EMBO reports

Co-inhibition of immunoproteasome subunits LMP2 and LMP7 is required to block autoimmunity

Michael Basler, Michelle M. Lindstrom, Jacob J. LaStant, J. Michael Bradshaw, Timothy D. Owens, Christian Schmidt, Elmer Maurits, Christopher Tsu, Herman S. Overkleeft, Christopher J. Kirk, Claire L. Langrish & Marcus Groettrup

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 June 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received 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, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript. As the reports are below, I will not detail them here. However, the referees state that the major weakness of the manuscript is the lack of a clear demonstration of LMP7/2-selectivity of the compounds employed, in particular of LMP7-selectivity for PRN1126, the lack of positive control experiments, and the indirect nature of the data showing that the therapeutic potential of immunoproteasome inhibitors is dependent on the combined inhibition of LMP2 and LMP7. I think that these points need to be addressed with further experiments.

After cross-commenting with the referees it became clear that the suggested mouse lines with point mutations in the active center of LMP2 and 7 would indeed be very useful to address these major concerns. However, the referees agree that this would be beyond the scope of the present manuscript. Nevertheless, alternative experiments to strengthen the claims of the manuscript would be needed. The referees suggest, in addition to what they state already in their reports, to

- show based on the data in Fig 3b (suggesting that a shift in Mw of single proteasome subunits indicates binding of an inhibitor) that PRN1126 induces a shift of LMP7 only (also the LMP2 inhibitors used need to be included in this experiment).
- address the presumed lack of inhibition of MECL-1 using fluorogenic substrates that are exclusively cleaved by MECL-1 in an experiment including all inhibitors used in this manuscript as well as an appropriate positive control.

Thus, a significant revision is required before publication of your manuscript can be considered, and I would also understand your decision if you chose to rather seek rapid publication elsewhere at this

stage. However, given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that the referee concerns must be addressed in the revised manuscript and 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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Important: All materials and methods should be included in the main manuscript file.

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Please also format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
http://embor.embopress.org/authorguide#statisticalanalysis

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

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.

REFEREE REPORTS

Referee #1:

Basler and coauthors have previously reported on an LMP7-selective inhibitor with anti-inflammatory function (ONX 0914). They here test a novel LMP7-selective inhibitor, PRN1126. Treatment with this inhibitor fails to mimic the effects of ONX 0914 treatment, both in cell culture and in mouse models of inflammatory / autoimmune disease. In a series of experiments the authors then show that ONX 0914 in fact inhibits both LMP7 and LMP2, and that inhibition of both these proteasome subunits is required to mediate the anti-inflammatory effects observed following treatment with ONX 0914.

The experiments are well performed and the need for concomitant inhibition of LMP7 and LMP2 in anti-inflammatory treatments is convincingly shown.

Nevertheless, LMP7-selectivity of PRN1126 is not convincingly demonstrated. In particular, the potential effects of this inhibitor on MECL-1 remain entirely unaddressed.

Referee #2:

In 2009, the authors of the manuscript "Co-inhibiting immunoproteasome subunits LMP2 and LMP7 is required to block autoimmunity" have published in a Nature Medicine publication that the "LMP7-specific" immunoproteasome inhibitor PR-957 can be applied in a therapeutic way in a number of different preclinical models of auto-immune diseases. In this manuscript, Basler et al. now inform us that the proteasome inhibitor PR-957/ONX 0914 used in these studies is actually not "LMP7-specific" but additionally may also inhibit LMP2. Thus, ONX 0914 should be considered to be "just" an "immunoproteasome inhibitor". Furthermore, in this manuscript, the authors introduce another inhibitor PRN-1126 - which the authors proclaim would now really be "LMP7-specific". Most interestingly, in most preclinical models PRN-1126 does not share the ameliorating effects of PR-957 and only in combination with other immunoproteasome inhibitors has a therapeutic effect on auto-immune diseases.

In general, to understand the molecular mechanism underlying the therapeutic effects of immunoproteasome inhibitors is rather important. Also, the experiments as such have been performed reasonably well - although for instance in the EAE model the overall, maximal severity score is rather low, hampering a conclusive interpretation of the data; furthermore lack most experiments positive controls that would document that PR-957 is actually functional in the performed experiments.

