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Dr. Fang-Fang Ji
Science Editor
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RE: 10716: Nuclear Factor κB (NF-κB) Represses the Expression of Latent Membrane Protein 1 in Epstein-Barr Virus Transformed Cells

Dear Dr. Ji,

Thanks for the extension for us to resubmit our manuscript. We appreciate the thoughtful reviews conducted by the Reviewers, and here are our responses:

Reviewer #1:

"Major 1.As the study suggested, NF-κB negatively regulated LMP1 expression in IB4 cells, but some reports got different conclusions, the reasons could be drawn as genetic differences in cell lines, type of assays, et al. Then what're the expression levels of LMP1 in other EBV-transformed cell lines with the same conditions? You'd better have a try."

Response: there are many reasons for observed discrepancies. EBV has several latencies and with varied LMP1 expression. We trust other reported results but offer our own explanations for the discrepancies. One of them is the LMP1 expression level: Other works are done in LMP1-low backgrounds, but ours are in EBV type III latency, a high LMP1 expression state. Because high LMP1 is detrimental for growth, the observed negative effects might make sense. In addition, both conclusions might be right as NFkB may have dual roles in LMP1 regulation in different environments. This might be the major reason for the discrepancy and we have put this point in the manuscript in the Discussion.

"*Minor 1.Some spelling errors could be found in the paper*. "

Response: We have revised the manuscript extensively.

"*2.How to determine the minimum time for the induction of IκB? What's the standard?*

Also **Reviewer #2**, point 2." *It is not clear why "the minimum time" for the induction of IkappaB was determined and how "this" was tested. The authors should clarify these points in the manuscript*."

Response: We used Western blot analysis for the detection of IkB. Increase of the expression as a standard. The induction at the three hours seems to be uncertain, six hours later, the induction is obvious (new Figure 1B). The bottom line is that we have established the correlation between IkB induction and LMP1 stimulation (Figure 1). The minimum time for induction of IkB seems irrelevant now. To avoid the confusion for the readers, we would like to delete sentences about the minimum time for induction in the manuscript.

Reviewer #2:
"1. It is well-known that NF-kappaB activity is fine-tuned by positive and negative regulators as well as by post-translational modifications. At least, levels of NF-kappaB proteins and their subcelullar location should be determined in IB4 cells and shown in Figs 1 and 2. Another protein (or other proteins) classically known to be regulated by NF-kappaB should be examined as control(s).

Response: Because the line has been characterized extensively, and we have examined the expression of IkB, it might not necessary to re-analyze the line.

"2. *It is not clear why "the minimum time" for the induction of IkappaB was determined and how "this" was tested. The authors should clarify these points in the manuscript*." see above responses to Reviewer #1.

"3. LMP1 expression decreases from 6 h in IB4 cells either grown with or without tetracycline (Fig. 1). An explanation to this issue should be given. What are the levels of LMP1 and IkappaB at time 0? The amount of IkappaB at different times should be shown, particularly because of the 24-h delay in NF-kappaB inhibition required for the induction of IkappaB as it has been reported elsewhere (Cahir-McFarland et al, Pro Natl Acad Sci USA, 2000, 97:6055-6060; Cahir-McFarland et al, J Virol, 2004, 78:4108-4119)."

Response: The line was offered by the Kieff's group. The detailed time courses for induction of IkB might not checked extensively. In addition, we have found that IkB expression is more sensitive than that determined by FLAG antibody (data not shown). The LMP1 expression is related to the NFkappaB activity.

"4. In Fig. 2, as was pointed out above, overexpression of NF-kappaB should be confirmed in IB4 cells. Whether the experiment was performed with or without tetracycline should be indicated. How long did it take the isolation of CD4-positive cells? Was it performed in the presence or absence of tetracycline? Under the conditions used, what is the actual amount of IkappaB? What effect would be expected if the expression of IkappaB were to be induced? "

Response: The activity of NF-kB had been confirmed by the reporter assay as shown in the revised Figure2. The IB4 line used here is the *parental* line that is NOT containing any tetracycline regulatory components. About 30 minutes for the selection of transfected cells. The experiments were performed in the absence of tetracycline because it is not applicable. We did not check the expression of IkB because NFkB levels are increased (new Figure 2). When IkB

were induced, as shown in the Figure 1, LMP1 should be increased. All those points are now in the text.

"5. Relative LMP1 and IkB levels should be quantify and shown in Figs 1 and 2."

Response: We found that the quantification of the Western is not always agree with the results seen. Because our major conclusions are aiming for increase or decrease, the Western blot film without quantification might be suffice. It is obvious the LMP1 is altered there.

" *6. To make the results of LMP1 promoter activity in 293T cells upon overexpression of LMP1, LMP-DM and NF-kappaB (Figs. 3 and 4) more comparable to those described by others, shorter promoter regions (or even larger) should be analyzed.* "

Response: We did several promoter reporter constructs, and we found the same results could be obtained. The results are shown in the revised Figure 3. It is hard to compare with others, as the reporter (CAT) and different lengths of promoter constructs were used.

"*7. Statistical analyses should be performed and described in the Materials and Methods section. Statistical significance should be indicated in Figs 3 and 4.* "

Response: We have added the section as suggested. And *p* values are shown in Figures 3 and 4.

"8. *The authors should also discuss their results considering those obtained by Cahir-McFarland et al (Pro Natl Acad Sci USA, 2000, 97:6055-6060), in which LMP1 expression was not affected by inhibition of NF-kappaB (using the same cell line).* "

Response: First of all, we have no idea about their observations. As mentioned above, the line was provided by the group listed above. Second, we did our best to explain the issue. We think the growth condition for the cells in various environments might be slightly different and in addition, passages numbers of the cells might be a factor too. The sentence is now in the Discussion.

"*9. Data obtained by using the NF-kappaB inhibitor BAY11-7082 are not reported in the Results section; therefore they should not be mentioned in the Discussion section as "Data not shown". Should they either be incorporated in the corresponding section or refer to them as "Zhang L, personal communication" or "unpublished results", or give an appropriate quotation.* "

Response: we refer them as "unpublished results" as suggested

Minor points: 1. Materials and Methods Section. The descriptions of the CD-4 and b-galactosidase expression plasmids are missing. 2. p 4, 3rd paragraph, line 3. Please indicate a brief description for NF-kappaB reporter construct or a reference. 3. p 5, 1st paragraph, first line. Quote the reference of tubulin antibody. 4. p 5, last line, p 6, first line "…while CD4-positive cells are attached to the wall of…" It should be indicated as "attached to Dynabeads CD4" instead. 6. p 6, 2nd paragraph. Used dilutions of primary antibodies should be given. References and dilutions of secondary antibodies should be given"

Response: CD4 and beta-galactosidase, NFkB-reporter plasmids are all described in the text now. Tubulin antibody were also described. Primary antibody dilutions were varied among experiments. Those points are all addressed in the manuscript.

We have extensively revised and made all necessary changes in the manuscript. We believe that eh manuscript is greatly improved.

Sincerely yours,

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