Intratumoral DNA-based delivery of checkpoint-inhibiting antibodies and interleukin 12 triggers T cell infiltration and anti-tumor response

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Running title: Combined gene transfer of checkpoint inhibitors and IL-12

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ABSTRACT

To improve the anti-tumor efficacy of immune checkpoint inhibitors, numerous combination therapies are under clinical evaluation, including with IL-12 gene therapy. The current study evaluated the simultaneous delivery of the cytokine and checkpoint-inhibiting antibodies by intratumoral DNA electroporation in mice. In the MC38 tumor model, combined administration of plasmids encoding IL-12 and an anti-PD-1 antibody induced significant anti-tumor responses, yet similar to the monotherapies. When treatment was expanded with a DNA-based anti-CTLA-4 antibody, this triple combination significantly delayed tumor growth compared to IL-12 alone and the combination of anti-PD-1 and anti-CTLA-4 antibodies. Despite low drug plasma concentrations, the triple combination enabled significant abscopal effects in contralateral tumors, which was not the case for the other treatments. The DNA-based immunotherapies increased T cell infiltration in electroporated tumors, especially of CD8+ T cells, and upregulated the expression of CD8+ effector markers. No general immune activation was detected in spleens following either intratumoral treatment. In B16F10 tumors, evaluation of the triple combination was hampered by a high sensitivity to control plasmids. In conclusion, intratumoral gene electrotransfer allowed effective combined delivery of multiple immunotherapeutics. This approach induced responses in treated and contralateral tumors, while limiting systemic drug exposure and potentially detrimental systemic immunological effects.

INTRODUCTION

Immune checkpoint inhibitors have emerged as a new cornerstone for cancer therapy, demonstrating durable responses in a variety of tumors. However, the fraction of patients that benefit from these treatments is still limited, with many factors that can lead to primary or acquired resistance[1]. The combination of monoclonal antibodies (mAbs) targeting cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) has been shown to increase response rates for some indications, but is also associated with high risks of grade 3/4 adverse events[2, 3]. To further exploit the potential of these checkpoint inhibitors, safer and more effective combination therapies are required. In view of this, up to 3,000 clinical trials are evaluating mAbs that block the PD-1 axis in combination with other cancer therapies[4], including with intratumoral immunotherapies[5]. These immunostimulatory agents, such as oncolytic viruses, cytokines and agonists of pattern recognition receptors, aim to promote an anti-tumor immune response by different modes of actions than checkpoint-inhibiting mAbs. Their intratumoral administration maximizes the drug concentration at the tumor site, while limiting systemic exposure and associated adverse events[5]. One specific therapy that is currently being tested in combination with systemic checkpoint blockade is intratumoral gene transfer of the cytokine interleukin 12 (IL-12, ClinicalTrials.gov: e.g. NCT02493361 (ref. 6), NCT03132675, NCT03567720, NCT04526730).

IL-12 is a pleiotropic pro-inflammatory cytokine with effects on both the innate and adaptive immune system. Although its therapeutic use has initially been limited due to severe immune-related toxicity following systemic administration[7], intratumoral delivery of plasmid DNA (pDNA) encoding IL-12 has been shown to avoid this toxicity and enable regressions of both treated and untreated lesions in patients with metastatic melanoma and Merkel cell carcinoma[8, 9]. In these studies, IL-12 pDNA was administered by means of electroporation, a technique already used in the clinic to improve the delivery of chemotherapeutic drugs to tumor cells.

Electroporation comprises the application of short electrical pulses to the pDNA administration site, which temporally increases the cell membranes' permeability and thereby enables targeted *in vivo* DNA transfection[10, 11]. The resulting local IL-12 expression stimulates immune cell infiltration in tumors, as well as T cell activation, antigen presentation and programmed cell death ligand 1 (PD-L1) expression[8], thereby making poorly inflamed tumors more sensitive to checkpoint blockade. Indeed, the combination of the anti-PD-1 mAb pembrolizumab and intratumoral IL-12 gene electrotransfer has led to a 41% objective response rate in melanoma patients who were unlikely to respond to anti-PD-1 therapy[6].

The favorable safety profile and clinical efficacy of locally expressed IL-12 demonstrate the potential of intratumoral gene electrotransfer, and suggest it can also be applied to deliver and combine other biological drugs. Indeed, DNA-based delivery enables transfected cells to produce the encoded drugs *in vivo* for a prolonged period of time. Compared to conventional protein-based treatments, this approach can allow for a reduced administration frequency and can overcome the complex and costly *in vitro* manufacturing of, for example, therapeutic mAbs[12]. In addition to tumor-targeting nanobodies[13], we previously evaluated intratumoral gene electrotransfer of anti-CTLA-4 and anti-PD-1 mAbs, demonstrating local and systemic anti-tumor responses in a subcutaneous (s.c.) mouse tumor model. Intratumoral expression of these checkpoint inhibitors resulted in only low and transient systemic mAb exposure, and can thereby potentially reduce the high toxicity risk associated with systemic delivery the corresponding mAb proteins[14]. The complete response rate, however, was moderate following gene transfer of both mAbs, leaving room for improvement.

The aim of the current study was to demonstrate proof of concept for the combined, simultaneous delivery of checkpoint-inhibiting mAbs and IL-12 by means of intratumoral DNA-based gene electrotransfer. The anti-tumor efficacy was evaluated in two s.c. syngeneic mouse tumor models: the highly immunogenic MC38 and poorly immunogenic B16F10 model.

We also explored the effects of the combined gene transfer approach on immune cells in tumors and spleens of MC38-bearing mice.

MATERIALS AND METHODS

Mice and tumor cell lines

C57BL/6J mice were purchased from Charles River Laboratories (Saint Germain Nuelles, France). All experiments were approved by the KU Leuven Animal Ethics Committee (P130/2017).

The MC38 cell line, derived from C57BL/6 colon adenocarcinoma cells, was purchased from Kerafast (ENH204-FP, Boston, MA, USA) in 2017. Cells were grown in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 50 U/ml penicillin/streptomycin (41965062, 10500064, 11140035, 11360039, 15630056, 15070063, Thermo Fischer Scientific, Waltham, MA, USA). The C57BL/6 melanoma cell line B16F10, purchased from ATCC (CRL-6475, Manassas, VA, USA) in 2017, was grown in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum and 1 mM sodium pyruvate. Both cells lines were maintained in a humidified incubator at 37°C and 5% CO_2. The master stocks, frozen after four and six passages, respectively, were shown to be free of *Mycoplasma* contamination (MycoAlert mycoplasma detection kit, LT07-218, Lonza, Basel, Switzerland).

For *in vivo* tumor experiments, 1 x 10^6 MC38 cells or B16F10 cells in 100 µl D-PBS (14190169, Thermo Fischer Scientific) were injected subcutaneously in the right flank of 6- to 7-week old female C57BL/6J mice, following four to seven additional passages after thawing. To study abscopal effects, 0.25 x 10^6 MC38 cells in 100 µl D-PBS were injected subcutaneously in the left flank four days after the first tumor cell injection. For B16F10 tumor rechallenge, cured mice received a s.c. injection of 1 x 10^6 B16F10 cells in 100 µl D-PBS in the left flank 92 days after the first tumor cell injection. Tumor growth was evaluated two to three times per week with an electronic caliper (500-712-20, Mitutoyo, Kawasaki, Japan). Tumor volumes were

calculated with the formula *length x width² x 0.5*. Mice were sacrificed when tumor volume exceeded 2000 mm³, or when they lost more than 10% of their weight.

pDNA constructs

The pDNA constructs consisted of an ampicillin resistance gene, a pUC origin of replication and an expression cassette with a CAG promoter and a TK poly(A) sequence[11, 13-17]. pDNA production and purification were performed as previously described[15].

To construct an IL-12-expressing pDNA [p(IL-12)], a cDNA sequence encoding the murine p35 subunit and murine p40 subunit linked by a picornavirus-derived self-cleaving 2A peptide was derived from literature[18]. This sequence was synthetized by Genewiz (Leipzig, Germany) and cloned in-house into the above-mentioned pDNA backbone. Proper cloning was verified by sequencing and restriction analyses. *In vitro* IL-12 expression was evaluated by ELISA and Western blot. An empty plasmid [pNull], which corresponds to the pDNA backbone devoid of the CAG-driven expression cassette, was provided by Icosagen (Tartu, Estonia) and served as a control for p(IL-12).

The DNA-based murinized IgG1 anti-mouse PD-1 mAb [p(aPD-1)] and DNA-based murine IgG2a^{b} anti-mouse CTLA-4 mAb [p(aCTLA-4)] were previously constructed and validated *in vitro* and *in vivo*[14]. DNA-based isotype controls [p(IgG1) and p(IgG2a^{b})] were established with the sequences of murine mAbs towards *Clostridium difficile* toxin A and B, respectively, which were generated in-house by PharmAbs. Cloning of the variable regions onto the appropriate heavy and light chain constant regions in CAG-driven pDNA constructs was performed by Icosagen for p(IgG1) and in-house for p(IgG2a^{b}). Proper in-house cloning was confirmed by sequencing and restriction analyses. *In vitro* mAb expression was validated by ELISA and SDS PAGE. For all DNA-based mAbs, heavy and light chain were expressed by separate plasmids.

