CD40 and CD80/86 signaling in cDC1s mediate effective neoantigen vaccination and generation of antigen-specific CX3CR1^{+} CD8^{+} T cells

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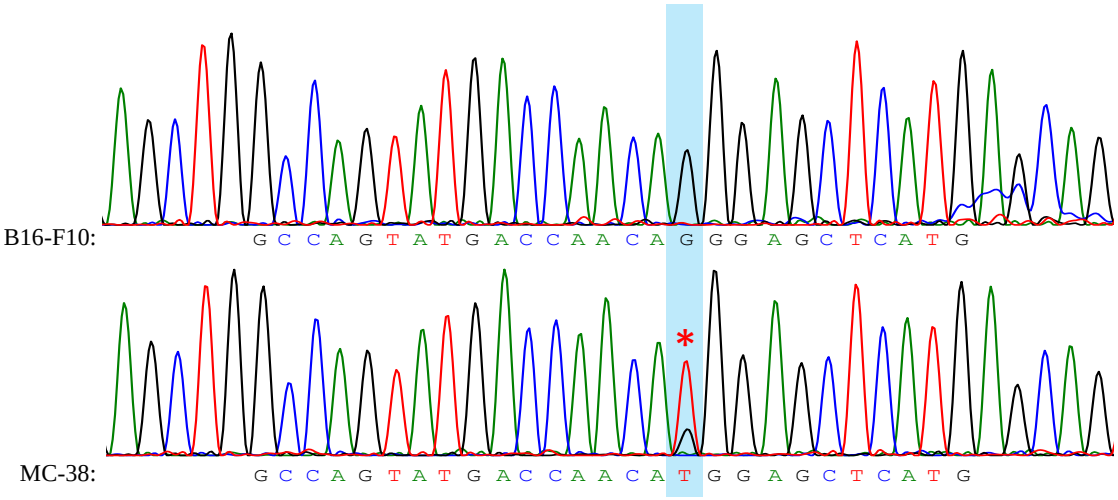
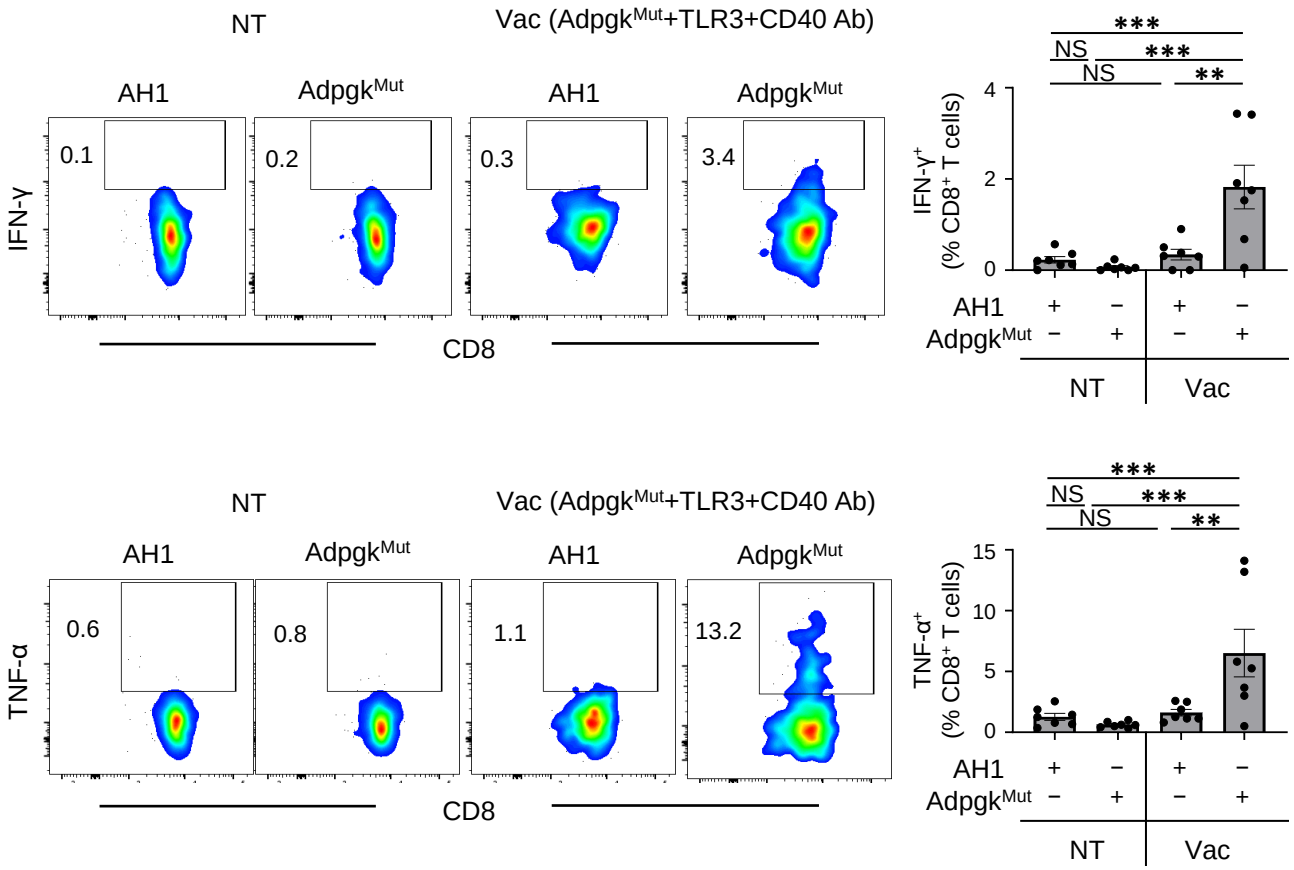
Supplementary Information

