Author’s response to reviews

Title: Respiratory Syncytial Virus and TNFalpha Induction of Chemokine Gene Expression Involves Differential Activation of Rel A and NF-kappaB1

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We would like to thank the reviewers for their excellent comments for the improvement of the manuscript. Indeed we were impressed that both reviews agreed that the major concern of the study seems to involve a lack of support for our claim that RSV and TNF activate chemokines through distinct NF-kB signaling pathways. Although our data is suggestive of distinct pathways we fully agree that we have not defined the NF-kB pathway induced by RSV and concur that based on our present evidence cannot state that the RSV and TNF induce distinct NF-kB pathways (we have changed the title of the paper). However, I do think it is fair to say based on our new data that RSV and TNF activate chemokine gene expression differently since the inhibitors had very different effects on chemokine gene expression and NF-kB binding activity depending whether induction was by TNF stimulation or RSV infection. Indeed to better address this concern we utilized a new transcription factor assay from Active Motif (Carlsbad, CA) in which the binding activity of p65 and p50 can be quantified independently by ELISA. As new Fig. 4, these data replace the unconvincing and confusing gel shift data and provide direct evidence that RSV and TNF differentially induce p65 and p50 in A549 epithelial cells. The results indicate that RSV induces primarily p65 whereas TNF induces both p65 and p50 and the inhibitor NAC inhibits RSV but not TNF induction of p65 which is consistent with the effects of NAC on chemokine induction by RSV and TNF.

Other points:

Dr. Rippe

1. With regard to AP-1. We actually did look at AP-1 binding activity but we didn't find an induction in response to RSV under our culture and infection conditions. This may be due to a large background binding activity associated with AP-1 in A549 cells. We are currently using the Active Motif assay kit for cJun and cFos to further elucidate the role of AP-1 in RSV induction of chemokine expression. The other reason we focussed primarily on NF-kB is it has been reported in the literature to be the major factor involved in chemokine gene transcription at least in response to TNFa, which was our comparison.

2. With regard to figure 2: All the data from the gels were quantified by STORM and normalized to the GAPDH (which is the very bottom band on the gel). From the gel in Fig. 2 MCP-1 and IL-8 at 24 hrs do appear to the eye to be somewhat less but that is in part due to the higher background in those lanes. But when compared to the untreated lane the fold induction based on the STORM reveals that there was indeed an increase in MCP-1 and IL-8 mRNA at 24 hrs.

3. With regard to Figs. 4 and 5: We have replaced the gel shifts with the Active Motif data on p65 and p50 binding activity to give a new Fig. 4.
Dr. Guo

1. With regard to figure 3. Although the high background makes it appear that the induction levels of chemokine by TNF are less than in Fig 2. STORM quantification reveals that after normalization they are approximately the same as in Fig. 2
2. With regard to binding complexes on the kB elements of chemokine promoter. We agree that different complexes could bind to the specific elements on the IL-8, MCP-1 and RANTES promoters. But for the immediate purposes of comparison between RSV and TNF induced complexes we chose to use the consensus kB elements which has been shown to mimic most of the kB elements in chemokine promoters in other studies. The new data in Fig. 4 using Active Motif ELISA for p65 and p50 binding activity provides new evidence for the differences between TNF and RSV induction of NF-kB.
3. With regard to the discrepancy in the induction kinetics. We agree that MCP-1 and IL-8 induction is rapid and appears to precede the translocation of NF-kB. We suggest that other elements and factors in addition to NF-kB (such as Sp1 or AP-1) may be important at least for the early induction of expression. However, at the same time NAC blocks NF-kB binding activity at 2 hrs and IL-8 and MCP-1 expression induced by RSV at 24 hrs suggesting that NF-kB does contribute to IL-8 and MCP-1 expression. Many other studies have shown that these chemokines are induced by NF-kB albeit the kinetics of activation has not been demonstrated. Although these are important experiments it is not clear to us how transfection experiments will fully resolve this issue since it is already been shown by transfection experiments that NF-kB and AP-1 are the major elements in IL-8 induction by RSV and NF-kB and NF-IL-6 are the major elements in TNFα induction of IL-8.