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Tilsotolimod with Ipilimumab Drives Tumor Responses in Anti-PD-1 Refractory Melanoma

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Authors' Contributions

C. Haymaker: Conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing. **D.H. Johnson:** Formal analysis, writing—original draft. **R. Murthy:** Supervision. **S. Bentebibel:** Methodology. **M.I. Uemura:** Clinical support and enrolled patients. **C.W. Hudgens:** Methodology. **H. Safa:** Investigation, methodology. **M. James:** Clinical Support and enrolled patients. **R.H.I. Andtbacka:** Writing—review and editing, clinical support and enrolled patients. **D.B. Johnson:** Writing—review and editing, clinical Support and enrolled patients. **M. Shaheen:** Writing—review and editing, clinical support and enrolled patients. **M.A. Davies:** Writing—review and editing, clinical support and enrolled patients. **S. Rahimian:** Conceptualization, resources, writing—review and editing. **S.K. Chunduru:** Conceptualization, resources, writing—review and editing. **D.R. Milton:** Formal analysis. **M.T. Tetzlaff:** Formal analysis, writing—review and editing. **W.W. Overwijk:** Methodology. **P. Hwu:** Writing—review and editing, clinical support and enrolled patients. **N. Gabrail:** Writing—review and editing, clinical support and enrolled patients. **S. Agrawal:** Writing—review and editing, designed and initially synthesized tilsotolimod and provided TLR expertise during the design and execution of the study. **G. Doolittle:** Writing—review and editing, clinical support and enrolled patients. **I. Puzanov:** Writing—review and editing, clinical support and enrolled patients. **J. Markowitz:** Writing—review and editing, clinical support and enrolled patients. **C. Bernatchez:** Writing—review and editing, oversaw the translational work. **A. Diab:** Conceptualization, writing—original draft, project administration.

Supplementary data for this article are available at Cancer Discovery Online (<http://cancerdiscovery.aacrjournals.org/>).

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Data and Materials Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Adi Diab (adiab@mdanderson.org).

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Abstract

Many patients with advanced melanoma are resistant to immune checkpoint inhibition. In the ILLUMINATE-204 phase I/II trial, we assessed intratumoral tilsotolimod, an investigational Toll-like receptor 9 agonist, with systemic ipilimumab in patients with anti-PD-1-resistant advanced melanoma. In all patients, 48.4% experienced grade 3/4 treatment-emergent adverse events. The overall response rate at the recommended phase II dose of 8 mg was 22.4%, and an additional 49% of patients had stable disease. Responses in noninjected lesions and in patients expected to be resistant to ipilimumab monotherapy were observed. Rapid induction of a local IFN α gene signature, dendritic cell maturation and enhanced markers of antigen presentation, and T-cell clonal expansion correlated with clinical response. A phase III clinical trial with this combination ([NCT03445533](#)) is ongoing.

SIGNIFICANCE: Despite recent developments in advanced melanoma therapies, most patients do not experience durable responses. Intratumoral tilsotolimod injection elicits a rapid, local type 1 IFN response and, in combination with ipilimumab, activates T cells to promote clinical activity, including in distant lesions and patients not expected to respond to ipilimumab alone.

INTRODUCTION

For patients with advanced melanoma that progressed on or after PD-1 inhibitors, subsequent treatment with the CTLA4 inhibitor ipilimumab produces a response for a small percentage of patients (10%–16% response rate; refs. 1-3). Thus, there is a pressing

need for rational combinations that potentiate T cell–mediated tumor regression. Increasing evidence suggests responses to checkpoint inhibitors (CPI) require preexisting endogenous tumor-specific T cells, which can be further potentiated by immune modulators (4–6). Multiple mechanisms of primary and secondary resistance have been described, including lack of adequate innate immune response. Manipulation of the innate immune system within the tumor using intratumoral (IT) immunotherapy may serve to prime antitumor T cells by activating dendritic cells (DC) at the tumor site, ensuring optimal local tumor antigen/neoantigen presentation while avoiding systemic toxicities. Toll-like receptor 9 (TLR9) is a pattern recognition receptor that recognizes unmethylated CpG motifs present in bacterial DNA and is expressed by plasmacytoid dendritic cells (pDC), B cells, neutrophils, and macrophages (7–9). Activation of TLR9 stimulates Th1-type immune responses, including high levels of type 1 IFNs, resulting in downstream activation and maturation of antigen-presenting cells (APC; refs. 10, 11).

Extensive structure-activity relationship studies led to creating tilsotolimod (IMO-2125), an investigational synthetic TLR9 agonist (12–14). Tilsotolimod IT injection in animal models produced an increase in local APC maturation, CD3⁺ T cells, and expression of multiple immune checkpoints, including PD-1 and CTLA4, in both injected and noninjected, distant tumors (15). Direct IT, but not systemic injection of other TLR9 agonists, augmented the response to checkpoint inhibitor treatment in animal models of poorly immunogenic melanoma (16), which has also been observed for IT tilsotolimod with checkpoint inhibitors in multiple tumor models (17–19). We hypothesize that the inhibition of CTLA4, a key suppressive pathway in recently activated T cells, by ipilimumab to potentiate the effect of the APC activation triggered by tilsotolimod will show similar benefits in human solid tumors. Here we report the final results of tilsotolimod in combination with ipilimumab in ILLUMINATE-204, a phase I/II clinical trial in patients with advanced melanoma who progressed on or after anti–PD-1 therapy. Detailed longitudinal immune analyses of biopsy specimens of injected and noninjected lesions further support the potential benefit of this combination.

RESULTS

In phase I, 18 patients received ipilimumab in combination with tilsotolimod at 4 mg ($n = 3$), 8 mg ($n = 9$), 16 mg ($n = 3$), and 32 mg ($n = 3$). In phase II, 44 patients received tilsotolimod at the recommended phase II dose (RP2D) of 8 mg in combination with ipilimumab. Patient demographics and baseline characteristics for all patients ($N = 62$) are described in Supplementary Table S1. The median age was 62.0 years (range, 32–91), and 61.3% were male. At baseline, 51.6% had visceral metastases, 43.5% had stage IV M1c disease, and 24.2% had lactate dehydrogenase (LDH) above normal levels. All patients had received prior therapy for advanced disease that included anti–PD-1 therapy. Most patients (40/62, 64.5%) received tilsotolimod injection into a deep or visceral lesion, under image guidance (Supplementary Table S2).

