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Haemophilus influenzae responds to glucocorticoids used in asthma therapy by modulation of biofilm formation and antibiotic resistance

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

Editor: Céline Carret

1st Editorial Decision 02 March 2015

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise substantial issues on your work, which should be convincingly addressed in a major revision of the present manuscript.

As you will see from the below reports, though the referees find the study to be of potential interest, they also raise a number of concerns about the conclusiveness of the results and the limited mechanistic insights provided. Referee 1 is rather unclear about the suitability of the model and more details and clarifications, including experimental ones, are needed all along the study. While more positive, referee 2 is also concerned about limited insights and questions the clinical relevance. We do not find the transcriptome analysis a prerequisite for revision but repeating key experiments using a more clinical strain/isolate would greatly improve the paper. Furthermore, the article should be written with a more general readership in mind. Referee 2 requests discussion at least on the mechanism, but we would like to encourage you to address it in an experimental way as much as you possibly can.

Overall, it is clear that publication of the manuscript cannot be considered at this stage. I also note that addressing the reviewer's concerns in full, including experimentally as mentioned above, will be necessary for further considering the manuscript in our journal and this appears to require a lot of additional work and experimentation. I am unsure whether you will be able or willing to address those and return a revised manuscript within the 3 months deadline (let us know if you think you

might need more time). On the other hand, given the potential interest of the findings, I would be willing to consider a revised manuscript with the understanding that the referee's concerns must be fully addressed and that acceptance of the manuscript would entail a second round of review. I should remind you that it is EMBO Molecular Medicine policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end I would strongly advise against returning an incomplete revision and would also understand your decision if you choose to rather seek rapid publication elsewhere at this stage.

I look forward to seeing a revised form of your manuscript as soon as possible.

Should you find that the requested revisions are not feasible within the constraints outlined here and choose, therefore, to submit your paper elsewhere, we would welcome a message to this effect.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

I am uncertain about the mouse model used.
There are to many messages in the same paper. It is difficult to follow.

Referee #1 (Remarks):

Earl et al. describes how the glucocorticoid beclomethasone promotes H. influenzae persistence by causing upregulation of a set of genes including regulation of the ECF sigma factor RpoE and its anti-sigma factor (Mc1A). Their data is supported by a mouse model consisting of cyclophosphamide-treated animals as well as analyses of sputa from patients suffering from asthma. Finally, the authors suggest that beclomethasone also makes the Haemophilus in biofilm more resistent against azithromycin both in vitro and in the mouse model. The manuscript contains an impressive amount of results describing several aspects of this intersting and clinically relevant phenomenon. Unfortuately, the paper is overloaded with data, contains several different observations, and hence is not fully logical in several parts. The work might be better of if divided into two separate stories.

Major comments

1. The authors treat mice with cyclophosphamide in order to induce granulocytopenia, and hereby prolong the study of H. influenzae survival in the lung. This is a model that has been used for pneumococci in the past. As correctly pointed out, several studies have shown influx of inflammatory cells in the mouse lung exposed to H. influenzae. However, here it is confusing for the reader. Neutrophilic mice are used, and then all the influx of cells are monitored at 3 and 7 days. When do the mice have restored cellular numbers? Why not test H. influenzae in another model excluding cyclophosphamide? Or was the purpose to use "clean" mice without an immune system to exclude the immunosuppresive effects of beclomethasone?

2. What role does mometasone and prednisolone play on H. influenzae survival in the cyclophosphamide-mouse model?

3. Clinical samples were obtained from patients that had been treated with either beclomethasone or mometasone. The difference between those two glucocorticoids and why either or is used should be clearly defined. In Table I, transcript levels are designated "+" or "++" - what does this mean; is it really a statistically significant difference?

4. P. 10, line 9: What is known about the clinical strains tested? Do strains isolated from patients on mometasone treatment respond similarly to beclomethasone as described for the selected H. influenzae strains?

5. It is not clear if authors suggest that the glucocorticosteroid acts directly on RpoE. How does beclomethasone activate RpoE? Please clarify in the Results section.

6. Authors speculate that glucocorticosteroid might have a receptor on the bacterial surface. How come this receptor was missed in the mutant library?

7. Do the authors think that beclomethasone has an intracellular target also in bacteria? Can it be checked?

8. Has it been excluded that beclomethasone does not change the MIC for azithromycin when analysed in broth, that is, not during biofilm formation?

