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The alarmin Mrp8/14 as regulator of the adaptive immune response during allergic contact dermatitis

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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 June 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting, but also consider that some more experimental data is needed to substantiate the findings reported. In particular better support for that MRP8/14 acts via TLR4 as a negative regulator of DC maturation is needed. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow a single round of major revision only and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

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):

This paper reports the rather surprising finding that Mrp14-/- mice show an INCREASED allergic contact dermatitis than wt mice, which is contrast with the known function of Mrp8/14 in activating the immune system.
The results explain this result by showing that exposure to Mrp8/14 restrains the ability of DCs to mature and to promote T cell proliferation.

The explanation provided by the authors is likely to be correct. However, the reported effects of Mrp8/14 on DC maturation are very small (although the capacity of DCs to promote T cell proliferation is clearly different based on their exposure to Mrp8/14 or not). This opens up the possibility that the explanation provided is not complete, or least that it should be carefully qualified.
In particular, these points should be carefully considered:

1. Fig1. I cannot see a "strong upregulation" of CD86 and MHC-II in iBMDCs treated with Mrp8 or Mrp8/14. The FACS shown are very noisy, show a very variable expression in cell populations and translate into a very modest upregulation.
I also do not like the presentation of results as "fold-increase" (panel B), because this obscures the variability among controls. The data should be presented as number of cells beyond a certain threshold, the threshold being the same in all animals; in this way the controls also would have a standard deviation.

2. I do not undertand Fig 2A. If the readout on the y axis is ear swelling, and is the difference between the right and left ear (as stated in Methods), these differences cannot come out exactly to zero before challenge, if only for the variability intrinsic in the measurement (ears have a slightly different thickness in different places) and the biological variability between the left and right ears of the same animal.
More important still, the authors should indicate how many animals there actually were in each group. The same number of animals should be used in the irritant dermatitis control (Fig 2D); even more important, the swelling in the irritant dermatitis experiment is so small that a different between groups may be difficult to detect anyway. This is an important control, and the authors should convince us that Mrp8/14 do not have an anti-inflammatory effect.
The same statistical considerations apply to other experiments reported in different figures.

3. The effect on iDC maturation is small, although the effect on the ability of DCs to promote T cell proliferation is fairly large. How can the authors exclude that the primary effect is actually on DC maturation, as opposed for example on their ability to establish a physical connection with T cells and to activate the correct signaling pathways?

4. In the discussion, the authors comment rather expansively on the possible implication for inflammatory bowel disease. These speculations are too far from the object matter of the paper, and they may well be wrong. I suggest that they should limit themselves to a much more generic indication that their finding may have implications for other aspects of the immune system's workings.
In contrast, a more detailed discussion of the implications for contact dermatitis is required. Are human individuals with mutations in the human equivalents of Mrp8/14 known? Is their phenotype consistent with the findings of this mouse work?

5. The title is too vague. The authors should choose a title that closely describes the object of their work (ie contact dermatitis) and not advertise unproven general significance for the whole of adaptive immunity.

6. The implications of this work for the "hygiene hypothesis" are not obvious to me and should either be clarified or removed (also in the abstract).

Referee #2 (Remarks to the Author):

The authors report small but reproducible differences in dendritic cell activation and T cell proliferation between Mrp14 wt and ko mice in a model of contact dermatitis. Most of the effect seems to be explained by inhibition of DC maturation in the presence of Mrp14 that is relieved in KO DCs. Adoptive transfer experiments convincingly show that dermal DCs are responsible for the phenotype. Although the mechanism is not fully identified, this is an interesting effect that is somewhat unexpected in view of the pro-inflammatory effect of Mrp8. Concerns regard experimental design, data interpretation and the discussion.

