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Intratumoral Delivery of Plasmid Interleukin-12 Via Electroporation Leads to Regression of Injected and Non-Injected Tumors in Merkel Cell Carcinoma

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Translational Relevance

Intratumoral immunotherapy is gaining momentum, but its applicability (and likely efficacy too) is limited by the need for repeated injections because of limited intratumoral persistence of the injected drugs. We utilized plasmid interleukin-12 DNA (pIL-12 or tavokinogene telseplasmid; 'tavo') and *in vivo* intratumoral electroporation (i.t.-tavo-EP) to acheive prolonged intratumoral IL-12 expression in 15 patients with Merkel cell carcinoma (MCC), an aggressive, immunogenic cancer. Systemic delivery of IL-12, a cytokine that facilitates T-helper-1 responses, had previously been associated with severe adverse events (AEs), including death. I.t.-tavo-EP administered on days 1, 5, and 8 resulted in sustained (day 22) intratumoral IL-12 expression without any systemic or severe AEs. Resultant local inflammation led to systemic immune responses, with regression of non-injected MCC lesions in 3 of 10 (30%) patients and an overall response rate of 25% (3/12) in patients with metastatic MCC. Gene electrotransfer, specifically i.t.-tavo-EP, warrants further investigation for immunotherapy of cancer.

Structured Abstract

Purpose: Interleukin-12 (IL-12) promotes adaptive type-1 immunity and has demonstrated anti-tumor efficacy, but systemic administration leads to severe adverse events (AEs), including death. This pilot trial investigated safety, efficacy, and immunologic activity of intratumoral delivery of IL-12 plasmid DNA (tavo) via *in vivo* electroporation (i.t.-tavo-EP) in patients with Merkel cell carcinoma (MCC), an aggressive virus-associated skin cancer.

Experimental Design: Fifteen MCC patients with superficial injectable tumor(s) received i.t.-tavo-EP on days 1, 5, and 8 of each cycle. Patients with locoregional MCC (Cohort A, N=3) received one cycle before definitive surgery in week 4. Patients with metastatic MCC (Cohort B, N=12) received up to 4 cycles total, administered at least six weeks apart. Serial tumor and blood samples were collected.

Results: All patients successfully completed at least one cycle with transient, mild (Grade 1, 2) AEs and without significant systemic toxicity. Sustained (day 22) intratumoral expression of IL-12 protein was observed along with local inflammation and increased tumor-specific $CD8^+$ T cell infiltration, which led to systemic immunologic and clinical responses. The overall response rate was 25% (3/12) in Cohort B, with two patients experiencing durable clinical benefit (16 and 55+ months respectively). Two Cohort A patients (one with pathologic complete remission) were recurrence-free at 44+ and 75+ months.

Conclusions: I.t.-tavo-EP was safe and feasible without systemic toxicity. Sustained local expression of IL-12 protein and local inflammation led to systemic immune responses and clinically meaningful benefit in some patients. Gene electrotransfer, specifically i.t.-tavo-EP, warrants further investigation for immunotherapy of cancer.

Introduction

Prior to the success of immune checkpoint inhibitors, immunomodulatory cytokines were the backbone of cancer immunotherapy for decades and provided proof-of-concept for successfully harnessing the immune system against cancer. Interleukin-2 (IL-2) and interferon (IFN) are approved by the United States Food and Drug Administration for advanced melanoma and renal cell carcinoma. Therapeutic utility of cytokines, however, has been limited by low response rates, significant treatment-associated systemic toxicities, and the paucity of predictive biomarkers. The pursuit of novel cytokines with better efficacy and of novel approaches to deliver cytokines without systemic toxicity remain areas of active investigation in cancer immunotherapy, both as monotherapy and in combination immunotherapy regimens.

IL-12 is a 70 kD heterodimeric, multifunctional protein discovered in 1989 and rapidly recognized as a master regulator of adaptive type 1 cell-mediated immunity (1,2). IL-12 production by innate immune cells (e.g. monocytes, macrophages, neutrophils, and dendritic cells) polarizes naïve $CD4^+$ T-cells towards the T-helper-1 (T_H1) phenotype; these cells then produce high levels of IFN-γ upon restimulation (3-5). IL-12 also induces the production of IFN-γ and increased proliferation and cytotoxicity of natural killer cells and $CD8^+$ T-cells (6,7). Additionally, IL-12 up-regulates the expression of human leukocyte antigen (HLA) class I and II, and intercellular adhesion molecule-1 on human cancer cells (melanoma), enhancing T cell extravasation and increasing antigen binding (8). These unique biological properties and promising preclinical data prompted active investigation of recombinant IL-12 (rIL-12) in the treatment of advanced solid tumors and hematologic malignancies. In early phase clinical trials using systemic administration of rIL-12, encouraging biological and clinical responses were observed, but were tempered by severe multi-organ toxicities that included dose-limiting

cytopenias, hepatitis, and even treatment-related deaths in two patients (9-11). These observations led to development of localized IL-12 delivery directly to the tumor in forms other than recombinant protein. This was attempted through direct injection of IL-12 plasmid; viral vectors; or modified fibroblasts, autologous tumor cells, or dendritic cells engineered to secrete IL-12 (12-19). These methods showed efficacy in preclinical models, but not as much in clinical trials, likely related to low gene transfer efficiency (20). In 2008, Daud *et al* reported a clinical trial using intratumoral injection of IL-12 plasmid DNA (tavo) followed by *in vivo* electroporation (i.t.-tavo-EP) to enhance the efficiency of transfection and thus local IL-12 protein expression in 24 patients with advanced melanoma (21). Treatment led to robust dose-proportional expression of IL-12 protein within injected lesions (21). I.t.-tavo-EP was safe and well-tolerated, with minimal systemic toxicity and no significant systemic IL-12 spillage. Four of 19 patients with distant disease, exhibited distant responses in non-injected lesions, including 3 complete remissions (CR). Six additional patients had stable disease (SD) at distant sites lasting from 4-20 months. Responses occurred over a span of 6-18 months and were associated with hypopigmentation and gradual regression of abscopal tumors suggesting immune-mediated mechanisms. Encouraged by this data, we investigated this approach in treating another aggressive and highly-immunogenic skin cancer, Merkel cell carcinoma (MCC).

