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ORIGINAL RESEARCH

The role of NK cells and CD39 in the immunological control of tumor metastases

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ABSTRACT

Tumor metastases are responsible for death in the majority of cancer patients. Here we have explored the role of the ectonucleotidase CD39 in select models of tumor metastases and further tested the therapeutic anticancer activity of the NTPDase inhibitor sodium polyoxotungstate (POM-1). CD39 was expressed on tumor-infiltrating regulatory T cells (Treg), myeloid cells and some NK cells, and it was upregulated on these cells within tumors early after inoculation in vivo. NK cell numbers and effector functions were increased in globally CD39-deficient mice and also in WT mice treated with POM-1. Dosing with POM-1 suppressed experimental and spontaneous metastases in four different tumor models and was well tolerated. This anti-metastatic activity was completely abrogated in mice, that were depleted of NK cells, had IFNγ neutralized or were deficient in CD39 expression in bone marrow-derived cells. POM-1 was highly effective in suppressing metastases when used in combination with BRAFi/MEKi or anti-PD-1/anti-CTLA-4 or IL-2. These data highlight the importance of the CD39 pathway in suppressing NK cell-mediated anti-tumor immunity and validate further the development of CD39-based therapies in the clinic.

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Introduction

Immune checkpoint blockade (ICB) (antibody antagonists of CTLA-4 or PD-1 or PD-L1) and adoptive cell therapies (ACT, eg. CAR-T cells) have generated unparalleled and at times durable responses in several cancers and have firmly established immunotherapy as a new pillar of cancer therapy.[1] Despite durable responses, many patients are innately resistant to immune-based therapies or develop acquired resistance, thus indicating the presence of additional immune regulatory or escape mechanisms in these groups.[2] Furthermore, CTLA-4 blockade alone or in combination causes many immune-related adverse events (iRAEs).[3] Thus, there is a need for new immunotherapies that reduce side effects and aid subgroups of cancer patients that fail contemporary ICB therapy where additional immune regulatory or escape mechanisms exist.

Purinergic (P1 and P2) signaling pathways are an important component of peripheral and central immune regulation.[4] Adenosine (via P1) and ATP (via P2) are normally present at very low levels in extracellular fluids,[5] but the cancer microenvironment can lead to the release of high levels of ATP through a variety of mechanisms, including cell destruction.[6] By signaling through P2 purinergic receptors on immune cells, extracellular ATP functions as a Danger-Associated Molecular Pattern (DAMP) to promote both innate and adaptive immune responses.[4,6] During inflammation, however, extracellular ATP undergoes phosphohydrolysis by ectonucleotidases (most prominently CD39 and CD73), culminating in the formation of high levels of adenosine within the tumor microenvironment (TME).[7] Adenosine is an immunosuppressive metabolite that regulates tumor immunity. Hypoxia, high cell turnover, inflammation, and expression of CD39 and CD73 are important factors in adenosine production. CD39 is a cell surface ectoenzyme that converts ATP into AMP, while CD73 converts AMP into adenosine. Thus, CD39 is pivotal within the ectoenzyme cascade that converts ATP into adenosine. We have previously extensively reviewed the impact of adenosine biology on cancers and contributed to the understanding of CD39, CD73 and adenosine receptors in cancer.[7–12] Limited clinical data are currently available for the efficacy of targeting adenosine A2AR receptor or CD73, but trials are in progress and preclinical studies have highlighted that targeting the adenosine pathway may provide therapeutic benefit alone or in combination with PD-1/PD-L1 blockade.[9,10] Targeting CD39 at the apex of the pathway, rather than downstream adenosine alone, would also have the potential to augment ATP-P2-mediated responses[12] and this is the focus of study.

CD39 is known to be highly expressed on tumor-infiltrating immune cells – particularly Treg, effector T cells, and myeloid cells.[12] Increased CD39$^+$ Treg were reported in head and neck squamous cell cancer patients with active disease, causing adenosine-mediated immune suppression which was reversible by A2AR or CD39 blockade.[13] A subset of tumor-infiltrating CD8$^+$

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T cells is marked by high expression of CD39[14] and the frequency of these cells increased with tumor growth, while expression was absent on $CD8^+$T cells isolated from secondary lymphoid tissues of these tumor-bearing mice. In specimens from breast cancer and melanoma patients, $CD39^+CD8^+$ T cells were present within tumors and these cells exhibited an exhausted phenotype with impaired production of IFNγ, TNF, IL-2, and high expression of coinhibitory receptors.[14] Comparable results showing altered levels of expression of CD39 in the stroma, vasculature and infiltrating immune cells in tumors have been found, by some of us in earlier work, examining pancreatic cancer and colorectal malignancy in both clinical and experimental samples.[15,16] Although T-cell receptor engagement appears to induce CD39 on human $CD8^+$ T cells, as does oxidant stress,[17] exposure to IL-6 and IL-27 also promoted CD39 expression on stimulated human and mouse $CD8^+$ T cells. These and other findings show that the TME drives the acquisition of CD39 as an immune regulatory molecule on $CD8^+$ T cells. But CD39 can also be expressed on tumor-associated endothelium, stroma and tumor cells.[7,18] In particular, melanomas are one of the few cancer types that might have a significant proportion of tumor cells expressing CD39, as defined by IHC.[19] Thus, CD39 is able to modulate the TME through actions on tumor, vasculature, stroma and in select cases immune cells, altogether favoring tumor growth and escape.

Early preclinical studies from some of us have shown that CD39-deficient mice are resistant to lung and liver metastases in the B16F10 mouse melanoma model.[20] Deletion of CD39 also impaired tumor angiogenesis and delayed the growth of LLC lung carcinoma and B16F10 melanoma in these mutant mice.[21] Additionally, pharmacological blockade of CD39 activity with the NTPDase inhibitor sodium polyoxotungstate (POM-1) improved antitumor immunity and decreased metastatic spread in the liver in several mouse tumor models.[22] Underscoring its importance, antibody blockade of CD39 also enhanced the survival of NOD mice in patient-derived sarcoma models.[23] Importantly, the role of NK cells and host CD39 in POM-1 activity and POM-1 activation alone and in combination with other promising targets that might regulate metastasis has not been evaluated in depth.

Here using different models of tumor metastases, we explore the mechanism of action of POM-1 and the role of host CD39, and the effectiveness of targeting CD39 using POM-1 compared to, and in combination with, other possible anti-metastatic therapies.