Supplementary Figures 1-9
Supplementary Table 1

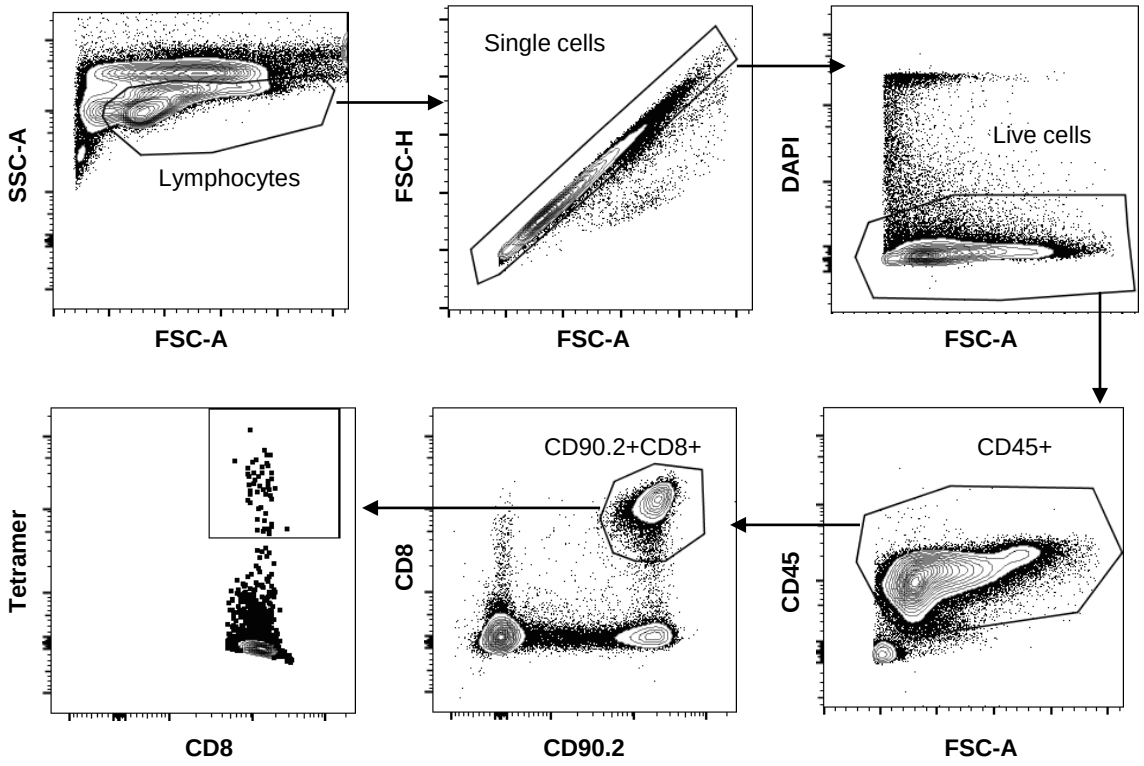


Supplementary Figure 1 cDNA sequencing of mutated Adpgk neoantigen in MC38 cells

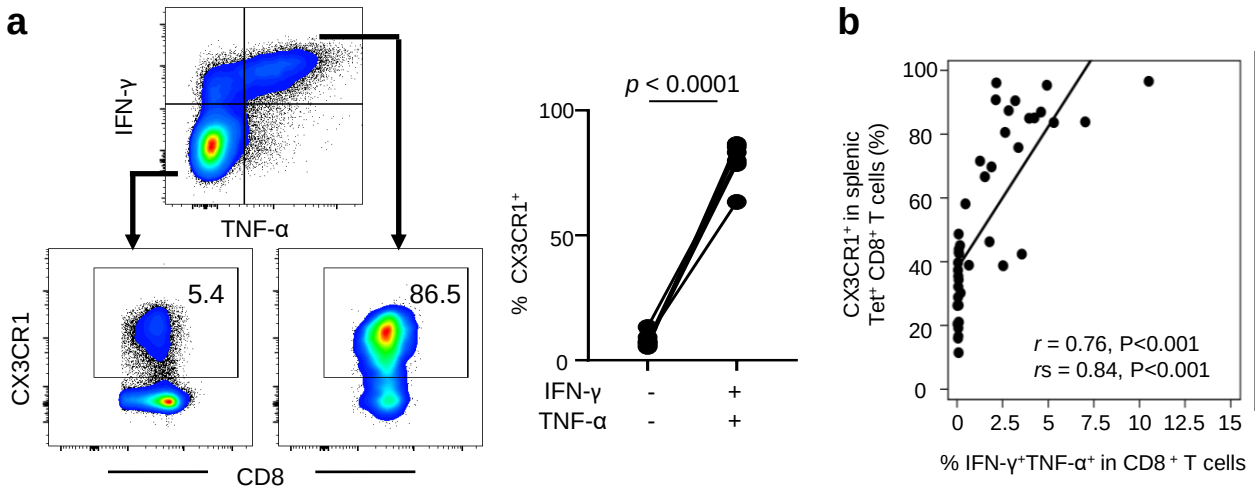
Total RNA was extracted from MC38 cells (harboring mutated Adpgk) along with B16F10 cells (harboring wild type Adpgk), and subjected to cDNA synthesis for Sanger DNA sequencing. An asterisk indicates the mutation of G→T, resulting in arginine (R) to methionine (M) transformation on 304th amino acid sequences of Adpgk gene.



Supplementary Figure 2 Neoantigen/TLR3/CD40 vaccination facilitates neoantigen-specific effector CD8⁺ T cell infiltration into tumors. Related to Fig. 1c.
Left shows representative FACS plots showing IFN-γ (upper) or TNF-α (lower) expression gated with CD8⁺ T cells in tumors of MC38-tumor bearing mice treated with PBS (NT) or Adpgk^{Mut}/TLR3/CD40 vaccinations (Vac) as described in Fig. 1a. Tumor cells were collected 1 week after 2nd vaccination, and co-cultured with AH1 or Adpgk^{Mut} peptide in the presence of brefeldin A for 5 hrs before intracellular staining. Numbers denote percentage of IFN-γ⁺ or TNF-α⁺ cells. Right panel shows the frequency of the IFN-γ⁺ or TNF-α⁺ cells in CD8⁺ T cells in each group (n = 7 mice per group). Data shown are representative from two independent experiments. NS not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparisons. Mean ± SEM.



Supplementary Figure 3 Gating strategy of circulating Adpgk^{Mut} neoantigen-specific CD8^{+} T cells in MC38-tumor bearing mice.



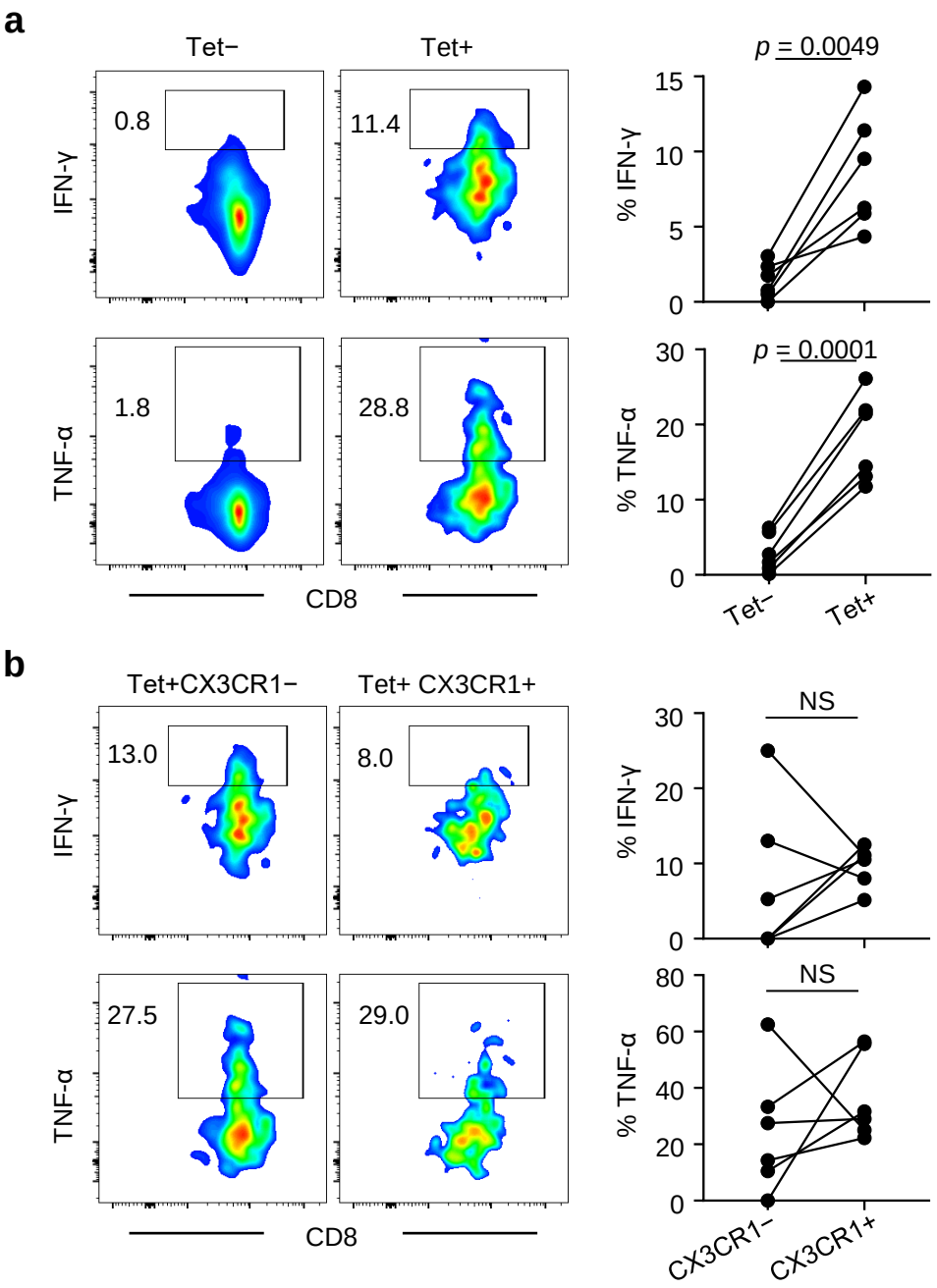
Supplementary Figure 4 High frequency of CX3CR1^{+} subset in neoantigen/TLR3/CD40 vaccination-induced peripheral CD8^{+} T cells that are capable of producing effector cytokines. Related to Fig. 2.

