

Supporting Information

Cancer Immunomodulatory Effect of *Bidens pilosa* L. in Mice: Suppression of Tumor-Associated Macrophages and Regulatory T cells

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Table S1. Chemical information of compounds **1-8**.

Compound	Chemical Name
1	8,3'-dihydroxy-3,7,4'-trimethoxy-6-O- β -D-glucopyranosyl flavone
2	3- β -D-Glucopyranosyloxy-1-hydroxy-6(E)-tetradecene-8,10,12-triyn
3	3, 5-dihydroxy-3', 5'-dimethoxyflavon-7-O- β -D-glucopyranoside
4	Trifolin
5	3, 6-O-dimethylquercetagenin-7-O- β -D-glucoside
6	3,4'-dimethoxy-7-O- β -glucopyranoside quercetin
7	hesperetin-7-O- β -D-glucopyranoside
8	luteolin

Table S2. Primer sequences used for qRT-PCR analysis.

Mouse Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
ARG1	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA
CD25	GAAGGCATACCAGAAAGGGTTGA	ACTCTGTCCTTCCACGAAATGAT
YM1	CATGAGCAAGACTTGCGTGAC	GGTCCAAACTCCATCCTCCA
INOS	GTTCTCAGCCCAACAATAACAAGA	GTGGACGGGTCGATGTCAC
TNFA	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
CCL2	ATGCAGGTCCCTGTCATGCTTCTG	CTAGTTCACTGTCACACTGGTCACTCC
CCL22	TCTTGCTGTGGCAATTCAGA	GAGGGTGACGGATGTAGTCC
IL-10	ATCGATTTCTCCCTGTGAA	TGTCAAATTCATTATGGCCT
IL-1B	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
CXCL10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
CCR7	CATTGCCTATGACGTCACCTACA	GAAGGCATACCAGAAAGGGTTGA
FOXP3	TTCCTCCCGCTCTCTGACTCT	AAGCGCCAGTTGTGTACAAATATC
TGFB	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC
CXCR3	AATGCCACCCATTGCCAGTAC	AGCAGTAGGCCATGACCAGAAG
CCR4	TGCACCAAGGAAGGTATCAAGG	GTACACGTCCGTCATGGACTT
CCR8	CGTGGGCTGCAAGAACTGA	AGAGACCACCTTACACATCGC
CCR10	CTGACCTTTTATTGGCCCTGAC	CGAAGACTGAAACCAAGTGCG
VEGFR2	CTGGAGCCTACAAGTGCTCG	GAGGTTTGAAATCGACCCTCG
18S rRNA	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGGTGT

Table S3. Antibody information utilized in the Western blot experiment

Antibody	Dilution	Company
CD206	1:6000	Proteintech, cat. #81525-1-RR
CD80	1:4000	Proteintech, cat. #66406-1-Ig
Foxp3	1:6000	Proteintech, cat. #22228-1-AP
CD25	1:5000	ABclonal, cat. #A2984
PD-1	1:10000	Proteintech, cat. #66220-1-Ig
PD-L1	1:15000	CUSABIO, cat. #CSB-MA878942A1m
GITR	1:5000	Proteintech, cat. #23899-1-AP
CD4	1:3000	Proteintech, cat. #19068-1-AP
VEGFR2	1:2000	Proteintech, cat. #26415-1-AP
GAPDH	1:10000	Proteintech, cat. #60004-1-Ig
HRP Alpha Tubulin Mouse Monoclonal	1:10000	Proteintech, cat. #HRP-66031
Antibody		
HRP-conjugated Affinipure Goat anti-	1:10000	Proteintech, cat. #RGAR001
Rabbit IgG(H+L)		
HRP-conjugated Affinipure Goat anti-	1:10000	Proteintech, cat. #RCAM001
Mouse IgG(H+L)		

