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Increased Numbers of Monocyte-Derived Dendritic Cells during Successful Tumor Immunotherapy with Immune-Activating Agents

Sabine Kuhn,*† Evelyn J. Hyde,* Jianping Yang,* Fenella J. Rich,* Jacquie L. Harper,* Joanna R. Kirman,*† and Franca Ronchese*

Local treatment with selected TLR ligands or bacteria such as bacillus Calmette–Guérin increases antitumor immune responses and delays tumor growth. It is thought that these treatments may act by activating tumor-associated dendritic cells (DCs), thereby supporting the induction of antitumor immune responses. However, common parameters of successful immune activation have not been identified. We used mouse models to compare treatments with different immune-activating agents for the ability to delay tumor growth, improve priming of tumor-specific T cells, and induce early cytokine production and DC activation. Treatment with polyinosinic-polycytidylic acid or a combination of monosodium urate crystals and *Mycobacterium smegmatis* was effective at delaying the growth of s.c. B16 melanomas, orthotopic 4T1 mammary carcinomas, and reducing 4T1 lung metastases. In contrast, LPS, monosodium urate crystals, or *M. smegmatis* alone had no activity. Effective treatments required both NK1.1+ and CD8+ cells, and resulted in increased T cell priming and the infiltration of NK cells and CD8+ T cells in tumors. Unexpectedly, both effective and ineffective treatments increased DC numbers and the expression of costimulatory molecules in the tumor-draining lymph node. However, only effective treatments induced the rapid appearance of a population of monocyte-derived DCs in the tumor-draining lymph node, early release of IL-12p70 and IFN-γ, and low IL-10 in the serum. These results suggest that the activation of existing DC subsets is not sufficient for the induction of antitumor immune responses, whereas early induction of Th1 cytokines and monocyte-derived DCs are features of successful activation of antitumor immunity.


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**Abbreviations used in this article:** ATCC, American Type Culture Collection; BCG, bacillus Calmette–Guérin; DC, dendritic cell; dLN, draining lymph node; DT, diphtheria toxin; LN, lymph node; MSU, monosodium urate crystal; PAMP, pathogen-associated molecular pattern; poly I:C, polyinosinic-polycytidylic acid.

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tumor growth, reduce metastatic load, and activate both CD8+ T cells and NK cells. Interestingly, DC activation in the draining LNs (dLNs) did not correlate with treatment success. Rather, the treatments that delayed tumor growth also induced a rapid release of Th1 cytokines, as well as the accumulation of monocye-derived DCs in the dLNs, suggesting a key role for these mechanisms in effective antitumor immune responses.

Materials and Methods

Mice

All mice were bred at the Malaghan Institute of Medical Research Biological Medical Research Unit and were matched for age and sex within experiments. The following strains were used: C57BL/6J and C571c-DTR (The Jackson Laboratory, Bar Harbor, ME), B6.SJL-Ptpcr and BALB/c (Animal Resources Centre, Australia), and RAG1-/- (Walter and Eliza Hall Institute of Medical Research, Australia). OTI mice expressing a transgenic TCR specific for Kb+OVA257–264 were from Melbourne University (Australia). CD11c-DTR bone marrow chimeras were generated by injecting 10^7 CD11c-DTR bone marrow cells into irradiated (2 × 550 rad) C57BL/6J hosts; mice were rested for ≥8 wk before being used in experiments. All experimental protocols were approved by the Victoria University of Wellington Animal Ethics Committee.

Tumor cell lines, tumor challenge, and assessments

The B16-F1 melanoma (American Type Culture Collection [ATCC]) and B16.OVA cell lines expressing a truncated OVA (22) were maintained in complete IMDM, as described (23). The 4T1 mammary carcinoma (ATCC) was maintained in ATCC-formulated RPMI 1640 medium supplemented with 10% FBS. Extended in vitro passaging was avoided for all cell lines, and expression of MHC and OVA Ag were verified. Mice were injected s.c. with 10^6 or 10^5 B16 melanoma cells into the flank or 10^4 4T1 carcinoma cells into the mammary fat pad. Tumor size and survival were calculated as described in (24).