a, b MC38-tumor bearing mice were treated with Adpgk^{Mut}/TLR3/CD40 vaccinations as described in Fig. 1a. Splenocytes were collected 1 week after 2nd vaccination, and co-cultured with Adpgk^{Mut} peptide in the presence of brefeldin A for 5 hrs before intracellular staining.

a Representative FACS plots showing IFN-γ and TNF-α expression gated with CD8^{+} T cells in splenocytes. Lower panels show CX3CR1 expression in IFN-γ^{-} TNF-α^{-} cells (left) and IFN-γ^{+} TNF-α^{+} cells (right). Numbers denote percentage of CX3CR1^{+} cells. Data shown are representative of three independent experiments. Frequency of CX3CR1 cells from the IFN-γ^{-} TNF-α^{-} or IFN-γ^{+} TNF-α^{+} subset is shown in the right panel (n = 7 mice per group). Two-tailed paired *t*-test.

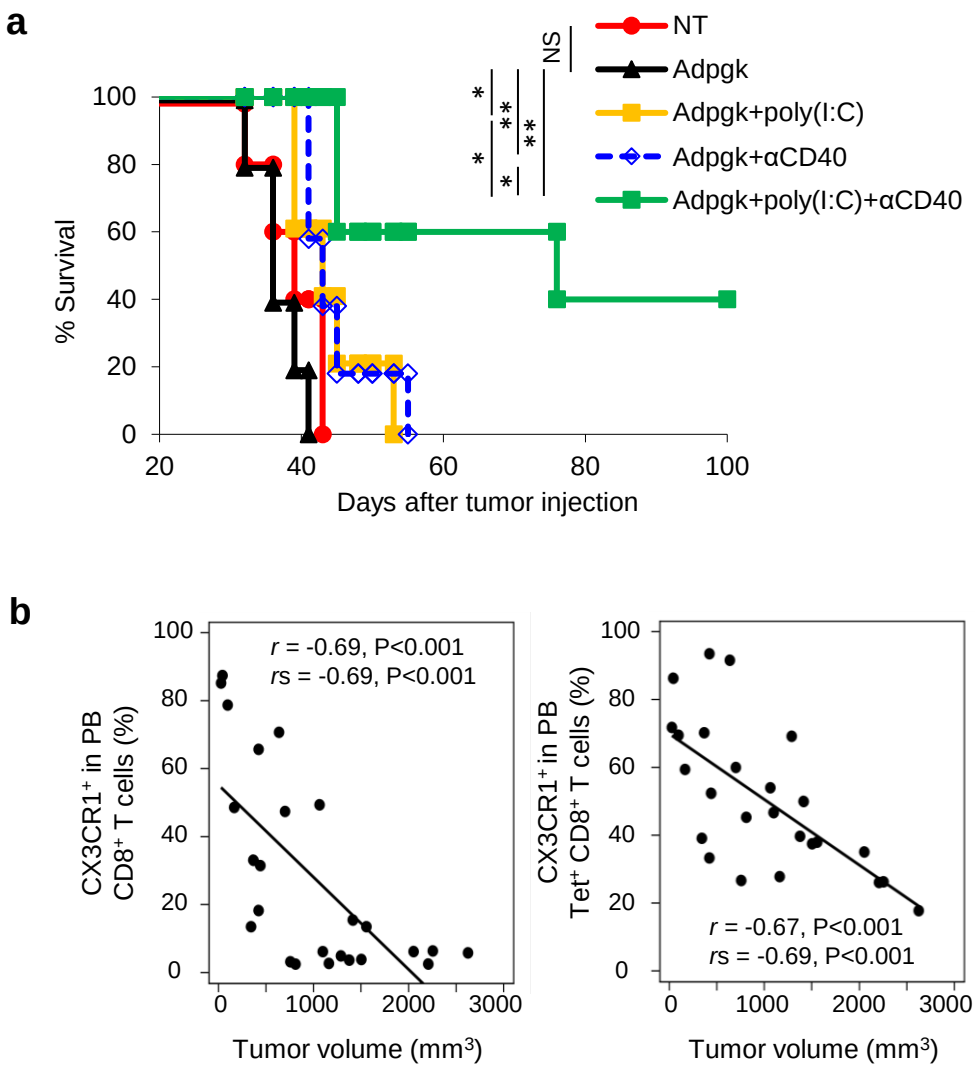
b Scatter plot of the frequency of IFN-γ^{+} TNF-α^{+} cells against the frequency of Adpgk^{Mut} tetramer (Tet)$^{+}$ CX3CR1^{+} CD8^{+} T cells in splenocytes. Correlation is shown using Pearson correlation (*r*) and Spearman correlation coefficients (r_s).

