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Stabilizing the VE-cadherin catenin complex blocks leukocyte extravasation and vascular permeability

Dörte Schulte, Verena Küppers, Nina Dartsch, Andre Broermann, Hang Li, Alexander Zarbock, Olena Kamenyeva, Friedemann Kiefer, Alexander Khandoga, Steffen Massberg, Schulte, Verena Küppers, Nina Dartsch, Andre Broermann, Hang Li, Alexander Zarbock, Olena Kamenyeva, Friedemann Kiefer, Alexander Khandoga, Steffen Massberg and Dietmar Vestweber

Corresponding author: Dietmar Vestweber, Max-Planck-Institute of Molecular Biomedicine

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(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

17 March 2011

Dear Dr. Vestweber,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express significant interest in your work, and are broadly in favour of publication. However, all three do raise a number of issues that would need to be addressed in a revised version of the manuscript first.

While I don't see the need to go into the reports in detail here, I do want to highlight one issue. The major criticism raised by referees 2 and 3 is the lack of mechanistic understanding of why the VE-cad:alpha-cat fusion protein stabilises junctions. Clearly this is a valid concern, but we do recognise that this is not the major point of the paper, and also that how cadherin links to the cytoskeleton and how this relates to junction stability is a complicated and controversial issue. The referees both make a number of suggestions for potential experiments that could help on this front, and we would encourage you to attempt to gain further insight here. However, we would not see a full understanding of the molecular basis for junctional stabilisation by the fusion construct as being essential for eventual publication here.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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 us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REREE REPORTS

Referee #1:

The manuscript addresses the consequences of replacing endogenous VE-cadherin with a VE-cadherin-alpha catenin fusion, thereby eliminating possible regulatory events that disrupt the cadherin-beta-catenin complex. The results are striking - although some animals die embryonically, those that survive exhibit remarkable resistance to agents that induce vascular permeability. Leukocyte transmigration across vessels is dramatically reduced. Overall, this is an exceptionally well done and interesting study that utilizes both mouse genetic models and in vitro cellular experimental approaches. One potential weakness is that the authors may be over-interpreting (or simplifying) the mechanism by which the VE-cad-alpha-catenin fusion regulates permeability and transmigration. Although it is most likely due to direct effects on the stability of the cadherin-mediated adhesive contact, it is also possible that the effects are indirect. This possibility was addressed to some extent by monitoring tight junction components and VEGFR association, but nonetheless, it is possible that transmigration is impacted indirectly. Examples include changes in actin dynamics or cell signaling upon leukocyte binding, or changes in vimentin organization that might be indirect consequences of expressing the chimera. With that said, this is a well-done study with broad significance to the vascular biology and cadherin field.

Referee #2:

In this elegant study, Schulte et al present in vivo evidence for the importance of the paracellular pathway in leukocyte transendothelial migration. Using a series of in vivo models, they show that constitutively linking VE-cadherin with alpha-catenin strongly reduces TEM by either neutrophils, T cells or HL60 cells in various tissues and in HUVEC. This effect was found not to affect the transcellular pathway. Finally, authors analyse the dynamics of VE-cad and the fusion protein by biochemical and FRAP analysis. The work provides an important in vivo complementation to the study by Noda et al, who used similar constructs in an in vitro analysis of cAMP-based stimulation of junctional localization of VE-cadherin.

The study of Schulte et al is of a high level and in particular the in vivo experiments are clearcut and compelling. I think this work settles the issue of whether the paracellular route of migration is important in vivo.

There are a few controls that could be included for this part, in particular analysis of the levels of endogenous alpha- and beta- and p120 catenin and even plakoglobin in the cells that express the different constructs should be shown. This would be in addition to the controls already in Fig 1C, but since this paper is also about catenin function, it is relevant to include

As to Fig 6, I wasn't entirely sure why the analysis of VEGFR was relevant here. Although VEGFR associates to VE-cad, do the authors suggest that they had anticipated reduced VEGFR signalling in the presence of the fusion construct?

I would have rather liked to see if regulation of VEcad by VE-PTP (association and/ or dephosphorylation) was altered by the fusion with a-cat. This appears more directly related to the issue of the regulation of the complex.

