EMBO reports

Pseudomonas aeruginosa LecB suppresses immune responses by inhibiting transendothelial migration

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Dear Dr. Mueller,

Thank you for the submission of your research manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, the referees have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, I will not detail them here.

EMBO reports emphasizes novel functional findings with strong in vivo relevance over detailed mechanistic insight. Thus, referee points regarding further mechanistic insight need not to be addressed (but of course, if you have already such data, it would be nice to include them); other major concerns (dealing with the main conclusions of the study) and all technical points need to be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The presented study is to investigate the role of a specific microbial lectin-LecB derived from P. aeruginosa on vascular and immune systems. Recognition of P. aeruginosa on glycoconjugates from epithelial and endothelial cells has been established earlier (Kirkeby et al., 2006; Kirkeby et al., 2007). The functional impact of LecB binding on epithelial and endothelial cells as well as immune responses has not been well-studied.

Using human skin explant culture and in vitro Transwell migration assays, the authors have demonstrated inhibitory effect of LecB on emigration of various immune cells from skin and in transwell migration. In parallel, prominent reduction and redistribution of adhesions junction protein VE-cadherin were observed in LecB treated endothelial cells.

However, the authors did not address the underlying mechanism, how such changes of EV-cadherin contribute to enhanced barrier function of endothelial cells? Reduced EV-cadherin and peri-nuclear localization are not expected when the endothelial barrier function is enhanced. Literature supports are needed for the transcellular tension hypothesis proposed by authors in discussion.

Even though the LecB binding to various leukocytes appears to be much lower than endothelial cells, such difference does not preclude the potential direct negative impact of LecB on leukocyte migration. Especially, LecB is shown to bind to beta1 integrin (Thuenauer et al., 2020), which is also critical for leukocyte migration. The migratory impairment observed in vivo and in vitro are likely to be a result of combined effects from dysfunctional endothelial cells and compromised migration of leukocytes.

DC emigration from skin upon LecB treatment was reduced in Fig 1, why the number of DCs in lymphatics of lymph nodes is so high in Fig. 4c? The authors speculate low dose and frequency of LecB injection in vivo may permit the migration of DCs from skin to lymph nodes contrasting continues exposure of skin explant to LecB.

It is also possible that high number of BMDCs were injected intradermally, which may promote the collective trans-endothelial migration. Skin painting experiment (such as FITC painting) to investigate the migration of endogenous dendritic cells from skin to lymph nodes in the presence or absence of LecB is recommended. The initial blockage of trans-endothelial migration through lymphatics caused by LecB treatment may be revealed in such experimental setting. BMDC model, which is necessary for DC OVA loading and OTII responses, can be used to support the second blockage at subcapsular lymphatics and impaired T cell activation.

Fig 2C is showing the cell numbers that are remained in the upper chamber. However, there are already more lymphocytes transmigrate to lower chamber upon LecB treatment, why there are still more lymphocytes remained in upper chamber? Are the input cell numbers different between cell types?

"Yet, in LecB-treated cells FAK was predominantly distributed on the cell surface. In light of the intercellular presence of VE-cadherin, suggestive of protein degradation" Not sure, how the location of FAK can suggest VE-cadherin degradation, if it's bases on previous publication, references need to be cited. Also from the image, FAK is rather concentrated on the intracellular junction in 2D culture, not overall cell surface. Similarly the distribution of VE-cadherin before LecB treatment is on the intercellular junction.

Details of human skin explant experiment are not provided, including protocol and source of samples and related IRB.

Overall, this manuscript is well written, straightforward, and interesting with some novelties. However, the presented data are mostly of descriptive nature lacking mechanistic explanation for observed changes. Such as how LecB treatment affects VE-cadherin distribution and how such changes inhibit trans-endothelial migration of leukocytes. The reviewer is not certain, if mechanistic insights are required for publication in EMBO reports.

Reference:
Kirkeby, S., Hansen, A.K., d'Apice, A., and Moe, D. (2006). The galactophilic lectin (PA-IL, gene LecA) from Pseudomonas aeruginosa. Its binding requirements and the localization of lectin receptors in various mouse tissues. Microb Pathog 40, 191-197. 10.1016/j.micpath.2006.01.006.

Kirkeby, S., Wimmerová, M., Moe, D., and Hansen, A.K. (2007). The mink as an animal model for Pseudomonas aeruginosa adhesion: binding of the bacterial lectins (PA-IL and PA-IIL) to neoglycoproteins and to sections of pancreas and lung tissues from healthy mink. Microbes Infect 9, 566-573. 10.1016/j.micinf.2007.01.025.

Thuenauer, R., Landi, A., Trefzer, A., Altmann, S., Wehrum, S., Eierhoff, T., Diedrich, B., Dengjel, J., Nyström, A., Imberty, A., and Römer, W. (2020). The Pseudomonas aeruginosa Lectin LecB Causes Integrin Internalization and Inhibits Epithelial Wound Healing. mBio 11. 10.1128/mBio.03260-19.