Flow cytometry

LNs or tumors were digested into single-cell suspensions using DNase I and Liberase TL (Roche), resuspended in FACS buffer (PBS with 10 nM EDTA, 2% FBS, and 0.01% NaN3), and incubated with anti-mouse CD16/32 (2.4G2) before staining with the following Abs: CD45 (30-F11), CD11c (HL3), CD11b (M1/70), CD8 (53-67), CD86 (GL1), CD40 (3/23), CD3e (2C11), Vn2 (B20.1), NK1.1 (PK136), B220 (RA36B2) (all from BD Biosciences), CD64 (X54-5/7.1), Ly6G (1A8), Ly6C (HK1.4) (Bio-Legend), Ly6B.2 (7/4) (Serotec), and MHCII (3JP) (prepared in-house). Streptavidin-PE or PE–Texas Red (BD Biosciences) were used where required. Cell viability was assessed using DAPI or Live/Dead Fixable blue (Invitrogen). For intracellular cytokine staining, cell suspensions were incubated for 6 h in Golgi Stop with no restimulation and stained with anti-IFN-γ (XMG1.2) and anti–TNF-α (MP6-XT22) Abs or the respective isotype controls (R3-34 or EBRG1) using the BD Cytofix/Cytoperm kit (all from BD Biosciences). At least 20,000 live, CD45+ events were collected for analysis of tumor infiltrates, and 10,000 live, CD11c+ MHCII+ events were collected for analysis of LN DCs. Events were acquired on a BD LSR II SORP (Becton Dickinson) and analyzed using FlowJo software version 9.3.1 (TreeStar). Cell sorting was performed on a BD FACSVantage DiVa (Becton Dickinson).

Analysis of cell morphology

Cell populations were sorted by FACS to >95% purity, spun onto microscopy slides (500rpm, 6min), and stained with Diff-Quick (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). Images were taken on an Olympus BX51 microscope using a 40× objective.

In vivo T cell–proliferation assay

CD8+ T cells were positively selected from spleens and LNs of OTI mice or OTI × B6.SJL-Ptpcr mice using anti-CD8 MicroBeads (Miltenyi Biotec) and labeled with CFSE, as described (26). A total of 1–2 × 10^6 cells was injected i.v. into C57BL/6J or CD11c-DTR BM chimera hosts bearing established B16.OVA tumors. OTI proliferation was assessed in tumor-draining LNs 3–5 d later.

Transfer of activated T cells

OTI T cells were activated in vitro with culture with SIINFEKL–loaded bone marrow DCs, as described (26). Purity and CD62LlowCD44high status were verified by flow cytometry, and 1 × 10^6 CTLs were transferred i.v. into B16.OVA-bearing recipients.

Depletion of cell populations in vivo

Depletion of CD8+ or NK1.1+ cells was by i.p. injection of 250 μg purified 2.43 (anti-CD8) or PK136 (anti-NK1.1) mAbs, respectively, on days −2 and 0 relative to tumor injection. Cell depletion in blood was consistently >90% throughout the experiment. For DC depletion, CD11c-DTR bone marrow chimeras were injected i.v. with 350 μg diphtheria toxin (DT; Sigma-Aldrich) on days −1, +2, and +4 relative to T cell transfer. Depletion was confirmed by flow cytometry of tumor-draining LNs.

FIGURE 1. Selected treatments delay tumor growth and reduce metastases. Mice bearing B16-F1 tumors (A) or orthotopic 4T1 tumors (B, C) were given peritumoral injections of the indicated treatments on days 7, 9, 11, and 13. Data in (A) and (B) are pooled from two to four independent experiments with five to seven mice/group; statistical evaluation was by two-way ANOVA with the Bonferroni post hoc test. Statistical evaluation in (C) was by the Kruskal–Wallis test with the Dunn multiple-comparison test. ***p < 0.001.
Analysis of serum cytokines

Peripheral blood was collected at the indicated times after peritumoral treatment and left to clot for 1–2 h. Serum was separated by centrifugation, and cytokines were measured using a Milliplex kit (Millipore) and a Bio-Plex reader (Bio-Rad), according to the manufacturer’s instructions.

Statistics

Statistical analyses were performed using Prism 5.0 software (GraphPad). Means ± SE are shown in all graphs. Where sample size from combined experiments was ≥10, normality was assessed using the D’Agostino–Pearson test. Normally distributed data were compared by one-way ANOVA with the Tukey test. In all other cases, the Kruskal–Wallis test with the Dunn multiple-comparison test was used. Tumor growth data were analyzed by two-way ANOVA with the Bonferroni post hoc test. Survival data were analyzed using the log-rank test with the Bonferroni correction. Differences of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were deemed significant, very significant, and extremely significant, respectively.

