

IgA EGFR antibodies mediate tumor killing in vivo

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Editor: Anneke Funk / Céline Carret

1st Editorial Decision

04 October 2013

Thank you for the submission of your manuscript "IgA EGFR antibodies mediate tumor killing in vivo" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript (please see the attached PDF for a formatted report of Reviewer 3). You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

Importantly, reviewer #2 highlights that the efficacy of the IgA antibodies has to be demonstrated in an immunocompetent mouse model.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The authors applied two different mouse models to study the activity of their anti-EGFR IgA antibodies in direct comparison to cetuximab (IgG1). This included a transgenic mouse model of Fc α R-transgenic mice as well as a syngeneic tumor mouse model, thus allowing to investigate the contribution of different effects (Fab- and Fc-mediated) on tumor killing.

Referee #1 (General Remarks):

This manuscript provides first data from *in vivo* experiments indicating that anti-EGFR IgA antibodies, especially IgA2, are of therapeutic use. They provide insights into the mechanisms of tumor cell killing by macrophages and PMNs. Furthermore, data on the pharmacokinetic properties of IgAs in comparison to cetuximab is shown. Here, a rather short half-life of the IgA antibodies was confirmed, likely in part mediated by clearance through ASGP receptors. In previous studies, the authors provided data on the expression and purification of recombinant IgAs. From their previous publications it is obvious that IgA2 is rather heterogeneous, which might be caused by differences in N- and O-glycosylation. Thus, a more detailed analysis of the glyco-pattern of the recombinant IgAs should be included, which might also help to interpret the PK data. In this respect, a table with all the PK data should be added. Effects of possible clearance through receptor (EGFR) mediated clearance should at least be discussed and compared to half-lives of therapeutic antibodies, e.g. data for cetuximab from clinical trials.

Surprising is the finding that cetuximab does not show strong tumor cell killing in the syngeneic tumor model. The authors should elaborate on the presence and effects of NK cells in the peritoneum.

Finally, the pros and cons of using IgA antibodies in comparison to IgG antibodies should be discussed in more detail, including also approaches for further optimization of therapeutic IgA antibodies.

Referee #2 (General Remarks):

The manuscript by Boross et al. entitled "IgA EGFR antibodies mediate tumor killing *in vivo*" presents evidence for the ability of a chimeric anti-EGFR antibody of the IgA isotype to inhibit the growth of tumors *in vivo*. Using two different tumor models in SCID mice and one in wildtype mice, the authors have demonstrated using whole body imaging techniques that IgA anti-EGFR antibodies are capable of decreasing tumor size either to the same or a greater extent than the IgG variant of the anti-EGFR antibody. Using *in vivo* antibody-mediated depletion and *in vitro* coculturing, the authors have identified macrophages as the key effectors to IgA anti-EGFR-mediated tumor inhibition. This study provides solid *in vivo* evidence for the ability of tumor targeted antibodies of isotypes other than IgG to confer tumor protection. While this is an important proof-of-concept study, the present work has several major limitations with respect to the application of IgA-based therapies in cancer patients.

Major Points:

1. The two main models utilized to assess the efficacy of IgA anti-EGFR were in SCID mice. The data generated in these models clearly show that IgA anti-EGFR is protective against the growth of tumors. However, it is unclear how effective IgA anti-tumor therapy would be in a fully immunocompetent host. While the authors have provided an additional model in WT mice, this protocol lasted only 16 hours and thus cannot be said to reflect the *in vivo* activity of a IgA-mediated therapy in patients with solid tumors. The authors' conclusions would be greatly strengthened if they could provide evidence of IgA-mediated tumor protection in immunocompetent mice. Several tumor lung models similar to the one presented have been shown to be effective in WT animals and contain defined tumor antigens which would enable the analysis of the efficacy of IgA-mediated protection in a more physiological setting.

2. The authors have attempted to compare the *in vivo* efficacy of IgG- and IgA-based therapeutics in

tumor protection. Utilizing a SCID based peritoneal model, they have concluded that IgA2-based therapy is as effective as treatment with the related anti-tumor IgG. However, it has been shown that IgG is capable of leading to the priming of cognate T cells and that some anti-tumor effects of IgG are mediated by the priming of such cells. Given the absence of these cells in the models used, it is likely that the observed therapeutic benefit of IgG-based therapies is an underestimation of the true efficacy of the anti-tumor IgG. It is thus unclear whether the performance of IgA-based treatments could match that of IgG-based treatments in immuno-competent hosts. Further evidence would need to be provided in WT mice in order to properly compare the efficacies of IgG- and IgA-based anti-tumor regimens.

3. As the authors have pointed out, the half-lives of IgG and IgA are vastly different and it is extraordinarily difficult to compare the efficacy of IgG- and IgA-mediated immunotherapies. In order to do so, the authors have utilized different injection regimens to attempt to normalize circulating therapeutic antibody concentrations but provide data that equivalent levels were never reached. Besides rendering it even more difficult to accurately compare the efficacy of IgG- vs IgA-based anti-tumor therapies, this point raises the issue of how cost-effective any IgA-based therapeutic antibody could ever be. Given that much higher doses and much more frequent administration would be required in order to attain circulating levels that would still be less than that for the equivalent IgG therapeutic, it can be expected that the cost of an IgA-based anti-tumor therapy would be prohibitive. In the absence of a direct demonstration that IgA anti-tumor therapeutics can outperform IgG anti-tumor treatments in an immuno-competent host, it would be difficult to justify the additional cost associated with IgA-based therapies. This is a critical point that the authors need to address.

4. Utilizing both in vitro systems and an in vivo peritoneal model of tumor growth, the authors have identified macrophages as the key anti-tumor effector cells of IgA anti-EGFR. Given the short time frame of the in vivo experiment (16 hours) which would preclude the involvement of adaptive immune cells, it is unclear whether macrophages would also be the key effector cells in long term anti-tumor therapy. Furthermore, the peritoneal cavity is a compartment naturally enriched in macrophages. In order to bolster their claims that macrophages would be expected to be the main effector cells in IgA-mediated therapy in cancer patients, the authors would need to provide convincing evidence in a solid tumor model elsewhere in the body that macrophages drive IgA anti-tumor responses.

Minor Points:

1. A more appropriate control for the macrophage depletion assay would be use liposomes that are not known to deplete this cell population rather than PBS.
2. Figure 5E is very unclear. It is unclear what the labeling refers to. This needs to be more adequately explained either in the figure itself or in the figure legend.

Referee #3 (Comments on Novelty/Model System):

Summary of major findings and referees overall impression

Antibodies have been successful so far for the therapy of some hematologic cancers, but only marginally so for the treatment of most solid tumors. There is a clear need to explore, whether they can be used with greater success to treat solid tumors. One strategy is to search for better targets allowing a better discrimination between solid tumor cells and normal cells, but this approach has not been very successful, and suitable targets appear to be very rare. In a second approach, antibody-drug conjugates (ADCs; immuno-toxins; immuno-nanoparticles), as well as biantid tri-specific recombinant antibody-derivatives are intensely pursued, but most of these are still in preclinical and early clinical development. A third and very important new idea is explored in this study. All currently approved antibodies for cancer therapy are of the IgG class and operate via effector mechanisms mediated by Fc receptors (Fc Rs). These are present on specific subsets of human effector cells, and all of these are recruited for anti-tumor activity by antibodies of the IgG type. The anti-tumor effects of therapeutic IgGs in oncology are mediated mostly by Natural Killer (NK) cells, neutrophilic granulocytes (polymorphnuclear cells; PMNs), macrophages and other mechanisms including secondary systemic immune responses as well as complement activation.