Data shown are representative of two independent experiments.



Supplementary Figure 5 Neoantigen/TLR3/CD40 vaccination facilitates infiltration of neoantigen-specific CD8⁺ T cells that have the capacity of producing effector cytokine against the cognate antigen. Related to Fig. 2.

a, b Left shows representative FACS plots showing IFN-γ (upper) or TNF-α (lower) expression gated with CD8⁺ T cells (**a**) or Adpgk^{Mut} tetramer (Tet)⁺ CD8⁺ T cells (**b**) in tumors of MC38-tumor bearing mice treated with Adpgk^{Mut}/TLR3/CD40 vaccinations as described in Fig. 1a. Tumor cells were collected 1 week after 2nd vaccination, and co-cultured with Adpgk^{Mut} peptide in the presence of brefeldin A for 5 hrs before intracellular staining. Numbers denote percentage of IFN-γ⁺ or TNF-α⁺ cells. Right panel shows the frequency of the IFN-γ⁺ or TNF-α⁺ cells in each group (n = 6 mice per group). NS not significant, two-tailed paired *t*-test.

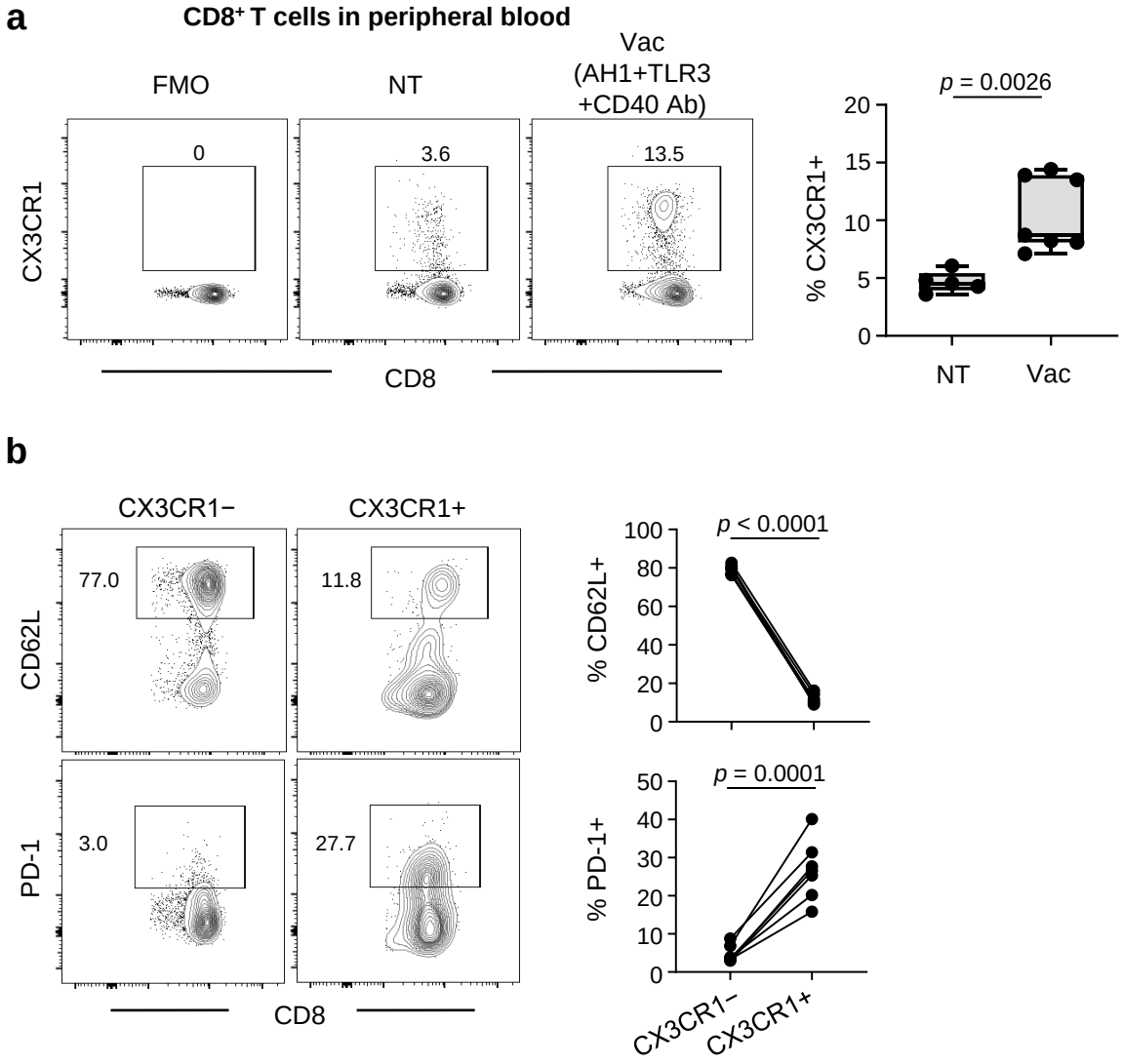


Supplementary Figure 6 Effective neoantigen/TLR3/CD40 vaccination correlates with generation of antigen-specific CX3CR1$^+$ CD8$^+$ T cells. Related to Fig. 2b.

a Survival curves in MC38-tumor bearing C57BL/6 mice in different treatment groups as indicated. (n = 5 mice per group). NS not significant, *P < 0.05, **P < 0.01, log-rank (Mantel-Cox) test.

b Scatter plots of tumor volumes against the frequency of the CX3CR1$^+$ subset in CD8$^+$ T cells (left) and Adpgk^{Mut} tetramer (Tet)$^+$ CD8$^+$ T cells (right) in peripheral blood (PB) one week after 2nd vaccination. Correlation is shown using Pearson correlation (r) and Spearman correlation coefficients (r_s).

Data shown are representative from two independent experiments.

