Clinical Cancer Research

Intratumoral G100, a TLR4 Agonist, Induces Antitumor Immune Responses and Tumor Regression in Patients with Merkel Cell Carcinoma

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Abstract

Purpose: G100 is a toll-like receptor 4 (TLR4) agonist that triggers innate and adaptive antitumor immune responses in preclinical models. This pilot study assessed the safety, efficacy, and immunologic activity of intratumoral (IT) administration of G100 in patients with Merkel cell carcinoma (MCC).

Patients and Methods: Patients with locoregional MCC ($n = 3$; cohort A) received neoadjuvant IT G100 (2 weekly doses at 5 μg/dose) followed by surgery and radiotherapy; patients with metastatic MCC ($n = 7$; cohort B) received 3 doses in a 6-week cycle and could receive additional cycles with/without radiotherapy.

Results: IT G100 was safe and feasible in both neoadjuvant and metastatic settings. Treatment-related adverse events were mostly grade 1 or 2 injection-site reactions. IT G100 led to increased inflammation in the injected tumors with infiltration of $CD8^+$ and $CD4^+$ T cells and activation of immune-related genes. These proinflammatory changes were associated with local tumor regression and appeared to promote systemic immunity. All 3 cohort A patients successfully completed therapy; 2 patients remain recurrence free at 44+ and 41+ months, including 1 with a pathologic complete response after G100 alone. In cohort B, 2 patients achieved sustained partial responses, both lasting 33+ months after 2 cycles of therapy.

Conclusions: In this first-in-human study, IT G100 induced antitumor immune responses, demonstrated acceptable safety, and showed encouraging clinical activity.

See related commentary by Marquez-Rodas et al., p. 1127

Introduction

Malignant tumors are a complex collection of cancer cells, stromal cells, and immune cells that exist in a supportive microenvironment built around a tumor-induced scaffold of fibrous matrix, blood, and lymphatic vessels (1). Cells within the tumor microenvironment (TME) inhibit antitumor immune function through multiple mechanisms, including production of suppressive cytokines, modulation of chemokines required for T-cell trafficking, inhibition of dendritic cells, induction of checkpoint regulators, and physical barriers such as fibrosis (1–3). Novel treatment approaches are needed to overcome the complex mechanisms of immune suppression within the TME and promote effective antigen presentation and immune system activation. Inducing a transformation from a "cold" (immunologically suppressed) to a "hot" (inflamed and immunologically active) environment within the TME could potentially induce primary local and systemic immune responses to previously unrecognized tumor antigens and boost existing immunity.

Several agents designed to modify the TME are currently being investigated, including cytokines, oncolytic viruses, and toll-like receptor (TLR) agonists. Toll-like receptors are a family of receptor molecules that can detect pathogen-associated molecular patterns (PAMP) present on bacterial, fungal, or viral pathogens and initiate innate and adaptive immune responses against these targets. Various TLR agonists are in clinical development for cancer immunotherapy, either as an adjuvant component of a cancer vaccine, or as monotherapy (4, 5). The current study evaluates the safety and efficacy of G100, a potent TLR4 agonist, administered as an intratumoral (IT) injection for the treatment of patients with Merkel cell carcinoma (MCC).

TLR4 is expressed on the cell surface of dendritic cells, monocytes, macrophages, T cells, and B cells, as well as some

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Translational Relevance

In this first-in-human investigation, intratumoral (IT) administration of G100, a synthetic toll-like receptor 4 agonist, induced inflammatory changes within the tumor microenvironment (TME) including increased infiltration of $CD8^+$ and $CD4^+$ T cells in the injected tumors and activation of immune-related genes, thus transforming the TME from a "cold" (immunologically suppressed) to a "hot" (inflamed and immunologically active) environment. These changes facilitated local and systemic immune responses against tumor-associated antigens that translated into clinically meaningful responses in patients with advanced Merkel cell carcinoma, including a pathologic complete remission in the neoadjuvant setting and durable objective responses in the metastatic setting. Administration of IT G100 as a neoadjuvant therapy prior to surgery and radiotherapy was safe and feasible. IT G100 had minimal systemic toxicity and no immune-related adverse events. These data indicate that IT G100 is a promising cancer immunotherapy warranting further investigation both as a monotherapy and in combination with other immunotherapies.

nonimmune cells, and recognizes lipopolysaccharide (LPS), a polysaccharide that is anchored in the outer bacterial membrane by lipid A (6–9). Activation of TLR4 can stimulate dendritic cells and promote Th1-type responses (10). G100 contains the TLR4 agonist glucopyranosyl lipid A (GLA), a synthetic analogue of naturally derived lipid A, formulated in a stable oil-in-water emulsion (SE). Naturally occurring lipid A exists in various chemical forms (e.g., with varying number of acyl chains) and this variation can affect its immune and other properties; the synthetic analogue GLA was developed to decrease product heterogeneity and minimize the potential for systemic toxicity.

Stimulation of TLR4 by GLA activates immune cells primarily by inducing the production of chemokines and cytokines that mediate adaptive immune responses through multiple signaling pathways (11–15). GLA rapidly stimulates the maturation of DCs (11, 12, 16) and is hypothesized to facilitate development of antitumor CD8 T-cell responses by enhancing cross-presentation of endogenous tumor antigens released by dead or dying cells. In preclinical models of lymphoma and other cancers, IT injection of G100 alone led to local and systemic tumor regression, including regression of distal noninjected/treated lesions (abscopal responses; ref. 17).

MCC is a highly immunogenic cancer. Approximately 80% of MCC tumors are associated with the Merkel cell polyomavirus (MCPyV; ref. 18). Viral antigens are expressed by tumor cells, and MCPyV-specific T cells and antibody responses can be detected in patients with MCC (19, 20). Indeed, IT infiltration of MCPyV-specific $CD8^+$ T cells correlates with longer survival (21). Unfortunately, MCC tumors are able to evade the immune system (22, 23). The MCC TME typically has reduced MHC-I expression, sparse IT $CD8^+$ T cells, and immune cells showing an exhausted phenotype with expression of programmed cell death protein 1 (PD-1/PDCD1) and Tim-3 (24). While the recent reports of PD-1 pathway blockade (with pembrolizumab, avelumab, and nivolumab) in metastatic MCC appear promising, approximately 50% of patients do not respond persistently to these agents (25–27). IT immunotherapy may overcome the local immunosuppressive mechanisms in the MCC TME and complement the activity of systemic immunotherapy.

