European Journal of Immunology

Peer review correspondence

Lysosomal phospholipase A2: A novel player in host immunity to *Mycobacterium tuberculosis*

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Handling Executive Committee member: Prof. Caetano Reis e Sousa

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

<u>First Editorial Decision – 8 January 2014</u>

Dear Dr. Schneider,

Manuscript ID eji.201344383 entitled "Lysosomal phospholipase A2: A novel player in host immunity to Mycobacterium tuberculosis" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

You should also pay close attention to the editorial comments included below. In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of
Prof. Caetano Reis e Sousa

Laura Soto Vazquez
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Reviewer: 1

Comments to the Author
In the current manuscript, Schneider and colleagues investigate the consequences of lysosome PLA2 deficiency in the protective immune response to M. tuberculosis. The authors show that LPLA2 deficient mice displayed higher bacterial burdens in the lungs and associate this phenotype with reduced Th1 cell responses. Furthermore, the authors show that the dissemination of M. tuberculosis to the lymph nodes of LPLA2 deficient mice occurred in a similar manner to that observed for WT ones, although the differentiation of CD4 IFN-gamma-producing T cells was affected. Despite the higher bacterial burden in the absence of LPLA2, the lung inflammation was found to be decreased in this situation and the animals only succumbed at late time points post infection with high dose of inoculum.
The paper is written in a clear way and the story is easy to follow. While the data presented is interesting and of potential interest to the field of mycobacterial research, it lacks on the mechanisms underlying the increased susceptibility of LPLA2 KO mice.
- The differences shown for bacterial load (Fig. 1A) and even in terms of CD4+IFN-gamma-producing cells (Fig. 4F) seem to be less pronounced with time. Is the observed phenotype transient? The authors mention in the Discussion that even for later time points the differences observed for bacterial burdens hold true. These data are of interest and should be shown.

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- Data in Figure 1E is non-statistically significant. Are the authors convinced that «… LPLA2 KO mice, though more susceptible to infection, only succumb very late even upon high dose M. tuberculosis infection» (Discussion page 14)?
- The authors show less inflammation in the lungs of LPLA2 deficient mice and also less cell numbers on day 43 post-infection. This is in line with decreased cell recruitment in this case. In addition to CD4 T cells, have the authors investigated the recruitment of other immune cells? Are the differences also observed at earlier time points?
- Is the absence of LPLA2 only affecting Th1 cell responses or are Th17 ones also affected? the observation that LPLA2 deficient mice has reduced T cell responses during the early stages of the chronic stages of the infection is rather interesting and needs to be further explored with the potential to provide important insights into the T cell activation and maintenance during M. tuberculosis infection.
- The authors show that the bactericidal activity of LPLA2 deficient macrophages is not affected (Figs. 3A and 3B), but then conclude that the in vivo functions of macrophages are impaired. This is on the basis that less TNF and CCL2 are produced in the lungs of LPLA2 infected mice as compared to WT ones (Fig. 5A). Is the production of these molecules affected in the in vitro system? If so, the author's argument would be a lot stronger. These data could be included as part of Fig. 3.
- Based on the data shown in Fig. 5, the authors claim that less IFN-gamma is produced in M. tuberculosis-infected LPLA2 mice than in WT ones. However, the differences shown appear to be non-statistically significant. The authors should clarify this issue. Could they support their argument for example by measuring mRNA?
- Figs. 6 and 7 could be merged as they both relate to what might be happening in the LNs of LPLA2 deficient animals upon M. tuberculosis infection. The authors show that the dissemination of M. tuberculosis to the LN is comparable in WT and LPLA2 deficient infected mice. However, the differentiation of Th1 cells appears to be impaired in the case of LPLA2 deficient mice, as is the production of TNF and CCL5. Did the authors also check the production of IL-12, a key determinant of Th1 cell differentiation? Are LPLA2 deficient DCs fully functional? Is the kinetics of DC migration to the LN similar in WT versus LPLA2 deficient mice? Is the proliferation of Th1 cells similar in WT or LPLA2 deficient mice? These are aspects that could elucidate the mechanism(s) underlying the impaired Th1 cell responses observed in LPLA2 deficient mice and should be considered by the authors. Reducing the phenotype to the decrease observed for CCL5 may be over simplistic.
- Figure legends: it is not clear how many times was each experiment performed, neither whether Mean±SD is shown in the graphs. Also, statements describing the data are not needed in the figure legends. For the figures showing experimental infections, it would be important to specifically show or refer in the figure legend the CFU obtained 24h post infection. The authors are vague on this referring to 200 CFUs in some cases and to approximately 200-250 CFUs in other. Most of the figures show one experiment representative of the 2 or 3 performed. The average of the 2 or 3 experiments should be plotted and the statistical analysis performed on these results, rather than on technical replicates. Why did the authors chose to perform T tests instead of ANOVA?

