Effective neoantigen vaccination correlates with generation of antigen-specific CX3CR1+CD8+ T cells mediated by CD40 and CD80/86 signaling in cDC1s

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Abstract

Background The use of tumor mutation-derived neoantigen represents a promising approach for cancer vaccines. Preclinical and early-phase human clinical studies have shown the successful induction of tumor neoepitope-directed responses; however, overall clinical efficacy of neoantigen vaccines has been limited. One major limitation of this strategy is the lack of a reliable blood-based surrogate marker for clinical response. Here, we investigated a role of CX3C chemokine receptor 1 (CX3CR1), a marker of T-cell differentiation in predicting response to neoantigen vaccination in a preclinical model.

Methods Mice bearing MC38 colon adenocarcinoma cells that harbor a single-epitope mutation within the Adpgk protein were treated with mutant Adpgk peptide ($Adpgk_{Mut}$) in combination with toll-like receptor 3 (TLR3) agonist poly(I:C) and/or anti-CD40 antibody (Ab). Antitumor efficacy of neoantigen vaccination and the frequency of peripheral blood $Adpgk_{Mut}$-specific $CX3CR1^{+}CD8^{+}$ T cells were assessed. Mechanisms underlying the generation of $CX3CR1^{+}CD8^{+}$ T cells were examined using knockout and bone marrow chimeric mice.

Results We found that CD40 stimulation significantly enhanced antitumor efficacy of neoantigen and TLR3 agonist vaccination, which was associated with an increased frequency of circulating $Adpgk_{Mut}$-specific effector $CX3CR1^{+}CD8^{+}$ T cells. Generation of neoantigen-specific $CX3CR1^{+}CD8^{+}$ T cells by $Adpgk_{Mut}$/CD40/TLR3 stimulation was dependent on CD40 and Batf3 in bone marrow-derived cells. Further mechanistic studies using mixed bone marrow chimeras identified key roles for CD40 and CD80/86, but not CD70 signaling in Batf3-dependent conventional type 1 dendritic cells (cDC1s) for generation of neoantigen-specific cytotoxic $CX3CR1^{+}CD8^{+}$ T cells and therapeutic efficacy of neoantigen vaccine.

Conclusions Taken together, our results underscore critical roles of cDC1s and an implication of dual CD40/TLR3 stimulation in neoantigen-based therapeutic vaccines, and demonstrate the potential utility of CX3CR1 as a circulating predictive T-cell biomarker in vaccine clinical trials.

List of Abbreviations

Ab: antibody

Batf3: basic leucine zipper transcription factor ATF-like 3

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

CX3CR1: CX3C chemokine receptor 1

cDC1s: conventional type 1 dendritic cells

GZMA: granzyme A

HEV: high endothelial venules

ICOS: inducible T cell co-stimulator

IFN-γ: interferon-γ

KLRG1: killer-cell lectin like receptor G1

NS: not significant

NT: no treatment

PD-1: programmed cell death protein 1

PB: peripheral blood

MHC: major histocompatibility complex

TLR3: toll-like receptor 3

WT: wild-type

83 # Background

84 Despite objective therapeutic benefit seen in several clinical trials, the overall clinical
85 success of the peptide-based vaccines has thus far been limited.[1 2] This is due, at least in part, to
86 the low immunogenicity of self-antigens, the incomplete understanding of immunological
87 mechanisms underlying effective priming of tumor-specific T cells, and the lack of a reliable
88 biomarker for clinical response. Somatically mutated genes within tumors can generate
89 neoantigens, which create de novo epitopes for T cells.[3] Since neoantigens have not undergone
90 central thymic selection, they can be deemed as highly immunogenic tumor-specific antigens.[3]
91 Recent advances of neoantigen identification by massive parallel sequencing and computational
92 prediction of neo-epitopes have demonstrated that individualized mutanome-based vaccinations
93 are promising.[4-6]

94 Intratumoral infiltration of $CD8^+$ T cells is an independent prognostic factor for survival
95 of patients with various cancer types.[7 8] In contrast, increases in peripheral blood (PB) tumor-
96 associated antigen-specific T cells following immunization do not necessarily translate into
97 antitumor effects.[9] Analysis of T-cell infiltrates in serially obtained tumor samples would be
98 ideal to evaluate response to vaccine therapy; however, it is invasive and especially challenging
99 for visceral tumors. Identification of circulating biomarkers that correlate with effectiveness of
100 vaccination and clinical outcome of patients would markedly improve current treatment
101 regimens.

102 The chemokine receptor, CX3CR1, was recently identified as a marker of effector T-cell
103 differentiation.[10 11] Unlike other proliferation, co-stimulatory and co-inhibitory molecules such
104 as Ki67, ICOS, PD-1, and CTLA-4 that are only transiently upregulated on T cells after
105 activation, CX3CR1 is stably expressed on virus- and tumor-specific $CD8^+$ T cells upon

106 differentiation via unidirectional differentiation from the CX3CR1- subset during the effector
107 phase.[10-12] Furthermore, our study and others have shown that CD8+ T cells expressing high
108 levels of CX3CR1 exhibit decreased expression of L-selectin (CD62L) and CXCR3,
109 trafficking receptors necessary for entry across lymphoid organ high endothelial venules
110 (HEV) and the tumor microvasculature,[13 14] respectively, and become dominant subsets of
111 tumor-specific CD8+ T cells in PB,[11 12] suggesting a potential advantage as a circulating T-cell
112 biomarker. These observations prompted us to hypothesize that effective vaccination is
113 associated with the increased frequency of antigen-specific CX3CR1+CD8+ T cells in PB.

114 Here, we used a syngeneic mouse model of colon adenocarcinoma with the neo-epitope
115 presented in major histocompatibility complex (MHC) class I H-2D^b molecules,[15] and
116 investigated the relationship between antitumor efficacy of neoantigen vaccination and the
117 frequency of neoantigen-specific CX3CR1+CD8+ T cells. Our results indicate that generation of
118 circulating neoantigen-specific CX3CR1+CD8+ T cells correlates with successful vaccination
119 with mutated peptide and CD40/toll-like receptor (TLR) 3 agonists. Mechanisms underlying the
120 generation of CX3CR1+CD8+ T cells were examined using knockout and bone marrow chimeric
121 mice. Our results support circulating CX3CR1+CD8+ T cells as a marker of effective T-cell
122 priming via Batf3-dependent conventional type 1 dendritic cells (cDC1s).