Intratumoral pDNA electroporation

Intratumoral pDNA electrotransfer was performed in palpable tumors, as previously described[14]. Mice were therefore randomized into groups based on tumor volume (ranging from 50 to 270 mm^3 across studies) and weight at the day of treatment. Ten mice per group were included for combination studies, five to eight mice per group for dose-response and flow cytometry studies. These numbers were based on our previous *in vivo* tumor experiments[13-15]. No blinding to the group allocation was performed.

In brief, five days after MC38 injection and seven or nine days after B16F10 injection, mice received a single intratumoral injection of 0.074-122.5 µg pDNA in 30 or 50 µl D-PBS immediately followed by electroporation. The electroporation protocol comprised of two series of four 5-ms square-wave pulses of 600 V/cm in perpendicular directions at a frequency of 1 Hz. Pulses were delivered by the preclinical NEPA21 Electroporator (Sonidel Limited, Dublin, Ireland) with CUY650P5 tweezer electrodes (Sonidel Limited) at a fixed width of 5 mm and covered with Eco Ultrasound Transmission Gel (G0066, Fiab, Vicchio, Italy). The current and total energy were verified with the NEPA21 readout.

For the DNA-based mAbs, heavy chain pDNA and light chain pDNA were administered at a 1:1 molar ratio. When the combination of DNA-based IL-12 and DNA-based checkpoint inhibitors was evaluated, the treatments of the individual and control pDNA groups were supplemented with equimolar amounts of pNull, p(IgG1) and/or p($IgG2a^b$) to substitute p(IL-12), p(aPD-1) and/or p(aCTLA-4), respectively. Of note, untreated mice received neither pDNA, buffer nor electroporation.

ELISA

Blood was collected by retro-orbital bleeding of mice, processed to plasma and stored at -20°C. Murine IL-12 concentrations were determined in plasma with the mouse IL-12 (p70) ELISA

MAX Deluxe Set (433604, BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Anti-PD-1 and anti-CTLA-4 mAbs were quantified in plasma with in-house developed ELISAs, as previously described[14]. To assess the specificity of the detected anti-PD-1 mAb levels, plasma samples were analyzed with and without a prior 1-hour incubation with an approximately 50-fold molar excess of the target PD-1 (1021-PD-100, R&D Systems, Minneapolis, MN, USA) at room temperature.

Flow cytometry

Eight days after intratumoral pDNA delivery, mice were sacrificed and spleens and tumors were harvested and weighed. Spleens were processed into single-cell suspensions by pressing them through a 70-µm cell strainer, followed by removal of the red blood cells with ACK lysing buffer (A1049201, Thermo Fischer Scientific). Tumors were cut into small pieces and digested in RPMI-1640 medium (52400041, Thermo Fisher Scientific) with 10 U/ml collagenase I, 400 U/ml collagenase IV and 30 U/ml DNase I (LS004214, LS004212, LS002058, Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C under continuous rotation for 25 minutes. The digested tissue was mechanically disrupted and filtered through a 70-µm cell strainer. After red blood cell lysis, the cells were passed through a second 70-µm cell strainer.

Single-cell suspensions were incubated with supernatant of 2.4G2 hybridoma cells (HB-197, ATCC) to block non-specific binding, and with fixable viability dye eFluor 780 (65-0865-18, Thermo Fischer Scientific) to label dead cells. Cells were then stained for CD45 (custom, BD Biosciences, San Jose, CA, USA), CD62L (741230, BD Biosciences) and CD69 (104510, BioLegend). After fixation and permeabilization with the Foxp3 staining kit (00-5523-00, Thermo Fischer Scientific), cells were stained with a panel containing antibodies against CD4, CD8a, CD19, CD103, CD25 (612952, 612898, 747332, 740238, 566120, BD Biosciences), CD127, CTLA-4, GITR, T-bet, GATA-3 (custom, BD Biosciences), CD3, PD-1, KLRG1,

ICOS, Ki-67, TCRβ, CD44, NK1.1 (100229, 135231, 138429, 313538, 652420, 109215, 103037, 108701, BioLegend), Neuropilin, ST2, Foxp3, Eomes (46-3041-82, 25-9335-82, 17-5773-82, 48-4875-82, Thermo Fischer Scientific) and Helios (130-112-636, Miltenyi Biotec, Bergisch Gladbach, Germany). Precision Count Beads (424902, BioLegend) were added to determine absolute cell counts. Flow data were acquired on a BD FACSymphony (BD Biosciences) and analyzed with Flowjo 10.7.1 (Becton, Dickinson and Company, Ashland, OR, USA). Compensation was done using AutoSpill[19]. Dead cells and doublets were gated out prior to downstream analysis.

Statistics

Statistical analyses were performed with GraphPad Prism 9.1.1 (GraphPad Software, San Diego, CA, USA). Data were presented as mean + standard error of the mean (SEM) or standard deviation and compared between all treatment groups with one-way ANOVA with a Tukey's test for multiple comparisons (or Šídák's test for multiple comparisons when selected groups were compared). Kaplan–Meier survival curves were analyzed with the log-rank (Mantel-Cox) test with a Holm's test for multiple comparisons. Two-sided P values below 0.05 were considered significant.

RESULTS

Combined DNA-based delivery of IL-12 and an anti-PD-1 antibody results in similar anti-tumor responses as the respective monotherapies in an MC38 tumor model

Combined intratumoral gene transfer was first evaluated for IL-12 and an anti-PD-1 mAb, based on the available clinical data with the mAb proteins[6]. We used mice bearing a s.c. MC38 tumor, an immunologically hot model sensitive to both IL-12 (ref. [20, 21]) and checkpoint-inhibiting mAbs[14, 22], and treated them with a single intratumoral pDNA administration followed by electroporation. For the DNA-based anti-PD-1 mAb p(aPD-1), we used the same dose as in our previous experiments (60 µg)[14], but only administered once instead of three times. The optimal dose of the IL-12-expressing plasmid p(IL-12) to evaluate in combination was determined by dose-response studies in MC38-bearing mice (2.5 µg, Supplementary Fig. S1). All mice received the same total pDNA dose by addition of an equimolar amount of an empty plasmid pNull or isotype control plasmid p(IgG1) to substitute p(IL-12) and p(aPD-1), respectively. Similarly, pDNA treatments in subsequent combination studies were supplemented with the appropriate control plasmids to equalize the pDNA dose administered to all mice within one experiment. To evaluate the effect of pDNA electroporation alone, one treatment group in each experiment just received the combination of control plasmids.

p(IL-12) and p(aPD-1) monotherapy both resulted in significant tumor growth delay compared to untreated mice ($P<0.0001$ for p(IL-12), $P<0.001$ for p(aPD-1)), but no complete tumor regressions were observed (Fig. 1, Supplementary Fig. S2). The combination of p(IL-12) and p(aPD-1) resulted in 10% complete regressions (Supplementary Fig. S2), but neither tumor growth (Fig. 1) nor survival (Supplementary Fig. S3) was significantly different compared to the respective individual treatments. On the other hand, p(IL-12), both separate and combined with p(aPD-1), did improve the anti-tumor response compared to the control plasmids pNull and p(IgG1) ($P<0.05$), which had no significant effect on tumor growth ($P>0.05$ *versus*

untreated, Fig. 1). Plasma concentrations of the expressed anti-PD-1 mAb were below 250 ng/ml, except for one mouse which had levels up to 650 ng/ml. IL-12 was not detectable in the plasma (i.e. < 20 pg/ml), further demonstrating the safety benefit of intratumoral gene transfer. In summary, combined intratumoral electrotransfer of DNA-based IL-12 and a DNA-based anti-PD-1 mAb induced moderate responses in MC38 tumors, yet comparable to those of the individual treatments.

Triple combination of DNA-based IL-12, anti-PD-1 and anti-CTLA-4 antibodies improves local and abscopal effects in a dual MC38 tumor model

To improve the efficacy of the combined gene transfer approach, the intratumoral treatment was expanded with a DNA-based anti-CTLA-4 mAb p(aCTLA-4) (60 µg). Local responses in treated tumors as well as systemic anti-tumor effects in contralateral untreated lesions were evaluated in mice bearing two s.c. MC38 tumors. Similar to p(IL-12) and p(aPD-1) (Fig. 1), the triple combination with p(aCTLA-4) led to 10% complete regressions in treated tumors (Fig. 2A, Supplementary Fig. S4A). However, the triple combination did significantly improve the anti-tumor response compared to p(IL-12) alone ($P<0.05$) and to the combination of both checkpoint inhibitors ($P<0.01$, Fig. 2A). In the contralateral untreated tumors, the combination of p(IL-12), p(aPD-1) and p(aCTLA-4) enabled a significant abscopal effect compared to untreated mice ($P<0.01$), with 20% complete regressions (Fig. 2B, Supplementary Fig. S4B). The individual treatments, on the other hand, had no significant effect on the distant tumors ($P>0.05$ *versus* untreated, Fig. 2B). Throughout follow-up, plasma levels of the corresponding proteins were low, suggesting that the observed abscopal effect was not mediated by circulating mAbs or IL-12 (IL-12: undetectable, i.e. < 40 pg/ml; anti-CTLA-4 mAb: < 40 ng/ml; anti-PD-1 mAb: < 100 ng/ml except for two mice with levels up to 550 ng/ml). In short, combined intratumoral gene transfer of IL-12 and checkpoint inhibitors outperformed the separate

treatments, improving both local and systemic anti-tumor effects with only limited systemic exposure to the expressed drugs.

