Author's response to reviews

Title: Growth and metastasis of B16-F10 melanoma cells is not critically dependent on host CD73 expression in mice

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Author's response to reviews: see over
Major points:

1. *It is unclear why the authors went to the trouble to repeat experiments in mice where CD73 was deficient in only endothelial cells or hematopoietic cells, given the negative results with mice in which CD73 was knocked out globally.*

Your point is well taken and the answer is rather simple. The reasons why we at all got started on this project were reports in literature [J. Immunol. 2012, 189:2226-2233; Cancer Res. 2011, 71:2892-2900; J. Clin. Invest. 2011, 121:2371-2382; Eur. J. Immunol. 2011, 41:1231-1241] that CD73 is important for tumor growth and we had the competitive advantage of having a floxed CD73 mouse and endothelial, T-cell, T_{reg} and other specific mutants available which we thought could help us to more closely delineate the underlying mechanism. However, to our own disappointment we were unable to replicate the original experiments reported in literature, despite we really have tried very hard, did many controls and discussed the matter with several melanoma experts in the field. The reasons we left the endothelial specific CD73 KO experiment in the manuscript was that this mouse at least showed a phenotype.

2. *The conditions for the ecto-5'-nucleotidase enzyme assay are not optimal. Normally, one would use a substrate concentration 10-fold higher than the K_{m}. However, the concentration of substrate is only about 2-fold higher than the K_{m} in this manuscript. Was the generation of substrate linear with time under these conditions?*

We agree! It would have been better to use a higher concentration of the substrate. However, in every experiment we measured the generation of etheno-adenosine at several time points (10, 20, 30, 40 and 50 min after starting the reaction). We found a linear generation of etheno-adenosine under all conditions and are therefore are confident to have measured AMPase activity.

3. *The authors should show the data that reveal the efficiency of Tie2-Cre mediated deletion of the CD73 gene in endothelial cells.*

We have now added immunohistochemical images of the carotid artery which clearly show successful deletion of CD73 on endothelial cells. This information is now given in Supplementary Figure S1. We also have genetic evidence for the successful deletion in endothelial cells. However, we would like to keep this information for another publication which we are currently preparing using the Tie2-Cre mediated deletion of the CD73 gene in endothelial cells in a different context.
4. The injection of B16-F10 melanoma cells i.v. is not really a model for metastasis.

We agree that intravenous injection of B16-F10 cells is in strict terms not a model for studying hematogenic metastasis to the lung. However this procedure as well as the term are well-established in literature and this model is widely used for the study of “metastasis” [Cancer Res. 2011, 71:2892-2900; PLoS ONE 2014, 9:e96141]. We therefore would like to maintain the term. In the revised manuscript we now indicate that this model studies pulmonary seeding of tumor cells after intravenous injection (page 5).

5. The authors conclude that their inability to see an effect of host CD73 deletion on B16-F10 melanoma cell growth is because of the low ecto-5’-nucleotidase on these cells. A more likely explanation may be that the B16-F10 melanoma is not very immunogenic. Deletion of CD73 slows tumor growth only in the presence of an intact immune system and is most convincing when the tumor cells express a model tumor antigen such as Ova or the SIY antigen in the case of B16-F10. In the earlier paper by Stagg et al. the growth of B16-F10 melanoma was only slightly decreased in CD73 KO mice [Cancer Res. 2011, 71:2892-2900] – results which are not so different than those presented by Burghoff et al.

Thank you for this comment. We agree that in literature tumor cell lines expressing an immunogenic epitope showed a higher susceptibility to CD73 deletion than their parent tumor cell line. However, by enhancing tumoral immunogenicity with an antigen normally not presented in the tumor context, one is creating an artificial setting in which it is difficult to decide whether or not the antitumoral immune response is directed towards the artificial epitope. On the other hand, one of the co-authors of our paper (Bernhard Homey) has published similar studies with B16-F10 cells which were also injected subcutaneously: they found significantly reduced leucocyte recruitment to the tumor site and enhanced tumor growth when the chemokine CCL27 was removed in a knockout model [Proc. Natl. Acad. Sci. U.S.A. 2007, 104:19055-19060]. In the adenosine context it has been reported that inhibition of CD73 leads to the progression of B16-F10 tumors by enhancing release of Th1- and Th17-associated cytokines [J. Immunol. 2012, 189:2226-2233]. Together this suggests a significant involvement of the antitumoral immune response in the growth of unaltered B16-F10 melanoma in mice.