However, IgA immunoglobulins are also very abundant in the human body and have a different distribution from IgGs. They are abundant at mucosal sites and in milk, blood, and body fluids, and play important roles in anti-microbial defenses. IgA receptors show a different tissue distribution and cellular expression patterns from IgG receptors, but the effector cells bearing these can also be recruited for antibody-mediated elimination of cancer cells. Until recently, a widely held expectation was, that IgA-receptors present on PMNs in circulating blood would most likely be the major class of effector cells mediating anti-tumor effects of IgA-type antibodies *in vivo*, should these ever be developed for therapeutic purposes. There was no great enthusiasm in the field to recruit PMNs as effectors, because PMNs trigger a number of pro-inflammatory reactions, and there was concern (albeit maybe unjustified), that this may lead to uncontrollable side effects such as cytokine storms and septic shock-like syndromes. It was known that macrophages also carried the IgA receptor Fc RI, but it was not clear, whether macrophages would be available in sufficient numbers at the site of solid tumors to be a therapeutically relevant population. It was known, that Tumor Associated Macrophages (TAMs) existed in many tumors, but it was thought, that these contributed more tumor-promoting than anti-tumor effects. Therefore, the idea to use IgA antibodies as anti-tumor agents has not been received with great enthusiasm so far.

The authors of this study and their collaborators however have consistently pursued this idea over the past 15 years, and made the seminal observation a few years ago, that in a direct comparison, bispecific antibodies (bs abs) recruiting Fc Rs on PMNs mediated even more potent cytolytic reactions than bs abs targeting the same tumor antigen, but recruiting PMNs via Fc RI, the high affinity receptor for IgG (CD64). In recent years, this team also generated chimeric antibodies targeting the tumor antigen EGFR with an Fc-domain from a human IgA. They showed, that in cytolytic assays in cell culture, using whole human blood as a source of effector cells, these chimeric proteins induced an even stronger lysis of EGFR-bearing cancer cells than Cetuximab, an approved EGFR-antibody with an Fc-portion from a human IgG.

However, until the present study, the cytolytic potential of antibodies recruiting Fc R-bearing effectors for the elimination of cancer cells has never been tested in an animal model. The authors of the present study and their collaborators have previously generated mice transgenic for the human Fc R1, and now studied for the first time, whether xenotransplanted human cancer cells would be eliminated in such mice by chimeric antibodies bearing an IgA-derived Fc-domain (Fc). Two different xenografted models were generated in the present study: in the first, the tumor cells engrafted in the lung, in the second they engrafted in the peritoneum. In both models, the chimeric IgA2 EGFR-antibody produced significant *in vivo* anti-tumor activity. In addition, the authors generated a third model, a syngeneic model using murine BaF3-EGFR cells transplanted into a syngeneic recipient. These are a marrow-derived immortalized pro-B cell line, however not a tumor-derived line as in the other two models, stably transfected with human EGFR. These cells engrafted and proliferated in the peritoneum, and in this hyperproliferation model, the chimeric IgA2-EGFR antibody produced even stronger cytolytic effects *in vivo* than the approved antibody Cetuximab. The surprising result was, that this cytolytic effect was not primarily mediated by PMNs, as one might have anticipated based on the impression prevailing in the literature, but by macrophages. When macrophages were abrogated by pretreatment of the mice with clodronate liposomes, then the cytolytic effect was no longer observed. Selective abrogation of the PMNs by pretreatment with a GR-1 antibody depleting the entire myeloid compartment had no effect on graft-cell lysis *in vivo*. The effect was strictly dependent on the presence of the transgenic human Fc R1 protein on the surface of the murine effectors, and only occurred, when the target cells were grafted into recipient mice transgenic for this receptor, but not after transplantation into their WT counterparts. These results are important and exciting, because they draw the attention of the community of drug-developers to the opportunity of using antibodies and antibody-derivatives recruiting Fc Rbearing effectors for the elimination of solid tumors. This opportunity had previously not been sufficiently appreciated, because the false impression prevailed, that the major effectors bearing these receptors would be PMNs, and there was some prejudice against the use of this population.

Now however, as a result of the present study, this misconception has been rectified, and we have learned, that the major effectors will likely be macrophages rather than PMNs, provided the situation can be extrapolated from mice to humans. This is not a foregone conclusion, because surprisingly, the different Fc-receptors perform somewhat different but overlapping tasks in mice and humans, and therefore, it is necessary to study, whether similar results as those obtained here in mice will also be found in humans. However, there is sufficient similarity between the two species to justify reasonable hope, that a similar situation will also prevail in humans.

This opens major new possibilities, because in the past few years macrophages have received great

new interest as an effector population for cancer therapy. Several groups have discovered, that many human tumor cells and in particular the highly invasive cells, carry a "don't eat-me" signal on their surface, the surface antigen CD47. This is a ligand for the counterstructure SIRP on the surface of macrophages. Binding of macrophages to tumor cells and formation of the CD47-SIRP couple induces an anti-phagocytic signal in the macrophage. CD47 antibodies block this signal, leading to enhanced tumor cell phagocytosis by macrophages. Therefore, it appears possible to reprogram macrophages from their tumor-promoting role as TAMs into anti-tumor effectors by blocking the CD47-SIRP signaling circuit. Together with the view presented in this paper, that macrophages eliminate tumor cells in vivo probably mostly by phagocytosis, these findings open major opportunities for the development of novel cancer therapeutics.

Major claims of the paper:

a) The chimeric antibody IgA2 EGFR, carrying an Fc-domain from an IgA2 antibody fused to the antigen binding domain of the EGFR-antibody Cetuximab, was tested in a mouse model xenografted with human epidermoid carcinoma-derived A431 cells, which carry a high surface density of EGFR. The mice were transgenic (tg) for human Fc R1 (CD89). Two models were studied, one with pulmonary, the other with peritoneal engraftment and proliferation of cancer cells.

Claim 1: In both models with different sites of cancer engraftment, the chimeric antibody produced statistically significant anti-tumor effects, comparable to the effects of Cetuximab, in the Fc R1-tg mice.

b) Claim 2: In the syngeneic hyperproliferation model of immortalized murine Ba/F3-EGFR cells transplanted into the peritoneum, an anti-transplant effect was also observed, which was stronger for the chimeric antibody than for Cetuximab.

c) Claim 3: The in vivo anti-transplant effect was mediated by macrophages, and was abrogated or significantly decreased in the absence of the transgenic Fc R1. Abrogation of the PMN compartment had no influence on the anti-transplant effect.

d) Claim 4: Agents recruiting effectors via Fc R1 have the potential to become effective anticancer therapeutics in the future.