MCC is a rare, highly aggressive skin cancer associated with the Merkel cell polyomavirus (MCPyV) and ultraviolet radiation exposure. MCC is considered immunogenic, with reports of spontaneous regression and responsiveness to immunotherapy (22-27). Strong $CD8^+$ T cell infiltration is associated with dramatically improved survival, independent of stage at presentation (28). The mechanisms of this immunogenicity, however, have only recently been understood (27). Virus-positive MCC (VP-MCC) accounts for ~80% of MCC cases in the United

States; MCPyV antigens are persistently expressed in VP-MCC and are immunogenic, which also allows for rigorous study of local and systemic anti-tumor immune responses in this subset. The remaining 20% of cases are virus-negative (VN-MCC) and are associated with high mutational burden from ultraviolet-radiation-induced damage, which likely facilitates expression of neoantigens. These insights have led to multiple successful trials of immunotherapies for VP-MCC and VN-MCC. Programmed death-1 (PD-1) axis checkpoint inhibitors (e.g. avelumab, pembrolizumab, and nivolumab) are now preferred front-line systemic therapy in eligible patients (including VP-MCC and VN-MCC) (29). These agents have impressive frequency, durability, and depth of objective responses, with some of the highest objective response rates (40-60%; ORR) reported among solid tumors treated in the first line (22,30,31). Unfortunately, approximately half of patients do not experience durable benefit, and 15-20% of patients experience severe Grade 3-4 immune-related AEs (22,30,31), indicating a need for novel therapeutic approaches that target the tumor microenvironment (TME) without excessive systemic immune toxicities and augment responses to checkpoint blockade.

Here, we report the results of a single-arm, open-label, pilot trial of i.t.-tavo-EP for advanced MCC patients who had at least one superficial (injectable) lesion.

Materials and Methods:

Study design

This was a single-arm, open-label, pilot trial of i.t.-tavo-EP in 15 patients with MCC (NCT01440816) evaluating the clinical efficacy, safety and tolerability, and immunologic changes in the TME, including changes in IL-12 protein expression in the TME after treatment. The study was conducted at the University of Washington in accordance with International

Conference on Harmonization guidelines for Good Clinical Practice and the code of Federal Regulations and guided by the ethical principles of the Belmont Report. The protocol was approved by the local Institutional Review Board. All patients provided written, informed consent at the time of screening.

Patients

Eligible patients were ≥18 years of age with histologically-confirmed MCC and at least one injectable lesion, defined as a superficial (cutaneous, subcutaneous, or nodal) tumor amenable to intratumoral injection in the outpatient setting. MCPyV-positive status was not required. Patients were required to have measurable disease per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (32), an Eastern Cooperative Oncology Group (ECOG) performance score of 0-2, and adequate hematologic, renal, and hepatic function. Patients were excluded if immunosuppressed. Patients were enrolled onto one of two cohorts: patients with locoregional MCC who were candidates for definitive therapy (surgery +/- radiation therapy) were enrolled in Cohort A (N=3) and patients with metastatic MCC in Cohort B (N=12).

Treatment

The treatment schema is provided in **Supplemental Fig. 1**. Patients received i.t.-tavo-EP on days 1, 5, and 8 of each cycle in an outpatient clinic setting following administration of a local anesthetic and under direct palpation of the tumor mass without radiologic guidance. Patients with locoregional MCC (Cohort A) received one cycle before planned definitive surgery in week 4, followed by adjuvant radiation therapy per clinician discretion. Patients with distant metastatic disease (Cohort B) could receive up to four cycles total, administered at least six weeks apart,

with restaging clinical and radiologic evaluation at week 6 of each cycle.

I.t.-tavo-EP was applied to specific treatment zones (corresponding to the tumor volume covered by a sterile electroporation applicator comprised of 6 stainless steel electrodes 1.5 cm long and arranged in a circular array of ~1 cm diameter) within the injected tumor(s) that were selected at the beginning of each cycle. Up to 4 treatment zones (in one or multiple tumors) could be treated within each cycle with no new zones added during a cycle. For patients in Cohort B, new treatment zones (in same or different tumors) could be selected at the beginning of each cycle. The pIL-12 dose for each treated zone was fixed at 0.5 mL or 0.25 mg (fixed concentration of 0.5 mg/mL). The maximum total dose of plasmid injected in one patient on a given day was capped at 2 mL or 1.0 mg. The plasmid injection in each zone was followed immediately by electroporation before moving onto the next zone. For electroporation, the applicator was inserted into the same zone in which the plasmid was injected, and six electrical pulses at a field strength (E+) 1300 volts/cm and pulse width of 100 μs at 400 msec intervals were administered as described (21).

Clinical assessments

Clinical endpoints included safety and tolerability, as well as efficacy, which measured both injected and non-injected lesion regression, recurrence-free survival in Cohort A, and objective response by RECIST v1.1 in Cohort B. AEs were graded according to National Cancer Institute Common Terminology Criteria for AEs (v4.03). Clinical assessment of tumor responses was performed for both injected and non-injected (distant) lesions according to RECIST v 1.1. For Cohort B patients with distant disease, assessment of overall response via radiologic imaging studies was carried out at baseline and at week 6 of each treatment cycle, and then as clinically

indicated. After completion of treatment, patients were followed at least annually for relapse, disease progression, and overall survival.

Biopsy and peripheral blood collection

Serial tumor biopsies and peripheral blood samples were collected from all patients to evaluate cellular immunologic changes, as well as changes in IL-12 p70 protein expression in treated tumors (**Supplemental Fig. 1**). Pre-treatment tumor biopsy was attempted on day 1 and post-treatment biopsy of the same tumors on day 22; the latter was substituted by surgical specimen collection during week 4 in Cohort A patients. Tumor samples were divided for biomarker studies: 1) formaldehyde-fixed paraffin embedded (FFPE) blocks for immunohistochemistry (IHC), multi-spectral IHC or gene expression, 2) flash-frozen for protein expression, and 3) processed immediately for isolation and culture of tumor-infiltrating lymphocytes (TIL) as previously described (33). Blood samples for immune response analysis were collected at baseline, day 22, and week 6 of each cycle. Peripheral blood mononuclear cells (PBMCs) were isolated using routine Ficoll gradient centrifugation and were immediately cryopreserved.

Immune response analyses

Tumor-MCPyV status was assessed using T-antigen IHC (CM2B4 antibody, Santa Cruz Biotechnology) and/or detection of MCPyV oncoprotein antibodies (AMERK test; **Table 1**) as previously described (34-36).

Immunohistochemistry analyses

Standard histology was performed on all biopsies. Monochromatic IHC staining with antibodies to CK20 (KS20.8, Dako), CD8 (C8/144B, Dako), CD4 (SP35, Cell Marque), CM2B4 (MCPyV T-antigen, sc-136172, Santa Cruz), MHC class I (EMR8-5, MBL), PD-L1 (E1L3N, Cell Signaling), and FoxP3 (14-5773-82, eBioscience) was performed. H&E and IHC-stained slides were reviewed for evidence of tumor and necrosis prior to performing further biomarker analyses. For multispectral IHC staining, FFPE specimens were deparaffinized and rehydrated, subjected to heat-induced antigen retrieval, and stained as described previously (37). Staining was performed using the following antibodies: PD-1 (EPR4877, AbCam), PD-L1 (SP142, Spring Bio), CD4 (RBT-CD4, BioSB), CD8 (C8/144B, Dako), and CD68 (PG-M1; Dako). Slides were imaged with a Vectra Automated Quantitative Pathology Imaging System (Perkin Elmer). Images were analyzed using inForm Software (Perkin Elmer) and were evaluated by a pathologist.

