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Gene electrotransfer of IL-2 and IL-12 plasmids effectively eradicated murine B16.F10 melanoma

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ABSTRACT

Gene therapy has become an important approach for treating cancer, and electroporation represents a technology for introducing therapeutic genes into a cell. An example of cancer gene therapy relying on gene electrotransfer is the use of immunomodulatory cytokines, such as interleukin 2 (IL-2) and 12 (IL-12), which directly stimulate immune cells at the tumour site. The aim of our study was to determine the effects of gene electrotransfer with two plasmids encoding IL-2 and IL-12 *in vitro* and *in vivo*. Two different pulse protocols, known as EP1 (600 V/cm, 5 ms, 1 Hz, 8 pulses) and EP2 (1300 V/cm, 100 μs, 1 Hz, 8 pulses), were assessed *in vitro* for application in subsequent *in vivo* experiments. In the *in vivo* experiment, gene electrotransfer of pIL-2 and pIL-12 using the EP1 protocol was performed in B16.F10 murine melanoma. Combined treatment of tumours using pIL2 and pIL12 induced significant tumour growth delay and 71% complete tumour regression. Furthermore, in tumours coexpressing IL-2 and IL-12, increased accumulation of dendritic cells and M1 macrophages was obtained along with the activation of proinflammatory signals, resulting in CD4 + and CD8 + T-lymphocyte recruitment and immune memory development in the mice. In conclusion, we demonstrated high antitumour efficacy of combined IL-2 and IL-12 gene electrotransfer protocols in low-immunogenicity murine B16.F10 melanoma.

1. Introduction

Currently, gene therapy shows considerable potential for the treatment of cancer [1]. In cancer gene therapy, several strategies are employed that rely on the expression of different types of therapeutic genes that induce apoptosis, block oncogene expression, replace defective tumour suppressor genes and enhance tumour immunogenicity [2]. The main obstacle for successful gene therapy is the delivery of transgenes to targeted tissues. Viral vectors are still the primary tool used for the introduction of therapeutic genes into the targeted tissue [3], and despite the good transfection efficiency, the main drawbacks are limited DNA packaging capacity, immune response to viral particles and off-target activity that leads to unwanted clinical outcomes [4,5]. A less expensive and safer alternative to viral vectors is the introduction of naked DNA (plasmids) into cells using electroporation [6,7].

Electroporation is a technique based on exposing cells and tissues to an external electric field to increase the permeability of the membrane, thereby allowing the introduction of therapeutic molecules and genetic material (DNA, RNA) inside the cells [8]. The most developed biomedical application of electroporation is electrochemotherapy, which uses electroporation as a safe and effective delivery method for the introduction of cytotoxic drugs and is currently recognized as local therapy for cutaneous tumours and metastases [9]. Clinical and preclinical studies also confirmed the applicability of electrochemotherapy for the treatment of certain deep-seated tumours that are not suitable for surgery or other local ablative therapies, such as colorectal liver metastases and hepatocellular carcinoma [10–12]. Another utilization of electroporation is gene electrotransfer, in which genetic material is

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introduced into the cell, which allows the cells to produce transgenes that have direct or indirect antitumour (immunomodulatory, cytotoxic, antiangiogenic, etc.) effects or specific tumour antigens that induce a specific antitumour immune response [13–16]. Many efforts are underway through *in vitro* and *in vivo* studies to optimize gene electrotransfer protocols to minimize tissue damage and improve gene transfection efficiency [17–19]. Several studies have been published in the field of immunomodulatory gene therapies using electroporation, and immunostimulatory cytokines interleukin 2, 12, 15 and GM-CSF were evaluated [20–26]. Preclinical studies showed no or minimal toxicity of plasmid IL-12 gene electrotransfer [27]. Therefore, this therapy has been applied in clinical studies as a single treatment or in combination with immune checkpoint inhibitors [28–30].

Interleukin 12 (IL-12), with its potent proinflammatory functions, is a suitable candidate for cancer immunotherapy. IL-12 signalling is responsible for the acquisition of cytotoxic functions by $CD8^+$ cells, differentiation of Th1 and induction of IFN-γ production, which enhances phagocytic functions and local inflammation [31]. The major drawbacks are the severe toxicities related to high-dose systemic recombinant IL-12 administration. With a gene electrotransfer approach, the systemic effect could be avoided [32]. Intratumoural IL-12 gene electrotransfer has already presented favourable results in preclinical and early-phase clinical studies [33]. In a phase I clinical trial in patients with advanced melanoma, electroporation of plasmid IL-12 was shown to elicit systemic cellular and humoural responses that resulted in melanoma lesion regression [22,34].

Another example of a cytokine with strong antitumour activity is interleukin 2 (IL-2), which was approved by the FDA in the 1990 s for the treatment of metastatic melanoma and renal cell carcinoma and is still in use today in the recombinant protein form [35]. IL-2 is important for the survival, proliferation and differentiation of different subsets of T cells and considered a potent mitogen. Similar to IL-12, high-dose toxicity is also a problem that can be avoided with local targeted delivery [36]. In a study using a murine melanoma model, intratumoural administration of IL-2 plasmid DNA appeared well tolerated and caused a reduction in tumour size [37]. Nevertheless, monotherapy using IL-2 appears to be insufficient to increase the survival rate due to the already mentioned high-dose related toxicity and dual functional proprieties on T cells (activation of Treg cells) [35]. However, the combined gene electrotransfer of IL-2 and IL-12 has not been evaluated to date.

Gene electrotransfer of IL-2 in combination with IL-12 could prevent the activation of Treg cells by IL-2 and further amplify the antitumour effectiveness of both cytokines [38]. IL-2 and IL-12 have separate signalling pathways that elicit different but complementary biological effects and induce the expression of IFN-γ predominantly by T and NK cells, which stimulates IL-12 production by dendritic cells and macrophages. High levels of IFN-γ and IL-12 also promote the activation of CD4+, CD8 + and NK cells that produce more IL-2, which in turn upregulates the IL-12 receptor on the surface of NK cells [39,40]. Furthermore, IL-12 expression at the tumour site recruits and activates T and NK cells onsite while IL-2 expression stimulates them and causes their proliferation. The cooperative actions of these cytokines could modify the tumour microenvironment by recruiting tumour-specific immune cells, thus leading to the activation of these immune cells and subsequent elimination of tumour cells. Therefore, the immunological proprieties of the tumour could be changed to increase their responsiveness to therapies. Tumours can be divided into "cold" tumours, which have low levels of cytokine production and T cell infiltration and usually do not respond well to immunotherapy, and "hot" tumours, which present the opposite characteristics [41]. Gene electrotransfer of these two mutually supportive interleukins has the potential to turn unresponsive cold tumours into responsive "hot" tumours.

Thus, the aim of our study was to evaluate combined IL-2 and IL-12 immunotherapy using electroporation to intratumourally introduce plasmids encoding IL-2 and IL-12. First, the viability and expression profile of IL-2 and IL-12 transcripts in the B16.F10 murine melanoma cell line after transfection with the aforementioned plasmids was determined. Then, the effect of gene electrotransfer of plasmids on tumour growth was determined in a murine B16.F10 melanoma model, which is considered a very aggressive and immunologically cold type of tumour [42]. Moreover, a protein expression profile analysis of both cytokines and histological analysis of tumour sections were performed to evaluate the underlying mechanisms of antitumour effectiveness.

2. Materials and methods

2.1. Cell lines

The B16.F10 murine melanoma cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in advanced minimum essential medium (AMEM; Gibco, Thermo Fisher Scientific, Waltham, USA) supplemented with 5% foetal bovine serum (FBS; Gibco), 10 mL/L L-glutamine (GlutaMAX; Gibco), 100 U/mL penicillin (Grünenthal, Aachen, Germany) and 50 μg/mL gentamicin (Krka, Novo mesto, Slovenia) in a 5% CO_2 humidified incubator at 37 °C. The cells were routinely tested and confirmed to be free from mycoplasma infection using the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza Group Ltd, Basel, Switzerland)

2.2. Plasmids

Three different plasmids were used in the experiments: pORFmIL-12 (p40 p35) (InvivoGen, Toulouse, France), which codes for mouse interleukin 12; pUNO1-mIL02 (InvivoGen, Toulouse, France), which codes for mouse interleukin 2; and pControl [43], which was used as a control plasmid (Table 1).

Plasmids were amplified in competent *E. coli* and purified using Endo Free Plasmid Mega Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol and dissolved in endotoxin-free water (Qiagen, Hilden, Germany) at a concentration of 1 mg/mL or 0.5 mg/mL. The concentration of plasmid was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA). The identity of each plasmid was verified by restriction analysis and subsequent agarose gel electrophoresis for the separation of formed bands [44]. Moreover, the purity of isolated plasmids was determined using an Epoch microplate spectrophotometer with a Take3™ Micro-Volume Plate (BioTek, Bad Friedrichshall, Germany), which measured the 260/230 and 260/280 absorbance ratios (Table 1).

2.3. In vitro electroporation

When B16.F10 cells reached approximately 80% confluence, they were collected and resuspended in cold electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \cdot 6H_2O$) at a concentration of 25×10^6 cells/mL. Afterwards, 11 μL of plasmid (at a concentration of 1 mg/mL) or cold electroporation buffer (control and EP group) was added to 44 μL of the cell suspension at the aforementioned concentration. Next, 50 μL of the plasmid and cell mixture was electroporated with an electric pulse generator (GT-01, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia). Pulses were delivered through two parallel stainless-steel plate electrodes, with a 2.4 mm gap in between. Two pulse protocols were used: EP1 consisted of eight

Table 1
Plasmid characteristics.

Plasmid	Base Pairs	Promotor	Antibiotic Resistance Gene	260/280 Ratio	260/230 Ratio	Restriction Enzyme Pairs
pORFmIL-12 (p40 p35)	4833	hEF1-HTLV	ampicillin	1.85	2.12	SacI + SalI NdeI + NcoI
pUNO1-mIL02	3973	hEF1-HTLV	blasticidin	1.92	2.09	NheI + NdeI PstI + NotI
pControl	3390	CMV	ampicillin	1.87	2.11	SacI + ApaLI ApaLI

square-wave electric pulses with a voltage-to-distance ratio of 600 V/cm, pulse duration of 5 ms and frequency 1 Hz, which are commonly used in gene electrotransfer protocols; and EP2 consisted of eight square-wave electric pulses with a voltage-to-distance ratio of 1300 V/cm, pulse duration of 100 μs and frequency of 1 Hz, which are commonly used in electrochemotherapy protocols. After electroporation, the cells were transferred to 24-well plates and incubated for 5 min before the addition of 1 mL of AMEM culture medium. Further tests were performed as described below.

Experimental groups were as follows: cold electroporation buffer alone (Control), individual plasmids alone (pControl, pIL-12, pIL-2) and combination of pIL-12 and pIL-2 plasmids (1:1 ratio, 5 μL each). All groups were treated with the two different electroporation protocols (EP1 and EP2) and without electroporation.

2.4. Cytotoxicity assay

After the treatment, 15 μL of B16.F10 cells from each experimental group were further diluted with 985 μL of medium and plated in 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 1500 cells per well. The cells were then incubated at 37 °C in a 5% CO_2 humidified incubator. Based on the growth rate of the B16.F10 tumour cells (doubling time, ~20 h), 3 days after electroporation, 10 μL of Presto Blue® viability reagent (Thermo Fisher Scientific) was added to the wells, and one hour later, fluorescence intensity was measured using a microplate reader (Cytation 1, BioTek Instruments, Winooski, VT, USA) with a 530/590 nm excitation/emission filter. The results were plotted as the fraction of cell survival compared to the electroporated (electroporated groups) and nonelectroporated (nonelectroporated groups) control groups. The experiments were performed in 3 biological replicates, with each consisting of 8 technical parallels.

2.5. qPCR

After the treatment, B16.F10 cells from each experimental group were seeded in T75 cell culture flasks (North America Laboratories San Diego, CA, USA). Cells treated with the EP2 electroporation protocol and without electroporation were seeded at a density of 1×10^6 cells per flask, while cells treated with the EP1 electroporation protocol were seeded at a density of 2×10^6 cells per flask. Cells were then incubated at 37 °C in a 5% CO_2 humidified incubator. After 2 days, the cells were trypsinized and total RNA was isolated using a peqGOLD Total RNA Kit (VWR, Vienna, Austria). After isolation, the concentration and purity (260/230 and 260/280 absorbance ratios) were measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and Epoch microplate spectrophotometer with a Take3™ Micro-Volume Plate (BioTek, Bad Friedrichshall, Germany), respectively. Total RNA (500 ng) was reverse-transcribed into cDNA using a SuperScript VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Afterwards, 1 ng of cDNA was amplified with a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using custom primers for transcripts of mouse *Il-2* (forward: 5'-GCAGGATGGAGAATTACAGGAA −3', reverse: 5'- GCAGAGGTCCAAGTTCATCTTC −3') and *Il-12* (forward: 5'-CTCTCGTTCTTGTGTAGTTCCA −3', reverse: 5'- ACAGATGACATGGTGAAGACG −3') and commercial primers for β-actin (*Actb*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) housekeeping genes (Integrated DNA Technologies, Coralville, IA, USA) and SYBR Green Master Mix (Thermo Fisher Scientific). Two housekeeping genes were used for more accurate data normalization [45]. The experiments were performed in 3 biological replicates, each consisting of 2 technical parallels. The results are plotted relative to the expression of the housekeeping genes *Actb* and *Gapdh* (ΔCt).

2.6. Animals and tumour induction

In the experiment, 3×10^5 B16.F10 murine melanoma cells resuspended in 100 μL saline solution were injected subcutaneously into the shaved right flank of 6–8-week-old female C57Bl/6 mice weighing 19–23 g (Charles River, Lecco, Italy). Therapies started when tumours reached a volume of approximately 50 mm^3 (day 0). Six mice per cage were housed in specific pathogen-free conditions in a carousel mouse IVC rack system (Animal Care Systems Inc., Revere Parkway, USA) at a relative humidity of 55 ± 10%, temperature of 20–24 °C and light/dark cycle of 12 h. Food and water were provided ad libitum. All procedures were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (Republic of Slovenia, Ministry of Agriculture, Forestry and Food, permission no. 34401–1/2015/43). The ARRIVE guidelines were followed for reporting the *in vivo* experiments [46].

2.7. In vivo electroporation

Mice were included in the treatment groups when their tumours reached approximately 50 mm^3 (day 0), 6 mm in diameter. EP1 pulses were delivered 10 min after intratumoural (i.t.) injection of 50 μL of plasmid DNA (0.5 mg/mL) or endotoxin-free water (control group and EP1 group). The pulses were generated by an electric pulse generator (ELECTRO cell B10, Betatech, Saint-Orens-de-Gameville, France) and delivered in two sets of 4 pulses delivered across two stainless-steel electrodes, with a 6 mm gap between them. To ensure good conductivity at the contact between the tumour and the electrodes, a water-based gel was applied (Ultragel, Budapest, Hungary). During the entire procedure, the animals were kept under inhalation anaesthesia with 1.5% isofluran (Izofluran Torrex para 250 mL, Chiesi Slovenia, Ljubljana, Slovenia) delivered with an oxygen concentrator (Supera Anaesthesia Innovations, Clackamas, USA) at a flow rate of 1 L/min.

The experimental groups were as follows: endotoxin-free water alone (Control); endotoxin-free water combined with electroporation (EP1); individual plasmids without electroporation (pIL-12 and pIL-2); individual plasmids coupled with the EP1 protocol (EP1 pControl, EP1 pIL-12, and EP1 pIL-2); pIL-12 + pIL-2 plasmid combination (ratio of 1:1, 25 μL each) coupled with the EP1 protocol (EP1 Comb).

2.8. Tumour growth measurements

After the therapy, tumour growth was monitored three times per week using Vernier callipers to determine and quantify the antitumour effects. The volumes were calculated using the following equation: $V = a \times b \times c \times \pi/6$, where a, b, and c represent the perpendicular diameters of the tumour. The animals were euthanized when the tumours reached a volume of 350 mm^3. The mice that obtained a complete response (tumour-free for 100 days) were rechallenged with a subcutaneous injection of 3×10^5 B16.F10 murine melanoma cells (resuspended in 100 μL saline solution) in the mouse left flank and monitored for tumour outgrowth. If the tumours did not develop, the mice were followed for another 100 days.

2.9. ELISA

For the *in vitro* part of the experiment, B16.F10 cells from each experimental group were grown in 2 mL of cell culture medium in 6-well plates (Corning Incorporated, Corning, NY, USA). Cells treated with the EP2 electroporation protocol and without electroporation were seeded at a density of 1×10^6 cells per flask, while cells treated with the EP1 electroporation protocol were seeded at a density of 2×10^6 cells per flask. Cells were incubated at 37 °C in a 5% CO_2 humidified incubator. After 2 days, the cell culture medium was collected, and then the cells were trypsinized and counted in a haemocytometer (Weber Scientific, Hamilton, NJ, USA). The presence of IL-12 and IL-2 proteins in culture medium was determined using commercial ELISA mouse IL-2 and IL-12 detection kits (Thermo Fisher Scientific) according to the manufacturers' instructions, and absorbance at 450 nm was measured using a microplate reader (Cytation 1, BioTek Instruments, Winooski, VT, USA). The experiments were performed in 3 biological replicates, each consisting of 4 technical parallels.

For the *in vivo* part of the experiment, ELISA was performed on days 3, 5 and 7. Because the human endpoint was reached in the control groups before day 7, the samples were taken at day 5. Blood was collected from the infraorbital sinus of isoflurane-anaesthetized mice using glass capillaries and transferred into 0.5 mL serum collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After blood collection, the mice were humanely euthanized. The blood was allowed to coagulate for 20 min, and the tubes were then centrifuged for 5 min at 2000 rcf to obtain the serum. The serum was kept at −20 °C until the analysis. The tumours were also excised, and half of the tumour was snap frozen in liquid nitrogen and stored at −80 °C until further analysis. The tumours were then homogenized using a mortar with a pestle, and the homogenized tumours were weighed and resuspended in 500 μL PBS and Protease Cocktail (Thermo Fisher Scientific). The suspension was then centrifuged for 10 min at 1500 rcf, and the supernatant was collected and stored at −80 °C until analysis. IL-2 and IL-12 detection was performed via ELISA (both Thermo Fisher Scientific) according to the manufacturers' instructions, and absorbance was measured using a microplate reader (Cytation 1, BioTek Instruments). The data were normalized to the cell number for the *in vitro* assay and tumour weight for the *in vivo* experiments.

2.10. Histological analysis

The other half of the tumours harvested on days 3 and 5–7 after the treatment were fixed and paraffin-embedded (FFPE). In detail, samples were left overnight in a formalin-free zinc fixative (BD Biosciences, San Jose, CA, USA) and then transferred into 70% ethanol until further analysis. Subsequently, each fixed tissue was embedded in paraffin. The paraffin-embedded blocks were cut into 2 μm-thick-sections for H&E (hematoxylin and eosin) staining and into 4 μm sections for immunohistochemical staining, with 6 sections per tumour. Three sections of each sample were stained using H&E according to standard procedures. Necrotic areas, which were pink-coloured, and viable parts, which were purple-coloured, were identified. Images of H&E-stained tumours were obtained using a DP72 CCD camera connected to a BX-51 microscope (Olympus, Düsseldorf, Germany) at 4× magnification.

2.11. Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick FFPE tissue sections using four different primary antibodies to detect the presence of macrophages (anti-F4/80 Ab, anti-MHCII Ab), DCs (anti-CD11c Ab), helper T lymphocytes (anti-CD4 Ab) and cytotoxic T lymphocytes (anti-CD8 Ab). Before the use of the CD11c antibody, the antigen retrieval procedure was performed in sodium citrate buffer (10 mM sodium citrate, pH = 6) for 60 min at 95 °C. For the other antibodies, no retrieval methods were used. Subsequently, sections were incubated in a humid chamber overnight at 4 °C with the following primary antibodies (used at a 1:100 dilution in blocking buffer): rat anti-mouse F4/80 clone BM8, rat anti-mouse MHC class II clone M5/114.15.2, Armenian hamster anti-mouse CD11c clone N418, rat anti-mouse CD4 clone 4SM95 and rat anti-mouse CD8 clone 4SM16 (eBioscience, San Diego, CA, USA). Incubation with the primary antibody was omitted for the negative controls. Next, to amplify the antibody signal, biotin-labelled secondary antibodies were used. Each section was incubated with anti-rat IgG and anti-hamster IgG secondary antibodies (Invitrogen/Life Technologies, Carlsbad, CA, USA, 1:1000) and HRP-conjugated avidin for 30 min at room temperature. To detect the antibody signal, a substrate/chromogen mixture consisting of 3,3′-diaminobenzidine (DAB) was employed, followed by haematoxylin counterstaining. The immunostained slides were observed under bright light by an Eclipse E600 photomicroscope equipped with a DMX1200 digital camera (Nikon Instruments SpA, Calenzano, Italy) at 40x and 20x magnification. Photos were taken under the same lighting conditions. The image data were analysed using ImageJ software [47]. For each immunohistochemical stain, five to nine fields (width: 58.254 square pixels corresponding to 384355.606 μm^2) per experimental group were chosen. The extension of the stained areas in a photographic field was taken as an indicator of the number of stained cells. The stained areas were detected by colour-thresholding the image, the saturation and brightness were adjusted to exclude the background colour. The resulting area was then measured in square pixels.

2.12. Statistical analysis

Differences between the experimental groups in *in vitro* experiments were analysed via a one-way analysis of variance (ANOVA) followed by Tukey's tests and Student's t tests for group comparisons. The differences between the experimental groups in the *in vivo* experiments were evaluated by Kruskal-Wallis tests followed by Dunn's and Mann-Whitney tests for group comparisons. Immunohistochemistry data are represented as the mean values and standard deviations that were computed and compared by ANOVA followed by resampling through a 10000-iteration bootstrap of the F-stat values. Following the ANOVA, post hoc pairwise comparisons were performed by Tukey's honestly significant difference [48] using the Real Statistics Resource Pack software (Release 6.2) [49] For graphical representation and other statistical analyses, GraphPad Prism (GraphPad, San Diego, CA, USA) software was used. A p value of<0.05 was considered to be significant.

3. Results

3.1. In vitro recombinant IL-2 and IL-12 protein cytotoxicity

First, the cytotoxicity of recombinant IL-2 and IL-12 to B16.F10 cells was determined to exclude the potential direct toxic effects of those interleukins on the tumour cells. The cells were treated with different concentrations of recombinant protein, and their survival was determined after three days. Over the wide range of tested concentrations, no direct effect of the cytokines on cell survival was observed (Fig. 1).

3.2. In vitro survival after electroporation with pIL-2 and pIL-12

The cytotoxicity of the two different electroporation protocols on B16.F10 cells without the addition of plasmids was first evaluated. The exposure of cells to the EP1 pulse protocol led to a significant reduction in survival compared to the nonelectroporated control group (for 0.4). In contrast, the exposure of cells to the EP2 protocol alone led to a minor reduction in survival compared to the nonelectroporated control group (for 0.2) (Fig. 2a). Afterwards, the cytotoxicity of three different plasmids (pIL-2, pIL-12 and pControl) alone and the combination of pIL-2 and pIL-12 (comb) coupled with two different electroporation protocols (EP1 and EP2) and without electroporation was determined. The addition of the plasmids alone without subsequent exposure to the electric field did not reduce the survival of cells (Fig. 2b). On the other hand, gene electrotransfer of plasmids using the EP1 pulse protocol led to a significant reduction in cell survival regardless of the plasmid DNA used ($p < 0.05$) (Fig. 2c). However, the survival of cells exposed to the EP2 pulse protocol and plasmids was in the same range as the cells exposed to the EP2 pulse protocol alone (Fig. 2d).

3.3. In vitro IL-2 and IL-12 transcripts expression

The expression of IL-2 and IL-12 in B16.F10 cells were evaluated via qRT-PCR two days after GET of the plasmids. In the control, IL-2 and IL-12 were not detected in untreated cells. The IL-2 and IL-12 transcripts were detected only in their respective groups and in the comb group treated with the EP1 and EP2 pulse protocols (Fig. 3a, b). Levels of IL-2 and IL-12 transcripts did not significantly differ between the cells treated with GET of a single IL-2 or IL-12 plasmid or with the combination of both plasmids. However, the level of either transcript was higher after the EP1 protocol than the EP2 pulse protocol ($p < 0.05$).

3.4. In vitro IL-2 and IL-12 protein expression

The production of the IL-2 and IL-12 protein in B16.F10 cells after gene electrotransfer were determined by ELISA of the cell culture media. As previously demonstrated for mRNA expression, IL-2 and IL-12 proteins were detected only in their respective groups and in the comb group treated with both pulse protocols (Fig. 4a, b). The IL-2 concentration was significantly higher in cells treated with the EP1 pulse protocol than in cells treated with EP2 (Fig. 4a), and the same result was also observed for IL-12 concentrations (Fig. 4b) ($p < 0.05$). Interestingly, the concentrations of IL-2 and IL-12 after gene electrotransfer of either pIL-2 or IL-12 using the EP1 pulse protocol were higher than the concentrations of the proteins in the EP1 comb group ($p < 0.05$).

Due to higher levels of IL-2 and IL-12 proteins after the EP1 protocol *in vitro* and the presence of IL-12 in the serum of mice treated with pIL12 in an *in vivo* pilot study (Supplementary Fig. 1), this pulse protocol was selected for the *in vivo* experiments.

3.5. Therapeutic effectiveness in vivo

A delay in tumour growth was observed in all groups treated with pIL-2, pIL-12 and their combination coupled with the EP1 pulse protocol compared to the control groups, where all the mouse tumours reached the endpoint of the experiment within 5–6 days (Fig. 5a). Furthermore, in the treatment group with the addition of the pIL-2 plasmid alone (EP1 pIL-2), all mice reached the endpoint of the experiment within 12 days (Fig. 5b). In contrast, treatment with the pIL-12 plasmid alone (EP1 pIL-12) resulted in a complete response in two out of seven mice. In the remaining five mice, tumours reached the endpoint between 20 and 50 days after the treatment (Fig. 5c). Two mice that achieved a complete response were reinjected with tumour cells on the opposite flank, and tumours developed within 11 and 30 days (Table 2). In contrast, treatment with a combination of pIL-2 and pIL-12 plasmids resulted in a complete tumour response in five mice, while in the remaining two mice, the tumour reached the

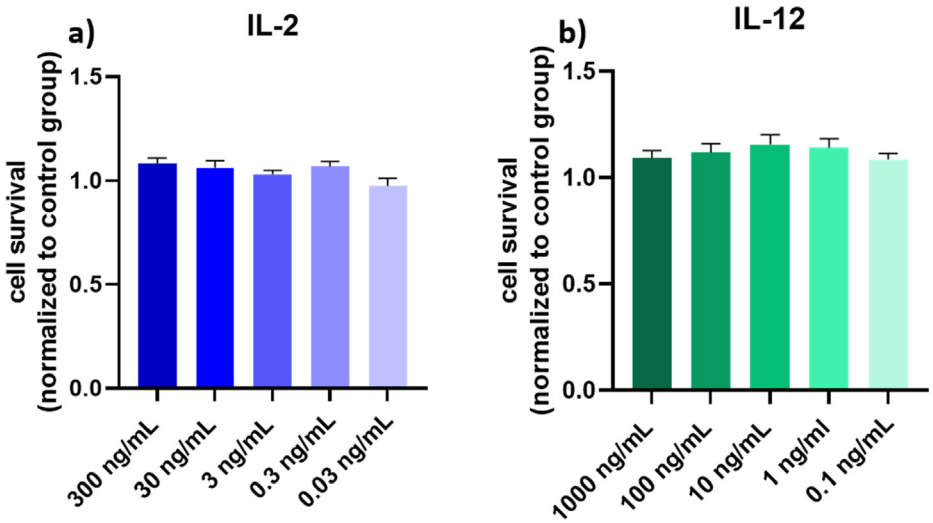

Fig. 1. *In vitro* cytotoxicity of recombinant IL-2 and IL-12 in the B16.F10 murine melanoma cell line. The results are shown as cell survival 72 h after the incubation of cells in five different concentrations of recombinant IL-2 a) and IL12 protein b). The experiments were performed in 3 biological replicates, each consisting of 8 technical parallels. The values are expressed as the AM ± SEM.

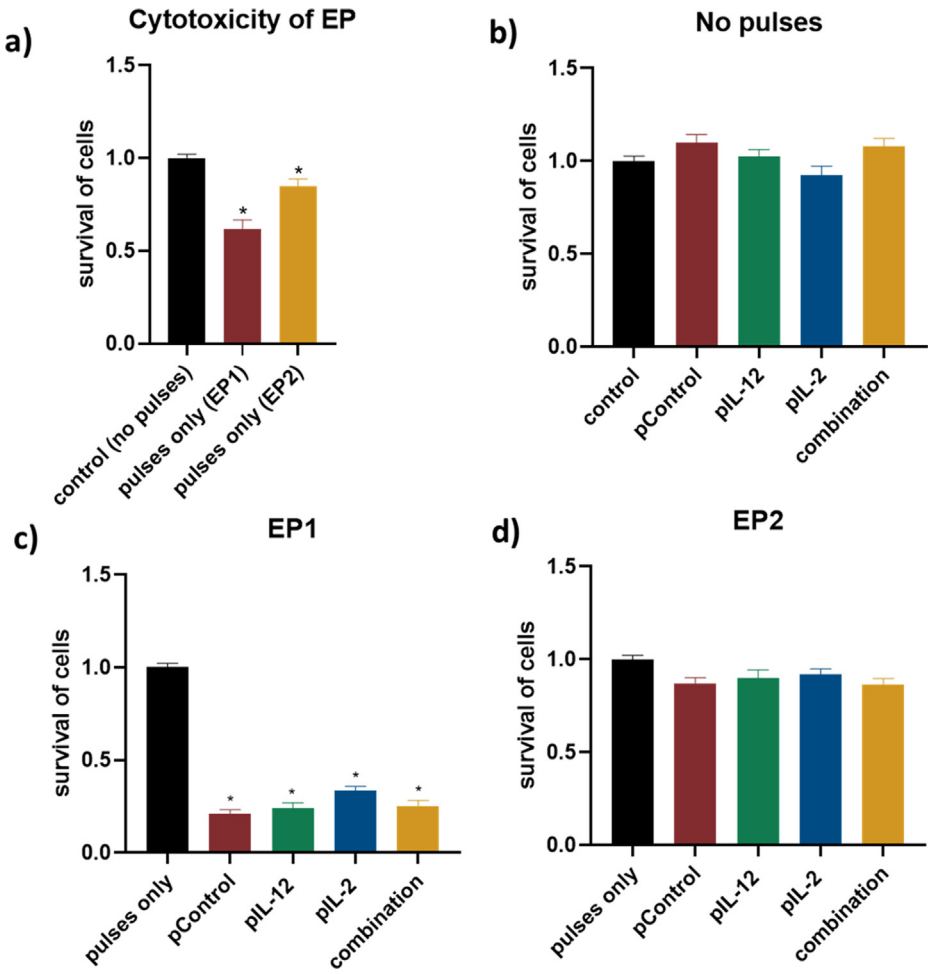

Fig. 2. Impact of the two electroporation protocols on the survival of B16.F10 cells without the plasmid addition (a). *p < 0.05 compared to the control and EP1 or EP2. *In vitro* cytotoxicity after plasmid addition coupled with two different electroporation protocols (c, d) and without electroporation (b) in B16.F10 cells. The results are shown as cell survival normalized to the pertinent control group (electroporated and nonelectroporated, without plasmid addition). The experiments were performed in 3 biological replicates, each consisting of 8 technical parallels. *p < 0.05 compared to pulses only. The values are expressed as the AM ± SEM.

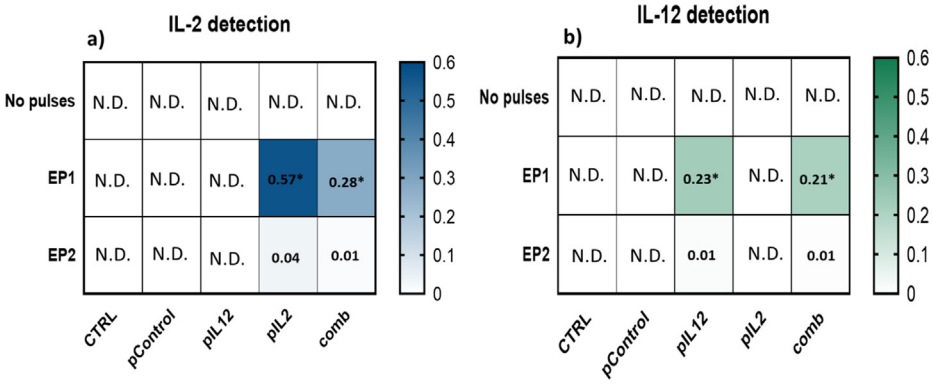

Fig. 3. *In vitro* mouse IL-2 (a) and IL-12 (b) mRNA expression after electroporation. a) Detection of IL-2 transcripts. b) Detection of IL-12 transcripts. The data are plotted as ΔCt (relative to the average of the two housekeeping *Actb* and *Gapdh* genes). The experiments were performed in 3 biological replicates, each consisting of 2 technical parallels. *p < 0.05 compared to EP1 or EP2. N.D. = not detected. The values are expressed as the AM ± SEM.

experimental endpoint within 22 and 35 days (Fig. 5d). Moreover, five mice that achieved a complete response were reinjected with tumour cells on the opposite flank and 4 showed no tumour growth for the period of 100 days after reinjection (Table 2). All cured mice in the EP1 comb group developed vitiligo, which is the result of the immune system acting against normal melanocytes. This phenomenon is mainly localized to the treatment area; however, the vitiligo sparsely spread to the whole coat over time. In addition, the mice gained weight and reached 37 ± 2 g at the end of the experiments (aged 8–9 months) (Fig. 5e).

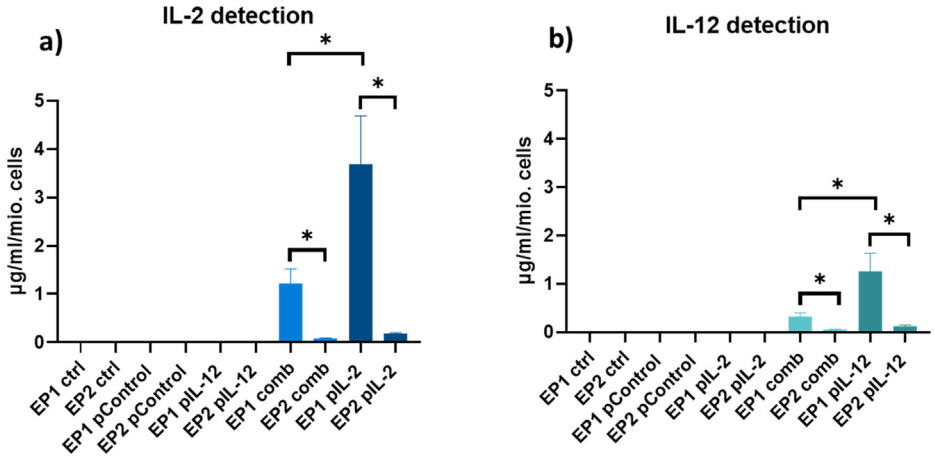

Fig. 4. *In vitro* evaluation of mouse IL-2 and IL-12 protein expression encoded by plasmid DNA after electroporation. a) Detection of IL-2 protein. b) Detection of IL-12 protein. The data are plotted as a concentration of the protein [µg/mL] per million cells. The experiments were performed in 3 biological replicates, each consisting of 4 technical parallels. *p < 0.05 values were considered statistically significant. The values are expressed as the AM ± SEM.

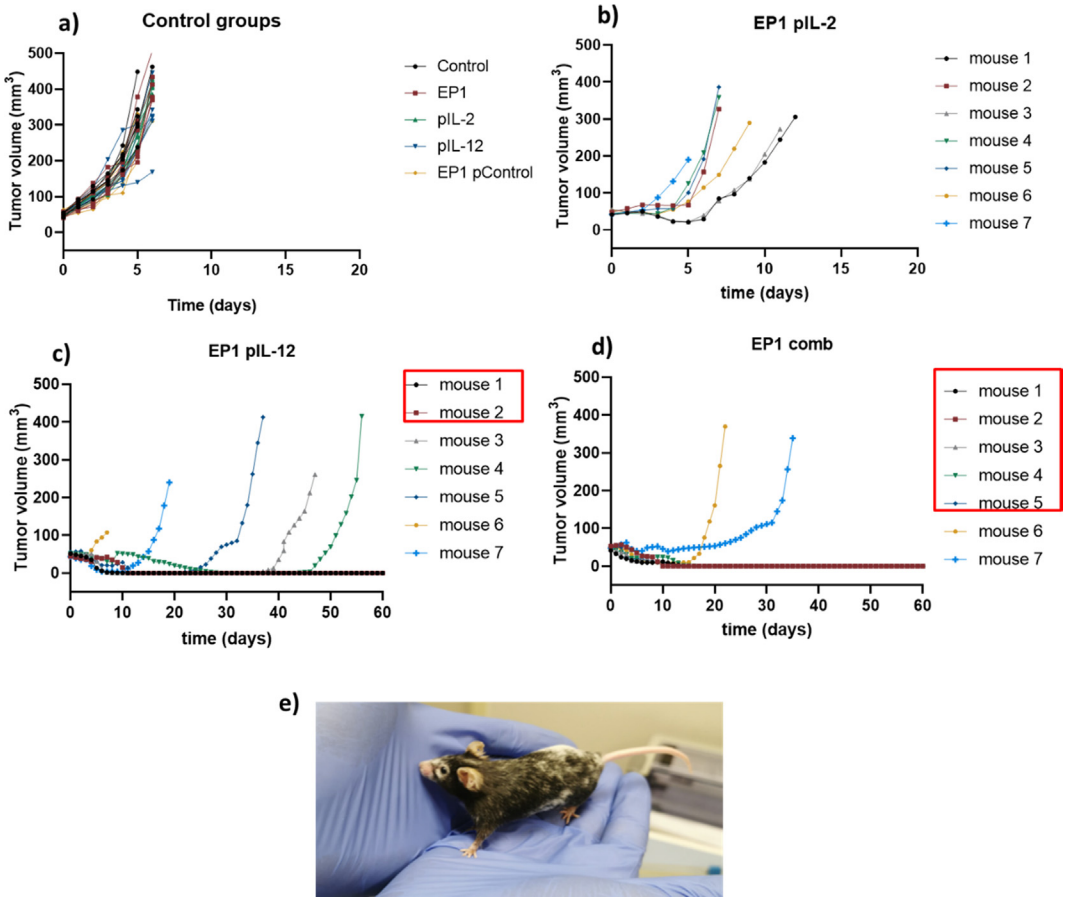

Fig. 5. Therapeutic effectiveness in B16.F10 murine melanoma tumours. a) Tumour growth curves of single tumours in the control groups. b), c), d) Tumour growth curves of the EP1 pIL-2 group (n = 7), EP1 pIL-12 group (n = 7) and EP1 comb group (n = 7), respectively, are represented individually per mouse. e) Vitiligo observed in all cured mice in the EP1 comb group. A tumour volume of 350 mm^3 was preset as the human endpoint of the experiment. Mice with complete response (tumour free 100 days after the treatment) are marked with a red square.

3.6. In vivo IL-2 and IL-12 protein expression

To confirm the effectiveness of the gene electrotransfer treatments, IL-2 and IL-12 protein expression in the treatment and control groups was determined. Tumour tissue and serum samples were taken 3 and 5–7 days after the treatment and analysed using ELISA.

The IL-2 analysis of the tumour and serum samples showed increased expression only in the EP1 pIL-2 and EP1 comb groups. A decrease in the IL-2 concentration in tumour tissue from day 3 to days 5–7 was observed in the EP1 pIL-2 group, where it dropped by 93%. Moreover, a decrease in the concentration of IL-2 over time was also observed in serum, where it dropped below the level of detection (Fig. 6a, b) ($p < 0.05$). The change in the concentration

Table 2
Number of mice with a complete response and survival rate after secondary rechallenge.

Therapeutic group	Complete response [N° of mice]	Tumour growth after secondary challenge [N° of mice]	Survival rate after secondary rechallenge [%]
EP1 pIL-12	2	2	0
EP1 comb	5	1	80

in the EP1 comb group from day 3 to days 5–7 was not statistically significant due to the high data variations in the therapeutic group.

The IL-12 analysis of the tumour and serum samples showed similar results as the IL-2 analysis. Increased expression of IL-12 was detected only in the EP1 pIL-12 and EP1 comb groups. A decrease in the IL-12 concentration over time was observed in the EP1 pIL-12 group in serum, where the concentration dropped by 95% (Fig. 6c, d) ($p < 0.05$). The concentration in the EP1 comb group at day 3 compared to days 5–7 remained approximately the same.

3.7. Intratumoural EP1 comb treatment induces necrosis and immune infiltrate in skin and tumours

To evaluate the effectiveness of the EP1 comb treatment in inducing tumour regression and immune infiltration, a histological analysis was performed on tumour sections from treated and untreated mice on days 3 and 7. H&E staining was performed to evaluate the impact of different treatments on tumour tissue by discriminating the necrotic areas from the viable areas. In particular, the EP1 comb and EP1 pIL-12 sections showed extensive necrosis distributed throughout the tumour as an effect of the treatment (Fig. 7). In contrast, the controls and EP1 pIL-2 tumours displayed necrotic areas mainly located centrally, which was due to the aggressive growth of the tumours (Fig. 7).

Treated tumours were generally smaller than control tumours. Notably, EP1 comb-treated tumours showed wider necrotic areas than that of any other experimental group (Fig. 7). As a positive control, LPS was subcutaneously injected into a small control mouse group (n = 3), and it promoted massive infiltration of immune cells at the injection site.

Concerning immune cell infiltration, in the LPS (positive control) group, H&E staining revealed massive infiltration in the region of the dermis (Fig. 7). The immune cells that infiltrated into the EP1 comb group were more concentrated in the tumour region, thus following the same trend of intensity of the LPS group. Immune cell infiltration was also observed in EP1 pIL-2 and EP1 pIL-12 tumours, albeit to a lesser extent. In the other groups, little or no infiltration of immune cells was observed (Fig. 7).

3.8. Intratumoural EP1 comb treatment enhances inflammatory cell recruitment in tumours

To evaluate the immune response and determine the infiltration of immune cells, IHC staining was performed using anti-F4/80, anti-MHCII and anti-CD11c antibodies specific for macrophages and dendritic cells as reported elsewhere [14]. In particular, dendritic cells were detected by CD11c staining, macrophages were detected by IHC staining with F4/80 as a generic marker and MHCII-based IHC was used to specifically evaluate the M1 macrophage i.t. infiltrate [50].

Significant ($p < 0.05$) increases in the stained areas with respect to negative controls resulted in all the immunostaining experiments, as detailed below.

IHC for F4/80 showed a relatively larger stained area in the EP1, EP1 pControl, EP1 pIL-2, EP1 pIL-12, EP1 comb, and LPS (positive

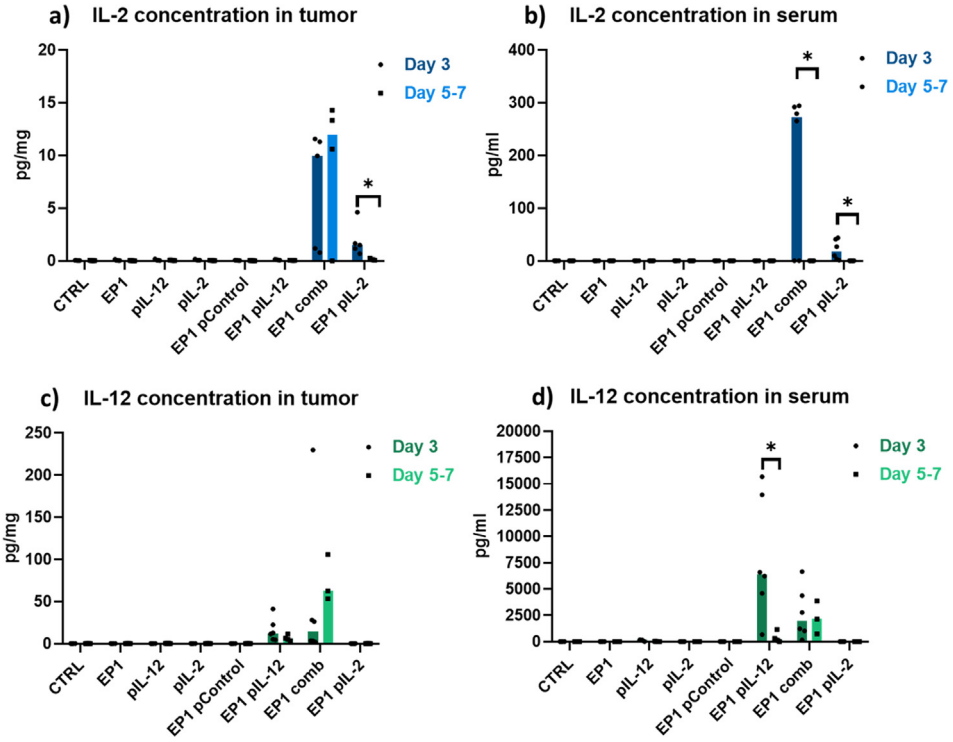

Fig. 6. *In vivo* evaluation of mouse IL-2 and IL-12 protein expression in mouse tumour and serum samples. a), b) Concentrations of IL-2 in tumour tissue and serum on days 3 and 5–7. c), d) Concentrations of IL-12 in tumour tissue and serum on days 3 and 5–7. The concentrations of IL-2 and IL-12 in tumour tissue are presented as pg of protein per mg of tumour tissue. The dots represent values for individual samples; bars represent the median values for 5–6 samples per experimental group. *$p < 0.05$ values were considered statistically significant.

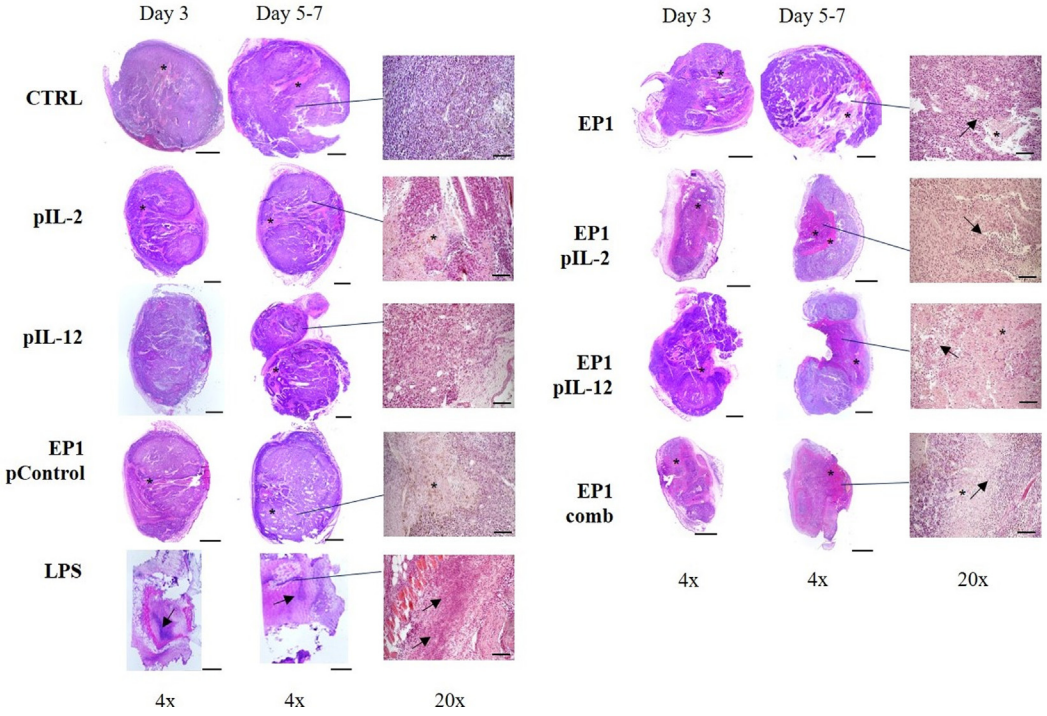

Fig. 7. Representative images of melanoma FFPE sections stained with haematoxylin and eosin on days 3 and 5–7 after treatment. Positive control samples were skins treated with LPS. Asterisk denotes necrotic areas. Arrows denote immune cell infiltration that is visible as intensely coloured red–purple cells. 4x magnification, scale bar = 1 mm; 20x magnification, scale bar = 100 μm.

control) groups, whereas no signal was observed in the negative control and pIL-2 and pIL-12 groups (Fig. 8a). The largest areas were observed in the EP1 comb, EP1 pIL-2, EP1 pIL-12, and LPS groups at days 5–7, while lower values were observed at both day 3 and days 5–7 for the EP1 and EP1 pControl groups as an effect of electroporation alone (Fig. 8a).

IHC for MHCII again showed larger stained areas in the EP1, EP1 pControl, EP1 pIL-2, EP1 pIL-12, EP1 comb, and LPS (positive control) groups, whereas in the negative control group and in the pIL-2 and pIL-12 groups, very low values were recorded (Fig. 8b). At days 5–7, the area of MHCII-positive staining was largest in the EP1 comb group, reducing from the LPS (positive control) towards the EP1, EP1 pControl, EP1 pIL-2 and EP1 pIL-12 groups (Fig. 8b). The positive staining of both F4/80 and MHCII in the EP1 comb, EP1 pIL-2, EP1 pIL-12, and LPS groups indicated a higher density of M1 macrophages in these groups.

Finally, IHC for CD11c demonstrated larger areas for the EP1 comb, EP1 pIL-2, EP1 pIL-12, and LPS groups, in which the extension increased from day 3 to days 5–7, and lower values for the EP1 pControl and EP1 groups, while the signal appeared almost null in the negative groups (Fig. 8c, Fig. 9c). These data indicate a small accumulation of CD11c + dendritic cells in tumours electroporated with or without the pControl plasmid and a strong infiltration of these cells in tumours injected with pIL-2 and pIL-12 in combination with electric pulses.

3.9. Intratumoural EP1 comb treatment enhances the recruitment of T lymphocytes in tumours

To determine whether the EP1 comb treatment could have an effect on T lymphocytes, IHC staining was performed. Melanoma sections obtained from treated and control mice were analysed at the same time points (3 days, 5–7 days) for the presence of CD4 + and CD8 + T cells. As shown in Fig. 11, low but significant T-cell mobilization was obtained 3 days following EP1, EP1 pIL-2, and EP1 pIL-12 treatment, while higher infiltration of CD4 + and CD8 + T lymphocytes was observed in the EP1 comb group ($p < 0.05$). Levels of infiltrating T-cells were higher after 5–7 days in all these groups. In particular, we detected the highest recruitment of CD4 + and CD8 + T cells in melanoma obtained from mice receiving the EP1 comb treatment after 5–7 days ($p < 0.000$), as detailed in Fig. 10.

4. Discussion

In this study, we demonstrated the feasibility and antitumour effectiveness of gene electrotransfer of IL-12 in combination with IL-2 in murine melanoma tumours. After gene electrotransfer of the therapeutic plasmids into melanoma tumours, the concentration of both interleukins in their specific treatments and combination groups significantly increased, thus leading to the polarization of macrophages into the antitumoural M1 phenotype, as demonstrated by intense MHCII staining. Moreover, the accumulation of CD11c + antigen-presenting cells in the tumour tissue was also observed. Complete response of tumours treated with this combination was obtained in 71% of mice, and mice developed immune memory, since a 80% rejection rate after the secondary challenge was obtained. The effects of the therapy were also marked by the manifestation of vitiligo, which was present throughout the animals' fur.

IL-2 and IL-12 are cytokines that exert their antitumour action via indirect effects on immune cells. IL-12 signalling is responsible for the acquisition of cytotoxic functions by $CD8^+$ cells and Th1 differentiation and for the induction of IFN-γ production, while IL-2 is important for the survival, proliferation and differentiation of different subsets of T cells, including $CD8^+$ [51]. Thus, to demonstrate that the action of cytokines is not directly related to their action of the tumour cells themselves, the potential cytotoxicity of the IL-2 and IL-12 proteins on B16.F10 cells *in vitro* was evaluated. The viability of the cells that were subjected to different concentrations of

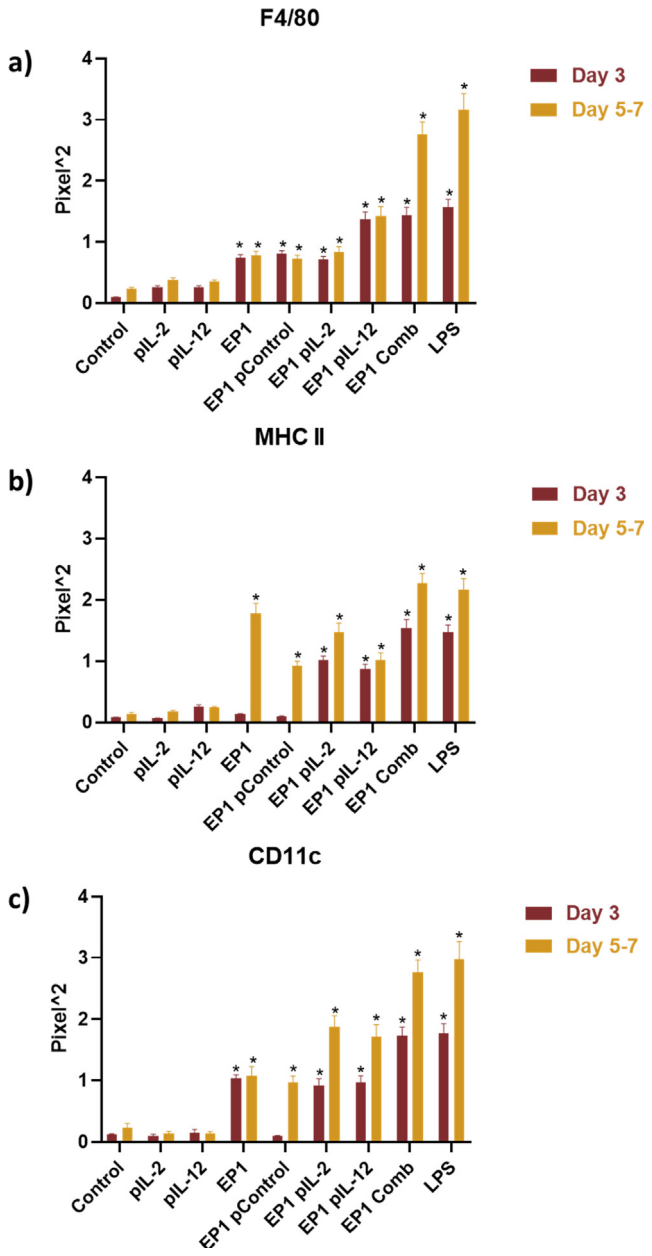

Fig. 8. Histogram of the mean width of areas immunostained with anti-F4/80 Ab a), anti-MHC-II Ab b), and anti-CD11c Ab c) expressed in squared pixels. *p < 0.05 significant difference of mean value with respect to negative controls. Legend: Day 3, sampling after three days; Days 5–7, sampling after 5–7 days. The values are expressed as the AM ± SD.

IL-2 and IL-12 was compared to the viability of the control group. Consistent with reports from the literature, we also demonstrated that neither alone nor in combination, IL-2 and IL-12 affect melanoma cell viability [52].

The cytotoxicity of plasmids that code for IL-2 and IL-12 delivered by EP1 and EP2 pulse protocols *in vitro* was also assessed. The results showed evident differences between the two pulse protocols. The survival of cells treated with the EP1 pulse protocol was noticeably reduced, whereas the treatment of cells with the EP2 pulse protocol led to a minor reduction in viability compared to the nonelectroporated control, as previously shown for other types of cells when applying pulses with shorter durations [13,53]. Furthermore, cell viability dropped significantly after the addition of plasmids coupled with the EP1 pulse protocol in comparison to cells that were exposed to EP1 only. On the other hand, the addition of plasmids to melanoma cells coupled with the EP2 pulse protocol did not affect cell viability. Since the cytotoxicity of the plasmid encoding IL-2 and IL-12 as well as the combination of both plasmids was in the same range as the cytotoxicity of the control plasmid (pControl), it can be further emphasized that the cytotoxic effect does not result from the proteins encoded by therapeutic plasmids but rather from the presence of foreign DNA in the cytosol of melanoma cells. These data are also consistent with those obtained by previous studies, where the survival of B16.F10 cells treated with different plasmids and the EP1 pulse protocol were lower than that of the electroporated control [54,55]. Furthermore, the mRNA and protein levels of IL-2 and IL-12 were measured after the cells were exposed to the different treatment combinations. Both interleukin transcripts and their protein forms were detected only in groups treated with the corresponding plasmid coupled with electroporation. Although B16.F10 cells produce IL-2 [56], in our set of experiments, we did not detect IL-2, and our results demonstrated that the level of proteins in the cells was due to the proteins that were encoded by introduced plasmid DNA. Moreover, the level of transcript expression was higher after EP1 pulses than after EP2 pulses. This finding was also confirmed by an ELISA analysis of the cell supernatants, where the levels of IL-2 and IL-12 were higher in groups exposed to the EP1 pulse protocol.

The cytotoxicity and transfection efficiency of a pulse treatment that is measured *in vitro* can be very different from the effect of the same protocol on a tumour *in vivo* [57-60]. Although the EP1 pulse protocol yielded lower cell survival, higher mRNA and protein expression was obtained *in vitro* and was thus selected for the *in vivo* part of the study because high transfection efficiency and consequently high production of the proteins are needed for successful targeted therapy. The EP2 protocol consists of short and high voltage pulses that can preserve viability but enable only moderate transfection efficiency [60]. This kind of pulse is commonly used in electrochemotherapy procedures to introduce small molecules (chemotherapeutic drugs) into tumour cells and cause cell death [57,61]. The EP1 protocol, however, consists of longer and lower voltage pulses that are more toxic to the cells but can drive large and electrophoretically active molecules, such as DNA, to the electropermeabilized membrane, thus maximizing uptake [57,62]. In our case, the high cytotoxicity of the EP1 protocol could also contribute to more efficient tumour eradication. Namely, harsh electroporation conditions can kill tumour cells that consequently release tumour antigens, which in return generate an inflammatory environment and cause immune cell infiltration [63,64]. Therefore, the choice of pulse parameters depends on what needs to be achieved. After the EP1 pulses, significant differences in mRNA expression were not observed between the single plasmid treatment groups (EP1 pIL-2 and EP1 pIL-12) and the combination group (EP1 comb); however, differences were observed in protein expression. The reason for this discrepancy is not known but could be related to the weak correlation between protein and mRNA expression (only 40%), and the majority of variation can be explained by posttranscriptional regulation [65].

Based on the *in vitro* results and pilot *in vivo* results, the EP1 pulse protocol was chosen for further *in vivo* experiments. In other *in vivo* studies, effective gene electrotransfer of IL-12 was carried out with pulse protocols similar to our EP2 protocol [66,67]. In a study by Shirley et al., 80% CR was obtained using a higher pulse amplitude (similar to our EP2), which was much higher than the amplitude used in our *in vivo* experiment. In addition to the selection of the pulse protocol, several other parameters, such as the plasmid composition, dose of the plasmid, strain of the mice, size of the tumours at the beginning of therapy, etc. can affect the antitumour effectiveness. Consequently, differences in tumour

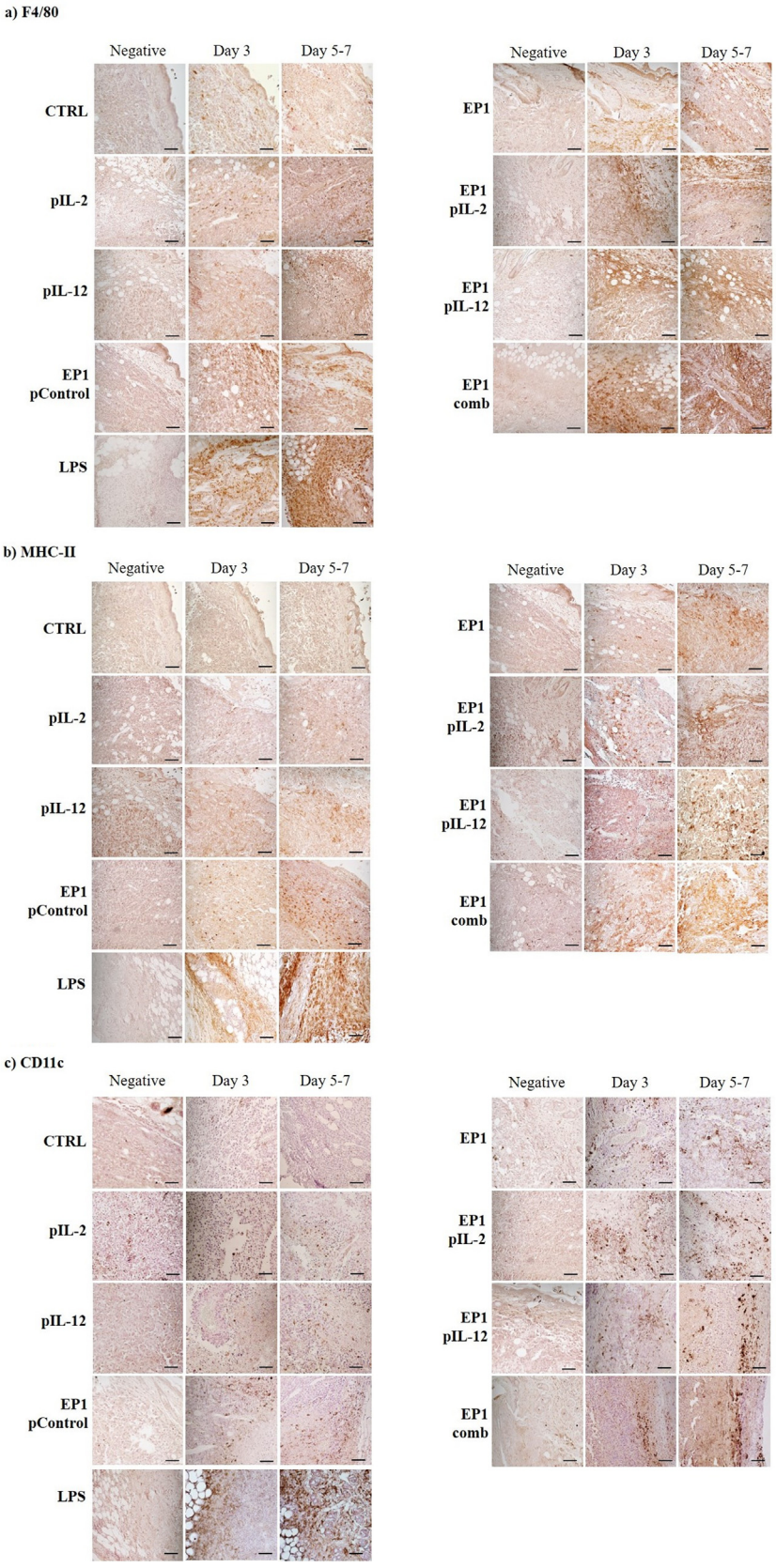

Fig. 9. Representative images of melanoma FFPE sections stained with a) F4/80 and b) MHC-II, which are monoclonal antibodies specific for macrophages and c) CD11c, which is an antibody specific for dendritic cells, on day 3 and days 5–7 after treatment. Positive control samples were skins treated with LPS. Immunopositive cells are stained brown. The negative images of all groups are not related to a specific day. 20x magnification, scale bar 100 μm.

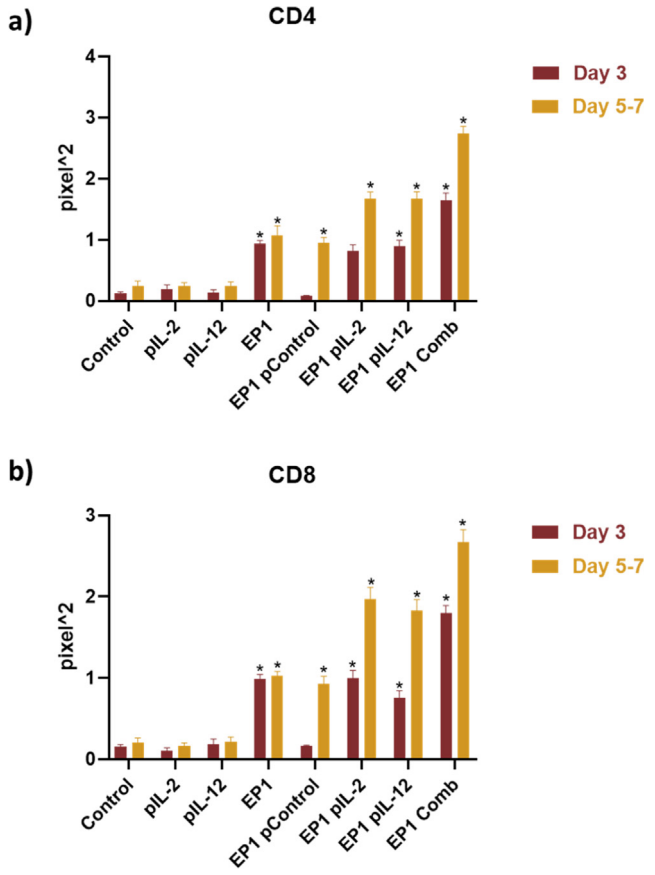

Fig. 10. Histogram of the mean width of areas immunostained with anti-CD4 Ab a) and anti-CD8 Ab b) expressed in squared pixels. *p < 0.05, significant difference of mean value with respect to negative controls. Legend: Day 3, sampling after three days; Days 5–7, sampling after 5–7 days. The values are expressed as the AM ± SD.

response between different research groups can be observed [33,68].

As shown in Fig. 5, tumours treated with EP1 pIL-12 and the EP1 comb achieved significantly delayed tumour growth. Furthermore, 29% of the mice in the EP1 pIL-12 group and 71% in the EP1 comb group also obtained complete responses. Moreover, in 4 out of 5 of the surviving mice in the EP1 comb group, rejection of tumour cells after secondary challenge was observed. In contrast, in the mice that were cured with EP1 pIL-12, the tumours regrew after secondary challenge. Furthermore, EP1 pIL-2 monotherapy showed a lower efficiency, with a delay of tumour growth of only 6–7 days, and it did not cure any of the mice. Interestingly, in the cured mice after the combination therapy, which were also resistant to rechallenge, vitiligo combined with an increase in body weight was observed. Vitiligo is a sign of an overacting immune system towards normal melanocytes and thus is recognized as an undesired adverse event, although in clinical trials of immunotherapy for melanoma, it was associated with a favourable response [69].

Although IL-2 as a recombinant protein has been used for the treatment of melanoma patients, only a small number of studies evaluated the gene electrotransfer of IL-2 in melanoma [37,70]. However, numerous studies have evaluated gene electrotransfer of IL-12 monotherapy and demonstrated its efficacy on a variety of histologically different tumours [26,71-73]. Although studies have explored viral and nonviral local delivery of a combination of IL-12 and IL-2 genes, to the best of our knowledge, this is the first study using electroporation as a delivery strategy for this combination of genes into tumour cells [74,75].

To determine the mechanisms of the observed antitumour effectiveness, several subsequent analyses were performed. First, the protein levels in tumours and serum after gene electrotransfer of pIL-2, pIL-12 and their combination were evaluated using ELISA. The results showed increased tumour and serum levels of both proteins in tumours treated with either of the plasmids and electroporation compared to the levels of proteins in their respective control groups. The increased IL-12 levels after intratumoural gene electrotransfer were also confirmed in other studies [76,77]. Furthermore, apart from the IL-12 concentration in serum samples, the IL-2 and IL-12 concentrations in tumour and serum samples were higher in the EP1 comb group than in the monotherapy groups (EP1 pIL-2 and EP1 pIL-12), although the differences were not statistically significant. High tumour concentrations of IL-2 and IL-12 in the EP1 comb group could be the result of endogenous expression caused by functional cooperation between them, which leads to a positive feedback loop. Moreover, the notable therapeutic effectiveness of the treatment with the combination compared to monotherapies could also be explained by such cooperation. At day 7, the serum concentration of IL-2 protein decreased while that of IL-12 did not. The decrease in IL-2 concentration in the serum samples can be explained by the low protein half-life in the serum [78], while the sustained IL-12 concentration in the serum could also be attributed to the cooperative action of these two interleukins.

Second, the antitumour effectiveness was evaluated by histological and immunohistological analyses. HE staining demonstrated larger necrotic areas in the EP1 pIL-12 and EP1 comb groups than in the other control groups and the EP1 pIL-2 group. These findings are linked to higher tumour antigen release and consequently facilitated antigen presentation [79]. Additionally, extensive immune cell infiltration was observed in tumours of the EP1 comb group that followed the same trend of intensity as that observed in skin treated with LPS, which served as a positive control. Immune infiltration was also observed in EP1 pIL-12- and EP1 pIL-2-treated tumours, although on a smaller scale, none was observed in control tumours. The results indicate that the highest infiltration of immune cells was induced by gene electrotransfer of therapeutic genes into tumours. These observations were in line with the tumour growth and ELISA results, which together further support the therapeutic effects of the combined interleukin treatment.

The investigation of the immune infiltrate by immunohistochemistry on paraffin-embedded tumour sections at day 3 and day 5 or 7 demonstrated the presence of dendritic cells, macrophages and lymphocytes. Positive staining for F4/80 was observed only in tumours treated with electroporation (EP1, EP1 pControl, EP1 pIL-2, EP1 pIL-12 and EP1 comb). Higher signals were seen in EP1 pIL-2, EP1 pIL-12 and EP1 comb tumours on days 5–7 compared to day 3 and other non-electroporated control tumours. MHC II staining was similar to F4/80 staining, with the largest positive area in tumours of the EP1 comb group at days 5–7 and the smallest on day 3 and in tumours that were not subjected to electroporation. As shown by CD11c staining, large dendritic cell recruitment was observed in the tumours of the EP1 comb group as well as in the tumours of the EP1 pIL-2 and EP1 pIL-12 groups on day 7, while the infiltrate was moderate in tumours of the EP1 pControl and EP1 groups both on day 3 and on day 7. Both macrophages and dendritic cells are a part of the innate immune system that can kill tumour cells directly or by the presentation of tumour antigens to T and B lymphocytes that represent the adaptive immune response [80]. Positive staining for F4/80 and MHC II suggests the presence of highly phagocytic M1 macrophages that arise in the presence of IFN-γ. M1 macrophages promote inflammation with the production of IL-12 and ROS, which help the adaptive immune system eradicate tumour cells [81]. Fur-

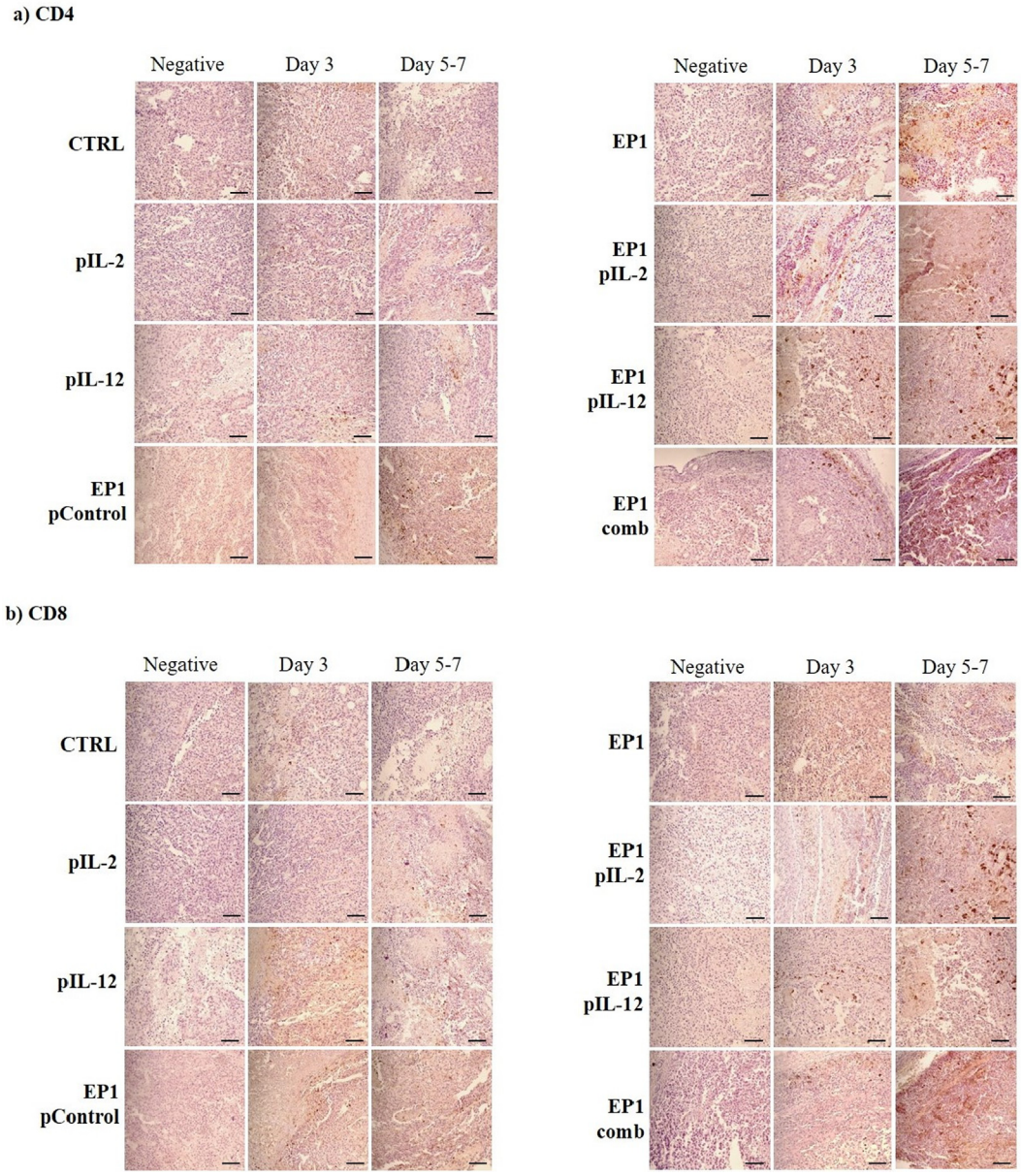

Fig. 11. Representative images of melanoma FFPE sections stained with a) CD4 and b) CD8, which are monoclonal antibodies specific for helper T cells and cytotoxic T cells, respectively, on days 3 and days 5–7 after treatment. Immunopositive cells are stained brown. The negative images of all groups are not related to a specific day. 20x magnification, scale bar 100 μm.

thermore, IFN-γ can increase MHC I expression in tumour cells, thus further facilitating antigen presentation to T cells [82]. In parallel, our IHC data demonstrated that the recruitment of helper and cytotoxic T cells was significantly impacted by the EP1 comb protocol and, to a lower degree, by EP1 pIL-2 and EP1 pIL-12. These findings confirmed that the increase in both T helper and T cytotoxic type-induced signals, such as MHC-II expression on APCs and increased MHC I expression in tumour cells, and a suitable proinflammatory context provided by the EP1 combination treatment are crucial for eliciting immune activation. Moreover, these data indicate a combined role of IL-2 and IL-12 proteins in recruiting immune cells, among which proinflammatory M1 macrophages, dendritic cells and lymphocytes were identified in our study. In the presence of the combination of the therapeutic proteins IL-2 and IL-12, antigen-presenting cell and lymphocyte migration at the tumour site supports tumour-associated antigen presentation and subsequent tumour eradication.

5. Conclusions

Currently, there is an increasing demand to develop new strategies or combine existing approaches to improve cancer treatment outcomes. In this study, the efficacy of gene electrotransfer of IL-12 in combination with IL-2 on B16.F10 murine melanoma was tested. The results confirmed the feasibility and effectiveness of this therapeutic approach in low immunogenic B16.F10 tumours.

Thus, our combined approach resulted in activation of the immune system, which in turn eradicated the tumour and led to immune memory. However, as strong vitiligo was observed in cured mice, a refinement of the therapy should be performed that would lead to milder side effects with the same antitumour effectiveness.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.107843.

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