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Inhibition of CD73 Improves B Cell-Mediated Anti-Tumor Immunity in a Mouse Model of Melanoma

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CD73 is a cell surface enzyme that suppresses T cell-mediated immune responses by producing extracellular adenosine. Growing evidence suggests that targeting CD73 in cancer may be useful for an effective therapeutic outcome. In this study, we demonstrate that administration of a specific CD73 inhibitor, adenosine 5′-(α,β-methylene)diphosphate (APCP), to melanoma-bearing mice induced a significant tumor regression by promoting the release of Th1- and Th17-associated cytokines in the tumor microenvironment. CD8+ T cells were increased in melanoma tissue of APCP-treated mice. Accordingly, in nude mice APCP failed to reduce tumor growth. Importantly, we observed that after APCP administration, the presence of B cells in the melanoma tissue was greater than that observed in control mice. This was associated with production of IgG2b within the melanoma. Depletion of CD20+ B cells partially blocked the anti-tumor effect of APCP and significantly reduced the production of IgG2b induced by APCP, implying a critical role for B cells in the anti-tumor activity of APCP. Our results also suggest that APCP could influence B cell activity to produce IgG through IL-17A, which significantly increased in the tumor tissue of APCP-treated mice. In support of this, we found that in melanoma-bearing mice receiving anti-IL-17A mAb, the anti-tumor effect of APCP was ablated. This correlated with a reduced capacity of APCP-treated mice to mount an effective immune response against melanoma, as neutralization of this cytokine significantly affected both the CD8+ T cell- and B cell-mediated responses. In conclusion, we demonstrate that both T cells and B cells play a pivotal role in the APCP-induced anti-tumor immune response. The Journal of Immunology, 2012, 189: 2226–2233.

Cancer cells are able to escape immune surveillance through multiple mechanisms, including the production of immunosuppressive factors in the tumor microenvironment that can impair immune cell function (1). Adenosine plays an important role in the mechanism of tumor escape (2, 3). Adenosine is an ATP-derived nucleoside, highly released during hypoxic conditions typical of tumor microenvironment (4). In this context, cancer cells rapidly degrade ATP into adenosine, which in turn accumulates in the tumor mass (5). Adenosine inhibits T cell proliferation (6) and critically impairs the cytokine production and the cytotoxicity of activated T cells (7, 8), protecting the tumor from immune-mediated destruction (2). Adenosine thus represents an important immunosuppressive molecule in the tumor microenvironment that limits the activation of the immune system to eradicate cancer cells (3, 9).

Extracellular adenosine is produced from the cells by two ectonucleotidases: CD39, which hydrolyzes ATP and ADP into AMP; and CD73, which catalyzes AMP conversion into adenosine. CD73 is the rate-limiting enzyme in this process (10) and is expressed on different cell types, including endothelial and epithelial cells (11), subsets of leukocytes (12), and Foxp3+ regulatory T cells (Tregs) (13). Notably, CD73 is upregulated in several types of cancers (14), and growing evidence suggests that CD73 plays a crucial role in the control of tumor progression. Indeed, it has been demonstrated that inhibition of CD73 activity (15) or CD73 knockdown on tumor cells (16) inhibits tumor growth by enhancing the anti-tumor T cell response. More recently, by using CD73-deficient mice, it has been shown that CD73 on hematopoietic cells (including Foxp3+ Tregs) impairs the anti-tumor T cell-mediated immune response (17, 18). These effects are attributed to the regulation of extracellular adenosine generated by CD73 within the tumor microenvironment (17, 18).

In the current study, we determined the therapeutic anti-tumor efficacy of a specific inhibitor of CD73, adenosine 5′-(α,β-methylene) diphosphate (APCP). We provide new insights into the mechanism(s) underlying the anti-tumor activity of APCP in a mouse model of melanoma. Our results indicate that administration of APCP inhibited tumor growth by promoting a Th1- and Th17-like immune response in the tumor environment. These effects are correlated with a higher presence of tumor-infiltrating CD8+ T cells. Moreover, we show that B cells are also required for the anti-tumor effects induced by APCP, as Ig-producing cells. Indeed, depletion of CD20+ B cells significantly reduced the anti-tumor effects of APCP and the production of APCP-induced IgG2b. Furthermore, we found that the anti-tumor activity of APCP is dependent on IL-17A, which in turn affects the APCP-induced...
cytotoxic immune response and the levels of IgG2b within the melanoma tissue.

