-detection reagent (Lonza, Verviers, Belgium) for 5 min at room temperature, bioluminescence measured by a luminometer (BD Monolight 3096 Microplate Luminometer, BD Biosciences, Heidelberg, Germany) and expressed as relative luminescence units (RLU). Maximum AK release was obtained by incubating target and effector cells with ToxiLight® 100% Lysis Reagent Set (Lonza, Verviers, Belgium), and baseline AK release with medium alone. Baseline release from T cells and tumor cells was <10% of maximal release in all experiments and subtracted from each value.

Cytokine ELISA

Serum of animals collected 14 days after tumor implantation, 4 weeks after first treatment procedure (e.g. RFA, CpG application or both) was used for cytokine analysis.

ELISAs were performed according the manufacturers' guidelines and were assessed at 450 nm with a wave length correction at 540 nm to substract background noise in an Genios plate reader (Tecan, Germany).

KIT	Positive control	Provider	Species
IL-8 ELISA KIT ABIN367974	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
IL-10-ELISA KIT ABIN365253	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
IL-12 ABIN628352	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
Interferon-gamma ABIN578993	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
TNF-alpha ABIN415985	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
VEGF ABIN431314	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
IL-6 ABIN365251	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit
IL-2 ABIN415968	Included in the KIT	Antibodies-online, Aachen Germany	Anti-rabbit

RFA Application

After a maturation period of 21 days leading to an average tumor size of 10 mm, animals of groups B, B*, D and D* (*n*=32) were treated with ultrasound-guided percutaneous RFA[14] using a RF generator (Elektrotom HF 106; Integra, Tuttlingen, Germany), equipped with a perfused RF needle applicator of 1.1mm outer diameter and a 10mm active electrode. The self-adhesive neutral electrode was applied on the animal's shaven back. The needle applicator was advanced into the focus of the tumor under ultrasound guidance and isotonic saline was continuously instilled into

the coagulation zone via microbores in the needle tip at a flow rate of 40ml/h. Treatments were performed at a power output of 20W for 4 min.