123

124 # Materials and Methods

125 **Mice**

126 Female C57BL/6 mice, CD40-/- mice (B6.129S2-*Cd40lg*^{tm1Imx}/J), CD80/86-/- mice
127 (B6.129S4-*Cd80*^{tm1Shr} *Cd86*^{tm2Shr}/J), and Basic leucine zipper transcription factor ATF-like 3

128 (Batf3)$^{-/-}$ mice (B6.129S(C)-*Batf3*$_{tm1Kmm}$/J) were purchased from the Jackson Laboratory, and
129 bred in-house (Roswell Park Comprehensive Cancer Center). CD70$^{-/-}$ mice on C57BL/6
130 background have been previously described.[16] All mice were 7 to 12 weeks old at the beginning
131 of each experiment, and were maintained under specific pathogen-free conditions at the Roswell
132 Park animal facility according to approved institutional guidelines.
133
134 **Cells**
135 A MC38 murine colon adenocarcinoma cell line was gift from Dr. Weiping Zou
136 (University of Michigan), and was maintained in RPMI (Gibco) supplemented with 10% FBS
137 (Sigma), 1% NEAA (Gibco), 2 mM GlutaMAX-1 (Gibco), 100 U/ml penicillin-streptomycin
138 (Gibco), and 55 μM 2-mercaptoethanol (Gibco). Cells were authenticated by morphology,
139 phenotype and growth, and routinely screened for *Mycoplasma*, and were maintained at 37°C in
140 a humidified 5% CO_2 atmosphere.
141
142 ***In vivo* vaccination and cancer immunotherapy studies**
143 Female C57BL/6 mice were injected s.c. with 5-8 × 10^5 MC38 cells. Mice were treated
144 with 100 μg soluble Adpgk$_{Mut}$ peptide (GenScript) with or without 50 μg poly(I:C) (InvivoGen)
145 and/or 50 μg agonistic CD40 antibody (Ab) (BioXCell, clone FGK45) twice one week apart. PB
146 was obtained for immune monitoring one week after the 2$_{nd}$ vaccination. Tumor volumes were
147 calculated by determining the length of short (l) and long (L) diameters (volume = $l^2 \times L/2$).
148 Experimental end points were reached when tumors exceeded 20 mm in diameter or when mice
149 became moribund and showed signs of lateral recumbency, cachexia, lack of response to noxious
150 stimuli, or observable weight loss.

151

152 **Generation of bone marrow chimeras**

153 To generate bone marrow chimeras, C57BL/6 mice were irradiated with 500 cGy
154 followed by a second dose of 550 cGy 3 hours apart. To obtain donor bone marrow, femurs and
155 tibiae were harvested and the bone marrow was flushed out. For CD40$^{-/-}$ bone marrow chimeras,
156 5×10^6 bone marrow cells from CD40$^{-/-}$ mice were injected per mouse to irradiated C57BL/6
157 wild-type (WT) mice. For Batf3$^{-/-}$/WT, Batf3$^{-/-}$/CD40$^{-/-}$, Batf3$^{-/-}$/CD80/86$^{-/-}$, and Batf3$^{-/-}$/CD70$^{-/-}$
158 mixed bone marrow chimeras, 5×10^6 bone marrow cells of a 1:1 mixture were injected to
159 irradiated C57BL/6 WT mice. After 8-12 weeks, recipients were used for the experiments.

160

161 **Flow cytometry and intracellular granzyme and IFN-γ assay**

162 Fluorochrome-conjugated antibodies are shown in Table S1. DAPI, LIVE/DEAD Fixable
163 Aqua (Thermo Fisher Scientific) staining cells were excluded from analysis. For tetramer
164 staining, PE-labeled MHC class I (H-2D^b) specific for mouse Adpgk 299-307
165 (ASMTNMELM)[15] was kindly provided by the NIH Tetramer Core Facility. Intracellular GZMA
166 and IFN-γ assay was performed as described before.[12 17] Intracellular IFN-γ in splenocytes was
167 assessed after stimulation with PMA (50 ng/ml) and ionomycin (1 ug/ml) (Sigma-Aldrich) in the
168 presence of Brefeldin A (BD Biosciences) for 5h *in vitro*. Samples were analyzed using an LSR
169 II or an LSRFortessa (BD Biosciences) with FlowJo software (TreeStar).

170

171 **Statistical Analysis**

172 Statistical analysis was performed using Student's *t*-test or Mann-Whitney *U* test for
173 comparisons between two groups, one-way ANOVA for multiple comparisons, or the Mantel-

174 Cox method (log-rank test) for survival analysis using GraphPad Prism 8.02 (GraphPad
175 Software). The p values for comparison of means were determined by two-tailed *t*-test and one-
176 way and two-way ANOVA. $P < 0.05$ was considered statistically significant. Data are presented
177 as mean ± SEM.

178

179 # Results

180 **Effective neoantigen/CD40/TLR3 agonists vaccination correlates with generation of**
181 **antigen-specific effector CX3CR1+CD8+ T cells.**

182 Neoantigen vaccination with a TLR3 agonist increases antigen-specific tumor-infiltrating
183 lymphocytes in a preclinical model[18] and patients.[4 5] CD40 signaling drives robust DC activation
184 to generate cytotoxic CD8+ T cells,[19-21] and synergizes with the TLR3 ligand in inducing an
185 expansion of peptide or DC vaccine-primed and adoptively transferred antigen-specific CD8+ T
186 cells.[22-25] To delineate a role of CD40 signaling in the context of neoantigen vaccination, we
187 used MC38 colon adenocarcinoma cells that harbor a single-epitope mutation within the Adpgk
188 protein (**Supplementary Fig. S1**).[15] C57BL/6 mice bearing MC38 tumors were treated with
189 different combinations of mutant Adpgk peptide ($Adpgk_{Mut}$), agonistic anti-CD40 Ab, and/or the
190 TLR3 agonist poly(I:C) **(Fig. 1A)**. $Adpgk_{Mut}$ peptide vaccination with either anti-CD40 Ab or
191 poly(I:C) improved survival compared to non-treatment and $Adpgk_{Mut}$ alone (**Fig. 1B**). However,
192 combined anti-CD40 Ab and poly(I:C) stimulation synergistically enhanced $Adpgk_{Mut}$ peptide
193 immunization (referred to as $Adpgk_{Mut}$/CD40/TLR3 hereafter), resulting in significantly
194 prolonged survival (**Fig. 1B**). We analyzed circulating neoantigen-specific CD8+ T cells one
195 week after the 2nd vaccination using $Adpgk_{Mut}$ tetramer (**Supplementary Fig. S2**), and found that
196 the frequency of $Adpgk_{Mut}$ neoantigen-specific CD8+ T cells expressing the chemokine receptor

