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BRIEF COMMUNICATION

Improving therapeutic efficacy of IL-12 intratumoral gene electrotransfer through novel plasmid design and modified parameters

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Abstract

The use of immunomodulatory cytokines has been shown effective in regressing a wide range of tumors. However, systemic delivery of recombinant cytokines results in serious, potentially life-threatening, adverse effects. By contrast, nucleic acid transfer via electroporation (EP) is a safe and effective method of delivering plasmid-encoded cytokines to tumors. Intratumoral delivery of IL-12 plasmid DNA by electroporation (IT-pIL12-EP) produced objective response rates in Phase 2 clinical trials in metastatic melanoma. However, only 17.9% of patients receiving IT-pIL12-EP show a complete therapeutic response. Here, we sought to improve the antitumor efficacy of our clinical IT-pIL12-EP plasmid electroporation platform. We evaluated multiple plasmid designs for IL-12 expression. IL-12 expression from a plasmid incorporating a picornavirus-derived co-translational P2A site was the most effective in expressing IL-12p70. In addition, modifying the electroporation parameters improved transfection efficiency and expression of plasmid-derived IL-12p70, as well as its downstream effector IFN-γ in vivo. Finally, using a murine melanoma model that is representative of the intended target patient population, we show that combining modified electroporation conditions with the pIL12-P2A plasmid expression enhances the systemic antitumor response. These improvements to the IT-pIL12-EP platform may improve patient clinical response rates and survival when translated to clinical trials.

Introduction

Gene electrotransfer (GET), a non-viral method to deliver nucleic acids to target cells via application of pulsed electrical fields, was first described in the early eighties using mouse lyoma cells [1]. Since then, electroporation (EP)-based technologies have expanded into biotechnological and medical applications [2, 3]. In the medical field, the delivery of plasmid DNA coding for therapeutic genes into solid tumors has shown promise both in preclinical and clinical settings. However, the therapeutic use of EP-mediated transfer of plasmid DNA to tumor tissues has been hampered by inefficient gene transfer leading to low protein expression.

Interleukin-12 (IL-12) is an immunomodulatory cytokine mainly produced by antigen-presenting cells (APCs) upon bacterial or viral infection [4, 5]. The IL-12-specific heterodimeric receptor complex IL-12R is found on natural killer (NK) cells, NK T cells and activated T cells [6]. IL-12 enhances cytotoxicity of effector T lymphocytes and NK cells, and induces proliferation and interferon-γ (IFN-γ) production [7, 8]. IFN-γ has long been associated with potent antitumor and angiostatic properties [9, 10]. In addition, IL-12 induces maturation of type 1T helper cells and enhances antigen-specific $CD8^+$ T-cell responses [8, 11].

Several approaches to deliver IL-12 intratumorally have been shown to invoke a strong localized immune response leading to regression of a wide range of established tumors while producing low or no systemic toxicity [12–15]. One method to deliver therapeutic levels of IL-12 is EP of

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plasmid DNA directly into the tumor. The delivered plasmid DNA behaves as an inactive molecule (prodrug) and only the transcribed and translated gene product, IL-12p70 protein, elicits a physiological response. GET of IL-12 plasmid DNA directly into the tumor (IT-pIL12-EP) has demonstrated an acceptable safety profile and has been shown effective in preclinical and clinical studies [15–19]. In clinical studies, 17.9% of metastatic melanoma patients who received IT-pIL12-EP showed complete regression of treated and untreated lesions [18]. Compared with the toxicity associated with systemic treatment [20], localized expression of IL-12 within the tumor microenvironment has proven to be very safe with minimal treatment-related adverse effects [18].

Bioactive IL-12 (IL-12p70) is a heterodimeric pro-inflammatory cytokine composed of IL-12 p35 and IL-12p40 subunits [21]. As optimal expression of IL-12p70 requires 1:1 molar expression of p35 and p40, multiple attempts have previously been made to optimize plasmid design to yield robust production of bioactive IL-12p70. These include co-translation of the two subunits with an internal ribosome entry sequence (IRES) [22], physically tethering the two subunits together via a linker sequence [23] or employing a 2A peptide skipping sequence derived from picornavirus virus [24].

Here, we sought to improve the efficacy and systemic antitumor response of our clinical IT-pIL12-EP platform [25] by (a) modifying in vivo EP conditions, and (b) enhancing plasmid-derived IL-12p70 expression. This improved IL-12 therapeutic platform was evaluated in vitro and in vivo using a murine syngeneic tumor model. We report that modifications to the EP parameters including lowering the electric field strength (low voltage) combined with a longer pulse length significantly increased the transfection efficiency of intratumoral EP. In addition, a plasmid design linking the IL-12 p35 and p40 subunits via a 2A peptide sequence (pIL12-P2A) leads to improved expression and secretion of IL-12p70 than the clinically used IL-12 expression plasmid (pIL12-IRES). Finally, we show that combining the novel plasmid design (pIL12-P2A) with low voltage EP conditions significantly improves survival in a murine tumor regression study when compared with the murine equivalent of our current clinical IT-IL-12-EP platform.

