Supplement

Supplement methods

Antibodies and reagents. Antibodies detecting p-IKKα/β (#2697S) and p-IRF3 (#4947S), p-JNK1/2 (#9251), p-STAT3 (#9145) and p-ERK1/2 (#4377S) were from Cell Signaling Technology. Anti-β-actin (ab49900), anti-STK4 (ab51134) and anti-CD68 (ab955) were from Abcam. Myc-tag (19C2) mouse Ab (M20002) was from Abmart and anti-TLR4 (BS3489) was from Bioworld. Anti-p65 (sc-372) and anti-IRAK1 (sc-7883) were from Santa Cruz Biotechnology. LPS, IRAK1/4 inhibitor (I5409-25MG), anti-HA (H3663) and anti-GAPDH (G9295) were from Sigma. APC-anti-F4/80 (17-4801-82), FITC-anti-CD11b (11-0112-82), FITC-anti-B220 (11-0452-82), APC-anti-CD3 (17-0031-81), PE-anti-CD14 (12-0149-42), FITC-anti-NK1.1 (11-5941-81), PE-anti-rabbit-IgG (12-4739-81), APC-anti-CD11b (17-0118-42), PerCP-Cyanine5.5-anti-Ly-6C (45-5932-80) anti-CD284/MD-2 complex (558293) and anti-F4/80 (14-4801-81) were from eBioscience. PerCP-Cy^{TM}5.5-anti-p-STAT3 (560114) and PE-Cy^{TM}7-anti-phospho-p65 (560335) were from BD Biosciences. FACS Calibur, LSR II, Aria II (BD Biosciences) and FlowJo software (Tree Star) were used for FACS assay. Poly(I:C) HMW and type B oligonucleotide CpG were from InvivoGen. Recombinant mM-CSF was from R&D Systems. Protein G beads were from GE healthcare. Elisa kits for IL-6, TNF, IL-1β or STK4 were from eBioscience or China Haling Biotech.

Plasmids, transfection and luciferase assays. Myc-tagged STK4 and K59R, STK4-ΔC were cloned into the pMX-IRES-GFP vector. IRF-7, IRAK1-HA and TBK1-HA were cloned into

the pCDNA3.1 vector. Myc-STK4 or its mutants and the retroviral packaging plasmid pCL-10A were cotransfected into HEK293T cells, and supernatants were collected to primary MEF cells or RAW264.7 cells to generate stable transfected cells. Plasmids expressing Flag-tagged TRIF or pRL-TK-Renilla were kindly provided by Drs. B. Sun, and R. Hu (Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academic of Sciences, Shanghai 200031, China). HEK293T cells were transfected with plasmids expressing STK4 or K59R mutant and TRIF, TBK1, IRF-7, or IRAK1, together with pRL-TK-Renilla and the IFN-β luciferase reporter plasmids. After 24 hrs, luciferase readings were determined with a Dual-Luciferase Reporter Assay (Promega).

RNA interference, Real-time PCR and Sequence analysis. siRNA oligos targeting mouse *Stk4* (sense: 5'-GGGACUAGAAUACCUUCAUTT-3'; antisense: 5'-AUGAAGGUAUUCUAGUCCCTT-3', GenePharma) or mouse IRAK1 (purchased from Dharmacon) were transfected into murine PEMs using Lipofectamine2000 (Invitrogen). Cellular RNA was extracted with Trizol reagent (Sigma) and cDNA was generated with M-MLV transcriptase (Promega) for quantitative real-time PCR (RT-PCR) assay using SYBR Green Dye (2043, DBI Bioscience) on a CFX-96 detection system (Bio-Rad). cDNA from human HCC samples was synthesized by the MMLV reverse transcriptase (Promega) using an oligo (dT) primer. Human p53 and STAT3 were then amplified from cDNA samples by PCR, followed by DNA sequence.

RNA microarray. RNA of liver tissue from HCC patients was extracted and prepared for Affymetrix RNA microarrays. Samples were divided into two groups according to the expression levels of IL6 and STK4, i.e., IL-6^{hi}STK4^{lo} and IL-6^{lo}STK4^{hi}. The expression scores of annotation files were supplied by the manufacturer and gene signature comparisons were performed between the two groups.

Immunoprecipitation, immunoblotting and Immunostaining. Cells were lysed in 1% NP-40 lysis buffer (50 mM Tris-HCl, 1% Nonidet-P40, 0.1% SDS, 150 mM NaCl) together with fresh prepared protease inhibitors NaF, Na_3VO_4, PMSF and cocktails followed by centrifugation. Supernatants were incubated with the indicated antibodies (1ug) and protein G beads. Proteins in the pull-down samples were separated by 8-12% SDS-PAGE, followed by immunoblotting analysis with the indicated antibodies. β-actin was used as an internal control. For immunostaining, LPS-stimulated MEFs were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, then stained with anti-p65 and Hoechst. Images were taken under Olympus BX-81 microscopy.

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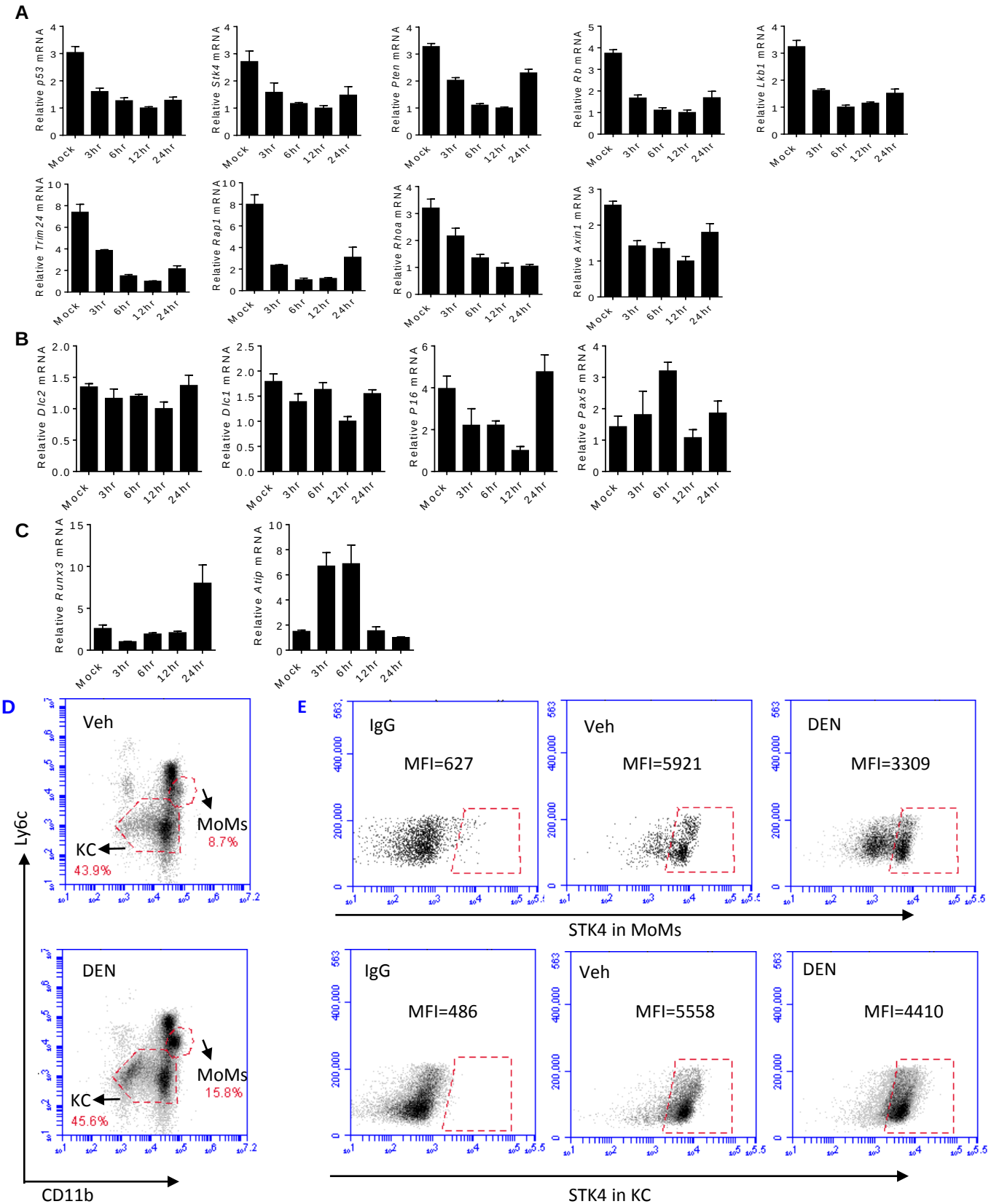
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Relative p53 mRNA
Relative Stk4 mRNA
Relative Pten mRNA
Relative Rb mRNA
Relative Lkb1 mRNA
Relative Trim24 mRNA
Relative Rap1 mRNA
Relative Rhoa mRNA
Relative Axin1 mRNA
Mock
3hr
6hr
12hr
24hr
B
Relative Dlc2 mRNA
Relative Dlc1 mRNA
Relative P16 mRNA
Relative Pax5 mRNA
C
Relative Runx3 mRNA
Relative Atip mRNA
D
Veh
MoMs
8.7%
KC
43.9%
DEN
MoMs
15.8%
KC
45.6%
Ly6c
CD11b
E
IgG
MFI=627
Veh
MFI=5921
DEN
MFI=3309
STK4 in MoMs
IgG
MFI=486
Veh
MFI=5558
DEN
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STK4 in KC