Intratumoral gene transfer of IL-12, anti-PD-1 and anti-CTLA-4 antibodies drives profound changes in tumor-infiltrating, but not splenic T cells

Next, we explored the immunological changes caused by intratumoral electroporation of p(IL-12), p(aPD-1) and p(aCTLA-4) to estimate the drivers of the associated anti-tumor responses and to evaluate if combined gene transfer can amplify effects on immune-cell level. In mice bearing a single MC38 tumor, treatment with the triple combination was compared to p(IL-12) and to the combination of p(aPD-1) and p(aCTLA-4). Similar to the previous experiments, the two control groups were either untreated or received control plasmids. Eight days after intratumoral pDNA delivery, tumors and spleens were harvested and immune cells in both tissues were analyzed by flow cytometry.

At the time of harvest, tumors treated with DNA-based immunotherapeutics were smaller than in the control groups, although not all differences reached statistical significance. No obvious difference was observed between the triple combination and the two other treatment groups ($P>0.990$). Likewise, tumor sizes of the control pDNA and untreated group were similar at the time of harvest ($P>0.900$, Supplementary Fig. S5).

In treated tumors, p(IL-12) monotherapy, the combination of p(aPD-1) and p(aCTLA-4), and the corresponding triple combination increased the percentage of infiltrating T cells compared to control mice ($P<0.05$ or $P<0.01$ *versus* untreated, $P<0.10$ or $P<0.01$ *versus* control plasmids), with no differences between these three groups. Electroporation of the control plasmids pNull, p(IgG1) and p($IgG2a^b$) had no effect on T cell infiltration ($P>0.999$ *versus* untreated, Fig. 3A). Overall, this corresponds to the differences in tumor size observed at the time of harvesting (Supplementary Fig. S5). Interestingly, the rise in T cells was mainly driven by cytotoxic T

cells (Fig. 3B), since no changes in helper T cells or regulatory T cells (Tregs) were observed between the treatment groups (Fig. 3C-3D). This translated in a significant decrease in the ratio of CD4+ Foxp3+ Tregs to CD8+ cytotoxic T cells in the tumors treated with p(IL-12) and/or p(aPD-1) and p(aCTLA-4) ($P<0.05$ or $P<0.01$ *versus* control plasmids or untreated, Fig. 3E), suggesting that an effective anti-tumor immune response was evoked by the DNA-based immunotherapies. None of the treatments significantly affected the percentage of natural killer (NK) cells or B cells within the tumor-infiltrating immune cell population, nor the absolute cell count of leukocytes, T cells or the different T cell subsets per milligram of tumor weight (Supplementary Fig. S6).

Besides the presence of the T cell subsets within the tumor, the expression of different markers on these cells was assessed. The triple combination of p(IL-12), p(aPD-1) and p(aCTLA-4), but not the other treatments, significantly increased the percentage of KLRG1+ CD8+ cytotoxic T cells ($P<0.05$ *versus* untreated, Supplementary Fig. S7), and more specifically the KLRG1+ CD127- short-lived effector cells ($P<0.05$ *versus* untreated, Fig. 3F). Following the triple therapy, also the percentage of ST2+ CD8+ T cells was increased compared to p(IL-12) ($P<0.05$) and, although not statistically significant, to untreated mice ($P=0.071$, Fig. 3G). Treatment with p(aPD-1) and p(aCTLA-4) significantly upregulated the expression of the proliferation marker Ki-67 ($P<0.01$ *versus* untreated, Fig. 3H) and the immune checkpoint PD-1 ($P<0.05$ *versus* control plasmids and untreated, Fig. 3I) on cytotoxic T cells, changes that were not significant for the treatments including p(IL-12). Effector CD8+ cells, identified as CD44+ CD62L-[23], were significantly enriched after treatment with the DNA-based checkpoint inhibitors ($P<0.05$ *versus* untreated), and a clear positive trend was present after the triple therapy ($P=0.068$ *versus* untreated, Fig. 3J). This was accompanied with a decrease in CD62L+ CD44- naive cytotoxic T cells in the tumor ($P<0.05$ and $P=0.083$ *versus* untreated, respectively, Supplementary Fig. S7). Interestingly, markers that were upregulated in the overall intratumoral

CD8+ T cell population were also increased in the effector subset (Supplementary Fig. S8). For intratumoral CD4+ Foxp3- helper T cells (Supplementary Fig. S9), a decrease in Eomes+ cells was observed in all electroporated groups, which reached statistical significance for the combination of p(aPD-1) and p(aCTLA-4) ($P<0.05$ *versus* untreated, Fig. 3K). Expression of the early activation marker CD69 was also decreased after treatment with p(aPD-1) and p(aCTLA-4) ($P<0.05$ *versus* untreated, Fig. 3L). No significant changes to untreated mice were observed for the studied markers on intratumoral CD4+ Foxp3+ Tregs (Supplementary Fig. S10). Observations in the CD44+ CD62L- effector subset of helper T cells and Tregs (Supplementary Fig. S11-S12) were mostly in line with those in the respective overall populations.

To assess if the local DNA-based treatments also evoked a major systemic immune activation, which might lead to adverse events[24], splenocytes of treated mice were evaluated with the same flow cytometry panel as used for the evaluation of the tumor-infiltrating immune cells. None of the treatments had an effect on the total number of immune cells or the percentage of specific immune cell subsets within the spleen (T cells, cytotoxic T cells, helper T cells, Tregs, NK cells and B cells, Supplementary Fig. S13). Changes in the expression of the studied markers were limited, and mainly observed in the case of intratumoral delivery of p(aPD-1) and p(aCTLA-4) (Supplementary Fig. S14-S16).

In summary, intratumoral electroporation of p(IL-12), p(aPD-1) and p(aCTLA-4) increased the fraction of infiltrating T cells in treated tumors. Especially CD8+ cytotoxic cells were enriched, which also had an upregulated expression of effector markers. Systemic immunological changes in the spleen were limited following intratumoral DNA-based immunotherapy. Overall, these effects suggest the generation of an effective tumor-specific immune response. However, no pronounced differences were detected between the combination of IL-12 and checkpoint inhibitors and the respective separate treatments eight days after treatment.

Strong responses to plasmid DNA electroporation hamper the evaluation of the triple combination in B16F10 tumors

Following the promising results in MC38-bearing mice, the intratumoral delivery of p(IL-12), p(aPD-1) and p(aCTLA-4) was evaluated in s.c. B16F10 tumors. This poorly immunogenic tumor model has repeatedly been used to evaluate IL-12 therapies[25, 26], but appears to be less responsive to checkpoint blockade[27]. Tumors were induced by injection of 1 million B16F10 cells, which resulted in rapid tumor progression in untreated mice yet allowed significant anti-tumor responses by IL-12 gene therapy. Based on dose-response studies (Supplementary Fig. S17), 0.22 µg p(IL-12) was selected for combination with the DNA-based checkpoint inhibitors, as this dose resulted in moderate tumor growth inhibition and a low complete response rate. Interestingly, this is more than tenfold lower than the dose used in MC38-bearing mice, indicating that B16F10 tumors are more sensitive to IL-12 gene therapy than the highly immunogenic MC38 tumors. The same doses of p(aPD-1) and p(aCTLA-4) were used as in our previous experiments (60 µg of each). Surprisingly, intratumoral electroporation of the control plasmids pNull, p(IgG1) and p($IgG2a^b$) led to strong anti-tumor responses ($P<0.0001$ *versus* untreated), with finally 50% complete regressions (Fig. 4A, Supplementary Fig. S18A). This was not observed in our p(IL-12) dose-finding studies in B16F10 tumors (Supplementary Fig. S17), likely because of the much lower intratumoral pDNA doses administered in these studies (total dose of control plasmids: 6.67-0.25 µg in dose-finding studies *versus* 119.58 µg in combination study). The intratumoral expression of IL-12 and checkpoint-inhibiting mAbs resulted in a significantly higher anti-tumor effect compared to the control plasmids ($P<0.05$ for p(IL-12) and the triple combination, Fig. 4A). However, no significant differences could be detected between the triple combination on the one hand and p(IL-12) and the DNA-based checkpoint inhibitors alone on the other hand, given that all treatments led to high complete

response rates (100%, 100% and 80%, respectively, Fig. 4A, Supplementary Fig. S18A and S19A). Similar to the experiments with MC38-bearing mice, plasma levels of the expressed drugs were low. IL-12 could not be detected in the plasma of the mice (i.e. < 100 pg/ml). Anti-PD-1 mAb levels were all below 200 ng/ml, except for one mouse with peak levels of 450 ng/ml two weeks after pDNA delivery. Anti-CTLA-4 mAb plasma concentrations peaked one week after pDNA delivery, with levels up to 180 ng/ml, and all dropped below 30 ng/ml two weeks later.