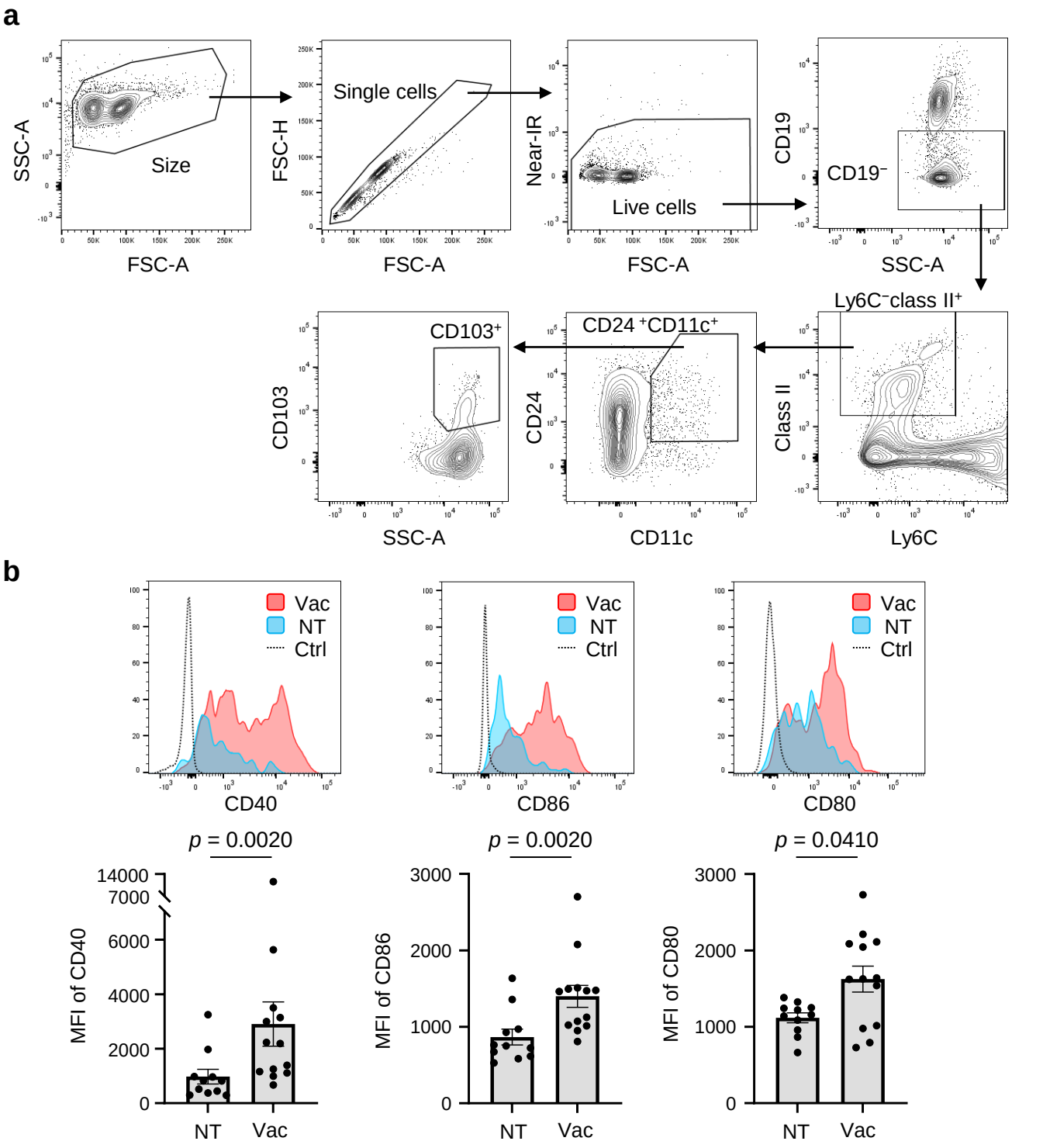


Supplementary Figure 7 Generation of CX3CR1$^+$ CD8$^+$ T cells in peripheral blood (PB) of MC38 tumor-bearing mice treated with irrelevant peptide and TLR3/CD40 vaccination. Related to Fig. 2

MC38-tumor bearing mice were treated with PBS (NT) or irrelevant AH1 peptide and TLR3/CD40 agonists (Vac) twice 1 week apart. PB was collected after 2nd Vac for flow cytometric analysis.

a, Representative FACS plots showing CX3CR1 expression gated with CD8$^+$ T cells. FMO, fluorescence minus one (CX3CR1). Numbers denote percentage of CX3CR1$^+$ cells. Frequency of CX3CR1$^+$ cells in CD8$^+$ T cells is shown in the right panel (n = 5-7 mice per group).

