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Effective Innate and Adaptive Antimelanoma Immunity through Localized TLR7/8 Activation

Manisha Singh,* Hiep Khong,**† Zhimin Dai,* Xue-Fei Huang,* Jennifer A. Wargo,** Zachary A. Cooper,** John P. Vasilakos,** Patrick Hwu,*,**† and Willem W. Overwijk*,*†

Intratumoral immune activation can induce local and systemic antitumor immunity. Imiquimod is a cream-formulated, TLR7 agonist that is Food and Drug Administration approved for the treatment of nonmelanoma skin cancers, but it has limited activity against melanoma. We studied the antitumor activity and mechanism of action of a novel, injectable, tissue-retained TLR7/8 agonist, 3M-052, which avoids systemic distribution. Intratumoral administration of 3M-052 generated systemic antitumor immunity and suppressed both injected and distant, un.injected wild-type B16.F10 melanomas. Treated tumors showed that an increased level of CCL2 chemokines and infiltration of M1 phenotype-shifted macrophages, which could kill tumor cells directly through production of NO and CCL2, were essential for the antitumor activity of 3M-052. CD8+ T cells, B cells, type I IFN, IFN-γ, and plasmacytoid dendritic cells were contributed to efficient tumor suppression, whereas perforin, NK cells, and CD4 T cells were not required. Finally, 3M-052 therapy potentiated checkpoint blockade therapy with anti–CTLA-4 and anti–programmed death ligand 1 Abs, even when checkpoint blockade alone was ineffective. Our findings suggest that intratumoral treatment with 3M-052 is a promising approach for the treatment of cancer and establish a rational strategy and mechanistic understanding for combination therapy with intratumoral, tissue-retained TLR7/8 agonist and checkpoint blockade in metastatic cancer. The Journal of Immunology, 2014, 193: 000–000.

Therefore, development of injectable, local-release formulations of TLR7 and TLR7/8 agonists are an area of intense study and drug development.

Growing evidence suggests that tumor-associated macrophages (TAMs) play an important role in tumor growth. TAMs can assume M1 or M2 phenotypes, with M1 TAMs producing IL-12 to promote tumoricidal responses, whereas M2 TAMs produce IL-10 and promote tumor progression (11, 12). One of the factors that may drive M1/M2 TAM ratios in tumors is the chemokine CCL2/MCP-1. Low CCL2 concentrations can promote accumulation of M2 TAMs and tumor growth whereas high CCL2 secretion results in predominant M1 TAM infiltration and tumor destruction (13). Therefore, shifting TAM phenotype from M2 toward M1 could be an important therapeutic strategy (14–17).

In this study, we report therapeutic activity of a novel TLR7/8 dual agonist, 3M-052, in a preclinical model of melanoma. 3M-052 is an injectable, lipid-modified imidazoquinoline that forms a tissue depot with gradual, sustained release, allowing local TLR triggering activity without systemic cytokine release (18). 3M-052 is currently under clinical development by the 3M Drug Delivery Systems Division for use in vaccines and cancer therapy. Intratumoral 3M-052 monotherapy induced local innate immune activation as well as systemic, Ag-specific CD8+ T cell responses that suppressed distant, un injected tumors. Mechanistically, the intratumoral macrophages shifted from an M2-dominant to M1-dominant phenotype, whereas CCL2 blockade or macrophage depletion abolished therapeutic activity. 3M-052 has promise as monotherapy or in combination with checkpoint blockades, anti–programmed death ligand 1 (PD-L1) or anti–CTLA-4, for the treatment of metastatic melanoma and other cancers.