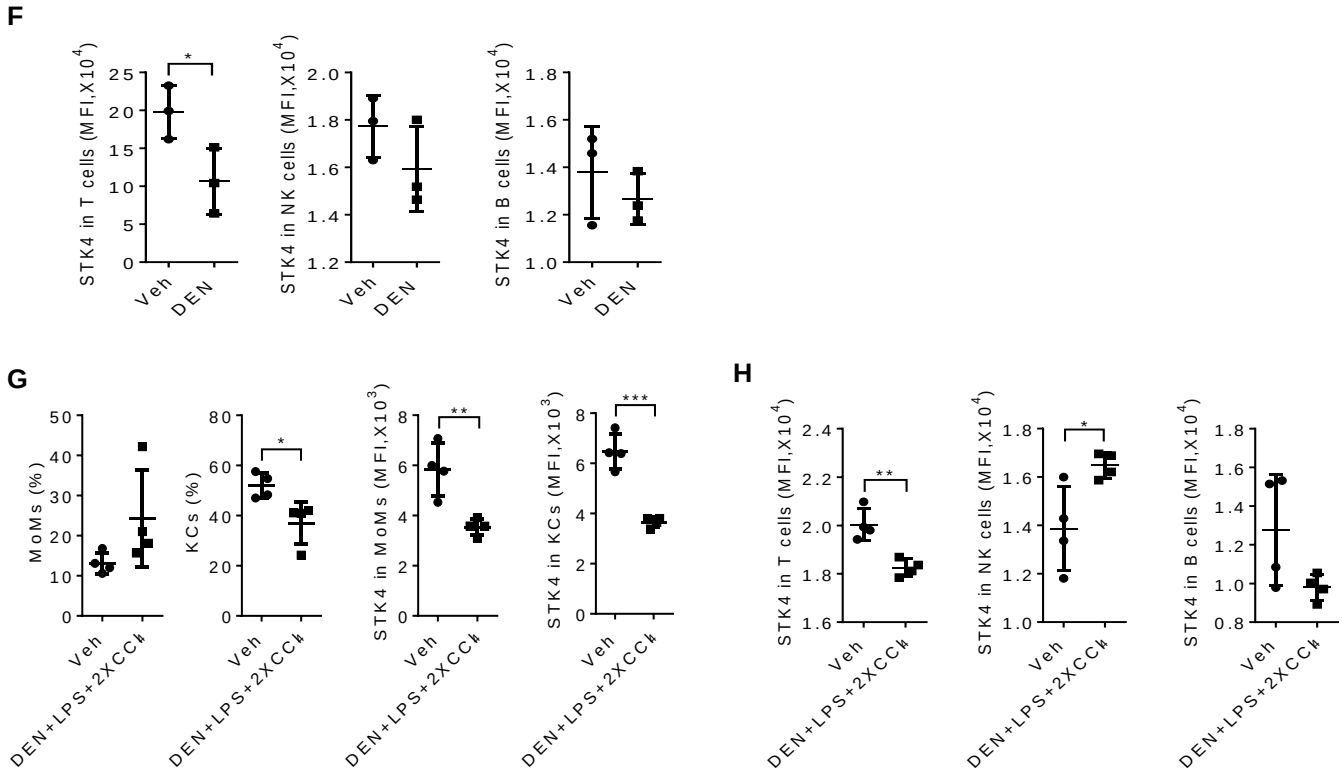


Figure S1, related to Figure 1. (A-C) Wild-type PEMs were infected with 1x10^9 CFU/ml *E.coli* for 30 mins. After removing *E.coli*, cells were further cultured for different time points to determine mRNA levels of tumor suppressor genes by qRT-PCR. Values were normalized for ***β-actin*** mRNA levels (n=2). (D, E) Percentages of intrahepatic MoMs (F4/80$^+$CD11b^{hi}Ly6c^{int}) or KCs (F4/80$^+$CD11b^{lo}Ly6c^{lo}), and STK4 expression levels were analyzed by FACS. (F) Wild-type male mice were treated with controls (Veh) or DEN for 11 months to induce HCC (n=3). STK4 expression in intrahepatic lymphocytes from Veh- and DEN-treated mice was examined by FACS. (G, H) Wild-type mice were treated with control (Veh) or DEN plus two weekly injections of CCl_4 and daily injection of LPS (300 μg/kg, starting 1 week before the first CCl_4 injection) to check percentages of immune cells in livers or STK4 expression levels (n>=4). *P $<$ 0.05 ; **P $<$ 0.01, 2-tailed, unpaired Student's t test.