IL-12 p70 protein expression

To determine whether i.t.-tavo-EP led to increased expression of the IL-12 protein in the TME, pre- and post-biopsies were collected, lysed, and analyzed for IL-12 p70 protein, a heterodimer comprised of p40 and p35 subunits. Pre- and post-treatment frozen paired biopsies were weighed before homogenization in lysis buffer, lysed, and subjected to centrifugation to sediment insoluble cellular material prior to determination of IL-12 p70 protein levels using a multi-analyte immunoassay platform (MAGPIX®, Luminex). Each immunoassay was performed in 96-well plates and according to the MILLIPLEX MAP High Sensitivity Human Cytokine Premixed Magnetic Bead Kit (EMD Millipore Billerica, MA cat#. SPRHSCYMG60PX13). Each assay included duplicate 50 μL homogenate samples, which were added to capture–bead-treated plates.

Detection beads were then added to the plates before quantifying the analyte. For analysis, replicate values below the lower limit of quantification (LLOQ) were adjusted to the run-specific LLOQ value. All values were then adjusted to pg/g of sample and averaged to produce an average expression value. No replicate values were adjusted or excluded from analysis on the basis of the upper limit of quantification.

Gene expression analysis

FFPE-extracted RNA (approximately 50 ng) was analyzed using the human Immunology V2 panel and the nCounter® platform (NanoString® Technologies, LabCorp, Seattle, WA). This panel profiles 594 immunology-related human genes as well as two types of built-in controls: positive controls (spiked RNA at various concentrations to evaluate the overall assay performance) and 15 negative controls (to normalize for differences in total RNA input). Total RNA was isolated using the AllPrep kit (Qiagen, Valencia, CA) from FFPE specimens after histologic confirmation of evaluable tumor. Sample preparation and hybridization was carried out according to the manufacturer's instructions. In brief, 5 µL (100 ng) of total RNA was hybridized at 96°C overnight and then digitally analyzed for frequency of each RNA species. Data were collected using the nCounter® Digital Analyzer, and data normalization and analysis were carried out using the nSolver™ software (V.3). Normalization factors were derived from the geometric mean of housekeeping genes, mean of negative controls, and geometric mean of positive controls.

RNA extraction (tissue pellets):

Tumor tissue samples procured by punch biopsy or fine needle aspirate (FNA) were homogenized in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (Life Technologies, Carlsbad, CA) with protease inhibitors (cComplete-mini, EDTA- free; Roche Life Science, Indianapolis, ID). Clarified supernatants and cell pellets were stored separately at -80°C. Total RNA was isolated from cell pellets using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. Total RNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and quality was assessed using the 2100 Bioanalyzer (Agilent, Santa Clara, CA) for both the standard and smear analyses by LabCorp (Seattle, WA). Samples were excluded from analysis if Bioanalyzer RIN score was less than 2.0 and smear analysis indicated <30% of RNA were less than 300 bp.

MCPyV-specific tetramer staining

Subjects were HLA-I typed at Bloodworks Northwest (Seattle, WA). PBMC and/or TIL from patients with HLA-I types that corresponded to available MCPyV-specific tetramers (A*02:01, A*24:02, B*35:02, B*3701, B*0702; n=13 patients) were incubated with 100 nM of dasatinib for 10 minutes at 37°C. Cells where then stained with appropriate tetramers and analyzed using flow cytometry. At least 2 million PBMC or TIL were stained with anti–CD8-FITC (clone 3B5, ThermoFisher), violet viability dye (Invitrogen), anti-CD14-Pacific Blue (clone M5E2, Biolegend), anti-CD19-Pacific Blue (clone HIB19, Biolegend), anti-CD4 PerCP and either A02/KLLEIAPNC-APC, A24/EWWRSGGFSF-APC, B07/APNCYGNIPL-PE, B35/FPWEEYGTL-PE tetramer (Immune Monitoring Lab at Fred Hutchinson Cancer Research Center) or B37/KEWWRSGGFSF-PE tetramer (Buus Lab, Denmark) as described (33,38-40).

Cells were acquired on an Aria II Cell sorter with BD-FACSDiva software, and were analyzed with FlowJo (v10.0.8) software. Cells of interest were viable and identified as $CD8^+$ $tetramer^+$ cells in the lymphocyte forward/side scatter region with >0.01% of $CD8^+$ T cells co-staining with tetramers. Cells staining positively for CD4, CD14, or CD19 were excluded. The frequency of MCPyV-specific CD8 T cells out of the total CD8 T cell fraction was determined from paired pre- and post-fresh tumor biopsies and PBMC. The fold change in tetramer-positive T cell frequency between pre- and post-treatment was calculated as the difference between the post-treatment tetramer-positive T cell frequency and pre-treatment tetramer-positive T cell frequency over the pre-treatment tetramer-positive T cell frequency. Fold change was used to evaluate changes in tetramer-positive T cell enrichment following treatment in the periphery and intratumorally.

T cell receptor sequencing

Total genomic DNA was extracted from whole flash-frozen tumor biopsies using the spin column method and the DNeasy kit (Qiagen). High-throughput deep sequencing was used to determine T cell receptor beta locus (*TRB*) complementarity-determining region 3 (CDR3) with the Illumina Genome Analyzer (Adaptive Biotechnologies, Seattle, WA) using the immunoSEQ immune-profiling system (41). Read normalization was performed as described (42). Identification of the Vβ, Dβ, and Jβ gene segments contributing to each TCRβ CDR3 sequence was performed using the published algorithm (41). The T cell fraction within MCC tumors was determined by calculating the percentage of a tumor sample that was composed of T cells. To determine whether there was a change in T cell enrichment between pre- and post-treatment specimens, the fold change in the T cell fraction was calculated as the difference between the

post-treatment T cell fraction and pre-treatment T cell fraction over the pre-treatment T cell fraction.

Statistical analyses

Descriptive statistics were used to summarize baseline patient characteristics, safety, clinical response, and immunological response variables. All injected and non-injected tumors were assessed and responses to treatment were classified as complete responses (CR), partial responses (PR), stable disease (SD), or progressive disease (PD) per RECIST v1.1 definitions (32). The overall objective response rate (ORR) per standard RECIST v1.1 guidelines is also reported for those patients with distant metastases in Cohort B. Progression-free survival and overall-survival were calculated from the day of treatment initiation to documented disease progression and/or death, respectively. All analyses and summaries were produced using SAS® version 9.3 (or higher).