6. The data on tumor infiltrating leukocytes are presented as % of CD45+ cells. If the data were presented in the same format as in the Wang paper [J. Clin. Invest. 2011, 121:2371-2382], it would be easier to compare the results. There may be many fewer tumor infiltrating leukocytes with the parental B16-F10 cells.

This is certainly a good idea, However, Stagg et al. [Cancer Res. 2011, 71:2892-2900] and Wang et al. [J. Clin. Invest. 2011, 121:2371-2382] presented their findings on infiltrating immune subpopulations as % of all cells, but did not include the gating strategy by which the cell suspensions from excised tumors were analysed. Due to lack of this information we are unable to present our data in a similar format to permit a direct comparison.
Minor points:

1. *The manuscript would benefit from being edited by a native English speaker. Some of the word choices do not follow standard English usage.*

We once again checked for standard English usage with help of an English speaking colleague.

2. *Figure 7 is described as being an IFN-γ-ELISPOT on “tumor cells.” However, the assay was done on a mixture of tumor cells and infiltrating host leukocytes.*

This is a valid point, thank you. We have made the appropriate changes (page 28-29).

3. *Were the tumors minced prior to enzymatic digestion?*

Yes, the tumors were excised from the animals, minced and then enzymatically digested. We have now added this information (page 9 and 27).
Dear reviewer,

We greatly appreciate your thoughtful comments on our manuscript. Below, you find a point-by-point reply to your comments.

1. Results: B16-F10 cells showed very little CD73 and negligible AP activity. Neither complete loss of host CD73 nor specific knockout of CD73 on endothelial cells or hematopoietic cells affected tumor growth after subcutaneous or intradermal tumor cell application. Also lung metastasis after intravenous B16-F10 injection was not altered in CD73^{-/-} mice. However, the major problem with B16-F10 cells is that most of its adhesion and growth factor profiles are unlike those of its human melanoma counterparts. Even the enzymes used for invasion into tissues, the ability of cells to overpower the immune system, the antiapoptotic mechanisms, and many other cancer cell hallmarks do not reflect the human disease [J. Investig. Dermatol. 2010, 130:911-912]. In fact in different human melanoma cells, upregulated expression of ecto-5'-nucleotidase is associated with a highly invasive phenotype. Therefore my first question is why the authors selected a murine model that appears far from the main molecular characteristics of human melanomas.

We agree that insights gained from a murine melanoma cell line cannot be directly transferred to human melanoma development. However, B16-F10 cells represent a well-established cell line to study in vivo tumor development in transgenic mice which has been used in many previous tumor studies [J. Immunol. 2012, 189:2226-2233; Cancer Res. 2011, 71:2892-2900]. This point is addressed in our introduction on page 5. We decided not to use a human melanoma cell line because injection into mice would create an even more artificial setting making the translation to either humans or mice even more difficult. We therefore used an established murine skin cell line such as B16-F10 melanoma and – given all the limitations pointed out by you - consider this to be an adequate animal model to study local tumor growth (by subcutaneous or intradermal injection) in the presence and absence of host CD73.

As to the role of tumor CD73, we have also used in separate experiments (not included in this study) Lewis lung carcinoma cells (LLC). LLC exhibit an about 4-fold higher CD73-activity compared to B16-F10 cells. But still, we did not observe statistically significant differences between the two subgroups (with and without CD73 in host) after inoculation of LLC. This suggests to us, that tumor CD73 activity – at least in the range of these two cell lines and in the mouse model – may not be an important variable for tumor growth.
2. A2A adenosine receptor protects tumors from antitumor T cells [Proc. Natl. Acad. Sci. U.S.A. 2006, 103:13132-13137]. Indeed, the inhibition of antitumor T cells via their A2AR in the adenosine-rich tumor microenvironment may explain the paradoxical coexistence of tumors and antitumor immune cells in some cancer patients (the "Hellstrom paradox"). ATP, ADP and AMP are present in the human blood stream. Therefore it appears that A2A/A2B receptors (if overexpressed) in tumor cells and not in host cells are the critical factors. A2A/A2B receptor antagonists were effective in reducing the metastasis of tumors expressing CD73 endogenously (4T1.2 breast tumors) and when CD73 was ectopically expressed (B16-F10 melanoma) [Proc. Natl. Acad. Sci. U.S.A. 2013, 110:14711-14716].


