Science Advances

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Supplementary Materials for

IFN-γ–dependent tumor-antigen cross-presentation by lymphatic endothelial cells promotes their killing by T cells and inhibits metastasis

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The PDF file includes:

> Figs. S1 to S10
> Legend for movie S1

Other Supplementary Material for this manuscript includes the following:

> Movie S1

Figure S1.

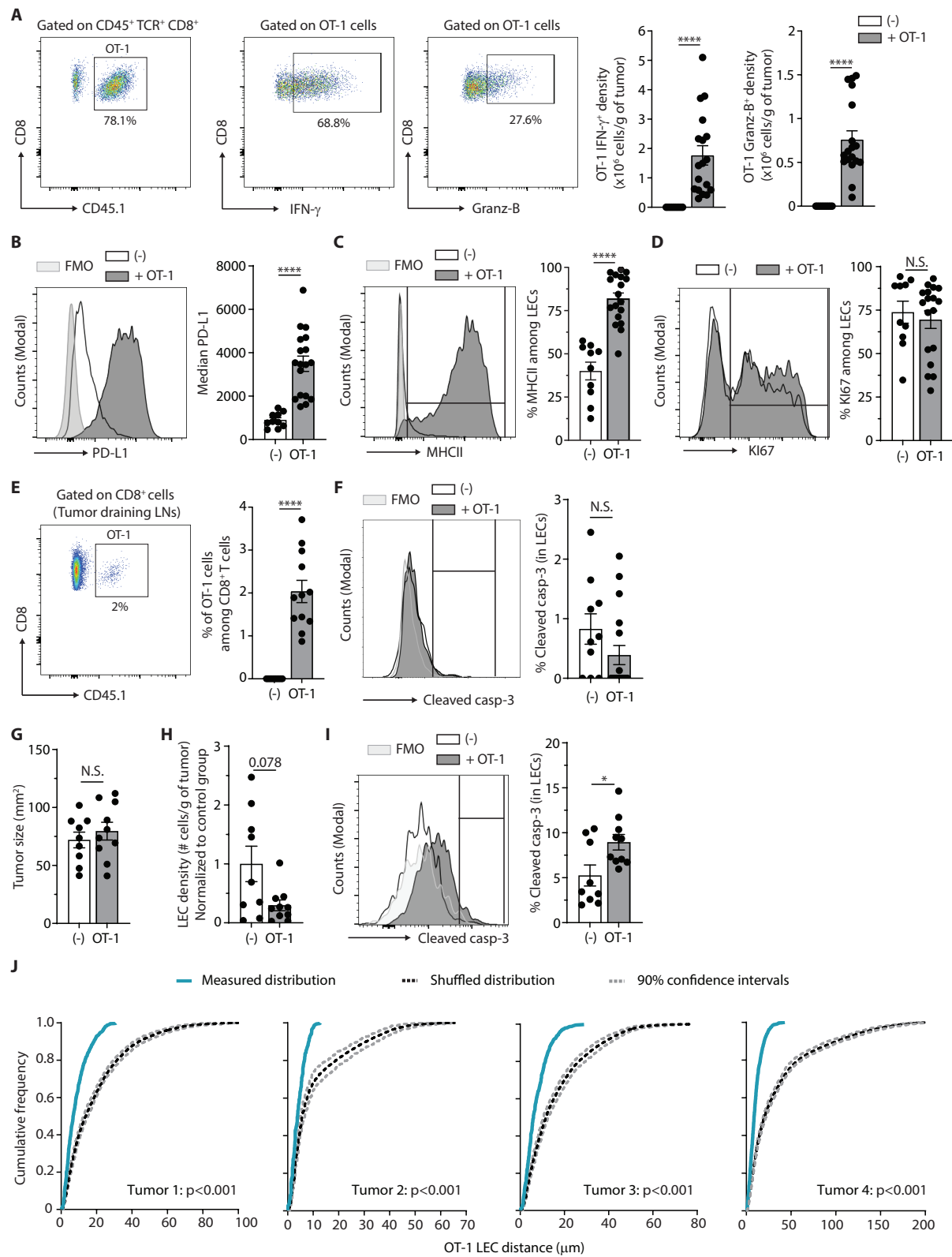**Fig. S1. Tumor LEC phenotype and density, and OT-1 distribution in B16F10-OVA$^+$VC$^+$ tumor bearing mice adoptively transferred with OT-1 effectors**

