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Generation of a syngeneic orthotopic graft murine model of peritoneal carcinomatosis for studying *in vivo* nsPEF anticancerous effects

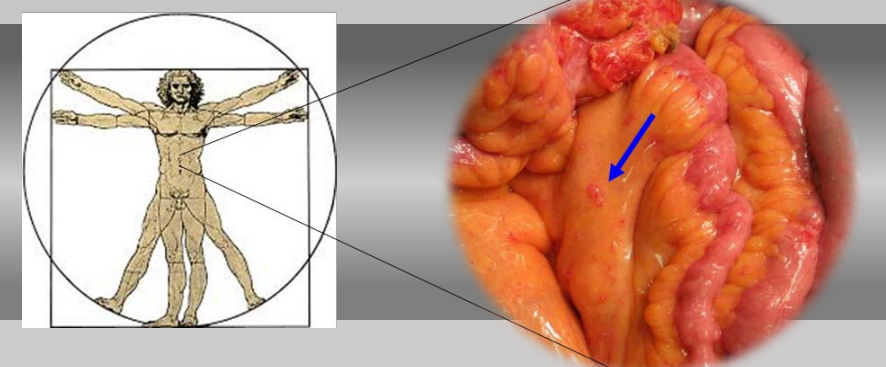
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Introduction



Colorectal cancer (CRC) causes around 700 000 deaths annually and more than 3.5 millions of patients living with it [1]. Indeed, peritoneal metastases are found in 10 to 25% of cases of CRC [2,3]. Until recently, oncologists and digestive surgeons have considered peritoneal carcinomatosis (CPC, the implantation of tumor cells throughout the peritoneal cavity) as an incurable disease [4]. Currently, the only treatment for validated CPC is based on a complete cytoreduction associated with intraperitoneal hyperthermia chemotherapy (HIPEC), supervised by systemic chemotherapy [5], only available to patients with resectable CPC. The majority of patients with CPC is considered unresectable at the time of diagnosis [8]. It is for these reasons that it is imperative to work on **therapeutic alternatives** to treat these many patients with unresectable peritoneal carcinomatosis.

High-intensity pulsed electric fields with nanosecond durations (3-300ns; nsPEFs) have emerged as a promising tool for tumor ablation [10-12]. The physical mechanisms and specific type of **cell death** that occurs following the application of nsPEFs is under investigation by several groups and initial studies have shown effects on cell signaling through calcium and cell death pathways [13-14], mitochondria [15] and cytoskeleton [17-19].

In order to develop a new model of colorectal peritoneal carcinomatosis in mouse, we grafted **murine colon carcinoma CT-26 cells** expressing luciferase in immunocompetent BALB-c mice by **intravenous, subcutaneous, intraperitoneal or intraperitoneal injection after peritoneum aggression (IV, SC, IP or A respectively)** and followed the tumoral growth by bioluminescence over 15 days post-grafting. The adequate model suitable for studies on nsPEF treatment was evaluated by **analyzing tumoral growth, histology and immunohistochemistry** (proliferation, T lymphocyte infiltration, vascularization...) and ***in vivo* multiphoton microscopy** of peritoneal tumoral tissue. *In vitro* CT-26 cells were analyzed by flow cytometry for their sensitivity to nsPEFs (survival, apoptosis) in a **cuvette exposure system**.

Methods

1. CT-26 cell culture

Firefly luciferase-expressing murine CT-26 colon carcinoma cells (M Pocard) were grown at 37 °C in a 5% CO₂ humidified atmosphere, in Modified Essential Medium with EARLES salts (Gibco) supplemented with 10% fetal bovine serum (Gibco), 0.2% Glucose (Gibco), 2 mM L-glutamine (Dominique Dutcher) and 100 U/ml penicillin 100 µg/ml streptomycin (Gibco). The viability of cells was greater than 90% as determined by visual counts using Trypan Blue exclusion on a Malassez cell.

