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A role for LFA-1 in delaying T lymphocyte egress from lymph nodes

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Editor: Karin Dumstrei

1st Editorial Decision 21 June 2012

Thank you for submitting your manuscript to the EMBO Journal. I have now had a chance to read your manuscript carefully and to discuss it with the other members of our editorial team. In addition, I have also sought external advice on the study from a good expert in the field. I have now heard back from the advisor and I am sorry to inform you that we find that the manuscript at present is not a good for fit for the EMBO Journal. However, with some additions - see below - we would be willing to look at a new submission.

Your analysis reports on at the role of the Integrin, LFA-1, in T lymphocyte migration in the Lymph Node (LN) using two-photon microscopy. The findings show that LFA-1 deficient T cells spend less time in the LN as compared to LFA-1 expressing T cells. LFA1-/- T cells can cross into lymphatic vessels, but reverse less frequently back into the LN as compared to LFA-1 expressing T cells. LFA1-/- T cells also migrate faster and have a more direct migration path as compared to LFA1+/+ cells.

Both the advisor and I appreciate that the analysis provides support for that LFA-1 is a 'retention receptor' that retains T cells in the LN. However we also find that the analysis at present is too preliminary for the EMBO Journal. We find that further data supporting that altering LN residence time affects the efficacy/ability of T cells to encounter APCs is needed for publication here. We also note that there is no clear quantification to support that reverse migration back to the LN affects total residence time. It would be good if a rough estimate could be provided to show that the reverse migration is actually what affects the residence time spent in the LNs.

Should you be able to add more data to address these points, we would be willing to look at a new submission.

I am sorry that I can't be more positive on this occasion, but I do hope that you find these comments helpful.

1st Resubmission 20 August 2012

In June we submitted our manuscript " A role for LFA-1 in delaying T cell egress from lymph nodes" (EMBOJ-2012-82353) and received an email from you suggesting that our study should be extended in two specific ways before you would consider sending it out for review. We now make a new submission which addresses both the issues raised by yourself and your advisor.
We provide a calculation showing that the differential in numbers of LFA-1+/+ and LFA-1-/- T cells that return to the T cell parenchyma rather than egressing could account for the difference in their "dwell time" in the LN presented in Fig. 1 (Results, page 15/16).
Secondly we have tested the functional significance of the LFA-1- mediated 'shuttling" back into the T cell parenchyma using a model of 3A9 transgenic T cells responding to HEL. We find 2 fold more reactive 3A9 T cells in control mice compared with mice where access to ICAM-1 on the LV is blocked by anti-ICAM-1 mAb (revised Fig. 8).
We very much hope that these new additions to our manuscript will provide sufficient support for sending it for wider review.

2nd Editorial Decision 28 September 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, all three referees find the analysis interesting and are supportive of publication in the EMBO Journal. They raise a number of specific and constructive comments and addressing those would strengthen the study. Given the comments provided, I would like to invite you to submit a suitably revised manuscript for our consideration. I should point out that it is the policy of the EMBO Journal to allow a major round of 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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know as I can grant an extension if needed.

Should you have any further questions regarding any of the suggested experiments please don't hesitate to contact me. I look forward to your revision.

REFEREE REPORTS

Referee #1

In their study "A role for LFA-1 in delaying T lymphocyte egress from lymph nodes", Reichardt et al investigate whether LFA-1 integrin expression on T cells plays a role in their lymph node egress behaviour. They present data that CD4 T cells deficient in LFA-1 egress the lymph node more quickly than wild type cells and that this is likely due to differences in the probability of crossing into the lymph at lymphatic sinuses - with LFA-1+/+ cells more likely to re-enter the lymph node parenchyma than LFA-1-/- cells. Thus far only CCR7 and S1PR1 expression have been shown to

influence egress probability, so the description of LFA-1 as influencing this process is an interesting finding that is well-substantiated by the data provided. However, some suggested revisions of the manuscript would clarify specific aspects of the data shown and solidify the conclusions drawn. In some instances, descriptions of results and conclusions are imprecise or not as clear as they might be and would thus benefit from careful rephrasing/editing. In addition, the manuscript includes a few pieces of data that do not seem relevant to the questions posed by the authors and some of the choices the authors made in their analyses (e.g., division of egress probability data into different locations) are not well explained. The weakest part of the manuscript provided is the final section that attempts to argue that the absence of LFA-1 expression has a functional consequence for recruitment into an immune response. Unfortunately the experimental design is inadequate to support the conclusions drawn, which are overstated in the discussion.

Specific issues:

Figure 1
• The x-axis labelling in Figure 1A seems somewhat misleading: "6, 10, 12 hours homing", but after 6 hours entry is blocked and thus homing no longer occurred after 6 hours. Correcting this would make the figure clearer. Also, do the authors have cell counts at the different time points following entry block for both transferred populations? Including this would enable a better quantitative understanding of the egress rates of the two populations. The change in ratio implies that within 4 hours twice as many LFA-/- cells have egressed as compared to WT cells. How many cells have left in this time and are the estimates the authors obtain for WT cells comparable with published literature on LN dwell times? Without the counts this is hard to assess. Also, because the number of WT vs. KO cells that enter the LN is grossly different, it is useful to have these numbers so that possible evaluate the likely accuracy of the counting and its fractional variability among animals. The latter (number of animals examined) should be stated.
• For Figure 1B, it would also be a substantial improvement to include the counts of the transferred T cell populations. How did lymph node entry efficiency of transferred cells in ICAM-1-/- compare to the transfers into WT recipients? How does egress rate of WT cells change in the absence of ICAM-1-/-? From the WT/KO ratios, this is cannot be determined, but if counts were shown for both Figure 1A and B, then the effect of ICAM1 on entry and egress would be apparent.
• In Figure 1C, counting WT vs. KO cells in the T cell area in multiple sections of lymph nodes from multiple mice and including this summarized data would be more convincing than showing a few images with 2-6 cells of each population.

Figure 2
• The definition of cortical LV versus medullary LV is not correct. For instance, see Bajenoff et al (2006) JEM 303:619, Figure 2, for images of the span of the medullary sinuses. Most of what the authors are calling "cLV", are actually medullary sinuses, while cortical sinuses are found between B cell and T cell zones (eg. see Grigorova et al (2009) Nat Immunol 10: 58, Figure 1).

Figure 4
• Figure 4A - the total CCR7 and S1PR1 expression as assessed by western blot is irrelevant to the question posed, as only surface expression of these receptors impact egress and thus these data should be removed. Surface CCR7 can be stained for quite easily by flow cytometry, and this should be done, while the chemotactic data is sufficient for S1PR1, given that no commercial antibodies are available to assess surface expression.
• Figure 4C - this assay merely proves that ICAM1 dependent migration requires LFA1-/- expression, which is known, but does not help in addressing the question of whether there are differences in S1PR1 or CCR7 expression/responsiveness between WT and KO.

