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The location of splenic NKT cells favours their rapid activation by blood-borne antigen

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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 02 February 2012

Thank you for submitting your manuscript to the EMBO Journal. Please find enclosed the comments of two of the three reviewers whom we had asked to evaluate your manuscript for EMBO Journal. We are still waiting for a third report, but given the present majority recommendation I can make a preliminary decision now to save time. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so.

As you can see below, both referees find the analysis interesting and appreciates that it advances out insight into NKT cell activation in the spleen. While referee #1 raises minor concerns, referee #2 raises a number of specific issues that has to be resolved for publication here. Given the comments provided, I would like to ask you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. I will forward the comments of the third referee to you as soon as we receive them, together with our final editorial decision.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Barral and coll undertake an elegant and complex study to determine the topographical distribution of iNKT cells in the spleen as a function of their response to a synthetic blood-born particulated antigen model.

Previous studies that attempted to define the topographical distribution of iNKT cells in secondary lymphoid organs, although not has the main goal of the study, did not reach univocal conclusions. Using methods differentfrom those utilized here by Barral et al, two distinct localization for iNKT cells were found: exclusion from the splenic T cell zone (PALS) and, by reflection, probable association with the B cell follicles and MZ (Stetson et al JEM 2003); inclusion mainly in the PALS and exclusion from the B cell areas, MZ and red pulp (RP) (Thomas et al JEM 2011).

Barral et al show with an acceptable degree of approximation that, at resting conditions, the majority of splenic iNKT cells are localized in the MZ (50%), followed almost equally by the RP (25%) and PALS (25%), but are essentially excluded from the B cell follicles, based on complementary experimental approaches: 1. Rapid iNKT cell labeling kinetics following injection of anti-CD45 mAb in vivo; 2. Histological (confocal microscopy) detection of endogenous TCRb+NK1.1+ cells and of adoptively transferred marked iNKT cells; 3. Time lapse multiphoton microscopy of adoptively transferred labeled iNKT cells from isolated spleens.
The authors go on by assessing the effective relationship between the preferential anatomical localization of iNKT cells in the MZ and RP of the spleen and their rapid response to blood-born particulated antigens. They indeed provide evidence that MZ/RP associated iNKT cells are rapidly activated within 2h from the injection of the particulated antigen into mice, and that this depends on the uptake and presentation of it by MZ/RP resident DCs and macrophages, with some intriguing residual antigen handling possibly by MZ/RP B cells.

The study by Barral et al has the merit to clarify the iNKT cell topography in the spleen, underscoring their function as innate-like sentinel of blood born infections owed to their anatomical localization.

Minor points
1. Does the injection of soluble aGalCer (not complexed to particles) elicits the same kind of differential activation in MZ- vs PALS-associated iNKT cells?
2. Figure 6. It would be useful to show the % of gated iNKT cells in panels B, D, F.

Referee #2

This concise report provides new insight regarding the location of NKT cell activation in the spleen. The low frequency of NKT cells and their lack of a single marker that distinguishes them from other T cells or NK cells has made determining their distribution difficult. Using in vivo antibody pulse labeling, multicolor confocal analysis of sections and adoptive transfer approaches, this study shows convincingly that the majority of NKT cells in the spleen are located in the marginal zone and red-pulp. It then shows that it is the blood exposed cells that are rapidly activated by intravenously injected lipid antigens. Using macrophage plus DC ablation approaches, NKT cell activation is shown to depend on these cells, and some evidence is provided that DCs contribute to antigen presentation. 2-photon microscopy on splenic explants shows that red pulp and MZ NKT cells migrate quite rapidly in the absence of their lipid antigen but undergo frequent migration arrest when antigen is present. Overall, these data advance our understanding of the site of NKT cell activation by systemic lipid antigen and they provide some initial information on the earliest dynamics of NKT cell activation in the spleen.

Specific concerns:

1. Liposomal clodronate in addition to depleting macrophages and DC's is known to cause a loss of marginal zone B cells from the spleen. In published work these authors have shown that B cells can form stable conjugates with NKT cells. This confounds interpretation of the data presented in Figure

6A-D. The authors should examine how the numbers of MZ B cells at different time points after clodronate treatment relates to the NKT cell response. Alternatively, they might determine whether DT administration to CD11c-DOG animals results in changes in splenic B cell populations to rule out this possibility.