This study represents the first investigation of G100 administered IT in a human tumor. In this pilot clinical trial, we evaluated the safety, efficacy, and immunologic effects of IT G100 alone and in combination with radiotherapy in patients with MCC.

Patients and Methods

Study design

This was an open-label pilot trial of IT G100 in 10 patients with MCC conducted at the University of Washington (UW, Seattle, WA) 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.

The primary objective was to assess the safety and feasibility of IT G100 as neoadjuvant therapy in local disease and both as monotherapy and in conjunction with radiotherapy in the metastatic setting. Secondary objectives were to assess the clinical efficacy and the immunologic effects of this treatment approach.

Patients

Eligible patients were $\geq$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 IT injection in the outpatient setting. MCPyV-positive status was not required. Patients were required to have measurable disease as per Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1; ref. 28), an Eastern Cooperative Oncology Group (ECOG) performance score 0 to 2, and adequate hematologic, renal, and hepatic function. Patients judged to be immunosuppressed or with any major comorbidities were excluded. Patients were enrolled onto one of two cohorts: patients with locoregional disease who were candidates for definitive therapy (surgery $\pm$ radiotherapy) were enrolled in cohort A ($n = 3$) and patients with metastatic disease in cohort B ($n = 7$).

Treatment

The treatment schema is provided in Supplementary Fig. S1. All IT injections were performed in an outpatient clinic setting following administration of a local anesthetic and under direct palpation of the tumor mass. No radiologic imaging or special services were used. The 5 μg dose was selected on the basis of acceptable safety in prior clinical studies of GLA administered as an adjuvant for standard protein-adjuvant vaccines (intramuscular or subcutaneous administration) for infectious diseases such as influenza (29).

In cohort A, patients with locoregional MCC received neoadjuvant G100 at 5 μg/day injected IT into superficial MCC tumor(s) on days 1 and 8 followed by definitive therapy starting in week 4. Definitive treatment included surgery and/or radiotherapy as per standard care guidelines as determined by the team of treating physicians. Tumor biopsies were collected at baseline and following G100 treatments on day 22 prior to surgery or at the time of definitive surgery in week 4.

In cohort B, patients with metastatic MCC received G100 at 5 μg/day injected IT into superficial MCC tumor(s) on days 1, 8,

and 22 of the first 6-week cycle. All patients had a pretreatment tumor biopsy on day 1 and a posttreatment biopsy on day 22 of cycle 1. If no significant toxicity was observed during cycle 1, cohort B patients could receive up to 3 additional treatments. In cycles 2 through 4, G100 was administered in combination with radiotherapy when feasible and indicated for local control/palliation. Patients received a single fraction of high-dose radiotherapy (usually 8 Gy) to the injectable tumor (and other tumors, if clinically indicated), followed by IT injection of 5 μg G100 within 72 hours of radiotherapy, then weekly IT G100 injections (at 5 μg/day) beginning on day 8 of a 6-week cycle (i.e., days 8, 15, 22, 29, and 36). The combination regimen was based on preclinical evidence of additive or synergistic effects of radiation in combination with G100 (30) and evidence that increasing from 3 to 6 doses led to increased abscopal tumor shrinkage in animal models (unpublished data).

Clinical assessments

Clinical assessment of tumor responses was performed for both injected and noninjected (distant) lesions according to RECIST v1.1. The first clinical response assessment of injected lesions in both cohorts was on day 22 of cycle 1, at the time of the posttreatment biopsy. For cohort B patients with distant disease, assessment of overall response via radiologic imaging studies was carried out at baseline and at week 6, and then as clinically indicated. In addition to site interpretation, radiologic imaging studies were reviewed independently. Adverse events (AE) were graded according to National Cancer Institute Common Terminology Criteria for Adverse Events (v4.03). After completion of treatment, patients were followed at least annually for relapse or disease progression and overall survival.

Immune response analyses

All 10 patients had a pretreatment tumor biopsy on day 1 and posttreatment tumor collection on day 22 (or at the time of surgery in week 4 in cohort A patients). Tumor samples were divided in three: (i) formaldehyde-fixed paraffin embedded (FFPE) blocks for IHC, (ii) flash-frozen for genomic and transcriptomic studies, and (iii) processed immediately for isolation and culture of tumor-infiltrating lymphocytes (TIL) as described previously (19). Whole blood samples for immune response analysis were collected at baseline, on day 22, and at week 6 of each cycle. Peripheral blood mononuclear cells (PBMC) were isolated at the above time points using routine Ficoll gradient centrifugation and were cryopreserved.

MCPyV status. Tumor-MCPyV status was assessed by T-Ag IHC (CM2B4 antibody, Santa Cruz Biotechnology; ref. 31) and T-Ag serology, as described previously (20, 32).

IHC analyses. Slides from the FFPE tumor blocks were stained with H&E, and with antibodies to CK20/KRT20 (KS20.8, Dako), CD8/CD8a (C8/144B, Dako), CD4 (SP35, Cell Marque), CM2B4 (MCPyV T-antigen staining; sc-136172, Santa Cruz Biotechnology), MHC class I/HLA-1/HLA-A,B,C (EMR8-5, MBL), PDL-1/CD274 (E1L3N, Cell Signaling Technology), FOXP3 (14-5773-82, eBioscience), and TLR4 (MAB14783, R&D Systems). The IT $CD8^+$ lymphocyte infiltrate was scored on a 0 (absent) to 5 (strong) scale (32).

For multispectral IHC staining, FFPE slides were deparaffinized and rehydrated, subjected to heat-induced antigen retrieval, and stained as described previously (33) with the following antibodies: PD-1 (EPR4877, AbCam), PD-L1/CD274 (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 evaluated by a pathologist.

