Supplementary information

Additional file 2

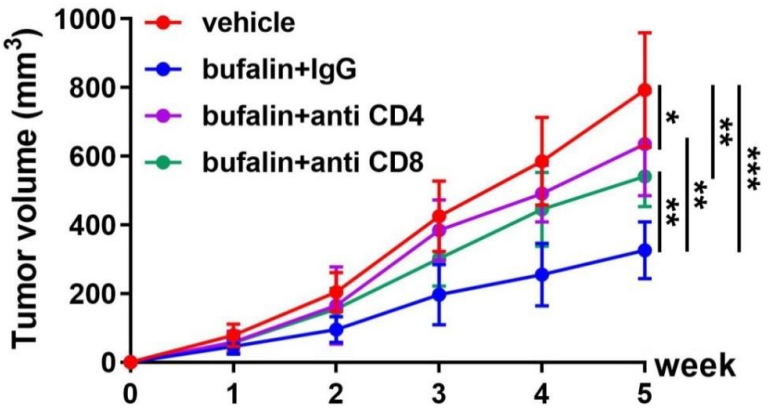

Figure S1. Bufalin stimulates T cell immune response to HCC. Tumor growth was assessed in HCC-bearing C57BL/6 mice upon the treatment with vehicle or bufalin accompanied with IgG, anti-CD4 and -CD8 neutralizing antibodies respectively. Mice were treated after 6 days of HCC cell inoculation (n=5 per group), and tumor volume were examined every week. The data were presented as mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

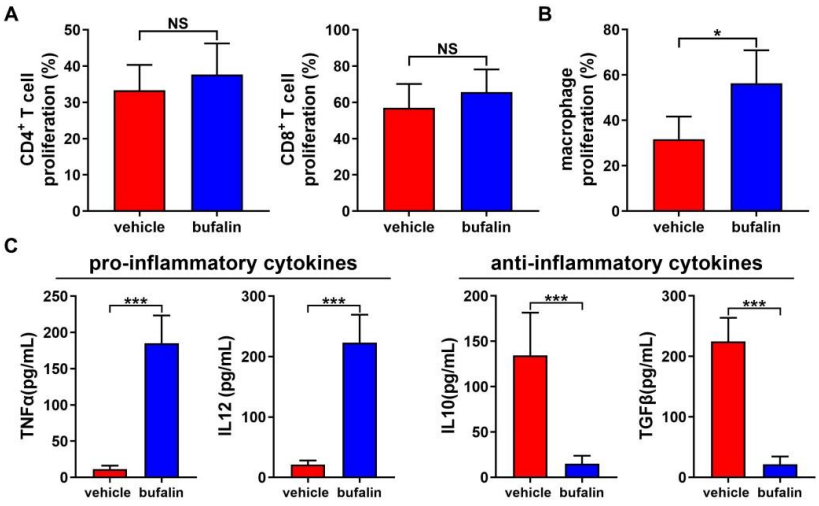

Figure S2. Bufalin promotes the proliferation of macrophage and production of pro-inflammatory cytokines in macrophage without affecting T cell proliferation. (A) CFSE-labelled splenic $CD4^+$ and $CD8^+$ T cells were stimulated with anti-CD3/CD28 antibody in the presence or absence of bufalin for 48h. Cell proliferation was measured by flow cytometry in triplicate. **(B)** CFSE-labelled macrophages were stimulated with PMA in the presence or absence of bufalin for 48h. Cell proliferation was measured by flow cytometry in triplicate. **(C)** Macrophages were stimulated with PMA in the presence or absence of bufalin for 48h, and supernatants were collected for cytokines analysis by ELISA in triplicate. The data were presented as mean±SEM. NS, no significant; **P*<0.05; ****P*<0.001.