Significance of the claims:

These claims are significant, because until now the false opinion dominated the field, that the major class of effector cells bearing Fc Rs likely to be recruited by cancer therapeutics addressing Fc Rs, would be PMNs. There was a prejudice against recruiting PMNs, and consequently, no major efforts have been made over the past 20 years in the field to develop IgA-type antibody agents for cancer therapy, at least no efforts comparable in scale to those made for IgG-based agents. The authors of the present study have been the rare exception. They did not become victims of this misconception and steadfastly continued to work on the idea, that agents recruiting Fc R-bearing effectors could become useful for cancer therapy. In this paper they show for the first time, that chimeric antibodies bearing an Fc -type Fc-domain can eliminate solid human tumors in vivo (in a xenotransplanted murine model), and that this effect was strictly dependent on the presence of a functional human Fc R1 on the mouse effector cells. This is significant, because it now becomes more obvious, that it is necessary to devote a major new effort to the development of Fc R-addressing therapeutic agents, and that such agents offer a realistic chance to make new progress in the treatment of solid tumors. Macrophages as effectors may attack the tumor cells by different mechanisms from the effector cells recruited by the agents explored so far, which mainly addressed NK-cells and other classes of effector mechanisms. As there are other important recent developments, allowing investigators to convert tumor associated macrophages (TAMs) from tumor-promoting into anti-tumor effectors, such as the blockage of the CD47-SIRP "don't-eat-me" signal circuits, these two approaches combined create a new wave of optimism. Therefore, it appears likely, that the publication of this manuscript will trigger a wave of new studies devoted to the question, of how we can recruit Fc R-bearing effectors, in particular macrophages, as a therapeutically useful effector cell population for the fight against solid tumors.

Are the claims novel and convincing?

The claims are novel and surprising, because they run counter the prevailing view in the field, which expected the major class of effector cells bearing FcRs recruited in vivo by agents addressing these triggers to be PMNs. The authors' claim is convincing, because abrogation of the macrophage

compartment with clodronate liposomes is a standard procedure in the field, and because the authors have presented convincing evidence, that this procedure has indeed abrogated the macrophage compartment in the peritoneum in their experiments by > 90%. The PMN compartment in the peritoneum was abrogated by > 99% by pretreatment of the mice with the GR-1 antibody (Fig. S3A), which is also a standard procedure in the field. Therefore, the data are trustworthy. If someone wanted to be very critical, he might argue, that the measurements presented here demonstrate, that in the whole mouse peritoneum the PMNs were strongly depleted by pretreatment with the GR-1 antibody, but that possibly a small fraction of PMNs could still have been produced, which may have been responsible for the residual killing by the total peritoneal cell population reported in Fig. 6D. However, if this had been the case, then abrogation of macrophages with clodronate liposomes should not have affected the elimination of BaF3-EGFR cells *in vivo* recorded in Fig. 6C, because this treatment had no significant effect on the number of Ly6G^{hi} PMNs in the peritoneum (Fig. S3B). Therefore, taken together, the set of mutually reinforcing data of this study presents convincing evidence for this reviewer, that indeed the major subset of effector cells bearing Fc Rs responsible for the anti-proliferative effects *in vivo* in the third model with mouse BaF3-cells were the macrophages. From there it is still an extrapolation to accept, that the same was also true for the xenotransplanted human tumor cells in the lung and peritoneum in models 1 and 2 (Figs. 4 and 5), where it was not yet shown, that here too the macrophages were the relevant effectors, because in these cases, this was technically more difficult to achieve. However, based on the strong data presented for the third model with murine BaF3 cells, this reviewer is willing to make this leap of faith. Still, the possibility remains, that macrophages may have been the dominant effector cell population in the third model, because they are abundant in the peritoneum, particularly after the irritation created by the transplant, whereas they may not be equally numerous and effective in a tumor-bearing lung (model 1).

Appropriate discussion of claims in the context of earlier literature:

The discussion of the novel claims in the context of the earlier literature is appropriate. The last claim (claim 4) however was, that recruitment of Fc R-bearing effector cells may become useful for clinical cancer therapy in the future. This claim is justified, in particular in view of the recent findings, that tumor associated macrophages (TAMs) can be converted from tumor-promoting into tumor-surveillance cells by blockage of the CD47-SIRPα "don't-eat-me" signal circuit (Jaiswal S et al. 2010. *Trends Immunol.* 31: 212-9; Willingham SB et al. 2012. *PNAS* 109: 6662-7; Chao MP, Weissman IL & Majeti R. 2012. *Curr Opin Immunol.* 24: 225-32). Therefore, it may be useful for the authors to include this aspect in the discussion, in order to allow a broader readership to better integrate the importance of the new data into the context of current knowledge in the field.

Is the study important to the field?

Yes, very important. It opens an entire new avenue in pursuing IgA-type antibodies and other agents recruiting Fc R-bearing effectors for cancer therapy, which may become even more relevant for the treatment of solid tumors than the IgG class antibodies and agents recruiting NK cells and/or T-cells as effectors, that have been tried so far with limited success. For different tumor types, different classes of effector cells infiltrate the tumor tissue, and it may be necessary in the future, to choose the therapeutic agent deliberately so, that it recruits the effectors, that are already infiltrating the particular tumor tissue at hand. As TAMs are present in many solid tumors and are now known to be convertible into anti-tumor effectors by blocking the CD47-SIRP circuit, it indeed appears to be a very important and promising new avenue to look into agents recruiting macrophages as a novel and potentially more effective class of effectors for the fight against solid tumors than the agents used to date.

Does the paper stand out from others in the field ?

Yes, because it shows for the first time, that agents recruiting Fc R-bearing effector cells, in particular macrophages, for the antibody-mediated elimination of cancer cells, can contribute to a significant elimination of human tumor cells *in vivo*, in a living mammalian organism.

Interest for more than a specialized audience ?

Yes, for most readers, because cancer at present is among the top 3 causes of death, and with the increased ageing of the world population, is moving up into the top 2 causes, together with malfunction of the cardiovascular system. Therefore, any new agents that might fight cancer more effectively than available agents are of interest for at least half of the world's population.

Other experiments that would strengthen the paper:

This reviewer would like to know, whether the cancer cell lines used in this study (A1207 and A431) as well as the Ba/F3 cells, are positive for CD47, and if so, whether addition of a blocking CD47 antibody to the ADCP reactions with macrophages would further enhance phagocytosis of these cells, as one would expect it from the recent discoveries in the field (Jaiswal 2010; Willingham 2012; Chao 2012; see above). However, this is an extension of the questions raised by the paper, and there is no need to address this issue for the paper to be publishable in EMBO MM as is. The major result of the paper is may be not so much to have provided new answers, but rather new questions. The fact, that this reviewer immediately has a burning new question going beyond the scope of the paper attests to the high quality of the paper by the reviewer's standards.