Functional analysis of TILs. Effector cytokine secretion from TILs in response to relevant MCPyV peptides was determined as described previously (19). TILs were derived from minced MCC tissue and nonspecifically expanded using phytohaemagglutinin (PHA; Remel), natural human IL2 (IL2R/nIL2; Hemagen Diagnostics), recombinant human IL15 (IL15R/rIL15; R&D Systems), and allogeneic-irradiated PBMC. nIL2 and rIL15 in fresh T-cell medium was added every second day for 14 to 20 days. TIL samples were stimulated with peptide pools in the presence of anti-CD28, anti-CD49d/ITGA4, brefeldin A, and autologous carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous PBMC as antigen-presenting cells (APC). After 12–18 hours, cells were stained for CD4, CD8, and intracellular IFNγ, and between 1×10^6 and 6×10^6 cells were assessed via flow cytometry. Each peptide pool contained approximately 25 peptides, each 13 amino acids (AA) in length, that overlapped by 9 AA and corresponded to the persistently expressed region of MCPyV (19). Specifically, Pool 1 covered the common T antigen (CT; AA 1-77), Pools 2 and 3 covered AA 69–181 and 173–281, respectively, of the large T antigen (LT), and Pool 10 covered AA 69–186 of the small T antigen (ST). Final concentration of each peptide was approximately 1 μg/mL. Samples with >0.01% $CD8^+$ or $CD4^+$ cells secreting IFNγ were considered positive.

MCPyV-specific tetramer staining. Subjects were HLA-I typed at Bloodworks Northwest. PBMCs and/or TILs from patients with HLA-I types corresponding to available MCPyV-specific tetramers [A^*0201 (peptide CT 15-23), A^*2402 (peptide LT 92-101), B^*3502 (peptide ST 83-91); $n = 8$ patients] were expanded, stained with appropriate tetramers, and analyzed by flow cytometry. At least 2×10^6 PBMCs or TILs were stained with anti-CD8-phycoerythrin (PE)–conjugated antibody (Clone 3B5, Life Technologies), 7-AAD viability dye (BioLegend), and the tetramers (as above) labeled with either APC or PE (Immune Monitoring Lab at Fred Hutchinson Cancer Research Center, Seattle, WA) and the percentage of cells in the $tetramer^+$, $CD8^+$ region was determined. Samples with >0.01% $CD8^+$ T cells costaining with tetramers were considered positive.

T-cell receptor sequencing and analysis. 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 analyze the *TCRβ/TCRB* complementarity-determining region 3 (CDR3) with the Illumina Genome Analyzer (Adaptive Biotechnologies) using the immunoSEQ immune-profiling system (34). In-frame unique sequences without stop codons, referred to as unique productive sequences, were used for the repertoire analysis. Identification of the Vβ, Dβ, and Jβ gene segments contributing to each TCRβ CDR3 sequence was performed using the published algorithm (34). To determine T-cell receptor (TCR) clonality of tumor samples, Shannon entropy was calculated on the estimated number of genomes of all productive TCRs and normalized by dividing

by the $\log_2$ of unique productive sequences in each sample. Clonality was calculated as 1 − normalized entropy. Computationally identified clones were analyzed for significantly different abundances between two samples using a binomial test with Benjamini–Hochberg corrected *P* values, such that false discovery rates were held at 5% (35).

Gene expression analysis. Total RNA was isolated using the AllPrep kit (Qiagen) from snap-frozen biopsy tissue. RNA concentration was quantified using UV spectroscopy with a Nanodrop device (NanoDrop Products). A total of 200 ng RNA was used for gene expression analysis using the human nCounter PanCancer Immune Profiling Panel (NanoString Technologies), which includes 770 genes. Sample preparation and hybridization was carried out using the nCounter Preparation Station according to the manufacturer's instructions. Data were collected using the nCounter Digital Analyzer and data normalization and analysis were carried out using the nSolver software.

Statistical analyses

All enrolled patients who received at least one dose of G100 were included in the safety analysis. Descriptive statistics were used to summarize baseline patient characteristics, safety, clinical response, and immunologic response variables. Objective response rate (ORR) was calculated as the proportion of evaluable lesions classified as a complete response (CR) or a partial response (PR). The overall ORR per standard RECIST v1.1 guidelines is also reported for those patients with distant metastases. PFS and time to next treatment were calculated from the day of treatment initiation.

Logistic regression analyses were performed to identify genes for which the posttreatment:pretreatment gene expression ratio was predictive of a positive response to G100 treatment. For each of the 770 genes, an OR for response was estimated from a logistic regression with response as the dependent variable and the $\log_2$ of fold change in gene expression as the explanatory variable. Significance was evaluated with a likelihood ratio test, and raw *P* values were adjusted for multiplicity using false discovery rates (35) as implemented in Proc Multtest in SAS 9.4. Because none of the false discovery rates were <0.05, the raw *P* values are reported to indicate potential signals for relevant genes warranting further study.

Results

Patients

Ten patients were enrolled between January 2014 and May 2015; 3 patients had locoregional MCC (cohort A) and 7 patients had metastatic disease (cohort B). Median patient age at enrollment was 67 years. All patients had an ECOG status of 0, except 1 patient (G7) in cohort B who had an ECOG status of 1. Most patients (80%) had undergone prior surgery, 60% had received prior radiotherapy, 50% had received prior systemic chemotherapy, and 40% had received prior biologic therapy (Table 1).

Safety and tolerability of IT G100

All 10 patients completed at least 1 cycle of IT G100. All three patients in cohort A received 2 doses of G100 and successfully completed definitive surgery and radiotherapy without any delays. Four of 7 patients with metastatic disease in cohort B received a second cycle of therapy (in conjunction with hypofractionated radiotherapy to the injected lesion). The median number of G100 doses administered in cohort B was 7 (range 3–8). No patients in either cohort required dose reductions, dose interruptions, or treatment discontinuation due to AEs.

Adverse events considered related to G100 are presented in Table 2. AEs consisted primarily of mild local toxicity [e.g., injection-site reactions (ISR)]; patients experienced minimal systemic toxicity. The majority of AEs were mild and transient. One patient in cohort A experienced a nonserious grade 3 ISR involving localized skin breakdown at the site of injection and biopsy. All other AEs were grades 1 to 2. No serious adverse events (SAE) were observed.

Clinical outcomes

All 10 patients were alive and in continued follow-up at the time of this analysis, with a median follow-up time of 33.7 months (range, 20.6–44.6 months). Treatment and clinical outcomes are summarized in Table 1 and Fig. 1. In cohort A, all 3 patients with locoregional MCC successfully completed surgery and adjuvant radiotherapy after neoadjuvant G100. Two of 3 patients continue to be recurrence free at 44+ months (patient G2) and 41+ months (patient G4) after initiation of study treatment; the third patient (patient G9) had disease recurrence at 5 months. Patient G2 achieved a pathologic CR from neoadjuvant IT G100. This patient, who had biopsy-proven MCC in an enlarged right inguinal lymph node (LN) without a known primary lesion developed clinical inflammation of the injected tumor after two doses of IT G100. The injected LN and a proximal draining LN were surgically removed in week 4, and upon pathologic assessment there was no evidence of residual MCC as demonstrated by histologic review and by IHC staining for cytokeratin 20 (CK20; Fig. 2).