In phase I, the maximum tolerated dose (MTD) was not reached for tilsotolimod in combination with ipilimumab. The selected RP2D for tilsotolimod was 8 mg based on acceptable safety at all dose levels, clinical activity including one durable complete

response (CR), immune effects in support of the proposed mechanism with plasmacytoid DC activation observed 24 hours after injection, and similar preliminary findings in the evaluable patients treated with 4 mg tilsotolimod.

Safety results are summarized in Table 1. In the safety population ($N = 62$), 48.4% of patients experienced grade 3 or 4 treatment emergent adverse events (TEAE), and 32.3% experienced at least one serious adverse event. Discontinuations of tilsotolimod and ipilimumab due to TEAEs were 4.8% and 9.7%, respectively. No TEAEs leading to death were reported. The most common TEAEs were fatigue (51.6%), nausea (40.3%), anemia (38.7%), diarrhea (37.1%), and pyrexia (35.5%), and the most common grade 3/4 TEAEs were diarrhea, increased alanine aminotransferase (ALT), and increased aspartate aminotransferase (AST; 6.5% each), as well as colitis and fatigue (4.8% each). Immune-related adverse events were reported in 25.8% of patients, most commonly hypophysitis and rash (8.1% each); increased ALT, increased AST, colitis, and diarrhea (6.5% each); and hepatitis (4.8%). Grade 3 or 4 immune-related adverse events (irAE) were reported in 15 patients (24.2%) and included colitis, increased ALT, and increased AST (4.8% each), as well as hypophysitis, hepatitis, and diarrhea (3.2% each). Four patients (6.5%) discontinued ipilimumab, and three patients (4.8%) discontinued tilsotolimod due to irAEs.

Results of overall response rate (ORR) analyses are summarized in Table 2. A Simon two-stage design was implemented for ipilimumab-naïve patients treated at the RP2D regardless of the phase in which they were enrolled (see Methods). During stage 1, responses were observed in 5 of 10 patients, and an additional 11 patients were enrolled. The ORR for these 21 patients (prespecified primary efficacy analysis) was 28.6% [Supplementary Fig. S1; P value vs. historical control (20) of 11% = 0.02].

For all evaluable patients who received the RP2D ($n = 49$), the ORR was 22.4% [CR, $n = 2$; partial response (PR), $n = 9$; Table 2]. The median duration of response for these patients was 11.4 months, with 7 of the 11 responses lasting at least 6 months. An additional 24 patients had stable disease (SD), for a disease control rate (DCR; CR + PR + SD) of 71.4%. Results were similar in the subset of patients who had received prior anti-CTLA4 for metastatic disease ($n = 7$; ORR, 28.6%; DCR, 57.1%). At least some degree of tumor reduction was observed in 49.0% of patients and could occur in both injected and noninjected tumors (Fig. 1A-C). Responses occurred generally at the first disease assessment and deepened over time in some cases (Fig. 1D and E).

At a median follow-up of 13.4 months (range, 3.0–47.0), the Kaplan–Meier estimated median overall survival (OS) for these patients was 21.0 months (95% CI, 9.8 months to not evaluable). OS rates were 87.5% at 6 months and 60.0% at 12 months (Fig. 1F). Median progression-free survival (PFS) was 5.1 months (95% CI, 3.7–7.0 months), with PFS rates of 44.2% at 6 months and 19.0% at 12 months (Fig. 1G).

Induction of a Type 1 IFN Response in Injected Lesions

Tumor tissue and blood were collected longitudinally prior to and during treatment from the first 25 evaluable patients treated with any dose of tilsotolimod + ipilimumab, which included patients with prior ipilimumab exposure for treatment of metastatic disease ($n =$

5; Fig. 2A). Twenty-three of 25 patients with biopsy samples passed assay-specific quality-control procedures (Supplementary Table S2). We employed NanoString gene expression profiling on biopsies taken before and 24 hours after tiltsotolimod IT administration (prior to ipilimumab treatment) to determine the early impact of the IT administration of tiltsotolimod alone on the local immune response (Supplementary Table S3). IT administration of tiltsotolimod induced a robust type 1 IFN response in the injected tumor in all tested patients, with multiple type 1 IFN-regulated genes significantly upregulated, including *IRF7*, *IL12A*, *IL1RN*, *CCL8*, and *CCL7* (adjusted $P < 0.01$, $n = 15$; Fig. 2B). Tiltsotolimod also induced genes associated with either a type 1 or type 2 IFN (IFN γ) response [e.g., *IDO* and *CD274 (PD-L1)*], but IHC showed no significantly upregulated PD-L1 expression on tumor cells across all patients at 24 hours postinjection ($P = 0.1126$; Supplementary Fig. S2). Induction of indoleamine 2,3-dioxygenase (IDO) in the tumor tissue by tiltsotolimod at 24 hours postinjection corresponded with the increase observed at the RNA level described above ($P = 0.0012$, $n = 13$; Fig. 2C). IT tiltsotolimod also upregulated expression of genes involved in antigen processing and presentation, including *TAP1* ($P = 0.0015$), *TAP2* ($P = 0.002$), and the inducible immune proteasome genes including *PSMB9* ($P = 0.0007$) but not the constitutive proteasome genes including *PSMB7* ($P = 0.987$; Supplementary Table S4). In the 24-hour biopsy, IT administration of tiltsotolimod did not directly result in the upregulation of classic IFN γ response genes (ref. 21; Supplementary Table S4).

