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

Haiyan Zhang*, Dipti Vijayan*, Xian-Yang Li, Simon C. Robson†, Nishamol Geetha, Michele W. L. Teng‡ and Mark J. Smyth*†

*Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, Australia; †Departments of Medicine and Anesthesia, Beth Israel Deaconess Medical Center, Boston, MA, USA; ‡Cancer Immunoregulation and Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Herston, Australia.

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/MElK 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.

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. 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. Furthermore, CTLA-4 blockade alone or in combination causes many immune-related adverse events (irAEs). 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. Adenosine (via P1) and ATP (via P2) are normally present at very low levels in extracellular fluids, but the cancer microenvironment can lead to the release of high levels of ATP through a variety of mechanisms, including cell destruction. 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. 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). 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. Limited clinical data are currently available for the efficacy of targeting adenosine A2AR receptor or CD39, 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. Targeting CD39 at the apex of the pathway, rather than downstream adenosine alone, would also have the potential to augment ATP-P2-mediated responses 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. 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. A subset of tumor-infiltrating CD8+ T cells were reported to be neutralized or were deficient in CD39 expression in bone marrow during inflammation, however, extracellular ATP functions as a Danger-Associated Molecular Pattern (DAMP) to promote both innate and adaptive immune responses. Despite this, but the cancer microenvironment can lead to the release of high levels of ATP through a variety of mechanisms, including cell destruction.

CONTACT: Mark J. Smyth
Mark.Smyth@qimrberghofer.edu.au
Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston 4006, Australia

*These authors contributed equally to this work
© 2019 Taylor & Francis Group, LLC

Supplemental data for this article can be accessed on the publisher’s website.
T cells is marked by high expression of CD39 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. Comparable results showed 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. Although T-cell receptor engagement appears to induce CD39 on human CD8+ T cells, as does oxidant stress, 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. 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. 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. Deletion of CD39 also impaired tumor angiogenesis and delayed the growth of LLC lung carcinoma and B16F10 melanoma in these mutant mice. Additionally, pharmacological blockade of CD39 activity with the NTPDase inhibitor sodium poloxamersulfate (POM-1) improved antitumor immunity and decreased metastatic spread in the liver in several mouse tumor models. Underscoring its importance, antibody blockade of CD39 also enhanced the survival of NOD mice in patient-derived sarcoma models. 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 (Figure 1). The second, LWT1, a metastatic variant of the Braf<sup>WT</sup> mutant SM1WT1 melanoma also controlled by NK cells (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 LWT1 (Figure 2(b)) mice or naïve mice, demonstrated CD39 expression on the most of the granulocytic myeloid-derived (Ly-6G<sup>+</sup>CD11b<sup>+</sup>) and Treg (CD4<sup>+</sup>FoxP3<sup>+</sup>) cells, as well as 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<sup>KO</sup>) 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 LWT1 was equivalent between WT and CD39<sup>KO</sup> mice (Supplementary Figure 1(a–c)). Expression of CD39 and CD73 was similar on SM1WT1 compared with LWT1 melanomas (Supplementary Figure 1(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 Figure 1(e)). A small fraction of CD8+ T cells (17–33%) were CD39+ and these CD8<sup>+</sup>CD39+ T cells expressed high levels of PD-1 (data not shown), consistent with previous published findings. 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<sup>KO</sup> 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<sup>KO</sup> 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<sup>KO</sup> mice 24 h after intravenous B16F10 inoculation, it was clear that an increase in the number CD45<sup>+</sup> was detected in the CD39<sup>KO</sup> 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<sup>KO</sup> mice inoculated with either B16F10 or LWT1, when compared with WT mice inoculated with B16F10 or LWT1, respectively (Figure 4(b–e)).
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+/CD39low, Supplementary Figure 2(b)) model where neoadjuvant therapy is given prior to surgical resection of the primary tumor. 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...
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\(^{-/}\) mice compared with WT mice (Figure 6(a,b), B). Furthermore, using CD45.1 congenic Ptprc\(^{-/}\) mice, we then created four different cohorts of BM chimeric mice (WT-WT (Ptprc)), WT-CD39\(^{-/}\), CD39\(^{-/}\)-WT, CD39\(^{-/}\)-CD39\(^{-/}\)), 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 Ifn\(\gamma\) function