Results:

Patient Characteristics

Fifteen patients (Pt) with locoregional (Cohort A, n=3) or metastatic (Cohort B, n=12) MCC were enrolled between January 2012 and January 2015; their baseline characteristics are summarized in **Table 1**. The pathological stage at enrollment, per AJCC 8th edition(43), of Cohort A patients was IIIA (n=1; T0 pN1b M0) or IIIB (n=2; T1 pN1b M0 and T1 pN3 M0), and for Cohort B patients was unresectable stage IIIB (n=1; T1 pN3 M0) or stage IV (n=11; M1). Median patient age at enrollment was 66 years. Patients had an ECOG status of 0 (n=11) or 1 (n=4). Most patients (86.7%) had undergone prior surgery, 66% had received prior radiation

therapy, 40% had received prior systemic chemotherapy, and 40% had received prior biologic therapy with none receiving prior PD-1/PD-L1 blockade (**Table 1**). The majority of patients (12/15; 80%) had MCPyV-positive tumors (VP-MC. C), correlating well with the expected rate of viral-positivity among MCC cases more broadly (44); all 3 VN-MCC patients were in Cohort B.

Safety and Tolerability

Overall, i.t.-tavo-EP was safe and well-tolerated (**Table 2**). In Cohort A, all 3 patients successfully completed neo-adjuvant i.t.-tavo-EP followed by the planned definitive therapy without any treatment-induced delays. In Cohort B, all 12 patients successfully completed at least one treatment cycle (range 1-4 cycles, median 1 cycle) of i.t.-tavo-EP. Treatment-related adverse events (TRAEs) were primarily mild to moderate (i.e., Grade $\leq$2); none were Grade 3 or higher. There were no deaths or treatment discontinuations due to TRAEs. All patients (15/15; 100%) experienced transient Grade 1 pain associated with the electroporation procedure that lasted a few seconds; this occured despite the use of lidocaine injections, but was manageable with patient education and did not require systemic analgesics or lead to treatment-discontinuation. The second most common TRAE was Grade 1 injection site reactions, which did not require medical intervention in most cases. There were no clinically notable systemic toxicities, including laboratory parameters associated with renal and hepatic function. Two patients total (2/15; 13.3%) had a serious adverse event (SAE); neither were considered related to the study treatment. One patient developed a Grade 3 urinary tract infection that resolved with treatment. Another patient (Pt#13) was diagnosed with hepatocellular carcinoma 9 months after

the last i.t.-tavo-EP treatment; this was diagnosed upon elective resection of a pre-existing liver lesion that had been presumed to be an MCC metastasis at the time of study entry.

Clinical outcomes

Treatment and clinical outcomes are summarized in **Table 3**. In Cohort A, two of three patients continue to be recurrence-free at 75+ months (Pt#6) and 44+ months (Pt#16) from treatment initiation; the third patient (Pt#9) had disease recurrence at 9 months. Pt#16, who had biopsy-proven recurrent MCC in an enlarged right inguinal lymph node, had pathologic CR after neo-adjuvant i.t.-tavo-EP with no detectable residual MCC on histologic review including IHC staining for CK20.

In Cohort B, three of 12 (25%) patients had an objective response (all 3 were PR), one patient had SD, and 8 patients had PD as the best response. The median time to progression for Cohort B was 1.5 months overall and was 7 months for the three responders. Two (Pt#2 and Pt#13) of the 3 responders had clinically meaningful benefit lasting 16 months and 55+ months respectively. Pt#2 was a 55-year-old man with primary VP-MCC on left ankle and multiple recurrences in the left lower extremity and regional lymph nodes over 2 years despite several prior therapies (including surgery, radiation therapy, chemotherapy, and intratumoral interferon). He received two cycles of i.t.-tavo-EP treatment and had an impressive PR (>70% regression) associated with regression of distant untreated lesions; this clinical response was accompanied by evidence for induction of a systemic immune response (described below). He was progression-free for 7 months and then received another 2 cycles of i.t.-tavo-EP with an additional progression-free period lasting 9 months. Pt#13 was a 82-year-old man with primary MCC on

the right proximal elbow and subsequent distant metastatic disease with 2 large abdominal wall subcutaneous nodules and a suspicious liver lesion. Prior therapies included surgery, chemotherapy (carboplatin plus etoposide) and intratumoral injections of a toll-like receptor 4 agonist on a clinical trial. He received 2 cycles of i.t.-tavo-EP with complete resolution of his bulky treated lesions over the next several months (NOTE: only a small proportion of the two lesions was treated and the majority of the bulky lesions were untreated); the suspicious liver lesion remained unchanged, was subsequently resected and found to be hepatocellular carcinoma. The patient has been MCC progression-free for 55+ months after starting study treatment. Pt#14 also had a PR lasting 3 months, but developed PD with a new brain metastasis. He received stereotactic radiosurgery to the brain lesion followed by pembrolizumab resulting in a CR lasting 44 months.

A high proportion (12/27; 44.4%) of measurable injected lesions exhibited major (defined as ≥30%) regression. Ten of 12 patients in Cohort B had distant untreated lesions that were response-evaluable; 3 of these 10 patients (30%) had major regression in at least one distant lesion, which suggests systemic anti-tumor immune responses from local therapy in these patients. These patients included Pt#2 and Pt#14 who had overall objective responses too. As mentioned above, both of the measurable lesions in Pt#13 were treated and hence he did not have measurable non-injected distant lesions. Another patient with distant regression of a non-injected lesion had overall PD.

Intratumoral IL-12 gene and protein expression

Elevated intratumoral IL-12 gene and protein expression were observed in post-treatment specimens (**Figures 1A and 1B**). Specifically, increased expression (by 2-fold or more) of IL-12

p70 protein was observed post-treatment (on day 22) versus baseline (day 1) in 6 out of 10 (60%) patients with evaluable paired tumor biopsies (**Figure 1B**). While we did not measure systemic IL-12 levels in the peripheral blood in this study, there was no evidence of clinically-significant systemic spillage of IL-12 beyond the local TME, based on a complete lack of systemic TRAEs in this study and similar to results reported by Daud *et al* in the Phase 1 study (21).

Increased peripheral and intratumoral MCPyV-specific T cells are associated with response

Using our library of MCPyV-specific HLA class-I tetramers (33,38-40), we tracked MCPyV-specific T cell responses, both locally in the treated lesions (**Figure 2A**), and also systemically, in the peripheral blood (**Figure 2B**) and in untreated lesions (**Figure 3A,B**). Thirteen of 15 patients expressed suitable HLA class-I alleles for tetramer screening (**Supplemental Table 1**). We performed MCPyV-specific tetramer staining on paired pre- and post-therapy TIL from five patients with MCC-VP tumors (**Figure 2A**). The frequency of MCPyV-specific tetramer+ TIL significantly increased (≥1.5 fold change in at least one MCPyV-specific T cell population) in three of five patients (Pt#2,8,13) following treatment, and decreased in one patient (Pt#7), with one patient (Pt#10) not having any detectable MCPyV-specific T cells in pre- or post-specimens. Interestingly, all 3 patients with increased frequency of MCPyV-specific TIL experienced clinical responses.