2. Related to 1, previous work has shown that SIGNR1+ macrophages reappear in the spleen more slowly than CD169+ macrophages. It would be useful to know if the full restoration of the NKT cell response correlates with the reappearance of the liposome capturing SIGNR1+ cells.

3. It is suggested that DCs and possibly macrophages in the splenic MZ are involved in presenting CD1d-lipid complexes to NKT cells. It would seem appropriate to show data for the CD1d expression on the SIGNR1+ macrophages and MZ DCs e.g. using the dual labeling confocal microscopy procedures used so effectively to locate the NKT cells.

4. Data are shown in Figure 2E-H and Suppl. Movies for NKT cell migration in the red pulp (under the splenic capsule of the whole explanted spleen) in the absence of lipid antigen. Then in Figure 5D-I and movies, data are shown for NKT cell movement in the MZ region of lipid exposed spleen after the organ has been sliced open. Minimally, data using the latter technique also need to be shown with spleens from mice that had not received the lipid antigen as it is possible that cell behavior will differ in the subcapsular red pulp and the MZ. More details on the spleen slice procedure should be included in the methods.

5. In order to verify that transferred NKT cells behave similarly to endogenous NKT, the authors should perform a pulse antibody labeling experiment of transferred NKT. Pulse-antibody labeling of the NKT transfer system could also be used after administration of lipid antigen to determine if there are quantifiable changes in exposure of these cells to blood and control for any downmodulation of the TCR that might occur after antigen exposure.

6. It would be valuable if the imaging analysis could be performed in tissue where the antigen presenting cells are also visible. This might be achieved using CD11c-reporter mice or perhaps by adding or injecting antibodies that selectively label one or other macrophage population prior to the imaging analysis.

Minor
1. The 2-photon microscopy is performed with splenic explants. Given that the NKT cells under investigation are located in the blood-rich parts of the spleen, the lack of blood flow in splenic explants may lead to alterations in cell behavior. This possibility should be noted in the manuscript.
2. In Figure 1A, B (left panels) it is perhaps surprising that there seems to be no CD45-PE labeling in the red pulp. Is this just a contrast issue?
3. In figure 2A first panel, the authors should explain the meaning of the white dots.
4. The authors do not say in MS how the microshperes are administered (they only say "injected", does this mean IV ?)

Additional Correspondence 06 February 2012

We have now received the third report on your paper, which I have provided below. As you can see, the referee supports publication in the EMBO Journal pending minor revisions. I would therefore like to ask you to submit a suitably revised manuscript that addresses the raised concerns.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee#3:

Natural killer T cells are relatively abundant in the spleen, and the authors have provided the first detailed description of the location of NKT cells there, showing the highest concentration in the marginal zone, with some cells in the red pulp and white pulp as well. Furthermore, they show that the minority population of NKT cells in the white pulp does not respond to particulate glycolipid antigen, because blood borne particulate antigens are trapped by macrophages in the marginal zone. They also use intravital microscopy to show that NKT cells patrol in the marginal zone and arrest after activation. The local, antigen dependent stimulation of NKT cells requires the presence of macrophages and probably DC as well, although the available technologies really do not provide a dissection of the roles of these two cell types. These data are novel, because there has been little information previously on the location and movement of NKT cells in the spleen, although there are now descriptions of NKT cell movements in liver, lung and lymph node. While the system used may not perfectly represent stimulation by bacterial infections, infection studies would be much more challenging, and the data presented here are novel and important.

Minor points
1. Although the authors state that the CD45+ (blood accessible) and CD45- NKT cells do not differ in phenotype (Fig. S1), the difference in CD69 expression appears to be significant-the authors should comment on this.

2. What is the number of repeats for the experiments in Fig. 3? Is the increase in NKT cells in the marginal zone after antigen statistically significant?

3. In Fig. 5, is there any significance to the apparently greater localization of particulate lipid antigen with SIGN-R1 and MARCO expressing cells compared to those expressing CD169?

4. The authors should note that for the residual activation of NKT cells in the absence of DC and macrophages the MFI for intracellular IFN appears to be lower-so not only are fewer cells activated, but they appear to be less activated.

