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*J Immunol* 2014; 193:4722-4731; Prepublished online 24 September 2014;
doi: 10.4049/jimmunol.1401160

http://www.jimmunol.org/content/193/9/4722
Effective Innate and Adaptive Antimelanoma Immunity through Localized TLR7/8 Activation

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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, uninjected 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: 4722–4731.

The online version of this article contains supplemental material.

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Received for publication May 6, 2014. Accepted for publication August 26, 2014.

This work was supported by National Institutes of Health Grants R01 CA143077 (to W.W.O.) and P01 CA128913 (to P.H. and W.W.O.), as well as by University of Texas MD Anderson Cancer Center Specialized Programs of Research Excellence in Melanoma Grant P50 CA093459 (to P.H.).

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Abbreviations used in this article: BP, branched; MDC, monocyte-derived cell; DC, dendritic cell; i.t., intratumorally; KO, knockout; pDC, plasmacytoid DC; PD-L1, programmed death ligand 1; TAM, tumor-associated macrophage; TDLN, tumor-draining lymph node.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1401160

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 Institute and the University of Texas Health Science Center at Houston Animal Care and Use Committee.
Center Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from the National Cancer Institute. Rag2 knockout (KO), B cell KO (IgH), Prf KO, IFN-γ KO, and Bdc2-DTR mice were from the 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 brafV600E (18) melanoma cell line (20) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, l-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 (NE NP, Super Refined; Croda Europa, East Yorkshire, U.K./ethanol. The formulation was filtered through a poly-ethersulfone 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 x 10^5 B16.F10 or B16.OVA melanoma cells on day −7 in the left flank, and for contralateral tumor experiments 3 x 10^5 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 x 10^5 cells in the left flank on day −9. Tumor size is expressed as the product of perpendicular diameters of tumors measured with calipers. Mice were sacrificed when tumor size reached ≥200 mm^2 in diameter.

### Cell depletion, chemokine neutralization, and checkpoint blockade experiments

Mouse Abs against CCL2 (2H5), CD4 (GK1.5), CD8 (2.43), NK.1.1, Ly6C cells, PD-L1 (10E9G20), CTLA-4 (9H10), and respective isotype controls were purchased from Bio X Cell. Anti–PD-L1 mAb (10F.9G20), CTLA-4 (9H10), and respective isotype controls were purchased from BioLegend and CD4, CD3, NK.1.1, CD49b, CD69 from BD Biosciences. Cells were stained with Abs against CD45, CD19, B220, CD11b, CD11c, CD220, Siglec-H, Ly6C, F4/80, and CD40 from BioLegend; CD11b, CD11c, CD4, CD3, NK.1.1, CD49b, and CD69 from BD Pharmingen. Data were acquired on a LSR II flow cytometer (BD Biosciences). A Cytofix/Cytoperm kit (BD Biosciences) was used to permeabilize cells for intracellular staining. Flow cytometric analysis

Leukocytes were isolated from mechanically disrupted tumors by lysis of red blood cells (RBC) and centrifuged and supernatant was collected. Cytokines/protein. Palpable 7-d tumors (≥20 mm^2) 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. Most animals that were treated with 3M-052 never reached a size of 200 mm^2 during the observation period, they developed dry ulceration (necrosis), requiring euthanasia. Because of animal welfare issues, 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 both B16.F10 and B16.OVA melanoma cells (6

### Flow cytometric analysis

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 non-responsive 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 mm^2) 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. Most animals that were treated with 3M-052 never reached a size of 200 mm^2 during the observation period, they developed dry ulceration (necrosis), requiring euthanasia. Because of 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 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 antitumor activity against established BP melanoma, derived from the Tyr::CreER, Braf^CA, 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^− B220^−) and decreased numbers of lymphoid DCs (CD11c^+CD11b^+ B220^+) 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+ (Fig. 3E) 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 mediate 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

**FIGURE 2.** 3M-052 activates innate immune cells. Seven-day B16.F10 tumors were treated with 35 μg 3M-052 or vehicle i.t. and after 24 h, CD45+ cells in tumor and TDLNs were analyzed for innate immune cells. Activated (CD40+CD86+) myeloid (CD11b+CD11c+B220+) and lymphoid (CD11b+CD11c+B220+) DCs in (A) TDLNs and (B) tumor. Activated (CD40+) B cells in (C) TDLNs and (D) tumor. Activated (CD69+) NK cells in (E) TDLNs and (F) tumor. (G) pDCs in tumor and TDLNs. (H) Total TAMs and (I) activated (CD40+) TAMs in tumor and TDLNs. Data are representative of at least two independent experiments with n = 4 in each group. Error bars are SEM. *p < 0.05, **p < 0.005 (unpaired two-tailed t test).
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.

FIGURE 5. CCL2 and macrophages mediate tumor suppression by 3M-052. B16.F10 tumors were treated with 3M-052. (A) Total macrophages and (B and C) CD206 expression in macrophages (CD11b^+F4/80^+CD206^+) were measured in tumor and TDLNs after 3 d of treatment by flow cytometry. (D) CCL2, CCL3, CCL4, and M-CSF in tumor lysate after 1 d of treatment. (E) B16.F10 tumor growth after 3M-052 treatment and CCL2 neutralization. (F) B16.F10 tumor growth after 3M-052 treatment and macrophage depletion with clodronate liposomes. (G) B16.F10 tumor growth after 3M-052 treatment and Ly6C^+ cells depletion. (H) Ly6C Ab treatment effect on macrophages and T cells in tumor; tumors were analyzed after 1 wk of Ly6C Ab treatment for indicated cell types. (I) 3M-052 or vehicle-activated macrophages and B16.F10 tumor cells were cocultured for 96 h in the presence or absence of L-NAME. (J) B16.F10 tumor cells were cultured in the presence of 3M-052 or vehicle-activated macrophage supernatant for 96 h. An MTT assay was performed to evaluate tumor cell lysis. Tumor growth is plotted as means ± SEM with n = 5 in each group. Data are representative of at least two independent experiments. *p < 0.05, **p < 0.005 (unpaired two-tailed t test).
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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**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 re-
fection, 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 imi-
dazoquinolines 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 ex-
press 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 imiqui-
mod 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 combina-
tion 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 concen-
trations 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 CD11b+Ly6C+ 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 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 in-
creased 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 com-
parisons 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 im-
une 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 TLR9 agonist therapy (2, 31). This is im-
portant, because a major goal of i.t. therapy is systemic tumor re-
gression, 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 set-
tings 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 microenvi-
ronment 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 im-
munogenic tumors. Our findings suggest that local i.t. treatment
with immunomodulatory compounds such as 3M-052 is a prom-
ising 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.