Results

Treatment with poly I:C and MSU + M. smegmatis delays primary tumor growth and reduces lung metastases

We compared different immune-activating agents for their ability to delay the growth of established B16-F1 melanomas in vivo. We used a protocol of four peritumoral treatments given on days 7, 9, 11, and 13 after tumor injection. This protocol was selected because it was superior to one to three treatments (data not shown) and to other routes of administration (27).

As shown in Fig. 1A and Supplemental Fig. 1, treatment with poly I:C or MSU + M. smegmatis significantly delayed tumor growth compared with PBS controls. The antitumor effect of poly I:C is in line with its activity in mesothelioma and ovarian cancer models (15, 28); in contrast, the combination of MSU + M. smegmatis has not been investigated before. Treatment with LPS, MSU, or M. smegmatis alone had no significant effect on tumor growth. Combined treatment with poly I:C, together with LPS (Fig. 1A, Supplemental Fig. 1), MSU, or M. smegmatis (data not shown), did not improve the effect seen with poly I:C alone. Notably, MSU was effective when given with live or heat-killed M. smegmatis but not with BCG (Fig. 1A, Supplemental Figs. 1, 2). Similar results were also observed using the more immunogenic E.G7.OVA thymoma model: treatment with poly I:C or MSU + M. smegmatis induced the greatest delay in tumor growth, whereas MSU or M. smegmatis had a weak effect, and LPS was ineffective (data not shown).

The effect of these treatments was also examined in the 4T1 mammary carcinoma model. 4T1 cells were injected orthotopically in the mammary fat pad, and treatments were given at the primary tumor site using the same protocol as for B16-F1. Again, primary tumor growth was delayed by treatment with poly I:C or MSU + M. smegmatis (Fig. 1B) but not by LPS (data not shown). In addition, the metastatic load in the lung was substantially decreased after poly I:C or MSU + M. smegmatis treatment (Fig. 1C). Thus, treatment of established tumors with selected immune-activating agents can delay tumor growth and reduce metastasis.

Delayed tumor growth after local immunotherapy requires both CD8⁺ T cells and NK cells

To investigate the contribution of innate and adaptive immune cells to the observed antitumor activity, we used RAG1⁻/⁻ mice, which lack mature T and B cells. Treatment of B16-F1 tumors with MSU + M. smegmatis was completely ineffective in RAG1⁻/⁻ mice, whereas poly I:C induced a weak response (Fig. 2A), suggesting a complete or partial dependence on adaptive immune cells, respectively.

To further define the immune cell populations involved in the antitumor effect, C57BL/6 mice were depleted of CD8⁺ or NK1.1⁺ cells prior to tumor inoculation and local immunotherapy. Depletion of either population completely abrogated the effect of adjuvant treatment (Fig. 2B), indicating that CD8⁺ T cells and NK1.1⁺ cells were both required for the response to poly I:C and MSU + M. smegmatis.

To assess whether CD8⁺ T cells and NK cells were present in tumors, immune cell infiltrates were examined by flow cytometry 2 d after the fourth treatment. Representative gating is shown in Supplemental Fig. 3. The frequencies of both CD8⁺ T cells and NK cells were increased in tumors treated with poly I:C or MSU + M. smegmatis compared with PBS controls (Fig. 3A). In contrast, treatment with LPS or MSU or M. smegmatis had no significant effect on CD8⁺ T cell or NK cell tumor infiltration.

To indirectly evaluate effector activity in the tumor, intracellular cytokine levels in CD8⁺ and NK cells were also determined. Treatment with MSU + M. smegmatis resulted in the most marked increase in the percentages of IFN-γ- and TNF-α-producing CD8⁺ and NK cells, followed by treatment with poly I:C (Fig. 3B, 3C). Subtle differences in the cytokines produced by each cell population after treatment with MSU + M. smegmatis or poly I:C were also observed; in particular, MSU + M. smegmatis was superior at inducing IFN-γ production by NK cells and TNF-α production by CD8⁺ T cells. However, the functional impact of these differences was not exami-
Local immunotherapy increases the proliferation of tumor-specific CD8$^+$ T cells in the dLN