In cohort B, 2 of 7 patients with metastatic disease had objective responses and 5 patients had progressive disease (PD). Patient G6 with palpable right inguinal lymphadenopathy and biopsy-proven distant disease (bone metastasis) received G100 to the enlarged inguinal LN in cycle 1. Following G100 treatment alone in cycle 1, the patient experienced a 28% regression in the size of the injected LN per RECIST v1.1. The patient then received a second cycle of treatment consisting of a single dose of 8 Gy radiotherapy to the inguinal LN and the bone lesion followed by weekly IT G100 injection (5 doses total) to the inguinal LN, after which a complete resolution of the injected inguinal LN was achieved. This patient remains in an ongoing remission 33+ months after initiation of study treatment, with maintained resolution of the target lesion, no new lesions, and bone scan showing no residual uptake in the biopsy-proven bone metastasis. Patient G8, who had confirmed disease progression after prior chemotherapy and immunotherapy with a 4-1BB agonist, also achieved a PR after treatment with G100 in combination with radiotherapy in cycle 2. G100 therapy was initiated after approximately 5 to 7 half-lives of the 4-1BB agonist (as per published pharmacokinetic data for this antibody; refs. 36, 37). This patient remains in an ongoing PR after 33+ months. In both of the cohort B patients with ongoing responses, no additional therapy has been given since G100.

Immune response analyses

The ability of G100 to alter the TME was evaluated in tumor samples obtained at baseline and post-G100 treatment (the sample obtained during definitive surgery during week 4 in cohort A or the day 22 biopsy in cohort B, prior to any radiotherapy).

Table 1. Baseline characteristics, treatment, and response in 10 patients with MCC treated with IT G100

				Viral status[c]		Study treatment				Response	
Patient No.	Gender/ age[a]	Primary lesion site	Prior treatment[b]	IHC	T-Ag serology	Treated/target[d] lesions, *n*	Cycles, *n*	G100 doses, *n*	Other planned treatment	Best response	Response duration, months
Cohort A											
G2	M/70y	Right inguinal lymph node	None	Positive	Positive	1/1	1	2	Surgery, adjuvant RT	Pathologic CR after G100 alone; NED after planned treatment	44+ (ongoing)
G4	M/67y	Lower right abdomen	Surgery	Negative	Positive	1/1	1	2	Surgery, adjuvant RT	NED after planned treatment	41+ (ongoing)
G9	M/55y	Left buttock	Surgery, RT	Positive	Positive	1/1	1	2	Surgery, adjuvant RT	NED after planned treatment	5
Cohort B											
G1	M/67y	Left buttock	Surgery, CT, RT, biologic	Positive	Positive	1/3	1	3	None	Progressive disease	N/A
G3	M/82y	Right elbow	Surgery, CT, RT	Positive	Positive	2/2	1	3	None	Progressive disease	N/A
G5	M/56y	Unknown[e]	RT	Positive	Positive	1/5	2	8	RT	Progressive disease	N/A
G6	M/67y	Unknown[e]	Surgery	Positive	Positive	1/1	2	8	RT	PR (28% regression in treated lesion after G100 alone and then PR following 2nd cycle G100 plus radiation)	33+ (ongoing)
G7	M/64y	Chest	Surgery, CT, RT, biologic	Positive	Negative	2/4	1	3	None	Progressive disease	N/A
G8	M/64y	Left abdominal wall	Surgery, CT, biologic	Positive	Positive	1/2	2	7	RT	PR (46% regression in tumor-involved lymph nodes following G100 plus radiation)	33+ (ongoing)
G10	M/80y	Left parietal scalp	Surgery, CT, RT, biologic	Negative	Negative	2/5	2	8	RT	Progressive disease	N/A

Abbreviations: CT, chemotherapy; M, male; NED, no evidence of disease; RT, radiotherapy; y, years.

[a]At enrollment.

[b]Eighty percent (80%) of patients had undergone prior surgery; 50% had received prior systemic CT, 60% had received prior radiotherapy, and 40% had received prior biology. Biologic therapy consisted of CD137/4-1BB agonist and IL12 electroporation in patient G1, anti-LAG-3 therapy in patient G7, CD137/4-1BB agonist (with confirmed progressive disease on the same) in patient G8, and anti-PD-1 therapy and IT interferon in patient G10.

[c]Viral status results by IHC with CM2B4 antibody and serology were discordant in 2 patients. Patient G4 in cohort A was IHC negative but was seropositive at multiple timepoints, and TILs from this patient contained $CD4^+$ and $CD8^+$ T cells reactive to MCPyV peptides; this patient was most likely virus positive. Patient G7 in cohort B was considered IHC positive but with a borderline score; serology was negative, and TILs were tetramer negative with no detectable MCPyV-reactive cells; this patient was most likely virus negative.

[d]Target lesions include both injected and noninjected (distant) lesions.

[e]No identified primary or occult.

Table 2. Treatment-emergent adverse events considered related to G100

	Patients with adverse event ($n = 10$)			
	Grade 1 *n* (%)	Grade 2 *n* (%)	Grade 3 *n* (%)	Any grade *n* (%)
Any treatment-related adverse event	6 (60)	2 (20)	1 (10)	9 (90)
ISR	6 (60)	1 (10)	1[a] (10)	8 (80)
Influenza-like illness	4 (40)	1 (10)	0	5 (50)
Bruising	2 (20)	0	0	2 (20)
Constipation	1 (10)	0	0	1 (10)
Fatigue	1 (10)	0	0	1 (10)
Pyrexia	1 (10)	0	0	1 (10)
Skin infection	0	1 (10)	0	1 (10)

Abbreviation: ISR, injection-site reaction.
[a]Grade 3 ISR consisted of localized skin breakdown at the site of injection and pretreatment biopsy.

