**T CELLS**

**MAP4K4 negatively regulates CD8 T cell–mediated antitumor and antiviral immunity**

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During cytotoxic T cell activation, lymphocyte function-associated antigen–1 (LFA-1) engages its ligands on antigen-presenting cells (APCs) or target cells to enhance T cell priming or lytic activity. Inhibiting LFA-1 dampens T cell–dependent symptoms in inflammation, autoimmune diseases, and graft-versus-host disease. However, the therapeutic potential of augmenting LFA-1 function is less explored. Here, we show that genetic deletion or inhibition of mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) enhances LFA-1 activation on CD8 T cells and improves their adherence to APCs or LFA-1 ligand. In addition, loss of Map4k4 increases CD8 T cell priming, which culminates in enhanced antigen-dependent activation, proliferation, cytokine production, and cytotoxic activity, resulting in impaired tumor growth and improved response to viral infection. LFA-1 inhibition reverses these phenotypes. The ERM (ezrin, radixin, and moesin) proteins reportedly regulate T cell–APC conjugation, but the molecular regulator and effector of ERM proteins in T cells have not been defined. In this study, we demonstrate that the ERM proteins serve as mediators between MAP4K4 and LFA-1. Last, systematic analyses of many organs revealed that inducible whole-body deletion of Map4k4 in adult animals is tolerated under homeostatic conditions. Our results uncover MAP4K4 as a potential target to augment antitumor and antiviral immunity.

**INTRODUCTION**

CD8 T cells play important roles in restraining tumor growth and eliminating virus-infected cells. Cytotoxic T lymphocytes (CTLs) are primed by dendritic cells (DCs) that present foreign peptides in complex with major histocompatibility complex (MHC) class I. Primed CTLs can kill target cells that are presenting MHC class I–foreign peptide complexes by depositing lytic granules at the contact area of T cell–target cell conjugates or by engaging the Fas–7–associated surface antigen (FAS) pathway (1). In most of the cancer patients, CTLs fail to curtail tumor growth because of both T cell–intrinsic and tumor-microenvironmental suppression mechanisms (2). Extensive efforts are being invested to boost anticancer immunity by targeting T cell–negative regulators.

At priming, naive T cells encounter DCs in the lymph node (LN) (3). Adhesion between these two cell types initiates immunological synapse (IS) formation (4). Lymphocyte function-associated antigen–1 (LFA-1) is the major integrin mediating this adhesion by binding to ligands on DCs, including intercellular adhesion molecule–1 (ICAM-1), ICAM-2, and ICAM-3 (5). Engagement of LFA-1 sensitizes CD8 T cells to lymphocytic choriomeningitis virus (LCMV) infection (6) and provides costimulation for optimizing naive T cell activation in response to T cell receptor (TCR) engagement (7, 8). The duration and strength of T cell–DC conjugation also affect CTL functions including cytokine production and proliferation (9–13). Furthermore, because IS is deployed when CTLs encounter target cells to exert their cytolytic activity, LFA-1 also directly contributes to the effector phase (14). Inhibiting LFA-1 decreases T cell activation in certain contexts and dampens T cell–dependent inflammatory responses, autoimmune diseases, and graft-versus-host disease (15). However, the therapeutic potential of augmenting LFA-1 function is less explored.

Another family of proteins involved in T cell–APC (antigen-presenting cell) conjugation is ERM (ezrin, radixin, and moesin) proteins, which serve as bridges between the plasma membrane and the cytoskeleton (16). Among the ERM proteins, moesin and ezrin are expressed in T cells and are rapidly dephosphorylated and inactivated within minutes of T cell activation (17). This inactivation increases T cell–APC conjugation (18). ERM proteins are known substrates of the serine/threonine protein kinase MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4) (19). Map4k4 is broadly expressed across different cell types, and its function is context dependent (20–23). Map4k4 is involved in the progression of many cancers (24, 25). It regulates endothelial cell migration (26) and promotes vascular inflammation and atherosclerosis (23). Loss of Map4k4 compromises macrophage function during lipopolysaccharide challenge in vivo (20) and attenuates CD4 T cell proliferation and activation in vitro (27). Whereas CD4 Cre–mediated constitutive T cell loss of Map4k4 leads to systemic inflammation and type 2 diabetes (21), inducible pan-tissue deletion of Map4k4 in adult mice improves insulin sensitivity in liver and adipose tissues (22) and protects mice from hyperinsulinemia and β-cell hypertrophy in response to long-term high-fat diet (23).

To date, the role of Map4k4 in CD8 T cells and the functional relationship between ERM and MAP4K4 in T cells have not been explored. Here, we report that pan-tissue Map4k4 inducible knockout (iKO) in adult mice increases CD8 T cell numbers and activity in tumors and in virus-infected hosts. Mechanistically, Map4k4 deletion reduces ERM phosphorylation in CD8 T cells upon activation and increases LFA-1 activation, thereby enhancing conjugation of CD8 T cells with APCs or antigen-loaded target cells, leading to

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increased activation of CD8 T cells in vitro and in vivo models. Our study links MAP4K4, ERM, and LFA-1 in CD8 T cell activation and reveals a potential strategy to harness the therapeutic potential of augmenting LFA-1 function.

