Targeting CD73 enhances the anti-tumor activity of anti-PD-1 and anti-CTLA-4 mAbs

Bertrand Allard¹, Sandra Pommey¹, Mark J. Smyth²,³,⁴,⁵,* and John Stagg¹,*

¹ Centre de Recherche du Centre Hospitalier de l’Université de Montréal, Faculté de Pharmacie et Institut du Cancer de Montréal, Montréal, Québec, Canada, H2L 4M1.
² Cancer Immunology Program, Trescowthick Laboratories, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria 3002, Australia.
³ Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Victoria 3010, Australia.
⁴ Immunology in Cancer and Infection Laboratory, Queensland Institute of Medical Research, Herston, Queensland 4006, Australia.
⁵ School of Medicine, University of Queensland, Herston, Queensland 4006, Australia.

*Equal senior authors

Running Title: Anti-CD73 synergizes with anti-PD-1 and anti-CTLA-4 mAbs

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Correspondence: John Stagg, Centre de recherche du Centre Hospitalier de l’Université de Montréal, Montréal, Québec, Canada, H2L. E-mail: john.stagg@umontreal.ca; TEL: 514-890-8000 ext :25170. FAX: 514-412-7661.
Translational relevance statement:

Some cancer patients show durable clinical responses to anti-CTLA-4 or anti-PD-1 mAbs. For this reason, immune-checkpoint inhibitors hold great potential as cancer therapeutics. Nevertheless, clinical benefits from immune-checkpoint inhibition are still modest. One potential explanation is that tumors employ non-overlapping immunosuppressive mechanisms to facilitate immune escape. One of these mechanisms consists at the accumulation of extracellular adenosine. In this study, we show that targeted blockade of CD73, the ecto-nucleotidase that catabolizes the generation of extracellular adenosine, significantly enhances the therapeutic activity of anti-PD-1 and anti-CTLA-4 mAbs. Our study strongly thus suggests that targeted blockade of CD73 can improve the antitumor activity of immune checkpoint inhibitors.
Abstract

Purpose: Monoclonal antibodies (mAb) that block programmed death (PD)-1 or cytotoxic T lymphocyte antigen (CTLA-4) receptors have been associated with durable clinical responses against a variety of cancer types and hold great potential as novel cancer therapeutics. Recent evidence suggest that targeted blockade of multiple immunosuppressive pathways can induce synergistic anti-tumor responses.

Experimental Design: In this study, we investigated whether targeted blockade of CD73, an ecto-nucleotidase that catabolizes the hydrolysis of extracellular adenosine monophosphate (AMP) to adenosine, can enhance the anti-tumor activity of anti-CTLA-4 and anti-PD-1 mAbs against transplanted and chemically-induced mouse tumors.

Results: Anti-CD73 mAb significantly enhanced the activity of both anti-CTLA-4 and anti-PD-1 mAbs against MC38-OVA (colon) and RM-1 (prostate) subcutaneous tumors, and established metastatic 4T1.2 breast cancer. Anti-CD73 mAb also significantly enhanced the activity of anti-PD-1 mAb against 3-methylcholanthrene (MCA)-induced fibrosarcomas. Gene-targeted mice revealed that single agent therapies and combinatorial treatments were dependent on host IFN-γ and CD8+T cells, but independent of perforin. Interestingly, anti-CD73 mAb preferentially synergized with anti-PD-1 mAb. We investigated the effect of extracellular adenosine on tumor-infiltrating T cells and demonstrated that activation of A2A adenosine receptor enhances PD-1 expression, but not CTLA-4 expression, on tumor-specific CD8+ T cells and CD4+ Foxp3+ T regulatory cells.
Conclusions: Taken together, our study revealed that targeted blockade of CD73 can enhance the therapeutic activity of anti-PD-1 and anti-CTLA-4 mAbs and may thus potentiate therapeutic strategies targeting immune checkpoint inhibitors in general.
Introduction

Tumor-infiltrating lymphocytes (TILs) control the clinical progression of various types of cancers (1–4). Nevertheless, most tumors persist despite being infiltrated with tumor-specific CD8+ T cells. This is in part due to the dysfunctional nature of TILs and the presence of immunosuppressive factors and regulatory cells in the tumor microenvironment (5,6). One of the major causes of TIL dysfunction is a process known as T cell exhaustion, which results in the expression of inhibitory receptors (7–9). Monoclonal antibodies (mAb) able to block these inhibitory receptors – also called immune-checkpoint inhibitors – have been associated with objective clinical responses against various types of cancer and hold great potential as novel cancer therapeutics (10–12).