Materials and Methods

Mice and cell lines

All animal experiments were performed in accordance with National Institutes of Health guidelines and approved by the MD Anderson Cancer Medicine...
Center Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from the National Cancer Institute. Rag2 knockout (KO), B cell KO (IgH), Pff KO, IFN-γ KO, and Bdc2-2-DTR mice were from The Jackson Laboratory, and IFNAR KO mice were provided by Dr. Paul W. Dempsey at the University of Zurich (Zurich, Switzerland) (19). All mice were used at 6–12 wk of age. B16.F10, B16.OVA, and the braf V600E cell line (20) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1-glutamine, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (all from Invitrogen Life Technologies). The TRAMP-C2 cell line was cultured in DMEM-HG (Life Technologies) supplemented with 5% Nu-serum IV, 5% FBS, 5 μg/ml insulin, 10 nM dihydrotestosterone, and penicillin-streptomycin.

3M-052 and its vehicle

An injectable formulation of 3M-052 and the vehicle were provided by 3M Drug Delivery Systems (3M Center, St. Paul, MN) (18). Briefly, 3M-052 was formulated in sesame oil (NF NP, Super Refined; Croda Europa, East Yorkshire, U.K./ethanol. The formulation was filtered through a polyethersulfone 0.2-μm filter (Millipore, Billerica, MA). Drug content was determined by HPLC, and the final concentration was 0.4–0.5 mg/ml.

Tumor induction, treatment, and monitoring

C57BL/6 or KO mice were s.c. inoculated with 3 × 105 B16.F10 or B16.OVA melanoma cells on day −7 in the left flank, and for contralateral tumor experiments 3 × 105 B16.F10 or B16.OVA tumor cells were also inoculated in to right flank on day −3. Only left flank palpable tumors were treated intratumorally (i.t.) on days 0 and 4 with 28–35 μg 3M-052 or vehicle and right flank tumors were left untreated. For BP and TRAMP-C2 models tumors were implanted by s.c. injection of 5 × 106 cells in the left flank on day −9. Tumor size is expressed as the product of perpendiccular diameters of tumors measured with calipers. Mice were sacrificed when tumor size reached ≥200 mm2 in diameter.

Cell depletion, chemokine neutralization, and checkpoint blockade experiments

Mouse Abs against CCL2 (2H5), CD4 (GK1.5), CD8 (2.43), NK1.1, Ly6C cells, PD-L1 (1096200), CTLA-4 (9H10), and respective isotype controls were purchased from Bio X Cell. Anti–PD-L1 mAb and anti–CTLA-4 were injected i.p. (200 μg) 1 h after 3M-052 treatment and repeated every 4 d. Others Abs were injected i.p. (200 μg) on days −1 and 0, and Ab injections were repeated every 4 d thereafter to maintain depletion or neutralization. Cell depletion efficiency was confirmed by flow cytometry on peripheral blood or tumor 2–3 d after Ab injection. In vivo depletion of pDCs was induced and maintained by diphtheria toxin injection (i.p.: 5 μg diphtheria toxin/g body weight every other day) in B6c2-2-DTR mice harboring B16 melanoma 1 d before the start of 3M-052 treatments and repeated every other day until the end of experiment. Clodronate and control liposome (anionic) were purchased from FormuMax and injected i.t. (40 μl 1:10 dilution in PBS) 2 d before the start of 3M-052 treatment and repeated every week.

Flow cytometric analysis

Leukocytes were isolated from mechanically disrupted tumors by lymphocyte separation medium (Coming cellgro). RBCC lysis was performed on spleens and blood. Ag-specific T cell responses were evaluated by OVA tetramer (Beckman Coulter) and CD8 (BioLegend) staining. Intracellular CD206 (BioLegend) and IFN-γ staining was performed using the Cytofix/ Cytoperm kit (BD Biosciences). Cells were stained with Abs against CD45, CD19, B220, CD11b, CD11C, B220, Siglec-H, Ly6C, F4/80, and CD40 from BioLegend, CD3, CD4, CD8, CD11c, CD68, and CD69 from BD Pharmingen. Data were acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star).

