

TRPA1 activation and Hsp90 inhibition synergistically downregulate macrophage activation and inflammatory responses *in vitro*.

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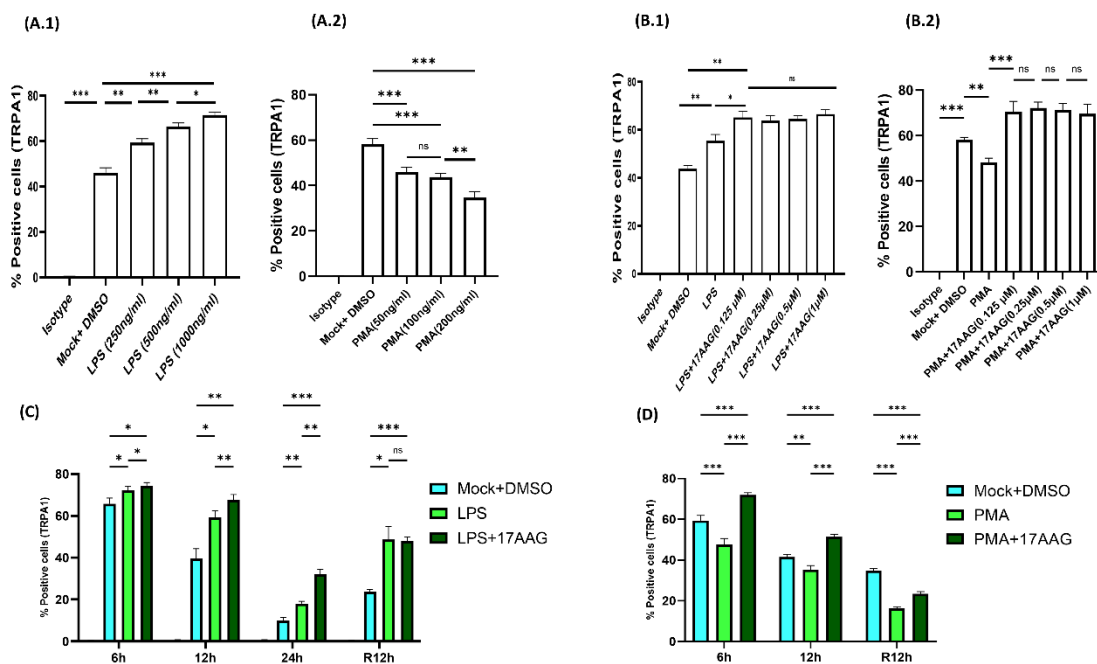
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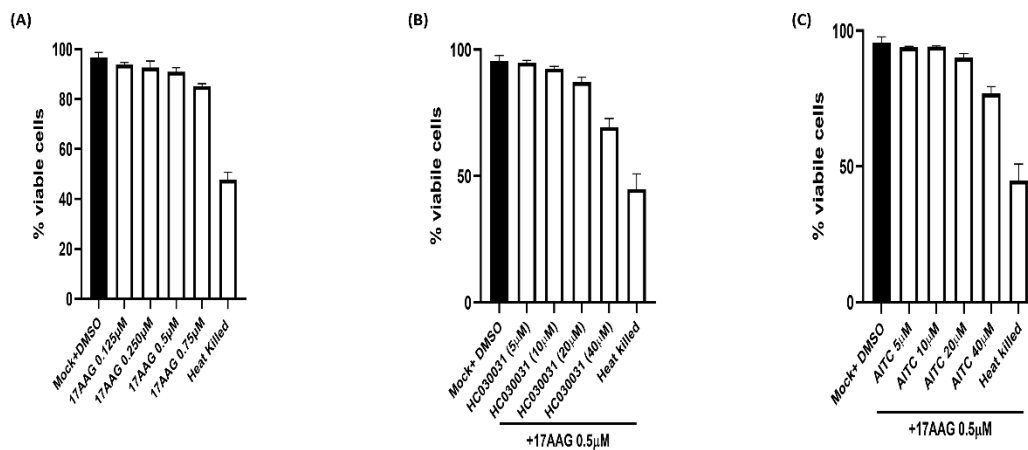
Equal contributions