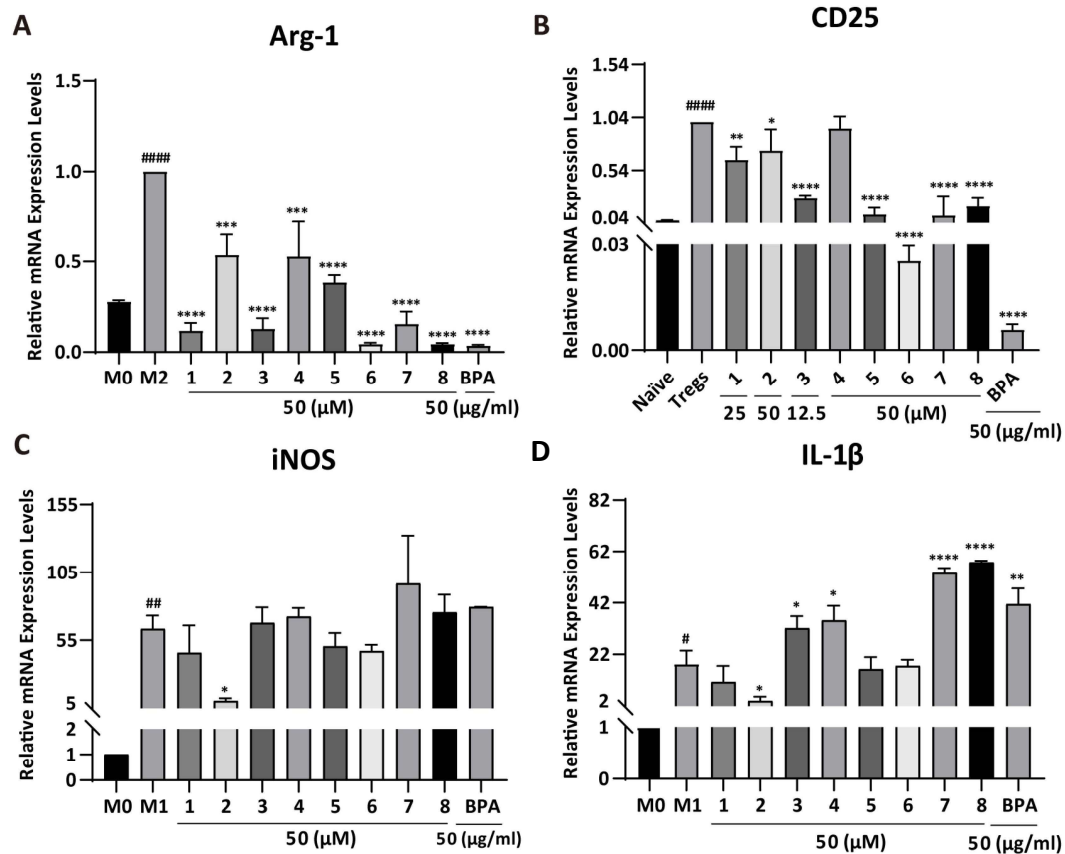


Figure S1. The effects of **BPA** and **1-8** on M1/M2 macrophages and Tregs analyzed by qRT-PCR. **(A)** Arg-1 mRNA expression in M2 macrophages; **(B)** CD25 mRNA expression in Tregs; **(C)** iNOS mRNA expression in M1 macrophages; **(D)** IL-1 β mRNA expression in M1 macrophages. Gene expression levels were quantified using qRT-PCR and normalized to 18S RNA as the reference gene. Statistical significance for all experiments is denoted as follows (n=3): compared to the M0 or Naïve group, # P <0.05, ## P <0.01 and #### P <0.0001; compared to M2/M1/Tregs group, * P <0.05, ** P <0.01, *** P <0.001, and **** P <0.0001.

