Supplementary Materials for

Tryptophan potentiates $CD8^+$ T cells against cancer cells by TRIP12 tryptophanylation and surface PD-1 downregulation

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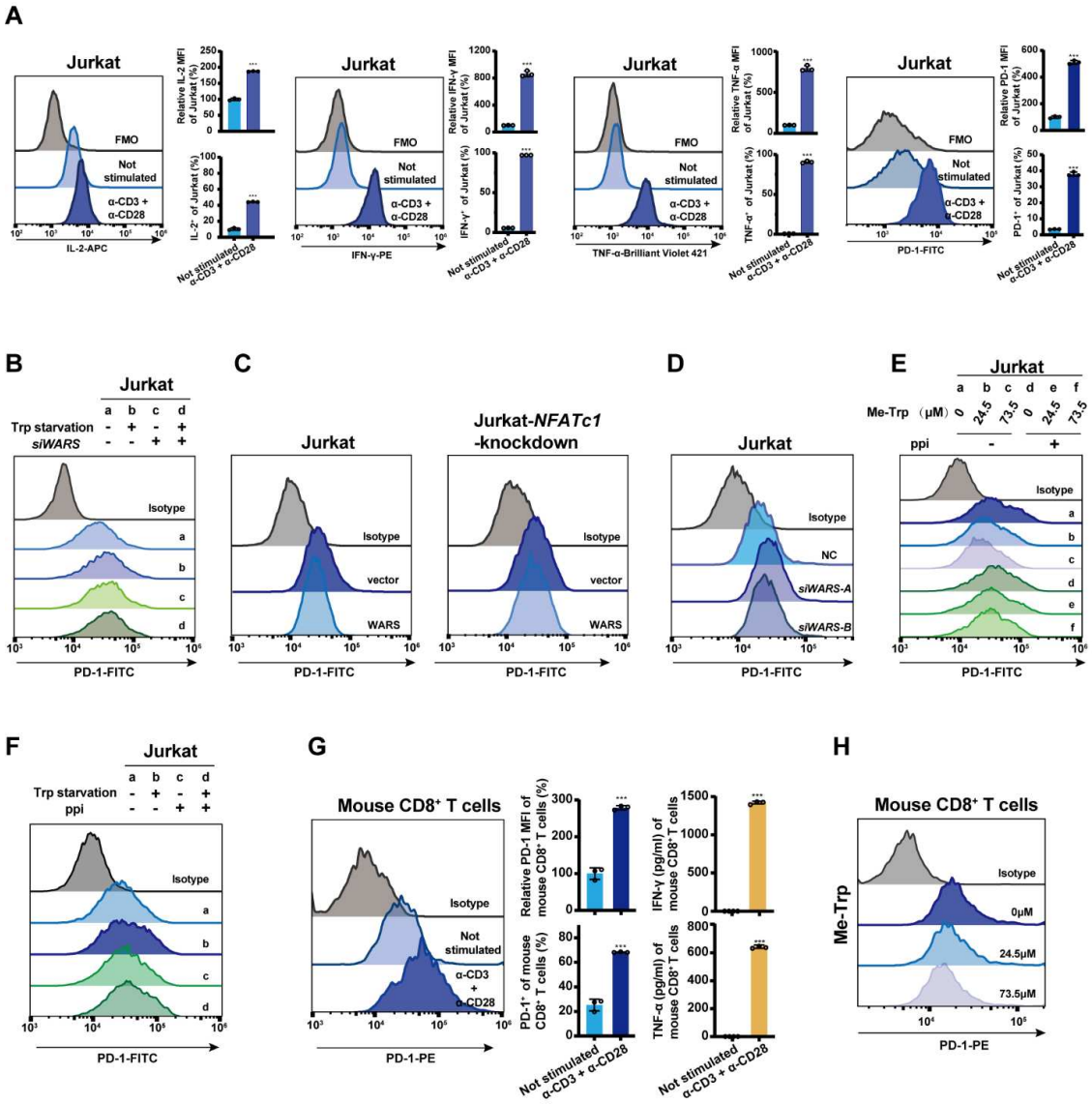

Figure. S1.

Tryptophan WARS-dependently downregulates PD-1 expression

S1A, anti-CD3 and anti-CD28 activated Jurkat T cells. Jurkat T cells were treated by anti-CD3 (5 μg/ml) and anti-CD28 (2.5 μg/ml) antibodies. The intracellular staining of IL-2, IFN-γ and TNF-α, cell surface PD-1 levels and percentage of positive cells were assessed to confirm the successful activation. n = 3, mean ± SD (herein after for all statistics in supplemental figures unless indicated otherwise).

S1B, knockdown WARS abrogated cell surface PD-1-regulation by Me-Trp. Representative flow cytometry results of Figure 1D.

S1C-S1D, Silencing NFATc1 diminished WARS ability to alter cell surface PD-1. Representative flow cytometry results of Figure 1E and 1F.