To evaluate if the treatments evoked a different anti-tumor immune memory, all mice with complete tumor regressions were rechallenged with B16F10 cells 13 weeks after the first tumor cell injection. While all naive control mice developed tumors, 40 to 75% of the rechallenged mice were resistant to tumor growth (Supplementary Fig. S18B), which resulted in a significantly lower average tumor volume ($P<0.0001$, Fig. 4B) and significantly improved survival ($P<0.001$ or $P<0.05$, Supplementary Fig. S19B) compared to the naive mice for all rechallenged groups. Similar as after the first tumor injection, no significant differences were observed between the mice treated with the triple combination, p(IL-12) alone or the DNA-based checkpoint inhibitors alone. Intratumoral electroporation of the control plasmids also led to a similar degree of protection against tumor rechallenge (Fig. 4B, Supplementary Fig. S18B and S19B). To sum up, intratumoral electroporation of control pDNA yielded strong responses in B16F10 tumors, which were further improved when the pDNA expressed IL-12 and/or checkpoint-inhibiting mAbs. Long-term systemic anti-tumor effects, however, were similar in complete responders of all evaluated treatments. In the current setup, no benefits of combined gene transfer of IL-12 and checkpoint inhibitors were observed, potentially masked by the strong effect of pDNA electroporation and the respective separate treatments in this model.

DISCUSSION

The high resistance rates to checkpoint inhibitors have fueled the search for more effective combination therapies[4]. One such example is the combination of systemic anti-PD-1 mAbs with intratumoral electroporation of plasmid-based IL-12. We hypothesized that delivery via intratumoral gene transfer could be further exploited for the development of safe and effective combination treatments.

First, we evaluated intratumoral DNA-based delivery of IL-12 and an anti-PD-1 mAb in a s.c. MC38 mouse tumor model. Despite prior dose-finding studies for p(IL-12), combined gene transfer did not outperform the respective DNA-based monotherapies. This is in contrast with a clinical trial in patients with cold melanoma tumors[6] and several preclinical studies that showed additive or synergistic effects of intratumorally expressed IL-12 and systemic anti-PD-1 mAb treatment[28-30]. These preclinical studies, however, used oncolytic viruses for IL-12 expression, which act as a third immunostimulating factor, in tumor models refractory to PD-1 blockade. Moreover, Garris *et al*. showed that anti-PD-1 mAb treatment as such can indirectly stimulate IL-12 expression in MC38 tumors by infiltrating dendritic cells[20]. In order to obtain an increased effect with the combined gene transfer approach, we included a second DNA-based checkpoint inhibitor, p(aCTLA-4). While anti-PD-1 mAbs unleash the brakes on activated T cells, anti-CTLA-4 mAbs intervene earlier in the immunity cycle during T cell priming[31], thereby adding a distinct mechanism to promote anti-tumor immunity in combination with IL-12. Indeed, the triple combination of p(IL-12), p(aPD-1) and p(aCTLA-4) did enhance tumor growth inhibition compared to p(IL-12) and the DNA-based checkpoint inhibitors, and also improved the systemic anti-tumor response, as demonstrated by significant regressions of distant untreated tumors. The absence of an abscopal effect with DNA-based IL-12 and the DNA-based checkpoint inhibitors alone, which has been observed in previous studies[14, 25, 32, 33], can probably be linked to the lower IL-12 pDNA dose and single pDNA

391 administration applied in the current study, respectively. Overall, this study demonstrates that
392 DNA-based gene electrotransfer allows effective and straightforward intratumoral delivery of
393 three immunomodulatory agents at once.

394 To further explore the mechanism behind the response to the DNA-based immunotherapies,
395 changes in tumor-infiltrating lymphocytes were studied eight days after treatment. As
396 previously reported for intratumoral IL-12 gene transfer[21, 33, 34] and systemic delivery of
397 checkpoint inhibitors[22, 27, 35], p(IL-12) and the combination of p(aPD-1) and p(aCTLA-4)
398 increased CD3+ T cells and cytotoxic CD8+ T cells in treated tumors. While this explains the
399 tumor growth delay compared to untreated mice in these groups, no further increase in T cell
400 infiltration was observed when the three DNA-based therapeutics were combined. This could
401 correspond to the lack of obvious difference in anti-tumor response among the three treatment
402 groups at the time that samples were harvested. Overall, none of the studied cell populations
403 was significantly altered between the triple combination and the individual treatments, except
404 for CD8+ T cells expressing the IL-33 receptor ST2. Since ST2 signaling has been shown to
405 enhance the anti-tumor activity of CD8+ T cells[36], the increased percentage of ST2+ cells might
406 have contributed to the improved anti-tumor response to the triple combination observed at later
407 time points. While p(aPD-1) and p(aCTLA-4) increased CD44+ effector cytotoxic T cells,
408 intratumoral KLRG1+ short-lived effector cells were only significantly enriched after delivery
409 of the triple combination. In a study of Mukhopadhyay *et al*., these KLRG1+ CD8+ effector
410 cells were proposed as an important mediator of the systemic anti-tumor effects of intratumoral
411 IL-12 gene electrotransfer[33]. Remarkably, intratumoral expression of the IgG2a^{b} anti-CTLA-4
412 mAb[14] did not influence the percentage of Tregs within the tumor. This suggests that the effects
413 of p(aCTLA-4) at the time of the analysis were mainly evoked by blockade of CTLA-4 rather
414 than depletion of Tregs[37]. Furthermore, intratumoral pDNA electroporation caused a decrease
415 in Eomes+ CD4+ helper T cells. Eomes expression has been associated with Th1 responses and

cytotoxic activity, but can also drive differentiation into immunosuppressive type 1 regulatory T (Tr1) cells in mice[38-40]. To determine the exact role of Eomes+ cells in our study, further analysis of e.g. the expressed cytokines is needed. Other changes in tumor-infiltrating T cells following electroporation of control plasmids were limited, which is in line with their poor effect on tumor growth at the time of sample harvesting. Interestingly, flow cytometric analyses of the spleen showed that the intratumoral expression of IL-12, the anti-PD-1 mAb and anti-CTLA-4 mAb did not result in a major systemic immune activation. Together with the low plasma levels of the expressed drugs, this suggests that intratumoral DNA-based gene transfer can reduce or avoid the immune-related adverse events observed with systemic drug administration[24], and can allow for the combination of other drugs that would be highly toxic when delivered systemically. More specialized mouse models could be used in future studies to confirm the improved biosafety compared to conventional protein-based treatments[24, 41, 42], in addition to comparisons in terms of efficacy. In contrast to our study, other reports of intratumoral immunotherapy did observe an increase in effector CD8+ T cells in the spleen and hypothesized that these cells correspond to disseminated tumor-specific T cells[23, 33]. Since we observed an abscopal effect with the triple combination in a dual MC38 tumor, we expect that also in our study tumor-specific T cells disseminated to the periphery and contralateral tumor. However, we hypothesize that these cells only present a small proportion of the immune cells within the spleen and therefore did not cause any detectable changes. To substantiate the observed systemic anti-tumor effects, specific analyses for tumor-specific T cells could be performed in future studies, in addition to characterization of immune cells infiltrating the contralateral tumors. Lastly, it is important to emphasize that this exploratory analysis was limited to one time point and had a limited number of mice per group. Characterization of the tumor-infiltrating and splenic immune cells at additional time points in more extended groups

could provide more insight in the time-dependent evolution of the observed effects and further validate the observed trends.

Finally, the triple combination of the DNA-based IL-12, anti-PD-1 mAb and anti-CTLA-4 mAb was evaluated in s.c. B16F10 tumors. We hypothesized that this poorly immunogenic tumor model would benefit more from (combined) immunotherapy than the immunologically hot MC38 model[43]. However, B16F10 tumors were highly sensitive to electroporation of pDNA, irrespective of the presence or absence of a therapeutic transgene, which made it impossible to distinguish the possible beneficial effect of combined gene transfer. Indeed, next to some physical damage caused by electroporation, pDNA can activate intracellular DNA sensors in the transfected cells, thereby stimulating an innate and subsequently adaptive immune response towards the tumor. This anti-tumor effect of pDNA has repeatedly been described in the literature, and varies in strength depending on the applied pulse protocol, pDNA dose and composition, and tumor model[44-46]. The stronger effect in B16F10 compared to MC38 tumors observed in this study is in contrast with the hypothesis that highly immune-inflamed tumors are more responsive to pDNA electroporation[44], and suggests that other factors may play an important role. Future studies can therefore focus on elucidating these factors and finding ways to reduce the effect of control plasmids in B16F10 tumors (e.g. by reducing the total pDNA dose, by adjusting the electroporation protocol), to be able to better evaluate the beneficial effect of combined gene transfer of IL-12 and checkpoint inhibitors in B16F10 tumors. Finally, this treatment approach can be tested in additional tumor models, to further assess its translatability.