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Overall, the paper is interesting and with potential interest to the field. However, in its current form and due to the lack of a mechanistic basis to explain the observations discussed, the current version of the manuscript does not provide any novel insight into the role of LPLA2 in the protective immune response to M. tuberculosis or into the pathophysiology of tuberculosis.

Reviewer: 2

Comments to the Author
Schneider et al. studied the course of M. tb infection and the induction of an immune response to infection in mice deficient in phospholipase A2. These animals are slightly more susceptible than wt mice and show impaired Th1 responses and inflammation.

The difficulty in this study is to distinguish what is due to the lack of the enzyme as a possible provider of a required metabolite from the over-abundance of phospholipids that remain undigested. It would be helpful to study in vitro what the effects are of a surplus of the major phospholipids. In particular, will an overload with surfactant-type phospholipids affect the antimycobacterial function of peritoneal macrophages (given that studying alveolar macrophages themselves would likely be rather difficult) or the priming of T cells?

In general, however, this is a careful study with a rather comprehensive immune phenotyping.

Finally, given the known role of lipoxins in promoting susceptibility of mice to TB, these eicosanoids should also be tested.

First Revision – authors' response – 14 April 2014

Reviewer: 1
Comments to the Author
In the current manuscript, Schneider and colleagues investigate the consequences of lysosome PLA2 deficiency in the protective immune response to M. tuberculosis. The authors show that LPLA2 deficient mice displayed higher bacterial burdens in the lungs and associate this phenotype with reduced Th1 cell responses. Furthermore, the authors show that the dissemination of M. tuberculosis to the lymph nodes of LPLA2 deficient mice occurred in a similar manner to that observed for WT ones, although the differentiation of CD4 IFN-gamma-producing T cells was affected. Despite the higher bacterial burden in the absence of LPLA2, the lung inflammation was found to be decreased in this situation and the animals only succumbed at late time points post infection with high dose of inoculum.

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The paper is written in a clear way and the story is easy to follow. While the data presented is interesting and of potential interest to the field of mycobacterial research, it lacks on the mechanisms underlying the increased susceptibility of LPLA2 KO mice.

- The differences shown for bacterial load (Fig. 1A) and even in terms of CD4+IFN-gamma-producing cells (Fig. 4F) seem to be less pronounced with time. Is the observed phenotype transient? The authors mention in the Discussion that even for later time points the differences observed for bacterial burdens hold true. These data are of interest and should be shown.

Thanks to the referee for pointing out this issue. The phenotype is not a transient one. Therefore, we added a graph showing CFU data from a late time point after infection, i.e. day 195 to Figure 1 (new Fig. 1 A), which illustrates perpetuation of the differences in bacterial loads between LPLA2 KO and WT mice throughout infection.
We have further rearranged Figure 1 and removed the early time point of CFU analysis since we show data on early lung CFU in Figure 6. We also removed the CFU from spleen (former Figure 1D) from this figure and included it as Figure 2E where it fits better with the data on spleen weight and cell counts.

- Data in Figure 1E is non-statistically significant. Are the authors convinced that «… LPLA2 KO mice, though more susceptible to infection, only succumb very late even upon high dose M. tuberculosis infection» (Discussion page 14)?

Due to the higher bacterial loads, we are convinced that LPLA2 KO mice fail to properly control M. tuberculosis infection. In order to argue along our data we rephrased the sentence accordingly:
"The fact that LPLA2 KO mice, despite having higher bacterial loads, succumb to infection earlier than WT mice but only at very late time points, even upon a high initial dose of M. tuberculosis, indicates that the limited T cell activation observed is still sufficient to keep the infection at bay for long time." (Discussion p. 15).