Multispectral IHC demonstrated IT T-cell infiltration post-G100 therapy in 2 patients with durable benefit (patients G2 and G6), but not in a clinical nonresponder (patient G1). As shown for patient G6 in Fig. 3A, the vast majority of $CD4^+$ and $CD8^+$ T cells in the TME were peritumoral prior to the treatment (in the tumor vasculature and at the tumor edge). After treatment with G100, increased infiltration of $CD4^+$ and $CD8^+$ T cells into the injected tumor was observed, which was accompanied by clinical reduction in tumor size. Multispectral IHC also demonstrated colocalization of $CD4^+$ T cells, $CD8^+$ T cells, CD68 (monocytes/macrophages), and PD-1/PD-L1–expressing cells within the TME (Fig. 3B). These data are consistent with an increased general inflammatory response within the TME and colocalization of immune cells required for effective antitumor immune responses. In addition, single antigen IHC for CD4 T cells, CD8 T cells, MHC-I, $FoxP3^+$, and PD-L1 expression was performed in all patients and while there was evidence for increased IT inflammation post-G100 in some other patients as well, there was no clear correlation between baseline expression status and clinical/pathologic responses in this small cohort (data not shown).

The expression profile of 770 immune response–related genes in baseline and posttreatment tumor biopsies were compared using mRNA hybridization gene expression analysis. Gene expression data demonstrated induction of multiple genes related to innate and adaptive immune responses in the TME of injected tumors following G100 therapy; an example of gene expression data is shown in Fig. 4A. Across all 10 patients, the genes with the greatest increase in expression after normalization included genes coding for chemokines/chemoattractants and immune-modulatory cytokines (Supplementary Table S1). Of note, these genes were not uniformly induced in all patients. To investigate differences in the upregulation of immune response–related genes between responders versus nonresponders, gene expression was analyzed by clinical outcome groups. Patients G2, G4, G6, and G8 were classified as "responders" for these analyses based on successful clinical and/or pathologic outcomes (prolonged relapse-free survival in cohort A and durable objective responses in cohort B). Specific differences were observed between responders and nonresponders (Supplementary Table S1). Comparison of gene induction (posttreatment:pretreatment expression ratio) in clinical responders versus nonresponders revealed a trend for greater induction ($\geq$2-fold higher) in responders for genes linked to macrophage and T-cell chemotaxis [*SPP1* (osteopontin)], adhesion of macrophages (*MSR1*), activation of T cells and monocytes (*ALCAM/CD166*), activation of dendritic cells (*TREM2*), neutrophil chemotaxis (*CXCL8/IL8*, *CXCL5*), and others (Supplementary Table S2). Because of the small number of patients, the fold difference in the induction of specific genes in responders versus nonresponders represent trends that do not reach statistical significance when adjusted for multiple testing. When analyzed statistically using logistic regression analyses with genes as predictors of response, immune response genes whose induction was most significantly associated with response (ranked according to unadjusted *P* value) are shown in Supplementary Table S3 and partially overlap with the genes that showed $\geq$2-fold higher induction in responders. Genes whose induction was most significantly associated with a lack of response are also shown. Overall, these data suggest the ability of IT G100 to induce broad activation of chemokine- and cytokine-related genes in injected tumors promoting increased immune cell infiltration in the TME, with greater activation of a specific set of genes related to T-cell, macrophage, and dendritic cell functions in the tumors of patients with clinical responses.

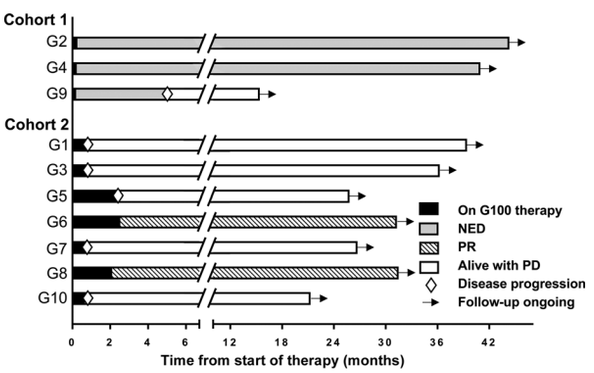

Figure 1.
Patient characteristics, treatment details, and clinical outcomes in 10 patients with MCC treated with IT G100. Each horizontal bar represents a patient. All patients were alive at last follow-up. All 3 patients with locoregional disease had no evidence of disease (NED) after definitive treatment with surgery/radiotherapy. Two patients with metastatic disease achieved a PR and 5 had progressive disease (PD).

In an attempt to identify pretreatment biomarkers that might predict clinical response to G100, we analyzed differentially expressed genes at baseline in clinical responders versus nonresponders. While broad immune activation was noted in the TME of responding patients post-G100, there were no clear predictors of clinical response identified in pretreatment biopsies. Baseline expression of TLR4 mRNA and that of its associated coreceptors, LY96 (MD2) and CD180, measured from all cells within the tumor biopsy, did not appear to correlate with response (Supplementary Fig. S2). Analyses of protein expression by IHC revealed high levels of TLR4 expression in the TME of all baseline MCC biopsy samples. Expression was noted in MCC cancer cells, inflammatory cells, endothelial cells, and to a lesser extent, fibroblasts.

Analyses of TILs by TCR sequencing were performed on pre- and posttreatment biopsies, including those from 3 responding patients (G2, G4, G6). In general, TCR sequencing results demonstrated an increase in TCR clones posttreatment, indicating the development of an inflammatory response following G100. In patient G6, who had metastatic disease and achieved an ongoing clinical PR, there was an overall shift toward greater

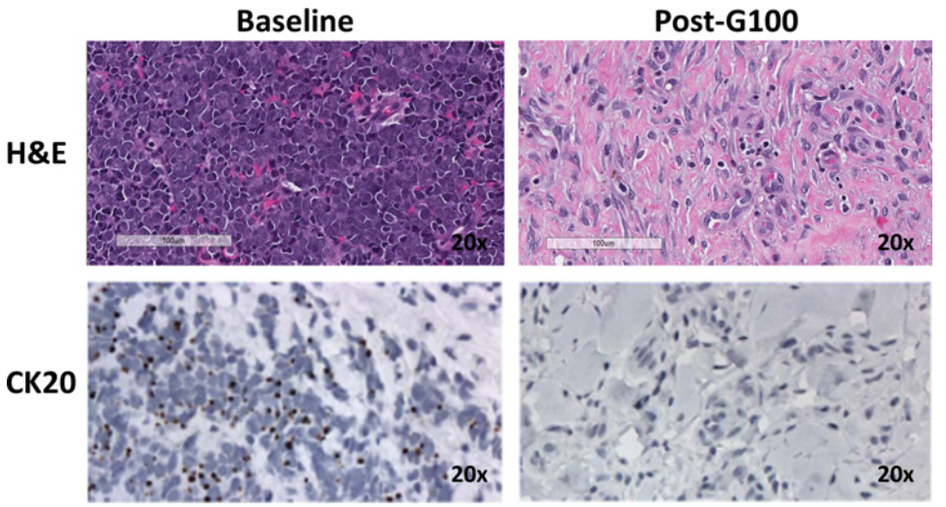

Figure 2.
Pathologic CR with single-agent G100 (patient G2). Representative H&E staining and IHC staining for CK20 are shown for pretreatment biopsy samples (baseline) and for the residual mass that was surgically removed posttreatment with 2 doses of G100. Absence of staining for CK20 posttreatment demonstrates pathologic CR after G100 alone. Scale bars denote a 100 μm region at 20× magnification.