Materials and Methods

Mice
C57BL/6j and Athymic Nude-Foxn1null mice were purchased from Harlan Laboratories (Udine, Italy). Mice were maintained in the National Cancer Institute “G. Pascale” Animal Facility (Naples, Italy), according to institutional animal care guidelines, Italian D.L. no.116 of January 27, 1992, and European Communities Council Directive of November 24, 1986 (86/ 609/EEC).

Cell culture and CD19+ B cell isolation
B16-F10 mouse melanoma cells were from American Type Culture Collection (Rockville, MD, Italy). The K1775 murine melanoma cells were kindly provided by Dr. Silvio Hemmi (University of Zurich, Zurich, Switzerland). Cells were cultured in complete DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma-Aldrich, Milan, Italy).

CD19+ B cells were purified from the spleens of naïve C57BL/6j mice by magnetic separation using a CD19+ cell isolation kit according to the manufacturer’s instructions (EasySep Stem Cell; Voden, Milan, Italy). The purity of CD19+ B cells was checked by flow cytometry by using anti-CD19 and anti-B220 Abs (eBioscience, San Diego, CA) and was routinely around 90%. CD19+ B cells were cultured in RPMI 1640 enriched with 10% FBS and treated with APCP (5 μg/ml; Sigma-Aldrich, Milan, Italy) for 24 h. Supernatants were analyzed for cytokine production by ELISA, and cells were stained with the markers MHC class I, MHC class II, and CD20 and analyzed by FACS.

Animal studies
Mice (female at 6–8 wk old) were injected s.c. on the right flank with 3 × 106 B16-F10 cells or with 5 × 104 K1775 cells. APCP (400 μg/mouse) was delivered to the mice by the peritumoral (i.p.) route on day 10 and day 12 after tumor injection. This time point was selected in preliminary studies as it achieved optimal anti-tumor effects. Tumor volume was monitored with a digital caliper and calculated using the formula V = 4/3 × π × (d1/2) × (d2/2), where V = volume (mm3), D = long diameter (mm), and d = short diameter (mm). Mice were sacrificed on day 13 after tumor cell implantation, and melanoma tissues and proximal lymph nodes were isolated for further analyses. In some experiments, an anti-CD73 mAb (TY/23, 10 μg/mouse, i.p.) was administered to melanoma-bearing mice as described for APCP.

In some experiments, an anti-CD20 mAb (rat IgG, 250 μg/mouse in 100 μl PBS; eBioscience) (19, 20) was injected i.p. on the same day that mice received APCP (day 10), and mice were sacrificed on day 13. The anti-CD20 mAb depleted splenic CD20+ B cells by 90% compared with IgG, as previously demonstrated in our laboratory (20).

In other experiments, a neutralizing mAb against IL-17A (clone eBioM17F3, mouse IgG2a, 20 μg/mouse; eBioscience) was injected i.p. every day starting from day 10 until day 13. The anti-IL-17A mAb reduced IL-17A release in the melanoma tissue by ~95% compared with IgG (data not shown).

Cell analysis
Tumors, lymph nodes, and spleens were digested with 1 U/ml collagenase A (Sigma-Aldrich, Milan, Italy). Cell suspensions were passed through 70-μm cell strainers, and RBCs were lysed. The cells were used for flow cytometric analyses (Becton Dickinson FACSCalibur, Milan, Italy). The following Abs were used: CD3–PECy5.5, CD8–allophycocyanin, CD4–allophycocyanin (eBioscience, San Diego, CA; R&D Systems, Abingdon, U.K.; Bethyl Laboratories, Montgomery, TX).

ELISA
IL-17A, TNF-α, IFN-γ, IL-10, TGF-β, and IgG2b were detected in melanoma tissues and supernatants of cultured cells by using mouse-specific ELISA kits (eBioscience, San Diego, CA; R&D Systems, Abingdon, U.K.; Bethyl Laboratories, Montgomery, TX).