Paired baseline and post-treatment peripheral blood samples from patients with MCPyV-positive tumors (n=10 evaluable patients) were stained with the same panel of five MCPyV tetramers as used to evaluate TIL. Only one patient (1/10; 10%) exhibited ≥1.5-fold increase of MCPyV-specific T cells (**Figure 2B**); this patient (Pt#13) did have a clinical response (PR). Five patients did not have tetramer+ T cells for one or two tetramers at baseline or post-treatment.

Taken together, these data suggest that i.t.-tavo-EP therapy can induce systemic and intratumoral expansion of MCPyV-specific T cells, which appear to be associated with a clinical response to treatment.

Abscopal responses and increased intratumoral T cell infiltration following i.t.-tavo-EP therapy

Additional evidence of systemic, abscopal immune responses was observed in Pt#2 who experienced an objective PR with regression of several treated as well as non-treated lesions (**Figure 3A**). Expanded TILs were analyzed from biopsies obtained pre-therapy from a treated lesion and eight months post-therapy from an untreated lesion. TILs from the untreated lesion showed greater MCPyV-specific tetramer staining (6-fold) relative to the baseline pre-treatment TIL isolated from a separate treated lesion; **Figure 3B**), suggesting abscopal induction of substantial MCPyV-specific T cell recruitment in untreated lesions by i.t.-tavo-EP.

To evaluate global changes in intratumoral T cell responses (not restricted to MCPyV-specific T cells), sequencing of T cell receptor beta chains was performed on pre- and post-treatment tumor specimens. In six of seven patients, the fraction of T cells (relative to the total tumor cell number) increased in post-treatment samples independent of clinical response (**Figure 3C**), suggesting that i.t.-tavo-EP can increase infiltration or expansion of T cells.

Multispectral immunohistochemical staining was used to evaluate spatial changes in intratumoral T cell infiltration and their expression of immunoregulatory molecules PD-1 and PD-L1 in a pre- and post-treatment lesion from Pt#10 (**Figure 3D,E**). I.t.-tavo-EP induced increased infiltration of $CD8^+$ and $CD4^+$ T cells, and also a concomitant increase in PD-1 and PD-L1 expression in this lesion. Despite evidence of increased T cell infiltration, this patient did not respond to i.t.-tavo-EP therapy. Taken together, these data indicate that i.t.-tavo-EP therapy

can increase intratumoral T cells, including those specific for MCPyV epitopes, but that additional mechanisms of local immune evasion may limit clinical responses.

Discussion

This pilot trial investigated i.t.-tavo-EP in 15 patients with advanced MCC. I.t.-tavo-EP administered on days 1, 5, and 8 resulted in sustained (day 22) intratumoral IL-12 expression without any systemic or severe AEs. Resultant local inflammation led to systemic immune responses, suggested by regression of non-injected MCC lesions in 3 of 10 (30%) patients and an overall response rate of 25% (3/12) in Cohort B patients with metastatic MCC. Neoadjuvant administration before surgery and radiation therapy in locoregional MCC was feasible; one of 3 Cohort A patients experienced a pathologic CR.

This trial validates the previously reported findings of the Phase 1 trial of i.t.-tavo-EP in melanoma patients (21). It highlights the immunogenicity of this approach that evokes clinically meaningful and durable responses in a subset of patients. Similar to the Phase 1 trial, this intratumoral treatment was safe and well tolerated; there were no severe TRAEs, suggesting lack of systemic spillage of IL-12 protein. This is remarkable, because systemic delivery of IL-12, a promising cytokine that facilitates T_H1 responses, has previously been associated with severe AEs, including death. Sustained local expression of IL-12 in the TME on day 22 with treatment only on days 1, 5, and 8 highlights the potential of gene electrotransfer (GET) to overcome a limitation of most intratumoral immunotherapy approaches, the requirement for frequent repeated injections due to limited intratumoral persistence of the injected drugs. The platform for GET is relatively flexible for inducing local intratumoral expression of plasmid DNA of choice

and also mitigates neutralizing antibodies and the risk of viral integration observed with gene delivery via viral vectors (20).

Due to shared viral antigens expressed by VP-MCC (12 of 15 patients within this study), study of cancer-specific T cell responses against conserved viral epitopes was possible. Using five MCPyV-specific HLA-peptide tetramers (33,38-40), we found detectable increases in MCpyV-specific T cells in the MCC TME following i.t.-tavo-EP in patients with clinical responses; with a concomitant increase of tetramer-positive T cells in the PBMC of one patient. Additionally, we found that i.t.-tavo-EP induced increased intratumoral infiltration or expansion of T cells independent of response to therapy. This suggests that additional mechanisms of immune evasion are involved and indeed, i.t.-tavo-EP treatment in one evaluable patient was associated with increased expression of PD-1 and PD-L1 within the TME (**Fig 3D**). Notably, IL-12 has been shown to significantly induce expression of IFN-γ, which can drive potent anti-tumor effects via enhanced immunogenicity and direct tumoral-static mechanisms, but this cytokine can also trigger upregulation of PD-L1 and IDO-1 in the TME (45). Consequently, combining i.t.-tavo-EP with PD-1/PD-L1 pathway blockade may provide an appealing therapeutic combination. Indeed, tumor regression after therapeutic PD-1 blockade may require pre-existing cancer-specific immune response (46). Therefore, the observation that i.t.-tavo-EP induces intratumoral infiltration and systemic expansion of T cells, and may increase PD-L1 expression suggests a potential for therapeutic synergy. Interestingly, intratumoral PD-L1^{+} tumor associated macrophages can also contribute to “adaptive resistance”, yet IL-12 has been reported to reduce the frequency of MDSC suppressor subsets (47). Further investigation into the role and potential modulation of this innate immune subset during i.t.-tavo-EP therapy is warranted. Several studies using IL-12 delivery in combination with PD-1 blockade have shown promising

efficacy in murine models (48,49). Intriguingly, five MCC patients in this trial received PD-1 blocking therapies (as monotherapy or in combination with other immunotherapies) at some point in their treatment course after i.t.-tavo-EP; all 5 had an objective response to PD-1/PD-L1 axis blockade (**Supplemental Table 2**). Based on these observations and other clinical trials in melanoma and metastatic triple-negative breast cancer, combined with the excellent safety profile of this approach, several clinical trials are currently investigating the combination of i.t.-tavo-EP with PD-1 blocking therapies (NCT03132675, NCT03567720).