† Corresponding author

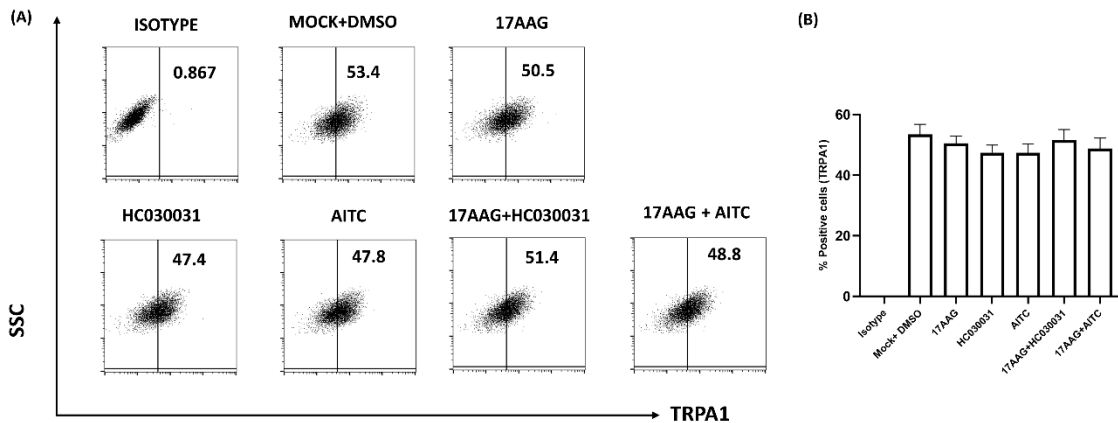
Supplementary information



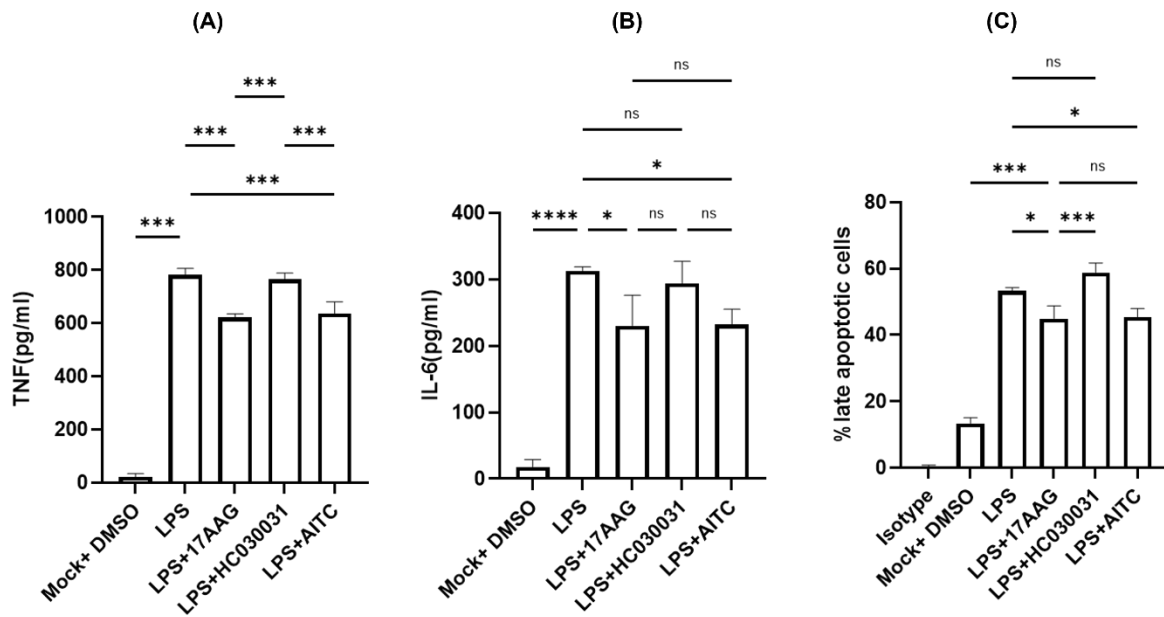
Supplementary fig.1 Dose and time kinetics of TRPA1 expression in LPS/PMA-stimulated and Hsp90-inhibited macrophages. RAW 264.7 cells were treated with different doses of LPS, PMA, and 17-AAG for 12 h. (A) Bar graph representing the percentage of positive cells for TRPA1 with different doses of LPS (A.1) / PMA (A.2). (B) Bar graph representing the percentage of positive cells for TRPA1 for differential doses of 17-AAG + LPS (500 ng) (B.1) / PMA (100 ng) (B.2). (C) Bar graph comparing the percentage of positive cells for TRPA1 in the presence of LPS or LPS + 17AAG condition or (D) PMA, PMA + 17-AAG at different time points. R represents time points after the reversal of conditions (replacing the existing culture media with fresh media containing only DMSO). The data represent the mean \pm SD of three independent experiments. One-way ANOVA was used for A and B, and two-way ANOVA was used for C. Differences between groups with a p-value less than 0.05 were considered statistically significant (ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