2. *In vivo* murine model of limited CPC

5-weeks old Balb-c mice (Charles River Laboratory, France) were grafted with 40 µl of 30 000 CT-26 cells by intravenous, subcutaneous, intraperitoneal injection only or intraperitoneal injection after peritoneum aggression (respectively IV, SC, IP, A) and followed the tumoral growth by bioluminescence over 15 days post-grafting under isoflurane anesthesia. In accordance with the guidelines of the institution, the experiments were approved by the committee for animal research at the institution (Carcinopulse, SM Bardet). First, mice were intravenously injected in the peritoneum cavity with 25 mg/kg of Xenolight D-Luciferin (Perkin Elmer) under isoflurane anesthesia and, immediately after injection, imaged with a cooled charge-coupled device (CCD) camera system (IVIS[®] Lumina System, Perkin Elmer). 10 mice per group (+ NaCl injected control group) were studied daily between day 3 and 15 post-grafting, and sets of imaging data were acquired. For quantitative analysis, a circular region of interest (ROI) was placed over the tumor, and the total signal (photons/s) in this ROI was determined using Image Analyst MKII software.

3. nsPEF exposure system

In previous studies we have developed *in vitro* and *in vivo* nsPEF exposure setup [20,21] composed of an nsPEF generator, a digital phosphor oscilloscope (DPO), a high-voltage measurement device (tap-off), an electrode-based delivery system. The nsPEF generator (FPG 20, FID Technology, Germany) delivered 10ns pulses with 50kV/cm amplitude. A 1GHz oscilloscope (DPO 4104, Tektronix, USA) using 30dB attenuator connected to the 40dB attenuation tap-off was used to display the time-domain measurements of each delivered electrical pulse. The numerical dosimetry of the nsPEF exposure setup was performed using numerical modeling and full-wave 3D simulations based on a discretization of Maxwell's equations in integral form [22,23].

Adherent cells were trypsinized, washed and suspended at 1 million/ml in complete medium in a volume of 30 µl in a wideband electroporation cuvette (BioRad, 165-2089) containing complete medium (conductivity of 1.5 S.m⁻¹). For nsPEF exposure, the biological cuvette (plastic chamber containing two opposing flat aluminum electrodes spaced with a 1 mm gap, electrode dimensions : 10 mm large and 10 mm high) was connected to the coaxial cable using a custom-made adapter with two plated electrodes soldered on an N-connector. The temperature variation inside the exposed sample in the cuvette at room temperature (22°C) was measured using a fluoro-optic probe (Luxtron, Lumasense, USA).

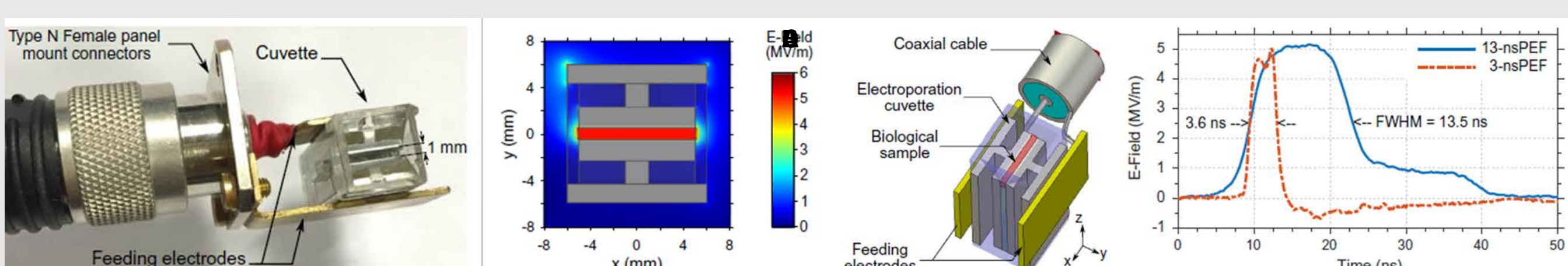


Fig 1. Electromagnetic field determination of the cuvette exposure system (A) by numerical modeling and full-wave 3D simulations based on a discretization of Maxwell's equations in integral form. The dosimetry of the *in vitro* delivery systems showed that the electric field in the central region between the electrodes had a good homogeneity, as based on 3D numerical simulations (B, C). The intensity of the electric field that can be delivered was around 50 kV/cm and the 10ns pulse had a 3.6ns rise time (D).