Cytokine/chemokine assay

Twenty-four hours after 3M-052 or vehicle treatment, tumors were mechanically disrupted and centrifuged and supernatant was collected. Cytokines/chemokines were measured using a Milliplex mouse cytokine/chemokine panel (Millipore) according to the manufacturer’s instructions. Fluorescence signal was measured on a Luminex 100/200 system, and data were analyzed using Excel software.

Cytolytic assay

Macrophages (CD11b F4/80+) were sorted from mouse splenocytes and cultured with 3M-052 (3 μg/ml) or vehicle for 48 h. B16-F10 melanoma cells (6 × 103) were cultured with stimulated macrophages at a 1:10 E/T ratio or with 200 μl macrophage culture supernatant for 96 h. Cytotoxicity was measured in 96-well plates using an EZ4U nonradioactive cell proliferation and cytotoxicity assay (Bio-medica) according to the manufacturer’s manual. Data were expressed as the percentage lysis or percentage of live cells calculated as follows: % lysis = [1 – (Ab coculture cells – Ab effector cells) / Ab target cells] × 100; % live cells = [Ab16 cells with 3M/V-stimulated macrophage supernatant – blank] / [Ab16 cells with 3M/V – blank] × 100. A value for absorbance.

Statistical analysis

All results are expressed as the means ± SEM. Mouse and sample group sizes were n = 5 unless otherwise indicated. Statistical analysis was performed with GraphPad Prism 4 software. Data were analyzed using unpaired two-tailed t tests, and differences were considered significant at p < 0.05. Survival experiments used log-rank Mantel–Cox test for survival analysis. All experiments were performed at least twice with comparable results.

Results

Intratumoral administration of 3M-052 suppresses local injected and distant uninjected melanoma growth

Most innate immune cells, including APCs in mice and humans, express TLR7 and/or TLR8 (21, 22). In C57BL/6 mice, TLR8 is nonresponsive to imidazoquinolines such as resiquimod and 3M-052, but both pDCs, myeloid DCs, and macrophages in mice express TLR7 and respond to TLR7 agonists (10, 23). Thus, activation of tumor-associated TLR7+ APCs with 3M-052 could generate a range of innate and adaptive antitumor immune responses. We tested the antitumor effect of 3M-052 against the poorly immunogenic, wild-type B16.F10 melanoma and the more immunogenic version B16.OVA, engineered to express the chicken OVA protein. Palpable 7-d tumors (∼20 mm2) were treated with i.t. 3M-052 or vehicle on days 0 and 4 (treatment schematic; Fig. 1A). Growth of both B16.F10 and B16.OVA tumors was suppressed after 3M-052 treatment, resulting in prolonged survival (Fig. 1B–D). However, the treatment efficacy of 3M-052 was more profound with B16.OVA than B16.F10 tumor. Although most tumors that were treated with 3M-052 never reached a size of 200 mm2 during the observation period, they developed dry ulceration (necrosis), requiring euthanasia. Because the goal of cancer therapy is typically the treatment of metastatic cancer, we tested the activity of i.t. 3M-052 on the growth of distant, uninjected tumors. 3M-052 treatment of an established tumor effectively suppressed the growth of metastatic cancer, we tested the activity of i.t. 3M-052 on the growth of distant, uninjected tumors. 3M-052 treatment of an established tumor effectively suppressed the growth of distant, uninjected B16.F10 and B16.OVA tumors, suggesting this local approach can have systemic efficacy (Fig. 1E, 1F). To ensure that antitumor activity of 3M-052 was not limited to B16 melanoma, we also tested activity against established melanoma, derived from the Tyr::CreER, BrafC50; Pten lox/lox mouse (20), and against TRAMP-C2 prostate cancer, and we found antitumor activity against these tumors as well (Fig. 1G, 1H).