S1E-S1F, Pyrophosphate inhibited Me-Trp supplemental and tryptophan-starvation to alter PD-1. Representative flow cytometry results of Figure 1G and 1H.

S1G, anti-CD3 and anti-CD28 activated mouse naïve CD8$^+$ T cells. Mouse naïve CD8$^+$ T cells were treated by anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies. Secretion of IFN-γ and TNF-α were detected by ELISA and cell surface PD-1 levels were measured by flow cytometry to confirm the successful activation. Percentage of PD-1 positive cells and PD-1 MFI were assessed.

S1H, Me-Trp administration decreased mouse CD8+ T cells surface PD-1. Representative flow cytometry results of Figure 1I.

*P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's t-test. (herein after)

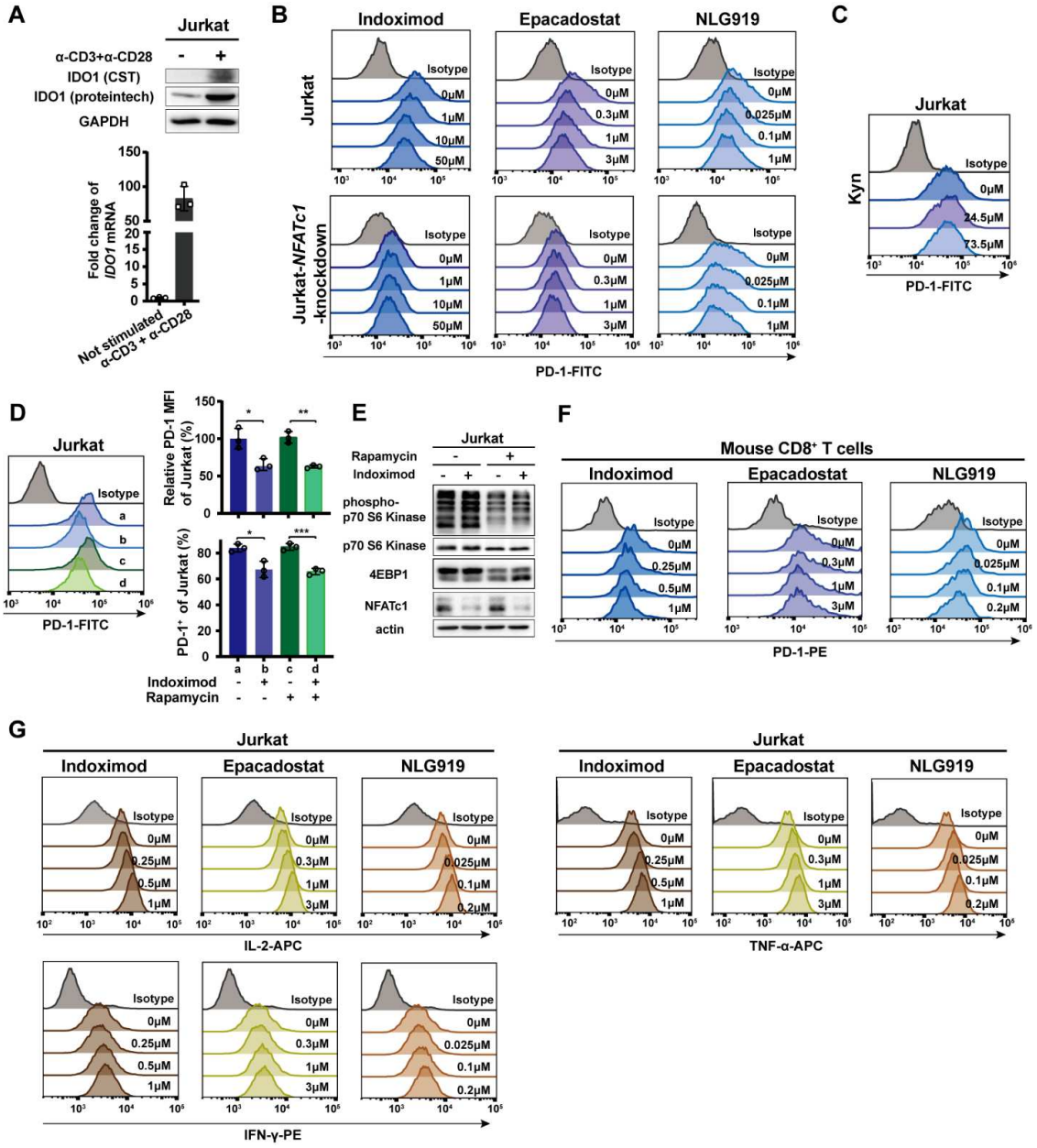

Figure. S2.

IDO inhibitors decreases PD-1 through accumulating intracellular tryptophan

S2A, Activated Jurkat cells had enhanced IDO1 expression. Jurkat cells and Anti-CD3 (5 μg/ml) and anti-CD28 (2.5 μg/ml) antibodies co-stimulated Jurkat T cells were analyzed for IDO1 protein with two antibodies (upper) and mRNA (lower) levels.