To assess whether increased infiltration of CD8$^+$ T cells in tumors was due to increased priming in vivo, we used a B16 melanoma expressing intracellular OVA. Tumor-bearing mice received CFSE-labeled OTI T cells on day 8 and one treatment on day 9; proliferation was assessed 3 d later in the tumor-draining LNs and contralateral, non-draining LNs. In all groups, OTI proliferation was restricted to tumor-draining LNs (Fig. 4A). All treatments increased the percentage of divided OTI T cells compared with PBS control, but this increase was statistically significant only in the group treated with MSU + M. smegmatis (Fig. 4A). Treatment with MSU + M. smegmatis or poly I:C also significantly increased the number of divided OTI T cells in dLNs (Fig. 4A). Little or no increase was observed in the other treatment groups.

To establish the identity of the cells presenting tumor Ag in the dLN, we used CD11c-DTR chimeras in which CD11c$^+$ cells, which include DCs and other APC populations, can be depleted by DT treatment. As shown in Fig. 4B, proliferation of tumor-specific CD8$^+$ T cells in the dLN was significantly decreased in mice depleted of CD11c$^+$ cells, whereas strong tumor-specific proliferation was observed in C57BL/6 mice treated with the same dose of DT. Together with the data in Fig. 4A, these results suggest that poly I:C and MSU + M. smegmatis improve CD8$^+$ T cell priming by modulating the function of CD11c$^+$ cells, most likely DCs.

LPS was shown to improve OVA-specific T cell priming in some studies (29), but it was ineffective in the tumor context used in our experiments. To determine whether the lack of LPS activity was due to an inability to support T cell priming, we used a model in which tumor-dependent T cell priming was bypassed by the transfer of in vitro–activated OTI CTLs. As shown in Fig. 4C, local LPS treatment or transfer of 1 million CTLs were unable to significantly delay tumor growth compared with mock-treated controls. In contrast, LPS in conjunction with CTL transfer markedly improved survival, indicating that LPS can potentiate tumor immune if activated T cells are present. Thus, LPS is unable to support T cell priming in a tumor context, but it can increase existing effector function at the tumor site.

Effective local immunotherapies induce accumulation of monocyte-derived DCs in the dLNs

The results in Fig. 4 suggest that local immunotherapy may act by increasing the function of DCs in tumor-draining LNs. Therefore, we examined DC populations in tumor-draining LNs 1–2 d after the first treatment. DCs were identified on the basis of CD11c and MHC class II expression (Fig. 5A) and were divided into B220$^+$ plasmacytoid DCs, CD8$^+$ DCs, and CD11b$^+$ DCs. The CD11b$^{hi}$ DCs also included a subpopulation expressing the monocyte markers Ly6C and Ly6B (30) and were provisionally identified as monocyte-derived DCs. The number of total DCs in dLNs increased markedly in response to poly I:C, MSU + M. smegmatis,
and LPS (Fig. 5B), thus did not correlate with treatment effect. The activation markers CD86 (Fig. 5C) and CD40 (data not shown) were upregulated on the total DC population and on the CD11b+MHCIIhigh DC population after LPS or poly I:C treatment and, likewise, did not correlate with treatment success. Analysis of individual DC subsets showed that the proportion of plasmacytoid DCs in dLN did not change in response to peritumoral treatment, whereas CD11b+ DCs and CD8+ DCs were affected only by LPS (Fig. 5D). However, a significant increase in both the frequency and number of Ly6C+Ly6B+ monocyte-derived DCs was observed exclusively in the groups treated with poly I:C or MSU + M. smegmatis (Fig. 5E). DCs in tumors were also examined at this same time point, but they did not increase in frequency (Fig. 5F) and did not upregulate activation markers (data not shown).

The number of monocyte-derived DCs in tumor-draining LNs was examined again 1 d after the fourth and last treatment. As shown in Fig. 6A, the number of monocyte-derived DCs remained high in mice treated with poly I:C, and it was further augmented in mice that received M. smegmatis with or without MSU. Monocyte-derived DCs from poly I:C- or M. smegmatis-treated mice also appeared activated and expressed significantly higher levels of CD86 and CD40 than did the corresponding DC populations in PBS controls (Fig. 6B). No increases in DC number or expression of activation markers were observed in mice treated with MSU alone or LPS.