Results

Expression of CD39 in tumor models

To examine the role of host CD39 in tumor control we first examined two experimental metastasis models of mouse melanoma. The first, B16F10, is a melanoma that has been used for numerous studies of NK cell-mediated control of metastasis[24,25] (Figure 1). The second, LWT1, a metastatic variant of the $Braf^{V600E}$ mutant SM1WT1 melanoma also controlled by NK cells[11,26] (Figure 2). In culture, both B16F10 and B16F10-GFP were determined as negative for cell surface CD73 and CD39 expression (Figure 1(a)), while LWT1 expressed CD73, but again was negative for surface CD39 expression (Figure 2(a)). Despite these melanomas being negative for CD39 expression in culture, using the readily identifiable B16F10-GFP melanoma, we were able to show CD39 upregulation on B16F10-GFP cells in the lungs of mice 24 h after intravenous injection (Figure 1(c)). Analysis of the lung mononuclear cells from tumor-bearing B16F10-GFP (Figure 1(d,e)) or LTW1 (Figure 2(b)) mice or naïve mice, demonstrated CD39 expression on the most of the granulocytic myeloid-derived ($Ly\text{-}6G^+CD11b^+$) and Treg ($CD4^+FoxP3^+$) cells, as well as on a proportion of NK cells (Figure 1(d)). CD73 expression was also on a proportion of all T cell subsets (Figures 1(d), 2(b)). Marginal increases in CD39 and CD73 mean fluorescence intensity (MFI) were seen in the NK cell population (Figures 1(d), 2(b)) and an increase in the percentage of the NK cell population expressing CD39 was observed (Figure 1(e)) at this early time point after tumor inoculation.

Role of host CD39 in tumor metastasis

We next examined the role of host CD39 in these two different models of experimental lung metastases (Figures 1(f), 2(c)). Clearly, CD39-deficient mice ($CD39^{KO}$) displayed significantly reduced B16F10 (Figure 1(f)) or LWT1 (Figure 2(c)) lung metastases compared with wild-type (WT) mice. By contrast, the subcutaneous growth of B16F10, SM1WT1 (the original parental line of LWT1), and LTW1 was equivalent between WT and $CD39^{KO}$ mice (Supplementary Figure1(a–c)). Expression of CD39 and CD73 was similar on SM1WT1 compared with LWT1 melanomas (Supplementary Figure1(d)), and in the TILs of SM1WT1 tumors at day 8 (Supplementary Figure 1 (e)). It was obvious that all T cells and myeloid cells examined displayed increased CD39 compared with splenic leukocytes from the same mice (Supplementary Figure1(e)). A small fraction of $CD8^+$ T cells (17–33%) were $CD39^+$ and these $CD8^+CD39^+$ T cells expressed high levels of PD-1 (data not shown), consistent with previous published findings.[14] So, despite an elevated CD39 level on the immune cells in the subcutaneous TME, loss of host CD39 had no major impact on local tumor growth under these experimental conditions.

Host CD39 promotes lung metastases by suppressing NK cells and IFNγ function

We next assessed the mechanism by which lung metastases were reduced in the $CD39^{KO}$ mice. Using both the B16F10 melanoma (Figure 3(a–c)) and LWT1 melanoma (Figure 3(d–f)) models, we demonstrated the critical importance of NK cells and IFNγ, but not T effector cells, in controlling experimental lung metastases. Notably, the $CD39^{KO}$ mice metastases resistance phenotype was lost after NK cell depletion and IFNγ neutralization.

When evaluating the immune infiltrates into the lungs of WT and $CD39^{KO}$ mice 24 h after intravenous B16F10 inoculation, it was clear that an increase in the number $CD45^+$ was detected in the $CD39^{KO}$ strain (Figure 4(a)). In addition, there was a trend towards increased NK cells and IFNγ-producing NK cell numbers in the lungs of $CD39^{KO}$ mice inoculated with either B16F10 or LTW1, when compared with WT mice inoculated with B16F10 or LWT1, respectively (Figure 4(b–e)).