Results

A P2A-linked pIL12 construct showed increased production of functional IL-12p70 in vitro

In an effort to identify the most efficient molecular design for plasmid-based IL-12 expression, we generated three murine IL-12 (mIL12) expression plasmids and evaluated them in relation to the murine homolog of the clinically used IL-12 plasmid, pIL12-IRES (mIL12-p35-IRES-p40) in vitro (Fig. 1a). Three of the tested constructs express the two mIL12 subunits p35 and p40 separately, whereas one construct coded for a fusion protein of the p35 and p40 subunit linked together with a GGGGS(x3) linker. The plasmids expressing both mIL12 genes separately either linked the two sequences via an IRES or a self-cleaving picornavirus 2A-like (P2A) sequence. Both these regulatory elements are well described and allow expression of two separate genes from a single transcript [26]. A similar version of the plasmid coding for a p40-p35 single-chain fusion protein has been described previously [23]. In an in vitro expression assay, all four IL-12 plasmids produced detectable levels of p70 protein. However, IL-12 levels from mIL12-p40-(G_4S)x3-p35 plasmid were barely above background levels, which is likely due to instability of the full-length fusion protein (Fig. S1). The highest level of IL-12p70 expression was achieved with the 2A-linked plasmid with IL-12 p35 in position one and p40 in position two (mIL12-p35-P2A-p40; Fig. 1b). This IL-12 expression plasmid will be referred to as pIL12-P2A. Given the improvement in mouse p70 expression from pIL12-P2A, we generated the equivalent human version of pIL12-P2A (Fig. 1c and Fig. S2) and compared it with its IRES-linked clinical counterpart pIL12-IRES. Similar to the murine version, human pIL12-P2A produced approximately three-fold more IL-12p70 than pIL12-IRES (Fig. 1c). A bioassay (HEK-Blue™ IL-12 sensor cells) was used to assess the biological activity of supernatants produced from human pIL12-P2A-transfected HEK293 cells. Consistent with the enzyme-linked immunosorbent assay (ELISA) data, pIL12-P2A supernatants reached the EC50 at lower concentrations than human pIL12-IRES, confirming that higher levels of functional IL-12p70 are produced from a P2A-linked construct (Fig. 1d). The plasmid design highlighted in Fig. 1a (green dotted box) was then chosen for further in vivo testing.

Modified GET conditions improved intratumoral transfection efficiency

Current EP conditions used in the clinic have a high field strength and short pulse based on previous applications for eletrochemotherapy [25]. We sought to improve intratumoral transduction of DNA plasmids by modifying EP parameters. Our goal was to lower the applied field strength with an accompanying increase in pulse length in order to potentially improve cell viability while maintaining sufficient expression of DNA-encoded genes. This approach is in agreement with Weaver's model suggesting that low voltage EP conditions can improve cell survival and hence