Major criticisms
none

Minor criticisms and comments; numbered;

1. Clarity of writing: Many of the results are described in the present tense. Use of the simple past tense throughout the manuscript to describe experiments and results is preferred by most journals. Example: Abstract, 7th line from top: "Using Fc R1 transgenic mice we demonstrate...". Should read: "...we demonstrated...", because the preceding and the following sentences both are in the past tense, and switching tenses is not indicated here.
2. Abstract needs to be reworked for grammar. The second sentence reads: "Previous studies showed that....to result in more effective tumor killing by myeloid effector cells compared to Fc R targeting". Should read: "Previous studies showed that....resulted in more effective elimination of tumor cells by myeloid effector cells than triggering via Fc R". At several positions in the text the authors use the comparative "...more....compared to..", which is often seen in internet English, whereas grammatically correct English would be: "...more...than..". Similarly, p.5 9th line from top reads: "superior ADCC activity compared to Cetuximab...". This is not very elegant. Why not simply "...stronger ADCC activity than Cetuximab..." ?
3. Throughout the text the authors use "tumor killing" and "anti tumor activity", when they describe the elimination of tumor cells in vitro, as in this sentence in the abstract. However, tumor cells in vitro strictly speaking are not the same as tumors, because they lack blood vessels, stroma cells, infiltrating leukocytes and other defining components of tumor architecture. Therefore, the authors need to distinguish between "tumor killing" and "tumor cell killing" in a more rigorous manner throughout the manuscript. Both are really not the same.
4. Tumor-derived cell lines need to be described in a little greater detail and a reason for their choice needs to be given. In the abstract and in the text the reader is confronted with A431 and A1207 tumor-derived cell lines, without being given any information, from which type of tumors these cells were derived, and for what reason they were chosen here. It would help the reader for better understanding, if the authors could explain, that A431 cells were derived from a human epidermoid carcinoma and were chosen, because they represent the type of carcinoma one would like to treat with an EGFR-specific therapeutic agent, and also, because this particular line carries an unusually high surface density of EGFR, and therefore is a frequently chosen target line to evaluate cytolytic activity and anti-tumor cell activity of a number of therapeutic agents intended for use against carcinomas. Similarly, it would help to explain, that A1207 cells are derived from a glioma, which is a different type of tumor than a carcinoma, and that they also carry high levels of EGFR. Therefore, the addition of these cells to the panel of target cells allows the investigators to ask, whether EGFR-directed agents are capable of eliminating not only carcinoma-, but also glioma-derived cells. If the answer is yes, then this speaks in favor of the agent, because it then can eliminate a broader range of EGFR-positive tumor cells than only carcinoma cells. These specifications should be given either in the materials and methods section or in the text. The average reader of the paper cannot be expected to have these details at his fingertips. The journal is asking, whether this paper is of interest for a broader readership than just experts in the field. The answer is: yes, but only if highly specialized terminology is explained to the general reader and not supposed to be known beforehand. If uncommon terminology is used without explanation, then non-expert readers from the general audience quickly stop reading. Similarly, the murine Ba/F3 cells are not explained, but it needs to be said somewhere, that they are a bone-marrow-derived, immortalized cell line, representing an early pro-B cell stage, which are IL-3 dependent.

In the syngeneic transplant model (pages 8, 9; Fig. 6), the authors want to evaluate the "antitumor" activity of the chimeric IgA2 EGFR antibody using EGFR-expressing Ba/F3 cells. However, nowhere in this manuscript is it specified, whether the transplanted Ba/F3 cells are tumorigenic or not. If they are not tumor-derived, but only immortalized, then they may be expected to give rise to a hyperproliferating mass of cells in the peritoneum of transplanted mice, which is not a tumor, but simply a mass of non-malignant hyperproliferating cells. Therefore, what would then be measured in this syngeneic model, would not be an "anti-tumor" activity, as is claimed in the header of this paragraph, but an "anti-proliferative" activity for non-malignant cells, or simply an "anti-transplant effect". This would not be the same at all as an anti-tumor effect. Therefore, to justify their claims, the authors need to specify the nature of the Ba/F3 cells and to be more rigorous in their use of scientific terminology. Also, they claim, that the elimination of target cells by macrophages was mostly by phagocytosis, but in the text they call this "tumor cell killing". Strictly speaking, "phagocytosis" is not the same as "tumor cell killing", because the phagocytosed cells inside the macrophage are still alive, and only later die for other reasons. In strict scientific terminology, this process should be referred to as "elimination" by phagocytosis, but not as "killing". The authors need to use more rigorous scientific terminology throughout the paper to remain within the tradition of EMBO MM as a rigorously scientific professional journal.

5. Fair representation of earlier literature? It would seem appropriate for the authors to mention the recent articles from the Weissman and Majeti labs, describing that tumor-associated macrophages found in many solid tumors can indeed be converted into tumor-surveillance cells, provided the CD47-SIRPa "don't-eat-me" signals present on many cancer cells and in particularly on the highly invasive subset thereof are blocked.

6. Unpublished data. At several places in the manuscript, the authors mention "unpublished data", which they refer to as "data not shown". Most journals prefer to refer to such data as "unpublished data", which means, the data are there, but simply have not been published yet. If the authors say: "data not shown", then this means: "we have the data, but we will not show them". This is not scientifically correct, because if the data are mentioned, then the authors must be prepared to show them upon request, otherwise they cannot be quoted as evidence. Evidence, which the authors are unwilling to show, is useless. It is an unnecessary insult to the intelligence of the reviewer, if the authors say: "we have the data, but we will not show them to you. You better take our word for it". This is not scientifically correct. Either you quote the data, and then you must be willing to show them, or you don't mention them. But to say: "we have the data, but we won't show them to you. Trust our words" is a non-scientific appeal to blindly believe because of someones authority and to switch off the readers own critical mind. This is scientific fundamentalism, but not the type of critical science, which is the culture of the EMBO journals.

7. Page 6: The asialoglycoprotein receptor is referred to as "a sugar receptor". This is not correct, because although the protein carries a carbohydrate moiety, it is not necessarily the glycan, which is the ligand for the receptor. It could be an epitope on the polypeptide backbone.

8. Page 8, bottom. The other paragraphs in the results section carried a final sentence summing up the essential findings of each section. This is very helpful for the reader, and it would be helpful to add such a sentence also at the end of this paragraph.

9. Last section on p.9. Here the effect of depletion of Ba/F3-EGFR cells was studied in the peritoneum, and the last sentence of the first paragraph states, that "...depletion of PMNs had no effect on tumor killing". However, as the Ba/F3 cells are not malignant, tumorigenic cells, but simply immortalized cells hyperproliferating in the peritoneum, this is not a "tumor", but a mass of benign, hyperproliferating cells. Therefore, it is inappropriate to call the effect "tumor killing". It is an "anti-proliferative effect" or "an anti-transplant effect". The wording of the statement needs to be scientifically rigorous. The data are so strong already, that the authors do not need to overstate their interpretation. They have nothing to win by this and win more by rigorously describing the facts.

10. Figure legends. P. 19. Fig. 1. Heading reads: "IgA EGFR mediate efficient tumor killing ex vivo". What is a tumor ex vivo? The tumor was not extirpated and cultured in an organ-culture

system. Text should read "tumor cell killing ex vivo", because the data do not demonstrate killing of tumors ex vivo, but of tumor cells ex vivo, which -as explained above- is not the same at all.

11. Fig. 6A. The text states on p. 6, 10th line from the bottom, that in the experiments with cells transplanted into mice, only the IgA2-EGFR antibody was used. However, in Fig. 6A the labelling indicates IgA1-EGFR. This needs to be clarified, because the figure legend also mentions IgA2-EGFR.

Rating of the manuscript:

technical quality: high

strength of evidence for the conclusions drawn: strong, with the exception of the confusion due to loose wording and the lack of distinction between "tumor killing" and "tumor-cell killing".

novelty: high; as explained above

medical impact: high in the intermediate range future. It will take a few years, until agents recruiting Fc R-bearing effectors will be developed sufficiently far to reach clinical testing, but they will come eventually, and probably will have an impact even for such solid tumors for which currently available therapeutic modalities are unsatisfactory.

adequacy of model system: models 1 and 2 are adequate to establish antitumor activity. The data achieved with model 3, the syngeneic model, are over-interpreted to mean "anti-tumor" activity, when all they really prove is an antagonistic effect against the peritoneal hyperproliferation of immortalized, non-malignant Ba/F3 cells. clarity and interest for the non-specialist: high, as explained above.

