Disruption of the CD39 immune checkpoint pathway increases the efficacy of various anticancer therapies in syngeneic mouse tumor models

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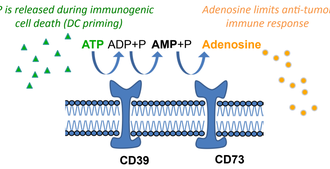
Abstract

The CD39-CD73-adenosine pathway is an emerging regulator of the immune antitumor response. CD39 is expressed within tumors and the tumor microenvironment by several cell populations including immune and cancer cells. In tumor tissues, the pathway leads to the accumulation of immunosuppressive adenosine together with decreased levels of immunoactivating peritumoral ATP. We reported previously that CD39 blockade increased T cell and NK cell-mediated cytotoxic activity in vitro and disclose, during this meeting, the development of the first human-CD39-blocking humanized antibody (S. Augier et al., Preclinical development of a humanized blocking antibody targeting the CD39 immune checkpoint for cancer immunotherapy). Here we demonstrated that this pathway is involved in tumor-induced resistance to various cancer therapies in syngeneic mouse melanoma, colon cancer and fibrosarcoma models. We used therapy-resistant mouse models or inefficacious treatment regimens in the CD39 knockout mice to assess the capacity of CD39 to affect the response to chemotherapies, tumor associated antigen (TAA)-targeting antibodies and immunomodulators such as anti-PD1 antibodies. We achieved increased response rates, increased response duration and some complete and long lasting tumor regressions in the CD39 deficient context. These preclinical proof-of-concept studies highlight the role of the CD39 immune checkpoint pathway in limiting the efficacy of various anticancer therapies in syngeneic mouse models and thereby support the potential clinical value of the humanized CD39-neutralizing antibody under development.

CD39 ecto-ATPase is functionally expressed in tumor microenvironment



CD39 is expressed by immune cells within tumors

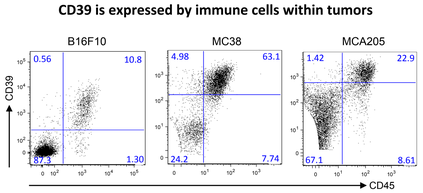


Figure 1: Percentages of CD39+CD45+ and CD39+CD45- cells isolated from tumors in WT mice assessed by flow cytometry are indicated in upper right and upper left quadrants.

CD39 (Entpd1) hydrolyzes ATP and ADP into AMP which is then processed into adenosine by the CD73 ecto-5'-nucleotidase (Nt5e).
ATP (immunoactivator) and adenosine (immunosuppressor) are critical regulators of both innate and adaptive immune responses.

Tumors display ATPase activity

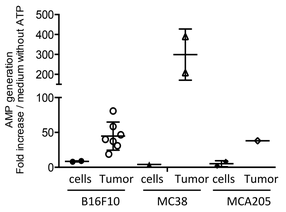


Figure 2: AMP generation in the supernatants from *in vitro* cultured cell lines (cells) or freshly dissociated tumors (Tumor) is determined by mass spectrometry.

TILs from mouse tumors co-express CD39 and PD-1

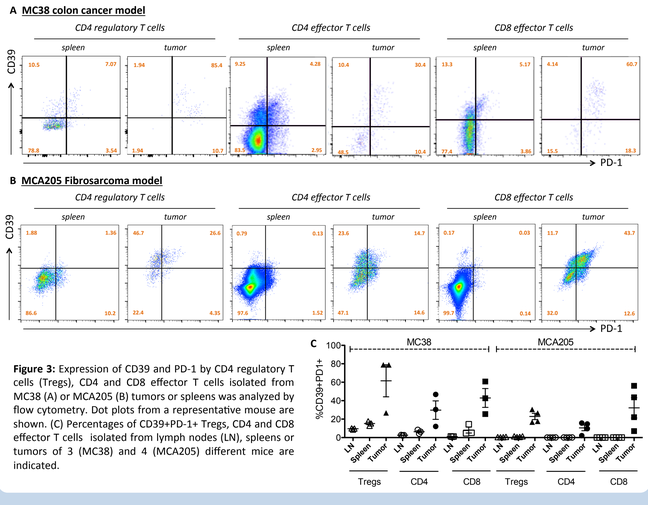
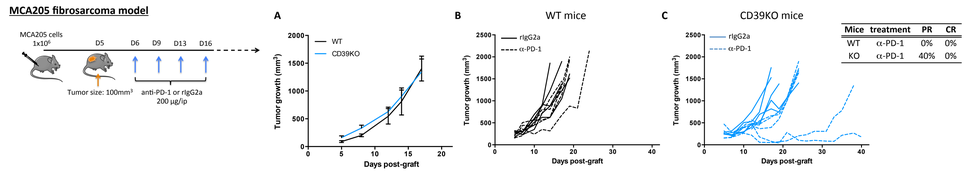


Figure 3: Expression of CD39 and PD-1 by CD4 regulatory T cells (Tregs), CD4 and CD8 effector T cells isolated from MC38 (A) or MCA205 (B) tumors or spleens was analyzed by flow cytometry. Dot plots from a representative mouse are shown. (C) Percentages of CD39+PD-1+ Tregs, CD4 and CD8 effector T cells isolated from lymph nodes (LN), spleens or tumors of 3 (MC38) and 4 (MCA205) different mice are indicated.