In conclusion, we demonstrated that intratumoral gene electrotransfer can be applied to efficiently combine IL-12, an anti-PD-1 mAb and an anti-CTLA-4 mAb in MC38-bearing mice. This triple combination improved local and systemic anti-tumor responses compared to the checkpoint inhibitors and IL-12 alone, and mediated an increase in effector CD8+ T cells in treated tumors. The treatment was associated with a favorable safety profile, as systemic drug

exposure was limited and major systemic immune activation was absent. Successful application of the combined gene transfer approach in other tumor models, such as B16F10, requires further adjustment of the treatment setup to differentiate its effect from that of pDNA electroporation as such, and a more in-depth evaluation of e.g. the systemic anti-tumor response to validate the potential advantage compared to the single DNA-based treatments. Overall, the results of the current study suggest that intratumoral DNA-based gene transfer can present an effective approach to facilitate the development of various other combinations of immunotherapeutics.

ACKNOWLEDGEMENTS

We are very grateful to Dr. Maya Imbrechts, Gerlanda Vella, Dr. Emanuela Pasciuto and Prof. Susan Schlenner (KU Leuven, Leuven, Belgium) for their help with the preparation and their advice regarding the design of the flow cytometry experiment described in the current manuscript. We would also like to thank Prof. Damya Laoui and Aleksandar Murgaski (VUB, Brussels, Belgium) for sharing their protocol for tumor dissociation. Finally, we would like to thank all staff members of the Laboratory for Therapeutic and Diagnostic Antibodies (KU Leuven) for their help with the tissue processing for flow cytometry.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

LJ, NG, KH and PD designed the overall study and interpreted the results. LJ performed the experiments and analyzed the data. LY, SJ and AL contributed to the design and the execution of the flow cytometry experiment and the interpretation of the respective results. LJ wrote the manuscript, which was reviewed and edited by KH and PD. All authors read and approved the manuscript for publication.

FUNDING

This research is supported by Research Foundation – Flanders (FWO: PhD mandate 1133220N to LJ, and research project G0E2117N to KH and PD) and CELSA (research project CELSA/19/030 to KH and PD).

REFERENCES

1. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science* 2018; **359**(6382)**:** 1350-1355.
2. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD *et al.* Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* 2019; **381**(16)**:** 1535-1546.
3. Kooshkaki O, Derakhshani A, Hosseinkhani N, Torabi M, Safaei S, Brunetti O *et al.* Combination of Ipilimumab and Nivolumab in Cancers: From Clinical Practice to Ongoing Clinical Trials. *Int J Mol Sci* 2020; **21**(12): 4427.
4. Upadhaya S, Neftelino ST, Hodge JP, Oliva C, Campbell JR, Yu JX. Combinations take centre stage in PD1/PDL1 inhibitor clinical trials. *Nat Rev Drug Discov* 2021; **20**(3): 168-169.
5. Middleton MR, Hoeller C, Michielin O, Robert C, Caramella C, Öhrling K *et al.* Intratumoural immunotherapies for unresectable and metastatic melanoma: current status and future perspectives. *Br J Cancer* 2020; **123**(6)**:** 885-897.
6. Algazi AP, Twitty CG, Tsai KK, Le M, Pierce R, Browning E *et al.* Phase II Trial of IL-12 Plasmid Transfection and PD-1 Blockade in Immunologically Quiescent Melanoma. *Clin Cancer Res* 2020; **26**(12)**:** 2827-2837.
7. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB *et al.* Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 1997; **90**(7)**:** 2541-2548.
8. Algazi A, Bhatia S, Agarwala S, Molina M, Lewis K, Faries M *et al.* Intratumoral delivery of tavokinogene telseplasmid yields systemic immune responses in metastatic melanoma patients. *Ann Oncol* 2020; **31**(4)**:** 532-540.

9. Bhatia S, Longino NV, Miller NJ, Kulikauskas R, Iyer JG, Ibrani D *et al.* Intratumoral Delivery of Plasmid IL12 Via Electroporation Leads to Regression of Injected and Noninjected Tumors in Merkel Cell Carcinoma. *Clin Cancer Res* 2020; **26**(3)**:** 598-607.
10. Heller R, Heller LC. Gene electrotransfer clinical trials. *Adv Genet* 2015; **89:** 235-262.
11. Jacobs L, De Smidt E, Geukens N, Declerck P, Hollevoet K. Electroporation outperforms in vivo-jetPEI for intratumoral DNA-based reporter gene transfer. *Sci Rep* 2020; **10**(1)**:** 19532.
12. Hollevoet K, Declerck PJ. State of play and clinical prospects of antibody gene transfer. *J Transl Med* 2017; **15**(1)**:** 131.
13. Vermeire G, De Smidt E, Casteels P, Geukens N, Declerck P, Hollevoet K. DNA-based delivery of anti-DR5 nanobodies improves exposure and anti-tumor efficacy over protein-based administration. *Cancer Gene Ther* 2021; **28**(7-8)**:** 828-838.
14. Jacobs L, De Smidt E, Geukens N, Declerck P, Hollevoet K. DNA-Based Delivery of Checkpoint Inhibitors in Muscle and Tumor Enables Long-Term Responses with Distinct Exposure. *Mol Ther* 2020; **28**(4)**:** 1068-1077.
15. Hollevoet K, De Smidt E, Geukens N, Declerck P. Prolonged *in vivo* expression and anti-tumor response of DNA-based anti-HER2 antibodies. *Oncotarget* 2018; **9**(17)**:** 13623-13636.
16. Hollevoet K, De Vleeschauwer S, De Smidt E, Vermeire G, Geukens N, Declerck P. Bridging the Clinical Gap for DNA-based Antibody Therapy through Translational Studies in Sheep. *Hum Gene Ther* 2019; **30**(11): 1431-1443.
17. Vermeire G, De Smidt E, Geukens N, Williams JA, Declerck P, Hollevoet K. Improved Potency And Safety Of DNA-encoded Antibody Therapeutics Through Plasmid Backbone And Expression Cassette Engineering. *Hum Gene Ther* 2021; e-pub ahead of print Sep 4 2021; doi:10.1089/hum.2021.105.

18. Campbell, J, Canton, DA, Pierce, RH. Plasmid constructs for heterologous protein expression and methods of use. Patent US20190153469A1; 2019.

19. Roca CP, Burton OT, Gergelits V, Prezzemolo T, Whyte CE, Halpert R *et al.* AutoSpill is a principled framework that simplifies the analysis of multichromatic flow cytometry data. *Nat Commun* 2021; **12**(1)**:** 2890.

20. Garris CS, Arlauckas SP, Kohler RH, Trefny MP, Garren S, Piot C *et al.* Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-γ and IL-12. *Immunity* 2018; **49**(6)**:** 1148-1161.e7.

21. Hewitt SL, Bailey D, Zielinski J, Apte A, Musenge F, Karp R *et al.* Intratumoral IL12 mRNA Therapy Promotes TH1 Transformation of the Tumor Microenvironment. *Clin Cancer Res* 2020; **26**(23): 6284-6298.

22. Wei SC, Anang NAS, Sharma R, Andrews MC, Reuben A, Levine JH *et al.* Combination anti-CTLA-4 plus anti-PD-1 checkpoint blockade utilizes cellular mechanisms partially distinct from monotherapies. *Proc Natl Acad Sci U S A* 2019; **116**(45)**:** 22699-22709.

23. Ishihara J, Fukunaga K, Ishihara A, Larsson HM, Potin L, Hosseinchi P *et al.* Matrix-binding checkpoint immunotherapies enhance antitumor efficacy and reduce adverse events. *Sci Transl Med* 2017; **9**(415): eaan040.

24. Pai CS, Simons DM, Lu X, Evans M, Wei J, Wang YH *et al.* Tumor-conditional anti-CTLA4 uncouples antitumor efficacy from immunotherapy-related toxicity. *J Clin Invest* 2019; **129**(1)**:** 349-363.

25. Burkart C, Mukhopadhyay A, Shirley SA, Connolly RJ, Wright JH, Bahrami A *et al.* Improving therapeutic efficacy of IL-12 intratumoral gene electrotransfer through novel plasmid design and modified parameters. *Gene Ther* 2018; **25**(2)**:** 93-103.

26. Momin N, Mehta NK, Bennett NR, Ma L, Palmeri JR, Chinn MM *et al.* Anchoring of intratumorally administered cytokines to collagen safely potentiates systemic cancer immunotherapy. *Sci Transl Med* 2019; **11**(498): eaaw2614.

27. Selby MJ, Engelhardt JJ, Johnston RJ, Lu LS, Han M, Thudium K *et al.* Preclinical Development of Ipilimumab and Nivolumab Combination Immunotherapy: Mouse Tumor Models, In Vitro Functional Studies, and Cynomolgus Macaque Toxicology. *PLoS One* 2016; **11**(9)**:** e0161779.