4. Survival and apoptosis analysis of CT-26

For each exposure parameter, a duplicate of 250 cells/well was reseeded on a glass coverslip in a 24 well plate in complete medium for 3 to 7 days. They were fixed 20 min with 4% paraformaldehyde and permeabilized 15 min with 0.1% Triton in PBS. After labelling with 20 µM DAPI (Sigma, D9542), fluorescent nuclei were imaged by fluorescent microscopy and counted in random fields with Fiji/ImageJ (NIH). Immediately after exposure, the cells (n=3 for each parameter) were transferred to Eppendorf tubes and volume was completed to 100 µl with complete medium. They were loaded with 100 µl of Muse[™] Annexin V & Dead Cell reagent (MCH100105, Millipore) for 20 min at room temperature. Determination of the apoptosis index (by Annexin V) and cell viability (by 7-AAD) was simultaneously measured by fluorescence using a flow cytometer (Muse[®] Cell Analyzer, Millipore) at 45 min and 3 hours post-exposure.

4. Multiphoton fluorescence of CPC nodules

Anesthetized mice and samples were positioned on a stage of a customized Olympus multiphoton microscope BX61WI/FV1200MPE with a 25X immersion objective (1.05NA, 2.0mm working distance) coupled with a tunable femtosecond Ti:Sapphire pulsed laser (Chameleon Ultra II, Coherent) for the excitation. Image stacks were taken at 2µm steps, acquired under 810nm excitation for second harmonic generation (SHG, collagen) and auto-fluorescence (elastin) wavelength with FluView FV1200 software (v4.1.1.5, Olympus). The different components of the emitted light from the sample were separated using a dichroic mirror (570nm) and detected by a pair of photomultiplier tubes preceded by fluorophore specific emission filters (BA 575-630 for elastin, 405/10 for SHG). The images obtained were analyzed with Imaaris software (Bitplane AG) or Fiji/ImageJ (NIH).

5. Histology and Immunohistochemistry

Tissues around the site of tumors (Intestine and peritoneum) were collected and embedded in paraffin. Sections (12 µm) were cut, stained with hematoxylin-eosin-safran (HES) and for immunohistochemistry (CD3, CD4, CD8) on an automate BenchMark XT (Roche[®], Meylan, France), to be analyzed for the presence of tumor nodules.

Results

Fig 2. All animals survived the operative procedures (IV, SC, IP or A) and presented diverse and limited in size tumor nodules in the peritoneal cavity (A and B, example of peritoneum aggression from group A at day 15 post-grafting). HES staining of a colorectal localized nodule reveals an enrichment of CT26+ cells (B)