Immunohistochemistry
For histological analyses, melanoma tissues were fixed in OCT medium (Pella, Milan, Italy) and cut in 7-μm cryosections. Frozen sections were stained with Ki67 (Abcam, Cambridge, U.K.) or Bcl-2 (Santa Cruz Biotechnology, DBA, Milan, Italy) and detected with FITC anti-rabbit or FITC anti-mouse secondary Abs, respectively. In all staining experiments, isotype-matched IgG and omission of the primary Ab was used as negative control. Slides were analyzed by a fluorescence microscope (Carl Zeiss, Milan, Italy) by means of the Axioplan Imaging Program (Carl Zeiss).

Immunoblot analysis
Tumor tissues were homogenized in RIPA buffer (RIPA Precipitation Buffer). Anti–Bcl-2 (Santa Cruz Biotechnology, DBA, Milan, Italy) or anti-tubulin Abs (Sigma-Aldrich, Rome, Italy) were used. Immunoreactive proteins were quantified by densitometry analysis (GelDoc Instrument).

Statistical analysis
Results are expressed as mean ± SEM. All statistical differences were evaluated by either Student t test or one-way ANOVA, followed by Bonferroni’s posttest as appropriate, and p values <0.05 were considered statistically significant.

Results
APCP-induced tumor regression is associated with increased release of Th17- and Th1-like cytokines
To investigate the effect of CD73 blockade on tumor growth, we used APCP, which has successfully been used in various murine models, including those for cancer (16, 18, 21). C57BL/6j mice were s.c. injected with 3 × 105 B16-F10 cells, and 10 d later mice were treated with APCP (400 μg/mouse, i.p.). The administration of APCP significantly reduced tumor growth in melanoma-bearing mice compared with PBS-treated mice (APCP 65.8 mm3 versus PBS 816.2 ± 259.2 mm3; p < 0.01) (Fig. 1A). To verify the effect of APCP on melanoma growth, we also evaluated the expression of Ki67, a proliferation marker (22). We observed a significant reduction in cells staining for Ki67 when mice were treated with APCP (Fig. 1B, 1C). In addition, expression of Bcl-2, an antiapoptotic protein (23), was reduced in tumor tissue harvested from mice treated with APCP compared with that in tissue sections harvested from mice treated with PBS (Fig. 1D, 1E). Thus, mice receiving APCP exhibited reduced tumor growth compared with control, consistent with previous studies (16, 18, 21). This effect was associated with a reduction in the number of proliferating cells within the tumor and increased susceptibility of cells to apoptosis.

CD73-derived adenosine can modulate the inflammatory response (24); therefore, we analyzed the levels of cytokines (IFN-γ, TNF-α, IL-17A, IL-10, TGF-β) in the homogenates of melanoma tissue harvested from the APCP-treated mice described earlier. Notably, we found that the levels of IL-17A, a proinflammatory cytokine, were significantly increased in the tumor tissue after APCP treatment (Fig. 1F). Mice receiving APCP also showed increased release of the Th1-associated cytokines TNF-α and IFN-γ (Fig. 1G and 1H, respectively), whereas the levels of both IL-10 and TGF-β were not elevated in the tissue of mice treated with APCP (Fig. 1I and 1J, respectively). APCP is a well-known inhibitor of CD73, and this effect is associated with reduced expression of CD73 on tumor cells (25). This effect was confirmed by FACS analysis of K1775 melanoma cells treated with APCP, showing a reduction in the percentage of CD73+ cells (Fig. 1K, 1L). The anti-tumor activity of APCP on melanoma-bearing C57BL/6j mice was accompanied by high production of Th1- and Th17-like cytokines within tumor tissue.