Overall rating: a highly exciting, high quality paper, which establishes an important new concept, namely that agents recruiting Fc R-bearing effector cells can be effective against human tumors in vivo. This is a qualitatively major new result in the field, which is well supported by the presented data. Therefore the manuscript merits publication in EMBO MM with high priority, after the minor cosmetic problems with rigorous scientific language as mentioned above have been addressed.

Referee #3 (General Remarks):

Congratulations, beautiful work; nice data; reviewer is convinced

1st Revision - authors' response

02 May 2013

Reviewer #1:

Comments of the reviewer:

This manuscript provides first data from in vivo experiments indicating that anti-EGFR IgA antibodies, especially IgA2, are of therapeutic use. They provide insights into the mechanisms of tumor cell killing by macrophages and PMNs. Furthermore, data on the pharmacokinetic properties of IgAs in comparison to cetuximab is shown. Here, a rather short half-life of the IgA antibodies was confirmed, likely in part mediated by clearance through ASGP receptors. In previous studies, the authors provided data on the expression and purification of recombinant IgAs. From their previous publications it is obvious that IgA2 is rather heterogeneous, which might be caused by differences in N- and O-glycosylation. Thus, a more detailed analysis of the glyco-pattern of the recombinant IgAs should be included, which might also help to interpret the PK data. In this respect, a table with all the PK data should be added.

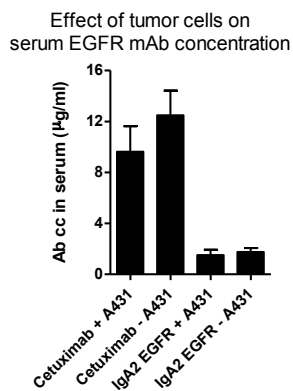
Reply:

The preparations of IgA1 and IgA2 EGFR are indeed heterogeneous. We performed additional glycoanalyses of the IgA EGFR antibodies, which confirmed their heterogeneity. The glycoanalyses revealed that IgA1 EGFR contains higher amounts of terminally galactosylated N-glycans and IgA2 EGFR contains more mono-sialylated-N-glycans. These differences could explain the difference in

PK in mice. The data of the analyses are now included in the paper as supplementary data (Figure S3 and Table S2). We also added a Table with the PK data (Table S1).

Effects of possible clearance through receptor (EGFR) mediated clearance should at least be discussed and compared to half-lives of therapeutic antibodies, e.g. data for cetuximab from clinical trials.

This study was limited to mice and our anti-human EGFR mAbs do not cross-react with mouse EGFR. However, A431 cells express human EGFR and could possibly act as sink for EGFR antibodies. We measured serum concentrations of cetuximab and IgA2 EGFR in mice injected with A431 tumor cells or PBS. This experiment revealed no difference in serum concentrations for both cetuximab and IgA2 EGFR with or without tumor.



In this experiment SCID mice were injected with 5×10^5 A431-luc2 cells intravenously. 25 µg cetuximab was injected on day 0 and 50 µg IgA2 EGFR daily between day 0 and 4. Alternated mice were bled daily between day 1 and 4. Pooled serum concentrations of EGFR mAbs between day 1 and 4 are plotted.

These data indicate that internalization via EGFR did not substantially influence serum levels of EGFR mAbs in this model. However, since the tumor mass in the first days is relatively low, this can be different under situations of higher tumor load. In humans, clearance by EGFR indeed influences the PK of EGFR mAbs. However, it is expected that an IgA EGFR would suffer similarly from this effect as an IgG1 EGFR does.

Surprising is the finding that cetuximab does not show strong tumor cell killing in the syngeneic tumor model. The authors should elaborate on the presence and effects of NK cells in the peritoneum.

It was indeed surprising that cetuximab did not mediate tumor cell killing in the syngeneic peritoneal model. However, we have previously found that NK cells are not recruited to the peritoneum (Boross P, Hematologica, 2011). The contribution of NK cells in mice to tumor cell lysis in vivo is questionable (Uchida, JEM, 2004; Gong J Immunol, 2005). A recent study showed that mouse NK cells express relatively low levels of FcγRIII (Biburger M & Nimmerjahn F, Immunology letters, 2012). This could explain why the role of NK cells in mouse studies is less important than in humans. This does not exclude the possibility that in humans NK cells do contribute to the effect of cetuximab.

Finally, the pros and cons of using IgA antibodies in comparison to IgG antibodies should be discussed in more detail, including also approaches for further optimization of therapeutic IgA antibodies.

We have now included a discussion on the pros and cons of using IgA in the manuscript. However, since the aim of the present manuscript was solely to provide a proof-of-concept for the use of IgA in mAb therapy of cancer, we kept it concise.

One of the strength of IgA lies in its ability to – next to macrophages - also efficiently activate PMNs, which are poorly activated by IgG mAbs. The in vivo action of IgA is not subjected to the inhibitory effects of FcγRIIB and not influenced by polymorphisms in activating FcγRs. Moreover, IgA is a natural antibody of the mucosa and therefore it may reach different anatomical locations than IgG. Because of these different properties it is expected that in humans IgA have a different activity compared to IgG.

Developing IgA as a therapeutic agent is obviously a major challenge and requires in improved understanding of the biology of IgA and FcαRI. Since IgA exists in humans in multiple forms (monomer, dimer, secretory), it would be desirable to first determine which of these formats can best be used for tumor therapy. The recombinant mAb could be further stabilized and / or effector functions could be improved / silenced through introduction of amino acid mutations in the IgA Fc part. The glycosylation of IgA is more complex than that of IgG and the effect of the glycans on the in vivo functions of IgA are incompletely understood. The production and purification of IgA clearly needs to be optimized. There are several strategies already used with success to extend the in vivo serum half-life of recombinant proteins, which could be applied to IgA as well.

An issue which should be carefully investigated is the potential toxicity of IgA in humans. Since PMNs express high FcαRI and they are abundantly present in blood, injection of high amounts of IgA may cause unwanted side effects.

IgA immune complexes were shown to be involved in IgA nephropathy. Importantly, kidney IgA deposits contain only antibodies of the IgA1 subclass. Therefore, one would expect that using IgA2 would avoid this possible side effect.

Induction of adaptive immunity after IgG mAb therapy was suggested to contribute to the therapeutic effects of IgG. It is possible that priming of T cells by IgA is less efficient than by IgG, however, this should be investigated experimentally.

IgG1 can efficiently activate the complement system. Whether complement is involved in the anti-tumor effects of IgG mAbs is currently controversial and may depend on the target antigen, animal model used or compartment investigated. Since IgA is a poor complement activator (it can only activate the lectin and alternative pathways of complement), it would perhaps not be suitable to employ against targets where complement was suggested to play a dominant role during mAb therapy. On the other hand, infusion reactions, the most commonly observed side effect during mAb therapy, are thought to be mediated by the activation of complement. Using IgA could avoid this common complication.