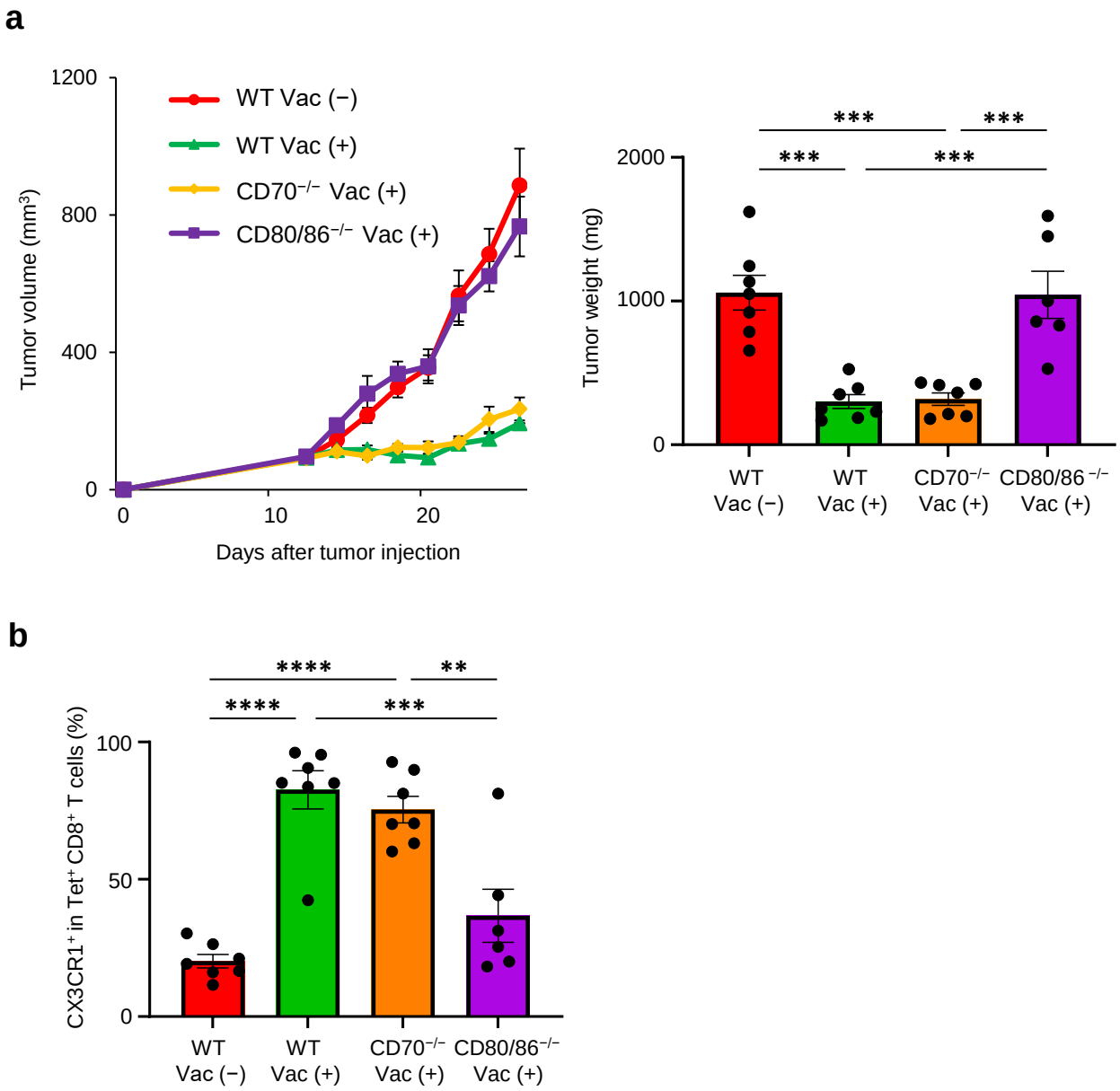
b, Representative FACS plots of CX3CR1$^-$ (left) or CX3CR1$^+$ (right) cells gated with CD8$^+$ T cells. Numbers denote percentage of each marker-positive cells. Frequency of marker-positive cells from the CX3CR1$^-$ or CX3CR1$^+$ subset is shown in the right panels for each marker (n = 7 mice per group). Two-tailed unpaired (**a**) and paired *t*-test (**b**). Box plots: dot, single PB; hinges, 25th and 75th percentiles; middle line, median; whiskers, minimum to maximum value (**a**).