RESULTS

**Map4k4 loss suppresses tumor growth in a CD8 T cell–dependent manner**

Previously, we revealed that Map4k4 iKO mice had impaired growth of both KPP1 pancreatic and TC1 lung tumors with a modest reduction on vascular function (26). We expand this finding to additional models and showed that Map4k4 iKO hosts also reduced tumor growth of the MC38 colorectal and KPR3070 pancreatic tumor models (Fig. 1A and figs. S1A and S2E). Given that Map4k4 is highly expressed in immune cells (fig. S3) (28), we evaluated the immune compartment’s contribution to antitumor efficacy. Increased numbers of intratumoral CD3 and CD3/CD8 T cells were seen in KPP1 (Fig. 1, B and C), TC1 (fig. S1B), and MC38 (Fig. 1D) tumors grown in Map4k4 iKO hosts. CD3 T cells appear to be distributed throughout the tumor and show no bias toward tumor vasculature detected by MECA-32 staining in KPP1 tumors (Fig. 1B). To understand the contribution of mature lymphocytes to reduced tumor growth in Map4k4 iKO hosts, we bred the Map4k4-floxed allele into the Rag2−/− background. MC38 tumors grew at similar rates in Rag2−/− Map4k4 iKO mice compared with Rag2−/− Map4k4 wild-type (WT) hosts, revealing that T and B cells are required for the Map4k4 deletion phenotype (Fig. 1E and fig. S1C). Depleting CD8 T cells, but not CD4 T cells, reversed inhibition of tumor growth for Map4k4 iKO hosts (Fig. 1F and fig. S1D), indicating a requisite role of CD8 T cells in mediating the Map4k4 iKO phenotype. To understand whether Map4k4−/− T cells are sufficient to suppress tumor growth, we adoptively transferred CD3 T cells isolated from Map4k4 WT and iKO spleens into Rag2−/− hosts. Although Map4k4−/− T cell counts in the blood of reconstituted mice were slightly less than WT T cells (fig. S1E), they were more effective than WT T cells in reducing MC38 tumor growth and increasing tumor necrosis (Fig. 1, G to I, and fig. S1F). Because more Map4k4−/− CD8 T cells, but not CD4 T cells, accumulated to higher numbers in tumors than their WT counterparts (fig. S1G), we also reconstituted Rag2−/− mice with only CD8 T cells from Map4k4 WT and iKO spleens and confirmed comparable T cell reconstitution in peripheral organs between both genotypes (fig. S1H). Similarly, Map4k4−/− CD8 T cells were better at reducing tumor growth than WT CD8 T cells (Fig. 1J and fig. S1I). Next, we analyzed tumor-infiltrating lymphocytes (TILs) to assess whether CD8 T cell functionality is affected. Among freshly isolated MC38 CD8 TILs from immune competent Map4k4−/− hosts without any in vitro stimulation, more cells expressed the cytolytic protein granzyme B (GZMB) (Fig. 1K) and fewer expressed negative regulators, programmed cell death protein–1 (PD-1), cytotoxic T lymphocyte–associated protein–4 (CTLA-4), and CD39 (fig. S2, A to D). These cells also produced more cytokines after ex vivo anti-CD3/28 stimulation (Fig. 1L). Together, these data suggest that Map4k4−/− CD8 T cells are more functional and are both necessary and sufficient to inhibit tumor growth.

Given the above findings, we asked whether inhibition of PD-1/PD-L1 signaling, a pathway that causes T cell dysfunction induced by chronic antigen stimulation (29), can further improve the antitumor activity afforded by Map4k4 loss. Loss of Map4k4 and anti–PD-L1 treatment alone showed comparable efficacy in the pancreatic KPR3070 tumor model, and the combination of these two treatments demonstrated an additive effect (fig. S2E), indicating that Map4k4 could be a potential target to combine with PD-L1/PD-1 inhibitors. The combination only resulted in tumor stasis and not tumor regression, but this is not surprising because tumor cell proliferation rates are generally higher in implanted mouse tumor models than in human cancers; therefore, achieving tumor regression in such models is challenging. Retrospective analysis of approved drugs and their preclinical performances showed that reducing tumor growth rates by ~60% in preclinical models predicted objective clinical responses in patients (30). Mean tumor growth rates are reduced by 60% in the iKO group, 57% in the anti–PD-L1 group, and 88% in the combination group relative to the control, suggesting that this combination might translate into objective response in the clinic.

**Map4k4 loss augments activation of naïve CD8 T cells**

Because CD8 T cell activation is a multistep process, alteration of any step could account for the observed phenotype with Map4k4-deleted CD8 T cells described above. To determine which step during T cell activation is affected by Map4k4 loss, we analyzed the early response to tumor challenge in draining LNs from mice harboring MC38 tumors for 10 days, a time point when tumor sizes were not substantially different between genotypes. Map4k4−/− hosts had enlarged draining LNs (Fig. 2A), increased frequency of CD8 T cells recognizing the tumor–specific retroviral antigen p15e (Fig. 2B), and Ki67+ CD8 T cells (Fig. 2C) in the draining LNs. Despite increased Ki67+ CD8 TILs at this time point, it had not yet led to increased TIL counts (Fig. 2D). We then investigated whether these early changes eventually led to enhanced T cell responses within tumors by examining TILs 16 days after tumor implantation. Total CD8 TILs (Fig. 2E) as well as tumor-specific p15e+ CD8 TILs and MC38 neoantigen-specific CD8 TILs (Fig. 2F) were increased in Map4k4−/− hosts at this time point. To assess the cytokine secretion potential of CD8 TILs, we restimulated MC38 tumor single-cell suspensions ex vivo with a combination of MC38 tumor–specific peptides. Increased numbers of interferon-γ–positive (IFN-γ+) CD8 TILs were detected, and these cells secreted more IFN-γ in Map4k4−/− hosts (Fig. 2G).

Together, these data suggest that Map4k4 loss enhanced the early antitumor response in draining LNs that precedes the intratumoral phenotype.