Nevertheless, to unequivocally make the point that the therapeutic potential of immunoproteasome inhibitors is dependent on the combined inhibition of LMP2 and LMP7 requires substantially more work. In its current form, all data presented are yet again indirect. Yet again the same assays are used as in the original publication from 2009 and their follow-ups. Most assays in the current manuscript are based on other "subunit-specific" inhibitors or LMP7 gene-deficient mice, which the authors themselves proclaim to affect the incorporation of other immunoproteasome subunits, such as LMP2. Thus, in order to make a convincing point of the specificity of the inhibitors, mouse strains would have to be established with CRISPR/Cas-mediated point mutations in the active center of LMP7 and/or LMP2. These mutations would resemble the treatment of subunit-specific inhibitors, but would not interfere with the formation of immunoproteasome complexes. Only such an approach would sufficiently well address this rather important issue.

Referee #3:

Basler et al. provide an excellent article on the mechanism behind the ability of ONX 0914 to ameliorate the effects of autoimmunity in preclinical models. ONX 0914 is an immunoproteasome

(IP) inhibitor that has been assumed to block autoimmunity through the specific inhibition of LMP7. PRN1126 is a compound synthesized by the authors and they provide evidence that it is an LMP7-specific inhibitor. However, in both in vitro and in vivo models, ONX 0914 and PRN1126 behave differently in their ability to downregulate MHC-I expression, IL-6 secretion, and autoimmunity in the DSS-colitis and EAE mouse models. Next, they provide evidence that while PRN1126 does not inhibit the LMP2 subunit, ONX 0914 does at higher concentrations. LMP2-specific inhibitors alone are unable to lower expression of MHC-I, IL-6 secretion, and block Th17 differentiation, but can in the presence of PR1126, similar to the effects of ONX 0914 alone. Lastly, the combinatory inhibition of both LMP2 and LMP7 slow the manifestations of DSS-colitis and EAE. This paper adds to the literature in that it provides an elucidation of the mechanism behind ONX 0914, however it does not explain how blockade of LMP2/LMP7 slow progression of autoimmunity.

Page 3; Authors write, "To improve pharmacological and toxicological profiles new proteasome inhibitors with immunoproteasome selectivity have been developed." There should be a comma between 'profiles" and "new".

Page 4: Authors write "On the cellular level, these effects were shown to involve two major pathways of disease development, namely cytokine secretion and T helper cell differentiation(Basler et al., 2013)." Place a space between "differentiation" and the reference.

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Page 6 and overall statement (for multiple pieces of in vitro data): did you test cell viability upon exposure of PRN1126? Can you provide this data? Can you also provide data that dual inhibition of both LMP7 (with PRN1126) and LMP2 (with ML604440 or LU-001i) does not affect viability?

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Figure 2a, 2b, & 2c: Are any of these changes statistically significant? I see no p-values or evidence of statistical testing.

Figure 2d: Why didn't the authors show absolute values for IL-6 secretion instead of showing relative reduction from 100% Was this due to IL-6 secretion variability amongst human donors? Also, y-axis should say "% of maximal IL-6 secretion".

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As above, please go through entire article and make sure there are space before your (references). Also, please be uniform throughout the manuscript for italicizing "et al." or not.

Materials & Methods: The authors should provide information on the antibodies they used for flow cytometry.

1st Revision - authors' response 14 August 2018

Point-to-point reply (Ms. EMBOR-2018-46512V1)

Comments Editor:
As you will see, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript. As the reports are below, I will not detail them here. However, the referees state that the major weakness of the manuscript is the lack of a clear demonstration of LMP7/2-selectivity of the compounds employed, in particular of LMP7-selectivity for PRN1126, the lack of positive control experiments, and the indirect nature of the data showing that the therapeutic potential of immunoproteasome inhibitors is dependent on the combined inhibition of LMP2 and LMP7. I think that these points need to be addressed with further experiments.