Migration and activation of innate immune cells

TLR7/8 triggering by 3M-052 is expected to activate murine TLR7+ innate leukocytes, including macrophages, pDCs, and cDCs, possibly resulting in tumor-specific T cell responses. We studied leukocytes (CD45+ in tumor and tumor-draining lymph nodes (TDLNs) 24 h after i.t. administration of 3M-052 or vehicle control. Significant accumulation of myeloid DCs (CD11c+CD11b+ B20+) and decreased numbers of lymphoid DCs (CD11c+ CD11b+ B20+) were found in TDLNs of 3M-052–treated mice, and both DC types had upregulated CD40 and CD86 activation markers. We did not find expansion and activation of DCs in the tumor (Fig. 2A, 2B). Similarly, activated B cells (CD19+CD40+) were present in TDLNs but not in the tumor (Fig. 2C, 2E), suggesting that i.t. 3M-052 induces APC activation and migration from tumor to TDLNs. NK cells were reduced in both tumor.
and TDLNs (data not shown) but showed increased expression of CD69 activation marker (Fig. 2D, 2F). Interestingly, we found greatly reduced numbers of pDCs (CD11C+Siglec-H+ and CD11C+Siglec-H+CD40+) in tumor and in TDLNs (Fig. 2G and data not shown). In contrast, macrophages (CD11b+F4/80+) were significantly increased in tumor and TDLNs 24 h after i.t. 3M-052 administration (Fig. 2H); however, CD40 upregulation on macrophages was seen only in TDLNs (Fig. 2I). These data indicate potent innate immune activation by 3M-052 demonstrated by accumulation, activation, and migration of innate immune cells.

**Induction of tumor-specific CD8+ T cells and mechanism of tumor suppression**

To investigate whether adaptive immunity is required for 3M-052–mediated tumor suppression, we treated both B16.F10 and B16.Ova tumors in C57BL/6 and Rag2 KO mice, deficient in T and B cells. Therapeutic efficacy of 3M-052 was significantly but not completely abrogated in Rag2 KO mice (Fig. 3A, 3B). Indeed, 8 d after i.t. treatment, we found more OVA257–264-specific CD8+ T cells in 3M-052 versus vehicle-treated tumors (Fig. 3C) and spleens (Fig. 3D). Depletion of CD4+ or CD8+ T cells in C57BL/6 mice bearing B16.Ova tumors revealed that tumor killing was partially dependent on CD8+ but not CD4+ T cells (data not shown), whereas B cell KO mice revealed a contribution of B cells as well (Fig. 3F). We also confirmed that CD8+ T cells were required for therapeutic efficacy against wild-type B16.F10 tumors (Supplemental Fig. 1A). The CD8+ T cell effector molecules, IFN-γ, contributed to the therapeutic efficacy in both B16.Ova and B16.F10 tumor models (Fig. 3G and data not shown) whereas perforin did not contribute (Fig. 3H). Thus, although adaptive immunity contributed to the antitumor activity of i.t. 3M-052, additional mechanisms also mediated tumor suppression.
Type I IFN, pDCs, and NK cells have been shown to play an important role in TLR9 and TLR7/8 agonist–mediated tumor suppression (8, 24), and thus we evaluated their importance in 3M-052–mediated B16.F10 and B16.Ova tumor suppression. NK cells, although suppressing tumor growth even without treatment, were not required for 3M-052–mediated antitumor activity (Fig. 4A, 4B). To establish the contribution of pDCs and type I IFNs, we treated conditionally pDC-deficient (Bdca-2-DTR) mice and type I IFN receptor (IFNAR) KO mice. Similar to results observed in IFN-γ KO mice, growth of vehicle-treated tumors was accelerated in mice lacking type I IFN or pDCs. However, i.t. 3M-052 still reduced tumor size in these settings (Fig. 4C–E), suggesting that pDCs and type I IFN are not major mediators of the antitumor activity of 3M-052 but are required for efficient tumor suppression.