28. Quetglas JI, Labiano S, Aznar M, Bolaños E, Azpilikueta A, Rodriguez I *et al.* Virotherapy with a Semliki Forest Virus-Based Vector Encoding IL12 Synergizes with PD-1/PD-L1 Blockade. *Cancer Immunol Res* 2015; **3**(5)**:** 449-454.

29. De Lucia M, Cotugno G, Bignone V, Garzia I, Nocchi L, Langone F *et al.* Retargeted and Multi-cytokine-Armed Herpes Virus Is a Potent Cancer Endovaccine for Local and Systemic Anti-tumor Treatment. *Mol Ther Oncolytics* 2020; **19:** 253-264.

30. Ge Y, Wang H, Ren J, Liu W, Chen L, Chen H *et al.* Oncolytic vaccinia virus delivering tethered IL-12 enhances antitumor effects with improved safety. *J Immunother Cancer* 2020; **8**(1): e000710.

31. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC *et al.* Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade. *Cell* 2017; **170**(6)**:** 1120-1133.e17.

32. Sin JI, Park JB, Lee IH, Park D, Choi YS, Choe J *et al.* Intratumoral electroporation of IL-12 cDNA eradicates established melanomas by Trp2(180-188)-specific CD8+ CTLs in a perforin/granzyme-mediated and IFN-γ-dependent manner: application of Trp2(180-188) peptides. *Cancer Immunol Immunother* 2012; **61**(10)**:** 1671-1682.

33. Mukhopadhyay A, Wright J, Shirley S, Canton DA, Burkart C, Connolly RJ *et al.* Characterization of abscopal effects of intratumoral electroporation-mediated IL-12 gene therapy. *Gene Ther* 2019; **26**(1-2)**:** 1-15.

34. Shi G, Edelblute C, Arpag S, Lundberg C, Heller R. IL-12 Gene Electrotransfer Triggers a Change in Immune Response within Mouse Tumors. *Cancers (Basel)* 2018; **10**(12): 498.

35. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* 2010; **107**(9)**:** 4275-4280.

36. Gao X, Wang X, Yang Q, Zhao X, Wen W, Li G *et al.* Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *J Immunol* 2015; **194**(1)**:** 438-445.

37. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M *et al.* Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res* 2013; **1**(1)**:** 32-42.

38. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A *et al.* Eomesodermin promotes the development of type 1 regulatory T (T_R1) cells. *Sci Immunol* 2017; **2**(10): eaah7152.

39. Mazzoni A, Maggi L, Siracusa F, Ramazzotti M, Rossi MC, Santarlasci V *et al.* Eomes controls the development of Th17-derived (non-classic) Th1 cells during chronic inflammation. *Eur J Immunol* 2019; **49**(1)**:** 79-95.

40. Roessner PM, Llaó Cid L, Lupar E, Roider T, Bordas M, Schifflers C *et al.* EOMES and IL-10 regulate antitumor activity of T regulatory type 1 $CD4^+$ T cells in chronic lymphocytic leukemia. *Leukemia* 2021; **35**(8)**:** 2311-2324.

41. Liu J, Blake SJ, Harjunpää H, Fairfax KA, Yong MC, Allen S *et al.* Assessing Immune-Related Adverse Events of Efficacious Combination Immunotherapies in Preclinical Models of Cancer. *Cancer Res* 2016; **76**(18)**:** 5288-5301.

42. Adam K, Iuga A, Tocheva AS, Mor A. A novel mouse model for checkpoint inhibitor-induced adverse events. *PLoS One* 2021; **16**(2)**:** e0246168.

43. Zhong W, Myers JS, Wang F, Wang K, Lucas J, Rosfjord E *et al.* Comparison of the molecular and cellular phenotypes of common mouse syngeneic models with human tumors. *BMC Genomics* 2020; **21**(1)**:** 2.

44. Bosnjak M, Jesenko T, Kamensek U, Sersa G, Lavrencak J, Heller L *et al.* Electrotransfer of Different Control Plasmids Elicits Different Antitumor Effectiveness in B16.F10 Melanoma. *Cancers (Basel)* 2018; **10**(2): 37.

45. Marrero B, Shirley S, Heller R. Delivery of interleukin-15 to B16 melanoma by electroporation leads to tumor regression and long-term survival. *Technol Cancer Res Treat* 2014; **13**(6)**:** 551-560.

46. Heller LC, Coppola D. Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. *Gene Ther* 2002; **9**(19)**:** 1321-1325.

FIGURE LEGENDS

Figure 1. Combined intratumoral delivery of DNA-based IL-12 and a DNA-based anti-PD-1 antibody in MC38 tumors. C57BL/6J mice received a single intratumoral pDNA electrotransfer (indicated by the arrow) five days after MC38 tumor cell injection. 2.5 µg p(IL-12) or an equimolar amount of pNull was administered in combination with 60 µg p(aPD-1) or an equimolar amount of p(IgG1) in 30 µl D-PBS. One group received no treatment. Tumor volumes, represented as mean + SEM, were compared with one-way ANOVA on day 17 after tumor cell injection, when the first mice had to be sacrificed. Asterisks in the figure legends, not accompanied by a square bracket, indicate the statistical difference compared to untreated mice (n = 10 mice per group, * $P<0.05$, *** $P<0.001$, **** $P<0.0001$, CR: complete responders).

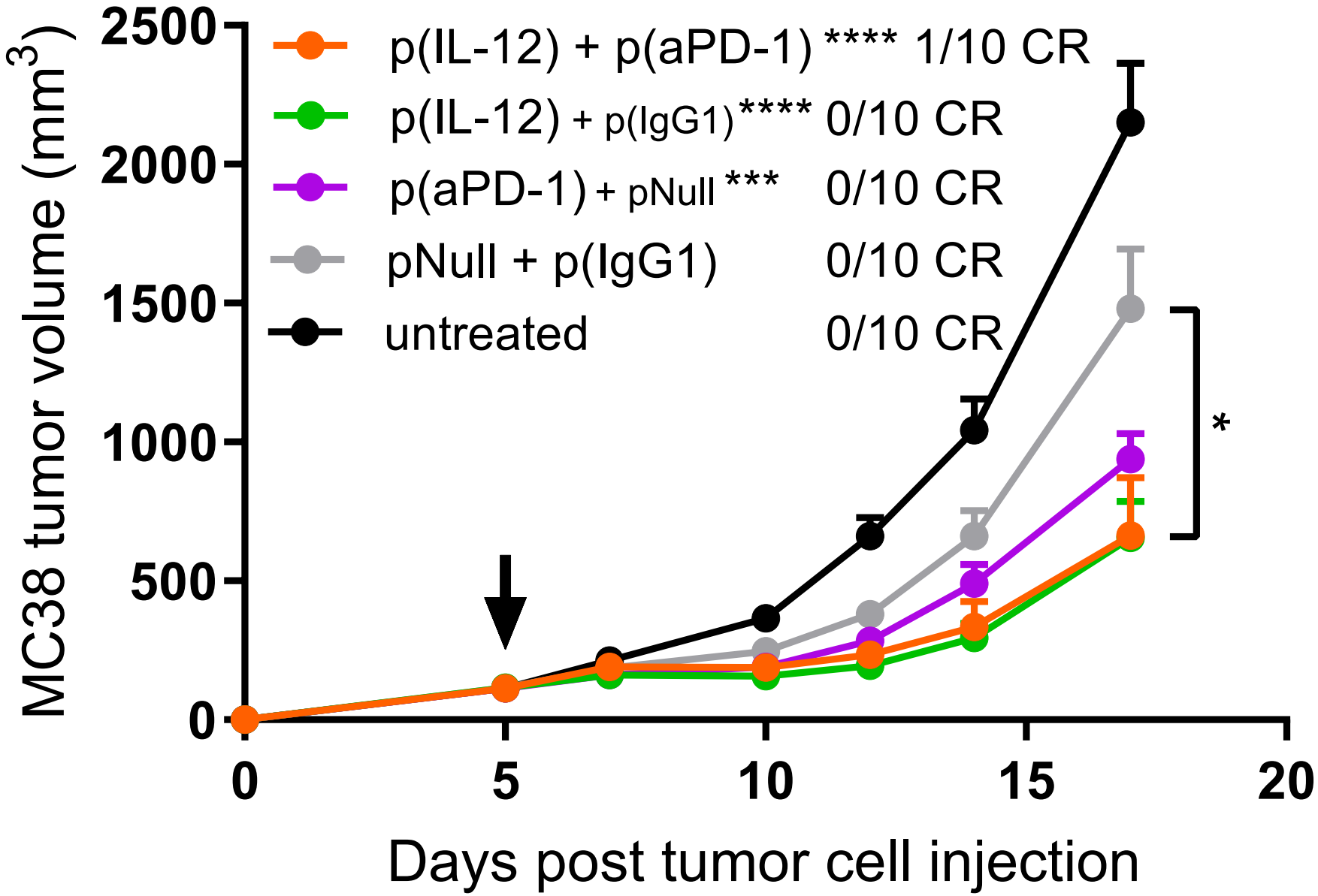
Figure 2. Combined intratumoral delivery of DNA-based IL-12 and DNA-based anti-PD-1 and anti-CTLA-4 antibodies in MC38 tumors. C57BL/6J mice were subcutaneously injected with 1 x 10^6 MC38 cells in the right flank (primary tumor), and 0.25 x 10^6 MC38 cells in the left flank four days later (contralateral tumor). The primary tumor received a single intratumoral electrotransfer (indicated by the arrow) of 2.5 µg p(IL-12) or an equimolar amount of pNull in combination with 60 µg p(aPD-1) and 60 µg p(aCTLA-4) or equimolar amounts of the DNA-based isotype controls in 50 µl D-PBS, five days after tumor injection. The contralateral tumor received no treatment. In one group, both tumors were left untreated. Tumor volumes, represented as mean + SEM, and the number of complete responders (CR) are separately shown for primary **(A)** and contralateral **(B)** tumors. If no complete regressions were observed within a treatment group, the number of CR is not mentioned. Tumor volumes were compared with one-way ANOVA on day 17 and day 19 after primary tumor cell injection, when the first untreated mice and the first mice of other treatment groups were sacrificed,