Further analysis of monocyte-derived DCs in MSU + M. smegmatis-treated mice (Fig. 6C) showed that these cells expressed high CD11b, Ly6C, and CD64 but not B220, Ly6G, or CD8. Unlike monocytes, they expressed high levels of CD11c and MHC class II. Cytospins of FACS-sorted monocyte-derived DCs revealed a DC-like morphology with prominent dendrites (Fig. 6D). When cultured with T cells in vitro, monocyte-derived DCs demonstrated a strong T cell stimulatory ability that was comparable to conventional DCs (data not shown). Together, these results suggest that effective local immunotherapies correlate with a rapid and sustained increase in the number of monocyte-derived DCs in dLNs.

Effective local immunotherapies induce IFN-γ and IL-12p70 release
To examine in more detail the factors involved in the early response to local immunotherapies, cytokine levels in serum were examined 1 and 3 h after the first treatment (Fig. 7). Only effective treatments induced significant release of the type 1 cytokines IFN-γ and IL-12p70 in serum. A variable increase in the levels of GM-CSF and IL-1b was also observed, especially in the poly I:C and MSU + M. smegmatis treatment groups, but this only reached significance for IL-1b after MSU + M. smegmatis treatment. Treatment with poly I:C and MSU + M. smegmatis also resulted in a moderate increase in the proinflammatory cytokines IL-6 and TNF-α (data not shown), with low levels of IL-10. In contrast, ineffective treatments either induced no detectable serum cytokines (MSU, M. smegmatis alone) or high levels of IL-6 together with high IL-10 and low IL-12p70 (LPS). Cytokine mRNA was also examined in the tumor tissue using real-time PCR. These results (data not shown) confirmed that...
LPS treatment was associated with high IL-6 and IL-10 expression in tumors, whereas treatment with poly I:C and MSU + M. smegmatis induced only moderate mRNA expression for these cytokines.

Discussion

In this study, we compared the ability of different treatments to activate tumor-associated DCs, induce adaptive antitumor immune responses, and retard tumor progression. We found that only two treatments, poly I:C and MSU + M. smegmatis, enhanced antitumor immune responses and delayed tumor growth. Interestingly, treatment success did not correlate with upregulation of maturation markers on “steady-state” DCs or inflammatory cytokine production. Rather, antitumor activity was associated with a rapid increase in the numbers of monocyte-derived DCs in the tumor-draining LN, suggesting that the presence of this DC population was an important determinant of successful antitumor immune responses. This observation is consistent with a recent report from our group (31) describing a similar DC population in the dLN of E.G7-OVA tumors successfully treated with M. smegmatis.

Our finding that T cells specific for the model tumor Ag OVA failed to proliferate in mice depleted of CD11c+ cells is consistent with a critical role for DCs in antitumor immune responses. Yet, relatively little research has examined the effects of immunostimulatory treatments on DCs in tumors and tumor-draining LNs. CpG oligodeoxynucleotides were shown to enhance IL-12 production in tumoral DCs (32), and, together with poly I:C, they can induce the activation of DCs in dLNs (33); however, the impact of this and other treatments on DC activation, migration to dLNs, Ag presentation, and the responding DC subsets has not been determined. In our experiments, DC numbers and activation status in dLNs did not correlate with increased T cell proliferation or the antitumor effect. A detailed examination of DC subsets in tumor-draining LNs revealed that only poly I:C and MSU + M. smegmatis induced increased numbers of a DC population expressing the monocyte marker Ly6B (30), together with Ly6C and CD64 (34), which are used routinely to identify monocyte-derived DCs. The DC-like morphology and ability to induce vigorous T cell proliferation in vitro (data not shown) also identify these cells as monocyte-derived DCs.

Monocytes that are recruited to sites of local inflammation can differentiate into DCs (35). These DCs are capable of cross-priming CTLs in vivo (36) and are critical for the initiation of immune responses against some pathogens (37, 38). The differentiation of monocytes into DCs was thought to be dependent on GM-CSF, because GM-CSF can induce DC differentiation from monocytes in vitro and is elevated during inflammation in vivo (39). However, recent data in GM-CSFR–deficient mice suggest that GM-CSF is dispensable (40), whereas IFN-γ produced by NK cells can drive monocyte differentiation into DCs (41). In our study, serum GM-CSF levels were variable, and elevated serum IFN-γ and production of IFN-γ by NK cells correlated well with increased numbers of monocyte-derived DCs in dLNs and a successful response to immunotherapy. In addition, serum IL-12 levels were elevated in treated mice. Increased intratumoral IL-12 was reported to improve the Ag-presenting function of all populations of myeloid cells in tumors, including DCs, macrophages, and myeloid-derived suppressor cells (42).