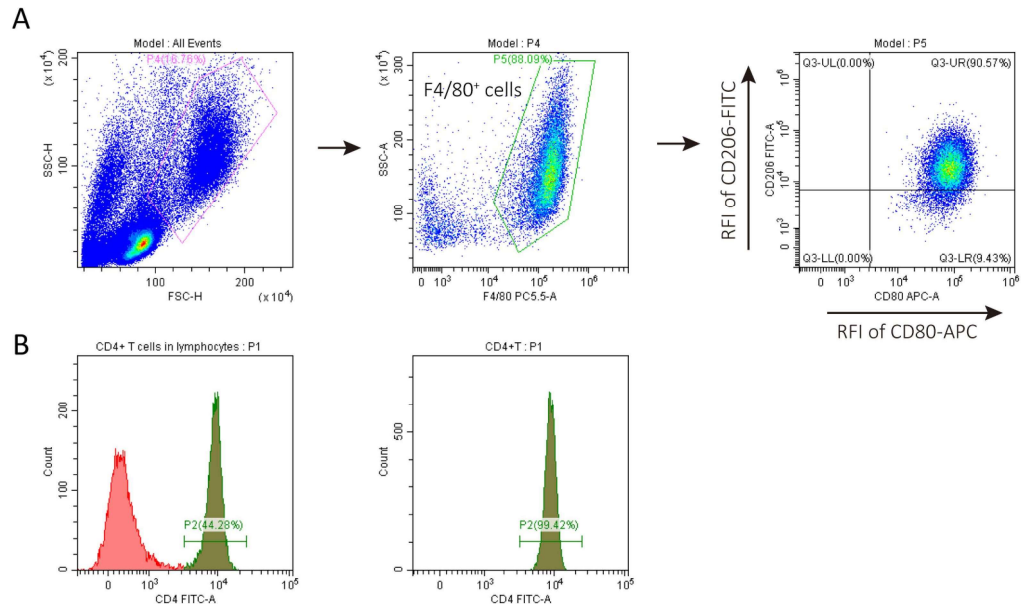


Figure S2. Flow cytometry plots for macrophage gating strategy and CD4⁺ T cell purity. (A) Gating strategy used for flow cytometric identification of macrophages. (B) Purity of CD4⁺ T cells. Left, CD4⁺ T cell purity among lymphocytes isolated from mouse lymph node and spleen. Right, CD4⁺ T cell purity after isolation using a CD4⁺ T cell isolation kit.