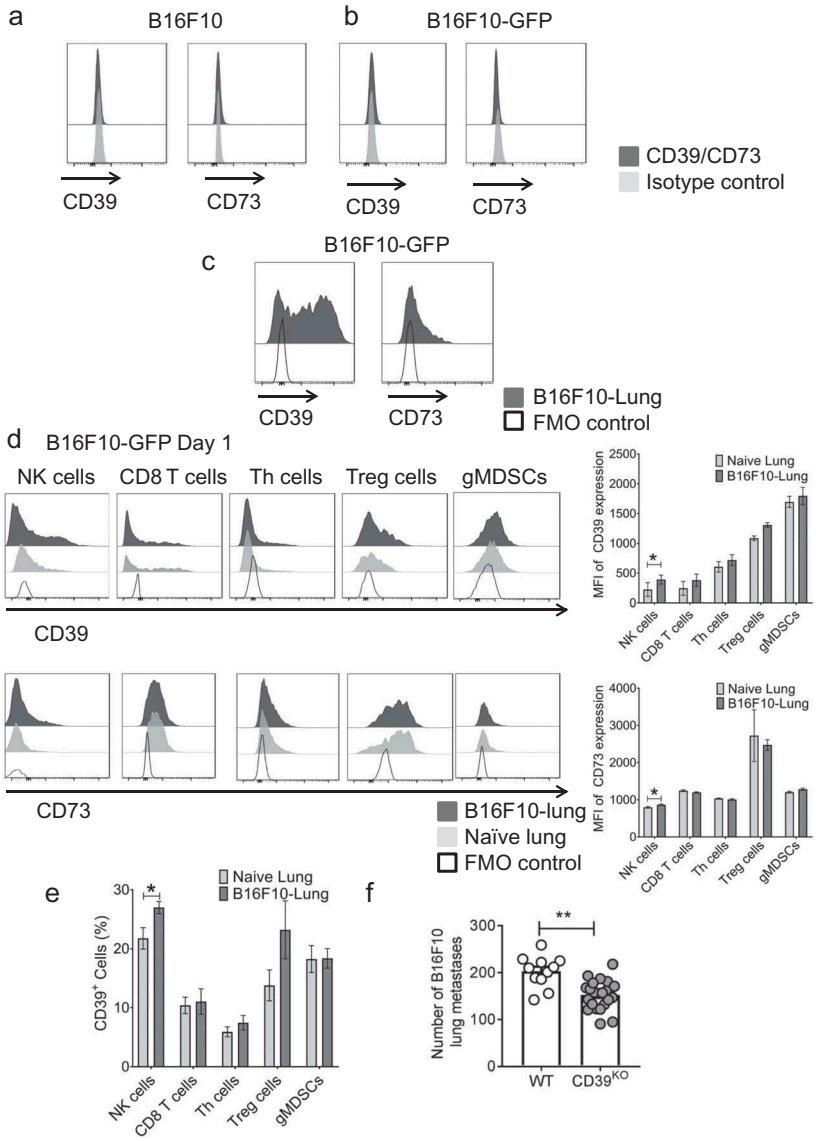

Figure 1. Reduced B16F10 experimental lung metastases in $CD39^{KO}$ mice compared to wildtype mice. (a,b) Flow cytometry histograms showing CD39 or CD73 expression on B16F10 (a) and B16F10-GFP (b) cell lines. (c) WT mice (n = 8 mice/group) were injected i.v. with 2×10^5 B16F10-GFP cells or PBS on day 0. After 24 h, lungs were harvested and single cell suspensions were made for flow cytometric analyses. (c) Representative overlaid CD39 and CD73 histogram plots of B16F10-GFP cells from B16F10-GFP bearing lung and FMO controls are shown. (d) Gating on live $CD45.2^+$ cells of lymphocyte morphology, representative CD39 or CD73 histogram plots of NK cells ($NK1.1^+$ $TCR\beta^-$), $CD8^+$ T cells ($CD8^+TCR\beta+$), Th cells ($CD4^+FoxP3^-TCR\beta^+$), Treg cells ($CD4^+FoxP3^+TCR\beta^+$) and gMDSCs ($NK1.1^-TCR\beta^-Ly\text{-}6G^+CD11b^+$) from B16F10-GFP bearing lung, PBS (naïve lung) and FMO control are shown (left panels). Statistical analysis of CD39 or CD73 MFI staining in NK cells, $CD8^+$ T cells, Th cells, Treg cells, and gMDSCs, mean ± SEM (*, $p < 0.05$) (right panel). (e) Further analysis of B16F10-GFP bearing lung, PBS and FMO control (from the same experiment as Figure 1(d)), percentages of $CD39^+$ cells amongst NK cells, CD8 T cells, Th cells, Treg cells, and gMDSCs are summarized, mean ± SEM (*, $p < 0.05$). (f) Groups of C57BL/6 WT or $CD39^{KO}$ mice (n = 11–23 mice/group, pooled from 3 experiments) were injected i.v. with 2×10^5 B16F10 melanoma cells (n = 11–23 mice/group) on day 0. On day 14, lungs were harvested and the metastatic burden was quantified by counting colonies on the lung surface. Data presented as mean ± SEM. Statistical significance between WT and $CD39^{KO}$ groups was determined by the Mann–Whitney test (**, $p < 0.01$).

POM-1 suppresses experimental and spontaneous metastasis

Given the potential impact of host CD39 on tumor metastasis, we next examined the therapeutic impact of targeting CD39 using POM-1. In the first three experimental metastasis models, POM-1 was administered in a 3-day schedule early after tumor inoculation because it is well recognized that NK cells are naturally most active in the first 1–4 days after intravenous tumor inoculation. Under these experimental conditions, POM-1 significantly suppressed B16F10 (Figure 5(a)) and LWT1 (Figure 5(b)) melanoma and RENCA renal carcinoma (Figure 5(c)) lung metastases. In culture, RENCA did not express CD39 or CD73 (Supplementary Figure 2(a)). To examine the potential therapeutic effect of POM-1 against spontaneous metastases we employed the orthotopic 4T1.2 mammary carcinoma ($CD73^+$ $CD39^{low}$, Supplementary Figure 2(b)) model where neoadjuvant therapy is given prior to surgical resection of the primary tumor.[3] All mice untreated or treated with PBS typically die of distant metastasis to the lung, bones and other organs within 25 to 40 days of surgery (Figure 5(d)). Here again, POM-1 alone displayed a significant minor therapeutic effect with increased

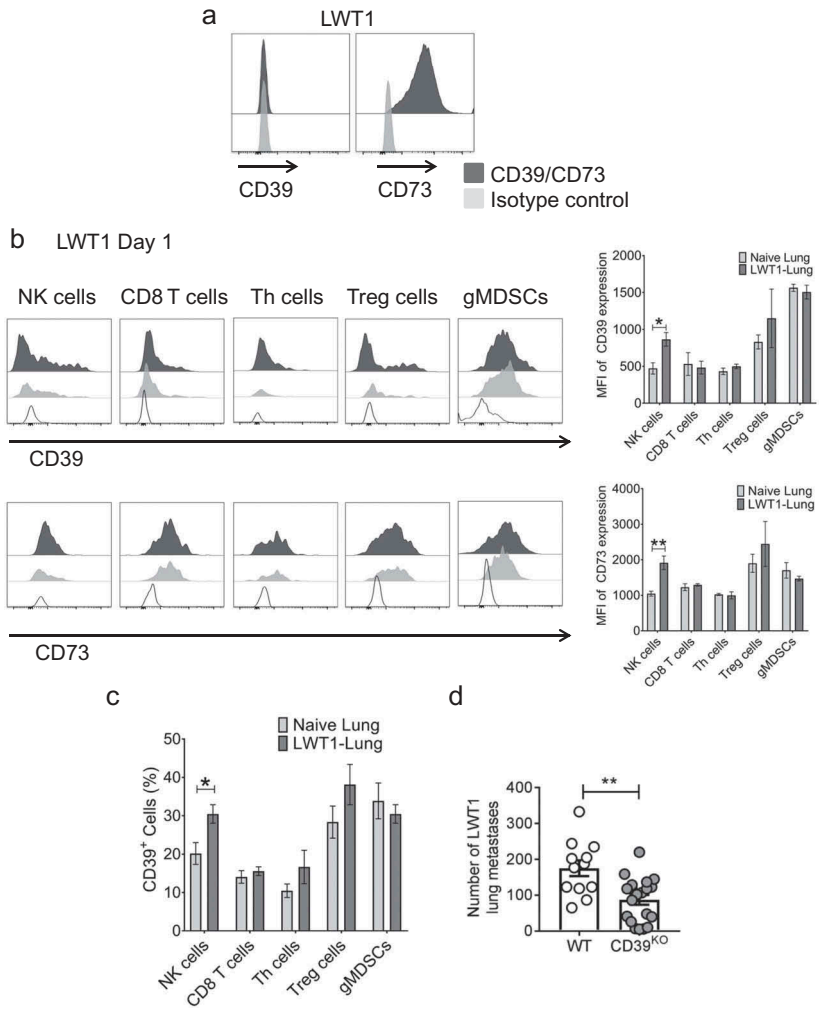

Figure 2. Reduced experimental LWT1 lung metastases in $CD39^{KO}$ mice compared to wildtype mice. (a) Flow cytometry histograms showing CD39 or CD73 expression on the LWT1 cell line. (b) WT mice (n = 8 mice/group) were injected i.v. with 5×10^5 LWT1 cells or PBS (naïve lung) on day 0. After 24 h, lungs were harvested and single cell suspensions were made for flow cytometric analyses. (b) Representative overlaid CD39 and CD73 histogram plots of NK cells ($NK1.1^+$ $TCR\beta^-$), CD8 cells ($CD8^+TCR\beta^+$), Th cells ($CD4^+FoxP3^-TCR\beta^+$), Treg cells ($CD4^+FoxP3^+TCR\beta^+$) and gMDSCs ($NK1.1^-TCR\beta^-Ly\text{-}6G^+CD11b^+$) from LWT1 bearing lung, naïve lung, and FMO control are shown. Statistical analysis of CD39 or CD73 MFI staining also shown mean ± SEM (*, $p < 0.05$, **, $p < 0.01$). (c) Statistical analysis of percentages of $CD39^+$ cells amongst NK cells, CD8 cells, Th cells, Treg cells, and gMDSCs, mean ± SEM (*, $p < 0.05$). (d) Groups of C57BL/6 WT or $CD39^{KO}$ mice (n = 12–20 mice/group, pooled from three experiments) were injected i.v. with 5×10^5 LWT1 melanoma cells on day 0. On day 14, lungs were harvested and the metastatic burden was quantified by counting colonies on the lung surface. Data presented as mean ± SEM. Statistical significance between WT and $CD39^{KO}$ groups was determined by Mann–Whitney test (**, $p < 0.01$).

survival, but no cure of mice (Figure 5(d)). In all the tumor models employed macroscopic observation of the POM-1 treated mice did not reveal any overt pathology, with mice remaining healthy throughout the experiment (data not shown).