Figure 5/6/7
• The section "Features of T cell migration..." seems unnecessary and the control experiments can be mentioned briefly under the next heading.
• For video 3, it might be helpful to indicate the LV outline for the entire movie, since it is quite dim or to show it in a different color other than dark blue for it to be more visible. Also, it might be helpful for the purpose of highlighting the scoring process to indicate for this entire movie how the scoring was done (as in Grigorova et al (2009), suppl movie 3) rather than just highlighting specific examples.
• Can the authors explain somewhere why they would expect egress of KO vs WT T cells from

different locations to be distinct? So far there is no evidence for this in the literature, so it is unclear why the authors are treating these areas as distinct for the purpose of their analysis. Since they are doing so, this would require some justification. Especially as the results they get are equivalent to what was shown in Figure 5. The number of figures and text length could be reduced significantly if the lack of an effect of location was summarized rather than laid out in such detail.
• In Suppl movie 4, the white outline drawn seems completely arbitrary. What is leading the authors to draw it there? Perhaps the staining is simply not transmitting well in the image shown?
• the playback speed (relative to actual time) of suppl movie 5 seems to be much slower than for the other movies. For comparative purposes, could this be made consistent among movies?

Figure 8
• it is not clear where the 1.6 fold estimate comes from (pg. 16). Is it from data pooled from movies at least 30 min long? Or is it really, as the text states, "a calculation" and if so, based on what?
• To draw the conclusion that enhanced egress of LFA1-/- 3A9 Tg T cells is responsible for their decreased numbers upon HEL injection in the absence of interactions with ICAM-1, rather than this being due to impaired entry, it would seem important to do a control to check that ICAM-1 antibody treatment does not also impair homing by providing the numbers of cells entering the LN at early times after transfer (2-4 hrs). A lack of staining of the HEV that is not shown to the readers is insufficiently convincing to be able to make this claim.
• The data during activation raises a number of additional questions:
• could the authors comment whether the difference in cell numbers is due to differences in cell division rather than recruitment/lack of egress?
• are LFA1-/- cells able to egress despite CD69 expression? Since during an immune response CD69 upregulation prevents cell egress, it is unclear why the authors expected to see differences in their experimental set up. If they were expecting to see differences in the antigen search strategy then perhaps 48 hours is too late and an earlier time point is more likely to reveal differences?

Discussion
• The authors state that T cells "use" LFA-1 as a retention receptor or describe the "use" of LFA-1 by T cells to "withstand exit cues", thus implying a desire of the T cell to stay within lymph nodes. Rather, their data and that of others suggests that egress upon reaching a lymphatic sinus is a stochastic process, with the expression of particular surface receptors, including LFA-1, impacting the probability of either (1) egressing into the lymph or (2) returning into the lymph node. Thus, the authors' phrasing is imprecise and implies intent when there is none. This should be re-phrased.
• The authors state: "we provide evidence that return T cell trips to the parenchyma of the T zone give these cells further opportunity to scan for antigen-loaded presenting cells and to become activated". While this may be true and is a reasonable hypothesis to propose, the authors have not, in fact, provided evidence for this. Quite the contrary, they show that the fraction of CD69+ cells is equivalent on LFA1 deficient cells and they have not assessed recruitment into an antigen-specific response in any other way. How do the authors explain the lack of a difference in CD69 expression, given their hypothesis?
• It also does not follow that "by aiding T cells to have more than one attempt to find antigen in the LN, use of LFA-1 is an additional factor amplifying their immune surveillance role." Surely "immune surveillance" is a process that has to optimize two competing events - staying within a single LN longer to search for antigen where egress delay would be helpful versus scanning for antigen in other LNs, for which egress is required. Thus, retention in a lymph node does not necessarily amplify immune surveillance - stating it this way is overly simplistic.

Additional questions:
= do the authors have any data that the changes in egress rate they see for CD4 T cells is also the case for CD8 T cells lacking LFA-1?
= Do anti-integrin αL antibodies previously used to block lymph node entry (eg. Lo et al (2005) JEM 201:291) have a similar effect on lymphocyte egress rates as deficiency of LFA-1? Given that these antibodies have been used to investigate egress, this would be useful for the field to know.

Minor points:
1) Some typos in the abstract: "T cells returned to the lymph node parenchyma with frequency" - seems to be a word missing here, eg. greater (also in last paragraph, intro); "whether to leave or return into the node" (rather than returning).

Referee #2

This is an elegant work which suggests a key role for the LFA-1-ICAM-1 pair at a post lymph node entry stage of naïve T cells. The authors provoke the idea that LFA-1 dependent adhesive interactions of these T cells with multiple stromal elements that express ICAM-1 in resting lymph nodes prolong T cell retention in the T cell zones of these nodes. Furthermore, in the central zone area (but not in the periphery) LFA-1 also contributes to faster motility and enhanced ability of lymphocytes to scan DCs. These findings are somewhat predictable given the high levels of ICAM-1 already demonstrated to be constitutively expressed by many stromal and non stromal cells in resting lymph nodes. A key new finding, however, is that wt LFA-1 expressing T cells reverse migrate back into the LN after probing the lymphatic vessel walls whereas LFA-1-/- T cells do it much less frequently. Consequently, LFA-1 null T cells cross and enter lymphatic vessels more efficiently than wt T cells, both in the peripheral and central areas of the T zone. Importantly, these two cell types behave similarly in ICAM-1 deficient lymph nodes.

While elegant and carefully performed, the work does not elucidate why this reverse migration is accomplished by normal T cells but much less so by LFA-1 deficient T cells. The authors speculate that high S1P juxtaposed to ICAM-1 activates LFA-1 adhesiveness to lymphatic vessels and thereby increase T cell retention nearby these vessels as well as reverse migration. However, enhanced haptotaxis to ICAM-1 is not proven and does not explain all the findings although the evidence is convincingly demonstrated by multiple means and in different regions of the lymph node.

Major comments

1. One explanation for this puzzle is that self antigens engage TCRs on naïve T cells entering lymph nodes at very low affinity and thereby alter their in vivo responsiveness to S1P. Naïve CD4 T cells have been shown to respond to MHC-II dependent signals during their scanning of lymph nodes. As CD69 is a negative regulator of the major S1P receptor, S1P1 (but not of CCR7!!), low TCR signals, prolonged by LFA-1-ICAM-1 engagements may upregulate CD69 on LFA-1 expressing but not on LFA-1 null T cells, decrease S1P1 levels, and keep CCR7 signaling intact. This will bias wt T cells to get attracted by CCR7 signals and increase T cell dwelling in expense of egress. The way to prove this hypothesis and substantiate this work is to replace all hematopoietic cells of the lymph node with hematopoietic cells deficient in MHC-II. If, in the absence of MHC-II on DCs, but with residual MHC-II expression on lymphatics, no difference between LFA-1 null and wt T cells is detected, this will indicate that the LFA-1 dependent dwelling of naïve T cells in lymph nodes is TCR driven and depends on DC expressed MHC-II rather than non DC expressed MHC-II. Since in this setting, lymphatic vessels and FRCs will remain intact, it will also allow the differentiation between the contribution of antigen presentation on these cells as opposed to DCs.

2. The indication that ex vivo, LFA-1 null and wt T cells share identical responsiveness to S1P is non informative and perhaps even misleading. It is possible that LFA-1-/- T cells in fact express higher S1P1 in vivo, as outlined in my earlier paragraph on CD69 upregulation and S1P1 downregulation. This possibility could explain the higher directionality of these T cells towards exit sites. Differences in S1P1 levels between LFA-1 null and wt T cells would be very important to detect. In addition, since S1P1 levels are reciprocal to CD69, it would be very interesting to confirm higher CD69 expression on wt T cells. At any rate, currently, this part of the data is misleading as it pertains to conserved S1P1 levels in LFA-1 null T cells.