9. P. 13, line 23-31: how can the deletion of rpoE and mclA, that govern genes in the opposite direction, both make the biofilms more resistant?

10. If the rpoE mutant's growth is not affected in rich or minimal medium, what is the explanation for the increased resistance to azithromycin seen in Figure 6? Is the outer membrane changed in some way; are the PBPs downregulated?

Minor comments

1. P. 6, line 19; "complex sBHi media" is difficult to understand for the common reader, and should be explained.

2. P. 7, line 4-6: the yfeA homolog, also called Haemophilus Protein F, has been implicated in many other virulence mechanisms, including adherence and host immune evasion. See Jalalvand et al., JID, 2013 and Su et al., Mol Micro 2013.

3. Table E3: RD KW20 is not an NTHi, but a serotype D that has lost the capsule.

4. P. 20, line 28 "at a dose" should be deleted.

5. Fig. E2: "Beclomethasone" is misspelt.

Referee #2 (Remarks):

Earl et al show nicely that glucocorticoids have an impact on the stress physiology of Haemophilus influenzae, leading to increased antibiotic resistance and biofilm production. The interesting findings of this manuscript is of particular interest, since NTHi isolates are widely recognized to participate and are leading causes of otitis media, COPD, or asthma associated infections. Especially, treatment regimes for COPD and asthma include usage of steroids and antibiotics, whereby any trigger, which will lead to increased antibiotic resistance and biofilm production within the pathogen bacterial flora is detrimental for the patients. Therefore, the impact of this story has high priority to the clinical and scientific community.

Discussion of the results:

1) Fig. 1) Shown are experiments addressing prototype H. influenzae strain Rd KW20. Animal experiments in mice show significantly enhanced persistence of bacteria in the lungs as a well in the spleens of mice treated with beclomethasone. Interestingly, no effect was observed for the recruitments of granulocytes in the BALF samples. Even more neutrophils are recruited at day 7, which actually is not expected.
Question: is it known from human studies, whether beclomethasone would behave similar? Pls provide background information.

2) Fig. 2) Steroids induce specific transcriptome in vitro. Among such genes are those up regulated for Fe-uptake, biofilm formation, general stress response and antibiotic resistance. These data are

convincing and were validated in part by qRT-PCR.

3) Fig 3) Steroids induce specific transcriptome in steroids treated mice. Transcriptome results obtained from mice were compared with in vitro cultured Rd strain with and without steroid treatment. There, a significant overlap of same genes up-regulated due to steroids in vitro and in the treated mice were observed.
Pls: indicate on page 8, line 9, the mouse model of infection includes treatment with steroids.

4) Steroid treated patients and steroid responsive NTHi derived from sputum of asthma patients. Three notably steroid responsive identified genes were tested in qRT-PCR and found to be up-regulated. Very importantly, here the authors show that patients treated with beclomethasone do induce NTHi genes, whereas patients treated with mometasone did not! For consequences for further clinical treatment directions this outcome is most interesting and valuable. The authors even show that differential steroid responsiveness of isolated clinical strains could be demonstrated. This experiment would have even higher impact, if NTHi strain isolates derived from the such sputums would have been used for such experiments.

5) Identifies of genes that mediate response of H. flu to steroids. What is the mechanism by which beclomethasone leads to increase expression of steroid responsive genes, such as HI1677. For that a reporter gene system comprising HI1677::lux was constructed. Then 4000 transposon insertion mutants were tested. Among the identified gene loci with altered expression of reporter gene system in the presence of beclomethasone, was also RpoE, the alternative sigma factor for periplasma and outermembrane stress response.

Generally to RpoE, also if it comes to the discussion part, the authors need to better cite and explain the function of the RpoE system to the reader! For example how is stress recognized, e.g. by Omp's shedding tripeptids from their C' terminus into the periplasm due to degradation, these will bind to DegS PDZ domain, then DegS triggers RseP, which finally proteolyse RseA/MclA the docking or anti-sigma protein of RpoE in the membrane to release RpoE into the cytosole. Then, RpoE can bind to RpoE promoters and will initiate transcription of RpoE depending genes. Where do the authors suggest that steroids such as beclomethasone cause stress which would be sensed by the RpoE system? On the OMPs level? How would outer membrane protein profile of beclomethasone treated NTHi cell look like? Would there signal tripeptides occur? This at least should be discussed, also in regard of what type of steroids are good and which one are not useful?

