Tumor cell–derived microparticles induced by methotrexate augment T-cell antitumor responses by down-regulating expression of PD-1 in neutrophils

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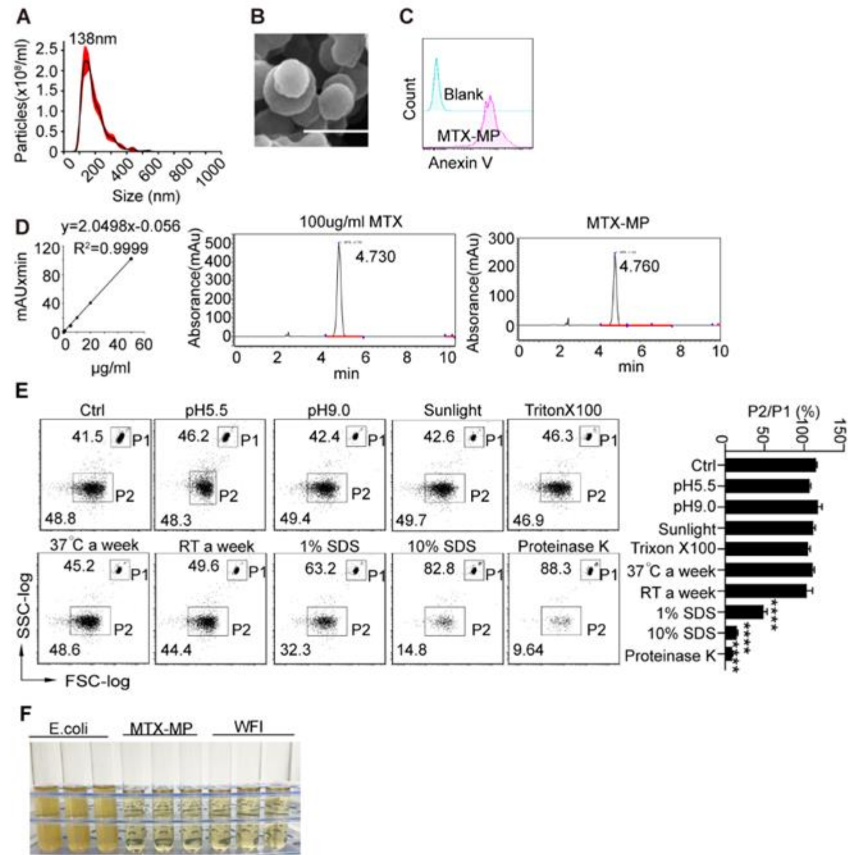
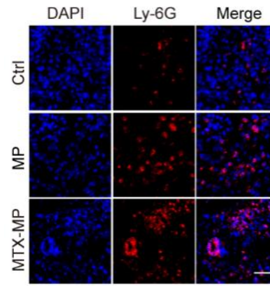
Supplementary Figures S1–S7 and supplementary table S1



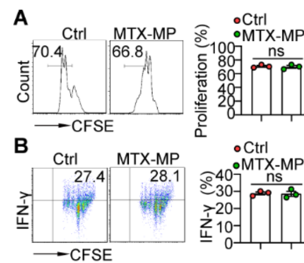
Supplementary Fig S1 related to Fig 1.

(A-B) MTX-MPs were isolated from H22 tumor cells, size distribution was measured by Malvern Zetasizer Nano ZS90 particle size analyzer **(A)**, morphology was analyzed by scanning electron microscope **(B)**, and annexin V **(C)** was analyzed by flow cytometry. **(D)** 5×10^7 H22 cells derived MTX-MPs were prepared and the drugs include were analyzed by HPLC. Drugs were quantified by using Ultimate 3000 HPLC system in a C18 column, and drugs concentration were calculated according to standard curve. **(E)** The stability of MTX-MPs was analyzed under different conditions including pH5.5, pH9.0, explored to sunlight and room temperature (RT) for a week, and SDS or proteinase K for 30 min. MTX-MPs were then recollected and mixed with 3μm beads for flow cytometric analysis, P1 represent beads and P2 represent MTX-MPs. **(F)** Sterility of MTX-MPs suspension were analyzed. MTX-MPs suspension were added to Trypticase Soy Broth (TSB) for bacteria growth, and E. coli as a positive control and Water For Injection (WFI) as negative control. For all graphs, data present mean ± SEM, $^{****}P<0.0001$, ns, not statistically significant by One way ANOVA (E).