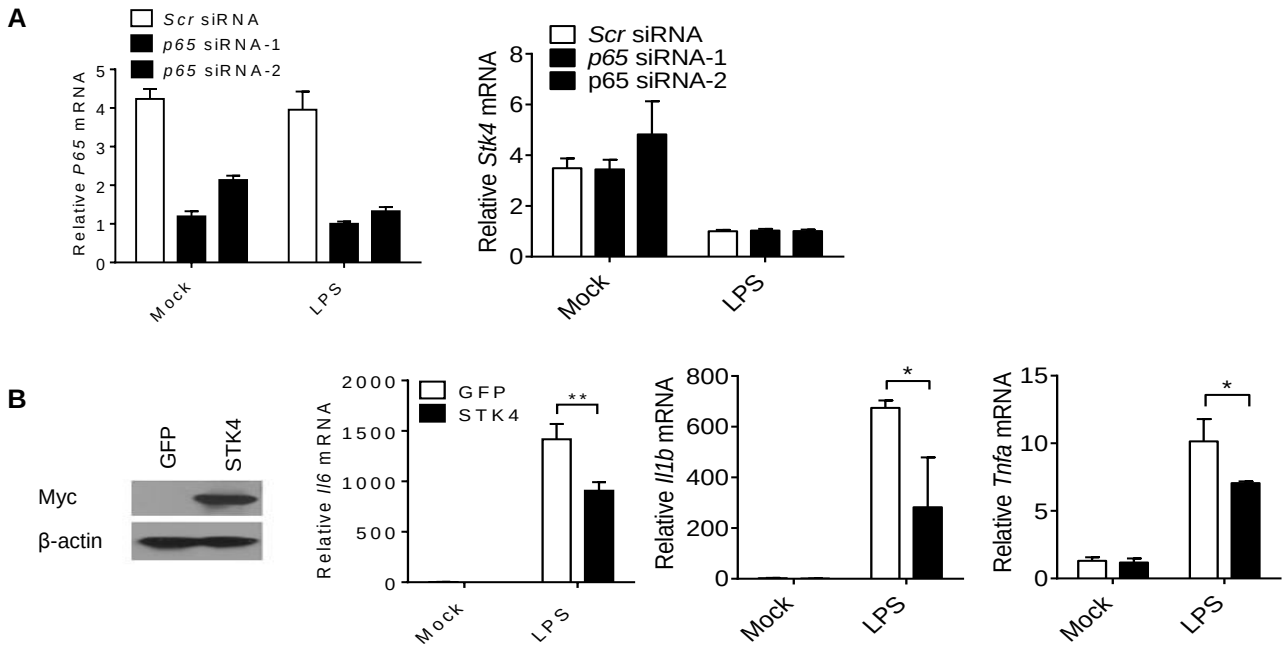


Figure S2 , related to Figure 2. (A) Peritoneal exudate macrophages (PEMs) were transfected with the *Scramble* control siRNA or two specific *p65* siRNAs, then treated with 1 μg/ml LPS for 6 hrs to evaluate mRNA levels of *p65* or *Stk4* by RT-PCR. (B) Myc-tagged STK4 or GFP was overexpressed in RAW264.7 cells followed by immunoblotting using anti-Myc antibody. After LPS treatment, mRNA level of *Il6*, *Il1b* and *Tnfa* were assessed. Data represent mean ± SD (n>=3). 2-tailed, unpaired Student's t test. *P < 0.05 ; **P < 0.01.

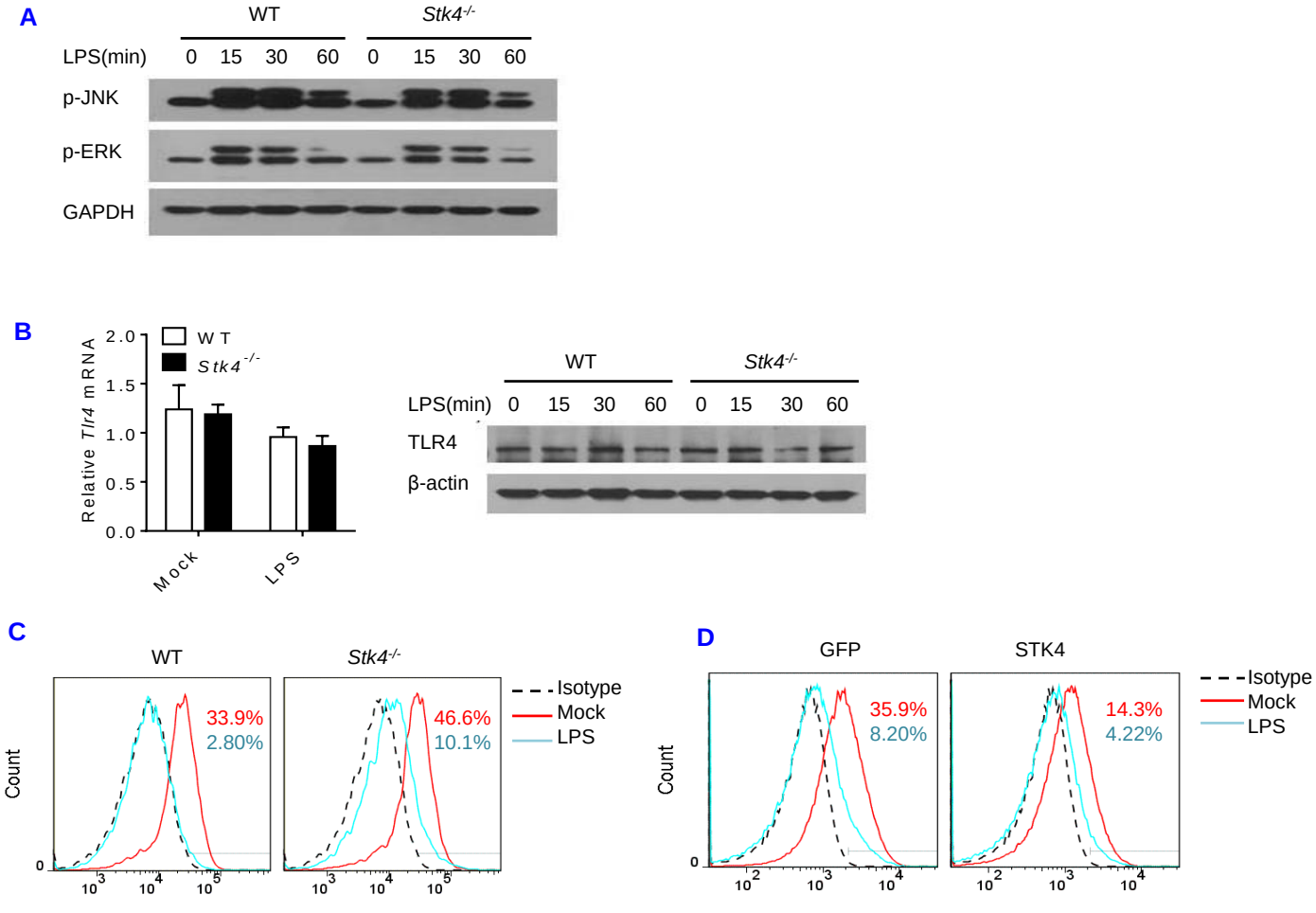


Figure S3, related to Figure 3. (A) The levels of phosphorylated JNK or ERK & (B) TLR4 expression were detected by immunoblotting or RT-PCR in LPS-stimulated wild-type and *Stk4*$^{-/-}$ macrophages (mean ± SD, n=4, *P < 0.05, 2-tailed, unpaired Student's t test). Surface TLR4 levels were detect by FACS in (C) LPS-treated wild-type and *Stk4*$^{-/-}$ PEMs, or in (D) LPS-treated RAW264.7 cells overexpressing GFP and STK4. Data represent the representative experiments from at least three independent experiments.