The first immune-checkpoint inhibitor approved for cancer treatment is the anti-CTLA-4 mAb ipilimumab (Yervoy) (13). CTLA-4 belongs to the immunoglobulin superfamily of receptors, which also includes PD-1, TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3), BTLA (B and T lymphocyte attenuator) and VISTA (V-domain immunoglobulin suppressor of T cell activation) (14). Ipilimumab is approved for treatment of unresectable or metastatic melanoma, either as initial therapy or after relapse. Anti-CTLA-4 mAb therapy enhances the anti-tumor function of CD8+ T cells, increases the ratio of CD8+ T cells to Foxp3+ T regulatory cells (Tregs) and inhibits the suppressive function of Tregs (15). Another promising immune checkpoint that can be targeted for cancer therapy is the PD-1/PD-L1 axis (16,17). PD-1 is expressed by
activated T cells, B cells, monocytes and NKT cells. It has two ligands, PD-L1 and PD-L2, with distinct expression profiles. Notably, PD-L1 expression on tumor cells has been associated with poor prognosis in cancer patients (18,19). Targeted blockade of PD-1 or PD-L1 enhances adaptive anti-tumor immunity by preventing T cell exhaustion and by promoting T cell homing to tumors (20,21). In two recent phase I clinical trials, targeted blockade of PD-1/PD-L1 was associated with objective clinical responses in 6-28% of cancer patients (16,17).

While some patients respond to anti-CTLA-4 or anti-PD-1 mAb therapy, the majority of patients show modest or no clinical benefits. One potential explanation is that tumors employ additional and non-overlapping immunosuppressive mechanisms to facilitate immune escape (5). Targeted blockade of multiple immunosuppressive pathways might thus be required to induce clinically relevant anti-tumor responses. This is supported by several recent studies demonstrating that targeted inhibition of multiple immunosuppressive pathways can induce synergistic anticancer activity in mice (21–23).

One of the most recently identified immunosuppressive pathways involved in tumor immune escape is the production of extracellular adenosine by CD73 (ecto-5’-nucleotidase) (24–26). CD73 is expressed on lymphocytes, endothelial and epithelial cells, where it participates in endothelial cell barrier function, protection from ischemia and regulation of immune responses. Importantly, CD73 has been found to be overexpressed in several types of human cancers, and high CD73 expression has been
associated with a poor prognosis (27). We and others have demonstrated that CD73 expressed on tumor cells as well as on host hematopoietic and non-hematopoietic cells promotes tumor immune escape and metastasis in mice (28–31). We have also established the proof-of-concept that targeted blockade of CD73 can induce potent anti-tumor immune responses (32) and synergize with chemotherapeutic drugs known to promote immunogenic responses (33). The inhibition of CD39, an ecto-enzyme operating upstream of CD73, was also shown to have therapeutic effects and to synergize with chemotherapy in preclinical cancer models (34).

Herein, we investigated whether targeted blockade of CD73 can enhance the therapeutic activity of the immune checkpoint inhibitors anti-CTLA-4 and anti-PD-1 mAbs. Our study reveals that anti-CD73 mAb enhances the therapeutic activity of both anti-CTLA-4 and anti-PD-1 mAbs. Interestingly, the greatest therapeutic effect was observed when anti-CD73 mAb was combined with anti-PD-1 mAb. Analysis of tumor-infiltrating T cells (TILs) revealed that CD73-derived adenosine enhances PD-1, but not CTLA-4 expression on TILs via activation of A2A adenosine receptor. Taken together, our study suggests that targeted blockade of CD73 can synergize with immune checkpoint inhibitors, especially those targeting the PD-1/PD-L1 axis.
Materials and Methods

Cell lines and animals

MC38-OVA (CD73low), RM-1 (CD73low) and 4T1.2 (CD73high) tumor cell lines have been previously described (28,29,32). Wild type C57Bl/6 or BALB/c mice were purchased from Charles River and maintained at the Centre de Recherche du Centre Hospitalier de l’Université de Montréal, or bred and maintained at the Peter MacCallum Cancer Centre. IFN-γ-deficient C57Bl/6 mice and perforin-deficient C57Bl/6 mice were bred and maintained at the Peter MacCallum Cancer Centre. CD73-deficient mice were kindly provided by Dr. Linda H. Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK) and were bred and maintained at the Centre de Recherche du Centre Hospitalier de l’Université de Montréal.