We next assessed the mechanism by which B16F10 lung metastases were decreased in POM-1-treated WT mice. Consistent with the CD39\(^{-/}\) phenotype, we observed an essential role for NK cells and IFN\(\gamma\), 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...
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\textsuperscript{V600E} mutant LWT1 line, so as to be able to additionally examine Braf/MEKi combination therapy, as previously described.\textsuperscript{11} IL-2 was the most effective monotherapy, but POM-1 alone was as active as a combination Braf/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.\textsuperscript{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.\textsuperscript{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.
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 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. 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. 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, 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. In that model, hepatic metastasis appeared to be promoted by either bone marrow or non-hematopoietic expressed CD39, 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 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<sup>−/−</sup> (CD45.1+) and C57BL/6 (CD45.2+) CD39-deficient mice (CD39<sup>KO</sup>) were bred in-house and maintained at the QIMR Berghofer Medical Research Institute. CD45.1<sup>+</sup> Ptprc<sup>−/−</sup> WT mice and CD45.2<sup>+</sup> CD39<sup>KO</sup> mice (as recipient mice 10 mice per group) were irradiated twice with a total dose of 1050 cGy as used previously described. Ten million BM cells from Ptprc<sup>−/−</sup> mice or CD39<sup>KO</sup> 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
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% CO₂. 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...
on day 14, and metastatic colonies on the surface of the lungs were counted using a dissecting microscope. For spontaneous metastasis and surgery, $2 \times 10^4$ 4T1.2 mammary carcinoma cells were injected into the fourth mammary fat pad as previously described. 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.

**Primary tumor growth**

For primary tumor growth experiments, B16F10 ($1 \times 10^5$), SM1WT1 ($1 \times 10^6$), or LWT1 ($1 \times 10^6$) 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$^{\text{KO}}$ 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-TCR$\beta$ (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
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 one-way ANOVA with Tukey multiple comparisons test (*, p < 0.05; **, p < 0.01, ***, p < 0.001).
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.

Acknowledgments

We thank Liam Town, Kate Elder and Brodie Quine for maintaining and genotyping our mouse colonies. We also thank Deborah Barkauskas and Juming Yan for technical assistance.

Competing financial interests

M.J. Smyth has research agreements with Bristol Myers Squibb, Aduro Biotech, and Tizona Therapeutics. S.C. Robson has a research agreement with Tizona Therapeutics.

Financial support

M.J.S. was supported by a National Health and Medical Research Council (NH&MRC) Senior Principal Research Fellowship (1078671) and Program Grant (1132519). S.C.R. was supported by NIH R21CA221702, R01DK108894, and DoD W81XWH-15-PRMRP-FPA. M. W. L. T. was supported by an NH&MRC Project Grant (1120887).

Funding

This work was supported by the National Institutes of Health [R21CA221702]; National Institutes of Health [R01DK108894]; National Health and Medical Research Council of Australia (NH&MRC) [1132519]; NH&MRC [1078671]; NH&MRC [1120887]; U.S. Department of Defense [W81XWH-15-PRMRP-FPA].

ORCID

Xian-Yang Li http://orcid.org/0000-0003-4163-5283
Mark J. Smyth http://orcid.org/0000-0001-7098-7240

References

1. Smyth MJ, Ngiow SF, Ribas A, Teng MW. Combination cancer immunotherapies tailored to the tumour microenvironment. Nat Rev Clin Oncol. 2016;13:143–158. doi:10.1038/nrclinonc.2015.209.
2. Restifo NP, Smyth MJ, Snyder A. Acquired resistance to immunotherapy and future challenges. Nat Rev Cancer. 2016;16:121–126. doi:10.1038/nrc.2016.2.
3. Liu J, Blake SJ, Yong MC, Harjunpää H, Ngiow SF, Takeda K, Young A, O’Donnell JS, Allen S, Smyth MJ, Teng MW. Improved efficacy of neoadjuvant compared to adjuvant immunotherapy to eradicate metastatic disease. Cancer Discov. 2016;6:1382–1399.
4. Cekic C, Linden J. Purinergic regulation of the immune system. Nat Rev Immunol. 2016;16:177–192. doi:10.1038/nri.2016.4.
5. Blay J, White TD, Hoskin DW. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. Cancer Res. 1997;57:2602–2605.
6. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol. 2013;31:51–72. doi:10.1146/annurev-immunol-032712-100008.
7. Young A, Mittal D, Stagg J, Smyth MJ. Targeting cancer-derived adenosine: new therapeutic approaches. Cancer Discov. 2014;4:879–888. doi:10.1158/2159-8290.CD-14-0341.
8. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. Nat Rev Cancer. 2017;17:709–724. doi:10.1038/nrc.2017.86.
9. Allard B, Pommye S, Smyth MJ, Stagg J. Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Clin Cancer Res. 2013;19:5626–5635. doi:10.1158/1078-0432.CCR-13-0545.
10. Mittal D, Young A, Stannard K, Yong M, Teng MW, Allard B, Stagg J, Smyth MJ. Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. Cancer Res. 2014;74:3652–3658. doi:10.1158/0008-5472.CAN-14-0957.
11. Young A, Ngiow SF, Madore J, Reinhardt J, Landsberg J, Chitsazan A, Rautela J, Bald T, Barkauskas DS, Ahern E, et al. Targeting adenosine in BRAF-mutant melanoma reduces tumor growth and metastasis. Cancer Res. 2017;77:4684–4696. doi:10.1158/0008-5472.CAN-17-0393.
12. Allard B, Longhi MS, Robson SC, Stagg J. The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immuno Rev. 2017;276:121–144. doi:10.1111/imr.12528.
Suppression of metastases using a new lymphocyte checkpoint target for cancer immunotherapy. Cancer Discov. 2016;6:446–459. doi:10.1158/2159-8290.CD-15-0944.