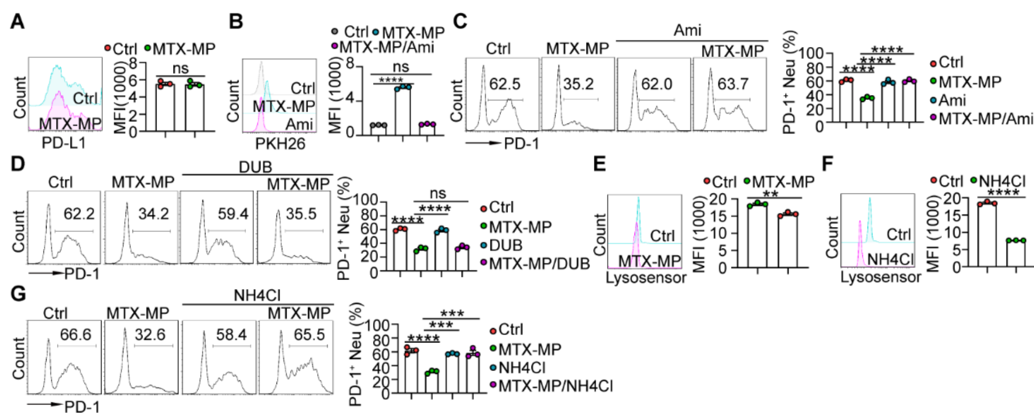
Supplementary Fig S2 related to Fig 1

3×10^5 H22 tumor cells were injected subcutaneously, when tumor size reach 5×5 mm^2, the mice were i.v injected with MTX-MPs daily for 3 times, the neutrophils in tumor tissue were analyzed using immunofluorescence staining. Scale bar 20 μm.



Supplementary Fig S3 related to Fig 1

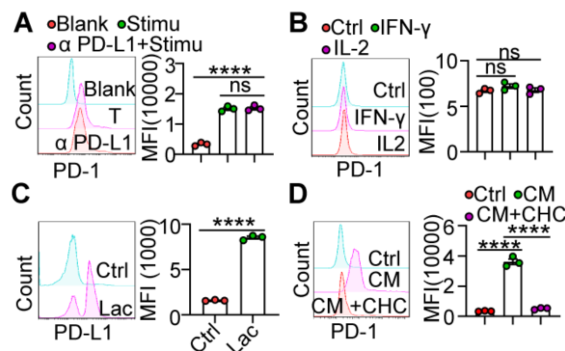
(A - B) H22 derived MTX-MPs were incubated with CFSE-labeled CD8+ T cells for 3 days, the proliferation **(A)** and IFN-γ releasing **(B)** were determined by flow cytometry. For all graphs, data present mean ± SEM, ns, not statistically significant by unpaired Student's t-test (A and B).



Supplementary Fig S4 related to Fig 2

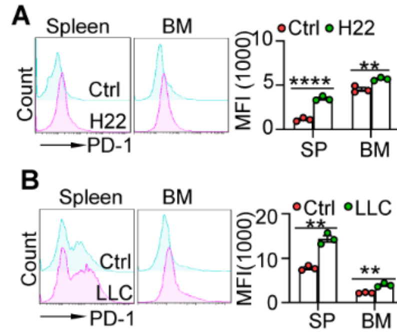
(A) Neutrophils were isolated from H22 ascites mice, and incubated with MTX-MPs for 6 hours, the expression of PD-L1 was analyzed by flow cytometry. **(B)** Same as (A),

