SUPPLEMENTARY MATERIAL

Supplementary Methods:

Proteasome Activity Assay:

Proteasome activity was evaluated in Jurkat and ILU-18 cells by employing a biotinylated proteasome activity profiling probe, Ada-Lys (biotinyl)-(Ahx) 3-(Leu) 3-vinyl sulphone (BIOMOL, PA), that specifically targets the catalytic sites of the proteasome. Cells, either untreated or following treatment with 0.25μM Aclacinomycin for 2h, were lysed in buffer containing 50mM Tris-HCl (pH 7.4), 5mM MgCl2, 250mM sucrose and 2mM ATP. Lysates equalized for protein (30μg) were labeled with 5μM biotinylated probe in a reaction buffer containing 50mM Tris-HCl (pH 7.4), 5mM MgCl2, 2mM ATP and 2mM DTT for 2h at 37 C. Labeling reactions were quenched by the addition of SDS-sample buffer and boiling for 5 min. Samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with streptavidin conjugated to horseradish peroxidase (HRP) and detected using Enhanced Chemiluminescence.

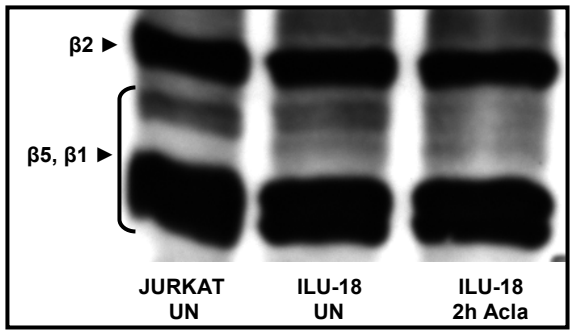
Re-ChIP:

ChIP procedure was performed as described in Materials and Methods, with the following modifications. ChIP dilution buffer used in immunoprecipitation was supplemented with 5mM NEM. Following initial immunoprecipitation with α-p65, beads were washed as described and eluted with Extraction buffer (TE Buffer, 12mM DTT, 5mM NEM, 1.6% SDS, protease inhibitor cocktail) by incubating at 37°C for one hour. Eluant was diluted 25X with ChIP Dilution buffer supplemented with 5mM NEM and 0.5mg of purified BSA and incubated overnight with FK2 Antibody. After precipitating the immune complexes with Salmon Sperm DNA/Protein G Agarose, beads were washed with High Salt wash buffer and TE buffer.

Immunoprecipitation of p65/RelA:

As described in Materials and Methods, treated and untreated ILU-18 cells were subjected to chromatin immunoprecipitation assay. Following the last wash of the IP procedure, p65-DNA complexes bound to protein G beads were resuspended in 30μL 2X SDS-Sample buffer. To reverse the crosslinks, the immunoprecipitates were boiled for 15 minutes. Boiled samples were resolved by SDS-PAGE followed by detection of p65 by Western blot. Immunoprecipitates obtained employing normal rabbit serum served as specificity control.

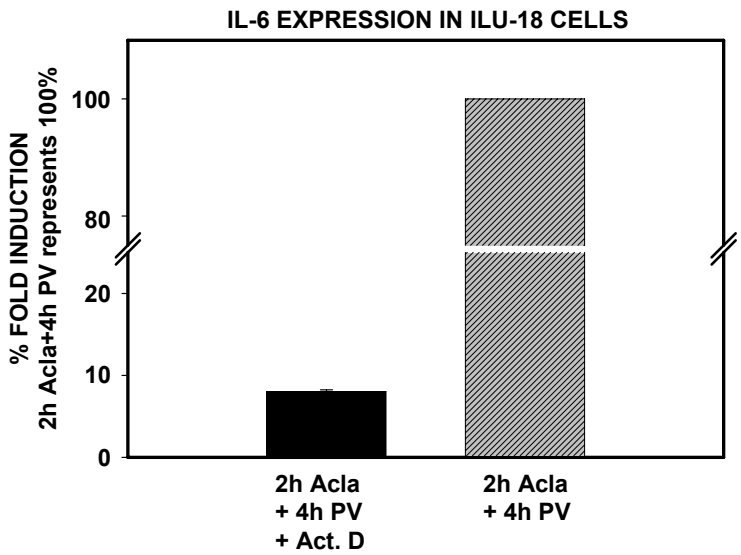
Figure S1



Proteasome inhibition by Aclacinomycin results in the loss of chymotryptic activity by specifically targeting the β5 subunit of the proteasome.

