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Original Article

Adenosine limits the therapeutic effectiveness of anti-CTLA4 mAb in a mouse melanoma model

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Abstract: Combination therapies for melanoma that target immune-regulatory networks are entering clinical practice, and more are under investigation in preclinical or clinical studies. Adenosine plays a key role in regulating melanoma progression. We investigated the effectiveness of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibody (mAb) in combination with either modulators of adenosine receptors (AR) activation or an inhibitor of adenosine production in a murine model of melanoma. We found that treatment with APCP, selective inhibitor of the adenosine-generating nucleotidase CD73, enhanced the activity of anti-CTLA4 mAb, by improving tumor immune response. Blockade of the adenosine A2a receptor (A2aR), which plays a critical role in the regulation of T-cell functions, significantly reduced melanoma growth. Most importantly, combination therapy including an A2aR antagonist with anti-CTLA4 mAb markedly inhibited tumor growth and enhanced anti-tumor immune responses. Targeting A3R and CTLA4 was not as effective in limiting melanoma growth as targeting A2aR. These data suggest that the efficacy of anti-CTLA4 melanoma therapy may be improved by targeting multiple mechanisms of immune suppression within tumor tissue, including CD73 or A2a receptor.

Keywords: CD73, adenosine receptor, CTLA4, melanoma, immunotherapy

Introduction

The co-inhibitory receptor Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) is expressed on the surface of T-cells. CTLA-4 interaction with members of the B7 family on antigen-presenting cells (APCs) modulates T-cell activation. Monoclonal antibodies (mAbs) that block CTLA-4 enhance T-cell proliferation and activation and induce long-term regression of melanoma [1-3]. CTLA4 is also expressed on T regulatory cells (Tregs) and antibodies anti-CTLA4 limit Tregs activity [4]. The anti-human CTLA4 mAb, ipilimumab [5-7], is currently Food and Drug Administration (FDA)-approved for patients with metastatic melanoma. However, the therapeutic benefit of ipilimumab is limited to a small subset of patients [7]. Recent studies demonstrate that the therapeutic outcomes in melanoma patients is improved by combining anti-CTLA-4 mAb with chemotherapy [8] or other immune-modulating agents [9-11]. Of note, the concomitant blockade of different immune-regulatory targets ("immune checkpoints") is a promising useful approach to increase the success of immunotherapy against melanoma. Accordingly, a large number of pre-clinical studies are focused on investigating the immune suppressive mechanisms in the tumor microenvironment that can limit the responsiveness to CTLA4 mAb therapy [2, 12, 13].

Adenosine is an ATP-derived nucleoside, produced in the extracellular compartment by two ectonucleotidases: CD39, which hydrolyzes ATP and ADP into AMP, and CD73, which converts AMP into adenosine. CD73 is expressed both on tumor cells and host immune cells, including Tregs and myeloid-derived suppressor cells [14, 15]. Adenosine is known to inhibit T-cell proliferation [16] and reduce cytokine production and cytotoxicity of activated T-cells [17, 18], via A2a receptor subtype activation, protecting the tumour from immune-mediated destruction [19]. Previously, we [20, 21] and others [22-24] have demonstrated that pharmacological inhi-

bition of CD73 significantly delayed melanoma growth in mice in a T-cell-dependent manner. This effect is most likely dependent on decreased adenosine-mediated effects via A2aR.

In recent years, investigations have focused on the role of the adenosine A3R in cancer progression. In contrast to A2aR, stimulation of A3R induces an efficient anti-tumor immune response dependent on T-cells and NK cells [25-27]. Indeed, A3R selective agonists have shown promising therapeutic effects in tumor-bearing animals, including melanoma [28].

This study investigated whether modulation of adenosine generation in the tumor tissue, by inhibition of CD73, can increase the anti-tumor activity of anti-CTLA4 mAb in a well-established mouse melanoma model. We found that pharmacological inhibition of CD73 in combination with anti-CTLA4 mAb significantly inhibit melanoma growth. Furthermore, we compared the therapeutic potential of targeting adenosine receptors A2a or A3, that play pivotal roles in the regulation of adenosine-mediated immunemodulatory effects in cancer, in combination with anti-CTLA4 mAb. Blocking of A2a adenosine receptor combined with anti-CTLA4 mAb significantly enhanced the response to anti-CTLA4 mAb therapy compared with control and mAb alone. These data are translationally relevant to the development of new combinatorial therapies against melanoma.