Host CD39 is critical for demonstration of therapeutic POM1 activity

It was important to establish the specificity and mechanism of action of POM-1. There was also a trend towards reduced B16F10 and LWT1 lung metastases when employing another NTPDase inhibitor, ARL67156 (Supplementary Figure 3). But more importantly, POM-1 was no longer active against B16F10 and LWT1 in $CD39^{KO}$ mice compared with WT mice (Figure 6(a,b), B). Furthermore, using CD45.1 congenic Ptprca mice, we then created four different cohorts of BM chimeric mice (WT-WT (Ptprca), WT-$CD39^{KO}$, $CD39^{KO}$-WT, $CD39^{KO}$-$CD39^{KO}$), and demonstrated that the effect of POM-1 was largely mediated by CD39 expression on hematopoietic cells (Figure 6(c)). Reconstitution of BM immune cells was equivalent in all chimeras with no obvious defects in immune cell populations (data not shown).

POM-1 anti-metastatic activity requires NK cells and ifny function

We next assessed the mechanism by which B16F10 lung metastases were decreased in POM-1-treated WT mice. Consistent with the $CD39^{KO}$ phenotype, we observed an essential role for NK cells and IFNγ, but not $CD8^+$T cells, in controlling experimental lung metastases (Figure 7(a)). When evaluating the immune infiltrates into the lungs of POM-1-treated WT mice 24 h after intravenous B16F10 or LWT1 melanoma inoculation, it was clear that there was an increase in the number of CD45+ cells (Figure 7(b–e)), number of NK

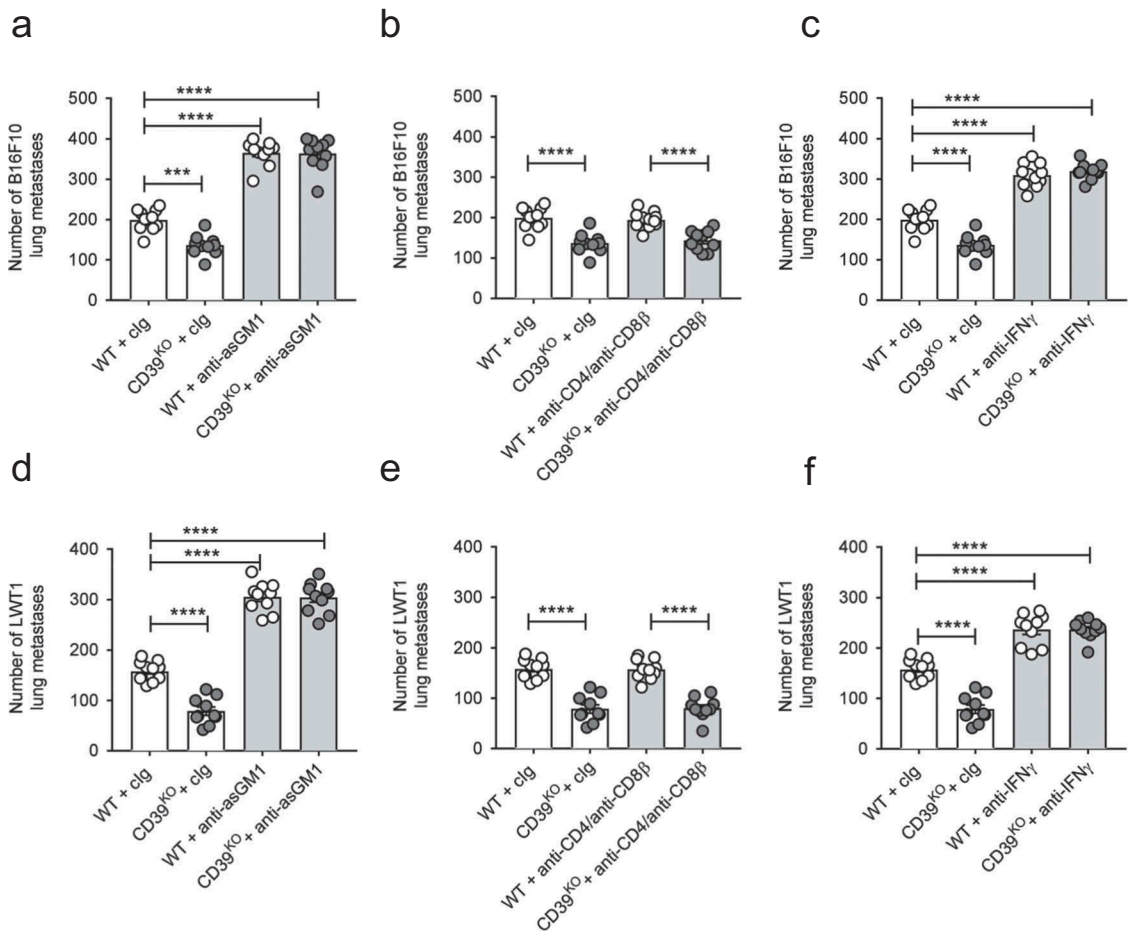

Figure 3. Suppression of lung metastases in CD39KO mice is NK cell- and IFNγ-dependent. Groups of C57BL/6 WT or $CD39^{KO}$ mice (n = 10–11 mice/group) were injected i.v. with (a–c) 2×10^5 B16F10 melanoma cells or (D-F) 5×10^5 LWT1 melanoma cells on day 0. In some groups, mice were either treated i.p. with (a–d) clg or anti-asialoGM1 (asGM1) (50 μg/mouse), (b–e) clg or anti-CD4 and anti-CD8β (each 100 μg/mouse) or (c–f) clg or anti-IFNγ (250 μg/mouse) on days −1, 0 and 7, relative to tumor cell inoculation. On day 14, lungs were harvested and metastatic burden determined by counting tumor nodules on the lung surface. Data presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey multiple comparisons test (***, $p < 0.001$; **** $p < 0.0001$).

cells (Figure 7(c–f)), and number of IFNγ-producing NK cells (Figure 7(d–g)) compared with PBS-treated WT mice.

POM-1 combines with other anti-metastatic therapies

Finally, given the strong anti-metastatic activity of POM-1 in two models of melanoma metastases, and the role of host CD39, we wanted to compare POM-1 activity alone and in combination with other contemporary and approved therapies for melanoma. These include antibody therapies targeting the immune checkpoint molecules PD-1 and CTLA-4, small molecule inhibitors targeting mutated Braf and MEK, and the cytokine IL-2. Combinations of these agents with an inhibitor of CD39 have not previously been reported to any significant extent, and particularly in the context of metastases. Here we used the $Braf^{V600E}$ mutant LWT1 line, so as to be able to additionally examine Brafi/MEKi combination therapy, as previously described.[11] IL-2 was the most effective monotherapy, but POM-1 alone was as active as a combination Brafi/MEKi and was more active than anti-PD1/anti-CTLA-4 (Figure 8(a)). Strikingly, POM-1 had at least additive activity in combinations with these other currently approved immunotherapies. All groups of mice gained weight post treatment (Figure 8(b)). These data warrant the future development of small molecule inhibitors of CD39 or antibodies targeting the ATPDase activity of CD39.