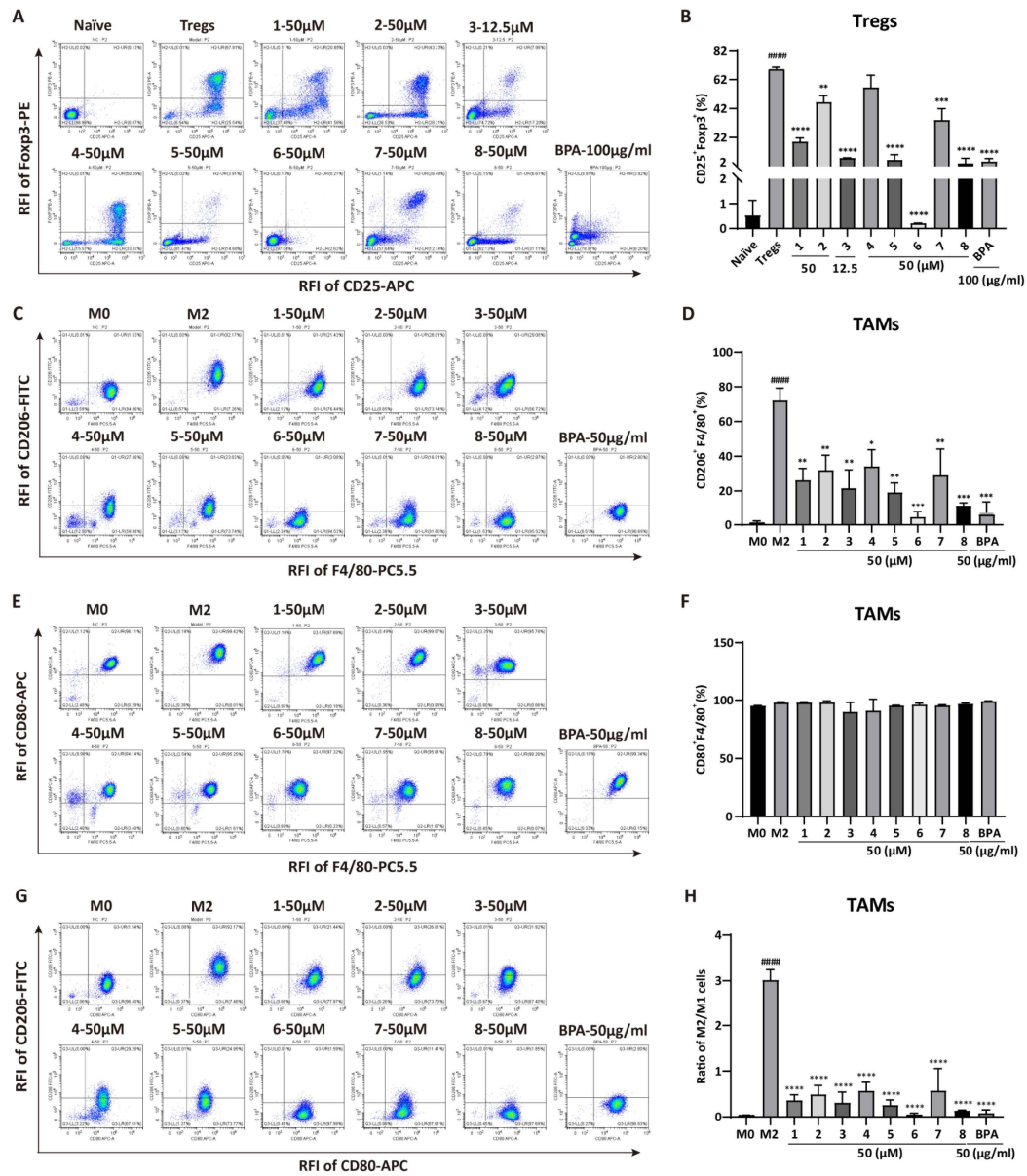


Figure S3. Effects of BPA and 1-8 on M0 differentiation into M2 macrophages and CD4⁺ T cell differentiation into Tregs. **(A)** Representative flow cytometry plots of CD25⁺Foxp3⁺. **(B)** Quantification of CD25⁺Foxp3⁺ frequency. **(C)** Representative flow cytometry plots of F4/80⁺CD206⁺. **(D)** Quantification of F4/80⁺CD206⁺ frequency. **(E)** Representative flow cytometry plots of F4/80⁺CD80⁺. **(F)** Quantification of F4/80⁺CD80⁺ frequency. **(G)** Representative flow cytometry plots of CD206⁺/CD80⁺. **(H)** Quantification of CD206⁺/CD80⁺ frequency. Statistical significance for all experiments is denoted as follows (n=3): compared to the M0/Naïve group, ####*p*<0.0001; compared to M2 /Tregs group, **p*<0.05, ** *p*<0.01, *** *p*<0.001, and **** *p*<0.0001. RFI means Relative Fluorescence Intensity.