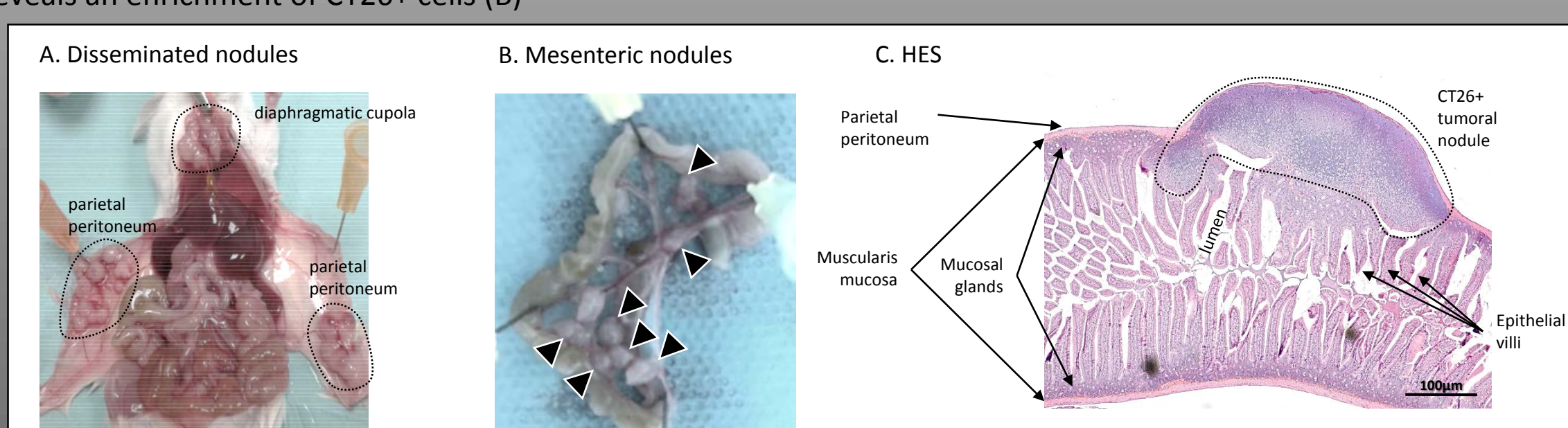


Fig 3. *In vivo* quantification of tumor growth by bioluminescence tracking of CT26 cells over 15 days reveals that the laparotomy surgery (group A : aggression of the peritoneum and intraperitoneal CT26 injection) presents a sufficient and adequate growth with a relevant Peritoneal Carcinosis Index (PCI) of Sugarbaker