5. In Fig. S4, why are the CD45- NKT cells (not blood accessible) responsive when there are no DC (after diphtheria toxin treatment) but also when the DC are partially recovered (day 6 after clodronate) but not at day 2 after clodronate?

6. In Fig. 6, are the particles up taken by MZ macrophages and/or DCs? This can be examined directly by FACS. Can MZ macrophages and DCs present antigens to NKT cells in vitro? Because the depletion experiments are undiscriminating, further experimentation along these lines should be done.

7. Figure 6E, why is there less particulate αGalCer staining (green color) comparing with 6A and 6C?

1st Revision - authors' response 29 February 2012

Reply to reviewers

We are very grateful for the positive comments by the reviewers and their thoughtful questions. We believe that the additional experiments performed have strengthened the manuscript and revealed important new insights into the behaviour and activation of splenic NKT cells

Reviewer #1 (R1)

*Barral and coll undertake an **elegant and complex study** to determine the topographical distribution of iNKT cells in the spleen as a function of their response to a synthetic blood-born particulated antigen model. Previous studies that attempted to define the topographical distribution of iNKT cells in secondary lymphoid organs, although not has the main goal of the study, did not reach univocal conclusions. Using methods different from those utilized here by Barral et al, two distinct localization for iNKT cells were found: exclusion from the splenic T cell zone (PALS) and, by reflection, probable association with the B cell follicles and MZ (Stetson et al JEM 2003); inclusion mainly in the PALS and exclusion from the B cell areas, MZ and red pulp (RP) (Thomas et al JEM 2011). Barral et al show with an acceptable degree of approximation that, at resting conditions, the majority of splenic iNKT cells are localized in the MZ (50%), followed almost equally by the RP (25%) and PALS (25%), but are essentially excluded from the B cell follicles, based on complementary experimental approaches: 1. Rapid iNKT cell labeling kinetics following injection of anti-CD45 mAb in vivo; 2. Histological (confocal microscopy) detection of endogenous TCRb+NK1.1+ cells and of adoptively transferred marked iNKT cells; 3. Time lapse multiphoton microscopy of adoptively transferred labeled iNKT cells from isolated spleens. The authors go on by assessing the effective relationship between the preferential anatomical localization of iNKT cells in the MZ and RP of the spleen and their rapid response to blood-born particulated antigens. They indeed provide evidence that MZ/RP associated iNKT cells are rapidly activated within 2h from the injection of the particulated antigen into mice, and that this depends on the uptake and presentation of it by MZ/RP resident DCs and macrophages, with some intriguing residual antigen handling possibly by MZ/RP B cells. The study by Barral et al has **the merit to clarify the iNKT cell topography in the spleen**, underscoring their function as innate-like sentinel of blood born infections owed to their anatomical localization.*

We would like to thank **R1** for their appreciation of the interest of our manuscript to the field and the contribution that it makes to the understanding of NKT cell biology.

R1 raised a number of **minor points** that we have addressed as follows:

1. Does the injection of soluble aGalCer (not complexed to particles) elicit the same kind of differential activation in MZ- vs PALS-associated iNKT cells?

In response to **R1's** suggestion, we have injected soluble αGalCer and analysed NKT cell activation. Indeed, we have obtained the same kind of different pattern of NKT cell activation to that observed with particulates. This new data has been included in the revised **Supplementary Figure S4C-S4F** and discussed in the revised version of the manuscript (**Pages 9 and 15**).

2. Figure 6. It would be useful to show the % of gated iNKT cells in panels B, D, F

We have included the requested quantifications in the revised version of the Figure.

Reviewer #2 (R2)

*This concise report **provides new insight regarding the location of NKT cell activation in the spleen**. The low frequency of NKT cells and their lack of a single marker that distinguishes them from other T cells or NK cells has made determining their distribution difficult. Using in vivo antibody pulse labeling, multicolor confocal analysis of sections and adoptive transfer approaches, this study shows convincingly that the majority of NKT cells in the spleen are located in the marginal zone and red-pulp. It then shows that it is the blood exposed cells that are rapidly activated by intravenously injected lipid antigens. Using macrophage plus DC ablation approaches, NKT cell activation is shown to depend on these cells, and some evidence is provided that DCs contribute to antigen presentation. 2-photon microscopy on splenic explants shows that red pulp and MZ NKT cells migrate quite rapidly in the absence of their lipid antigen but undergo frequent migration arrest when antigen is present. Overall, **these data advance our understanding of the site of NKT cell activation by systemic lipid antigen** and they provide some initial information on the earliest dynamics of NKT cell activation in the spleen.*

We would like to thank **R2** for their assessment of our work and in particular its significance and novelty regarding the location of NKT cell activation in the spleen.