25. Roman Aguilera A, Lutzky VP, Mittal D, Li XY, Stannard K, Takeda K, Bernhardt G, Teng MWL, Dougall WC, Smyth MJ. CD96 targeted antibodies need not block CD96-CD155 interactions to promote NK cell anti-metastatic activity. Oncoimmunology. 2018;7:e1424677. doi:10.1080/2162402X.2018.1424677.

26. Ferrari de Andrade L, SF N, Stannard K, Rusakiewicz S, Kalimutlu M, Kk K, Tey S-K, Takeda K, Zitvogel L, Martinet L, et al. Natural killer cells are essential for the ability of BRAF inhibitors to control BRAFV600E-mutant metastatic melanoma. Cancer Res. 2014;74:7298–7308. doi:10.1158/0008-5472.CAN-14-1339.

27. Simoni Y, Becht E, Fehlings M, Loh CY, Kared H, et al. Bystander CD8(+) T cells are abundant and phenotypically distinct in human tumour infiltrates. Nature. 2018;557:575–579. doi:10.1038/s41586-018-0130-2.

28. Sade-Feldman M, Yizhak K, Bjorgaard SL, Ray JP, de Boer CG, Jenkins RW, Lieb DJ, Chen JH, Frederick DT, Bartzily-Rokni M, et al. Defining T cell states associated with response to checkpoint immunotherapy in melanoma. Cell. 2018;175:998–1013 e20. doi:10.1016/j.cell.2018.10.038.

29. Guillelery C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. Nat Immunol. 2016;17:1025–1036. doi:10.1038/ni.3518.

30. Young A, Ngio SF, Gao Y, Patch AM, Barkauskas DS, Messaoudene M, Lin G, Coudert JD, Stannard KA, Zitvogel L, et al. A2AR adenosine signaling suppresses natural killer cell maturation in the tumor microenvironment. Cancer Res. 2018;78:1003–1016. doi:10.1158/0008-5472.CAN-17-2826.

31. Tang F, Zheng P. Tumor cells versus host immune cells: whose PD-L1 contributes to PD-1/PD-L1 blockade mediated cancer immunotherapy? Cell Biosci. 2018;8:34. doi:10.1186/s13578-018-0232-4.

32. Hirata Y, Furushashi K, Ishii H, Li HW, Pinho S, Ding L, Robson SC, Frenette PS, Fujisaki J. CD150(high) bone marrow treys maintain hematopoietic stem cell quiescence and immune privilege via adenosine. Cell Stem Cell. 2018;22:445–53 e5. doi:10.1016/j.stem.2018.01.017.

33. Li XY, Das I, Lepleiter A, Addala V, Bald T, Stannard K, Barkauskas D, Liu J, Aguilera AR, Takeda K et al. CD155 loss enhances tumour suppression via combined host and tumor-intrinsic mechanisms. J Clin Invest. 2018;128:2613–2625. doi:10.1172/JCI98769.

34. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngio SF, Rautela J, Straube J, Waddell N, Blake SJ et al. Tumor immunoevasion by the conversion of effector NK cells into type I innate lymphoid cells. Nat Immunol. 2017;18:1004–1015. doi:10.1038/ni.3800.

35. Chan CJ, Martinet L, Giffillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, Ritchie DS, Colonna M, Andrews DM, Smyth MJ. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. Nat Immunol. 2014;15:431–438. doi:10.1038/ni.2850.