S2B, NFATc1 knockdown prevented IDO inhibitors to decrease PD-1. Representative flow cytometry results of Figure 2B.

S2C, Kynurenine had negligible effects on surface PD-1 expression. Representative flow cytometry results of Figure 2C.

S2D, Indoximod regulates PD-1 in the presence of rapamycin in Jurkat cells. The expression of PD-1 were detected in stimulated Jurkat T cells that were cultured in media supplemented with Indoximod under absence or presence of rapamycin. n = 3, mean ± SD.

S2E, **Indoximod regulates NFATc1 in the presence of rapamycin in Jurkat cells.** The expression of NFATc1 were detected in stimulated Jurkat T cells that were cultured in media supplemented with Indoximod under absence or presence of rapamycin.

S2F, IDO inhibitors decreased PD-1 in mouse $CD8^+$ T cells. Representative flow cytometry results of Figure 2D.

S2G, IDO inhibitors increased IL-2, IFN-γ and TNF-α level in T cells. Representative flow cytometry results of Figure 2E.

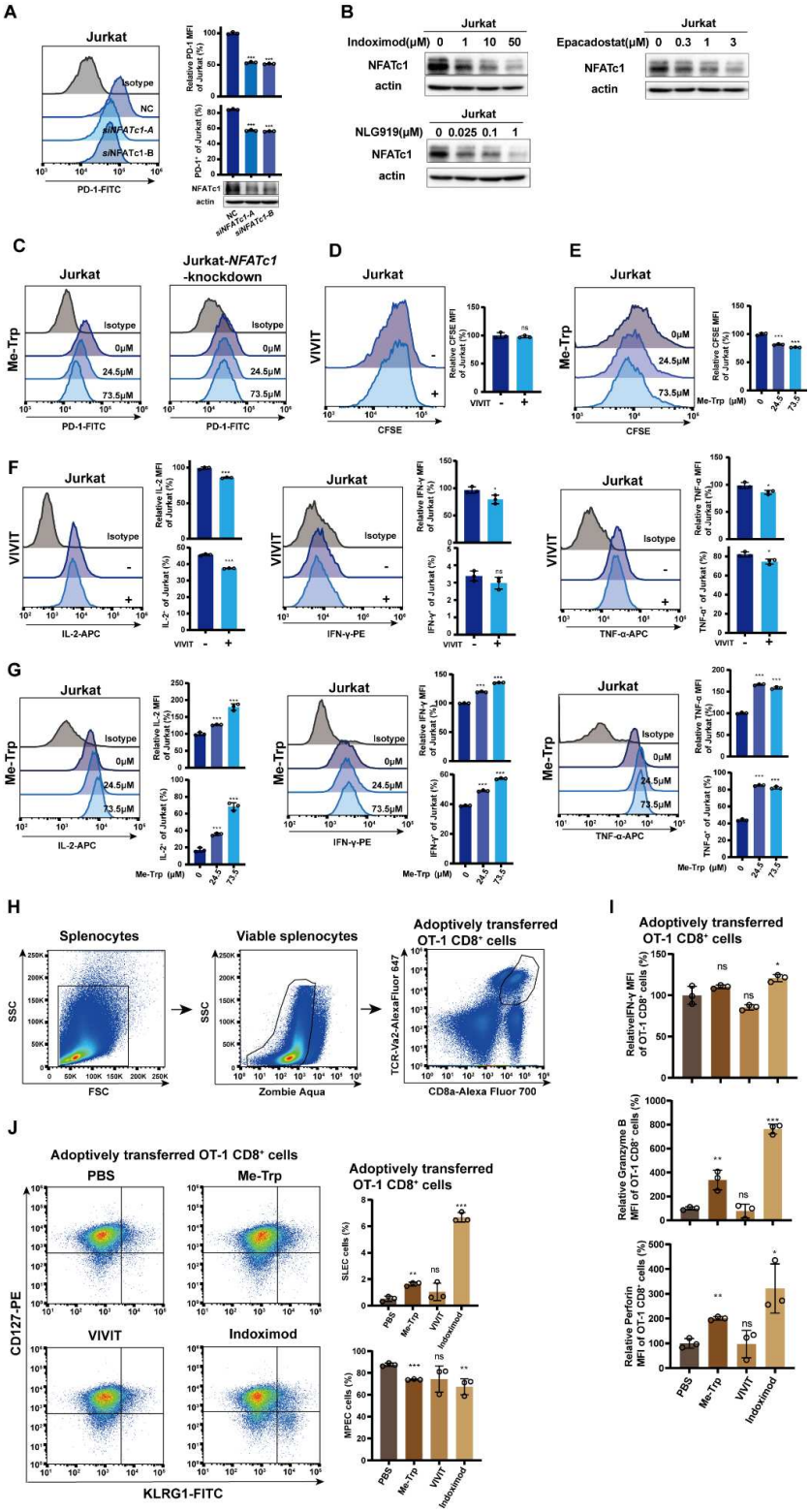
A
Jurkat
Isotype
NC
siNFATc1-A
siNFATc1-B
PD-1-FITC
Relative PD-1 MFI of Jurkat (%)
PD-1+ of Jurkat (%)
NFATc1
actin
B
Jurkat
Indoximod(μM) 0 1 10 50
NFATc1
actin
Epacadostat(μM) 0 0.3 1 3
NLG919(μM) 0 0.025 0.1 1
C
Jurkat
Jurkat-NFATc1 -knockdown
Me-Trp
0μM
24.5μM
73.5μM
D
VIVIT
CFSE
Relative CFSE MFI of Jurkat (%)
E
Me-Trp (μM)
F
IL-2-APC
IFN-γ-PE
TNF-α-APC
G
H
Splenocytes
Viable splenocytes
Adoptively transferred OT-1 CD8+ cells
SSC
FSC
Zombie Aqua
TCR-Va2-AlexaFluor 647
CD8a-Alexa Fluor 700
I
Relative IFN-γ MFI of OT-1 CD8+ cells (%)
Relative Granzyme B MFI of OT-1 CD8+ cells (%)
Relative Perforin MFI of OT-1 CD8+ cells (%)
J
PBS
Me-Trp
VIVIT
Indoximod
CD127-PE
KLRG1-FITC
SLEC cells (%)
MPEC cells (%)