1. The title does not correctly represent the contents of the manuscript. First, Mrp14 has no effect per se. Second, Mrp8 appears to dampen (rather than regulate) T cell proliferation.
2. MDSCs are a group of poorly defined monocyte-derived cells and maybe even neutrophils. It is not fair to call them immature DCs. It is unclear whether the characterization as CD11b+Gr1+ is sufficient to identify MDSCs.
3. One explanation for the apparent discrepancy between the data in figure 1 and the dermatitis data is that dermal DCs are very different from BMDCs. This possibility should be explored.
4. Figure 3B indeed shows that exogenous recombinant Mrp8 impairs BMDC differentiation. At the same time, the data suggest that endogenous Mrp8 has no effect, because the differentiation of Mrp14 deficient and sufficient BMDCs is the same. This raises two questions: Is recombinant Mrp8 contaminated with LPS? Second, is the level of recombinant Mrp8 much higher than the Mrp8 secreted from BMDCs? Seems to be 3-fold higher.
5. Throughout the manuscript, it seems to make no difference whether T cells are from Mrp14-/- or wt mice. A likely explanation is that Mrp8 is not expressed by T cells. Is this so?
6. The effect of MDSCs on T cell CFSE dilution is small (if any) and not convincing. Figure 5A: how many experiments? 5B: is 30 to 40 really different from 39 to 46? Stats? Figure 5 could be eliminated. The critical experiment is shown in figure 6.
7. The dermal DCs were injected iv. A tacit assumption is that they home correctly. Any evidence for this?
8. The first four paragraphs of the discussion largely repeat the results. Is there evidence of more autoimmune disease in Mrp14 KO mice?
9. The T cell proliferation assays are not well described. Some are apparently allogeneic. What mouse strain? Figure 1 says Balb/c. Others? Or was a TCR transgenic used?
10. The CD86 and MHC-II differences reported in figure 1 are very small and likely incorrectly calculated. Inspection of the histograms suggests that the upregulation by LPS was at least 10-fold for CD86, whereas the effect of Mrp8 was very modest. The negative control (isotype?) is not specified.
11. Figure 3, 8 g/ml is three times the maximum endogenous Mrp8. This should be repeated with a more reasonable dose.
12. The data in figure 4 is uninterpretable, since both the dDCs and T cells were wt or Mrp14-/-. Most likely, the T cell source makes no difference, but this needs to be formally shown.

Minor
1. Page (not numbered), line 1: should "native" be "naÔve"?

Referee #3 (Remarks to the Author):

The manuscript "The alarmins Mrp8 and Mrp14 are potent regulators of adaptive immunity" by Petersen and colleagues describes a negative role for these proteins in the development of allergic contact dermatitis (ACD). Based on their well documented roles as alarmins regulating positively a variety of immune recognition and response systems, this is unexpected and the authors attempt to dissect this observation. They provide hypothesis at the level of DC maturation and activation and in vitro and in vivo experimentation in support of this hypothesis. Overall the logic is solid and experiments are in par well done. There are several places where the hypothesis demonstration falls short of robust experimental support.

Major concerns:

1. The entire logic is based on the fact that MRP8/14 through TLR4 act as a negative regulator of DC maturation/activation during the development of a dermal T cell activation response. While this is possible it could all be simply a classic and simple desensitization that was well described for the TLR as well as other activatory pathways. In this scenario, induction of endogenous TLR ligands would dampen normally the response while in the absence of these ligands response can be exacerbated. While this is not a minus in the story, it has to be actively presented as well as tested for from a pathway point of view at the level of the signaling cascade. One would want to show - in vitro BMDCs having the TLR signal partially desensitized and describe the level at which this is happening. Also in TLR4 ko animals this should not happen. Additionally, phenotypes in TLR4 ko mice should be discussed and considered in this context as they have been very well described in similar models (example: J Exp Med. 2008 Sep 1;205(9):2151-62).
2. Dermal DCs which are the cells instigating the response being read in vivo are not wel characterized phenotypically; the functional characterization is indeed difficult and some of the readouts (t cell proliferation) are marginal but in line with the phenotype. Despite this, the authors used very string decisive language around the phenotype, which is minor and the MRP8/14 have relatively small effects; additionally little is mentioned about other immune instances that could test this hypothesis. Similar experiments are relatively easier done in the lung. Also immune responses could deconstruct this system better. This should be also reflected in the title which should be focused on the exact systems one can prove the biology in.
3. The last major experimental line in which ko dermal DCs are being used in transfers to recapitulate the phenotype is missing significant controls to show identical cellular homing before initiation of the immune response. While this is challenging it is necessary in order to be able to interpret this major line of experiments.

