Supplemental Tables and Figure Legends

Supplemental Table 1. Antibodies

Antibody	Clone/Catalog#	Source
Phopho-p65 (S536)	3033	Cell Signaling Technology
Nucleophosmin	3542	Cell Signaling Technology
p100/52	4882	Cell Signaling Technology
RelB	SC-226	Santa Cruz Biotechnology
RelA/p65	SC-372	Santa Cruz Biotechnology
IκBα	SC-371	Santa Cruz Biotechnology
p300	SC-584	Santa Cruz Biotechnology
RNA polymerase II	SC-899	Santa Cruz Biotechnology
NIK	SC-7211	Santa Cruz Biotechnology
LTßR	4H8 WH2	Axxora
ß-tubulin	TUB 2.1	Sigma
γ-tubulin	GTU-188	Abcam

The antibodies were used at the indicated concentrations as described by the manufacturer, except for the RelB (1/1000), IκBα (1/1000), NIK (1/1000) and p65 (1/5000) antibodies.

Supplemental Table 2. Primer sequences for Semi-quantitative RT-PCR

Gene	Forward	Reverse
Csf2	5'-GCCATCAAAGAAGCCCTGAA-3'	5'-GCGGGTCTGCACACATGTTA-3'
Ccl2	5'-AGGTCCCTGTCATGCTTCTG-3'	5'-TCTGGACCCATTCCTTCTTG-3'
RelB	5'-GTCTTTCCCCACGAGGCTAT-3'	5'-CCGTACCTGGTCATCACAGAG-3'
Map3k14	5'-CACTCGCTTGTTTGAGGACA-3'	5'-GATCTCCCACTTCCCACAGA-3'
Distal	5'-GAGCTTCTGGAGAGGGAGGT-3'	5'-TCCCAGGCTTAGTCTGTTGC-3'
Proximal	5'-GCCTGACAACCTGGGGGAAG-3'	5'-TGATTAATGGTGACCACAGAACTC-3'

Primer sequences were from reference # 10 and # 18.

Supplemental Table 3. Oligonucleotides for Reporter synthesis

ID	Sequence
R0	CGAGatgacgctacgaca
F0	tgtcgtagcgtcatCTCGAGGGGCCTTTAAAGCAG
R18	CTGAGGAGGATGCGGGCTGCTTTAAAGGCCCCT
F35	CCCGCATCCTCCTCAGGACCTTAGCCAGGCCT
R51	CTGCGGGCTGCTTTAAAGGCCTGGCTAAGGTC
F67	TTAAAGCAGCCCGCAGGTGGGCTGCCAGTTCTT
R83	GGGTTTTCATTAATAAGCCCTTCCAAGAACTGGCAGCCCAC
F100	GGAAGGGCTTATTAATGAAAACCCCCCAAGCCTGACAACCT
R124	CCAGTGAGCCTTCCCCCAGGTTGTCAGGCTTGGG
F141	GGGGGAAGGCTCACTGGCCCCATGTATAGCTGATAAGGG
R158	GAGTTGTGGAATCTCCTGGCCCTTATCAGCTATACATGGGG
F180	CCAGGAGATTCCACAACTCAGGTAGTTCCCCCGCCC
R199	GACCACAGAACTCCAGGGGGGCGGGGGAACTACCT

F216 CCCTGGAGTTCTGTGGTCACCATTAATCATTTCCTCTAACTG
R234 ACTGCAAAAGAGCTCTTATATACACAGTTAGAGGAAATGATTAATGGT
F258 TGTATATAAGAGCTCTTTTGCAGTGAGCCCAGTACTCAGAGAG
R282 TCCTCAGGACCTTAGCCTTTCTCTCTGAGTACTGGGCTC
F301 AAAGGCTAAGGTCCTGAGGAGGAAAGCTTgccaagga
F321 tccttggcAAGCTTTCC

XhoI and HindIII sites added to the front and end of sequence
Primer sequences were from reference # 13 and # 18.

Supplemental Figure Legends

Supplemental figure 1. Induction of Csf2 transcripts by LTßR MAb treatment following TNF priming. Cells were treated with the following conditions

1. TNF, 25 ng/ml for 1 hour
2. LTßR, 0.5 µg/ml for 4 hours
3. TNF', primed with TNF for 1 hour and then rested for 4 hours
4. TNF'/LTßR, primed with TNF for 1 hour and then LTßR for 4 hours

Complementary DNA was generated from total RNA samples and then analyzed for Csf2 transcripts by qPCR.