After cross-commenting with the referees it became clear that the suggested mouse lines with point mutations in the active center of LMP2 and 7 would indeed be very useful to address these major concerns. However, the referees agree that this would be beyond the scope of the present manuscript. Nevertheless, alternative experiments to strengthen the claims of the manuscript would be needed. The referees suggest, in addition to what they state already in their reports, to

- show based on the data in Fig 3b (suggesting that a shift in Mw of single proteasome subunits indicates binding of an inhibitor) that PRN1126 induces a shift of LMP7 only (also the LMP2 inhibitors used need to be included in this experiment).
Reply: Enriched lymphocytes from mouse spleens were incubated with PRN1126, ML604440, and LU-001i and a shift in Mw of single proteasome subunits was analysed by immunoblotting. LU-001i leads to a shift of LMP2 but not LMP7 bands to higher apparent molecular weights in western blot, demonstrating that this inhibitor is LMP2- but not LMP7-specific. In contrast to the expoxyketone LU-001i, ML604440 and PRN1126 do not lead to a shift of immunoproteasome subunit bands to higher apparent molecular weights in western blot. Thus, the epoxyketone inhibitor class induces the shifts most likely due to stable covalent modification and shifts to apparent higher molecular weight are not a hallmark of LMP7 or LMP2 subunit inhibitors in general. These data were included in Fig EV4A.

- address the presumed lack of inhibition of MECL-1 using fluorogenic substrates that are exclusively cleaved by MECL-1 in an experiment including all inhibitors used in this manuscript as well as an appropriate positive control.
Reply: β2c and MECL-1 are the subunits responsible for the trypsin-like activity in the constitutive proteasome (CP) and the immunoproteasome (IP), respectively. To address the impact of PRN1126 and ML604440 on the trypsin-like activity, mouse and human CP and IP were incubated with PRN1126 or ML604440, and the trypsin-like activity was assessed with the fluorogenic substrate Bz-VGR-AMC. PRN1126 and ML604440 did not affect the trypsin-like activity, indicating that these inhibitors, in contrast to the positive control MG132, do not

inhibit β2c in the CP and MECL-1 in the IP, the proteolytically active subunits in the proteasome responsible for the trypsin-like activity. These data were included in Fig EV1B-E and Fig EV4B-E. The lack of inhibition of MECL-1 by LU-001i using fluorogenic substrates were already shown previously by us (Basler et al. (2018), Br J Pharmacol, 175(1):38-52.) Furthermore, to investigate the impact of PRN1126 on the caspase-like activity, which is evoked by β1c, human and mouse CPs were incubated with different inhibitor concentrations and assayed with the fluorogenic substrate z-LLE-βNA. PRN1126 did not reduce the caspase-like activity below 10^{-6} M. This data was included in Fig EV1F-G in the revised manuscript. Taken together, we now investigated all subunits of the constitutive and immunoproteasome and clearly show that PRN1126 is only inhibiting LMP7 in the mouse and human immunoproteasome at the used concentrations.

Referee #1:

Basler and coauthors have previously reported on an LMP7-selective inhibitor with anti-inflammatory function (ONX 0914). They here test a novel LMP7-selective inhibitor, PRN1126. Treatment with this inhibitor fails to mimic the effects of ONX 0914 treatment, both in cell culture and in mouse models of inflammatory / autoimmune disease. In a series of experiments the authors then show that ONX 0914 in fact inhibits both LMP7 and LMP2, and that inhibition of both these proteasome subunits is required to mediate the anti-inflammatory effects observed following treatment with ONX 0914.

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Nevertheless, LMP7-selectivity of PRN1126 is not convincingly demonstrated. In particular, the potential effects of this inhibitor on MECL-1 remain entirely unaddressed.

<u>Reply:</u> To address this issue mouse and human immunoproteasomes were incubated with PRN1126, and the trypsin-like activity was assessed with the fluorogenic substrate Bz-VGR-AMC. PRN1126 did not affect the trypsin-like activity, demonstrating that PRN1126 does not inhibit β2c in the CP and MECL-1 in the IP, the proteolytically active subunits in the proteasome responsible for the trypsin-like activity. These data were included in Fig EV1B-E in the revised manuscript.
Furthermore, to investigate the impact of PRN1126 on the caspase-like activity, which is evoked by β1c, human and mouse CPs were incubated with different inhibitor concentrations and assayed with the fluorogenic substrate z-LLE-βNA. PRN1126 did not reduce the caspase-like activity below 10^{-6} M. This data was included in Fig EV1F-G in the revised manuscript. Taken together, we now investigated all subunits of the constitutive and immunoproteasome and clearly show that PRN1126 is only inhibiting LMP7 in the mouse and human immunoproteasome at the used concentrations.

