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TLR4 mediated skin carcinogenesis is dependent on immune and radioresistant cells

Deepak Mittal, Fabiana Saccheri, Tobias Pusterla, Marco Bianchi, Emilie Vénéreau, Maria Rescigno

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the delay in getting back to you with a decision, which was owed to initial difficulties in finding a sufficient number of suitable experts available to review at this time. We have now finally received the reports and recommendations of four expert referees, and as you will see, all of them find your results interesting in principle and also potentially important. At the same time, they however also raise a varying number of substantive concerns with the study in its current form. Among the more significant issues here is the repeatedly mentioned problem that the requirement of HMGB1 for actual skin carcinogenesis is not clearly demonstrated; other major issues in need of clarification are a more definitive exclusion of bacterial TLR triggers, as well as the currently little-understood link of a transient inflammatory response to tumorigenesis with different outcomes.

In light of these various important concerns, it appears that full revision of the paper would likely require a substantial amount of further work to provide the required clarification and additional experimental validation; this also means that we unfortunately do currently not see ourselves in the position to make strong commitments with regard to potential publication of a revised manuscript. Given the importance of the topic and the potential interest of your findings, I would nevertheless be inclined to allow you the opportunity to address the various criticisms in the form of a revised manuscript. Thus, should you feel confident that you might be able to satisfactorily respond to the most salient points raised by the reviewers, we would be willing to consider a revised version further for publication. As it is our policy to allow only one round of major revision, please however make sure to diligently answer to all the points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website:

http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision, or if you would like to discuss the importance and/or feasibility of addressing certain specific points raised by the reviewers.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Mittal et al. show that mice lacking TLR4 are resistant to the development of skin cancer induced by DMBA combined with croton oil (CO) treatment. They also provide evidence suggesting that CO treatment leads to the release of HMGB1 by keratinocytes, which then acts to initiate skin inflammation in a TLR4-dependent manner. Based on this evidence, they suggest that HMGB1 regulates skin carcinogenesis by directly binding and activating TLR4. The findings showing that TLR4, but not TLR2 and TLR9, knockouts are resistant to skin carcinogenesis similarly to the MyD88 knockouts are novel and important. Also the observation that HMGB1 seems to play a role in CO induced skin inflammation are interesting, but the data presented do not provide convincing evidence that HMGB1 controls skin carcinogenesis by activating TLR4.
Specific comments.
1. The authors show (fig. 1) that, unlike TLR2 and TLR9 KO mice, TLR4 KO mice are resistant to skin tumorigenesis similarly to MyD88 knockouts. Based on this evidence the authors conclude that TLR4 promotes skin tumorigenesis via MyD88 signaling. However, considering the fact that TLR4 signals not only via MyD88 but also through TRIF, and that MyD88 functions as a proximal adaptor for IL-1 and IL-18 receptors, it is difficult to rule out a possibility that the absence of TRIF-dependent TLR4 signaling events in TLR4 KO animals and the impaired IL-1/18 mediated MyD88 signaling in MyD88 KO animals coincidentally result in a similar degree of tumor resistance between these two strains. In support of this possibility, Chen et al showed that in a sterile inflammation model dependent on HMGB1 and MyD88, IL1R but not TLR4 played a critical role in triggering inflammation (Chen et al, Nat. Med. 2007 vol. 13 (7) pp. 851-6).
2. In fig. 6 A and B, topical EtOH treatment may (or may not...) efficiently kill skin commensal bacteria but the TLR agonists from dead bugs will still remain in skin. Therefore, one can argue that CO treatment introduces TLR4 agonists from killed skin bacteria in dermal cells by breaching the skin barrier. Also, some TLR4 agonists derived from bacteria may not be eliminated by polymyxin B, which is efficient only for neutralization of LPS. To convincingly exclude the role of bacteria-induced TLR4 signaling in skin carcinogenesis, the authors would need to study CD14-deficient or germ-free mice.
3. In fig. 6 C and D, the authors describe that CO or TPA treatment induces extracelluar release of HMGB1 in skin. In contrast to the two stage tumor model, TLR4-deficient C3H/HeJ mice were shown to be more susceptible to DMBA only mediated skin cancer model (Cancer Res. 2008 Jan 15; 68(2):615-22). It will be interesting to see if DMBA treatment also induces HMGB1 in skin.
4. In fig.7, the authors demonstrated that neutralization of HMGB1 indeed reduces inflammation in CO treated mouse. However, they did not show the role of HMGB1 in the context of tumor progress. Does anti- HMGB treatment in wild type mice manifest a similar level of resistance to skin tumor development that was seen in TLR4 or MyD88 KO mice?