Supplemental figure 2. LTßR priming does not increase TNF-mediated Ccl2 (A) and RelB (B) induction. Complementary DNA samples from figure 2 were analyzed for Ccl2 and RelB transcripts by qPCR.

1. TNF, 25 ng/ml for 1 hour
2. LTßR, LTßR MAb 0.5 µg/ml for 4 hours
3. LTßR'/TNF, primed with LTßR MAb for 4 hours and then TNF for 1 hour
4. TNF', primed with TNF for 1 hour and then rested for 4 hours
5. TNF'/LTßR, primed with TNF for 1 hour and then LTßR for 4 hours

The p values from student T test analyses for supplemental figure 2B were 0.053 (A) and 0.6 (B).

Supplemental Figure 3. NIK (Map3k14) expression was required for p100 processing and LTßR-MAb priming of TNF-mediated Csf2 expression. (A) The processing of p100 following LTßR-treatment was inhibited in NIK knockdown cells. p100 levels in cells transfected with either the control (pLKO.1) or NIK (Map3k14 shRNA) shRNA plasmid were measured following LTßR-MAb treatment. The results from three separate experiments were summarized in supplemental figure 3A. (B) The amount of Csf2 expression following TNF or LTßR-MAb treatment in cells transfected with either control (pLKO.1) or NIK (Map3k14 shRNA) shRNA plasmid was measured by RT-PCR . Transfection of the human NIK (MAP3K14) expression plasmid restored LTßR MAb priming in NIK knockdown cells. (C) Lysates from NIK knockdown cells transiently transfected with either the control (pCMV-SPORT6) or NIK (pCMV-SPORT6/MAP3K14) expression plasmid for 48 hours were immunoprecipitated and immunoblotted with the

NIK antibody. (D) The expression of the human NIK (MAP3K14) expression plasmid restored the synergistic effect of LTßR MAb priming in TNF-mediated Csf2 transcription in NIK knockdown cells.

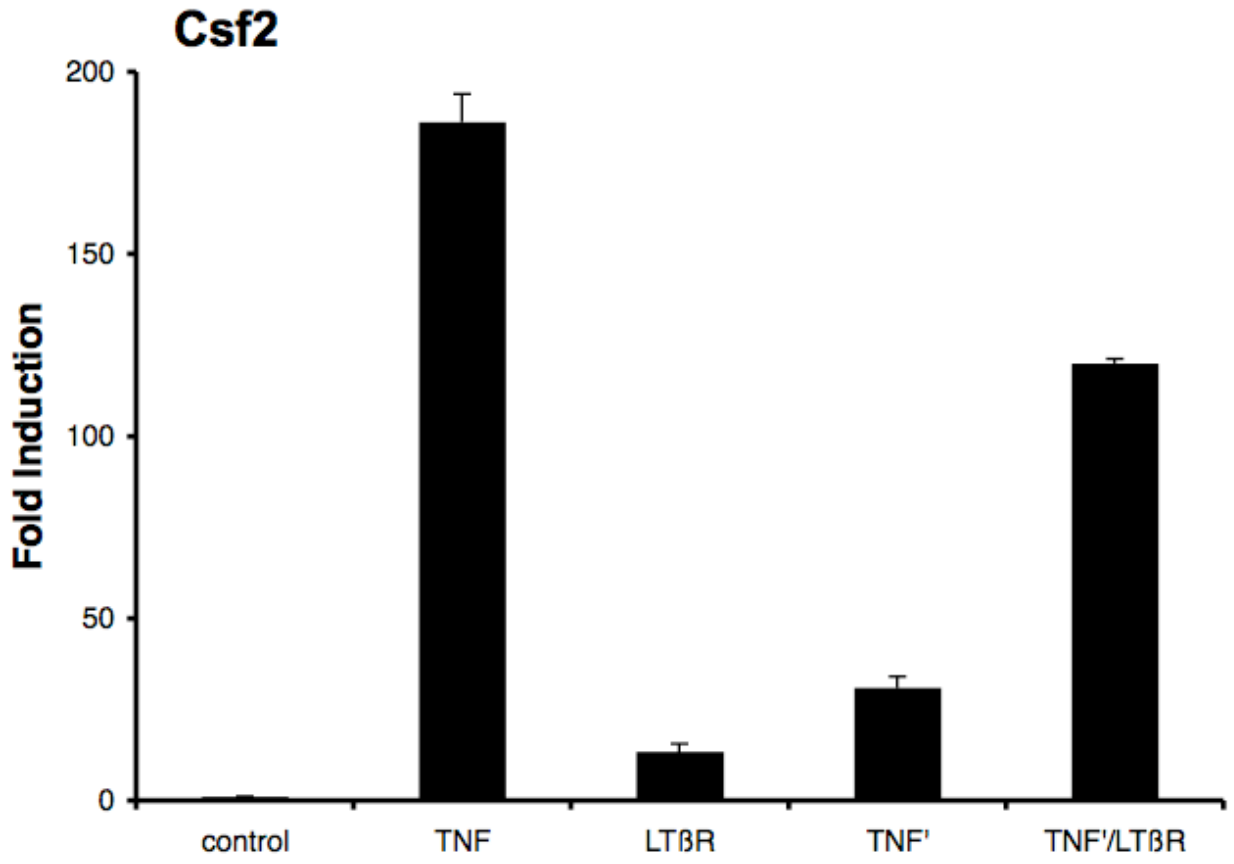
Supplemental figure 4. The detection of p100, p52, p65, and RelB in the cytoplasmic and nuclear extracts from cells treated with either vehicle (medium), TNF (25 ng/ml), or LTßR MAb (0.5 μg/ml) for the indicated time period. Cytoplasmic and nuclear extracts from treated cells were fractionated as described in the Materials and Methods section. The extracts were probed with the indicated antibodies. NPM and ß tubulin served as the nuclear and cytoplasmic loading controls, respectively.