3. Other potential mechanisms should be taken into account. For instance, as recently proposed, antagonism of adenosine A2A receptor expressed by lung adenocarcinoma tumor cells and cancer associated fibroblasts inhibits their growth [Cancer Biol. Ther. 2013, 14:860-868]. Not only could there be prevention of negative signaling in T cells within the tumor microenvironment and inhibition of angiogenesis, but also an inhibitory effect on tumor-promoting, immunosuppressive cancer associated fibroblasts and a direct inhibitory effect on the tumor cells themselves. Besides, and closely related, extracellular ATP (which highly increases in fast growing tumors or hyperinflamed tissues) exerts opposite effects on activated and regulatory CD4$^{+}$ T cells via purinergic P2 receptor activation [J. Immunol. 2012, 189:1303-1310].

We consider it well possible that CD73-derived adenosine may have activated A2A receptors of lung adenocarcinoma cells and tumor associated fibroblasts and thereby stimulated tumor growth in vitro and in vivo [Cancer Biol. Ther. 2013, 14:860-868]. This mechanism appears unlikely to be operative in our experiments, since we found that in our model with melanoma cells, lack of CD73-derived adenosine on host cells did not influence tumor growth. However, in our study we did not analyse the expression adenosine receptors on tumor cells and fibroblasts. If the mechanism you proposed is operative, adenosine must have been derived from the melanoma cells, which, however, have only negligible CD73 activity.

Trabanelli et al. [J. Immunol. 2012, 189:1303-1310] describe that high ATP concentrations within the tumor stimulates regulatory T cell proliferation and immunosuppressive functions. These results are in line with results in which injected melanoma cells showed retarded growth in A2A$^{-/-}$ mice and when A2A antagonists were applied [Proc. Natl. Acad. Sci. U.S.A. 2006, 103:13132-13137]. The problem we are facing with this hypothesis is how ATP is broken down to adenosine: in our knockout the CD73 is lacking in the host and melanoma cells have only negligible AMPase activity.

Again a well taken point. As reported by many groups the ATP concentration within the tumor is very high. In most publications ATP is assumed to show a proinflammatory and tumor damaging effect. By degrading ATP to adenosine, tumors are able to create a microenvironment with immunosuppressive and proangiogenetic properties that favors tumor growth. This has been well covered in reviews on the significance of CD73 and adenosinergic signalling in the tumor microenvironment [J. Biomed. Biotechnol. 2012, 2012:473712; Oncogene 2010, 29:5346-5358].

Estrela et al. [Nat. Med. 1995, 1:84-88] proposed a pathway where ATP is degraded to adenosine extracellularly before entering tumor cells thereby inhibiting tumor growth by diminishing intracellular GSH levels. This mechanism to be operative would require significant enzymatic activity to breakdown ATP extracellularly to adenosine. Clearly, in our setting with melanoma cells, CD73 - the main ectoenzyme which breaks down AMP to adenosine - did not play a role.
Reviewer: Sirpa Jalkanen

Dear reviewer,

Thank you for your critical reading of our manuscript and your thoughtful comments. We have revised the manuscript according to your suggestions. In the following, please find a detailed response to each of your comments.

Major point:

As discussed in this manuscript, the reasons may be the different knockout line, environmental aspects (microbiota), a different method for measuring the volume. There is also a possibility that the B16 melanoma used in this paper has mutated to a direction that is not evoking CD73 dependent responses in the host. The lack of any changes in tumor infiltrating leukocytes would support this idea. To find out, whether the reason is in the B16 melanoma or in the knockout strain another tumor model should be added.

Like other groups working in the field [J. Immunol. 2012, 189:2226-2233; Neoplasia 2011, 13:365-373] we purchased B16-F10 melanoma cells from ATCC and only used them freshly prepared. We also took great care using cells at a very low passage number, at same passage for all animals and by checking them regularly for cell viability and presence of mycoplasma. Another reason why we believe that the B16-F10 melanoma cells are not the source of error is the following. One of the co-authors of our paper (Bernhard Homey) has published studies with the same B16-F10 cell line which were also injected subcutaneously: they found significantly reduced leucocyte recruitment to the tumor site and enhanced tumor growth when the chemokine CCL27 was removed in a knockout model [Proc. Natl. Acad. Sci. U.S.A. 2007, 104:19055-19060]. We therefore think that the problem is not with the melanoma cells but rather with the host CD73.