Discussion

There has been much recent focus on the expression of CD39 on tumor-reactive and infiltrating T cells.[27,28] Importantly, CD39 can be expressed by a variety of cells in the TME and immune control can be mediated by a number of lymphoid and myeloid cells depending upon the context. In particular, tumor metastases are often lethal for the patient and control of tumor metastases is often mediated by NK cells.[29] Here we have extended the knowledge about CD39 as a target molecule in approaches to suppress or prevent experimental and spontaneous tumor metastases. CD39 was expressed on regulatory T cells, myeloid cells, and some NK cells in naïve mouse lungs but was upregulated on these same cell types early after melanoma inoculation in vivo. NK cell numbers and effector functions were enhanced in CD39-deficient mice or WT mice treated with the POM-1. POM-1 suppressed experimental and spontaneous metastases in four different tumor models, and its anti-metastatic activity was completely abrogated in mice

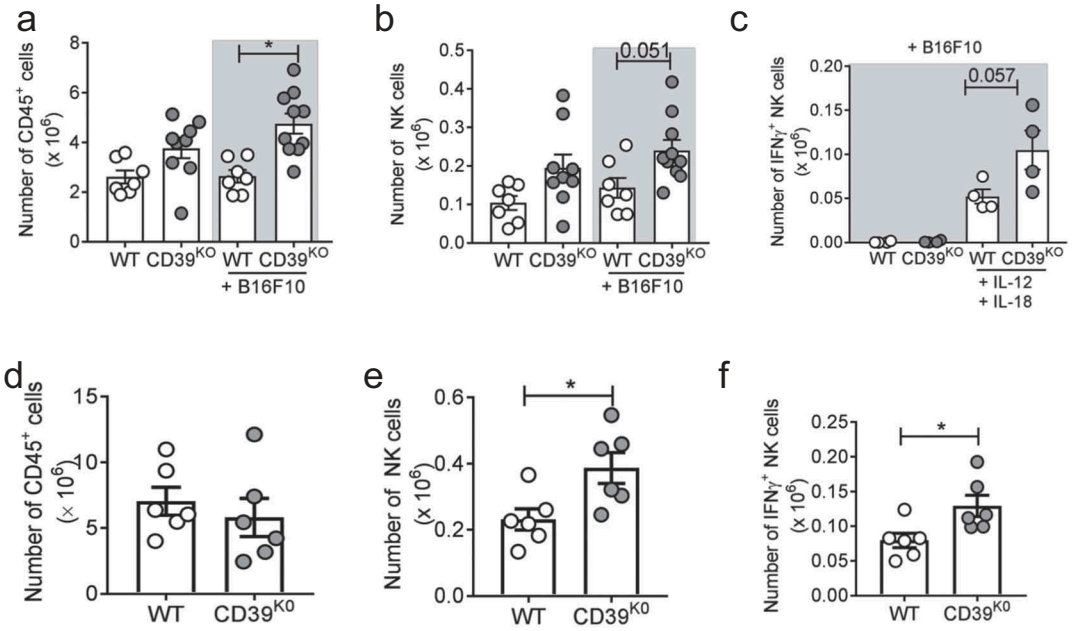

Figure 4. Increased infiltration of $CD45^+$ immune cells and NK cells in the tumor-burdened lungs of $CD39^{KO}$ mice. C57BL/6 WT or $CD39^{KO}$ mice (n = 7–10 mice/group) were injected i.v. with (a–c) 2×10^5 B16F10 or (D-F) 5×10^5 LWT1 melanoma cells on day 0. Twenty-four hours later, lungs were harvested and single cell suspensions were prepared. The absolute numbers of (a–d) total $CD45.2^+$ immune cells and (b–e) NK cells were determined by flow cytometry. Lung suspensions were treated with recombinant mouse IL-12 (1 ng/ml) and mouse IL-18 (50 ng/ml) for 4 h to determine differences in the numbers of (c–f) $IFN\gamma^+$ NK cells. Data presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey multiple comparisons test (*, $p < 0.05$).

depleted of NK cells, neutralized for IFNγ or deficient for CD39 amongst bone marrow cells. By contrast, CD39 expressed by non-hematopoietic cells was not critical for POM-1-mediated anti-metastatic activity.

Importantly, targeting CD39 with POM-1 was effective in suppressing metastases in combination with all the current contemporary targeted and immunotherapies for melanoma, including anti-PD-1 with or without anti-CTLA-4, a combination of inhibitors for Braf and MEK, or IL-2. New inhibitors of CD39 are being generated as are monoclonal antibodies that block the ATPDase function of CD39[18] and these are likely to be significantly superior in half-life to POM-1. Despite the clear limitations of POM-1, and potential for toxicity with longer-term exposure, these data highlight the importance of the CD39 pathway in suppressing NK cell-mediated anti-tumor immunity and the need to translate new CD39 targeting agents into clinical trials.

Melanoma is one cancer where CD39 may be expressed on the tumor cells, and certainly tumor endothelial cells can also express significant levels of CD39.[18] Despite the ability of the mouse melanomas to upregulate CD39 upon injection in vivo, the effect of POM-1 appeared to be more dependent upon the host BM-derived cells expressing CD39 rather than the tumor or the endothelium. Amongst the BM-derived cells expressing CD39, NK cells, Treg and myeloid/granulocyte cell types are all possible candidates for mediating the majority of the immunosuppressive function of CD39. In our previous work, we illustrated in naïve mice that the most terminally differentiated NK cells express CD39, but many NK cells did not express CD39.[30] Further assessment of $CD39^+$ and $CD39^-$ NK cells will be required to determine any distinctions in their function. Like many other immunosuppressive pathways, such as PD-L1,[31] the major site of action of CD39 may be very model and disease context dependent. Our previous studies have highlighted the potential role of CD39 on Treg suppressing NK cells and thus permitting hepatic metastasis.[20] In that model, hepatic metastasis appeared to be promoted by either bone marrow or non-hematopoietic expressed CD39,[20] whereas this current data in lung models of disease suggested that with or without POM-1 treatment, CD39 expressed by bone marrow-derived cells was most critical. Future studies using recently derived CD39 floxed strains of mice[32] will be required to discern the distinct and specific role of CD39 on NK cells, regulatory T cells, myeloid cells and endothelial cells in the setting of tumor metastasis and which of these are most relevant to the anti-metastatic activity of POM-1.