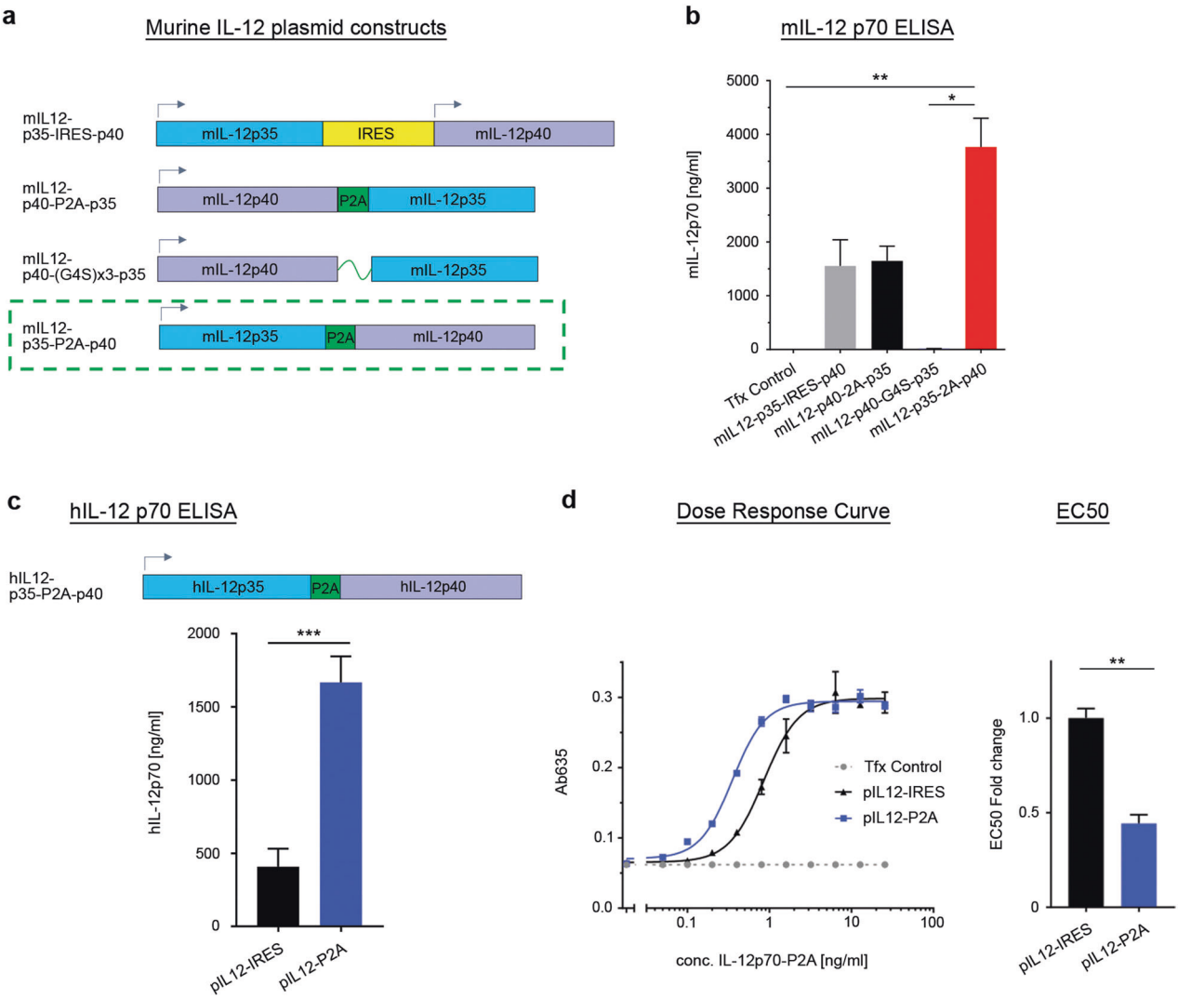


Fig. 1 A P2A-linked pIL12 construct produces high levels of IL-12p70 in vitro. **a** Schematic diagrams of different mIL12 expression plasmid designs that were generated and tested in vitro are shown. The green dashed line denotes the construct chosen for further analysis based on expression profiles. **b** pIL12 constructs depicted in **a** were transfected into HEK293 cells and supernatants harvested 48 h later for ELISA analysis for IL-12p70 heterodimers. The mIL12-p35-P2A-p40 construct produced the highest amount of IL-12p70. (Kruskal–Wallis H test; $*p < 0.05$, $**p < 0.01$; representative example of $n = 3$). **c** A human equivalent of mIL12-p35-P2A-p40 was generated and tested in the HEK293 expression system. This human pIL12-P2A plasmid showed more than threefold higher IL-12p70 expression than its IRES equivalent ($n = 3$). Shown concentrations are normalized to transfection control values (Mann–Whitney; $***p < 0.001$). **d** Culture supernatants from cells transfected with human pIL12-P2A and human pIL12-IRES were tested in an IL-12 bioassay. Different dilutions of culture supernatants from HEK293 cells transfected with the same amount of either human pIL12-P2A or pIL12-IRES were tested. A representative dose-response curve out of three independent experiments is shown. The mean EC50 was twofold lower in pIL12-P2A samples ($n = 3$, Mann–Whitney; $**p < 0.01$)

allow increased expression of introduced gene products [27]. To test this, we electroporated a luciferase reporter construct Luc2p-P2A-mCherry (RFP-Luc) into B16.F10 tumors using low field strength ("low voltage": defined as 400 V/cm, 8 pulses × 10 ms duration) or high field strength ("high voltage": defined as 1500 V/cm, 6 pulses × 0.1 ms duration). Forty-eight hours after EP, luciferase activity was measured in vivo using a Lago imaging system. We found that low voltage EP conditions produced significantly higher luciferase activity than either RFP-Luc plasmid only or RFP-Luc + high voltage conditions (Fig. 2a; $p < 0.05$). Similar results were achieved in MC38 tumors (Fig. S3). Next, we compared low voltage with high voltage conditions using a flow cytometry-based assay to determine percentage of transfected cells isolated from the excised tumor. For this experiment, B16.F10 tumors were electroporated with the RFP-Luc plasmid and 48 h after treatment, tumors were excised and single-cell suspensions analyzed for RFP expression by flow cytometry. We saw an increase in mean percentage of RFP^+ cells from 2 to 8% when using