The enhanced early antitumor immune response upon Map4k4 deletion implies its role in T cell priming. To evaluate this possibility, we examined the outcome of Map4k4 deletion in naïve CD8 T cells. We first confirmed Map4k4 protein loss in Map4k4 iKO CD8 T cells by Western blot (fig. S4A). Map4k4−/− splenic naïve CD8 T cells proliferated more in response to stimulation with anti-CD3/CD28 antibodies assessed by increased live cell counts and 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 3A). In addition, more Map4k4−/− CD8 T cells expressed IFN-γ and tumor necrosis factor–α (TNF-α) as well as secreted more IFN-γ and interleukin-2 (IL-2) in response to anti-CD3/28 stimulation (Fig. 3B and fig. S4B).

Consistent with increased proliferation and IFN-γ/TNF-α frequency, more iKO CD8 T cells displayed activation markers, CD69 and CD44, after 48 hours of stimulation (fig. S4, C and D). The frequency of early T cell activation marker ICAM-1 and both frequency and mean fluorescence intensity (MFI) of NUR77 were also elevated in Map4k4−/− CD8 T cells after 4 hours of anti-CD3/28 stimulation (fig. S4E). Because NUR77 is not only an early activation marker but also an
Fig. 1. Enhanced CD8 T cell activity mediates the antitumor effect of Map4k4 deletion. (A) MC38 tumor growth kinetics (top) and tumor weights at end point (bottom) in Map4k4 WT and iKO mice (n = 8 to 9 mice per group). (B) Representative images of KPP1 tumors grown in Map4k4 WT and iKO hosts and stained with CD3, CD8, MECA-32, and DAPI (4′,6-diamidino-2-phenylindole). Bottom panels are higher magnifications of boxed areas. (C) Quantification of CD3+ and CD3+/CD8+ cell counts in KPP1 whole tumor sections normalized to tumor tissue area. (D) FCM analysis of TIL counts in MC38 tumors. (E) MC38 tumor growth kinetics in Rag2−/− Map4k4 WT or iKO hosts (n = 9 to 11 mice per group). (F) MC38 tumor growth kinetics in Map4k4 WT or iKO hosts using control versus CD8-depleting (left) or CD4-depleting (right) antibodies (n = 7 to 13 mice). (G to I) MC38 tumor growth kinetics (G), tumor weights (H), and representative images of hematoxylin and eosin–stained MC38 tumor sections and associated necrosis scores (I) at end point of Rag2−/− hosts reconstituted with Map4k4 WT or iKO T cells (n = 15 mice per group). (J) MC38 tumor growth kinetics of Rag2−/− hosts reconstituted with Map4k4 WT or iKO CD8 T cells (n = 17 mice per group). (K) Representative FCM plots (left) and analysis (right) of intracellular GZMB staining of MC38 CD8 TILs on day 24 after tumor inoculation. FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein; Cy5.5, cyanine5.5. (L) Representative FCM plots (left) and analysis (right) of intracellular IFN-γ and TNF-α staining of MC38 CD8 TILs on day 24 after tumor inoculation either unstimulated (Uns.) or stimulated (Stim.) with anti-CD3/CD28 in vitro for 5 hours (gated on live/CD45/CD3/CD8 cells). PE, phycoerythrin; Cy7, Cyanine7; APC, allophycocyanin. (n) indicates starting animal number. “/+” indicates animals taken down due to the institution-defined tumor size limit or poor health condition. d, days after tumor inoculation. In dot plots, each dot represents a mouse. g, gram.) For all figures: Mann-Whitney test was run to determine P values for experiments with two groups, and ordinary one-way analysis of variance (ANOVA) or two-way ANOVA was used for multiple groups. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001; ns, not significant. Error bars indicate SEM.
indicator of TCR signal strength (31, 32), increased NUR77 supports elevated TCR signaling in Map4k4−/− CD8 T cells. We then stimulated CD8 T cells with lower concentrations of anti-CD3 along with a fixed concentration of anti-CD28 to examine whether these cells have a lower activation threshold. At a lower concentration of anti-CD3 (0.5 μg/ml), WT CD8 T cells lacked IFN-γ secretion, whereas iKO CD8 T cells readily secreted IFN-γ, produced more IL-2, and increased CD69 frequency (Fig. 3C and fig. S4F). These data suggest that Map4k4 deletion enhances activation of naïve splenic CD8 T cells in response to polyclonal stimulation. To determine whether kinase activity is required for enhanced activation, WT naïve CD8 T cells were exposed to GNE-220, a Map4K4 kinase inhibitor (26), or to the vehicle control, dimethyl sulfoxide (DMSO), during anti-CD3/28 stimulation. Mouse splenic naïve CD8 T cells treated with GNE-220 secreted more IFN-γ and IL-2 and had a higher frequency of CD69 positivity and more live cell counts indicative of proliferation (fig. S4, G to I). We also stimulated human peripheral CD8 T cells with low or high concentrations of anti-CD3/28 in the presence of 1 μM GNE-220, which increased IFN-γ and IL-2 secretion compared with control (fig. S4I). These data indicate that inhibition of MAP4K4 kinase activity accounts for the effect of Map4k4 deletion.
Map4k4 loss enhances antigen-driven CD8 T cell activation

To determine how Map4k4−/− CD8 T cells respond to antigen-driven stimulation, we crossed Map4k4fl/fl:Rosa26-CreERT2 (fl, floxed) mice to OT-1 TCR transgenic (Tg) mice. OT-1 CD8 T cells specifically recognize the ovalbumin-derived SIINFEKL peptide (33). Naïve Map4k4−/− OT-1 CD8 T cells secreted more IFN-γ and IL-2 when stimulated with SIINFEKL-pulsed APCs (Fig. 3D and fig. S5A). Preactivated Map4k4−/− OT-1 CD8 T cells killed more ovalbumin-overexpressing
MC38 tumor cells in vitro (fig. S5B). We also evaluated in vivo priming of splenic CD8 T cells 18 hours after injection with different doses of SIIIFKEL peptide (0, 0.1, and 10 µg per mouse) in Map4k4 WT and iKO OT-1 Tg mice. More Map4k4−/− CD8 T cells were positive for CD69, GZMB, and Ki67, even at lower peptide doses (Fig. 3E), demonstrating that Map4k4-deleted CD8 T cells have enhanced activation at both lower and higher antigen challenge.