Supplemental figure 5. LTßR'/TNF induced p65 DNA binding activity was specific for the NF-κB consensus sequence. (A) As described in the kit protocol (Active Motif), two micrograms of the nuclear extract from cells primed with LTßR and then treated with TNF (LTßR'/TNF) were incubated with either wild type or mutated NF-κB consensus sequence during the DNA binding process. (B) p65 DNA binding curves. Various amounts of nuclear extracts from treated cells were assessed for p65 DNA binding activity. The slopes of TNF and LTßR'/TNF were 0.09 and 0.12, respectively, which suggested that the difference in p65 DNA binding activity between the two treatments was nominal.

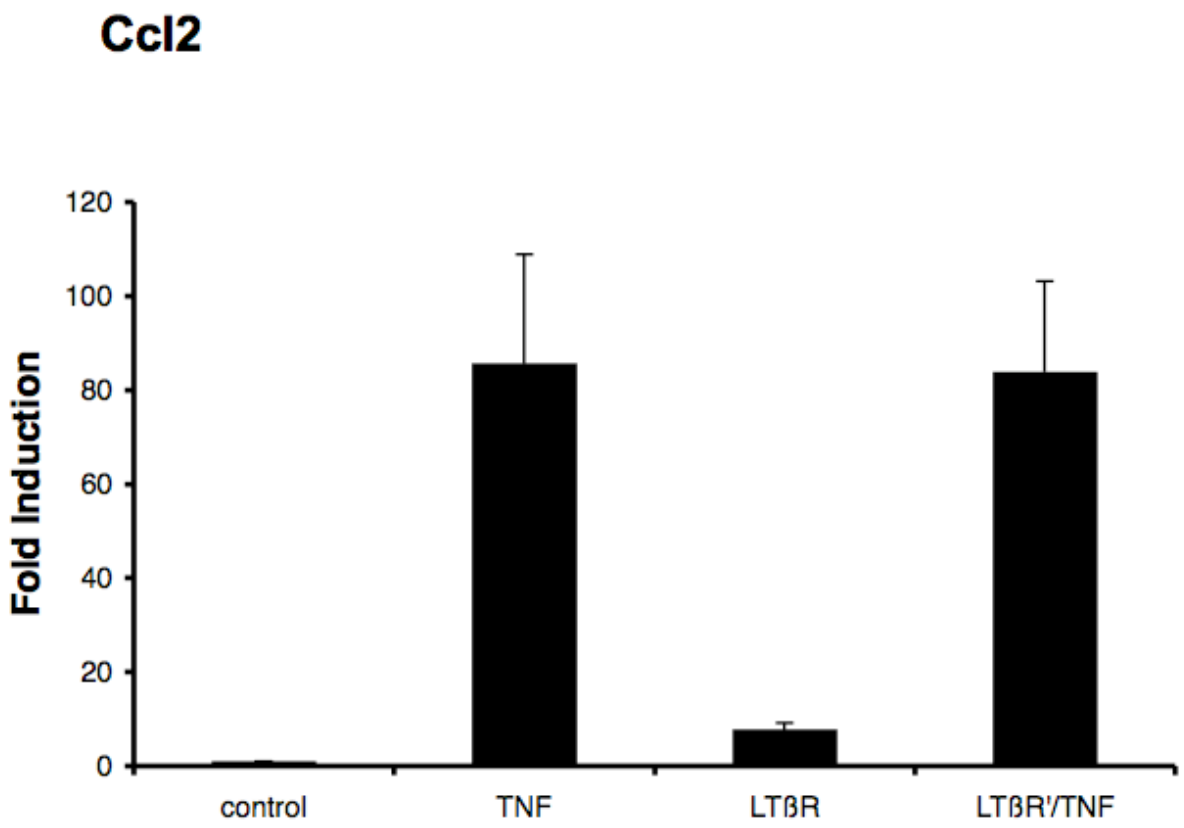
Supplemental Figure 6. (A)Nuclear RelB and p65 do not associate with each other. One hundred micrograms of nuclear extract from cells primed with LTßR-MAb (4 hr), washed, and treated with TNF (1 hr) was initially immunoprecipitated with normal rabbit Ig (2 μg) overnight at 4°C (lane 2). Protein A/G PLUS beads (Santa Cruz Biotechnology) were added to the sample and rotated for an additional hour. The samples were centrifuged to remove the supernatant (unbound sample). The unbound sample was subsequently immunoprecipitated with two micrograms of RelB antibody for 4 hours at 4°C (lane 3). As described earlier, Protein A/G PLUS beads were added to the sample and rotated for an additional hour. The unbound sample was subsequently immunoprecipitated with two micrograms of RelB antibody at 4°C overnight (lane 4). This process was repeated however with two micrograms of p65 antibody for 4 hr (lane 5) and overnight (lane 6). The beads were washed three times with RIPA buffer and the immunoprecipitated samples were eluted from the beads with SDS sample buffer and boiling. The immunoprecipitated RelB (top) and p65 (bottom) was detected by immunoblot analysis as described in the Material and Methods section. Ten microgram of the original nuclear sample was included (input, lane 1). (B) Recruitment of p65 to the Csf2 promoter was independent of RelB. Chromatin immunoprecipitation samples (as described in figure 8C) from cells expressing either the control (pGIPZ) or RelB shRNAmir plasmid were analyzed for p65 recruitment to the Csf2 promoter after LTßR-MAb priming and then treated with TNF.

Supplemental Figure 7. Recruitment of p65 and RelB to the same DNA region. An immunodepletion ChIP assay was performed as described previously (see reference 14