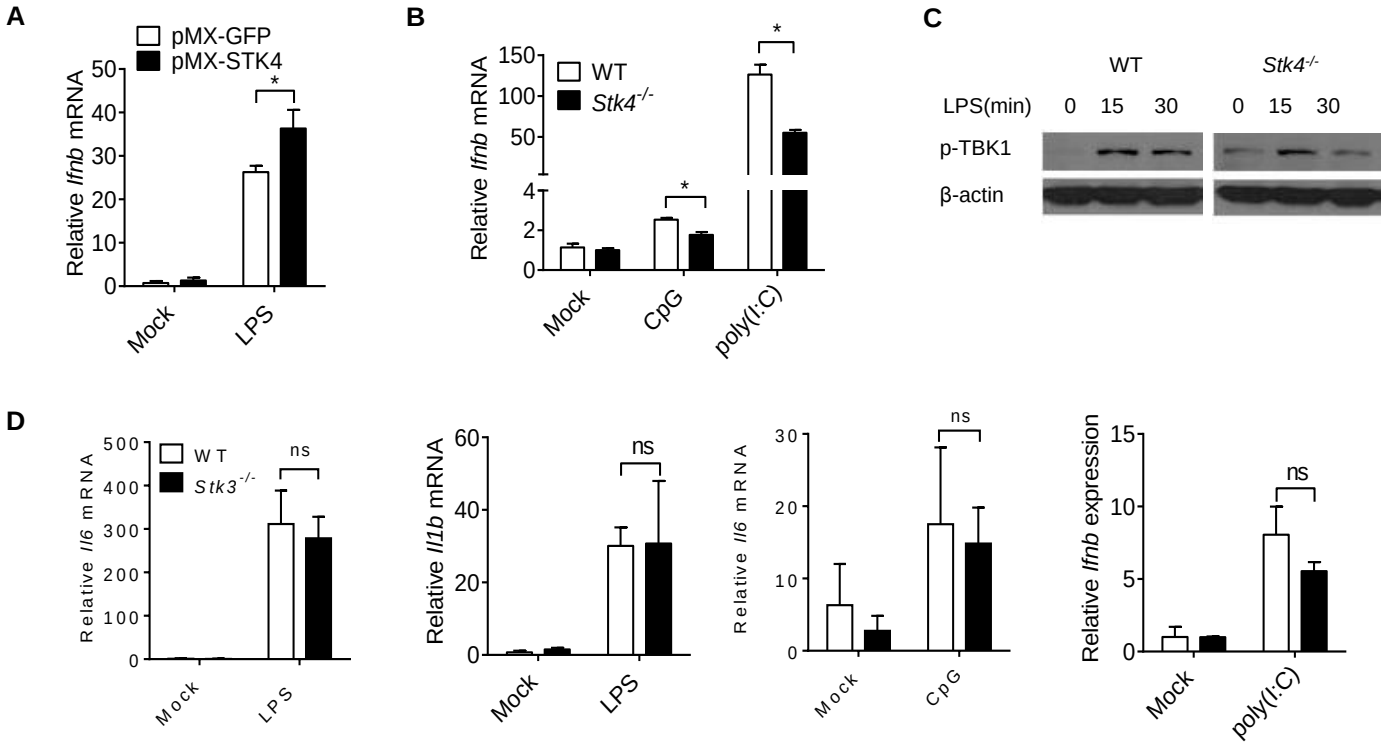


Figure S4, related to Figure 4. *Ifnb* mRNA levels were assessed in (A) LPS-treated RAW264.7 cells overexpressing GFP or STK4 & in (B) 2μg/ml CpG ODN-stimulated or 10 μg/ml poly(I:C)-stimulated wild-type or *Stk4*$^{-/-}$ macrophages. (C) The phosphoTBK1 levels in LPS-stimulated wild-type or *Stk4*$^{-/-}$ BMMs were checked by immunoblotting analysis. Samples were run on the same gel but were not contiguous as indicated by the black line. n=2 (D) Wild-type and *Stk3* deficient macrophages were stimulated with 1 μg/ml LPS, 2μg/ml CpG ODN and 10 μg/ml poly(I:C) for 6 hrs to analyze mRNA levels of *Il6*, *Il1b* and *Ifnb*.
(A, D) Data represent mean ± SD (n>=3). *P < 0.05, 2-tailed, unpaired Student's t test.

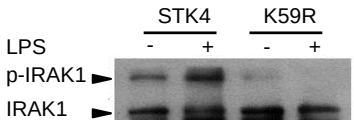


Figure S5, related to Figure 5. Immunoprecipitation with anti-IRAK1 was performed from LPS-stimulated RAW264.7 cells which over-expressed STK4 or K59R, followed by immunoblotting with anti-IRAK1 antibody. Data represent of the representative experiments from at least two independent experiments.