Supplementary Figure 8 Neoantigen/TLR3/CD40 vaccination increases CD40, CD80 and CD86 expression in CD103⁺ DCs in TdLN. Related to Fig. 5.

MC38-tumor bearing mice were treated with PBS (NT) or Adpgk^{Mut} peptide and TLR3/CD40 agonists (Vac) twice 1 week apart as described in Fig. 1a. TdLN was collected after 2nd Vac for flow cytometric analysis.

a, Gating strategy of identifying CD103⁺ DCs in TdLN. **b**, Representative histograms (upper) and median fluorescence intensity (MFI) (lower) of CD40, CD86 and CD80 expression in CD103⁺ DCs . (n = 11-13 mice per group). Two-tailed unpaired *t*-test (**b**). Ctrl (control), expression of each marker in fluorescence minus three (CD40, CD80 and CD86). Mean ± SEM. Data shown are representative of two independent experiments.



Supplementary Figure 9 CD80/86 but not CD70 signaling mediates antitumor efficacy of neoantigen/TLR3/CD40 vaccination (Vac) and generation of antigen-specific CX3CR1^{+}CD8^{+} T cells. Related to Fig. 6.

a MC38 tumor-bearing wild type C57BL/6 mice (WT), CD70$^{-/-}$, or CD80/86$^{-/-}$ mice were treated with Adpgk^{Mut} peptide and TLR3/CD40 agonists (Vac) or PBS (Vac (−)) twice 1 week apart as described in Fig. 1a. Tumor volume curves (left) and tumor weight (mg) at day 28 are shown (n = 6-7 mice per group).

b Frequency of CX3CR1^{+} subset in Adpgk^{Mut} Tet^{+} CD8^{+} T cells in peripheral blood (PB) of MC38 tumor-bearing wild type WT, CD70$^{-/-}$, or CD80/86$^{-/-}$ mice treated with or without Vac (n = 6-7 mice per group). PB were harvested 1 week after 2nd Vac.

NS not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, one-way ANOVA with Tukey's multiple comparisons. Mean ± SEM.

Table S1: Fluorochrome-conjugated antibodies used in flow cytometry

Antibody	**Clone**	**Manufacturer**
CD8	53-6.7	BioLegend
CD19	1D3	BD Biosciences
CD40	1C10	Thermofisher
CD80	L307.4	BD Biosciences
CD86	2331 (FUN-1)	BD Biosciences
CD90.1	OX-7	BioLegend
CD90.2	53-2.1	BioLegend
CX3CR1	SA011F11	BioLegend
CD45	30-F11	BD Biosciences
CD62L	MEL-14	BioLegend
CD11c	HL3	BD Biosciences
CD24	M1/69	BD Biosciences
CD103	2E7	BioLegend
I-A/I-E (class II)	M5/114.15.2	BioLegend
Ly6C	HK1.4	BioLegend
4-1BB	17B5	BioLegend
KLRG1	2F1	BD Biosciences
CXCR3	CXCR3-173	BioLegend
PD-1	29F.1A12	BioLegend
Granzyme A	GzA-3G8.5	Thermofisher
IFN-γ	XMG1.2	BD Biosciences
TNF-α	MP6-XT22	BioLegend