Referee #2:

The authors have examined effects of Lec-B, a virulence factor of bacterium, P. aeruginosa, on leukocyte transmigration and T-cell activation. They have demonstrated that Lec-B inhibited emigration of dendritic cells (DCs) from human skin explants. However, Lec-B did not significantly affect T-cell emigration from skin explants, suggesting that Lec-B affects transmigration through lymphatic vessels. Lec-B inhibited PBMC and monocyte migration in in vitro transepithelial migration assays. In the same assay, Lec-B also inhibited migration of T-cells to a lesser extent. Using adoptive transfer of OT-II T-cells in mice, the authors showed that Lec-B reduced T-cell activation initiated by OVA-immunized DCs. The authors also found that Lec-B caused disappearance of cadherin in the endothelial cell junctions (perhaps via endocytosis), and reduced stress fiber assembly. The authors proposed that these changes in the cell junctions of endothelial cells may impair diapedesis, resulting in the inhibition of DC migration into lymph nodes and adaptive immunity. The experiments are well done. The work is significant because the effects of Lec-B on immunity has not been fully understood. I have the following concerns:

1. Lec-B has been shown to cause integrin internalization and inhibit epithelial wound healing (Thuenaur et al., doi: 10.1128/mBio.03260-19). Because Lec-B shows binding to DCs (Appendix S3), inhibition of integrin signaling by Lec-B may directly alter migration of DCs . Transwell migration assays without epithelial cell barrier may clarify this possibility.
2. Fig. 1 is confusing: Fig.1B shows that Lec-B insignificantly affects migration of T-cells, yet Fig 1C shows very low p values of *** with T-cells. Also why are p values between Fig. 1B and 1C so different? I assumed that they are mislabeled. Also Fig. 1C has linked lines, indicating that they are from the same human skin. Linked lines should also be shown in Fig. 1B, which will help us to see the difference in significance.
3. Degradation of VE-cadherin shown in the appendix Fig S1 should be moved to Fig. 2.
4. Fig. 2E. FAK/DAPI double staining must be triple staining with F-actin.
5. Fig. 2E. Actin staining is not consistent with the videos shown in the appendix. The videos show much less actin staining in cell junctions.
6. Page 10, line 4. The description of "with little actin polymerization activity" is an overstatement. I believe "Low levels of polymerized actin" is more appropriate for this situation.
7. The loss of stress fibers by Lec-B may suggest reduced actomyosin contractility. Simple staining or Western blots may reveal whether Lec-B alters phosphorylation of myosin regulatory light chain. If the contractility is inhibited by Lec-B, it explains the inhibition of transmigration by Lec-B.

Referee #3:

The authors of a manuscript, "Pseudomonas aeruginosa LecB suppresses the immune response by inhibiting trans-endothelial migration" present evidence to suggest that a bacterial lectin (LecB) binds to and deranges the endothelial cell cytoskeleton, compromising the ability of the endothelium to support migration of antigen presenting cells (APC) across the barrier. This impairment in facilitating trans-endothelial migration is suggested to limit the emigration of antigen presenting cells into the central paracortical T cell zone of the lymph nodes and thereby reduce T cell expansion through reduced ability to engage APCs. The study employs human in vitro and mouse in vivo models to examine their hypothesis and the study includes convincing data with robust statistical analysis for many of the results presented. Major concerns raised include: (1) the omission of important controls (including for LecB and the various inhibitory approaches) and (2) failure to address or discuss the impact of LecB during an actual infectious process. These issues are highlighted below along with other points needing further clarification.

1. In the majority of experiments, the author chose to control for the exposure to LecB with no treatment at all. This decision raises several questions that remain unaddressed yet are critical toward understanding the potential relevance of their observations involving LecB. Would other lectins cause similar results? In this regard, might a different lectin with an alternative specificity represent an important control? Are authors purporting that their observations represent a broad response to many

types of lectins or are unique to lecB (and perhaps a narrow family of additional bacterial lectins)? Is there anything known about the lectin binding domain of LecB and if this were mutated would the effects described be lost? What are receptors for LecB and would antagonizing or deleting these on endothelial cells negate the impact that LecB has on endothelial changes and transmigration of leukocytes?
2. In attempts to interfere with LecB, the approaches are variable and nonoverlapping. In Figure 1, fucose is used to block effects of LecB in certain cases (1C) whereas inhibitor DH445 is used in others (1E). Is there a reason for the selectivity in approach to inhibition depending on the assay or would the hypothesis be that both treatments would be inhibitory in each situation? Further there are no control for these inhibitory treatments such as an alternative sugar or structurally related molecule that does not target LecB. For immunofluorescent experiments, did the authors consider using excess unlabeled LecB as an additional specificity control?
3. The method for verifying endothelial barrier integrity through establishment of a tight monolayer of cells prior to initiating assays was not made clear.
4. During the time frame of LecB exposure to endothelial cells, the authors did not provide any cell viability data. Is it possible that the inflicted changes to the cytoskeleton are not direct, but secondary to LecB induced toxicity?
5. The image data is of high quality but lacks quantitative analysis in certain key figures (S1A, 2D-E, S2).
6. The authors do not address, either experimentally or through their discussion, how LecB would access endothelial cells during an infection. The authors do not discuss or consider whether the effect of LecB on endothelial is observed during an infectious process, as their experiment focus on delivery of a purified protein. Is LecB universally expressed by P. aeruginosa and how is it delivered by pathogen to host in the context of a disease process? Without additional evidence it remains possible that LecB effects on endothelial cells and subsequent impairment of leukocyte trafficking may not represent a dominant or even significant pathogenic feature during infection. As the authors point out in the Introduction, LecB is also involved in bacterial adherence, biofilm formation, and has mitogenic potential. Alternatively, LecB-endothelial interaction may only be relevant in certain types of P. aeruginosa infections. These important considerations were not adequately discussed.
7. The source of human material (PBMCs and skin) as well as accompanying assurances associated with working with this material was not made clear in the methods.
8. The authors describe Figures 4D-F in the text, where they confusingly refer to as Figures 3D-F.