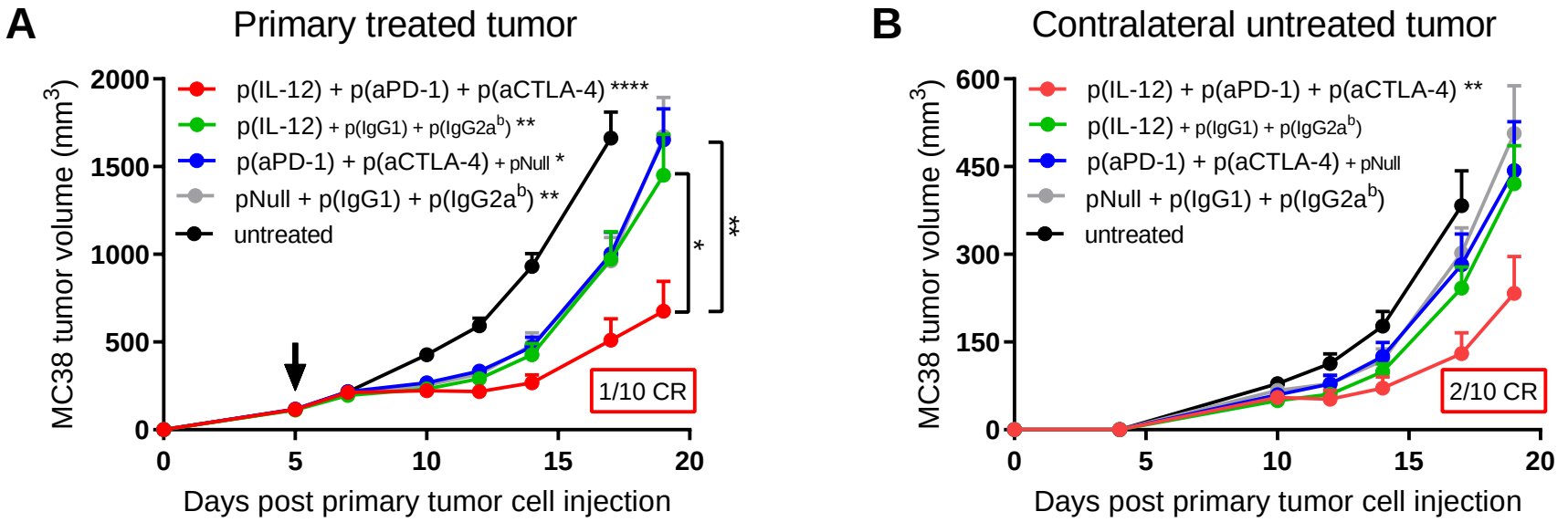
respectively. Asterisks in the figure legends, not accompanied by a square bracket, indicate the statistical differences with untreated mice on day 17 (n = 10 mice per group, * P<0.05, ** P<0.01, **** P<0.0001).

Figure 3. Effect of intratumoral delivery of DNA-based IL-12, anti-PD-1 and anti-CTLA-4 antibodies on MC38-infiltrating T lymphocytes. C57BL/6J mice bearing one s.c. MC38 tumor received a single intratumoral pDNA electrotransfer five days after tumor cell injection. 2.5 µg p(IL-12) or an equimolar amount of pNull was administered in combination with 60 µg p(aPD-1) and 60 µg p(aCTLA-4) or equimolar amounts of the DNA-based isotype controls in 50 µl D-PBS. One group received no treatment. Eight days after treatment, tumors were harvested, processed into single-cell suspensions and analyzed by flow cytometry. **(A-D)** The percentage of T cells (CD3+ TCRβ+, **A**), cytotoxic T cells (CD3+ TCRβ+ CD8+, **B**), helper T cells (CD3+ TCRβ+ CD4+ Foxp3-, **C**) and Tregs (CD3+ TCRβ+ CD4+ Foxp3+, **D**) within the population of tumor-infiltrating leukocytes (CD45+). **(E)** The ratio of the number of Tregs to the number of cytotoxic T cells within the tumor. **(F-J)** The percentage of short-lived effector cells (KLRG1+ CD127-, **F**), ST2+ cells (**G**), Ki-67+ cells (**H**), PD-1+ cells (**I**) and effector cells (CD44+ CD62L-, **J**) within the tumor-infiltrating cytotoxic T cells. **(K-L)** The percentage of Eomes+ cells (**K**) and CD69+ cells (**L**) within the tumor-infiltrating helper T cells. Scatter plots show mean + standard deviation. On each plot, data of all treatment groups were compared with one-way ANOVA (n = 6-8 mice per group, * P<0.05, ** P<0.01, P values between 0.05 and 0.10 are also shown).

Figure 4. Combined intratumoral delivery of DNA-based IL-12 and DNA-based anti-PD-1 and anti-CTLA-4 antibodies in B16F10 tumors. (A) C57BL/6J mice received a single intratumoral pDNA electrotransfer (indicated by the arrow) seven days after B16F10 tumor cell

injection. 0.22 µg p(IL-12) or an equimolar amount of pNull was administered in combination with 60 µg p(aPD-1) and 60 µg p(aCTLA-4) or equimolar amounts of the DNA-based isotype controls in 50 µl D-PBS. One group received no treatment. Tumor volumes were compared with one-way ANOVA on day 14 and day 19 after tumor cell injection, when the first untreated mouse and the first mouse of the pNull + p(IgG1) + p($IgG2a^b$) group had to be sacrificed, respectively. Asterisks in the figure legends, not accompanied by a square bracket, indicate the statistical differences with untreated mice on day 14 (n = 10 mice per group, CR: complete responders). **(B)** Mice that showed complete regressions after intratumoral gene transfer were rechallenged with B16F10 tumor cells, 92 days after the first tumor cell injection. A group of age-matched naive mice was included as controls. Tumor volumes were compared with one-way ANOVA on day 13 and day 18 after tumor rechallenge, when the first naive mouse and the first mouse of the pNull + p(IgG1) + p($IgG2a^b$) group had to be sacrificed, respectively. Asterisks in the figure legends, not accompanied by a square bracket, indicate the statistical differences with naive mice on day 13 (n = 5-10 mice per group, TF: mice that became tumor-free after rechallenge). All data are represented as mean + SEM (* $P<0.05$, **** $P<0.0001$).


MC38 tumor volume (mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
Days post tumor cell injection
p(IL-12) + p(aPD-1) **** 1/10 CR
p(IL-12) + p(IgG1) **** 0/10 CR
p(aPD-1) + pNull *** 0/10 CR
pNull + p(IgG1) 0/10 CR
untreated 0/10 CR
*