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In 2009, the authors of the manuscript "Co-inhibiting immunoproteasome subunits LMP2 and LMP7 is required to block autoimmunity" have published in a Nature Medicine publication that the "LMP7-specific" immunoproteasome inhibitor PR-957 can be applied in a therapeutic way in a number of different preclinical models of auto-immune diseases. In this manuscript, Basler et al. now inform us that the proteasome inhibitor PR-957/ONX 0914 used in these studies is actually not "LMP7-specific" but additionally may also inhibit LMP2. Thus, ONX 0914 should be considered to be "just" an "immunoproteasome inhibitor". Furthermore, in this manuscript, the authors introduce another inhibitor PRN-1126 - which the authors proclaim would now really be "LMP7-specific". Most interestingly, in most preclinical models PRN-1126 does not share the ameliorating effects of PR-957 and only in combination with other immunoproteasome inhibitors has a therapeutic effect on auto-immune diseases.

In general, to understand the molecular mechanism underlying the therapeutic effects of immunoproteasome inhibitors is rather important. Also, the experiments as such have been

performed reasonably well - although for instance in the EAE model the overall, maximal severity score is rather low, hampering a conclusive interpretation of the data; furthermore lack most experiments positive controls that would document that PR-957 is actually functional in the performed experiments.

Reply: PR-957 (ONX 0914) is already included in most experiments as positive control (Fig 2, 3, 4, and 5) and shows the same effects as previously published, and thus is functional. Since we already showed in Fig 2 that ONX 0914 is reducing EAE score and weight loss in colitis (as previously published in Basler et al. (2014), EMBO Mol. Med., 6:226-238, Figure 2A; and Basler et al. (2010), J. Immunol., 185:634-641, Figure 5B) we have decided not to include ONX 0914 in Fig 6.

Nevertheless, to unequivocally make the point that the therapeutic potential of immunoproteasome inhibitors is dependent on the combined inhibition of LMP2 and LMP7 requires substantially more work. In its current form, all data presented are yet again indirect. Yet again the same assays are used as in the original publication from 2009 and their follow-ups. Most assays in the current manuscript are based on other "subunit-specific" inhibitors or LMP7 gene-deficient mice, which the authors themselves proclaim to affect the incorporation of other immunoproteasome subunits, such as LMP2. Thus, in order to make a convincing point of the specificity of the inhibitors, mouse strains would have to be established with CRISPR/Cas-mediated point mutations in the active center of LMP7 and/or LMP2. These mutations would resemble the treatment of subunit-specific inhibitors, but would not interfere with the formation of immunoproteasome complexes. Only such an approach would sufficiently well address this rather important issue.

Reply: In our opinion, in the revised version of this manuscript containing further specificity controls as outlined in our reply to Referee #1 above, together with previously published results, we clearly demonstrated that PRN1126, ML604440, and LU-001i are subunit specific inhibitors. The suggested mouse lines with point mutations in the active center of LMP2 and LMP7 would be an additional possibility to address the specificity of these inhibitors. However, we agree with the editor that this is beyond the scope of the present manuscript. Furthermore, it has been shown that point mutations in the active center of LMP2 (Thr to Ala) results in inhibition of processing of the subunits leading to incompletely processed subunits (Schmidtke et al. (1996), EMBO J., 15(24):6887–6898). Proteasome complexes with partially processed LMP2 subunits behaved essentially like proteasomes containing wild type LMP2 with respect to catalytic activity. Hence, the interpretation of results using the suggested cells containing point mutations in the active center would be rather difficult.