clone frequencies posttreatment, with multiple new (not detectable pretreatment) unique T-cell clones detected and multiple preexisting (identified at baseline) clones increased within the TME following G100 therapy (Fig. 4B). These data suggest that G100 increases the inflammatory response both by expanding reactive TILs as well as by inducing or attracting newly reactive T-cell responses.

A detailed TCR clone analysis was performed on patient G2 who had a pathologic CR in the injected lesion. When TCR clones were analyzed in tumor and blood samples, 601 independent TCR clones that were initially not detected in the baseline pre-G100 tumor biopsies and PBMCs were detected for the first time in post-G100 tumor and blood, indicating that many previously undetected T-cell clones were likely being induced as newly reactive T cells (or were not initially present due to sampling; Supplementary Fig. S3). Of these TCR clones that were not detected in baseline tumor or blood, 225 were also found in the draining normal lymph node of this patient post-G100. In addition to these apparently newly induced T cells, there were 865 TCR clones that were detectable in the blood but not in the tumor at baseline and increased in blood and became detectable in tumor post-G100. These data indicate an expansion of preexisting clones that now appear to have been drawn into the tumor. Similar results were seen for patient G6.

Specific immune responses against the tumor associated MCPyV virus were measured by MCPyV-specific tetramer staining and by intracellular cytokine staining of T cells from pre- and post-G100 tumor and blood samples exposed to viral antigens (Supplementary Table S4). In patient G2, who achieved a pathologic CR and has remained recurrence free for 44+ months, IFNγ secretion following stimulation with MCPyV peptides was detected in $CD8^+$ T cells from the draining noncancerous lymph node (Supplementary Fig. S4A), suggesting the presence of an antitumor immune response beyond the injected TME. Similarly, in patient G4, MCPyV-specific $CD4^+$ T-cell reactivity in the injected tumor was detected post-G100 treatment but not pretreatment (Supplementary Fig. S4B). In contrast, MCPyV-specific T-cell reactivity was not detected in patient G9, who had no evidence of disease after definitive treatment but experienced disease recurrence 5 months after initiation of study treatment. Thus, reactivity to tumor-specific antigens was induced in some patients after treatment with G100.

Discussion

This pilot study represents the first-in-human investigation of IT administration of G100, a novel synthetic TLR4 agonist. The results highlight the ability of this approach to overcome immune suppressive mechanisms of the TME, to stimulate immune responses against tumor-associated antigens (i.e., *in situ* immunization), and to improve clinical outcomes in patients with MCC. G100 administered IT at the 5 μg dose was well-tolerated in patients with MCC, with mostly mild local injection-site reactions and no serious systemic toxicity. Neoadjuvant administration prior to surgery and radiotherapy in locoregional MCC was feasible, and the combination with radiotherapy in metastatic MCC was well tolerated. Repeated weekly administration did not lead to cumulative toxicity or to any reports of autoimmune events.

IT G100 resulted in the reversal of immune suppression and reestablishment of an active immune response within the TME as evidenced by the induction of immune response–related genes, the induction of MCPyV-reactive T cells, the colocalization of $CD4^+$ T cells, $CD8^+$ T cells and macrophages in the TME, and the increased number of unique clones observed within the tumor and PBMC following treatment. Differences in posttreatment gene expression were observed in the 4 patients with clinical responses versus nonresponders, and some differences were statistically significant (Supplementary Table S3); however, these data represent the outcome of a small group of patients and a number of factors may underlie the likelihood of response, including differences in the TME, other unexplained immune suppression, or TLR4 responsiveness (e.g., polymorphisms). Immune responses in the TME were associated with meaningful clinical activity including a pathologic CR in an injected tumor mass after just two G100 injections, which was accompanied by an ongoing recurrence-free survival lasting 44+ months following surgery and radiotherapy, and ongoing objective responses lasting 33+ months in 2 patients with metastatic disease. The durability of systemic disease control