(A-F) C57BL/6 mice were injected or not with *in vitro* activated CD8$^+$ OT-1 cells (1x10^6 cells) 9 days post B16F10-OVA$^+$VC$^+$ inoculation, and tumors **(A-D)** and TdLNs **(E, F)** were analysed 5 days later. **(A)** Representative flow cytometry dot plot of intratumoral OT-1 cells (alive, CD45$^+$, TCR$^+$, CD8$^+$, CD45.1$^+$) producing IFN-γ and granzyme-B (Granz-B). Histograms show IFN- γ and granzyme-B producing OT-1 densities. **(B)** PD-L1 median, **(C)** MHCII$^+$ frequency and **(D)** KI67$^+$ frequency among tumor LECs analysed by flow cytometry. Graphs represent a pool of 2 experiments with 10-18 mice per group. **(E)** Representative flow cytometry dot plot and histogram showing OT-1 cell frequency in TdLNs (alive, CD8$^+$, CD45.1$^+$). **(F)** Frequency of Cleaved-casp3$^+$ LECs (CD45^{neg} CD31$^+$ GP38$^+$) analysed in TdLNs by flow cytometry. Graphs are pooled from 2 experiments with 10-12 mice per group. **(G-I)** C57BL/6 mice were inoculated with a mix of B16F10-VC$^+$ and B16F10-OVA$^+$VC$^+$ (ratio 9:1) and transferred with *in vitro* activated OT-1 cells 9 days later. **(G)** Tumor size 5 days post OT-1 transfer. **(H)** Intratumoral lymphatic vessel density and **(I)** frequency of Cleaved casp3$^+$ LECs. Graphs represent a pool of 2 experiments with 9-10 mice per group. **(J)** C57BL/6 mice were injected or not with *in vitro* activated CD8$^+$ OT-1 cells (1x10^6 cells) 9 days post B16F10-OVA$^+$VC$^+$ inoculation. Cumulative frequency distribution of measured distances between OT-1 and LECs at day 4 post OT-1 injection. Randomization (shuffled distribution) of acquired images was performed to assess statistical significance of co-localization within individual tumor sections (tumor 1-4). (A, B, C, D, E, F, G, H and I) Mann-Whitney tests, Error bars show mean ± SEM.
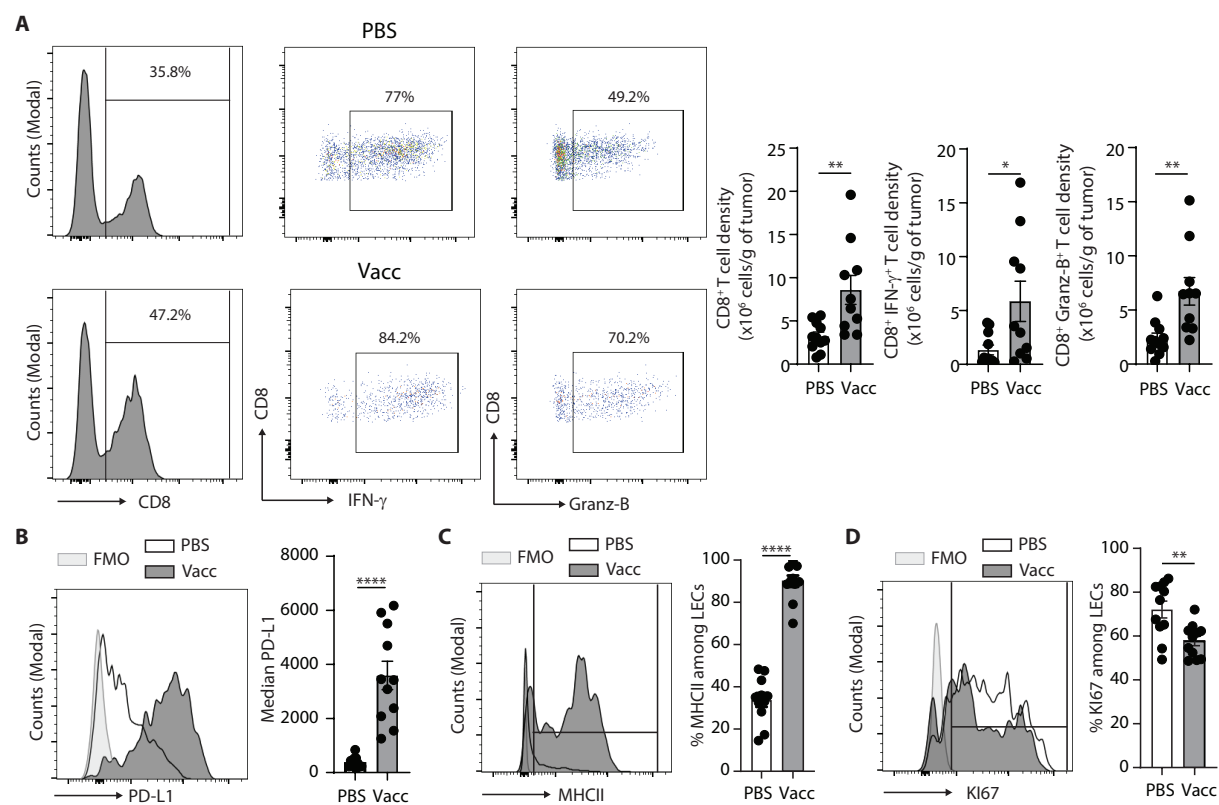
Figure S2.