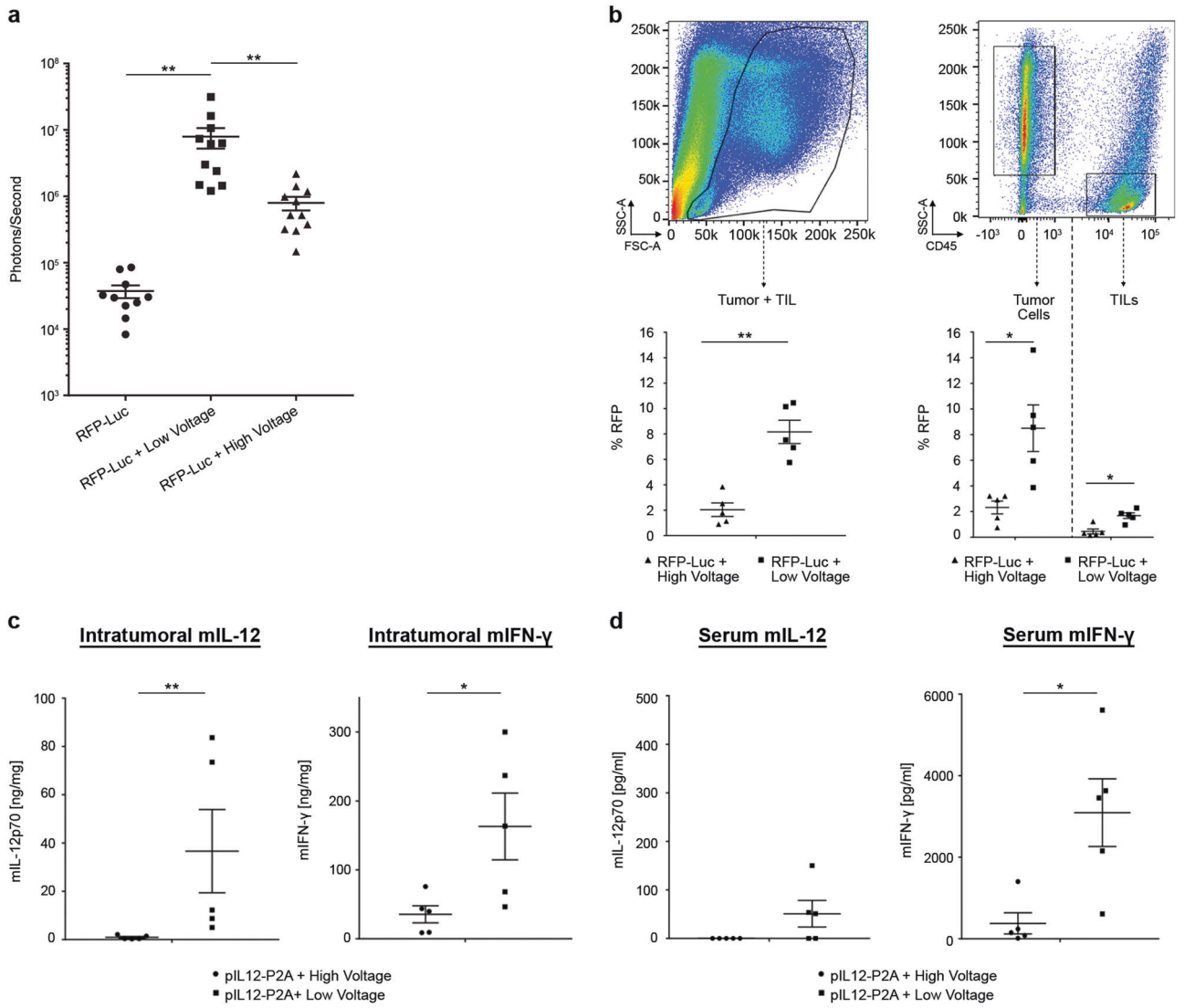


Fig. 2 Modification of electroporation parameters leads to improved intratumoral transfection efficiency and increased IL-12 expression. **a** B16.F10 tumor-bearing mice received intratumoral injections of Luc2p-P2A-mCherry (RFP-Luc) followed up with or without electroporation. Forty-eight hours after EP, mice were injected intraperitoneally with D-luciferin substrate and intratumoral luciferase activity was measured using the Lago in vivo imaging system. Data shown as scatter plot ± SEM with RFP-Luc $n = 10$, and each of the other treatment groups $n = 11$ (one-way ANOVA; $^{**}p < 0.01$). **b** B16.F10 tumor-bearing mice were treated intratumorally with RFP-Luc plasmid. Two treatment groups were analyzed: RFP + high voltage ($n = 5$) and RFP + low voltage ($n = 5$). Forty-eight hours after plasmid injection and electroporation with high or low voltage, tumors were excised and single-cell suspensions prepared for FACS analysis. Transfection efficiency was determined by % RFP^+ cells of live cells and the data shown are normalized to background RFP signals produced by injection of RFP plasmid without electroporation. A representative dot plot was chosen to show the applied gating strategy (Mann–Whitney; $^*p < 0.05$; $^{**}p < 0.01$). **c** IT-pIL12-EP with low voltage conditions leads to elevated mIL-12p70 and mIFN-γ levels in the tumor. Tumors were treated with pIL12-P2A + high voltage, or pIL12-P2A + low voltage conditions. Lysates from treated tumors ($n = 5$ per treatment group) were analyzed by mIL-12p70 and mIFN-γ MAGPIX®. Data shown are normalized to total protein extracted from tumors (Mann–Whitney; $^*p < 0.05$; $^{**}p < 0.01$). **d** Serum from mice analyzed in C) was also tested for mIL-12p70 and mIFN-γ protein levels by MAGPIX® (Mann–Whitney; $^*p < 0.05$)

low voltage conditions (Fig. 2b, left panel). When looking at %RFP^+ within the tumor cell or tumor-infiltrating lymphocyte (TIL) population, a similar three- to fourfold increase was observed in both populations (Fig. 2b, right panel) suggesting that the improvement in transfection efficiency was consistent between these two cell populations. Approximately 2% of the TILs recovered from the tumor 48 h after treatment express the electroporated reporter gene under low voltage conditions.

Next, we wanted to determine how the different EP conditions affect expression of intratumorally administered pIL12. Five mice with implanted B16.F10 tumors were injected with pIL12-P2A and electroporated using either low or high voltage conditions. Forty-eight hours after

treatment, the tumors were harvested, lysed, and analyzed by MAGPIX® for mIL-12p70 and mIFN-γ protein expression. Intratumoral mIL-12p70 and mIFN-γ levels were significantly elevated when low voltage conditions were applied (Fig. 2c; $p < 0.05$). Serum from sacrificed animals was also analyzed by MAGPIX®. No significant mIL-12p70 levels were detected in the serum, suggesting that expression of plasmid-derived mIL12 is restricted to the electroporated tumor (Fig. 2d). Substantial levels of the downstream effector IFN-γ could be detected in the tumor and serum under both low and high voltage conditions but was significantly increased under low voltage EP conditions.

IT-pIL12-P2A-EP produced higher intratumoral and serum IFN-γ levels than its IRES-driven counterpart

We used a syngeneic B16.F10 tumor model to compare in vivo expression levels of pIL12-P2A with the pIL12-IRES construct. The implanted tumors grew over 9–11 days to a volume of 80–150 mm^3, and were then injected with 10 µg plasmid DNA and subsequently electroporated with low voltage conditions. At the study endpoint 48 h post treatment, tumors were extracted and homogenized. Tumor lysates and serum samples were analyzed for mIL-12p70 and mIFN-γ protein levels using the MAGPIX® system. Intratumoral protein levels of mIL-12p70 and its downstream effector molecule mIFN-γ were increased in pIL12-P2A treated mice (Fig. S4A) suggesting that improved IL-12p70 expression from pIL12-P2A leads to physiologically relevant downstream signaling. Even with increased intratumoral IL-12 protein levels from pIL12-P2A, IL-12 serum levels were only slightly above background levels suggesting that the favorable toxicology profile described for pIL12-IRES [17] is maintained (Fig. S4B). The increased bioactivity of pIL12-P2A was also reflected in elevated mIFN-γ serum levels at 48 h post treatment.