Minor points:
1. The sources and quality control for MRP8 and MRP14 proteins used in in vitro assays should be well documented in material and methods.
2. Figure 4 C is confusing as it could open the possibility for a T cell intrinsic component of the MRP8/14 defect.
3. Fig 5B axes are not labeled.
4. Discuss the significant amount of literature on TLR2/4 and RAGE in similar systems.

1st Revision - authors' response 30 September 2012

Reviewer #1
We thank the reviewer for encouraging and critical comments. Our responses are given below.

1) Fig1. I cannot see a "strong upregulation" of CD86 and MHC-II in iBMDCs treated with Mrp8 or Mrp8/14. The FACS shown are very noisy, show a very variable expression in cell populations and translate into a very modest upregulation. I also do not like the presentation of results as "fold-increase" (panel B), because this obscures the variability among controls. The data should be presented as number of cells beyond a certain threshold, the threshold being the same in all animals; in this way the controls also would have a standard deviation.

We agree with the reviewer and replaced the term “strongly up-regulated” by “up-regulated” in the text on page 5. Additionally, we revised figure 1 and now presented mfi-shifts instead of fold-increase including the standard deviation of the controls (see revised figure 1A).

2) I do not understand Fig 2A. If the readout on the y axis is ear swelling, and is the difference between the right and left ear (as stated in Methods), these differences cannot come out exactly to zero before challenge, if only for the variability intrinsic in the measurement (ears have a slightly different thickness in different places) and the biological variability between the left and right ears of the same animal. More important still, the authors should indicate how many animals there actually were in each group. The same number of animals should be used in the irritant dermatitis control (Fig 2D); even more important, the swelling in the irritant dermatitis experiment is so small that a different between groups may be difficult to detect anyway. This is an important

control, and the authors should convince us that Mrp8/14 do not have an anti-inflammatory effect. The same statistical considerations apply to other experiments reported in different figures.

We agree and as suggested by the reviewer we now calculated the differences in ear thickness between the right and the left ears prior to challenge. We added these data in the revised figures 2A and D and also to figure 6. Furthermore, we performed three additional experiments including additional 5 animals per group of irritant contact dermatitis (ICD) using slightly higher doses of croton oil (3% instead of 2.5%). Now the more pronounced ear swelling clearly indicates that there is indeed no difference in the outcome of ICD in wt and Mrp14 -/- mice as shown in the revised Figure 2D. With this clear negative result it is not allowed in Germany to perform additional, identical experiments just to adapt the numbers of ACD and ICD. A general anti-inflammatory effect of MRP8/MRP14 is now clearly excluded as already shown previously in several other inflammatory models (Vogl et al, 2007, *Nat. Med.* 13, 1042-1049; Van Lent et al, 2008, *Ann. Rheum. Dis.* 67, 1750-1758; Van Lent et al, 2008, *Arthritis Rheum.* 58, 3776-3787; Loser et al, 2010, *Nat. Med.* 16, 713-717; Yonekawa et al, 2011, *Atherosclerosis* 218, 486-492; Schelbergen et al, 2012, *Arthritis Rheum.* 64, 1477-1487; Van Lent et al, 2012, *Arthritis Rheum.* 64, 1466-1476).

3) The effect on iDC maturation is small, although the effect on the ability of DCs to promote T cell proliferation is fairly large. How can the authors exclude that the primary effect is actually on DC maturation, as opposed for example on their ability to establish a physical connection with T cells and to activate the correct signaling pathways?

We agree with the reviewers comment and it was not our intention to suggest that expression of surface receptors presented in our manuscript is the only or dominant mechanism responsible for the observed differences in as much as effects on T-cell proliferation are much more impressive. We obeyed the suggestion of the reviewer and analysed the number of physical contacts of BMDCs from C57BL/6 wt and Mrp14 -/- mice with allogenic T cells from BALB/c mice using time-lapse microscopy of cells embedded in a three-dimensional collagen matrix. We found an increased ability of Mrp14 -/- BMDCs for physical contacts with T cells in comparison to wt BMDCs as an additional mechanism. Moreover, the up-regulation of the T cell activation marker CD69 was more pronounced on T cells after contact with Mrp14 -/- BMDCs than with wt BMDCs. These findings and methods related to staining are now included on pages 7 and 16 of the text respectively, and shown in revised Figure 3H.