Tumor suppression requires CCL2 and tumor-associated macrophages

Suppression of B16.F10 and B16.Ova melanoma in Rag2 KO mice in response to 3M-052 indicates the importance of innate immune cells. The relative abundance of TAMs 1 d after i.t. 3M-052......
pointed to a possible role in the antitumor activity of i.t. 3M-052. Indeed, 3 d later we found more TAMs (CD11b+ F4/80+) in tumor and TDLNs (Fig. 5A). TAMs can play a dual role in tumor development; that is, M1 TAMs can suppress and M2 TAMs can promote tumor growth (13, 17, 25). Using CD206 (mannose-binding receptor), a definitive marker for M2 macrophages (26), we found that i.t. 3M-052 induced the accumulation of M1 over M2 macrophages in tumor and TDLNs (Fig. 5B, 5C). Thus, 3M-052 treatment increases the M1/M2 TAM ratio in tumor, possibly implicating them in its mechanism of tumor control.

Macrophage-related chemokines such as CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, and M-CSF were also highly upregulated in the tumor after 1 d of treatment (Fig. 5D), with CCL2 being the most dramatically increased. Because we had found strong induction of the macrophage chemotactic chemokine CCL2 in 3M-052–injected tumors, we studied the relationship between CCL2 and TAM accumulation by systemically neutralizing CCL2 with CCL2-specific Ab. Mice bearing B16.F10 or B16.Ova tumors were treated with 3M-052 or vehicle with or without repeated anti-CCL2 treatments. Remarkably, CCL2 neutralization completely abolished the therapeutic effect of 3M-052 against B16.F10 or B16.Ova tumors (Fig. 5E, Supplemental Fig. 1B) with reduced infiltration of TAMs (data not shown). These data indicate that the antitumor activity of 3M-052 against B16.F10 was dependent on CCL2. To further confirm that TAMs are responsible for tumor suppression, we depleted >80% of TAMs in tumor-bearing mice by clodronate
liposome and found that the antitumor effect of 3M-052 was lost (Fig. 5F). However, clodronate liposomes also appeared to deplete small fractions of other leukocytes (data not shown). To confirm the direct importance of TAMs for the antitumor effect of 3M-052, we depleted the macrophage precursor subset (27), CD11b^+Ly6Chi monocytes, which were enriched in tumor and TDLNs after 3M-052 treatment (data not shown). Systemic administration of anti-Ly6C Ab completely abrogated therapeutic efficacy of 3M-052 (Fig. 5G) and greatly reduced the number of TAMs but not T cells (Fig. 5H).

3M-052–activated macrophages induce direct tumor killing via NO

We next analyzed whether macrophages could directly kill tumor cells in response to 3M-052 treatment. Because a major mechanism of tumor killing by macrophages is through production of large quantities of NO (28), we determined the involvement of NO in 3M-052–mediated tumor killing. B16.F10 tumor cells were cocultured with 3M-052–activated splenic macrophages in the presence or absence of L-NAME (an NO synthase inhibitor). 3M-052 increased macrophage cytotoxicity against tumor cells, and this killing was completely abolished by addition of L-NAME (Fig. 5I), indicating that NO from 3M-052–activated macrophages was a major mediator of direct tumor killing. Indeed, we found that culture supernatants from 3M-052–activated macrophages killed B16.F10 tumor cells (Fig. 5J).