Material and methods

Mice and cells

Female C57Bl6j mice (6-8 weeks old) were purchased from Harlan (Harlan Laboratories, Udine, Italy). All the experiments were conducted according to Institutional animal care guidelines, Italian D.L. no.116 of 27 January 1992 and European Communities Council Directive of 24 November 1986 (86/609/ECC). The protocol was approved by the Committee on the Ministero della Salute, DG Sanità Animale e Farmaci Veterinari. All procedures were performed under gaseous anesthesia and mice were sacrificed by cervical dislocation. All efforts were done to minimize suffering. B16-F10 murine melanoma cell line was purchased from American Type Culture Collection (LGC Standards S.r.l., Milan, Italy).

Antibodies and chemicals

Affinity purified anti-mouse CTLA-4 mAb (clone 9H10) was purchased from eBioscience (eBioscience, San Diego, CA, USA).

For cell staining the following anti-mouse antibodies were used: CD3-PeCy5.5; CD8-allopycocyanin or CD8-PE; CD4-allopycocyanin or CD4-FITC; CD25-PE; FoxP3-PeCy5.5 (all eBioscience). Anti-mouse CD16/CD32 (eBioscience) was used to block non-specific Fc-mediated interactions.

Adenosine 5'-(α,β-methylene) diphosphate (APCP) and ZM241365 were purchased from Sigma-Aldrich, Milan, Italy. Cl-IB-MECA was purchased from Tocris Cookson Ltd., London, UK.

Experimental in vivo procedures

B16-F10 murine melanoma cells ($2x10^5$/mouse) were subcutaneously injected on the right flank of anesthetized mice at day 0 and treated at day 7, 9 and 11 with anti-CTLA4 Ab (100 μg/mouse) [2, 29] or APCP (400 μg/mouse) [20] or ZM241365 (40 μg/mouse) [30, 31] or Cl-IB-MECA (20 ng/mouse) [26, 27]. Phosphate-buffered saline alone was used as vehicle control for all drugs. Hamster IgG (eBioscience) was used as control. Anti-CTLA4 mAb was delivered to mice intraperitoneally (i.p.). APCP, ZM241365 or Cl-IB-MECA was injected peritumorally (p.t.). Tumor growth was monitored by measuring perpendicular diameters, as previously reported [20, 26, 27]. Mice were sacrificed at day 14 to isolate melanoma tissues for further analyses. For long-term experiments, mice were euthanized according to the animal care protocol when the tumor volume reached ~1000 mm^3.

Tumor infiltration analysis

To assess tumor-infiltrating cells by flow cytometric analysis, tumor tissues harvested from mice 14 days after tumor cells implantation were digested with 1 U/ml collagenase A (Sigma-Aldrich) and red blood cells were lysed. Cell suspensions were then passed through 70 μm cell strainers and blocked with anti-mouse CD16/CD32 antibody. Cells were stained with mouse-specific antibodies as reported above. For intracellular staining cells were incubated with antibodies after fixation/permeabilization (eBioscience). Data were acquired with FACS-Calibur flow cytometer (BD Biosciences).