3. Is the motility of LFA-1-/- T cells slower than of wt T cells, even when both T cells are sequestered in lymph nodes in the presence of the S1P1 antagonist, FTY720?

4. T cell crossing of HEVs is mainly LFA-1 dependent (as also shown by the Hogg lab). Since the few LFA-1 null T cells that do enter lymph nodes do so via VLA-4-VCAM-1 interactions (a previous study by the Hogg's lab), it is possible that these few cells express higher b1 integrins, in particular VLA-4. It is mandatory to test if this is the case in the present setting because if LFA-1 null T cells inside lymph nodes are much higher in their VLA-4 content, they may use FRC expressed VCAM-1 for their motility and this may also alter explain some of the findings.

5. The rationale for the use of shear in the experiments testing the behavior of T cells on S1P and ICAM-1 is unclear. Where will T cells about to leave the T zone experience shear flow? This

experiment must be repeated using transwells in which either bare or ICAM-1-coated filters are used and chemotaxis to S1P is determined. Under these conditions, more LFA-1 null T cells may get chemoattracted by S1P. This may provide further evidence for the higher tendency of LFA-1 null T cells to respond to S1P exit signals (In addition to the CD69-S1P1 axis discussed in my previous comments).

Minor comments
1. ICAM-1 deficient mice contain higher levels of Tregs. A comment on this possibility and its implications in this model should be discussed.

Referee #3

This is an interesting paper analyzing the effect of LFA-1 deficiency on T cells dynamics in the lymph node. The authors showed that LFA-1-/- T cells display a minor defect in interstitial migration but an important reduction in their dwell time in the lymph node. Using intravital two-photon imaging, they provide evidence that WT T cells that probe the lymphatic network often return to the lymph node parenchyma. This was much less pronounced for LFA-1-/- T cells that appeared to egress more rapidly. The authors conclude that LFA-1 act as a 'retention factor' in the lymph node.

Overall, the study is technically well performed and provides novel information regarding the T cell journey in lymph nodes. However, several issues need to be addressed

1) The idea that LFA-1 act as a 'retention factor' and 'has a major role in T cell choices' to exit lymph node is, to my point of view, an overstatement.
This would imply an 'active role' for LFA-1 for example because the activity of LFA-1 is regulated at the time of egress which is not demonstrated here. In the WT situation, LFA-1 is always expressed on T cells so it is not clear how LFA-1 would contribute to any kind of choice.
I would recommend to remove this terminology and to stick to what is clearly demonstrated here, ie that T cells probe lymphatic vessels and return to the parenchyma with a contribution of LFA-1 mediated adhesion in this process.

2) The authors should show the results of the critical experiments after swapping the vital dyes to ensure of the absence of a dye-specific effect. While the authors mentioned the dyes are 'routinely swapped' it would be helpful to see the actual data in a supplemental figure
Also difference in brightness of the dyes (CFSE-stained cells seem brigher than SNARF-labeled cells, based on their apparent size) should impact on the calculation of the T cell shape or the occurrence of T cell disappearance from the field of view (shown in Fig.8D-E).

3) The authors state that there is no difference between velocities of WT and LFA-1-/- T cells in ICAM-1-/- hosts when the difference is exactly in the same range (11-13%) of that observed in WT hosts (with $p=0.08$). Overall, this point is not very clear.

4) It seems counterintuitive that LFA-1 is required to respond to S1P (Fig. 4C) when WT cells have an increased dwell-time in the lymph node compared to LFA-1-/- T cells. Could this be discussed?

5) In the experiment in Fig. 8E aimed to show that blocking ICAM-1 on lymphatics impact on T cell activation, how do the authors exclude that the anti-ICAM-1 Ab does not also reduce T cell homing to the lymph node (which should lead to the same effect)? Also, does ICAM-1 LV block also modify the time of residence in the lymph node in the absence of antigen ?
The fact that all T cell become CD69+ after treatment with anti-ICAM-1 seems to contradict the hypothesis proposed by the author that reverse migration help T cell find antigen. Maybe it is important for sustained antigen recognition?

Referee #1

Specific issues:
Figure 1
• “The x-axis labelling in Figure 1A seems somewhat misleading: "6, 10, 12 hours homing", but after 6 hours entry is blocked and thus homing no longer occurred after 6 hours. Correcting this would make the figure clearer. Also, do the authors have cell counts at the different time points following entry block for both transferred populations? Including this would enable a better quantitative understanding of the egress rates of the two populations. The change in ratio implies that within 4 hours twice as many LFA-/- cells have egressed as compared to WT cells. How many cells have left in this time and are the estimates the authors obtain for WT cells comparable with published literature on LN dwell times? Without the counts this is hard to assess. Also, because the number of WT vs. KO cells that enter the LN is grossly different, it is useful to have these numbers so that possible evaluate the likely accuracy of the counting and its fractional variability among animals. The latter (number of animals examined) should be stated.”

We have simplified the x-axis labeling of Fig. 1A to better reflect the timing of LN blockade. The CD4 adoptive transfer experiments used to calculate the WT/KO ratios were collected over a period of time where there was some variation in LN yields. However the ratio of WT/KO CD4 T cells was a significant constant and it is for this reason that we chose to illustrate the data in this way in Fig.1. This ratio which suggested a shorter dwell time for the LFA-$1^{-/-}$ T cells in the LN is then further investigated in the subsequent text. The numbers of mice used in these experiments is included in the Fig. Legend.

• “For Figure 1B, it would also be a substantial improvement to include the counts of the transferred T cell populations. How did lymph node entry efficiency of transferred cells in ICAM-1-/- compare to the transfers into WT recipients? How does egress rate of WT cells change in the absence of ICAM-1-/-? From the WT/KO ratios, this is cannot be determined, but if counts were shown for both Figure 1A and B, then the effect of ICAM1 on entry and egress would be apparent.”

See above comment.

• “In Figure 1C, counting WT vs. KO cells in the T cell area in multiple sections of lymph nodes from multiple mice and including this summarized data would be more convincing than showing a few images with 2-6 cells of each population.”

We have counted the numbers of adoptively transferred LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells in tissue sections at 6 h and now include this data. An average of 95.71±16.57 *LFA-$1^{+/+}$* and *LFA-$1^{-/-}$* 16.71±2.78 CD4 T cells/LN tissue slice were obtained from 7 mice giving an WT/KO ratio of 7/1 which is similar to the ratio obtained by flow cytometry of whole LNs.

Figure 2
• “The definition of cortical LV versus medullary LV is not correct. For instance, see Bajenoff et al (2006) JEM 303:619, Figure 2, for images of the span of the medullary sinuses. Most of what the authors are calling "cLV", are

actually medullary sinuses, while cortical sinuses are found between B cell and T cell zones (eg. see Grigorova et al (2009) Nat Immunol 10: 58, Figure 1)".

The reviewer questions the terminology used to describe cortical/ medullary sinuses in Fig. 2. The Bajenoff paper (J. Exp. Med. 303:619, Figure 2) shows ear-draining LN imaged in what appears to be a vertical slice. In contrast, our image in Fig 2 shows the inguinal LN as seen *in situ*, i.e. with the top (convex, follicular side) of the LN in the downward position while the observer looks directly onto the concave, medullary aspect.

