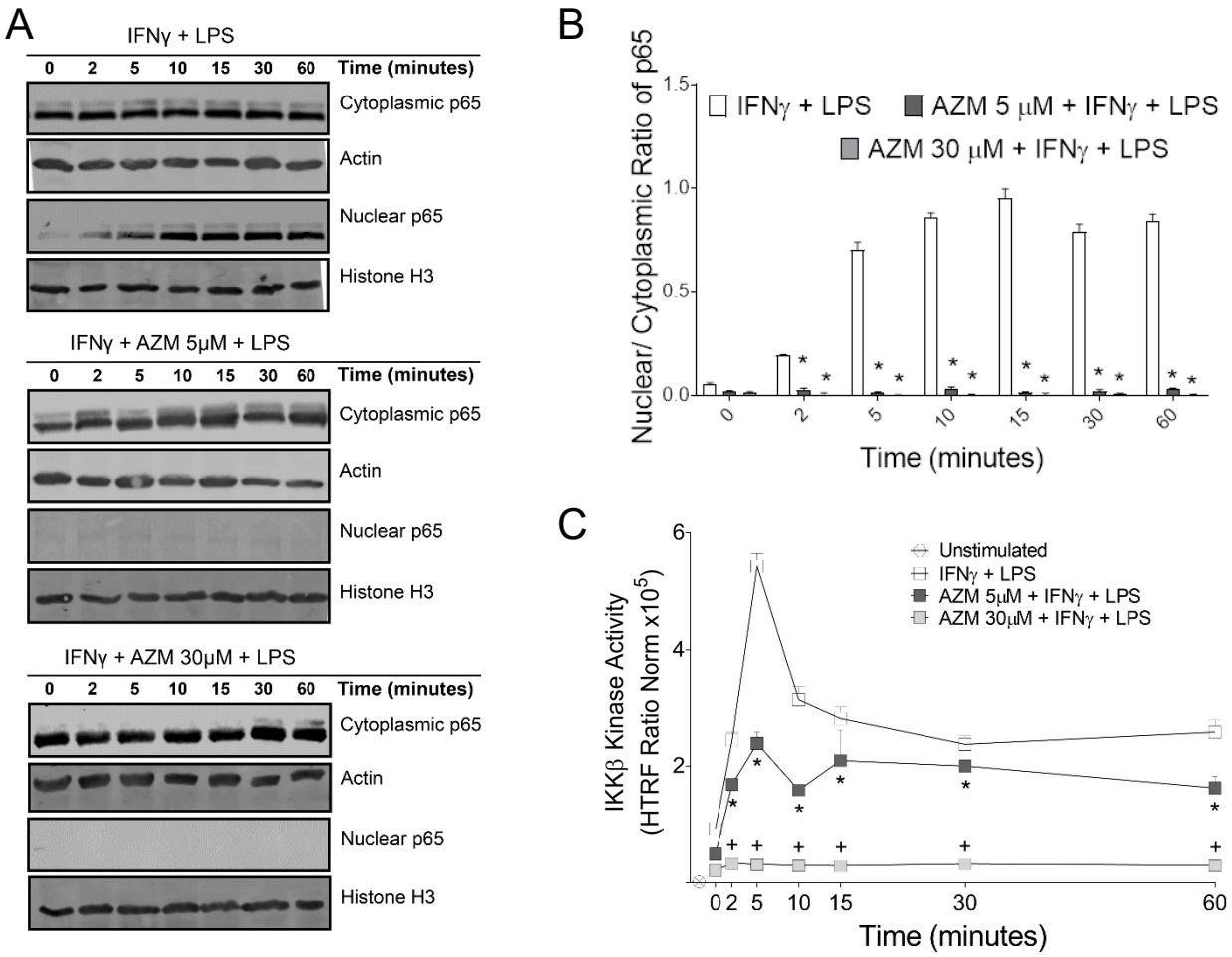
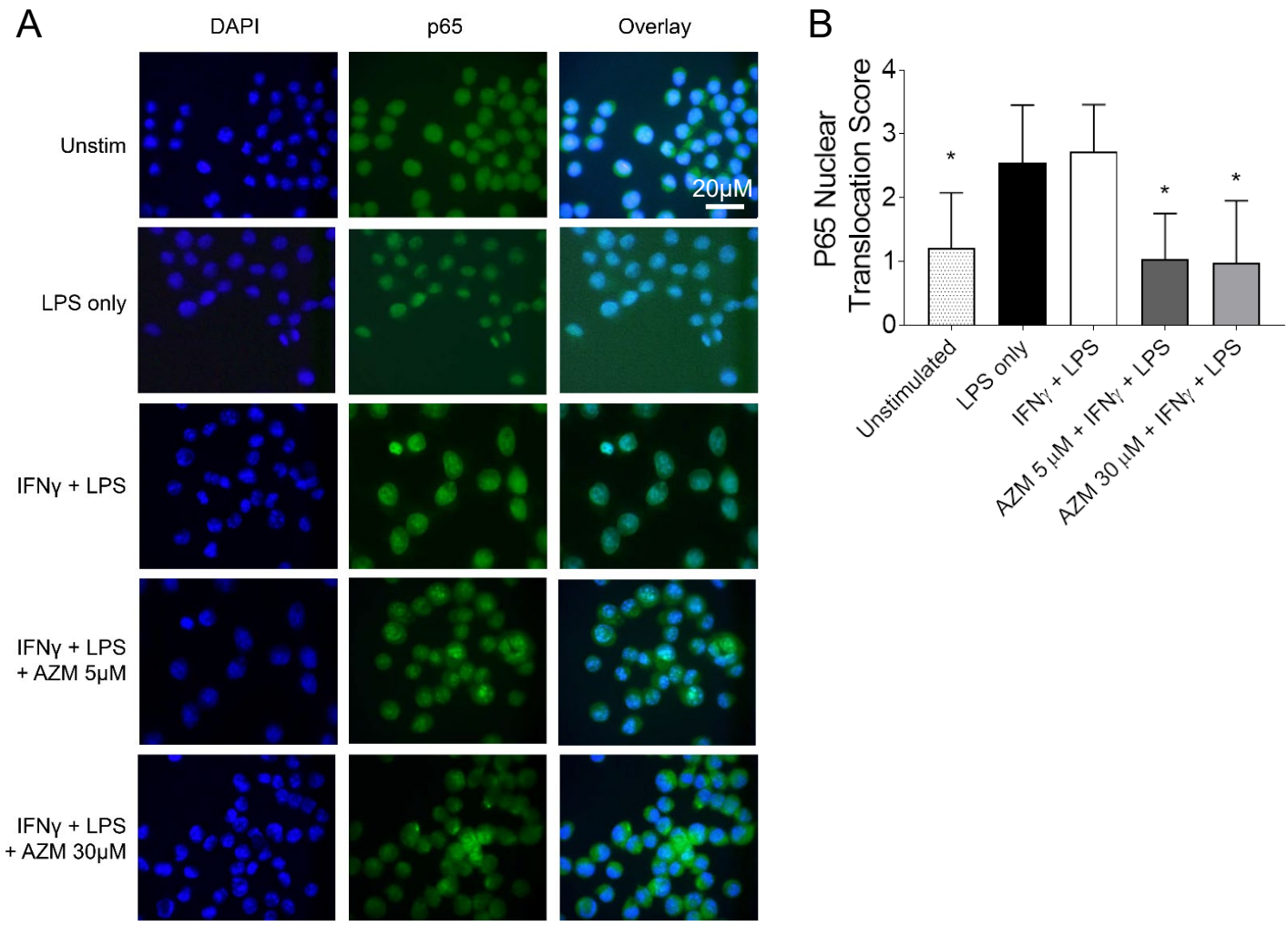
SUPPLEMENTAL FIGURE 1