Referee #2 (Remarks to the Author):

The paper by Mittal et al. contains potentially interesting information on the contribution of HMGB1 and TLR4 to chemically induced skin carcinogenesis. The authors should consider

ameliorating their paper in the following points:

Major
It is not true that TLR4 and its downstream effector MyD88 are upregulated "during tumor progression" as this is stated in the abstract and in the main text of the paper when the authors analyze the expression level of TLR3 and MyD88 by immunohistochemistry on melanoma and colon cancer specimen. There is an increase in expression level of these proteins in (pre-)neoplastic lesions as compared to normal cells, but there is no clear increase when melanomas progress. This must be reworded accordingly.

Minor
- The authors should be more explicit when they comment on the tumor types induced by crton oil and DMBA. Are there any melanomas?
- "Significantly" on page 5, line 10 (comment of Fig. 1B) refers to which statistical test?
- "Statically" on page 9 presumably refers to "statistically".
- In Fig. 1C, the data presentation is very hard to follow. It would be better to give a "percentage of mice with carcinoma", in form of a column, with the number of mice in each group indicated for each group, below each column. The same remark applies to fig. 4D.
- A size bar is missing in Fig 3A.
- The data in Fig. 5 should be subjected to some kind of quantification (number of Gr-1 positive cells per square mm etc.). The same remark applies to Fig. 6D and 7B.

Referee #3 (Remarks to the Author):

This MS provides reasonable evidence that "cell-damage"associated HMGB1 functions as endogenous ligand for TLR4 dependent "Inflammatory responses" conditioning in a 2 step model chemically induced skin carcinogenesis.

Questions:
1. With the exception of TNFalpha (Fig 3A) the inflammatory parameters tested appear rather short lived. How does this mechanistically impact tumorgenesis?

2. Given that DMBA administration can protect via contact hypersensitiviy, TLR4 could "help" to generate contact hypersensitivity (Not tested)

3. What is the molecular basis that drives developments of either papillomas or carcinomas?

4.That HMGB1 can act as endogeneous ligand is established in the literature. Here the authors extend this finding to a skin tumorgenesis model. However, Taniguchi recently provided evidence (nature,2009) that members of the HMGB family function as universal sentinels for nucleic acid driven activation of innate immune cells .If so TLR7,TLR3 and RIG like receptor k.o.mice ought to be tested.

Referee #4 (Remarks to the Author):

Review of EMBO J. MS
The authors of this report examined the role of TLR signaling in two stage skin carcinogenesis. The show that induction of squamous cell carcinomas (SCC) requires signaling through TLR4 via MyD88. They also show rather convincingly that TLR4 and MyD88 are required for induction of skin inflammation in response to application of croton oil (CO). These results are rather unexpected as CO contains phorbol esters which are supposed to act through protein kinase C (PKC) which functions downstream to TLR4 and MyD88 and not upstream to them. The authors explain these results by showing that phorbol ester and CO treatment induces the release of HMGB1. Neutralization of HMGB1 prevents CO-induced skin inflammation.
By-and-large, the results are convincing and novel. Although I believe that this manuscript is suitable for publication in the EMBO J, I strongly recommend that the title be changed not to include HMGB1, as the authors never demonstrate that HMGB1 is required for skin carcinogenesis. They only show that it is needed for induction of skin inflammation.