Antibodies and chemicals

Purified anti-mouse CD73 mAb (clone TY/23), purified anti-mouse PD-1 mAb (clone RMP1-14), purified anti-mouse CTLA-4 mAb (clone 9H10 and UC10-4F10), anti-mouse CD4 (clone GK1.5), anti-mouse CD8β (clone 53.5.8) and purified control Ig (MAC4) were purchased from BioXCell (West Lebanon, NH). V450-conjugated anti-mouse CD8α, PE-CF594-conjugated anti-mouse PD-1, Alexa488-conjugated anti-mouse CTLA-4, Alexa647-conjugated anti-mouse TCRβ, Alexa700-conjugated anti-mouse CD4, PE-conjugated anti-mouse Foxp3, PE- or Alexa647-conjugated anti-mouse CD73 (clone TY/11.8) were purchased from BD Bioscience. PE-conjugated MHC (major
histocompatibility complex) class I/SIINFEKL tetramer was obtained from Université de Montréal. 5′-(N-ethylcarboxamido) adenosine (NECA) and SCH58261 were purchased from Sigma.

**In vivo treatments**

MC38-OVA tumor cells (10⁶ cells) or RM-1 tumor cells (5 x 10⁵ cells) were injected subcutaneously into syngeneic C57Bl/6 mice. Where indicated, mice were treated with anti-CD73 mAb (TY/23; 100 µg i.p.), anti-PD-1 mAb (RMP1-14; 100 µg i.p.), anti-CTLA-4 mAb (9H10 or UC10-4F10; 100 µg i.p) or control Ig (MAC4; 100 µg i.p). To assess the role of T cells, mice were also injected weekly i.p. with anti-CD4 mAb (100 µg clone GK1.5) and/or anti-CD8β mAb (100 µg clone 53.5.8) or control Ig (100 µg clone MAC4). For survival experiments, 5 x 10⁴ 4T1.2 tumor cells were inoculated into the fourth mammary fat-pad of BALB/c mice. On day 25, mice were anesthetized, the primary tumor was surgically removed, the wound was closed with surgical clips and treatments commenced 3 d later. To assess PD-1 and CTLA-4 expression levels in TILs, MC38-OVA tumor cells (10⁶ cells) were injected subcutaneously into syngeneic wild type or CD73-deficient C57Bl/6 mice, treated at day 12 and 15 with SCH58261 (1 mg/kg) and/or NECA (0.05 mg/kg i.p + 0.05 mg/kg s.c.) and tumors isolated at day 18 for flow cytometry analysis.
Flow cytometry of tumor-infiltrating lymphocytes

MC38-OVA tumors were excised, minced with scissors, and incubated 1 hour at 37°C for
in PBS containing collagenase type 4 (Worthington Biochemical) and DNase I (Roche).
Tumor cell suspensions were passed through a 70-μm cell strainer, washed twice in PBS,
and resuspended in PBS 2% serum. Anti-CD16/32 mAb (clone 2.4G2) was used to block
Fc receptors. To assess ovalbumin-specific CD8+ T cells, TCRβ+CD8α+ cells were pre-
gated and percentages of tetramer-reactive cells evaluated. Flow cytometry was
performed on a Fortessa (BD Bioscience) and analyzed using the software program
FlowJo.