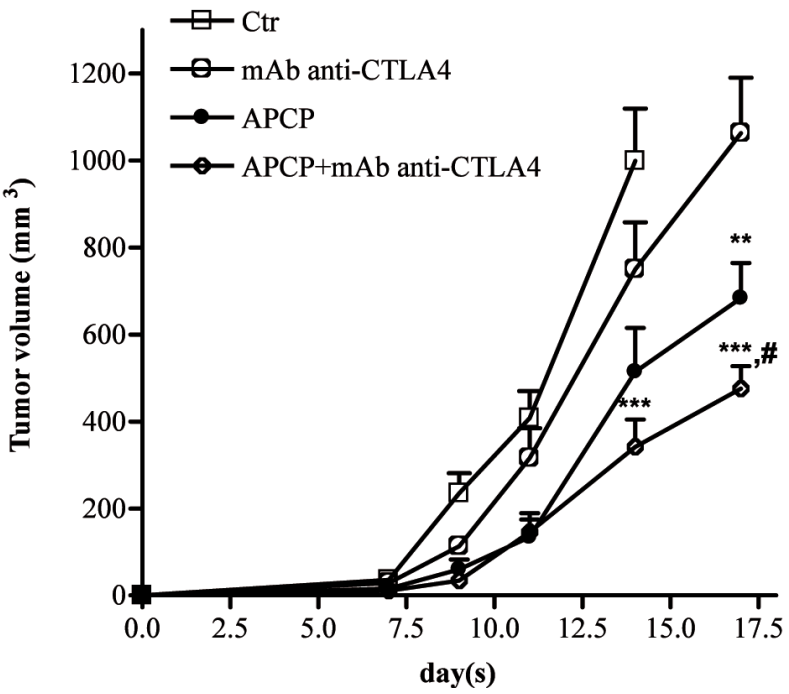

Figure 1. Anti-tumor activity of anti-CTLA4 mAb in combination with APCP. Melanoma-bearing mice were treated on day 7, 9 and 11 with APCP (400 μg/mouse, p.t.) and/or anti-CTLA4 mAb (100 μg/mouse, i.p.). Data are from three independent experiments and represent mean ± SEM (n=8-16). **p<0.01; ***p<0.001 versus anti-CTLA4 mAb; #p<0.05 versus APCP as determined by two way ANOVA analysis.

Cytokine analysis

IFN-γ and Granzyme B were analyzed by ELISA kits (R&D Systems, Abingdon, UK and eBioscience) in melanoma tissue homogenates obtained after digestion.

Statistical analysis

Data are from at least two independent experiments and results are expressed as mean ± SEM (n=8-16). All statistical differences were evaluated by either two-tailed Student's *t* test or one way ANOVA analysis or two way ANOVA analyses as appropriate. *P* values <0.05 were considered statistically significant.

Results

Inhibition of CD73 increases the anti-tumor activity of anti-CTLA4 mAb in melanoma-bearing mice

To study the effects of adenosine on the anti-tumor activity of anti-CTLA4 mAb, we used C57Bl6j mice subcutaneously injected with B16.F10 melanoma cells. Melanoma-bearing mice were treated with the selective CD73 inhibitor APCP (400 μg/mouse, p.t.) and/or anti-CTLA4 mAb (100 μg/mouse, i.p.). Our previous study showed that inhibition of CD73 with APCP in the tumor tissue significantly reduced melanoma growth [20]. Anti-CTLA4 mAb did not affect tumor growth in the B16.F10 melanoma model (**Figure 1**), consistent with previous studies [12, 13, 32]. However, mice treated with both APCP + anti-CTLA4 mAb displayed significantly decreased tumor growth compared with control, and APCP or anti-CTLA4 alone (**Figure 1**).

To acquire more insight about the mechanism of the anti-tumor effect of APCP in combination with anti-CTLA4 mAb, we analyzed T-cells in tumor tissue by flow cytometry. In APCP-treated mice the percentage of tumor-infiltrating CD8+T-cells increased compared with control mice (**Figure 2A**) and it was similar to those observed in mice treated with both blockers (**Figure 2A**). Combination therapy with APCP and anti-CTLA4 mAb increased the percentage of tumor-infiltrating CD4+T-cells (**Figure 2B**); whilst the levels of Tregs were markedly reduced in all treated groups (**Figure 2C**). Accordingly, the intratumoral CD8+T-cells to Tregs ratios were significantly enhanced in mice treated with combined therapy APCP/anti-CTLA4 mAb, compared to control (**Figure 2D**). CD4+T-cells to Tregs ratios in the tumor were also increased in combination regimen (**Figure 2E**), due to both decrease of Tregs and increase of CD4+T-cells after treatment with APCP + anti-CTLA-4 mAb. Cytokine analysis by ELISA revealed increased levels of IFN-γ in melanoma tissue of mice treated with APCP or APCP in combination with anti-CTLA4 mAb compared to control or anti-CTLA4 mAb alone (**Figure 2F**).