4) In the discussion, the authors comment rather expansively on the possible implication for inflammatory bowel disease. These speculations are too far from the object matter of the paper, and they may well be wrong. I suggest that they should limit themselves to a much more generic indication that their finding may have implications for other aspects of the immune system's workings. In contrast, a more detailed discussion of the implications for contact dermatitis is required. Are human individuals with mutations in the human equivalents of Mrp8/14 known? Is their phenotype consistent with the findings of this mouse work?

We shortened our discussion section according to the reviewer's suggestion. We discussed the relevance of our findings for ACD in more detail in the discussion section on page 12. Mutations of MRP8 or MRP14 genes in humans have not been described so far.

5) The title is too vague. The authors should choose a title that closely describes the object of their work (ie contact dermatitis) and not advertise unproven general significance for the whole of adaptive immunity.

We agree and specified the title accordingly.

6) The implications of this work for the "hygiene hypothesis" are not obvious to me and should either be clarified or removed (also in the abstract).

We agree with the reviewer that our work has no direct implications for the hygiene hypothesis. However, in our opinion it is a striking finding that lack of an endogenous TLR4 ligand is associated with a phenotype which at least is in accordance with the major conclusion of the so called hygiene hypothesis that continuous subclinical stimulation of TLR4 dampens allergic processes. In our opinion it should be allowed to raise this point in the discussion section. However, according to the

suggestion of the reviewer we shortened this part and clarified our intention in the revised discussion section on page 12.

Reviewer #2
We thank the reviewer for encouraging and critical comments. Our responses are given below.

1) The title does not correctly represent the contents of the manuscript. First, Mrp14 has no effect per se. Second, Mrp8 appears to dampen (rather than regulate) T cell proliferation.

The dominant form in vivo is the Mrp8/14 complex. Our data indicate that Mrp8 is the active form in the complex while Mrp14 has regulatory properties. Mrp8 has certainly the capacity to activate already maturated dendritic cells but we now show that long term stimulation during early development leads to a 'suppressed' phenotype of dendritic cells. Therefore our data do not indicate that Mrp8 simply dampens T cell proliferation However, according to the reviewer's suggestion we specified the title of our revised manuscript (See also response to comment 5 of reviewer 1).

2) MDSCs are a group of poorly defined monocyte-derived cells and maybe even neutrophils. It is not fair to call them immature DCs. It is unclear whether the characterization as CD11b+Gr1+ is sufficient to identify MDSCs.

We apologize not having explained our characterization of MDSCs in more detail. In agreement with the general accepted nomenclature we characterized monocytic MDSCs as $CD11b^+$ $Ly\text{-}6C^+$ cells. In contrast to this, granulocytic MDSCs are $CD11b^+$ $Ly\text{-}6G^+$ cells. $CD11b^+$ $Ly\text{-}6C^+$ MDSCs are known to play a prominent role in autoimmunity and inflammation and we focussed on $CD11b^+$ $Ly\text{-}6C^+$ MDSCs. Our data indicate that we see reduction of so called MDSCs in MRP14 -/- mice as described earlier in a tumour model (Ref. Cheng et al, 2008 of our manuscript) but our transfer experiments revealed that MDSCs are not significantly involved for the observed effects in ACD in vivo. It was not our intention to say that MDSCs are immature DCs. To clarify this, we improved our description of MDSCs in the results section (page 4).

3) *One explanation for the apparent discrepancy between the data in figure 1 and the dermatitis data is that dermal DCs are very different from BMDCs. This possibility should be explored.*

The reviewer is right that there are differences in the properties of dermal DCs and BM derived DCs. However, numerous DC subsets from Mrp14-/- mice, such as BM-derived DC, dermal DC, epidermal Langerhans cells or LN-resident DC showed a higher capacity to induce T-cell proliferation (Fig. 4A and data not shown) clearly suggesting a rather general effect of Mrp8/14 on the DC phenotype and function.