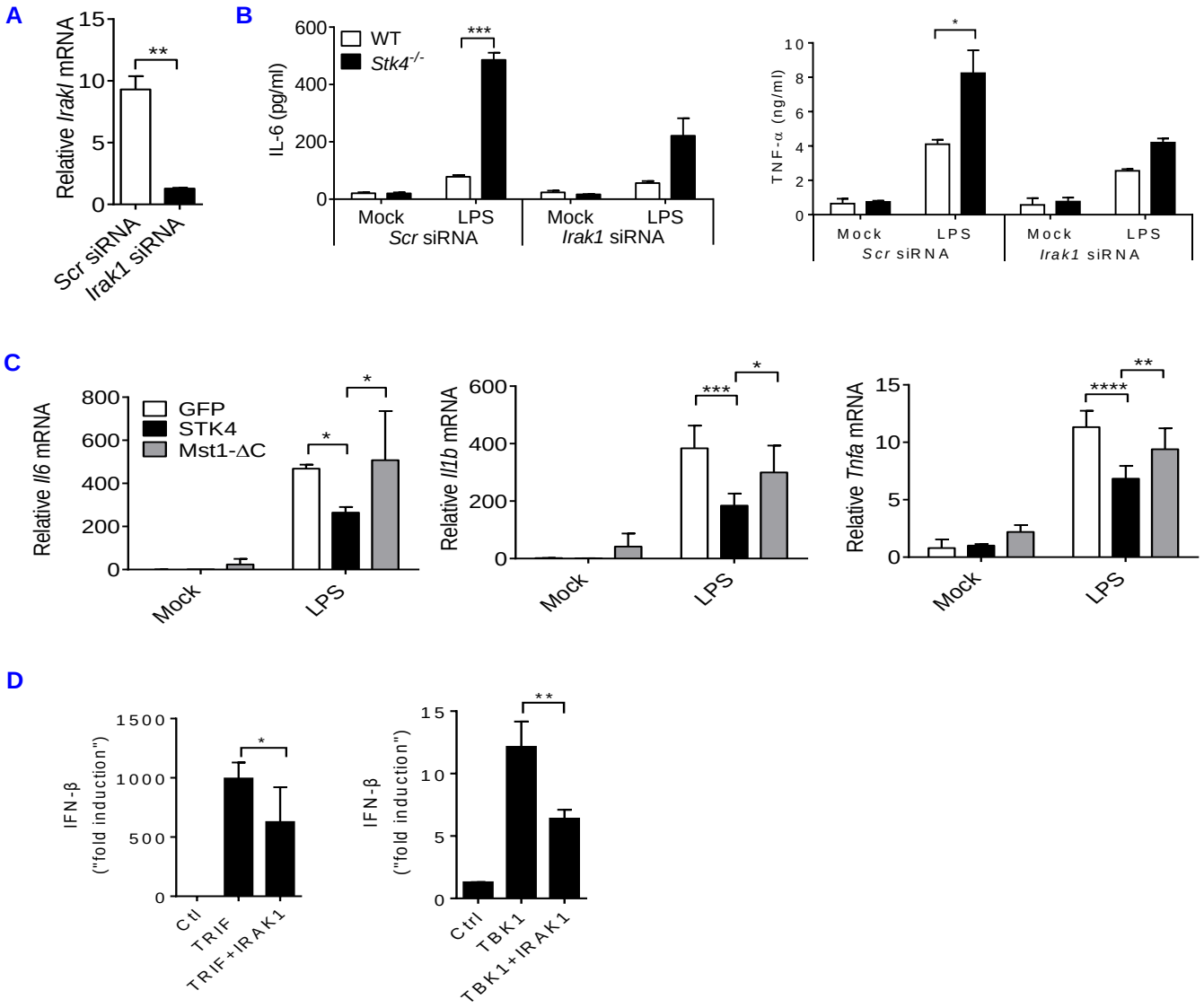


Figure S6, related to Figure 6. (A) PEMs were transfected with the *Scramble* siRNA or *Irak1* siRNA and the knockdown efficiency was determined with RT-PCR. (B) Wild-type and *Stk4*$^{-/-}$ PEMs were transfected with the scramble siRNA or *Irak1* siRNA, then stimulated with or without LPS for 6 hrs. Concentrations of IL-6 and TNF-α in supernatants were checked by ELISA. 2-tailed, unpaired Student's t test (n>=2). (C) mRNA levels of *Il6*, *Il1b* and *Tnfa* were measured by RT-PCR in LPS-stimulated RAW264.7 cells overexpressing GFP, STK4 or STK4-ΔC. 2-way ANOVA with Holm-Sidak's multiple comparisons test (n=4). (D) HEK293T cells were transfected with the IFN-β luciferase reporter and plasmids expressing TRIF, TBK1 or IRAK1 to measure luciferase activity. Renilla was cotransfected as an internal control. 1-way ANOVA with Holm-Sidak's multiple comparisons test (n=4) .*P $<$ 0.05 ; **P $<$ 0.01; ***P $<$ 0.001.

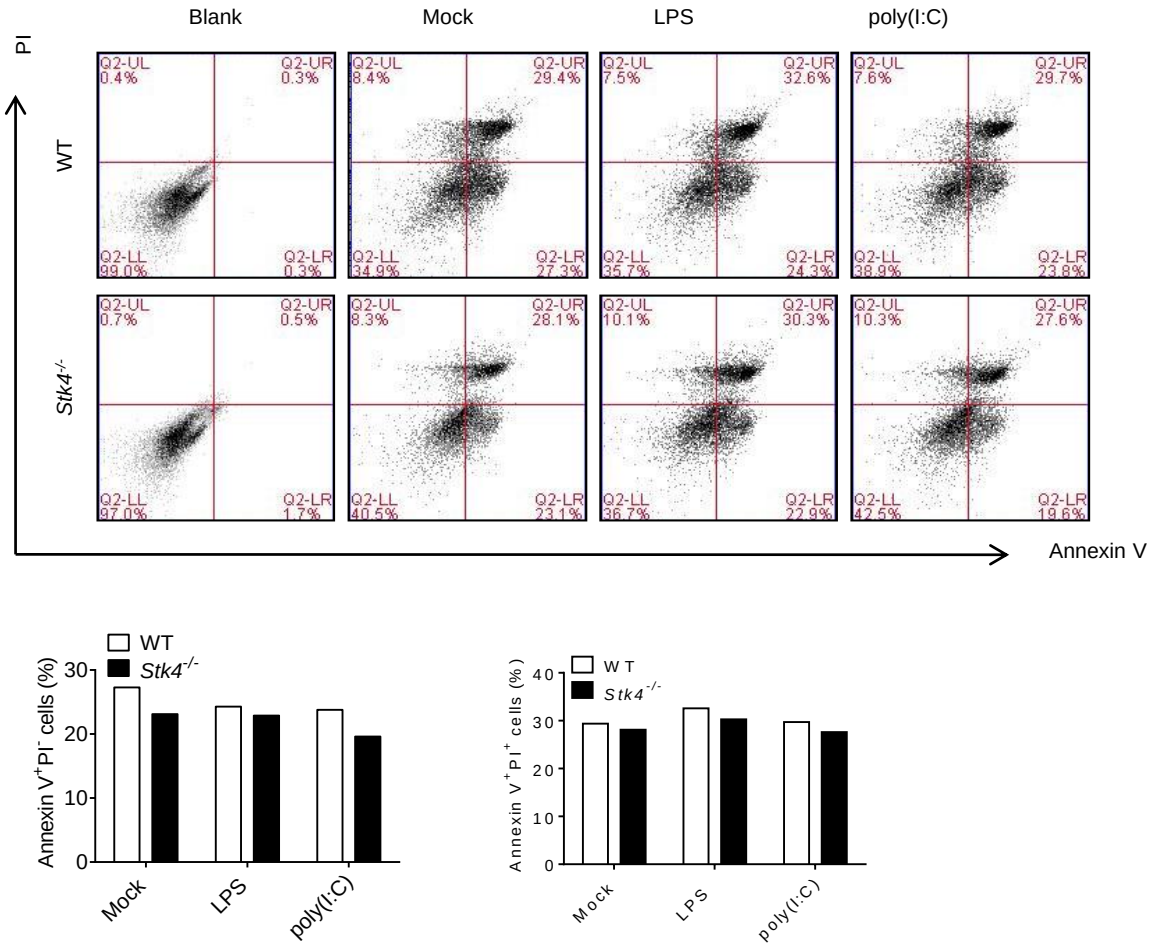


Figure S7, related to Figure 7. Apoptosis assay was carried out by Annexin V and PI staining in untreated, LPS- or poly(I:C)-treated wild-type and *Stk4* deficient PEMs. Data represent of the representative experiments from at least three independent experiments.