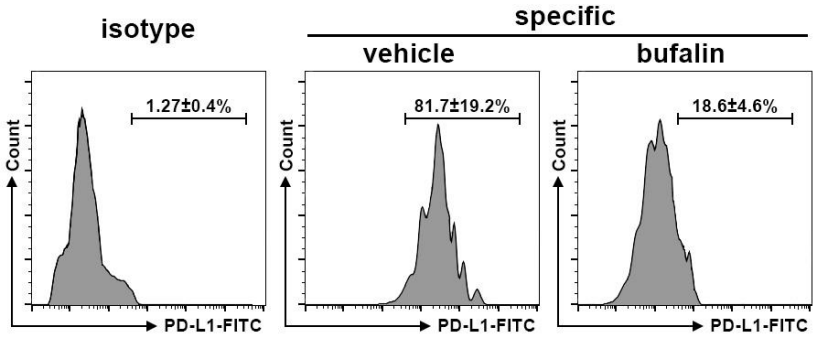

Figure S3. Bufalin suppresses PD-L1 expression on macrophage. The expression of PD-L1 was detected by FACS in the TIMs isolated from liver tumor in HCC-bearing mice upon vehicle or bufalin treatment (n=5 mice). Representative histograms show the downregulation of PD-L1 in the bufalin-treated TIMs compared with vehicle-treated ones.

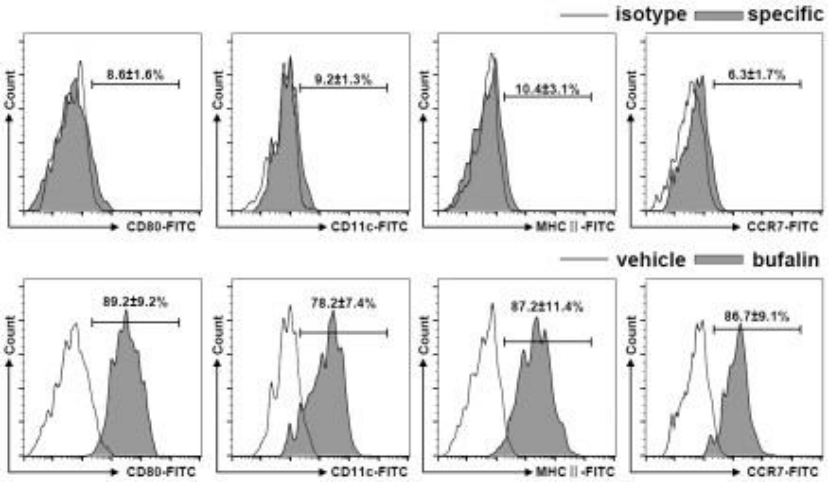

Figure S4. Bufalin stimulates M1 polarization of BMDM. The expression of M1-associated stimulatory molecules was detected by FACS in the BMDMs upon vehicle or bufalin treatment. Representative histograms show increased CD80, CD11c, MHC-II and CCR7 surface expression in the BMDMs upon bufalin treatment compared with vehicle.

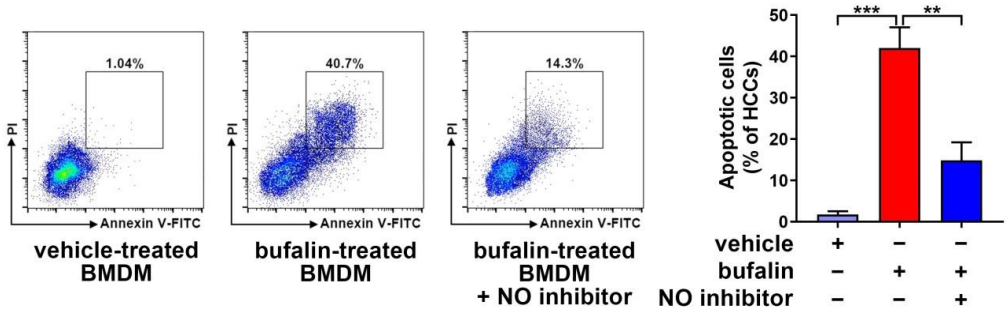

Figure S5. Inhibition of NO expression impairs the cytotoxicity of bufalin-treated BMDMs. HCC cells were co-cultured with vehicle- or bufalin-treated BMDMs in the absence or presence of NO inhibitor, and the percentage of apoptotic HCC cells labeled with PI and Annexin V were detected by FACS. Data are shown as mean±SEM. ***P*<0.01, ****P*<0.001.