Quantitative real-time PCR

Tumor tissues were disrupted by a rotor-stator homogenizer followed by RNA isolation
using RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA
reverse transcription was carried out using Superscript VILO cDNA synthesis kit
(Invitrogen). Real-time PCR was carried out using a Step One Plus Thermal cycler using
oligonucleotides and Taqman probe mix specific (gene expression assays) for mouse
IFN-γ (Mm01168134_m1), Tbx21 (Mm00450960_m1), and GADD45A
(Mm00432802_m1) (all from Applied Biosystems). Samples were normalized relative to
GADD45A transcript expression levels.
MCA-induced fibrosarcomas

Groups of 15 male C57Bl/6 mice were inoculated s.c. in the hind flank with 400 µg of 3-methylcholanthrene (MCA; Sigma-Aldrich) in 0.1 ml of corn oil. Once the tumors were established (0.24-0.39 cm²) mice were treated with intraperitoneal injections of cIg (100 µg), anti-CD73 mAb (100 µg), anti-PD-1 mAb (100 µg) or combination anti-CD73/PD-1 mAbs (100 µg each) twice weekly for 6 weeks. Development of fibrosarcomas was monitored weekly over the course of 300 days. Measurements were made with a caliper and tumor sizes were calculated using the product of two perpendicular diameters (mm²).
Results

CD73 blockade enhances anti-PD-1/CTLA-4 mAb therapy via IFN-γ and CD8+ T cells.

We investigated whether targeted blockade of CD73 could enhance the therapeutic activity of anti-PD-1 and anti-CTLA-4 mAbs. In a first set of experiments, MC38 mouse colon cancer cells expressing ovalbumin (MC38-OVA) were injected subcutaneously to syngeneic mice and treated on day 12, 16 and 20 with anti-CD73 and/or anti-PD-1 mAbs. Monotherapy with anti-CD73 or anti-PD-1 mAb significantly delayed tumor growth, consistent with previous studies (35,28). Strikingly, the combined administration of anti-CD73 and anti-PD-1 mAbs induced complete tumor rejection in all treated mice (Fig. 1A). We investigated the importance of IFN-γ and perforin responses in this anti-tumor effect using gene-targeted mice and observed that the anti-tumor activity of anti-PD-1 and anti-CD73 mAbs, administered as single agents or in combination, was dependent on IFN-γ (Fig. 1B) and independent of perforin (Fig 1C).

We next investigated the effect of combining anti-CD73 mAb with anti-CTLA-4 mAb. As shown in Fig. 1D, anti-CD73 mAb significantly enhanced the therapeutic activity of anti-CTLA-4 mAb (Fig. 1D). However, it did not induce complete tumor regression. Similar to anti-PD-1 mAb, the anti-tumor activity of anti-CTLA-4 and anti-CD73 mAbs, administered as single agents or in combination, was dependent on IFN-γ (Fig. 1E) and independent of perforin (Fig 1F). Similar to what we observed in MC38-OVA tumors, anti-CD73 mAb significantly enhanced the anti-tumor activity of anti-PD-1 and anti-
CTLA-4 mAbs against subcutaneous RM-1 prostate tumors (suppl. Fig. S1A-B). We further investigated the role of CD8$^+$ T cells and CD4$^+$ T cells in the activity of anti-CD73 mAb as single agent or in combination with anti-CTLA-4 or anti-PD-1 mAb. Our data showed that treatment with anti-CD73 mAb as a single agent or in combination with anti-CTLA-4 or anti-PD-1 mAb is dependent on CD8$^+$ T cells, independently of CD4$^+$ T cells (suppl. Fig. S2).

**Anti-CD73 mAb potentiates Th1 responses and downregulates CD73 expression on TILs.**

We next investigated the effect of the combinatorial treatments on tumor-infiltrating antigen-specific CD8$^+$ T cells. Consistent with previous studies (21,32), monotherapy with anti-CD73, anti-CTLA-4 or anti-PD-1 mAb increased tumor infiltration with antigen-specific CD8$^+$ T cells compared to control-treated mice (Fig. 2A). When anti-CD73 mAb was combined with anti-PD-1 or anti-CTLA-4 mAb, tumor-infiltrating antigen-specific CD8$^+$ T cells further increased compared to single agent treatments (Fig. 2A). Notably, this increased accumulation of antigen-specific CD8$^+$ T cells following combinatorial treatments was associated with increased expression of the Th1 immune genes Tbx21 (also called T-bet) and its target IFN-γ (Fig. 2B). We had previously shown that anti-CD73 mAb can downregulate CD73 expression on tumor cells (32). We now investigated whether anti-CD73 mAb therapy downregulated CD73 expression on TILs. As shown in Figure 2C-D (and suppl. Fig. S3), anti-CD73 mAb therapy significantly
downregulated CD73 expression levels on CD8+ and CD4+ TILs, irrespective of tumor-specificity or Foxp3 expression.