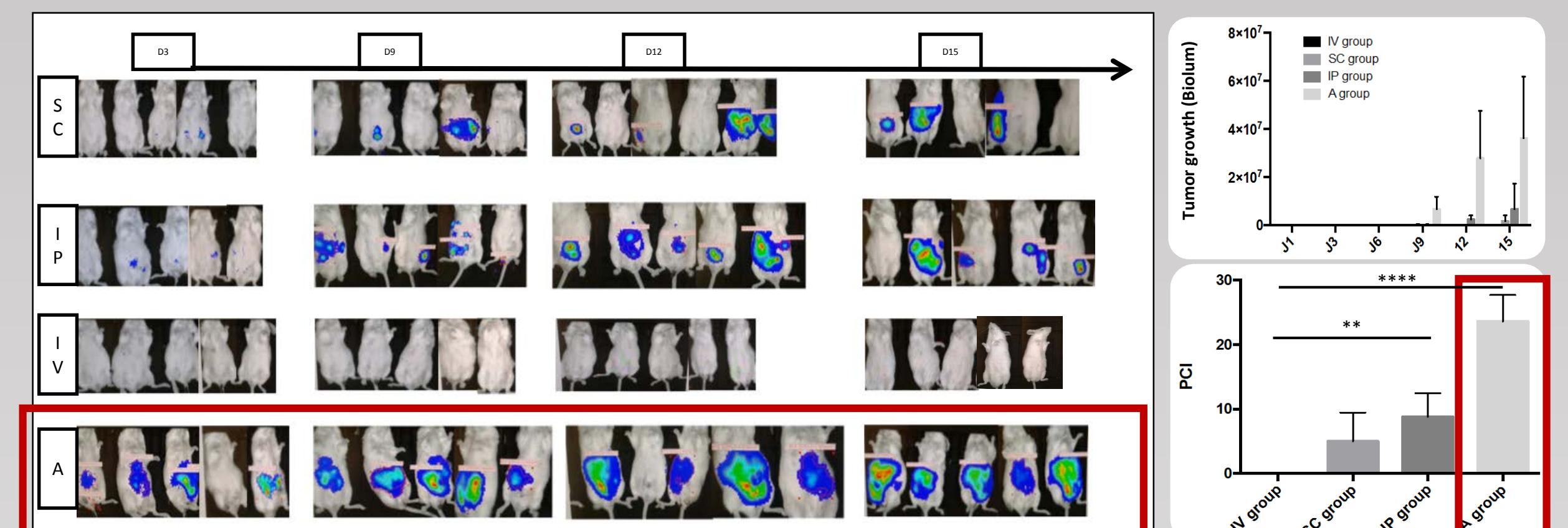


Fig 4. Grafted CPC nodules reveal high number of cells including Tumor infiltrating Lymphocytes (TILs) among a complex tumoral micro-environment (high vascularization, poor fibrosis, dense tissue). Scale bar in A applied to C,E,F,G and in B applied to D