4) *Figure 3B indeed shows that exogenous recombinant Mrp8 impairs BMDC differentiation. At the same time, the data suggest that endogenous Mrp8 has no effect, because the differentiation of Mrp14 deficient and sufficient BMDCs is the same. This raises two questions: Is recombinant Mrp8 contaminated with LPS? Second, is the level of recombinant Mrp8 much higher than the Mrp8 secreted from BMDCs? Seems to be 3-fold higher*

We agree with the reviewers´ comment that possible endotoxin contaminations in the Mrp8 preparations are a major issue. All Mrp-proteins used in this study were prepared and qualitatively controlled as published earlier (Vogl et al, 2007, *Nat. Med.* 13, 1042-1049). The maximal endotoxin contamination is below 2 pg LPS / µg Mrp protein as determined by LAL. All effects observed could not be inhibited by addition of PolymyxinB but were abolished by heat denaturation of proteins. We apologize for not having mentioned the exclusion of LPS in our protein preparations in our Material and Methods section. We have now added this information to materials and methods on page 14.
Furthermore, we missed to note that BMDCs do neither express and secrete Mrp8 nor Mrp14. During differentiation of bone marrow cells to dendritic cells or macrophages Mrp8/14 is rapidly down regulated at early stages of differentiation. Thus, one cannot expect 'endogenous' effects of MRP8 in a culture models of pure BMDCs.

5) *Throughout the manuscript, it seems to make no difference whether T cells are from Mrp14-/- or wt mice. A likely explanation is that Mrp8 is not expressed by T cells. Is this so?*

That's correct T cells do neither express Mrp8 nor Mrp14. We added this information in our revised manuscript on page 8. However, T-cells express TLR4 under some conditions which theoretically could influence T-cell differentiation or activation by MRP8.

6) *The effect of MDSCs on T cell CFSE dilution is small (if any) and not convincing. Figure 5A: how many experiments? 5B: is 30 to 40 really different from 39 to 46? Stats? Figure 5 could be eliminated. The critical experiment is shown in figure 6.*

We agree with the comment of the reviewer that the critical and more important experiment is shown in figure 6.
However, we would like to show figure 5 for completeness of our findings. We agree the overall contribution of MDSCs on T cell proliferation is small but still significant. It illustrates that Mrp8/14 induces a shift from immunogenic myeloid cells like mature DCs to rather immature myeloid cells like MDSCs as shown in a tumour model before. We replaced the term "…which finally results in a inhibitory effect on adaptive immunity…" by "…which finally results in a minor but still significant inhibitory effect on adaptive immunity…" in the text on page 8.
Our data indicate that we see this effect in ACD as well but, as the reviewer suggested, has no relevant effect in vivo as confirmed by our transfer experiments.
We performed four independent experiments in figure 5A which is as now described more clearly in the figure legend. We added figure 5C as a new figure showing summarized MDSC data including statistics.

7) *The dermal DCs were injected iv. A tacit assumption is that they home correctly. Any evidence for this?*

We thank the reviewer for this valuable comment and added additional experimental work. To address this question we checked if the injected DCs home correctly. Therefore, we stained isolated dermal DCs with "cell tracker orange" (CTO) prior to injection. 48 hours after i.v. application we analyzed the draining lymph nodes for CTO-positive cells by FACS analysis. In another set of experiments, we applied FITC on the ears of wt and Mrp14 -/- mice and after 18 hours we analysed the draining lymph nodes for FITC-positive cells. In both experimental settings we did not find differences in homing between wt and Mrp14 -/- mice indicating that the migratory properties are not altered in Mrp14 -/- dendritic cells. These results and methods related to them are indicated in the text on page 9 and we now added these data as new supplementary figure S2.