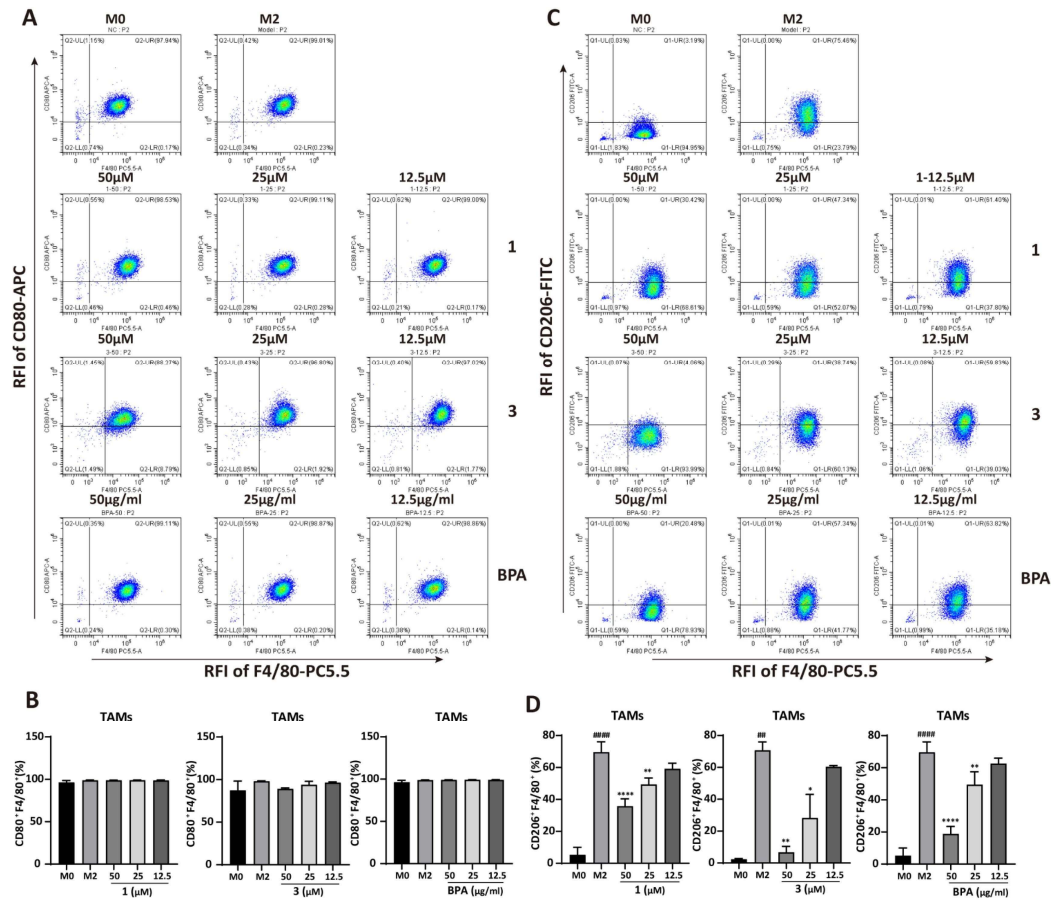


Figure S4. Dose-dependent effects of **BPA**, **1**, and **3** on M2 macrophages differentiation. **(A)** Flow cytometry analysis of F4/80⁺CD80⁺ under increasing concentrations of BPA (12.5, 25, 50 μ g/ml) or **1** and **3** (12.5, 25, 50 μ M). **(B)** The histograms for the quantified results of F4/80⁺CD80⁺ cells. **(C)** Analysis of F4/80⁺CD206⁺ cell population using flow cytometry. **(D)** The histograms for the quantified results of F4/80⁺CD206⁺ cells. Statistical significance for all experiments is denoted as follows (n=3): compared to the M0 group, # p <0.05, ### p <0.001, and #### p <0.0001; compared to M2 group, * p <0.05, ** p <0.01, and **** p <0.0001. RFI means Relative Fluorescence Intensity.