**CD73 blockade prolongs survival of mice with metastatic cancer when combined with anti-PD-1/CTLA-4 mAbs.**

We next assessed the therapeutic activity of combining anti-CD73 mAb with anti-PD-1 or anti-CTLA-4 mAb in a mouse model of established metastatic cancer. Syngeneic mice were injected subcutaneously with 4T1.2 mouse breast cancer cells (which have the potential to spontaneously metastasize), primary tumors were surgically removed at day 25 and immunotherapy was initiated at day 28. As shown in Fig. 3, tumor-bearing mice undergoing surgery alone, with subsequent control treatment, died of metastatic disease by day 41 (median survival of 36 days). Post-surgery monotherapy with anti-CD73 mAb, anti-PD-1 mAb or anti-CTLA-4 mAb each significantly prolonged survival compared to control treatment (median survival of 46.5, 55 and 49 days, respectively). When anti-CD73 mAb was combined with anti-CTLA-4 mAb, the median survival was increased to 63 days. When anti-CD73 mAb was combined with anti-PD-1 mAb, the median survival was increased to 70 days (Fig. 3; significance of differences in survival between groups estimated by Log-rank Mantel-Cox test, p < 0.0001).
Combined treatment with anti-CD73/anti-PD-1 mAbs induces tumor regression in a chemically-induced sarcoma model.

We further assessed whether targeted blockade of CD73 could enhance the therapeutic activity of immune checkpoint inhibition with anti-PD-1 mAb in a more stringent model of carcinogenesis. We used the MCA-induced fibrosarcoma model, where host immunity has been shown to suppress tumor initiation and progression, and where CD73 has been shown to promote tumor immune escape (29). C57Bl/6 mice were inoculated with 400 μg MCA subcutaneously and were treated with anti-CD73 mAb, anti-PD-1 mAb or combination anti-CD73/anti-PD-1 mAbs for 6 weeks. As shown in Figure 4, while single agent treatments had modest anti-tumor effects, combining anti-CD73 mAb with anti-PD-1 mAb significantly delayed MCA-induced tumor progression. Comparing individual tumor growth rates (in mm²/day) also revealed greater anti-tumor activity with the combination treatment (p < 0.0001 by ANOVA or Kruskal-Wallis test; suppl. Fig. S4). Notably, 2/15 mice treated with the combination anti-CD73/anti-PD-1 mAbs showed complete tumor regression, and 6/15 mice showed a partial response.

Activation of A2A adenosine receptor upregulates PD-1 expression on TILs.

Because extracellular adenosine often accumulates in solid tumors and that TILs often express PD-1 and/or CTLA-4, we investigated whether CD73-derived adenosine could regulate PD-1 and/or CTLA-4 expression in TILs. For this purpose, we treated tumor-bearing mice with the pan-adenosine receptor agonist NECA and analyzed cell surface expression of PD-1 and CTLA-4 on TILs. PD-1 and CTLA-4 expression was also
assessed on TILs of CD73-deficient mice. As shown in Figure 5, administration of NECA to tumor-bearing mice significantly increased PD-1 expression levels on antigen-specific CD8^+ TILs and CD4^+Foxp3^+ TILs, but had no effect on CTLA-4 expression. Conversely, in tumor-bearing CD73-deficient mice, CD8^+ TILs showed decreased expression of PD-1, but not CTLA-4 (Fig. 5B), while CD4^+Foxp3^+ TILs showed unaltered PD-1 and CTLA-4 expression (Fig. 5C). Because high affinity A2A adenosine receptor is upregulated on activated T cells, we investigated whether A2A adenosine receptor signalling was involved in upregulating PD-1 expression on TILs. As shown in Fig. 5B, NECA-mediated upregulation of PD-1 on TILs was inhibited when mice were co-treated with an A2A adenosine receptor antagonist (SCH58261). We further investigated the increase in PD-1 expression levels on T cells induced by A2A adenosine receptor signalling in vitro. As shown in Fig. 5D, adenosine receptor activation with NECA following in vitro T cell activation significantly increased PD-1, but not CTLA-4, expression levels on both CD4^+ and CD8^+ T cells, and this was completely inhibited by the A2A adenosine receptor antagonist SCH58261. Taken together, our data strongly suggest that CD73-derived adenosine enhances PD-1 expression on activated T cells via A2A adenosine receptor signalling.