The reviewer is correct that the central lymphatic vessels (LV) that we observe do not represent typical cortical LV as these are usually found at the follicular side of the LN in between follicles (inter-follicular) as represented in Fig. 1 of Grigorova *et al* Nat Immunol 10:58 2009. However that figure also shows several LV leading from the central paracortical T zone that end on the medullary side of the LN. These are the LV that we see and describe in our study. These central LV resemble cortical rather than medullary LV in various ways. First, they start out in areas dominated by T cells (paracortical T zone). More importantly, they show a continuous tubular structure and a clear lumen, i.e. free of macrophages. However, we agree that the more superficial these LV become, the more appropriate the term "medullary" becomes for these LV based on their localization. They gradually become sparsely macrophage-associated but remain tubular in appearance. Thus we will refer to the LV as "central" or "paracortical" and compare them with the "peripheral" LV that end distally in the medulla.

Figure 4

• "Figure 4A - the total CCR7 and S1PR1 expression as assessed by western blot is irrelevant to the question posed, as only surface expression of these receptors impact egress and thus these data should be removed."

We would like to retain this data as the blot shows that the total level of CCR7 and S1P1 is the same in LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells even if one can't tell whether or not the expression is on the surface. This seems relevant.

'Surface CCR7 can be stained for quite easily by flow cytometry, and this should be done, while the chemotactic data is sufficient for S1PR1, given that no commercial antibodies are available to assess surface expression."

Flow cytometry data showing the expression of CCR7 on gated LFA-$1^{-/-}$ and LFA-$1^{+/+}$ T cells is now included as Fig. 4B. The majority of both types of CD4 T cell express CCR7.

• "Figure 4C - this assay merely proves that ICAM1 dependent migration requires LFA1-/- expression, which is known, but does not help in addressing the question of whether there are differences in S1PR1 or CCR7 expression/responsiveness between WT and KO."

This Fig. 4C (now 4D) experiment shows that S1P and CCL21 can activate LFA-1 and Fig. 4B (now 4C) gives the information that the general chemotactic responsiveness to S1P and CCL21 is the same for LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells. We hope the reviewer agrees that these are important points to establish.

Figure 5/6/7

• “The section "Features of T cell migration..." seems unnecessary and the control experiments can be mentioned briefly under the next heading.”

We have removed this section but have briefly introduced our intravital microscopy parameters where appropriate in connection with Fig. 2 (Suppl. Fig. 2A) and Fig. 5 (Suppl. Fig. 2B, Video 1 and 2).

• “For video 3, it might be helpful to indicate the LV outline for the entire movie, since it is quite dim or to show it in a different color other than dark blue for it to be more visible. Also, it might be helpful for the purpose of highlighting the scoring process to indicate for this entire movie how the scoring was done (as in Grigorova et al (2009), suppl movie 3) rather than just highlighting specific examples.”

Video 3 has been altered as the reviewer has suggested with the scoring now indicated in Grigorova *et al*. (2009).

• “Can the authors explain somewhere why they would expect egress of KO vs WT T cells from different locations to be distinct? So far there is no evidence for this in the literature, so it is unclear why the authors are treating these areas as distinct for the purpose of their analysis. Since they are doing so, this would require some justification. Especially as the results they get are equivalent to what was shown in Figure 5. The number of figures and text length could be reduced significantly if the lack of an effect of location was summarized rather than laid out in such detail.”

The reviewer is correct in pointing out that the LV interaction data presented in previous Figs. 5&6 was similar. We have now combined the data from these 2 separate analyses (new Fig. 5). The reason for originally separating this data was because 1. the tubular LV are macrophage-free in the central zone but then gradually become sparsely macrophage-associated and 2. the LFA-$1^{+/+}$ T cells have increased velocity in the central zone but then reduce their velocity the more superficial to the LN were the observations. These changes occur in a gradual manner with no sharp boundary towards the periphery of the LN. However in spite of these differences, the interaction with the LV seems constant so it is reasonable to combine all tubular LV observations (see new Fig. 5 and Suppl. Fig. 5).

• “In Suppl movie 4, the white outline drawn seems completely arbitrary. What is leading the authors to draw it there? Perhaps the staining is simply not transmitting well in the image shown?”

We have now eliminated this video associated with previous Fig. 6.

• “the playback speed (relative to actual time) of suppl movie 5 seems to be much slower than for the other movies. For comparative purposes, could this be made consistent among movies?”

The ftp rate of Suppl Video 5 (now 4) has been changed so as to be comparable to the other videos.

Figure 8

• “it is not clear where the 1.6 fold estimate comes from (pg. 16). Is it from data pooled from movies at least 30 min long? Or is it really, as the text states, "a calculation" and if so, based on what?”

A question was whether the 2.3 fold decrease in LN dwell time of *LFA-$1^{-/-}$* compared with *LFA-$1^{+/+}$* T cells (Fig. 1A) could be accounted for by the use of LFA-1 to reverse migrate back into the LN. We have reconsidered this matter after discussion with a computational colleague and now present the

calculation following on from the measurements in Fig. 5 that have been used to arrive at an estimate (page 12, para.2). To investigate this issue, the comparative proportion of *LFA-1*$^{+/+}$ or *LFA-1*$^{-/-}$ T cells leaving the LN at 6 h was determined by calculating the percentage of each T cell type in contact with LV that subsequently exited over 30 min following adoptive transfer (WT-11.62x0.65=7.55%; KO-29.73x0.65=19.32%). Thus over this time period 2.6 fold more *LFA-1*$^{-/-}$ than *LFA-1*$^{+/+}$ T cells left the LN indicating that their distinctive behaviour at the point of LV exit could account for effect of LFA-1 on T cell LN dwell time. This is a very rough estimate as it is unknown whether the reverse migration versus exiting is the same at all time points and at all lymphatic vessel contacts within the LN.

• "To draw the conclusion that enhanced egress of LFA1-/- 3A9 Tg T cells is responsible for their decreased numbers upon HEL injection in the absence of interactions with ICAM-1, rather than this being due to impaired entry, it would seem important to do a control to check that ICAM-1 antibody treatment does not also impair homing by providing the numbers of cells entering the LN at early times after transfer (2-4 hrs). A lack of staining of the HEV that is not shown to the readers is insufficiently convincing to be able to make this claim.
• The data during activation raises a number of additional questions:
• could the authors comment whether the difference in cell numbers is due to differences in cell division rather than recruitment/lack of egress?
• are LFA1-/- cells able to egress despite CD69 expression? Since during an immune response CD69 upregulation prevents cell egress, it is unclear why the authors expected to see differences in their experimental set up. If they were expecting to see differences in the antigen search strategy then perhaps 48 hours is too late and an earlier time point is more likely to reveal differences?"