Other comments:
- It is unlikely that glycyrrhizin is a specific inhibitor of HMGB1. Furthermore, both this inhibitor and BoxA, which is likely to act more specifically, are only shown to be capable of reducing skin inflammation. Their effect on tumor promotion is never examined.
- The mechanism of CO or phorbol ester induced HMGB1 release is not explained. If this is the key event responsible for tumor promotion, it should be explored.
Thank you very much for your decision letter on our manuscript n. EMBOJ-2009-72918. We can answer most of the reviewers' comments, but we have some concerns in performing experiments on carcinogenesis as the protocols that are used are very long (8 -12 months to reach statistical significance). Besides the fact that we have only 90 days to respond, we think that delaying so long the publication of our manuscript may eventually lead to a scoop by other laboratories. In addition, the requested experiments of carcinogenesis in TLR7, TLR3 and RIG like receptor k.o. mice are beyond the scope of our manuscript.

Additional correspondence 23 December 2009

Thank you very much for your decision letter on our manuscript n. EMBOJ-2009-72918. We can answer most of the reviewers' comments, but we have some concerns in performing experiments on carcinogenesis as the protocols that are used are very long (8 -12 months to reach statistical significance). Besides the fact that we have only 90 days to respond, we think that delaying so long the publication of our manuscript may eventually lead to a scoop by other laboratories. In addition, the requested experiments of carcinogenesis in TLR7, TLR3 and RIG like receptor k.o. mice are beyond the scope of our manuscript. On the other hand, we can respond to all of the issues that you highlighted in your decision letter that are important to be addressed

1. the repeatedly mentioned problem that the requirement of HMGB1 for actual skin carcinogenesis is not clearly demonstrated;

2. other major issues in need of clarification are a more definitive exclusion of bacterial TLR triggers,

3. as well as the currently little-understood link of a transient inflammatory response to tumorigenesis with different outcomes.'

Point 1:The clear demonstration that HMGB1 is involved in the carcinogenesis is difficult to achieve as mice deficient for HMGB1 are embryonically lethal and a continuous addition of HMGB1 inhibitors throughout the experiment is not feasible as pointed out by Reviewer 1, while reviewer 4 simply asks for deleting in the title the involvement of HMGB1 in carcinogenesis, which is a more than justified request.

Point 2:As we were also worried to have underestimated a role for the indigenous flora, we have coadministered croton oil with LPS thus expecting to observe an increase in tumorigenesis. By contrast we found that LPS is protective against carcinogenesis. This demonstration clearly argues against a possible involvement of LPS-associated to bacteria in TLR-4-mediated carcinogenesis. LPS would rather be protective. These results will be added to the manuscript.

Point 3:Since the inflammatory response is transient, croton oil is applied twice a week for 34 weeks. If treatments are delayed or are not protracted over time, tumorigenesis is not induced, but this has been clearly demonstrated in previous reports from other laboratories. Hence the reviewer is raising a point that has been already addressed previously in the literature.

Thus, we ask you whether you would be willing to receive a revised version of our manuscript that takes into consideration all of the points raised by the reviewers, but without carrying out additional experiments on carcinogenesis.

Additional correspondence (reply) 23 December 2009

I have now had a chance to look closer at the query you sent regarding the requirements for the revision of your manuscript, EMBOJ-2009-72918. With regard to the main question, whether lengthy additional carcinogenesis experiments in other knock-out backgrounds would be essential, I agree that this would probably be beyond the scope of the current study. Where further carcinogenesis experiments would have likely been helpful is of course in an HMGB1 deletion background, but I understand the problems with this from your response. So with regards to your responses to the points I specifically reiterated in my letter, I cannot predict whether the referees will consider them sufficiently convincing, but I think it is worth trying at this stage.

Hoping this was of help, and looking forward to reading your revised manuscript

1st Revision - authors' response 03 April 2010

First of all we would like to thank the reviewers for their helpful comments that we think have improved our manuscript. We now have a new figure (Fig. 6) and 6 additional supplementary figures. Changes are highlighted as underlined. Below is a point-by-point response to refereesí comments.