Supplementary fig.2 Cell viability assay for TRPA1 modulators (AITC and HC-030031) in Hsp90-inhibited macrophages. RAW 264.7 cells were treated with different doses of 17-AAG or AITC and HC-030031 in the presence of 0.5 µM of 17-AAG. Cells were harvested at 24 h and immediately stained with 7-AAD and analyzed via FC. (A) Bar graph showing the percentage of viable RAW 264.7 cells at different doses of 17-AAG. (B) Bar graph representing the percentage of viable RAW 264.7 cells at different doses of AITC (B) and (C) HC-030031 in Hsp90-inhibited macrophages. The data represent the mean ± SD of three independent experiments. One-way ANOVA has been performed for statistical significance analysis. Differences between groups with a p-value < 0.05 were considered statistically significant (***, p < 0.001).



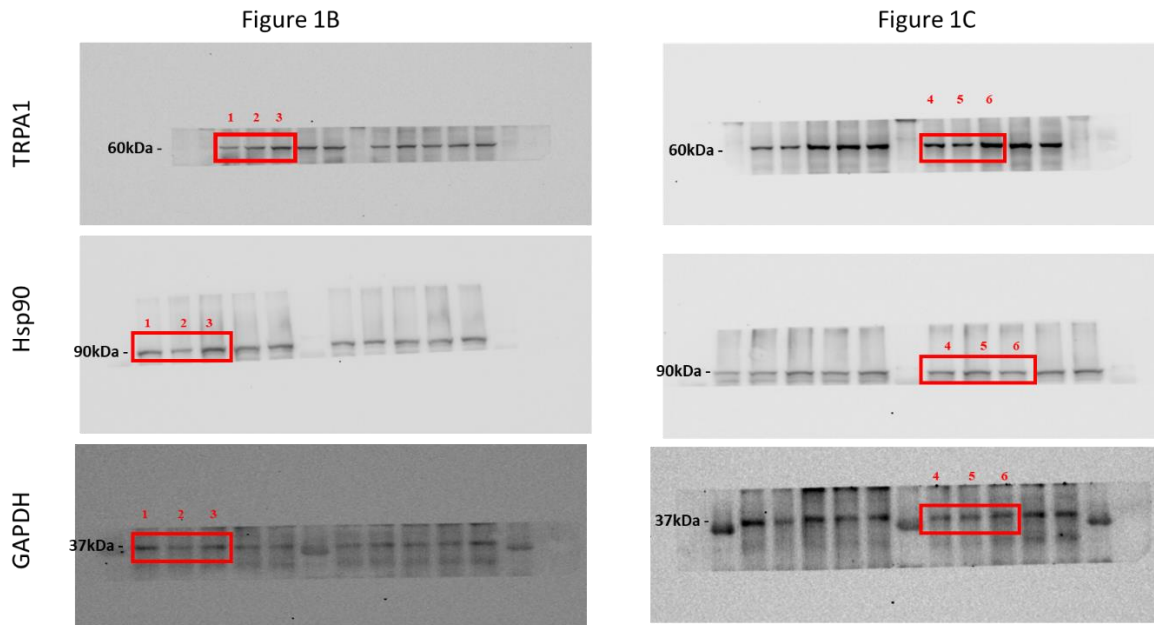
Supplementary fig.3 Expression levels of TRPA1 in the presence of TRPA1 modulators alone or in combination with 17-AAG in RAW 264.7 cells. Supplementary fig.3 Expression levels of TRPA1 in the presence of TRPA1 modulators alone or in combination with 17-AAG in RAW 264.7 cells. (A) FC dot-plot depicting the percentage of positive cells for TRPA1 in differential concentrations of AITC and HC-030031 alone or with 17-AAG and (B) representative bar graph showing the same. The data represent the mean ± SD of three independent experiments. One-way ANOVA has been performed to find statistical significance among groups. Differences between groups with a p-value < 0.05 were considered statistically significant.