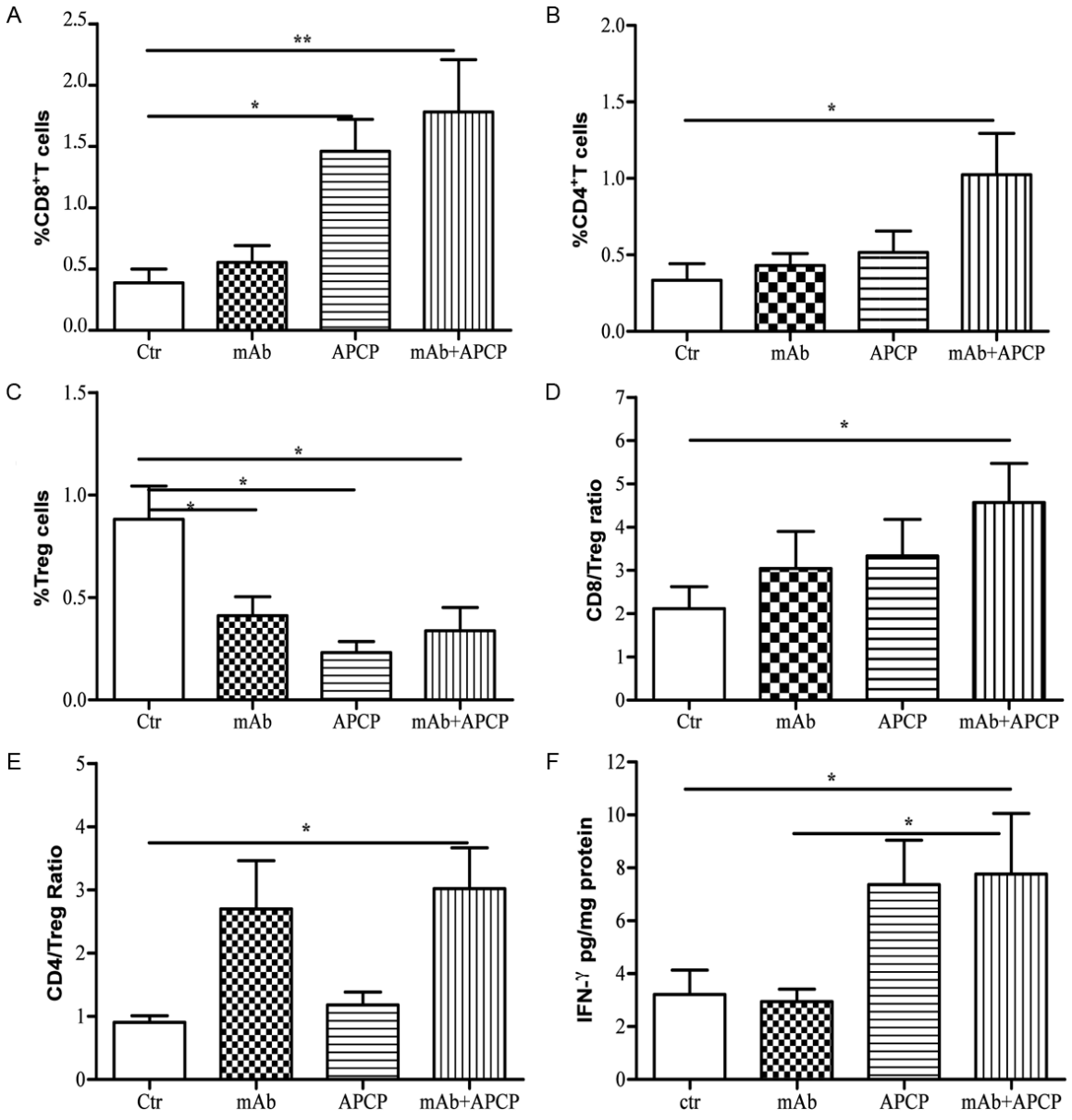

Figure 2. Analysis of T-cells and cytokines in mice treated with APCP and/or anti-CTLA4 mAb. Percentage of tumor-infiltrating CD8+T-cells (identified as CD3+CD8+T-cells) (A), CD4+T-cells (as CD4+Foxp3-cells) (B) and Treg cells (identified as CD25+CD4+Foxp3+cells) (C). (D) CD8+T cells and (E) CD4+T-cells to Treg ratios. (F) Levels of IFN-γ measured in the tumor tissue by ELISA. Data are from two independent experiments and represent mean ± SEM (n=8-12). *P<0.05 and **p<0.01 as determined by one way ANOVA analysis.

Together, these results show that the combination of APCP and anti-CTLA4 mAb is effective in limiting tumor growth in B16 melanoma model.