Referee #1 (Remarks to the Author):

Mittal et al. show that mice lacking TLR4 are resistant to the development of skin cancer induced by DMBA combined with croton oil (CO) treatment. They also provide evidence suggesting that CO treatment leads to the release of HMGB1 by keratinocytes, which then acts to initiate skin inflammation in a TLR4-dependent manner. Based on this evidence, they suggest that HMGB1 regulates skin carcinogenesis by directly binding and activating TLR4. The findings showing that TLR4, but not TLR2 and TLR9, knockouts are resistant to skin carcinogenesis similarly to the MyD88 knockouts are novel and important. Also the observation that HMGB1 seems to play a role in CO induced skin inflammation are interesting, but the data presented do not provide convincing evidence that HMGB1 controls skin carcinogenesis by activating TLR4.

Specific comments.
1. The authors show (fig. 1) that, unlike TLR2 and TLR9 KO mice, TLR4 KO mice are resistant to skin tumorigenesis similarly to MyD88 knockouts. Based on this evidence the authors conclude that TLR4 promotes skin tumorigenesis via MyD88 signaling. However, considering the fact that TLR4 signals not only via MyD88 but also through TRIF, and that MyD88 functions as a proximal adaptor for IL-1 and IL-18 receptors, it is difficult to rule out a possibility that the absence of TRIF-dependent TLR4 signaling events in TLR4 KO animals and the impaired IL-1/18 mediated MyD88 signaling in MyD88 KO animals coincidentally result in a similar degree of tumor resistance between these two strains. In support of this possibility, Chen et al showed that in a sterile inflammation model dependent on HMGB1 and MyD88, IL1R but not TLR4 played a critical role in triggering inflammation (Chen et al, Nat. Med. 2007 vol. 13 (7) pp. 851-6).

--- We do not rule out a possible involvement of IL-1/18 mediated MyD88 signaling pathways in skin tumorigenesis. We have added a sentence in the discussion to clear this point and have quoted the reference indicated by the reviewer (page 12, line 5).

2. In fig. 6 A and B, topical EtOH treatment may (or may not...) efficiently kill skin commensal bacteria but the TLR agonists from dead bugs will still remain in skin. Therefore, one can argue that CO treatment introduces TLR4 agonists from killed skin bacteria in dermal cells by breaching the skin barrier. Also, some TLR4 agonists derived from bacteria may not be eliminated by polymyxin B, which is efficient only for neutralization of LPS. To convincingly exclude the role of bacteria-induced TLR4 signaling in skin carcinogenesis, the authors would need to study CD14-deficient or germ-free mice.

--- The reviewer is absolutely right. To assess the role of LPS in skin tumorigenesis, we coadministrated LPS (recognized by CD14, TLR4 complex) with croton oil on the skins of mice. We hypothesized that although LPS is not involved in skin inflammation, it may still potentiate croton oil induced skin tumorigenesis. However, to our surprise, we found that LPS has a protective rather than a worsening effect in tumor development (New Supp Fig. 6). This result argues against a possible role of bacterial TLR4 agonist (LPS) in skin tumorigenesis. This could be somehow explained by the insensitiveness of keratinocytes to LPS (not shown) but the reason for the protection remains to be established.

3. In fig. 6 C and D, the authors describe that CO or TPA treatment induces extracellular release of HMGB1 in skin. In contrast to the two stage tumor model, TLR4-deficient C3H/HeJ mice were shown to be more susceptible to DMBA only mediated skin cancer model (Cancer Res. 2008 Jan 15; 68(2):615-22). It will be interesting to see if DMBA treatment also induces HMGB1 in skin.

--- We analyzed HMGB1 release after DMBA treatment and we found some induction of HMGB1 that was minimal as compared to that induced after CO treatment (New Suppl. Fig. 7A). We have also added new data showing that CO-dependent HMGB-1 release is accompanied by LDH release and keratinocyte necrosis (New Fig. 6C) while DMBA never induces keratinocyte necrosis (New Suppl. Fig. 7).