As to another tumor cell line, we have in separate experiments also used Lewis lung carcinoma cells (LLC). LLC exhibited an about 4-fold higher CD73-activity compared to B16-F10 cells (27.7 ± 0.2 compared with 7.0 ± 1.8 nmol × h⁻¹ × 10⁶ cells). But still, we did not observe statistically significant differences between the two subgroups (with and without CD73 in the host; n = 5) after inoculation of LLC. This suggests to us, that tumor CD73 activity – at least in the range of these two cell lines – is not an important determinant factor for tumor growth. Because our paper is mostly on B16-F10 cells, we decided not to include the data on LLC.
Minor points:

1. The CD4/CD25 positive cells cannot be called regulatory T cells without FoxP3. The CD4⁺/CD25⁺/FoxP3⁺ cells are just a minor population among CD4/CD25 positive T cells.

This is certainly correct. We have replaced the term T⁰reg cells by CD4⁺ CD25⁺ T cells (page 13 and 27).

2. Likewise, the analyses do not include macrophage subtypes. Thus, using CD11b and c markers possible differences in immunosuppressive macrophages may go unnoticed. This should be mentioned in discussion.

Thank you. We have now commented on this in the discussion on page 13.

3. In the methods it is written that ‘cells were intravenously injected (tail vein) into male host animals’ and in the results that ‘in all cases bone marrow from male mice was transplanted into female mice’. Please, correct.

Thank you for making us aware of this mistake. Correction has been made (page 11).

4. It is said that the subcutaneous injection were done to hindlimb. Footpad? Please, specify. Did the tumors metastasize to the draining lymph?

Injection was done into the hindlimb right beneath the hip. We now specified this in the methods on page 8.

In another series of experiments – not included in the present study - we analysed the inguinal lymph nodes - which are the draining lymph nodes - for the presence of metastasis derived from B16-F10 melanoma cells. We found in the ipsilateral lymph node of CD73⁻/⁻ animals a slightly higher melan-A mRNA whereas in the contralateral lymph node of CD73⁻/⁻ animals melan-A mRNA was decreased. Since these differences did not reach the level of significance, we did not include this information into the manuscript.
Reviewer: John Stagg

Dear reviewer,

Thank you for your critical reading of our manuscript. In the following, please find a point-by-point response to your comments.

Major Points:

1. Intriguingly, the authors report that upon intradermal tumor cell injection, peritumoral edema was significantly reduced in CD73$^{-/-}$ mice. The cause of this phenotype was not investigated. Does this reflect decreased inflammation? The authors performed bone marrow chimeras in which WT mice were transplanted with WT or CD73$^{-/-}$ bone marrow. These BM chimeras did not show decreased peritumoral edema, suggesting that CD73 on non-hematopoietic cells mediated the observed decreased peritumoral edema. However, no experimental data is shown to support this. To confirm a role for non-hematopoietic CD73, the authors must perform the appropriate chimeras by transplanting CD73$^{-/-}$ mice with WT or CD73$^{-/-}$ bone marrow. The authors further proposed that endothelial-CD73 is involved in the decreased peritumoral edema observed in CD73$^{-/-}$ mice. Surprisingly, however, they did not perform the experiment in endothelial-specific CD73-deficient mice.

In experiments in which B16-F10 melanoma cells were applied intradermally, we found a significant reduction of tumor edema in CD73$^{-/-}$ mice only when using MR imaging techniques whereas tumor volume was not altered. However the difference in tumor edema was not mirrored by respective changes in the intratumoral immune response when comparing FACS data from digested tumors of WT und CD73$^{-/-}$ mice. Since tumor edema was only visible with MRI, it was not possible to precisely identify this area after removal of the tumor to further investigate the cause of this effect. We showed that CD73 on hematopoietic cells did not mediate this effect. However, we found this information to be only of minor relevance for the overall message of our paper and therefore did not further investigate which type of non-hematopoietic cells was involved.

In fact, one of the reviewers rightly asked why we went to the trouble to repeat experiments in mice where CD73 was deficient in only endothelial cells or hematopoietic cells given the negative results with mice in which CD73 was knocked out globally. The answer is rather simple. The reasons why we at all got started on this project was the report from the reviewers lab [Cancer Res. 2011, 71:2892-2900] and others [J. Immunol. 2012, 189:2226-2233; J. Clin. Invest. 2011, 121:2371-2382; Eur. J. Immunol. 2011, 41:1231-1241] that CD73 is important for tumor growth and we had the competitive advantage of having a floxed CD73 mouse and endothelial, T cell, T$_{reg}$ and other specific mutants available which we thought could help us to delineate the underlying mechanism. However, we were unable to replicate the original experiments reported in the literature, despite we really have tried hard, did many controls and discussed the matter with several melanoma experts in the field.
2. The FACS data of tumor-infiltrating immune cells show minimal T cell infiltration in both WT and CD73\(^{-}\) mice. FITC levels make the analysis difficult to interpret, i.e. there is no clear CD4 or CD8 T cell population visible. Analysis at earlier time-points, optimization of T cell staining, and absolute CD45 counts is recommended.