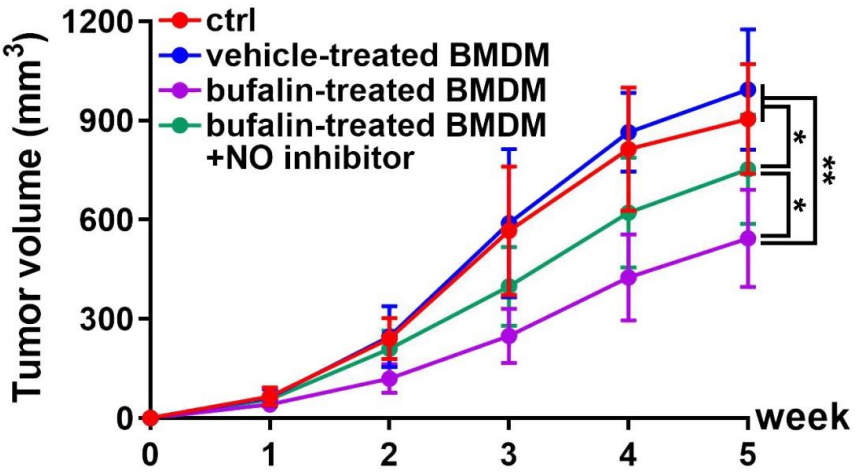

Figure S6. Inhibition of NO expression abrogated the suppressive effect of bufalin-treated BMDMs on HCC tumorigenesis. Nude mice were treated by vehicle-primed BMDMs, bufalin-primed BMDMs, bufalin-primed BMDMs accompanied with NO inhibition, respectively, after one week of the subcutaneous inoculation of Hepa1-6 HCC cells (n=5 per group), and tumor growth was assessed in 5 weeks. **P*<0.05, ***P*<0.01.