These results indicate that the anti-tumor effect of APCP in melanoma-bearing C57BL/6j mice was accompanied by high production of Th1- and Th17-like cytokines within tumor tissue.
APCP treatment increased tumor-infiltrating B cells

Previous studies showed that tumor growth is inhibited in CD73-deficient mice because of the improved T cell-mediated response (17, 18). Our results described above show that inhibition of tumor growth by APCP administration in melanoma-bearing mice correlated with cytokines associated with Th17- and Th1-like immune responses in the melanoma. Consistent with previous reports (18), the percentage of tumor-infiltrating CD3+CD8+ T cells was increased after APCP treatment (Fig. 3A, 3B), whereas the percentage of CD4+ T cells, NK1.1+ cells, NKT cells, and Foxp3+ Tregs were not altered (Supplemental Fig. 1A–D). Surprisingly, we found that APCP increased the number of infiltrating B cells (CD19+ B220+ cells) within the melanoma tissue (Fig. 3C, 3D). This was associated with increased levels of the IgG2b in the tumor tissue (Fig. 3E), whereas the levels of IgM (PBS 0.155 ± 0.02 ng/mg protein versus APCP 0.113 ± 0.01 ng/mg protein, n = 11) and IgG2a (PBS 1.38 ± 0.21 ng/mg protein versus APCP 1.41 ± 0.35 ng/mg protein, n = 7) were unaltered, and IgG1 and IgG3 were not detectable.

These results indicate that the tumor regression observed in mice receiving the CD73 inhibitor APCP is associated with an increased percentage of tumor-infiltrating CD8+ T cells. Moreover, the data suggest that APCP administration increased the numbers of B cells and the production of IgG2b within the melanoma tissue.
B cell-depleted mice, we analyzed the tumor-infiltrating cells. Neither the percentage of APCP-induced IFN-\(\gamma\)+CD8+ T cells (Fig. 4C) nor IFN-\(\gamma\) levels (Fig. 4D) were significantly affected in B cell-depleted mice after APCP administration. The percentage of IFN-\(\gamma\)+CD4+ T cells, which was similar in all groups, is also shown (Fig. 4C). CD20+ B cell depletion, however, prevented APCP-induced levels of IgG2b within the melanoma compared with IgG-treated mice (Fig. 4E). This suggests that IgG2b-producing B cells significantly contributed to the anti-tumor effects induced by APCP in melanoma-bearing mice.

**FIGURE 2.** Administration of anti-CD73 mAb, TY/23 (10 \(\mu\)g/mouse, p.t.), reduced tumor growth in B16-F10 melanoma-bearing mice (A) and increased the levels of IFN-\(\gamma\) (B) and IL-17 (C) in the tumor tissue. APCP treatment reduced tumor volume in mice bearing K1735 tumors (D) and increased the levels of IFN-\(\gamma\) (E) and IL-17 (F) in the tumor tissue. Results are expressed as mean ± SEM (n = 5). *p < 0.05, **p < 0.01 (one-way ANOVA or Student t test, as appropriate).

**FIGURE 3.** APCP administration promotes both the recruitment of CD8+ T cells and B cells within tumor lesion. Percentage of CD8+ T cells (A) and B cells (C) in tumor tissue by gating on CD3+CD8+ T cells and CD19+B220+, respectively. Representative dot plots are shown in (B) and (D). (E) IgG2b levels detected by means of ELISA in tumor tissue homogenates. Results are from three independent experiments and are expressed as mean ± SEM, n = 10. *p < 0.05 (Student t test).
In B cell-depleted animals, we observed that although anti-CD20 mAb treatment can affect IL-17A production (25, 26), APCP treatment increased the levels of IL-17 in the tumor tissue (Fig. 4F). Moreover, we found that APCP-treated mice had increased tumor-infiltrating IL-17+CD4+ T cells (Fig. 4G). The number of tumor-infiltrating IL-17+CD8+ T cells was similar in all treated groups (Supplemental Fig. 3).

APCP-induced anti-tumor effect is dependent on IL-17A
To understand the role of IL-17A in APCP-induced tumor growth regression, B16-F10–implanted C57BL/6j mice were injected with a neutralizing Ab for IL-17A (20 μg/mouse, i.p.) or IgG control (mouse IgG) every day starting from day 10 after tumor cell implantation (Fig. 5A). Mice were treated with APCP or PBS on day 10 and 12 and sacrificed on day 13 as described earlier (Fig. 5A). Administration of the IL-17A mAb did not alter tumor growth in melanoma-bearing mice (Fig. 5B). In contrast, IL-17A neutralization significantly blocked the anti-tumor effect of APCP (anti–IL-17A mAb plus APCP 704.18 ± 69.8 mm3 versus IgG plus APCP 379.73 ± 78.9 mm3; p < 0.05) (Fig. 5B).