We now include a new experiment (new Fig. 8) to test the influence of LFA-1 interactions with the LV on immune responsiveness that eliminates the concern that anti-ICAM-1 is preventing T cell entry into the LN. An important factor in such an experiment was to remove from consideration any interaction between T cells and dendritic cells (DCs) that might make use of LFA-1 in order to focus on the non-APC interactions of the T cells. We have chosen the model of pre-injecting OVA-laden DCs into WT and ICAM-1$^{-/-}$ host mice that then received transgenic OT-2 T cells that are sensitive to OVA peptide on APC. In this model, the OT-2 cells respond to the APC and we have investigated the extent of their proliferation in each host LN. After assessing parameters of peptide dose, experimental timing and DC uptake (Mempel *et al*, Nature,427,154, 2004; Lammerman *et al*, Nature, 453, 51 2008) and experimental timing, we have recorded the extent of proliferation at 72 h and found that the OT-2 T cells in the WT host proliferated 1.5 fold over the ICAM-1$^{-/-}$ host. Although the main focus of this manuscript is on the transit of non-primed CD4 T cells through the LN, we hope that the OT-2 experiment serves to give some indication of how the use of ICAM-1 on the lymphatic vasculature also influences T cell activity when antigen is encountered.

Discussion
• "The authors state that T cells "use" LFA-1 as a retention receptor or describe the "use" of LFA-1 by T cells to "withstand exit cues", thus implying a

desire of the T cell to stay within lymph nodes. Rather, their data and that of others suggests that egress upon reaching a lymphatic sinus is a stochastic process, with the expression of particular surface receptors, including LFA-1, impacting the probability of either (1) egressing into the lymph or (2) returning into the lymph node. Thus, the authors' phrasing is imprecise and implies intent when there is none. This should be re-phrased."

The reviewer's comments are well-taken and we have altered the text in the Discussion to more precisely reflect the action of the T cells and the role of LFA-1 in the exit process.

• "The authors state: "we provide evidence that return T cell trips to the parenchyma of the T zone give these cells further opportunity to scan for antigen-loaded presenting cells and to become activated". While this may be true and is a reasonable hypothesis to propose, the authors have not, in fact, provided evidence for this. Quite the contrary, they show that the fraction of CD69+ cells is equivalent on LFA1 deficient cells and they have not assessed recruitment into an antigen-specific response in any other way. How do the authors explain the lack of a difference in CD69 expression, given their hypothesis?"

In a new experiment (Fig. 8) dealing with antigen responsiveness, we show that the proportion of OT-2 T cells able to undergo proliferation is decreased by 1/3 in *ICAM-1*$^{-/-}$ compared with WT hosts. While not the main focus of the manuscript, this experiment provides some information as to why there might be a beneficial effect on an immune response for T cells to shuttle back and forth into the node parenchyma. More detail as to whether there are differences between the "shuttling" T cells and those making a first passage through the node would be a topic for future work.

• "It also does not follow that "by aiding T cells to have more than one attempt to find antigen in the LN, use of LFA-1 is an additional factor amplifying their immune surveillance role." Surely "immune surveillance" is a process that has to optimize two competing events - staying within a single LN longer to search for antigen where egress delay would be helpful versus scanning for antigen in other LNs, for which egress is required. Thus, retention in a lymph node does not necessarily amplify immune surveillance - stating it this way is overly simplistic."

The reviewer again makes a relevant point that we now include comment on in the Discussion (page 20, para. 2). It is correct that the term "immune surveillance" has been taken to imply the scanning by T cells of a number of lymph nodes making the search for antigen more comprehensive. It seems reasonable to suggest that "rescanning" an individual lymph node may also contribute to increased efficiency in detection of antigen-laden APC by T cells although at a local level.

Additional questions:

"do the authors have any data that the changes in egress rate they see for CD4 T cells is also the case for CD8 T cells lacking LFA-1?"

We have routinely investigated only the behavior of CD4 T cells. Interestingly a recent study reveals that CD4 and CD8 T cells have quite different LN transit characteristics so analysis of LFA-1 usage by CD8 T cells would certainly be of interest (Mandl *et al* PNAS 109, 18036, 2012).

“= Do anti-integrin αL antibodies previously used to block lymph node entry (eg. Lo *et al* (2005) JEM 201:291) have a similar effect on lymphocyte egress rates as deficiency of LFA-1? Given that these antibodies have been used to investigate egress, this would be useful for the field to know.“

Unfortunately we have not performed experiments comparing LFA-$1^{-/-}$ T cells in host mice with WT T cells in mice treated with anti-LFA-1 mAbs.

Minor points:
1) “Some typos in the abstract: "T cells returned to the lymph node parenchyma with frequency" - seems to be a word missing here, eg. greater (also in last paragraph, intro); "whether to leave or return into the node" (rather than returning)”.
Corrected.

Referee #2

Major comments

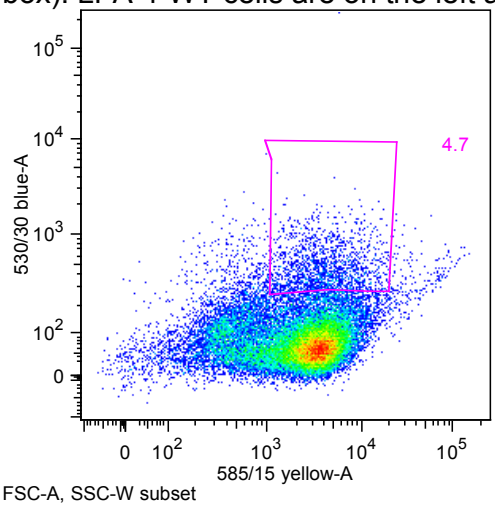
1. “One explanation for this puzzle is that self antigens engage TCRs on naïve T cells entering lymph nodes at very low affinity and thereby alter their in vivo responsiveness to S1P. Naïve CD4 T cells have been shown to respond to MHC-II dependent signals during their scanning of lymph nodes. As CD69 is a negative regulator of the major S1P receptor, S1P1 (but not of CCR7!!), low TCR signals, prolonged by LFA-1-ICAM-1 engagements may upregulate CD69 on LFA-1 expressing but not on LFA-1 null T cells, decrease S1P1 levels, and keep CCR7 signaling intact. This will bias wt T cells to get attracted by CCR7 signals and increase T cell dwelling in expense of egress. The way to prove this hypothesis and substantiate this work is to replace all hematopoietic cells of the lymph node with hematopoietic cells deficient in MHC-II. If, in the absence of MHC-II on DCs, but with residual MHC-II expression on lymphatics, no difference between LFA-1 null and wt T cells is detected, this will indicate that the LFA-1 dependent dwelling of naïve T cells in lymph nodes is TCR driven and depends on DC expressed MHC-II rather than non DC expressed MHC-II. Since in this setting, lymphatic vessels and FRCs will remain intact, it will also allow the differentiation between the contribution of antigen presentation on these cells as opposed to DCs.”