8) *The first four paragraphs of the discussion largely repeat the results. Is there evidence of more autoimmune disease in Mrp14 KO mice?*

The Mrp14 -/- mice are completely healthy, fertile and show no phenotype in the absence of any disease, which was published by us and the group of Nancy Hogg a couple of years ago (see also ref. Manitz et al, 2003 and Hobbs et al, 2003). Therefore there is no evidence for development of spontaneous autoimmune diseases in healthy Mrp14 -/- mice.

9) The T cell proliferation assays are not well described. Some are apparently allogeneic. What mouse strain? Figure 1 says Balb/c. Others? Or was a TCR transgenic used?

We apologize not having described these experimental details clearer. The Mrp14 -/- mouse was backcrossed to C57Bl/6 background (F10 generation) and for allogenic T-cell proliferation assays we used T-cells from Balb/c mice. We now clarified it in the figure legends if allogenic or syngenic T cell proliferation assays were performed. We did not use TCR transgenic models.

10) *The CD86 and MHC-II differences reported in figure 1 are very small and likely incorrectly calculated. Inspection of the histograms suggests that the upregulation by LPS was at least 10-fold for CD86, whereas the effect of Mrp8 was very modest. The negative control (isotype?) is not specified.*

We agree with the criticism of the reviewer and therefore replaced the term “strong up-regulated” by “up-regulated” in the text section on page 5 as also mentioned by the first reviewer. The effects of Mrp8 on surface expression of CD86 and MHC-II are certainly moderate albeit significant. However, the increase in the T cell proliferation rates are within the same range as observed for the LPS condition indicating relevance of our findings. Differences in contact times between MRP14 -/- and wt DCs may be another relevant factor (See also response to comment 3 of reviewer 1). The negative control in figure 1 represents the surface expression of CD86 and MHC-II on unstimulated iBMDCs. We modified figure 1 and now presented mfi-shifts instead of fold-increase including the standard deviation of the controls (see revised figure 1A).

11) *Figure 3, 8 µg/ml is three times the maximum endogenous Mrp8. This should be repeated with a more reasonable dose.*

In this point we do not agree with the reviewer. It is right that 8 µg/ml Mrp8 is higher than the systemic serum Mrp8/14 concentration during the sensitization phase of the mice only indirectly reflecting concentrations at local sites of release. However, local Mrp-levels are typically 10 to 30 times higher than corresponding systemic levels during disease (Van Zoelen et al, 2009, *Am. J. Respir. Crit. Care Med.* 180, 1098-1106; Frosch et al, 2003, *Arthritis Rheum.* 48, 2622-2626; Frosch et al, 2000, *Arthritis & Rheum.* 41, 628-637). Therefore, doses used in our experimental settings are of physiological relevance in vivo.

12) The data in figure 4 is uninterpretable, since both the dDCs and T cells were wt or Mrp14-/-. Most likely, the T cell source makes no difference, but this needs to be formally shown.

We agree with the reviewers´ comment and included more statistics in figure 4C to make the point clear that the T cell source does not affect proliferation rates. In addition figure 4C was not labelled clearly which we corrected accordingly.

Minor point: Page (not numbered), line 1: should "native" be "naive"?.
Correct, we changed it accordingly.

Reviewer #3
We thank the reviewer for encouraging and critical comments. Our responses are given below.

Major concerns:
1) *The entire logic is based on the fact that MRP8/14 through TLR4 act as a negative regulator of DC maturation/activation during the development of a dermal T cell activation response. While this is possible it could all be simply a classic and simple desensitization that was well described for the TLR as well as other activatory pathways. In this scenario, induction of endogenous TLR ligands would dampen normally the response while in the absence of these ligands response can be exacerbated. While this is not a minus in the story, it has to be actively presented as well as tested for from a pathway point of view at the level of the signaling cascade. One would want to show - in vitro BMDCs having the TLR signal partially desensitized and describe the level at which this is happening. Also in TLR4 ko animals this should not happen. Additionally, phenotypes in TLR4 ko mice should be discussed and considered in this context as they have been very well described in similar mode ls (example: J Exp Med. 2008 Sep 1;205(9):2151-62).*