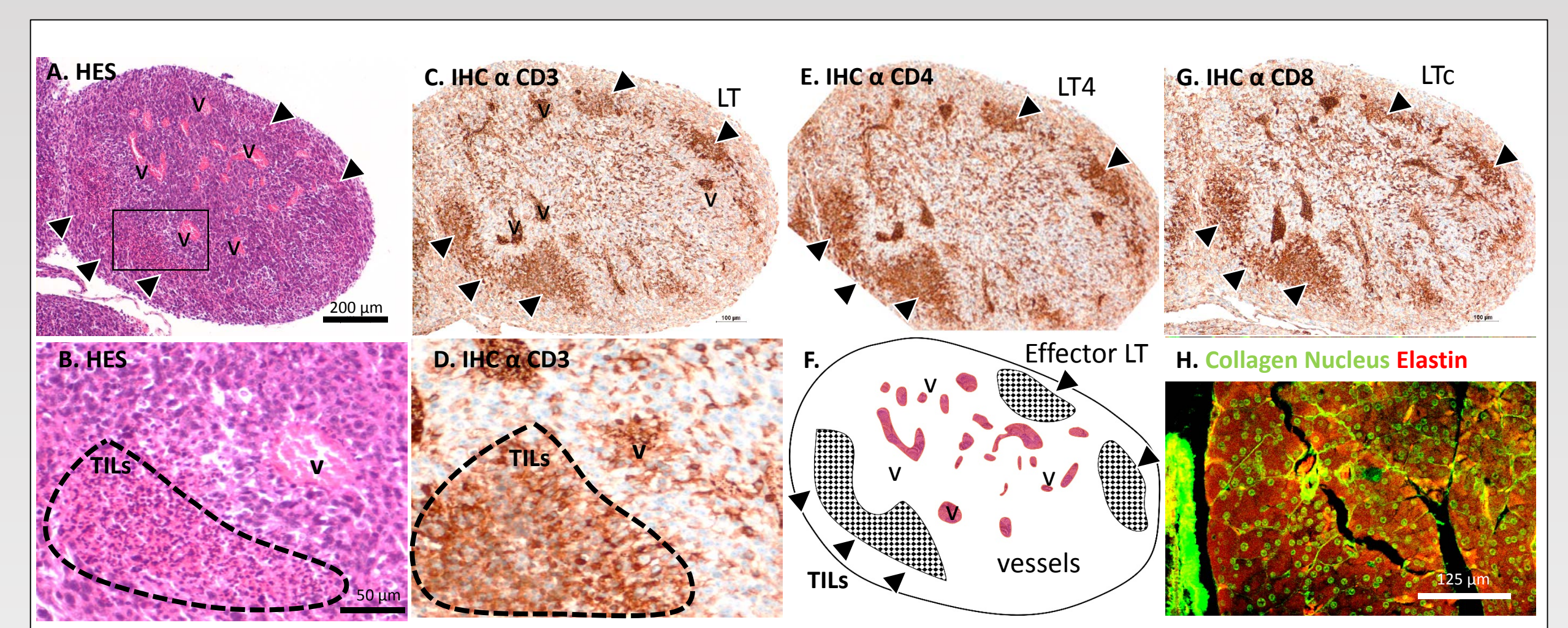
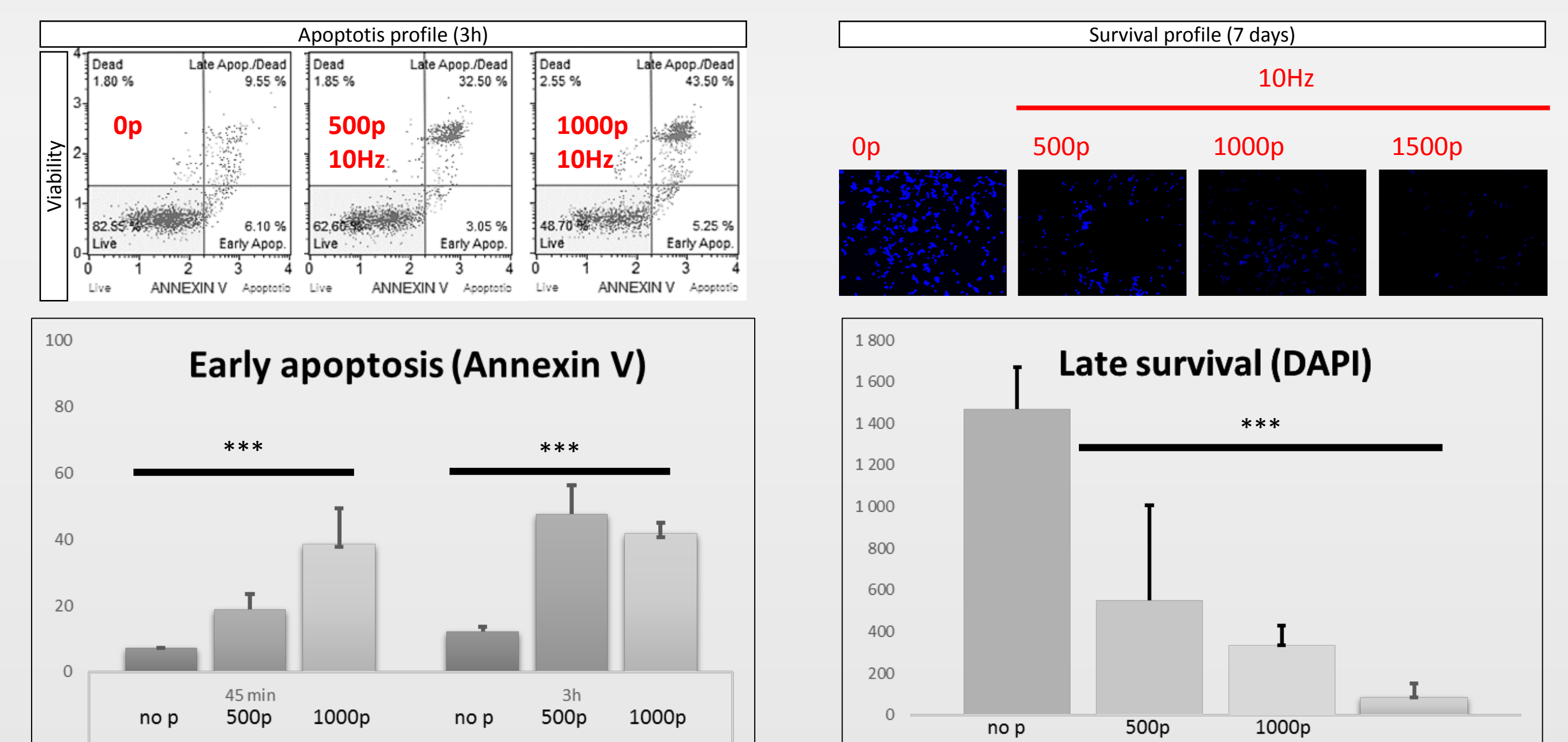