Materials and methods

Mice

Wild-type (WT) C57BL/6 and BALB/c mice were purchased from Walter and Eliza Hall Institute for Medical Research or bred in house. C57BL6 $Ptprc^a$ (CD45.1+) and C57BL/6 (CD45.2+) Cd39-deficient mice ($CD39^{KO}$) were bred in-house and maintained at the QIMR Berghofer Medical Research Institute. $CD45.1^+$ *Ptprc*^{a} WT mice and $CD45.2^+$ $CD39^{KO}$ mice (as recipient mice 10 mice per group) were irradiated twice with a total dose of 1050 cGy as used previously described.[33] Ten million BM cells from $Ptprc^a$ mice or $CD39^{KO}$ mice were then i.v. injected to the irradiated mice to construct BM chimera mice. Neomycin water was given to these mice for three weeks. After confirming the BM

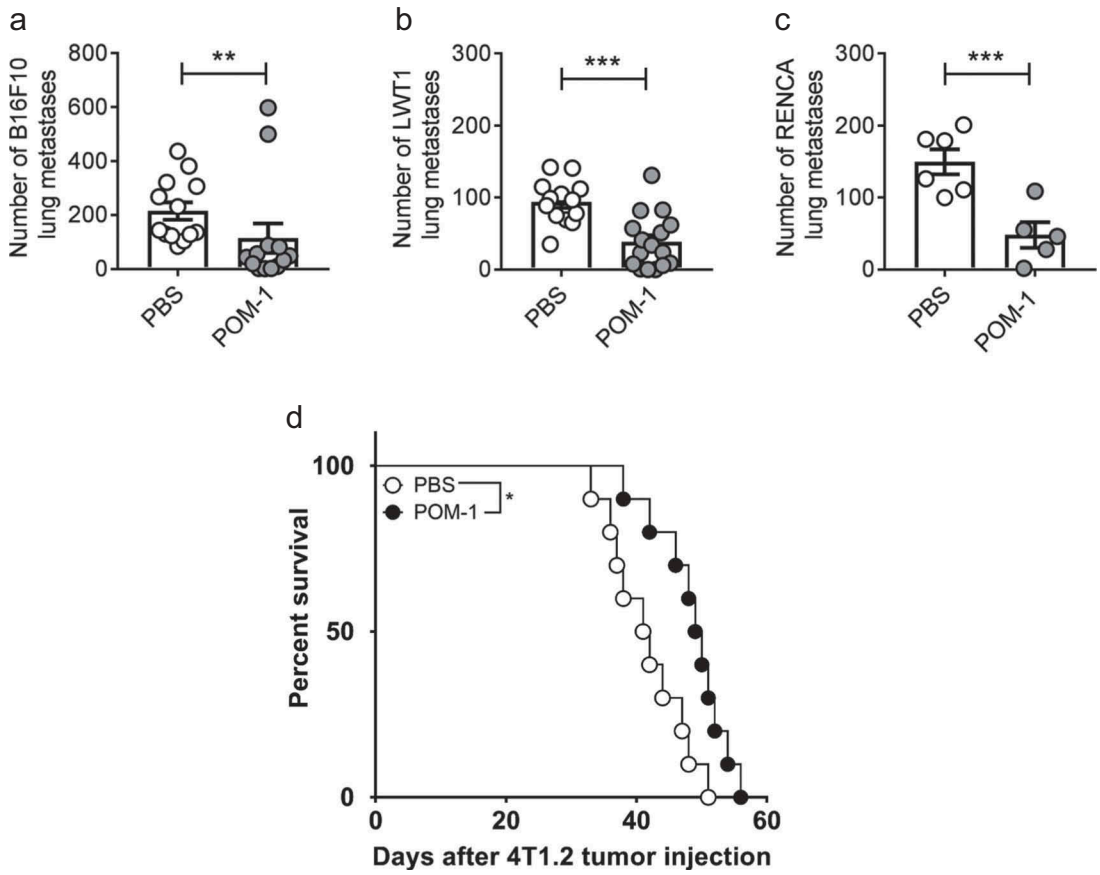

Figure 5. POM-1 decreases experimental and spontaneous metastases in mice. (a) Groups of 5–16 WT mice were injected i.v. with 2×10^5 B16F10 melanoma cells, (b) 5×10^5 LWT1 melanoma cells or (c) 2×10^5 RENCA renal carcinoma cells on day 0. Some mice were either given PBS or POM-1 (250 µg i.p.) on days 0, 1 and 3. On day 14, lungs were harvested and the metastatic burden was quantified by counting colonies on the lung surface. Data presented as mean ± SEM. Statistical significance was determined by Mann–Whitney test or one-way ANOVA with Tukey multiple comparisons test (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). (d) Groups of 9–10 BALB/c mice were injected into the fourth mammary fat pad with 2×10^4 4T1.2 mammary carcinoma cells. Mice were then treated with PBS or POM-1 (250 µg i.p.) on days 8, 9 and 10 and the primary mammary gland tumor was resected on day 12. Mice were then monitored for survival. Survival curves for each group are plotted, and statistical significance between PBS and POM-1-treated groups was calculated using a Mantel Log rank test (*, $p < 0.05$).

reconstruction by flow cytometry of peripheral blood, B16F10 cells were i.v. injected (2×10^5) into the BM chimeric mice. No mice were excluded based on pre-established criteria in all studies, and no active randomization was applied to any experimental group. The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome. Experiments were conducted as approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee.

Cell culture

Mouse B16F10 and B16F10-GFP melanoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (Bovogen), 1% Glutamine (Gibco), 1% HEPES (Gibco) and 1% Penicillin/Streptomycin (Gibco). SM1WT1 melanoma, SM1WT1 LWT1 melanoma, RENCA renal carcinoma, and 4T1.2 mammary carcinoma cells were cultured in RPMI 1640, supplemented with 10% Fetal Calf Serum (Bovogen), 1% Glutamine (Gibco), and 1% Penicillin-Streptomycin (Gibco). All cell lines were maintained at 37°C, 5% CO_2. Cell injection and monitoring procedures were described in previous studies.[24,34,35] All cell lines were routinely tested negative for Mycoplasma, but cell line authentication was not routinely performed.

Experimental and spontaneous tumor metastasis models

B16F10 melanoma (2×10^5), LWT1 melanoma (5×10^5), or RENCA renal carcinoma (2×10^5) cells were injected intravenously into the tail vein of mice. On days 0, 1 and 3 after tumor inoculation, some mice were treated intraperitoneally (i.p.) with PBS or POM-1 (250 µg, Santa Cruz Biotechnology) or ARL 67156 (5 mg/kg, Sigma Aldrich). Depletion of NK cells, $CD4^+$ T cells and/or $CD8^+$T cells or IFN-γ, were done by i.p. treatment on days −1, 0 and 7 with anti-asGM1 (50 µg/mouse), anti-CD4 (GK1.5, 100 µg/mouse), anti-CD8β (53.5.8, 100 µg/mouse) or anti-IFN-γ antibody (H22, 250 µg/mouse). An appropriate isotype control was also used in these experiments. Some groups of mice were treated with additional therapies alone or in combination with POM-1 including anti-PD1 (RMP1-14, 250 µg i.p. days 0 and 3) with or without anti-CTLA-4 (UC104F10, 250 µg i.p. days 0 and 3); Brafi (PLX4720 Plexxicon Inc., 200 µg i.p. on days 0 and 3) and MEKi (GSK1120212, 1.2 µg gavage on days 0 and 3); or IL-2 (100,000 i.p. on days 0, 1, 2, and 3). Lungs were harvested