We followed the reviewer’s advice and explored if the effect of Mrp8/14 on DC differentiation might be a simple desensitization. We could exclude this possibility since short time preincubation of DCs with Mrp8 (from day 5 to day 6 of culture) prior to LPS activation (from day 6 to day 8 of culture) had no suppressing effect confirming that MRPs induce a lasting effect on early DC-differentiation different from classical ‘LPS tolerance’. We included these data to our revised figure 3D and E.
Furthermore, we performed experiments with BMDCs of TLR-4 -/- mice and could demonstrate that the early DC differentiation was not suppressed by Mrp8 in TLR4 -/- cells (new figure 3C) demonstrating that MRP-effects on early DC differentiation are TLR4 dependent as well. The paper mentioned by the reviewer is an excellent example for a role of TLR-dependent signalling in allergic contact hypersensitivity.

This information is now added to the text on pages 3 and 10.

2) *Dermal DCs which are the cells instigating the response being read in vivo are not well characterized phenotypically; the functional characterization is indeed difficult and some of the readouts (t cell proliferation) are marginal but in line with the phenotype. Despite this, the authors used very string decisive language around the phenotype, which is minor and the MRP8/14 have relatively small effects; additionally little is mentioned about other immune instances that could test this hypothesis. Similar experiments are relatively easier done in the lung. Also immune responses could deconstruct this system better. This should be also reflected in the title which should be focused on the exact systems one can prove the biology in.*

We agree that dermal dendritic cells are not a homogenous population and phenotypical characterization not complete. However, we could observe in principal identical effects of Mrp8/14 or Mrp8 on isolated dermal dendritic cells as well as dendritic cells derived from the bone marrow. So at least a subpopulation of dermal dendritic cells is sufficient to mediate the immunosuppressive effects induced by the Mrp´s. We changed our 'very string decisive language' as suggested by the reviewer. Our MRP14 -/- mouse is currently investigated by more than 50 groups including labs focussing on pulmonary inflammation. However, to our knowledge no published data are available so far.
As suggested also by both other reviewers we changed the title accordingly.

3) *The last major experimental line in which ko dermal DCs are being used in transfers to recapitulate the phenotype is missing significant controls to show identical cellular homing before initiation of the immune response. While this is challenging it is necessary in order to be able to interpret this major line of experiments.*

That's a really valuable comment which was also raised by the first reviewer. To address this question we checked if the injected DCs home correctly. Therefore, we stained isolated dermal DCs with "cell tracker orange" (CTO) prior to injection. After 48 hours we analyzed the draining lymph nodes for CTO-positive cells by FACS analysis. In another set of experiments, we applied FITC on the ears of wt and Mrp14 -/- mice and after 18 hours we analysed the draining lymph nodes for FITC-positive cells. In both experimental settings we did not find differences in homing between wt and Mrp14 -/- mice indicating that the migratory properties are not altered in Mrp14 -/- dendritic cells. These results and methods related to them are indicated in the text on page 9 and we now added these data as new supplementary figure S2.

Minor points:

1. The sources and quality control for MRP8 and MRP14 proteins used in in vitro assays should be well documented in material and methods.

We agree with the reviewers´ comment to rule out effects of possible endotoxin contaminations in the Mrp8 preparations as mentioned in our comment to reviewer 2, comment 4. We apologize for not having mentioned the exclusion of LPS in the used protein preparations in our Material and Methods section. This information is now added to the text on pages 14.

2. Figure 4 C is confusing as it could open the possibility for a T cell intrinsic component of the MRP8/14 defect.

We agree with the comment of the reviewer that results in Figure 4C are hard to understand. We apologize that we did not label bars correctly in the old Figure 4C. We now corrected legends and labels in the revised Figure 4C. Figure 4 comprises all combinations of dendritic cells/T-cells from wt and MRP14 -/-. In addition, T cells do not express Mrp proteins, therefore the possibility of intrinsic effects on T-cell differentiation can be ruled out. We added this information in our revised manuscript on page 8. Furthermore, we included more statistics in the revised figure 4C to make the point clear that the T cell source does not affect proliferation rates induced by wt- or Mrp14 -/- - dendritic cells isolated from the draining lymph nodes or bone marrow, respectively.