Modifications to plasmid design and EP parameters lead to improved antitumor response in a B16.F10 contralateral tumor model

The experimental model depicted in Fig. 3 was used to test if the combination of improvements made to our IT-pIL12-EP platform result in a therapeutic advantage in vivo. This two-tumor model allows us to evaluate the effects of pIL12 both on the treated primary tumor and on a distant, non-treated contralateral lesion. First, we wanted to define the minimal effective dose of our IT-pIL12-P2A-EP platform combining the pIL12-P2A plasmid with low voltage EP conditions. Here, we show that our novel IT-pIL12-P2A-EP platform suppresses the primary and contralateral tumor in a

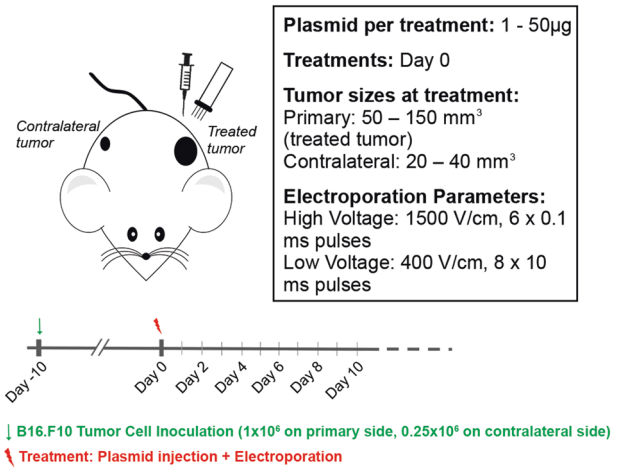
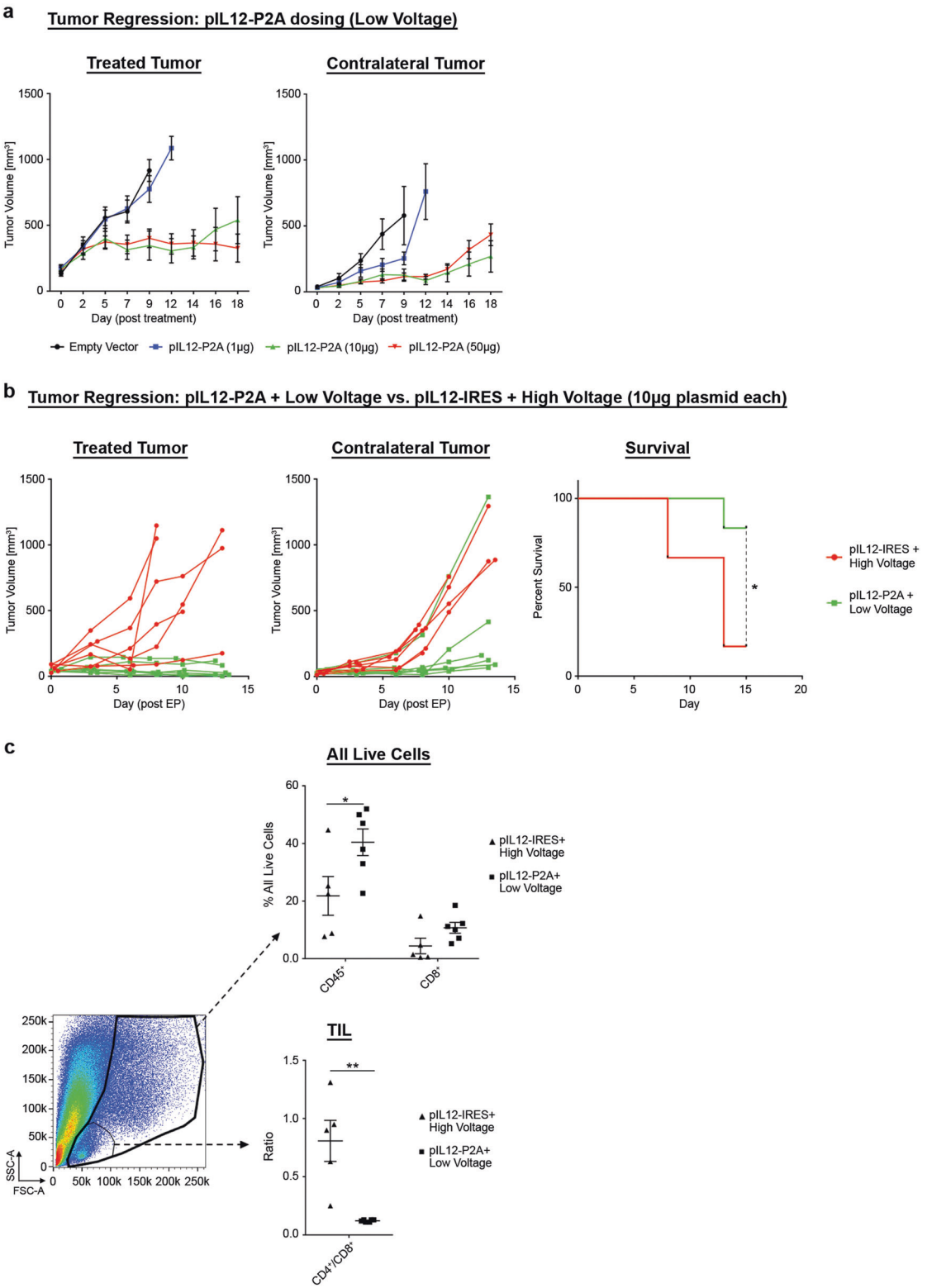