Referee #3:

Basler et al. provide an excellent article on the mechanism behind the ability of ONX 0914 to ameliorate the effects of autoimmunity in preclinical models. ONX 0914 is an immunoproteasome (IP) inhibitor that has been assumed to block autoimmunity through the specific inhibition of LMP7. PRN1126 is a compound synthesized by the authors and they provide evidence that it is an LMP7-specific inhibitor. However, in both in vitro and in vivo models, ONX 0914 and PRN1126 behave differently in their ability to downregulate MHC-I expression, IL-6 secretion, and autoimmunity in the DSS-colitis and EAE mouse models. Next, they provide evidence that while PRN1126 does not inhibit the LMP2 subunit, ONX 0914 does at higher concentrations. LMP2-specific inhibitors alone are unable to lower expression of MHC-I, IL-6 secretion, and block Th17 differentiation, but can in the presence of PR1126, similar to the effects of ONX 0914 alone. Lastly, the combinatory inhibition of both LMP2 and LMP7 slow the manifestations of DSS-colitis and EAE. This paper adds to the literature in that it provides an elucidation of the mechanism behind ONX 0914, however it does not explain how blockade of LMP2/LMP7 slow progression of autoimmunity.

Page 3; Authors write, "To improve pharmacological and toxicological profiles new proteasome inhibitors with immunoproteasome selectivity have been developed." There should be a comma between 'profiles" and "new".
Reply: We inserted a comma between "profiles" and "new".

Page 4: Authors write "On the cellular level, these effects were shown to involve two major pathways of disease development, namely cytokine secretion and T helper cell differentiation(Basler et al., 2013)." Place a space between "differentiation" and the reference.
Reply: We placed a space before the reference.

Page 4, last paragraph: You state on page 3 that ONX 0914 does not bind LMP2 due to "steric hindrance by Phe31" and hence ONX 0914 is "used as the prototype LMP7-selective inhibitor in many studies." So, at this point, it's not quite clear why PRN1126, a LMP7-specific inhibitor, was developed. Later on, on Page 8, the question of their differences finally comes up and the authors show that PRN1126 is not inhibiting LMP2, but ONX 0914 is. I would have liked this issue to be discussed up front, likely in the last paragraph of the introduction. That is, introduce the point of the article in the introduction.
Reply: As suggested by the Referee we discussed this issue in the last paragraph of the introduction of the revised manuscript.

Page 6 and overall statement (for multiple pieces of in vitro data): did you test cell viability upon exposure of PRN1126? Can you provide this data? Can you also provide data that dual inhibition of both LMP7 (with PRN1126) and LMP2 (with ML604440 or LU-001i) does not affect viability?
Reply: We tested cell viability of splenocytes treated with PRN1126, ML604440, LU-001i, PRN1126+ML604440, or PRN1126+LU-001i overnight; the same set-up we used for all in vitro assays using splenocytes. Flow cytometry analyses of different immune cells incubated with propidium iodide did not show increased cell death using these inhibitors compared to control cells. These data were included in Fig EV5 in the revised manuscript.

Page 6, in "PRN1126 affects the presentation of an LMP7-dependent epitope" paragraph. In addition to the PRN1126 data, the authors provide nice negative controls, such as the female mouse and the LMP7-/- mouse, and how they don't present UTY246-254 on the surface. What I would like to see is a positive control, specifically a decrease in UTY246-254 expression with a known LMP7 inhibitor (ie ONX 0914). And, again, how does increasing concentrations of PRN1126 affect cell viability.
Reply: In this experiment (Fig 2A) we clearly demonstrate that PRN1126 reduces the presentation of UTY246-254 in a dose dependent manner. We have previously shown that ONX 0914 reduces the presentation of UTY246-254 (Muchamuel et al. (2009), Nat Med, 6(2):226-38; Basler et al. (2018), Br J Pharmacol, 175(1):38-52.). Furthermore, LU-005i, an inhibitor of the immunoproteasome, also reduced the presentation of UTY246-254 (Basler et al. (2018), Br J Pharmacol, 175(1):38-52.). Cell viability (see comment above) was addressed in a new experiment and included in Fig EV5.

Figure 2a, 2b, & 2c: Are any of these changes statistically significant? I see no p-values or evidence of statistical testing.
Reply: P-values were included for Figure 2B. Due to the clear results, experiments 2A and 2C were only performed twice. In our opinion, it is not appropriate to perform statistical test on experiments with n=2. However, similar samples as used in Fig 2C are shown in Fig 5A for which p-values are shown in the Figure.