The paper clearly has two sides: (1) showing that junctions are key to TEM in vivo and (2) an attempt to explain the observed findings with the fusion protein. The latter point relates to the FRAP studies and the increased connection to F-actin (ie participation in the insoluble fraction)

The animals that do get born are healthy, which is remarkable, given the strong phenotype and the reduction of induced permeability. One would predict that this would severely impair formation of blood vessels as well. It suggests that during formation of new junctions, other mechanisms may be important than during transient loss of permeability or TEM, that is based on changes in established cell-cell contacts. This may relate to the fact that the fusion construct is suggested to link better to F-actin, as claimed by the authors. It appears that the authors conclude that this construct cannot dissociate from F-actin as well as the normal VEcad can. This in turn suggests that the regulation of the complex is not at the level of the a-cat-F-actin link, but at the level of the a-cat-VEcad connection. This does suggest that under normal conditions, the complex dissociates during TEM. This aspect is briefly addressed during the discussion, but could have been discussed in more detail.

At the same time, I am not convinced the study goes far enough to explain the effect on junctions by the fusion protein. I do not see why a-cat would bind better to F-actin if it is bound to VE cad constitutively vs dynamically. Is it the truncation itself? Does the isolated, truncated a-cat protein bind better to F-actin compared to full-length a-cat?

The FRAP studies do show some differences but these are not very large and are hard to reconcile with a 60% inhibition of TEM. I therefore doubt whether the FRAP assays are fit to explain the biological consequences of the fusion protein.

I think that what the authors have done with this approach is dramatically increase the local concentration of a-cat in the junctions of the cells expressing the fusion as compared to the controls. Given the fact that a-cat is an actin-remodelling protein, this will also very likely affect the actin cytoskeleton. - The analysis of the F-actin distribution in these cells is therefore an obvious one to include.

Moreover, this may also explain the phenotype of the linear junctions, which resembles much the effect of cAMP elevation, which ultimately is also based on the remodelling of cortical actin.

To address the issue of local a-cat concentration at junctions, authors should double stain cells such as in 7B (expressing similar levels of VE-cad at the cell surface) with antibodies to both VE-cad and a-cat (using an antibody that recognizes the truncated a-cat protein) . Since the ratio of the proteins in the fusion is 1:1, the corresponding ratio of VE-cad : a-cat in the control cells can be estimated by quantifying a series of confocal images/junctions.

This may allow the authors to conclude that although the 'plasticity' of the complex is key to the regulation during TEM, the reason for this lies primarily in the local concentration of its components.

Referee #3:

In the manuscript by Schulte et al., the authors generated mice with a VE-cadherin- α -catenin fusion construct replacing VE-cadherin. These mice were resistant to the induction of vascular leaks by VEGF or histamine, whereas these mice displayed decreased leukocyte recruitment into inflamed cremaster, lung and skin. VE-cadherin- α -catenin associated more intensely with the actin cytoskeleton. This is an interesting and potentially important paper. The in vivo findings are convincing. More mechanistic insights would be helpful.

Specific comments:

- 1) The authors demonstrate increased numbers of peripheral leukocytes in Table S2, however they do not explain this important finding. How are the numbers of lymphocytes and monocytes in the spleen or lymph nodes?
How are the numbers of different myeloid cells and progenitors in the bone marrow? The latter point is important as some hematopoietic cells may have endothelial origin (Cell Stem Cell. 2008 Dec 4;3(6):625-36.). I.e. is the difference in numbers of peripheral leukocytes due to a developmental alteration or due to reduced transmigration of leukocytes through the endothelium?
- 2) The in vitro experiments with transfected HUVEC in figure 5 are difficult to interpret due to the presence of endogenous VE-cadherin in the cells. These experiments should be repeated with primary endothelial cells from the respective mice or with the endothelioma lines presented in fig. 6. In addition, in vitro permeability assays should be shown with primary endothelial cells from the respective mice or with the endothelioma lines.
- 3) Fig. 3: Given the lower extravasation of leukocytes one would expect to see higher levels of adherent cells. If one also takes into account the higher numbers of circulating neutrophils (as shown in Table S2) in the VEC-a-C mice, it seems that even adhesion was inhibited in the VEC-a-C mice. This should be analysed in detail and explained.
In addition, the authors should provide analysis of the expression of ICAM-1, VCAM-1, E-selectin etc. in VEC-a-C and VEC-WT primary endothelial cells and tissues from these mice.
- 4) Fig. 7: Are G-actin and F-actin levels changed in VEC-a-C and VEC-WT endothelial cells? How about stress fiber formation (constitutively and upon stimulation with e.g. histamine)?
Moreover, a recent paper showed that VE-cadherin regulates ROCK activity and MLC phosphorylation (Curr Biol. 2009 Apr 28;19(8):668-74). The authors should compare ROCK and MLC activity in VEC-a-C and VEC-WT endothelial cells.
- 5) Further mechanistic insights regarding the function of the fusion protein should be provided. Such a fusion may induce a significant conformational change in VE-cadherin and affect its function. Is p120 or beta-arrestin binding to VE-cadherin affected by the fusion of alpha-catenin? Is the accessibility of the Y658 phosphorylation site and thus phosphorylation of these sites the same in VEC-a-C and VEC-WT? Are there any differences in the kinetics of VEC internalization between the VEC-a-C and VEC-WT? These are important points in order to completely characterize the fusion protein.
- 6) The authors conclude that VEC-a-C association with the actin cytoskeleton is enhanced, although they do comment on the findings of the Yamada paper (Cell 2005) that alpha-catenin binding to actin or to the cadherin-beta-catenin complex is mutually exclusive. However, the VEC-a-C fusion protein does not interact with beta-catenin anymore, which would allow its enhanced association with actin. Thus, the conclusion of the authors is very likely to be correct. A more detailed discussion here would be appropriate.

1st Revision - Authors' Response

28 June 2011

Thank you very much for organizing the review process of our manuscript. We were very pleased about the constructive comments of the reviewers and have extensively revised our manuscript.

We have addressed basically each point of the reviewers by additional new experiments (see new Suppl tables S2, S3, S5, Suppl fig. S1; fig. 4C, fig. 5E, fig. 6D and E, fig. 7, fig. 8) as outlined in detail below.

We think that the revised version of our paper has been considerably improved and hope that you will consider the paper in its present form for publication in The EMBO Journal.

Detailed response:

Referee #1:

The manuscript addresses the consequences of replacing endogenous VE-cadherin with a VE-cadherin- α -catenin fusion, thereby eliminating possible regulatory events that disrupt the cadherin-beta-catenin complex. The results are striking - although some animals die embryonically, those that survive exhibit remarkable resistance to agents that induce vascular permeability. Leukocyte transmigration across vessels is dramatically reduced. Overall, this is an exceptionally well done and interesting study that utilizes both mouse genetic models and in vitro cellular experimental approaches. One potential weakness is that the authors may be overinterpreting (or simplifying) the mechanism by which the VE-cad- α -catenin fusion regulates permeability and transmigration. Although it is most likely due to direct effects on the stability of the cadherin-mediated adhesive contact, it is also possible that the effects are indirect. This possibility was addressed to some extent by monitoring tight junction components and VEGFR association, but nonetheless, it is possible that transmigration is impacted indirectly. Examples include changes in actin dynamics or cell signaling upon leukocyte binding, or changes in vimentin organization that might be indirect consequences of expressing the chimera. With that said, this is a well-done study with broad significance to the vascular biology and cadherin field.

We agree with the reviewer: Although VE-cadherin- α -catenin affects permeability and transmigration most likely by directly enhancing the stability of the cadherin-mediated adhesive contacts, it is indeed important to rule out indirect effects. Therefore we have done the following additional control experiments:

- 1) We show that the F-actin/G-actin ratio is similar in endothelioma cells established from VECWT and VE-C- α -C mice (New figure 8C)
- 2) Distribution of F-actin and vimentin in both cell lines is similar (New Fig. 8A).
- 3) Myosin light chain-phosphorylation levels are not affected (new figure 8B)
- 4) p120 binding and endocytosis are unaltered (new figure 8D)
- 5) Wnt induced accumulation of β -catenin in the nucleus are similar (new figure 7B)
- 6) VE-PTP association with VE-cadherin is similar in both cells types (new parts D + E of figure 6)
- 7) Expression levels of a panel of adhesion molecules and receptors on endothelial cells is unaffected by the VEC- α -C fusion protein (supplementary figure S1).