R2 raised a number of specific concerns that we have addressed as follows:

*1. Liposomal clodronate in addition to depleting macrophages and DC's is known to cause a loss of marginal zone B cells from the spleen. In published work these authors have shown that B cells can form stable conjugates with NKT cells. This confounds interpretation of the data presented in Figure 6A-D. The authors should examine how the numbers of MZ B cells at different time points after clodronate treatment **relates** to the NKT cell response. Alternatively, they might determine whether DT administration to CD11c-DOG animals results in changes in splenic B cell populations to rule out this possibility.*

As suggested by **R2** we have analyzed the different splenic B cell populations in CD11c-DOG mice after administration of DT. The B cell populations examined were found to be unchanged in comparison to untreated mice. This data has been included in the revised **Supplementary Figure S5O**.

2. Related to 1, previous work has shown that SIGNR1+ macrophages reappear in the spleen more slowly than CD169+ macrophages. It would be useful to know if the full restoration of the NKT cell response correlates with the reappearance of the liposome capturing SIGNR1+ cells.

In response to **R2's** suggestion we have performed new clodronate (CLL) treatment experiments to analyze the effect of the recovery of different macrophage populations in NKT cell activation. Sixteen days after CLL treatment we found that the population of $F4/80^+$ and $MARCO^+$ macrophages started to recover with cells appearing scattered through the splenic red pulp. After antigen injection both cell populations colocalize with particulate lipids, however the extent of NKT cell activation was still comparable to the one detected 6 days after CLL treatment suggesting that the correct positioning of macrophages in the MZ is critical for efficient antigen retention and subsequent NKT cell activation. Indeed, at day 22 after CLL treatment we observed full recovery of NKT cell activation that correlates with the repopulation the splenic MZ by macrophages. This new data has been included in **Figure 7E-H**, **Supplementary Figure S5I-S5K** and discussed in the revised manuscript (**Page 12**).

3. It is suggested that DCs and possibly macrophages in the splenic MZ are involved in presenting CD1d-lipid complexes to NKT cells. It would seem appropriate to show data for the CD1d expression on the SIGNR1+ macrophages and MZ DCs e.g. using the dual labeling confocal microscopy procedures used so effectively to locate the NKT cells.

We have analyzed CD1d expression in DCs and SIGN-$R1^+$ MZ macrophages showing that both cell types express high levels of CD1d. This new data has been included in the new **Figure 6C and 6D**

4. Data are shown in Figure 2E-H and Suppl. Movies for NKT cell migration in the red pulp (under the splenic capsule of the whole explanted spleen) in the absence of lipid antigen. Then in Figure 5D-I and movies, data are shown for NKT cell movement in the MZ region of lipid exposed spleen after the organ has been sliced open. Minimally, data using the latter technique also need to be shown with spleens from mice that had not received the lipid antigen as it is possible that cell behavior will differ in the subcapsular red pulp and the MZ. More details on the spleen slice procedure should be included in the methods.

As suggested by **R2** we have analyzed the dynamic behavior of NKT cells in the absence of antigen after the slicing open the organ. Under these imaging conditions NKT cells showed identical dynamic parameters as those measured by imaging the whole spleen through the collagen capsule. This data has been included in the revised version of the manuscript and in the new figure **Supplementary Figure S3** and new **Movie S3**. A detailed procedure regarding the imaging technique has been included in the revised manuscript (**Pages 7 and 21-22**)

5. In order to verify that transferred NKT cells behave similarly to endogenous NKT, the authors should perform a pulse antibody labeling experiment of transferred NKT. Pulse-antibody labeling of the NKT transfer system could also be used after administration of lipid antigen to determine if there are quantifiable changes in exposure of these cells to blood and control for any down-modulation of the TCR that might occur after antigen exposure.