Blockade of A2aR enhances anti-CTLA4 mAb efficacy

Adenosine A2aR plays a pivotal role in mediating immune-suppressive effects in cancer [19, 33-38]. To evaluate the role of A2aR in anti-CTLA4 therapy, we examined the anti-tumor activity of A2aR antagonist, ZM241365 (40 μg/mouse, p.t.), in combination with anti-CTLA4 mAb. Melanoma-bearing mice treated with ZM241365 alone showed a marked tumor growth inhibition compared with controls (**Figure 3**). The combination therapy showed significant tumor growth delay compared with control or either agent alone (**Figure 3**). This effect was associated with increased levels of

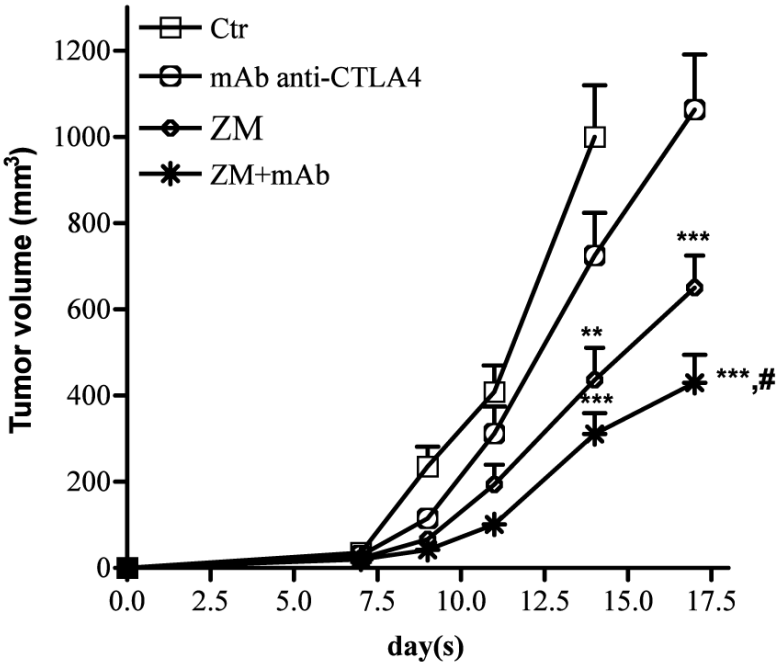

Figure 3. Anti-tumor activity of anti-CTLA4 mAb in combination with ZM241365. Melanoma-bearing mice were treated on day 7, 9 and 11 with ZM241365 (40 μg/mouse, p.t.) (referred in figure as ZM) and/or anti-CTLA4 mAb (100 μg/mouse, i.p.). Data are from three independent experiments and represent mean ± SEM (n=8-14). **p<0.01; ***p<0.001 versus anti-CTLA4 mAb; #p<0.05 versus ZM241365 as determined by two way ANOVA analysis.

tumor-infiltrating CD8+T-cells (**Figure 4A**) and reduced accumulation of Tregs in tumor tissue (**Figure 4B**). Importantly, mice treated with ZM241365 alone showed increased infiltration of CD8+T-cells (**Figure 4A**) and reduced levels of Tregs within melanoma tissue (**Figure 4B**). CD8+T cells to Tregs ratios were elevated in mice treated with both ZM241365 and anti-CTLA4 mAb (**Figure 4C**). The levels of CD4+T-cells in treated mice were not significantly altered compared with control (data not shown).

Cytokine analysis showed that the levels of both IFN-γ and granzyme B were elevated in tumor tissue after combination therapy with ZM241365 and anti-CTLA4 mAb (**Figure 4D** and **4E**, respectively). These data show that a combination therapy including CTLA4 blockade with ZM241365 significantly retarded melanoma growth compared to single-agent regimens. This effect was associated with an increase in the frequency of CD8+T-cells in tumors, while tumor infiltration of Tregs significantly decreased. Treatment with anti-CTLA4 mAb and ZM241365, which was administered peritumorally, did not show any systemic toxic effect in mice, such as drug-related death and body weight loss (data not shown).