Figure. S3.

Tryptophan and WARS downregulates PD-1 transcription factor NFATc1

S3A, Knockdown *NFATc1* results in decreased levels of PD-1. The MFI and PD-1 positive cells were determined in both Jurkat T cells and in *NFATc1* knockdown Jurkat T cells. n = 3, mean ± SD.

S3B, IDO inhibitors decrease NFATc1. The protein levels of NFATc1 were examined in Jurkat cells that were treated with IDO inhibitors (indoximod, epacadostat and NLG919 analogue).

S3C, NFATc1 knockdown abrogated Me-Trp to decrease the cell surface PD-1. Representative flow cytometry results of Figure 3J.

S3D-S3E, VIVIT peptide and Me-Trp effects on proliferation of Jurkat cells. The proliferation of Jurkat cells were measured under absence and presence of VIVIT peptide (15μM, 24h) (**S3D**) or Me-Trp (**S3E**). n = 3, mean ± SD.

S3F, VIVIT peptide decreased cytokine levels. The IL-2, IFN-γ and TNF-α levels were detected in stimulated Jurkat T cells, VIVIT peptide-treated Jurkat T cells by flow cytometry. n = 3, mean ± SD.

S3G, Trp supplementation increased cytokine levels. The IL-2, IFN-γ and TNF-α levels were detected in stimulated Jurkat T cells, Trp-treated Jurkat T cells by flow cytometry. n = 3, mean ± SD.

S3H, Sorting OT-1 $CD8^+$ T cells from adoptively transferred C57BL/6 mice. $CD8^+$ cells were isolated from OT-1 mice and adoptively transferred to C57BL/6 mice. Splenic leukocytes of C57BL/6 mice were collected, viable T cells were distinguished by Zombie Aqua staining, then a $CD8^+$ TCR-$Va2^+$ cell gate was used to define adoptively transferred $CD8^+$ T cells.

S3I, Adoptively transferred $CD8^+$ T cell cytokine production in C57BL/6 mice is altered by Trp and IDO inhibitor. $CD8^+$ cells isolated from OT-1 mice were adoptively transferred to Trp-, VIVIT-, Indoximod-treated C57BL/6 mice. The IFN-γ, Granzyme and Perforin levels of sorted $CD8^+$ T cells were detected by flow cytometry. n = 3, mean ± SD.

S3J, Adoptively transferred $CD8^+$ T cell differentiation is altered by Trp and IDO inhibitor in C57BL/6 mice. $CD8^+$ cells isolated from OT-1 mice were adoptively transferred to Trp-, VIVIT-, Indoximod-treated C57BL/6 mice. $CD8^+$ T cells were sorted eight days after

adoptive transfer, and KLRG1 and CD127 levels were detected by flow cytometry. n = 3, mean ± SD.