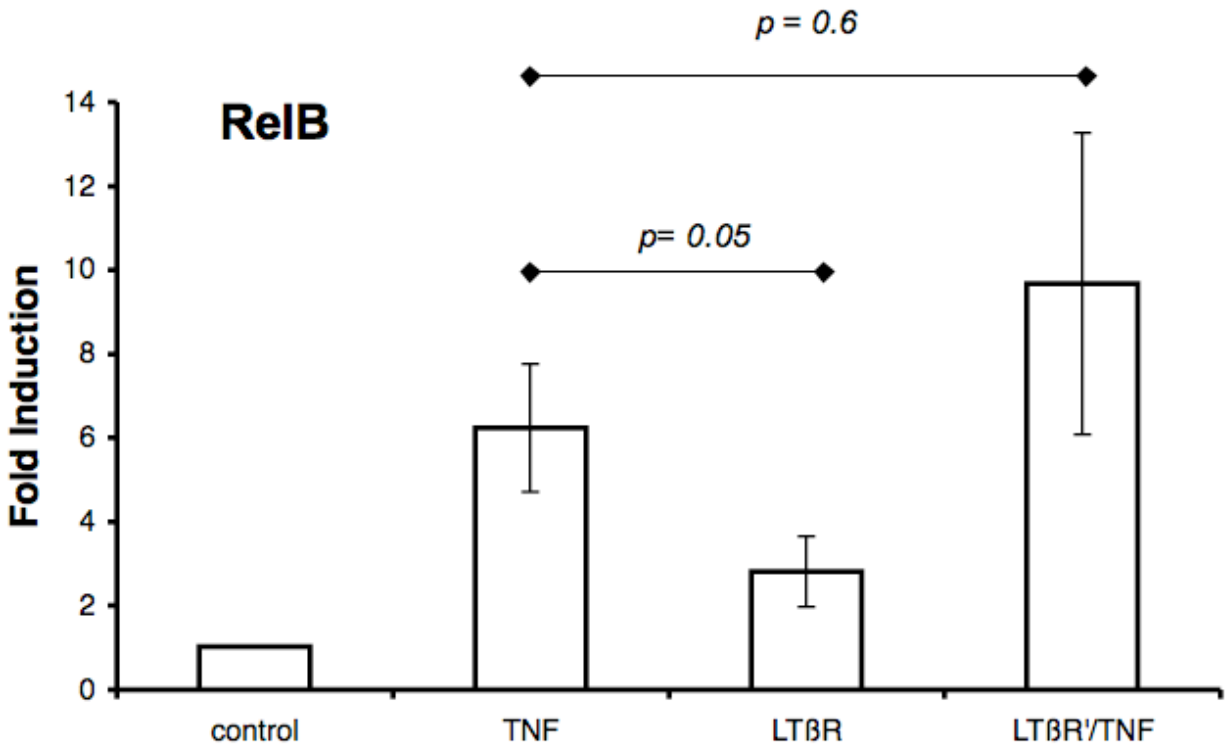
in text) with the following modifications. Sheared chromatin from cells primed with LTßR MAb and then treated with TNF was initially incubated with either normal Ig, anti-p65, or anti-RelB overnight. The immunoprecipitated chromatin was depleted from the sample with the addition of Protein A/G PLUS beads (Santa Cruz). The immunodepleted chromatin samples were subsequently immunoprecipitiated with the indicated antibody. The precipitated DNA was used in a PCR reaction and the PCR products were run through a DNA agarose gel.