Effect of A3 adenosine receptor stimulation on anti-tumor activity of anti-CTLA4 mAb

The peritumoral administration of the selective agonist of A3R, Cl-IB-MECA, significantly inhibits tumor growth in melanoma-bearing mice [27]. Prompted by these results, we analyzed whether anti-tumor activity of CTLA4 blockade could benefit from Cl-IB-MECA administration. However, in contrast with the promising results of the combinations of APCP or ZM241365 with anti-CTLA4 mAb, the combination of Cl-IB-MECA with anti-CTLA4 mAb did not cause any additional benefit in limiting melanoma growth compared with Cl-IB-MECA alone (**Figure 5**). CD8+T-cells to Tregs ratios and CD4+T-cells to Tregs ratios were unchanged in mice treated with combination therapy Cl-IB-MECA + anti-CTLA4 mAb, compared with control or single agents (data not shown). These results suggest that Cl-IB-MECA administration efficiently inhibits melanoma growth by improving anti-tumor T-cell response, but does not enhance the therapeutic response to anti-CTLA4 mAb in this melanoma model.

Discussion

Melanoma is a potentially lethal tumor, highly resistant to most chemotherapeutics. Immunotherapy against metastatic melanoma has shown encouraging results. The recently FDA-approved anti-CTLA4 antibody ipilimumab improves overall survival in patients with metastatic melanoma [7, 39]. However, cures remain rare and unpredictable. Targeting multiple immune checkpoints in tumor microenvironment may further improve the effectiveness of melanoma immunotherapy, improving response rates in patients [9-11].