A
Primary treated tumor
MC38 tumor volume (mm3)
2000
1500
1000
500
0
0
5
10
15
20
Days post primary tumor cell injection
p(IL-12) + p(aPD-1) + p(aCTLA-4) ****
p(IL-12) + p(IgG1) + p(IgG2ab) **
p(aPD-1) + p(aCTLA-4) + pNull *
pNull + p(IgG1) + p(IgG2ab) **
untreated
*
**
1/10 CR
B
Contralateral untreated tumor
MC38 tumor volume (mm3)
600
450
300
150
0
0
5
10
15
20
Days post primary tumor cell injection
p(IL-12) + p(aPD-1) + p(aCTLA-4) **
p(IL-12) + p(IgG1) + p(IgG2ab)
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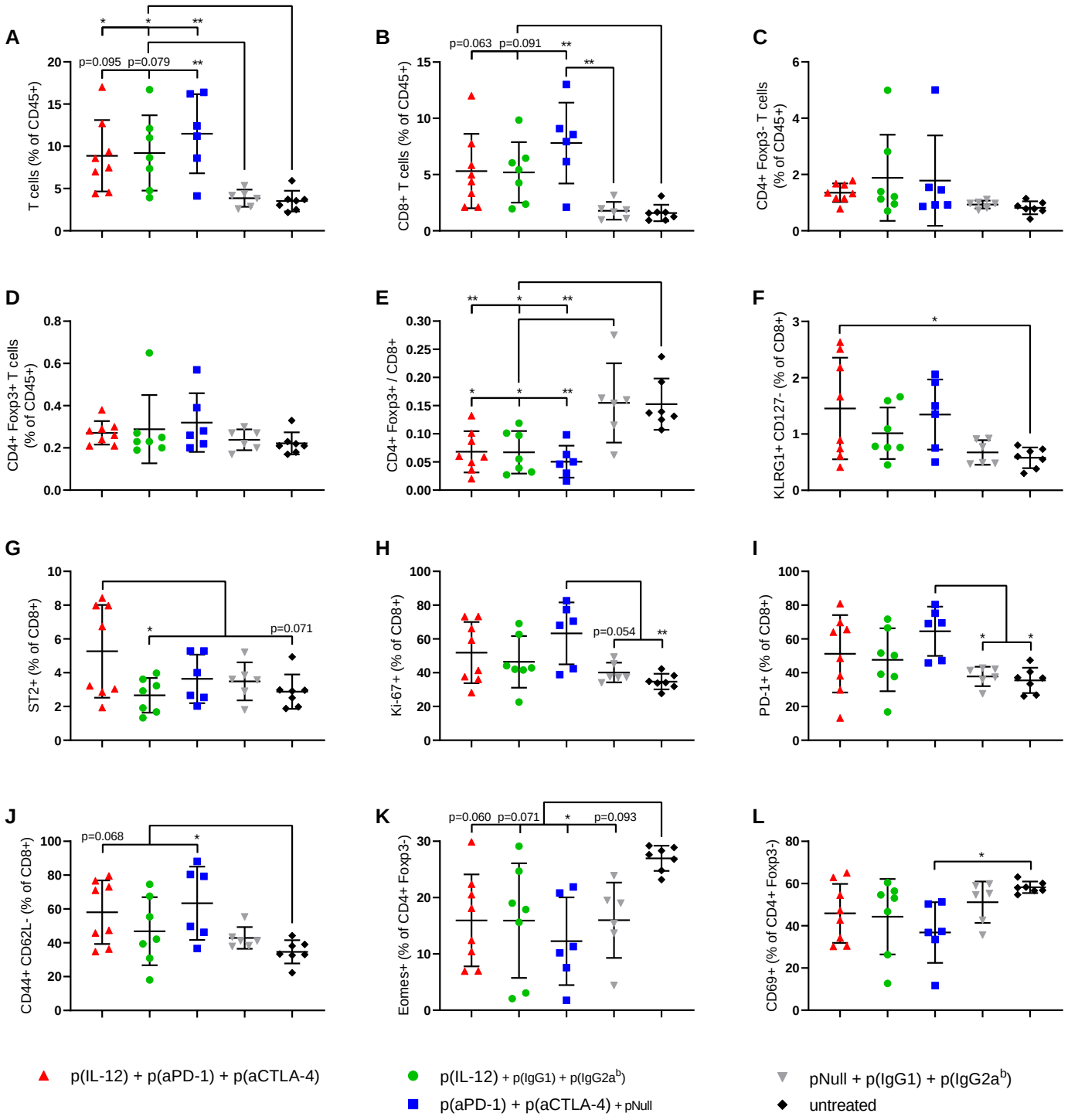
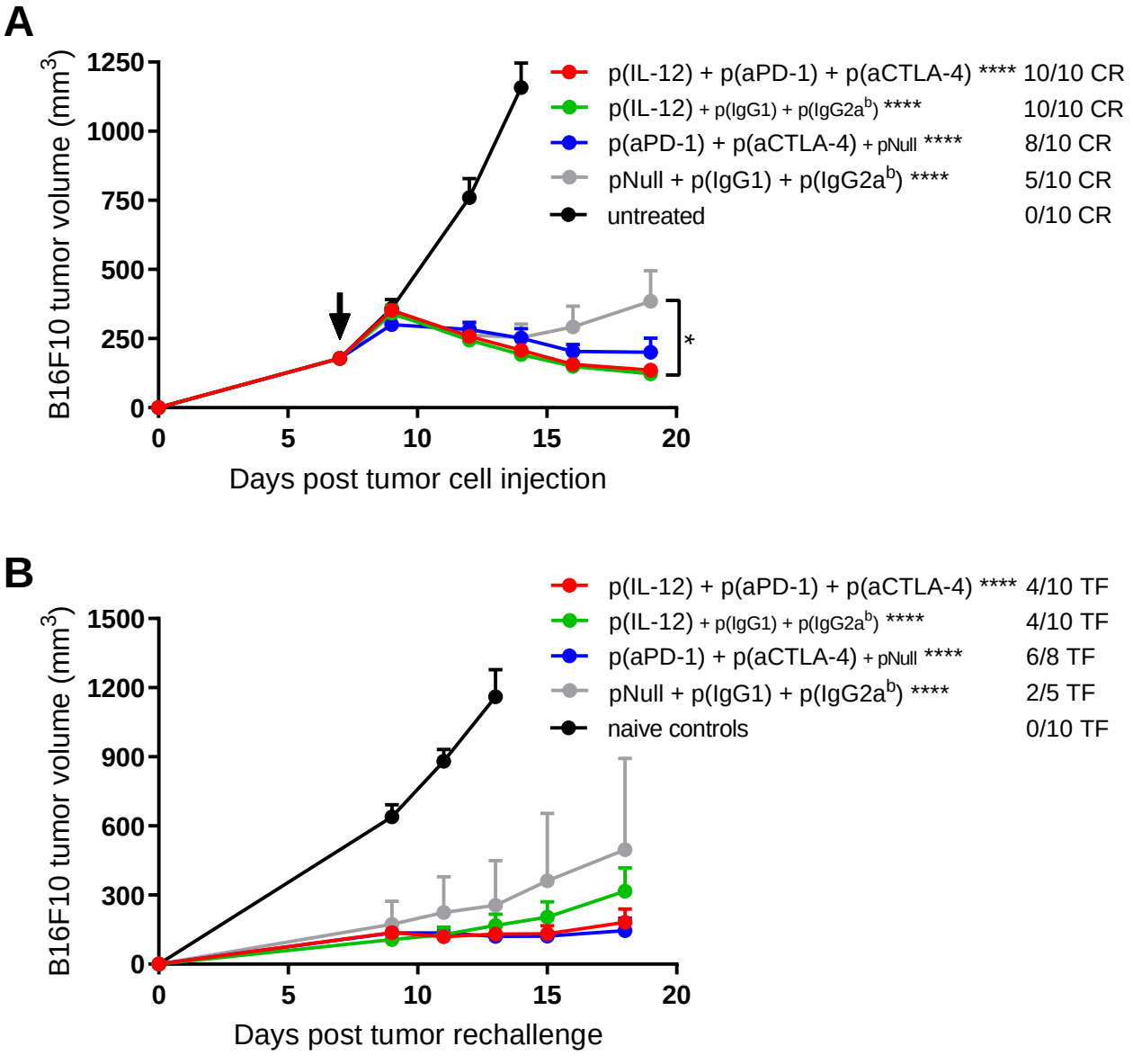
pNull + p(IgG1) + p(IgG2ab)
untreated
2/10 CR


A
T cells (% of CD45+)
p=0.095
p=0.079
B
CD8+ T cells (% of CD45+)
p=0.063
p=0.091
C
CD4+ Foxp3- T cells (% of CD45+)
D
CD4+ Foxp3+ T cells (% of CD45+)
E
CD4+ Foxp3+ / CD8+
F
KLRG1+ CD127- (% of CD8+)
G
ST2+ (% of CD8+)
p=0.071
H
Ki-67+ (% of CD8+)
p=0.054
I
PD-1+ (% of CD8+)
J
CD44+ CD62L- (% of CD8+)
p=0.068
K
Eomes+ (% of CD4+ Foxp3-)
p=0.060
p=0.071
p=0.093
L
CD69+ (% of CD4+ Foxp3-)
p(IL-12) + p(aPD-1) + p(aCTLA-4)
p(IL-12) + p(IgG1) + p(IgG2a^b)
p(aPD-1) + p(aCTLA-4) + pNull
pNull + p(IgG1) + p(IgG2a^b)
untreated


A
B16F10 tumor volume (mm3)
1250
1000
750
500
250
0
0
5
10
15
20
Days post tumor cell injection
*
p(IL-12) + p(aPD-1) + p(aCTLA-4) **** 10/10 CR
p(IL-12) + p(IgG1) + p(IgG2ab) **** 10/10 CR
p(aPD-1) + p(aCTLA-4) + pNull **** 8/10 CR
pNull + p(IgG1) + p(IgG2ab) **** 5/10 CR
untreated 0/10 CR
B
B16F10 tumor volume (mm3)
1500
1200
900
600
300
0
0
5
10
15
20
Days post tumor rechallenge
p(IL-12) + p(aPD-1) + p(aCTLA-4) **** 4/10 TF
p(IL-12) + p(IgG1) + p(IgG2ab) **** 4/10 TF
p(aPD-1) + p(aCTLA-4) + pNull **** 6/8 TF
pNull + p(IgG1) + p(IgG2ab) **** 2/5 TF
naive controls 0/10 TF