Pseudomonas aeruginosa LecB suppresses the immune response by inhibiting transendothelial migration

Sponsel et al.

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Response to the reviewers' comments

Reviewer 1.

We thank Reviewer 1 for the critical reading of the manuscript and the questions raised. We have replied to all comments to improve the manuscript that we hope now satisfies the Reviewer making it acceptable for publication.

R1. However, the authors did not address the underlying mechanism, how such changes of EV-cadherin contribute to enhanced barrier function of endothelial cells? Reduced EV-cadherin and peri-nuclear localization are not expected when the endothelial barrier function is enhanced. Literature supports are needed for the transcellular tension hypothesis proposed by authors in discussion.

We acknowledge that our observations are unexpected in the context of increased endothelial barrier function and cite its literature in the discussion. We do not exclude other mechanism and now discuss this further in light of the additional data on the myosin regulatory chain phosphorylation and the integrin expression, asked for by Reviewer 2 and integrin expression (see below). "One possible explanation for diminished leukocyte transmigration is that LecB impairs leukocyte diapedesis by preventing the development of transcellular tension necessary to create the intercellular openings. This model is supported by loss of stress fibres and reduced myosin regulatory chain phosphorylation, which would have a negative impact on cell contractility. However, reduction in integrin cell surface expression by LecB such as CD11c/ITGaX and CD18/ITGb2 of dermal DCs could also participate, given the importance of integrins in leukocyte migration. Further work is required to clarify the contribution of dysfunctional endothelial cells and compromised leukocyte-endothelial interaction in LecB-mediated inhibition of leukocyte migration. "

R1. Even though the LecB binding to various leukocytes appears to be much lower than endothelial cells, such difference does not preclude the potential direct negative impact of LecB on leukocyte migration. Especially, LecB is shown to bind to beta1 integrin (Thuenauer et al., 2020), which is also critical for leukocyte migration. The migratory impairment observed in vivo and in vitro are likely to be a result of combined effects from dysfunctional endothelial cells and compromised migration of leukocytes.

To reply to this question that was also posed by Reviewer 2, we tested for LecB-induced changes in integrins on human skin DC subsets by flow cytometry. We found CD11c (ITGaX) and CD18 (ITGb2) to be reduced on dermal DCs. This data is presented in Fig EV1, described in the text and discussed (see above). Results section: "In light of the role of integrins (ITGs) in leukocyte migration and the loss of

ITGb1 from epithelial cells by LecB (Thuenauer *et al.*, 2020), we assessed the cell surface expression of CD18/ITGb2, CD11c/ITGaX and CD11b/ITGaM. There was a reduction in the CD18/ITGb2-CD11c/ITGb2 heterodimer on dermal DCs but not Langerhans cells (**Fig EV1**)."

R1. DC emigration from skin upon LecB treatment was reduced in Fig 1, why the number of DCs in lymphatics of lymph nodes is so high in Fig. 4c? The authors speculate low dose and frequency of LecB injection in vivo may permit the migration of DCs from skin to lymph nodes contrasting continues exposure of skin explant to LecB. It is also possible that high number of BMDCs were injected intradermally, which may promote the collective trans-endothelial migration. Skin painting experiment (such as FITC painting) to investigate the migration of endogenous dendritic cells from skin to lymph nodes in the presence or absence of LecB is recommended. The initial blockage of trans-endothelial migration through lymphatics caused by LecB treatment may be revealed in such experimental setting. BMDC model, which is necessary for DC OVA loading and OTII responses, can be used to support the second blockage at subcapsular lymphatics and impaired T cell activation.

We acknowledge the explanations for the presence of BMDCs in the lymphatics and may also suggest that the syringe pressure with which the cells are injected enhances lymphatic drainage and thus may contribute to their accumulation in the lymph node subcapsular space.

We have therefore rephrased our discussion: "By providing two doses of LecB, we tried to optimize the experimental conditions to anticipate rapid and late DC arrival. […] Alternatively, the injection of high BMDC numbers may have contributed to overcome LecB-blockage of trans-endothelial migration."

As suggested, we have explored the impact of LecB on endogenous DCs using imiquimod-containing Aldara cream to induce inflammation of the skin. The data show that LecB prevents the inflammation-dependent rise in endogenous, skin-derived dermal DCs and Langerhans cells. In addition, we have targeted the skin DC with anti-DEC-205 coupled to a fluorochrome and see a reduction of the DEC-205 subsets among the migratory DCs. As suggested, the data is presented in main Fig 4 as first panel and in Figure EV6. "First, we determined whether DCs were inhibited by LecB in their migration to LNs by exposing mouse ear skin to the TLR-7 agonist imiquimod (Aldara cream) in the absence or presence of two intradermal injections of LecB. In spite of a strong increase in LN cellularity owing to the inflammatory stimulus, LecB prevented the entry of the migratory skin DC subsets and Langerhans cells into the draining LN (**Fig 4A**). Similar observations were made when targeting the DEC-205^{+} skin DCs (**Fig EV6**)." We also refer to this data in the discussion: "In mice, our data showed that endogenous and injected DCs were restrained in entering the LN, which consequently resulted in a lower T cell response."

R1. Fig 2C is showing the cell numbers that are remained in the upper chamber. However, there are already more lymphocytes transmigrate to lower chamber upon LecB treatment, why there are still more lymphocytes remained in upper chamber? Are the input cell numbers different between cell types?