4. In fig.7, the authors demonstrated that neutralization of HMGB1 indeed reduces inflammation in CO treated mouse. However, they did not show the role of HMGB1 in the context of tumor progress. Does anti- HMGB1 treatment in wild type mice manifest a similar level of resistance to skin tumor development that was seen in TLR4 or MyD88 KO mice?

--- The reviewer is right. We have not shown a role for HMGB-1 in skin tumorigenesis but only in inflammation. Treatment with HMGB1 inhibitors for skin tumorigenesis for 1 year is not feasible. Hence, we have changed the title and text accordingly.

Referee #2 (Remarks to the Author):

The paper by Mittal et al. contains potentially interesting information on the contribution of HMGB1 and TLR4 to chemically induced skin carcinogenesis. The authors should consider ameliorating their paper in the following points:

Major
It is not true that TLR4 and its downstream effector MyD88 are upregulated "during tumor progression" as this is stated in the abstract and in the main text of the paper when the authors analyze the expression level of TLR4 and MyD88 by immunohistochemistry on melanoma and colon cancer specimen. There is an increase in expression level of these proteins in (pre-)neoplastic lesions as compared to normal cells, but there is no clear increase when melanomas progress. This must be reworded accordingly.

--- Thank you for the comment, the text has been reworded.

Minor
- The authors should be more explicit when they comment on the tumor types induced by croton oil and DMBA. Are there any melanomas?
--- Tumor refers to carcinoma, we have now made this clear (page 5 line 10). There were few melanomas. Only two melanomas appeared in twenty wild type mice. None of the TLRs KO or MyD88 KO mice had melanoma.
- "Significantly" on page 5, line 10 (comment of Fig. 1B) refers to which statistical test?
--- It refers to WT versus KO mice (now explained in the text page 5 line 11).

- "Statically" on page 9 presumably refers to "statistically".
--- Thank you, the text has been reworded.

- In Fig. 1C, the data presentation is very hard to follow. It would be better to give a "percentage of mice with carcinoma", in form of a column, with the number of mice in each group indicated for each group, below each column. The same remark applies to fig. 4D.
--- fig 1C and 4D have been replaced with percentage of mice with carcinoma.

- A size bar is missing in Fig 3A.
--- added

- The data in Fig. 5 should be subjected to some kind of quantification (number of Gr-1 positive cells per square mm etc.). The same remark applies to Fig. 6D and 7B.
--- quantification results are now added. Fig. 5 has been moved to Suppl. Fig. 5 together with data quantification. Quantification of Fig. 7B is in 7C. Regarding quantification of Fig. 6D it was very difficult to be quantified, but the difference with TLR4 KO was striking.

Referee #3 (Remarks to the Author):

This MS provides reasonable evidence that "cell-damage"associated HMGB1 functions as endogenous ligand for TLR4 dependent "Inflammatory responses" conditioning in a 2 step model chemically induced skin carcinogenesis.

Questions:
1. With the exception of TNFalpha (Fig 3A) the inflammatory parameters tested appear rather short lived. How does this mechanistically impact tumorgenesis?

--- Besides the fact that TNF-alpha is the main inflammatory cytokine involved in skin tumorigenesis (Moore et al, 1999), many reports have shown that in order to have tumorigenesis, the tumor promoter (CO) needs to be repetitively administered. Only in this way a ëchronic inflammatory statusí can be generated (Slaga, 1983).

2. Given that DMBA administration can protect via contact hypersensitiviy, TLR4 could "help" to generate contact hypersensitivity (Not tested)

--- In the manuscript by Yusuf et al. they were repeatedly administering DMBA and this is probably why they induced contact hypersensitivity. In our case we administered DMBA only twice. Thus, even though some CHS may have been induced with the second treatment, it cannot be responsible for tumor development as never rechallenged with DMBA. In addition, Yusuf et al. found an opposite result: i.e. TLR4 was a tumor promoter.

3. What is the molecular basis that drives developments of either papillomas or carcinomas?

This is a very interesting question that deserves a whole study to be answered. Not much is known on the transition from papilloma to carcinoma.