Referee #2:

In this elegant study, Schulte et al present in vivo evidence for the importance of the paracellular pathway in leukocyte transendothelial migration. Using a series of in vivo models, they show that constitutively linking VE-cadherin with α -catenin strongly reduces TEM by either neutrophils, T cells or HL60 cells in various tissues and in HUVEC. This effect was found not to affect the transcellular pathway. Finally, authors analyse the dynamics of VE-cad and the fusion protein by biochemical and FRAP analysis. The work provides an important in vivo complementation to the study by Noda et al, who used similar constructs in an in vitro analysis of cAMP-based stimulation of junctional localization of VE-cadherin.

The study of Schulte et al is of a high level and in particular the in vivo experiments are clearcut and compelling. I think this work settles the issue of whether the paracellular route of migration is important in vivo.

There are a few controls that could be included for this part, in particular analysis of the levels of endogenous α - and β - and p120 catenin and even plakoglobin in the cells that express the different constructs should be shown. This would be in addition to the controls already in Fig 1C, but since this paper is also about catenin function, it is relevant to include.

We have now analyzed the association of VE-PTP with VEC-WT and VEC- α -C in the respective endothelioma cells and found the same levels of co-precipitation (Fig. 6D). In addition we tested in triple transfected cells whether VE-PTP would dephosphorylate VEGFR-2-phosphorylated VEC-WT or VEC- α -C with similar efficiency. Again we found similar results for both constructs (Fig. 6 E)

The paper clearly has two sides: (1) showing that junctions are key to TEM in vivo and (2) an

attempt to explain the observed findings with the fusion protein. The latter point relates to the FRAP studies and the increased connection to F-actin (i.e. participation in the insoluble fraction).

The animals that do get born are healthy, which is remarkable, given the strong phenotype and the reduction of induced permeability. One would predict that this would severely impair formation of blood vessels as well. It suggests that during formation of new junctions, other mechanisms may be important than during transient loss of permeability or TEM, that is based on changes in established cell-cell contacts. This may relate to the fact that the fusion construct is suggested to link better to F-actin, as claimed by the authors. It appears that the authors conclude that this construct cannot dissociate from F-actin as well as the normal VEcad can.

This in turn suggests that the regulation of the complex is not at the level of the α -cat-F-actin link, but at the level of the α -cat-VEcad connection. This does suggest that under normal conditions, the complex dissociates during TEM. This aspect is briefly addressed during the discussion, but could have been discussed in more detail.

At the same time, I am not convinced the study goes far enough to explain the effect on junctions by the fusion protein. I do not see why α -cat would bind better to F-actin if it is bound to VE cad constitutively vs dynamically. Is it the truncation itself? Does the isolated, truncated α -cat protein bind better to F-actin compared to full-length α -cat?

The FRAP studies do show some differences but these are not very large and are hard to reconcile with a 60% inhibition of TEM. I therefore doubt whether the FRAP assays are fit to explain the biological consequences of the fusion protein.

Indeed we believe that the most likely explanation of our results is that VEC- α -C enhances the efficiency of VE-cadherin/F-actin interactions by irreversibly increasing the stability of the VEcadherin-catenin complex, which implies that the dissociation of the normal VE-cadherin-catenin complex (either at the connection between VE-cadherin with β - or γ -catenin, or at the connection between β - or γ -catenin with α -catenin) is a major step in destabilizing VE-cadherin mediated adhesive interactions. Examples for a dissociation of β -catenin from VE-cadherin induced by VEGF have been published (Monaghan-Benson et al. 2009) and were discussed.

Our FRAP results show that at steady state conditions a larger fraction of VEC- α -C molecules than of VEC-WT molecules (50% increase) is actin-associated and therefore immobile. If one assumes that it is the actin-associated fraction of VE-cadherin molecules that is mainly relevant for the stability of endothelial junctions, it is not unlikely that a 50% increase in actin association is of major importance for a 60% reduction in leukocyte transmigration. In addition, in the light of experiments by Noda et al. (2010) it is well possible that stimuli that open endothelial cell contacts (such as VEGF, histamine or leukocytes) might further increase the difference in actin association between VEC-WT and VEC- α -C.

We do not think that our VEC- α -C construct binds more avidly to actin than VEC-WT via its associated catenin-complex. Instead, we believe that not all VEC-WT molecules at endothelial cell contacts are catenin-associated and therefore not all VEC-WT molecules have a chance to bind to the actin cytoskeleton. In contrast, VEC- α -C molecules have a higher chance to be linked to actin. This would provide an alternative explanation for the differences in membrane mobility and detergent extraction that we observed.

I think that what the authors have done with this approach is dramatically increase the local concentration of α -cat in the junctions of the cells expressing the fusion as compared to the controls. Given the fact that α -cat is an actin-remodelling protein, this will also very likely affect the actin cytoskeleton. - The analysis of the F-actin distribution in these cells is therefore an obvious one to include.

Moreover, this may also explain the phenotype of the linear junctions, which resembles much the effect of cAMP elevation, which ultimately is also based on the remodelling of cortical actin.

To test this possibility we have analyzed the distribution of F-actin in endothelioma cells from VEC-WT and VEC- α -C mice. However, as shown in the new figure 8A, we found no obvious difference in stress fiber formation or in the formation of cortical actin between these cells.

To address the issue of local α -cat concentration at junctions, authors should double stain cells such as in 7B expressing similar levels of VE-cad at the cell surface) with antibodies to both VE-cad and α -cat (using an antibody that recognizes the truncated α -cat protein) . Since the ratio of the proteins in the fusion is 1:1, the corresponding ratio of VE-cad : α -cat in the control cells can be estimated by quantifying a series of confocal images/junctions. This may allow the authors to conclude that although the 'plasticity' of the complex is key to the regulation during TEM, the reason for this lies primarily in the local concentration of its components.

We have now analyzed whether endothelioma cells from VEC-WT and VEC- α -C mice differ in the ratio of α -catenin and VE-cadherin at their cell contacts. As shown in the new figures 7D+E, we found no such differences (we used antibodies against α -catenin that recognize its truncated form). Thus, the local concentration of α -catenin was not dramatically enhanced by VEC- α -C.

Referee #3:

In the manuscript by Schulte et al., the authors generated mice with a VE-cadherin- α -catenin fusion construct replacing VE-cadherin. These mice were resistant to the induction of vascular leaks by VEGF or histamine, whereas these mice displayed decreased leukocyte recruitment into inflamed cremaster, lung and skin. VE-cadherin- α -catenin associated more intensely with the actin cytoskeleton. This is an interesting and potentially important paper. The in vivo findings are convincing. More mechanistic insights would be helpful.

Specific comments:

1) The authors demonstrate increased numbers of peripheral leukocytes in Table S2, however they do not explain this important finding. How are the numbers of lymphocytes and monocytes in the spleen or lymph nodes? How are the numbers of different myeloid cells and progenitors in the bone marrow? The latter point is important as some hematopoietic cells may have endothelial origin (Cell Stem Cell. 2008 Dec 4;3(6):625-36.). I.e. is the difference in numbers of peripheral leukocytes due to a developmental alteration or due to reduced transmigration of leukocytes through the endothelium?

As requested we have now determined the number of B220+, CD4+ and CD8+ lymphocytes and the number of Gr-1+ myeloid cells and 7/4+ neutrophil granulocytes in spleen and inguinal lymph nodes of VEC-WT and VEC- α -C mice. However, we found no difference (new supplementary tables S4, S5). Due to this result we tested whether short term homing of naïve lymphocytes into lymph nodes would be reduced in VEC- α -C mice. Surprisingly, we found that in strong contrast to the entry of neutrophils and lymphocytes into inflamed tissue, entry of primary isolated, [51Cr] labeled naïve lymphocytes into lymph nodes was not reduced in VEC- α -C mice. These results are now shown in the new Fig. 4C. These new and unexpected results suggest that the entry of naïve lymphocytes through specialized high endothelial venules (HEV) is not inhibited by VEC- α -C, in strong contrast to the entry of leukocytes into inflamed tissue. This could argue for a transcellular route of diapedesis in HEV, or for a different mechanism for the opening of endothelial junctions in HEV. These results are now mentioned in abstract and discussion.

In addition, our results suggest that it is not reduced homing of lymphocytes into secondary lymphoid organs that would explain the elevated peripheral leukocyte counts. We then tested the number of lymphocytes, myeloid cells, and c-kit+, lin- cells in the bone marrow. Again we found no difference between both types of mice (Supplementary table S3, results section page 7). Thus we have presently no evidence for developmental, hematopoietic alterations that would cause the elevated levels of peripheral leukocyte counts in adult VEC- α -C mice.

2) The in vitro experiments with transfected HUVEC in figure 5 are difficult to interpret due to the presence of endogenous VE-cadherin in the cells. These experiments should be repeated with primary endothelial cells from the respective mice or with the endothelioma lines presented in fig. 6. In addition, in vitro permeability assays should be shown with primary endothelial cells from the respective mice or with the endothelioma lines.

We have now performed in vitro transmigration assays with primary mouse PMNs and endothelioma cells isolated from VEC-WT and VEC- α -C mice, and find again strong inhibition of transmigration by VEC- α -C. This is now shown in Fig. 5E. Permeability assays could not be performed with the endothelioma cells established from VEC-WT and VEC- α -C mice, since in vitro transendothelial permeability could not be stimulated.

3) Fig. 3: Given the lower extravasation of leukocytes one would expect to see higher levels of adherent cells. If one also takes into account the higher numbers of circulating neutrophils (as shown in Table S2) in the VEC- α -C mice, it seems that even adhesion was inhibited in the VEC- α -C mice. This should be analysed in detail and explained. In addition, the authors should provide analysis of the expression of ICAM-1, VCAM-1, E-selectin etc. in VEC- α -C and VECWT primary endothelial cells and tissues from these mice.

We agree with the reviewer that at first glance one could have expected that reduced diapedesis would lead to an increase in adherent cells. However, integrin activation known to be required for leukocyte adhesion to the vessel wall is transient, and leukocytes unable to extravasate can indeed dissociate from the vessel wall and get washed away by the blood stream. A precedent for this are

mice deficient for the endothelial tight junction protein ESAM (Wegmann et al., J Exp. Med., 2006), where extravasation is inhibited although leukocyte adhesion and rolling are not affected. Peripheral neutrophil counts, which are indeed higher in VEC- α -C mice than in VECWT mice under non-inflammatory conditions (table S2) are irrelevant for the cremaster results, since peripheral neutrophil counts, enhanced under inflammatory conditions (IL-1 β), are similar between VEC-WT and VEC- α -C mice. This had been documented before in (old) supplementary table S3 (now S6), and is now more explicitly mentioned at the end of the legend of figure 3. Nevertheless, we have now compared (by FACS analysis) the expression levels of constitutively expressed adhesion molecules and signaling receptors such as ESAM, PECAM-1, VE-cadherin, Tie-2, VEGFR-2 and VE-PTP, and of cytokine inducible adhesion molecules such as ICAM-1, VCAM-1, P-selectin, and E-selectin on endothelioma cells established from VECWT and VEC- α -C mice. In addition, we analyzed primary isolated endothelial cells from IL-1 β stimulated cremaster tissues of both type of mice by FACS for the expression levels of ICAM-2, VCAM-1, ICAM-1, E-selectin, and P-selectin. No differences were found (shown now in the new supplementary figure S1).

4) *Fig. 7: Are G-actin and F-actin levels changed in VEC-a-C and VEC-WT endothelial cells? How about stress fiber formation (constitutively and upon stimulation with e.g. histamine)? Moreover, a recent paper showed that VE-cadherin regulates ROCK activity and MLC phosphorylation (Curr Biol. 2009 Apr 28;19(8):668-74). The authors should compare ROCK and MLC activity in VEC-a-C and VEC-WT endothelial cells.*

We determined G-actin and F-actin levels in VEC- α -C and VEC-WT endothelioma cells and found no difference between both cell types (shown now in new Fig. 8C). In addition we found similar staining pattern for vimentin and F-actin in VEC- α -C and VEC-WT endothelioma cells and similar phosphorylation levels of MLC (New fig. 8A, B)

5) *Further mechanistic insights regarding the function of the fusion protein should be provided. Such a fusion may induce a significant conformational change in VE-cadherin and affect its function. Is p120 or beta-arrestin binding to VE-cadherin affected by the fusion of alphacatenin? Is the accessibility of the Y658 phosphorylation site and thus phosphorylation of these sites the same in VEC-a-C and VEC-WT? Are there any differences in the kinetics of VEC internalization between the VEC-a-C and VEC-WT? These are important points in order to completely characterize the fusion protein.*

We have analyzed the association of p120 with VEC- α -C and found no difference when compared to VEC-WT (shown now in new Fig. 8D). The accessibility of Y658 for phosphorylation could not be analyzed, since the commercially available antibodies against Y658-P are not specific for this tyrosine. (We immunoprecipitated wt VE-cadherin and a VEcadherin-Y658F mutant protein from peroxyvanadate-treated, transfected cells and found that the commercial "anti Y685-P-specific" antibodies recognized both forms of VE-cadherin in immunoblots equally well.) We also compared endocytosis of VEC-WT and VEC- α -C in HUVEC and found no difference (new figure 8E+F).

6) *The authors conclude that VEC-a-C association with the actin cytoskeleton is enhanced, although they do comment on the findings of the Yamada paper (Cell 2005) that alpha-catenin binding to actin or to the cadherin-beta-catenin complex is mutually exclusive. However, the VEC-a-C fusion protein does not interact with beta-catenin anymore, which would allow its enhanced association with actin. Thus, the conclusion of the authors is very likely to be correct. A more detailed discussion here would be appropriate.*

The question whether or how α -catenin links cadherins to the actin cytoskeleton is presently very controversial. As we pointed out in our discussion, the fact that α -catenin may not be able to bind to actin and to β -catenin at the same time does not argue against an association of the cadherin catenin complex with actin via α -catenin and additional linker molecules. We agree with the reviewer that it is even possible that VEC- α -C might directly bind to F-actin, in contrast to β -catenin associated α -catenin. We have now mentioned this possibility in the discussion (page 18), but made clear at the same time that it is simply not known whether the fusion

Editorial Decision

18 July 2011

Dear Dr. Vestweber,

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-77321R. It has now been seen again by referees 2 and 3, whose comments are enclosed below. As you will see, both agree that you have addressed the concerns from the previous round of review well, and are now fully supportive of publication here. I am therefore pleased to be able to tell you that we will be able to accept your manuscript to be published in EMBOJ. There are just a couple of points from the editorial side that need to be dealt with first:

- Please can you include an Author Contributions statement (below the Acknowledgments)?
- Please can you include details of the statistical tests used and state what the error bars represent in the figure legends for all relevant graphs.
- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a single PDF file comprising the original, uncropped and unprocessed scans of all gels used in the figures? These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. This PDF will be published online with the article as a supplementary "Source Data" file. Please let me know if you have any questions about this policy.

If you could send a revised version of the manuscript text that we can upload in place of the current version, that would be great. Also, if you are willing to provide the source data (which I hope you will be!), could you send this to us so that we can upload it? I look forward to receiving this final revision.

Many thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2:

The authors have adequately addressed the various issues raised and I have no further comments.

Referee #3:

The authors are highly commended for their great work revising the manuscript. Altogether, this is an excellent piece of work. The difference between extravasation upon inflammatory conditions and lymph node homing presented in the revised manuscript is exciting.