3. Fig 5B axes are not labeled.

We followed the reviewer's advice and corrected figure 5B.

4. Discuss the significant amount of literature on TLR2/4 and RAGE in similar systems.

We addressed this point in our revised version as suggested by the reviewer. This information and references are now added to the discussion on page 10.

2nd Editorial Decision 15 October 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by referees #1 and 3 and their comments are provided below. As you can see, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore pleased to accept the paper for publication here. Before doing so, referee #1 has a few suggestions to improve the clarity of the text and the figures - no new experiments are needed. The referee suggests to improve the discussion concerning the hygiene hypothesis and to make this argument more clear. Also the figures need a few amendments.

Once we get the revised version in, we will go ahead with its acceptance.

Yours sincerely

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised version of the ms by Petersen et al is clearly improved.

My greatest reservation, which I see was more or less shared by the other reviewers, was that the results were not clear-cut enough to eliminate all doubts or possible alternative explanations. From this point of view, some experiments were redone and gave clearer results, and some alternative explanations were tried and found wanting.

Yet some more polishing is required. The main point, as I see it, is that Mrp8/14 stimulates DCs, yet prolonged exposure to Mrp8/14 retards the maturation of DCs, so that effectively Mrp8/14 acts as a proinflammatory mediator on the short term but reduces adaptive responses on the long term. The authors are right in pointing out that this recalls the hygiene hypothesis: activation of TLR4 by pathogens gives inflammation on the short term, but reduces allergy on the long term. The interesting part is that the activator of TLR4 is a self protein in this case, and not a pathogen.
It is then a pity that their text does not argue their interpretation in clearer terms. I completely missed what they meant with the hygiene hypothesis when I first read the paper, and only grasped it now. What is needed is a clearer argument, especially in the discussion. The title, as well, is much improved but somehow misses the point that the authors want to make.

The individual experiments that I had criticized have now been fixed. However, the figures are still more difficult to read than need be. For example, in figure 1A the y axis is labeled "change in surface expression (mfi-shift)" whereas a more intuitive label would be "surface expression (mfi)". Their label laso makes on wonder why the unstimulated cell should have a "change" of nearly 5, whereas by definition they should have no change since they are the reference. Fig 1B is nearly unreadable because too small, and so on.

All in all, these are minor problems that can be fixed with no additional experiments.

Referee #3 (Remarks to the Author):

The revised manuscript and changes made answers the prior criticism and I evaluate it suitable for publication.

2nd Revision - authors' response 27 October 2012

Reviewer #1
We thank the reviewer for the encouraging and critical comments. Our responses are given below.

1) The main point, as I see it, is that Mrp8/14 stimulates DCs, yet prolonged exposure to Mrp8/14 retards the maturation of DCs, so that effectively Mrp8/14 acts as a pro-inflammatory mediator on the short term but reduces adaptive responses on the long term. The authors are right in pointing out that this recalls the hygiene hypothesis: activation of TLR4 by pathogens gives inflammation on the short term, but reduces allergy on the long term. The interesting part is that the activator of TLR4 is a self protein in this case, and not a pathogen. It is then a pity that their text does not argue their interpretation in clearer terms. I completely missed what they meant with the hygiene hypothesis when I first read the paper, and only grasped it now. What is needed is a clearer argument, especially in the discussion.

We improved our discussion according to the reviewer's suggestion. We now illustrated the parallels of our data and the 'hygiene hypothesis' in clearer terms in the discussion section on page 12.

2) The figures are still more difficult to read than need be. For example, in figure 1A the y axis is labeled "change in surface expression (mfi-shift)" whereas a more intuitive label would be "surface expression (mfi)". Their label also makes on wonder why the unstimulated cell should have a "change" of nearly 5, whereas by definition they should have no change since they are the reference. Fig 1B is nearly unreadable because too small, and so on.

We followed the reviewer's advice and improved the figures. We enlarged the figures (especially figure 1 B, figure 3, and figure 5) as well as all labels. The label "change in surface expression (mfi-shift)" has been changed to "surface expression (mfi-shift)" as suggested. In addition we described the calculation of mfi-shifts in the Material and Methods section on page 16.