Fig. S2. OVA+CpG-B vaccination enhances PD-L1 and MHCII expression on tumor LECs
C57BL/6 mice were vaccinated or not with OVA+CpG-B at day 5 post B16F10-OVA$^+$VC$^+$inoculation. **(A)** Representative flow cytometry dot plot and densities of intratumoral CD8$^+$ T cells producing IFN-γ and granzyme-B densities at day 12. **(B)** PD-L1 median, **(C)** MHCII$^+$ frequency and **(D)** KI67$^+$ frequency among tumor LECs analysed by flow cytometry 7 days post vaccination. Graphs represent a pool of 2 experiments with 10-11 mice per group. Mann-Whitney test. Error bars show mean ± SEM.
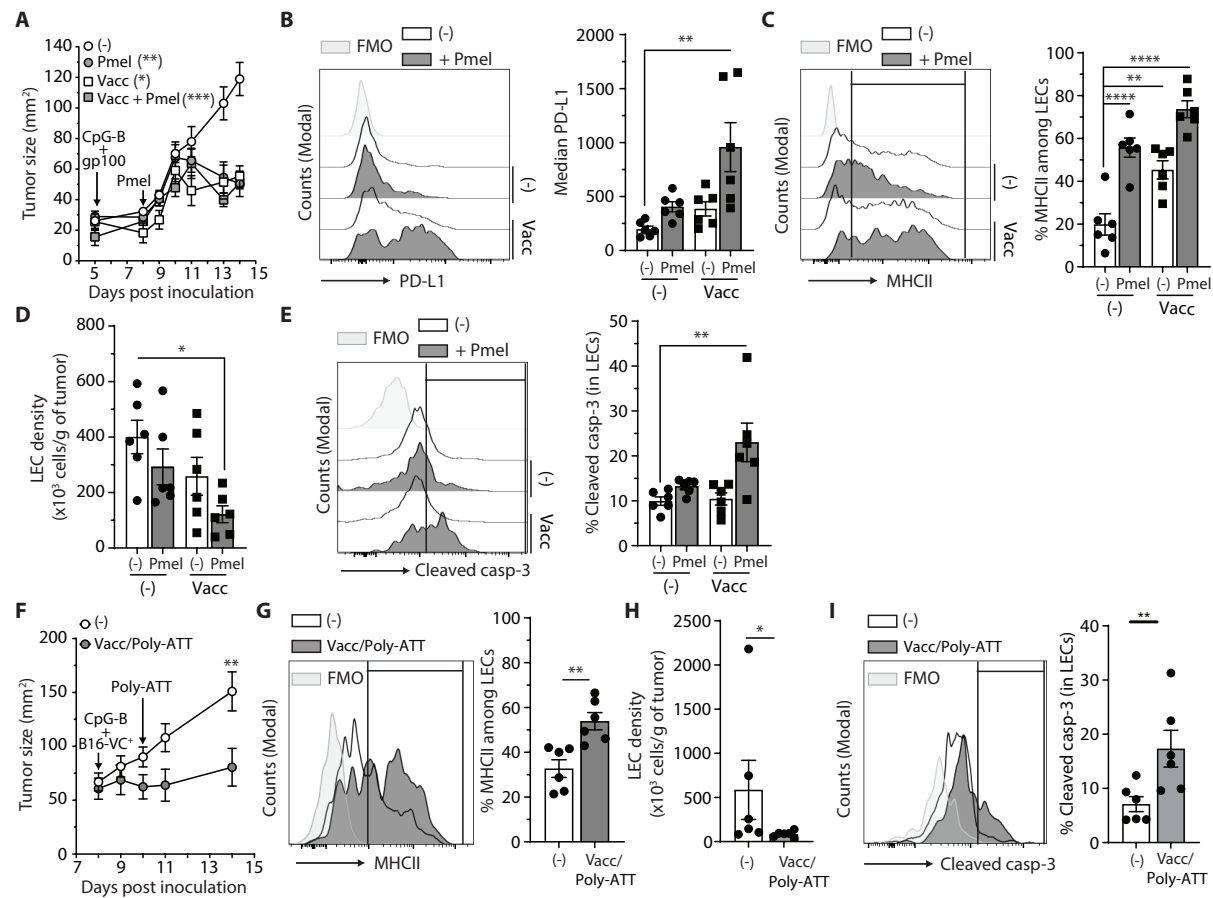
Figure S3.

Fig. S3. Tumor specific T cell transfer and irradiated tumor cell+CpG-B vaccination reduce tumor LV density by promoting tumor LEC apoptosis

(A-E) Mice were vaccinated or not with hgGp100+CpG-B at day 5 and injected or not with *in vitro* activated CD8$^+$ Pmel-1 cells (12x10^6 cells) at day 8 post B16F10-OVA$^+$VC$^+$ inoculation. **(A)** Tumor growth was followed until day 14. **(B)** PD-L1 median, **(C)** MHCII$^+$ frequency **(D)** LEC density, and **(E)** Cleaved casp-3 frequency among tumor LECs analysed by flow cytometry 9 days post vaccination. Graphs are from 1 experiment with 6 mice per group. **(F-I)** Mice were vaccinated or not with irradiated B16F10-VC$^+$ cells+CpG-B at day 8 and injected or not at day 10 post B16F10-VC$^+$ inoculation with CD8$^+$ T cells (1x10^6 cells) activated *in vitro* with DCs loaded with irradiated B16F10-VC$^+$cells. **(F)** Tumor growth was followed until day 14. **(G)** MHCII$^+$ frequency **(H)** LEC density, and **(I)** Cleaved casp-3 frequency among tumor LECs analysed by flow cytometry 6 days post vaccination. Graphs are from 1 experiment with 6 mice per group. (A, F) Two-way ANOVA test, (B-E and G-I) One-way ANOVA test. Error bars show mean ± SEM.
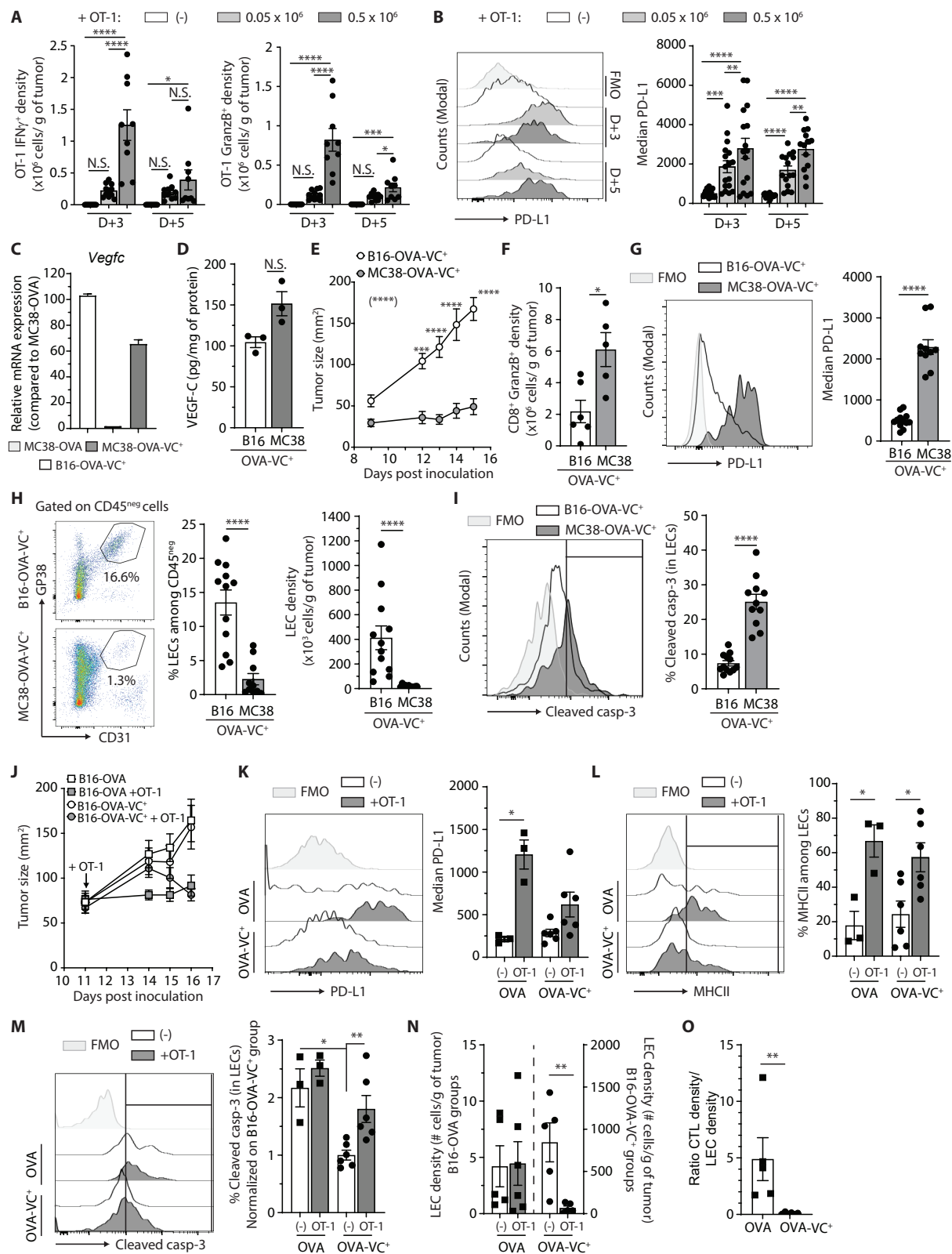
Figure S4.