197 CX3CR1 correlated with tumor response (**Fig. 1B**). To gain mechanistic insight into the
198 generation of this subset, we evaluated the frequency of neoantigen-specific CX3CR1^{+}CD8^{+} T
199 cells in mice treated with different combination of vaccination. Interestingly, CD40 but not
200 TLR3 stimulation contributed to the generation of the CX3CR1^{+} subset (**Fig. 1C**). Phenotypical
201 and functional evaluation of the Adpgk$_{Mut}$-specific CX3CR1^{-} and CX3CR1^{+} subsets revealed the
202 CX3CR1^{+} subset contained significantly more CD62L^{-}, 4-1BB^{+}, PD-1^{+}, KLRG^{+} and granzyme A
203 (GZMA)$^{+}$ populations with the ability to produce IFN-γ (**Fig. 1D, E**), suggesting recently-
204 activated effector T cells.
205
206 **Generation of antigen-specific CX3CR1^{+}CD8^{+} T cells by neoantigen/CD40/TLR3 agonists**
207 **vaccination is dependent on CD40 expression in hematopoietic cells.**
208 Since the above results implicate the agonistic anti-CD40 Ab to the costimulatory
209 receptor CD40 as a major driver for the generation of Adpgk$_{Mut}$-specific CX3CR1^{+}CD8^{+} T cells
210 in the periphery, we next investigated the requirement of host CD40 molecule using CD40$^{-/-}$
211 mice. The lack of increase in the frequency of Adpgk$_{Mut}$-specific CX3CR1^{+}CD8^{+} T cells in
212 CD40$^{-/-}$ mice denoted the requirement of CD40-CD40 ligand pathway in this process (**Fig. 2A**).
213 Since CD40 molecule is not only expressed by antigen-presenting cells, but also by non-
214 hematopoietic component such as endothelial cells,[26] we generated bone marrow chimeras to
215 further confirm the direct immunomodulatory effect of CD40 signaling in hematopoietic lineage
216 cells. Lethally irradiated C57BL/6 mice were reconstituted with bone marrow cells from either
217 CD40$^{-/-}$ or WT controls. After 8 weeks, the chimeras were implanted with MC38 tumors,
218 followed by Adpgk$_{Mut}$/CD40/TLR3 stimulation. We found that mice reconstituted with CD40$^{-/-}$

219 bone marrow did not generate the CX3CR1+CD8+ T-cell subset, indicating an essential role of
220 CD40 signaling in hematopoietic cells (**Fig. 2B**).
221
222 **Critical roles of CD40 signaling in cDC1s for antitumor efficacy of neoantigen/CD40/TLR3**
223 **agonists vaccination and generation of antigen-specific CX3CR1+CD8+ T cells.**
224 cDC1s specialize in cross-presentation of exogenous antigens on MHC class I molecules,
225 and play a key role in antitumor immunity and response to immunotherapy.[18 27-30] To assess
226 whether cDC1s are necessary for driving the formation of CX3CR1+CD8+ T cells, we employed
227 mice deficient of Batf3 transcription factor, which is required for the maturation of cDC1s.[31] A
228 significant decrease of CX3CR1+CD8+ T cells was observed in Batf3-/- mice (**Fig. 3A**),
229 suggesting the requirement of cDC1s for generation of CX3CR1+CD8+ T cells.
230 cDC1s express high levels of TLR3, and TLR3 agonist Poly-ICLC has been used in
231 personalized neoantigen vaccine trials with vaccine-related objective responses.[5 6] However, the
232 role of CD40 signaling in cDC1s in vaccine-based immunotherapy remains elusive. To address a
233 direct role of CD40 signaling in cDC1 lineage for CX3CR1+CD8+ T cell development, we
234 generated CD40-/-/Batf3-/- or WT/Batf3-/- mixed bone marrow chimeras into irradiated C57BL/6
235 WT mice. CD40-/-/Batf3-/- to WT mice (CD40-/- plus Batf3-/-) lack CD40 expression only in
236 Batf3-dependent bone marrow-derived cells while WT/Batf3-/- to WT mice have an intact CD40
237 signaling. In CD40-/- plus Batf3-/- mixed chimeras, CX3CR1+CD8+ T cell responses remained
238 minimal compared to WT plus Batf3-/- mixed bone marrow chimeras, indicating that CD40
239 expression on cDC1s is crucial for the CX3CR1+CD8+ T-cell induction (**Fig. 3B**). In line with
240 these results, mice reconstituted with CD40-/- plus Batf3-/- mixed bone marrow chimeras had

241 significantly decreased antitumor efficacy of Adpgk$_{Mut}$/CD40/TLR3 vaccination compared to
242 mice with WT plus Batf3$^{-/-}$ mixed bone marrow chimeras (**Fig. 3C**).
243
244 **Non-redundant requirement of CD80/86 but not CD70 signaling in cDC1s for antitumor**
245 **efficacy of neoantigen/CD40/TLR3 agonists vaccination and generation of antigen-specific**
246 **CX3CR1^{+}CD8^{+} T cells.**
247 In addition to CD40, both CD70 and CD80/86 have been reported to play roles in DC-
248 mediated T cell activation and persistence;[32] however, the role of these molecules on cDC1s for
249 T-cell differentiation remains elusive. Therefore, the requirements of CD70 and CD80/86
250 signaling in cDC1s were assessed in mixed chimeric mice of CD70$^{-/-}$ plus Batf3$^{-/-}$ and CD80/86$^{-/-}$
251 plus Batf3$^{-/-}$. There was no difference in the frequency of Adpgk$_{Mut}$-specific CX3CR1^{+}CD8^{+} T
252 cells between WT plus Batf3$^{-/-}$ and CD70$^{-/-}$ plus Batf3$^{-/-}$ mixed bone marrow chimeras (**Fig. 4A**)
253 with similar tumor control (**Fig. 4B**), suggesting a redundant role of CD70 signaling in cDC1s. In
254 sharp contrast, generation of Adpgk$_{Mut}$-specific CX3CR1^{+}CD8^{+} T cells (**Fig. 4C**) and antitumor
255 efficacy of Adpgk$_{Mut}$/CD40/TLR3 vaccination (**Fig. 4D**) were abrogated in the CD80/86$^{-/-}$ plus
256 Batf3$^{-/-}$ compared to the control WT plus Batf3$^{-/-}$ mixed bone marrow chimeras. Thus, the
257 CD80/86-CD28 pathway in cDC1s plays crucial roles in mediating antitumor efficacy and
258 generating antigen-specific effector CX3CR1^{+}CD8^{+} T cells in the context of neoantigen
259 vaccination.
260

261 # Discussion

262 In this study we have demonstrated that the addition of agonistic anti-CD40 Ab enhances
263 cDC1-mediated therapeutic efficacy of neoantigen/poly(I:C) vaccination, and identified a novel

264 link between antitumor efficacy of neoantigen vaccine and an increased frequency of circulating
265 neoantigen-specific CX3CR1+CD8+ T cells. Moreover, using mixed bone marrow chimeras from
266 Batf3-/-, CD40-/-, CD70-/-, and CD80/86-/- mice, we have shown critical roles of CD40 and
267 CD80/86 signaling in cDC1s for generation of neoantigen-specific CX3CR1+CD8+ T cells and
268 for the *in vivo* antitumor efficacy of neoantigen/CD40/TLR3 agonists vaccination.