- The authors show less inflammation in the lungs of LPLA2 deficient mice and also less cell numbers on day 43 post-infection. This is in line with decreased cell recruitment in this case. In addition to CD4 T cells, have the authors investigated the recruitment of other immune cells? Are the differences also observed at earlier time points?

This is indeed also true for other immune cells. As depicted in Figures 4, we also analyzed recruitment and activation of CD8 T cells. Like CD4 T cells, the numbers of CD8 T cells recruited to the lung were already reduced at day 20 post infection. In addition, we investigated the recruitment of innate cells and found reduced infiltration of CD11b+Ly6G+ inflammatory monocytes into the LPLA2 KO lungs compared

to WT lungs. These data have now been included as Figure 4 A. The results and discussion have been amended accordingly (Results p. 7; Discussion p. 12).

- Is the absence of LPLA2 only affecting Th1 cell responses or are Th17 ones also affected? the observation that LPLA2 deficient mice has reduced T cell responses during the early stages of the chronic stages of the infection is rather interesting and needs to be further explored with the potential to provide important insights into the T cell activation and maintenance during M. tuberculosis infection.

We measured IL-17A levels in lungs of WT and LPLA2 KO mice 20 and 45 days after M. tuberculosis infection. Since IL-17A concentrations did not differ between WT and LPLA2 KO lungs, we concluded that IL-17A is not affected by the LPLA2 KO phenotype and included the data as supporting information (Suppl. Fig. S1C) and mentioned them in the text (Results p. 8; Discussion p.12).

- The authors show that the bactericidal activity of LPLA2 deficient macrophages is not affected (Figs. 3A and 3B), but then conclude that the in vivo functions of macrophages are impaired. This is on the basis that less TNF and CCL2 are produced in the lungs of LPLA2 infected mice as compared to WT ones (Fig. 5A). Is the production of these molecules affected in the in vitro system? If so, the author’s argument would be a lot stronger. These data could be included as part of Fig. 3.

We thank the referee for pointing out the putative effect of LPLA2 deficiency on TNFa production as a measure of macrophage activation in vitro. Indeed, TNFa production upon infection of KO macrophages with M. tuberculosis is significantly reduced when compared to WT ones. These data have been added as Fig. 3D and are mentioned in the text (Results p.7; Discussion p. 12).

- Based on the data shown in Fig. 5, the authors claim that less IFN-gamma is produced in M. tuberculosis-infected LPLA2 mice than in WT ones. However, the differences shown appear to be non-statistically significant. The authors should clarify this issue. Could they support their argument for example by measuring mRNA?

This claim originates from the data shown in Figures 4 and 6 depicting reduced frequencies of IFN-□ producing CD4 and CD8 T cells in lungs and lymph nodes upon in vitro restimulation. Together with the reduced IFN-□ levels in organ lysates as shown in Figure 5 - though statistically not significant - it can be stated that production of this Th1 hallmark cytokine is limited in KO when compared to WT mice upon Mtb infection. We rephrased the sentence in the Results and Discussion part accordingly (page 8; page 12).

- Figs. 6 and 7 could be merged as they both relate to what might be happening in the LNs of LPLA2 deficient animals upon M. tuberculosis infection. The authors show that the dissemination of M. tuberculosis to the LN is comparable in WT and LPLA2 deficient infected mice. However, the

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differentiation of Th1 cells appears to be impaired in the case of LPLA2 deficient mice, as is the production of TNF and CCL5. Did the authors also check the production of IL-12, a key determinant of Th1 cell differentiation?

We follow the suggestion of the referee and merged Figures 6 and 7 in order to make it easier for the reader to correlate lymph node bacterial loads and cytokine production.
IL-12 levels were below the detection limit and therefore not shown.

Are LPLA2 deficient DCs fully functional? Is the kinetics of DC migration to the LN similar in WT versus LPLA2 deficient mice?
Is the proliferation of Th1 cells similar in WT or LPLA2 deficient mice? These are aspects that could elucidate the mechanism(s) underlying the impaired Th1 cell responses observed in LPLA2 deficient mice and should be considered by the authors. Reducing the phenotype to the decrease observed for CCL5 may be over simplistic.