Supplementary fig.4 Effect of TRPA1 modulators in the absence of Hsp90 inhibitor in regulating LPS-induced inflammation. Bar graph representing the quantity of TNF (A) and IL-6 (B) production in RAW 264.7 cells under differential conditions of TRPA1 modulator and LPS at 12 h. (C) bar graph representing the % of late apoptotic cells from RAW 264.7 cells under differential conditions of TRPA1 modulator and LPS at 24 h. The data represent the mean \pm SD of three independent experiments. One-way ANOVA has been performed to find statistical significance among groups. Differences between groups with a p-value less than 0.05 were considered statistically significant (ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Western blot RAW images:

➤ **Figure 1 of the manuscript:**

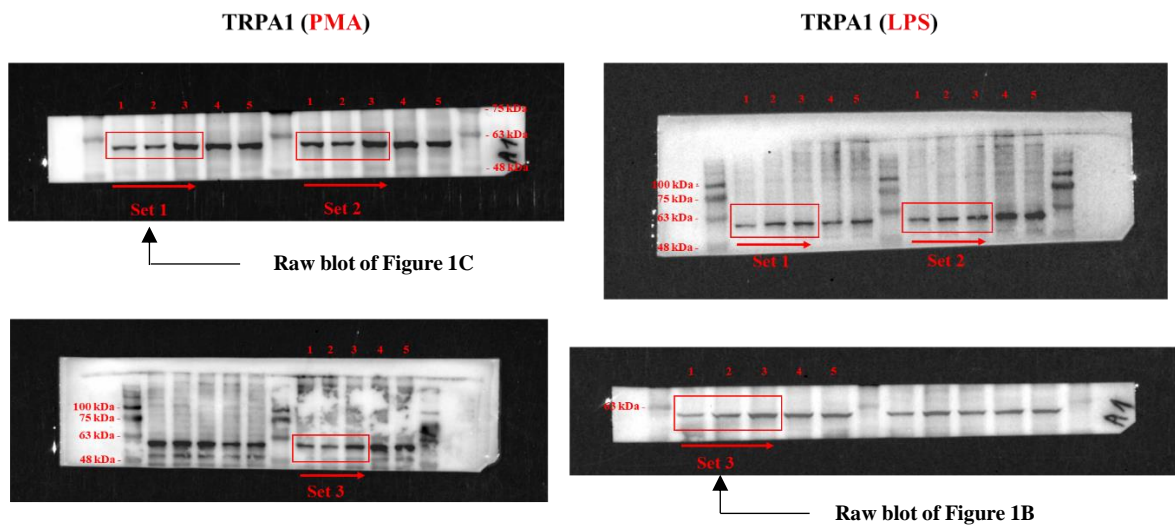


Lanes:

1 – Mock, 2 – LPS, 3 – LPS+ 17-AAG, 4 – Mock, 5 – PMA, 6 – PMA+ 17-AAG

Fig: Raw images for Figures 1 B and 1 C.