In vivo electroporation of tumors also has several limitations, many of which are shared with other intratumoral approaches. It has primarily been investigated in patients with superficial, clinically accessible tumors; administration to deeper-seated (e.g., visceral) lesions, while possible and in development, will have additional challenges. I.t.-tavo-EP monotherapy is less suitable for patients with bulky visceral lesions and/or rapidly progressive disease due to the relatively low proportion of treated tumor burden that is unlikely to overcome the immune evasion mechanisms in rapidly growing distant lesions. It will be ideal to combine this approach with other systemic therapies in such patients. Nevertheless, the current study provides proof-of-concept of this approach in enhancing immunogenicity in MCC and is worthy of further exploration in MCC and other cancers.

In conclusion, i.t.-tavo-EP was safe and feasible in MCC patients without severe systemic toxicities. It led to sustained local expression of IL-12 protein, which facilitated local and systemic immunity and clinically meaningful responses in a subset of patients. Gene electrotransfer warrants further investigation for immunotherapy of cancer, either as monotherapy or in combination with agents such as PD-1 blockade therapy.

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Table 1. Patient Baseline Characteristics

	Cohort A (N = 3)	Cohort B (N = 12)	Overall (N = 15)
Age in years			
Median [range]	59 [50-73]	69 [53-86]	66 [50-86]
Sex			
Male (%)	3 (100%)	9 (75.0%)	12 (80.0%)
Female (%)	0 (0.0%)	3 (25.0%)	3 (20.0%)
Ethnicity			
White (%)	3 (100%)	11 (92%)	14 (93%)
Other (%)		1 (8%)	1 (7%)
ECOG[a] Performance Status			
Score of 0 (%)	3 (100%)	8 (67.7%)	11 (73.3%)
Score of 1 (%)	0 (0.0%)	4 (33.3%)	4 (26.7%)
Pathologic Stage at Enrollment per AJCC 8th Edition (43)			
IIIA (%)	1 (33.3%)	0 (0.0%)	1 (6.7%)
IIIB(%)	2 (66.7%)	1 (8.3%)	3 (20.0%)
IV (%)	0 (0.0%)	11 (91.7%)	11 (73.3%)
Prior Therapies			
Surgery (%)	1 (33.3%)	12 (100%)	13 (86.7%)
Radiation (%)	1 (33.3%)	9 (75.0%)	10 (66.7%)
Systemic Therapy (%)	0 (0.0%)	6 (50.0%)	6 (40.0%)
Chemotherapy		5	5
Immunotherapy[b]		6	6
Targeted Therapy		1	1
MCPyV[c] status[d]			
Positive (%)	3 (100%)	9 (75.0%)	12 (80.0%)
Negative (%)	0 (0.0%)	3 (25.0%)	3 (20.0%)

[a] ECOG: Eastern Cooperative Oncology Group
[b] Prior immunotherapies included interferon-beta, 4-1BB agonistic antibody, and a toll-like receptor agonist; none had received prior PD-1 blockade.
[c] MCPyV: Merkel Cell Polyomavirus
[d] Viral status was determined by MCPyV oncoprotein serological status and CM2B4 immunohistochemistry staining.

Table 2. Adverse Events

Adverse Events[a]	All Grades n (%)	≥ Grade 3 n (%)
Related to Study Treatment		
Procedural pain	7 (46.7)	0
Treatment site inflammation	3 (20.0)	0
Treatment site cellulitis	2 (13.3)	0
Treatment site bruising	1 (6.7)	0
Pain (tumor)	1 (6.7)	0
Pain (lymph node)	1 (6.7)	0
Peripheral edema[b]	1 (6.7)	0
Pyrexia	1 (6.7)	0
Unrelated to Study Treatment		
Occurring in ≥2 patients		
Fatigue	3 (20.0)	0
Severe (Grade 3)		
Urinary tract infection (SAE[c])	—	1 (6.7)
Hepatocellular carcinoma	—	1 (6.7)

[a]Treatment-emergent adverse events were all adverse events that began on or after the first administration of study treatment.
[b]Included lymphedema.
[c]SAE: Serious adverse event.

Table 3. Summary of Clinical Outcomes[a]	
Progression-Free Survival[b]	**Median, in months [range]**
Cohort A	NR[c] [9–75+]
Cohort B (all; N = 12)	1.5 [1.5–55+]
Cohort B responders (N = 3)	7 [3–55+]
Objective Response Rate (Cohort B only; N = 12)	**n (%)**
Partial Response	3 (25.0)
Stable Disease	1 (8.3)
Progressive Disease	8 (66.7)
Local Lesion Response Rate[d] (Response-evaluable local lesions; N = 27[c])	**n (%)**
Major regression (≥30%)	12 (44.4)
Distant Lesion Response Rate (Response-evaluable Cohort B patients[e]; N = 10)	**n (%)**
Patients with regression of ≥1 distant lesion(s)[f]	3 (30.0)

[a] Clinical outcomes in patients with potential clinical benefit are described in detail in the manuscript text.
[b] Interval is defined as the duration between the date of treatment initiation (Study Day 1) to the date of documented objective or clinical disease progression (Cohort B)/relapse (Cohort A).
[c] NR: Not Reported.
[d] Proportion of treated lesions with major (≥30%) regression.
[e] These patients had at least one distant tumor that was defined as a non-injected Merkel cell carcinoma tumor, clearly distinct from treated lesions.
[f] Proportion of patients with at least one lesion left untreated that reduced in size by ≥30% suggesting systemic immune response.