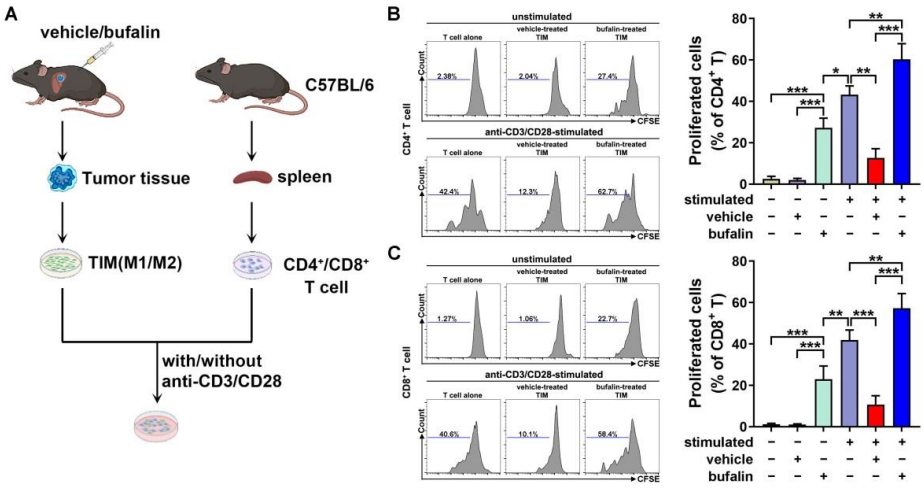

Figure S7. Bufalin-treated TIMs promote the proliferation of CD4$^+$ and CD8$^+$ T cells *ex vivo*. (A) Schematic diagram showed the evaluation of CD4$^+$ or CD8$^+$ T cells proliferation affected by bufalin- or vehicle-treated TIMs. The CD4$^+$ or CD8$^+$ T cells isolated from mice spleen, and TIMs isolated from liver tumor in HCC-bearing mice upon bufalin treatment or vehicle (n=3 per group), were co-cultured in the presence or absence of anti-CD3/-CD28 antibodies for 48h. **(B and C)** CFSE-labelled CD4$^+$ or CD8$^+$ T cells treated with bufalin- or vehicle-treated TIMs in the presence or absence of anti-CD3/-CD28 antibodies were collected, and the percentage of CFSE^{low} proliferative CD4$^+$ or CD8$^+$ T cells were determined by FACS. Data were presented as mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

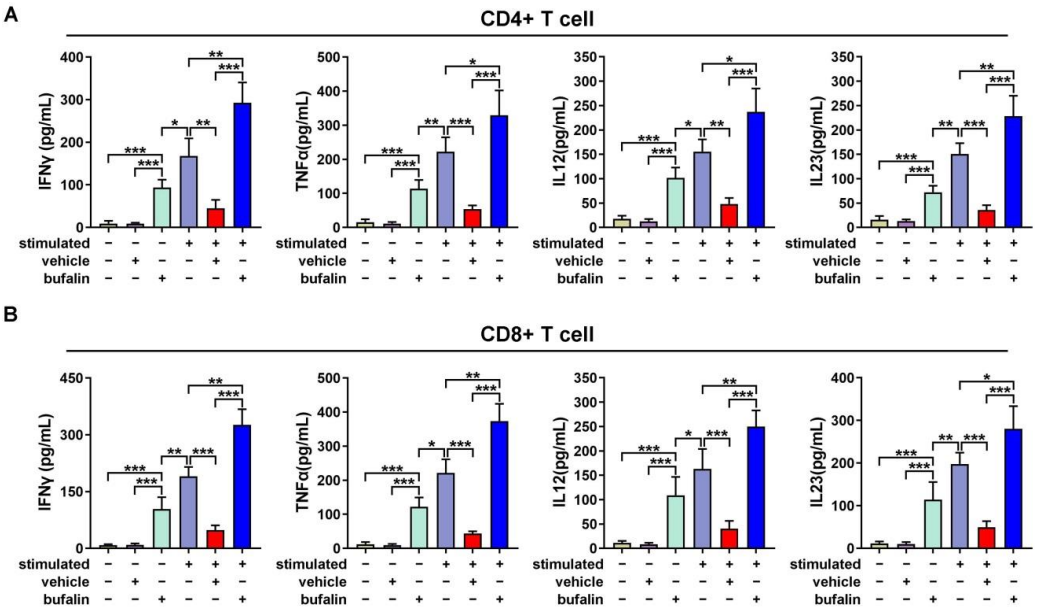

Figure S8. Bufalin-treated TIMs promote the production of immunostimulatory cytokines in CD4$^+$ and CD8$^+$ T cells. (A) The supernatants from the co-culture of CD4$^+$ or **(B)** CD8$^+$ T cells with bufalin- or vehicle-treated TIMs in the presence or absence of anti-CD3/-CD28 antibodies, were collected for the analysis of the production of IFN-γ, TNF-α, IL-12 and IL-23 by ELISA (n=3 per group). Data were presented as mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