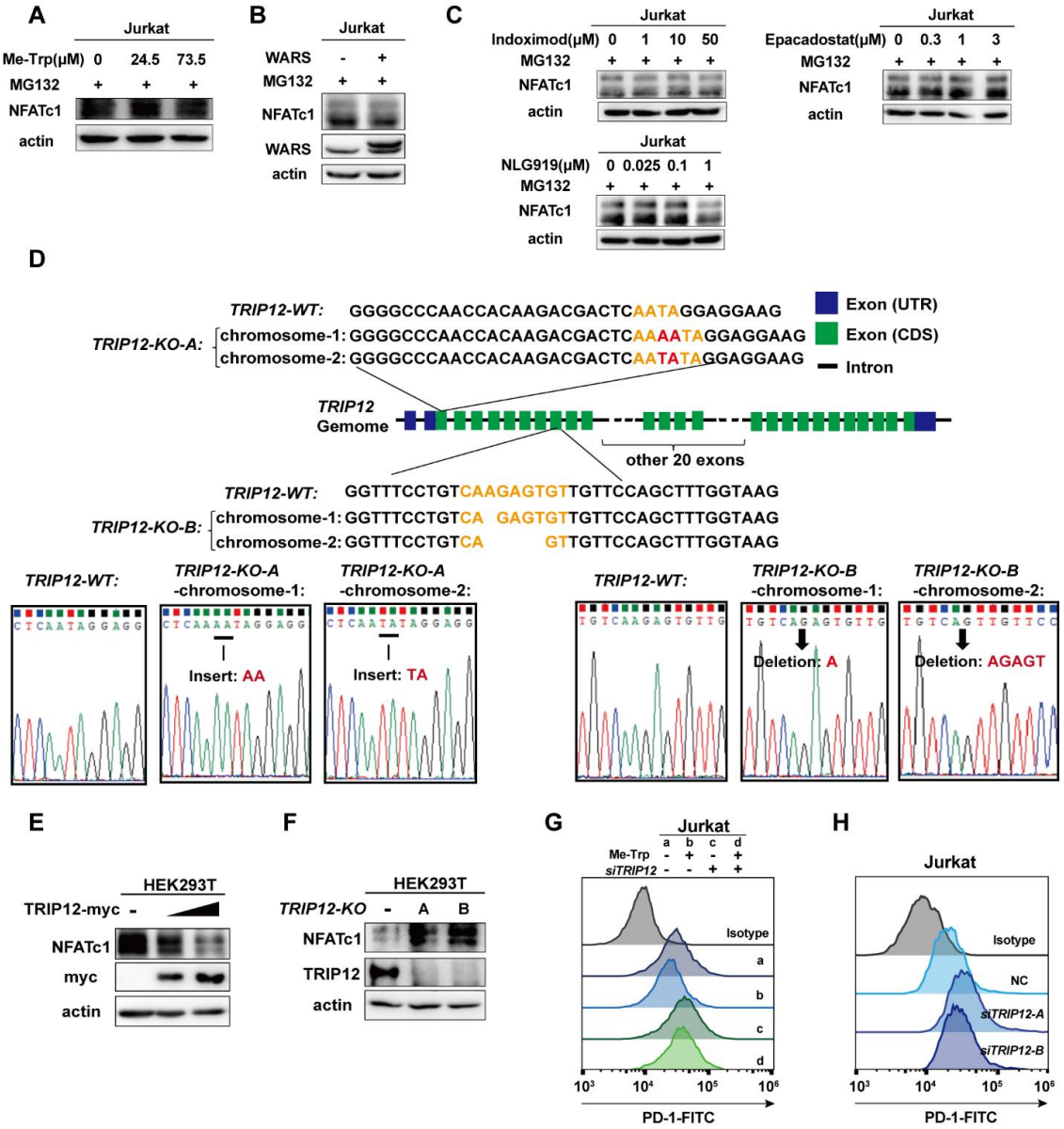

Figure. S4.

NFATc1 is subject to degradation by E3 ligase TRIP12

S4A-S4C, Tryptophan, WARS and IDO inhibitors could not decrease NFATc1 in the present of MG132. The protein levels of NFATc1 were examined in Jurkat cells that were

treated with Me-Trp (**S4A**), WARS overexpression (**S4B**) or IDO inhibitors (indoximod, epacadostat and NLG919 analogue) (**S4C**).

S4D, Knockout *TRIP12* in HEK293T cells. Schematic diagram of CRISPR/Cas9-mediated knockout of TRIP12 (TRIP12-KO) in HEK293T cells with two sgRNAs (upper). The success of *TRIP12* knockout was confirmed by sequencing (lower).

S4E-S4F, TRIP12 controlled NFATc1 protein levels. The endogenous NFATc1 levels were detected in HEK293T cells when either TRIP12 was overexpressed (**S4E**) or *TRIP12* was knockout (**S4F**) in HEK293T cells.

S4G, *TRIP12* knockdown abrogated Me-Trp to decrease the cell surface PD-1. Representative flow cytometry results of Figure 4I.

S4H, Silencing *TRIP12* elevated cell surface PD-1 protein. Representative flow cytometry results of Figure 4K.