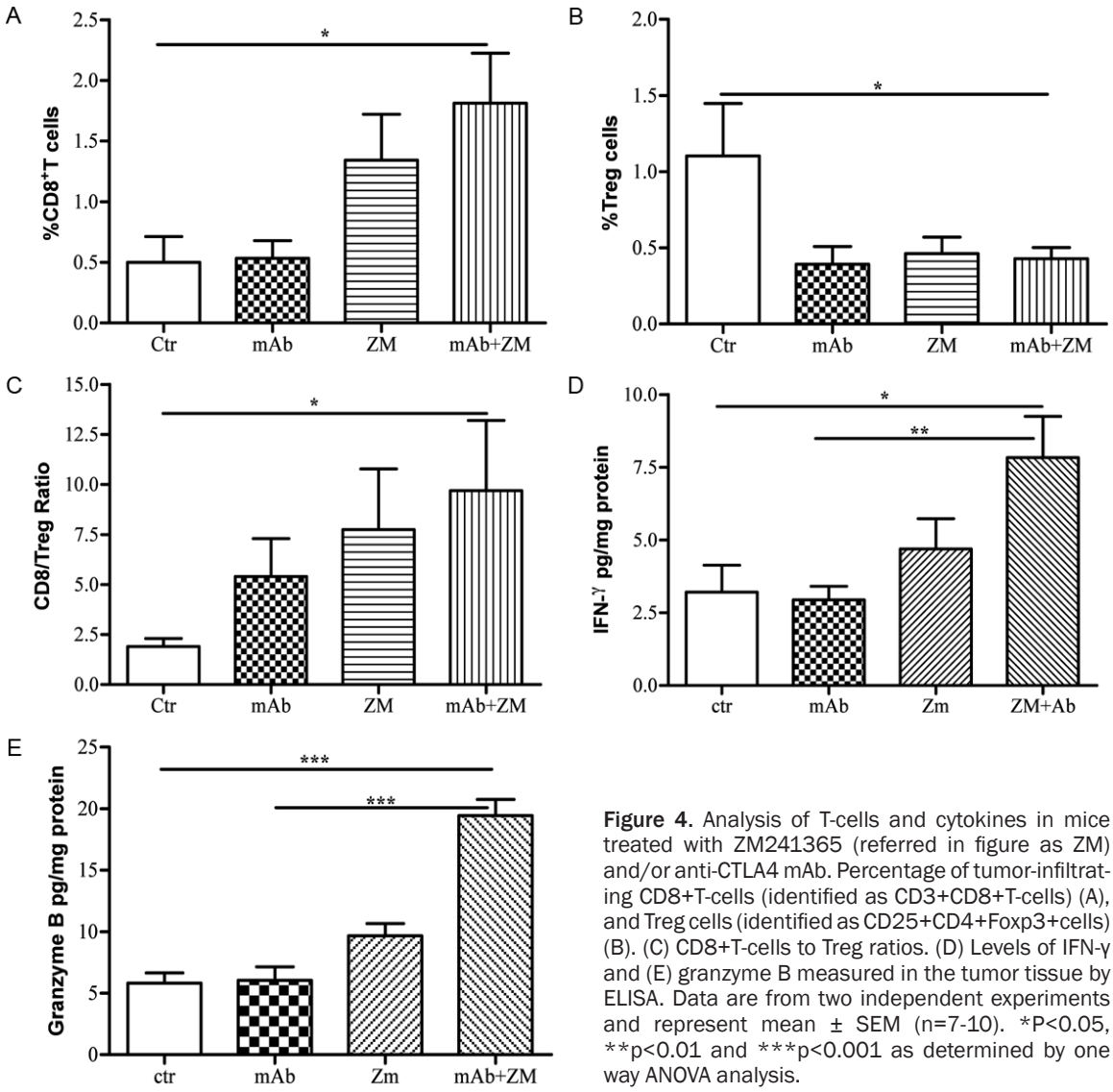

Figure 4. Analysis of T-cells and cytokines in mice treated with ZM241365 (referred in figure as ZM) and/or anti-CTLA4 mAb. Percentage of tumor-infiltrating CD8+T-cells (identified as CD3+CD8+T-cells) (A), and Treg cells (identified as CD25+CD4+Foxp3+cells) (B). (C) CD8+T-cells to Treg ratios. (D) Levels of IFN-γ and (E) granzyme B measured in the tumor tissue by ELISA. Data are from two independent experiments and represent mean ± SEM (n=7-10). *P<0.05, **p<0.01 and ***p<0.001 as determined by one way ANOVA analysis.

In this study we examined the anti-melanoma effects of simultaneously blocking CTLA4 and modulators of the adenosinergic system. Both pathways are critically involved in the regulation of T-cell effector functions. CD73 inhibitor APCP proved to significantly limit tumor growth in mice. Combination of APCP with CTLA4 blockade resulted in an enhanced melanoma growth delay compared to either single agent. Increasing evidence indicate that CD73, expressed on tumor cells, promotes tumor growth by producing adenosine [22, 24]. Adenosine is also generated by Tregs, which highly express CD73 on their surfaces [14]. CD73-deficient mice are tumor-resistant and show an increased influx of CD8+T-cells [22] and low numbers of Tregs within tumor tissue [22]. Our data show that tumor growth inhibition by APCP in combination with anti-CTLA4 mAb was associated with elevated levels of IFN-γ and enhanced infiltration of CD8+T-cells and CD4+T-cells within tumors. We also found that combination therapy significantly reduced the number of Tregs. As a result, the intra-tumor ratio of effector T-cells to Tregs was also increased. These results suggest that adenosine generated by CD73 may limit the therapeutic effectiveness of CTLA4 blockade, by hampering tumor infiltration by effector T-cells, while favoring that of Tregs. Consistent with our results, a paper published while we were preparing our manuscript shows that in other mouse tumor models blockade of CD73 enhances the anti-tumor activity of anti-CTLA4