Together these results suggest that blockade of CD73 is associated with high IL-17A production in the tumor environment. Moreover, this cytokine is critical for the observed anti-tumor effect of APCP. Indeed, the results suggest that APCP-induced IL-17A could positively influence both CD8+ T cell- and B cell-mediated responses within the tumor.

APCP did not affect tumor growth in nude mice
We further investigated the effect of APCP on tumor growth in athymic nude mice, which lack T cells. Nude mice were injected with B16-F10 cells, and 10 d later mice were twice administered with APCP as described earlier for C57BL/6j mice. Tumor growth in nude mice was not affected by APCP treatment (APCP 990.4 ± 414.7 mm3 versus PBS 1066.8 ± 520.4 mm3) (Fig. 6A). These results confirm that T cells are required for the APCP-induced regression of melanoma. Additionally, APCP treatment did not modulate B cell activation or IgG2b levels in nude mice (Fig. 6B). These data further support the concept that APCP could indirectly influence B cell activity to produce IgG by inducing inflammatory T cell-associated cytokines, such as IL-17A, which we could not detect in these mice (data not shown).
Discussion

In this study, we provided new insights into the mechanism underlying the anti-tumor activity of APCP, a CD73 inhibitor, in a mouse model of melanoma. Administration of APCP facilitated a local Th1- and Th17-associated cytokine release, which in turn affects tumor cell growth. Similar results were observed using an anti-CD73 mAb. Importantly, we observed that the anti-tumor activity of APCP in mice is mediated, at least in part, by B cells producing IgG2b within the tumor lesion.

Several studies have shown that CD73 via adenosine generation can promote tumor growth in mice. Adenosine derived from CD39 in concert with CD73, expressed both on tumor cells and on host cells (including Tregs), accumulates within tumor tissue dampening anti-tumor T cell immunity (13, 15, 16). Moreover, tumor-associated Tregs, which highly express CD39 and CD73, inhibit Th17 cell development through the adenosinergic pathway (27). The tumor resistance of CD73-deficient mice is associated with an increased influx of CD8+ T cells (18) and low numbers of Tregs within the tumor (21). Of note, anti-CD73 mAb therapy or blockade of CD73 significantly inhibits tumor growth (15, 18) and enhances the efficacy of adoptive T cell therapy (18). In our study, we found that the anti-tumor effect of APCP was associated with a greater presence of melanoma-infiltrating CD8+ T cells. These data further indicate that the anti-tumor activity of APCP in immune-competent mice, bearing B16-F10 melanoma, is T cell-dependent. Accordingly, in nude mice APCP failed to reduce tumor growth. This study is the first, to our knowledge, to demonstrate that B cells are also involved in the anti-tumor effect of APCP in mice.

Several studies have shown that B cells play an important role in the anti-tumor immunity. For example, B cell-deficient mice (28, 29) or mice depleted of B cells (30–32) are protected from tumor proliferation (including Tregs), accumulates within tumor tissue dampening anti-tumor T cell immunity (13, 15, 16). Moreover, tumor-associated Tregs, which highly express CD39 and CD73, inhibit Th17 cell development through the adenosinergic pathway (27). The tumor resistance of CD73-deficient mice is associated with an increased influx of CD8+ T cells (18) and low numbers of Tregs within the tumor (21). Of note, anti-CD73 mAb therapy or blockade of CD73 significantly inhibits tumor growth (15, 18) and enhances the efficacy of adoptive T cell therapy (18). In our study, we found that the anti-tumor effect of APCP was associated with a greater presence of melanoma-infiltrating CD8+ T cells. These data further indicate that the anti-tumor activity of APCP in immune-competent mice, bearing B16-F10 melanoma, is T cell-dependent. Accordingly, in nude mice APCP failed to reduce tumor growth. This study is the first, to our knowledge, to demonstrate that B cells are also involved in the anti-tumor effect of APCP in mice.