Our data suggested that anti-CD73 mAb may decrease PD-1 levels in TILs. In order to test this hypothesis, we analyzed PD-1 and CTLA-4 expression levels in MC38-OVA TILs after treatment with anti-CD73 mAb. Treatment with anti-CD73 mAb did not alter PD-1 or CTLA-4 levels in TILs (suppl. Fig. S5). Thus, while extracellular adenosine positively regulates PD-1 levels on TILs, blocking CD73 with mAb clone TY/23 is
insufficient to affect this process, suggesting a non-redundant mechanism of synergy between TY/23 and anti-PD-1 mAb.
Discussion

In this study, we report that targeted blockade of CD73 can enhance the therapeutic activity of the immune-checkpoint inhibitors anti-PD-1 and anti-CTLA-4 mAbs. Blocking immune checkpoints with monoclonal antibodies is associated with objective clinical responses in a variety of cancer types (10,13,16,17). The anti-CTLA-4 mAb ipilimumab is approved for treatment of metastatic melanoma and is being evaluated for other cancer indications (clinicaltrial.gov identifiers: NCT01693783, NCT01688492, NCT01611558, NCT01285609). While ipilimumab is associated with clinical responses, it is limited by the generation of autoimmune toxicities due to on-target effects. Accordingly, up to 25% of patients treated with ipilimumab develop serious grade 3-4 adverse events (36). Targeting PD-1 or its ligand PD-L1 is as another promising cancer immunotherapy. Remarkably, in phase 1 clinical trials (16,17), anti-PD-1/anti-PD-L1 mAbs were associated with objective clinical responses in a variety of cancer types, including poorly immunogenic cancers such as non-small cell lung cancer. Anti-PD-1/anti-PD-L1 mAb therapy caused drug-related grade 3 or 4 adverse events in 14% and 9% of patients, respectively (16,17). Thus, a major challenge in the use of anti-CTLA-4 and anti-PD-1/PD-L1 mAbs is to define favorable clinical settings that strike an optimal balance between tumor immunity and autoimmunity.

One potential avenue is to target non-overlapping immunosuppressive mechanisms that facilitate immune escape. Theoretically, targeting immunosuppressive pathways specifically expressed in the tumor microenvironment will favor the activation of anti-
tumor over autoimmune responses. Targeted blockade of multiple immunosuppressive pathways might thus be required to induce clinically relevant anti-tumor responses. Indeed, several studies have demonstrated the synergistic anti-tumor activity of combinatorial immunotherapies (22,23,37,38). Curran et al. (21) demonstrated that combined blockade of CTLA-4 and PD-1 induces synergistic anti-tumor activity in mice. Concurrent blockade of CTLA-4 and PD-1 has also shown encouraging results in a phase I clinical trial (38). Studies have also tested the potential benefit of combining anti-PD-1 mAb with tumor vaccines (39,40) and low-dose Treg-depleting cyclophosphamide (41). In the latter setting, anti-PD-1 mAb was found to prolong the inhibition of Tregs by low-dose cyclophosphamide. Anti-PD-1 mAb therapy has also been shown to act synergistically with adoptive T cell therapy (42,43).

Our data demonstrated that both anti-PD-1 and anti-CTLA-4 mAbs can significantly benefit from targeted blockade of CD73. In the subcutaneous MC38-OVA tumor model, we observed that combining anti-CD73 mAb with anti-PD-1 mAb was associated with complete responses. Consistent with previous work, the anti-tumor effects of both anti-CD73 and anti-PD-1 mAbs were dependent on IFN-γ, and independent of perforin (32,44). Whether this is an idiosyncratic feature of mouse models or whether IFN-γ is also the preferred mechanism-of-action in humans remains to be determined.