Because both the abundance and TCR ligand strength determines the quality of T cell responsiveness, we investigated whether MAP4K4 inhibition affects naïve CD8 T cell priming in response to varying strengths of antigens by using different affinity peptides (34, 35) at a range of concentrations. MAP4K4 inhibition enhanced CD25 and CD69 expression (fig. S6, A and B) and IFN-γ and IL-2 secretion (fig. S6, C and D) of naïve CD8 T cells after they were cocultured with DCs pulsed with high-affinity SIIIFKEL (fig. S6, A to D, first column) or medium-affinity SIIIFKEL (fig. S6, A to D, T4, second column) peptides in a concentration-dependent manner. However, MAP4K4 inhibition had a weak or negligible effect on CD69 expression or cytokine production after stimulation with low-affinity peptide SIIGFEKL, although it had a modest effect on CD25 expression with peptide (100 µg/ml; fig. S6, A to D, G4, third column). These data suggest that MAP4K4 inhibition enables CD8 T cells to respond to low antigen density and further enhances their activation at high antigen density, but does not alter their sensitivity to antigen affinity.

We then validated the role of MAP4K4 in the early CD8 T cell antigen response in the LCMV–Armstrong strain (LCMV-ARM) infection model that heavily depends on rapid CD8 T cell priming and activation in an antigen-dependent manner (36). We infected Map4k4 WT and iKO mice with LCMV-ARM and analyzed spleens at 5, 7, 8, and 15 days after infection. Spleen weights and CD8 T cell counts were increased in iKO mice at days 7 and 8 (Fig. 4, A and B, and fig. S7, A and B). We analyzed other major cell types in the spleens at day 7 and found no alterations except increased neutrophil numbers (fig. S7, C, D, and F). At day 8, there were more LCMV-antigen–specific (GP33 and NP396) (Fig. 4C) and IFN-γ− CD8 T cells (Fig. 4D) in the iKO spleens without ex vivo stimulation. In addition, more iKO CD8 T cells produced IFN-γ in response to NP396 peptide restimulation ex vivo (fig. S7E). We also analyzed cytolytic activity of CD8 T cells by CD107a surface expression that is associated with release of lytic granule proteins perforin and granzymes (37). MFI of CD107a in the positive population was increased in iKO CD8 T cells (Fig. 4E). On day 15 after infection, we found no difference between Map4k4 WT and iKO spleens (Fig. 4, A and B), indicating that the action of MAP4K4 occurred during the early steps of the antiviral response. Together, these results suggest that antigen-specific acute activation and expansion of CD8 T cells are enhanced upon Map4k4 deletion in the LCMV-ARM infection model.

**MAP4K4 regulates LFA-1 and ERM activation during CD8 T cell priming**

Next, we evaluated whether MAP4K4 controls IS formation between CD8 T cells and DCs during priming. We found that Map4k4-deleted or Map4k4-inhibited OT-1 CD8 T cells formed more conjugates with SIIIFKEL-pulsed WT DCs compared with WT OT-1 CD8 T cells (Fig. 5A and fig. S8, A and B) and LFA-1−/− T cell–DC contact areas were increased with Map4k4−/− CD8 T cells (Fig. 5B). On resting T cells, LFA-1 exists in a low-affinity conformation. Upon TCR stimulation, LFA-1 switches to a high-affinity (HA) state that strengthens the contact between T cells and APCs (38). We asked whether Map4k4 deletion affects LFA-1 status. More Map4k4−/− splenic CD8 T cells displayed HA–LFA-1 on the surface after 30 min (fig. S9A) or 48 hours of anti-CD3/28 stimulation than WT cells (Fig. 5C), with total surface LFA-1 levels being comparable between genotypes (fig. S9C). HA–LFA-1 MFI was also higher among HA–LFA-1 Map4k4−/− CD8 T cells at 48 hours after stimulation (fig. S9B). More naïve and activated Map4k4−/− splenic CD8 T cells adhered to mouse ICAM-1–Fc protein–coated plates than WT CD8 T cells (Fig. 5D), whereas adhesion to plates coated with an irrelevant mouse immunoglobulin G (IgG)–Fc protein did not differentiate between T cell genotypes (fig. S9D). These data suggest that MAP4K4 regulates LFA-1 status during priming of CD8 T cells.