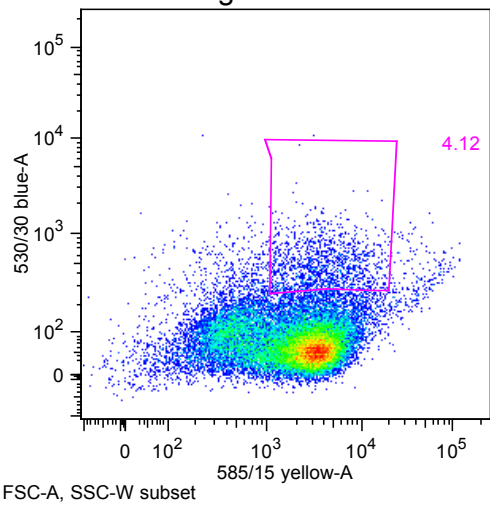
The interesting experiment suggested by the reviewer would allow separation of the contribution of LFA-1 contact to ICAM-1 on APC within the LN parenchyma from the contact to ICAM-1 on the lymphatic vasculature. However, our measurements of CD69 levels on LFA-1+/+ versus LFA-1-/- T cells (see next point below) argue against a preferred upregulation of CD69 by self antigens on LFA-$1^{+/+}$ cells as opposed to LFA-$1^{-/-}$ T cells. In fact, the levels of CD69 in both cells proved to be remarkably similar. Furthermore, we do not argue that LFA-1 interactions do not occur within the node parenchyma as opposed to the lymphatic vasculature which is the focus of our study. The fact that the velocity of LFA-$1^{+/+}$ T cells is greater in some areas of the T zone and slower in others compared with LFA-$1^{-/-}$ T cells, indicates that the behaviour of the WT cells is influenced by expression of LFA-1. A role for LFA-1 within the T zone is in keeping with the findings of Woolf *et al* (Nat. Immunol. 8, 1076-85, 2007) and Schumann *et al* (Immunity, 32, 703-13, 2010)

but potentially not with Park *et al* (Blood 115, 1572-81, 2010). The reason for this discrepancy is not clear.

2. “The indication that ex vivo, LFA-1 null and wt T cells share identical responsiveness to S1P is non-informative and perhaps even misleading. It is possible that LFA-1-/- T cells in fact express higher S1P1 in vivo, as outlined in my earlier paragraph on CD69 upregulation and S1P1 downregulation. This possibility could explain the higher directionality of these T cells towards exit sites. Differences in S1P1 levels between LFA-1 null and wt T cells would be very important to detect. In addition, since S1P1 levels are reciprocal to CD69, it would be very interesting to confirm higher CD69 expression on wt T cells. At any rate, currently, this part of the data is misleading as it pertains to conserved S1P1 levels in LFA-1 null T cells.”

We have assessed CD69 level on Macs bead-selected CD4 LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells prepared directly from the pLN and find it to be low and not significantly different between the two cell types. Following please see CD4 T cells double labeled for CCR7 (x axis) and CD69 (y axis, see pink box). LFA-1 WT cells are on the left and KO cells on the right.





3. “Is the motility of LFA-1-/- T cells slower than of wt T cells, even when both T cells are sequestered in lymph nodes in the presence of the S1P1 antagonist, FTY720? “

The reviewer asks about the T cell motility following administration of FTY720, an antagonist of S1P receptors, expression of which governs exit of lymphocytes from peripheral LN. We now include experiments measuring T cell motility in the paracortical T zone (depth 150 µm) of the inguinal LN following application of FTY720 based on a published treatment regimen (Cinamon *et al* Nat. Immunol. 5, 713-20 2004; Brown *et al*, J. Immunol. 185, 4873-82, 2010). Recipient mice were treated with FTY720 (Cayman Chemical Company, Ann Arbor, MI, USA) i.p. at 1 mg/kg, a regime previously shown to be effective for at least 20 hr. Using this route and dosage, we performed intravital imaging at the 8 hr time point. Our data reveal that the treatment with FTY720 had no immediate effect on the T cell motility (see new Suppl. Fig. 4A). Both LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells showed speed of motility comparable

to the “no treatment” situation. FTY720 was active at the time of imaging, as we additionally monitored lymphocyte counts in blood gained via cardiopuncture immediately after the mice were sacrificed post-imaging (see new Suppl. Fig. 4B). There was strong induction of lymphopenia in the presence of FTY720 affecting total lymphocyte counts, B cells and CD4+ T cells, all suggesting efficiency of the drug treatment. Thus, our data are similar to those of Halin *et al* (Blood 106, 1314-1322, 2005) and suggest unaltered interstitial T cell motility in peripheral LN in the presence of FTY720.

4. “T cell crossing of HEVs is mainly LFA-1 dependent (as also shown by the Hogg lab). Since the few LFA-1 null T cells that do enter lymph nodes do so via VLA-4-VCAM-1 interactions (a previous study by the Hogg's lab), it is possible that these few cells express higher b1 integrins, in particular VLA-4. It is mandatory to test if this is the case in the present setting because if LFA-1 null T cells inside lymph nodes are much higher in their VLA-4 content, they may use FRC expressed VCAM-1 for their motility and this may also alter explain some of the findings.”

We have reported that $\alpha 4$ and $\alpha 4\beta 7$ are expressed at the same level on adoptively transferred LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells (Berlin-Rufenach *et al*, J. Exp. Med., 189, 1467, 1999). This finding has been checked since that time and the result has remained the same. A differential use of $\alpha 4$ integrin does not therefore seem to provide an obvious explanation for the intra-nodal difference in velocity between LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells.

5. “The rationale for the use of shear in the experiments testing the behavior of T cells on S1P and ICAM-1 is unclear. Where will T cells about to leave the T zone experience shear flow? This experiment must be repeated using transwells in which either bare or ICAM-1-coated filters are used and chemotaxis to S1P is determined. Under these conditions, more LFA-1 null T cells may get chemoattracted by S1P. This may provide further evidence for the higher tendency of LFA-1 null T cells to respond to S1P exit signals (In addition to the CD69-S1P1 axis discussed in my previous comments).”

We now include chemotaxis experiments showing the migration of LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells migrating towards CXCL21 and S1P over ICAM-1-coated Transwell filters (Suppl. Fig. 3). The chemotaxing T cells were collected at 1.5 h in an effort to maximize any difference between LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells. However, the results mimic the findings with the uncoated filters in that the proportion of migrating CD4 T cells is similar for both T cell types. It has previously been reported that freshly isolated primary T cells require a source of stress to induce LFA-1 activation (Woolf *et al* 8, 1076, 2007) so this result was not unexpected, but it raises the question as to where this source of stress might come from in a LN. Flow is detected in lymphatic vessels in our experiments (Suppl. Fig 2, Suppl. Video 2). We speculate that T cell processes (or filopodia) potentially penetrate the lymphatic vasculature, as they penetrate HEV (Heasman *et al* J. Cell Biol. 190, 553-63, 2010; Shulman *et al*, Immunity, 30, 1-13, 2009), and are exposed to shear stress delivered by passing lymph. Another possibility is that the organization of ICAM-1 on the LV surface (density, clustering) provides conditions for LFA-1 activation. We have now added these speculations to the Discussion.

Minor comments

"1. ICAM-1 deficient mice contain higher levels of Tregs. A comment on this possibility and its implications in this model should be discussed."

As far as we could discover from the literature, induction of CD4 Tregs is very LFA-1/ICAM-1 dependent and is much reduced in ICAM-$1^{-/-}$ mice or wildtype mice receiving anti-ICAM-1 treatment following immune stimuli (Windish *et al* J. Leuk. Biol. 86, 713-725, 2009; Deane *et al*, J Immunol. 188, 2179-2188, 2012). If this were to be a factor in terms of the responses of naïve T cells, one might expect to observe lower CD69 expression on the recirculating LN resident LFA-$1^{-/-}$ T cells but this is not the case (see response to comment 2).