➤ **TRPA1 Replicates:**



Lanes:

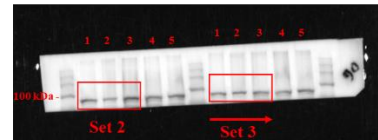
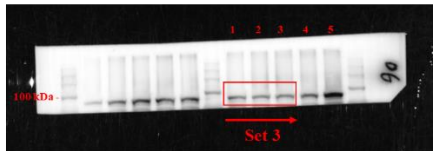
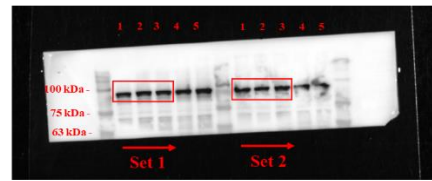
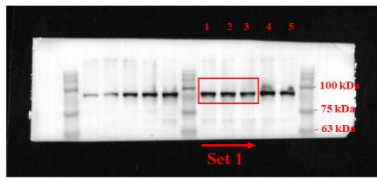
1 – Mock, 2 – LPS/PMA, 3 – LPS/PMA + 17-AAG, 4 – LPS/PMA + 17-AAG + HC, 5 – LPS/PMA + 17-AAG + AITC

Fig: Raw images of TRPA1 replicates.

➤ **Hsp90 Replicates:**

Hsp90 (PMA)

Hsp90 (LPS)



Raw blot of Figure 1C

Raw blot of Figure 1B

Lanes:

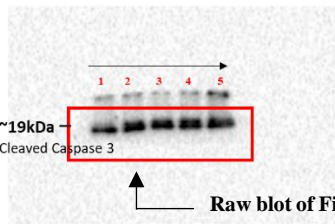
1 – Mock, 2 – LPS/PMA, 3 – LPS/PMA + 17-AAG, 4 – LPS/PMA + 17-AAG + HC, 5 – LPS/PMA + 17-AAG + AITC

Fig: Raw images of Hsp90 replicates.

➤ **Figure 6 of the manuscript:**

Figure 6 D (LPS)

Figure 6 E (PMA)

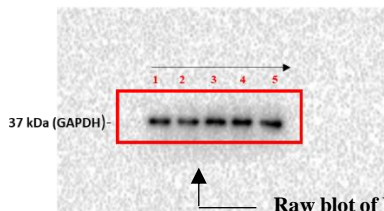


Cleaved Caspase 3

~19kDa-
Cleaved Caspase 3

Raw blot of Figure 6D

Raw blot of Figure 6E



GAPDH

37 kDa (GAPDH)-

Raw blot of Figure 6D

Raw blot of Figure 6E

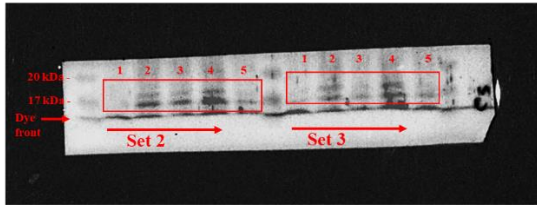
Lanes:

1 – Mock, 2 – LPS/PMA, 3 – LPS/PMA + 17-AAG, 4 – LPS/PMA + 17-AAG + HC, 5 – LPS/PMA + 17-AAG + AITC

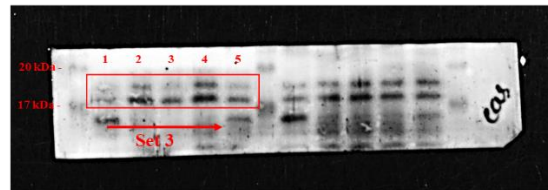
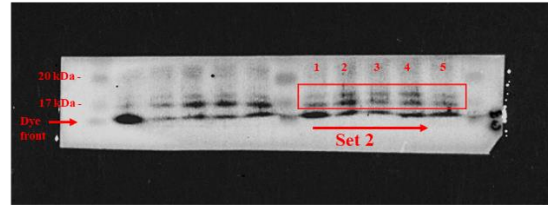
Fig: Raw images of Figures 6 D and 6E.

➤ **Cleaved Caspase 3 Replicates:**

Cleaved caspase 3 (PMA)



Cleaved caspase 3 (LPS)



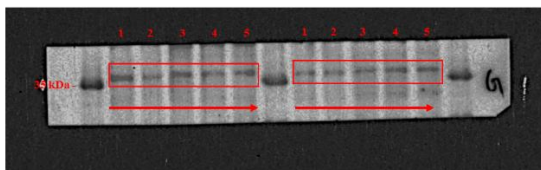
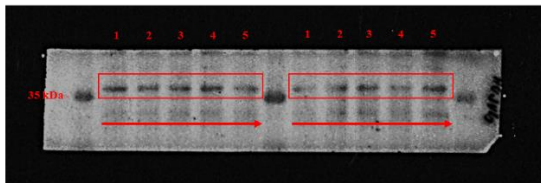
Lanes:

1 – Mock, 2 – LPS/PMA, 3 – LPS/PMA + 17-AAG, 4 – LPS/PMA + 17-AAG + HC, 5 – LPS/PMA + 17-AAG + AITC

Fig: Raw images of cleaved caspase 3 replicates.