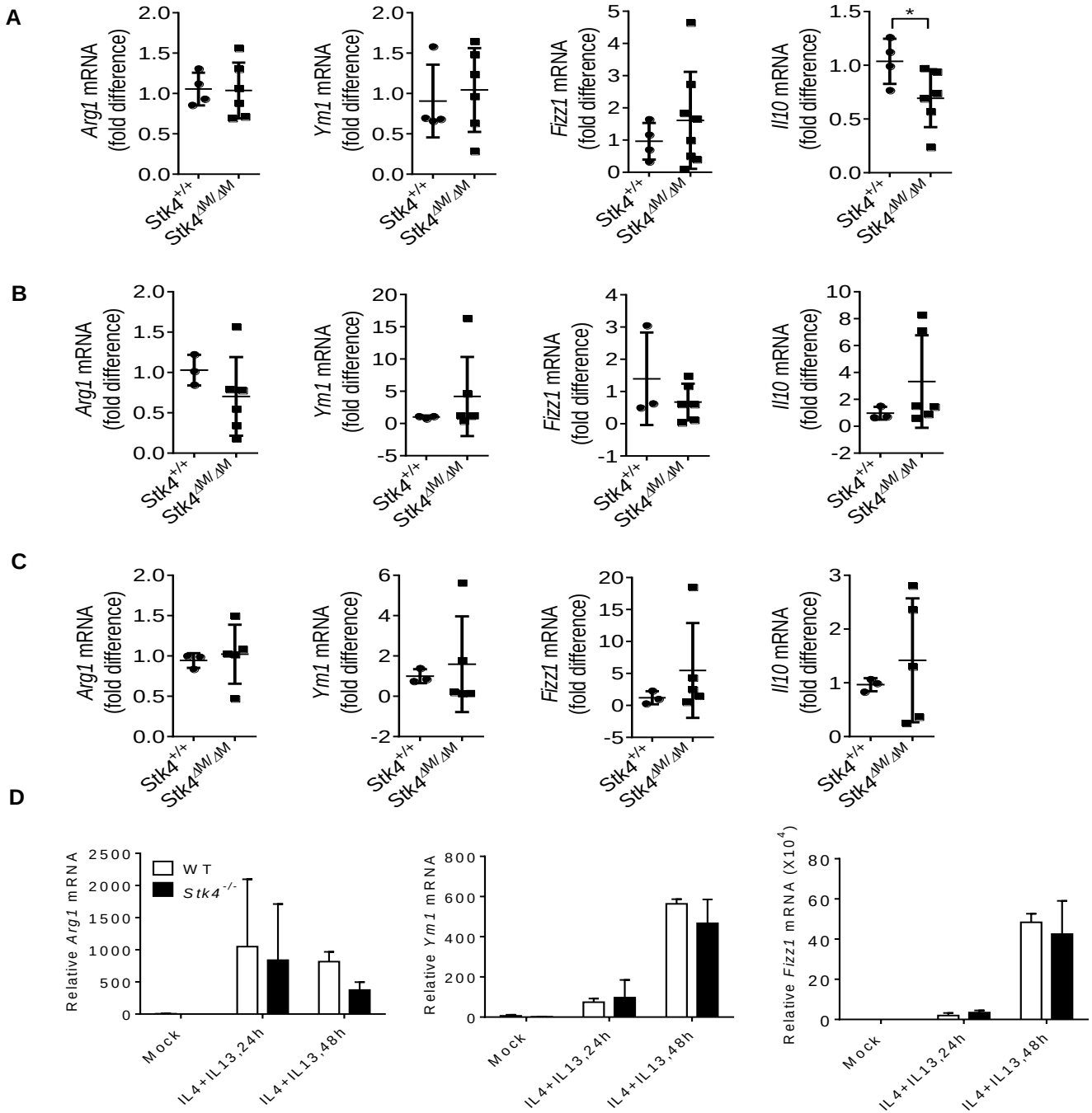


Figure S8, related to Figure 8. mRNA levels of *Arg1*, *Ym1*, *Fizz1* and *Il10* were determined in liver infiltrated macrophages from $Stk4^{+/+}$ (n=3) and $Stk4^{\Delta M/\Delta M}$ (n=6) mice after the treatment with DEN plus weekly CCl4 injection and daily LPS injection for 5 weeks (A), 7 month (B) or 9 month (C) (n>=3) . (D) $Stk4^{+/+}$ and $Stk4^{-/-}$ BMMs were stimulated with IL-4 and IL-13 (20 ng/ml) to evaluate mRNA levels of *Arg1*, *Ym1*, *Fizz1.* Values are mean ± SD from two independent experiments. *P < 0.05, 2-tailed, unpaired Student's t test.

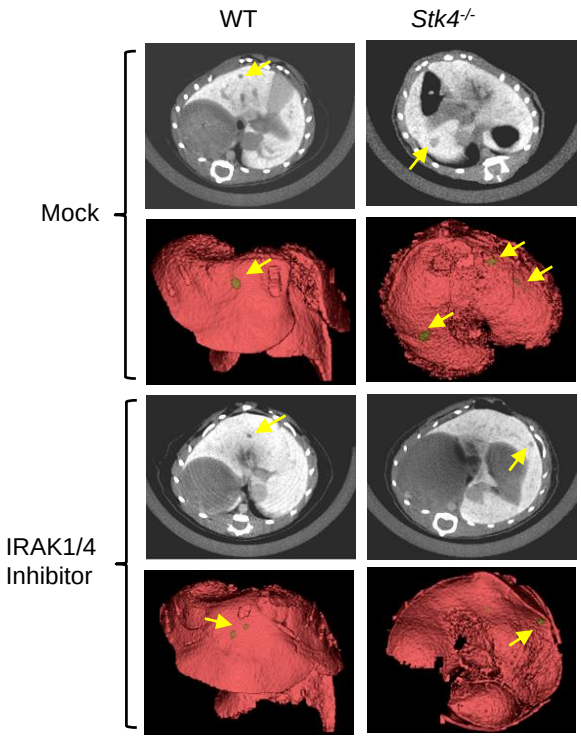
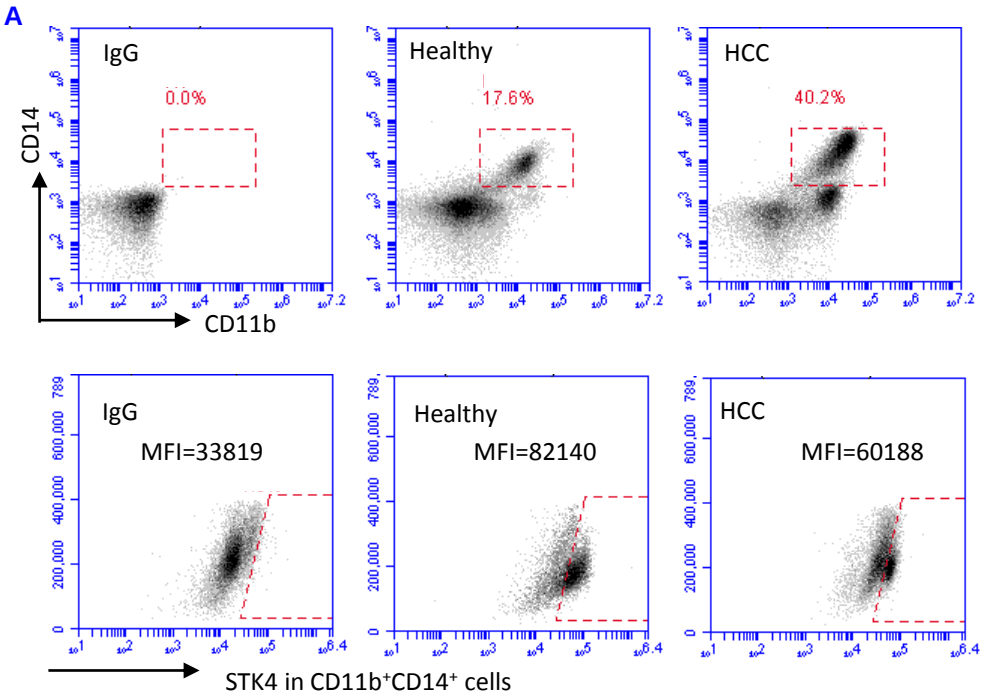
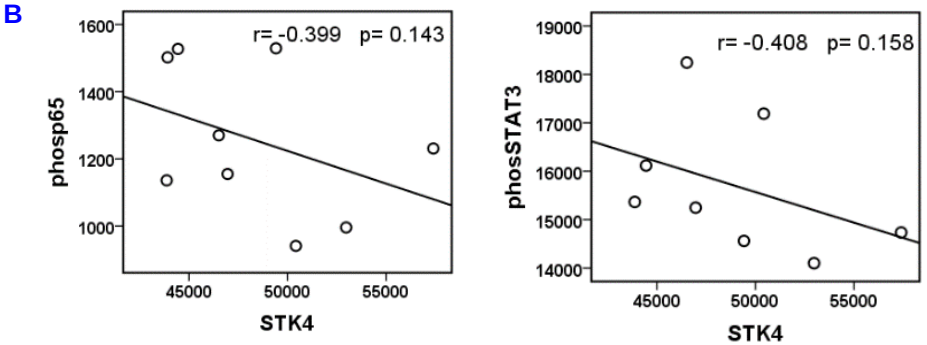