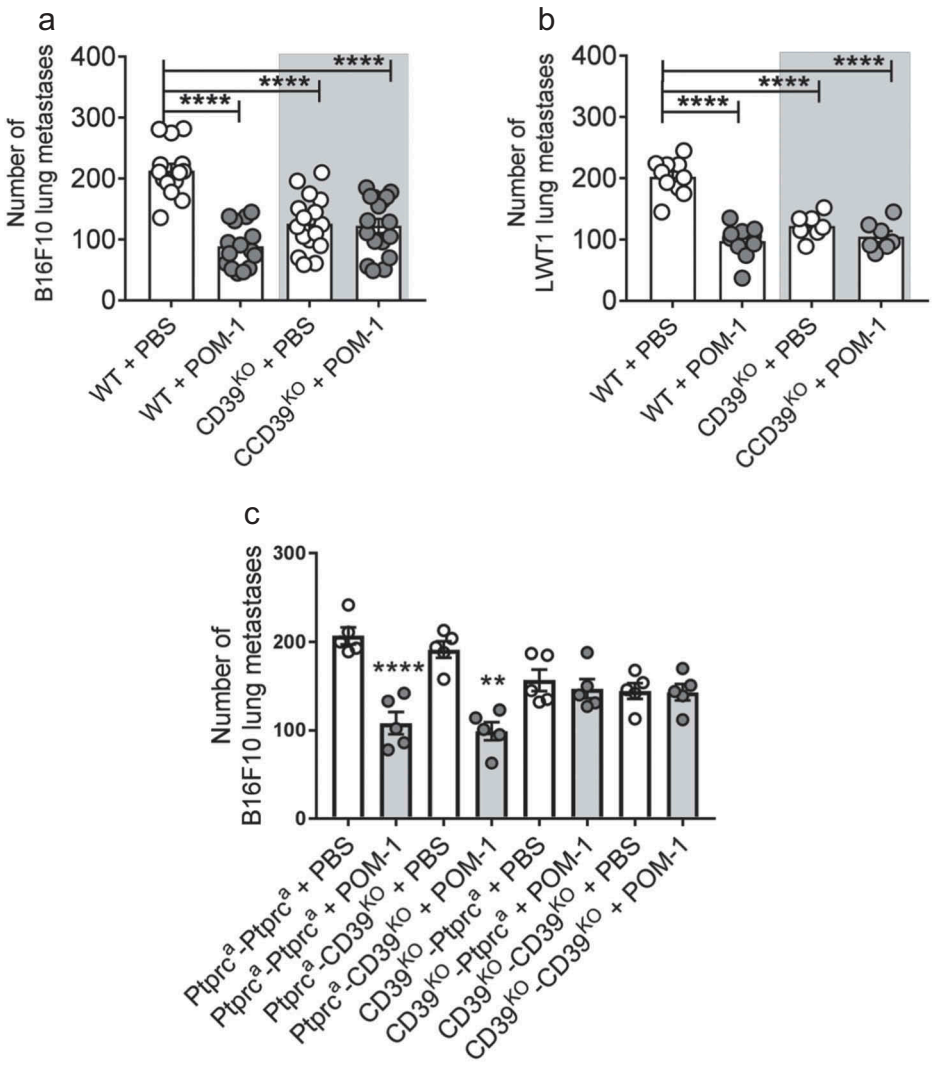

Figure 6. Role of hematopoietic CD39 in the anti-metastatic activity of POM-1. (a,b) Groups of C57BL/6 WT (or congenic $Ptprc^a$), $CD39^{KO}$, or BM chimeric mice (constructed from congenic $Ptprc^a$ and $CD39^{KO}$ mice as described in the Materials and Methods donor-recipient as labeled) (n = 5–10 mice/group) were injected i.v. with (a–c) 2×10^5 B16F10 melanoma cells or (B) 5×10^5 LWT1 melanoma cells on day 0. Some mice were either given PBS or POM-1 (250 µg i.p.) on days 0, 1 and 3. On day 14, lungs were harvested and the metastatic burden was quantified by counting colonies on the lung surface. Data presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey multiple comparisons test (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

on day 14, and metastatic colonies on the surface of the lungs were counted using a dissecting microscope. For spontaneous metastasis and surgery, 2×10^4 4T1.2 mammary carcinoma cells were injected into the fourth mammary fat pad as previously described.[3] Mice were then treated with PBS or POM-1 on days 8, 9 and 10 and the primary mammary gland tumor was resected on day 12. Mice were then monitored for survival as previously described.[3]

Primary tumor growth

For primary tumor growth experiments, B16F10 (1×10^5), SM1WT1 (1×10^6), or LWT1 (1×10^6) cells were s.c. injected into mice in a final volume of 100 µl (day 0). Subcutaneous primary tumor growth was measured using digital calipers, and tumor sizes were recorded.

Flow cytometry

Lungs, tumors, and spleens were harvested from WT and $CD39^{KO}$ mice and treated mice as indicated. Lungs and tumors were minced and digested with 1 mg/mL collagenase IV (Worthington Biochemical) and 0.02 mg/mL DNaseI (Roche) and homogenized to prepare single cell suspensions. Spleens were homogenized and red blood cells (RBCs) lysed in preparation for flow cytometry. For surface staining, single cell suspensions were stained with BUV737 anti-CD45.2 (104; BD biosciences), Brilliant Violet 605 anti-CD4 (RM4-5; Biolegend), BV711 anti-CD8a (53–6.7; Biolegend), PercpCy5.5 anti-TCRβ (H57-597; Biolegend), APC/Cy7 anti-CD11b (M1/70; Biolegend), fluorescein isothiocyanate (FITC) and APC anti-Ly6G (1A8; Biolegend and eBioscience), BUV395 anti-NK1.1 (PK136; BD biosciences), PE-Cy7 anti-CD39 (Duha59; Biolegend), PE anti-CD73 (TY/23; BD Bioscience) and respective isotype antibodies. Zombie Aqua (Biolegend) was used to

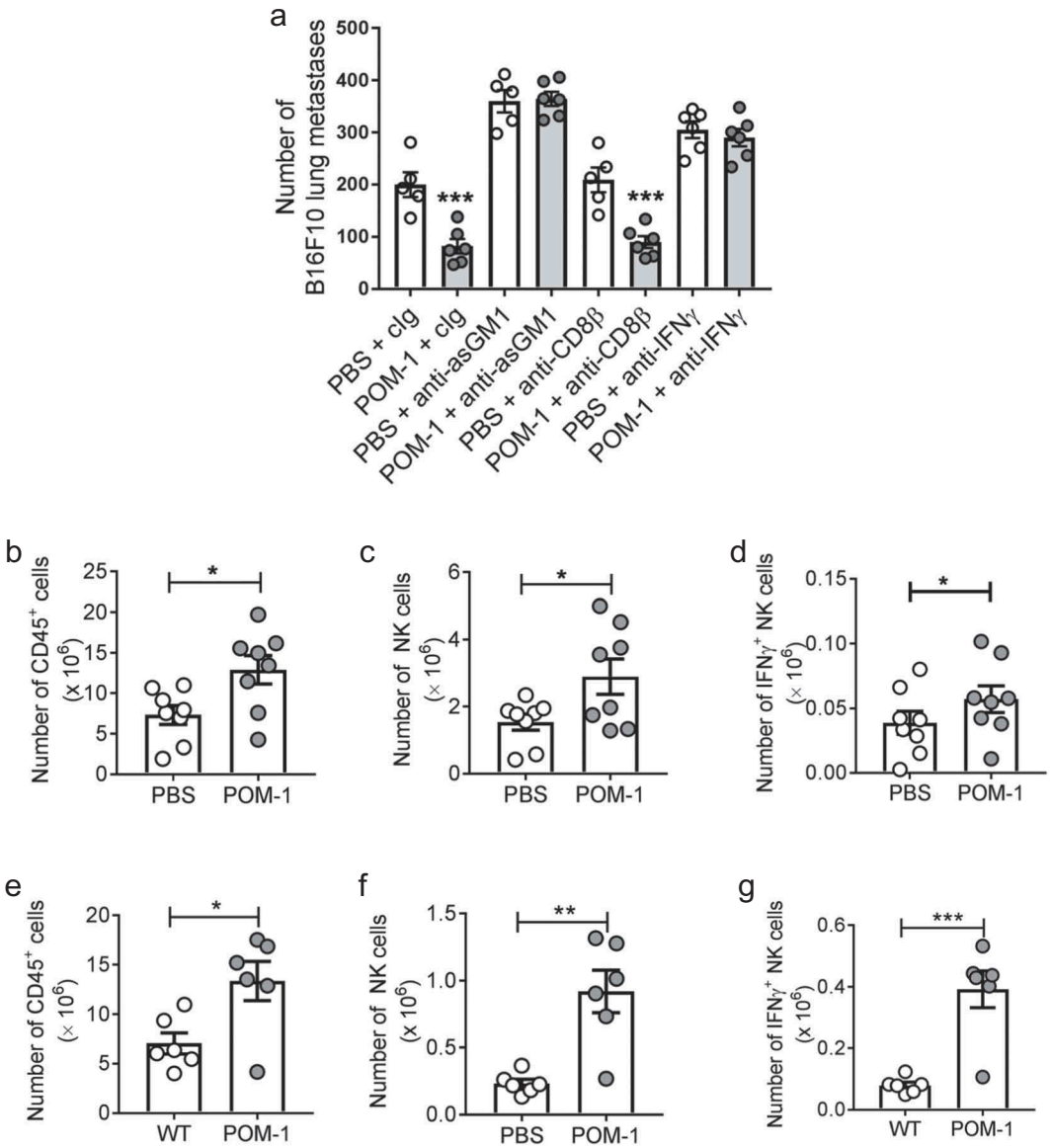

Figure 7. Role of NK cells and IFNγ in anti-metastatic activity of POM-1. (a) Groups of C57BL/6 WT (n = 5–6 mice/group) were injected i.v. with 2×10^5 B16F10 cells on day 0. In some groups, mice were either treated i.p. with clg, anti-asGM1 (50 µg/mouse), anti-CD8β (100 µg/mouse) or anti-IFNγ (250 µg/mouse) on days −1, 0 and 7, relative to tumor cell inoculation. Some mice were either given PBS or POM-1 (250 µg i.p.) on days 0, 1 and 3. On day 14, lungs were harvested and metastatic burden determined by counting tumor nodules on the lung surface. In the clg treatment group, *** means PBS + clg vs. POM-1 + clg. In the anti-CD8β treatment group, *** means PBS + anti-CD8β vs. POM-1 + anti-CD8β. WT mice (n = 8 mice/group) were injected i.v. with (b–d) 2×10^5 B16F10 or (E-G) 5×10^5 LWT1 melanoma cells on day 0. Some mice were either given PBS or POM-1 (250 µg) injected intraperitoneally on day 0 as indicated. The following day, lungs were harvested, and single cell suspensions were made. The absolute numbers of (b–e) total $CD45.2^+$ immune cells and (c–f) NK cells were determined by flow cytometry. Lung suspensions were treated for 3 h with eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) to determine differences in the numbers of (d–g) $IFN\gamma^+$ NK cells. Data presented as mean ± SEM. Statistical significance was determined by Mann–Whitney test or one-way ANOVA with Tukey multiple comparisons test (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$).

exclude dead cells. For intracellular transcription factor staining, surface-stained cells were fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol and stained using eFluor450-anti-Foxp3 (FJK-16s, eBioscience) and respective isotype antibodies. For intracellular staining of IFNγ, cells were stimulated in vitro with recombinant mouse IL-12 (1 ng/ml, Sigma Aldrich) and mouse IL-18 (50 ng/ml, Sigma Aldrich) for 4 h in the presence of GolgiPlug (BD Biosciences) or eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) for 3 h, and then surface stained as aforementioned. Surface stained cells were then fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's protocol and stained with APC anti-mouse IFN-γ (XMG1.2; Biolegend), and respective isotype antibodies. Cells were acquired on the BD LSR Fortessa V (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

Statistical analysis

Statistical analyses were determined using GraphPad Prism software. Data were compared by the Mann–Whitney test or

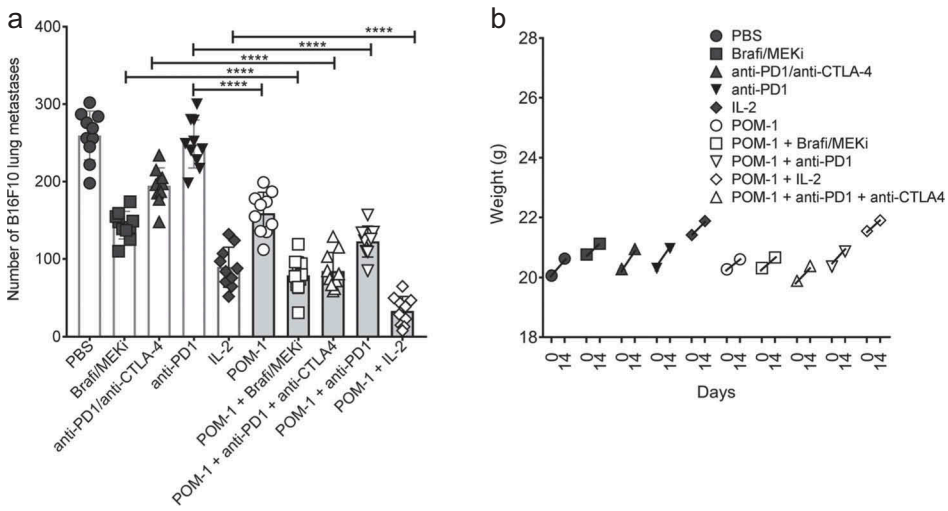

Figure 8. POM1 suppresses metastases in combination with other immunotherapies. Groups of C57BL/6 WT (n = 9–10 mice/group) were injected i.v. with 7.5×10^5 LWT1 cells on day 0. Mice then received one or more of PBS or POM-1 (250 µg i.p. on days 0, 1 and 3); anti-PD-1 with or without anti-CTLA-4 (250 µg i.p. on days 0 and 3); Brafi (PLX4720, 200 µg i.p.) with MEKi (GSK1120212, 1.2 µg gavage on days 0 and 3), or IL-2 (100,000 I.U. i.p. on days 0,1, 2, and 3). On day 14, lungs were harvested and the metastatic burden was quantified by counting colonies on the lung surface. Data presented as mean ± SEM. (B) Weights of mice were measured in the different treatment groups between tumor inoculation (day 0) and harvest (day 14). Statistical significance was determined by one-way ANOVA with Tukey multiple comparisons test (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

one-way ANOVA followed by Tukey post hoc analysis test to compare across groups. *p*-value less than or equal to 0.05 were considered significant.

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Competing financial interests

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