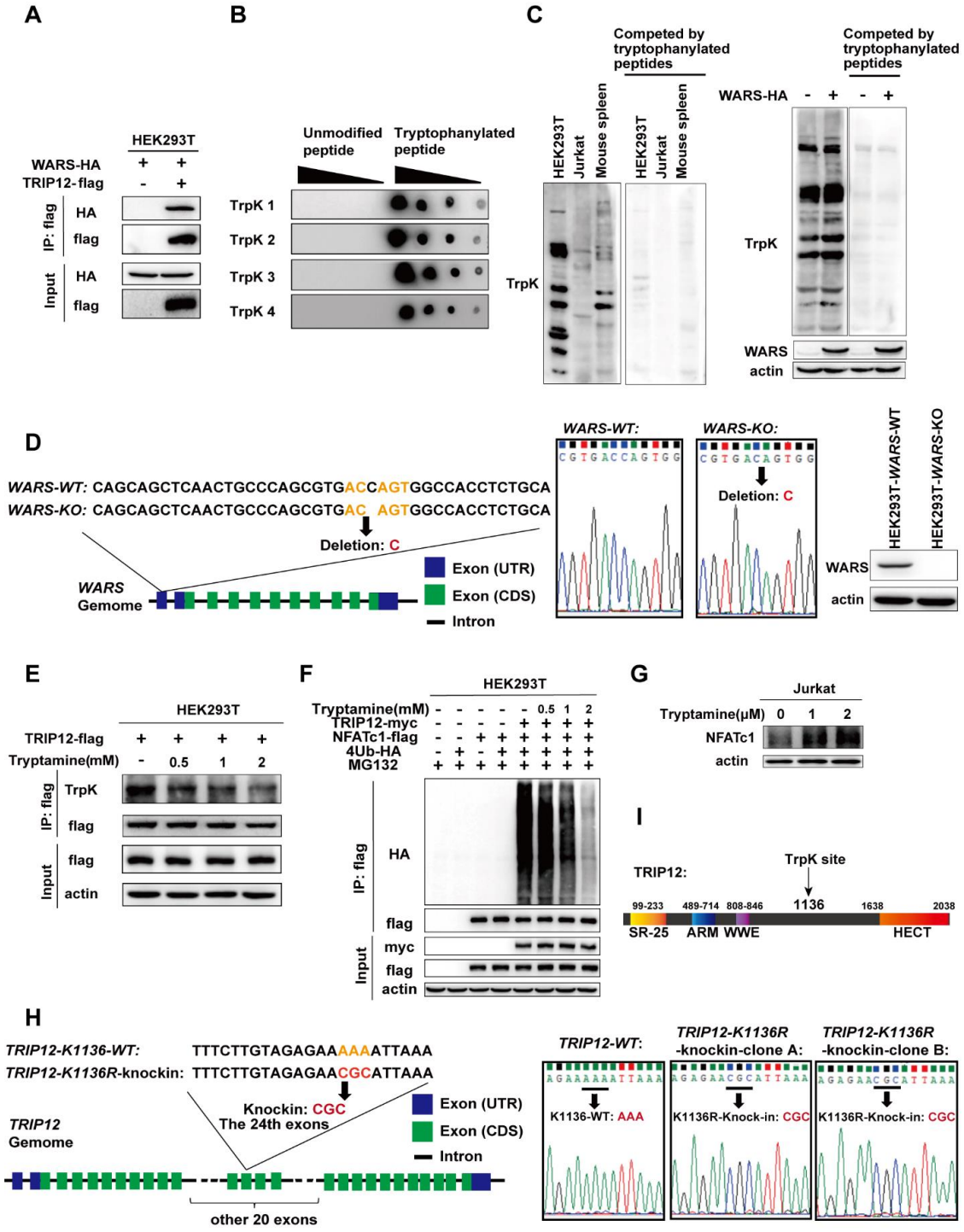

Figure. S5.

Tryptophanylation activates the E3 ligase activity of TRIP12

S5A, WARS interacted with TRIP12. Flag-tagged TRIP12 was co-overexpressed with WARS-HA in HEK293T cells. Interaction between TRIP12 and WARS was assayed by co-immunoprecipitation.

S5B-S5C, Validation of anti-tryptophanylation (TrpK) antibody. TrpK antibodies were verified by dot blotting. Tryptophanylated peptide and the corresponding unmodified peptide were serial diluted in 1:3 series and subject to dot-blot analysis **(S5B)**. To confirm the specificity of the antibody, TrpK antibodies signals of cell/tissue lysates were competed with Tryptophanylated peptide **(S5C)**.

S5D, Generation of *WARS* knockout. Schematic of CRISPR/Cas9 strategy to knockout *WARS* in HEK293T cells (left). The success of knockout was confirmed with sequencing (right).

S5E-S5F, WARS inhibitor tryptamine decreased TRIP12 tryptophanylation levels and NFATc1 ubiquitination. Flag-tagged TRIP12 was overexpressed in HEK293T cells that were treated with tryptamine at doses as indicated. TRIP12 tryptophanylation levels **(S5E)** and NFATc1 ubiquitination levels **(S5F)** were detected.

S5G, Tryptamine increased endogenous NFATc1 protein levels. Jurkat T cells were treated with tryptamine at doses as indicated. Endogenous NFATc1 levels were detected.

S5H, Constructing TRIP12^{K1136R} knockin HEK293T cells. Schematic of CRISPR/Cas9 strategy (left) and the sequencing verification of success of the construction (right) were shown (clone A and clone B were constructed from different sgRNAs).

S5I, K1136 is located between WWE and HECT domain. The position of tryptophanylation site K1136 in TRIP12 is shown.