DC frequency in tumors was not increased after one treatment with immune-activating agents. In contrast, after four treatments, the frequency of intratumoral Ly6C+Ly6B+ DCs was increased in all groups with the exception of the MSU-treated one (data not shown). Thus, intratumoral monocyte-derived DCs appear neces-

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**FIGURE 6.** Characterization of monocyte-derived DCs after repeat treatments. Monocyte-derived DCs in tumor-draining LNs were analyzed 1 d after the fourth and last treatment. (A) Total number of monocyte-derived DCs, gated as in Fig. 5A. (B) Expression of CD86 and CD40 on monocyte-derived DCs as median fluorescence intensity (MFI). Data are pooled from two independent experiments with five mice/group and were analyzed by ANOVA with the Tukey test. (C) Monocytes and monocyte-derived DCs from MSU + M. smegmatis–treated mice were identified as shown (upper left panel) and examined for a number of markers. (D) Morphology of FACS-sorted cell populations. Scale bar, 10 μm. Data are from one of two independent experiments that gave undistinguishable results. *p < 0.05, **p < 0.01, ***p < 0.001.
In addition, the different routes of LPS administration, as well as the effects of the tumor, could also explain the differential effects of LPS in the two studies.

In this study, successful immunotherapies resulted in activation of both CD8+ T cells and NK cells, thus may have broad efficacy against tumors that are variably sensitive to CD8+ T cell– or NK cell–mediated killing. Strikingly, depletion of either NK cells or CD8+ T cells completely abrogated the antitumor activity of poly I:C or MSU + M. smegmatis immunotherapy in C57BL/6 mice. This suggests that CD8+ T cells and NK cells are not merely cooperating at the effector phase but are both required to generate a response. In contrast to the complete lack of activity in CD8+ T cell–depleted animals, poly I:C induced a weak antitumor response in RAG1−/− mice. The reasons for this discrepancy were not directly investigated; however, they may be due to the suppressive function of regulatory T cells, which are not found in RAG1−/− animals but can suppress innate antitumor immune responses, including those mediated by NK cells, in C57BL/6 mice (45). NK cells are known to enhance T cell priming by stimulating DC activation and secretion of IL-12 and IFN-γ; thus, polarizing the response toward a Th1 phenotype (46, 47). Activated DCs, in turn, can recruit NK cells to dLNs (48) and enhance their cytotoxic activity and IFN-γ production (49). NK cell–derived IFN-γ may also contribute to DC and macrophage activation and increase the susceptibility of tumor cells to CTL killing via upregulation of MHC class I expression (50). A similar cooperation between CD8+ T cells and NK cells was reported in other models of local immunotherapy with immune-activating agents (14, 18, 51).

To our knowledge, this is the first report that local administration of MSU + M. smegmatis is an effective form of tumor immunotherapy. M. smegmatis was proposed as a useful low-risk alternative to pathogen-derived BCG (52). In addition, we report that M. smegmatis activity was not affected by heat killing, making it suitable for use in immunocompromised patients. In combination with MSU, M. smegmatis rapidly induced monocyte-derived DCs in the dLN. BCG, in contrast, was shown to activate DCs weakly and with delayed kinetics (53), possibly accounting for its lack of antitumor activity when used with MSU. It remains to be determined why MSU and M. smegmatis were both required for the antitumor effect. On its own, MSU was shown to promote rejection of immunogenic tumors (21). MSU can also activate the NLRP3 inflammasome, but it requires TLR ligands, such as those provided by mycobacteria, to induce pro–IL-1β production. Although signal integration from MSU and M. smegmatis for immune cell activation is an attractive possibility, additional explanations are also possible (e.g., improved stability of the combined adjuvant formulation or improved uptake by immune cells). Further studies are necessary to explain the cooperative effect between MSU and M. smegmatis.

In conclusion, comparing responses to a number of immune-activating agents enabled us to identify common features of successful antitumor immunity, namely, the rapid release of Th1 cytokines and the early induction of monocyte-derived DCs. The identification of these common features of effective antitumor immune responses will enable the design of treatments that selectively activate these mechanisms for successful cancer immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

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