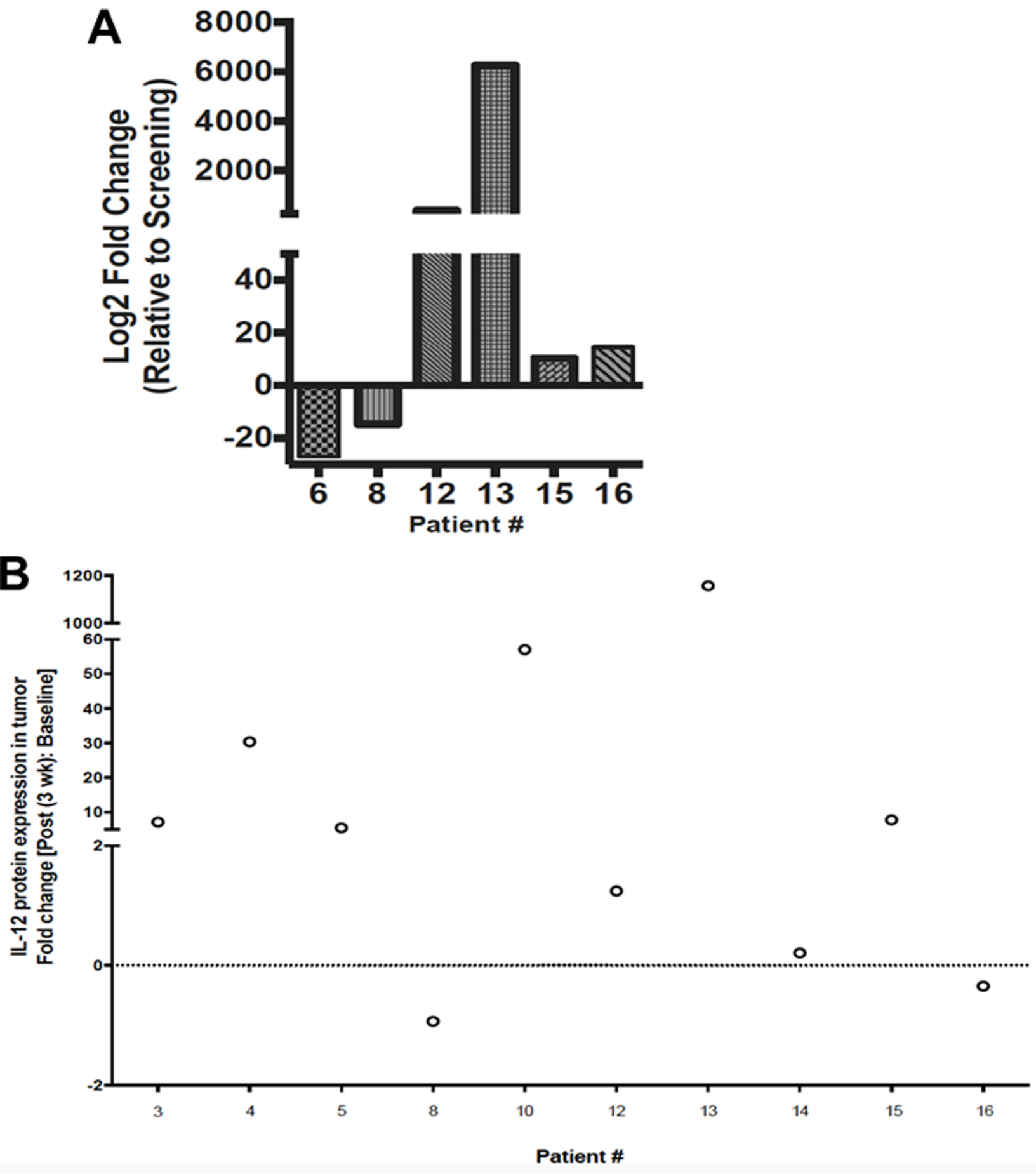
Figure Legends

Figure 1: Increased IL-12 gene and protein expression post therapy in electroporated lesions. A: IL-12 gene expression (*IL12A* subunit) was elevated in several tumors post treatment compared with pre-treatment. Changes in gene expression were analyzed using NanoString® nCounter platform. **B:** Paired pre- and post- biopsy samples from ten patients were evaluable for IL-12 p70 protein levels by MAGPIX®. Data are presented as fold change (post-/pre-treatment). The majority (6 of 10; 60%) had greater than 2-fold increase in IL-12 protein levels suggesting sustained expression (at day 22) after treatment on days 1, 5, and 8.

Figure 2: I.t.-tavo-EP induces intratumoral infiltration and systemic MCPyV-specific T cell expansion. MCPyV-specific CD8 T cells were evaluated intratumorally (**A**) and in peripheral blood mononuclear cells (PBMC) (**B**), using MCPyV-specific tetramers. **A:** Paired pre- and post-fresh tumor biopsies were cultured in the presence of IL-2 and IL-15 to obtain tumor infiltrating lymphocytes (TIL). **B**: Systemic evaluation of MCPyV-specific CD8 T cells from PBMCs was performed as in (A). Fold changes, post- versus pre-IL-12 therapy, in the MCPyV-specific tetramer-positive population, as described in Materials and Methods, are shown (the dotted line indicates a 2-fold change). Responders (R) are in green, non-responders (NR) are in red. Tetramer human leukocyte antigen (HLA) type is denoted on the X axis, and stars denote samples that were negative for tetramer positive cells at both pre- and post-time points.

Figure 3: Regression of treated and untreated lesions is associated with robust T cell responses and infiltration. A: Schema of treatment and response in Patient 2 with baseline (red circles), regressed lesions (green circles), a persistent lesion (yellow circle). Lesions treated with i.t.-tavo-EP are shown as lightning bolts (blue: current cycle; white: prior cycles). Two untreated lesions that regressed are shown in top right panel (blue arrows) **B:** Tetramer staining of MCPyV-specific CD8 T cells from TIL from pre- and post-therapy from Pt#2. Tetramer positive CD8 T cells are circled in each panel. Negative control is an HLA mismatched donor. **C:** T cell receptor (TCR) beta chain sequencing was used to determine TCR variable beta chain diversity using ImmunoSeq V3 (Adaptive Biotechnology) from pre- and post-treatment tumor specimens (patient number is shown on X axis, and * denotes patients in which the pre and post-treatment samples were from different lesions). Data are presented as fold change (a ratio of the difference between the post-treatment T cell fraction and pre-treatment T cell fraction over the pre-treatment T cell fraction). Bars are colored in correlation with clinical response, responder (R, green) and non-responder (NR, red). The dotted line denotes a greater than or equal to 2-fold change. **D & E**: Multispectral immunohistochemistry images of pre-treatment (**D**) show an MCC tumor (CK20, white/light blue) with very few infiltrating T cells (CD8 in green and CD4 in red). Three weeks after treatment (**E**), there is a large infiltrate of CD8 T cells (green), accompanied by increased PD-1 (magenta) and PD-L1 (orange) and loss of tumor (CK20, white/light blue). *Abbreviations*: RT: Radiation therapy; C1: Cycle 1; C2: Cycle 2 ; Tx: treatment

Figure 1 A and B, Bhatia



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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 2 A and B, Bhatia