3M-052 enhances antitumor activity of checkpoint blockade

Anti–CTLA-4- and anti–PD-L1-blocking Abs have shown impressive T cell–mediated clinical efficacy against human melanoma, but a sizeable fraction of patients does not respond to these therapies (29, 30). It is possible that induction of tumor-specific CD8^+ T cells, for example by i.t. TLR ligation, could provide a better T cell substrate for checkpoint blockade to act on. To test this hypothesis, we combined 3M-052 therapy with checkpoint blockade in a setting of established, rapidly progressing wild-type B16.F10 melanoma.
where checkpoint blockade therapy is ineffective. Interestingly, addition of 3M-052 therapy converted therapeutic failure to PD-L1 or CTLA-4 blockade into tumor suppression that was stronger than suppression observed after 3M-052 monotherapy (Fig. 6A, 6C), resulting in prolonged survival (Fig. 6B, 6D). Although 90% of tumors that were treated with anti–CTLA-4 plus 3M-052 or anti–PD-L1 plus 3M-052 never reached a size of 200 mm² during the observation period, they developed dry ulceration, requiring euthanasia. Next, we determined whether combination therapy is also superior for contralateral tumor growth suppression.
Anti–CTLA-4 plus 3M-052 or anti–PD-L1 plus 3M-052 were more effective than anti–CTLA-4 or anti–PD-L1 alone in inhibiting the growth of the injected tumor. Triple combination of 3M-052, anti–CTLA-4, and anti–PD-L1 showed superior activity against injected (data not shown) and distant, uninjected tumors (Supplemental Fig. 2A), and antitumor activity required CD8+ T cells (Supplemental Fig. 2B). To evaluate systemic immunity, B16.Ova tumors were treated with 3M-052 alone or with combination therapy and PBMCs were analyzed for the presence of tumor Ag-specific CD8+ T cells. Indeed, mice treated with 3M-052 plus anti–CTLA-4/anti–PD-L1 had more circulating Ova-specific IFN-γ+CD8+ T cells than did mice treated with either agent alone (Fig. 6E). Taken together, these data suggest that 3M-052 synergizes with checkpoint blockades in the T cell–mediated, systemic suppression of established B16.F10 melanoma.

**Discussion**

To our knowledge, this is the first report on the antitumor activity and mechanism of action of 3M-052, a new dual TLR7/8 agonist designed to overcome the limitation of imiquimod (TLR7) and resiquimod (TLR7/8) agonists by forming a depot at the site of injection and preventing systemic immune activation and toxicity (18). We and others have previously shown that i.t. administration of the TLR9 agonist CpG oligonucleotide is more effective than systemic administration owing to the induction of local innate immune activation, resulting in systemic adaptive antitumor immunity (2, 31). In the present study, we found that i.t. 3M-052 exerts antitumor activity against the aggressive wild-type B16.F10 melanoma through a dual mechanism of local activation of innate immunity, primarily through TAMs, as well as systemic, adaptive immunity. We also found systemic suppression of uninjected, distant B16.F10 as well as B16.OVA tumors; however, this effect

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**B16.F10**

**FIGURE 6.** Synergic effect of 3M-052 plus checkpoint blockade on B16.F10 tumor growth. Mice bearing s.c. B16.F10 tumors were treated on days 0 and 4 with 35 µg 3M-052 or vehicle i.t. and received 200 µg anti–CTLA-4 (A and B) and anti–PD-L1 (C and D) i.p. for every 4 d. Graphs depict tumor growth (A and C) and mouse survival (B and D). Tumor growth is plotted as means ± SEM with n = 5 in each group. (E) After 21 d of indicated treatment, PBMCs were isolated and cultured with OVA peptide for 4 h, surface and intracellular staining for CD8 T cells and IFN-γ were performed, and cells were analyzed by flow cytometry. *p < 0.05 versus anti–CTLA-4 (all anti–PD-L1-treated mice were dead from tumor at this time). Data are representative of at least two independent experiments. *p < 0.05, **p < 0.005, ***p < 0.005 (unpaired two-tailed t test).
was more profound against B16-OVA, which caused tumor rejection, likely due to the presence of the highly immunogenic model Ag, chicken egg OVA. We suspect that 3M-052 will be more potent in activating immune cells from humans than from mice, because in mice TLR8 does not signal in response to imidazoquinolines such as 3M-052. Because this nonfunctional TLR8 (and not the functional TLR7) is expressed on CD8α+ cDCs, the most efficient cross-primers of CD8+ T cells in mice (32), 3M-052 is unable to activate CD8α+ cDCs, and therefore cross-priming is inefficient unless the Ag is highly potent, such as OVA. However, BDCA3+ cDCs, the human counterpart of CD8α+ cDCs, do express TLR8 and respond to imidazoquinolines. We therefore speculate that 3M-052 will be more efficient at inducing cross-priming of tumor-specific CD8+ T cells in humans than in mice. The dual mechanism we report in the present study of direct tumor killing by macrophage and T and B cell–dependent tumor killing after treatment with TLR7 and TLR7/8 agonists may help explain earlier controversies regarding the contributions of innate and adaptive immunity to the antimalanoma activity of resiquimod or imiquimod (8, 9, 33–35). Broomfield et al. (36) injected imiquimod into malignant mesothelioma tumors and showed tumor suppression that included distant, un.injected tumors when anti-CD40 mAb was added to the regimen. These results, together with our data on the use of i.t. TLR7/8 agonist with anti–CTLA-4 and anti–PD-L1 checkpoint blockade, suggest several combination approaches to the treatment of metastatic cancer.