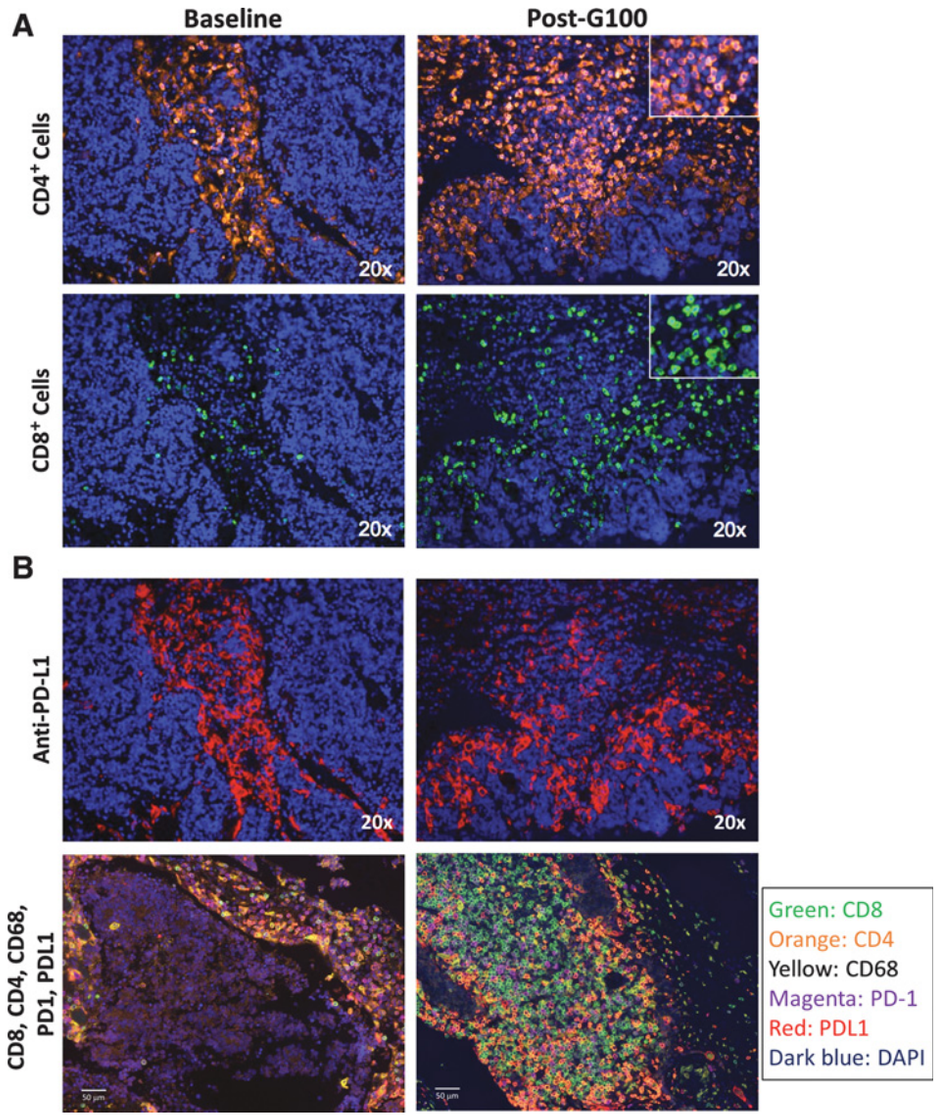

Figure 3.
IHC pre- and posttreatment with IT G100 in a patient with MCC (patient G6). **A,** Fluorescent IHC staining for $CD4^+$ and $CD8^+$ T cells demonstrates restriction of T cells to tumor vasculature and tumor edges prior to treatment, contrasted with diffuse tumor infiltration after treatment with G100. **B,** Multispectral IHC staining for $CD4^+$ T cells, $CD8^+$ T cells, CD68 (monocytes/macrophages), and PD-1/L1–expressing cells within tumors before and after treatment demonstrates increased inflammatory response within the tumor environment posttreatment. Scale bars denote a 50 μm region at 20× magnification.

in these 2 patients with documented metastatic disease is clinically meaningful, given the typical aggressive clinical course of patients with metastatic MCC (38). The results highlight the ability of local immunotherapy to overcome immune suppression and induce antitumor immune and clinical responses. The results of the current study are supported by initial results of a larger, ongoing study in patients with non-Hodgkin lymphoma (NHL), where G100 therapy in combination with low-dose local radiation to the injection site significantly increased CD8 TILs in posttreatment tumor biopsies compared with pretreatment biopsies and induced objective clinical responses as well as tumor inflammation and shrinkage in abscopal tumor sites (39). In the NHL study, a significant correlation between CD8 TILs post G100 compared with baseline was associated with the development of an objective tumor response.

While it is not feasible to fully discern the relative contributions of radiotherapy and G100 to the overall responses in this small study, the pathologic CR (patient G2) and tumor regression (patient G6) that occurred following G100 alone highlight the activity of G100 monotherapy. Furthermore, the G100 dose of 5 μg was chosen as an initial dose for this pilot study based on its use as an adjuvant for standard antigen-based vaccines such as for influenza (29) and is considered a low dose. In patients with NHL, G100 doses of 10 μg and 20 μg appeared to induce higher rates of objective and abscopal responses with a similar safety profile, although only a small number of patients were treated at 5 μg (39, 40).

In this study, there was no apparent correlation between clinical responses and baseline TLR4 mRNA expression. When analyzed by IHC, all baseline MCC tumor samples expressed high levels of TLR4, and so the ability to discriminate between responders and nonresponders was not feasible with this assay. In contrast, recently presented data demonstrated that TLR4 expression on follicular B cell lymphomas, as detected by IHC, was significantly associated with the development of objective clinical responses