269 Engagement of CD40 on DCs induces costimulatory molecules on their surface,
270 promotes their cytokine production, facilitates the cross-presentation of antigen,[26] and
271 synergistically augments expansion of both endogenous and adoptive transferred T cells.[22-24 33]
272 Our data further demonstrated that cDC1s play a critical role in antitumor efficacy of
273 TLR3/CD40 stimulation. Although Poly-ICLC (TLR3 agonist) has been used in neoantigen
274 vaccine clinical studies,[5 6] these findings underscore the implication of agonistic anti-CD40 Ab
275 in combination with Poly-ICLC to for the maximal engagement of cDC1 in vaccine-based
276 therapy.

277 The molecular and cellular mechanisms underlying differentiation of CX3CR1- to
278 CX3CR1+CD8+ T cells remain elusive. Recent studies have indicated that CD4+ T cell help or
279 agonistic anti-CD40 Ab facilitates generation of CX3CR1+CD8+ T cells.[34 35] Our results further
280 demonstrated the non-redundant requirement of CD40 signaling in cDC1s for the generation of
281 neoantigen-specific CX3CR1+CD8+ T cells. The observation that CD70 signaling in cDC1s was
282 dispensable for generation of the CX3CR1+ subset was unexpected given the several lines of
283 evidence suggesting that CD40 signaling relays the CD4+ T-cell help signal by the induction of
284 CD70 expression on DCs.[36] However, not all molecules regulated by CD4+ T cell help are
285 regulated by CD27, and cytokine signaling may have contributed to the generation of the
286 CX3CR1+ subset.[35] In support of this notion, IL-21 was found to modulate T-cell differentiation

287 and generation of CX3CR1+CD8+ T cells.[34 37] Additional mechanistic studies are needed to
288 further determine the signaling required for generation of the CX3CR1+ subset.

289 The lack of a reliable surrogate marker for clinical response remains the major limitation
290 of vaccine clinical trials. Mere presence of vaccine-primed tumor-specific T cells might not
291 correlate with effective regression of the tumor, and cannot be used as a standalone biomarker
292 for vaccine efficacy.[9] Our findings of an increased frequency of neoantigen-specific
293 CX3CR1+CD8+ T cells that have the ability to produce IFN-γ and GZMA after successful
294 vaccination align with the evidence from previous clinical studies showing that functional
295 assessment of T cells such as IFN-γ production and/or cytolytic activity was correlated with
296 response in patients treated with peptide vaccines.[38-40] Furthermore, this observation suggests the
297 potential utility of CX3CR1 as a blood-based predictive T-cell biomarker for other
298 immunotherapies such as immune checkpoint inhibitor therapy. Indeed, an increased frequency
299 of PB CX3CR1+CD8+ T cells has been observed in patients who responded to immune
300 checkpoint inhibitors.[41 42]

301 In summary, our study revealed cDC1-mediated potent antitumor efficacy of neoantigen
302 vaccine with dual CD40/TLR3 stimulation, identified a correlation between antitumor response
303 and the frequency of antigen-specific PB CX3CR1+CD8+ T cells, and provided the mechanistic
304 insight into the generation of CX3CR1+CD8+ T cells. These data suggest the maximal
305 engagement of cDC1s with CD40/TLR3 stimulation for the development of more effective
306 neoantigen vaccine therapy, and the potential implications of CX3CR1 as a circulating T-cell
307 biomarker for response in future clinical studies.

308

309

Declarations

Ethics approval and consent to participate

All performed animal experiments and procedures were reviewed and approved by the Roswell Park Comprehensive Cancer Center (RPCCC) IACUC. No human subjects were enrolled in any aspect of this study.

Consent for publication

N/A

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Author contributions

T.Y. contributed development of methodology, acquisition of data, analysis and interpretation of data, writing, review, and revision of the manuscript.

T.H, contributed development of methodology, acquisition of data, analysis and interpretation of data, review, and revision of the manuscript.

T.O. contributed acquisition of data, analysis and interpretation of data, review, and revision of the manuscript.

X.C. provided CD70$^{-/-}$ mice, review, and revision of the manuscript.

F.I. contributed conception and design, development of methodology, acquisition of data, analysis and interpretation of data, writing, review, and finalizing the manuscript.