Figure S9, related to Figure 9. Mice were i.p. injected the IRAK1/4 inhibitor for 9 weeks (3 mg/kg, twice a week, starts one week before the first CCl_4 injection). Serum IL-6 levels were checked 6 weeks after DEN treatment (Left panel) and liver tumor formation was detected by micro-CT imaging 18 weeks after DEN treatment (Right panel) (n=3).


A
IgG
0.0%
Healthy
17.6%
HCC
40.2%
CD14
CD11b
IgG
MFI=33819
Healthy
MFI=82140
HCC
MFI=60188
STK4 in CD11b+CD14+ cells


B
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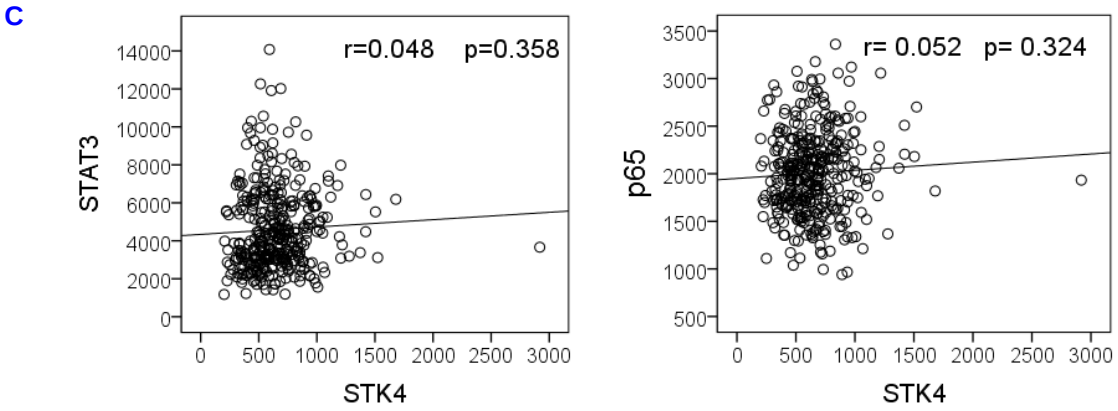
phosp65
STK4
r= -0.408 p= 0.158
phosSTAT3
STK4


C
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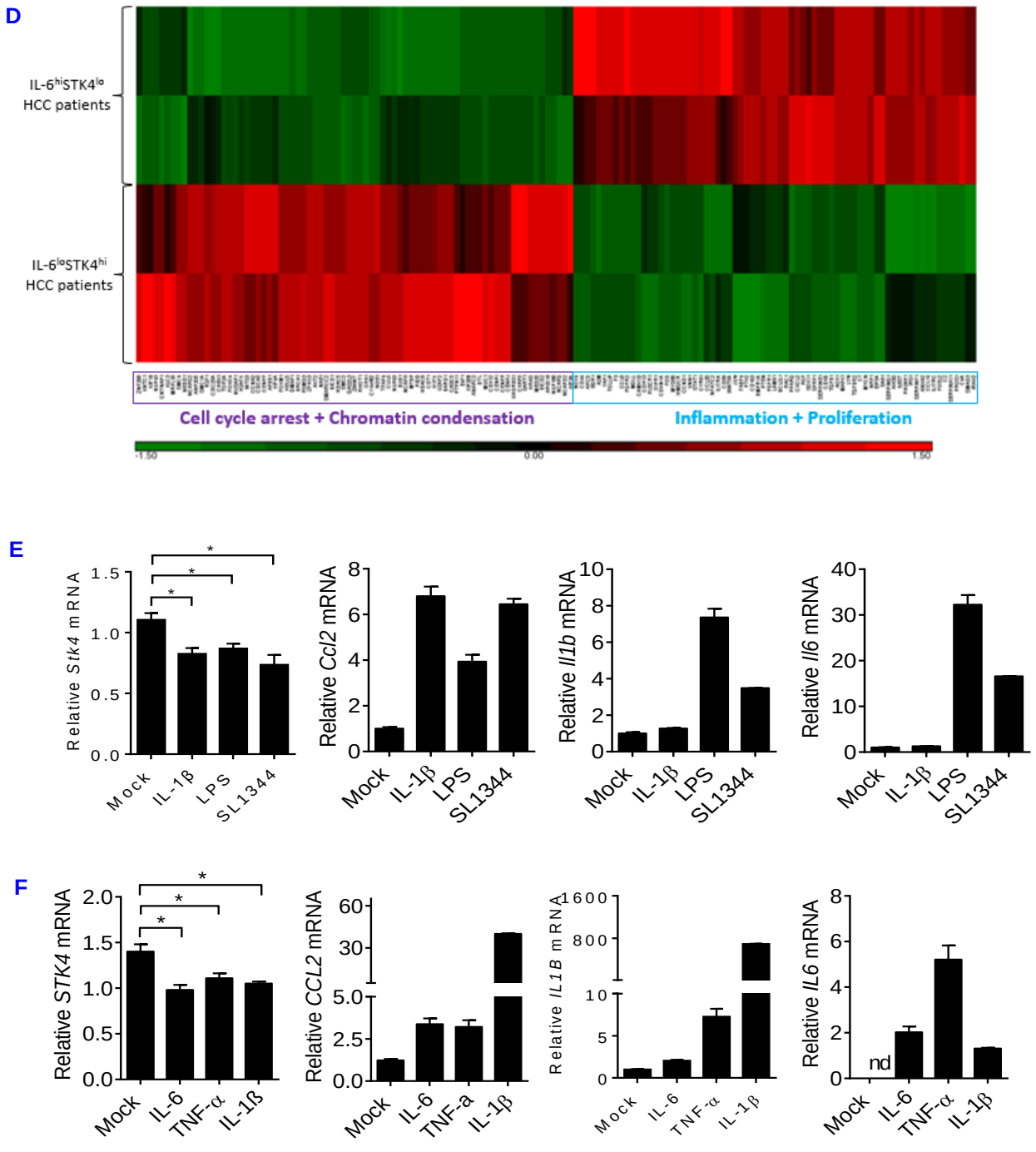
STAT3
STK4
r= 0.052 p= 0.324
p65
STK4


D
IL-6hiSTK4lo
HCC patients
IL-6loSTK4hi
HCC patients
Cell cycle arrest + Chromatin condensation
Inflammation + Proliferation
-1.50
0.00
1.50
E
Relative Stk4 mRNA
Relative Ccl2 mRNA
Relative Il1b mRNA
Relative Il6 mRNA
Mock
IL-1β
LPS
SL1344
F
Relative STK4 mRNA
Relative CCL2 mRNA
Relative IL1B mRNA
Relative IL6 mRNA
Mock
IL-6
TNF-α
IL-1ß
TNF-a
IL-1β
nd