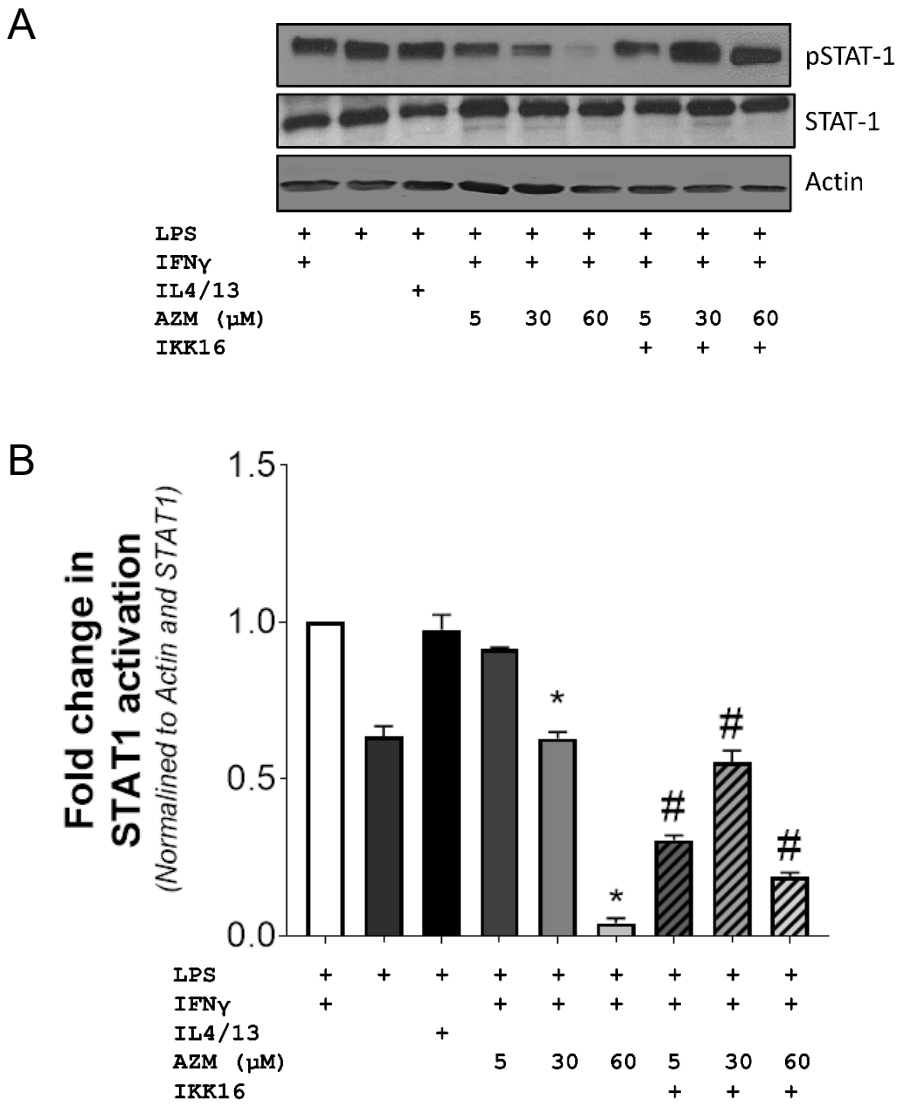
Azithromycin effects of the NF-κB signaling pathway in primary macrophages. Bone marrow derived macrophages (BMDM) were cultured in L929 media for 7 days. BMDMs were then plated at 2.5 x 10^5 cells per 1 ml of media in 24 well plates. Cells were allowed to attach for 8 hours and were then polarized overnight with IFNγ (50U/ml) alone or with azithromycin over a range of concentrations. Cells were then stimulated with LPS (10 nM) for durations ranging from 0 to 60 minutes. (A) Cells were harvested by scrapping and the nuclear and cytoplasmic fractions were separated. Western blots were performed to detect p65 in nuclear and cytoplasmic fractions. (B) Bar graph represents the ratio of nuclear to cytoplasmic fractions of p65 from (A). Nuclear fractions are normalized to Histone H3 and cytoplasmic fractions normalized to actin. Data is depicted as mean ± SD (p-value < 0.05 (*)). (C) Cells were lysed and proteins collected for HTRF assay. Graph represents change in IKKβ kinase activity over time post LPS stimulation normalized to total IKKβ levels. HTRF ratios were calculated from the fluorescense signals at 665nm and 620nm. Data represents mean ± %CV (p-value < 0.05 (*) AZM 5 μM; (+) AZM 30 μM compared to the IFNγ + LPS control) and is representative of 3 independent experiments. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test.