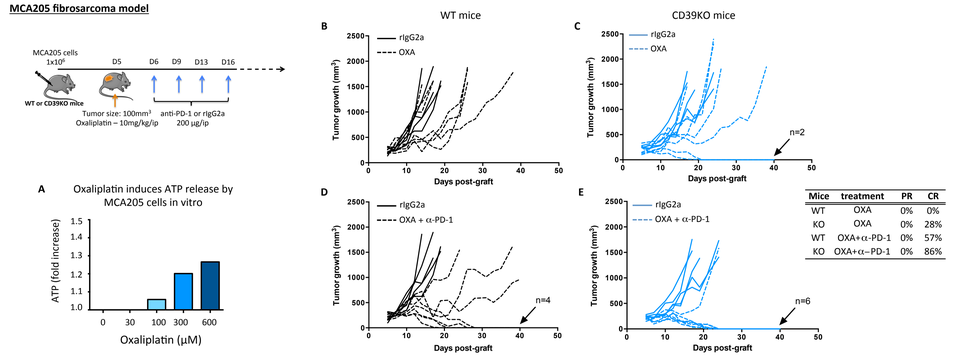
CD39 disruption sensitized to anti-PD-1 treatment in PD-1 resistant mouse tumor model



Mice	treatment	PR	CR
WT	α-PD-1	0%	0%
KO	α-PD-1	40%	0%

Figure 5: WT or CD39 KO mice were grafted subcutaneously with MCA205 fibrosarcoma cells. Mice were either untreated (A) or received 4 i.p. injections of anti-PD-1 or rIgG2a control isotype antibody at 200µg (B and C). Tumor growth as mean volumes + /- SEM (A) or for each individual mouse (B and C) are indicated. Percentages of partial (PR) and complete (CR) regressions are indicated in the table.

CD39 disruption leads to complete tumor regressions when combined with immunogenic chemotherapy and synergized with anti-PD-1 treatment to promote tumor eradication



Mice	treatment	PR	CR
WT	OXA	0%	0%
KO	OXA	0%	28%
WT	OXA+α-PD-1	0%	57%
KO	OXA+α-PD-1	0%	86%

Figure 6: (A) MCA205 fibrosarcoma cells were cultured in the presence of oxaliplatin at the indicated concentrations for 24h and concentration of extracellular ATP in the cell culture supernatant was determined. (B to E) WT or CD39 KO mice were grafted subcutaneously with MCA205 fibrosarcoma cells. At day 5, mice were treated or not with oxaliplatin (10mg/kg/ip) and then received 4 i.p. injections of anti-PD-1 or rIgG2a control isotype antibody at 200µg. Tumor growth for each individual mouse is indicated. Percentages of partial (PR) and complete (CR) regressions are indicated in the table.

Methods summary

Tumor cell lines: B16F10 melanoma cells, MC38 colon cancer cells and MCA205 fibrosarcoma cells were obtained from ATCC. Cells were cultivated in RPMI or DMEM medium supplemented with 10% fetal calf serum (FCS).

Tumor models: 5.10^4 B16F10 cells, 5.10^5 MC38 cells or 10^6 MCA205 cells were subcutaneously inoculated in the dorsal flank of 6-9 week-old C57BL/6 wild-type (WT) or CD39KO mice. Tumor volumes were evaluated every 2 days after initial detection using caliper. Treatments with rat IgG2a control isotype (rIgG2a, BioXCell, clone 2A3), rat anti-PD-1 (BioXCell, clone RMP1-14), mouse IgG2a control isotype (IgG2a, BioXCell, clone C1.18.4), mouse anti-gp75 (BioXCell, clone TA99) antibodies or oxaliplatin were administrated intraperitoneally (ip) at doses and kinetics indicated in figures.

Tumor dissociation: Tumor specimens were minced into 2mm pieces and digested during 1h using the gentle MACS Dissociator at 37°C in presence of 0.2mg/mL Collagenase type IV and 30 U/mL DNAse. Cell suspension was filtered through a 45µm mesh and washed twice with PBS before flow cytometry or mass spectrometry analyses.

Flow cytometry: Tumor cell suspensions were stained with PE Cyanine 7-coupled anti-CD39, FITC-coupled anti-PD-1, Alexa Fluor 700-coupled anti-CD45 and LIVE/DEAD® fixable dead cell stains to distinguish between live and dead cells, for 30min at 4°C. Cells were washed and resuspended in PBS containing 2% FCS and 0.2% NaN3. Cells were then analyzed using a FACSCanto cytometer, CD45, PD-1 and CD39 expression was analyzed using Flow Jo software.

AMP measurement by mass spectrometry MALDI-TOF: Tumor cell were resuspended in PBS supplemented with 50 µM ATP for 30 min at 4°C. After centrifugation, AMP and adenosine levels were analyzed in cell supernatants by mass spectrometry as previously described (Bastid J et al. Cancer Immunology Research, 2014).

