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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

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Abbreviations used in this article: APCP, adenosine 5'-(α,β -methylene)diphosphate; p.t., peritumoral; Treg, regulatory T cell.

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cytotoxic immune response and the levels of IgG2b within the melanoma tissue.

Materials and Methods

Mice

C57BL/6j and Athymic Nude-Foxn1tm 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/ECC).

Cell culture and CD19⁺ B cell isolation

B16-F10 mouse melanoma cells were from American Type Culture Collection (LGC Standards S.r.l., Milan, Italy), and K1735 mouse 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 naive 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 μ M; 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 3×10^5 B16-F10 cells or with 5×10^5 K1735 cells. APCP (400 μ g/mouse) was delivered to the mice by the peritumoral (p.t.) 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 $V = 4/3 \pi \times (D/2) \times (d/2)^2$, where V = volume (mm^3), 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 μ g/mouse, p.t.) was administered to melanoma-bearing mice as described for APCP.

In some experiments, an anti-CD20 mAb (rat IgG, 250 μ g/mouse in 100 μ l 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 eBioMM17F3, mouse IgG, 20 μ g/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- μ m cell strainers, and RBCs were lysed. The cells were used for flow cytometric analyses (Becton Dickinson FACSCalibur, Milan, Italy). The following Abs were used: CD8-PE, CD3-PECy5.5, CD4-FITC, CD25-PE, Foxp3-PECy5.5, NK1.1-PE, CD11c-FITC, CD19-PECy5.5, and B220-PE (eBioscience). Further characterization was performed by using the following Abs: CD3-PECy5.5, CD8-allophycocyanin, CD4-allophycocyanin, IFN- γ -PE, and IL-17-PE (eBioscience).

ELISA

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

Immunohistochemistry

For histological analysis, melanoma tissues were fixed in OCT medium (Pella, Milan, Italy) and cut in 7- μ m 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 (RIA 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 \pm 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 3×10^5 B16-F10 cells, and 10 d later mice were treated with APCP (400 μ g/mouse, p.t.). The administration of APCP significantly reduced tumor growth in melanoma-bearing mice compared with PBS-treated mice (APCP $254.4 \pm 65.8 \text{ mm}^3$ versus PBS $816.2 \pm 259.2 \text{ mm}^3$; $p < 0.01$) (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 tissue sections 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 CD73 inhibitor, and the possibility of off-target effects in vivo cannot be ruled out. However, similar results were obtained in mice administered with the anti-CD73 mAb TY/23 (Fig. 2A–C).

The anti-tumor activity of APCP was also evaluated in the K1735 tumor model. C57BL/6j mice were s.c. injected with K1735 melanoma cells, and 8 d later APCP was administered as previously described. APCP treatment significantly reduced tumor growth (Fig. 2D). This effect was associated with increased levels of IFN- γ (Fig. 2E) and IL-17A (Fig. 2F) in the 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.