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491

Figure 1

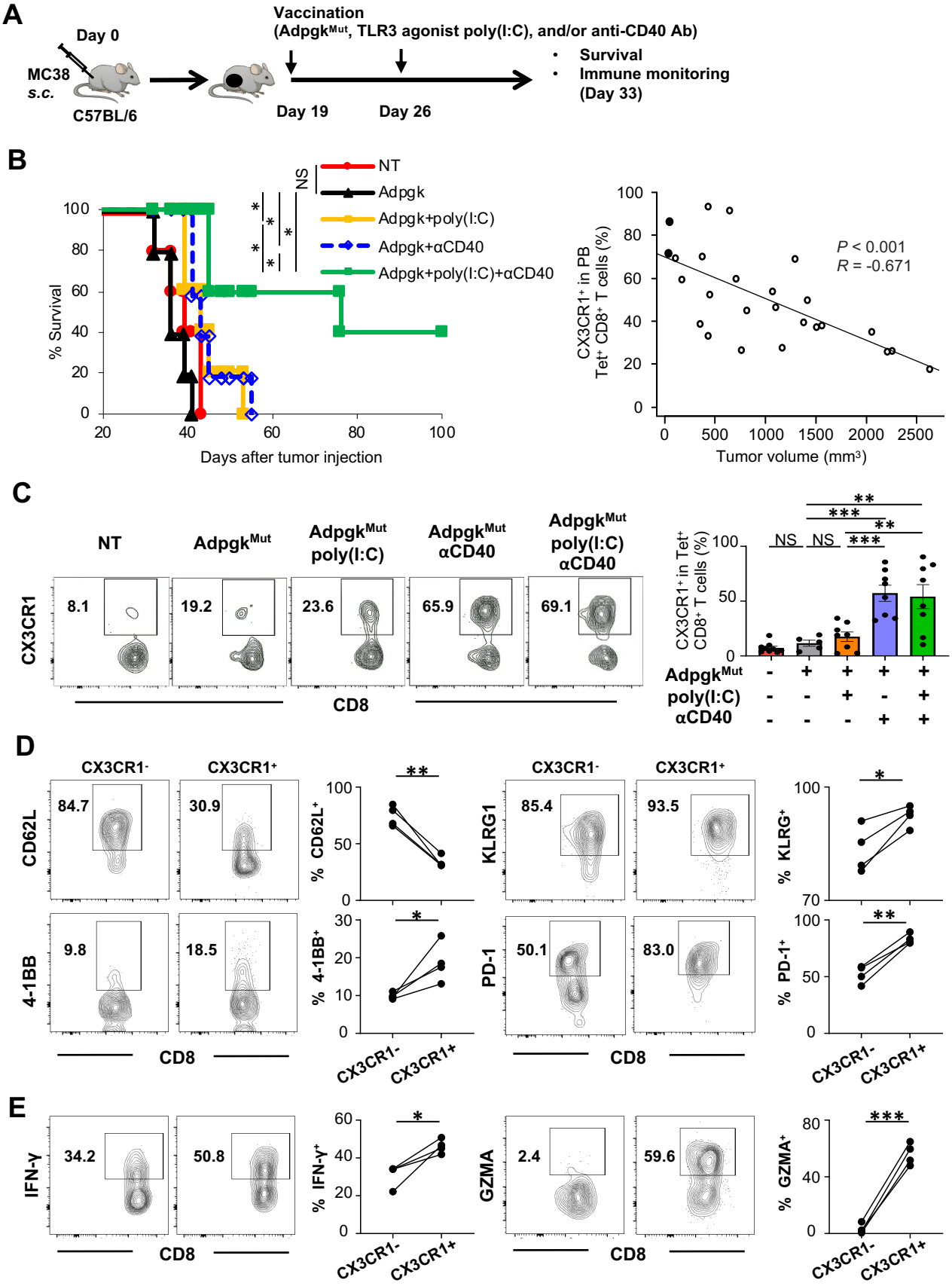
A
Day 0
MC38
s.c.
C57BL/6
Vaccination
(Adpgk^Mut, TLR3 agonist poly(I:C), and/or anti-CD40 Ab)
Day 19
Day 26
Survival
Immune monitoring
(Day 33)
B
NT
Adpgk
Adpgk+poly(I:C)
Adpgk+αCD40
Adpgk+poly(I:C)+αCD40
NS
% Survival
Days after tumor injection
CX3CR1+ in PB
Tet+ CD8+ T cells (%)
P < 0.001
R = -0.671
Tumor volume (mm3)
C
NT
Adpgk^Mut
Adpgk^Mut poly(I:C)
Adpgk^Mut αCD40
Adpgk^Mut poly(I:C) αCD40
CX3CR1
CD8
8.1
19.2
23.6
65.9
69.1
CX3CR1+ in Tet+ CD8+ T cells (%)
NS
Adpgk^Mut
poly(I:C)
αCD40
D
CX3CR1-
CX3CR1+
CD62L
4-1BB
KLRG1
PD-1
84.7
30.9
9.8
18.5
85.4
93.5
50.1
83.0
% CD62L+
% 4-1BB+
% KLRG+
% PD-1+
CD8
E
IFN-γ
GZMA
34.2
50.8
2.4
59.6
% IFN-γ+
% GZMA+

Figure 1 Effective neoantigen/CD40/TLR3 agonists vaccination correlates with generation of antigen-specific effector CX3CR1$^+$ CD8$^+$ T cells

(**A**) Experimental scheme for evaluation of neoantigen/anti-(α)CD40 antibody (Ab)/toll-like receptor (TLR3) agonist poly(I:C) vaccinations in an MC38 tumor model.

(**B**) Left shows survival curves in MC38-tumor bearing C57BL/6 mice in different treatment groups as indicated (n = 5 mice per group). Data shown are representative of two independent experiments. Right shows a scatter plot of tumor volumes against the frequency of Adpgk^{Mut} tetramer (Tet)$^+$ CX3CR1$^+$CD8$^+$ T cells in peripheral blood (PB) one week after 2nd vaccination. Black filled dots represent cured mice. Correlation is shown using Pearson correlation coefficients (R) and significance was determined using Spearman correlation.

(**C**) Representative FACS plots (left) and the frequency of the CX3CR1$^+$ subset (right) in Adpgk^{Mut}Tet$^+$CD8$^+$ T cells in PB of MC38-tumor bearing mice treated with various regimens as indicated (NT: no treatment). Numbers denote percentage of the CX3CR1$^+$ subset in Adpgk^{Mut} Tet$^+$CD8$^+$ T cells. (n = 5-9 mice per group). Data shown are pooled from two independent experiments.

(**D** and **E**) Representative FACS plots of CX3CR1$^-$ (left) or CX3CR1$^+$ (right) cells gated with Tet$^+$CD8$^+$ T cells in PB (**D**) and spleen (**E**) of MC38-tumor bearing mice after Adpgk^{Mut}/CD40/TLR3 agonists vaccinations. 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 = 4 mice per group). PB was harvested one week after 2nd vaccination. NS not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by log-rank (Mantel-Cox) test (B for survival), one-way ANOVA test with Tukey's multiple comparisons (**C**), or paired two-tailed *t*-test (**D** and **E**). Mean ± SEM.