Induction of the type 1 IFN signaling cascade has been shown to promote maturation of APCs and their recruitment to the inflamed site (21, 22). Markers of DC activation such as *CD80* and *IL12A* and chemoattractants *CCL7* and *CCL8* were upregulated at 24 hours after tiltsotolimod injection (Supplementary Table S4), corresponding with an increase in the macrophage gene expression score (*CD163*, *CD68*, *CD84*, *MS4A4A*; $P = 0.0003$, $n = 12$ paired samples; Fig. 2D). Maturation of the CD1c⁺ DC subset, as demonstrated by upregulation of MHC class II (HLA-DR) by flow cytometry on fresh tumor tissue, was found in a subset of patients ($P = 0.07$, $n = 12$; Fig. 2E). Taken together, these data support that IT injection of tiltsotolimod preferentially induced a type 1 IFN response gene expression signature, macrophage infiltration, and local DC maturation, potentially providing a pool of APCs ready and adequate for subsequent T-cell stimulation.

Presence of Local DCs at Baseline Correlates with Clinical Response to Combination Therapy

As TLR9 is expressed across multiple cell types (7-9) and a consistently strong type 1 IFN response was observed following treatment with tiltsotolimod, gene expression profiling was used to assess the expression of TLR9 and the presence of cell types associated with downstream clinical response in the injected lesion at baseline. Although baseline TLR9 gene expression in the injected tumor lesion was not an indicator of clinical response (Supplementary Fig. S3A), the DC score (*CCL13*, *CD209*, *HSD11B1*) was higher at baseline in tumors of responding patients (CR + PR; $n = 6$) compared with patients who progressed (PD; $n = 4$; $P = 0.017$; Fig. 3A). *CD40* but not *CD86* or *CD80* expression was also found to be significantly higher in tumors of responding patients as compared with patients with a best overall response (BOR) of SD or patients who progressed ($P = 0.018$ and 0.022 , respectively; Fig. 3B). Expression of CD123, a surrogate for pDCs, was

numerically increased in tumors of responding patients at baseline ($P = 0.067$ CR + PR vs. PD, Supplementary Fig. S3B). In addition, there was a trend toward higher levels of soluble PD-L2 in the plasma of responding patients compared with those with PD ($n = 15$; Fig. 3C). Conversely, a centered scoring of immune cell signatures indicated a relatively higher neutrophil score and greatly reduced mast cell score at baseline in the injected lesions in patients who did not respond to therapy (Fig. 3D; $n = 14$). Therefore, despite a uniform type 1 IFN response gene signature across all analyzed patients, response to this combination therapy correlates with the type of innate cell responding to IT tilsotolimod.

Presence of T Cells and Level of T-cell Activation in Baseline Tumor Tissue Do Not Correlate with Response to Therapy

Resistance to CPIs may be caused by an immunologically “cold” tumor microenvironment, wherein T cells are either excluded or rendered inactive (23, 24). Given the impact of IT tilsotolimod on the innate immune response and the correlation of DC presence with response, we quantified and characterized the tumor-infiltrating lymphocyte (TIL) at baseline. Responses were observed in patients who did not have immunologically “hot” tumors. Specifically, IHC assessment of baseline tumor biopsy specimens did not reveal a clear association of CD3⁺ and CD8⁺ TIL numbers with clinical response (Supplementary Fig. S4A and S4B). Similarly, NanoString gene expression profiling demonstrated that some patients with low baseline T-cell functional/IFN γ gene signature subsequently responded to treatment (Fig. 3E and F; Supplementary Table S5).

Lack of antigen presentation at the tumor site is another major mechanism of resistance to ipilimumab (25). As we were unable to identify specific mutations such as *B2M* or *Jak1* due to tissue limitations, we prioritized assessing the expression pattern of MHC-I (*HLA-A, B, C*) at baseline in both the injected and noninjected biopsied lesions. Hierarchical clustering demonstrated that, in contrast to MHC-II expression, expression of *HLA-A, B, C* was variable and not associated with response (Fig. 3G; Supplementary Fig. S5A-S5C; Supplementary Table S5). In addition, subanalyses of clinical parameters, including elevated LDH, BRAF mutation status, and prior systemic therapies, did not show a clear correlation with response to treatment.

Taken together, these data suggest that proper activation of local APCs by IT tilsotolimod can overcome inhibitory properties of immunologically “cold” tumors that may lack effective antigen presentation and have poor TIL functionality prior to therapy.

On-Treatment Biopsy Specimens Show Induction of T-cell Function and Proliferation in Both Local and Distant Lesions

To assess the impact of combination therapy on TIL function and the ability to convert a tumor from functionally “cold” to functionally “hot,” gene expression profiling of the local and distant tumor lesions was assessed at baseline and week 8 (Supplementary Table S5 and S6). We compared changes in gene expression profiles by response criteria as well as over time ($n = 13$; Fig. 4A-C; Supplementary Fig. S6). On treatment, there was a robust induction of *CD8a*, T-cell functional genes (*IL2*, *CD27*), and APC activation (*CD80*, *CD86*) and genes associated with response to IFN γ (*PD-L1*) in responding patients at week 8 in both local

and distant lesions that was not seen in nonresponding patients ($n = 13$; Supplementary Fig. S6A). Stratification based on response demonstrated a significant expression of *CD8a*, *CD27*, *FOXP3*, *CXCR3*, *IL2*, *TNFA*, and *CTLA4* in tumors from responding patients as compared with tumors from patients with SD or PD, whereas *IL17a* and *IL10* remained unaffected ($n = 13$; Fig. 4C; Supplementary Fig. S6B-S6D). Furthermore, other types of cellular functions, including immunoproteasome gene expression and macrophage function, increased by week 8 and were more enriched in responding patients (Supplementary Figs. S7A-S7E and S8A and S8B).

Similar to findings with single-agent ipilimumab (26), we observed an increased frequency of $CD8^+$ T_{EM} in the periphery (Supplementary Fig. S9A-S9D). In addition, we observed no significant change in $CD8^+$ T-cell proliferation by Ki-67 staining in the peripheral blood between responding and nonresponding patients on treatment as compared with baseline levels ($P > 0.05$ Fig. 4D and E). However, in responding patients, there was a burst of $CD8^+$ TIL proliferation in local and distant tumor lesions at week 8 as compared with baseline ($P = 0.0071$, Fig. 4D).