Csf2
Fold Induction
200
150
100
50
0
control
TNF
LTßR
TNF'
TNF'/LTßR

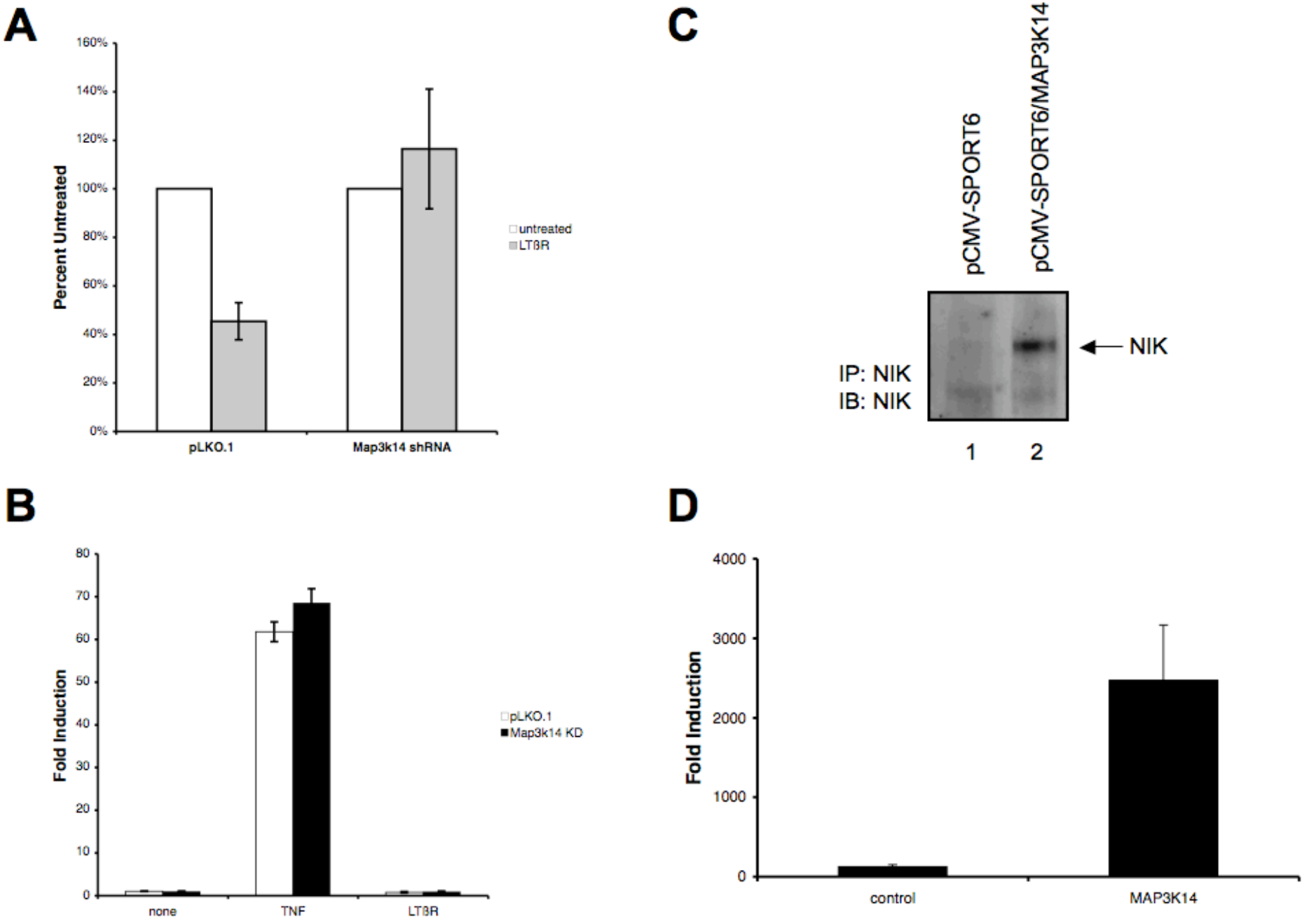
Suppl. Fig. 1


Ccl2
Fold Induction
120
100
80
60
40
20
0
control
TNF
LTβR
LTβR'/TNF

Suppl. Fig. 2A


p = 0.6
RelB
p= 0.05
14
12
10
8
6
4
2
0
Fold Induction
control
TNF
LTßR
LTßR'/TNF