Figure 2

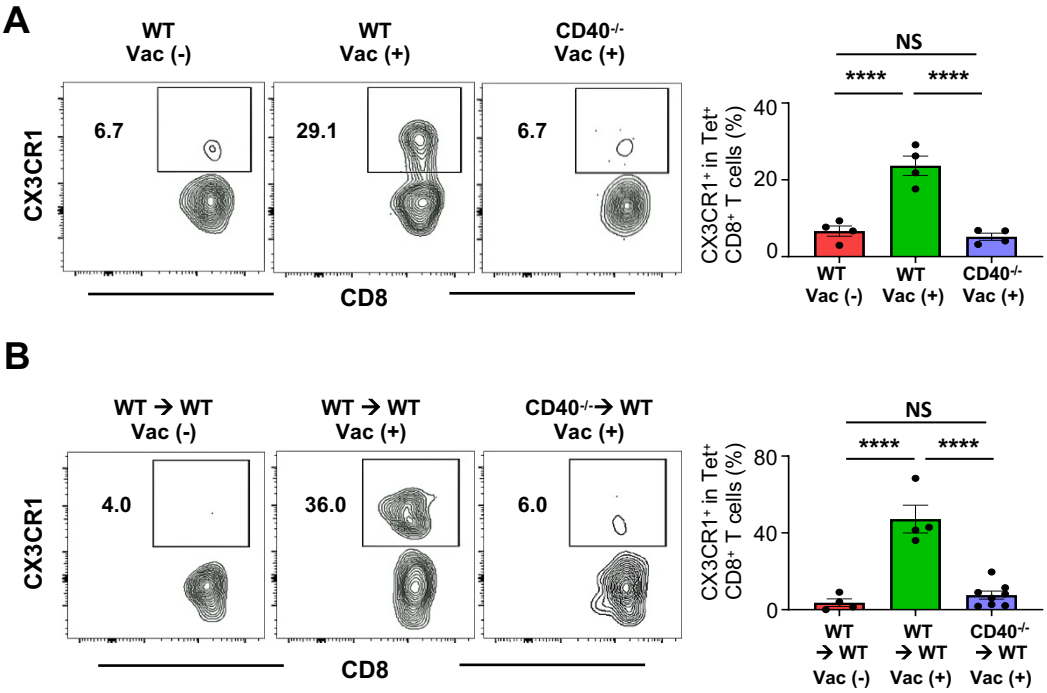

Figure 2 CD40-dependent generation of antigen-specific CX3CR1^{+} CD8^{+} T-cell subsets upon neoantigen/CD40/TLR3 stimulation

(**A**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing WT or CD40 $^{-/-}$ mice treated with or without Adpgk^{Mut}/CD40/TLR3 agonists vaccination (Vac) (n = 4 mice per group). Numbers denote percentage of the CX3CR1^{+} subset.

(**B**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing bone marrow chimeric mice (WT→WT or CD40 $^{-/-}$→WT mice) treated with or without Vac (n = 4-8 mice per group). (**A** and **B**) PB was harvested one week after 2nd Vac. ****P <0.0001 by one-way ANOVA test with Tukey's multiple comparisons. Mean ± SEM.

Figure 3

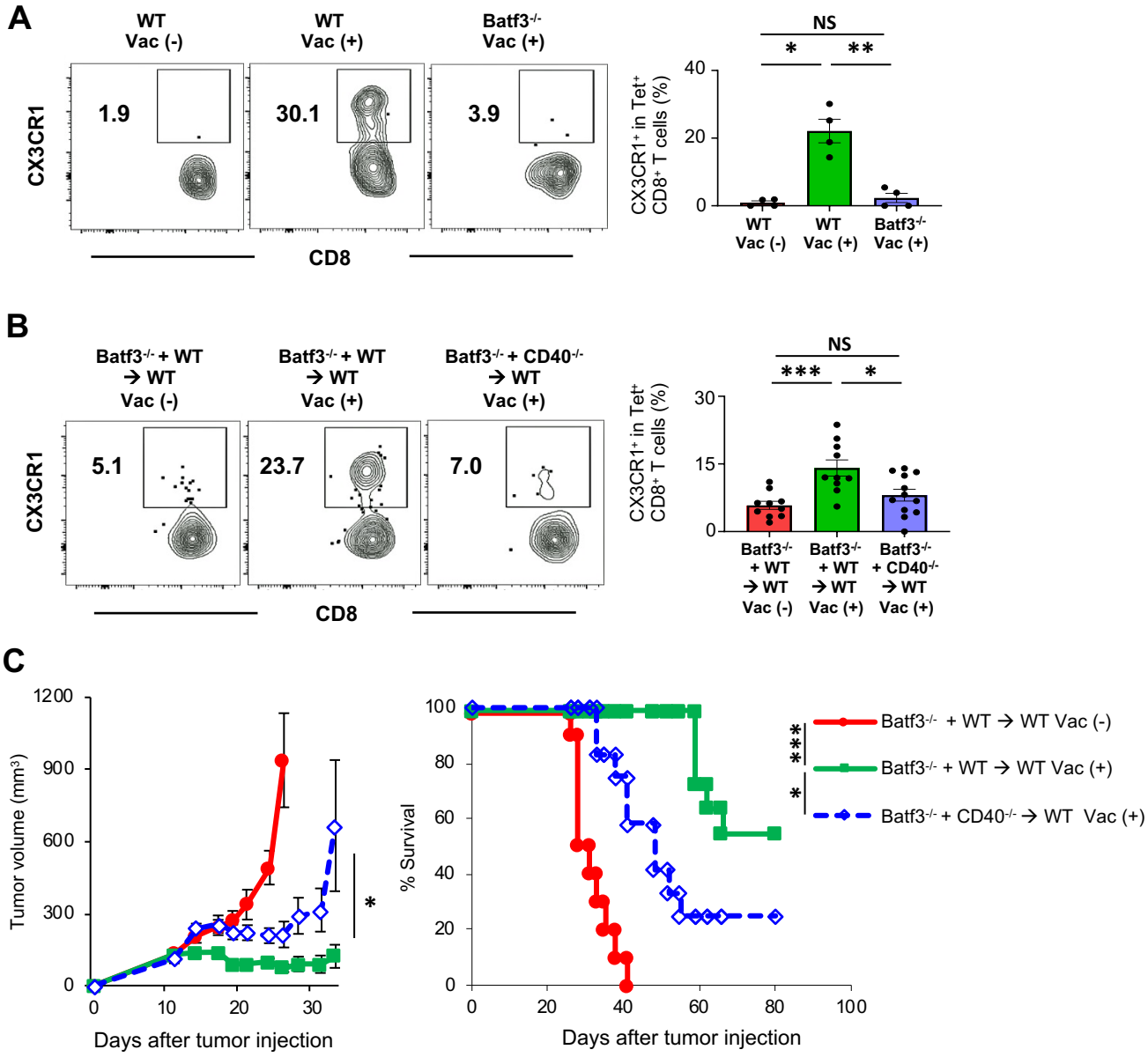
A
WT Vac (-)
WT Vac (+)
Batf3-/- Vac (+)
CX3CR1
1.9
30.1
3.9
CD8
NS
*
**
CX3CR1+ in Tet+ CD8+ T cells (%)
40
20
0
WT Vac (-)
WT Vac (+)
Batf3-/- Vac (+)
B
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD40-/- → WT Vac (+)
CX3CR1
5.1
23.7
7.0
CD8
NS
***
*
CX3CR1+ in Tet+ CD8+ T cells (%)
30
15
0
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD40-/- → WT Vac (+)
C
Tumor volume (mm3)
1200
900
600
300
0
0
10
20
30
Days after tumor injection
*
% Survival
100
80
60
40
20
0
0
20
40
60
80
100
Days after tumor injection
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD40-/- → WT Vac (+)
***
*

Figure 3 Critical roles of CD40 signaling in cDC1s for antitumor efficacy of neoantigen/CD40/TLR3 agonists vaccination and generation of antigen-specific CX3CR1^{+}CD8^{+} T cells.