Referee #3

1) "The idea that LFA-1 act as a 'retention factor' and 'has a major role in T cell choices' to exit lymph node is, to my point of view, an overstatement.
This would imply an 'active role' for LFA-1 for example because the activity of LFA-1 is regulated at the time of egress which is not demonstrated here. In the WT situation, LFA-1 is always expressed on T cells so it is not clear how LFA-1 would contribute to any kind of choice.
I would recommend to remove this terminology and to stick to what is clearly demonstrated here, ie. that T cells probe lymphatic vessels and return to the parenchyma with a contribution of LFA-1 mediated adhesion in this process."

As this reviewer has advised we have altered wording about the role of LFA-1 in terms of the LV interaction to more accurately reflect the data that we provide (see Discussion). LFA-1 would need to undergo activation in order to firmly bind ICAM-1, but we do not explore how this might happen.

2) "The authors should show the results of the critical experiments after swapping the vital dyes to ensure of the absence of a dye-specific effect. While the authors mentioned the dyes are 'routinely swapped' it would be helpful to see the actual data in a supplemental figure
Also difference in brightness of the dyes (CFSE-stained cells seem brigher than SNARF-labeled cells, based on their apparent size) should impact on the calculation of the T cell shape or the occurrence of T cell disappearance from the field of view (shown in Fig.8D-E/Fig. 7DE)."

Dye swapping was routinely done with no conflicting effects. We now include Suppl. Fig. 7 as an example of a dye swapping experiment that complements Fig. 6D-E to show that exchange of dyes from Red to Green does not alter the results obtained. As the reviewer correctly comments, it is important to be sure that the dye difference was not affecting perceived morphological differences between the LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells.

3) "The authors state that there is no difference between velocities of WT and LFA-1-/- T cells in ICAM-1-/- hosts when the difference is exactly in the same range (11-13%) of that observed in WT hosts (with p=0.08). Overall, this point is not very clear."

The reviewer points out that the range in velocities between LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells in WT host versus ICAM-1 hosts is similar though not significant in ICAM-1 hosts (Fig. 1D). As these findings are based on counting >100 cells per T cell type per host (n=3 expts), we have reported them as

being different. A similar small difference in velocity of wildtype versus CD18$^{-/-}$ T cells in WT but not in ICAM-1 hosts has also been reported by others (Woolf *et al* Nat. Immunol. 2007).

4) “It seems counterintuitive that LFA-1 is required to respond to S1P (Fig. 4C) when WT cells have an increased dwell-time in the lymph node compared to LFA-1-/- T cells. Could this be discussed?”

The speed of migration of T cells within the LN parenchyma appears to involve a form of active LFA-1 potentially influenced by the factors in the local microenvironment. At the point of egress through the lymphatic vasculature, both LFA-1$^{-/-}$ and LFA-1$^{+/+}$ T cells can respond to S1P but the outcome can differ, as the LFA-1$^{-/-}$ T cells have a tendency to egress whereas the LFA-1$^{+/+}$ T cells attach and migrate with a proportion return back into the node parenchyma. Our speculation would be that the transient activation (and de-activation) of LFA-1 as the T cell migrate T cell in the LN parenchyma is a separate phenomena from the role of LFA-1 at the point of exit. We have rewritten parts of the Discussion to better reflect these points.

5) “ In the experiment in Fig. 8E aimed to show that blocking ICAM-1 on lymphatics impact on T cell activation, how do the authors exclude that the anti-ICAM-1 Ab does not also reduce T cell homing to the lymph node (which should lead to the same effect)? Also, does ICAM-1 LV block also modify the time of residence in the lymph node in the absence of antigen?
The fact that all T cell become CD69+ after treatment with anti-ICAM-1 seems to contradict the hypothesis proposed by the author that reverse migration help T cell find antigen. Maybe it is important for sustained antigen recognition?”

We now include a new experiment (new Fig. 8) to test the influence of LFA-1 interactions with the LV on immune responsiveness that eliminates the concern that anti-ICAM-1 is preventing T cell entry into the LN. An important factor in such an experiment was to remove from consideration any interaction between T cells and dendritic cells (DCs) that might make use of LFA-1 in order to focus on the non-APC interactions of the T cells. We have chosen the model of pre-injecting OVA-laden DCs into WT and ICAM-1$^{-/-}$ host mice that then received transgenic OT-2 T cells that are sensitive to OVA peptide on APC. In this model, the OT-2 cells respond to the APC and we have investigated the extent of their proliferation in each host LN. After assessing parameters of peptide dose, experimental timing and DC uptake (Mempel *et al*, Nature,427,154, 2004; Lammerman *et al*, Nature, 453, 51 2008) and experimental timing, we have recorded the extent of proliferation at 72 h and found that the OT-2 T cells in the WT host proliferated 1.5 fold over the ICAM-1$^{-/-}$ host. Although the main focus of this manuscript is on the transit of non-primed CD4 T cells through the LN, we hope that the OT-2 experiment serves to give some indication of how the use of ICAM-1 on the lymphatic vasculature also influences T cell activity when antigen is encountered.

3rd Editorial Decision 24 January 2013

Thank you for submitting your revised manuscript to the EMBO journal. Your revision has now been re-reviewed by referees #1 and 2. As you can see below both referees appreciate the introduced changes. However they also have a few remaining comments that I would like to ask you to take into consideration in a final revision. If you would like to discuss any of the comments further please don't hesitate to contact me.

REFEREE REPORTS

Referee #1

The authors have improved the manuscript over the previous version, but some important data remain absent. In particular:

(1) In Figure 1 the authors have chosen not to show the requested count data for LN egress and as such they do not address whether egress rates are faster in ICAM1-/- hosts than in wild type mice, as would be presumed from the absence of change in ratio between wild type and LFA-1-/- T cells. For completion and additional validation of their conclusion it would have been nice to include these data in the figure. In addition, the count data would allow better comparison to other estimates of this parameter in the literature (eg. Mandl et al PNAS 2012), which would be useful.
(2) The authors have reworked the sections where they had defined lymphatic sinuses as being cortical vs. medullary. This part is much improved.
(3) For the CCR7 expression data shown in Figure 4, LFA-1+/+ and -/- cells are shown in separate histograms and a % of positive cells are given. This is not the relevant comparison here. Given that the expression level of CCR7 can impact egress rates (see Pham et al, 2008), the more relevant comparison would be the CCR7 mean fluorescent intensity accompanied by overlaid histograms so that it can be determined whether expression levels are indeed the same.
(4) The experiment in the final figure is improved compared to the earlier one shown. However, it is not clear whether homing of the OT2 T cells is impaired in the ICAM1-/- recipients, which could contribute to differences in responses. Showing the data only as percent proliferated does not address this. It would be useful to show OT2 cell counts as well as showing the CFSE plots.