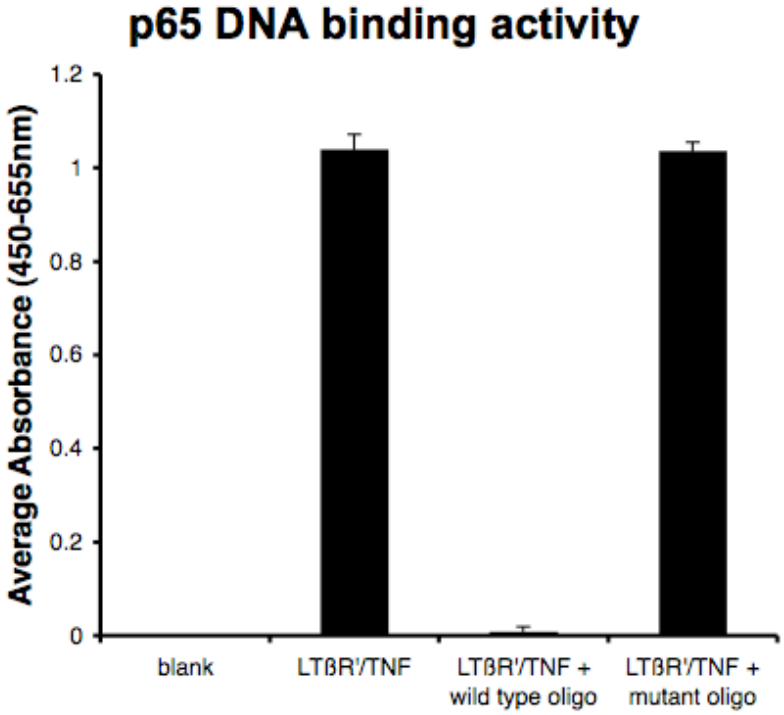
Suppl. Fig. 2B

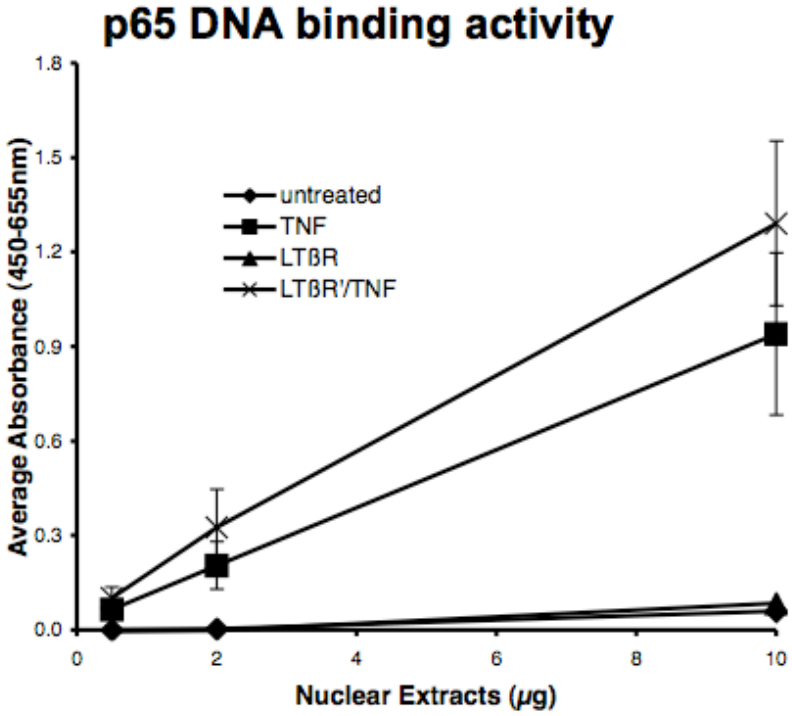


Suppl. Fig. 3A-D

Suppl. Fig. 4

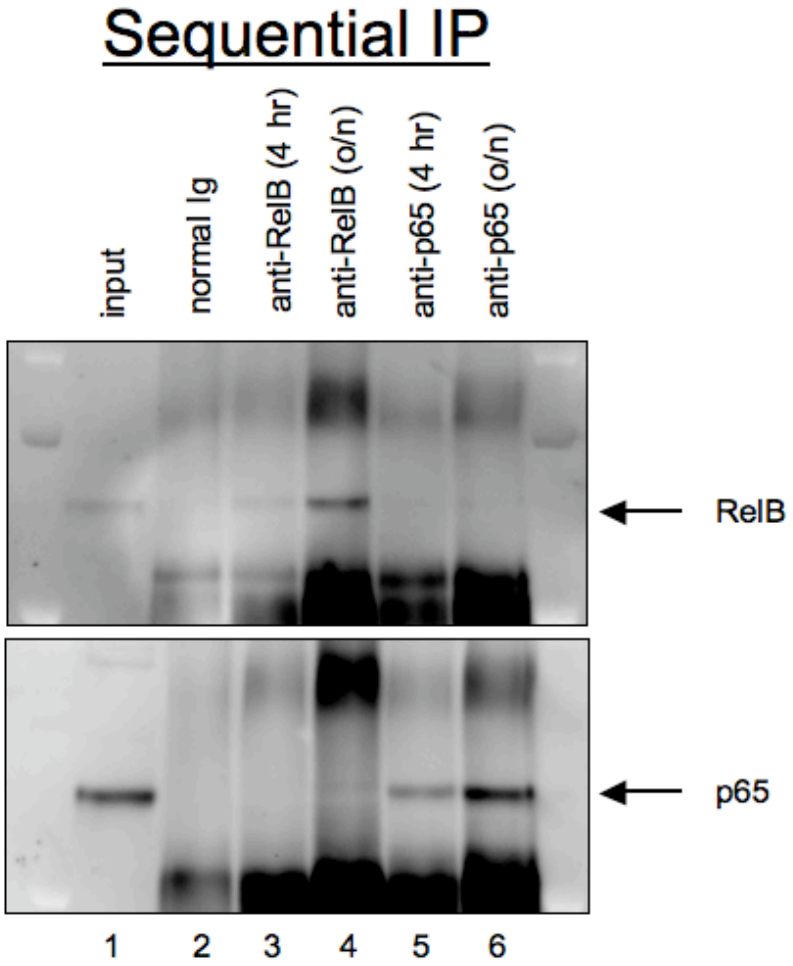
Cytoplasmic
Nuclear
Control
TNF (1 hr)
LTβR (1 hr)
LTβR (2 hr)
LTβR (4 hr)
LTβR' (4 hr) + vehicle (1 hr)
LTβR' (4 hr) + TNF (1 hr)
p65
RelB
p100
p52
ß-tubulin
NPM
1 2 3 4 5 6 7
8 9 10 11 12 13 14


p65 DNA binding activity
Average Absorbance (450-655nm)
1.2
1
0.8
0.6
0.4
0.2
0
blank
LTβR'/TNF
LTβR'/TNF + wild type oligo
LTβR'/TNF + mutant oligo