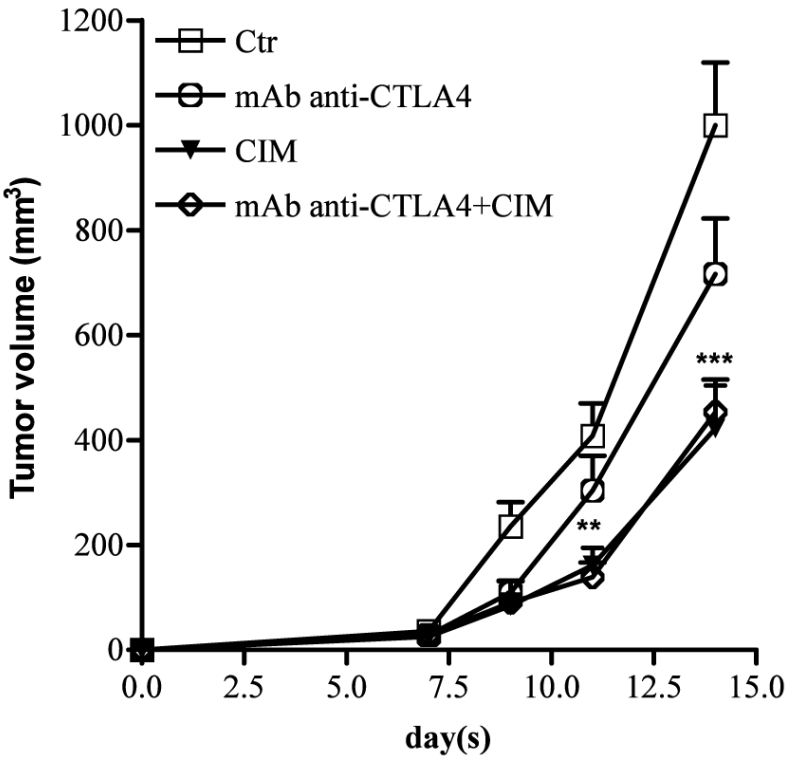

Figure 5. Anti-tumor activity of anti-CTLA4 mAb in combination with Cl-IB-MECA. Melanoma-bearing mice were treated on day 7, 9 and 11 with Cl-IB-MECA (20 ng/mouse, p.t.) and/or anti-CTLA4 mAb (100 μg/mouse, i.p.). Data are from three independent experiments and represent mean ± SEM (n=13). **p<0.01 as determined by two way ANOVA analysis.

and anti-PD-1 mAbs [40]. In the clinic, CTLA4 mAbs are particularly effective in melanoma, a tumor where immunotherapy is one of the most promising treatment modalities. Therefore, it was important to test whether CD73 inhibition increases the effectiveness of CTLA4 blockade is a well-established, highly aggressive melanoma model that mimics advanced metastatic disease. Our results strongly confirm that targeting CD73 can potentiate the anti-tumor activity of immunotherapeutic agents, including anti-CTLA4 mAb. The mechanism whereby adenosine blockade potentiates melanoma immunotherapy is still incompletely understood. Our results indicate that pharmacological modulation of the adenosine receptor A2a can increase the activity of anti-CTLA4 mAb. Blockade of A2aR in melanoma-bearing mice significantly reduced tumor growth. Importantly, combination therapy with CTLA4 blocking mAb and ZM241365 exhibited the best therapeutic results, suggesting an alternate therapeutic modality to CD73 inhibition. In combination-treated mice we observed increased infiltration of CD8+T-cells, inflammatory cytokine production and enhanced ratios of CD8+T-cells relative to Tregs in tumors. Adenosine generation by CD73 mediates immune suppression, mainly mediated by the activation of A2aR, which has the highest affinity for adenosine and is up-regulated on effector T-cells [41]. A2aR stimulation of T-cells inhibits T-cell receptor (TCR)-triggered effector functions, including proliferation, expansion and secretion of cytokines [16-18]. Moreover, A2aR activation suppresses CD8+T-cell cytolytic activity [33]. A2aR-deficient mice reject tumor cells in a T-cell-dependent manner and show increased responsiveness to T-cell adoptive transfer [19] and tumor vaccination [36]. Our study supports the therapeutic potential of A2aR antagonists to increase the effectiveness of melanoma immunotherapy. It is important to note that the A2aR antagonist ZM241385 likely blocks also A2bR. A2bR may contribute to the immune-suppressive effect of adenosine in cancer [42]. A2bR activation causes the release of pro-angiogenic factors that facilitate tumor progression [43]. However, in our hands A2bR blockade with a highly selective A2bR antagonist in combination with anti-CTLA4 mAb was not more effective than either agent alone (unpublished results, manuscript in preparation).

In contrast to the results obtained combining CTLA4 blockade with APCP or ZM241365, the combination with Cl-IB-MECA, a selective A3R agonist, did not show any therapeutic benefits compared with Cl-IB-MECA alone. The therapeutic potential of Cl-IB-MECA as anti-cancer agent has been examined both *in vitro* and *in vivo* studies [28]. We have recently demonstrated that the anti-tumor activity of Cl-IB-MECA in melanoma-bearing mice is dependent on CD8+T-cells and NK cells [26]. Cl-IB-MECA also

improved the activity of T-cell adoptive transfer [27]. Surprisingly, our results show that administration of Cl-IB-MECA did not improve the activity of CTLA4 blockade. These results suggest that targeting A2aR, an inhibitory receptor on T-cells, rather than A3R in tumor stroma may be a promising strategy to increase the effectiveness of CTLA4 mAb in melanoma. Systemic immune stimulation is toxic, and toxicity limits the clinical usefulness of CTLA4 mAb. Thus, it is imperative that combination strategies do not increase the toxicity of CTLA4 inhibition. We did not observe cumulative toxicity at the doses used in our study.

In conclusion, our data support the hypothesis that inhibition of adenosine production in tumors or inhibition of A2aR are promising strategies to increase the effectiveness of melanoma immunotherapy.

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Disclosure of conflict of interest

None.

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