Combination Therapy Drives Expansion of Dominant T-cell Clones That Are Shared between Injected and Noninjected Lesions in Responding Patients

To assess whether the T-cell receptor (TCR) repertoires in the injected and noninjected biopsied lesions had an influx of new T cells post therapy that has been observed in ipilimumab monotherapy (27), we performed CDR3 sequencing of the TCR β chain on DNA extracted from baseline and week 8 tumor biopsy specimens and peripheral blood mononuclear cells (PBMC) collected at baseline, week 5, and week 8. Cumulative frequency of the top 50 clones present in noninjected distant tumor tissue (identified at week 8) consistently increased following therapy in responding patients compared with nonresponders, including in a patient (Pt 8) who had previously received ipilimumab (Fig. 5A). A high frequency of the clones found in distant lesions at week 8 was observed at both baseline and week 8 in the injected lesion in responding patients as well as some nonresponding patients (Fig. 5B; Supplementary Fig. S10A). To determine whether this same expansion was observed systemically, the frequency of the top 50 clones from the distant lesion was tracked over time in the blood. Indeed, across some responding, SD, and PD patients, a dominant clone from the tissue could be found at high frequency in the blood (Fig. 5C; Supplementary Fig. S10B). However, most clones were only found at low frequency or not detected within the circulating T-cell population based on the depth of sequencing and the time points assessed.

Overall, these data demonstrate a tumor tissue-specific T-cell proliferation that is present predominantly in responding patients and correlates with an increased frequency of the top 50 T-cell clones present in the tumor lesions. These expanding clones are shared between local and distant lesions and between baseline and on-treatment time points, indicating recognition of tumor antigen(s) shared between lesions.

DISCUSSION

Most patients treated with CPIs face either primary resistance or disease recurrence that arises due to a number of resistance mechanisms (23). It may be possible to overcome this by IT administration of innate immune cell-activating agents that can ultimately stimulate the generation or expansion of preexisting antitumor T cells. Results of the phase I/II ILLUMINATE-204 clinical trial suggest that the combination of tilsotolimod, a novel TLR9 agonist, and ipilimumab is generally well tolerated and demonstrates evidence of clinical activity in anti-PD-1 refractory patients with advanced melanoma. Tilsotolimod in combination with ipilimumab had an irAE profile similar to ipilimumab alone. The most common toxicities associated with tilsotolimod were fatigue and pyrexia, which were primarily grade 1 or 2.

The protocol-defined primary efficacy analysis was ORR in the first 21 ipilimumab-naïve patients enrolled at the 8-mg RP2D of tilsotolimod in combination with ipilimumab. The ORR in this population was 28.6%, whereas the ORR for the entire efficacy-evaluable population at this dose was 22.4%. Responses were observed in patients with prior ipilimumab, prior targeted therapies, and LDH above normal limits, a long-established poor prognostic factor in patients with melanoma treated with ipilimumab (28-31) that was present in 24.2% of patients. Responses observed here compare favorably with retrospective observations of ORR (10%–16%; refs. 1-3) for ipilimumab monotherapy following anti-PD-1 in advanced melanoma. Limited data from prospective studies in similar patient populations are available, with a 7.4% ORR in one study of ipilimumab combined with intratumoral HF10, an oncolytic virus (32).

To our knowledge, this is the largest set of patients with advanced melanoma to have deep nodal and visceral tumors injected using interventional radiology. The ability to inject deep lesions suggests that tilsotolimod could be used for the treatment of other tumor types that would not be otherwise accessible. In addition, for the first time, this study design incorporated extensive longitudinal biopsy specimens, including 24 hours post-IT injection, allowing for assessment of early changes in innate immune response that would not otherwise be detected.

Importantly, the similar rate of tumor reduction in noninjected and injected lesions suggests a potential abscopal effect from tilsotolimod. Although it is possible that responses in noninjected lesions are driven by systemic treatment with ipilimumab, several observations here suggest that systemic responses are due to the combination therapy. First, several genes involved in enhanced antigen presentation were upregulated within 24 hours of intratumoral tilsotolimod injection and sustained at week 8. This includes the immunoproteasome genes PSMB8, PSMB9, and PSMB10 but not the constitutive proteasome genes PSMB7 and PSMD7. Importantly, these immunoproteasome genes remained elevated at week 8 primarily in responders. Along with a higher ORR than expected from ipilimumab alone, responses in noninjected lesions and in patients with known ipilimumab resistance indicators, including low MHC expression or low T-cell infiltration, who would be expected to be resistant to ipilimumab monotherapy, were observed (25). The observed increase in macrophages and the presence of immune chemotactic factors such as *CCL7* and *CCL8*

may suggest that the tilsotolimod + ipilimumab combination overcomes low HLA-ABC expression by both activating and recruiting APCs, consistent with recently published data (30). One limitation of this study is that tumor mutation burden, which has been associated with response to ipilimumab (33), was not assessed. Furthermore, expanding T-cell clones were shared between local and distant lesions. However, in a recently reported randomized study of combination ipilimumab plus talimogene laherparepvec (T-VEC) versus ipilimumab alone, in which T-VEC was injected into one or more superficial lesions, there was no increase in responses in distant lesions (31). These data further support our hypothesis that TLR9 activation by tilsotolimod has a direct impact on adaptive immune activation that may lead to clinical responses in patients who received combination therapy that would not be expected from ipilimumab alone and provided the rationale to ultimately assess this hypothesis in a randomized phase III trial.

Because the presumed target cells of tilsotolimod are TLR9⁺ local APCs that upregulate the costimulatory molecule CD86 and produce type 1 IFN upon stimulation, the combination with ipilimumab has the potential benefit of protecting the expression of CD86 from transendocytosis by its ligand CTLA4, possibly potentiating the antitumor effect (28). The positive correlation of DC presence with response supports this rationale, as DCs are known to most effectively provide costimulation to antitumor T cells. In addition, as CTLA4 upregulation is an early, inhibitory mechanism following T-cell activation, blockade of this axis may allow for optimal T-cell antitumor response (29). At baseline, *CTLA4* gene expression was significantly higher in the patients who would go on to respond to combination therapy, which to our knowledge has not previously been reported as a biomarker for ipilimumab response in solid tumors. The complex post-translational regulation of CTLA4 activation and cellular localization (34) make it difficult to draw strong conclusions from these findings but are worthy of further consideration in subsequent studies. The specific DC subtype(s) that correlate with response were unable to be directly assessed in this study, as the frequency of most subsets was below the limit of detection for flow cytometry and is not available as a validated score via NanoString. However, this is an area of active investigation using more sensitive technologies.