Fig. S4. An elevated CTL/LEC ratio is required to promote tumor LEC killing

(A-B) C57BL/6 mice were injected or not with different doses of *in vitro* activated OT-1 cells (0.05×10^6 or 0.5×10^6 cells) 9 days after B16F10-OVA$^+$VC$^+$ inoculation and sacrificed 3 or 5 days later. **(A)** IFN-γ and granzyme-B producing OT-1 densities and **(B)** expression levels of PD-L1 by LECs in tumors. Data are pooled from 2 experiments, N= 9-11 mice per group. **(C)** *Vegfc* mRNA expression levels in B16F10-OVA$^+$VC$^+$, MC38-OVA$^+$ and MC38-OVA$^+$VC$^+$ cell cultures. **(D-I)** C57BL/6 mice were inoculated with 0.5×10^6 B16F10-OVA$^+$VC$^+$ or MC38-OVA$^+$VC$^+$ cells. **(D)** VEGF-C protein levels were measured in tumors at day 15. **(E)** Tumor growth, **(F)** granzyme-B producing CD8$^+$ T cell densities, **(G)** expression levels of PD-L1 by LECs, **(H)** tumor LEC frequencies and densities and **(I)** frequency of Cleaved casp-3$^+$ tumor LECs were evaluated by flow cytometry at day 15. (D, G, H, I) are pooled from 2 experiments, N=11 mice per group. (F) Data are from 1 experiment, N= 5 mice per group. **(J-P)** C57BL/6 mice were injected or not with *in vitro* activated CD8$^+$ OT-1 CD45.1$^+$ cells (0.5×10^6 cells) 11 days post B16F10-OVA$^+$ or B16F10-OVA$^+$VC$^+$ inoculation. **(J)** Tumor growth was followed until day 16. **(K)** PD-L1 median, **(L)** frequencies of MHCII$^+$, **(M)** frequencies of Cleaved casp-3$^+$, **(N)** densities of LECs and **(O)** densities of CTLs were analysed in tumors at day 16 by flow cytometry. **(P)** CTL/LEC ratios were analysed in tumors in absence of OT-1 transfer. Graphs represent 1 experiment with 6 mice per group. (A, B) One-way ANOVA test. (D, F-I, K-N) Mann-Whitney test. (E, J, O-P) Two-way ANOVA test. Error bars show mean ± SEM.
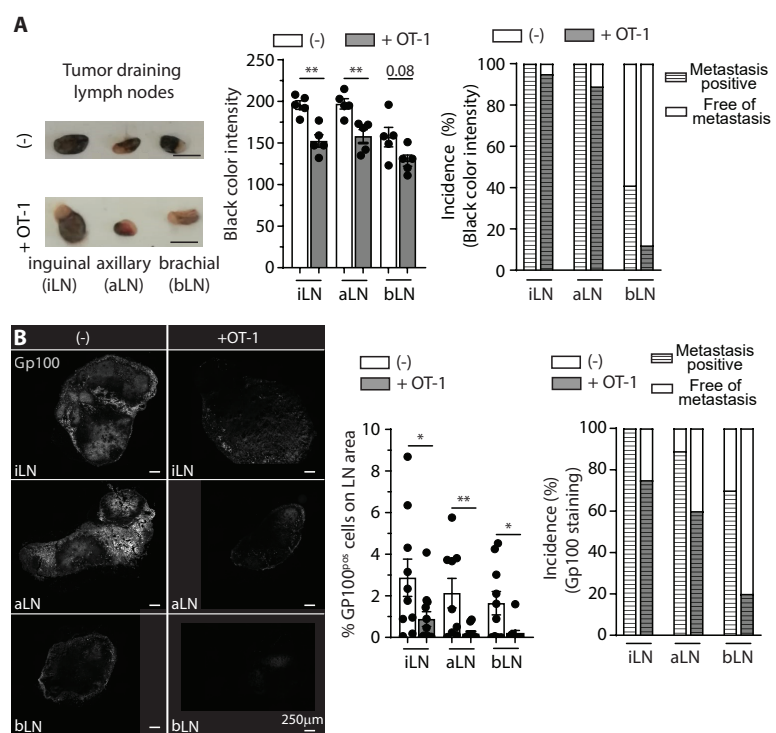
A

Tumor draining
lymph nodes

(-)

+ OT-1

inguinal axillary brachial
(iLN)   (aLN)   (bLN)

☐ (-) ▨ + OT-1

Black color intensity

** ** 0.08

iLN aLN bLN

☐ (-) ▨ + OT-1

Incidence (%)
(Black color intensity)

▤ Metastasis positive
☐ Free of metastasis

iLN aLN bLN

B

(-)   +OT-1

Gp100

iLN   iLN

aLN   aLN

bLN   bLN   250µm

☐ (-) ▨ + OT-1

% GP100^{pos} cells on LN area

* ** *

iLN aLN bLN

☐ (-) ▨ + OT-1

Incidence (%)
(Gp100 staining)

▤ Metastasis positive
☐ Free of metastasis

iLN aLN bLN

Figure S5.