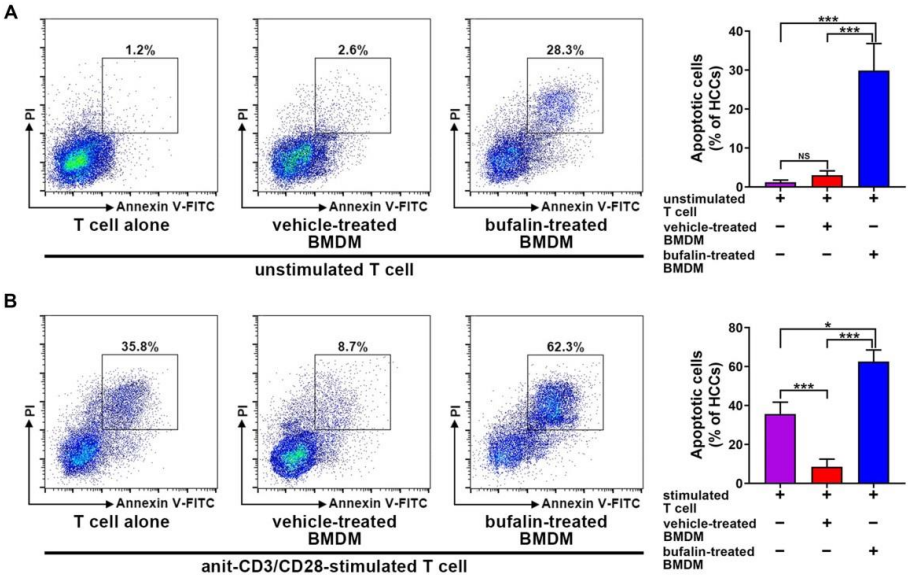

Figure S9. Bufalin-primed BMDM provokes the cytotoxic effect of CD8⁺ T cells on HCC cells. (A) In the absence of anti-CD3/-CD28 stimulation or **(B)** in the presence of anti-CD3/-CD28 stimulation, CD8⁺ T cells were incubated with vehicle- or bufalin-primed BMDMs, and then isolated to treat HCC cells. The percentage of apoptotic HCC cells labeled with PI and Annexin V were detected by FACS. Data are shown as mean±SEM. NS, no significant; **P*<0.05, ****P*<0.001.

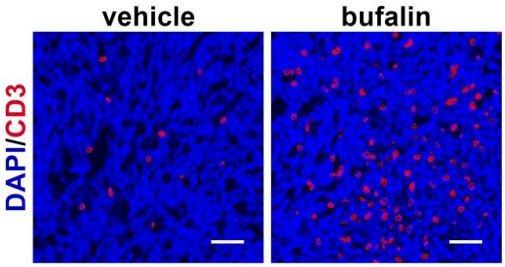

Figure S10. Bufalin promotes T cells to migrate and accumulate into tumor tissues. Representative immunofluorescence images (×200 fold) show the recruitment of T cells

with the expression of CD3 in the tumor tissues of HCC-bearing mice. DAPI was used to counterstain the nuclei. Scale bar = 50 μm.

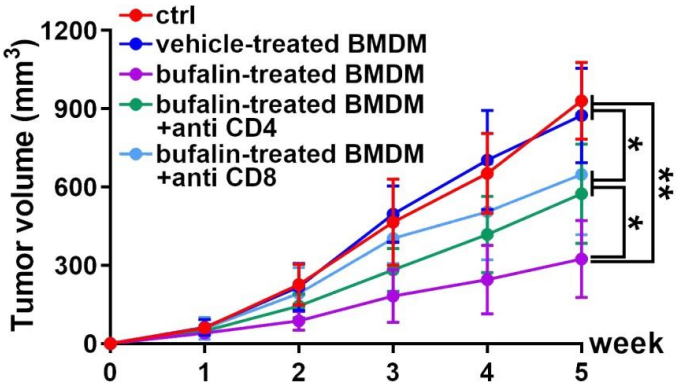

Figure S11. Bufalin-primed macrophages provoke the cytotoxic effect of T cells on inhibiting HCC growth. C57BL/6 mice were treated with vehicle- or bufalin-primed BMDMs after one week of the subcutaneous inoculation of Hepa1-6 cells. Anti-CD4 or -CD8 neutralizing antibodies were given concomitantly in bufalin-primed BMDMs groups, respectively (n=5 per group). Tumor growth was assessed in 5 weeks. **P*<0.05, ***P*<0.01.