As suggested by **R2** we have performed a pulse-labeling experiment after transfer of sorted NKT cells. Transferred and endogenous NKT cells exhibited identical antibody labeling *in vivo*, confirming their similar distribution in the tissue as observed by confocal microscopy (Figure 2A-D). This new data has been included in the revised **Figure 2E.**

6. It would be valuable if the imaging analysis could be performed in tissue where the antigen presenting cells are also visible. This might be achieved using CD11c-reporter mice or perhaps by adding or injecting antibodies that selectively label one or other macrophage population prior to the imaging analysis.

We agree with **R2** that visualization of antigen presenting cells during the process of NKT cell activation will be extremely interesting. However, multiphoton imaging of the spleen carries major technical difficulties reflected particularly in the extremely low penetrance during the imaging process. Despite our best efforts we haven't find a reagent that allowed us to specifically label splenic populations with enough efficiency and brightness to be directly visualized by multi-photon microscopy.

Minor points

1. The 2-photon microscopy is performed with splenic explants. Given that the NKT cells under investigation are located in the blood-rich parts of the spleen, the lack of blood flow in splenic explants may lead to alterations in cell behavior. This possibility should be noted in the manuscript.

This issue has been discussed in the revised version of the manuscript (**Page 14**)

2. In Figure 1A, B (left panels) it is perhaps surprising that there seems to be no CD45-PE labeling in the red pulp. Is this just a contrast issue?

Although we have observed some staining in the splenic red pulp after administration of CD45-PE, the antibody was found mainly concentrated in the MZ, which explains the increase of the fluorescent signal in this region.

3. In figure 2A first panel, the authors should explain the meaning of the white dots.

White dots depict NKT cells in the tissue sections. This has been clarified in the revised version of the manuscript.

4. The authors do not say in MS how the microshperes are administered (they only say "injected", does this mean IV ?)

In all the cases antigen has been administered iv. This has been stated through the revised manuscript.

Reviewer #3 (R3)

Natural killer T cells are relatively abundant in the spleen, and the authors have provided the first detailed description of the location of NKT cells there, showing the highest concentration in the marginal zone, with some cells in the red pulp and white pulp as well. Furthermore, they show that the minority population of NKT cells in the white pulp does not respond to particulate glycolipid antigen, because blood borne particulate antigens are trapped by macrophages in the marginal zone. They also use intravital microscopy to show that NKT cells patrol in the marginal zone and arrest after activation. The local, antigen dependent stimulation of NKT cells requires the presence of macrophages and probably DC as well, although the available technologies really do not provide a dissection of the roles of these two cell types. ***These data are novel, because there has been little information previously on the location and movement of NKT cells in the spleen****, although there are now descriptions of NKT cell movements in liver, lung and lymph node. While the system used may not perfectly represent stimulation by bacterial infections, infection studies would be much more challenging, and* ***the data presented here are novel and important.***

1. Although the authors state that the CD45+ (blood accessible) and CD45- NKT cells do not differ in phenotype (Fig. S1), the difference in CD69 expression appears to be significant-the authors should comment on this.

This has been pointed out in the revised version of the manuscript (**Page 5**)

2. What is the number of repeats for the experiments in Fig. 3? Is the increase in NKT cells in the marginal zone after antigen statistically significant?

As suggested by **R3** we have included statistic values in this experiment in the revised **Figure 3C and 3D.**

3. In Fig. 5, is there any significance to the apparently greater localization of particulate lipid antigen with SIGN-R1 and MARCO expressing cells compared to those expressing CD169?

R3 points out a very interesting question. Indeed, particulate lipids seem to be, to some extent, more efficiently retained by MARCO/SIGN-R1$^+$ MZ macrophages than by CD169$^+$ metallophillic macrophages. To date, the phenotypic and functional differences between those cell populations have been not fully addressed. Nevertheless, it is clear that metallophillic and MZ macrophages express a diverse set of scavenger receptors (reviewed in Taylor et al. Ann Rev Immunol 2005; 23:901–44), which may lead to their different ability to interact or retain several types of antigens or be infected by a plethora of infectious agents. It is therefore possible that different macrophage populations present distinctive capacity to retain lipid antigens in their arrival to the spleen, although the molecular mechanisms involved in this process will require further investigation.