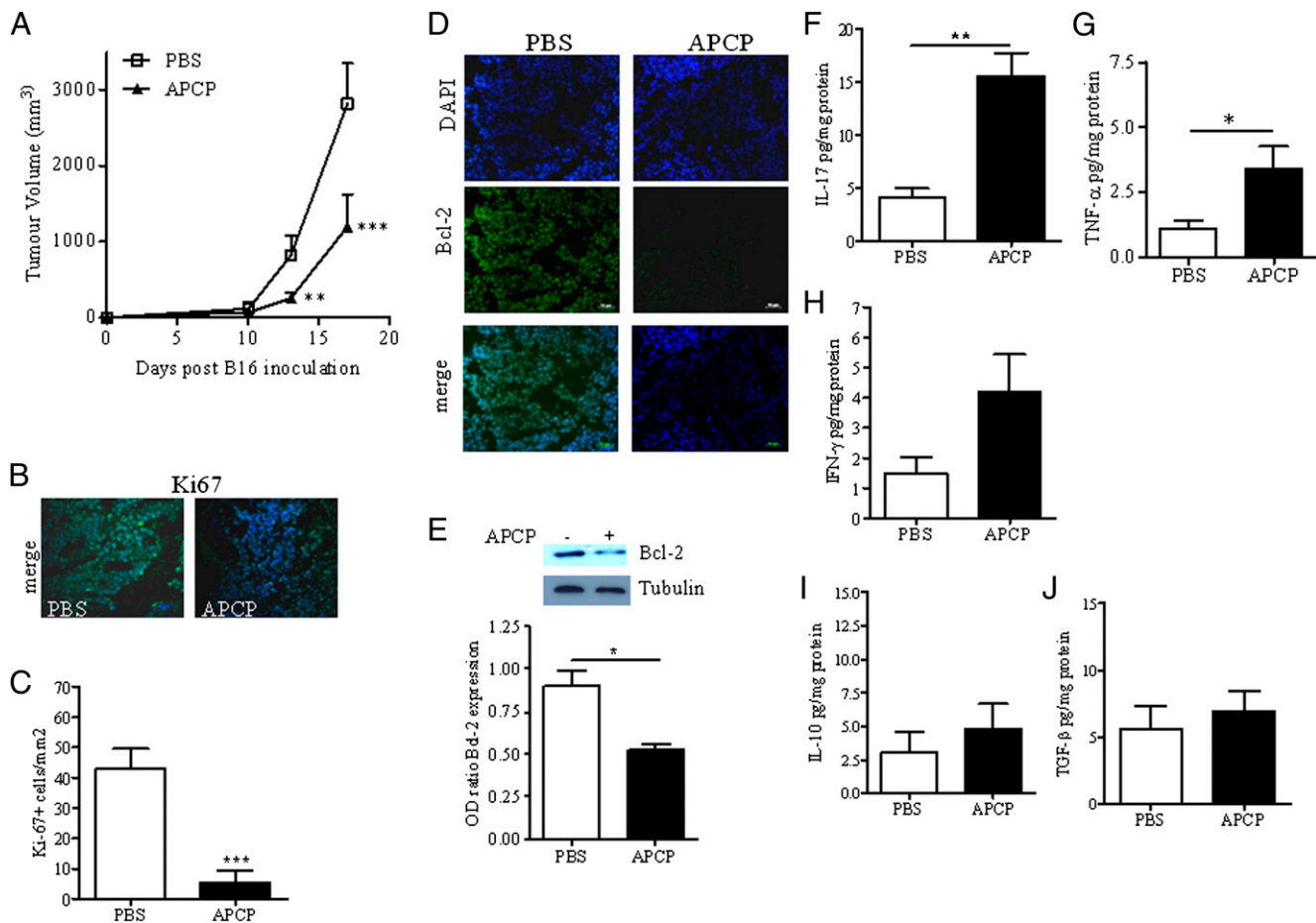


FIGURE 1. APCP administration significantly reduced tumor growth in melanoma-bearing C57BL/6j mice. **(A)** Tumor volume (mm^3) was significantly reduced in mice receiving APCP (400 $\mu\text{g}/\text{mouse}$, p.t.) compared with that in control mice (PBS) ($n = 13$). **(B and C)** Ki67 expression was determined by immunofluorescence staining in melanoma cryosections harvested from PBS- and APCP-treated mice (original magnification $\times 20$) (B) and quantified as number of Ki67⁺ cells per mm^2 of melanoma section by using ImageJ Software (National Institutes of Health) ($n = 6$) (C). **(D and E)** The expression of the antiapoptotic protein Bcl-2 in melanoma cryosections by immunofluorescence staining and in tissue lysates by Western blotting, respectively, of mice treated with PBS or APCP. Images are representative of $n = 5$ (original magnification $\times 20$). **(F–J)** Levels of IL-17A, TNF- α , IFN- γ , IL-10, and TGF- β , respectively, measured in the tissue homogenate of mice treated with PBS or APCP ($n = 13$). Results are from three independent experiments and are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA or Student t test, as appropriate).

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 cells contribute to the anti-tumor effects induced by APCP in melanoma-bearing mice

To determine whether administration of APCP could directly regulate B cell function, we performed in vitro experiments on isolated B cells. CD19⁺ B cells were isolated from spleen of naive C57BL/6j mice and cultured for 24 h with APCP (5 μM) or PBS. APCP treatment did not affect either B cell production of IL-10, IL-17A, and TNF- α (Supplemental Fig. 2A, 2B, and 2C, respectively) or MHC class I, MHC class II, and CD20 expression on B cells in vitro (Supplemental Fig. 2D, 2E, and 2F, respectively). Thus, although APCP was unable directly to influence B cell function in vitro, data obtained in mice indicate that inhibition of CD73 in the tumor environment may affect the humoral immune response.

To assess the role of B cells in the anti-tumor effect of APCP in vivo, we treated mice with APCP or PBS (on day 10 and 12 after B16-F10 injection) after B cell depletion using an anti-CD20 mAb injected i.p. on day 10 (Fig. 4A). The anti-CD20 mAb treatment alone did not significantly affect tumor growth in melanoma-bearing mice (Fig. 4B). The anti-tumor effect of APCP was partially reduced in CD20⁺ B cell-depleted mice compared with IgG plus APCP-treated mice (anti-CD20 mAb plus APCP 617.75 ± 107.1 mm^3 versus IgG plus APCP $299.54.24 \pm 71.37$ mm^3 ; $p < 0.01$) (Fig. 4B). To examine further the effect of APCP in CD20⁺