Given the correlation of DC presence, a type 1 IFN response gene signature, and macrophage infiltration in the injected lesions, we hypothesized that tilsotolimod was acting as an *in situ* vaccine leading to a priming process and the emergence of new T-cell clones in the tumor lesion. However, we observed that patients responding to tilsotolimod + ipilimumab showed (1) an expansion of relatively high-frequency clones in distant lesions and (2) that most of the dominant expanding clones are shared between lesions indicating a potential abscopal effect dependent on a preexisting immune response. In contrast, single-agent ipilimumab broadens the peripheral T-cell repertoire but promotes oligoclonal expansion in tumor tissue (27). Thus, the intratumoral adaptive immune response triggered by this combination is distinct from the immune response to ipilimumab alone. Our findings could possibly suggest that the antitumor T-cell response was driven by activation of preexisting response to antigen(s) shared between the injected and noninjected tumor lesions, as opposed to priming a new T-cell response. In support of this hypothesis, we also found that the expression of the chemoattractant CXCL10 was decreased on treatment biopsy specimens compared with baseline. Expression of additional IFN γ -related genes

was similarly decreasing or remained stable. This is in contrast with recently reported findings (35), possibly due to differences in the biopsy timing; we collected on-treatment biopsy specimens 7 weeks after initiating ipilimumab, compared with 2 or 4 weeks in the Checkmate 038 study. Recent data demonstrated a plateau of cell proliferation after two doses of combination ipilimumab/nivolumab, suggesting week 7 may be beyond the peak of immune activation (36).

On the basis of our data, we strongly believe that CTLA4 blocking is essential for optimal activation of both innate and adaptive immune arms by intratumoral TLR agonists, although other groups have reported clinical benefit of combination of intratumoral TLR agonists in combination with PD-1 inhibitors in patients with PD-1 blockade-refractory disease (37, 38). The TLR9 agonist SD-101 in combination with pembrolizumab increased IFN α -responsive genes in the blood and immune activation genes in the tumor (37). Although the mechanisms are not fully understood, the Ribas group reported that intratumoral TLR9 activation can overcome resistance to PD-1 inhibition that is due to the loss of IFN γ signaling and chemokines CXCL9/10, which occurred secondary to loss of function of the JAK1/2 genes (19). Furthermore, elegant studies by Melero, Ribas, and colleagues (39, 40) found that the TLR3 agonist BO-112 in combination with PD-1 blockade was able to produce antitumor responses in refractory patients or animal models. Again, the preclinical immune analyses suggest that BO-112 upregulation of IFN signaling and antigen presentation were able to overcome genetic resistance to checkpoint inhibitor therapy due to JAK1 loss (40). The benefit in combining a TLR9 agonist with PD-1 inhibition can be further supported by recent work showing that elimination of PD-L1 expression on DCs led to tumor inhibition and augmented CD8 T-cell responses, highlighting the important role of DCs in the PD-1/PD-L1 pathway (41). Altogether, these data highlight the importance of the TLR9 pathway in resistance to PD-1 blockade and may suggest that the optimal treatment approach will require combination of an intratumoral TLR9 agonist such as tilisotolimod with both systemic anti-CTLA4 and anti-PD-1.

Overall, this study demonstrates that the simultaneous stimulation of the innate and adaptive immune systems can trigger a productive antitumor response in heavily pretreated patients with advanced melanoma refractory to anti-PD-1 therapy. ILLUMINATE-301, a randomized phase III clinical trial of tilisotolimod at 8 mg in combination with ipilimumab compared with ipilimumab alone in patients with PD-1 refractory advanced melanoma, is ongoing ([NCT03445533](#)).

METHODS

Patients

The study enrolled patients from eight medical centers in the United States. The study was approved by the Food and Drug Administration and the institutional review boards and ethics committees at each center. This study was written and conducted in accordance with the principles of the Declaration of Helsinki. All patients granted a written informed consent prior to treatment initiation.

Eligible patients were 18 years of age or older and had histologically confirmed advanced melanoma (cutaneous, mucosal, or acral) with stage III or stage IV disease. Phase I patients must have had at least two measurable tumor lesions ≥ 1.0 cm that were accessible to biopsy. Phase II patients must have had at least one measurable lesion (per RECIST v1.1), which may have been the same site used for the intratumoral injections. All patients had symptomatic or confirmed radiographic progression by RECIST v1.1 during or after treatment with a PD-1 inhibitor, administered either as monotherapy or in combination. BRAF mutation–positive patients were eligible, and prior BRAF/MEK inhibitor treatment was not required. All patients had an Eastern Cooperative Oncology Group performance status score of 0 to 2. Key exclusion criteria were exposure to prior TLR agonists (excluding topical agents), patients with active autoimmune disease requiring disease-modifying therapy or ≥ 7.5 mg/d of prednisone, and ocular melanoma.

Study Design and Treatment Administration

The study design is shown in Fig. 2A. In patients receiving tilsotolimod in combination with ipilimumab, ipilimumab was administered at 3 mg/kg intravenously every 3 weeks beginning at week 2 of cycle 1 for four doses. An additional cohort received tilsotolimod in combination with pembrolizumab, which will be reported independently. IT tilsotolimod was administered once weekly for 3 consecutive weeks in the first (4-week) cycle, followed by injections on day 1 of every 3-week cycle for three cycles. In March 2017, the protocol was amended to include three additional maintenance doses every 6 weeks, for a total of nine doses. The addition of maintenance doses was intended to provide continued innate immune stimulation in response to neoantigens that may emerge in response to treatment (42).

Tilsotolimod was administered as a series of IT injections into a single tumor. The injected tumor for each patient was selected in order of priority from pathologic draining lymph nodes, superficial or subcutaneous metastases, or deep (visceral) metastases—the latter requiring interventional radiology support (Supplementary Fig. S11A). In the event that complete tumor regression occurred in all accessible lesions prior to completion of therapy, remaining injections were to be administered into another accessible lesion or into the tumor bed, except in the case of visceral lesions, for which remaining doses were to be injected subcutaneously to minimize procedural risks. Tilsotolimod was administered using a fanning method, injecting at several angles to maximize tilsotolimod throughout the lesion while avoiding necrotic areas. The total injected dose remained constant, whereas injection volume was adjusted depending on the size of tumor.