Remarkably, the combination anti-CD73/anti-PD-1 mAbs was significantly more effective than the combination anti-CD73/anti-CTLA-4 mAbs, against both subcutaneous
tumors and metastatic disease. This may be due to several non-exclusive factors. Firstly, this may be a consequence of the superior anti-tumor activity of anti-PD-1 versus anti-CTLA-4 mAb, when administered as single agents. Secondly, it could be due to synergistic effects of anti-CD73 and anti-PD-1 mAbs on Tregs. Accordingly, we have previously shown that Foxp3+ Tregs heavily rely on CD73 to promote MC38-OVA tumor growth (28) and several studies demonstrated that PD-1/PD-L1 interactions are important for differentiation of inducible Foxp3+ Tregs (45,46). Thus, the combination anti-CD73/anti-PD-1 therapy might effectively abrogate tumor-promoting effects of Tregs. Alternatively, anti-CD73 mAb might block non-enzymatic functions of CD73 (T cell adhesion for instance), which might potentiate immune checkpoint blockade. The mechanism behind anti-CD73/anti-PD-1 mAb combination thus remains unclear and requires further investigation.

In summary, the relatively low complete response rates currently observed in clinical trials with immune checkpoint inhibitors as single agent therapy highlights the importance of new combinatorial treatments able to strike an optimal balance between tumor immunity and autoimmunity. The production of extracellular adenosine by CD73 has recently been shown to be an important immunosuppressive pathway contributing to tumor immune evasion (24–26). Because extracellular adenosine accumulates in hypoxic tumors (47,48), we believe that targeting this pathway will synergize with immune checkpoint inhibitors. As was recently shown (49), combined blockade of non-redundant immunosuppressive pathways – i.e. indoleamine 2,3-dioxygenase (IDO) and CTLA-4 – can induce potent synergistic antitumor activity. Similar to IDO, CD73-derived
extracellular adenosine is a potent suppressor of effector T cells in tumors, especially those associated with Th1 responses. Our study suggests that targeted blockade of CD73 is another promising strategy in order to enhance the therapeutic activity of CTLA-4 and PD-1 checkpoint inhibition. In conclusion, it is our contention that targeted blockade of the immunosuppressive effects of extracellular adenosine may constitute an effective means to enhance the antitumor activity of immune checkpoint inhibitors.
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Figure Legends

Figure 1. Anti-CD73 mAb enhances anti-tumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Wild type (A and D), IFN-γ-deficient (B and E) or perforin-deficient (C and F) C57Bl/6 mice were injected subcutaneously with MC38-OVA tumor cells (10^6 cells) and treated on day 12, 16 and 20 with intraperitoneal (i.p.) injections of anti-CD73 mAb (100 μg clone TY/23), anti-PD-1 mAb (100 μg clone RMP1-14), anti-CTLA-4 mAb (100 μg clone UC10-4F10), anti-CD73/anti-PD-1 mAbs (100 μg i.p each; A-C), anti-CD73/anti-CTLA-4 mAbs (100 μg i.p each; D-F) or control Ig (200 μg i.p). Means ± standard errors of 5 mice per group are shown (*: p<0.05 by Mann-Whitney between combination and either single agent therapy). A representative of 2 experiments is shown.

Figure 2. Anti-CD73 mAb enhances Th1 responses generated by anti-PD-1 and anti-CTLA-4 mAbs. A. Wild type C57Bl/6 mice were injected subcutaneously with MC38-OVA tumor cells (10^6 cells) and treated on day 12 and 15 with intraperitoneal (i.p.) injections of anti-CD73 mAb (100 μg clone TY/23), anti-PD-1 mAb (100 μg clone RMP1-14), anti-CTLA-4 mAb (100 μg clone 9H10), anti-CD73/anti-PD-1 mAbs (100 μg each), anti-CD73/anti-CTLA-4 mAbs (100 μg each) or control Ig (200 μg). On day 18, tumor single cell suspensions were analysed by flow cytometry for CD8 expression and tetramer-reactivity (Tet). Means ± standard errors of 5 mice per group are shown (*: p<0.05 by Mann-Whitney test). B. Real-time PCR analysis of IFN-γ and T-bet (Tbx21) mRNA expression in tumors after treatment. Means ± standard errors of 4 mice per group are shown (*: p<0.05 by Mann-Whitney test). C-D. Percentages (C) and mean fluorescent
Intensity (MFI) (D) of CD73 expression in gated TIL populations from MC38-OVA tumor-bearing mice treated i.p. with anti-CD73 mAb or control Ig. Different anti-CD73 mAbs were used for blockade (clone TY/23) and flow cytometry (TY/11.8). Means ± standard errors of 5 mice per group are shown (***: p<0.001 by Mann-Whitney test).