Alternatively, instead of using engineered full IgA molecules, new generations of mAb formats (bispecific Abs or Ab fragments) that target FcαRI could be used to efficiently recruit FcαRI-positive effector cells for tumor cell killing.

We would like to thank this reviewer for his/her helpful comments and we hope we could answer his/her questions satisfactory.

Reviewer #2

The manuscript by Boross et al. entitled "IgA EGFR antibodies mediate tumor killing in vivo" presents evidence for the ability of a chimeric anti-EGFR antibody of the IgA isotype to inhibit the growth of tumors in vivo. Using two different tumor models in SCID mice and one in wild type mice, the authors have demonstrated using whole body imaging techniques that IgA anti-EGFR antibodies are capable of decreasing tumor size either to the same or a greater extent than the IgG variant of the anti-EGFR antibody. Using in vivo antibody-mediated depletion and in vitro coculturing, the authors have identified macrophages as the key effectors to IgA anti-EGFR-mediated tumor inhibition. This study provides solid in vivo evidence for the ability of tumor targeted antibodies of isotypes other than IgG to confer tumor protection. While this is an important proof-of-concept study, the present work has several major limitations with respect to the application of IgA-based therapies in cancer patients.

Major Points:

1. The two main models utilized to assess the efficacy of IgA anti-EGFR were in SCID mice. The data generated in these models clearly show that IgA anti-EGFR is protective against the growth of tumors. However, it is unclear how effective IgA anti-tumor therapy would be in a fully immuno-competent host. While the authors have provided an additional model in WT mice, this protocol lasted only 16 hours and thus cannot be said to reflect the in vivo activity of a IgA-mediated therapy in patients with solid tumors. The authors' conclusions would be greatly strengthened if they could provide evidence of IgA-mediated tumor protection in immuno-competent mice. Several tumor lung models similar to the one presented have been shown to be effective in WT animals and contain defined tumor antigens which would enable the analysis of the efficacy of IgA-mediated protection in a more physiological setting.

Meanwhile, we have performed a long-term model in immunocompetent mice as requested by this reviewer. We now included these newly obtained data using the theB16F10 lung metastasis model in immunocompetent C57BL/6 mice with a duration of 29 days. We used B16F10-luc2 cells transfected to express human EGFR that showed reliable outgrowth and could be treated with cetuximab. Using this model we demonstrate significant anti-tumor effect of IgA2 EGFR both using bioluminescent imaging as well as using the traditional method by counting the metastases in the lungs. We also found that in this model IgA2 EGFR's effect was entirely dependent on the presence of FcαRI (page 9-10, Figure 7).

2. The authors have attempted to compare the in vivo efficacy of IgG- and IgA-based therapeutics in tumor protection. Utilizing a SCID based peritoneal model, they have concluded that IgA2-based therapy is as effective as treatment with the related anti-tumor IgG. However, it has been shown that IgG is capable of leading to the priming of cognate T cells and that some anti-tumor effects of IgG are mediated by the priming of such cells. Given the absence of these cells in the models used, it is likely that the observed therapeutic benefit of IgG-based therapies is an underestimation of the true efficacy of the anti-tumor IgG. It is thus unclear whether the performance of IgA-based treatments could match that of IgG-based treatments in immuno-competent hosts. Further evidence would need to be provided in WT mice in order to properly compare the efficacies of IgG- and IgA-based anti-tumor regimens.

Data on IgA2 EGFR's efficacy using an immunocompetent model are now included in the revised manuscript. The results show that IgA2 EGFR is as efficient in FcαRI Tg C57BL/6 mice as cetuximab. The experiments suggest that it is even more efficient at later time points (page 9-10, Figure 7).

Anti-tumor IgG mAbs could indeed induce anti-tumor T cell response, which may contribute to therapeutic effect of these agents. To what extent this contributes to the anti-tumor effects is currently not clear. IgA immune complexes may engage FcαRI on dendritic cells and induce adaptive anti-tumor immunity similar to anti-tumor IgG antibodies. However FcαRI-mediated antigen presentation on mouse DCs was shown to be relatively inefficient in vitro (Otten MA, Immunobiology, 2006).

3. As the authors have pointed out, the half-lives of IgG and IgA are vastly different and it is extraordinarily difficult to compare the efficacy of IgG- and IgA-mediated immunotherapies. In order to do so, the authors have utilized different injection regimens to attempt to normalize circulating therapeutic antibody concentrations but provide data that equivalent levels were never reached. Besides rendering it even more difficult to accurately compare the efficacy of IgG- vs IgA-based anti-tumor therapies, this point raises the issue of how cost-effective any IgA-based therapeutic antibody could ever be. Given that much higher doses and much more frequent administration would be required in order to attain circulating levels that would still be less than that for the equivalent IgG therapeutic, it can be expected that the cost of an IgA-based anti-tumor therapy would be prohibitive. In the absence of a direct demonstration that IgA anti-tumor therapeutics can outperform IgG anti-tumor treatments in an immuno-competent host, it would be difficult to justify the additional cost associated with IgA-based therapies. This is a critical point that the authors need to address.

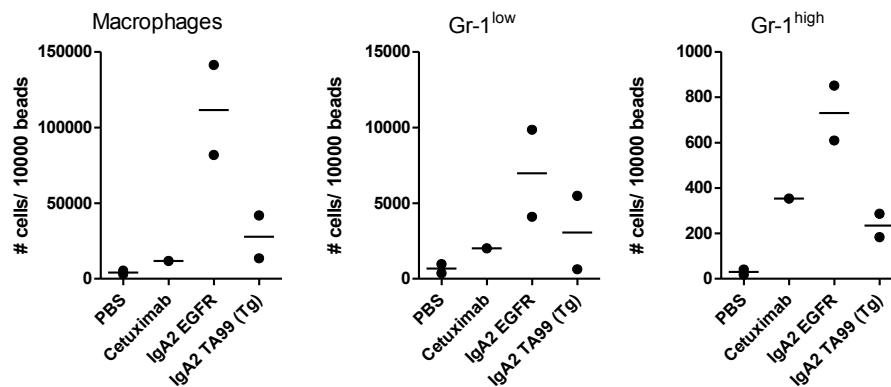
We indeed showed that the in vivo serum half-life of IgA EGFR is shorter than that of cetuximab. However, as shown by experiments using ASGPR blockage, the incomplete glycosylation of our IgA EGFR antibodies contributes greatly to the short half-life. The additional N-glycan analysis of IgA EGFR mAbs - now included in the manuscript – confirms this (page 6, Table S2). The expectation is that IgA that is similarly glycosylated as IgA found in humans will have a serum half-life comparable to IgA in humans.

In addition, we would like to stress that the half-life of human IgG1 in the mouse is longer than in humans because of the better binding to mouse FcγRn. The half-life of IgA in humans was estimated to be around 5 days (Monteiro R, Ann Rev Imm, 2003), compared to 15 hours in mice (present manuscript), whereas the half-life of human IgG1 is around 21 days in humans and is somewhat longer in mice. Thus it can be expected that the difference in serum half-life between IgG and IgA in humans is smaller than in mice. In addition, protein and / or glyco-engineered variants of IgA could extend serum half-life as seen with human IgG1. Several strategies are known to extend serum half-life of protein biologics, which could also be applied to IgA. Anti-tumor antibodies need to reach tumors, for which serum half-life may not even be that relevant.