(**A**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing WT or Batf3 $^{-/-}$ mice treated with or without Adpgk^{Mut}/CD40/TLR3 agonists vaccinations (Vac) (n = 4 mice per group). Numbers denote percentage of the CX3CR1^{+} subset.

(**B**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing bone marrow chimeric mice (Batf3$^{-/-}$ and WT→WT or Batf3$^{-/-}$ and CD40$^{-/-}$→WT mice) treated with or without Vac. Data shown are pooled from two independent experiments. (**A** and **B**) PB was harvested one week after 2nd Vac.

(**C**) Tumor growth and survival curves in MC38 tumor-bearing mixed bone marrow chimeric mice (Batf3$^{-/-}$ and WT→WT or Batf3$^{-/-}$ and CD40$^{-/-}$→WT mice) treated with or without Vac (n = 10-11 mice per group).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA test with Tukey's multiple comparisons (A, B), Mann-Whitney *U* test (C for tumor volume), or log-rank (Mantel-Cox) test (C for survival). Mean ± SEM (A, B).

Figure 4

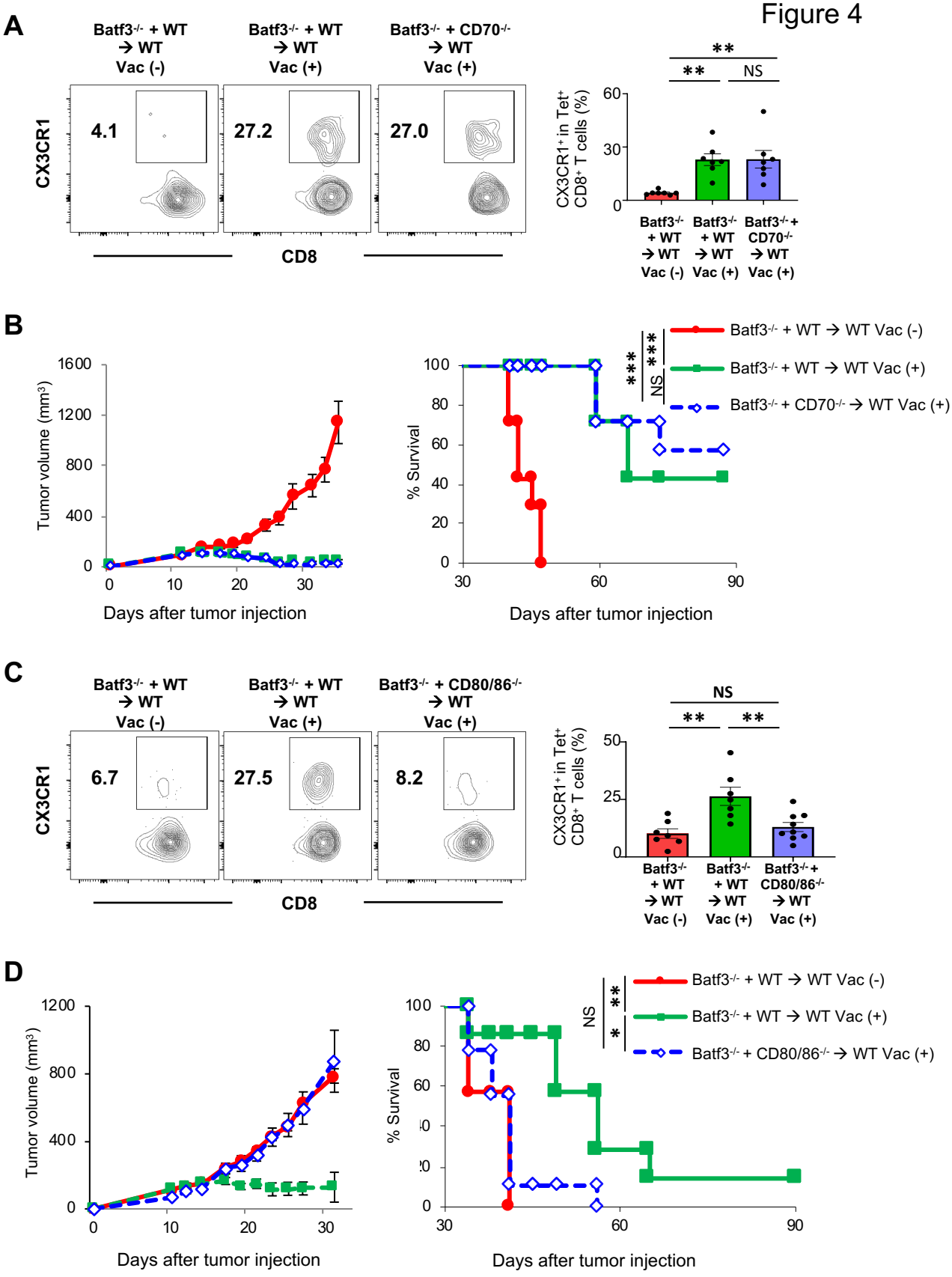
A
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD70-/- → WT Vac (+)
CX3CR1
CD8
4.1
27.2
27.0
CX3CR1+ in Tet+ CD8+ T cells (%)
**
**
NS
B
Tumor volume (mm3)
Days after tumor injection
% Survival
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD70-/- → WT Vac (+)
***
***
NS
C
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD80/86-/- → WT Vac (+)
6.7
27.5
8.2
NS
**
**
D
Batf3-/- + WT → WT Vac (-)
Batf3-/- + WT → WT Vac (+)
Batf3-/- + CD80/86-/- → WT Vac (+)
NS
**
*

Figure 4 Non-redundant requirement of CD80/86 but not CD70 signaling in cDC1s for antitumor efficacy of neoantigen/CD40/TLR3 agonists vaccination and generation of antigen-specific CX3CR1^{+}CD8^{+} T cells.

(**A**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing mixed bone marrow chimera (Batf3$^{-/-}$ and WT→WT or Batf3$^{-/-}$ and CD70$^{-/-}$→WT mice) mice treated with or without Adpgk^{Mut}/CD40/TLR3 agonists vaccinations (Vac) (n = 7 mice per group).