Figure 3. Therapeutic activity of combining anti-CD73 mAb with anti-PD-1 or anti-CTLA-4 mAb for treatment of established metastatic breast cancer. Fifty thousand 4T1.2 tumor cells were inoculated into the mammary fat-pad of wild type BALB/c mice, primary tumors were surgically removed on day 25 and mice were treated with 100 μg anti-CD73 mAb (TY/23), anti-PD-1 mAb (RMP1-14), and/or anti-CTLA-4 mAb (UC10-4F10) on day 28, 32, 36 and 40. Significance of differences in survival between all groups was estimated by Log-Rank (Mantel-Cox) test (p < 0.0001). Comparing anti-CD73 vs anti-PD-1/anti-CD73 or anti-CD73/anti-CTLA-4, p = 0.0002 by Log-Rank and p = 0.002 by Gehan-Breslow-Wilcoxon (GBW) test. Comparing anti-CTLA-4 vs anti-CD73/anti-CTLA-4, p=0.0027 by Log-Rank and p = 0.0043 by GBW. Comparing anti-PD-1 vs anti-PD1/anti-CD73, p=0.0027 by Log-Rank and p = 0.0043 by GBW. Comparing anti-PD-1/anti-CD73 vs anti-CD73/anti-CTLA-4, p = 0.0018 by Log-Rank and p=0.0039 by GBW.

Figure 4. Combined anti-CD73/anti-PD-1 mAb therapy inhibits carcinogen-induced tumorigenesis. C57Bl/6 mice were inoculated s.c. in the hind flank with 400 μg of 3-methylcholanthrene (MCA). Once tumors were established (0.24-0.39 cm²), mice were treated with (A) intraperitoneal (i.p.) injections of control Ig (100 μg), (B) anti-CD73
mAb (100 µg TY/23), (C) anti-PD-1 mAb (100 µg RMP1-14) or (D) combination anti-CD73/PD-1 mAbs (100 µg each) twice weekly for 6 weeks. Development of fibrosarcomas was monitored weekly over the course of 300 days. Tumor sizes of individual mice are represented.

Figure 5. CD73 and A2A adenosine receptor signaling enhances PD-1 expression on tumor-infiltrating T cells. Wild type or CD73-deficient C57Bl/6 mice were injected subcutaneously with MC38-OVA tumor cells (10^6 cells) and treated at day 12 and 15 with the pan-adenosine receptor agonist NECA (0.05 mg/kg i.p + 0.05 mg/kg s.c.) and/or the A2A adenosine receptor antagonist SCH58261 (1 mg/kg). At day 18, tumor cell suspensions were analyzed. A. FACS dot plots of tumor-infiltrating CD8^+ T cells (left) and histograms (right) of PD-1 expression levels on gated CD8^+Tet^+ T cells. B-C. Mean fluorescent intensity (MFI) of PD-1 (left) and CTLA-4 (right) expression on ovalbumin-specific CD8^+ T cells (B) and CD4^+Foxp3^+ T cells (C). Means ± standard errors of 5 mice per group are shown (*: p<0.05 by Mann-Whitney test). D. Splenic C57Bl/6 T cells were activated in vitro and treated 48 h later (indicated by the arrow) with NECA ± SCH58261. PD-1 and CTLA-4 expression levels (MFI) were measured by flow cytometry on CD4^+ and CD8^+ T cells at 24 h, 48 h and 72 h post-activation.
Figure 3

![Graph showing survival rates after 4T1.2 injection]

- clg
- αCD73
- αCTLA-4
- αPD-1
- αPD-1/αCD73
- αCTLA-4/αCD73

Percent survival vs. Days after 4T1.2 injection
Clinical Cancer Research

Targeting CD73 enhances the anti-tumor activity of anti-PD-1 and anti-CTLA-4 mAbs

Bertrand Allard, Sandra Pommey, Mark J. Smyth, et al.

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