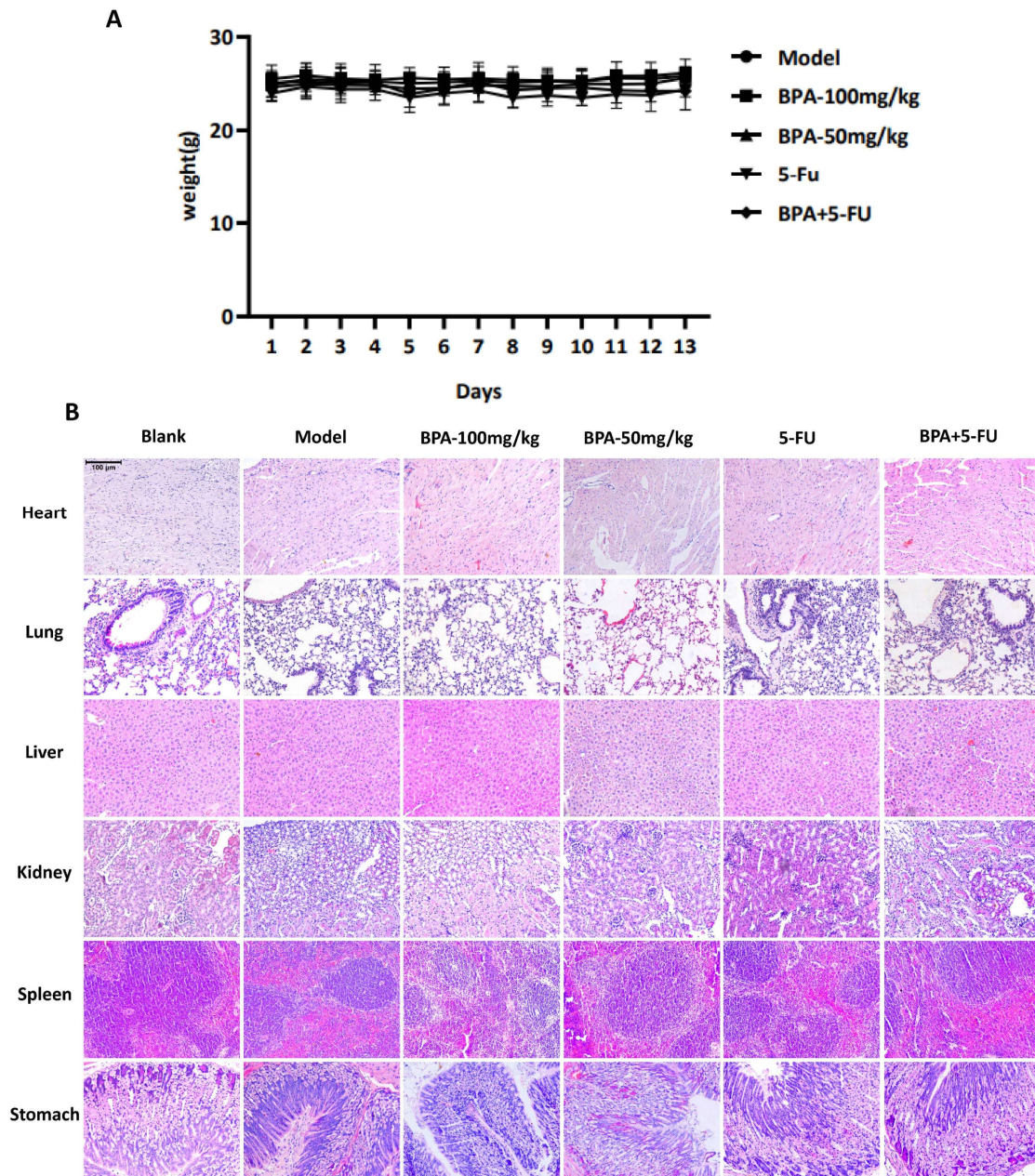


Figure S5. Body weight changes and representative H&E-stained images of mouse organs. **(A)** Statistical analysis of mouse body weight across all treatment groups. **(B)** Histopathological analysis of mouse visceral tissues (heart, liver, spleen, lungs, kidneys, and stomach) using H&E staining, indicating the absence of significant toxicity in the treatment groups. Magnified 200 times.