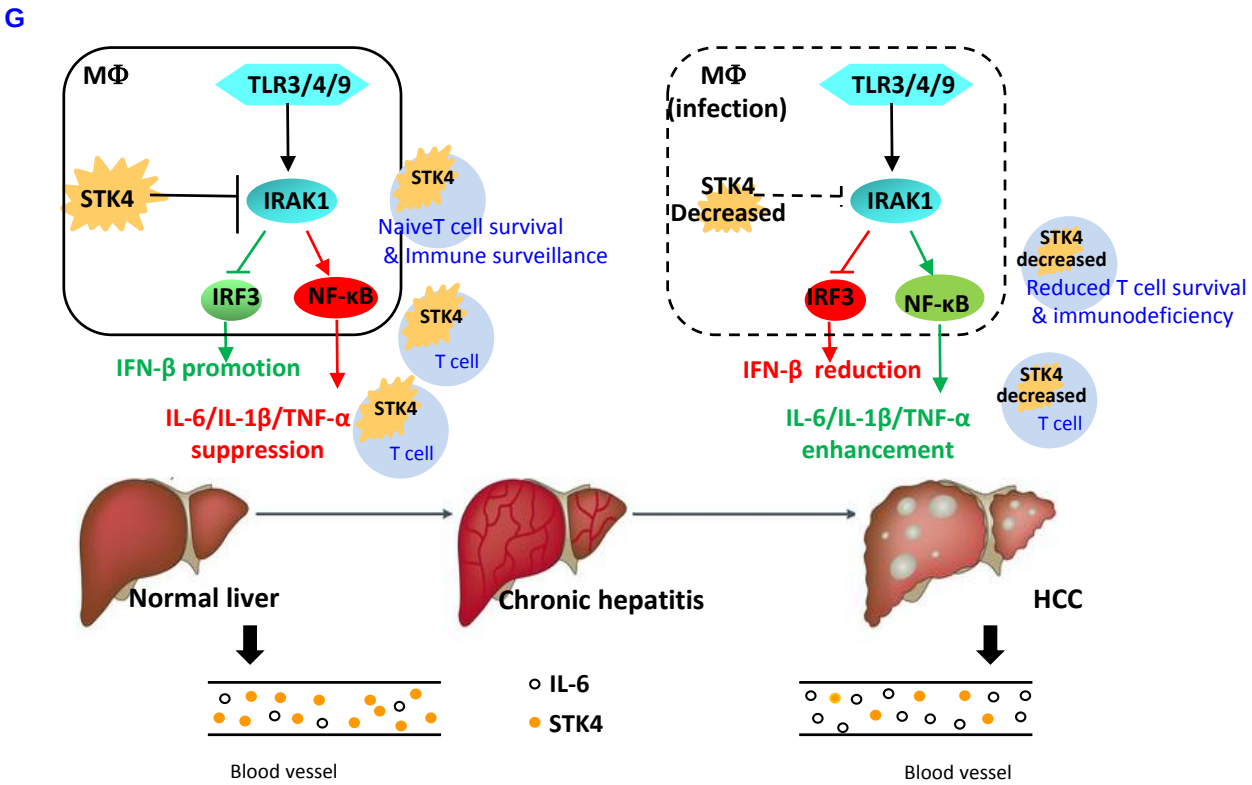


Figure S10, related to Figure 10. (A) PBMCs from healthy (n=7) and HCC patients (n=12) were stained with anti-CD11b, anti-CD14, anti-STK4 for FACS analysis. (B) Intrahepatic lymphocytes were separated from HCC patients' tumor tissue (n=8), followed by immunostaining with anti-CD11b, anti-STK4 or anti-phospho65, anti-phosphoSTAT3 for FACS assay. The correlation between STK4 expression and p65 or pSTAT3 levels was determined by Pearson test. (C) The mRNA levels of p53, STAT3 and STK4 in human HCC samples (n=369) were provided by the TCGA program and their correlation was determined by Pearson test. (D) The levels of genome transcripts from IL-$6^{lo}STK4^{hi}$ versus IL-$6^{hi}STK4^{lo}$ human liver tumors were examined using Affymetrix RNA microarray. Genes related to cell cycle arrest and chromatin condensation or inflammation and proliferation were listed. (E, F) mRNA levels of *Stk4*, *Ccl2*, *Il1b* and *Il6* were determined in primary mouse hepatocytes or human HepG2 cell line after exposed to the indicated cytokines, LPS or SL1344. Values represent mean ± SD. 1-way ANOVA with Tukey's and Holm-Sidak's multiple comparisons test. (G) Model: The anti-inflammation role in macrophages and anti-apoptosis role in naïve T cells of STK4 during the development of chronic inflammation-associated HCC. *P $<$ 0.05.

Table 1. Expression change of tumour suppressor genes in PEMs with *E.coli* infection

Reduction (< -2)	No change (-2 ~ 2)	Enhancement (> 2)
P53	*Pax5*	*Runx3*
Stk4	*Dlc1*	*Atip*
Rap1	*Dlc2*	
Axin1	*P16*	
Rb		
Pten		
Trim24		
Lkb1		

Wild-type PEMs were infected with $1x10^9$ CFU/ml *E.coli* for 30 min. After removing *E.coli*, cells were cultured for 12 hr to determine mRNA levels of tumor suppressor genes by qRT-PCR. Values were normalized for *β-actin* mRNA levels.

Table 2. Clinical pathologic characteristics and p53, stat3 status in patients with HCC

				mRNA		p53 statue		Stat3 statue	
No.	Age/sex	Stage	HBV	IL-6	STK4	DNA sequence	Consequence	DNA sequence	Consequence
2T	35/M	I	+	3.26	1.34	215 C → G 796 G → A	72 P → R 266 A → R	NS	
3T	51/M	II	+	38.66	1.38	695 T → A	232 I → N	/	WT
4T	37/F	I	+	76.15	1.44	215 C → T 503 A → G	72 P → R 168 H → R	/	WT
5T	56/F	I	+	2.13	1.77	215 C → G 469 G → C	72 P → R 157 V → L	/	WT
6T	73/M	IIIB	+	2.82	1.15	215 C → G 824 G → T	72 P → R 275 C → F	NS	
7T	59/M	I	+	25.21	1.32	215 C → G	72 P → R	/	WT
8T	70/M	II	+	20.32	1.90	215 C → G	72 P → R	/	WT
9T	57/F	II	+	15.36	2.39	215 C → G 747 G → T	72 P → R 249 R → S	/	WT
11T	49/M	IIIB	+	4.45	1.49	215 C → G 747 G → T	72 P → R 249 R → S	NS	
12T	46/M	I	+	15.18	1.65	475 G → C	159A → P	/	WT
13T	38/M	I	+	12.48	1.76	193 A → T	65 A → stop	/	WT
14T	42/M	ii	+	20.40	1.00	/	WT	/	WT
15T	56/M	I	+	7.94	1.31	215 C → G 452 C → G	72 P → R 151 P → R	/	WT
16T	59/F	I	+	178.9	2.74	215 C → G	72 P → R	NS	

WT: Wild type; Stop: stop codon; NS: no sequencing signaling