Fig. S5. Tumor draining lymph nodes metastasis are reduced after OT1 transfer

C57BL/6 mice were injected or not with 0.5×10^6 *in vitro* activated OT-1 9 days after B16F10-OVA$^+$VC$^+$ inoculation and sacrificed 5 days later. **(A-B)** Black color intensity **(A)** and immunofluorescent GP100 staining **(B)** of inguinal, axillary and brachial TdLN of mice analysed with Image J or QuPath respectively. Pictures show representative images (Scale bar = 0.25mm), and left histograms the quantification of the intensity for individual LNs per group. Right histograms represent the percentage of metastasis positive and negative (free of metastasis) LNs. (A) Data are representative of two independent experiments, N= 5 mice per group. (B) Data are a pool of 2 experiments, N=10 per group.
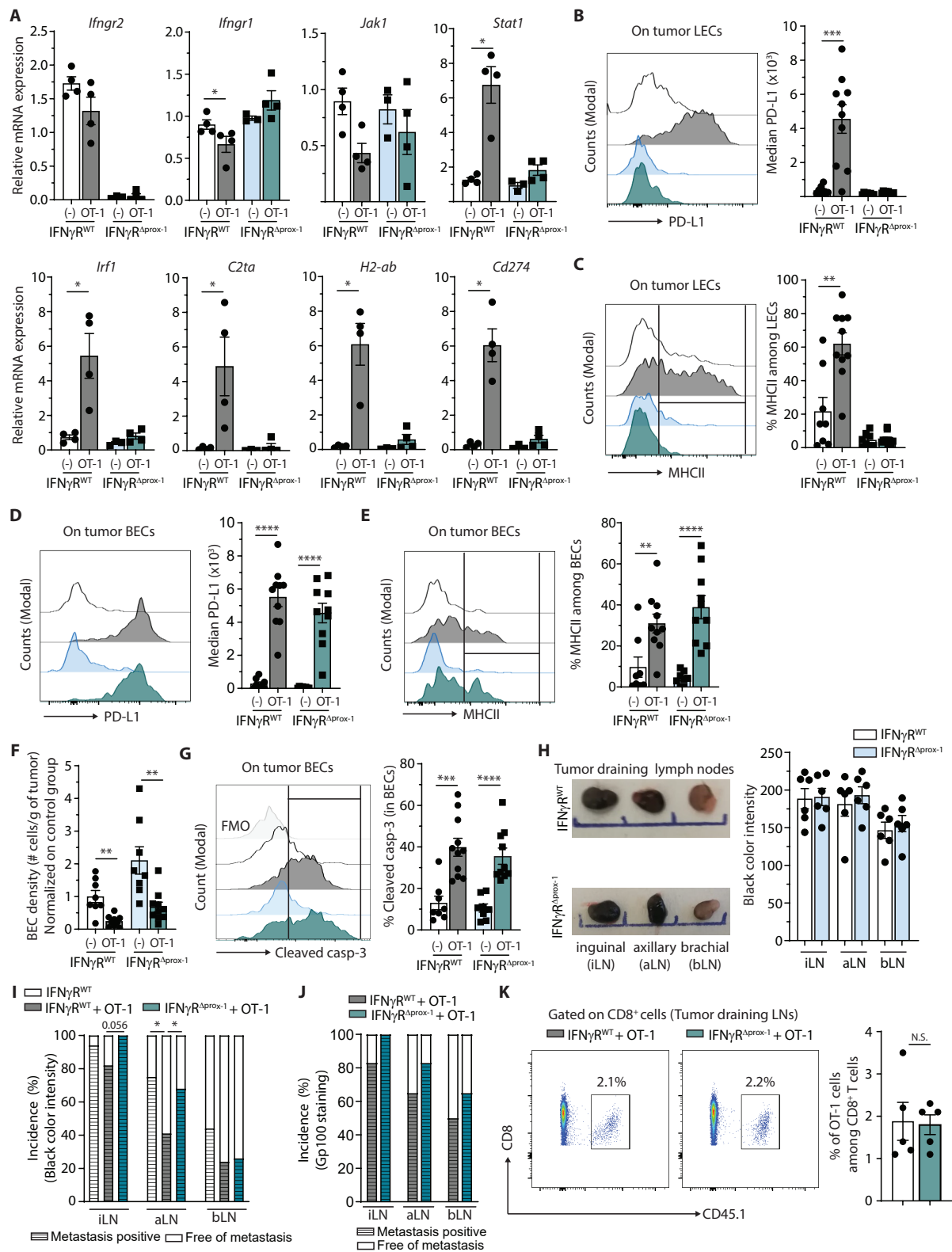
Figure S6.