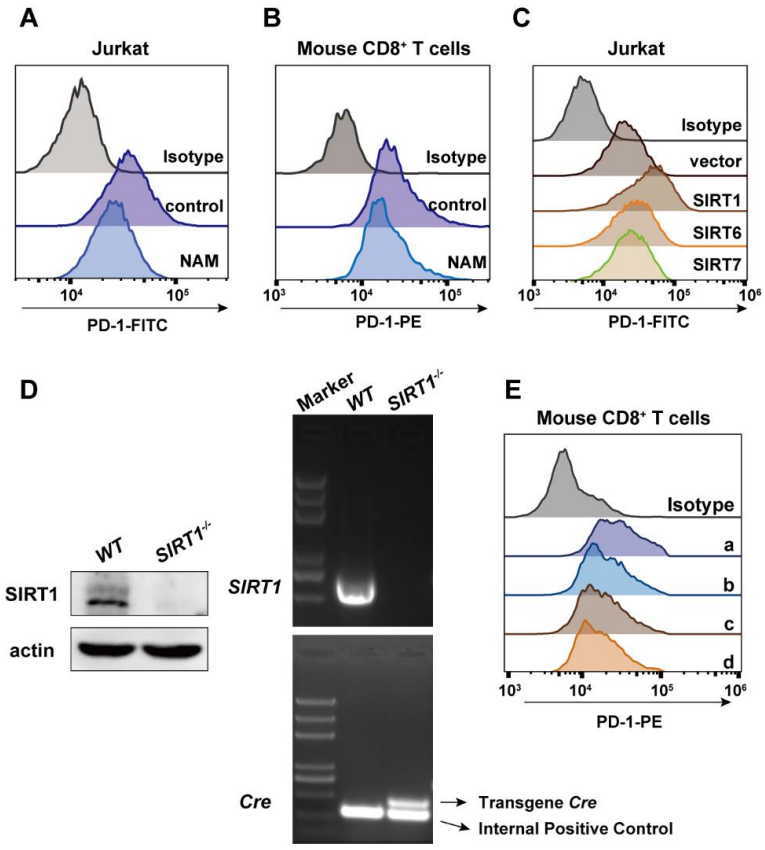

Figure. S6.

SIRT1 decreases TRIP12 tryptophanylation

S6A-S6B, NAM decreased cell surface PD-1 in Jurkat and mouse CD8⁺ T cells.

Representative flow cytometry results of Figure 6a **(S6A)**, Figure 6c **(S6B)**.

S6C, SIRT1 increased cell surface PD-1 expression. Representative flow cytometry results of Figure 6F.

S6D, Validation of *SIRT1*$^{-/-}$ knockout in mice. The absence of SIRT1 in *SIRT1*$^{-/-}$ knockout mice was confirmed by both western blot for protein (left) and PCR for gene (right).

S6E, *SIRT1* ablation in mice prevented NAM to decrease surface PD-1 expression.

Representative flow cytometry results of Figure 6G.