(**B**) Tumor growth curves in different treatment groups as indicated. (n = 7 mice per group)

(**C**) Representative FACS plots (left) and the frequency of the CX3CR1^{+} subset (right) in Adpgk^{Mut} Tet^{+}CD8^{+} T cells in PB of MC38-tumor bearing mixed bone marrow chimera (Batf3$^{-/-}$ and WT→WT or Batf3$^{-/-}$ and CD80/86$^{-/-}$→WT mice) mice treated with or without Vac (n = 7-9 mice per group).

(**D**) Tumor growth curves in different treatment groups as indicated (n = 7-9 mice per group).

(**A** and **C**) PB was harvested one week after 2nd Vac. NS not significant, **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001 by one-way ANOVA test with Tukey's multiple comparisons (A, C) or Mann-Whitney *U* test (B, D). Mean ± SEM.

Figure S1

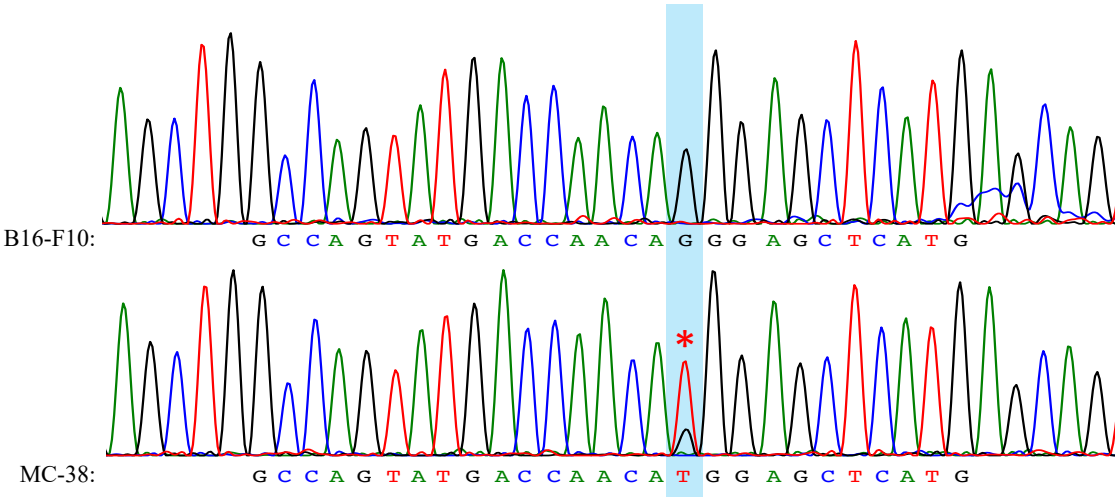

Fig. S1 cDNA sequencing of mutated Adpgk neoantigen in MC38 cells

Total RNA was extracted from MC38 cells (Mutated Adpgk) along with B16F10 cells (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.

Figure S2

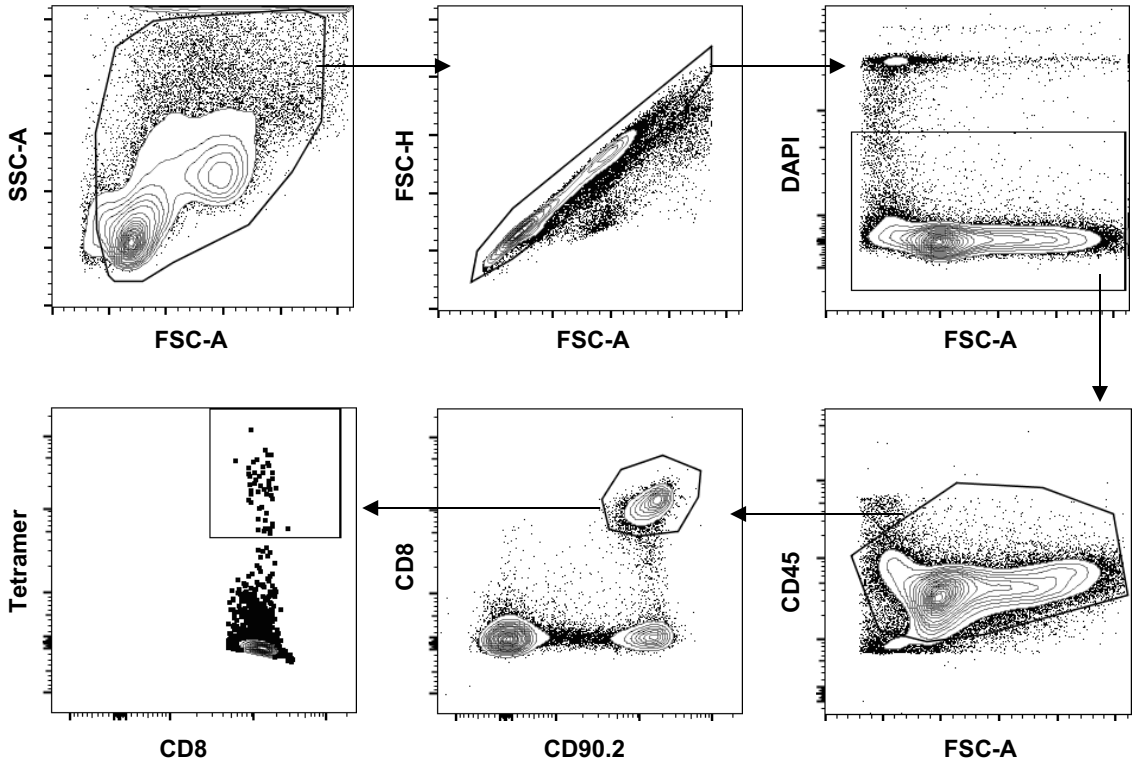

Fig. S2 Flow cytometry and gating of circulating Adpgk^{Mut} neoantigen-specific CD8^{+} T cells in MC38-tumor bearing mice. Representative of greater than 5 independent experiments.

Table S1: Fluorochrome-conjugated antibodies used in flow cytometry

Antibody	**Clone**	**Manufacturer**
CD8	53-6.7	Biolegend
CD90.1	OX-7	BioLegend
CD90.2	53-2.1	BioLegend
CX3CR1	SA011F11	BioLegend
KLRG1	2F1	BD Biosciences
CD62L	MEL-14	Biolegend
4-1BB	17B5	BioLegend
IFN-γ	XMG1.2	BD Biosciences
Granzyme A	GzA-3G8.5	Thermofisher