6) RpoE regulates expression of a subset of genes influenced by glucocorticosteroids. rpoE and mclA knockout mutants have been constructed and used for transcriptome analysis. Conclusively, some genes did not respond to presence of beclomethasone anymore, and rpoE and mclA did cause reverse action on some of these genes, as expected from knockout rpoE and knockout of mclA an anti-rpoE factor. To note here is that rpoE knockout mutants are actually lethal in E. coli, thereby survivors of rpoE knockout constructs are suppressor mutants. Whether this is the case for H. influenzae I don't know, but for future studies this should be under considerations.

7) Steroids have impact in biofilm and antibiotic tolerance. In general, if biofilm is produced antibiotic tolerance/resistance is enhanced! This was shown by many labs for many bacteria species. So it is even more interesting to see that beclomethasone altered biofilm structure, but not biomass, but showed increased azithromycin tolerance. Why is that, is there an explanation, are there other examples known? Again, very important, they showed this with azithromycin, which is often described in asthma patients. rpoE and mclA mutants behave similar showing reduced biofilm but enhanced azithromycin tolerance, what could be a scenario for that observation, even if as in part shown that rpoE and mclA should act in opposite ways, may be there are suppressor mutants involved, see also argument 5 for rpoE knockout mutations in general?

8) Last not least the authors showed in vivo relevance by treating mice with and without beclomethasone and azithromycin. Pls give attention to: CFU in text is mentioned 1x108, in figure legend is 1x 109 cells used for infection. There, the authors provided very convincing data, showing that untreated with beclomethasone all bacteria were basically killed by antibiotic in lung and spleen, however not in beclomethasone treated mice! Also rpoE knockout was not responding to beclomethasone, and showed intermediate azithromycin sensitivities.

Conclusions:
Pros: Very convincing data, important for future clinical treatment directions, many additional important information for the research field.
Cons: Would have been important to see how NTHi strains would respond to beclomethasone in transcriptome analysis, since many virulence factors are found in NTHi which are not present in Rd KW20 strain, the latter really is a laboratory adapted strain.

1st Revision - authors' response 30 March 2015

Point by point rebuttal to comments made in the decision letter:

Referee #1
1. The authors treat mice with cyclophosphamide in order to induce granulocytopenia, and hereby prolong the study of H. influenzae survival in the lung. This is a model that has been used for pneumococci in the past. As correctly pointed out, several studies have shown influx of inflammatory cells in the mouse lung exposed to H. influenzae. However, here it is confusing for the reader. Neutrophilic mice are used, and then all the influx of cells are monitored at 3 and 7 days. When do the mice have restored cellular numbers? Why not test in another model excluding cyclophosphamide? Or was the purpose to use "clean" mice without an immune system to exclude the immunosuppresive effects of beclomethasone?

Author response: The mouse model deployed in this study for *H. influenzae* pneumonia using cyclophosphamide has been used in the past by several labs with great effect (see for example Sekiya et al., 2008). However, we can understand the referee's concerns. To address this we carried out the *H. influenzae* mouse model of pulmonary infection without inclusion of the cyclophosphamide treatment step. The same trend was seen where beclomethasone treatment enhanced *H. influenzae* persistence in the airway but in this case, no dissemination to the spleen was seen. Furthermore, more neutrophils were recruited on day 3 as with the cyclophosphamide-treated mice but had returned to normal levels by day 4.

We have now included a statement in the main text of the article and an additional supplementary Figure (now Figure E2) to indicate this to the reader. Please see Page 6 lines 8-33.

2. What role does mometasone and prednisolone play on H. influenzae survival in the cyclophosphamide-mouse model?

Author response: We have carried out the experiment examining the impact that mometasone and prednisolone plays in *H. influenzae* survival in the cyclophosphamide-mouse model. Treatment with prednisolone gave a similar increased persistence phenotype as was observed for beclomethasone treatment. Mometasone also appeared to promote *H. influenzae* persistence, however its influence was considerably less than the other two steroids tested. We have now included statement to this effect in the main text and an additional supplementary Fig E1 to illustrate this to the reader. Please see Page 5 lines 26-33.