There is likely some variations in numbers between the different cell types. We have noted this in the description of the results: "The differences in numbers between the cell types in the control or the LecB conditions are likely due to unequal cell number input."

R1 "Yet, in LecB-treated cells FAK was predominantly distributed on the cell surface. In light of the intercellular presence of VE-cadherin, suggestive of protein degradation" Not sure, how the location of FAK can suggest VE-cadherin degradation, if it's bases on previous publication, references need to be cited. Also from the image, FAK is rather concentrated on the intracellular junction in 2D culture,

not overall cell surface. Similarly the distribution of VE-cadherin before LecB treatment is on the intercellular junction.

We rephrased this to avoid confusion and, in addition, have extended the imaging studies and quantified the subcellular locations of VE-cadherin, FAK and F-actin with respect to intracellular versus perimembranous positions. We find differences between control and LecB-treated cells with a redistribution between the two conditions. The text now reads: "FAK, which plays a central role in initiating and integrating various signaling pathways with effect on cytoskeleton (Quadri, 2012), is frequently displaced from a predominantly perimembranous location to an intracellular position after LecB."

R1. Details of human skin explant experiment are not provided, including protocol and source of samples and related IRB.

A similar comment was made by Reviewer 3 and we have added this information to the Materials and Methods section (heading: Human skin).

R1. Overall, this manuscript is well written, straightforward, and interesting with some novelties. However, the presented data are mostly of descriptive nature lacking mechanistic explanation for observed changes. Such as how LecB treatment affects VE-cadherin distribution and how such changes inhibit trans-endothelial migration of leukocytes. The reviewer is not certain, if mechanistic insights are required for publication in EMBO reports.

We thank Reviewer 1 for the comments and hope to have replied in a satisfactory manner.

Reviewer 2.

We thank Reviewer 2 for the critical reading of the manuscript and the questions raised. We have replied to all comments to improve the manuscript that we hope now satisfies the Reviewer making it acceptable for publication.

R2. Lec-B has been shown to cause integrin internalization and inhibit epithelial wound healing (Thuenaur et al., doi: 10.1128/mBio.03260-19). Because Lec-B shows binding to DCs (Appendix S3), inhibition of integrin signaling by Lec-B may directly alter migration of DCs . Transwell migration assays without epithelial cell barrier may clarify this possibility.

We acknowledge this possible effect of LecB on the DCs that may contribute to the observed inhibition of cell migration. This comment was also made by Reviewer 1. We have assessed the implication of integrin in LecB-mediated inhibition of DC migration by measuring CD11c (ITGaX) or (ITGaM) - CD18 (ITGb2) expression by flow cytometry on the different types of human skin DCs. We find a reduction of CD11c and CD18 on dermal DCs. This data is presented in Fig EV1C, described and commented in the text. Results section: "In light of the role of integrins (ITGs) in leukocyte migration and the loss of ITGb1 from epithelial cells by LecB (Thuenauer *et al.*, 2020), we assessed the cell surface expression of CD18/ITGb2, CD11c/ITGaX and CD11b/ITGaM. There was a reduction in the CD18/ITGb2-CD11c/ITGb2 heterodimer on dermal DCs but not Langerhans cells (**Fig EV1**)." Discussion: "However, reduction in integrin cell surface expression by LecB such as CD11c/ITGaX and CD18/ITGb2 of dermal DCs could also participate, given the importance of integrins in leukocyte migration. Further work is required to clarify the contribution of dysfunctional endothelial cells and compromised leukocyte-endothelial interaction in LecB-mediated inhibition of leukocyte migration."

R2. Fig. 1 is confusing: Fig.1B shows that Lec-B insignificantly affects migration of T-cells, yet Fig 1C shows very low p values of *** with T-cells. Also why are p values between Fig. 1B and 1C so

different? I assumed that they are mislabeled. Also Fig. 1C has linked lines, indicating that they are from the same human skin. Linked lines should also be shown in Fig. 1B, which will help us to see the difference in significance.

There has been a mistake when formatting the graph for which we apologize. As suggested also by Reviewer 3, Figure 1B has been modified to show the different consequences of LecB and LecA treatments, and Figure 1C now focuses on the effect of the synthetic, high-affinity LecB inhibitor DH445, rather than L-fucose. For clarity, statistical tests in the latter graph compares migration index, normalized to untreated conditions, for LecB versus LecB + DH445. "While T cells were not significantly affected by either lectins, there was a significant reduction in dermal $CD14^+$ and $CD14^-$ DCs as well as in epidermal Langerhans cells with LecB but not LecA (**Fig 1B**). Adding the LecB glycomimetic inhibitor DH445 (Sommer *et al.*, 2018) restored cell migration (**Fig 1C**)."

R2. Degradation of VE-cadherin shown in the appendix Fig S1 should be moved to Fig. 2.

This data has been moved to main Figure 2 as panel F. "We analyzed VE-cadherin protein levels by Western blot and found a time-dependent decrease (**Fig 2F**)."

R2. Fig. 2E. FAK/DAPI double staining must be triple staining with F-actin

We have provided additional images where we show double staining of FAK and F-actin and also verified by Caspase-3 staining that cells were not undergoing death, as asked for by Reviewer 3 (Fig EV3A-C). In addition, as asked for by Reviewer 3, we have quantified the perimembranous versus intracellular position of VE-cadherin, F-actin and FAK (Appendix Fig S2B). The new data is described in the Results section. "The alterations in protein expression and localizations were not due to LecB toxicity as assessed by cell viability by MTT and caspase-3 staining (**Fig EV3A-C**)."