To explore what acts downstream of MAP4K4, we examined ERM protein phosphorylation (pERM) in CD8 T cells because they are reportedly a direct substrate of MAP4K4 (19) and their activation is linked to T cell priming (17, 18). As expected, the fraction of pERM-high CD8 T cells decreased after 5 min of stimulation with anti-CD3/28 antibodies or specific antigen, and Map4k4 loss enhanced...
**Fig. 5.** Map4k4 deletion enhances APC–T cell conjugation, increases LFA-1 activation, and reduces ERM phosphorylation in CD8 T cells. (A) Conjugation of Map4k4 WT or iKO OT-1 CD8 T cells with WT APCs pulsed with increasing concentrations of SIINFEKL peptide (n = 5 per group, representative data of two experiments). (B) Confocal images of LFA-1 staining of Map4k4 WT or iKO OT-1 CD8 T cells conjugated to SIINFEKL-pulsed WT APCs (left) and quantification of LFA-1+ contact length between CD8 T cells and APCs (middle and right). Middle, per doublet quantification; right, per animal quantification. (C) Representative FCM plots (left) and analysis (right) of CD8 T cells expressing HA–LFA-1 without (Uns.) and with (Stim.) anti-CD3/CD28 treatment for 48 hours. Representative data of four experiments. (D) Adhesion of unstimulated and stimulated mouse splenic CD8 T cells on mouse ICAM-1–Fc–coated surfaces. (E) FCM analysis of pERMhigh CD8 T cells upon 5 min of anti-CD3/CD28 stimulation. Representative data of three experiments. (F) Representative FCM plots (left) and analysis (right) of pERMhigh in OT-1 CD8 T cells cocultured with unpulsed (Un.) or SIINFEKL-pulsed (Pulsed) WT APCs for 5 min. Representative data of three experiments. BMDCs, bone marrow-derived dendritic cells. (G) Adhesion of primary human CD8 T cells from two different donors nucleoprated with indicated small interfering RNAs (siRNAs) on human ICAM-1–Fc–coated surfaces. (H) Adhesion of TALL-1 cells overexpressing constitutively active forms of MAP4K4 (T181E) or moesin (T558E) on human ICAM-1–Fc–coated surfaces. Representative data of two experiments. Each dot in the dot plots represents a mouse in (B) to (F) and technical replicate in (G) and (H).
this effect by ~25 to 30% (Fig. 5, E and F). Total ERM levels were comparable between Map4k4 WT and iKO CD8 T cells with and without stimulation (fig. S9E). Because MSN (gene that encodes moesin) is the most abundantly expressed ERM member in CD8 T cells and mediates the inhibitory effect of MAP4K4 on endothelial cell integrin activation (26), we knocked down MSN and MAP4K4 in Jurkat cells, a commonly used T cell line (fig. S9F), and cocultured them with unpulsed or superantigen-pulsed Raji cells. Under both conditions, knocking down MSN was sufficient to increase HA–LFA-1 on Jurkat cell surfaces (fig. S9G) and enhanced adhesion to human ICAM-1–Fc similar to knocking down MAP4K4 (fig. S9H), whereas MSN or MAP4K4 knockdown did not alter adhesion to the negative control human IgG1–Fc protein (fig. S9I). We confirmed ICAM-1–Fc binding results with MSN or MAP4K4 knockdown in human peripheral blood CD8 T cells (Fig. 5G). Conversely, overexpression of constitutively active forms of MAP4K4 and moesin in CD8a-positive TALL-1 cells or Jurkat cells reduced adhesion to human ICAM-1–Fc (fig. S5H and fig. S9I). Therefore, moesin and MAP4K4 are necessary and sufficient for regulating LFA-1 status and interaction with its ligand ICAM-1. Last, we knocked down MAP4K4 and MSN together in primary human CD8 T cells and Jurkat cells to assess additive effects on adhesion and observed equivalent adhesion to single knockdown (Fig. 5G and fig. S9K), indicating that they most likely function in a linear pathway.

The activity of MAP4K4 in CD8 T cells requires LFA-1

We next asked whether LFA-1 activity mediates the phenotype observed with Map4k4 loss. Elevated conjugation of Map4k4−/− or MAP4K4-inhibited OT-1 CD8 T cells with SIINFEKL-loaded WT APCs was reversed with increasing concentrations of anti–LFA-1 blocking antibody (Fig. 6A). Blocking LFA-1 during antigenic stimulation of CD8 T cells with SIINFEKL-loaded DCs also diminished the effect of Map4k4 iKO on IFN-γ and IL-2 secretion (Fig. 6B). Increased adhesion of MAP4K4−/− or MSN-knockdown Jurkat cells on human ICAM-1–Fc-coated surfaces was also reversed by a small-molecule inhibitor that blocks LFA-1–ICAM-1 interactions (Fig. 6C). Furthermore, blocking LFA-1 during in vivo priming with SIINFEKL peptide attenuated activation markers CD69, GZMB, and KI67 in Map4k4−/− splenic OT-1 CD8 T cells (Fig. 6D). Last, blocking LFA-1 during LCMV-ARM infection restored Map4k4−/− splenic NPF396-tetramer1 CD8 T cell counts and IFN-γ frequency among the NPF396-tetramer+ CD8 T cells to near WT levels (Fig. 6E). Thus, LFA-1 function is necessary for the phenotypes associated with Map4k4 loss in CD8 T cells, supporting the notion that LFA-1 acts downstream of MAP4K4. To strengthen this conclusion, we tested whether the MAP4K4 inhibitor and LFA-1 agonistic antibody would have similar effects on CD8 T cell activation. We stimulated mouse splenic CD8 T cells with anti-CD3 alone or together with an LFA-1 cross-linking antibody (TS1/22) in the presence of DMSO or GNE-220. Cross-linking LFA-1 and GNE-220 treatment had comparable effects on T cell activation and IFN-γ expression (Fig. 6F). Except for a slight increase in CD25 frequency, MAP4K4 inhibition did not further enhance the impact of cross-linking LFA-1.