Nesbit et al. (13) found that low concentrations of CCL2 recruit M2 TAMs and promote tumor growth whereas high concentrations of this chemokine mostly attract tumoricidal M1 TAMs. Likewise, we found that B16 tumor produced low levels of CCL2 and contained mostly M2 TAMs, whereas i.t. 3M-052 increased levels of CCL2 and M1 TAMs and induced tumor destruction. 3M-052 also increased numbers of CD11bLy6Ch monocytes, which are known precursors for TAMs; indeed, their depletion strongly reduced TAMs and antitumor activity. To our knowledge, we show for the first time that i.t. TLR7/8 agonist shifts the i.t. M1/M2 macrophage ratio and, importantly, that these macrophages and their chemokine CCL2 are important for tumor control. M2 to M1 polarization of TAMs by 3M-052 holds promise for clinical application, because high M2/M1 TAM ratios are considered as a poor prognostic factor in multiple cancers including cutaneous and uveal melanoma and lung cancer (15, 37–40). In contrast, O’Sullivan et al. (41) reported that even in the absence of adaptive immunity, M1 TAMs can still mediate immunoediting and regression of sarcomas, underlining the importance of TAMs as therapeutic targets. Because M2 TAMs can also mediate resistance to other therapeutic modalities such as chemotherapy, the manipulation of the M1/M2 ratio in tumors with TLR agonists such as 3M-052 may hold promise beyond its direct antitumor activity (14, 42, 43).

It is interesting that 3M-052 converted nonresponsiveness to PD-L1 or CTLA-4 checkpoint blockade into T cell–dependent responsiveness. This suggests that 3M-052 induces tumor-specific T cells, which then benefit from PD-L1 and CTLA-4 blockade (44). It will be interesting to see whether melanoma patients who do not respond to checkpoint blockade alone will benefit from the addition of i.t. 3M-052 therapy.

Several reports show that TAM infiltration and tumor growth can be reduced by CCL2 inhibition (45–48). However, these reports evaluated the role of TAMs in untreated tumors. We found that upon immunotherapy with 3M-052, M1 TAMs increased in the tumor, and these TAMs were critical for tumor control. Similarly, inactivation of CCL2 by TAM-derived free NO radicals severely inhibited the activity of T cell–based immunotherapy due to the dependency of tumoricidal CD8+ T cells on CCL2 to infiltrate the tumors. Therefore, before instituting anti–CCL2 therapy, it is critical to determine whether CCL2 plays a tumor-promoting or tumor-suppressing role, depending on the types of pre-existing or treatment-induced innate and adaptive immunity.

We were surprised to find that pDCs and type I IFN were not indispensable for the antitumor activity of 3M-052, in contrast to previous studies on TLR7/8 agonists (8, 33, 34). This difference may be due to the difference between the widely used cream-based formulation, which would primarily impact the skin, versus our i.t. injected and retained formulation, which mostly impacts the tumor tissue.