Fig. S6: IFN-γR signaling is selectively abolished in LECs from IFNγR$^{\Delta prox-1}$ mice

(A-K) Tamoxifen-treated IFNγR^{WT} and IFNγR$^{\Delta prox-1}$ mice were injected or not with 0.5×10^6 OT-1 cells 9 days post B16F10-OVA$^+$VC$^+$ inoculation. **(A)** Tumor LECs (CD45^{neg}gp38$^+$CD31$^+$) were sorted by flow cytometry 4 days after OT-1 transfer, and mRNA levels on indicated genes were measured by qPCR. Data are representative of 1 experiment, N=3-4 mice per group. **(B)** PD-L1 and **(C)** MHCII expression on tumor LECs, and **(D)** PD-L1 and **(E)** MHCII expression **(F)** BEC density and **(G)** Frequencies of Cleaved casp-3$^+$ tumor BECs were analysed by flow cytometry 5 days post transfer. Data represent a pool of 2 experiments, N=6-10 mice per group. **(H)** Black color intensity of inguinal, axillary and brachial TdLNs of mice in absence of OT-1 transfer was analysed with Image J. Data represent one experiment with 6 mice per group. **(I-J)** Proportion of metastatic positive or negative (free of metastasis) calculated based on black color **(I)** or Gp100$^+$ staining **(J)**. **(K)** Representative flow cytometry dot plot and histogram of OT-1 cell frequency in TdLNs (alive, CD8$^+$, CD45.1$^+$) from IFNγR^{WT} and IFNγR$^{\Delta prox-1}$ mice. (A-G) Mann-Whitney test. (I) Chi^2 test. Error bars show mean ± SEM.
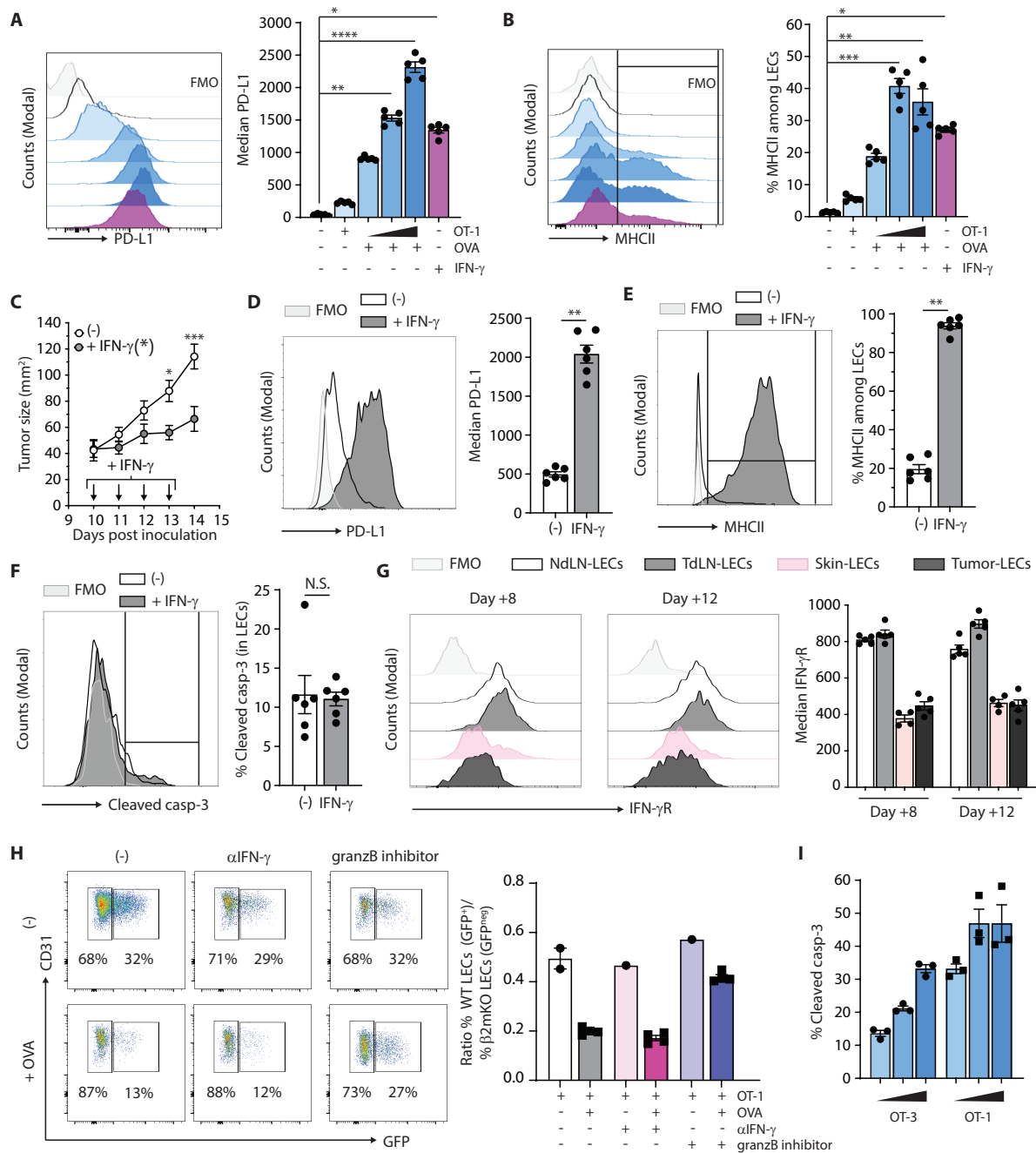
Figure S7.

Fig. S7: Tumor LECs killing is not directly mediated by IFN-γ but requires antigen-specific interactions with CTLs