4. The authors should note that for the residual activation of NKT cells in the absence of DC and macrophages the MFI for intracellular IFN appears to be lower-so not only are fewer cells activated, but they appear to be less activated.

We agree with **R3** in his comment. We have quantified the MFI values for IFN-γ producing NKT cells in clodronate treated mice and included this data in the revised version of the manuscript (**Page 11**).

5. In Fig. S4, why are the CD45- NKT cells (not blood accessible) responsive when there are no DC (after diphtheria toxin treatment) but also when the DC are partially recovered (day 6 after clodronate) but not at day 2 after clodronate?

These differences are actually not significant and only due to the variability between different animals. To avoid confusion we have included quantifications with the percentages of IFN-γ producing cells in the CD45$^+$ and CD45$^-$ populations for the different conditions tested in the revised Supplementary **Figure S5E, S5H, S5K and S5N.**

6. In Fig. 6, are the particles up taken by MZ macrophages and/or DCs? This can be examined directly by FACS. Can MZ macrophages and DCs present antigens to NKT cells in vitro? Because the depletion experiments are undiscriminating, further experimentation along these lines should be done.

As suggested by **R3** we have analyzed particulate lipid uptake by FACS and showed that around 50% of splenic DCs and SIGN-R1$^+$ MZ macrophages are retaining lipids 2 h after their administration. This data has been included in the new **Figure 6B** and discussed in the revised manuscript (**Page 10**).
To understand the ability of DCs and macrophages to mediate lipid presentation to NKT cells we have sorted primary DCs and MZ SIGN-R1$^+$ macrophages from the spleen and assayed their ability to stimulate NKT cell activation in vitro. This new data has been included in the new **Figure 6E and 6F** and discussed in the revised manuscript (**Page 10-11**).

7. Figure 6E, why is there less particulate αGalCer staining (green color) comparing with 6A and 6C?

We agree with **R3** that the amount of particulate antigen in the spleen of CD11c-DOG mice after DT treatment seem to be less than in CLL treated animals. Variations in the amount of antigen retained in the spleen after CLL/DT treatment could be due to several reasons, such as an effect on the splenic homeostasis due to the presence of apoptotic cells induced by these treatments. However, the differences in the amount of antigen present in the spleen after CLL/DT treatment cannot explain the lack of NKT cell activation. In fact, the extent of NKT cell activation was comparable after DT treatment and 2 days after CLL treatment despite the differences in antigen levels.

2nd Editorial Decision 13 March 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Referee #1 and 2 were available to review the revised version and I have now received their comments. As you can see below both appreciate the introduced changes. Referee #2 has a few remaining comments. S/he finds that it would be good to add CD1d and SIGN-R1 co-staining in tissue sections to the paper. I don't know if you have data on hand to address this or if this is easily done, if so then it would be good to include it. If not, then we will go ahead with paper without it. The last issue is to better describe the splenic slice method in the materials and methods section.

Once we get these last issues resolved then we will proceed with the acceptance of the paper for publication here.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Authors replies to my queries are satisfactory.
The manuscript is acceptable for publication.

Referee #2

In their revised manuscript the authors have addressed several of the earlier concerns and the manuscript is found to be improved. Data have been added to Figure 6 to show the CD1d expression on SIGN-R1+ MZ Møs by FACS. However, given that the isolated cells have not been well characterized (to formally establish that they correspond to the SIGN-R1+ cells under study in the MZ of the spleen) there data are not considered conclusive and it would still be valuable to show data on CD1d and SIGN-R1 costaining in tissue sections.
The splenic slice method needs to include information about the method used to generate the slice, the thickness of the slice and its orientation.

2nd Revision - authors' response 14 March 2012

As suggested, we have included a more detailed protocol for the imaging of fresh spleen sections together with an extra reference for this procedure (pages 21-22, find attached the revised version of the manuscript). Regarding the section staining, I reviewed our stainings with Patricia. We find that the CD1d antibody works much better in flow cytometry than it does in frozen sections (where it gives a extremely weak signal). This is why we decided to include in our study only the FACS data which we feel is more conclusive.