We then examined whether LFA-1 mediates the antitumor effect in Map4k4 iKO hosts. Consistent with the finding that LFA-1–deficient mice were not primed by tumor antigens and failed to reject immunogenic tumors (39), anti–LFA-1 treatment increased MC38 tumor growth rates and abolished the differences between Map4k4 iKO and WT hosts (Fig. 7A), indicating that LFA-1 is required for MAP4K4 function. Map4k4 loss increased CD8 TIL reactivity to the MC38 antigen p15e or a combination of MC38 neoantigens that was abrogated upon LFA-1 blockade (Fig. 7, B and C). To assess the cytolytic potential of autologous CD8 TILs, MC38 tumor single-cell suspensions were restimulated ex vivo with a combination of MC38 tumor-specific peptides, and membrane CD107a on CD8 TILs was measured. Map4k4−/− CD8 TILs increased the percentage and MFI of membrane CD107a to a greater extent than WT CD8 TILs, an effect that was disrupted by LFA-1 blockade (Fig. 7D). Last, we examined whether inhibition of MAP4K4 during ex vivo restimulation is sufficient to enhance CD107a membrane expression. MAP4K4 inhibitor GNE-220 treatment increased the percentage and MFI of CD107a on CD8 TILs above vehicle control upon tumor antigen restimulation, and the effect of GNE-220 was abolished by the anti–LFA-1 antibody (Fig. 7E). Although anti–LFA-1 increased tumor growth in Map4k4 WT hosts, it did not consistently affect CD8 TILs in the ex vivo studies. We hypothesize that this difference likely reflects functions of LFA-1 in other cell type(s) with antitumor activity such as natural killer cells and CD4 T cells. The role of MAP4K4 in other type(s) of intratumoral leukocytes remains to be determined in future studies.

MAP4K4 is down-regulated upon TCR engagement

Because MAP4K4 negatively regulates CD8 T cell priming and activation, we examined how MAP4K4 itself is regulated during IS formation. Phosphorylation of a critical residue in the MAP4K4 kinase domain, threonine-181 (T181), was rapidly reduced upon anti-CD3/28 stimulation in Jurkat cells and primary human CD8 T cells. The kinetics of MAP4K4 dephosphorylation mirrored the rapid phosphorylation of proximal TCR components such as SLP76, PLCγ, ERK, ZAP70, and NF-κB p65 (fig. S10, A and B). Total MAP4K4 protein levels decreased gradually after activation in both Jurkat and primary human CD8 T cells (fig. S10, A and B). MAP4K4 was detected in the cortex of Jurkat cells and OT-1 CD8 T cells and at the synapse between pairs of Jurkat-Raji cells or OT-1 CD8-DCs (fig. S11, A and D). When Jurkat cells were cocultured with superantigen (SEE)–loaded Raji cells, or OT-1 CD8 T cells were cocultured with SIINFEKL-loaded DCs for 60 min, MAP4K4 levels were reduced at the synapse and the cortex (fig. S11, A, B, D, and E). Random contacts between T cells and unpulsed APCs did not reduce MAP4K4 levels compared with isolated single T cells (fig. S11, A, B, D, and E), suggesting that this process is dependent on TCR engagement. Quantification of Jurkat-Raji and OT-1 CD8-DC doublets in the presence of antigen showed that co-occurrence of CD3 and MAP4K4 was reduced at the synapse upon IS formation (fig. S11, C and F). Together, these data indicate that Map4k4 is down-regulated upon TCR engagement, and this mode of regulation is consistent with MAP4K4 being a negative regulator of CD8 T cell priming and activation.

Pan-tissue deletion of Map4k4 is tolerated in adult mice

As MAP4K4 is broadly expressed and regulates different biological processes in many cell types (20–24, 26, 27), we evaluated the consequences of deleting Map4k4 in adult mice under normal physiological conditions 5 weeks or 5 months after tamoxifen administration. Hematological analyses of WT and iKO tissues showed no or modest alterations (fig. S12, A and B) at 5 weeks after tamoxifen injection. Because platelet counts were slightly decreased in iKO mice, we performed a panel of coagulation measurements. Map4k4−/− mice exhibited a slightly enhanced coagulation potential indicated by a
Naïve splenic CD8 T cells were used for all in vitro experiments. (A) FCM analysis of conjugation between unpulsed (Un.) or SIINFEKL-pulsed (Pulsed) WT APCs and Map4k4 WT or iKO OT-1 CD8 T cells (left) and DMSO- or GNE-220-treated WT OT-1 CD8 T cells (right) in the presence or absence of αLFA-1 antibody. (B) IFN-γ and IL-2 secretion by Map4k4 WT or iKO OT-1 CD8 T cells cocultured with SIINFEKL-pulsed WT APC in the presence or absence of αLFA-1 antibody. Representative data of three experiments. (C) Adhesion of Jurkat cells transfected with the indicated siRNAs on ICAM-1–Fc–coated surfaces in the presence of DMSO or LFA-1 inhibitor. Representative data of three experiments. (D) Representative FCM plots (left) and analysis (right) of CD69 (top), GZMB (middle), and Ki67 (bottom) staining of OT-1 splenic CD8 T cells from Map4k4 WT or iKO mice 18 hours after injection with αLFA-1 (500 µg/kg) or control antibodies with SIINFEKL peptide (10 µg; n = 11 to 13 mice per group). (E) Numbers of LCMV-antigen NP396-tetramer+ CD8 T cells per spleen (left) and % IFN-γ+ cells in NP396-tetramer+ CD8 T cells (right) from spleens of Map4k4 WT or iKO mice treated with daily injection of αLFA-1 (500 µg/kg) or control antibody starting 1 day before LCMV-ARM infection. Analysis was done on day 7 after LCMV infection. (F) Representative FCM plots (left) and analysis (right) of CD25 (top), CD69 (bottom), and intracellular IFN-γ (middle) frequencies in CD8 T cells cultured on plates coated with anti-CD3 alone or anti-CD3 plus an agonistic LFA-1 cross-linking antibody for 48 hours. [*αLFA-1: anti–LFA-1 blocking antibody; iso, isotype; MSN, moesin.]