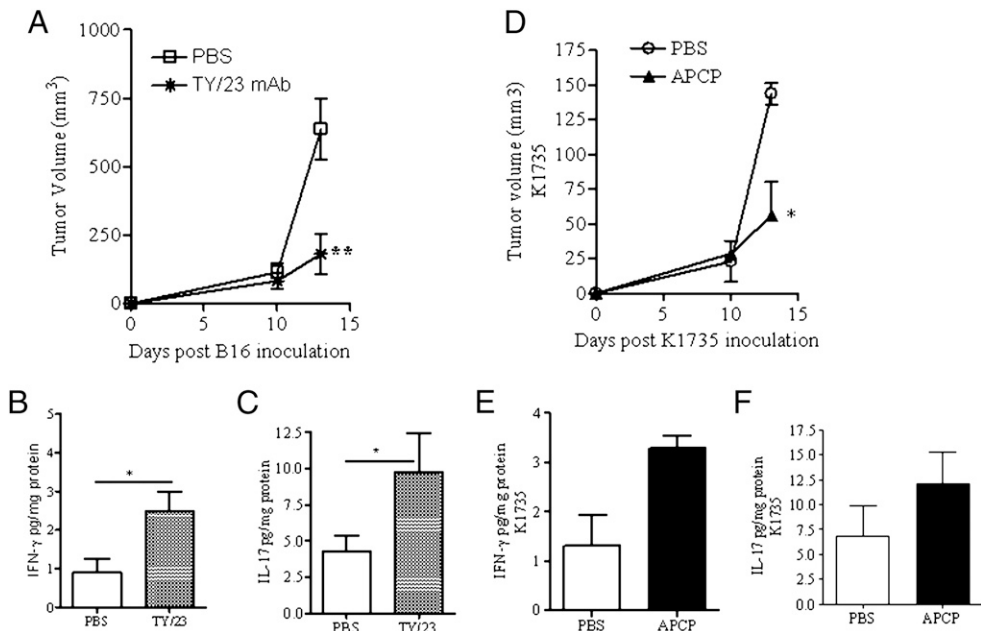


FIGURE 2. Administration of anti-CD73 mAb, TY/23 (10 μ g/mouse, p.t.), reduced tumor growth in B16-F10 melanoma-bearing mice (A) and increased the levels of IFN- γ (B) and IL-17 (C) in the tumor tissue. APCP treatment reduced tumor volume in mice bearing K1735 tumors (D) and increased the levels of IFN-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

B cell-depleted mice, we analyzed the tumor-infiltrating cells. Neither the percentage of APCP-induced IFN- γ ⁺CD8⁺ T cells (Fig. 4C) nor IFN- γ levels (Fig. 4D) were significantly affected in B cell-depleted mice after APCP administration. The percentage of IFN- γ ⁺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.

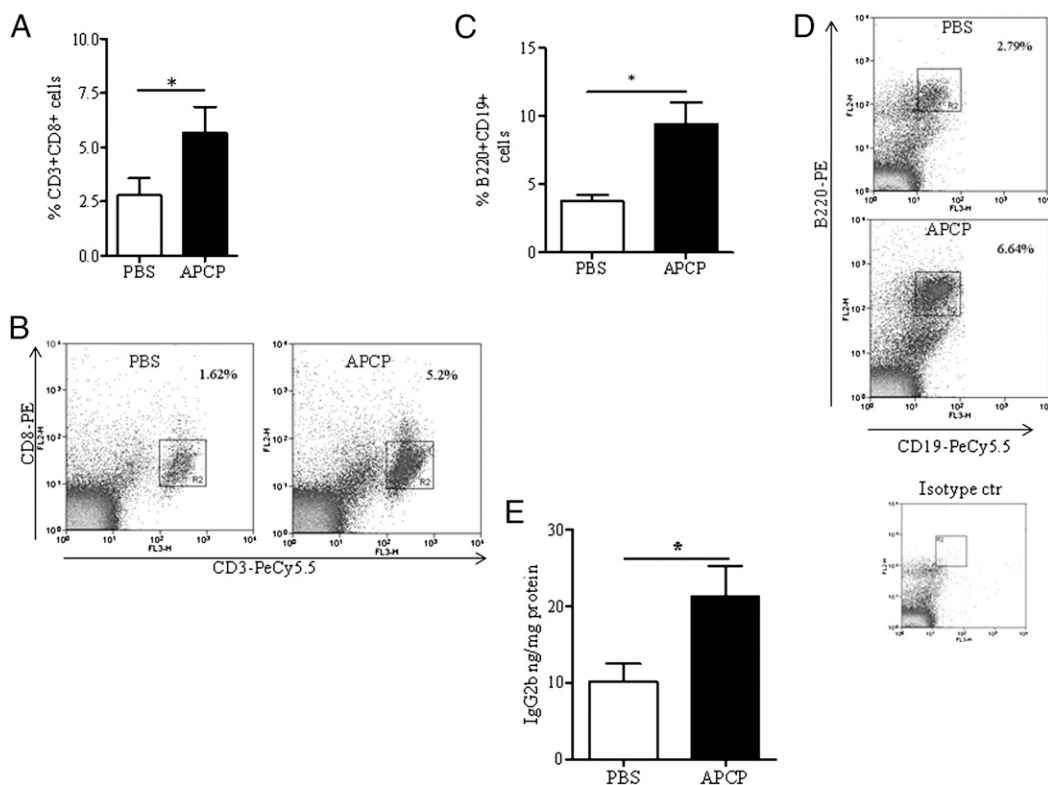


FIGURE 3. APCP administration promotes both the recruitment of CD8⁺ T cells and B cells within tumor lesion. Percentage of CD8⁺ T cells (A) and B cells (C) in tumor tissue by gating on CD3⁺CD8⁺ T cells and CD19⁺B220⁺, respectively. Representative dot plots are shown in (B) and (D). (E) IgG2b levels detected by means of ELISA in tumor tissue homogenates. Results are from three independent experiments and are expressed as mean \pm SEM, $n = 10$. * $p < 0.05$ (Student t test).

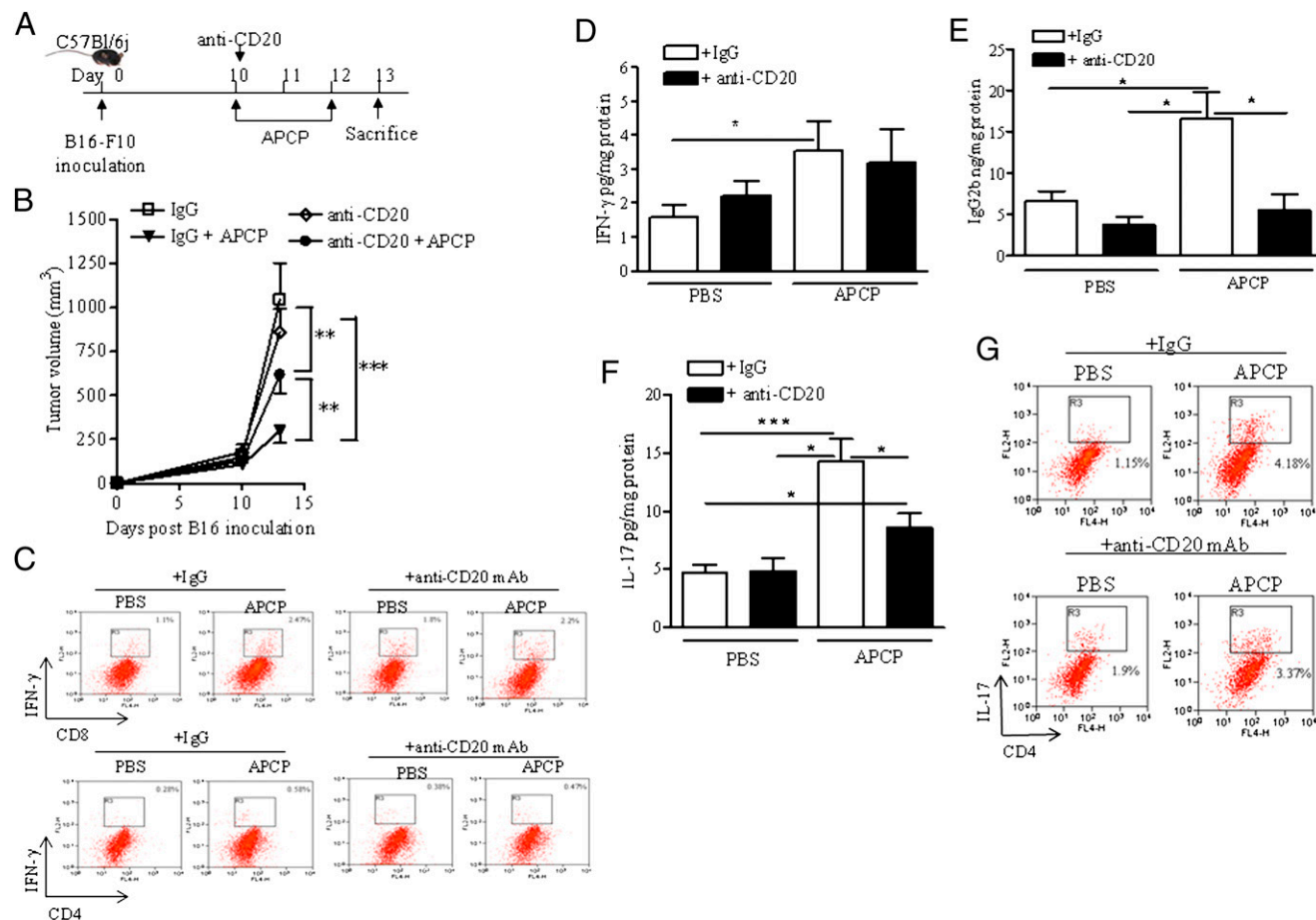


FIGURE 4. Depletion of CD20⁺ B cells reduced the anti-tumor effect of APCP in melanoma-bearing mice. **(A)** Experimental protocol: anti-CD20 mAb (250 $\mu\text{g}/\text{mouse}$, i.p.) was administered on day 10 after B16-F10 tumor cell implantation, when mice received APCP (400 $\mu\text{g}/\text{mouse}$). **(B)** Tumor volume (mm^3) in mice receiving anti-CD20 mAb or isotype control IgG after APCP or PBS administration. Representative dot plots of IFN- γ^+ cells gated on CD3⁺ CD8⁺ T cells or CD3⁺ CD4⁺ T cells are shown **(C)**. **(D-F)** Levels of IFN- γ , IgG2b, and IL-17, respectively, in the melanoma tissue of mice receiving anti-CD20 mAb (black bar) or IgG (white bar) after APCP or PBS administration. **(G)** Representative dot plots for IL-17⁺ gated on CD3⁺ CD4⁺ T cells in the tumor tissue are shown. Results are from three independent experiments and are expressed as mean \pm SEM, $n = 10$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA).

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 $\mu\text{g}/\text{mouse}$, 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 $704.18 \pm 98.4 \text{ mm}^3$ versus IgG plus APCP $379.73 \pm 78.9 \text{ mm}^3$; $p < 0.05$) (Fig. 5B). In addition, both the percentage of tumor-infiltrating CD8⁺ T cells (Fig. 5C) and the production of IFN- γ within tumor tissue (Fig. 5D, Supplemental Fig. 4) were significantly reduced after APCP treatment in IL-17A-depleted mice. Blockade of IL-17A also

significantly reduced both the percentage of B cells (Fig. 5E) and the levels of APCP-induced IgG2b within the tumor mass (Fig. 5F).

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 $990.4 \pm 414.7 \text{ mm}^3$ versus PBS $1066.8 \pm 520.4 \text{ mm}^3$) (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).

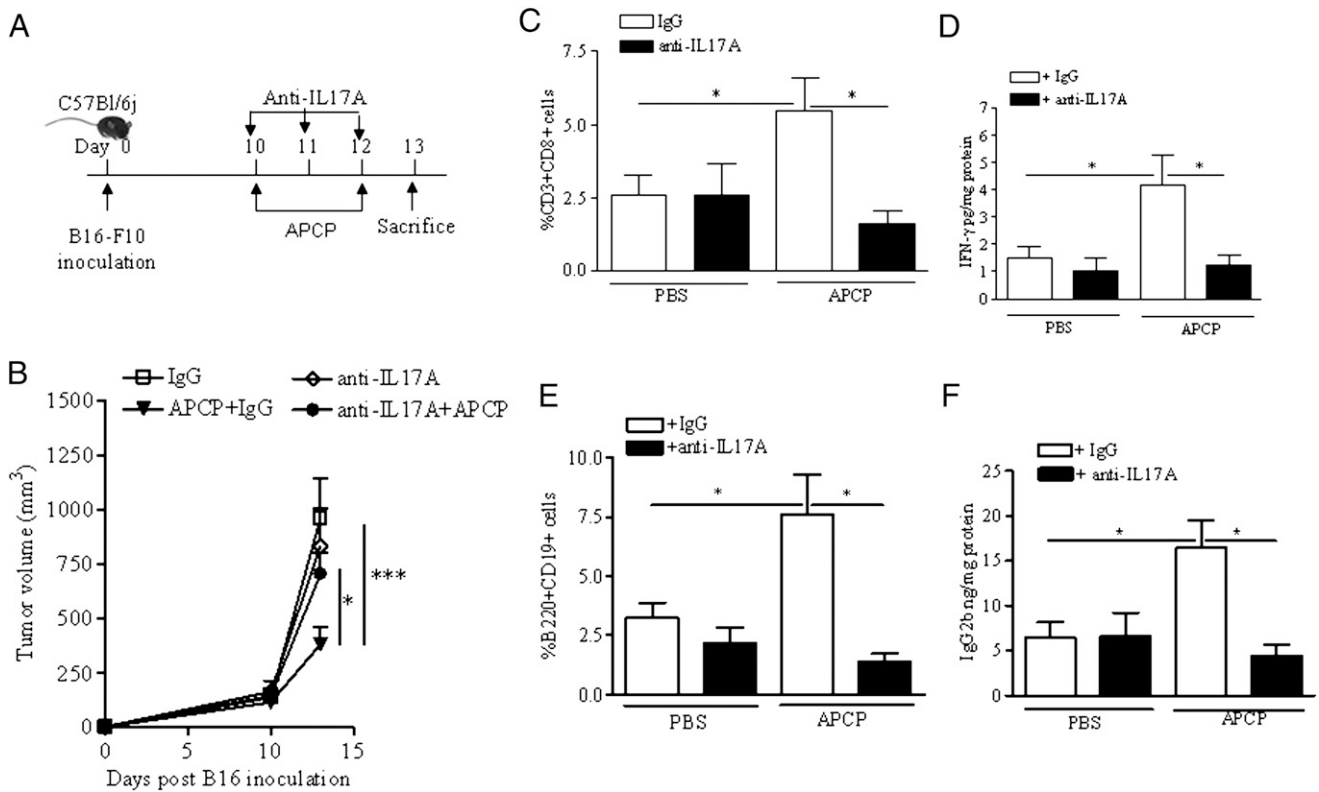


FIGURE 5. The anti-tumor effect of APCP in melanoma-bearing C57BL/6j mice is IL-17A mediated. **(A)** Experimental protocol: anti-IL-17A mAb (20 μ g/mouse, i.p.) was administered every day starting from day 10 after B16-F10 tumor cell implantation, when mice received APCP (400 μ g/mouse). **(B)** Tumor volume (mm^3) in mice receiving anti-IL-17A mAb or isotype IgG control and treated with PBS or APCP. Percentage of CD8⁺ T cells recruited in the tumor tissue **(C)** and levels of IFN- γ in the tumor mass **(D)** of mice receiving isotype IgG control (white bar) or anti-IL-17A mAb (black bar) and treated with PBS or APCP. In **(E)** and **(F)** are reported the percentage of tumor-infiltrating B cells and the tissue levels of IgG2b, respectively, in mice receiving isotype IgG control (white bar) or anti-IL-17A mAb (black bar). Results are from three independent experiments and are expressed as mean \pm SEM, $n = 9$. * $p < 0.05$, *** $p < 0.001$ (one-way ANOVA).

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. 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-

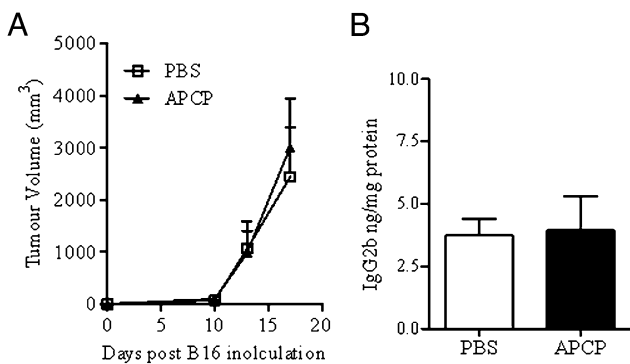


FIGURE 6. APCP administration did not affect tumor growth in nude mice. Athymic nude mice bearing melanoma B16 cells were treated with APCP 10 d after tumor cell implantation. **(A)** Tumor growth after APCP or PBS treatment. **(B)** IgG2b levels in the tumor tissue harvested from nude mice treated with APCP or PBS. Results are from two independent experiments and are expressed as mean \pm SEM, $n = 6$.

mor regression in an IgG2b-dependent manner (20, 35). These latter studies highlight the effector function of B cells as a source of IgG2b that are highly cytotoxic toward tumor cells (35).

Many studies report that the majority of B cells do not express CD73 (12), although some authors have demonstrated that CD73 is expressed on a subset of memory B cells (36), suggesting that CD73-derived adenosine could regulate B cell function (36, 37). However, as yet the role of CD73 in regulating B cell function has not been clearly defined. In the current study, we observed increased numbers of B cells in the melanoma tissue of APCP-treated mice. This result is associated with enhanced production of IgG2b in the tumor mass. Depletion of CD20⁺ B cells markedly reduced the anti-tumor effect of APCP and the level of IgG2b enhanced by APCP, further supporting the notion that B cells mediated the activity of APCP in reducing tumor growth as Ig-producing cells. Further work is needed to assess the importance of the IgG2b-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 *IL17a*^{-/-} 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 (27).

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 (43), 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.

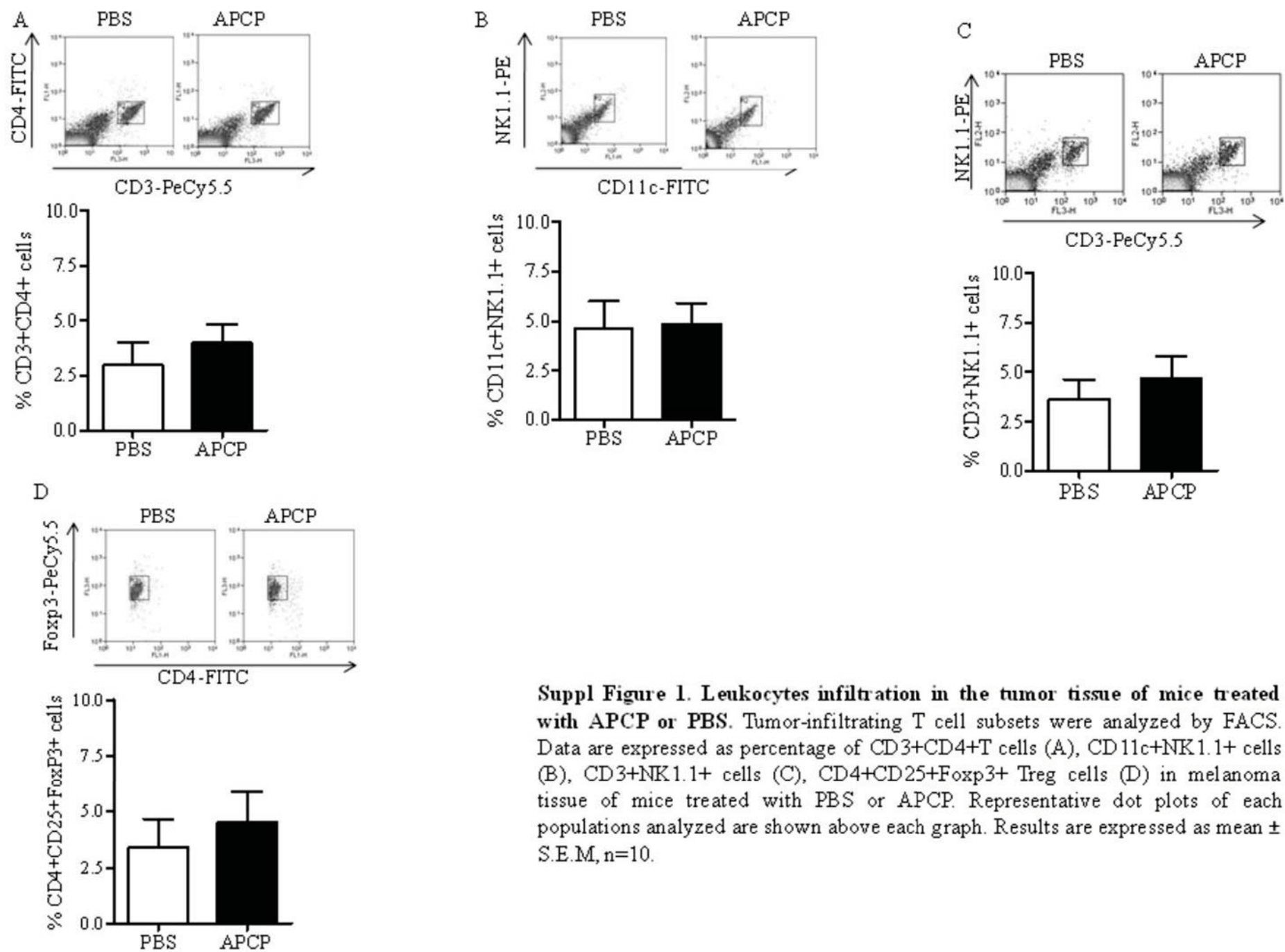
Disclosures

The authors have no financial conflicts of interest.

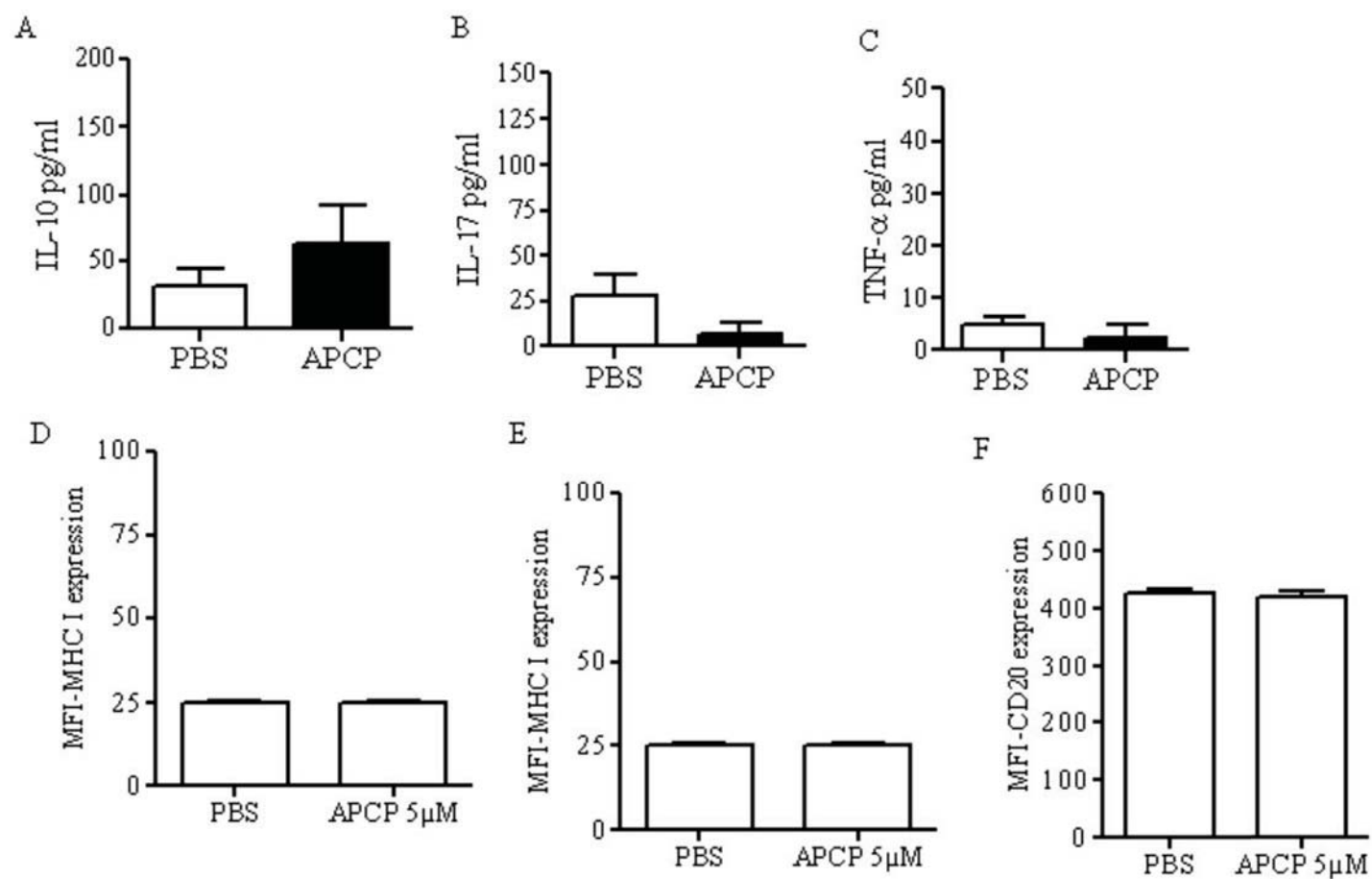
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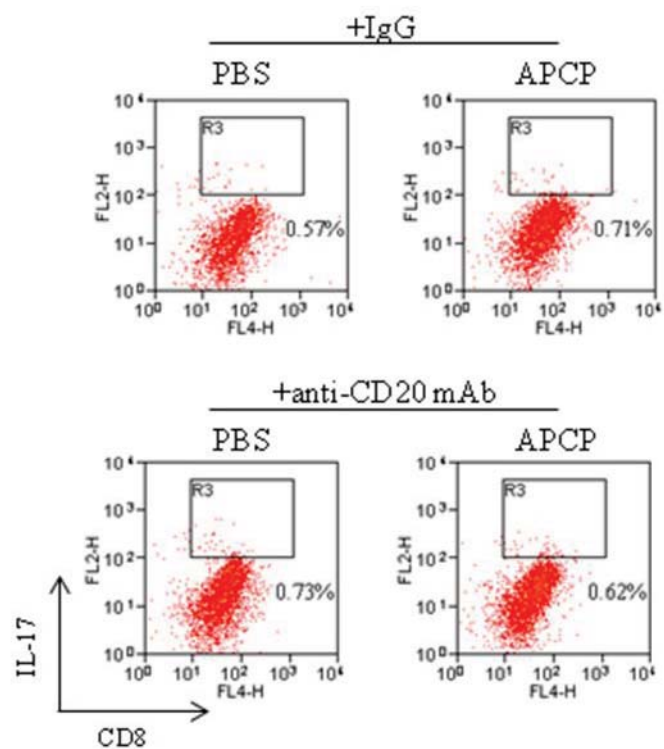
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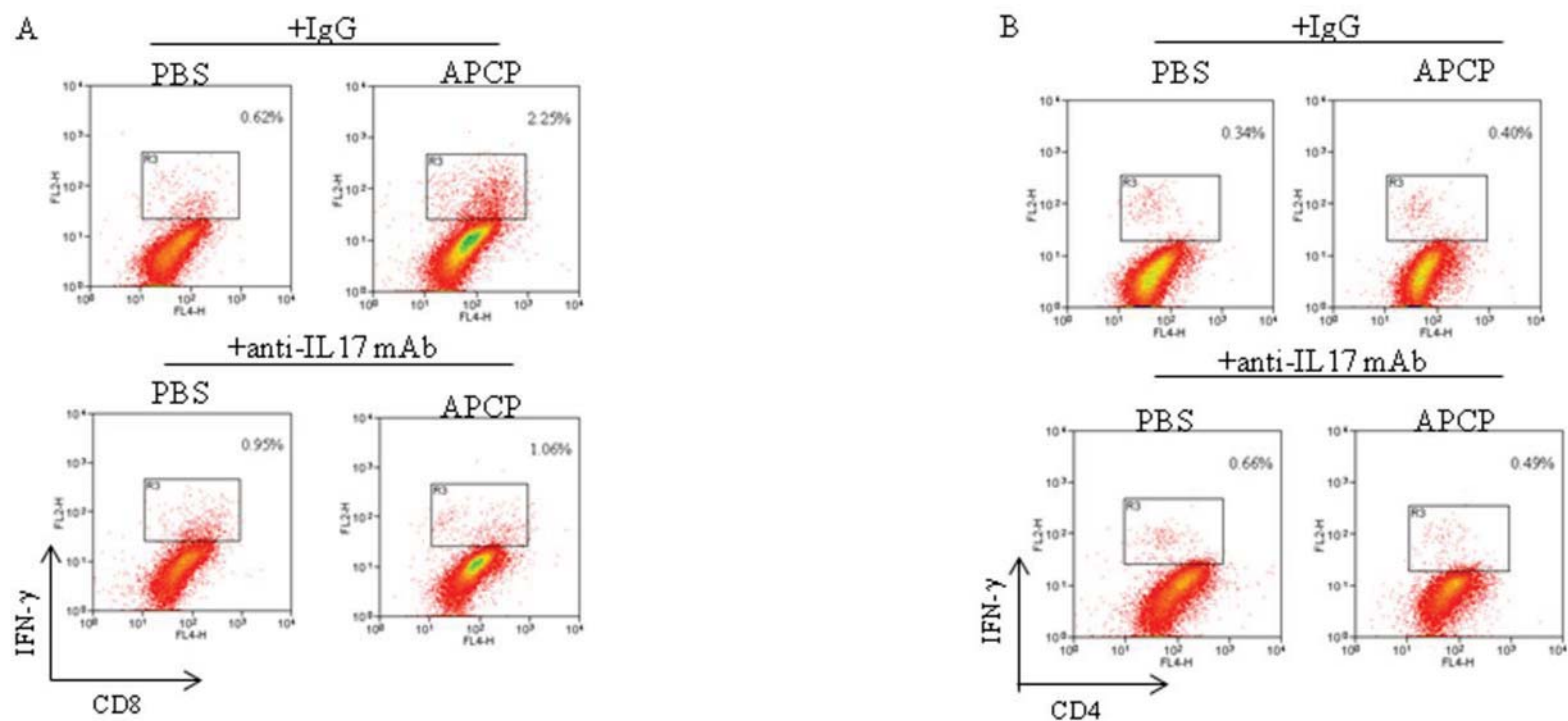
Suppl Figure 1. Leukocytes infiltration in the tumor tissue of mice treated with APCP or PBS. Tumor-infiltrating T cell subsets were analyzed by FACS. Data are expressed as percentage of CD3+CD4+T cells (A), CD11c+NK1.1+ cells (B), CD3+NK1.1+ cells (C), CD4+CD25+Foxp3+ Treg cells (D) in melanoma tissue of mice treated with PBS or APCP. Representative dot plots of each populations analyzed are shown above each graph. Results are expressed as mean \pm S.E.M, n=10.



Suppl. Figure 2. A), B) and C) IL-10, IL-17A and TNF- α levels, respectively, measured by means of ELISA in the supernatant of isolated CD19+ cells treated with APCP or PBS. D), E) and F) MHC class I, MHC class II and CD20 expression, respectively, detected by FACS analysis on isolated CD19+ B cells after treatment with APCP or PBS. Results are expressed as mean \pm S.E.M, n=5.



Suppl. Figure 3. Representative dot plots for IL17+ cells gated on CD3+CD8+ T cells in the tumor tissue of mice treated with APCP or PBS, and injected with an anti-CD20 mAb or isotype IgG control.



Suppl. Figure 4 Representative dot plots of IFN- γ + cells gated on CD3+CD8+T cells (A) and CD3+CD4+T cells (B) analyzed in tumor tissue from mice treated with PBS or APCP and injected with a mAb anti-IL-17 or isotype IgG control.