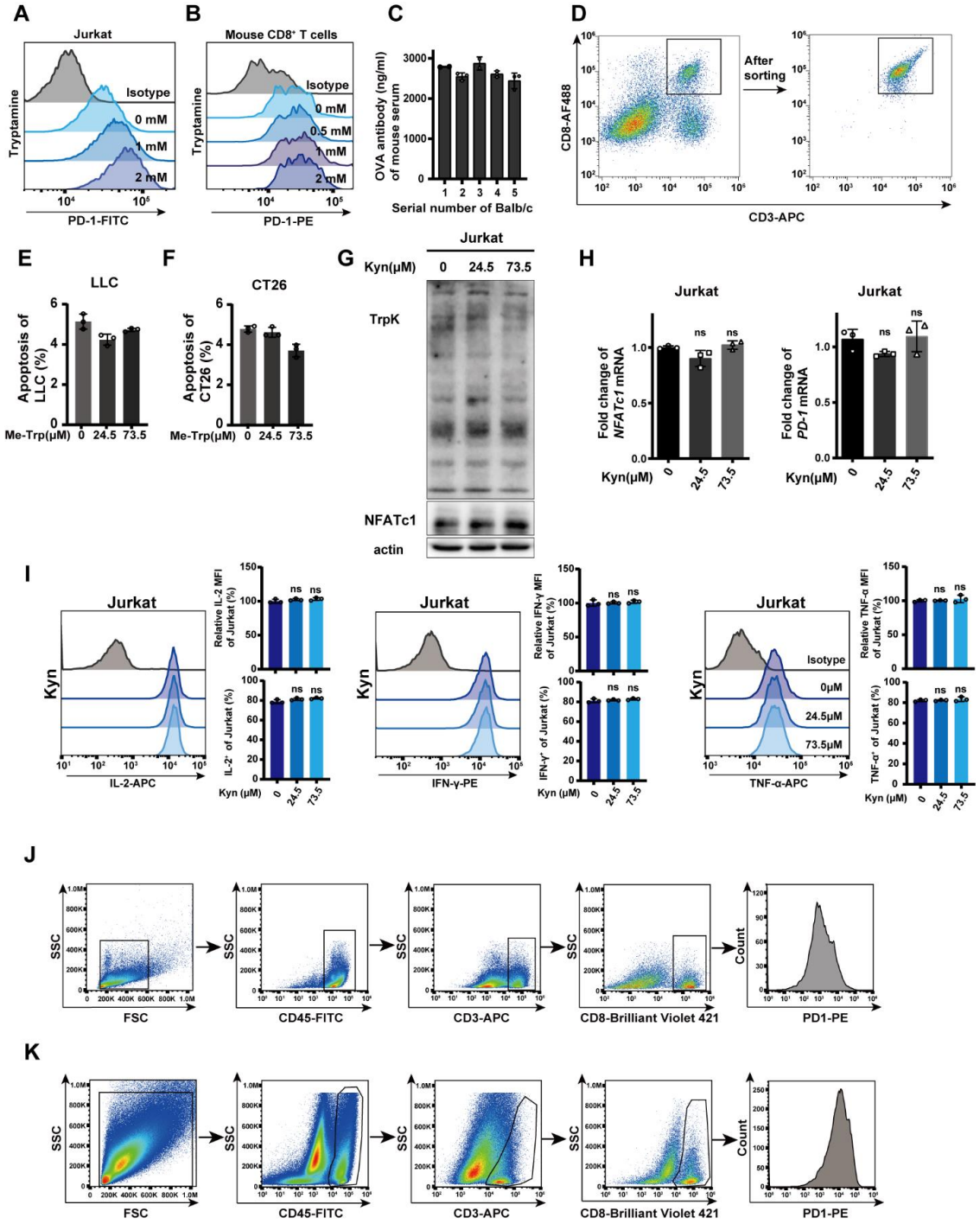

Figure. S7.

Increased tryptophanylation potentiates T cells to eliminate cancer cells.

S7A, Tryptamine increased cell surface PD-1 in Jurkat cells. Representative flow cytometry results of Figure 7A.

S7B, Tryptamine increased cell surface PD-1 in mouse CD8$^+$ T cells. Representative flow cytometry results of Figure 7C.

S7C, The concentration of OVA antibodies in serum of OVA-immuned BALB/c mice. Orbital blood from OVA-immuned BALB/c mice was collected, the concentrations of OVA antibodies were obtained by ELISA.

S7D, Sorting CD8$^+$ T cells from OVA immuned BALB/c splenocytes. Splenic leukocytes were distinguished by CD3$^+$ and CD8$^+$ cell gates to define CD8$^+$ T cells.

S7E-S7F, Tryptophan had negligible effect on the apoptotic rate of LLC and CT26 cells. Percentages of apoptosis of LLC or CT26 cells were analyzed for LLC **(S7E)** or CT26 **(S7F)** cells when they were cultured under Me-Trp levels as indicated. n = 3, mean ± SD.

S7G, Kynurenine didn't affect tryptophanylation and NFATc1 levels in Jurkat T cells. Jurkat T cells were cultured in media supplemented with indicated levels of kynurenine. Global tryptophanylation levels and NFATc1 levels were detected.

S7H, Kynurenine didn't affect NFATc1 and PD-1 mRNA levels in Jurkat T cells. The NFATc1 and PD-1 mRNA levels of anti-CD3 and anti-CD28 antibodies co-stimulated Jurkat T cells were detected with RT-qPCR when the cells were treated with different levels of kynurenine. n = 3, mean ± SD.

S7I, Kynurenine didn't affect IL-2, IFN-γ and TNF-α level in Jurkat T cells. The IL-2, IFN-γ and TNF-α levels were detected in stimulated Jurkat T cells, kynurenine-treated Jurkat T cells by flow cytometry. n = 3, mean ± SD.

S7J-S7K, Sorting CD8$^+$ T cells from tumor-bearing mice. Splenic leukocytes **(S7J)** and tumor-infiltrating lymphocytes **(S7K)** were first distinguished by a CD45+ cell gate, then T cells were distinguished by a CD3$^+$ cell gate, then a CD8$^+$ cell gate was used to define CD8$^+$ T cells.

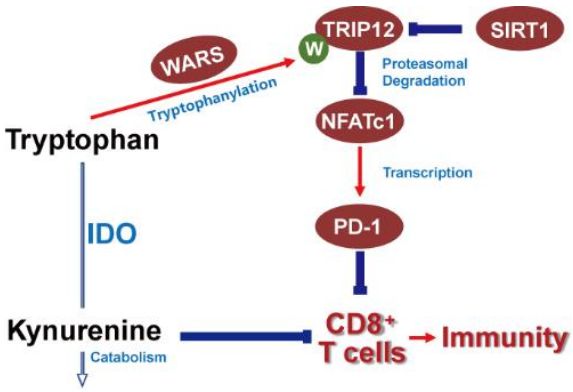

Figure. S8.

Both tryptophan and its catabolites are regulators of immunity

S8, A diagram that illustrates how tryptophan and tryptophan catabolites regulate immunity is shown.