increase in activated partial thromboplastin time (fig. S12C); however, this was not associated with evidence of overall hypercoagulability/disseminated intravascular coagulation as noted by the comparable fibrinogen levels, prothrombin time, and previous platelet counts between the genotypes. Therefore, the significance of these marginal changes is unclear. Furthermore, platelet counts of Map4k4 iKO mice went back to WT levels at 5 months (fig. S12D). At this time point, flow cytometric (FCM) analysis of thymus showed no alterations in
Fig. 7. Blocking LFA-1 diminishes the Map4k4 loss phenotype in the MC38 tumor model. (A) MC38 tumor growth kinetics by group (left) or individuals (middle) and disease progression measured by time for tumor size doubling (right) in Map4k4 WT and iKO mice treated with control or αLFA-1 antibody (n = 16 to 17 mice per group). */ indicates animals taken down due to the institution-defined tumor size limit or poor health condition. (B) Representative FCM plots (left) and analysis (bottom right) of tumor antigen p15e–specific CD8 TILs from groups in (A). (C) CD8 TILs that recognize mixed peptide–MHC-I tetramers representing five MC38 tumor-specific neoantigens. (D) CD107a staining of CD8 TILs in dissociated tumors from groups in (A) restimulated with control or a mixture of six MC38-specific peptides (p15e plus five neoantigens) for 12 hours. (E) CD107a staining of CD8 TILs from dissociated MC38 tumors restimulated with control or mixed MC38-specific peptides in the presence of DMSO or GNE-220 with control or αLFA-1 antibody. Each dot represents a mouse. In (B) to (E), left panels are representative FCM plots and right panels are quantitative analysis. Statistical analysis for (A) (right) was done by log-rank Mantel-Cox test.
the major cell populations (fig. S12E). FCM analysis of spleen and mesenteric LNs showed a modestly decreased frequency of naïve (CD62L+ CD44+) CD8 T cells with a slightly increased frequency of effector/memory (CD44+ CD62L−) CD8 T cells in Map4k4 iKO mice (fig. S12, F and G). Therefore, MAP4K4 deletion does not lead to overt abnormalities in mice.

**Higher MAP4K4 expression correlates with poorer outcome in cancer patients**

The relationship between MAP4K4 expression reportedly correlates negatively with patient outcomes in several cancer types (24, 40, 41). We substantiated these findings with data from six clinical trials that evaluated the effects of the anti–PD-L1 antibody atezolizumab: four trials for lung cancer (FIR, POPLAR, BIRCH, and OAK) and two urothelial bladder cancer trials (IMvigor210 and IMvigor211). In three lung cancer trials, high MAP4K4 expression correlated with poorer overall survival irrespective of treatment (fig. S13, A to C). A much smaller lung cancer trial showed a similar trend (fig. S13D, FIR). In two urothelial bladder cancer trials, a similar correlation was seen in IMvigor210, but not in IMvigor211 (fig. S13, E and F). Our data, along with published literature, are consistent with MAP4K4 being a negative regulator of several anticancer biological processes.

**DISCUSSION**

In this study, we demonstrated that MAP4K4 acts as a negative regulator of CD8 T cell activation during priming of naïve CD8 T cells or restimulation of effector CD8 T cells. MAP4K4 exerts this function by decreasing LFA-1 activation during IS formation. Although MAP4K4 was previously shown to inactivate β1 integrin in endothelial cells in the context of cell–extracellular matrix adhesion, its role in CD8 T cells and in the context of cell–cell interaction was not explored. This study reveals that the link between MAP4K4 and integrin inactivation is a general mechanism that operates in different cell types and biological processes.

T cells require adhesion at multiple steps during an immune response. These steps involve adhering to blood vessel walls for extravasation, scanning tissues for immune surveillance, and adhesion to targets for IS formation (5, 42). LFA-1 is critical at each step of these adhesion events, and its functional plasticity relies on the regulation of avidity and kinetics of binding to its ligands (43); therefore, modulating LFA-1 activity remains a viable strategy to enhance or dampen immune responses. Recently, detaching tumor-produced galecrtin from lymphocytes was shown to increase LFA-1 lateral diffusion and affinity that boosted effector functions in CD8 TILs (44). This mechanism is only applicable to tumors that overexpress galecrtin and is an indirect way to enhance LFA-1–mediated T cell activation. In this study, we identify a CD8 lymphocyte–intrinsic molecular mechanism to increase LFA-1 activation that enhances T cell function, leading to augmented antitumor and antiviral responses in vivo. Our approach represents a more general mechanism independent of tumor intrinsic phenotypes and uncovers a kinase as a druggable target to augment LFA-1 function.

The physiological importance of LFA-1 is context dependent. LFA-1–deficient lymphocytes failed to respond to immunization with low concentration of peptide but responded normally at 10× higher concentration of the same peptide in vivo (6). Likewise, LFA-1–deficient mice mounted normal responses to LCMV and vesicular stomatitis virus infection but could not be primed by tumor antigens and failed to reject immunogenic tumors (39). These studies implied that baseline LFA-1 is functionally necessary only when lymphocytes respond to weaker stimuli. Our data with LFA-1 inhibitors in vivo (Figs. 6, D and E, and 7A) are consistent with these reports. Different from the role of baseline LFA-1 activity defined by loss-of-function studies, our data with Map4k4 loss revealed an additional role of LFA-1 gain of function. We are excited to find that enhancing LFA-1 function is relevant even in the presence of a strong stimulus such as LCMV-ARM infection (Fig. 6).