Fig. 3 Contralateral B16.F10 tumor regression model to assess therapeutic effect of IT-pIL12-EP. Contralateral B16.F10 tumor model: mice are injected at two sites with different numbers of B16.F10 tumor cells: 1×10^6 cells on the primary side and 0.25×10^6 cells on the contralateral side. Once the mean primary tumor size reached a defined size range, it was injected with DNA and electroporated; tumor volume for primary and contralateral (non-treated) tumors was measured over time with digital calipers. Treatment conditions, including plasmid concentration and electroporation parameters, are shown and described in more detail in the Materials and methods section. A schematic representation of the experimental time-course is shown

dose-dependent manner (Fig. 4a). The minimum effective dose of pIL12-P2A plasmid was 10 µg. Using this 10 µg plasmid dose, we then compared pIL12-P2A + low voltage with pIL12-IRES + high voltage in our B16.F10 tumor regression model. Both the primary (treated) and the contralateral (untreated) tumor in pIL12-P2A + low voltage-treated mice showed enhanced suppression of tumor growth (Fig. 4b). Using this suboptimal dose of pIL12-IRES plasmid DNA, tumor growth in mice treated with high voltage conditions was similar to empty vector control animals (Fig. S5). The improved therapeutic effect of IT-pIL12-P2A-EP was also reflected in a statistically significant survival advantage (Fig. 4b, right panel). Finally, we analyzed immune cell infiltrates in tumors treated with these two IT-IL-12-EP platforms. Electroporated tumors were harvested 6 days post treatment and single-cell suspensions were analyzed by flow cytometry. Increased influx of $CD45^+$ leukocytes and $CD8^+$ T cells ($CD45^+$, $CD3^+$, $CD8^+$) was observed in the pIL12-P2A + low voltage group (Fig. 4c) but the difference in intratumoral $CD8^+$ T cells did not reach statistical significance. We also looked at the $CD4^+/CD8^+$ ratio within the TIL population because a low $CD4^+/CD8^+$ ratio indicates a potent immunological response to immunotherapy [28, 29]. A more than fourfold reduction in the $CD4^+/CD8^+$ ratio was observed in pIL12-P2A + low voltage-treated mice (Fig. 4c), demonstrating an enhanced antitumor immune response under these conditions.



Discussion

The ultimate goal of cancer therapy is the complete eradication of the disease. Although local treatments (e.g., surgery) can be curative without inducing a systemic antitumor response, in the setting of advanced disease, the bar is higher for local therapy: they should induce effective, systemic anticancer immune responses. Local cancer

◀ **Fig. 4** The novel IT-pIL12-P2A-EP platform (pIL12-P2A plasmid combined with low voltage EP conditions) improves antitumor immune response and systemic tumor growth suppression. **a** pIL12-P2A + low voltage regressed primary and contralateral tumors in a dose-dependent manner. Experimental set up, tumor staging, and treatment schedule are depicted in Fig. 3. The minimum effective dose was reached at 10 µg of pIL12-P2A plasmid DNA. Data plotted as mean ± SEM for each time point ($n = 10$ per treatment group). **b** Head-to-head comparison of 10 µg pIL12-P2A + low voltage ($n = 6$) with 10 µg pIL12-IRES + high voltage ($n = 6$) in a contralateral tumor regression model. Experimental set up, tumor staging, and treatment schedule are depicted in Fig. 3. Tumor growth curve for each individual mouse is graphed as spaghetti plots. Kaplan–Meier survival curve showed a statistically significant survival advantage of 10 µg pIL12-P2A + low voltage-treated mice (*$p < 0.05$). **c** Increased leukocyte infiltration and low intratumoral $CD4^+/CD8^+$ ratio in pIL12-P2A + low voltage-treated mice. B16.F10 tumor-bearing mice were treated intratumorally with 10 µg pIL12-IRES + high voltage ($n = 5$) or 10 µg pIL12-P2A + low voltage ($n = 6$). On day 6 post treatment, tumors were excised and single-cell suspensions prepared for FACS analysis. All live cells (outer gate with bold line) were analyzed for CD45 (Live/$CD19^-/CD45^+$) and CD8 (Live/$CD19^-/CD45^+/CD3^+/CD8^+$). T cells (Live/$CD19^-/CD45^+/CD3^+$) within the TIL gate were analyzed for CD4 and CD8 to determine the intratumoral $CD4^+/CD8^+$ T-cell ratio. A representative dot plot was chosen to show the 'Live cell' and 'TIL' gate (Mann–Whitney; *$p < 0.05$, **$p < 0.01$)

immunotherapy such as intratumoral EP of pIL12 can achieve this goal by acting as an "in-situ vaccine", priming the immune system within the tumor microenvironment and leading to the generation of systemic, tumor antigen-specific T cells [30, 31]. Treatment of metastatic melanoma patients with IT-pIL12-EP monotherapy (pIL12-IRES + high voltage) produced a pathologic complete response rate of 17.9% [18]. Although this represents a clear indication of the clinically meaningful immunologic activity of IT-pIL12-EP, these initial response rates leave room for improvement. Similar success rates have been described in murine melanoma models [15, 30]. One of the drawbacks of intratumoral vaccination via EP is the rather low transfection efficiency and low transgene expression. Here, we demonstrate enhanced systemic effect of IT-IL-12-EP by optimization of each component of our clinical platform. The resulting improvement in transfection efficiency and expression of IL-12 may reduce the overall number of treatments necessary for therapeutic effect, which will be beneficial for patients.

Functional IL-12 protein is a heterodimer comprised of two subunits, which requires simultaneous expression of both genes. One way to achieve this is the generation of a bicistronic vector harboring an IRES placed between the two desired genes. Although this element can initiate internal translation with high efficiency and is well studied [32], there are some disadvantages associated with IRES-linked bicistronic constructs [33, 34]. For instance, IRES-initiated translation is less efficient than cap-dependent initiation often leading to differences in expression levels of proteins encoded in the first and second reading frame. In the case of pIL12-IRES, this would result in unbalanced translation of p35 and p40 subunits thereby limiting production of bioactive IL-12p70. The 2A self-cleaving sequence found in many picornaviruses, however, can be used to generate two or more protein products from a single reading frame at the approximate ratio of 1: 1 [35]. In accordance with other reports testing 2A efficiency linking different reporter genes [36], we saw at least a twofold increase in total IL-12p70 protein expression in constructs that employed a 2A sequence as compared with an IRES (Figs. 1a, 2a). Thus, the novel 2A-based plasmid expresses elevated levels of functional IL-12 in vitro and in vivo. Moreover, this self-cleaving 2A sequence also provides a mechanism for generation of plasmids that express more than two genes in equal molar ratio. In addition, when using 2A peptides in polycistronic vectors the resulting N-terminal protein retains a partial 2A peptide, which can be utilized to detect and distinguish a vector-derived protein from its endogenous equivalent (Fig. S2). We currently do not understand why IL12p35-2A-p40 is more efficient at generating p70 than the inverse construct, IL-12p40-2A-p35. However, we hypothesize that early translation of p35 either promotes co-translational protein folding, or avoids production of inhibitory p40 homodimers [37]. Future studies should be directed at understanding the importance of subunit positionality as related to production of IL-12p70.