Several studies have shown that B cells play an important role in the anti-tumor immunity. For example, B cell-deficient mice (28, 29) or mice depleted of B cells (30–32) are protected from tumor proliferation. These results may be due to the activation status of B cells (33) and/or the immune-regulatory function of B cells (B10 cells), which produce IL-10 (34). In contrast, recent studies demonstrate that B cells facilitate T-mediated responses, which in turn impair tumor development (19, 20). These observations indicate that B cells can significantly contribute to control tumor growth. In addition, activated B cells can mediate significant tu-
CD8+ T cells (Tc17) inhibit B16-F10 melanoma growth (45) and NK cells (43, 44). Similarly, IL-17 produced by cytotoxic Th17 development. It has been reported that hydrolysis of ATP polarization toward Th17-producing cells. This hypothesis is tumor proliferation by facilitating the activation of CD8+ T cells 42). Recent studies also show that Th17 cells protect mice from competent mice through enhanced anti-tumor immunity (41, 45). Further work is needed to assess the importance of the ILg2b-mediated response in the therapeutic activity of APCP. APCP could indirectly affect the in vivo B cell activity to produce IgG by inducing the release of cytokines, such as IL-17, into the tumor microenvironment. IL-17A is a proinflammatory cytokine implicated in the pathogenesis of autoimmunity (38); however, the role of IL-17A in tumor immunity is controversial, as both pro- and anti-tumor effects have been described. In immune-deficient mice, IL-17A overexpressed in tumor cells enhanced tumor growth by promoting angiogenesis (39). Similar results have been obtained in IL7ar+− mice (40). In contrast, other studies demonstrated that IL-17A inhibits tumor growth in immune-competent mice through enhanced anti-tumor immunity (41, 42). Recent studies also show that Th17 cells protect mice from tumor proliferation by facilitating the activation of CD8+ T cells and NK cells (43, 44). Similarly, IL-17 produced by cytotoxic CD8+ T cells (Tc17) inhibit B16-F10 melanoma growth (45).

The current study shows that APCP administration leads to enhanced production of T cell-derived IL-17A within tumor tissue, suggesting that inhibition of CD73 could condition CD4+ T cell polarization toward Th17-producing cells. This hypothesis is supported by previous data on adenosine-induced suppression of Th17 development. It has been reported that hydrolysis of ATP to adenosine or adenosine analogues reduces IL-17 production by CD4+ T cells (46). Notably, Th17 cells in the tumor microenvironment are negatively associated with the presence of Tregs, which suppress Th17 cells through adenosine induction (27). Inhibition of ectonucleotidases, highly expressed on Tregs, recovered T cell IL-17 production (28).

We found that IL-17A blockade prevents the ability of APCP to inhibit tumor growth. This effect was correlated with a reduced presence of CD8+ T cell and reduced IFN-γ production in the melanoma tissue of APCP-treated mice. Although previous data indicate that IL-17A drives T cell recruitment (47), the effects on proliferation and/or survival may also be important. Notably, our results also suggest that APCP-induced IL-17A facilitates the presence of B cells within the tumor tissue and the production of IgG2b. Recent data indicate that IL-17A can positively regulate the humoral immune response. IL-17A promotes germinal center formation and class switch recombination to IgG subclasses (47, 48). Moreover, IL-17A sustains the proliferation of B cells and their differentiation into Ig-secreting cells in systemic lupus erythematosus (49). This supports our concept that APCP-induced IL-17A within the tumor tissue is essential for the regulation of the local B cell response. It is currently unclear what is the relative role of the other cytokines such as TNF-α and IFN-γ, which were elevated in melanoma tissue, in comparison with that of IL-17A in regulating the T cell and B cell recruitment, proliferation, and survival in response to APCP treatment in this model. Further work in this area is needed to elucidate these aspects of the anti-tumor effect of APCP.

In conclusion, our data demonstrate that in addition to T cells, B cells also contribute to the anti-tumor activity of APCP in mice via an IL-17A-mediated process. Thus, pharmacological inhibition of CD73 in the tumor tissue exerts a beneficial therapeutic effect by mounting a protective B cell- and T cell-mediated anti-tumor response.

Disclosures

The authors have no financial conflicts of interest.

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