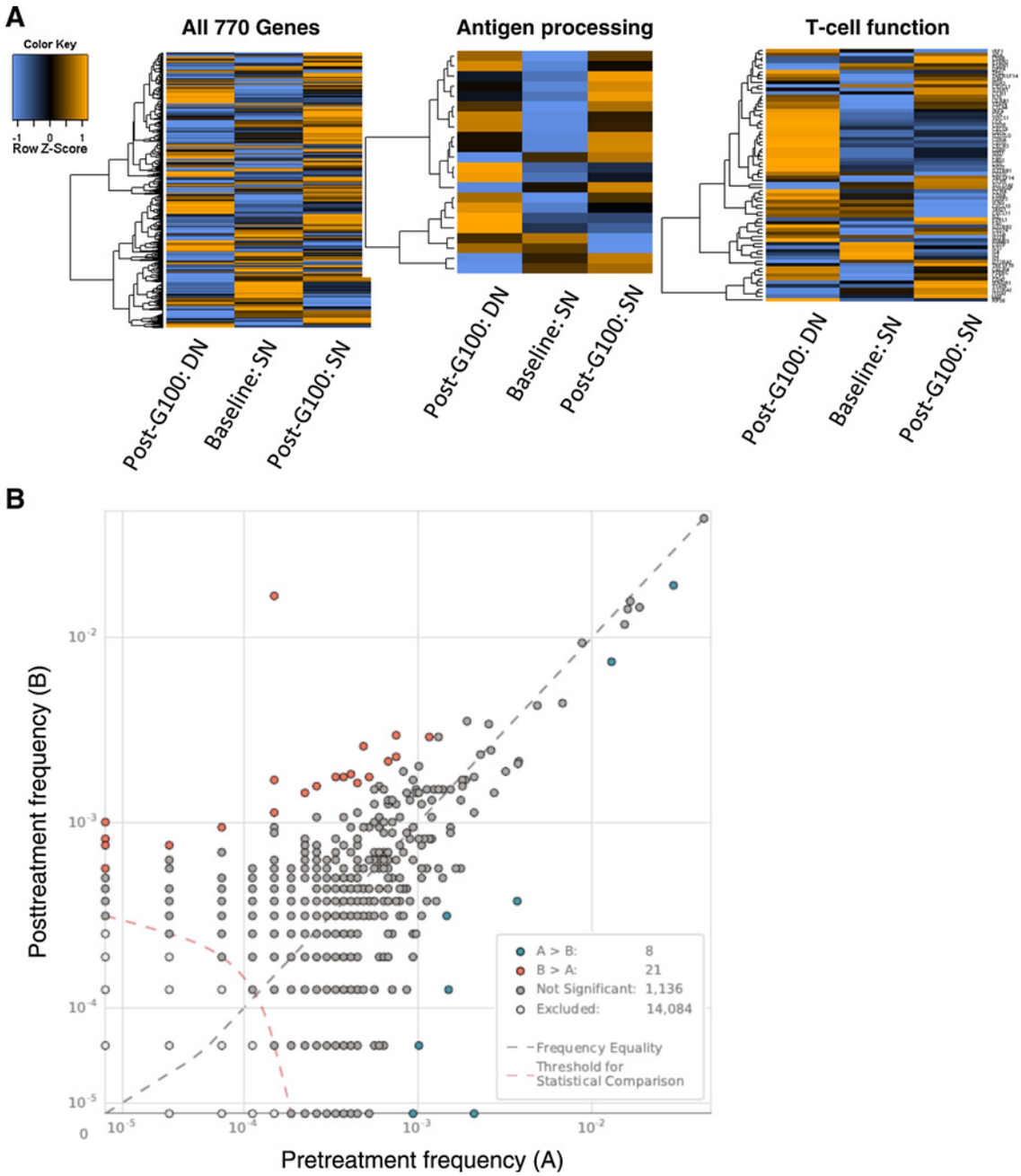

Figure 4.

Treatment-inducted changes in gene expression and TCR clonotypes. The heatmap in **A** depicts gene expression levels for 770 genes related to innate and adaptive immune responses in biopsy samples from a responding patient (patient G2). Expression levels in the tumor mass (biopsy-proven, tumor-involved right superficial inguinal lymph node; SN) at baseline and post-G100 treatment are shown as well as posttreatment levels in a deeper, untreated draining lymph node (DN). **B,** The frequency of clonotypes in pretreatment (*x*-axis) and posttreatment (*y*-axis) tumor biopsies from patient G6, who achieved an overall PR to therapy. Both newly identified clones and expansion of previously detected TCR clones were observed in posttreatment tumor biopsies compared with pretreatment biopsies. New clones are shown along the *y*-axis (undetected in the pretreatment sample but present posttreatment). Clones that were present in the pretreatment sample but were expanded posttreatment are shown above the 45° line. TCR clones with similar or decreased frequency posttreatment are shown on or below the 45° line. Clones shown on the *x*-axis indicate T cells that were below the limit of detection posttreatment. There was an overall shift toward greater clone frequencies posttreatment, with 21 clonotypes significantly enriched in the posttreatment sample compared with 8 in the pretreatment sample.

(40). The different findings in the lymphoma study and the current study could reflect differences in the biology of these malignancies. It is important to note that exposure to GLA can increase the expression of co-stimulatory molecules (e.g., CD40, CD80, CD86) on the cell surface of B-cell lymphoma cells, which can increase their ability to be effective APC as well as immune targets (17).

The excellent safety profile of IT G100 makes it an attractive partner for combination with other emerging immunotherapies as well as standard cancer therapeutics including cytokines, checkpoint inhibitors, and other novel biologic agents. Recently, PD-1 pathway blockade has emerged as a promising therapeutic option for patients with metastatic MCC, but unfortunately over half of patients do not persistently respond to PD-1/PD-L1 blockade (25, 26, 41). The overlap of the observed immune cell infiltration with PD-1/L1 expression in our study indicates a potential for synergy of G100 with anti-PD-1/L1 agents. G100 appears to increase the inflammatory response by inducing or attracting new T-cell clones into the TME as well as by expansion of preexisting reactive TILs; this may increase the activity of anti-PD-1 agents, which are thought to act predominantly on preexisting T-cell populations.

The ability of G100 to favorably alter the tumors towards a "hot" inflammatory environment has important implications not just for MCC, but for other tumor types as well. This treatment can be administered in the outpatient setting, does not have the biosafety concerns associated with other emerging IT approaches (such as oncolytic viruses), and appears suitable for combination therapy with systemic immunotherapy and radiotherapy. On the basis of the encouraging results of this pilot trial, additional studies of G100, both as a single agent and in combination with radiotherapy and checkpoint inhibitors are ongoing in several indications, including sarcoma (NCT02180698) and non-Hodgkin lymphoma (NCT02501473).

Investigations are ongoing with G100 to understand the optimal dose and treatment schedule, the synergistic effect of combining with radiation or other immune modulators, the ability to induce long-lasting immune memory, and the effectiveness in other cancer indications. While IT administration is a relatively simple procedure in superficial cancers like MCC or melanoma, other cancers where deep-seated tumors are more common may require ultrasound or CT-guided injections; clinical studies are ongoing to demonstrate the feasibility of G100 treatment in such tumors. Despite these remaining questions, this pilot trial confirms IT G100 is capable of stimulating an effective antitumor immune response that is potentially applicable to a wide variety of cancers.

This first report of clinical efficacy and safety of IT administration of G100, a synthetic TLR4 agonist, indicates this therapy was well-tolerated with minimal systemic toxicity and no immune-related adverse events. Administration of IT G100 as a neoadjuvant therapy prior to surgery and radiotherapy was safe and feasible. IT G100 induced inflammatory changes within the TME, which appeared to induce systemic antitumor immune responses and were correlated with clinically meaningful responses in patients with advanced MCC. The data suggest IT G100 to be a promising agent for further investigation in cancer therapy, either as a monotherapy or in combination with other immunotherapies.

Disclosure of Potential Conflicts of Interest

S. Bhatia is a consultant/advisory board member for Bristol-Myers Squibb, EMD Serono, and Genentech, and reports receiving commercial research support from Bristol-Myers Squibb, EMD Serono/Pfizer, Immune Design, Merck, and Oncosec. N.J. Miller is a co-inventor with the University of Washington and Fred Hutch Cancer Research Center of a patent on T-cell receptors. H. Lu, J. ter Meulen, and F.J. Hsu are employees of and have ownership interests at Immune Design. D.M. Koelle reports receiving commercial research support from Merck. No potential conflicts of interest were disclosed by the other authors.

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