(A-B) LEC/FRC cultures were loaded or not with OVA peptide (OVA) and incubated with either different ratio of *in vitro* activated OT-1 cells (1:10, 1:3 and 3:1 OT1:LEC/FRC) or soluble IFN-γ. **(A)** PD-L1 and **(B)** MHCII expression by LECs were analysed by flow cytometry after 14 h. Data are representative of two independent experiments. **(C-F)** C57BL/6 mice were injected or not intratumorally with 1.5μg of IFN-γ (twice a day during 4 days), 10 days after B16F10-OVA$^+$VC$^+$ inoculation. **(C)** Tumor growth was followed until day 14. **(D)** PD-L1, **(E)** MHCII expression and **(F)** frequencies of Cleaved casp-3$^+$ tumor LECs were analysed by flow cytometry at day 14. **(G)** B16F10-OVA$^+$VC$^+$ cells were inoculated into C57BL/6 mice. IFN-γR expression was analysed on LECs from non-draining LNs (NdLN-LECs), tumor draining LNs (TdLN-LECs), skin (Skin-LECs) and tumor (Tumor-LECs) by flow cytometry 8 and 12 days post-inoculation. **(H)** WT (LC3-GFP$^+$) or β2m^{KO} (GFP^{neg}) LEC/FRC cultures were loaded or not with OVA, and incubated or not with anti-IFNγ antibodies and granzyme B inhibitor. *In vitro* activated OT-1 cells were added in the cultures, and the ratio of frequencies of the percentage of WT and β2m^{KO} LECs (gated on CD45^{neg}CD31$^+$gp38$^+$ cells) were analysed 14 h later by flow cytometry. Data are from one experiment. **(I)** LEC/FRC cultures, loaded with OVAp, were incubated with different ratio of *in vitro* activated OT-3 or OT-1 cells (1:10, 1:3 and 3:1 T cell:LEC/FRC) and the frequency of Cleaved casp-3$^+$ LECs was analysed by flow cytometry. Data are from one experiment. (A, B) One-way ANOVA test. (C) Two-way ANOVA test, (D, E, F) Mann-Whitney test. Error bars show mean +/- SEM.
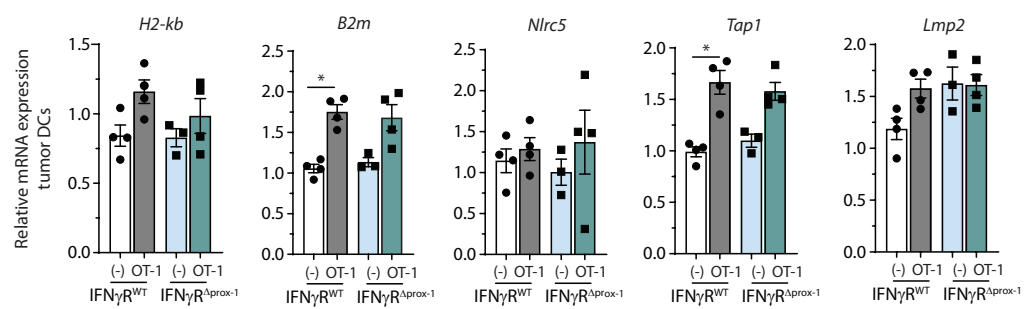
Figure S8.

Fig. S8: MHCI-restricted antigen (cross)-presentation pathways in tumor DCs.
Tamoxifen-treated IFNγR^{WT} and IFNγR$^{\Delta prox-1}$ mice were injected or not with 0.5x10^6 OT-1 cells 9 days post B16F10-OVA$^+$VC$^+$ inoculation. Tumor DCs (CD45^{pos}CD11c^{hi}MHCII$^+$) were sorted by flow cytometry 4 days after OT-1 transfer, and mRNA levels on indicated genes were measured by qPCR. Data are representative of 1 experiment, N=3-4 mice per group.
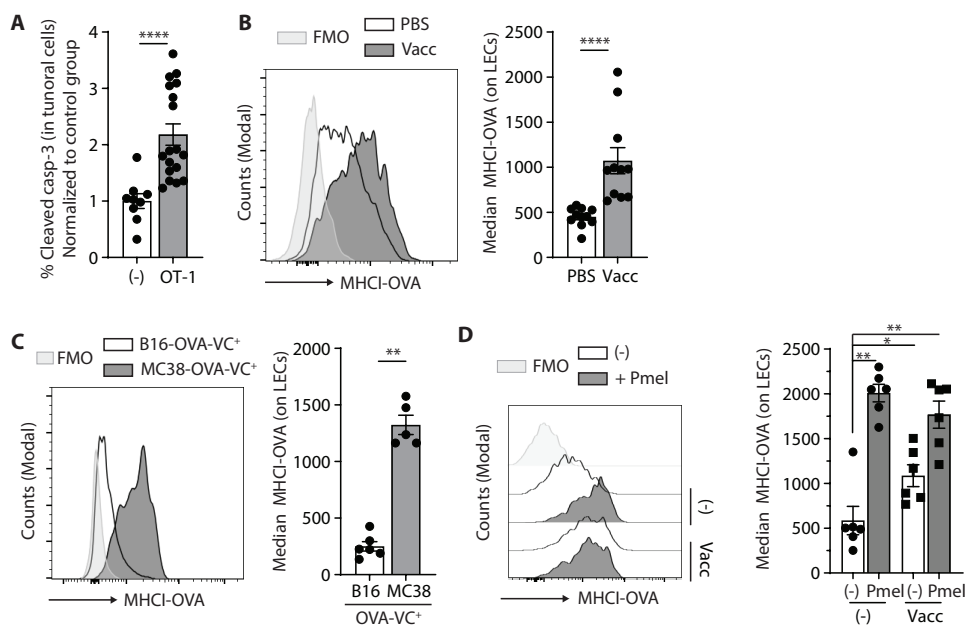
A

B

C

D

Figure S9.