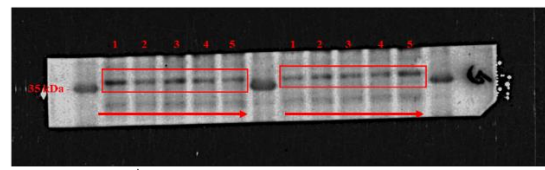
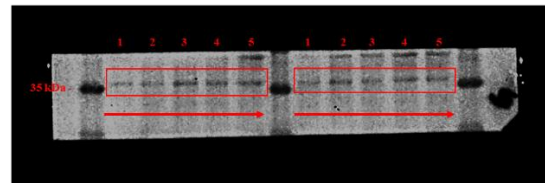
➤ **GAPDH Replicates:**

GAPDH (PMA)



Raw blot of Figure 1C

GAPDH (LPS)



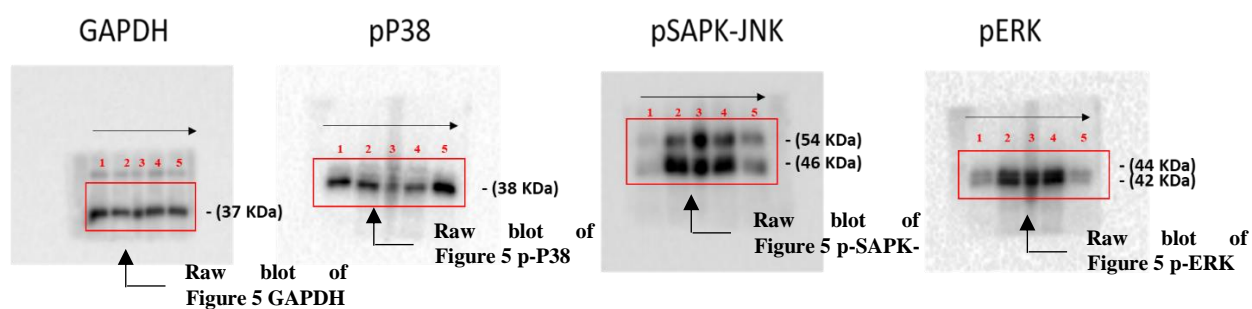
Raw blot of Figure 1B

Lanes:

1 – Mock, 2 – LPS/PMA, 3 – LPS/PMA + 17-AAG, 4 – LPS/PMA + 17-AAG + HC, 5 – LPS/PMA + 17-AAG + AITC

Fig: Raw images of GAPDH replicates.

➤ **Figure 5 of the manuscript:**



Lanes:

1 – Mock, 2 – LPS/PMA, 3 – LPS + 17-AAG, 4 – LPS + 17-AAG + HC, 5 – LPS + 17-AAG + AITC

Fig: Raw images of p-P38, p-SAPK-JNK and p-ERK.

➤ **p-P38 Replicates:**

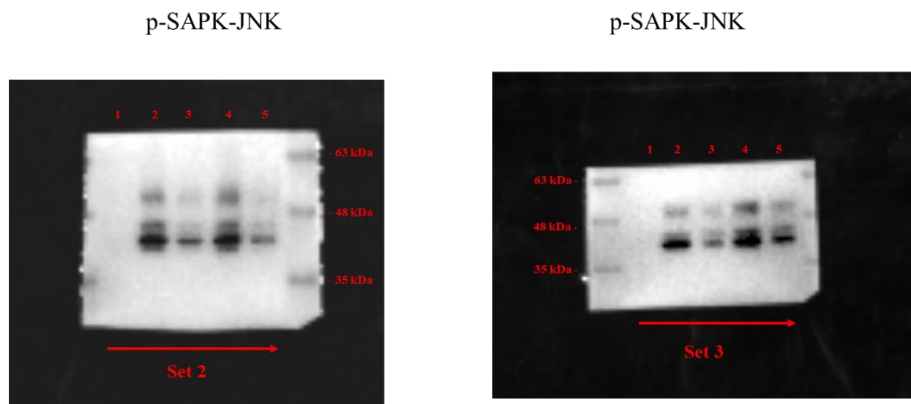


Lanes:

1 – Mock, 2 – LPS, 3 – LPS + 17-AAG, 4 – LPS + 17-AAG + HC, 5 – LPS + 17-AAG + AITC

Fig: Raw images of p-P38 replicates.

➤ **p-SAPK-JNK Replicates:**



Lanes:
1 – Mock, 2 – LPS, 3 – LPS + 17-AAG, 4 – LPS + 17-AAG + HC, 5 – LPS + 17-AAG + AITC

Fig: Raw images of p-SAPK-JNK replicates.

➤ **p-ERK Replicates:**



Lanes:
1 – Mock, 2 – LPS, 3 – LPS + 17-AAG, 4 – LPS + 17-AAG + HC, 5 – LPS + 17-AAG + AITC

Fig: Raw images of p-ERK replicates.

➤ **GAPDH Replicates:**



Lanes:

1 – Mock, 2 – LPS, 3 – LPS + 17-AAG, 4 – LPS + 17-AAG + HC, 5 – LPS + 17-AAG + AITC

Fig: Raw images of GAPDH replicates.

➤ **Table S1: Change in Fluo-4 AM intensity in RAW 264.7 cells with time in presence of different experimental treatments.**

Time(s)	Mock	LPS	Ionomycin	LPS+17AAG	LPS+17AAG+HCO 30031	LPS+17AAG+AITC	LPS+HC030031	LPS+AITC
	Mean ± standard deviation							
0-20	1.00 ± 0.000	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 0.00	1.00 0.00
20-40	0.98 ± 0.020	2.38 ± 0.58	1.41 ± 0.02	0.94 ± 0.12	0.91 ± 0.12	1.09 ± 0.12	1.03 0.07	1.59 0.15
40-60	0.98 ± 0.052	1.73 ± 0.24	1.58 ± 0.07	0.98 ± 0.07	0.90 ± 0.10	1.15 ± 0.17	1.11 ± 0.12	1.59 ± 0.12
60-80	0.94 ± 0.040	1.37 ± 0.38	1.44 ± 0.04	0.95 ± 0.06	0.83 ± 0.06	1.22 ± 0.07	1.09 ± 0.12	1.48 ± 0.10
80-100	0.97 ± 0.043	1.27 ± 0.37	1.38 ± 0.03	0.95 ± 0.06	0.84 ± 0.07	1.26 ± 0.08	1.12 ± 0.12	1.43 ± 0.19
100-120	0.98 ± 0.020	1.25 ± 0.40	1.21 ± 0.14	0.95 ± 0.03	0.82 ± 0.03	1.30 ± 0.11	1.09 ± 0.13	1.32 ± 0.25
120-140	0.97 ± 0.035	1.24 ± 0.32	1.22 ± 0.21	0.96 ± 0.07	0.84 ± 0.07	1.29 ± 0.10	1.12 ± 0.18	1.14 ± 0.11
140-160	0.92 ± 0.058	1.31 ± 0.25	1.21 ± 0.20	0.95 ± 0.04	0.83 ± 0.05	1.29 ± 0.10	1.12 ± 0.16	1.24 ± 0.01
160-180	0.95 ± 0.025	1.14 ± 0.15	1.26 ± 0.05	0.97 ± 0.02	0.90 ± 0.04	1.30 ± 0.11	1.13 ± 0.21	1.35 ± 0.18
180-200	0.95 ± 0.020	1.07 ± 0.11	1.21 ± 0.02	1.00 ± 0.02	0.85 ± 0.03	1.34 ± 0.10	1.10 ± 0.15	1.14 ± 0.11

Table S1: Fluo-4 AM MFI values obtained from RAW 264.7 cells under each experimental conditions and time interval