Referee #2

I am still puzzled as for the mechanism(s) that account for the key finding of this work i.e. that LFA-1 transmits retention/haptotactic signals which restrict T cell egress from lymph nodes and make a subset of T cells return to the parenchyma after reaching lymphatic vessels. Nevertheless, I believe the revised text includes sufficient reservations which leave open several possibilities to explain this overall interesting set of data.
My minor comments:
1. In order to make a strong argument that not only CD69 but also S1P1 and other S1P receptors are normal, I would suggest to determine S1P1 mRNA (due to lack of mAb to these key receptors) in wt and LFA-1 null T cells isolated after adoptive transfer.
2. The authors claim in response to my comment that "measurements of CD69 levels on LFA-1+/+ versus LFA-1-/- T cells argue against a preferred upregulation of CD69 by self antigens on LFA-1+/+ cells as opposed to LFA-1-/- T cells". If this indeed rules out a role of LFA-1-ICAM-1 stop signals in the accelerated egress of their LFA-1 null T cells, they should discuss this important point in the revised discussion. They should also display the CD69 FACs data as histograms and in MFI units and include it as a supplementary material.
3. The authors should discuss the conservation of a4 and of b1 (and not only of b7) integrins on adoptively transferred wt and LFA-1 null T cells.
4. "An average of 95.71{plus minus}16.57 LFA-1+/+ and LFA-1-/- 16.71{plus minus}2.78 T cells were counted in LN sections of the T cell zone". Please round up the numbers.

2nd Revision - authors' response 29 January 2013

Referee #1

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(1) "In Figure 1 the authors have chosen not to show the requested count data for LN egress and as such they do not address whether egress rates are faster in ICAM1-/- hosts than in wild type mice, as would be presumed from the absence of change in ratio between wild type and LFA-1-/- T cells. For completion and additional validation of their conclusion it would have been nice to include these data in the figure. In addition, the count data would allow better comparison to other estimates of this parameter in the literature (eg. Mandl et al PNAS 2012), which would be useful."

When these experiments were carried out, the data was collected and analyzed as a ratio between LFA-$1^{+/+}$ and LFA-$1^{-/-}$ T cells. This seemed a valid way to proceed as it overcame variables inherent in a multi-stage experiment such as preparation of T cells, sample loss and uptake into LNs. These preliminary findings were then extended in the rest of the manuscript. It seems that the reviewer is not insisting on the repetition of this work that would be a big undertaking. Thus we would like to leave the data as they are.

(2) "The authors have reworked the sections where they had defined lymphatic sinuses as being cortical vs. medullary. This part is much improved."

This was an important issue so it is gratifying that the reviewer is now happy with it.

(3) "For the CCR7 expression data shown in Figure 4, LFA-1+/+ and -/- cells are shown in separate histograms and a % of positive cells are given. This is not the relevant comparison here. Given that the expression level of CCR7 can impact egress rates (see Pham et al, 2008), the more relevant comparison would be the CCR7 mean fluorescent intensity accompanied by overlaid histograms so that it can be determined whether expression levels are indeed the same."

In Fig. 4B, the CCR7 staining of LFA-$1^{+/+}$ and LFA-$1^{-/-}$ CD4 T cells are displayed directly under one another so the similarity in expression levels can be easily observed. We would prefer to leave the Figure as it is, but to include the MFI±SD data in the text (page 10).

(4) "The experiment in the final figure is improved compared to the earlier one shown. However, it is not clear whether homing of the OT2 T cells is impaired in the ICAM1-/- recipients, which could contribute to differences in responses. Showing the data only as percent proliferated does not address this. It would be useful to show OT2 cell counts as well as showing the CFSE plots. "

We will include in the text the numbers (mean±SD) of OT2 cells undergoing proliferation in the WT versus ICAM-$1^{-/-}$ hosts showing that the cell numbers are equivalent (page 15). We would also include reference to the studies of Lehmann *et al* (J. Immunol. 171, 2588, 2003) and Boscacci *et al* (Blood 116, 915, 2010) that have looked in detail at the role of ICAM-1 (and ICAM-2) in T cell homing to LN. Both studies show a lack of difference in PLN size between WT, ICAM-1 and ICAM-2 KO mice, suggesting over-lapping roles for the LFA-1 ligands. In terms of detail, Boscacci *et al* show a short delay in T cell attaching and crossing the vasculature of ICAM-$1^{-/-}$ mice (30 -45 mins), but this does not affect overall homing (both studies). In the Fig. 8 experiments, we are looking at proliferation after 72 hr when the OT-2 T cells would have had a similar period of exposure, generally speaking, to Ag-laden DCs in either WT or ICAM-$1^{-/-}$ hosts.

Referee #2

"I am still puzzled as for the mechanism(s) that account for the key finding of this work i.e. that LFA-1 transmits retention/haptotactic signals which restrict T cell egress from lymph nodes and make a subset of T cells return to the parenchyma after reaching lymphatic vessels. Nevertheless, I believe the revised text includes sufficient reservations which leave open several possibilities to explain this overall interesting set of data.

My minor comments:

1. In order to make a strong argument that not only CD69 but also S1P1 and other S1P receptors are normal, I would suggest to determine S1P1 mRNA (due to lack of mAb to these key receptors) in wt and LFA-1 null T cells isolated after adoptive transfer."

We show by Western blotting using a credited Ab (Fig. 4A) that total T cell expression of S1P1 is similar in both WT and KO T cells. We would argue that measuring protein levels is a better indicator of receptor expression than looking at mRNA. What is not generally available is an

Ab/mAb that measures membrane expression of S1P1 by flow cytometry. That would be most convincing but unfortunately is not doable.

2. “The authors claim in response to my comment that "measurements of CD69 levels on LFA-1+/+ versus LFA-1-/- T cells argue against a preferred upregulation of CD69 by self antigens on LFA-1+/+ cells as opposed to LFA-1-/- T cells". If this indeed rules out a role of LFA-1-ICAM-1 stop signals in the accelerated egress of their LFA-1 null T cells, they should discuss this important point in the revised discussion. They should also display the CD69 FACs data as histograms and in MFI units and include it as a supplementary material.”

The generation of T cell “stop signals” within the lymph node is certainly an interesting topic. The reviewer may be thinking that the fact that CD69 expression levels are similar between WT and KO rules out LFA-1/ICAM-1-induced stop signals *in vivo* and might suggest either no integrin involvement or potentially alpha 4 integrins in CD69 upregulation (see next comment). We included the CD69 data in the last review for the reviewer’s interest, but would be unhappy about commenting further on this topic which needs to be separately investigated in a study looking directly at MHC-II interactions on presenting and other cells and most appropriately done in an *in vivo* study. We feel that this is beyond the scope of this present manuscript.

3. “The authors should discuss the conservation of a4 and of b1 (and not only of b7) integrins on adoptively transferred wt and LFA-1 null T cells.”

It is an interesting question to ask whether the alpha 4 integrins might be playing a role in T cell migration within the LN, but, again, this is another study. We know that alpha 4 integrin expression is not altered on the LFA-$1^{-/-}$ T cells (compared with WT) but this doesn’t allow one to say anything about the actual activity of alpha 4 integrins within the node. As stated in the previous response to the reviewer, we do have some information (not published) that the adhesion characteristics of alpha 4 integrins expressed by LFA-$1^{-/-}$ and LFA-$1^{+/+}$ T cells are similar, but this is *in vitro* data and we do not wish to speculate about what might be occurring *in vivo*.

4. "An average of 95.71{plus minus}16.57 LFA-1+/+ and LFA-1-/- 16.71{plus minus}2.78 T cells were counted in LN sections of the T cell zone". Please round up the numbers.”

Easily done (page 6).