Fig. S9: Tumor LECs upregulate MHCI-OVA complexes in immunogenic tumors.
(A) C57BL/6 mice were injected or not with *in vitro* activated OT-1 cells (0.5×10^6 cells) 9 days after B16F10-OVA$^+$VC$^+$ inoculation and sacrificed 5 days later. The frequency of Cleaved casp-3$^+$ tumor cells was analysed by flow cytometry. Graphs represent a pool of 2 experiments, N= 10-18 mice per group. **(B)** C57BL/6 mice were vaccinated or not with OVA+CpG-B at day 5 post B16F10-OVA$^+$VC$^+$ inoculation, and the expression levels of MHCI-OVA complexes by tumor LECs was analysed by flow cytometry at day 12. Graphs represent a pool of 2 experiments, N=10-11 mice per group. **(C)** C57BL/6 mice were inoculated with 0.5×10^6 B16F10-OVA$^+$VC$^+$ or MC38-OVA$^+$VC$^+$ cells, and the frequency of MHCI-OVA complexes by tumor LECs was analysed by flow cytometry at day 15. Data are from 1 experiment, N=6 mice per group. **(D)** Mice were vaccinated or not with hGp100+CpG-B at day 5 and injected or not with *in vitro* activated CD8$^+$ Pmel-1 cells (12×10^6 cells) at day 9 post B16F10-OVA$^+$VC$^+$ inoculation. The frequency of MHCI-OVA complexes by tumor LECs was analysed by flow cytometry at day 12. (A, B, C) Mann-Whitney test. (D) One-way ANOVA test. Error bars show mean ± SEM.
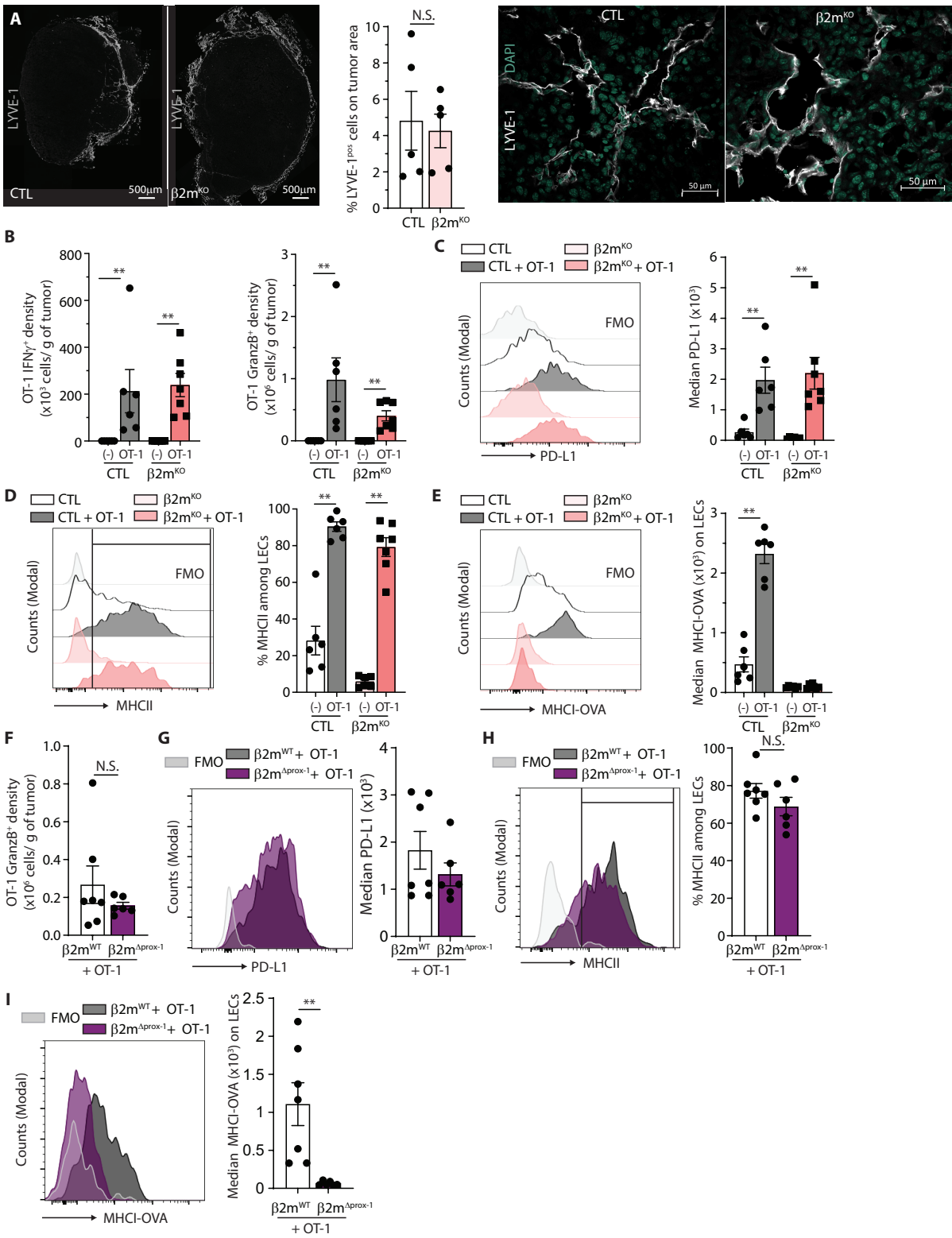
Figure S10.

Fig. S10: PD-L1, MHCII, and MHCI-OVA complex expression by tumor LECs lacking or not MHCI molecules.

(A) LYVE-1 immunofluorescent staining on tumor sections (day 12) from C57BL/6 and β2m^{KO} injected with B16F10-OVA$^+$VC$^+$ cells. **(B-E)** C57BL/6 and β2m^{KO} were injected or not with 0.5x10^6 OT-1 cells 8 days post B16F10-OVA$^+$VC$^+$ inoculation. **(B)** Density of IFN-γ and granzyme-B producing OT-1 cells, and **(C)** PD-L1, **(D)** MHCII, and **(E)** MHCI-OVA complex expression on tumor LECs were analysed by flow cytometry 4 days after OT-1 transfer. Data are representative of 2 independent experiments, N=6-7 mice per group. **(F-I)** Tamoxifen-treated β2m^{WT} and β2m$^{\Delta prox-1}$ mice were injected with 0.5x10^6 OT-1 cells 9 days post B16F10-OVA$^+$VC$^+$ inoculation. **(F)** Density of granzyme-B producing OT-1 cells, and **(G)** PD-L1, **(H)** MHCII, and **(I)** MHCI-OVA complex expression on tumor LECs were analysed by flow cytometry 5 days after OT-1 transfer. Data are from 1 experiment, N=6-7 mice per group. Mann-Whitney test. Error bars show mean ± SEM.

Movie S1.

Time-lapse confocal spinning disk video microscopy showing specific killing of WT LEC upon co-incubation with activated OT-1 *in vitro*. OT-1 (Bright field), co-culture of WT and β2mKO LEC (All LECs, grey), WT (LC3-GFP$^+$, green) and caspase-3 activity (pseudocolored). WT LEC are highlighted by outlines in all channels.