We thank referee for bringing this important question to our attention. Indeed, we measured T cell proliferation in WT and LPLA2 KO mice using ovalbumin as model antigen to test the general ability of LPLA2 KO mice to promote T cell proliferation. This was tested both, in naïve and in M. tuberculosis infected animals. In either case we observed comparable proliferation rates of adoptively transferred CFSE labeled OT1 (CD8) or OT2 (CD4) T cells in WT and KO mice. These data suggest that T cell proliferation in general is not hampered in the absence of LPLA2 and furthermore, that antigen presentation and DCs are fully functional. These data further support our hypothesis that impaired T cell responses in LPLA2 deficient mice during M. tuberculosis infection are due to a failure in immune cell recruitment. We have added T cell proliferation data in M. tuberculosis infected mice as Suppl. Figure 3. The data are also described and discussed in the text (Results p. 7/8 and Discussion p. 13).

- Figure legends: it is not clear how many times was each experiment performed, neither whether Mean±SD is shown in the graphs. Also, statements describing the data are not needed in the figure legends.

We have amended the figure legends accordingly.

For the figures showing experimental infections, it would be important to specifically show or refer in the figure legend the CFU obtained 24h post infection. The authors are vague on this referring to 200 CFUs in some cases and to approximately 200-250 CFUs in other.

Thanks to referee for pointing out this lack in information. The exact CFUs are now included in the figure legends. It should be emphasized that the phenotype observed was independent of the inoculum size.

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Most of the figures show one experiment representative of the 2 or 3 performed. The average of the 2 or 3 experiments should be plotted and the statistical analysis performed on these results, rather than on technical replicates.

For in vivo experiments, we show individual mice, i.e. biological replicates. For in vitro data in Figure 3, we indeed showed technical replicates. Unfortunately, some of the data are very difficult to pool or to average, e.g. CFU data because the growth characteristics in vitro can vary considerably between individual experiments. We therefore now show two experiments when plotting the averages was not possible. We included averaged data for LPLA2 enzyme activity, NO and TNF-a production.

Why did the authors chose to perform T tests instead of ANOVA?

Because we compared only two groups (WT versus LPLA2) for each time point analyzed.

Overall, the paper is interesting and with potential interest to the field. However, in its current form and due to the lack of a mechanistic basis to explain the observations discussed, the current version of the manuscript does not provide any novel insight into the role of LPLA2 in the protective immune response to M. tuberculosis or into the pathophysiology of tuberculosis.

Reviewer: 2
Comments to the Author
Schneider et al. studied the course of M. tb infection and the induction of an immune response to infection in mice deficient in phospholipase A2. These animals are slightly more susceptible than wt mice and show impaired Th1 responses and inflammation.

The difficulty in this study is to distinguish what is due to the lack of the enzyme as a possible provider of a required metabolite from the over-abundance of phospholipids that remain undigested. It would be helpful to study in vitro what the effects are of a surplus of the major phospholipids. In particular, will an overload with surfactant-type phospholipids affect the antimycobacterial function of peritoneal macrophages (given that studying alveolar macrophages themselves would likely be rather difficult) or the priming of T cells?

We did not observe differences in M. tuberculosis growth when comparing LPLA2 and WT macrophages independent of whether the macrophages were resting or activated (in which case they can restrict mycobacterial growth). This is shown in Figure 3 for peritoneal macrophages. Therefore, the phospholipidosis phenotype of KO macrophages does not affect anti-mycobacterial activity. Therefore, it is

also not expected that feeding lipid membranes to KO macrophages, would affect their anti-mycobacterial activity.

In general, however, this is a careful study with a rather comprehensive immune phenotyping.

Finally, given the known role of lipoxins in promoting susceptibility of mice to TB, these eicosanoids should also be tested.

According to the referee's suggestion, we measured Lipoxin A4. Similar to what we have found for LTB4, levels of LXA4 were not different between WT and KO LN and lungs but were increased in spleens of LPLA2 KO mice. Since we do not believe that this is of relevance for the phenotype observed in lung and LN of LPLA2 KO mice, we only added the data to the Ssupplementary Figure S2 (S2) and mentioned them in the results (p. 8) and discussion (p. 12).

Second Editorial Decision – 2 May 2014

Dear Dr. Schneider,

It is a pleasure to provisionally accept your manuscript entitled "Lysosomal phospholipase A2: A novel player in host immunity to Mycobacterium tuberculosis" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Laura Soto Vazquez

on behalf of Prof. Caetano Reis e Sousa

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