Figure 2d: Why didn't the authors show absolute values for IL-6 secretion instead of showing relative reduction from 100% Was this due to IL-6 secretion variability amongst human donors? Also, y-axis should say "% of maximal IL-6 secretion".
Reply: Due to variability amongst human donors we show relative reduction from 100%. We changed the y-axis to "% maximal IL-6 secretion".

Page 6, bottom: place a space between "expression" and "(Fehling et al., 1994).
Reply: We placed a space before the reference.

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Reply: We deleted one of the "in".

Figure 3a: x-axis has inhibitor spelled wrong.
Reply: We corrected inhibitor.

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Reply: PRN1126 does not induce electrophoretic mobility shifts of either LMP7 or LMP2. Thus, the epoxyketone inhibitor class induces the shifts most likely due to stable covalent modification and shifts to apparent higher molecular weight are not a hallmark of LMP7 or LMP2 subunit inhibitors in general. However, LU-001i (which was also included in this experiment) leads to a shift of LMP2 but not LMP7 bands to higher apparent molecular weights in western blot, demonstrating that this inhibitor is LMP2- but not LMP7-specific. This data was included in Fig EV4A.

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Page 14: authors write "Using an active-site ELISA, Muchamuel et al. showed an LMP2 inhibition by ONX 0914 of approximately 35% at 200 nM and 75% at 500 nM." Can you provide a reference for "Muchamuel et al." and write it in the proper referencing format?
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Reply: We broke this sentence up to make it more readable and placed a space before the reference.

As above, please go through entire article and make sure there are space before your (references). Also, please be uniform throughout the manuscript for italicizing "et al." or not.
Reply: We placed a space before all references and italicized all “et al.”.

Materials & Methods: The authors should provide information on the antibodies they used for flow cytometry.
Reply: We included the clone number, the company, and the dye of all antibodies used for flow cytometry.

2nd Editorial Decision 28 August 2018

Thank you for the submission of your revised manuscript to EMBO reports.

We have now received the full set of referee reports that is copied below. As you will see, all referees are very positive about the study and request only minor changes to clarify the conclusions made on caspase-like activity. Please also revisit Figure EV1 and add the missing data.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please provide up to five keywords in the system and on the first page of the manuscript.

- Please add the running title to the first page of the manuscript.

- Our data editors have already inspected the figure legends for completeness and accuracy. Please see the attached Word file with their suggested changes.

- In addition, I noticed the following issues regarding the statistical analysis in the manuscript that will need your attention:

+ Fig 2C: the legend states that the data are obtained from three technical replicates. Since the number of independent experiments is thus only 1, the statistical power is not sufficient to warrant the application of statistical tests. Please remove the p-values and preferentially show the individual data points.
+ Fig. 2E: Since you did the experiment in triplicate I suggest to combine the data from all three experiments to increase statistical power since currently n = 1 and again, the application of statistical tests is not recommended.

- I took the liberty to make some changes to the title and the abstract. Please have a look at the attached word file and amend as you see best fit.

- I noticed that Figures EV2 and EV3 consist of only one panel each. Could these two be combined into one figure?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS
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Referee #1:

In this revised version, the authors have added new data to demonstrate LMP7 specificity of PRN1126. While LMP7 selectivity is sufficiently shown, the conclusion at p. 5 (caspase -like activity is not reduced below 10 -6 M) is confusing. Caspase-like activity is not reduced at any concentration (Fig EV1), while trypsin-like activity in IP (MECL-1) is inhibited at PR1126 concentrations above 10 -6 M, which is not acknowledged.
In addition, the author reply claims to have included ML604440 in the experiments shown in Fig EV1. These data however have not been added to the Figure.

Referee #2:

The authors have addressed all requested aspects sufficiently well. In its current form the manuscript appears suitable for publication in the journal EMBO Reports.

Referee #3 (supports publication without further revision)

2nd Revision - authors' response 4 September 2018

We have now received the full set of referee reports that is copied below. As you will see, all referees are very positive about the study and request only minor changes to clarify the conclusions made on caspase-like activity. Please also revisit Figure EV1 and add the missing data.
Reply: See below: Reply to Referee#1

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please provide up to five keywords in the system and on the first page of the manuscript.
Reply: The following keywords were provided in the revised manuscript: proteasome/immunoproteasome/autoimmune disease/immunoproteasome inhibitor design

- Please add the running title to the first page of the manuscript.
Reply: The following running title was provided in the revised manuscript: IP co-inhibition blocks autoimmunity

- Our data editors have already inspected the figure legends for completeness and accuracy. Please see the attached Word file with their suggested changes.
Reply: We made all the suggested changes.