The aim of the present manuscript was to provide a proof-of-concept for the in vivo targeting of FcαRI for anti-tumor therapy. Clearly, the potency of anti-tumor IgA mAbs needs to be investigated further in different tumor models. At the same time we need to learn more about the biology of IgA and FcαRI, the optimal format to use, the production and purification, the distribution in vivo etc. in order to optimally exploit IgA for tumor therapy. We are confident that cost-effectivity of IgA can substantially be improved in the future to a level comparable to that for IgG.

4. Utilizing both in vitro systems and an in vivo peritoneal model of tumor growth, the authors have identified macrophages as the key anti-tumor effector cells of IgA anti-EGFR. Given the short time frame of the in vivo experiment (16 hours) which would preclude the involvement of adaptive immune cells, it is unclear whether macrophages would also be the key effector cells in long term anti-tumor therapy. Furthermore, the peritoneal cavity is a compartment naturally enriched in macrophages. In order to bolster their claims that macrophages would be expected to be the main effector cells in IgA-mediated therapy in cancer patients, the authors would need to provide convincing evidence in a solid tumor model elsewhere in the body that macrophages drive IgA anti-tumor responses.

We now included data obtained with the B16F10 lung metastasis model. In this model, IgA2 EGFR was able to reduce tumor growth in FcαRI Tg mice. At the end point of the experiment, selected lungs were collected, homogenized and analyzed for the presence of effector cells. We could detect increased amounts of effector cells (both F4/80+, Gr-1^{low} and Gr-1^{high}) in the lungs. Since only two mice were analyzed per treatment group we cannot statistically compare the different treatment groups.



Unfortunately, at the time of the analyses (day 29) in IgA2 EGFR-treated FcαRI Tg group almost no metastases were observed in the lungs and therefore this group could not be analyzed. Additionally, it is possible that effector cells are recruited at an earlier time point and by the time of the analyses they have already left or died. Since macrophages are constantly present in the lungs and in the tumor environment in general it is very difficult to track their contribution to mAb therapy.

We considered including experiments concerning the depletion of effector cell populations in this long term model, as we did in the short term model, to answer the question of the reviewer. However, long term depletion of effector cells is difficult to achieve. In case of chlodronate liposomes, macrophages return after 5-7 days. Using multiple administrations it is possible to keep their numbers low, however macrophages regulate functional aspects of various non-phagocytic cells. As a consequence, their long term absence may ultimately lead to the functional inactivity or even the disappearance of these non-phagocytic cells. Prolonged administration of anti-PMN mAb induces the production of mouse neutralizing antibodies, which renders the depletion no longer effective. We, therefore, decided that this experiment was not feasible, and attempted to analyze the local effector cells instead.

Minor Points:

1. A more appropriate control for the macrophage depletion assay would be use liposomes that are not known to deplete this cell population rather than PBS.

We respectfully disagree with the reviewer on this point, as we think that PBS liposomes do not represent the right control. The control animals should have normal, non-blocked, non-suppressed and non-activated phenotype. Liposomes, as most other particulate compounds, may block phagocytosis for a certain period of time.

2. Figure 5E is very unclear. It is unclear what the labeling refers to. This needs to be more adequately explained either in the figure itself or in the figure legend.

The text referring to Figure 5 and the Figure legend is extended to provide better explanation of what is presented. We hope that the presentation is clear now.

We would like to thank this reviewer for his/her helpful comments and we hope we now answered his/her questions satisfactory.

Reviewer #3

We thank this reviewer for his/her positive comments and thoroughly reading our manuscript, for his/her elaborate comments and for sharing his/her thoughts on our study. His/her comments are answered below.

Minor criticisms and comments; numbered;

1. Clarity of writing: Many of the results are described in the present tense. Use of the simple past tense throughout the manuscript to describe experiments and results is preferred by most journals. Example: Abstract, 7th line from top: "Using FcalphaR1 transgenic mice we demonstrate..."

Should read: "...we demonstrated...", because the preceding and the following sentences both are in the past tense, and switching tenses is not indicated here.

The text is corrected accordingly.

2. Abstract needs to be reworked for grammar. The second sentence reads: "Previous studies showed that...to result in more effective tumor killing by myeloid effector cells compared to FcgammaR targeting". Should read: "Previous studies showed that...resulted in more effective elimination of tumor cells by myeloid effector cells than triggering via FcgammaR". At several positions in the text the authors use the comparative "...more....compared to..", which is often seen in internet English, whereas grammatically correct English would be: "...more...than..".

Similarly, p.5 9th line from top reads: "superior ADCC activity compared to Cetuximab...". This is not very elegant. Why not simply "...stronger ADCC activity than Cetuximab..."?

The text is corrected accordingly.

3. Throughout the text the authors use "tumor killing" and "anti-tumor activity", when they describe the elimination of tumor cells in vitro, as in this sentence in the abstract. However, tumor cells in vitro strictly speaking are not the same as tumors, because they lack blood vessels, stroma cells, infiltrating leukocytes and other defining components of tumor architecture. Therefore, the authors need to distinguish between "tumor killing" and "tumor cell killing" in a more rigorous manner throughout the manuscript. Both are really not the same.

The text is corrected accordingly.

4. Tumor-derived cell lines need to be described in a little greater detail and a reason for their choice needs to be given. In the abstract and in the text the reader is confronted with A431 and A1207 tumor-derived cell lines, without being given any information, from which type of tumors these cells were derived, and for what reason they were chosen here. It would help the reader for better understanding, if the authors could explain, that A431 cells were derived from a human epidermoid carcinoma and were chosen, because they represent the type of carcinoma one would

like to treat with an EGFR-specific therapeutic agent, and also, because this particular line carries an unusually high surface density of EGFR, and therefore is a frequently chosen target line to evaluate cytolytic activity and anti-tumor cell activity of a number of therapeutic agents intended for use against carcinomas. Similarly, it would help to explain, that A1207 cells are derived from a glioma, which is a different type of tumor than a carcinoma, and that they also carry high levels of EGFR. Therefore, the addition of these cells to the panel of target cells allows the investigators to ask, whether EGFR-directed agents are capable of eliminating not only carcinoma-, but also glioma-derived cells. If the answer is yes, then this speaks in favor of the agent, because it then can eliminate a broader range of EGFR-positive tumor cells than only carcinoma cells. These specifications should be given either in the materials and methods section or in the text. The average reader of the paper cannot be expected to have these details at his fingertips. The journal is asking, whether this paper is of interest for a broader readership than just experts in the field. The answer is: yes, but only if highly specialized terminology is explained to the general reader and not supposed to be known beforehand. If uncommon terminology is used without explanation, then non-expert readers from the general audience quickly stop reading. Similarly, the murine Ba/F3 cells are not explained, but it needs to be said somewhere, that they are a bone-marrow-derived, immortalized cell line, representing an early pro-B cell stage, which are IL-3 dependent.

In the syngeneic transplant model (pages 8, 9; Fig. 6), the authors want to evaluate the "anti-tumor" activity of the chimeric IgA2 EGFR antibody using EGFR-expressing Ba/F3 cells. However, nowhere in this manuscript is it specified, whether the transplanted Ba/F3 cells are tumorigenic or not. If they are not tumor-derived, but only immortalized, then they may be expected to give rise to a hyperproliferating mass of cells in the peritoneum of transplanted mice, which is not a tumor, but simply a mass of non-malignant hyperproliferating cells. Therefore, what would then be measured in this syngeneic model, would not be an "anti-tumor" activity, as is claimed in the header of this paragraph, but an "anti-proliferative" activity for non-malignant cells, or simply an "anti-transplant effect". This would not be the same at all as an anti-tumor effect. Therefore, to justify their claims, the authors need to specify the nature of the Ba/F3 cells and to be more rigorous in their use of scientific terminology.

Also, they claim, that the elimination of target cells by macrophages was mostly by phagocytosis, but in the text they call this "tumor cell killing". Strictly speaking, "phagocytosis" is not the same as "tumor cell killing", because the phagocytosed cells inside the macrophages are still alive, and only later die for other reasons. In strict scientific terminology, this process should be referred to as "elimination" by phagocytosis, but not as "killing". The authors need to use more rigorous scientific terminology throughout the paper to remain within the tradition of EMBO MM as a rigorously scientific professional journal.

We thank the reviewer for the correction, the text is changed accordingly. We now use tumor cell killing in the text.

5. Fair representation of earlier literature? It would seem appropriate for the authors to mention the recent articles from the Weissman and Majeti labs, describing that tumor-associated macrophages found in many solid tumors can indeed be converted into tumor-surveillance cells, provided the CD47-SIRPα "don't-eat-me" signals present on many cancer cells and in particularly on the highly invasive subset thereof are blocked.

We now included a reference on the Weissman study on the importance of CD47-SIRPα interaction in our discussion (page 12). Both A431 and A1207 cells express CD47 (Timo van den Berg, Sanquin, Amsterdam, personal communication) and therefore it can be expected that blocking CD47-SIRP1a interactions would enhance phagocytosis of these cells.

6. Unpublished data. At several places in the manuscript, the authors mention "unpublished data", which they refer to as "data not shown". Most journals prefer to refer to such data as "unpublished data", which means, the data are there, but simply have not been published yet. If the authors say: "data not shown", then this means: "we have the data, but we will not show them". This is not scientifically correct, because if the data are mentioned, then the authors must be prepared to show

them upon request, otherwise they cannot be quoted as evidence. Evidence, which the authors are unwilling to show, is useless. It is an unnecessary insult to the intelligence of the reviewer, if the authors say: "we have the data, but we will not show them to you. You better take our word for it". This is not scientifically correct. Either you quote the data, and then you must be willing to show them, or you don't mention them. But to say: "we have the data, but we won't show them to you. Trust our words" is a non-scientific appeal to blindly believe because of someones authority and to switch off the readers own critical mind. This is scientific fundamentalism, but not the type of critical science, which is the culture of the EMBO journals.

We changed the term “data not shown” to “unpublished data” throughout the text.

7. Page 6: The asialoglycoprotein receptor is referred to as "a sugar receptor". This is not correct, because although the protein carries a carbohydrate moiety, it is not necessarily the glycan, which is the ligand for the receptor. It could be an epitope on the polypeptide backbone.

The term “sugar receptor” is changed to receptors recognizing glycoproteins.

8. Page 8, bottom. The other paragraphs in the results section carried a final sentence summing up the essential findings of each section. This is very helpful for the reader, and it would be helpful to add such a sentence also at the end of this paragraph.

An additional sentence is added (page 9).

9. Last section on p.9. Here the effect of depletion of Ba/F3-EGFR cells was studied in the peritoneum, and the last sentence of the first paragraph states, that "...depletion of PMNs had no effect on tumor killing". However, as the BaF3 cells are not malignant, tumorigenic cells, but simply immortalized cells hyperproliferating in the peritoneum, this is not a "tumor", but a mass of benign, hyperproliferating cells. Therefore, it is inappropriate to call the effect "tumor killing". It is an "anti-proliferative effect" or "an anti-transplant effect". The wording of the statement needs to be scientifically rigorous. The data are so strong already, that the authors do not need to overstate their interpretation. They have nothing to win by this and win more by rigorously describing the facts.

We fully agree with the reviewer and in the revised version of the manuscript we no longer use the term “tumor killing” with regard to the Ba/F3-EGFR cells throughout the manuscript.

10. Figure legends. P. 19. Fig. 1. Heading reads: "IgA EGFR mediate efficient tumor killing ex vivo". What is a tumor ex vivo? The tumor was not extirpated and cultured in an organ-culture system. Text should read "tumor cell killing ex vivo", because the data do not demonstrate killing of tumors ex vivo, but of tumor cells ex vivo, which -as explained above- is not the same at all.

The text is changed accordingly.

11. Fig. 6A. The text states on p. 6, 10th line from the bottom, that in the experiments with cells transplanted into mice, only the IgA2-EGFR antibody was used. However, in Fig. 6A the labeling indicates IgA1-EGFR. This needs to be clarified, because the figure legend also mentions IgA2-EGFR.

We apologize for this inconsistency; in this case the figure legend incorrectly stated IgA2 EGFR, because in this experiment indeed IgA1 EGFR was used. We used IgA2 EGFR to assess the anti-tumor effects of IgA antibodies. In that particular experiment we used IgA1 EGFR to assess recruitment of effector cells by IgA antibodies. However, since recruitment of effector cells is mainly triggered by the tumor cells and not by IgA antibodies, we expect that using IgA2 EGFR would lead to similar effector cell recruitment.

We would like to thank this reviewer again for his/her helpful comments and we hope we now answered his/her questions satisfactory.

Conflict-of-interest statement WB, PWHIP and JGJW are employees of Genmab and own warrants and/or stocks. LR and LPL are employees of Ludger Ltd.

We hope that our manuscript in the revised form is now acceptable for publication in EMBO Molecular Medicine.

2nd Editorial Decision

21 May 2013

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

-Thank you for providing The Paper Explained. However, we were wondering whether you would agree to discuss your findings in a little bit more details. Please see any TPE published on any of our research article online (www.embomolmed.org)

-Please reformat the references in our journal style as described in our online guidelines.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (General Remarks):

The manuscript is suitable for publication.

Referee #2 (General Remarks):

The manuscript by Boross et al. entitled "IgA EGFR antibodies mediate tumor killing in vivo" presents evidence for the ability of a chimeric anti-EGFR antibody of the IgA isotype to inhibit the growth of tumors in vivo. Using a variety of mouse models, the authors have demonstrated using whole body imaging techniques that IgA anti-EGFR antibodies are capable of decreasing tumor size either to the same or a greater extent than the IgG variant of the anti-EGFR antibody. This study provides solid in vivo evidence for the ability of tumor targeted antibodies of isotypes other than IgG to confer tumor protection.

The authors have satisfactorily addressed many of the comments of the reviewers. In addition to additional analysis of the glycoforms of IgA contained within their therapeutic preparation, the authors have also added relevant details pertaining to the cell lines used in their tumor models. Furthermore, they have added an additional model which utilizes immunocompetent mice and a

long term tumor metastasis assay and which is much more reflective of the situation seen in human cancer patients. It is unclear why the mice in this study were injected with an agent to increase PMN when the authors have demonstrated in other studies that macrophages are the key cell type responsible for IgA-mediated tumor killing. However, this concern does not negate the findings pertaining to the effectiveness of the therapeutic treatment and does not preclude publication of the study. The reviewers believe that the current work is an important proof-of-principle study and that it is now suitable for publication in EMBO Molecular Medicine.