ATP release: MCA205 ($2.5x10^5$) were plated in complete medium. After overnight incubation, cells were treated with oxaliplatin for 24h and concentration of extracellular ATP in supernatant was determined using the ATPlite luminescence ATP Detection Assay System (Perkin Elmer).

CD39 disruption enhanced anti-PD-1 efficacy in PD-1 sensitive mouse tumor model

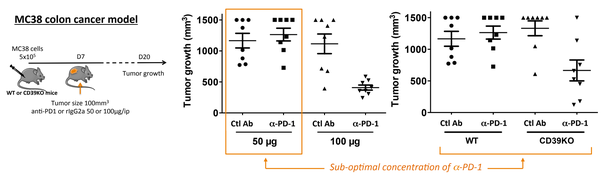


Figure 4: WT or CD39 KO mice (n = 8 per group) were grafted subcutaneously with MC38 colon cancer cells. Mice received on day 7 one i.p. injection of anti-PD-1 antibody or rIgG2a control antibody at 50 or 100µg as indicated. Tumor volumes at day 20 are indicated.

CD39 disruption increased tumor-targeted Ab therapy efficacy

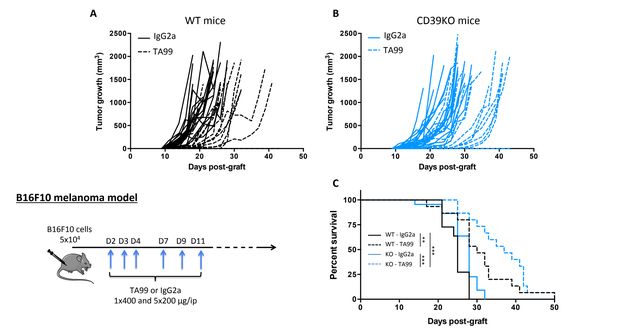


Figure 8: WT or CD39 KO mice were grafted subcutaneously with B16F10 melanoma cells. Mice received TA99 (anti-GP75) or IgG2a control antibody (i.p. injections, 400 µg/mouse on D2 and 200 µg/mouse on D3, D4, D7, D9 & D11). Tumor growth for each individual mouse (A and B) and survival (C) was followed up to day 50.

Conclusions & Perspectives

We demonstrated here that **CD39 is functionally expressed by immune cells from the tumor microenvironment** in 3 different syngeneic tumor mouse models. We further showed that a high percentage of **tumor infiltrating regulatory and effector lymphocytes co-express CD39 and PD-1**, a particular phenotype not seen in spleens and lymph nodes from the same animals.

Using both PD-1 sensitive (MC38) and resistant (MCA205) tumor models we demonstrated that **CD39 deficiency sensitizes to anti-PD-1 treatment**. We further demonstrated that the **antitumor efficacy of CD39 disruption is improved when combined with an immunogenic chemotherapy, leading to complete tumor regression and specific anti-tumor immunity**. In animals presenting PD-1 insensitive tumors, combination of immunogenic chemotherapy, anti-PD-1 antibody and CD39 disruption led to complete and durable responses in most animals.

These results strongly support the development of our humanized CD39 specific blocking antibodies for cancer immunotherapy (Augier S et al., AACR 2016 #3222).

Complete responses mediated by CD39 disruption combined with oxaliplatin are associated with specific anti-tumor immunity and long term protection

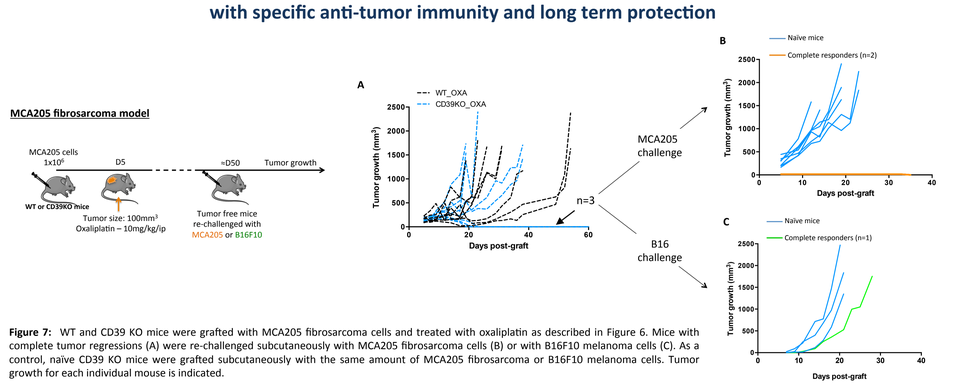


Figure 7: WT and CD39 KO mice were grafted with MCA205 fibrosarcoma cells and treated with oxaliplatin as described in Figure 6. Mice with complete tumor regressions (A) were re-challenged subcutaneously with MCA205 fibrosarcoma cells (B) or with B16F10 melanoma cells (C). As a control, naïve CD39 KO mice were grafted subcutaneously with the same amount of MCA205 fibrosarcoma or B16F10 melanoma cells. Tumor growth for each individual mouse is indicated.