In the phase I portion, patients were enrolled into four dose-level cohorts of tilsotolimod at 4, 8, 16, or 32 mg in combination with ipilimumab or three dose-level cohorts of tilsotolimod at 8, 16, or 32 mg in combination with pembrolizumab. Dose-limiting toxicities (DLT) were defined as grade 3 or higher hematologic or nonhematologic toxicity. Decisions regarding dose escalation were based only on DLTs that occurred during the first cycle of treatment (weeks 1 through 4); however, DLTs that occurred throughout the observation period were considered when determining the MTD or the RP2D. All patients in the safety

population who continued participation in the study for the entire DLT evaluation period or who discontinued prematurely due to a DLT met MTD or RP2D evaluation criteria.

The phase II portion was designed to assess preliminary efficacy of tilsotolimod in combination with ipilimumab or pembrolizumab at the MTD or RP2D. Prior to phase II, the tilsotolimod + pembrolizumab cohort was discontinued at the discretion of the study sponsor.

Safety and Efficacy Assessments

Clinical and laboratory safety assessments were conducted at baseline, weekly during cycle 1, and then on day 1 of subsequent cycles. Adverse events were categorized according to the Medical Dictionary for Regulatory Activities (MedDRA) and graded using the CTCAE version 4.03. The irAEs included predefined events and any additional events deemed by the investigator to be immune related. Efficacy evaluations occurred at weeks 8, 17, and 29, then every 3 months, and included clinical examination and computed tomography or magnetic resonance imaging of known sites of disease. Tumor response was assessed using RECIST v1.1. All response assessments of CR, PR, and PD were confirmed by imaging 4 weeks after the initial documentation of response.

Tumor Biopsy Specimens

Biopsy specimens of injected and noninjected lesions were collected. For phase I, biopsies of the injected lesion were performed at the screening visit (within 21 days prior to first treatment), within 24 to 48 hours following tilsotolimod injection on week 1 of cycle 1, optionally during week 2 of cycle 1, during week 8 (cycle 3), optionally during week 13 (cycle 4), and at the time of disease progression or during week 23, whichever was later. Biopsies of the designated noninjected lesion were performed at the screening visit, optionally prior to the first dose of ipilimumab or pembrolizumab, and during week 8. For phase II, tumor biopsies were optional at all specified visits. Repeat biopsies were performed only if feasible for those patients with visceral metastases injected with tilsotolimod.

Flow Cytometry

Fresh tumor tissue was disaggregated using a BD Medimachine System (BD Biosciences) and subsequent filtering to generate a single-cell suspension for staining. PBMCs were thawed, washed, and resuspended for staining. Surface staining was performed in FACS Wash Buffer (1× DPBS with 1% BSA) for 30 minutes on ice using fluorochrome-conjugated monoclonal antibodies from BD Biosciences, BioLegend, and eBioscience as described previously (43). Cells were fixed in 1% paraformaldehyde solution for 20 minutes at room temperature following surface staining. For panels containing transcription factors, cells were fixed and permeabilized using the BD Transcription Factor Kit according to the manufacturer's instructions. A complete list of the antibodies, catalog numbers, companies, and clones used is available. Gating strategies are shown in Supplementary Fig. S11B and S11C. Samples were acquired using the BD FACSCanto II or BD Fortessa X20 and analyzed using FlowJo Software v 7.6.5 (Tree Star). Dead cells were stained using AQUA live/dead dye (Invitrogen) and excluded from the analysis.

NanoString

RNA was extracted from core needle biopsy specimens that were preserved in RNAlater using the Qiagen AllPrep Universal Kit (cat. 80224) according to the manufacturer's instructions. Purity and concentration were assessed using NanoDrop. RNA was assayed using the NanoString PanCancer Immune Profiling Panel and analyzed using the nSolver Advanced Analysis Software.

Immunohistochemistry

IHC studies were performed using a Leica Bond autostainer as previously described, using the following antibodies and 3,3'-diaminobenzidine chromogen: CD3 (Dako A0452; 1:100), CD8 (Life Sciences Technologies MS457s; 1:25), FOXP3 (BioLegend 320102; 1:50), IDO (Cell Signaling 86630; 1:100), PD-1 (ABCAM ab137132; 1:250), and PD-L1 (Cell Signaling 13684S; 1:100; ref. 44).

Image Analysis

Slides were scanned at 20× magnification (Aperio ScanScope AT Turbo; Leica Biosystems). Image analysis software (Aperio Image-Scope) tabulated the number of IHC-positive cells within designated areas. The relatively small size of immune cells enabled a modified version of the Nuclear v9 algorithm to be applied as a basis for detecting immune marker positivity, and the intensity thresholds were adjusted manually to remove background artifacts and to account for variable differences in cell size (especially for PD-L1). CD3, CD8, and PD-1 expression were assessed in lymphocytes, whereas PD-L1 expression was counted in tumor cells.

TCR V β CDR3 Sequencing

Tumor core needle biopsy specimens were banked in RNAlater and stored at -20°C. PBMCs were banked in liquid nitrogen. Total DNA was isolated using the Qiagen AllPrep Kit according to the manufacturer's instructions. TCR β CDR3 sequencing was performed using the Adaptive Biotechnology pipeline. Survey-level sequencing was performed on DNA extracted from tumor tissue, and deep-level sequencing was performed on DNA extracted from PBMCs. Clonality was determined using ImmunoSEQ as previously described (45). Tracking data were extracted from ImmunoSEQ and analyzed at MD Anderson Cancer Center.

Plasma Analysis

Plasma from treated patients collected at baseline and on treatment was assessed using Invitrogen ProcartaPlex multiplex immunoassays (Thermo Fisher Scientific).