Fig 5. *In vitro* exposition of CT-26 cells to 10ns pulses induces cell death by apoptosis (A) and poor survival after 7 days post exposure (B). Three microscopic fields were arbitrarily chosen and the average DAPI+ cell number was determined (*** p<0.01). Experimental data are expressed as mean values ± SEM

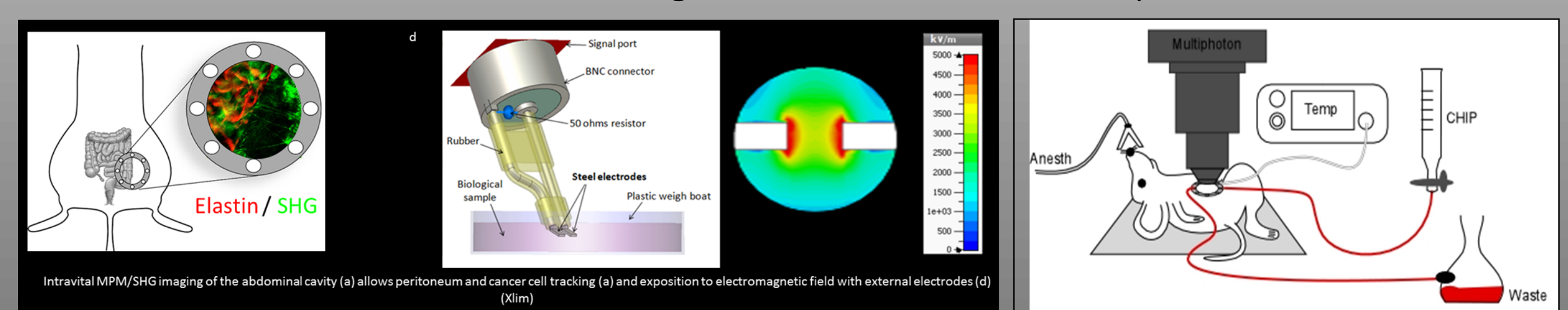


Thanks to conventional *in vivo* bioluminescence imaging using wild-type green-emitting luciferase, we characterized and compare *in vivo* the growth rate and dissemination of luc CT-26 tumors between the four different grafting methods (Fig.3). Upon implantation, intravenously injected cells did not demonstrate a significant growth rate when subcutaneous site displayed a limited carcinosis in the peritoneal cavity. Both intraperitoneal injection after peritoneum aggression or intraperitoneal injection alone showed elevated growth rates at 15 days post-surgery. Anatomic and histologic analysis corroborated the existence of various tumoral nodules, and we described by multiphoton microscopy tumoral fibrosis (SHG) infiltrated tumoral cells, in comparison with non-pathologic peritoneum (Fig.4).

In vitro data are also consistent with our previous published results on 5 tumoral cell lines [21], we observed a strong effect of nsPEF on cell viability, in a dose-dependent manner.

Conclusions - Future studies

- We have developed a limited but extensive model of **colorectal peritoneal carcinomatosis** in mouse suitable for nsPEF studies based on grafting murine colon carcinoma CT-26 cells expressing luciferase in immunocompetent BALB-c mice by intravenous, subcutaneous, intraperitoneal or intraperitoneal injection after peritoneum aggression.
- We have demonstrated that these CT-26 cells are sensitive *in vitro* to 10ns nsPEF.
- As an optimal *in vivo* model, we have chosen the intraperitoneal injection after peritoneum aggression as this technique provided a fast but limited tumoral growth, ideal to combine fluorescent and bioluminescent reporters to measure tumour regression and to visualize the signaling pathways activated in the hours and days following nsPEF application.
- Future *in vivo* electrodes have to be designed in order to facilitate the exposure.



-nsPEF exposure can be studied in combination with HIPEC therapy to evaluate the anti-tumoral synergic effects and the immune response.

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