the neutrophils were pretreated with amiloride hydrochloride (Ami, 100 μM) for 1 hour and then incubated with PKH26-labeled MTX-MPs for 6 hours, the uptake of MTX-MPs was analyzed by flow cytometry. **(C-D)** Neutrophils were isolated from H22 ascites mice, and incubated with MTX-MPs in the presence of Ami **(C)** or DUB-IN-1 (DUB, 1 μM) **(D)** for 6 hours, the expression of PD-1 was analyzed by flow cytometry. **(E-F)** Bone-marrow neutrophils were isolated from H22 tumor-bearing mice, and incubated with MTX-MPs **(E)** or NH4Cl (1mM) **(F)**, the pH changed was analyzed using lysosensor green. **(G)** Neutrophils were isolated from H22 ascites mice, and incubated with MTX-MPs in the presence of NH4Cl for 6 hours, the expression of PD-1 was analyzed by flow cytometry. For all graphs, data present mean ± SEM, ***P<0.001, ****P<0.0001, ns, not statistically significant by Student's t-test (A, E and F) or One way ANOVA (C, D and G).



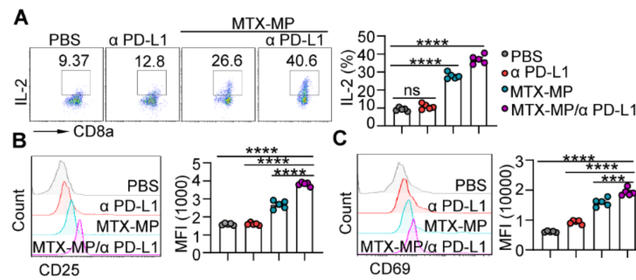
Supplementary Fig S5 related to Fig 3

(A-B) Neutrophils were isolated from bone marrow of healthy mice and cultured with CM of CD3/CD28 antibody-activated $CD8^+$ T cells in the presence of PD-L1 antibody **(A)** or IFN-γ (20 ng/mL), IL-2 (20 ng/mL) **(B)** for 12 hours, the expression of PD-1 in neutrophils was analyzed by flow cytometry. **(C-D)** Neutrophils were isolated from bone marrow of healthy mice and cultured with lactic acid for 12 hours, the expression of PD-L1**(C)** were determined by flow cytometry, or cultured with LLC CM, or LLC CM plus CHC for 12 hours, the expression of PD-1 **(D)** was determined by flow cytometry. For all graphs, data present mean ± SEM, ****P<0.0001, ns, not statistically significant by Student's t test (C) or One way ANOVA (A, B and D).



Supplementary Fig S6 related to Fig 5

(A-B) 1×10^5 H22 tumor cells were i.p injected to BALB/c mice **(A)**, or 1×10^6 LLCs were i.v injected to C57BL/6 mice **(B)** for 10 days, the expression of PD-1 in neutrophil in spleen and bone marrow were determined by flow cytometry. For all graphs, data present mean ± SEM, ***P*<0.01, **** *P*<0.0001, by unpaired Student's t-test (A and B).



Supplementary Fig S7 related to Fig 6

(A-C) 1×10^6 LLCs were i.v injected to C57BL/6 mice for 7 days, then PBS, MTX-MPs, anti-PD-L1 (20 μg/g, daily for 4days) or MTX-MPs (daily for 5 days) combined with anti-PD-L1 antibody were i.v injected, IL-2 **(A)**, CD25 **(B)** and CD69 **(C)** expressed in tumor-infiltrating $CD8^+$ T cells were analyzed by flow cytometry. *****P*<0.0001, ns, not statistically significant by One way ANOVA.

Supplementary Table S1 Primer sequences

Gene	Primer	Primer sequence
Mouse *Arg1*	FW	CAAGACAGGGCTCCTTTCAG
	RV	TGGCTTATGGTTACCCTCC
Mouse *Nos2*	FW	GCAGCTTGTCCAGGGATTCT
	RV	GCAGCTTGTCCAGGGATTCT
Mouse *Actb*	FW	GGCTGTATTCCCCT-CCATCG
	RV	CCAGTTGGTAACAATGCCATGT
Mouse *Il10*	FW	TGGACTCCAGGACCTAGACA
	RV	CGGAGAGAGGTACAAACG
Mouse *Elane*	FW	CATGGCCCTTGGCAGACTAT
	RV	AGTTCCTGGCAATGAGGGTG