Statistical Analysis

The following protocol-defined study populations were analyzed:

- Primary ipilimumab + IMO-2125 efficacy evaluable (PIEE) population: all patients who were ipilimumab-naïve on study entry and who were treated at the RP2D for the tilsotolimod + ipilimumab combination, regardless of which phase of the study they received it, and who received at least one dose of each

study drug. Patients who received ipilimumab only in the adjuvant setting were included in the PIIIE population.

- Secondary ipilimumab + IMO-2125 efficacy evaluable (SIIIE) population: all patients who had previously received ipilimumab prior to study entry and who were treated at the RP2D for the tilsotolimod + ipilimumab combination, regardless of which phase of the study they received it, and who received at least one dose of each study drug. Patients who received ipilimumab only in the adjuvant setting were not included in the SIIIE population.
- Safety population: all patients who received at least one dose of tilsotolimod.
- DLT evaluable population: all patients in the safety population who participated in the entire DLT evaluation period (weeks 1 to 4) or who discontinued prematurely due to a DLT.

The primary objective of phase I was to evaluate safety and to determine an RP2D. Secondary objectives were to determine plasma pharmacokinetics and to evaluate any preliminary antitumor activity.

The primary objective of phase II was investigator assessment of ORR, using RECIST v1.1. The primary hypothesis tested was that the ORR for the PIIIE population (ipilimumab-naïve patients treated with tilsotolimod + ipilimumab at the RP2D) was significantly greater than a historical control. A Bonferroni correction was applied to the α to control the type I error rate for the trial. The primary efficacy analysis of ORR for tilsotolimod in combination with ipilimumab was performed on the first 21 patients in the PIIIE population and tested against the null hypothesis of 11% ORR for ipilimumab as monotherapy, estimated using historical data for ipilimumab monotherapy in previously treated patients with metastatic melanoma (20). A sample size of 21 patients would achieve 77% power to detect a 24% difference in response rates using a one-sided significance level of 2.5%. A two-stage design was used, with an ORR of 2 of 10 patients required during stage 1 in order to enroll an additional 11 patients in stage 2. If at least 6 of the total 21 patients had a RECIST v1.1 response, then the null hypothesis would have been rejected in favor of the alternative hypothesis.

Secondary objectives for phase II included assessment of duration of response, durable response rate at 6 months, DCR, OS, OS at 6 and 12 months, PFS, PFS at 6 and 12 months, and safety and tolerability.

Efficacy analyses in the PIIIE population and the combined PIIIE and SIIIE populations (all patients treated with tilsotolimod + ipilimumab at the RP2D) were also completed and summarized using descriptive statistics.

Follow-up was calculated from the time of study enrollment to the last contact with the patient or death. OS is defined as the number of months from initiation of treatment to death from any cause. Patients who were alive as of the date of their last contact were censored as of that date.

Continuous variables were summarized using descriptive statistics such as mean, standard deviation, median, and range. Categorical variables were summarized by count and proportion and, if specified, with 95% confidence intervals for the proportion.

Analysis of DLTs was performed using the DLT-evaluable population; all other safety analyses were performed using the safety population and are summarized descriptively; no formal statistical hypotheses were tested.

Statistical analysis of flow cytometry data was performed using a Mann–Whitney test, and soluble factors were assessed using a two-sided unpaired Student *t* test through GraphPad prism version 7.03 (GraphPad Software). NanoString data were analyzed using the nSolver Advanced Analysis tool. Changes in individual gene expression from baseline were tested for significance using a parametric paired *t* test or parametric unpaired *t* test as indicated in the corresponding figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Tumor reduction. **A**, Maximum percent reduction from baseline in individual sum of the longest diameters (SLD) of target lesions. **B**, Maximum percent decrease in target lesion diameters in injected and noninjected measurable lesions. **C**, Baseline and post-therapy imaging of injected lesion (yellow arrow) and noninjected, distant lesions (red arrows) of a patient with complete response to therapy. **D**, Individual percent change from baseline in the SLD of target lesions. **E**, Time to and duration of clinical benefit. Patients with CR, PR, or SD are included, and each bar represents one patient. **F**, Kaplan–Meier estimates (8 mg tilsotolimod + ipilimumab population) of overall survival. **G**, Progression-free survival. CI, confidence interval; NE, not evaluable. † Patient had a 34.5% decrease in tumor burden from baseline but had progressive disease at the subsequent scan. # Patient had a 38.5% decrease in tumor burden from baseline but had progressive disease at the subsequent scan.

Figure 2. Tilsotolimod induces an early, local type 1 IFN response gene signature, macrophage influx, and DC1 maturation. **A**, Study design for tilsotolimod + ipilimumab combination. **B**, Volcano plot of RNA extracted from the local injected lesion at 24 hours after tilsotolimod compared with baseline ($n = 15$ unpaired samples). The adjusted P value is indicated. **C**, IDO expression was assessed in the tumor tissue using chromogenic IHC ($n = 13$ paired samples). **D**, The macrophage score was determined using the nSolver advanced analysis tool and is shown in a log₂ scale ($n = 12$ paired samples). **E**, Live, lineage-negative, CD1c⁺ myeloid DC1 (mDC1) cells were assessed for HLA-DR expression in the tissue at baseline and 24 hours after tilsotolimod injection. A minimum of 100 events was required for subgating ($n = 12$ paired samples).

Figure 3.

Correlations of local cell types with clinical activity. **A**, Dendritic cell score was determined using the nSolver advanced analysis tool and is shown in a log₂ scale according to type of response ($n = 14$). **B**, nSolver expression of myeloid markers ($n = 14$). **C**, Concentration of soluble PD-L2 (sPD-L2) prior to treatment in patient plasma according to type of response ($n = 15$). **D**, Cell-type centered score of each major cell type at baseline in injected lesions as determined by the nSolver advanced analysis tool is shown according to type of response ($n = 14$). **E**, Heat map by hierarchical clustering using the T-cell functionality gene set at baseline in both local and noninjected, distant lesions ($n = 14$). **F**, nSolver expression of select T-cell functionality and cytotoxicity genes ($n = 14$). P value indicates significance using an unpaired Student t test. **G**, Heat map by hierarchical clustering using the cytotoxicity gene set at baseline in both local and noninjected, distant lesions ($n = 14$).

