Author's response to reviews

Title: The NF-kB p65 and p50 Homodimer Cooperate with IRF8 to Synergistically Activate iNOS Transcription

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Author's response to reviews: see over
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Editors
BMC Cancer

Dear Editors:

We first would like to thank you and the reviewers for reviewing our manuscript and for the constructive comments regarding the revision of the manuscript. We have performed new experiments and revised the manuscript as advised.

New data are in figures 3C, 3D, 3E, 4B, 4C, and 5C.

Our point-to-point response to the reviewer's

Reviewer 1:

Comment 1: Several complementary experiments are required to conclude that both colon carcinoma and myeloid cells share the same regulatory mechanism for iNOS expression. a) In Figure 4C, the NF-κB/DNA complex in TNFα+IFNγ-treated T84 cells seems to be totally supershifted by a p65 antibody (although due to the red line, the bands cannot be seen well). This would suggest that unlike in myeloid cells, this complex in tumor cells does not contain a p50/p50 homodimer. To conclude that the NF-κB complex in tumor cells is also the NF-κB “homodimer” (in this case p65/p65) like in myeloid cells, a p50-specific antibody should be tested to show that the band is not shifted. Please include a positive control for supershift by the p50 antibody.

Revision 1: We thank the reviewer for this advice, and have performed new experiments. Although our EMSA studies in the myeloid cells show clear p50/p50 and p65/p65 homodimers, the NF-κB complex associated with the human iNOS promoter DNA in the human colon carcinoma cannot be clearly identified because there is binding of non-specific proteins to the same DNA sequence (Fig. 4C). In the last two months, we have repeated this EMSA at least 5 times. We used new cell cultures and made new DNA probes, but we could not determine whether there are p50/p50 or p65/p65 complexes that bind to the human iNOS promoter DNA. However, we can reproducibly show that: 1) TNFα treatment induces a protein complex associated with the NF-κB DNA probe (human iNOS promoter); 2) positive control NF-κB probe (Santa Cruz Biotech) showed that TNFα induces both p65 and p50 complexes in the human colon carcinoma cells; 3) anti-p65 and anti-p50 antibodies displaced the DNA-protein complexes at the human iNOS promoter (Fig. 4C); and 4) the cold probe competes away the protein binding to the DNA probe.

In the revised manuscript, we specifically state that the p50/p50 and p65/p65 homodimers bind to the nos2 promoter in myeloid cells.
Comment 2: b) In Figure 7A, the authors found that IFN# induced IRF8 expression in J774 cells. Whether IFN# induces IRF8 in T84 cells should be examined.
Revision 2: The reviewer’s point is well taken. We performed these new experiments as advised. Data is presented in Fig. 3D & E.

Comment 3: c) In Figure 7B, in order to conclude that IRF8 is required for the induction of iNOS expression in myeloid cells, the authors should show that the re-introduction of IRF8 into CL-2 cells can restore the induction of iNOS. This is because J774 and CL-2 cells are not siblings and it is unclear whether the observed difference is indeed due to IRF8. In addition, to conclude that IRF8 is also required for the induction of iNOS expression in colon carcinoma cells, IRF8 should be knocked-down or knocked-out in T84 cells.
Revision 3: We thank the reviewer for this advice. As the reviewer advised, we silenced the IRF8 expression in the colon carcinoma cell line using an IRF8-specific siRNA. Our results indicated that IFNγ regulates iNOS through IRF8 (Fig. 3E). The IRF8 deficient CL-2 therefore becomes a secondary approach. We feel that the overexpression experiment is not as physiologically relevant as the silencing experiment.

Comment 4: Several controls should be included to ensure the results from ChIP or IP experiments.
a) p50-ChIP without TNF# treatment to show that the binding is TNF#-dependent (Figure 4B)
b) ChIP-PCR for known a STAT1-binding site(s) to show that pSTAT1-ChIP itself is working (Figure 4B and 6B)
c) p65-ChIP without LPS stimulation to show that the binding is LPS-dependent (Figure 6B)
d) p50-IP without LPS stimulation to show whether the p65/p50 association is LPS-dependent (Figure 6C)
Revision 4: We thank the reviewer for this advice. A technical challenge is that NF-κB is constitutively expressed in many types of cells, especially in tumor cells. Therefore, the difference is quantitative. We have repeated the ChIP and have included the p65 antibody (Fig. 4B). As for pSTAT1, our new data indicate that pSTAT1 activates IRF8 to up-regulate iNOS. pSTAT1 does not directly regulate iNOS. This explains the negative results of pSTAT1 ChIP and EMSA (old Fig. 4B &C). Therefore, we have removed all pSTAT1 ChIP and EMSA from the manuscript.

Comment 5: Minor Essential Revisions
1. In line 169, “Table S1” should be “Table 1”.
2. In line 272, “p50-specific” should be “p50-specific”.
3. In lines 321-322, “has been show to” should be “has been shown to”.
4. In line 581 (the legend of Fig. 3B), “analyzed by real time RT-PCR” should be “analyzed by Western blotting”.
5. In Figure 7B, “FN-#” should be “IFN-#”.
6. Inconsistent spellings:
a. IFN# and IFN-
b. “anti-” and “Anti-” for the prefix of antibodies in the Figures.
c. “p65/DNA” and “p65-DNA”
Revision 5: Revised as advised. Thanks!
Reviewer 2:

Comment 1: In result section "IFN# and TNF# synergistically induce iNOS expression in human colon carcinoma cells", due to the complex signaling network TNF# treatment can activate pathways other than NFkB. Did the author validate their data by inhibiting NFkB activity with expectation of blocking of iNOS expression?

Revision 1: We thank the reviewer for this advice and agree with the reviewer that TNFα can activate pathways other than NF-κB. However, there is no NF-κB-specific inhibitor that will specifically block the p65- or p50- subunit.

Comment 2: In Fig 4C right panel, anti-p65 antibody incubation did not show super-shift band. But in Fig 6D both p65 and p50 antibodies show super-shift bands. How do the authors explain the difference? Another concern is that the authors did not show that unlabeled competing consensus DNA sequence can block the shift band.

Revision 2: We have repeated this experiment more than five times now, it seems there is a non-specific protein binding at this site. Therefore, we cannot clearly detect the p65 and p50 subunit. We have revised the manuscript to indicate that the p50/p50 and p65/p65 homodimers bind to the iNOS promoter in myeloid cells. As advised, we have used the cold probe for the competition experiment.

Comment 3: In Fig 3A lower panel, the authors should show statistic significance of the synergistic effect.

Revision 3: We thank the reviewer for this advice. We do not see a synergistic effect by statistical means in the colon carcinoma cells. Therefore, we remove the term “synergistic” in the revised manuscript.

Comment 4: In Fig 3C and 5C, can the authors add total STAT blot?

Revision 4: New experiments have been performed as advised (Fig. 3C and 5C)

Comment 5: There is a typo in Fig 7B side legend of “IFN#” : the "I" is missing.

Revision 5: Corrected. Thanks!

The editor initially gave us one month for the revision. We thank the editor for granting us one month extension. We have performed the new experiments as advised, and hope that the revision has addressed the reviewers’ comments.

Thanks again for your time to review our manuscript!

Sincerely,

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