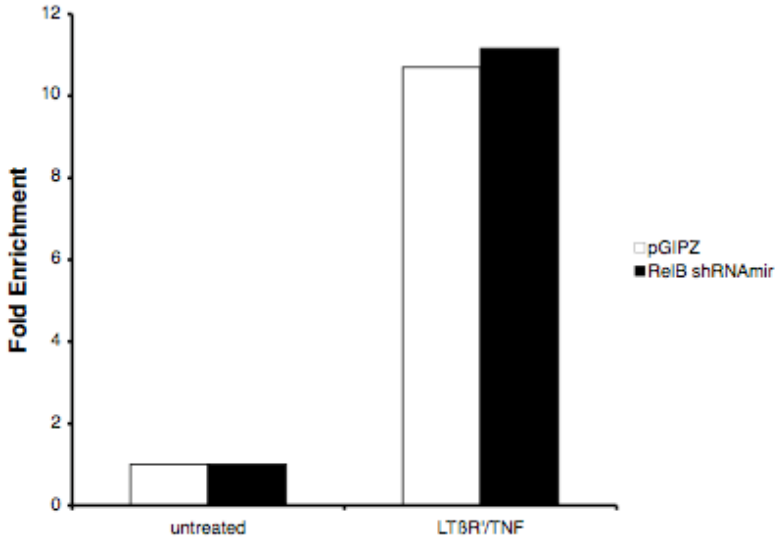

p65 DNA binding activity
Average Absorbance (450-655nm)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0
2
4
6
8
10
Nuclear Extracts (μg)
untreated
TNF
LTβR
LTβR'/TNF

Suppl. Fig. 5

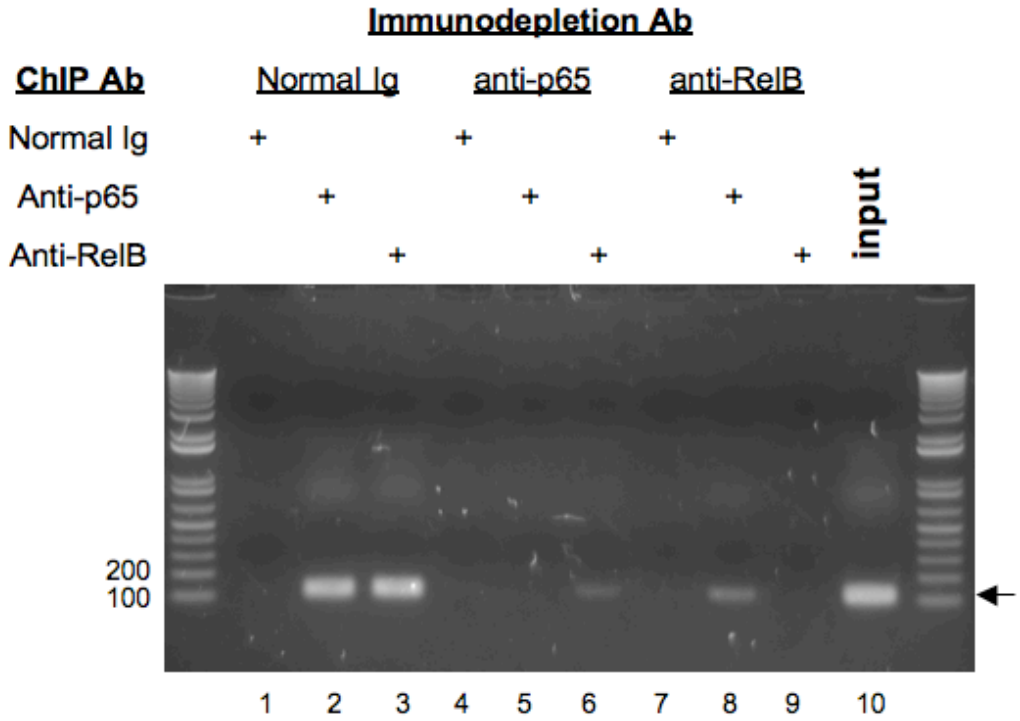
A



B



Suppl. Fig. 6A & B


Immunodepletion Ab
ChIP Ab
Normal Ig
anti-p65
anti-RelB
Normal Ig + + +
Anti-p65 + + +
Anti-RelB + + +
input
200
100
1 2 3 4 5 6 7 8 9 10

Suppl. Fig. 7