Map4k4 is broadly expressed in different cell types and exerts a variety of functions via distinct molecular mechanisms (20, 22, 26, 27). Broadly expressed factors could have pro- and antitumor effects depending on the cellular contexts. For example, components of the Hippo pathway reportedly have opposing functions in oncogenesis depending on the cell type (45, 46). For Map4k4, our studies together with previously published literature (24, 25) point to an overall prooncogenic function in multiple cell types that is further corroborated by the finding that higher MAP4K4 levels correlate with worse outcome in multiple cohorts of cancer patients [fig. S13 and (40)].

On the other side of the coin, the tolerability of systemically inhibiting a broadly expressed multifunctional factor warrants careful evaluation. Whereas knocking out Map4k4 in specific cell types is an important approach to uncover its multifaceted biological functions, the whole-body knockout in an adult animal is a more relevant means to evaluate the feasibility of systemic inhibition. Our findings that inducible whole-body knockout of Map4k4 does not lead to overt defects (fig. S12), together with the reports that inducible pan-tissue deletion of Map4k4 in adult mice improves insulin sensitivity (22) and protected mice from long-term high-fat diet (23), open up the possibility to inhibit MAP4K4 systemically. However, given the finding that constitutive loss of Map4k4 in CD4 T cells resulted in systemic inflammation and type 2 diabetes (21) and the limited ability of preclinical studies such as ours to predict human responses, the safety profile of inhibiting MAP4K4 in humans must be thoroughly evaluated in carefully designed clinical trials. In addition, our studies relied on inducible genetic deletion of Map4k4 that represents persistent loss of MAP4K4 function in all adult tissues, which is not possible to achieve in human. Because it is unclear whether in vivo pharmacological inhibition of MAP4K4 kinase activity will produce equivalent effects as genetic deletion, both the antitumor efficacy and safety profiles in cancer patients have to be evaluated in human clinical trials.

In summary, our study contributes to a body of literature that collectively points to the protumor role of MAP4K4. The therapeutic potential of MAP4K4 awaits further investigation in cancer patients.

**MATERIALS AND METHODS**

**Study design**

The aim of this study was to define the role of MAP4K4 in CD8 T cell priming and activation. We evaluated the effect of Map4k4 knockout on CD8 T cells using syngeneic tumor models, antigen stimulation of OT-1 Tg mice and the LCMV-ARM acute infection model. We used in vitro assays to dissect the cellular and molecular functions of MAP4K4. Immunophenotyping was achieved by FCM analysis, enzyme-linked immunosorbent assay (ELISA), Luminex assays, Western blot, quantitative polymerase chain reaction (qPCR), and confocal microscopy. The number of replicates is presented as dot plots in the figures, and experimental repeats are indicated in the figure legends. No animals were excluded except when health
issues require euthanasia before the end of study per institutional policy. Animals were randomly assigned for treatment groups. Statistical methods are outlined in the figure legends and in the “Statistical analysis” section in the Supplementary Materials. For further details, see Supplementary Materials and Methods.

SUPPLEMENTARY MATERIAL
immunology.scienceeagm.org/cgi/content/full/5/45/eaa2245/DC1
Materials and Methods
Fig. S1. Map4k4 loss reduced tumor growth and increased TILs.
Fig. S2. Map4k4 loss reduced inhibitory receptor expression and enhanced anti-PD-L1 efficacy. Fig. S3. Human Map4k4 expression in different cell types based on microarray study GSE1133.
Fig. S4. Map4k4 deletion or inhibition enhances T cell activation.
Fig. S5. MAP4K4 deletion enhances antigen-driven CD8 T cell activation and tumor cell killing.
Fig. S6. MAP4K4 inhibition enhances CD8 T cell activation in response to antigenic peptides with varying affinities.
Fig. S7. Analysis of Map4k4 WT and iKO spleens after LCMV-ARM infection.
Fig. S8. MAP4K4 deletion enhances CD8 T cell–APC conjugate formation.
Fig. S9. Map4k4 deletion increases LFA-1 activation and reduces ERM phosphorylation.
Fig. S10. TCR engagement inactivates and down-regulates MAP4K4 deletion increases LFA-1 activation and reduces ERM phosphorylation.
Fig. S11. TCR engagement down-regulates MAP4K4 in T cells.
Fig. S12. Pan-tissue iKO of Map4k4 does not result in overt changes under basal condition.
Fig. S13. Lower MAP4K4 expression in tumors correlates with better survival outcome in multiple populations of cancer patients.
References (47–55)
View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES
15. M. E. Anderson, T. J. Siahaan, Targeting ICAM-1/LFA-1 interaction for controlling Fig. S13. Lower MAP4K4 expression in tumors correlates with better survival outcome in various cell types based on microarray study GSE1133.


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MAP4K4 negatively regulates CD8 T cell–mediated antitumor and antiviral immunity

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Activation enhancer

CD8+ T cell activation is dependent on encounters with antigen-presenting cells (APCs) through immune synapse formation, which involves binding interactions mediated by multiple molecules on T cells, including lymphocyte function-associated antigen–1 (LFA-1). Here, Esen et al. show that mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) suppresses LFA-1 activation. CD8+ T cell activation is increased upon genetic deletion of Map4k4, which is caused by reduced phosphorylation of ERM (ezrin, radixin, and moesin) proteins that leads to enhanced LFA-1 activation, greater T cell–APC conjugation, and increased CD8+ T cell functionality. Together, these findings define MAP4K4 as a negative regulator of CD8 T cell activation.