Lysates were obtained either from ILU-18 cells treated with 0.25μM Aclacinomycin for 2 hours (ILU-18, 2h Acla) or from ILU-18 and Jurkat cells left untreated (ILU-18, UN; Jurkat, UN). A two hour labeling experiment was then carried out with lysates containing 30μg protein and the proteasome active site probe (Biomol, PA). Determination of proteasome catalytic subunits targeted by Aclacinomycin was determined by SDS-PAGE and immunoblotting.

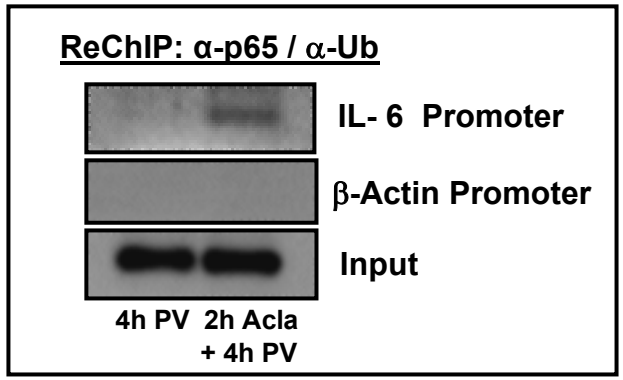
Figure S2



Proteasome-associated up-regulation of IL-6 expression by PV depends on *de novo* synthesis of IL-6 mRNA.

ILU-18 cells underwent pretreatment with Acla (0.25μM) for 2h, followed by PV treatment for 4h. Two hours into PV-stimulation, cells were either treated with Actinomycin D (10μg/mL) or left untreated. Impact of Actinomycin D treatment was assessed by isolation of total RNA and subsequent analysis by real-time PCR. Data are from two independent experiments. Values are represented as % Fold Induction. Expression of β-actin was used as an internal control for the analyses.

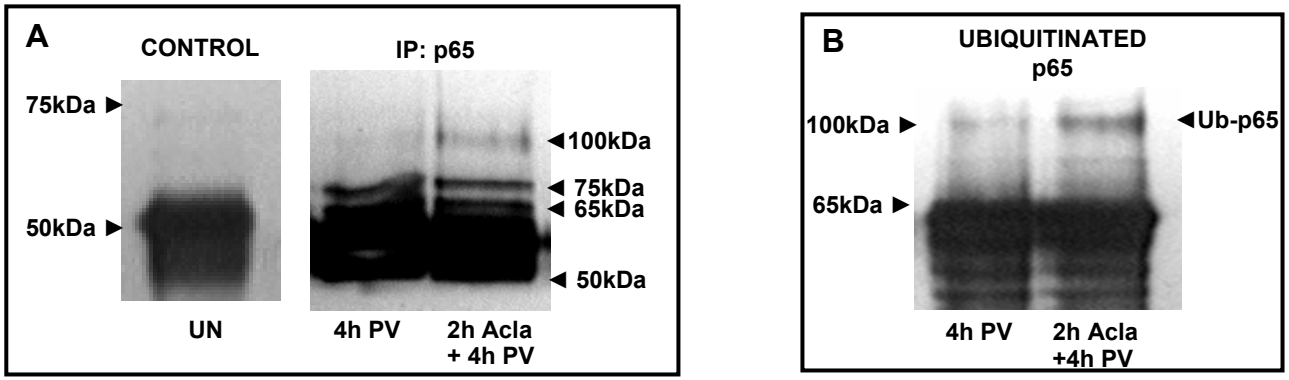
Figure S3



IL- 6 Promoter-associated p65/RelA is ubiquitinated.

ILU-18 cells were treated with PV (100μM) for 4 hours, with or without pretreatment with Acla (0.25μM) for 2 hours. Re-ChIP analysis employing p65 and FK2 antibodies was performed using immunoprecipitated DNA amplified with primers specific for a region of the *IL-6* promoter. To demonstrate ubiquitinated p65/RelA specificity to the *IL-6* promoter, immunoprecipitated DNA was also amplified with primers encompassing a region of the *β-Actin* promoter. Input samples are representative data generated from sonicated DNA fragments not subjected to immunoprecipitation and analyzed by PCR amplification with IL-6 promoter primers.

Figure S4



Proteasome inhibition results in the accumulation of ubiquitinated p65/RelA induced by PV treatment.

(A). ILU-18 cells were treated with 100μM pervanadate for 4 hours, with or without pretreatment with 0.25μM Aclacinomycin for 2 hours. By ChIP assay, p65-DNA complexes were obtained and then boiled for 15 minutes in 2X SDS-Sample buffer to reverse the crosslinks. The p65 subunit was then resolved by SDS-PAGE, followed by immunoblotting with an antibody specific to p65 and detection by ECL. Untreated cells were subjected to similar procedure and served as a control for the assay (UN).

(B). p65 obtained as in Panel A was analyzed by Western blot using antibody to Ubiquitin and detected by ECL.