Figure 4.

TIL activation and proliferation on treatment correlates with response to combination therapy. Unsupervised hierarchical clustering using NanoString gene expression profiling ($n = 13$) of (A) T-cell functional gene signature, (B) cytotoxicity gene signature, and (C) nSolver expression of select T-cell functional signature genes ($n = 13$). P value indicates significance using an unpaired Student t test. **D** and **E**, Proliferation assessed using Ki-67 staining through flow cytometry of CD8⁺ tumor-infiltrating lymphocytes at baseline, 24 hours after tilsotolimod injection, and at week 8 in tumor lesions (black hashed line) as well as in PBMCs (red hashed line) at baseline, 24 hours post tilsotolimod injection (when available), and week 8. Patients are grouped by response status.

Figure 5.

T-cell response is suggestive of shared antigen(s) and abscopal effect. **A**, Frequency of the top 50 clones in the noninjected, distant tumor lesions at week 8 and baseline for CR + PR (left graph), SD (middle graph), and PD (right graph) patients. **B**, Individual T-cell clones (top 50) identified at week 8 in the noninjected, distant lesions of individual responding patients assessed for presence at baseline and week 8 in the local, injected lesion and at baseline in the noninjected, distant lesion. Each image represents individual patients, with each circle representing an individual T-cell clone. Clones shared between lesions at all time points are shown in blue. The size of the circle indicates relative frequency at week 8 and the numbers indicate the frequency relative to initial baseline presence. **C**, Frequency of individual T-cell clones identified at week 8 in the noninjected, distant lesion in the blood at baseline and on treatment (week 5 and/or week 8) as indicated. Each color indicates an

individual T-cell clone. The number indicates the number of the 50 clones found in the blood at each time point tested. PD, progressive disease.

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Table 1.Adverse events to tilsotolimod + ipilimumab (*N* = 62)

Characteristic	Patients, <i>n</i> (%)
Patients with at least one TEAE	61 (98.4)
Patients with at least one grade 3/4 TEAE	30 (48.4)
Patients with at least one SAE	20 (32.3)
Patients with at least one immune-related TEAE	16 (25.8)
Patients discontinuing tilsotolimod due to TEAEs	3 (4.8)
Patients discontinuing ipilimumab due to TEAEs	6 (9.7)
Patients discontinuing tilsotolimod due to immune-related TEAEs	4 (6.5)
Patients discontinuing ipilimumab due to immune-related TEAEs	3 (4.8)
Most common TEAEs ^a	
Fatigue	32 (51.6)
Nausea	25 (40.3)
Anemia	24 (38.7)
Diarrhea	23 (37.1)
Pyrexia	22 (35.5)
ALT increased	20 (32.3)
Vomiting	19 (30.6)
Headache	18 (29.0)
Rash	17 (27.4)
AST increased	16 (25.8)
Chills	16 (25.8)
Most common grade 3/4 TEAEs ^b	
Diarrhea	4 (6.5)
ALT increased	4 (6.5)
AST increased	4 (6.5)
Colitis ^c	4 (6.5)
Fatigue	3 (4.8)
Most common immune-related TEAEs ^d	

Characteristic	Patients, n (%)
Hypophysitis	5 (8.1)
Rash	5 (8.1)
Colitis ^c	4 (6.5)
ALT increased	4 (6.5)
AST increased	4 (6.5)
Diarrhea	4 (6.5)
Hepatitis ^e	3 (4.8)
Most common grade 3/4 immune-related TEAEs ^d	
Colitis ^c	3 (4.8)
ALT increased	3 (4.8)
AST increased	3 (4.8)
Hepatitis ^e	2 (3.2)
Hypophysitis	2 (3.2)
Diarrhea	2 (3.2)

Abbreviation: SAE, severe adverse effect.

^aTEAEs that occurred in 25% of patients are shown and are listed in order of decreasing frequency.

^bGrade 3 or 4 TEAEs that occurred in 3 patients are shown and are listed in order of decreasing frequency.

^cIncludes preferred terms of colitis, autoimmune colitis, and enterocolitis.

^dIncludes events that occurred in 3. Immune-related AEs were identified during a sponsor review following database lock that was confirmed with the investigators.

^eIncludes patients with immune-related hepatitis or autoimmune hepatitis.

Table 2.

Summary of responses (8 mg tilsotolimod + ipilimumab population)

Characteristic	Primary efficacy analysis ^a (n = 21)	Ipilimumab exposed ^b (n = 7)	Ipilimumab naïve ^c (n = 42)	Total ^d (N = 49)
BOR, n (%) ^e				
CR	2 (9.5)	0 (0.0)	2 (4.8)	2 (4.1)
PR	4 (19.0)	2 (28.6)	7 (16.7)	9 (18.4)
SD	10 (47.6)	2 (28.6)	22 (52.4)	24 (49.0)
PD	5 (23.8)	3 (42.9)	11 (26.2)	14 (28.6)
ORR, % ^f	28.6 ^g	28.6	21.4	22.4
95% CI, %	11.3-52.2	ND	10.3-36.8	11.8-36.6
DCR, % ^h	76.2	57.1	73.8	71.4
95% CI, %	52.8-91.8	ND	58.0-86.1	56.7-83.4
Median DOR, mo	ND	ND	11.4	11.4
Interquartile range			10.2-NE	4.2-NE

Abbreviations: BOR, best overall response; CI, confidence interval; DOR, duration of response; ND, not determined; NE, not estimable; PD, progressive disease.

^aThe primary efficacy analysis was ORR assessed in the first 21 patients who did not receive ipilimumab for metastatic disease enrolled at the RP2D of tilsotolimod in combination with ipilimumab.

^bIn the metastatic disease setting.

^cAll patients who had not received ipilimumab for metastatic disease on study entry and treated at the RP2D of tilsotolimod in combination with ipilimumab.

^dAll patients treated at the RP2D of tilsotolimod in combination with ipilimumab.

^eAssessed using RECIST v1.1.

^f(CR + PR)/number of patients × 100.

^gP = 0.02 versus ORR for historical control of 11% (20).

^h(CR + PR + SD)/number of patients × 100.