3. Clinical samples were obtained from patients that had been treated with either beclomethasone or mometasone. The difference between those two glucocorticoids and why either or is used should be clearly defined. In Table I, transcript levels are designated "+" or "++" - what does this mean; is it really a statistically significant difference?

Author response: We note from the literature that inhaled beclomethasone is prescribed much more frequently (in 90% of cases) than mometasone, probably because it is effective but of lower cost than mometasone. However, mometasone only needs to be administered once every 24 hours (whereas beclomethasone is administered twice/three times in the same period). We have now included statement in the article to inform the reader what guides the clinicians' choice between beclomethasone and mometasone (cost, administration frequency, past experience) and have included a several citations to support this. Please see Page 10 lines 4-10.

As for the gene expression data, the reviewer is correct in pointing out that we had not reported specific fold changes in gene expression in Table 1. We have now revamped Table 1 to include numbers for fold changes (and standard deviation) for each of the analyzed genes. It is clear from this that the changes that we highlight are statistically significant. Please see amended Table 1.

4. P. 10, line 9: What is known about the clinical strains tested? Do strains isolated from patients on mometasone treatment respond similarly to beclomethasone as described for the selected H. influenzae strains?

Author response: The origins of the clinical strains tested in this study are described in Table E3 and Table E4. These nontypeable *H. influenzae* strains have been isolated from otitis media and asthma patient infections. Of the five strains that came from asthma patients all were exposed to steroid treatment (four were beclomethasone treated patients and one mometasone treated patient). We do not wish to draw attention to the steroid treatment regime of the patients from which the strains were isolated, given that the numbers are small. Nevertheless, regardless of their origin, these clinical isolates transcriptionally respond to prednisone, beclomethasone and mometasone in a similar fashion to our model strain (e.g. elevated expression of *HI0359, HI0360* and *HI0361* in response to prednisone and beclomethasone but no alteration in these genes when exposed to mometasone). This is reported in Table E3. It is also important to note that we have gone one step further and shown that mutation of the gene encoding *rpoE* in a subset of these isolates has the same effect of altered antibiotic resistance as in the model laboratory strain. These data are now reported in supporting Fig E8 and we have included a statement in the text to this effect. Please see Page 14 lines 30-33 and page 15 lines1-2.

5. It is not clear if authors suggest that the glucocorticosteroid acts directly on RpoE. How does beclomethasone activate RpoE? Please clarify in the Results section.

Author response: As we state in the text, the detailed mechanisms by which beclomethasone exerts its influence on *H. influenzae* gene expression and phenotypes such as biofilm formation and antibiotic resistance remain obscure. Although we suggest that glucocorticoids suppress the action of RpoE, we do not wish to imply that this is a direct action. We have addressed the possibility that glucocorticosteroids act directly on RpoE through determination of the capacity of purified RpoE to bind beclomethasone as assessed by isothermal titration calorimetry. The experimental data show that the binding affinity is low and therefore do not support a direct-binding model. This data is now reported in supporting Fig E9 and statement is included in the text, which we hope will clarify this point. Please see Page 18 lines 1-10.

6. Authors speculate that glucocorticosteroid might have a receptor on the bacterial surface. How come this receptor was missed in the mutant library?

Author response: One plausible reason for the inability to identify an interacting protein or receptor for glucocorticosteroid is that such a protein is either an essential gene product or that it contributes significantly to bacterial fitness. In such a case, a genetic approach would not work to reveal this.

7. Do the authors think that beclomethasone has an intracellular target also in bacteria? Can it be checked?

Author response: As we state in the text and in the response to point 5, the detailed mechanisms by which beclomethasone exerts its influence on *H. influenzae* gene expression and phenotypes such as biofilm formation and antibiotic resistance remain obscure. Hence we cannot exclude the possibility that beclomethasone has an intracellular target although the ITC experiments described in response to point 6 indicate that this target is not RpoE itself. We consider that identification of such a target (if indeed there is one) is beyond the scope and focus of the current work.

8. *Has it been excluded that beclomethasone does not change the MIC for azithromycin when*

analysed in broth, that is, not during biofilm formation?

Author response: We have examined azithromycin tolerance in *H. influenzae* strains (laboratory model and clinical isolates) grown planktonically in the presence and absence of beclomethasone. No difference in tolerance to azithromycin is seen. This data is now reported in supporting Fig E6 and statement is included in the article regarding this. Please see Page 14 lines 14-18.

9. *P. 13, line 23-31: how can the deletion of rpoE and mclA, that govern genes in the opposite direction, both make the biofilms more resistant?*

Author response: Although the reviewer is correct in the assertion that RpoE and MclA govern the expression of many genes in opposite directions, there are a small number that are regulated in the same fashion (see Fig. 3B), or that change in one mutant but not the other (Table E2). We also cannot exclude that multiple mechanisms can contribute to resistance to azithromycin but that different mechanisms are activated in the *rpoE* and *mclA* strains when grown in micro-chambers.

10. *If the rpoE mutant's growth is not affected in rich or minimal medium, what is the explanation for the increased resistance to azithromycin seen in Figure 6? Is the outer membrane changed in some way; are the PBPs downregulated?*

Author response: As the reviewer states, the data in Figure 6 show that the *rpoE* mutant when in mice is more tolerant than the wild type to azithromycin. In a similar fashion, biofilms formed by the *rpoE* mutant are more tolerant to azithromycin than those formed by the mutant. We speculate that within the mice the bacteria adopt largely a biofilm lifestyle.

Minor comments

1. P. 6, line 19; "complex sBHi media" is difficult to understand for the common reader, and should be explained.

Author response: sBHI refers to brain heart infusion agar plates supplemented with 10 µg/ml of hemin and 10 µg/ml of NAD. This is detailed in the Materials and Methods section. We have now modified the text at this point to make this clearer "…..by high throughput cDNA sequencing. For these experiments, bacteria were grown in complex sBHi medium, which is brain heart infusion agar supplemented with hemin and NAD (see Materials and Methods)."

2. P. 7, line 4-6: the yfeA homolog, also called Haemophilus Protein F, has been implicated in many other virulence mechanisms, including adherence and host immune evasion. See Jalalvand et al., JID, 2013 and Su et al., Mol Micro 2013.

Author response: We are aware that YfeA has been implicated in virulence previously and have cited several of the earlier manuscripts reporting this. We have now included the Jalalvand et al., (2013) JID, 2013 and Su et al., (2013) Mol Micro as suggested.

3. Table E3: RD KW20 is not an NTHi, but a serotype D that has lost the capsule.

Author response: We have amended this typo.

4. P. 20, line 28 "at a dose" should be deleted.

Author response: We have amended this typo.

5. Fig. E2: "Beclomethasone" is misspelt.

Author response: We have amended this typo.

Referee #2 (Remarks):

1) Fig. 1) Shown are experiments addressing prototype H. influenzae strain Rd KW20. Animal experiments in mice show significantly enhanced persistence of bacteria in the lungs as a well in the spleens of mice treated with beclomethasone. Interestingly, no effect was observed for the recruitments of granulocytes in the BALF samples. Even more neutrophils are recruited at day 7, which actually is not expected. Question: is it known from human studies, whether beclomethasone would behave similar? Pls provide background information.

Author response: The observed continued recruitment of neutrophils at day 7 in the cyclophosphamide-mouse model *H. influenzae* survival assay was unexpected. However, this observation is not unprecedented in the clinical setting given that neutrophilic asthma patients examined showed neutrophil numbers increase in patients undergoing inhaled corticosteroid treatment (please see Simpson et al., (2007) Thorax 62:211-218). We have now included statement the article and an additional references to indicate this to the reader. Please see Page 6 lines 27-33.

2) Fig. 2) Steroids induce specific transcriptome in vitro. Among such genes are those up regulated for Fe-uptake, biofilm formation, general stress response and antibiotic resistance. These data are convincing and were validated in part by qRT-PCR.

Author response: We are glad the referee finds these data convincing.

3) Fig 3) Steroids induce specific transcriptome in steroids treated mice. Transcriptome results obtained from mice were compared with in vitro cultured Rd strain with and without steroid treatment. There, a significant overlap of same genes up-regulated due to steroids in vitro and in the treated mice were observed.
Pls: indicate on page 8, line 9, the mouse model of infection includes treatment with steroids.

Author response: We have now included statement mouse model of infection includes treatment with steroids to indicate this to the reader. Please see Page 8 lines 11-13.

4) Steroid treated patients and steroid responsive NTHi derived from sputum of asthma patients. Three notably steroid responsive identified genes were tested in qRT-PCR and found to be up-regulated. Very importantly, here the authors show that patients treated with beclomethasone do induce NTHi genes, whereas patients treated with mometasone did not! For consequences for further clinical treatment directions this outcome is most interesting and valuable. The authors even show that differential steroid responsiveness of isolated clinical strains could be demonstrated. This experiment would have even higher impact, if NTHi strain isolates derived from such sputum's would have been used for such experiments.

Author response: As the referee reports, we have taken the clinical nontypeable *H. influenzae* strains from asthma patients (described in Table E3) and shown that they respond transcriptionally to the presence of mometasone, prednisone and beclomethasone by elevated expression of *HI0359, HI0360* and *HI0361*. We have gone one step further and shown that mutation of the *rpoE* gene in a subset of these isolates leads to the same antibiotic resistance phenotype as in the laboratory strain. We feel that the demonstration that the clinical nontypeable *H. influenzae* strains respond phenotypically and transcriptionally in the same way as our model laboratory strain is strong evidence of the broad clinical relevance of the findings.

This data is now reported in supporting Fig E8 and included statement the article regarding this. Please see page 14 lines 30-33 and page 15 lines1-2.

5) Identifies of genes that mediate response of H. flu to steroids. What is the mechanism by which

beclomethasone leads to increase expression of steroid responsive genes, such as HI1677. For that a reporter gene system comprising HI1677::lux was constructed. Then 4000 transposon insertion mutants were tested. Among the identified gene loci with altered expression of reporter gene system in the presence of beclomethasone, was also RpoE, the alternative sigma factor for periplasma and outermembrane stress response.

Generally to RpoE, also if it comes to the discussion part, the authors need to better cite and explain the function of the RpoE system to the reader! For example how is stress recognized, e.g. by Omp's shedding tripeptids from their C' terminus into the periplasm due to degradation, these will bind to DegS PDZ domain, then DegS triggers RseP, which finally proteolyse RseA/MclA the docking or anti-sigma protein of RpoE in the membrane to release RpoE into the cytosole. Then, RpoE can bind to RpoE promoters and will initiate transcription of RpoE depending genes. Where do the authors suggest that steroids such as beclomethasone cause stress which would be sensed by the RpoE system? On the OMPs level? How would outer membrane protein profile of beclomethasone treated NTHi cell look like? Would there signal tripeptides occur? This at least should be discussed; also in regard of what type of steroids are good and which one are not useful?

Author response: On reflection we agree with the reviewer that inclusion of some discussion of the RpoE system might be warranted. However we must point out that the available data cannot be fully explained by a model in which beclomethasone causes stress that can be sensed leading to activation of the RpoE system in the pathway succinctly described by the reviewer. Specifically, addition of beclomethasone has the same effect as a knockout of RpoE on the transcription of many genes and on phenotypes such as increased tolerance of biofilms to azithromycin. We have included some statements in the text in the Discussion to explain the function of the RpoE system to the reader in a little more detail (pointing to several excellent reviews on the topic) as well as to highlight the points made above, where we suggest that the action of beclomethasone is in negative modulation of the RpoE system. In the revised manuscript, we have addressed some possible mechanisms. We examined the possibility that glucocorticosteroid acts directly on RpoE, by assessment of the ability of purified RpoE to bind beclomethasone, as monitored by isothermal titration calorimetry. RpoE was unable to bind beclomethasone effectively (only low affinity), which does not support a direct interaction model. This data is now shown in Fig. E10. We also assessed the influence of beclomethasone on the outer membrane protein profile of the wild-type strain grown in sBHI broth at 37°C by SDS-PAGE and silver staining. There was no difference in major outer membrane protein profiles between the wild-type model strain with or without beclomethasone or a clinical isolate with or without beclomethasone. We have included the data on the outer membrane protein profile as supporting Fig E9 and a statement describing this outcome in the main text of the article (page 18 lines 1-11).

6) RpoE regulates expression of a subset of genes influenced by glucocorticosteroids. rpoE and mclA knockout mutants have been constructed and used for transcriptome analysis. Conclusively, some genes did not respond to presence of beclomethasone anymore, and rpoE and mclA did cause reverse action on some of these genes, as expected from knockout rpoE and knockout of mclA an anti-rpoE factor. To note here is that rpoE knockout mutants are actually lethal in E. coli, thereby survivors of rpoE knockout constructs are suppressor mutants. Whether this is the case for H. influenzae I don't know, but for future studies this should be under considerations.

Author response: The growth of the *rpoE* mutant in *H influenzae* is unaffected in rich or minimal medium. The mechanism of RpoE regulation in *H. influenzae* has been describe and shown not be essential making it less like RpoE from *E. coli* and other Gram-negative bacteria. Several laboratories have reported this finding (please see Humphreys et al., 1999; Craig et al., 2001; 2002). However, we do understand the referee's concern here and have included a statement to highlight this issue in the text. Please see Page17 lines 13-33.

7) Steroids have impact in biofilm and antibiotic tolerance. In general, if biofilm is produced antibiotic tolerance/resistance is enhanced! This was shown by many labs for many bacteria species. So it is even more interesting to see that beclomethasone altered biofilm structure, but not biomass, but showed increased azithromycin tolerance. Why is that, is there an explanation, are there other examples known? Again, very important, they showed this with azithromycin, which is

often described in asthma patients. rpoE and mclA mutants behave similar showing reduced biofilm but enhanced azithromycin tolerance, what could be a scenario for that observation, even if as in part shown that rpoE and mclA should act in opposite ways, may be there are suppressor mutants involved, see also argument 5 for rpoE knockout mutations in general?

Author response: It is known that that different mutations in the same organism as well as growth in different media can lead to alteration in biofilm structure, which may reflect the composition of the different polymers of the matrix. Bacteria within altered biofilm structures may also have different physiology from the wild type, because of alteration in parameters such as nutrient availability and oxygen tension. Thus although the general statement that biofilm formation enhances antibiotic tolerance as expressed by the reviewer is certainly true, variations in the level of tolerance within biofilms formed in different conditions or by different mutants are not unexpected. Please also see detailed response to point 5.

8) Last not least the authors showed in vivo relevance by treating mice with and without beclomethasone and azithromycin. Pls give attention to: CFU in text is mentioned 1x108, in figure legend is 1x 109 cells used for infection. There, the authors provided very convincing data, showing that untreated with beclomethasone all bacteria were basically killed by antibiotic in lung and spleen, however not in beclomethasone treated mice! Also rpoE knockout was not responding to beclomethasone, and showed intermediate azithromycin sensitivities.

Author response: We are glad the referee finds this data convincing. The issue that CFU in the text is mentioned 1x108, and in the figure legend is 1x 109 cells used for infection has been resolved.

2nd Editorial Decision 13 April 2015

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees who were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending final editorial amendments:

Please submit your revised manuscript within two weeks. I look forward to reading your revised article soon.

***** Reviewer's comments *****

Referee #1 (Remarks):

I think the Authors in a most admirable way have tackled the comments by the Reviewers. This has resulted in a more clear and easily read manuscript. The authors are congratulated to a very interesting study.

Referee #2 (Remarks):

Addressing revised version and rebuttal letter of Earl et al.,
After reading the revised version and the rebuttal letter, this version may have convinced referee #1. There mayor points were addressed by adding considerable amount of new data: see new Figure E1/ influence on H. flu persistence; E2/ no cyclophophamide treatment; revised Table 1/including quantity and statistics; Fig E8/ demonstration of response to beclomethasone of H. flu strains after isolating from patients treated with mometasone; Fig. E9/ binding of RpoE to beclomethasone; Fig. E6/ tolerance versus azithromycin in planktonical cells treated +/- beclomethasone;

As to referee #2: Issues 1) fulfilled; 2) fulfilled; 3) fulfilled; 4) See Fig. E8 fulfilled; 5) Fig. E) and E10/fulfilled. Even though if the action of beclomethasone is negative modulating the RpoE system, I found it valuable to inform the reader about what is known of the input regulatory cascade behind the RpoE system, so the reader can come up with own imaginations. Maybe the equilibrium of bound versus soluble RpoE is shifted in the presence of e.g. beclomethasone leading to increased binding of RpoE to MlcA in the membrane, therefore negatively regulating RpoE response. Or with other words, in the absence of beclomethasone the RpoE system is in higher alert conditions. This is provocative and of course a subject of future characterizations. A suggestion for the future work would be to actually sequence rpoE knockout strains and compare with parental strain and see whether suppressor mutations have occurred (see Davis and Waldor, 2009, Nucl Acid Res 37:5757); 6) fulfilled; 7) fulfilled; 8) fulfilled.