In both preclinical models and clinical trials, EP has been used successfully to deliver therapeutic genes via non-viral vectors (GET) [16, 19, 38] or to increase uptake of chemotherapeutic drugs into tumor cells (electrochemotherapy) [39–42]. The treatment goal and the design of the electrode array dictate the applied electric field strength in order to ensure optimal delivery of therapeutic agent and survival of electroporated cells [43]. Our IT-pIL12-EP therapy combines targeted tumor treatment and plasmid-based GET with the goal of evoking a strong systemic immune response leading to eradication of the treated tumor and any existing metastases. By lowering the applied field strength to 400 V/cm for treatment of B16.F10 tumors, we saw a significant increase in the percentage of transfected cells 48 h post treatment (Fig. 2b). The highest transduction efficiency achieved using low voltage conditions was approximately 10% of all tumor cells. This is only slightly less than what can be achieved with intratumoral viral transduction, which is in the range of 15% [44]. Considering the risk of insertional mutagenesis or development of neutralizing antibodies to virus-based therapies in humans, plasmid-based gene therapy with comparable transfection efficiency offers a distinctly advantageous safety profile. Interestingly, the observed increase in transfection efficiency is not restricted to tumor cells but also affects TILs. One of the reasons for this significant improvement in transfection efficiency may

be increased cell survival at these low voltage conditions [27]. As IT-pIL12-EP produces secreted IL-12 cytokine, which can induce IFN-γ in adjacent cells leading to production of more IL-12, a therapeutic effect can be observed with only a small percentage of cells being transfected due to this paracrine feed-forward effect. In addition, we hypothesize that for a secreted molecule it may not be important which cell type is electroporated within the tumor as the transfected cells solely function as factories to produce and secrete IL-12 into the tumor environment. As our data also show that EP of B16.F10 tumors with low voltage conditions can successfully transfect lymphocytes (Fig. 2b), targeting secondary lymphoid organs with GET may be a viable future direction to manipulate T cells (or other immune cells) in vivo. Electroporating these organs with plasmids coding for therapeutically relevant molecules, such as T-cell costimulatory molecules or chimeric T-cell receptors, may generate a physiologically relevant response. Design changes to the EP applicator and/or handle with the purpose to improve co-localization of DNA injectant and electric field may further increase intratumoral transfection efficiency. Finally, combining these improvements with catheter-based EP devices may enable us to target visceral lesions in future clinical trials.

In summary, the newly developed IT-pIL12-P2A-EP platform marks a significant improvement of EP-based cancer immunotherapy: (1) low voltage EP conditions lead to a significant improvement of expression of the therapeutic gene and systemic antitumor response, and (2) the P2A-based vector system leads to increased expression of IL-12p70 and allows for simultaneous expression of multiple genes. Testing the pIL12-P2A plasmid with low voltage EP conditions in our murine tumor model demonstrated increased influx of $CD8^+$ T cells into the tumor environment (Fig. 4c) and enhanced systemic antitumor response (Fig. 4b). This novel IT-pIL12-P2A-EP platform can potentially open the field of GET to a new set of therapeutically active molecules that previously could not be expressed sufficiently using in vivo EP.

Materials and methods

Tumor cell lines and mice

B16.F10 melanoma cells (CRL-6475), obtained from the American Type Culture Collection (ATCC), were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, and 1% gentamicin at 5% CO_2 and 37 °C. MC38 murine colon carcinoma cells (a generous gift from Dr. Holbrooke Kohrt, Stanford University) were maintained in DMEM, supplemented with 10% fetal bovine serum, and 1% Pen/Strep at 5% CO_2 and 37 °C. In preparation for injection, the cells were trypsinized, washed with sterile Dulbecco's phosphate-buffered saline (DPBS) (Gibco) and counted using a Cellometer (Cellometer Auto 2000, Nexcelom) or hemocytometer. Depending on the experiment, between 0.25 and 1×10^6 live cells in 100 µl of sterile DPBS were injected into the shaved flank of a C57BL/6J mouse. Tumors were measured using a digital caliper. Treatment began when the primary tumor volume reached ~ 100 mm^3, 9–11 days after cell inoculation. At this time, mice with primary tumors >50 and <150 mm^3 were randomized into study groups. Tumor volume (V_T) was calculated using the formula $V_T = a^2 \times b / 2$, where a = smallest diameter and b = perpendicular diameter. The animal technician measuring the tumors was blinded to the study. Female C57BL/6J mice between 6 and 8 weeks of age were ordered from Jackson Laboratory, and all animal housing and husbandry was performed by Explora BioLabs, San Diego. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Explora BioLabs. In accordance with the Animal Welfare Act, the number of animals per treatment group was reduced to the lowest possible number to still achieve an estimated statistical power of 80%.