4.That HMGB1 can act as endogeneous ligand is established in the literature. Here the authors

extend this finding to a skin tumorgenesis model. However, Taniguchi recently provided evidence (nature,2009) that members of the HMGB family function as universal sentinels for nucleic acid driven activation of innate immune cells .If so TLR7,TLR3 and RIG like receptor k.o.mice ought to be tested.

--- With our data we are not excluding the possibility that other TLRs than TLR2 or TLR9 may be involved as well, but we know that TLR4 deletion is sufficient to inhibit tumor development. We wrote a sentence to discuss this in the manuscript and have quoted the manuscript indicated by the reviewer (page 13 line 12).

Referee #4 (Remarks to the Author):

The authors of this report examined the role of TLR signaling in two stage skin carcinogenesis. The show that induction of squamous cell carcinomas (SCC) requires signaling through TLR4 via MyD88. They also show rather convincingly that TLR4 and MyD88 are required for induction of skin inflammation in response to application of croton oil (CO). These results are rather unexpected as CO contains phorbol esters which are supposed to act through protein kinase C (PKC) which functions downstream to TLR4 and MyD88 and not upstream to them. The authors explain these results by showing that phorbol ester and CO treatment induces the release of HMGB1. Neutralization of HMGB1 prevents CO-induced skin inflammation.
By-and-large, the results are convincing and novel. Although I believe that this manuscript is suitable for publication in the EMBO J, I strongly recommend that the title be changed not to include HMGB1, as the authors never demonstrate that HMGB1 is required for skin carcinogenesis. They only show that it is needed for induction of skin inflammation.

--- The reviewer is right and the title has been rephrased.

Other comments:
- It is unlikely that glycyrrhizin is a specific inhibitor of HMGB1. Furthermore, both this inhibitor and BoxA, which is likely to act more specifically, are only shown to be capable of reducing skin inflammation. Their effect on tumor promotion is never examined

--- The reviewer is right we have clarified in the text that HMGB1 is involved in skin inflammation and this may eventually lead to tumorigenesis, but as the reviewer is pointing out we have no direct proof of its involvement in tumorigenesis. On the other hand a treatment of 1 year with an HMGB1 inhibitor is not feasible.

- The mechanism of CO or phorbol ester induced HMGB1 release is not explained. If this is the key event responsible for tumor promotion, it should be explored.

--- We think that CO or TPA treatment induces keratinocyte cell death and this results in HMGB-1 release. To address this possibility we tested LDH release after CO treatment. Data is now presented in new Fig 6. As shown there is a correlation between LDH and HMGB-1 release suggesting a cell death-induced mechanism. This is further supported by the following results:

1) HMGB1 is released by mouse-differentiated keratinocytes after stimulation with CO; this correlates with LDH release and keratinocytes cell death (New Fig. 6C).
2) Culture supernatants from CO-treated keratinocytes activate bone marrow derived dendritic cells to release TNF-a in a TLR4 dependent fashion (New Suppl. Fig 8)
Together these results suggest that skin application of TPA or CO results in tissue damage (keratinocytes necrosis) and release of HMGB1 and LDH in a TLR4-independent way. CO-treated keratinocytes release HMGB1 that activates BM-derived cells to release inflammatory cytokines in a TLR4 dependent fashion.

Cited literature:
Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, Holdsworth H, Turner L, Rollins B, Pasparakis M, Kollias G, Balkwill F (1999) Mice deficient in tumor necrosis factor-alpha are

resistant to skin carcinogenesis. Nat Med 5(7): 828-831

Slaga TJ (1983) Overview of tumor promotion in animals. Environ Health Perspect 50: 3-14

Acceptance letter 07 April 2010

Thank you for submitting your revised manuscript for our consideration. I have now carefully evaluated your response, and it has now been seen once more by one of the original referees. I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we shall be able to proceed with formal acceptance of the paper, I would however need to ask you to send us (via email) a modified version of the main article text and of the supplementary information where the changes are not highlighted/underlined. Please also transmit the signed license forms at this stage (in case you should not have already done so). Once we will have received this final version, we should then be able to swiftly process the manuscript for publication.

Yours sincerely,

Editor
The EMBO Journal