SUPPLEMENTAL FIGURE 2



NF-κB p65 subunit accumulates in the cytoplasm around the nuclear membrane in azithromycin treated macrophages. BMDMs were plated at 2.5 x 10^5 cells on round glass coverslips. Cells were allowed to attach to the glass and then polarized overnight with IFNγ (50U/ml) alone or along with azithromycin. Cells were then stimulated with LPS (10 nM) for 30 minutes. (A) Immunofluorescence staining for the p65 subunit of NF-κB at 100X. Images show NF-κB p65 subunits stained in green (FITC) overlayed with DAPI nuclear staining with a scale of 10 μm. (B) Bar graphs represent the nuclear vs cytoplasmic fractions of p65 quantified using a scoring system as follows: p65 signal in cytoplasm only (score 0), evenly distributed between the nucleus and cytoplasm (score 1), mostly nuclear with faint cytoplasmic signal (score 2), or nuclear signal only (score 3). Data depicts mean ± SD and is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA. AZM treated groups were compared to IFNγ treated group ((*) p-value < 0.05).

SUPPLEMENTAL FIGURE 3



Azithromycin effect on STAT-1 activation in primary macrophages. BMDMs were plated at 2.5 x 10^5 cells per 1 ml of media in 24 well plates. Cells were then polarized with IL4 and IL13 (10 nM each), IFNγ (50U/ml) alone, or with IFNγ plus azithromycin (5, 30, and 100 μM) with or without IKK-16 (100 nM). After overnight polarization cells were stimulated with LPS (10 nM) for 15 minutes and proteins were harvested by cell lysis. (A) Western blots for the active form phospho-STAT-1 and inactive STAT-1. (B) Bar graph represents fold change in STAT-1 phosphorylation under different polarization conditions compared to IFNγ and LPS stimulated macrophages (normalized to actin and STAT-1 levels). Data depicts mean ± SD and is representative of 3 independent experiments. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test ((*) denotes significant difference compared to IFNγ+LPS; (#) denotes significant difference compared to the corresponding AZM concentration with no IKK16 treatment; p-value < 0.05).