R2. Fig. 2E. Actin staining is not consistent with the videos shown in the appendix. The videos show much less actin staining in cell junctions. Page 10, line 4. The description of "with little actin polymerization activity" is an overstatement. I believe "Low levels of polymerized actin" is more appropriate for this situation.

We aimed to compare the mobility and actin polymerization of the cells under the two conditions. It is possible that a difference between the videos and the fixed images is related to cell densities. As suggested, we have changed the wording. "While untreated cells exhibited actin dynamics concomitant with cell motility, the 3 h LecB exposure left the cells sessile with low levels of polymerized actin (**videos EV1,2**)."

R2. The loss of stress fibers by Lec-B may suggest reduced actomyosin contractility. Simple staining or Western blots may reveal whether Lec-B alters phosphorylation of myosin regulatory light chain. If the contractility is inhibited by Lec-B, it explains the inhibition of transmigration by Lec-B.

Indeed, because non-muscle myosin is stabilized by phosphorylation of its regulatory light chain we performed western and immunofluorescence analysis of p-myosin light $chain2^{Ser19}$ on untreated and LecB-treated cells. The western blot showed a reduction in p-myosin light $chain2^{Ser19}$. Further, we stained cells for p-myosin light $chain2^{Ser19}$ by immunofluorescence and quantified the signal in the perimembranous area versus the intercellular space. We saw a reduction for the perimembranous localization and a difference between perimembranous area versus the intercellular space for the 3h LecB time point. These findings are presented in additional panels of Fig EV3 and are described in the manuscript. "To further explore reduced cell contractility, we determined phosphorylation of the myosin regulatory light chain 2 that stabilizes myosin (Vicente-Manzanares *et al*, 2009). Western blot showed diminished myosin light chain2 phosphorylation at Ser19, and quantification of

immunofluorescence revealed a loss in the perimembranous area after 3h of LecB exposure (**Fig EV3D**). In conclusion, LecB reorganizes the VE-cadherin$^+$ adherens junction and the associated FAK and F-actin cytoskeleton with reduced myosin regulatory light chain phosphorylation, which would have a negative impact on cell contractility and therefore on leukocyte diapedesis."

We thank Reviewer 2 for the comments and hope to have replied in a satisfactory manner.

Reviewer 3.

We thank the Reviewer 3 for the critical reading of the manuscript and the questions raised. We have replied to all comments to improve the manuscript that we hope now satisfies the Reviewer making it acceptable for publication.

R3. 1. In the majority of experiments, the author chose to control for the exposure to LecB with no treatment at all. This decision raises several questions that remain unaddressed yet are critical toward understanding the potential relevance of their observations involving LecB. Would other lectins cause similar results? In this regard, might a different lectin with an alternative specificity represent an important control? Are authors purporting that their observations represent a broad response to many types of lectins or are unique to lecB (and perhaps a narrow family of additional bacterial lectins)? Is there anything known about the lectin binding domain of LecB and if this were mutated would the effects described be lost? What are receptors for LecB and would antagonizing or deleting these on endothelial cells negate the impact that LecB has on endothelial changes and transmigration of leukocytes?

We appreciate these questions and have therefore repeated the key experiment of the inhibition of human dendritic cell migration from human skin with LecA, the other *P. aeruginosa* lectin. The data shows no significant reduction in dendritic cell emigration from human skin with LecA. This finding is presented together with LecB in a modified graph shown as Figure 1B. It is described: "While T cells were not significantly affected by either lectins, there was a significant reduction in dermal CD14$^+$ and CD14$^-$ DCs as well as in epidermal Langerhans cells with LecB but not LecA (**Fig 1B**). " We also make this point in the discussion: "However, we found that LecA did not inhibit human DC skin emigration, indicating that obstructing leukocyte migration is not a general property of bacterial lectins."

R3. 2. In attempts to interfere with LecB, the approaches are variable and nonoverlapping. In Figure 1, fucose is used to block effects of LecB in certain cases (1C) whereas inhibitor DH445 is used in others (1E). Is there a reason for the selectivity in approach to inhibition depending on the assay or would the hypothesis be that both treatments would be inhibitory in each situation? Further there are no control for these inhibitory treatments such as an alternative sugar or structurally related molecule that does not target LecB. For immunofluorescent experiments, did the authors consider using excess unlabeled LecB as an additional specificity control?

The reason for using fucose in Figure 1C was the late availability of the synthetic, high-affinity inhibitor DH445. We have therefore repeated this experiment with DH455, which now replaces L-fucose. As noted by Reviewer 2, we have clarified the presentation of the data by focusing on the comparison between LecB versus LecB + DH445.

R3. 3. The method for verifying endothelial barrier integrity through establishment of a tight monolayer of cells prior to initiating assays was not made clear.

We have used transendothelial electrical resistance (TEER) measures to assess the cell monolayer tightness. As this information was not given in the text, we have provided it in the material and

methods section. "The tightness of the cell monolayer was verified by transendothelial electrical resistance."

R3. 4. During the time frame of LecB exposure to endothelial cells, the authors did not provide any cell viability data. Is it possible that the inflicted changes to the cytoskeleton are not direct, but secondary to LecB induced toxicity?

To reply to this question, we have tested for cell toxicity of LecB using MTT assay together with positive controls Staurosporine and serum-free medium culture. While, as expected, we found reduced cell viability in both control conditions, there was no significant impact of LecB after 1h or 3h. In addition, we detected cell apoptosis by caspase-3 immunofluorescence by staurosporine but not in the presence of LecB. We repeated immunofluorescence staining of VE-cadherin, F-actin and FAK with caspase-3 detection to assure that the cells imaged were not caspase-3 positive. These findings are presented in Figure EV2, panels B, C and D and described in the text. "The alterations in protein expression and localizations were not due to LecB toxicity as assessed by cell viability by MTT and caspase-3 staining (**Fig EV3A-C**)."

R3. 5. The image data is of high quality but lacks quantitative analysis in certain key figures (S1A, 2D-E, S2).

We have quantified the expression of VE-cadherin, F-actin and FAK with respect to a perimembranous or intracellular localization of key Figure 2E and present this data in Fig EV2B. We saw significant changes in protein expression and localization when cells were exposed to LecB. We have referred to the quantification of the Figure 2E images: "HUVECs were then left untreated or exposed to LecB for 1 h or 3 h, stained for VE-cadherin, filamentous (F)-actin, focal adhesion kinase (FAK), nuclei were colored with DAPI (**Fig 2E**) and images quantified for subcellular localization (**Fig EV2B**)." As for Figure EV2 (now Figure EV4), these images are complementary to the analysis of high endothelial venules by flow cytometry presented in Figure 3C that are quantified in Figure 3D.

R3. 6. The authors do not address, either experimentally or through their discussion, how LecB would access endothelial cells during an infection. The authors do not discuss or consider whether the effect of LecB on endothelial is observed during an infectious process, as their experiment focus on delivery of a purified protein. Is LecB universally expressed by P. aeruginosa and how is it delivered by pathogen to host in the context of a disease process? Without additional evidence it remains possible that LecB effects on endothelial cells and subsequent impairment of leukocyte trafficking may not represent a dominant or even significant pathogenic feature during infection. As the authors point out in the Introduction, LecB is also involved in bacterial adherence, biofilm formation, and has mitogenic potential. Alternatively, LecB-endothelial interaction may only be relevant in certain types of P. aeruginosa infections. These important considerations were not adequately discussed.

We appreciate these remarks and have therefore integrated them into our discussion: "An important question is the dynamics of LecB production during an infection and whether it remains associated with the bacterium. Of consideration is also the role of LecB in biofilm formation that may -in the light of our results- protect the bacteria from immunological surveillance and attack. "Our study employs purified LecB and calls for further studies using live *P. aeruginosa* expressing or lacking LecB."

R3. 7. The source of human material (PBMCs and skin) as well as accompanying assurances associated with working with this material was not made clear in the methods.

A similar comment was made by Reviewer 1 and we have added this information to the Materials and Methods section (headings: Human skin and Transmigration).

R3. 8. The authors describe Figures 4D-F in the text, where they confusingly refer to as Figures 3D-F.

We apologize for this error. We have carefully verified the correct numbering in the revised manuscript.

We thank Reviewer 3 for the comments and hope to have replied in a satisfactory manner.

Dear Dr. Mueller,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referee #1 has remaining concerns and suggestions to improve the manuscript, I ask you to address in a final revise manuscript. Please also provide a final p-b-p-response addressing the remaining points.

Moreover, I have these editorial requests I also ask you to address:

- I would suggest a slightly modified title:
Pseudomonas aeruginosa LecB suppresses immune responses by inhibiting transendothelial migration

- We plan to publish your manuscript in the Report format (as also indicated by you in the submission system). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

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See also the guidelines for figure legend preparation:
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- Please order the manuscript sections like this (using these names as headings):
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- Please add scale bars of similar style and thickness to the microscopic images (main and EV), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, many scale bars are rather thin and hard to see.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.

- Please make sure that all figure panels are called out separately and sequentially (main and EV figures). Presently, callouts for panels EV5A-C and EV6A&B are missing. Please check.

- Please name the movie files ‘Movie EVx’ and add callouts for these using these names to the manuscript text. Please upload the movie files ZIPed together with a text file containing a title and a legend. Finally, please remove the movie legends from the manuscript text file.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text and comments. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

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- two to four short bullet points highlighting the key findings of your study (two lines each).

- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The additional data have further strengthened this manuscript. Especially, the imiquimod treatment induced leukocyte migration is likely to reflect the true migratory defects caused by LecB treatment, which agrees with the data obtained from human skin explant culture. In contrast the data obtained from footpad injected BMDC, did not show the initial migratory defects of skin DCs, likely due to large number of injected DCs and collective migration. Similar phenotypic masking was also observed in talin1-deficient BMDCs1,2. The authors may want to discuss the discrepancy in their data by citing these earlier publications. The BMDC data shown in this manuscript however also demonstrate additional blockade in entering T cell zone once they arrive LNs. These are actually elegant results obtained by using these complementary experimental methods.
Fig 1C: As the data are showing the increase of emigrating cells upon inhibitor treatment, it is OK to normalize to untreated sample. However, it would be clearer to show the raw data as in 1B first before summarizing changes in the format as presented in 1C.

The Fig EV1 data showed that integrin expressions on dermal DCs are reduced by LecB treatment, but not Langerhans cells. However, the emigration of both dermal DCs and Langerhans cells are impaired by LecB treatment. Any explanations?
It's better to present data in Fig 2C in ratio as Fig 2B. It is also more consistent in figure format. In such case, there is no need for this extra statement. "The differences in numbers between the cell types in the control or the LecB conditions are likely due to unequal cell number input."
One should not speculate if the amount of input cells is different, they are either the same or different. The numbers of cells seeded for each cell types should also be provided in the methods.

Why do the lymphocytes show a significant reduction in migration across the monolayer of blood endothelial cells in Fig 2B, whereas Fig 1B showed no difference in migrating out of skin explant? Please provide explanation here. Is it due to purify of lymphocytes used in trans-well migration assays, as they were defined as CD14- cells from PBMC in the methods? What are the markers used to define lymphocytes in Fig 1B.

The authors stated that both VE-cadherin and LecB are found in peri-nuclear vesicles, however the author did not co-staining of these two molecules. The question is, are they co-localized and is LecB interacting with VE-cadherin directly?

Other minor comments:
It is clearer to say "by LecB treatment" than just say "by LecB" through out of this manuscript.
Fig 1, the p value for *** should be stated in the legends.
In Fig 1A, FS-A should be FSC-A.
Fig 2, typo "Visualizing F-actin with fluorescent phalloidin uncovered that LecB provoked the reduction in of cellular stress fibres and the formation of a cortical actin rims."
Fig EV2B, the method for identifying the perimembranous area should be described.

References:
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2. Lim, T.J.F., Bunjamin, M., Ruedl, C. & Su, I.H. Talin1 controls dendritic cell activation by regulating TLR complex assembly and signaling. J Exp Med 217(2020).

While the phenotypical description of this manuscript is convincing and clear. LecB induced endothelial barrier enhancement may be contributed by reduced myosin regulatory chain phosphorylation and reduced cell contractility, however some basic issues remain unanswered, such as if LecB binds to EV-cadherin directly, if they colocalized in peri-nuclear vesicles (see comments above). It would make sense to make this aspect the central focus of this manuscript and provide further supporting data.

The additional factors contributing to defective diapedesis of leukocytes potentially via direct effect of LecB on leukocytes are described, which may not be complete, but sufficient to support the observation.
The reviewer appreciates and is satisfied with the additional data provided in this revised manuscript. This manuscript is a step closer to be acceptable for publication in EMBO reports.

Referee #2:

The reviewers responded well to my concerns.

Referee #3:

The authors have satisfactorily addressed the previous critiques.

Pseudomonas aeruginosa LecB suppresses the immune response by inhibiting transendothelial migration

Sponsel et al.

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Emails: c.mueller@ibmc-cnrs.unistra.fr

winfried.roemer@bioss.uni-freiburg.de

Response to the reviewer's comments

Reviewer

We thank the Reviewer for the critical reading of the manuscript and the questions raised. We have replied to all comments to improve the manuscript that we hope now satisfies the Reviewer.

Reviewer: The additional data have further strengthened this manuscript. Especially, the imiquimod treatment induced leukocyte migration is likely to reflect the true migratory defects caused by LecB treatment, which agrees with the data obtained from human skin explant culture. In contrast the data obtained from footpad injected BMDC, did not show the initial migratory defects of skin DCs, likely due to large number of injected DCs and collective migration. Similar phenotypic masking was also observed in talin1-deficient BMDCs1,2. The authors may want to discuss the discrepancy in their data by citing these earlier publications. The BMDC data shown in this manuscript however also demonstrate additional blockade in entering T cell zone once they arrive LNs. These are actually elegant results obtained by using these complementary experimental methods.

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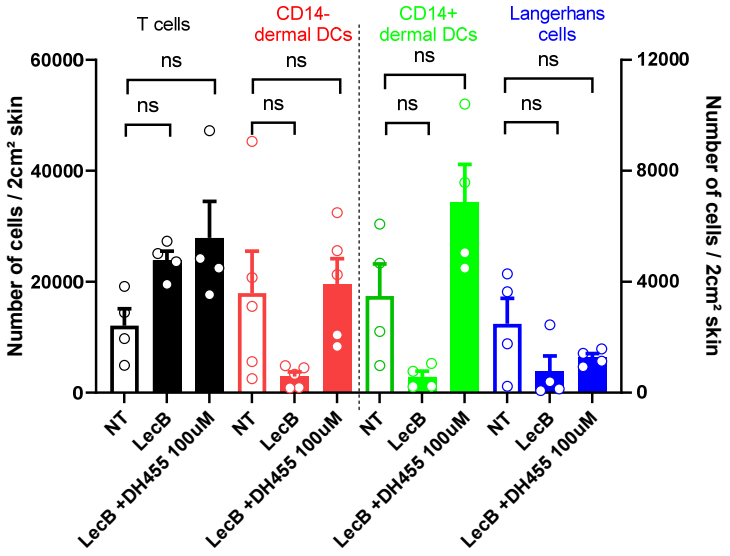
2. Lim, T.J.F., Bunjamin, M., Ruedl, C. & Su, I.H. Talin1 controls dendritic cell activation by regulating TLR complex assembly and signaling. J Exp Med 217(2020).

We thank the Reviewer for the reminder and we have re-read the publications. Lämmermann *et al.* showed that the absence of integrins on DCs did not affect migration of BMDC into the T cell area of LNs. Lim *et al.* showed that Talin-deficient mouse skin LCs or dDCs were affected in their migration to LNs under steady-state or LPS-activated conditions. The study did not provide detailed distribution of DCs within the LNs and focused on the role of Talin in cell activation for which, among other cells, BMDCs were used. While the questioning and experimental strategies of Lim *et al.* are different to ours and may confuse the reader, we have discussed the paper by Lämmermann *et al.* by adding to the discussion "…CD11c/ITGaX and CD18/ITGb2 may affect migration, although BMDC homing to LNs was not found to be dependent on integrins (Lämmermann *et al*, 2008). "

Reviewer: Fig 1C: As the data are showing the increase of emigrating cells upon inhibitor treatment, it is OK to normalize to untreated sample. However, it would be clearer to show the raw data as in 1B first before summarizing changes in the format as presented in 1C.