Plasmids and reagents

pUMVC3 and pUMVC3-mIL12 (mIL12-p35-IRES-p40) were purchased from Aldevron. The genes for constructing mIL12-p40-2A-p40, mIL12-p35-2A-p40, mIL12-p4-GAS-p35, and hIL12-p35-2A-p40 were PCR amplified from the corresponding geneblock fragments Integrated DNA Technologies (IDT) introducing the necessary restriction sites (details provided in supplement). For generation of Luc2p-P2A-mCherry, Luc2P was PCR amplified from pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega) and mCherry was amplified from a gene block fragment (IDT). Amplified DNA fragments were purified, digested, and ligated into pUMVC3. Positive clones were identified via restriction enzyme digests and verified with DNA sequencing. The following reagents were used for flow cytometric analysis: APC/Cy7 CD45, BV605 CD4, BV421 CD3, BV650 CD8, BV510 CD19 (all anti-mouse and all Biolegend), and Live/Dead Fixable Aqua Dead Cell Stain kit (Thermo Fisher). Antibodies used for western blotting: the antibody against the 2A cleavage scar was purchased from EMD Millipore; the antibody against mouse IL12A (p35 subunit) was purchased from Abcam.

Intratumoral treatment

C57BL/6J mice were anesthetized using 97% oxygen and 3% isoflurane. Tumors were injected with 50 µl (0.02–1 mg/

ml) plasmid DNA in sterile saline using a tuberculin syringe with a 27-gauge needle. Either a rectangular applicator containing four electrodes spaced 0.5 cm apart or a circular applicator containing six electrodes with a diameter of 0.5 cm apart was utilized by inserting the electrodes into the tumor. After administration of the DNA solution, six rotating (high voltage: 1500 V/cm, 0.1 ms pulse width, 300 ms delay) or eight unidirectional (low voltage: 400 V/cm, 10 ms pulse width, 300 ms delay) pulses were delivered. High voltage conditions were either delivered with the MedPulser (OncoSec Medical Inc.) or the ECM 830 pulse generator (BTX). Low voltage conditions were delivered using the ECM 830 pulse generator (BTX) or the GENESIS low voltage generator (OncoSec Medical Inc.; patent application WO 2016/161201 A3).

In vivo imaging

An optical imaging system (Lago, Spectral Instruments) was used to quantify luminescence of tumors that were previously treated with a Luc2p-P2A-mCherry plasmid. The mice were imaged at different time-points. To perform imaging, animals were anesthetized by exposure to 2% isoflurane in 500 ml/min of oxygen. Once anesthetized, 200 µl of a 15 mg/ml solution of D-luciferin (Gold Bio) prepared in sterile DPBS was administered by intraperitoneal injection with a 27-gauge syringe. Animals were then transferred to an anesthesia manifold on a 37 °C heated stage, where they continued to receive 2% isoflurane in 500 ml/min of oxygen. Luminescent images were acquired 20 min after injection using a 5-s exposure to a CCD camera cooled to −90 °C. Total photons emitted from each tumor was determined by post-processing using a region of interest with a 0.5 cm radius (AmiView, Spectral Instruments).

Dissociation of tumors for flow cytometric analysis

Single-cell suspensions were prepared from B16.F10 tumors. Mice were sacrificed with CO_2 and tumors were carefully excised leaving skin and non-tumor tissue behind. The excised tumors were then stored in ice-cold Hank's balanced salt solution (HBSS; Gibco) for further processing. Tumors were minced and incubated with gentle agitation at 37 °C for 20–30 min in 5 ml of HBSS containing 1.25 mg/ml collagenase IV, 0.125 mg/ml hyaluronidase and 25 U/ml DNase IV. After enzymatic dissociation, the suspension was passed through a 40 µm nylon cell strainer (Corning) and red blood cells removed with ACK lysis buffer (Quality Biological). Single cells were washed with PBS Flow Buffer (PFB: PBS without Ca^{++} and Mg^{++} containing 2% fetal calf serum and 1 mM EDTA) pelleted by centrifugation and resuspended in PFB for immediate flow cytometric analysis.

Tumor lysis for protein extraction

Fresh or frozen tumors were transferred to a 5 ml polystyrene tube containing 300 µl protein lysis buffer (150 mM NaCl, 50 mM TRIS pH 7.5, 1 mM EDTA, 0.5% Triton X-100) and placed on ice. The tumors were then homogenized for 30 s on ice using a LabGen 710 homogenizer (Cole Palmer). Homogenates were transferred to 1.5 ml Eppendorf tubes, spun down at 4 °C at full speed (19,000 *g*), and supernatants transferred to a fresh 1.5 ml Eppendorf tube for immediate analysis or storage at −80 °C.

Cytokine analysis by ELISA or MAGPIX

Tumor extracts or sera were analyzed for mIL12 and murine IFN-γ protein levels. This was either done by two separate ELISAs (DuoSet, R&D Systems) or multiplexed using the Milliplex mouse cytokine panel (Millipore Sigma). Multiplex plates were analyzed with the Luminex® MAGPIX® system. HEK293 cells (ATCC) were transfected in six-well plates with Mirus TransIT LT-1 reagent (Mirus Bio). Supernatants were collected 48–72 h post-transfection, cleared by centrifugation, and quantitated by ELISA (DuoSet, R&D Systems).

IL-12 bioassay

HEK-Blue™ cells (InvivoGen), designed to detect bioactive human or mouse IL-12, were used to quantitate the amount of plasmid-derived, functional IL-12 as described by the manufacturer. The bioassay was performed using a twofold dilution series of IL-12p70 ranging from 0 to 25.6 ng/ml.

Statistical analysis

Data were expressed as mean ± SEM and statistical analyses were performed by using Graphpad Prism (Graphpad Software Inc., La Jolla, CA, USA). Methods used were Mann–Whitney *U*-test or Kruskal–Wallis H test, depending on the number of data sets to be analyzed. Kaplan–Meier survival analysis were analyzed by the log-rank test.

Compliance with ethical standards

Conflict of interest All authors are past or present employees of Oncosec Medical Inc. Dr. Canton, Dr. Connolly, Dr. Campbell, Dr. Bahrami, and Dr. Pierce are inventors of patents, pending and published.

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