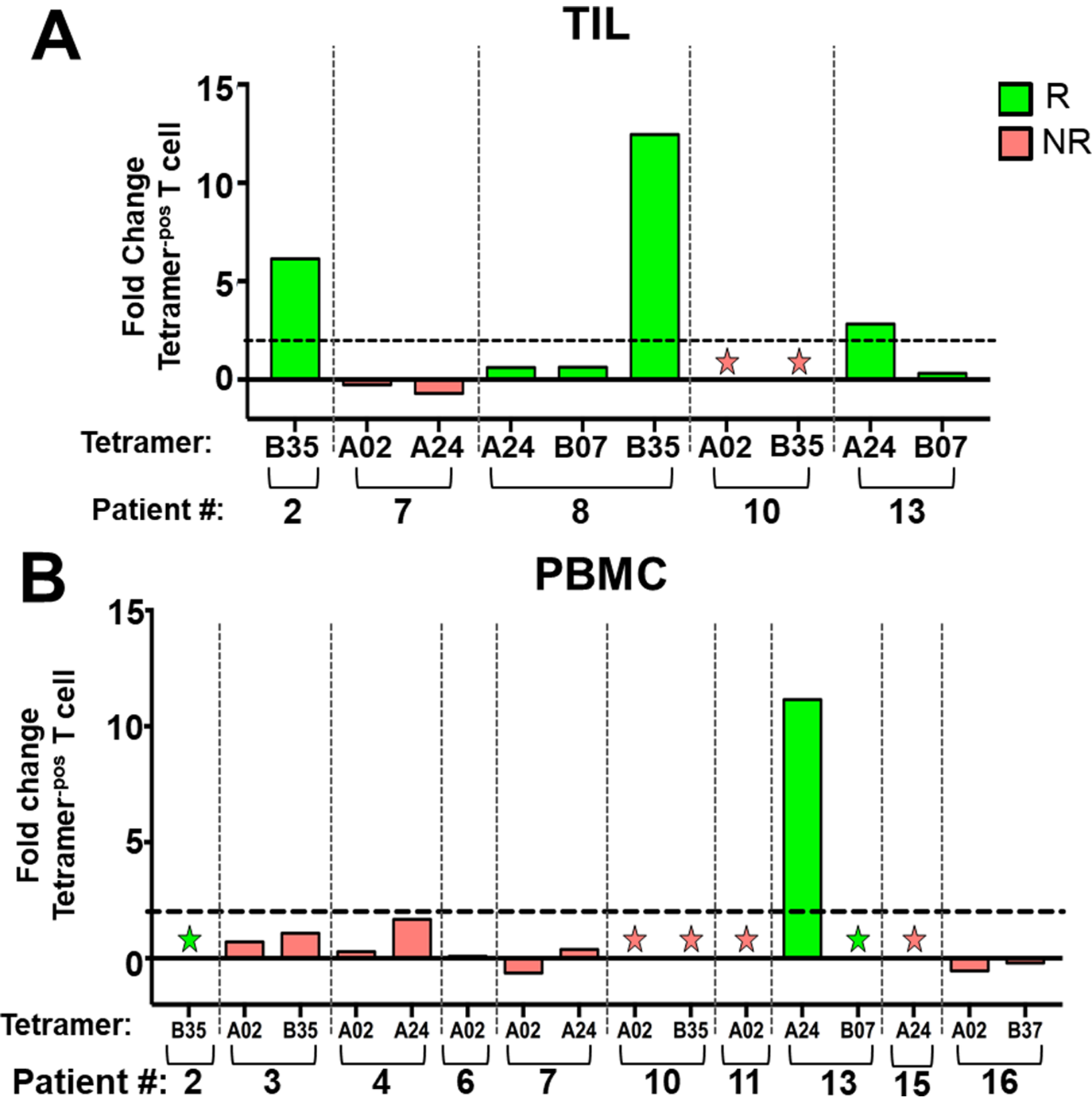
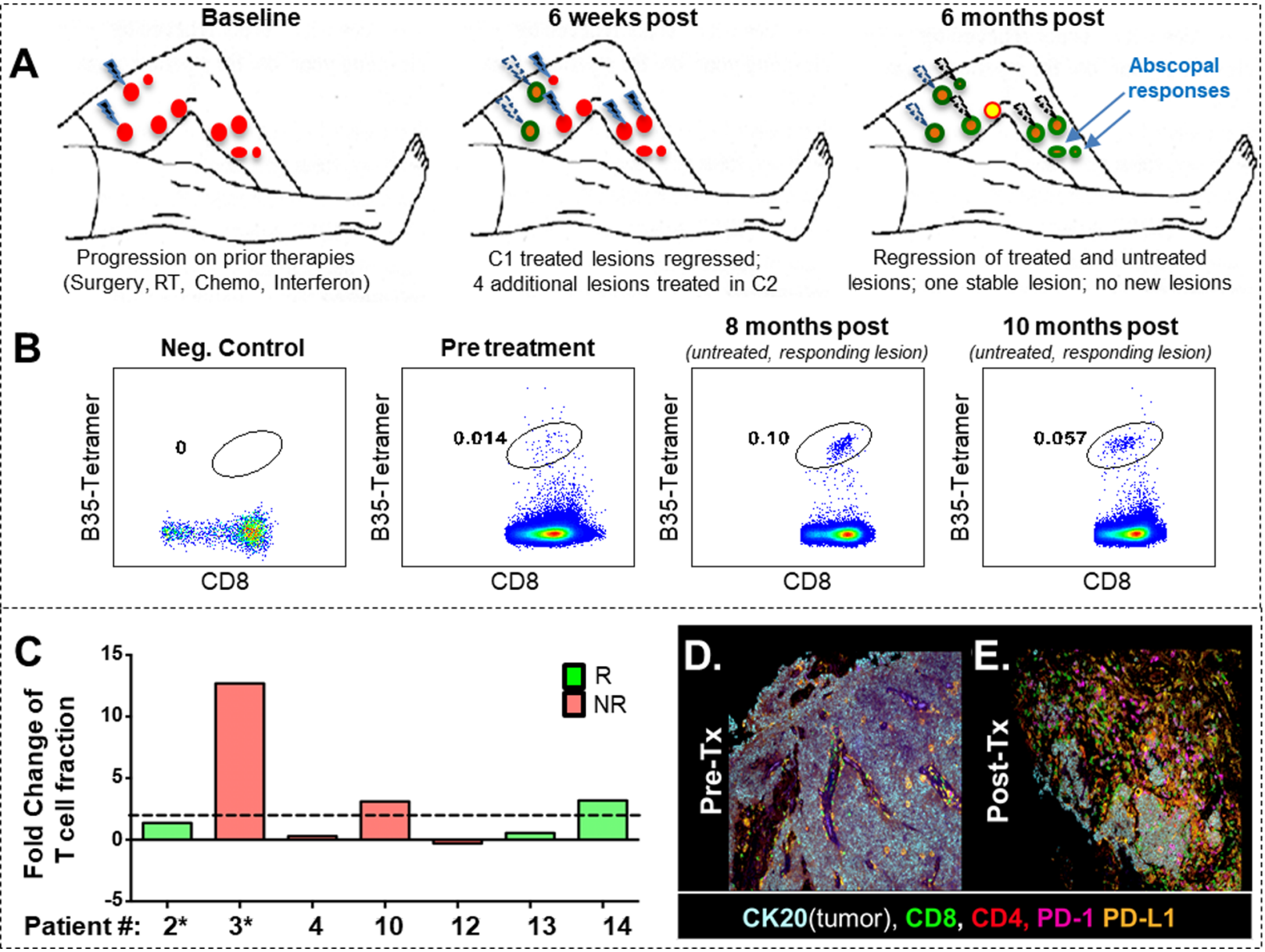


Figure 3 A, B, C, D, and E, Bhatia



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Intratumoral delivery of plasmid interleukin-12 via electroporation leads to regression of injected and non-injected tumors in Merkel cell carcinoma

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