We found abundantly more TAMS than pDCs in B16 tumors both before and after therapy with 3M-052, possibly explaining the dominance of macrophages in the antitumor effect. Our results are in accordance with earlier findings where pulmonary infection with Newcastle disease virus (a natural TLR7 agonist) resulted in increased numbers of alveolar macrophages and cDCs but not pDCs. IFN-α production by pDCs was only initiated when alveolar macrophages were depleted, suggesting a possible competition or cross-inhibition between macrophages and pDCs (49).

Clinically, the TLR7 agonist imiquimod is formulated as a cream and applied on the skin where it is partially absorbed. In contrast, 3M-052 is directly injected into the tumor, making dose comparisons difficult. In this study, 0.06 mg 3M-052 was injected, compared with 6.25 mg imiquimod gel topically applied in a previous mouse study (8). On a milligram per kilogram basis, 3M-052 was used at an ~6-fold greater amount than imiquimod as typically used in humans.

Consistent with a previous report on imiquimod (8), NK cells were not involved in 3M-052–mediated tumor killing; however, it has been demonstrated that NK cells are indispensable in CpG-mediated antitumor immunity (24), suggesting that TLR9 and TLR7/8 agonist suppress tumor growth by partially different immune mechanisms. B cells did contribute, raising the possibility of contribution of Abs to the antitumor effect of 3M-052 (50).

We and others previously reported systemic induction of CD8+ T cell immunity after TLR7/9 agonist therapy (2, 31). This is important, because a major goal of i.t. therapy is systemic tumor regression, including distant, un injected lesions. In the present study, we show that systemic CD8+ T cell immunity is also induced after TLR7/8 treatment, and this immunity is strong enough to reject distant, un injected tumors. Tumor-specific CD8+ T cells can have potent antitumor effects, but recent studies indicate that CD8+ T cells and IFN-γ induce a T cell–resistant environment within the tumor (51, 52). It will be interesting to see whether in such settings macrophages or/and other innate immune cells activated by TLR agonists, including 3M-052, may be less affected by these mechanisms and could continue to kill tumor cells.

In summary, 3M-052 not only generated systemic tumor-specific CD8+ T cell immunity but also modified the tumor microenvironment from tumor-promoting to tumor-inhibiting by shifting the phenotype of i.t. macrophages from a predominant M2 to M1 phenotype. M1 macrophages, T cells, and B cells all contributed to suppression of tumor growth, which was further enhanced by combination with PD-L1 or CTLA-4 checkpoint blockade. The effective induction of both innate and adaptive immunity makes 3M-052 useful for the treatment of both poorly and strongly immunogenic tumors. Our findings suggest that local i.t. treatment with immunomodulatory compounds such as 3M-052 is a promising approach for the treatment of metastatic cancer.

Disclosures

J.P.V. is an employee of 3M and is a beneficiary of 3M’s employee stock plan. The remaining authors have no financial conflicts of interest.
Fig.S1. Mechanism of tumor suppression by 3M-052. Tumor growth after 3M-052 treatment in (A) B16.F10 in CD8-depleted or WT mice (B) B16.Ova in CCL2 neutralized mice or WT mice. Tumor growth is plotted as mean ± SEM, n=5 in each group. *P<0.05, **P<0.005 (unpaired two-tailed t test).
Fig. S2. **Synergistic effect of triple therapy and its mechanism of action.** Mice bearing 7-day and 3-day subcutaneous B16.F10 tumors on opposite flanks were treated only in the 7-day tumor on day 0 and 4 with 35ug 3M-052 or vehicle, i.t. and received 100 ug each of anti-CTLA-4 and anti-PD-L1 or IgG isotype control i.p. every 4 days. (A) Growth of uninjected, distant tumor is shown. (B) Survival of mice; with or without CD8 T cells depletion mean ± SEM, n= 4 or 5. *P<0.05,**P<0.005.