As the reviewer can appreciate from the Figure below provided as raw data, there was a considerable inter-donor variability in the total number of cells emigrating from the skin explants. On the other hand, normalizing to untreated conditions shed light on the consistent effect of DH445 on relieving LecB-driven inhibition, with the exception of LCs.
We therefore prefer to show the Figure of normalized data.



Reviewer: The Fig EV1 data showed that integrin expressions on dermal DCs are reduced by LecB treatment, but not Langerhans cells. However, the emigration of both dermal DCs and Langerhans cells are impaired by LecB treatment. Any explanations?

While integrins may participate in cells crossing an endothelial barrier, our data strongly supports a role of LecB on modifying endothelial cells in a way that antagonizes diapedesis. By studying HUVECs in more detail, we found that LecB triggered the endocytic degradation of VE-cadherin, changes in FAK subcellular location and the formation of a cortical F-actin rim.

Reviewer: It's better to present data in Fig 2C in ratio as Fig 2B. It is also more consistent in figure format. In such case, there is no need for this extra statement. "The differences in numbers between the cell types in the control or the LecB conditions are likely due to unequal cell number input."

One should not speculate if the amount of input cells is different, they are either the same or different. The numbers of cells seeded for each cell types should also be provided in the methods.

After re-verification with the person in charge of these experiments, the number of input cells is equal for all cell types. Indeed, graphs 2B and 2C cannot be directly compared because the data is acquired in different ways: 2B is obtained from bulk analysis of the bottom chamber using a luminescent cell viability assay (and is normalized to NT), whereas Fig 2C is derived from microscopic imaging of the endothelial monolayer and shows raw, not normalized, values. The sum of 2B and 2C cannot be equal to the same number in all conditions, because the cells that have not adhered are unaccounted for. From the data one could hypothesize that lymphocytes in average adhere more to endothelia than monocytes or PBMCs, however, this remains speculative and specific experiment should be done to assess this.

For these reasons and in accord with the Reviewer's suggestion, we have omitted the statement: "The differences in numbers between the cell types in the control or the LecB conditions are likely

due to unequal cell number input" but have added to the Material and Methods sections that the input of all cells was 250 000: "…250 000 cells were added into the top chamber…".

Reviewer: Why do the lymphocytes show a significant reduction in migration across the monolayer of blood endothelial cells in Fig 2B, whereas Fig 1B showed no difference in migrating out of skin explant? Please provide explanation here. Is it due to purify of lymphocytes used in trans-well migration assays, as they were defined as CD14- cells from PBMC in the methods? What are the markers used to define lymphocytes in Fig 1B.

The review correctly cautions the absence of positive markers to define the lymphocyte populations better. For PBMC the CD14- populations comprise different types of lymphocyte whereas in the skin the HLA-DR cells it is mostly tissue-resident memory T cells. We have therefore modified the respective text with "… were identified as HLA-DR- lymphocytes…", and "…$CD14^-$ lymphocyte transmigration…".

Reviewer: The authors stated that both VE-cadherin and LecB are found in peri-nuclear vesicles, however the author did not co-staining of these two molecules. The question is, are they co-localized and is LecB interacting with VE-cadherin directly?

We thank the Reviewer for this interesting question that should be addressed in future studies to explore in more detail the molecular impact and mechanisms of LecB treatment on endothelial cells with respect to biochemical and cell-biological changes.

Other minor comments:

Reviewer: It is clearer to say "by LecB treatment" than just say "by LecB" through out of this manuscript.

We have added "treatment" to some sentences throughout the manuscript.

Reviewer: Fig 1, the p value for *** should be stated in the legends.

This has been corrected.

Reviewer: In Fig 1A, FS-A should be FSC-A.

This has been corrected.

Reviewer: Fig 2, typo "Visualizing F-actin with fluorescent phalloidin uncovered that LecB provoked the reduction in of cellular stress fibres and the formation of a cortical actin rims."

This has been corrected to: "Visualizing F-actin with fluorescent phalloidin uncovered that LecB provoked the reduction in of cellular stress fibres and the formation of a cortical actin rim."

Reviewer: Fig EV2B, the method for identifying the perimembranous area should be described.

We have clarified this by noting in the Figure legend: "The pixels of fluorescence of VE-cadherin, F-actin, FAK in these subcellular areas were determined for each cell based on phase contrast images using Fiji (ImageJ software) and normalized for cell surface area."

The method is also described in the section Material and Methods.

Reviewer: While the phenotypical description of this manuscript is convincing and clear. LecB induced endothelial barrier enhancement may be contributed by reduced myosin regulatory chain phosphorylation and reduced cell contractility, however some basic issues remain unanswered, such as if LecB binds to EV-cadherin directly, if they colocalized in peri-nuclear vesicles (see comments above). It would make sense to make this aspect the central focus of this manuscript and provide further supporting data.

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The reviewer appreciates and is satisfied with the additional data provided in this revised manuscript. This manuscript is a step closer to be acceptable for publication in EMBO reports.

We thank the Reviewer for critically reading the manuscript and hope to have answered in a satisfactory manner.

Christopher Mueller
Laboratory CNRS UPR3572 / I²CT - Immunologie, Immunopathologie et Chimie Thérapeutique
France

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The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Matherial and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Results section
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Material and methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	