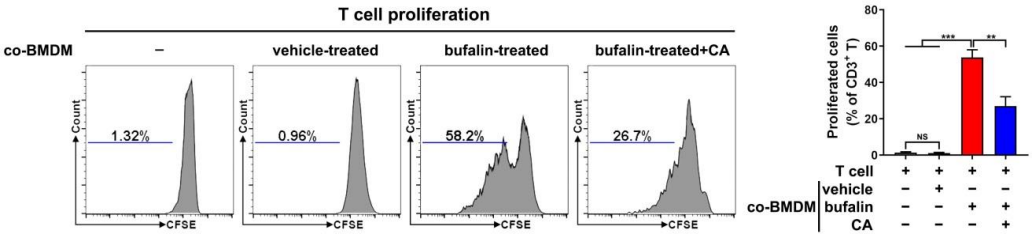

Figure S12. Inhibition of NF-κB activity dampens the effect of bufalin-primed BMDMs on promoting T cell proliferation. CFSE-labeled T cells were co-cultured with vehicle- or bufalin-primed BMDMs treated with or without CA respectively, and T

cell proliferation was assessed by flow cytometry. CA: cardamonin, NF-κB inhibitor. Data are shown as mean±SEM. NS, no significant; ***P*<0.01, ****P*<0.001.

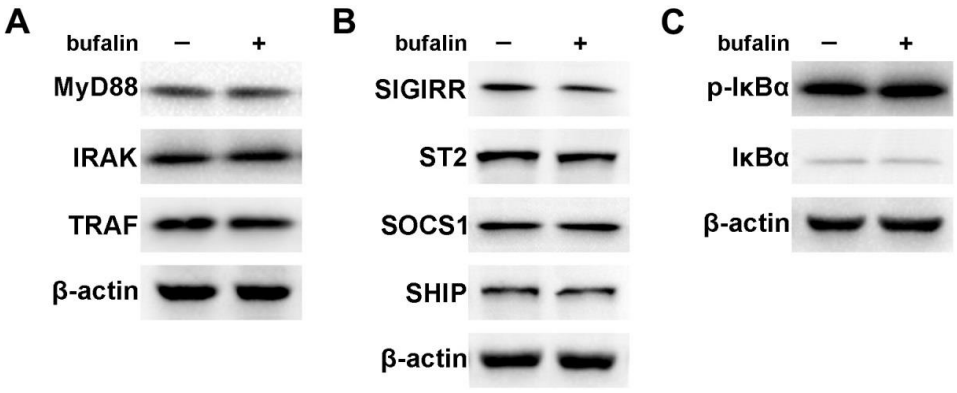

Figure S13. Bufalin has no effect on the expression of regulator proteins in the NF-κB signaling in BMDMs. BMDMs were treated with vehicle or bufalin for 48h, and the expression of diverse protein was detected by western blot. The expression of **(A)** MyD88, IRAK and TRAF, **(B)** SIGIRR, ST2, SOCS1 and SHIP, the negative regulators in the NF-κB signaling, and **(C)** IκBα and phosphorylated IκBα (p-IκBα), was detected, and no change of protein expression was observed in the condition of bufalin treatment compared with vehicle. β-actin was used as loading control.

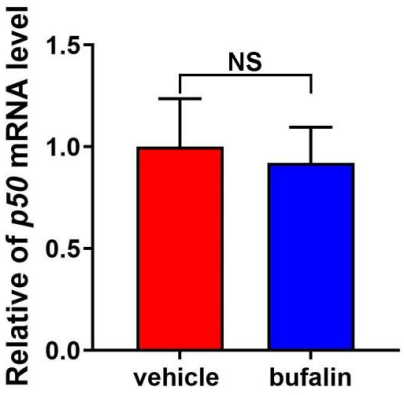

Figure S14. Bufalin does not affect the mRNA level of *p50* in BMDMs. The transcription level of *p50 NF-κB* was detected by quantitative RT-PCR in the BMDMs upon vehicle or bufalin treatment. No changes of the mRNA level of *p50 NF-κB* was observed in the condition of bufalin compared with vehicle. *Gapdh* was used to normalize the value. The data were presented as mean±SEM. NS, no significant.