FACS analysis in cell suspensions derived from tumor tissue is known to be rather difficult to perform due to the mixture of intact cells (tumor cells and infiltrating immune cells) together with cell debris under conditions where immune cells only represent a minor faction. That FACS staining in cell mixtures from tumor tissues is difficult has also been reported by other groups [J. Immunol. 2012, 189:2226-2233; J. Clin. Invest. 2011, 121:2371-2382]. We have used a FACS staining protocol optimized by our group which even permitted the analysis of all resident immune cell subsets in a “difficult” tissue such as the heart [PLoS ONE 2012, 7:e34730]. We therefore do not see the need to further optimize the protocol.

3. The authors report that complete loss of host CD73 has no effect on B16-F10 lung mets after i.v. injection, in contrast to previous studies. In their experiments, only one dose of B16-F10 cells was tested. The authors must test several doses in order to conclude.

In our lung metastasis experiments we relied on a publication of this reviewer in which also only one dose of injected cells was used [Cancer Res. 2011, 71:2892-2900]. As to the cell dosage we decided to use 2.5\(\times\)10\(^5\) B16-F10 cells and finished the experiment after 10 days. Interestingly the reviewer [Cancer Res. 2011, 71:2892-2900] also used 2\(\times\)10\(^5\) B16-F10 cells and terminated the experiment after 14 days. Since variation of cell number in all our experiments did not change the outcome we had no reasons to expect dose dependent changes.

4. While the reasons for such discrepancy are unclear, the study would benefit from a detailed phenotype analysis of MHC molecules and NK cell ligands on the B16-F10 cells used. The difference in tumor growth in WT versus control LoxP mice is particularly worrisome. As pointed out by the authors, it suggests genetic differences favouring B16 tumor growth in the CD73 LoxP mice. This could be the result of impaired NK cell activity. This could explain the discrepancy between this study and previous published study.

The difference observed between loxP mice compared to WT mice is not worrisome and does not come as a surprise on what is known in literature [Am. J. Physiol. Lung Cell Mol. Physiol. 2012, 302:L485-97]. Since genetic background and positional effects may play an important role it is important to always compare the mutant mice with their loxP counterparts. This is what we have done.

Since we did not find any differences in the immune cell composition we consider it very speculative to postulate impaired NK cell activity to be responsible. The aim of the present study was to further define the influence of host CD73 on tumor progression and not an in-depth analysis of the immune system in loxP mice. We therefore see no reason to carry out additional experiments along this line.
Minor Points:

1. “Targeted deletion of CD73 on endothelium was confirmed by histochemistry and qRT-PCR analysis.” – Please show data.

We added immunohistochemical data on the carotid artery in Supplementary Figure S1. We also have confirmed deletion of CD73 on mRNA level in endothelial cells by qRT-PCR. However, we would like to keep this information for another publication which we are currently preparing using the Tie2-Cre mediated deletion of the CD73 gene in endothelial cells in a different context.

2. In the result section, the authors write: “However, tumor growth and edema formation was significantly reduced when compared with the respective experiments in global (Fig. 2C-D) and endothelium specific CD73 mutants”. This sentence is not clear.

Thank you for the advice. We rephrased this sentence for better understanding (page 11).

3. The authors write: “it should emphasized that clinical studies testing CD73 expression on tumor cells as a prognostic marker have yielded contradictory results”. Reference to primary research articles (instead of reviews) is recommended.

Thank you for the comment. We now cited two clinical studies in which opposite results were observed when CD73 expression on breast cancer cells was tested as a marker for disease progression and prognosis [Mol. Cell Proteomics 2009, 8:1436-1449; Appl. Immunohistochem. Mol. Morphol. 2012, 20:103-107].

4. Peritumoral edema was found to be significantly attenuated in CD73-deficient mice. Could this explain the previously reported reduced tumor growth of primary B16F10 tumors in CD73-deficient mice?

No, the peritumoral edema can only be seen using MRI. Macroscopically, only the highly pigmented tumor is visible, which has been measured by other groups using caliper [J. Immunol. 2012, 189:2226-2233; J. Clin. Invest. 2011, 121:2371-2382]. The size of the primary tumor is not altered, it is just the tissue around the tumor. Therefore, the previously reported differences in B16-F10 tumors cannot be the result of different edema formation.