- In addition, I noticed the following issues regarding the statistical analysis in the manuscript that will need your attention:
+ Fig 2C: the legend states that the data are obtained from three technical replicates. Since the number of independent experiments is thus only 1, the statistical power is not sufficient to warrant the application of statistical tests. Please remove the p-values and preferentially show the individual data points.
Reply: We removed the p-values and show the individual data points.

+ Fig. 2E: Since you did the experiment in triplicate I suggest to combine the data from all three experiments to increase statistical power since currently n = 1 and again, the application of statistical tests is not recommended.
Reply: In this experiment we used 5 individual mice per group (thus, n = 5). However, as suggested we now combined the three experiments into one experiment (n=15).

- I took the liberty to make some changes to the title and the abstract. Please have a look at the attached word file and amend as you see best fit.
Reply: We changed title and the abstract according to your suggestions.

- I noticed that Figures EV2 and EV3 consist of only one panel each. Could these two be combined into one figure?
Reply: We combined former Figures EV2 and EV3 to the new Figure EV2A and B

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.
Reply: We now provide a short summary, bullet points, and a synopsis image.

****
Referee #1:

In this revised version, the authors have added new data to demonstrate LMP7 specificity of PRN1126. While LMP7 selectivity is sufficiently shown, the conclusion at p. 5 (caspase -like activity is not reduced below 10 -6 M) is confusing. Caspase-like activity is not reduced at any concentration (Fig EV1), while trypsin-like activity in IP (MECL-1) is inhibited at PR1126 concentrations above 10 -6 M, which is not acknowledged.
Reply: We apologize for this error. Caspase-like activity is not reduced at any concentration and trypsin-like activity in mouse immunoproteasome is inhibited at PR1126 concentrations above 10^{-6} M. We changed this in the revised manuscript.

In addition, the author reply claims to have included ML604440 in the experiments shown in Fig EV1. These data however have not been added to the Figure.
Reply: We apologize for not having made this sufficiently clear. The data regarding ML604440 were included in former Fig EV4 (now Fig EV3 in the revised manuscript) and not in Fig EV1.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michael Basler
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2018-46512V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 $\chi 2$ 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Not specified.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size estimates were defined prior to the experiments based on preliminary data and literature for the control group. Estimates of expected differences were used to calculate sample size using the the GPower3.0.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were randomly assigned to groups based on the number given by in-house breeding facility. Mice between groups were sex- and age-matched.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly assigned to groups based on the number given by in-house breeding facility.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In colitis experiments the weight is not biased. In EAE, the scores are well defined.
5. For every figure, are statistical tests justified as appropriate?	Yes. Due to low sample size no statistical test was performed for Fig 2A and 2C.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	As all sample sizes were low, the expected power of normal distribution tests was too low. Thus, single data points were plotted and assessed visually for normal distribution.
Is there an estimate of variation within each group of data?	yes.
Is the variance similar between the groups that are being statistically compared?	If variances of two groups were significantly different appropriate corrections were used. For t-Test we used the Welsh-Correction in case of unequal standard deviations.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provide in materials and methods clone number, company and dye used for all antibodies used in flow cytometry. We provide in material and methods a clone number and company or a refenrence for all antibodies used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines (reference) is provided in material and methods. Cells were not tested for mycoplasma contamination (not important for the purpose of the use of the used cell lines).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Reported in materials and methods (Mice, cell lines, and media).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were approved by the Review Board of Regierungspräsidium Freiburg in accordance with German Animal Protection Law. The study went through a process of ethical review (Regierungspräsidium Freiburg) prior to its commencement.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Animal studies are reported in compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Blood donation for research purposes was approved by the local ethics committee (Kantonale Ethikkommission, Kt. Thurgau, Switzerland)
12. 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.	Individual donors gave written consent and the experiments conformed to the principles set out in the WMA Declaration of Helsinki.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. 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.	NA
17. 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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA